NEW INSIGHTS INTO THE COMPLEXITY OF TUMOR IMMUNOLOGY IN B-CELL MALIGNANCIES: TUMOR IMMUNOLOGY AND IMMUNOTHERAPY

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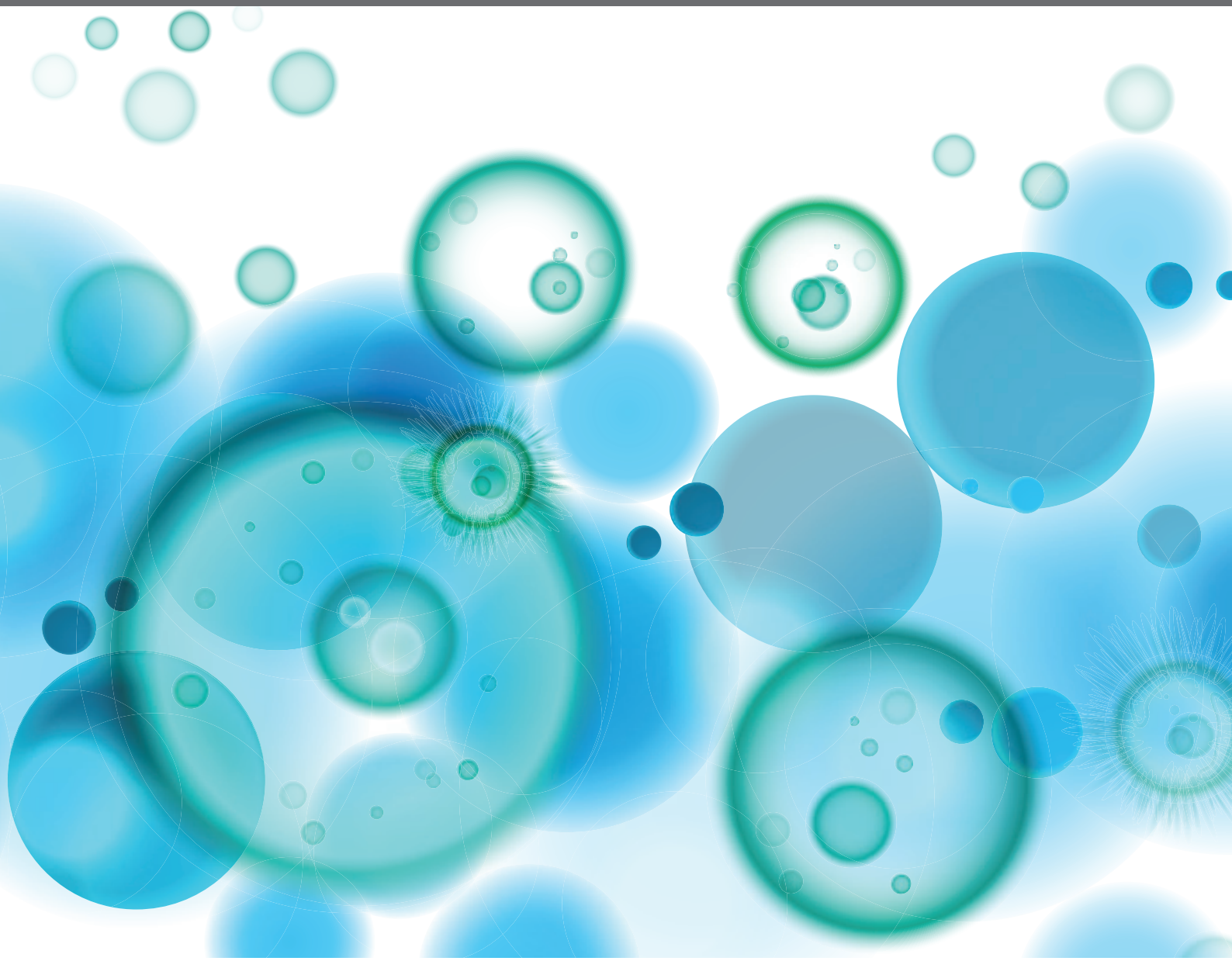
PUBLISHED IN: Frontiers in Immunology and Frontiers in Oncology



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ISSN 1664-8714
ISBN 978-2-88974-206-6
DOI 10.3389/978-2-88974-206-6

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NEW INSIGHTS INTO THE COMPLEXITY OF TUMOR IMMUNOLOGY IN B-CELL MALIGNANCIES: TUMOR IMMUNOLOGY AND IMMUNOTHERAPY

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Topic Editor MS received funding from Bayer AG.

Citation: Paggetti, J., Moussay, E., Seiffert, M., eds. (2022). New Insights Into the Complexity of Tumor Immunology in B-cell Malignancies: Tumor Immunology and Immunotherapy. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88974-206-6

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frontiers in Oncology

EDITORIAL
published: 03 February 2022
doi: 10.3389/fonc.2022.853620

Editorial: New Insights into the Complexity of Tumor Immunology in B-cell Malignancies: Tumor Immunology and Immunotherapy

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Keywords: leukemia, lymphoma, tumor microenvironment, stromal cells, chronic lymphocytic leukemia (CLL), immunotherapy, anti-tumor immunity

Editorial on the Research Topic

New Insights into the Complexity of Tumor Immunology in B-cell Malignancies: Tumor Immunology and Immunotherapy

With the recent advances in cancer immunotherapy, great expectations have been raised for the successful implementation of these novel treatment concepts for patients with B-cell malignancies. The malignant cells in these cancer entities are in constant interaction with non-malignant immune cells and stromal cells in primary and secondary lymphatic tissues, and many B-cell malignancies are associated with immune defects. Even though treatment with immune checkpoint inhibitors or CAR T-cell therapies showed efficacy in some patients, the majority did not respond, for so far unclear reasons. To improve response rates, a better understanding of the cross-talk between the cancer cells and their microenvironment, and of immune escape and treatment resistance mechanisms are urgently needed. The collection of reviews, opinion, method, and original research articles in this Research Topic focuses on the complexity of the tumor microenvironment (TME) in B-cell malignancies and on novel concepts of immunotherapy for patients with these deadly diseases.

In a detailed and timely review, Apollonio et al. summarize current knowledge on tumor intrinsic and extrinsic mechanisms critical to anti-tumor immune responses, as well as sensitivity to immunotherapies in B-cell lymphomas. They discuss the current understanding concerning the role of T cells and inflammatory signaling in these processes. The article further focuses on co-evolving stromal cells and their regulation of immune responses within the TME.

Similarly, Delahaye et al. discuss in a mini review article the current knowledge about protective niches in B-cell acute lymphoblastic leukemia (B-ALL) and the development of therapies targeting the crosstalk between leukemic cells and their microenvironment.

To improve immunotherapy approaches for patients with chronic lymphocytic leukemia (CLL), a better understanding of their defective immune system is required. Griggio et al. focus in their informative review on the main immune defects affecting patients with CLL, also describing the complex networks leading to immune evasion and tumor progression. They further summarize the evolution of immune-based therapeutic approaches, including immunomodulatory drugs, monoclonal antibodies, and immunotherapeutic strategies aiming at activating or administering leukemia-reactive immune effector cells.

Considering the recent advances in our understanding of T-cell activity and defects in CLL, a timely review by Vlachonikola et al. focuses on the binary, contradicting role of T lymphocytes in

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Specialty section:
This article was submitted to Hematologic Malignancies, a section of the journal Frontiers in Oncology

Received: *12 January 2022*
Accepted: *14 January 2022*
Published: *03 February 2022*

Citation:
Seiffert M, Moussay E and Paggetti J (2022) Editorial: New Insights into the Complexity of Tumor Immunology in B-cell Malignancies: Tumor Immunology and Immunotherapy. Front. Oncol. 12:853620. doi: 10.3389/fonc.2022.853620

CLL. They describe the imbalanced composition of the T-cell compartment in CLL and how it is driven by antigen selection. Further, they focus on T-cell defects and perspectives of novel immunotherapy approaches to overcome them.

One of the main immune defects described for patients with CLL, is the failure to develop proper immune synapses between malignant B cells and effector T cells which was linked to altered cytoskeleton properties. In an original research article, Wurzer et al. analysed immune synapse formation between CLL and NK cells and suggest a novel immune escape mechanism in CLL. They show that fast actin remodeling in CLL cells upon contact with NK cells prevents their effective NK-cell-mediated killing. These results highlight the critical role of the actin cytoskeleton in CLL cells in driving resistance against NK cell cytotoxicity and provide a new potential therapeutic point of intervention to target CLL immune escape.

Due to the increasing interest in the TME, extensive analyses revealed a tremendous complexity and heterogeneity of the cellular microenvironment in cancer. The development of novel technologies for single-cell analysis, like mass cytometry, enhances the number of cellular features that can be surveyed simultaneously and allows for resolving complex cellular systems. In a detailed method article by Gonder et al., the use of mass cytometry for a better characterization of cells in the TME of CLL is described. This includes a protocol for antibody panel design and validation, sample preparation and acquisition, machine set-up, quality control, and analysis of cells. Additionally, advantages and pitfalls of this technique are discussed.

Richter Syndrome (RS) is a highly aggressive B-cell malignancy that arises due to the transformation of CLL, and the development of effective treatment options for RS patients is an urgent clinical need. In a very informative review article, Augé et al. provide an extensive overview of genomic aberrations associated with RS. The article further focuses on the role of the PD-1/PD-L1 immune checkpoint axis in RS and discusses the potential of targeting this checkpoint as novel therapeutic option for patients with this aggressive disease.

Multiple myeloma (MM) is an aggressive malignancy of plasma cells associated with pathological changes in the bone marrow microenvironment. Due to these, the majority of patients with MM develop anemia. Immunomodulatory agents like pomalidomide or lenalidomide are used for the treatment of MM and associated with the recovery from anemia. Verma discusses in an interesting opinion article the potential mode-of-action underlying this observation.

Inhibitors of phosphoinositide 3-kinase (PI3K) are approved for the treatment of CLL and other B-cell lymphoma. But the PI3K signaling pathway is not only limited to cancer cells but also crucial for many components of the TME which has to be considered when treating patients with PI3K inhibitors. Aydin et al. address this important issue in their detailed review article. They summarize published data that show an impact of PI3K inhibition on the TME with a specific focus on CLL, and discuss how a better understanding of these effects of PI3K inhibitor-based therapies can serve as a rationale for the development of improved drugs or of novel combinatory treatment strategies in CLL.

Novel immunotherapies, like immune checkpoint blockade or CAR T-cell therapy showed efficacy in some patients with B-cell lymphoma, but for the majority of cases, this treatment approach failed. As a potential resistance mechanisms in Hodgkin and Non-Hodgkin lymphoma, Albakova et al. explore in their interesting review article the involvement of heat shock proteins (HSPs). As HSPs are highly expressed in lymphoma cells and are known to modulate immune responses and inhibit apoptosis, a better understanding of their role in anti-tumor responses may help in the development of more effective immunotherapy in B-cell lymphoma.

Activation of Toll-like receptor (TLR) signaling *via* synthetic agonists is an immunomodulatory approach currently tested in several clinical trials for cancer. In an original research article, Lu et al. show that treatment of established A20 B-cell lymphoma in mice with a synthetic TLR4 agonist controlled tumor development. This effect was associated with T-cell inflammation and dependent on $CD8^+$ T cells. Interestingly, the treatment response depended on TLR4 expression in B-cell lymphoma cells. TLR4 agonist treatment of lymphoma cells led to their enhanced antigen-presentation and increased their apoptosis. It was further sufficient to induce protective $CD8^+$ T-cell responses. Therefore, the therapeutic potential of TLR4 activation in B-cell lymphoma should be further explored.

A novel interesting treatment approach for primary central nervous system lymphomas (PCNSL) harboring B-cell receptors that recognize the common auto-antigens neurabin-I and SAMD14 is presented in the original research article by Bewarder et al. They generated a full-length IgG1 or Fab antibody format containing the PCNSL-reactive epitope of SAMD14/neurabin-I and showed specific binding of these constructs to lymphoma cells as well as their effective killing in coculture with PBMC.

Altogether, the collection of articles in this Research Topic provides a comprehensive overview of the immune microenvironment in B-cell malignancies and the potential of new immunotherapy approaches for these diseases, thereby supporting the development of improved treatment regimens for the patients.

AUTHOR CONTRIBUTIONS

All authors edited the Research Topic. MS wrote the editorial. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the Fonds National de la Recherche Luxembourg to JP and EM (C20/BM/14592342 and C20/BM/14582635).

ACKNOWLEDGMENTS

The topic editors would like to acknowledge all the authors who participated in this Research Topic.

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frontiers in Oncology

ORIGINAL RESEARCH
published: 19 August 2020
doi: 10.3389/fonc.2020.01438

Toll-Like Receptor 4 Expression on Lymphoma Cells Is Critical for Therapeutic Activity of Intratumoral Therapy With Synthetic TLR4 Agonist Glucopyranosyl Lipid A

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OPEN ACCESS

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Oncology

Received: *24 April 2020*
Accepted: *07 July 2020*
Published: *19 August 2020*

Citation:
Lu H, Betancur A, Chen M and ter Meulen JH (2020) Toll-Like Receptor 4 Expression on Lymphoma Cells Is Critical for Therapeutic Activity of Intratumoral Therapy With Synthetic TLR4 Agonist Glucopyranosyl Lipid A. Front. Oncol. 10:1438. doi: 10.3389/fonc.2020.01438

Intratumoral (IT) injections of Glucopyranosyl lipid A (G100), a synthetic toll-like receptor 4 (TLR4) agonist formulated in a stable emulsion, resulted in T-cell inflammation of the tumor microenvironment (TME) and complete cure of 60% of mice with large established A20 lymphomas. Strong abscopal effects on un-injected lesions were observed in a bilateral tumor model and surviving mice resisted a secondary tumor challenge. Depletion of CD8 T-cells, but not CD4 or NK cells, abrogated the anti-tumor effect. Unexpectedly, TLR4 knock-out rendered A20 tumors completely non-responsive to G100. *In vitro* studies showed that GLA has direct effect on A20 cells, but not on A20 cells deficient for TLR4. As shown by genotyping and phenotyping analysis, G100 strongly activated antigen presentation functions in A20 cells *in vitro* and *in vivo* and induced their apoptosis in a dose dependent manner. Similarly, the TLR4 positive human mantle cell lymphoma line Mino showed *in vitro* activation with G100 that was blocked with an anti-TLR4 antibody. In the A20 model, direct activation of B-lymphoma cells with G100 is sufficient to induce protective CD8 T-cell responses and TLR4 expressing human B-cell lymphomas may be amenable to this therapy as well.

Keywords: TLR4, GLA, B-cell lymphoma, intratumoral treatment, tumor microenvironment

INTRODUCTION

Only a minority of cancer patients currently benefits from immunotherapeutic interventions aimed at rescuing functional T-cells responses through modulation of immune check points, and this resistance is at least partly be due to the immunosuppressive nature of the tumor microenvironment (TME) (1). Immune recognition of tumor cells and priming of CD8 T-cell responses in the tumor draining lymph node requires processing and presentation of tumor antigens by cross-presenting dendritic cells, coupled with their activation by danger signals, and secretion of type 1 interferons (2, 3). Therefore, therapies that are capable of inducing proinflammatory changes in the TME, activating antigen presenting cells and inducing immune responses should have significant therapeutic potential on their own and in combination with other immunotherapies (4, 5). To this end, several agents designed to modify the TME are currently under clinical investigation, including cytokines, oncolytic viruses, toll-like receptor (TLR) 3, 7/8 and 9 agonists, RIG-I agonists and others (6, 7). While some of these treatment modalities have shown impressive levels of efficacy in murine

models, notably agonists of the stimulator of interferon genes (STING) pathway, activity in the clinic has been rather modest to date, especially in solid tumors with low levels of pre-existing T-cell inflammation.

Toll-like receptors (TLRs) belong to the families of pattern recognition receptors (PRRs), and recognize conserved microbial components, which are referred to as pathogen-associated molecular patterns (PAMPs), as well as endogenous danger signals, known as damage-associated molecular patterns (DAMPs). Common TLR-activating PAMPs include viral and bacterial nucleic acids (signaling trough TLR3, TLR7/8, or TLR9), flagellin (a TLR5 agonist), as well as lipopolysaccharide (LPS), lipoteichoic acid, and mannans (which signal through TLR2 or TLR4). Endogenous TLR-activating DAMPs are nucleic acids and the nuclear non-histone protein high mobility group box 1 (HGMB1). TLR control the activation, maturation and immunological functions of immune cells and are as a family either located in the plasma membrane (e.g., TLR2, TLR4, TLR5) or in endosomal membranes (e.g., TLR3, TLR7/8, and TLR9), of macrophages, dendritic cells (DCs), B cells, and natural killer (NK) cells, as well as some non-immune cells including epithelial cells, fibroblasts and malignant cells. Clinical trials are currently focused on evaluating systemic treatment with TLR3 agonists (Hiltonol™, Ampligen™, BO-112™) in combination with standard of care, or with immune checkpoint inhibitors, vaccines, cytokines or other treatments in a variety of solid tumors (colorectal carcinoma, breast, lung, prostate cancer and others), as well as hematological malignancies (MM, AML). TLR7/8 agonists (Imiquimod, Motolimod) are being used topically in a number of solid tumors (squamous cell carcinoma, melanoma, anal carcinoma, cutaneous T-cell lymphoma, and others), and a TLR9 agonist (SD-101) is being evaluated for intratumoral treatment of hematological malignancies (follicular lymphoma) and some solid tumors (prostate and others) (8). Toll-like receptor 4 (TLR4) is part of a cell surface receptor complex that recognizes lipopolysaccharide (LPS) and is expressed on professional antigen-presenting cells (APCs), such as dendritic cells (DC), monocytes, macrophages, and activated B cells, as well as some non-immune cells, including epithelium, endothelium, and smooth muscle (9). Activation of TLR4 on APC results in stimulation of antigen presentation, upregulation of costimulatory molecules such as CD40 and CD80, and secretion of inflammatory cytokines, including IL6 and IL12, and type I interferons (IFN) (10). In addition, TLR4 agonists have recently been shown to signal through an intracellular pathway which leads to activation of the non-canonical inflammasome with secretion of interleukin 1 beta (IL-1β) and IL-18 (11). Glucopyranosyl lipid A (GLA) is a synthetic TLR4 agonist that potently activates dendritic cells and has shown a very good safety and efficacy profile as an infectious disease vaccine adjuvant in several phase 1 and 2 studies (12, 13). Two formulations of GLA have been tested in preclinical and clinical studies, GLA-stable emulsion (SE) and GLA-aqueous formulation (AF). Both formulations have been shown to be potent vaccine adjuvants stimulating both antibody and T cell responses (14, 15). For intratumoral injection, GLA-SE is preferred as the emulsion retains GLA locally at the injection site. Intratumorally injected GLA-SE inflamed the TME in murine melanoma and glioma models, "pulling" antigen-specific, vaccine primed or passively transferred CD8 T-cells into tumors, which resulted in their regression (16). A recent clinical trial showed that intratumoral injection of GLA-SE (termed G100) inflamed the TME of Merkel cell carcinoma patients, with antitumor immune responses and objective tumor responses observed (17).

Using the murine A20 B-cell lymphoma, we investigated the effect of IT G100 on the TME and its dependency on TLR4 expression of the tumor cells.

MATERIALS AND METHODS

Mice

Female Balb/c mice (7∼8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME) and were maintained under specific pathogen free conditions. All the procedures described in this study were performed in compliance with the local Institutional Animal Care and Use Committee guidelines.

Reagents and Cell Lines

Fluorochrome-conjugated antibodies targeting mouse CD3, CD4, CD8, B220, NKp46, Foxp3, IFNγ, TNFα, IL2, CD40, CD80, CD86, and TLR4 were obtained from eBiosciences (San Diego, CA). The antibodies used for *in vivo* depletion, anti-CD4 (clone GK1.5) and anti-CD8 (clone 56.3) were purchased from BioXcell (West Lebanon, NH). The A20 cell line, originally derived from B lymphocytes of a naturally occurring reticulum cell sarcoma from an old Balb/c mouse, was obtained from the American Type Culture Collection (ATCC® TIB-208). The A20 cells were expanded in complete RPMI medium (RPMI with 10%FBS, pen/strep, and glutamine) before each tumor inoculation. A biallelic TLR4 knockout A20 cell line was generated using the CRISPR/Cas9 system at GenScript (Piscataway, NJ) and the biallelic gene knockout was confirmed by sequencing analysis. Glucopyranosyl lipid A (GLA, G100) was manufactured and formulated by Immune Design using proprietary methods. For preclinical work, two formulations of GLA were used. For *in vivo* studies, a stable oil-in-water formulation (G100) containing 2 mg/mL GLA in 2% squalene (SE) was adjusted to various GLA concentrations (1, 5, 10 or 20 μg GLA) in 2% SE. GLA-AF (aqueous formulation), which contained the surfactant dipalmitoyl phosphatidylcholine instead of squalene, was adjusted to 5 μg GLA/ml. For *in vitro* experiments, cells were exposed to GLA-AF for 48 h before being analyzed by Flow cytometry for expression of surface markers or analysis for RNA expression profiling. The Mino cell line is a human blood/Mantle cell lymphoma (B cell non-Hodgkin's lymphoma) that was obtained from ATCC (ATCC® CRL-3000).

A20 Tumor Model

Five million A20 murine lymphoma cells were implanted subcutaneously (s.c.) into Balb/c mice on the right flank (for unilateral tumor model) or on both sides (for a bilateral

tumor model, only one tumor injected). Tumor take was close to 100% using this inoculation method. Tumor growth was monitored using a digital caliper every 2–3 days and the tumor size was expressed as surface area (length x width). Mice were sacrificed when the tumor size reached over 200 mm^2. Intratumoral (IT) injection of G100 or control PBS or SE started on Day 7∼9 when the average tumor size was 30∼50 mm^2. The treatment was administered three times per week for a total of 7–9 doses. To investigate the direct effect of GLA without the emulsion on A20 cells with respect to tumor rejection, A20 cells were treated *in vitro* with GLA-AF (5 μg/mL) for 48 h before the cells were harvested and inoculated s.c. into Balb/c mice.

CD4 and CD8 T Cell Depletion

For selective depletion of CD4 or CD8 T cells, mice received intraperitoneal injection of the depletion antibody (100 μg) for two times at the week before IT G100 treatment and then once per week during treatment. FACS analysis confirmed that the depletion efficiency was more than 95% for both CD4 and CD8 T cells (data not shown).

Flow Cytometry

Staining of splenocytes was performed as described previously (18). TILs were isolated by centrifugation over Histopaque-1083 (Sigma-Aldrich, St. Louis, MO). For staining of T regulatory cells, splenocytes or TIL were first stained with anti-CD3-eF450/anti-CD4-FITC/anti-CD8-PerCP, and then stained with anti-FoxP3-PE after fixation and permeabilization. For the staining of activation markers and TLR4 expression on A20 cells, the cells were cultured in RPMI medium with or without GLA-AF (5 μg/mL) for 48 h before the cells were harvested for flow cytometry analysis. The cells were then stained with antibodies against CD80, CD86, and CD40, and TLR4 using surface staining. For evaluation of apoptosis and necrosis, cells were stained with an Annexin V (AV) and propidium iodide (PI) staining kit with binding buffer (Invitrogen, Carlsbad, CA). Data acquisition was done on a FACS LSRII flow cytometer (BD Biosciences, San Jose, CA). List mode data were analyzed using the FlowJo software (Tree Star, Ashland, OR).

Cell Growth Inhibition

The effects of GLA or other TLR agonists on growth of murine or human lymphoma cell lines were evaluated using different methods. In initial studies, cell viability and cell number were enumerated using a Nucleocounter, then a FACS-based high throughput method using LIVE-DEAD-NIR dye (ThermoFisher, Waltham, MA) was employed. For the evaluation of apoptosis and necrosis, cells were stained with AV and PI as described above. To compare the direct effect of different TLR agonists

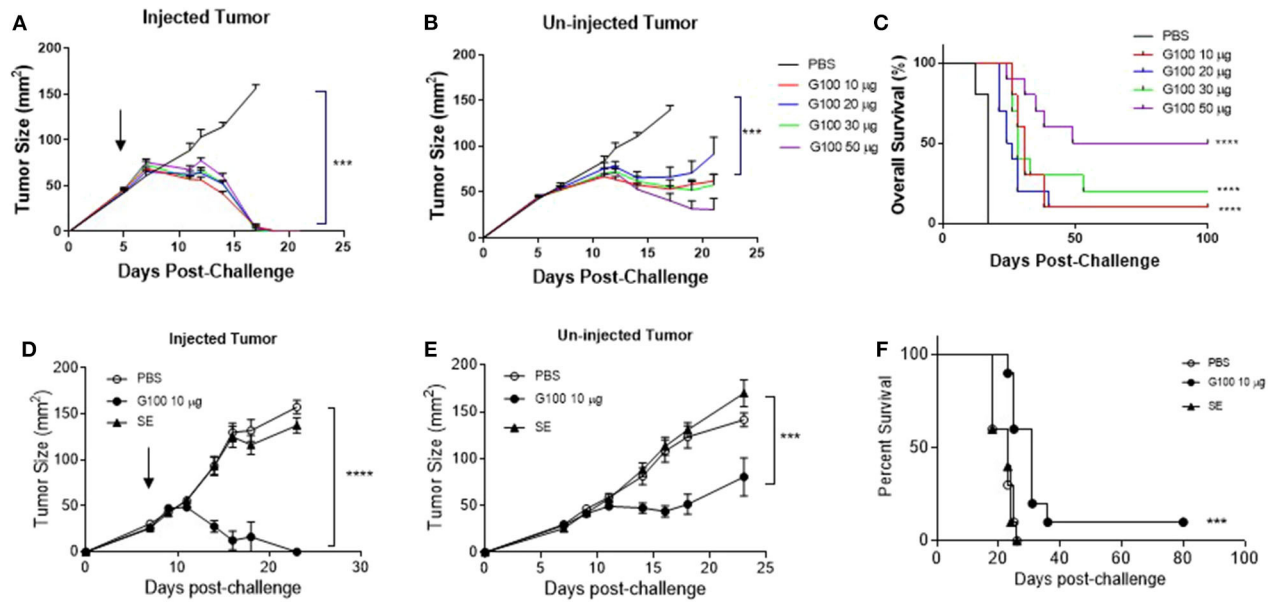

FIGURE 1 | G100 cures established murine A20 lymphomas and the anti-tumor effect depends on tumor cell TLR4 expression. **(A,B)** Tumor growth curves of G100-injected or contralateral un-injected A20 tumors. Five million A20 were implanted subcutaneously (s.c.) into Balb/c mice ($n = 5$/group) on both flanks. Intratumoral (i.t.) injection of G100 (10, 20, 30, or 50 μg GLA-SE) or PBS started on day 5 [indicated by the arrow on **(A)**] when the tumors were palpable (average size ∼40 mm^2), three times per week, for a total of 9 injections. Mice were sacrificed when any tumor size reached over 150 mm^2. **(C)** Survival curves in mice with bilateral A20 tumors treated with different doses of GLA-SE and PBS control. All the G100-treated mice (10, 20, 30, or 50 μg GLA groups) had significant longer survival than control (PBS treated) mice ($p < 0.0001$, adjusted p-values using Bonferroni method). The survival in the high dose (50 μg GLA) G100 group was significantly better than that in low dose group (10 μg) ($p = 0.03$). **(D,E)** Tumor growth curves in mice treated with G100 and two controls, PBS and SE, for both **(D)** injected tumors, and **(E)** un-injected tumors. $^{***}p < 0.001$, $^{****}p < 0.0001$ based on tumor size on day 18. There was no difference in growth kinetics between PBS and SE groups for both the injected and un-injected tumors. The arrow in **(D)** indicate treatment start time. **(F)** Survival curves in mice treated with both PBS and SE controls. $^{***}p < 0.001$ between G100 and SE or PBS. Again, there was no difference between SE and PBS.

in A20 cells, we cultured A20 cells in 96-well plates (10,000 cells/well) and treated the cells in duplicate with serial dilutions of GLA, TLR3 agonist poly I:C, and TLR9 agonist CpG ODN2006 (InvivoGen, San Diego). After 96 h of incubation, the cells were stained with LIVE-DEAD-NIR dye and analyzed on a flow cytometer. The percentage of viable cells as well as total number of viable cells in each well were analyzed in FlowJo.

Intracellular Staining (ICS)

ICS was performed using similar method as previously described with modifications (18). The spleens from G100 treated Balb/c mice that rejected A20 tumors and control naïve mice were harvested and homogenized using the AutoMACS tissue dissociator (Miltynei Biotec, San Diego, CA). Red blood cells were lysed by using the ACK lysis buffer from eBiosciences (San Diego, CA). For measurement of cytokine production, splenocytes were stimulated with γ-irradiated A20 tumor cells at a 10:1 ratio in 96-well plates with ∼ 500,000 cells per well in complete RPMI medium. Medium containing PMA and ionomycin (eBiosciences) was used as positive control. After 1 h of stimulation, brefeldin A was added to the culture wells and the plate was incubated overnight at 37°C in a humidified CO_2 incubator. The next day, the cells were stained with anti-CD4-AF-700, anti-CD3-PerCP, anti-CD8-PB, and anti-B220-V500. After surface staining, the cells were fixed with Cytofix buffer (BD Biosciences, San Jose, CA) and then permeabilized with Perm/Wash buffer containing 5% rat serum. Cells were then stained with anti-TNFα-Alexa 488, anti-IFNγ-PE, and anti-IL2-APC for intracellular cytokines. Cells were

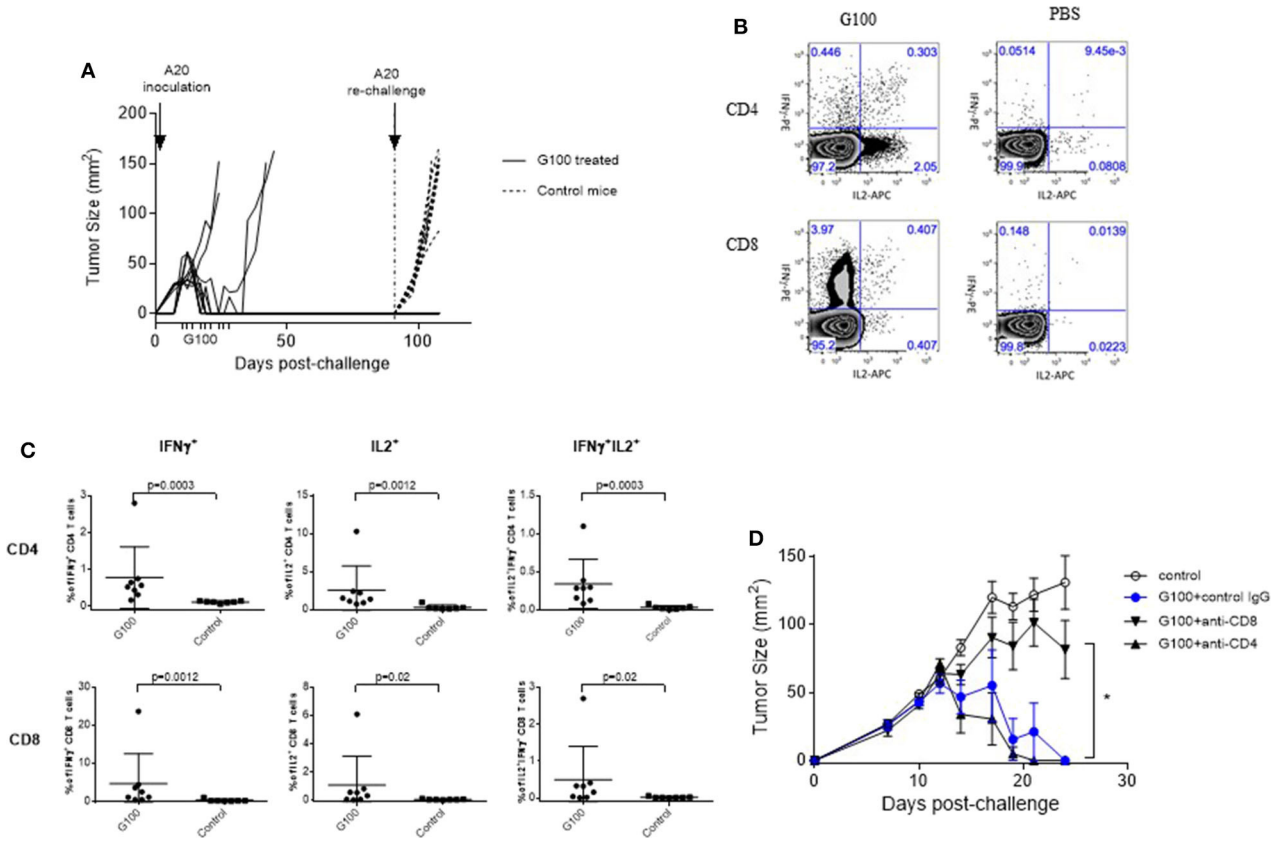

FIGURE 2 | G100 induces CD8 T cell-mediated tumor protection and memory responses. **(A)** Rejection of A20 tumor re-challenge in G100-treated mice. On Day 7 after A20 tumor cell inoculation, mice with palpable A20 tumors started receiving GLA-SE treatment (10 μg, 3x/week, for 3 weeks), as indicted on the x-axis. Six out of 10 treated mice had complete tumor regression and received a second tumor challenge on the opposite side of the original tumor at 3 months after the initial tumor inoculation (indicated by vertical dotted line on day 90). All these mice rejected the secondary tumor challenge, demonstrating the development of long-term immunity. Untreated mice developed tumor rapidly after A20 challenge, as shown in tumor growth curves with dotted lines. **(B)** Splenocytes were collected from G100-treated mice and naïve Balb/c mice ($n = 8$ per group) and stimulated with irradiated A20 tumor cells at a 10:1 ratio with the addition of brefeldin A and cultured overnight. Then the cells were then either surface stained or permeabilized and stained with antibodies against IFNγ, IL2, and TNFα and analyzed by FACS. Shown are representative dot plots showing IL-2 and IFNγ production in CD4 and CD8 T cells in splenocytes co-cultured with A20 tumor cells. For data analysis, cells were first gated as singlets based on FSC and SSC. Then the singlet cells were gated based on a viability dye. The live cells were gated for CD3+ T cells, which were further separated into CD4 and CD8 T cells. **(C)** Summary graphs showing the levels of cytokine positive cells in CD4 and CD8 T cells from GLA-treated or control naïve mice. **(D)** Tumor growth curves (mean ± sem) in mice that were treated with PBS, G100 (10 μg GLA, 2%SE /mouse, 3 times/week for 3 weeks), or G100 plus CD4 or CD8 T cell depletion. The depletion efficiency was more than 95% as measured by FACS (data not shown). Depletion of CD8 T cells but not CD4 T cells significantly abrogated the anti-tumor effects of G100. *$p < 0.05$ between G100 and G100 + CD8 depletion group.

washed with FACS buffer and analyzed on a 3-laser LSRII cytometer. List mode data were analyzed using the FlowJo 9.0 software.

Immunohistochemistry (IHC) Analysis

A20 tumors were treated with intratumoral G100 (10 μg) or control PBS every other day. After 3 treatments, tumors

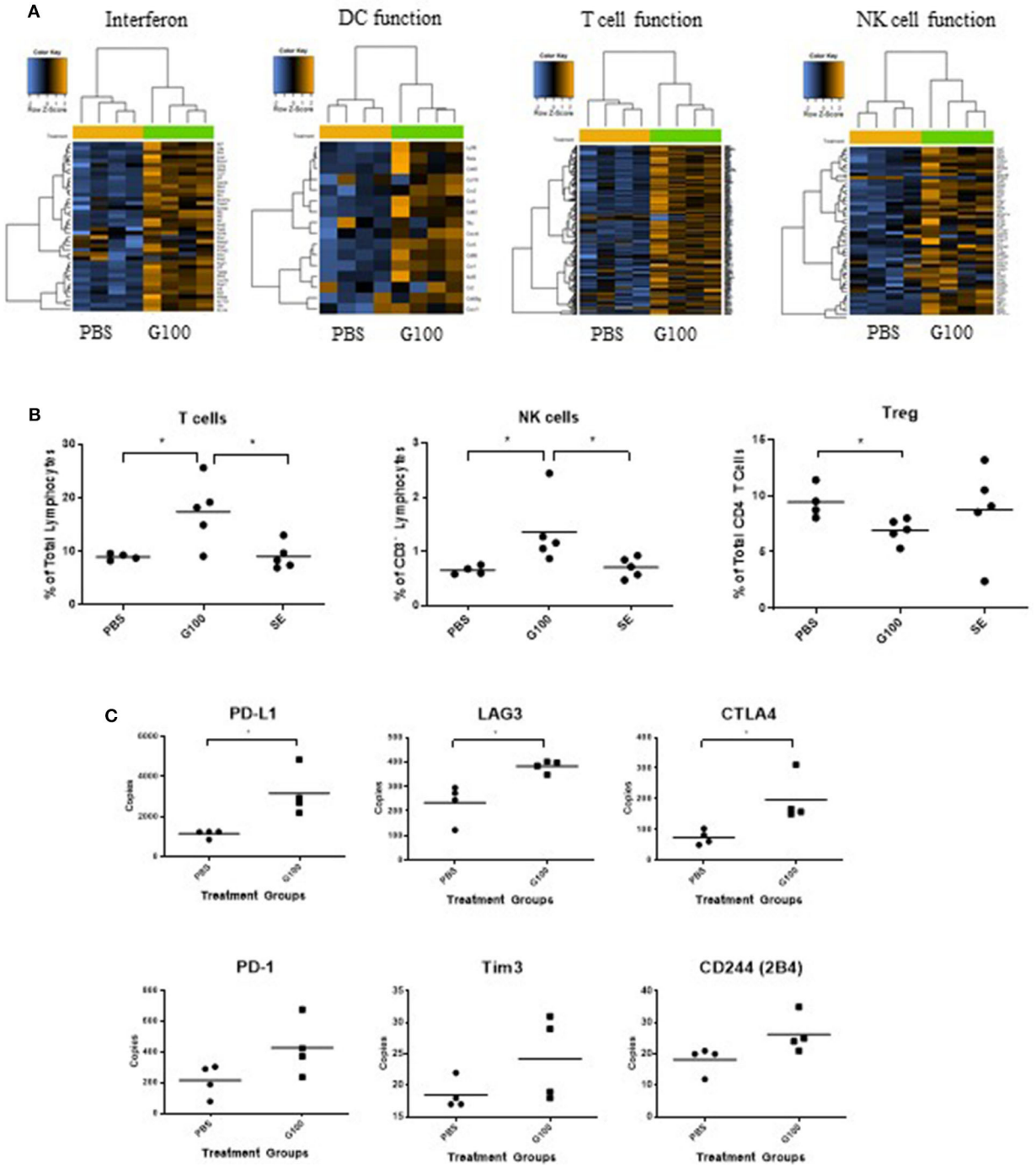

FIGURE 3 | G100 inflames TME of A20 tumors. **(A)** A20 tumors ($n = 4$ mice/group) treated with G100 (10 μg/ml GLA, 2%SE) for 1 week (3 injections, every other day) were collected for gene expression analysis by Nanostring. Multiple immune response pathways, including type 1 interferon signaling, DC function, T cell, and NK cell functions, were all activated. **(B)** Tumor infiltrating lymphocytes (TIL) were collected for Flow cytometry analysis and stained with antibodies for CD3, CD4, CD8, Foxp3, and NKp46. G100 treatment induced significant increase in T cells and NK cells and a decrease in T_{reg} among CD4 T cells. SE treatment was no different from PBS control in any of the markers. **(C)** Gene expression levels of T cell exhaustion markers in G100-treated and non-treated tumors ($n = 4$ mice/group). *$p < 0.05$ by student *t*-test.

were harvested and fixed in 10% formalin. Paraffin embedding, sectioning, and IHC analysis were performed at the Experimental Histology center at Fred Hutchinson Cancer Research Cancer (Seattle, WA). Anti-cleaved caspase 3 antibody (clone D3E9) and anti-Ki67 antibody (clone D3B5) were from Cell Signaling Technology (Danvers, MA).

RNA Expression Analysis of Murine and Clinical Specimens

A20 tumors were treated with G100 or control PBS every other day for a total of 3 times. Tumors were collected at 2 h after the last treatment and immediately stored in RNAlater (Ambion, Austin, TX). RNA was extracted using the DNA/RNA Allprep kit from Qiagen (Valencia, CA) for analysis with the nCounter® mouse PanCancer Immune Profiling Panel of Nanostring (Seattle, WA), a multiplex gene expression analysis with 770 genes from 24 different immune cell types, common checkpoint inhibitors, CT antigens, and genes covering both the adaptive and innate immune response. The hybridization reaction and scanning of slides was performed by the Genomic Center at Fred Hutchinson Cancer Center, Seattle, WA. The data were normalized and analyzed using nSolver software (Nanostring, Seattle, WA). To study the direct effect of GLA on A20 tumor cells, the cells were cultured in RPMI medium with or without GLA (5 μg/mL) for 48 h before the cells were harvested for RNA extraction and subsequent gene expression analysis using the same Nanostring panel, nCounter® mouse PanCancer Immune Profiling Panel. The expression level of different TLRs in A20 cells was compared by using Nanostring gene expression data normalized with house-keeping genes. Data analysis was done in nSolver using the Advanced Analysis tool with the following parameter setting: remove genes below specified threshold (TRUE); threshold count value (20); covariate (TimePoint); variable type (categorical); reference level (pre-Tx); perform normalization (TRUE); auto-select number of housekeepers (TRUE); perform differential expression testing (TRUE); predictors (TimePoint). TimePoint refers to whether a sample is from pre-treatment or post-treatment with G100.

Statistical Analysis

Statistical analysis was performed using SAS version 9.4 & GraphPad Prism (GraphPad software, San Diego, CA). Two-tailed Student *t*-test was used to analyze differences between treatment groups in tumor size (last measurement shown in figure) and tumor infiltrating lymphocytes (FACS). ANOVA was used to analyze the treatment time- and dose- dependent response of GLA on cell growth inhibition and induction of apoptosis and necrosis in A20 cells. Kaplan-Meier plots were used to analyze the mouse survival data in A20 lymphoma model. Comparison between survival curves was done using the log-rank test. Conclusions from sensitivity analysis using Fleming-Harrington test with parameters (1, 0) to address late effect are consistent with the main analysis using Log rank test (in **Figure 1C**). Bonferroni adjustment was used to control type 1 error for multiple comparisons. A $p < 0.05$ was considered statistically significant.

RESULTS

Anti-tumor Effects of G100 in the A20 Model Depend on Tumor Cell TLR4 Expression

As shown in **Figures 1A,B**, G100 treatment (10–50 μg, intratumoral 3x/week, 7–9 doses total) inhibited the growth of both injected (treated) and un-injected tumors (abscopal lesions) in the A20 murine lymphoma model. The overall survival (OS) of bilaterally challenged mice was significantly prolonged in all treatment groups receiving G100 (10, 20, 30, or 50 μg GLA/2%SE), as compared to PBS treated mice ($p < 0.0001$, **Figure 1C**), with a dose response observed and greatest survival (∼50%) in the 50 μg group. To investigate whether the stable emulsion (SE) formulation we used for G100 had any effects on its own, mice were treated with G100 (10 μg in 2%SE), SE (2%), or control PBS. As shown in **Figures 1D–F**, injection of SE alone had no tumor inhibitory effect and showed no difference from PBS. All surviving mice rejected tumor re-challenge 3 months after primary challenge, demonstrating a robust memory response (**Figure 2A**). Mice treated with G100 also had significantly higher levels of tumor antigen-specific

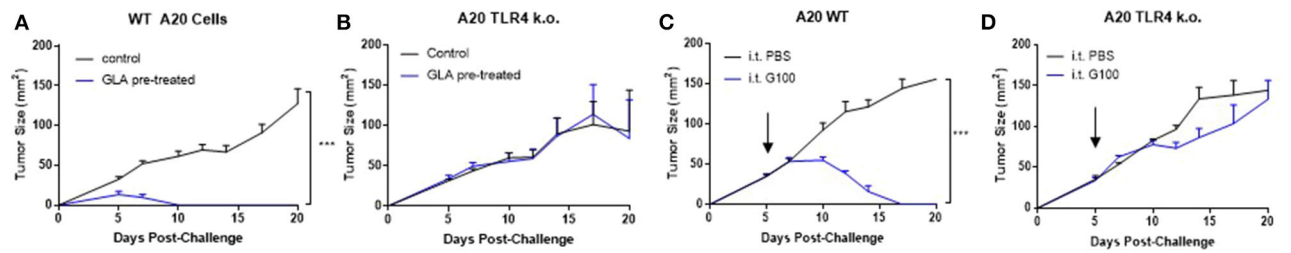

FIGURE 4 | GLA has anti-tumor effects in A20 WT but not A20 TLR4 ko tumor cells *in vitro* and *in vivo*. **(A,B)** Pre-treatment with GLA-AF (incubating cells in medium containing GLA-AF (5 μg /ml) *in vitro* for 48 h prior to subcutaneous implantation in mice inhibited the growth of wild type (WT), but not TLR4 knockout (k.o.) A20 tumor growth after inoculation. **(C,D)** IT G100 treatment inhibited the growth of WT but not TLR4 k.o. A20 tumors. IT injection of G100 (10 μg GLA-SE, 3x/week) started on Day 7 after the tumors were established and lasted for 3 weeks. Shown are average tumor sizes (mean ± sem) for each treatment groups ($N = 5$/group). ***$p < 0.001$ by student *t*-test.

CD4 and CD8 T cells that secrete IFNγ and IL2 after *in vitro* stimulation with A20 cells (**Figures 2B,C**). Selective depletion studies further demonstrated that the anti-tumor effect was dependent on CD8 T cells (**Figure 2D**). G100 activated multiple genes related to DC function, T cell and NK cell function in the A20 TME (**Figure 3A** and **Supplementary Table 1**) and FACS analysis of tumor infiltrating lymphocytes (TIL) showed significantly ($p < 0.05$) increased (∼2-fold) percentages of T cells and NK cells, with decreased (∼30%) Tregs in G100-treated mice (**Figure 3B**). Most exhaustion markers tested (PD-1, PDL1, LAG3, TIM3, CD244, CTLA4) were upregulated in A20 tumors after G100 treatment (**Figure 3C**).

Since A20 is derived from murine B cells which are known to express TLR4, we investigated the importance of TLR4 expression on tumor cells by generating a biallelic TLR4 knockout A20 cell line. To separate direct GLA effects on tumor cells from effects of GLA on immune cells in the TME, we pretreated A20 WT cells or A20 TLR4 k.o. cells with GLA-AF (5 μg /ml) *in vitro* for 48 h prior to subcutaneous implantation. As shown in **Figures 4A,B**, GLA-pretreated A20 WT cells did not establish tumors, whereas the TLR4 k.o. cell line was not affected by GLA pretreatment and established tumors with the same growth kinetics as A20 WT cells. A side-by-side comparison of IT G100 effect on WT A20 tumor vs. TLR4 k.o. tumors showed that

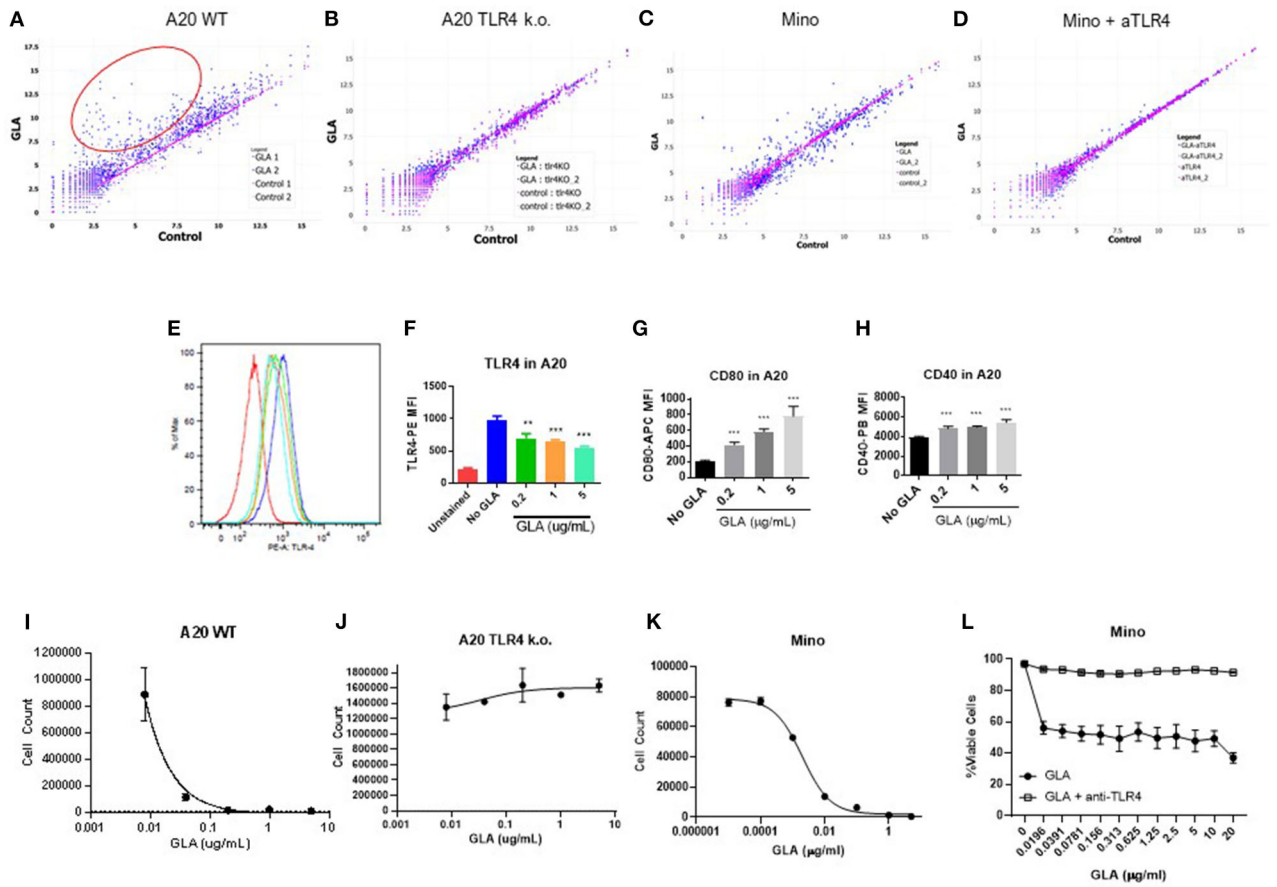

FIGURE 5 | GLA has direct effects on murine and human lymphoma cells by inducing activation and cell death. **(A–D)** Scatter plot of gene expression analysis in murine or human lymphoma cells. Each dot represents the expression level of a particular gene in the control group (x-axis) or GLA-treated (y-axis) group. The full list of genes with differential expression is available in **Supplementary Tables 2–5**. **(A,B)** A20 WT or TLR4 k.o. cells were treated with GLA-AF (5 μg/mL, 48 h) before gene expression analysis by Nanostring using the mouse panCancer immune profiling panel. **(A)** Scatter plot of gene expression levels in GLA- or control PBS- treated WT A20 cells. **(B)** Scatter plot of gene expression levels in GLA- or PBS- treated TLR4 k.o. A20 cells. The red circle marks genes that are significantly induced by GLA treatment. Two independent samples are included in each treatment group. **(C)** Mino lymphoma cells were treated with GLA-AF (5 μg/mL, 48 h) before gene expression analysis by Nanostring using the human panCancer immune profiling panel. **(D)** TLR4 was blocked by adding anti-TLR4 mAb (10 μg/mL) 15 min before GLA treatment and gene expression analysis. **(E–H)** Direct effects of GLA in A20 tumor cells *in vitro*. A20 tumor cells were treated with GLA (0.2, 1, or 5 μg/ml GLA-AF, 48 h) before staining with anti-TLR4/MD2, anti-CD80, or anti-CD40. Shown are overlay histogram of TLR4 staining in A20 cells **(E)**, and summary graphs of mean fluorescent intensity (MFI) of TLR4, CD80, and CD40 expression in GLA-treated or control A20 cells **(F–H)**. Treatment was done in duplicate. Each column indicates mean ± sem of duplicate wells. **(I,J)** Dose-dependent inhibition of the growth of WT but not TLR4 k.o. A20 cells by GLA. The cells were treated with serial dilution of GLA (0.01–5 μg/mL) for 96 h before the cells were stained with Live/Dead-NIR and evaluated by FACS. Shown are mean ± sem of cell numbers in two duplicate treatment wells. **(K)** Dose-dependent inhibition of the growth of Mino lymphoma cells. The cells were treated with serial dilution of GLA for 96 h before the cells were stained with Live/Dead-NIR and evaluated by FACS. **(L)** The addition of anti-TLR4 mAb blocked the growth inhibitory effects of GLA in Mino cells. $^{**}p < 0.01$, $^{***}p < 0.001$.

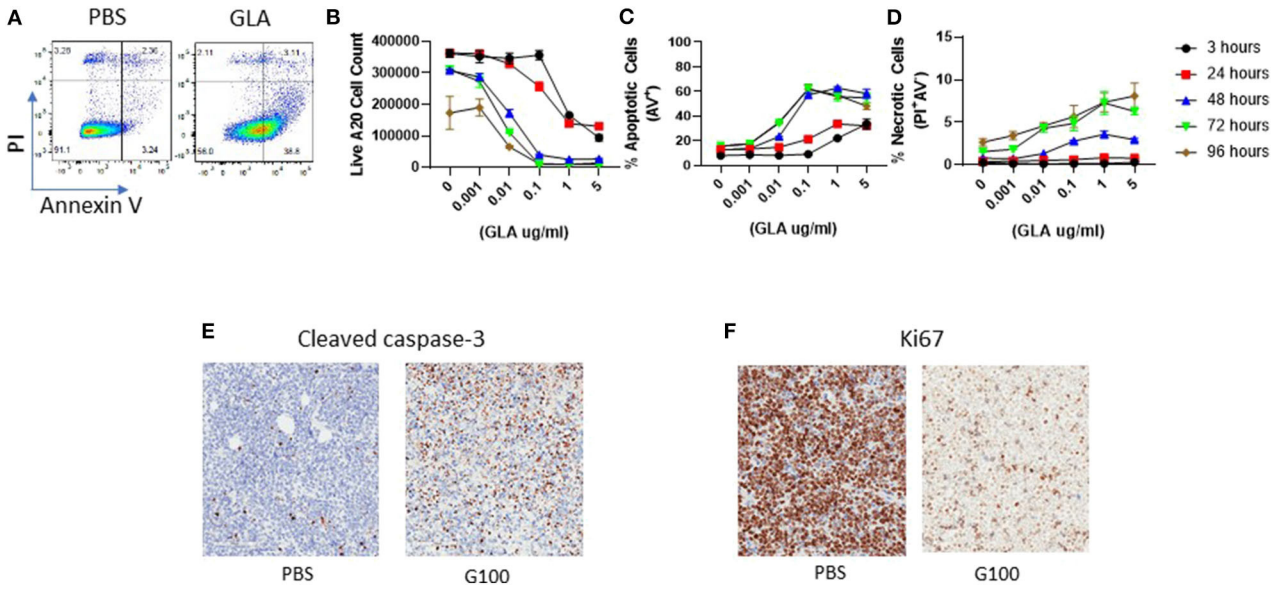

FIGURE 6 | Induction of A20 tumor cell apoptosis by GLA *in vitro* and *in vivo*. **(A)** Representative FACS graphs showing Annexin-V and PI staining in control or GLA-treated A20 cells. A20 cells were treated with GLA (5 μg/mL) for 48 h before staining with Annexin-V and PI. **(B–D)** Dose and time- dependent effect of GLA on the number of live A20 cells **(B)** and induction of apoptosis **(C)** and necrosis **(D)**. A20 cells were treated with serial dilutions of GLA-AF for 3, 24, 48, 72, or 96 h before the cells were stained with Annexin-V/PI and analyzed by flow cytometry. Each data point represents the mean ± sem of cell number or percentage of apoptotic or necrotic cells in two replicate wells. **(E,F)** *in vivo* G100 treatment induced cleaved caspase-3, an apoptosis marker, and decreased the expression of Ki67, a proliferation marker, in A20 tumor cells. A20 tumors were collected after three intratumoral injections of G100 (10 μg) or control PBS for IHC analysis. Brown dot indicates tumor cells that are positive for cleaved caspase-3 **(E)** or Ki67 **(F)**.

the *in vivo* anti-tumor effects of G100 were entirely dependent on expression of TLR4 (**Figures 4C,D**).

GLA Induces APC Functions and Apoptosis in A20 Cells

To address the potential direct effect of GLA on tumor cells, we examined both gene expression and phenotypic changes in WT and TLR4 k.o. A20 cells after GLA treatment. As shown in **Figures 5A,B**, treatment with GLA-AF (5 μg/mL, 48 h) resulted in induction of multiple immune-related genes in WT, but not TLR4 k.o. A20 cells. The upregulated genes include co-stimulatory molecules (CD40, CD80, CD86) and MHCII (H2-DMb2, H2-Eb1, H2-Dma, **Supplementary Tables 2, 3**). Flow cytometry analysis showed a dose-dependent reduction of TLR4 expression (**Figures 5E,F**) and dose-dependent induction of CD40 and CD80 by GLA-AF in WT A20 cells (**Figures 5G,H**). The induction of genes related to B cell activation and APC function was also observed in human Mino cells, a TLR4 expressing mantle cell lymphoma cell line (**Figure 5C** and **Supplementary Table 4**). Gene induction was blocked when cells were pretreated with an anti-human TLR4 mAb (**Figure 5D** and **Supplementary Table 5**). Prolonged exposure to GLA-AF (0.01–5 μg/mL, 96 h) resulted in dose-dependent decrease in cell number in WT but not TLR4 k.o. A20 cells (**Figures 5I,J**). The decrease in cell number and viability by GLA treatment was also seen in Mino cells and the effect was blocked by anti-TLR4 mAb (**Figures 5K,L**). The decrease in cell number in A20 cells after GLA treatment is consistent with induction of apoptosis (**Figure 6A**). Time course studies further showed that the induction of apoptosis and to a lesser degree also necrosis by GLA-AF in A20 cells was both dose- and time- dependent (**Figures 6B–D**). The IC_{50} at 48 h was ∼ 0.01 μg/mL. The ability of GLA to induce apoptosis was also observed *in vivo*. As shown in **Figures 6E,F**, after three injections of G100 or control PBS, G100-treated A20 tumor had significantly increased expression of cleaved caspase-3, an apoptosis marker, and decreased expression of Ki67, a proliferation marker.

To evaluate whether the direct effect on A20 tumor cells could also be induced by other TLR agonists currently used for intratumoral therapy, we treated A20 cells side-by-side with the TLR3 agonist poly I:C, TLR9 agonist ODN2006, or GLA-AF. As shown in **Figure 7A**, direct cytotoxicity on A20 cells was observed after treatment with GLA and CpG ODN2006, but not after treatment with poly I:C. Dose-response curves showed that A20 cells were most sensitive to GLA as compared to other TLR agonists (**Figure 7B**). Gene expression analysis showed that untreated A20 cells mainly expressed TLR4 and TLR9, but not other TLRs (**Figure 7C**). Overall, results obtained with human Mino cells were very similar (**Figures 7D–F**).

DISCUSSION

B-cells can play an important role function as antigen-presenting cells (APC), for example in the setting of prophylactic

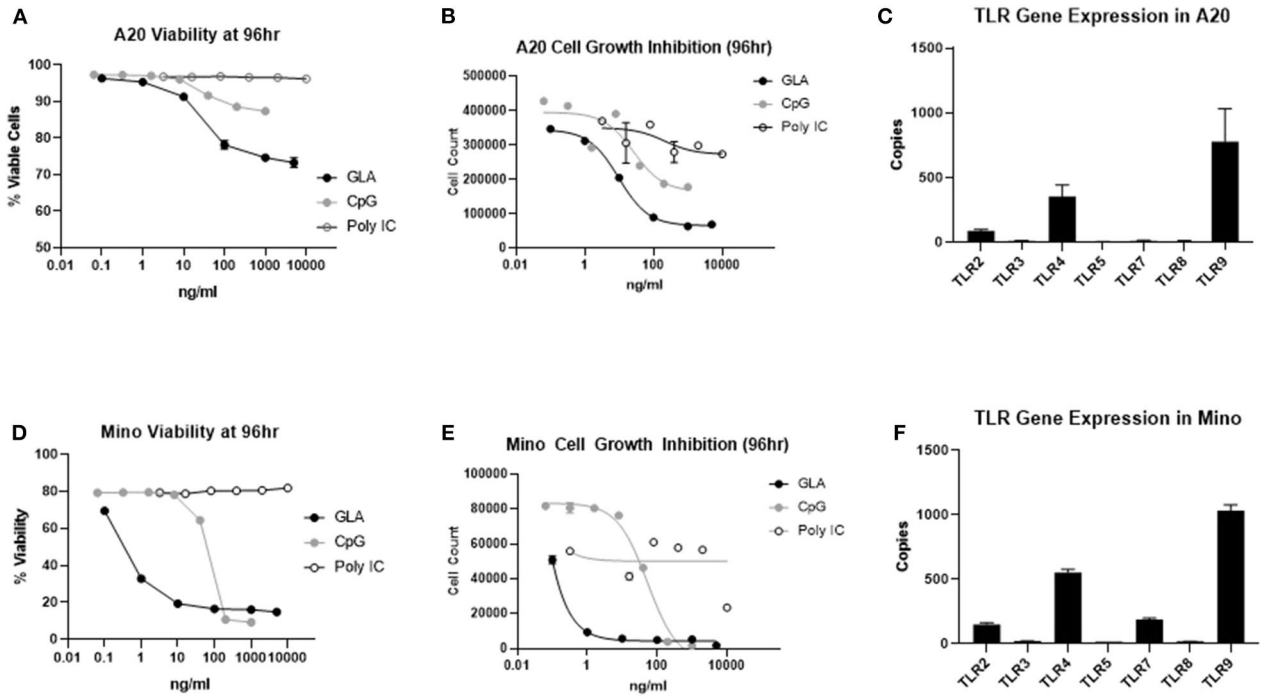

FIGURE 7 | A20 murine lymphoma and Mino human lymphoma cell lines are more sensitive to TLR4 agonist than TLR3 and TLR9 agonists. **(A)** GLA significantly inhibited the viability of A20 cells *in vitro*. Shown is the average viability of A20 cells (from two duplicate wells) at the given concentrations of GLA, CpG, or poly I:C. **(B)** GLA is more potent than poly I:C and CpG in inhibiting the growth of A20 cells. Shown are the average number of viable cells (y-axis) from two duplicate treatment wells at the given concentrations of GLA, CpG, or poly I:C (x-axis). **(C)** Expression level of different TLRs in A20 cells as determined by Nanostring gene expression assay using the panCancer Immune Profiling panel. Each bar indicates the expression level (mean ± sem) of a specific TLR gene in two independent RNA samples from A20 cells. **(D)** GLA significantly inhibits the viability of Mino cells. Shown is the average viability of Mino cells (from two duplicate wells) at the given concentrations of GLA, CpG, or poly I:C. **(E)** GLA is more potent than poly I:C and CpG in inhibiting the growth of Mino cells. Shown are the average number of viable cells (y-axis) from two duplicate treatment wells at the given concentrations of GLA, CpG, or poly I:C (x-axis). **(F)** Expression level of different TLRs in Mino cells as determined by Nanostring gene expression assay using the panCancer Immune Profiling panel. Each bar indicates the expression level (mean ± sem) of a specific TLR gene in two independent RNA samples from Mino cells.

vaccination, and it is currently being investigated whether B-tumor cells possess APC function that can be exploited therapeutically to induce clinically relevant anti-tumor responses (19, 20). To this end, stimulation with anti-CD40 antibody, CD40 ligand, or CpG has been shown to enhance the antigen presenting capacity of murine lymphoma cell lines and human tumor B cells via enhancing the expression of MHC-II, costimulatory molecules (mostly CD86 but also CD80) and adhesion molecules (CD54) (21–24).

Toll-like receptor 4 (TLR4) is widely expressed on immune cells in the mouse, especially macrophages and dendritic cells, as well as on B-cells and B-cell lymphomas. Dendritic cells (DC) are activated and matured by TLR4 agonists, and TLR4 engagement has been shown to promote cross-presentation in CD103+ murine DC (25). Stimulation of TLR4 also plays an important role in B-cell development, proliferation and induction of effector functions, such as secretion of antibodies and cytokines, immunoglobulin class-switch recombination, upregulation of co-stimulatory molecules (CD40, CD80), and activation of antigen presentation in the lymph node (26). Because we have previously shown safety, immunological activity and clinical benefit of IT G100 treatment in two small phase 1 trials in soft tissue sarcoma and Merkel cell carcinoma patients, we sought to generate preclinical data in the A20 model that support clinical studies in follicular lymphoma patients (17, 27).

We demonstrate here in a bilateral model of A20 lymphoma that IT treatment of only one tumor with the synthetic TLR4 agonist GLA at the highest dose evaluated (50 μg) cured ∼ 50% of mice, indicating the induction of a systemic immune response. This was confirmed by showing that the anti-tumor effects were mediated by tumor-specific splenic CD8 T-cells and that mice surviving a primary A20 challenge were completely protected against re-challenge 3 months later. A20 cells have been shown to be phenotypically similar to mature human B-cell lymphomas, expressing high levels of MHC class I and class II molecules and moderate levels of the costimulatory molecule B7-2 (CD86), and to present both exogenous and endogenous antigen (28). However, despite their intrinsic antigen-presenting capabilities, A20 tumors fail to be eliminated spontaneously and tumor-specific T cells are tolerized rather than activated *in vivo* (29). In contrast, G100-treated A20 tumors showed upregulation of genes related to DC activation, antigen presentation, NK cell activation, T cell

activation and other innate and adaptive immune functions, in line with previous reports characterizing in detail the innate immune activation with GLA in muscle and draining lymph nodes of mice (12, 30). Indicative of IFNγ-mediated adaptive immune resistance in the TME, upregulation of multiple checkpoint molecules, such as PD1, LAG3, TIM3, CTLA4, and PD-L1 in the TME was also observed in G100 treated tumors.

To investigate the possibility that G100-stimulated B-tumor cells would participate in the induction of antitumor responses, we first determined that the cells had an intermediate level of TLR4 expression at baseline as measured by flow cytometry. Next, we showed that *in vivo* GLA treatment of A20 cells upregulated the costimulatory markers CD80, CD86, and CD40, as well as MHCII, indicating the induction of APC functions. Interestingly, prolonged GLA incubation (48–96 h) also induced genes involved in apoptosis and cell death (caspase-1, caspase-3, Fas) and a significant reduction in cell numbers. Pre-treatment of A20 cells with GLA resulted in failed tumor establishment when implanted into mice. Taken together, these results suggested that GLA-mediated signaling induced APC functions as well as direct cytotoxicity, which likely both contributed to the observed antitumor effects. To test this hypothesis, we generated an A20 cell line with biallelic knockout of the TLR4 gene. Strikingly, both the direct effect of GLA on tumor cells (upregulation of activation markers, induction of apoptosis) and the clinical effect of intratumoral G100 injection (tumor shrinkage) were completely abrogated in the TLR4 k.o. model. This is unequivocal evidence that immune functions of A20 lymphomas cells can be harnessed directly to induce immune responses that eliminate tumors, without the need for additional therapeutic modalities to induce tumor cell death or cross-presentation. Previously, treatment of A20 tumors with intratumorally injected synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs (CpG), which are strong activators of toll-like receptor 9, was shown to have similar clinical efficacy as G100, however, knockout of TLR9 in tumor cells or the host animal reduced sensitivity to CpG but did not eliminate it completely (31). We quantified the expression of toll-like receptors 2, 3, 4, 5, 7, 8, and 9 in A20 tumors by RNA profiling, showing that TLR9 was expressed highest, followed by TLR4 and TLR2, whereas no TLR3 expression was detectable. This would explain why a recent publication reported that using a three-component *in situ* vaccine (ISV), consisting of the DC-activator Flt3 ligand, low-dose gamma irradiation and the toll-like receptor 3 (TLR3) agonist poly-ICLC, A20 tumors could only be cured if cross-presenting CD103+ dendritic cells were activated intratumorally (32). In summary, our experiments show that the effects of IT treatment of A20 tumors with a TLR4 agonist depends critically on the direct activation of the tumor cells themselves, in particular of antigen presenting pathways, which presumably turns them into target cells with increased susceptibility for T cell mediated killing.

There are limitations to our current study. It is recognized that although we have shown that both the direct effect of TLR4 agonist on tumor cells and CD8 T cell-mediated immune effects are necessary for the anti-tumor effects of GLA, a clear distinction between the two needs further investigation. To this end, when CD8 T cells isolated from tumor rejection mice are simultaneously transferred with A20 tumor cells to naïve recipient mice, no tumors formed (data not shown). Additional studies analyzing contralateral rejection of TLR4 KO A20 tumors upon treatment of WT A20 tumors, or implantation of WT A20 tumors in TLR4 k.o. mice would also help further delineate the role of immune-mediated effects. Additional studies on the pharmacokinetics (PK) of GLA after *in vivo* administration will also be informative to guide further optimization on formulation (oil concentration) and dosing regimen for anti-tumor effects. Preliminary studies have shown that after a single dose of intratumoral GLA-SE injection (10 μg), GLA was detectable in tumors at 96 h post-injection, but how the concentration in tumor compares to concentration used in our *in vitro* studies need further evaluation. Whether increasing the percentage of SE, which has known Th2-type immunomodulatory effects, will impact anti-tumor effect also remains to be investigated.

TLR4 staining by immunohistochemistry (IHC) has been examined in a number of hematologic malignancies and increased expression can be demonstrated on tumor cells in diffuse large B-cell lymphoma (DLBCL), multiple myeloma, and B acute lymphoblastic leukemia (B-ALL) (33, 34). We show here that the TLR4 positive human B-lymphoma cell line Mino responds to TLR4 stimulation in a very similar fashion to the murine A20 tumor cell line. TLR4 expression on tumor cells may therefore be biomarker for identifying patients with B-cell lymphomas susceptible to IT G100 treatment. To this end, we are currently performing a phase 1/2 trial of IT G100 in patients with follicular low-grade non-Hodgkin's lymphoma, with or without concurrent systemic anti-PD1 (Pembrolizumab) treatment (NCT02501473).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: the NCBI Gene Expression Omnibus (GSE150457).

ETHICS STATEMENT

The studies involving animals were reviewed and approved by the IACUC committee of the Infectious Disease Research Institute, Seattle.

AUTHOR CONTRIBUTIONS

HL and JM designed the study and wrote the paper. HL and AB performed the experiments. MC performed the statistical analysis. All authors contributed to the article and approved the submitted version.

FUNDING

The entire study was funded by Immune Design Corporation, Seattle, WA, USA, a wholly owned subsidiary of Merck & Co., Kennilworth, NJ.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.01438/full#supplementary-material

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Conflict of Interest: HL, JM, AB, and MC were full time employees and shareholders of Immune Design at the time of performing the study.

The authors declare that this study was fully funded by Immune Design. The funder fully designed and executed the study.

frontiers in Oncology

OPINION
published: 27 August 2020
doi: 10.3389/fonc.2020.01490

Molecular Pathways Engaged by Immunomodulatory Agents in Monoclonal Gammopathy-Associated Pure Red Cell Aplasia Rescue

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Keywords: MGUS, multiple myeloma, IMiDs, immunomodulation, ikaros (IKZF1), CRBN

INTRODUCTION

Anemia remains a challenge for most cancer patients treated with therapies that include chemotherapy to current immunotherapy or chemo-immunotherapy treatments. Multiple myeloma (MM) is a plasma cell neoplasm marked by the clonal proliferation of malignant plasma cells. Preceding stage in MM includes asymptomatic stages including monoclonal gammopathy of undetermined significance or MGUS before the disease progression to clinical MM. One of the clinical features of MM at diagnosis includes anemia, presenting in a majority of MM patients. Pathological changes in bone marrow microenvironment in monoclonal gammopathy/MGUS and MM contribute to the pathological imbalance in erythroid cell production, leading to lowered downstream mature erythroid cells. Molecular events and pathways that contribute to this pathological remodeling of erythroid pathway-elements remain unclear and need further investigations.

ANEMIA AND MYELOMA PROGRESSION

Anemia is among the key clinical hallmarks of myeloma diagnosis and also requires clinical management in patients with uncontrolled active progressive disease (1). Presentation of clinical myeloma involves preceding asymptomatic stages including monoclonal gammopathy of undetermined significance. Data on monoclonal gammopathy or MGUS associated pure red cell aplasia (PRCA) has remained obscure and requires more attention beyond a few recent reports (2). Pure red cell aplasia is marked by absence of precursor erythroid cells or erythroblasts, eventually leading to erythroid hypoplasia in the bone marrow of these patients. Novel targets including **R**egulator of **H**emoglobinization and **E**rythroid cell e**X**pansion or RHEX in the erythropoietin (EPO)/EPO receptor (EPOR) pathway that play key roles in erythroid cell maturation and development exclusively in humans were recently reported (3). Molecular mechanisms underlying the development of PRCA remain largely unidentified with description of immune-mediated either humoral or cellular ablation of erythroid precursors in the bone marrow. This translates into inefficient erythropoiesis leading to requirement of frequent blood transfusions to rescue anemia in these MGUS and MM patients.

Immunomodulatory (IMiD) agents like pomalidomide have recently been reported to induce increased *in vivo* γ-globin levels in erythrocytes of multiple myeloma patients (4). These studies have revealed the molecular mechanisms by which IMiDs like pomalidomide and lenalidomide reactivate fetal hemoglobin in myeloma patients. These pathways further help translate this

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Edited by:
Dimitar G. Efremov, International Centre for Genetic Engineering and Biotechnology, Italy

Reviewed by:
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Specialty section:
This article was submitted to Hematologic Malignancies, a section of the journal Frontiers in Oncology

Received: *03 April 2020*
Accepted: *13 July 2020*
Published: *27 August 2020*

Citation:
Verma R (2020) Molecular Pathways Engaged by Immunomodulatory Agents in Monoclonal Gammopathy-Associated Pure Red Cell Aplasia Rescue. Front. Oncol. 10:1490. doi: 10.3389/fonc.2020.01490

activation of fetal hemoglobin into recovery from anemia clinically, while still maintaining the anti-myeloma effects of this class of IMiD^R drugs. Although the molecular mechanisms underlying the anti-myeloma activity of IMiDs^R have been reported to involve the Cullin 4A (CUL4A)- Cereblon (CRBN) E3 ligase complex mediated proteasomal degradation of downstream targets (5). These CRBN targets include transcription factors like IKAROS (IKZF1), while additional molecular pathways underlying recovery from anemia in myeloma remain IKAROS or IKZF1 independent (4–6). Specifically, transcriptional modules affected by pomalidomide or lenalidomide has been reported to involve γ-globin repression included BCL11A, SOX6, IKZF1, KLF1, and LSD1. But IKAROS (IKZF1) was not identified as the key effector of this program, as IKZF1 ablation was not sufficient to phenocopy pomalidomide treatment (4). These findings point to the existence of additional pathways that may include EPO/EPOR dependent mechanisms driving the onset and recovery from anemia in MGUS and MM patients (**Figure 1**).

It is to be noted that monoclonal gammopathy-associated PRCA might be a paraprotein-related phenomenon. It can be further speculated that there could be a functional relationship between altered plasma cell and erythroid precursor, with a possible causal relationship between M-protein response and hematological response including PRCA reversal in the PRCA bone marrow after treatment with pomalidomide or IMiDs (2). A number of intriguing questions regarding the underlying molecular and pathogenetic mechanisms still remain unanswered. Modulation of tumor microenvironment is aptly speculated via possible roles of IgG monoclonal protein for erythroid inhibition. But based on the roles of IKZF1 and CRBN for eliciting anti-myeloma effects for any possible hematological response accompanying reduction in the disease burden (M-protein), it remains unclear if recovery of erythroid progenitors is CRBN or IKZF1 independent. Additionally, possible changes in the γ-globin levels can be further speculated in patients with hematological recovery after anti-myeloma therapies (IMiDs^R based) as reported by Dulmovits et al. (4).

DISCUSSION

Detailed molecular mechanisms describing the additional targets of IMiD action remain an active area of research (7) and these additional pathways may contribute to the recovery of erythroid elements in the bone marrow of MM patients. This is further validated by the data on absence of expected JAK2 V617F and CALR Type 1/2 type mutations in the monoclonal gammopathy-associated PRCA cohort reported by Korde et al. (2) eliminating the dysfunctional EPOR mediated JAK2-STAT5 pathway circuitry.

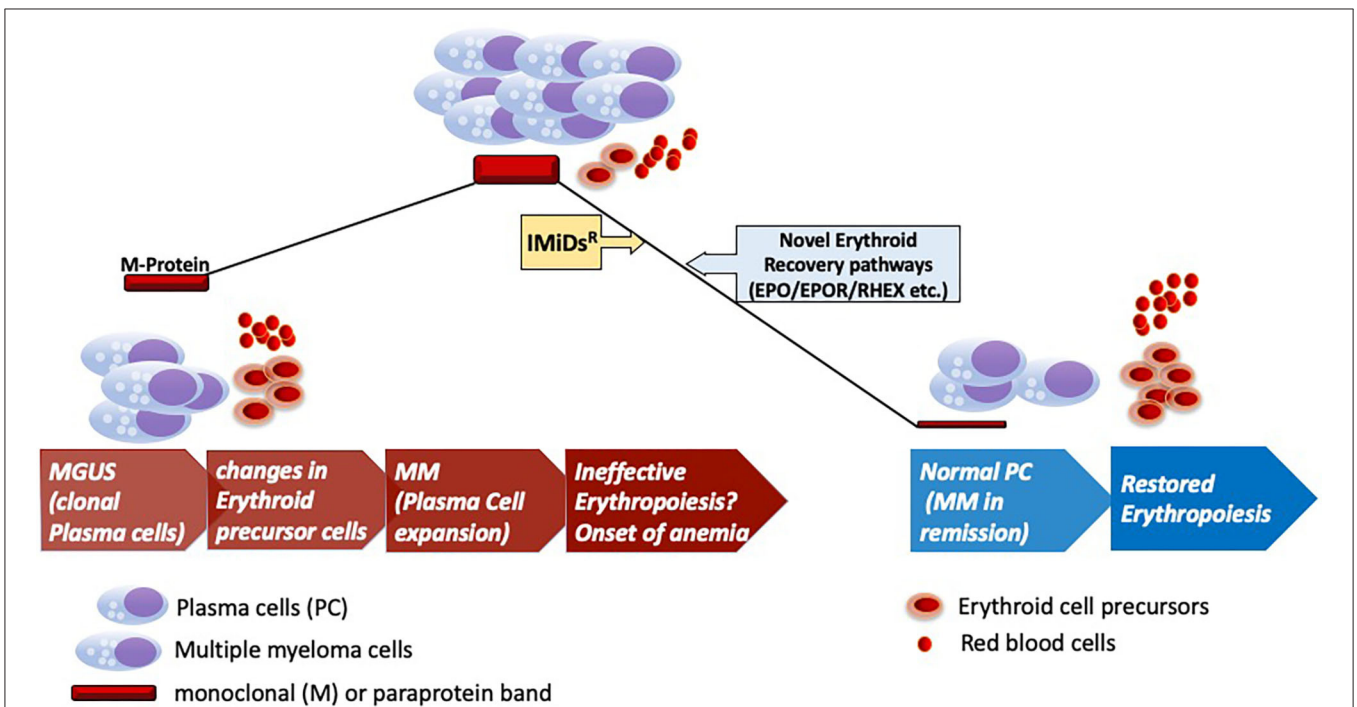

FIGURE 1 | Erythroid recovery in MGUS and MM. Development of clinical myeloma (MM) from preceding MGUS stage of the disease marking onset of pathological changes in the bone marrow for the onset of anemia. Anemia marks of the key features of clinical myeloma as shown with infiltration of clonally expanded malignant plasma cells. Therapeutic intervention with IMiDs^R leading to disease remission and recovery of the erythroid elements in the bone marrow with accompanied changes in the microenvironment. Anemic recovery might potentially engage pathways that are dependent on factors implicated in erythroid maturation (RHEX) and EPO/EPOR pathways. *IMiDs^R, Immunomodulatory drugs*; *EPO, Erythropoietin; EPOR, Erythropoietin Receptor*; *RHEX, **R**egulator of **H**emoglobinization and **E**rythroid cell e**X**pansion*.

Monoclonal gammopathy-associated PRCA illustrates an open ended clinical challenge to describe the underlying possible pathogenetic mechanisms by future mechanistic studies (**Figure 1**). Anti-myeloma drugs like IMiDs^R drive the erythroid recovery in this pathology but independent of currently known molecular pathways that exclusively drive the action of these drugs on myeloma cells and potentially some immune cell subsets. Absence of RTK mutations further makes a strong case for an alternative mechanistic active in the progenitor cells under the action of novel agents like pomalidomide or lenalidomide for erythroid recovery in MM. Recent discovery of novel regulators like RHEX implicated in the human erythroid cell maturation combined with the findings of Dulmovits et al. proposes further investigations of erythroid maturation pathways in MM and Monoclonal gammopathy or MGUS associated PRCA to uncover the underlying molecular mechanism. With the progress of cell therapies like the BCMA targeting CAR-T cells for late stage or relapsed/refractory MM (8), these insights into anemic recovery would benefit the ultimate advancement of advanced CAR-T based therapies for early stages of MM. Future detailed mechanistic studies can help identify alternative treatment strategies as compared to blood transfusions for these MGUS/MM patients for efficient anemic recovery.

AUTHOR CONTRIBUTIONS

RV prepared and wrote the manuscript.

ACKNOWLEDGMENTS

RV would like to extend sincere thanks to the staff at Yale Cancer Center, Section of Hematology and Yale Immunobiology for their kind support. RV would like to acknowledge Prof. Daniel DiMaio, MD, PhD (Genetics) and Prof. Richard Flavell, PhD, FRS (Immunobiology) at Yale University School of Medicine for their support.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

METHODS
published: 20 October 2020
doi: 10.3389/fimmu.2020.578176

Method for the Analysis of the Tumor Microenvironment by Mass Cytometry: Application to Chronic Lymphocytic Leukemia

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: 30 June 2020
Accepted: 29 September 2020
Published: 20 October 2020

Citation:
Gonder S, Fernandez Botana I, Wierz M, Pagano G, Gargiulo E, Cosma A, Moussay E, Paggetti J and Largeot A (2020) Method for the Analysis of the Tumor Microenvironment by Mass Cytometry: Application to Chronic Lymphocytic Leukemia. Front. Immunol. 11:578176. doi: 10.3389/fimmu.2020.578176

In the past 20 years, the interest for the tumor microenvironment (TME) has exponentially increased. Indeed, it is now commonly admitted that the TME plays a crucial role in cancer development, maintenance, immune escape and resistance to therapy. This stands true for hematological malignancies as well. A considerable amount of newly developed therapies are directed against the cancer-supporting TME instead of targeting tumor cells themselves. However, the TME is often not clearly defined. In addition, the unique phenotype of each tumor and the variability among patients limit the success of such therapies. Recently, our group took advantage of the mass cytometry technology to unveil the specific TME in the context of chronic lymphocytic leukemia (CLL) in mice. We found the enrichment of LAG3 and PD1, two immune checkpoints. We tested an antibody-based immunotherapy, targeting these two molecules. This combination of antibodies was successful in the treatment of murine CLL. In this methods article, we provide a detailed protocol for the staining of CLL TME cells aiming at their characterization using mass cytometry. We include panel design and validation, sample preparation and acquisition, machine set-up, quality control, and analysis. Additionally, we discuss different advantages and pitfalls of this technique.

Keywords: mass cytometry, chronic lymphocytic leukemia, lymphoma, microenvironment, immunosuppression

INTRODUCTION

In chronic lymphocytic leukemia (CLL), the microenvironment is crucial. CLL cells need to interact with their neighboring cells for their survival and proliferation. This is true for all the organs were CLL cells can be detected: in the blood, bone marrow, spleen, and lymph nodes. In humans, the very specific nodal microenvironment is an important site for activation of the B-cell receptor (BCR) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB), which directly drives cell proliferation and disease progression (1). The leukemic microenvironment (LME) is highly

immunosuppressive, and these immune cells represent crucial targets for immunotherapy. However, the development of such therapies is limited due to the lack of knowledge in the composition of the LME and to the singularity of this environment for each cancer type. In addition, it is composed of a high variety of stromal and immune cells, which often display phenotypes exclusive to the LME, with the appearance of populations unique to this very specific environment. In this context, the use of high dimensional techniques is crucial to unravel the LME in its full diversity. Mass cytometry was developed in the late 2000s/early 2010s in order to push forward the number of cellular parameters that could be analyzed in parallel compared to conventional Flow Cytometry (FC) analysis. Whereas in the early 70s, the detection of a single color by FC was a revolution, it appeared clearly that conventional cytometry was unable to reach the contemporary requirement and the willingness to decipher highly heterogeneous cell populations. Whereas mass cytometry technology allows the analysis of up to 50 parameters in parallel, it is still associated with a relatively high throughput compared to conventional FC. This was made possible by combining FC platform with mass spectrometry analysis. Mass spectrometry is able to precisely separate unique stable metal isotopes based on their atomic weight. These isotopes can be coupled to antibodies and used to stain cells. Contrary to conventional fluorophores-based cytometry, virtually no overlap between the different isotopes is observed, which enables to have up to 50 parameters detected simultaneously presently. The number of parameters analyzed, combined with the high throughput give rise to many advantages, among those, the detection of rare cell subsets and the discovery of novel cell populations or sub-populations, as markers which would not have been analyzed together in a normal FC panel can now be grouped. It must be noted that novel flow cytometers (e.g., spectral flow cytometers) can detect a similar amount of parameters.

Recently, we took advantage of the power of mass cytometry to decipher the splenic LME of the Eμ-TCL1 mouse, the canonical model for CLL, which allowed us to identify specific immune cell populations and the immune checkpoints PD1 and LAG3 as potential targets in CLL (2). Other groups applied this technology to study either LME or directly the diversity of the leukemic cells themselves in the context of B cell malignancies (3–6). However, these studies are still very rare in this field. It is worth noting that mass cytometry is classified as a tier 1 assay for cell profiling in the Cancer Immune Monitoring and Analysis Centers (CIMACs), which highlights the advantage of this technique.

Here, we provide a stepwise protocol for the staining of cells prior to acquisition on a mass cytometer, preceded by explanations, tips and troubleshooting for panel design, samples preparation, acquisition, and analysis, which are useful for new users of this technology.

METHODS

The experimental design in mass cytometry requires thoughtful planning as some experimental factors can have an impact on the acquisition, data analysis and the results. These factors include panel design, sample preparation, storage, fixation, and sample stimulation. Thus, the verification and standardization of the assay is highly important to avoid misleading interpretations.

Panel Design

The association of the antibody or probe with corresponding heavy-metal isotope to achieve the best possible signal intensity for the detection of specific targets on single cells is one of the most precious steps in the experimental design using mass cytometry (7). In general, the rules of panel design for FC are applicable to mass cytometry, with some adaptations regarding isotopic mass and contamination. Moreover, it is possible to adapt the antibody clones used for FC also for the mass cytometry panel as specificity and affinity to the matching antigens are already known.

First, it is crucial to understand the capabilities of the instrument. For example, the "Helios" version of the mass cytometry instruments is able to detect ions with atomic masses between 75 and 209 Da. It provides 135 channels out of which only approximately 50 are available today due to the current limitations of the heavy metal isotopes availability. In mass cytometry the sensitivity of the channels is dependent on the mass window of the detector which has a maximum sensitivity in the upper-middle channels (~157Gd to 170Er) (**Figure 1**). A combination of two factors explains the difference in sensitivity of the channels. First, because of collisions, ions with lower mass have more probability to be ejected from the ion beam during acquisition. Secondly, as ions with a mass-to-charge ratio (*m/z*) lower than 80 are filtered out (ions that are naturally found in the cells that need to be removed) and the quadrupole mass filter dedicated to this

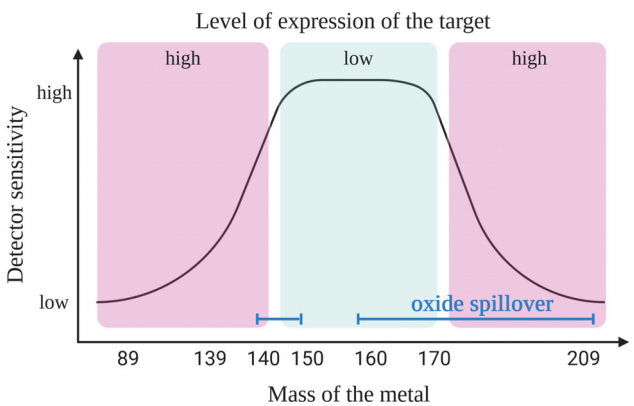

FIGURE 1 | Coupling target with an appropriate heavy metal. Primary targets (high expression) should be labeled with heavy metal isotope-coupled antibodies that are detected with a lower sensitivity (low or high masses). The most sensitive detectors should be reserved for the tertiary targets (low expression level). Due to oxide formation the pairing of antibodies with isotopes is not as simple as shown here. Oxide interference (blue line) are seen in masses >157 Gd but also in the range of lanthanide elements (140s), and this should be taken into consideration when designing the panel, as explained in the main text. Created with BioRender.com.

function has a maximum of efficacy for ions with a medium mass (8, 9).

In order to design a mass cytometry panel, the markers that need to be analyzed are first classified as primary (to identify major backbone subsets, such as CD45, CD3, CD4, and CD19; highly expressed and well defined), secondary (to fine-tune subsets, such as CD44, CD62L, CD23, and CD27; medium and variable expression) or tertiary antigens (low, unknown or variable expression, such as IL-10, LAG3, T-bet, and HELIOS). Then, the primary targets are associated to weak channels and the tertiary markers to the channels with the highest sensitivity. When designing the panel, it is important to have information on the expression level of considered marker in the specific context of the study. Indeed, the cell types, the type of sample and organs from where the cells originate may affect the level of expression for some markers. For example, the CD5 molecule is expressed on T and B cells including CLL cells; however, B cells usually express this molecule 2- to 5-fold less compared to T cells (10). In addition, CLL B cells show a high heterogeneity of CD5 expression (11) and patients with lower level of CD5 show a more aggressive disease (12). All these information will have an impact on choosing the correct channel for detection depending on the cell of interest. This first step of panel design needs adaptation according to the next steps described below, where three sources of crosstalk among channels need to be considered.

Mass cytometry is used for the detection of ~30–50 markers as it has the advantage compared to classical flow cytometry, to have no spillover between the heavy metal isotopes used as labeling. However, it is important to know the sources of crosstalk between channels. First, "isotopic impurity" is a contamination of the heavy metal with one of its isotopes. Even though a 100% pure isotope preparation is desirable for the use in mass cytometry, this is not feasible for all the metals used. Secondly, "abundance sensitivity" is explained by an error in the detection of the ions during acquisition. Abundance sensitivity can be detected in the channels with a mass higher or lower of 1 ("M+1", "M-1"). Finally, after ionization some metals have a tendency to oxidize, which might lead to an increase in the element mass by 16 (^{16}O, "M+16", **Figure 2**). La, Ce, Pr, Nd, and Gd metals have high levels of oxide formation (13). These oxidations cannot be eliminated but can be decreased by optimal setting of the make-up gas (14).

In general, when possible, it is recommended to design a panel with heavy metals separated by a mass difference of more than 1, and to avoid metals with M+16 mass. For panels with a high number of detected markers, this is not possible. To reduce the crosstalk, markers that are less expressed can be selected for less-pure metals. Hence, the contamination of this isotope will be minimum. If these metal-labeled antibodies are selected for primary markers, avoid adding a tertiary target at the M+16 and M +1/−1 positions. In addition, it is recommended to select markers that label well identified cell subsets for less-pure isotopes as their M+16 and M +1/−1 contamination can be easily spotted (for example CD4 and CD8 are virtually not co-expressed outside of the thymus). Furthermore, in case of expected high contamination in one channel, the latter should be used for an exclusion marker (cells that need to be gated out, for example CD19+ cells if the interest resides in T cells, **Figure 3**).

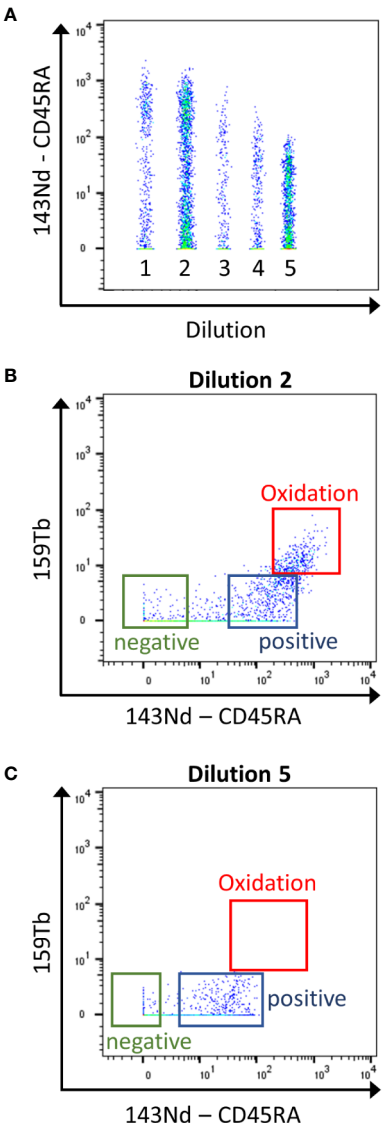

FIGURE 2 | Determination of the contamination due to metal oxidation during the titration. **(A)** The samples are concatenated in order to display the 5 dilutions for the CD45RA antibody in the same graph (1 to 5: range from 1:1 to 1:81 dilutions) **(B)** Using the second dilution (1:3), the CD45RA antibody gives a signal in the empty 159Tb channel, corresponding to oxidation of the metal (red box). **(C)** This signal is clearly reduced using the 5th dilution (1:81). This dilution will be used as it has the lowest contamination, still giving a good positive signal over negative one (Blue and green boxes).

Some strategies have been developed to correct any crosstalk between channels. Chevrier et al. have proposed a compensation method using a bead-based strategy and R-based software (15). This new method provides the first steps into mass cytometry compensation to avoid artifacts and improve sensitivity, although, a good panel design is still required to minimize crosstalk between channels.

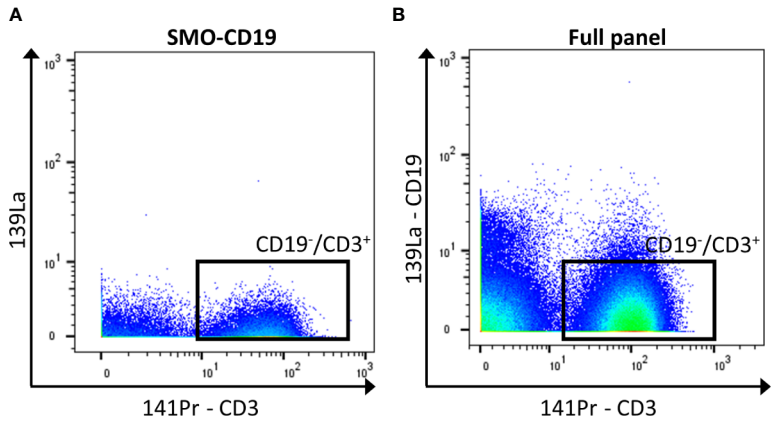

FIGURE 3 | Exclusion of CD19$^+$ cells. **(A)** The negative control for CD19 (SMO-CD19) is used to set the gate of the CD19$^-$/CD3$^+$ cells (black box). **(B)** The few remaining CD19+ cells are removed from the analysis by gating on CD19$^-$/CD3$^+$ cells in the full panel (black box).

In contrast to flow cytometry, cells are not detected by their size and granularity using forward scatter and side scatter as the cells are vaporized. In order to track cells, after permeabilization they are labeled with rhodium- (103Rh) or iridium-based (191Ir) DNA intercalators for nucleated cells (**Figure 4A**) and specific probes to characterize non-nucleated cells (16). In addition, the mass cytometer is unable to measure information of height, area and width as in the fluorescence FC to discriminate doublets from singlets. However, there are possibilities to reduce the amount of doublets by slowing down the acquisition rate, filtering and diluting the sample, bar coding and gating out events that show a higher DNA intercalator signal (**Figure 4B**). However, this limits the information available in term of DNA content of the cells, if the interest resides in cell cycle. In this case, 5-iodo-2-deoxyuridine (IdU; atomic mass of 127) detects cells which underwent DNA synthesis (17).

The viability staining is performed before the permeabilization step, by using cisplatin which enters the disrupted cell membrane of dead cells more easily than of live cells. Cisplatin binds nonspecifically to intracellular DNA and proteins and is detected in 195Pt channel (**Figure 4C**).

Having these rules in mind, the use of dedicated software is highly recommended to help for the design of specific panels. First, Fluidigm supplies already preconfigured screening panels for comprehensive phenotyping and functionality of certain cell populations, which will save time and simplify the experiment. Secondly, Fluidigm provides the online Maxpar Panel Designer (https://dvssciences.com) that calculates and visualizes predicted signal overlaps by the selection of available pre-conjugated antibodies from Fluidigm and custom antibodies/probes.

Heavy metal-labeled antibodies and kits are commercially available. However, they can be very limiting in regard to the panel design. This limitation demands in-house conjugating of the antibodies with the heavy metal-labeled isotopes. Guojun Han et al. provide a detailed protocol about conjugating antibodies with heavy-metal isotopes which requires different methods due to their chemistry and stability (18). Another method to overcome the issue of unavailability is the two-step staining. This consists in detecting the targets with primary monoclonal antibodies labeled with fluorophores followed by detection of this fluorophore by secondary antibodies conjugated with the desired metal isotopes.

In case of a very weak signal, it is possible to amplify it by either two-step staining or a second antibody that recognizes a different epitope on the target (14).

Panel Titration and Test

After designing the panel, it is crucial to titrate the antibodies, test the full panel and the experimental workflow before moving forward to the final samples.

Titration of Antibodies

As in flow cytometry, the titration of heavy metal-labeled antibodies is the key to optimize staining conditions, reduce nonspecific binding of antibodies and to validate the contamination in M+/−1 and M+16 channels.

In general, a serial dilution strategy of at least 5 dilutions (up to 7) is recommended for the selection of the appropriate concentration. Based on our experience, we recommend testing also higher concentrations than those suggested from the antibody data sheets. In our laboratory, we mostly perform the titration with a 1:3 dilution factor. First, we titrate the antibodies of primary markers. Afterward, we perform the titration of the secondary and tertiary targets, expressed only on specific subsets, by adding the known concentration of the primary markers to gate the lineage populations (context titration). For example, after the titration of CD3, CD4, CD8, and CD19, the antibodies for FoxP3, CD25, PD1, and LAG3 can be titrated. This has two advantages: to titrate the secondary in the context of the population of interest, but also, to enrich the signal by gating the cells which are known to express the secondary/tertiary markers. For further information, Van Vreden et al. provide a detailed protocol for the titration of new antibodies (19).

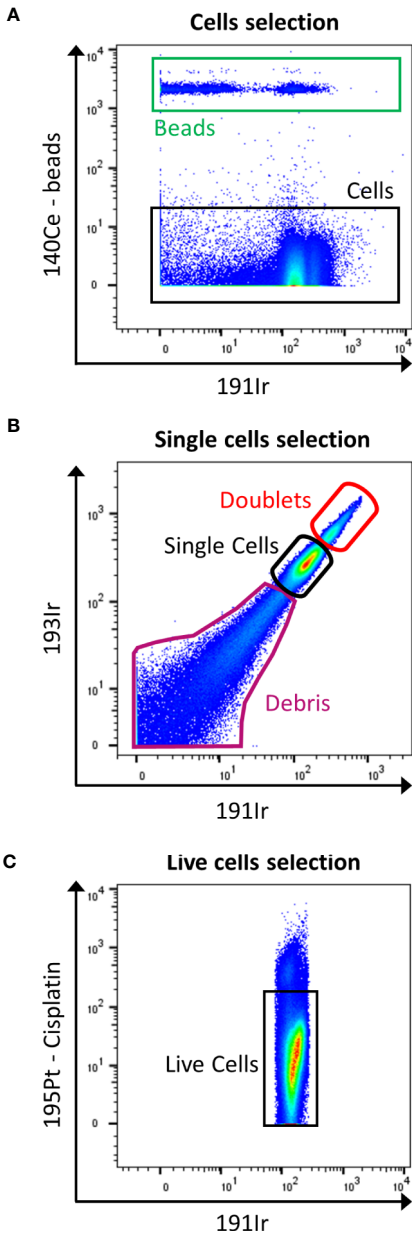

FIGURE 4 | Gating strategy for live single cells events. **(A)** The calibration beads (red box) are gated out by selecting 140Ce$^-$ population (black box). **(B)** Then, singlets are discriminated from doublets by displaying 193Ir vs. 191Ir (DNA intercalator). The single cells are the well-defined population in the middle part of the dot plot (black box). The population with a higher signal in Iridium are the doublets (red box), and the one with lower signal are the debris (purple box). **(C)** Finally, the live cells are gated as negative for cisplatin staining (black box).

If using probes after long-term storage (more than 6 months), it is important to re-titrate them. In addition, we experienced that storing the antibodies in an auto-defrost fridge induces a higher evaporation compared to a classic fridge, greatly affecting the concentration of the stock. Thus, we recommend storing the antibodies in a fridge without auto-defrost mechanism.

Figure 5 displays the selection of the correct concentration for antibodies. Here, we use the tool to concatenate samples in Flowjo10 (Becton, Dickinson & Company) to display all the dilutions in one plot. There should be a good separation between the negative and positive population. To help define this separation factor, the staining index can be calculated. It is the ratio of the separation between positive and negative population

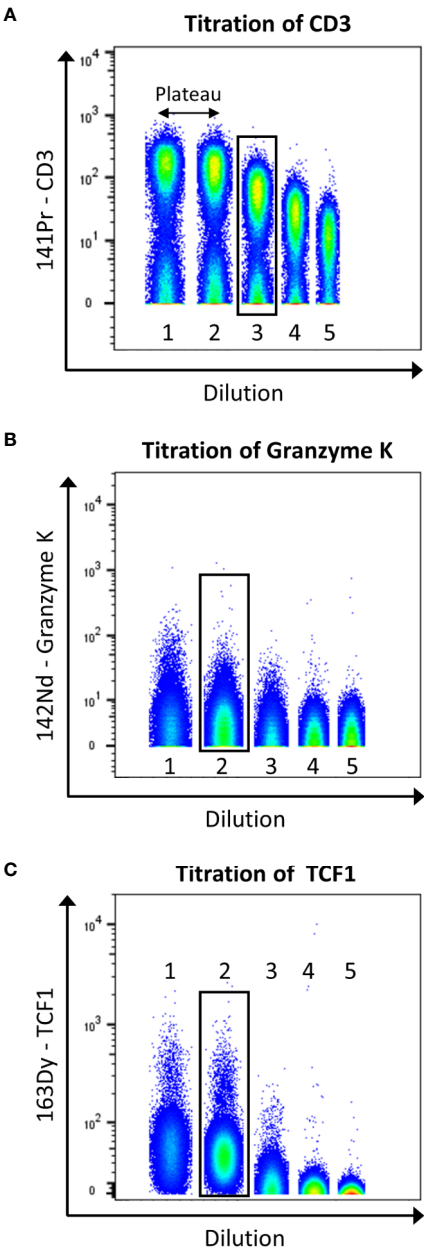

FIGURE 5 | Example of titration for the antibodies anti-CD3 **(A)**, -granzyme K **(B)**, and -TCF1 **(C)**. Data were uploaded in Flowjo and for each titration sample, and singlets live cells were gated as described before. Then, samples were concatenated to display on the same plot the five dilutions (range from 1:1 to 1:81). The dilution showing the best separation between negative and positive signals, before the appearance of a plateau was selected (black box).

divided by two times the standard deviation of the negative population (the higher, the better). If a plateau is observed, the first dilution before this plateau should be selected. In addition, it is also important to take into consideration the signal in the contaminated channels (M+16 and M+/−1). The selected dilution should have the better separation, before the appearance of the plateau, and giving as less contamination as possible.

Full Panel Test

After titration, testing the full panel on an appropriate control sample is essential. As some experiments use rare samples which are too precious to be used for the tests (BM or LN biopsies), the type of sample requires a careful consideration. For example, PBMCs from healthy donors are easy to obtain, however, stimulation or treatments should be considered, in order to induce expression of markers that are not found in PBMCs from healthy donors (such as exhaustion markers, specific cytokines, transcription factors…). In addition, these samples need to be handled in the same way as the experimental samples (e.g., isolation method, cell enrichment, storage…).

Assessing the impurity and oxidation rate of antibodies is also an important step during the full panel test. If it is not possible to reduce the contaminations by tuning the instrument or reducing concentration of the antibody, adjustments of the panel need to be considered.

During this test, we recommend to acquire an unstained sample, in order to validate the potential contamination due to reagents used for cell preparation. For example, barium is one of the most abundant elements, and is found in laboratory dish soaps. Distilled water can contain low levels of mercury, lead or tin or even iodine (14). Small adaptions in the daily laboratory life help to minimize the environmental contamination, such as using reagents with a high purity, new plastic or glass container for the storage of the buffers and testing these for contaminations on the mass cytometer. High purity reagents are commercially available.

Controls Used in Mass Cytometry

Panel Controls

In flow cytometry experiments, isotype controls can be useful. Unfortunately, until now, isotypes with the corresponding metal are not commercially available (16). Fluorescence minus one (FMO) controls are used in high multiparameter flow cytometry to account for the residual spillover following proper compensation. In mass cytometry, the cross-talk between channels is minimal (see *Panel Design*) and can be further attenuated by proper panel design. Therefore the signal-minus-one or metal-minus-one controls (SMO/MMO, respectively) are useful for unknown markers (mainly tertiary ones) or for panels with sub-optimal design. In addition, metal-minus-many (MMM) can also be used, where several targets are subtracted to the main panel (16). An additional control that can be used to determine gate boundaries is to consider positive and negative cell populations within the sample. For example, outside of the thymus, virtually all $CD4^+$ cells are $CD8^-$ (14). When using a two-step staining, it is necessary to include controls such as secondary antibody with and without a primary antibody, in order to examine the unspecific binding properties of the secondary antibody (14).

Control to Reduce Variability During Longitudinal Studies

Sample barcoding of the samples is a strategy that increases the efficiency of the process and decreases technical variability. Heavy metal-labeled cellular barcoding enables running multiple samples (multiplexing) in one tube by staining each sample with a unique combination of isotopes before pooling the samples. Bodenmiller and colleagues published a method for barcoding (20). A probe, which recognizes thiol groups in the cells (maleimido-mono-amide-DOTA (mDOTA), non-specific labeling of cells), is conjugated with 7 different lanthanide isotopes. This results into 128 (2^7) possible combinations. Zunder et al. further improved this method (21). Indeed, lanthanide can be conjugated to antibodies, thus the previous barcoding method restricts the use of such antibodies. Then, lanthanide are a source of contamination (abundance sensitivity) and oxidation. Here, they used palladium isotopes, which cannot be used for antibody conjugation (chemical incompatibility with the conjugation tools) so far, and have limited oxidation rate. In this method, they chelated 6 palladium (Pd) isotopes with isothiocyano-benzyl-EDTA, which labels proteins through their amine groups, allowing 64 combinations. Accordingly, the Cell-ID 20-Plex Pd is commercially available by Fluidigm providing 20 combinations (**Figure 6**). A drawback of these protocols is the need of staining on fixed and permeabilized cells. Lai et al. developed a multiplexing protocol using CD45 to barcode PBMCs (22). As the result of the acquisition of multiplexed samples, it reduces technical and instrumental variations. This being said, it is important to keep in mind that if one or more low-quality samples (poor viability, high amount of debris) are added in the pool of samples, the background will increase, reagent titer problems will occur, and the recovery can be reduced due to sample clumping, jeopardizing all the samples (7). Pipetting errors of the large antibody panels is an important source of variability. Therefore, lyophilization of the antibody mixes can be adopted (23).

For longitudinal studies, which involve separate acquisition of samples that need to be compared and cannot be pooled, it is important to implement a technical control. In this case, three types of controls are suggested and combination of those is possible. First, the use of a unique sample that will be stained and acquired at every run of acquisition is advised. This requires freezing of enough aliquots to cover the period of the study and will allow taking into consideration the variability in the staining. Another method is to include labeled spike-in samples as an internal reference to monitor variability between batches concerning the machine itself. These spike-in controls can be labeled with an isotope which is usually not used in the analysis of mass cytometry, such as Tantalum (23). Finally, Polystyrene bead standards containing known concentrations of the metal isotopes are used to normalize data using a mathematical algorithm that corrects for decrease in sensitivity of the instrument over the time of the study (further discussed in *Acquisition*).

Sample Preparation and Staining

Even though clinicians perform the sample collection of patient material and thus it is often not controllable, it is crucial to

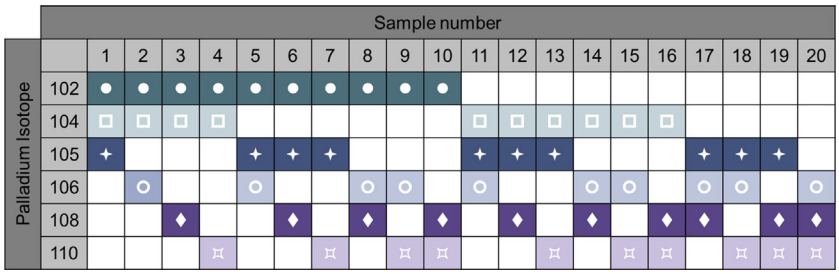

FIGURE 6 | Example of 6-choose-3 barcoding matrix with 6 palladium isotypes. Heavy metal-labeled cellular barcoding enables running multiple samples in one tube by staining each sample with a unique combination of isotopes before pooling the samples. Here, up to 20 samples can be combined by different staining pattern. Following oftware debarcoding, samples can be analyzed individually. For example, sample 1 will be positive for Palladium 102, 104, 105, but negative for the 106, 108, and 110 isotopes. Adapted from Fluidigm.

develop a clean sample preparation for a reliable data acquisition and analysis. Testing different protocols on healthy donor material before moving forward to the actual precious samples is highly suggested if not already established in the laboratory. Leelatian and colleagues published different protocols generating viable single cell suspension derived from human peripheral blood (24) and from a variety of human tissues and tumors (25) which are useful to obtain samples with a high cell viability and little amount of debris.

It is often necessary to enrich the samples for the cell of interest. For example, in the case of CLL, if the focus of the study lies on T cells, $CD19^+$ depletion to remove CLL cells is advised, whereas, if the leukemic cells are the one to study, negative selection of $CD19^+$ cells is preferred. The idea is to leave the cells of interest untouched. If considering a pre-enrichment with magnetic beads it is important to choose one that does not interfere with the detection of the mass cytometry. Thus, testing the reagents for background detection on the mass cytometer is recommended. In our experience, the use of the MACS Cell separation system (Miltenyi) does not interfere with the detection on the mass cytometer. Other methods to enrich cell populations are Fab-based Traceless affinity cell selection (IBA lifesciences) and fluorescence-activated cell sorting. In any case, we suggest to include antibodies in the panel that target cells which should have been removed by the pre-enrichment step, to be able to exclude the remaining cells during the analysis (**Figure 3**). If the sort consist of negative selection using multiple markers (for exclusion of T cells: CD3, CD4, and CD8), it is possible to use several antibodies with the same labeling isotope. Nevertheless, the user must be aware that cell enrichment will necessarily distort the frequencies of the different populations within the sample and can have an impact on the results. Consequently, adopting or not an enrichment step should be considered based on the biological question. An advantage of traditional FC over the CyTOF technology is its lower acquisition time. As a result, fluorophore-based cytometers can generally acquire larger samples without the need of enriching the population of interest.

Concerning sample handling, it is important to have in mind that freezing steps can greatly affect the expression of some markers, especially cytokines. Thus, it is recommended to test the effect of freezing on targets by conventional FC beforehand (**Figure 7**). If required, stimulation of the samples, by PMA and ionomycin, can be performed shortly before the staining. In order to hinder the transportation of the produced cytokines outside the cells, a reagent that stops the Golgi activity has to be added after stimulation, for example brefeldin A. However, the stimulation of the samples is artificial, and will reveal the maximum capacities of a cell to produce cytokines, but does not necessarily reflect this production in the microenvironment that would have been detected before freezing.

As for classical FC, it is recommended in mass cytometry experiments to use blocking reagents to reduce the staining of antibodies *via* their constant Fragment crystallizable region (Fc) domain on Fc receptors, which are mainly found on monocytes, macrophages, dendritic cells and B cells. However, considering other blocking methods is important if the CD16 or CD32 markers are of interest.

Extracellular staining for mass cytometry can be performed at room temperature, as it appears that internalization of antigens does not change the detection on the mass cytometer (which is important for flow cytometry, where staining is usually performed at 4°C). However, one should consider performing the fixation and permeabilization as recommended when using commercially available kits, which is mostly done at 4°C. After the surface and intracellular staining, the cells are incubated with the intercalator which will allow the cell detection. Before acquisition, it is possible to store the samples in the fridge for up to one week in the intercalator buffer. However, it is recommended to inject the samples to the mass cytometer as soon as possible, because long-term storage can have an effect on the detection of markers.

For storing of the samples, the use of polystyrene tubes/plates is preferred, however the recovery of cells on the mass cytometer is higher in polypropylene tubes. Thus, it is recommended to filter the cells into a polypropylene tube just before acquisition or for their storage.

Concerning the number of cells to prepare, it is important to know that in mass cytometry, only 50%–60% of the sample can be recovered, the rest of the sample will be lost due to the aggregation on the walls of the spray chamber and injector (7). An additional cell loss of 20%–30% should be taken into account

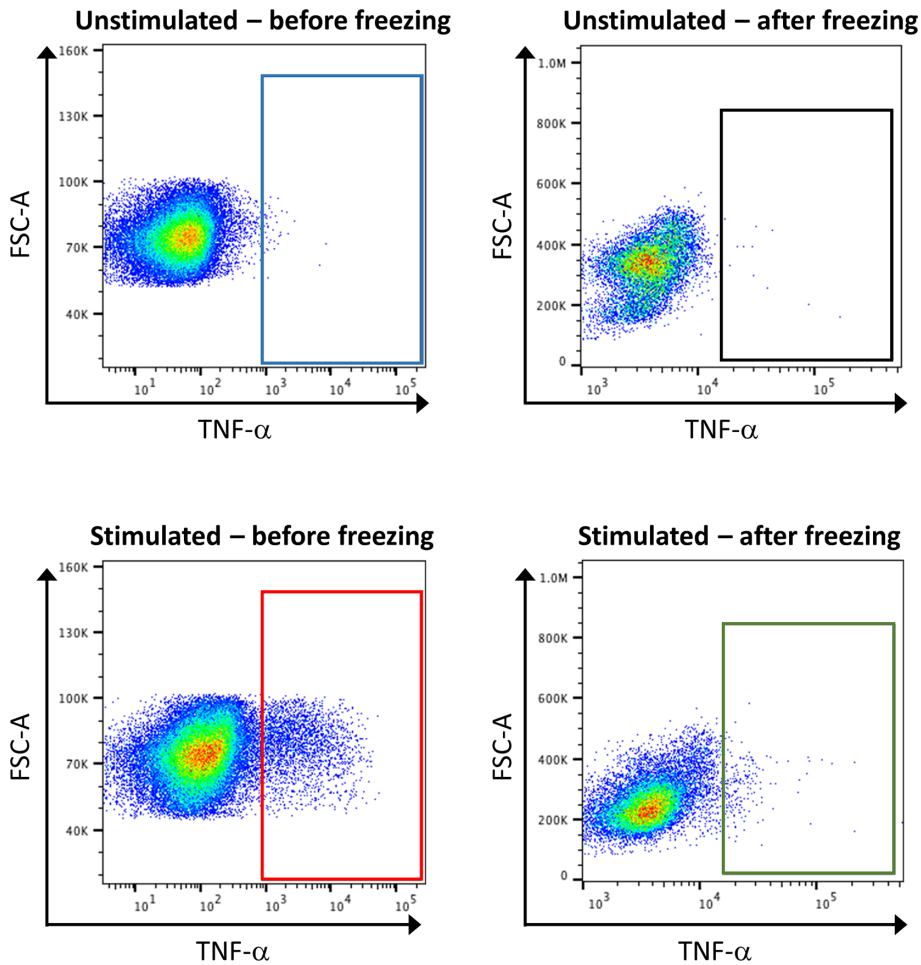

FIGURE 7 | Consequence of stimulation and sample freezing on cytokine production and detection. Healthy control PBMCs were stimulated or not with PMA, and either directly subjected to classical FC, or frozen for several day before being subjected to FC. The analysis of TNF-α expression shows that stimulation leads to an increase of its expression (blue box vs. red box). However, the signal is lost after cell freezing (green box vs. red box).

as cells will be lysed and lost during the sample preparation, staining procedure and washing steps. Performing the staining and washing steps in a 96-well plate helps to reduce cell loss. In addition, resuspending samples in high purity water removes any contamination before acquiring the samples. Here, also a commercially available running buffer can be used to reduce cell breakdown and antibody dissociation (13).

Acquisition

The CyTOF machinery is an inductively coupled plasma (ICP) time-of flight (TOF) mass spectrophotometer (MS). Samples are injected into the mass cytometer, manually or *via* an auto-sampler, and introduced into a nebulizer through a narrow capillary. Once in the nebulizer, the cell suspension is aerosolized into single-cell droplets by argon gas-based pneumatic nebulization and released into the spray chamber. Argon gas (also known as make-up gas) transports the cell droplets to the ICP torch along the heated spray chamber, subsequently shrinking them by evaporation. The sample introduction system has a cell transmission efficiency of approximately 60%–70% (7). Cells are then delivered into the plasma core wherein they are atomized, and the metal ions ionized, leading to the formation of a cloud of charged metal ions corresponding to single cells. The ion cloud passes through a quadrupole filter which removes low mass ions ($m/z < 80$) derived from naturally found elements in cells, such as carbon and oxygen, while allowing the flow of ions of analytical interest to proceed to the TOF chamber. Here, the reporter ions are accelerated at a fixed potential and thus travel at a speed proportional to the square root of their masses. Each mass ordered ion pulse is detected by an electrode multiplier, and the resulting signal recorded as a digitalized waveform by the detector *via* an analog-to-digital converter.

Continuous operation of the CyTOF machinery leads to loss of sensitivity due to several factors; including and build-up of cellular debris in nebulizer and cones. Even regular cleaning and maintenance can cause significant day-to-day signal variation. This is why it is crucial to use appropriate controls during

longitudinal studies (see *Control to Reduce Variability During Longitudinal Studies*).

Data Analysis

As discussed in previous sections, mass cytometry emerged as an alternative to traditional fluorescent-based flow cytometry, enabling the simultaneous detection of over 50 cellular parameters by using heavy metals as antibody/probes labeling reagents, and hence avoiding fluorescent spectral overlap.

The exponential increase in phenotypic and functional characteristics that can be analyzed, allows for the dissection of cellular diversity and heterogeneity with unprecedented resolution, and favors the discovery of new cell subpopulations by visualizing previously inaccessible marker combinations. The ability to perform such detailed single-cell profiling is particularly valuable for the dynamic characterization of immune cell subsets within the TME. This holds true for a wide range of solid and hematological tumors, including, as per our experience, B cell malignancies. However, the elevated number of parameters analyzed in parallel requires careful data processing in order to fully benefit from this technology. Here, we give a brief review of different analysis options, which, will provide the novel mass cytometry users with a better understanding of the distinct possibilities available. For further reading, more detailed reviews on data analysis are published by Kimball et al. and Pedersen and Olsen (26, 27).

Manual Gating

Mass cytometry data is saved as standard flow cytometry FCS 3.0 format, and consequently can be analyzed using traditional flow cytometry data analysis software such as FlowJo. In fact, manual gating can provide key insights on cellular abundance and expression and is particularly useful when it comes to user guided data analysis. However, interpretation of such complex data *via* manual gating of bivariate plots can become an overwhelming task, as the number of parameter pairs increases exponentially with the number of parameters analyzed. Additionally, meaningful multivariate relationships are lost as they cannot be discerned in two dimensions, and unanticipated cell populations can be unintentionally excluded given the subjective nature of manual gating. The immense complexity of the data generated demands exhaustive organization. Structured and consistent file nomenclature, as well as appropriate data storage, greatly facilitates downstream data processing and analysis. We recommend using manual gating for validation of the experiment (e.g., looking at specific cell populations that are known to be enriched or lost during treatment or between samples), to clean-up the data (gate out beads, dead cells, doublets of cells) and to exclude non-relevant cells (e.g., $CD19^+$ cells if interest resides in T cells) before proceeding to algorithm-based analysis.

High Dimensional Data Analysis Using Algorithms

Different algorithms were developed during the past decade for high-dimensional data. Some of the most widely used software kits include viSNE (28), SPADE (29), Phenograph (30), Citrus (31), and X-shift (32). These computational tools use a variety of languages (e.g., Mathlab, R or Python), clustering methods (e.g., parametric vs. non-parametric), and dimensionality reduction approaches to generate a comprehensive depiction of multiparametric single cell measurements. Nevertheless, the data retrieved is highly impacted by the choice of the algorithm, which, as a result must be chosen based on the nature of the biological question.

Even though these algorithms are developed by bioinformaticians at the forefront of mass cytometry research, their use is directed toward bench scientists working on a wide variety of fields. viSNE and SPADE were among the first algorithms developed for multiparametric data analysis, and, in our experience, represent a user-friendly option for recent adopters of mass cytometry. After gating on the population of interest, the data selected are exported and uploaded to a cloud-based computational platform or to a specific software (such as Cytobank or Cytosplore, respectively). These allow the user to run several algorithms to interpret high-dimensional data, including viSNE, SPADE, Citrus, and FlowSOM. The user can run them in the Cytobank platform or as single standing algorithms using the respective R packages or Bioconductor-based tools (e.g., Cytofast) (33). We hereby present a brief introduction to viSNE and SPADE followed by a practical guide on CyTOF data analysis using these algorithms in the context of LME analysis in B cell malignancies.

viSNE is a nonlinear dimensionality reduction technique based on the t-distributed stochastic neighbor embedding (tSNE) algorithm which enables the analysis of high-dimensional data on a two-dimensional map. The resulting viSNE plot, reminiscent of a traditional scatter plot, shows a continuum of cellular phenotypes distributed by the parameters tSNE1 and tSNE2; wherein phenotypically related cells cluster together leading to the formation of phenotypic islands. viSNE maintains single-cell resolution and takes into account high-dimensional similarities between all cell pairs, providing information about nearby and distant cells while preserving the geometry and non-linearity of the data. Cells are colored according to the expression of a chosen parameter in order to identify the cellular identity of the island by co-expression of lineage markers. When analyzing a complex mixture of cells, we find particularly useful the creation of a grid of viSNE plots organized per sample for different lineage defining parameters. Manual gating within the viSNE plots can be performed while in cytobank to obtain information such as population cell number and percentage in each experimental sample.

The global overview of the sample provided by viSNE facilitates the identification of known cell types, the distinction of phenotypic diversity within these populations, as well as the discovery of unexpected cell subsets. One of the main limitations of viSNE is the need for random down-sampling of cells to avoid event overcrowding in the 2D scatter plot. With a limited number of cells to be displayed, cell number can be reduced equally or proportionally, depending on the similarities in event number among the different samples. Finally, it is important to consider that independent viSNE runs on the same dataset will

produce different plots, and, as a result, it is only possible to compare experimental groups when subjected to the same viSNE run.

SPADE (Spanning-tree progression analysis of density-normalized events) is another unsupervised clustering algorithm which allows the visualization of high-dimensional single cell data as a 2D minimum spanning tree of interconnected nodes. Each node comprises a group of phenotypically related cells, with the size of the node indicating cell number, and the color quantifying the median intensity of a parameter of interest. The number of nodes in the SPADE tree is set by the user based on the expected cell populations to be found within the sample. We recommend launching the algorithm several times for each parental population using a different node number in order to find the most appropriate settings for the experiment in question. One aspect that must be taken into consideration is that the relative distance between two nodes is not proportional to the phenotypic similarities or differences among these populations, and thus, the SPADE tree structure can be slightly modified to suit the needs of the user. The specific phenotype of each cluster can be further characterized by creating a heat map including lineage marker MSI values derived from the multiparametric analysis. MeV, an open multiomics experimental viewer, is available for this purpose.

The schematic visualization of multiparametric data provided by SPADE facilitates the analysis of cellular heterogeneity and is particularly helpful when assessing changes in population structure. Nevertheless, given the agglomeration of events in nodes of phenotypically related cells, single cell resolution is lost.

In our experience, running both algorithms for the same dataset provides key complementary insights in order to analyze and interpret complex immunological data, viSNE gives a general overview of the high dimensional data while SPADE assesses any changes in population structure or marker expression within distinct cellular phenotypes.

The development of software kits for multiparametric data analysis is a rapidly evolving field. An increasing number of algorithms, which introduce novel visualization methods and overcome previous software restrictions are now being published and made available. In our laboratory, we continuously review and use new algorithms of interest as a complementary tool to viSNE and SPADE. A recent example would be H-SNE (Hierarchical Stochastic Neighbor Embedding), an algorithm available in Cytosplore software (34). H-SNE is able to represent multiparametric single cell data while maintaining non-linear relationships and, unlike viSNE, is not affected by overcrowding and hence not limited by cell number. A number of novel clustering algorithms have also been developed, and provide valuable insights when launched alongside SPADE. X-shift, for example, is a population finding algorithm available in the online platform VorteX. This algorithm utilizes multiparametric data to construct a weighted k-nearest-neighbor density estimation (kNN-DE) graph, followed by clustering based on cell event density (32). However, unlike SPADE, X-hit finds the optimal number of clusters in a data-driven manner. This reduces the potential over or under fragmentation of cell populations resulting from the estimated number of nodes when using SPADE. A common approach involves the integration of both algorithms: X-shift is used initially to define the number of cellular phenotypes, followed by the generation of a SPADE tree with an informed number of nodes.

Data Visualization

Downstream data analysis of CyTOF data can be performed using different software kits. Excel is commonly used for the analysis of single cell cytometry data, and hence is applicable to CyTOF data as well. However, the immense complexity of multiplex single cell technologies renders this method archaic and practically prohibitive. In order to effectively visualize the data, we find that Tableau, a data visualization tool, is the ideal program for downstream processing and visualization of high-dimensional CyTOF data (35).

For this purpose, Tableau offers two separate software kits, Tableau prep and Tableau desktop, designed for data preparation and visualization respectively. Initially, the data generated after running the high dimensional algorithms of interest are exported as a text file from the online platform or software (Cytobank, Cytospore). The text files are then imported to Tableau prep, which enables the user to combine, shape, and clean up data prior to analysis and visualization. Tableau Prep provides the data in a visual way by showing a row and column profile—whereas rows show every single cell and columns illustrate markers, sample ID- and further it displays every step during data preparation. The output of Tableau Prep is a table of the high-dimensional data organized in a format that fit to the researchers needs. Once the data has been appropriately prepared, it is exported to Tableau desktop, which allows visual analytics and data exploration and thus gives quick answers to specific research question (e.g., differences in expression of one marker between different conditions). The software is user-friendly and does not require advanced computational skills. With Tableau desktop, one can create and display different features, such as: create groups (e.g., healthy vs. tumor) and heatmaps, implement calculations with existing data (e.g., calculate the frequencies of cluster/total), display information (e.g., MSI values, cluster frequencies) and rebuild dot plots, viSNE plots and SPADE trees. Regarding the statistical analysis, Tableau desktop can be used to perform descriptive statistics (e.g., mean, median, percentile, standard deviation…). Nevertheless, complex statistical operations are not readily available. As a result, we easily export the needed data and perform our advanced analysis using statistic software such as Graph Pad Prism.

Stepwise Procedure for the Staining

The following protocol is applied to murine splenocytes from control and leukemic (Eu-TCL1) mice. Isolation and purification of cells of interest are not a topic of this methods article. Chosen methods should ensure cell viability and preserve antigen integrity as for FC. The following can be performed on fresh

or previously frozen cells, some restrictions applying to the latter (see *Sample Preparation and Staining*). A schematic overview is shown in **Figure 8**.

1. If required, thaw your cells in a water bath (37°C)
2. Transfer the cells in 10 ml of preheated Fetal Bovine Serum (FBS, Sigma Aldrich) or full media in a 50ml conical tube
3. Centrifuge at 500 x g for 5 min at room temperature (RT)
4. Remove supernatant (by discarding)
5. Resuspend cells in 1 ml of phosphate-buffered saline (PBS, without Mg^{2+}/Ca^{2+} Life Technology) containing 10% of FBS (PBS-10% FBS)
6. Prepare a dilution and count the cells
7. Transfer the desired amount of cells (typically between 5.10^5 and 3.10^6) in a 15-ml conical tube, wash by adding PBS
8. Centrifuge at 500 x g, 5 min, RT
9. Re-suspend the cells in 100 ul of 5 µM Cell-ID Cisplatin (Cis-Pt, Fluidigm)
10. Mix and incubate for 5 min at RT
11. Wash with 5 volume of PBS-10% FBS, centrifuge (500 x g, 5min, RT) and discard supernatant
12. In the meanwhile, prepare extracellular antibodiy mix
13. Re-suspend the cells in 45ul PBS-10% FBS with 5ul of Fc Blocker (purified anti-mouse CD16/32 antibody, Biolegend)
14. Transfer 50µl of cell suspension in U-bottom 96-well plate (Corning)
15. Incubate for 10 min at RT
16. Add extracellular antibody mix (50 µl/sample)
17. Pipet up and down the samples and incubate for 30 min at RT
18. Stop the reaction by adding 100 µl of PBS-10% FBS, centrifuge 500 x g, 5 min, RT and discard the supernatant by reverting the plate
19. Wash by adding 200 µl PBS-10% FBS per well, centrifuge 500 x g, 5 min, RT and discard the supernatants
20. Add 100µl of 1× FoxP3 Fix/Perm buffer (from FoxP3/transcription factor staining buffer set, eBioscience) per well
21. Pipet up and down and incubate your samples for 45 min at 4°C
22. Meanwhile, prepare the intracellular antibody mix
23. Wash each well with 100 µl of 1× Permeabilization buffer (from FoxP3/transcription factor staining buffer set)
24. Centrifuge (800 x g, 5 min, 4°C) and discard supernatant
25. Re-suspend cells in 50 µl of PBS-10% FBS
26. Add the intracellular antibody mix (50 µl/sample)
27. Incubate the samples for 30 min at RT
28. Add 100µlper well, centrifuge 800 x g, 5 min, 4°C and discard supernatant
29. Wash the cells by adding 200µl PBS-10% FBS, centrifuge 800 x g, 5 min, 4°C and discard supernatant
30. Repeat step 28
31. In the meanwhile, prepare a solution of 50 nM Cell-ID Intercalator-Iridium (Fluidigm) in Maxpar Fix & Perm buffer (Fluidigm)
32. Re-suspend cells in 200 µl of the Iridium-Intercalator solution
33. Pipet up and down and incubate overnight at 4°C.
34. The next day, centrifuge the 96-well plate at 800 x g, 5 min, 4°C, and discard supernatant
35. Wash cells by adding 200 µl of serum/protein free PBS, centrifuge (800 x g, 5 min, 4°C), and discard supernatant
36. Repeat step 34
37. Wash the cells with 200 µl of milliQ water, centrifuge (800 x g, 5 min, 4°C), and discard supernatant
38. Repeat step 36

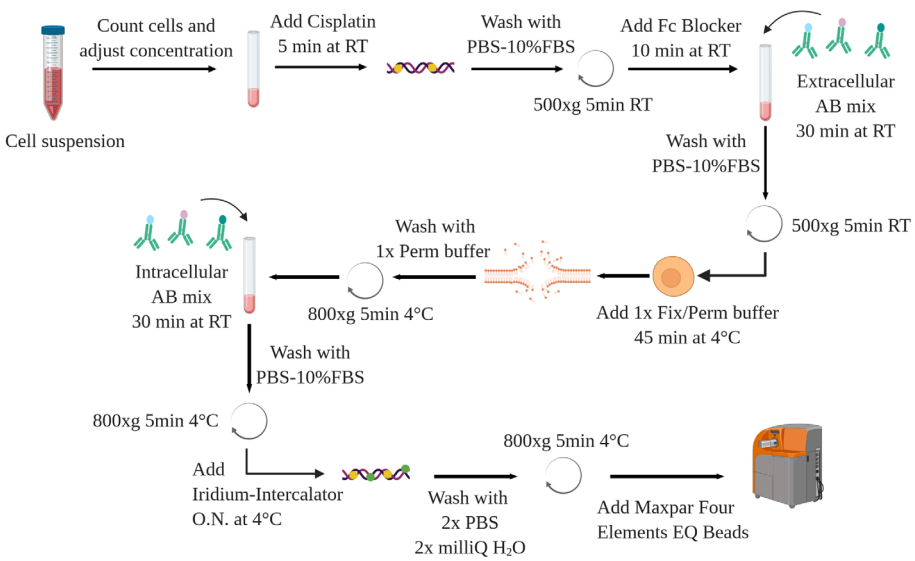

FIGURE 8 | Schematic overview of the staining protocol. AB, antibody. Created with BioRender.com.

39. Re-suspend the cells in 200 µl of milliQ water and transfer through the cell strainer of the Round-Bottom Tubes with Cell Strainer Snap Cap (Falcon) into polypropylene tubes
40. Wash the cells with 200 µl of milliQ water and add on the filter
41. Repeat step 38 with 100 µl of milliQ water, turn the filter and take the residual volume (final volume in the 5ml round bottom tube should be 500 µl)
42. Determine the cell number
43. Adjust the volume to 90% of final volume (final concentration 1.5×10^6 cells/ml: 900 µl for $1.5x10^6$ cells)
44. Add 10% Maxpar Four Elements EQ Beads (Fluidigm) before acquisition (for 1.5×10^6 cells 100 µl of beads)

EXPECTED RESULTS

It is widely admitted that the microenvironment plays a crucial role in the development and maintenance and progression of leukemia. However, in the context of CLL, this microenvironment is still understudied. In this context, we decided to decipher the immune cell landscape in a murine model of CLL, using mass cytometry few years ago (2, 36). We performed adoptive transfer (AT) of splenocytes from terminally diseased Eµ-TCL1 mice, which overexpress TCL1 exclusively in B cells and develop CLL within 8 to 12 months, into C57BL/6 recipient mice. Few weeks after the transfer, the recipient mice (AT-TCL1) developed the leukemia. The splenocytes from these diseased mice and from healthy control C57BL/6 mice were then subjected to CyTOF staining with a custom panel of 35 antibodies, following the above protocol. This panel was designed to study T lymphocytes, myeloid cells, and associated immune checkpoints. Following acquisition, the single live $CD19^-$ cells were selected (manually gated) using FlowJo. Data were reanalyzed for the purpose of this methods article, therefore cluster numbers and number of associated cells differs from our original report (2). The respective FCS files were uploaded to Cytobank and SPADE was launched using all the markers except the one used for the initial gating strategy, with a target number of 50 nodes and 10% targets events downsampling. Then, viSNE was run on the SPADE data using the same markers, a maximum of 1.3 millions of cells, with the following parameters: iteration of 2,000, perplexity of 30 and Theta of 0.5. The data were then exported from Cytobank, uploaded to TableauPrep where they were organized, and subsequently analyzed with TableauDesktop.

After creating two groups (healthy control HC and CLL samples), the viSNE plots can be visualized for the two groups separately. In addition, the plots for the two groups can be overlaid in order to easily identify cell populations which are highly enriched (red arrow) or depleted (blue arrow) in the CLL samples (**Figure 9A**). By allocating colors to each Spade cluster, they can be visualized on the viSNE plot (**Figure 9B**). In order to easily and quickly understand the composition of the viSNE plot, the expression of each marker can be displayed. This allows to get familiarize with the plot, and to know where the main cell populations sit (CD4+ T cells, CD8+ T cells, NK cells…). The cluster which was highly enriched in the CLL sample (**Figure 9A**, red arrow) is expressing high levels of CD8 (**Figure 9C**), whereas the one which was highly depleted (**Figure 9A**, blue arrow) expresses F4/80 (**Figure 9C**). After this first step in the understanding of the viSNE plot, the percentage of each cluster can be calculated in order to identify the clusters which are either enriched or depleted (**Figures 10A–C**, left panels). In addition, the MSI of each marker for each cluster can be extracted, and corresponding heatmaps can be created (e.g., in excel by conditional formatting) in order to define each cluster (**Figures 10A–C** upper right panels), which finally allows to precisely define each cluster, based on the expression of all the markers. Finally, the clusters can be highlighted on the viSNE plots (**Figures 10A–C**, lower right panels). In **Figure 10A**, three different clusters of Tregs are depicted, showing that clusters 10 and 35 are enriched in CLL samples compared to HC. The three clusters express CD3, CD4, FOXP3, CD25, and CTLA4 which are characteristic of Treg populations. The proper identification of Treg cells require the co-expression of these markers, which can have variable expression depending on the subsets (37). The three clusters express high levels of PD-L1, LAG3. Clusters 10 and 35 express higher levels of PD1 compared to cluster 19, and cluster 10 shows the highest expression of KLRG1, representing fully activated and highly suppressive Tregs. In **Figure 10B**, the cluster 30, which is virtually not found in HC, is highly enriched in CLL samples. This cluster is composed of $CD8^+$ T cells expressing high levels of inhibitory immune checkpoints KLRG1, LAG3, PD1 and CTLA4, characteristic for exhausted $CD8^+$ T cells. By highlighting cluster 30 on the viSNE plot, we can clearly see that this cluster corresponds to the cells we identified as highly enriched in **Figure 10A** (red arrow). Concerning the monocytes, 3 clusters were enriched, with clusters 13 and 36 which represent patrolling monocytes expressing high levels of PD-L1 and LAG3 and being associated with CLL progression (**Figure 10C**). From these data, we concluded that the development of CLL is associated with the establishment of a very immunosuppressive microenvironment. In addition, the identified immunosuppressive cells in the CLL splenic microenvironment express high levels of immune checkpoints, particularly PD1 and LAG3. We next sought to validate if these immune checkpoints could represent potential therapeutic targets for an immunotherapeutic approach. To this end, we treated AT-TCL1 with blocking antibodies directed against PD1 and LAG3. This therapy led to a control of CLL development, with the restoration of a normal immuno-competent splenic microenvironment (2). Altogether, the study of the splenic CLL LME allowed us to better understand the immunosuppression found in CLL, to define clusters of cells highly enriched in CLL, and to identify potential targets for immunotherapy.

DISCUSSION

In addition to be costly, mass cytometry experiments tend to be time consuming (from panel design to sample staining, acquisition, and analysis). In order to make sure to fully benefit from this technology, all the steps must be carefully planned. Even though clearly limited compared to FC, contaminations between channels need to be scrupulously considered and eliminated as much as possible to avoid

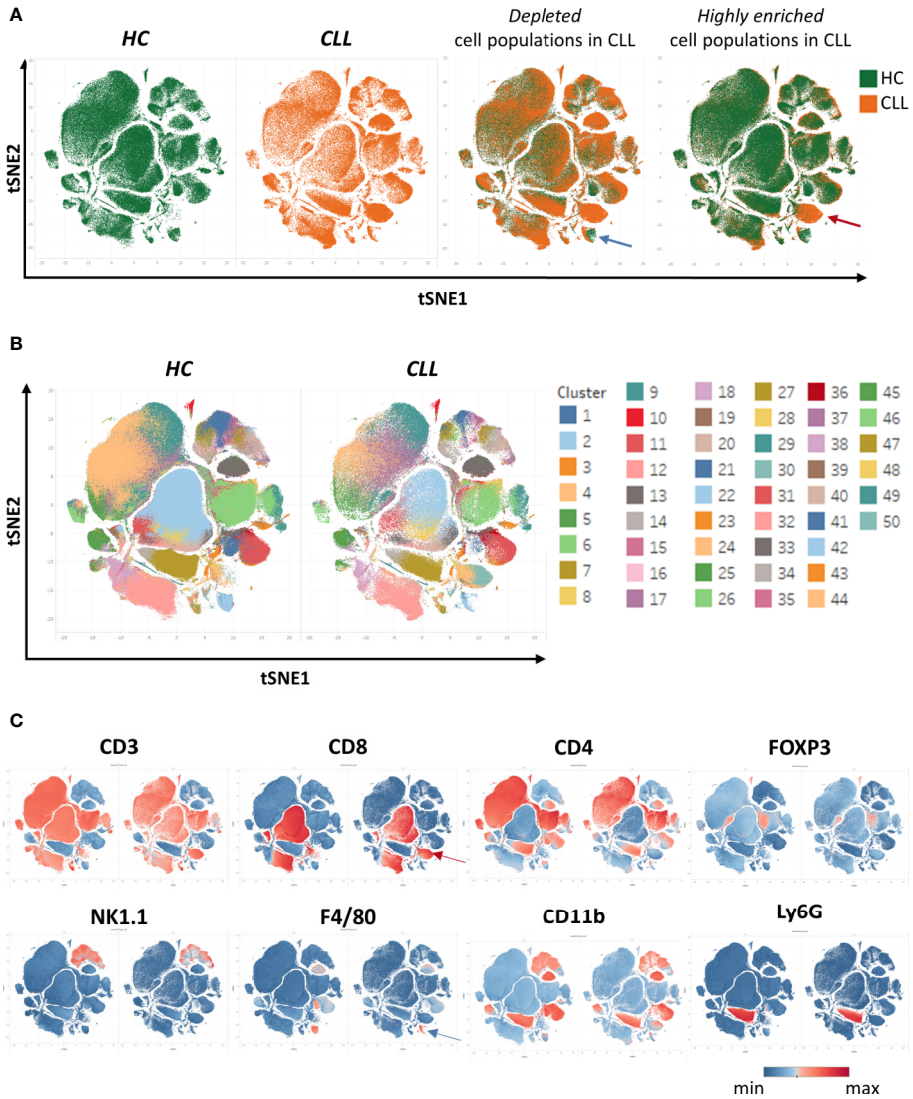

FIGURE 9 | Clustering of cells using SPADE and viSNE, visualized through the Tableau software. **(A)** The viSNE plots are displayed for each sample separately (first and second panels). On the third and fourth panels, the overlay of the plots is shown, displaying the same amount of cells in each sample, which allows to easily detect the clusters which are depleted (e.g., blue arrow) or enriched (e.g., red arrow) in the CLL samples. **(B)** In order to identify the clusters, each one was associated to a color and displayed on the plot. **(C)** The intensity of the different markers are displayed, allowing to quickly get insights into the identity of each cluster. Here, the cluster enriched in CLL identified in **(A)** expresses CD8 (red arrow), and the one depleted (blue arrow) expresses F4/80.

misinterpretation of the data. In addition to classical protein level quantification, mass cytometry offers a large range of detection of other biological parameters by the use of different probes. Post-translational modification, proteolysis, DNA synthesis, hypoxia, enzymatic activity, and chromatin modification can be assessed by this technique, opening up the possibilities for phenotypic analysis and discoveries of novel cell populations and unsuspected mechanism. However, the technology is currently limited to approximately the use of 50 channels, although the machines contains around 130 detectors. This is because the purity of some isolated metal isotopes is not high enough or it is not possible to couple those metals to antibodies/probes, although they could be detected by the mass cytometer. Effort is made to improve this pitfall, and the number of parameters analyzed in parallel will certainly increase in the coming years. The analysis of mass cytometry data is moving fast, with the development of new tools, not requiring bioinformatic skills. Mass cytometry is facing harsh competition with the development of fluorophore-based cytometers which can reach up to 30–50 parameters detected in parallel (such as the BDSymphony or bio-rad ZE5), and the emergence of spectral cytometers (Sony SP6800 and Cytek Aurora) positioning both technologies at the forefront of immunology research.

However, the advantages of mass cytometry still hold true, such as simpler panel design, multiplexing, minimal crosstalk among channel, no need of compensation. One main drawback

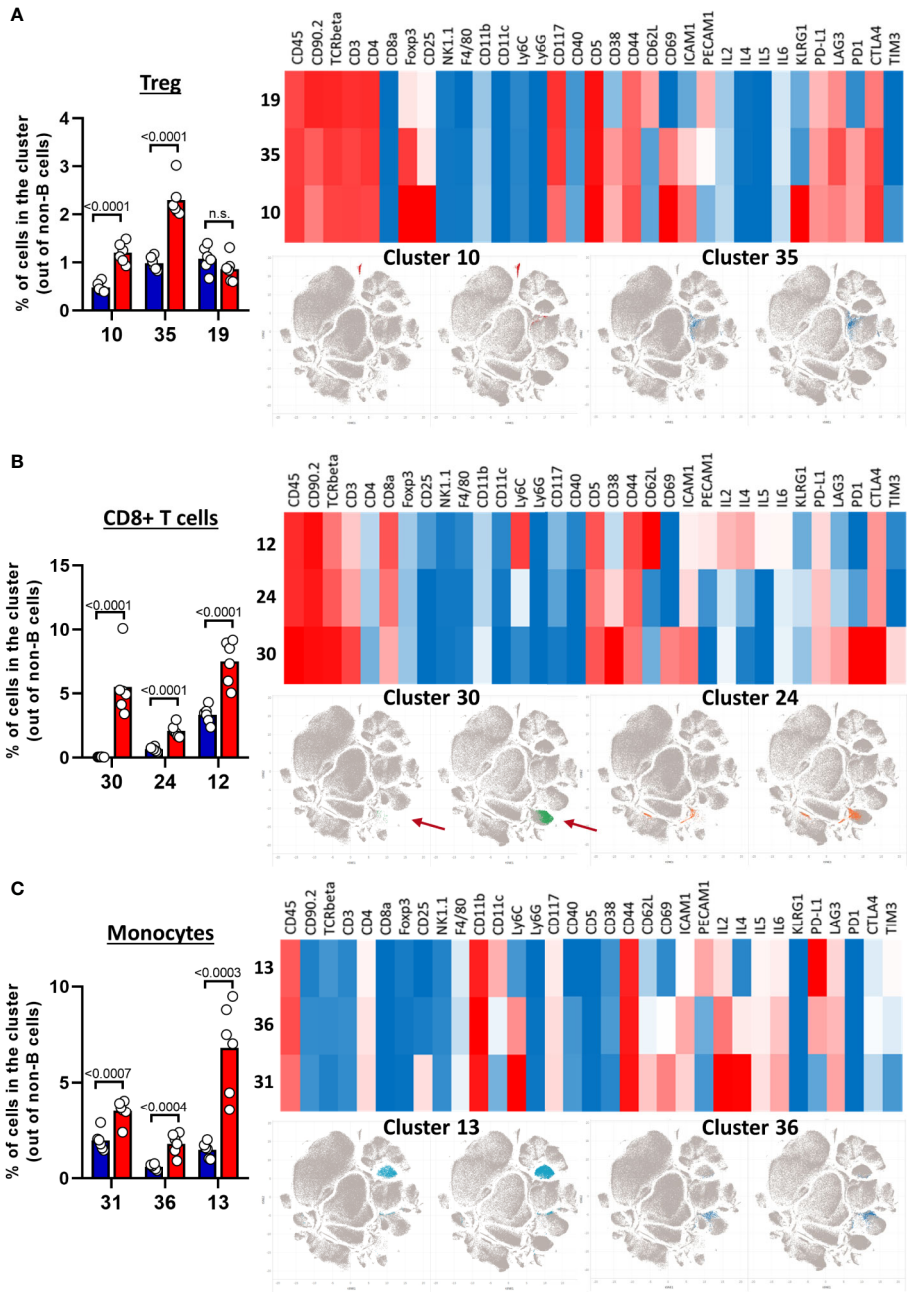

FIGURE 10 | Characterization of cell populations enriched in CLL. **(A–C)** The left panels show the percentage of clusters in HC and CLL samples. The upper right panels show the heatmap for the expression of selected markers, the lower right panels highlight the position of enriched clusters on the viSNE plots. **(A)** Treg clusters. **(B)** CD8+ T cells clusters. **(C)** Monocytes clusters.

of the technique, is the slower acquisition rate compared to classical FC, which can be overcome by performing a pre-enrichment of the cells of interest, in order to reduce the number of cells to acquire. However, it has to be noted that pre-enrichment can affect the final results.

In the recent years, a novel imaging technology has been developed (38), Imaging Mass Cytometry (IMC), which enables now the detection of up to 40 markers in a single imaging scan on tissue and tumor sections. This technology is provided by the same equipment as the mass cytometer, with adaptation for imaging. Complementary to mass cytometry, it allows the analysis of interaction between different cell types, but can also give insights on the activity of the cells, depending on their localization within the LME and interactions with other cells.

Mass cytometry represents a perfect asset for the study of LME in B cell malignancies. We applied this technology to the

splenic microenvironment in the Eμ-TCL1 mouse model and identified a potential immunotherapeutic approach for the treatment of CLL. Other groups applied this technology to B malignancies, and these studies allowed to obtained new and innovative information concerning malignant B cells themselves or immune cells found in the LME. Maity et al., detected clusters of B cells specific to a subset of CLL patients bearing the R110 mutation in the particular light-chain allele IGLV3-21, and observed that these patients are phenotypically closer to unmutated (UM) patients, independently of their hypermutation status (6). In Follicular Lymphoma (FL), mass cytometry analysis allowed to understand that the malignant cells have a unique phenotype, not found in healthy donors. Contrary to what was shown based on gene expression profiling, the B malignant cells are not similar to germinal center (GC) B cells (3). By the study of immune cells in the context of Hodgkin Lymphoma, M. Shipp's lab identified an enrichment of distinct regulatory T cells (Treg) populations with a T helper 1 (Th1) polarization phenotype (5). Finally, in FL, the presence of a specific T cell population expressing PD1 is associated with poor prognosis, whereas, the expression of PD1 on general T cells is not of prognostic value, highlighting the interest of having multiparametric analysis (4).

In conclusion, mass cytometry is an excellent tool to get phenotypical information at the single cell level, on protein expression, and the generalization of its use should lead to new discoveries, paving the road for the development of more specific and efficient therapies, targeting either directly the malignant cells or in the cells found in the LME.

MATERIAL AND EQUIPMENT

Equipment

The Helios system (Cytometry by Time of Flight, CyTOF; Fluidigm) equipment was used for the data shown in this study. In addition, controls, for the expression of some markers, were acquired on conventional CytoFLEX (Beckam Coulter) flow cytometer.

Cells and Reagents

All the reagents and cells used are described in *Stepwise Procedure for the Staining*.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article. Data presented in "Expected Results" were originally published in Blood2 [(2), Blood], and were reanalysed for the purpose of this article. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

SG, IF, MW, and AL wrote the manuscript. SG and MW performed the experiments, analyzed the data, and created the figures. GP, EG, and AC helped in writing, figures design, and for some experiments. EM, JP, and AL finalized the writing and supervised the team. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from FNRS "Télévie" to SG, IF, MW, GP, and AL (7.4502.19, 7.4529.19, 7.6504.18, 7.4501.18, 7.4502.17, and 7.4503.19), from FNR Luxembourg to EG and JP (PRIDE15/10675146/CANBIO and INTER/DFG/16/11509946) and from Plooschter Projet.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 12 November 2020
doi: 10.3389/fonc.2020.580364

Integration of the B-Cell Receptor Antigen Neurabin-I/SAMD14 Into an Antibody Format as New Therapeutic Approach for the Treatment of Primary CNS Lymphoma

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OPEN ACCESS

Edited by:
Jérôme Paggetti, Luxembourg Institute of Health, Luxembourg

Reviewed by:
Martina Deckert, University of Cologne, Germany
Etienne Moussay, Luxembourg Institute of Health, Luxembourg

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Specialty section:
This article was submitted to Hematologic Malignancies, a section of the journal Frontiers in Oncology

Received: 05 July 2020
Accepted: 15 October 2020
Published: 12 November 2020

Citation:
Bewarder M, Kiefer M, Moelle C, Goerens L, Stilgenbauer S, Christofyllakis K, Kaddu-Mulindwa D, Fadle N, Regitz E, Neumann F, Hoth M, Preuss K-D, Pfreundschuh M and Thurner L (2020) Integration of the B-Cell Receptor Antigen Neurabin-I/SAMD14 Into an Antibody Format as New Therapeutic Approach for the Treatment of Primary CNS Lymphoma. Front. Oncol. 10:580364. doi: 10.3389/fonc.2020.580364

Recently, neurabin-I and SAMD14 have been described as the autoantigenic target of approximately 66% of B-cell receptors (BCRs) of primary central nervous system lymphomas (PCNSL). Neurabin-I and SAMD14 share a highly homologous SAM domain that becomes immunogenic after atypical hyper-N-glycosylation (SAMD14 at ASN339 and neurabin-I at ASN1277). This post-translational modification of neurabin-I and SAMD14 seems to lead to a chronic immune reaction with B-cell receptor activation contributing to lymphoma genesis of PCNSLs. The selective tropism of PCNSL to the CNS corresponds well to the neurabin-I and SAMD14 protein expression pattern. When conjugated to Pseudomonas Exotoxin A (ETA′), the PCNSL reactive epitope exerts cytotoxic effects on lymphoma cells expressing a SAMD14/neurabin-I reactive BCR. Thus, the reactive epitopes of SAMD14/neurabin-I might be useful to establish additional therapeutic strategies against PCNSL. To test this possibility, we integrated the PCNSL-reactive epitope of SAMD14/neurabin-I into a heavy-chain-only Fab antibody format in substitution of the variable region. Specific binding of the prokaryotically produced SAMD14/neurabin-I Fab-antibody to lymphoma cells and their internalization were determined by flow cytometry. Since no established EBV-negative PCNSL cell line exists, we used the ABC-DLBCL cell lines OCI-Ly3 and U2932, which were transfected to express a SAMD14/neurabin-I reactive BCR. The SAMD14/neurabin-I Fab antibody bound specifically to DLBCL cells expressing a BCR with reactivity to SAMD14/neurabin-I and not to unmanipulated DLBCL cell lines. Eukaryotically produced full-length IgG antibodies are well established as immunotherapy format. Therefore, the PCNSL-reactive epitope of SAMD14/neurabin-I was cloned into a full-length IgG1 format replacing the variable domains of the light and heavy chains. The IgG1-format SAMD14/neurabin-I construct was found to specifically bind to target lymphoma cells

expressing a SAMD14/neurabin-I reactive B cell receptor. In addition, it induced dose-dependent relative cytotoxicity against these lymphoma cells when incubated with PBMCs. Control DLBCL cells are not affected at any tested concentration. When integrated into the Fab-format and IgG1-format, the PCNSL-reactive epitope of SAMD14/neurabin-I functions as **B**-cell receptor **A**ntigen for **R**everse targeting (BAR). In particular, the IgG1-format BAR-body approach represents a very attractive therapeutic format for the treatment of PCNSLs, considering its specificity against SAMD14/neurabin-I reactive BCRs and the well-known pharmacodynamic properties of IgG antibodies.

Keywords: B-cell receptor antigens, primary central nervous system lymphoma, neurabin-I/SAMD14, BAR-body, auto-antigens

INTRODUCTION

Primary diffuse large B-cell lymphoma (DLBCL) of the central nervous system (CNS), also called primary central nervous system lymphoma (PCNSL) (1), is a rare disease with an annual incidence rate of 0.47 cases per 100.000 population (2), accounting for <1% of all non-Hodgkin lymphomas (3). Genetic profiling studies suggest that the normal counterpart of PCNSL cells are late germinal center (GC) exit B-cells with a gene expression profile characteristic of both GC B-cells and activated B-cells (ABC) (1, 4). In accordance with this, PCNSLs show rearranged immunoglobulin genes, ongoing somatic hypermutation, and persistent B cell lymphoma 6 (BCL6) activity but lack terminal B-cell differentiation resulting in a fixed IgM/IgD phenotype (5–8). Frequent mutations in the B-cell receptor (BCR) and Toll-like receptor 9 (TLR9) pathways (9, 10) with transcriptional upregulation of NF-kB, overrepresentation of the autoimmunity-linked immunoglobulin gene VH4-34 (5, 11) and persistent expression of functional BCRs despite ongoing somatic hypermutation (7), all indicate an important role of antigenic BCR stimulation in the pathogenesis of PCNSL (12). This seems particularly true for primary vitreoretinal lymphomas which display an even more restricted immunoglobulin gene repertoire (11). PCNSLs are confined to the CNS, limited exclusively to the brain, spinal cord, leptomeninges, and eyes. This selective tropism may be caused by proteins that are expressed exclusively in the CNS stimulating only B-cells residing in the CNS (12, 13). Importantly, the CNS counts as immune sanctuary along with eyes and testes, allowing for lymphoma development under reduced immunosurveillance (14, 15).

First-line treatment of PCNSL is divided into induction therapy followed by a consolidation regimen (16). High initial response rates of up to 86% can be achieved with induction therapies incorporating the agents methotrexate, rituximab, thiotepa, and cytarabine (MATRix) (17). With the results of the randomized phase 3 trial "HOVON 105/ALLG NHL 24," which found no clear benefit of the addition of rituximab to chemotherapy, the role of rituximab in first-line treatment of PCNSLs is controversial (18). High-dose chemotherapy with autologous stem cell transplantation (ASCT) is thought to be an effective, safe, and feasible consolidation strategy (19, 20). Whole brain radiotherapy (WBRT) is another effective treatment option but associated with long-term side effects like impairment of higher cognitive functions (20, 21). Even though initial response rates are high, almost half of responders are bound to relapse and about one-third of the patients are primary refractory (22–24). Additionally, conventional immunochemotherapy is accompanied by many side effects like cytopenia and immunoglobulin deficiency, putting patients at high risk of infections and bleeding events. New and specifically targeted strategies are therefore urgently needed as treatment options for PNCSL.

We recently identified the sterile α-motif domain containing protein 14 (SAMD14) and the neural tissue-specific F-actin binding protein I (neurabin-I) as antigenic targets of the BCR from 66% (8/12 recombinant BCRs, cloned from primary tissue biopsies) of PCNSLs (12). SAMD14 and neurabin-I are primarily expressed in the CNS and share a highly homologous domain (SAM) in which the BCR binding epitope was identified. This SAM-domain was found to be post-translationally modified (N-hyperglycosylated) only in PCNSL patients with BCRs of SAMD14 and neurabin-I reactivity. It is speculated that this posttranslational modification results in a chronic immune reaction leading to lymphoma development over time (12). When coupled to the pseudomonas exotoxin A (ETA), the PCNSL-binding epitope of SAMD14/neurabin-I (hereafter referred to only as neurabin-I) confers cytotoxic effects against DLBCL cells transfected with neurabin-I-specific BCRs *in vitro*. This therapeutic approach has been termed "BAR" for "**B**-cell receptor **A**ntigen for **R**everse targeting" (12, 25).

The aim of this work is to integrate the PCNSL-specific BAR neurabin-I into an antibody format to generate a neurabin-I BAR-body and to test its functionality. To achieve this, neurabin-I was first integrated into an antibody fragment (antigen binding fragment = Fab) and later into a full length IgG1 antibody for assessment of binding properties and therapeutic potential.

MATERIALS AND METHODS

Bacteria, Cell Lines, and Cell Culture

The DLBCL cell line U2932 was kindly provided by the Dr. Senckenberg Institute of Pathology of the Goethe University Hospital Frankfurt. HEK 293T and OCI-Ly3 cells were purchased from DSMZ (Braunschweig, Germany). For authentication, concordance of the VH gene sequences with published sequences

was demonstrated by PCR analysis and sequencing. Cells were cultured in RPMI 1640 medium (Pan Biotech, Aidenbach, Germany), supplemented with 4 mmol glutamine and 10% FCS. Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll density centrifugation (1,500 rpm for 30 min) and used on day 2 after preparation without stimulation. DH5α competent *Escherichia coli* (*E. coli*) were obtained from Thermo Scientific™ (Waltham, MA, USA) and used for general cloning and sub-cloning. TG1 *E. coli* were used for expression of the heavy-chain-only Fab-format neurabin-I BAR-body.

Expression of a Neurabin-I Reactive BCR in OCI-Ly3 and U2932 Cells

To our knowledge there are no EBV-negative PCNSL cell lines which could be tested for neurabin-I autoreactivity and used for further studies. As substitute, two DLBCL cell lines, OCI-Ly3 and U2932, were transfected with a BCR with neurabin-I specificity cloned from a DNA sample of a PCNSL patient. For cloning we used a modified pRTS-1 expression vector which comprised following components: a heavy chain variable region (VH), heavy chain constant regions CH1-CH4, TM1, and TM2 as transmembrane regions, a cytoplasmic tail, a furin + 2A sequence, a light chain variable region (VL), and the light chain constant region (12, 26). VH and VL genes were derived from the neurabin-I reactive BCR of a PCNSL patient and obtained by digesting cryosections with 2 µl of proteinase K (Roche PCR grade) for 4 h at 55°C, followed by heat-inactivation at 95°C for 10 min. The subsequent semi nested PCRs were performed as described previously (12, 27, 28). PCR products of the PCNSL-derived VH and VL were re-extended according to corresponding immunoglobulin germline genes.

For transfection, 2×10^7/ml OCI-Ly3 and U2932 cells were washed three times with FCS-free RPMI-1640. A Gen Pulser (Biorad) with a 0.2 cm cuvette was used to transfect 2×10^6 cells with 5 µg plasmid DNA (140 V, 30 ms pulses). Cells were then put on ice and cultured in RPMI-1640 medium with 20% FCS. Selection of stably transfected cell lines was done with hygromycin at 250 µg/ml. Variable region gene PCRs were used to confirm successful transfection. Western blot and flow cytometry with anti-Flag antibodies were used to show expression of the Flag-tagged BCR.

Cloning Strategy for the Heavy-Chain-Only Fab-Format Neurabin-I BAR-Body

A modified pCES1 vector was used for the assembly of recombinant heavy-chain-only Fab-format neurabin-I BAR-bodies (29), comprising neurabin-I in substitution for the heavy chain variable domain (VH) and a CH1 domain. The light chain region was removed from the pCES1 vector by digesting 2 µg vector with 10 U of the restriction enzymes HindIII and AscI (Thermo Scientific™, Waltham, MA, USA) for 1 h at 37°C. For DNA blunting, the vector was incubated with DNA Polymerase I, Large (Klenow) fragment (Thermo Scientific™, Waltham, MA, USA) for 1 h at 37°C and blunt ends were ligated with 5 U T4-DNA ligase (Thermo Scientific™, Waltham, MA, USA) at 37°C over-night. The construct was coupled to a modified exotoxin A of *Pseudomonas aeruginosa* (ETA') by digesting the processed pCES1 vector with the restriction enzymes NotI and EcoRI following ligation of the accordingly prepared ETA' DNA fragment as described previously (30).

Three versions of the heavy-chain-only Fab-format neurabin-I BAR-body were cloned differing in the neurabin-I sequence selected to replace the variable region: version A (aa 1204–1324 of neurabin-I), version B (aa 1168–1285 of neurabin-I), version C (aa 1131–1250 of neurabin-I). Primers used are listed in **Table 1**. All versions contained the PCNSL binding neurabin-I epitope of 26 amino acids (aa 1226–1251) (12) at different positions from 5' to 3'.

Expression, Purification, and Detection of the Heavy-Chain-Only Fab-Format Neurabin-I BAR-Body

TG1 *E. coli* bacteria were transformed with the new vector comprising the heavy-chain-only Fab-format neurabin-I BAR-body coupled to ETA'. Recombinant soluble heavy-chain-only Fab BAR-bodies were expressed and purified as described previously (29, 31). In short, 50 µl of IPTG was added to 50 ml of TG1 bacteria (TY medium, cell density of 0.6 to 0.8 measured at 600 nm) for 4 h at 30°C and then centrifuged at 4,000 rpm for 15 min. After lysing, His-tagged and ETA'-coupled heavy-chain-only Fab-format neurabin-I BAR-bodies were purified by immobilized metal affinity chromatography (IMAC) using Talon beads (Takara Bio USA, Inc., Mountain View, CA, USA). Proteins were eluted with 150 mM imidazole for 5 min at room temperature and detected by western blot analysis using Mouse Anti-His Tag Recombinant Antibody (Qiagen, Hilden, Germany) and Goat Anti-Mouse IgG (H + L)-HRP Conjugate (Bio-Rad, Munich, Germany).

Cloning Strategy for the IgG1-Format Neurabin-I BAR-Body

To assemble the sequence the IgG1-format neurabin-I BAR-body a pSfi FLAG-Tag expression vector was used. The pSfi FLAG-Tag vector is derived from the pEGFP-C1 vector of Clontech (Mountain View, CA, USA) from which the eGFP ORF was removed and

TABLE 1 | Primers used for heavy-chain-only Fab-format neurabin-I BAR-bodies.

Heavy-chain-only Fab-format BAR-Body	Name	Sequence (5′-3′)
A	NRBI AA1204-NcoI-sense	CCA TGG CCA ATT TTA CCT TCA ATG AT
	NRBI AA1324-BstEII-antisense	GGT GAC CAT TTC CTT GAG TTT CTT
B	NRBI AA1168-NcoI-sense	CCA TGG CCA ACA CAT GGA TTA CAA AA
	NRBI AA1285-BstEII-antisense	GGT GAC CCC GAA TTC AGA TAC ATA CTG
C	NRBI AA1131-NcoI-sense	CCA TGG CCA ATG ACA GCC GGA AAG GA
	NRBI AA1251-BstEII-antisense	GGT GAC CCC AGA CTG TCC ATC ATC AAG

replaced by a FLAG-Tag. A pSfi-cloned IgG1 sequence consisting of a VH, the heavy chain constant regions CH1-CH3, a Furin + 2A sequence, a VL, and the light chain constant region (26) served as template. VH and VL were exchanged with a sequence of similar length (approximately 120 amino acids) of neurabin-I (aa 1168–1285) containing the PCNSL reactive epitope (aa 1226–1251). Primers used are listed in **Table 2**. Restriction enzymes MunI and BstEII (Thermo Fisher Scientific, Waltham, USA) were used for insertion of the neurabin-I fragment into the VH region whereas AgeI and SmaI were used for insertion of the neurabin-I fragment into the VL region.

Expression, Purification, and Detection of the IgG1-Format Neurabin-I BAR-Body

The completely assembled IgG1-format neurabin-I BAR-body was transfected into HEK 293T cells for production. HEK 293T cells were cultivated to a confluence of about 50–70%. One microgram plasmid DNA was diluted in 100 µl RPMI1640, mixed with 3 µl of X-tremeGENE™ HP DNA Transfection Reagent (Sigma-Aldrich Chemie GmbH, Munich, Germany) and incubated at room temperature for 10 min. Afterwards the preparation was added to HEK 293T cell cultures in a drop by drop fashion.

Purification of the IgG1-format neurabin-I BAR-body from the supernatant was performed *via* anti-FLAG antibody affinity chromatography using the ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich, St. Louis, USA). To purify 50 ml supernatant, 200 µl anti-FLAG M2-Affinity Gel was washed once with 1 ml PBS and then incubated with supernatant overnight at 4°C. The mixture was then centrifuged for 10 min at 2,500 rpm which was repeated a second time after discarding the supernatant in a new vessel for 3 min at 1,800 rpm. Three washing steps followed, each with 1 ml PBS and centrifugation was performed at 10,000 rpm for 30 s. Flag-tagged IgG1 neurabin-I BAR-bodies were eluted from the gel by adding 250 µl glycine pH 3 for 2 min with subsequent centrifugation at 10,000 rpm for 30 s. Supernatant containing the isolated IgG1 BAR-bodies was supplemented with 25 µl Na2HPO4. To exchange the IgG1 BAR-bodies into a different buffer system overnight dialysis against PBS was performed.

The BAR-body was detected by western blot analysis. Concentrated IgG1-format neurabin-I BAR-bodies were loaded on to a 10% SDS-PAGE and transferred to PVDF membrane using a transblot semi-dry transfer cell (Bio Rad). After blocking overnight at 4°C in PBS supplemented with 10% nonfat dry milk, transferred proteins were incubated with murine Monoclonal ANTI-FLAG® M2 antibody (Sigma-Aldrich, St. Louis, USA) at 1:2,500 for 45 min at room temperature. The membrane was rinsed with TBS 5 times for 2 min each before and after it was incubated with Goat Anti-Mouse IgG (H+L)-HRP conjugate (Bio-Rad, Feldkirchen) at 1:3,000 for 45 min. The chemiluminescence reagent LumiGLO® Reagent (Cell Signaling Technology, Frankfurt, Germany) was used for immunoblot detection.

Flow Cytometric Binding Assays

All flow cytometric analyses were performed using a BD FACS Canto Flow Cytometer and data was analyzed with WinMDI Software version 2.3 (Purdue University Cytometry Laboratories). Binding of heavy-chain-only Fab-format neurabin-I BAR-bodies to neurabin-I reactive BCRs was tested as follows: 5×10^6 OCI-Ly3 cells transfected with a neurabin-I reactive BCR were incubated with 5 µg/ml of each BAR body (versions A, B, and C) for 30 min at 4°C, washed with PBS and stained with 5 µl of Penta-His-Allophycocyanin-Antibody (Qiagen, Hilden, Germany) for 30 min at 4°C. OCI-Ly3 cells not transfected with the recombinant neurabin-I reactive BCR served as control cells and MAZ, the BCR target of the leukemic cells from a CLL patient, as control antigen (12).

Internalization assays were performed for version B of the Fab-format neurabin-I BAR-bodies (32). 5×10^6 OCI-Ly3 cells transfected with a neurabin-I reactive BCR were incubated with 5 µg/ml of the Fab-format neurabin-I BAR body (version B) for 30 min at 4°C. After a washing step in PBS, cells were incubated at 37°C for 30 min, fixed with 500 µl of 2% paraformaldehyde (PFA) for 15 min at 4°C, and permeabilized with 800 µl of 0.5% saponin. Intracellular staining of internalized Fab-format BAR-body was performed using 5 µl of Penta-His-APC-Antibody (Qiagen, Hilden, Germany) for 30 min at 4°C.

To determine binding properties of IgG1-format neurabin-I BAR-bodies to target cells, 5×10^6 U2932 cells transfected with a neurabin-I reactive BCR were incubated with 10 µg/ml IgG1-format neurabin-I BAR-bodies for 30 min, washed in PBS, and stained with murine Monoclonal ANTI-FLAG® M2 antibody for 30 min at 4°C (Sigma-Aldrich, St. Louis, USA). After another washing step in PBS, final staining was performed with goat anti-mouse IgG (H+L), APC for 30 min at 4°C (Thermo Scientific™, Waltham, MA, USA). IgG1 format BAR-bodies incorporating LRPAP1, an irrelevant BCR antigen of mantle cell lymphomas, served as controls.

Cytotoxicity Assays

Cytotoxicity of the constructs was determined by LDH release assay ("Cytotoxicity Detection Kit" by Roche). 5×10^3 OCI-Ly3 and U2932 lymphoma cells with or without neurabin-I reactive BCR were placed in each well.

Version B of ETA'-coupled Fab-format neurabin-I BAR-body and IgG1-format neurabin-I BAR-body were applied at

TABLE 2 | Primers used for the IgG1-format neurabin-I BAR-body.

IgG1-format BAR-Body	Name	Sequence (5′-3′)
Heavy chain	NRBI AA1168-MunI-sense	CAA TTG AAC ACA TGG ATT ACA AAA
	NRBI AA1285-BstEII-antisense	GGT GAC CCC GAA TTC AGA TAC ATA CTG
Light chain	NRBI AA1168-AgeI-sense	ACC GGT AAC ACA TGG ATT ACA AAA
	NRBI AA-1285-SmaI-antisense	CCC GGG GAA TTC AGA TAC ATA CTG

concentrations of 10, 5, 2.5, and 1.25 μg/ml. As Controls the constructs MAZ-ETA' and an IgG1-format BAR-body incorporating the B-cell receptor antigen LRPAP1 (25) or no construct were used.

To facilitate antibody-dependent cell-mediated cytotoxicity (ADCC), PBMCs were added to target cells and IgG1-format BAR-bodies at an effector/target ratio of 10:1, corresponding to 5×10^4 PBMCs per well.

Specific lysis was determined as (experimental lysis – spontaneous lysis)/(maximum lysis – spontaneous lysis) × 100. Maximum lysis was determined after adding 10% Triton X-100. Lactate dehydrogenase (LDH) was measured according to the protocol of the LDH assay kit (Roche, Mannheim, Germany). ELISA read-out was done using a Victor II microplate reader (PerkinElmer, Rodgau, Germany).

All experiments were performed in triplicate. Values (OD at 490 nm or specific lysis in %) are given as statistical mean of three experiments ± standard error of the mean.

RESULTS

Cloning and Expression of the Heavy-Chain-Only Fab-Format Neurabin-I BAR-Bodies

We aimed to clone and express three versions of a BAR-body construct that integrates the BCR-binding epitope of neurabin-I into a heavy-chain-only Fab-format. The variable region of a Fab-antibody is replaced by the BCR-binding epitope of neurabin-I (aa 1226–1251) to create the BAR region of the Fab-format BAR-body (**Supplementary Figures 1** and **2**). In order to mimic the molecular mass and conformation of a normal Fab-antibody, the epitope region was complemented by amino acids either at the NH2 (version A), the COOH (version C), or both ends (version B). The heavy-chain-only Fab-format neurabin-I BAR bodies in their versions A, B, and C were coupled to ETA' to mediate cytotoxicity to target cells (**Supplementary Figure 1**). The amino acid sequence enumeration refers to isoform 3 of neurabin-I (uniprot accession number: Q9ULJ8-3). Western blot analysis shows successful expression of heavy-chain-only Fab-format neurabin-I BAR bodies after expression in TG1 *E. coli* bacteria. Versions A and B were produced as three clones each (1–3) and BAR-Body C was produced as single clone (clone 4). All Fab-format BAR bodies had the expected molecular weight of approximately 67 kDa corresponding to 628 amino acids (**Figure 1**).

Identification of a Fab-Format BAR-Body Version With Highly Selective Binding Properties to Lymphoma Cells Expressing Neurabin-I Reactive BCRs

All three Fab-format BAR-body versions were tested for binding to OCI-ly3 cells transfected to express a patient-derived neurabin-I reactive BCR. As a control, all BAR-bodies were first tested against unmanipulated OCI-Ly3 cells; no binding could be observed (**Figure 2A**). All BAR- bodies were then tested against OCI-ly3 cells expressing a neurabin-I reactive BCR. As a positive control, the immunotoxin NRB-I/ETA was measured since it was previously shown to specifically bind to neurabin-I reactive BCRs (12). NRB-I/ETA bound indeed to OCI-ly3 cells expressing a neurabin-I reactive BCR (**Figure 2**). Of the BAR body clones, clone 2 of version B bound to OCI-ly3 cells expressing a neurabin-I reactive BCR, whereas all other clones did not bind (**Figure 2B**).

We tested the binding of clone 2 version B in a second independent set of experiments against an irrelevant antigen

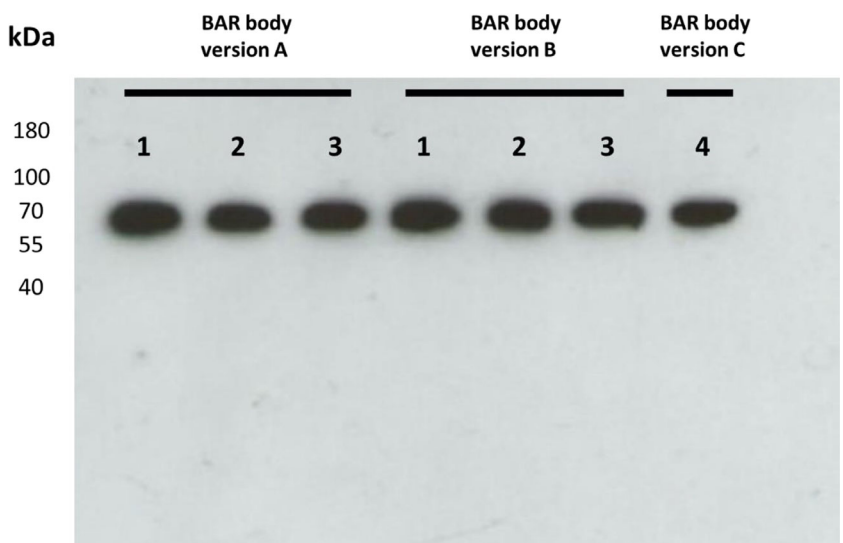

FIGURE 1 | Western blot of heavy-chain-only Fab-format neurabin-I BAR bodies after expression in TG1 E. coli bacteria. Fab-format neurabin-I BAR-bodies versions A, B [three clones each (1–3)] and C (single clone 4) were produced. All Fab-format BAR bodies had the expected molecular weight of approximately 67 kDa corresponding to 628 amino acids (1,884 base pairs).

(MAZ) coupled to the pseudomonas exotoxin A (MAZ/ETA). In the control experiments, no binding could be observed against unmanipulated OCI-Ly3 cells (**Supplementary Figure 3A**) but again we found specific binding of clone 2 version B but not the irrelevant antigen MAZ against OCI-ly3 cells expressing a neurabin-I reactive BCR (**Supplementary Figure 3B**).

Finally, surface (**Supplementary Figure 4A**) and intracellular (**Supplementary Figure 4B**) staining of OCI-ly3 cells transfected to express neurabin-I reactive BCRs with heavy-chain-only Fab-format neurabin-I BAR-bodies shows internalization of Fab-format BAR-bodies after 30 min incubation at 37°C. MAZ/ETA was used as control (left histograms of **Supplementary Figures 4A, B**).

We conclude that clone 2 version B binds specifically to OCI-ly3 cells expressing a neurabin-I reactive BCR. Therefore, the Fab-format BAR-body of clone 2, version B was selected for further evaluation and a new batch of constructs was produced (**Supplementary Figure 5**).

Heavy-Chain-Only Fab-Format Neurabin-I BAR-Body Mediates Killing of Lymphoma Cells Expressing a Neurabin-I Reactive BCR

Heavy-chain-only Fab-format neurabin-I BAR-body-induced specific cytotoxicity was measured by LDH release. No construct, MAZ/ETA and unmanipulated OCI-Ly3 cells were used as controls (**Figure 3**). Incubation of heavy-chain-only Fab-

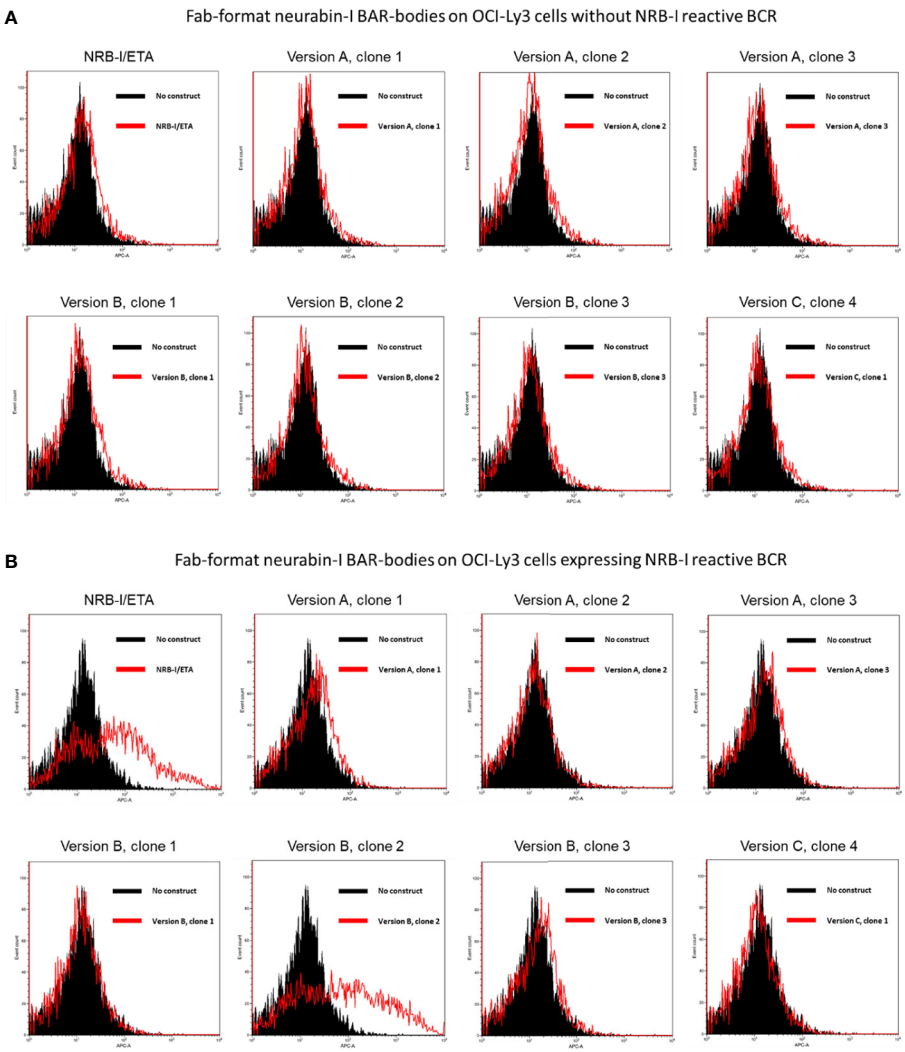

FIGURE 2 | Identification of a Fab-format BAR-body incorporating neurabin-I with binding capacity to DLBCL cells expressing a neurabin-I reactive BCR. Fab-format BAR bodies were generated in three different versions (version A, version B, and version C; see **Supplementary Figure 1**). **(A)** All three versions were tested for binding to OCI-ly3 cells that were not transfected with a patient-derived neurabin-I reactive BCR. No unspecific binding was detected. **(B)** Clone 2 of version B showed binding to OCI-ly3 cells transfected to express a neurabin-I reactive BCR similar to positive controls consisting of the immunotoxin NRB-I/ETA that has previously been shown to specifically bind to neurabin-I reactive BCRs.

format neurabin-I BAR-bodies (of clone 2 version B) with untransfected OCI-ly3 DLBCL cells at different concentrations (1.25–10 μg/ml) resulted in no LDH release indicating no unspecific Fab-format BAR-body-induced cytotoxicity (**Figure 3A**). In contrast, heavy-chain-only Fab-format neurabin-I BAR-bodies of clone 2 version B conferred cytotoxicity to OCI-ly3 DLBCL cells transfected with neurabin-I reactive BCRs. Fab-format BAR-body concentrations from 1.25 to 10 μg/ml were tested. Maximum LDH release was reached at approximately 10 μg/ml (**Figure 3B**). Fab-format BAR-body-induced lysis in relation to minimal and maximal lysis (specific lysis) is dose dependent, starting with approximately 30% at 1.25 μg/ml and reaching >90% at 10 μg/ml (**Figure 3C**) compared to maximal lysis by triton.

Generation of an IgG1-Format Neurabin-I BAR-Body

After determining the appropriate conformation of the BAR-region in Fab-format antibodies, we applied these findings to the generation of an IgG1-format BAR-body. We exchanged the variable regions of an IgG1 antibody for neurabin-I and adjacent amino acids as performed in the BAR-region of the Fab-format BAR-body, version B (**Supplementary Figure 6**). IgG1-format BAR-bodies were detected by Western Blot analysis using anti-Flag antibodies (**Figure 4A**) and by Coomassie Blue staining (**Figure 4B**). Both analyses confirmed the estimated molecular weight of the IgG1-format BAR-body of approximately 150 kD which is comparable to the molecular weight of IgG antibodies.

Highly Selective Binding of IgG1-Format Neurabin-I BAR-Bodies to Lymphoma Cells Expressing Neurabin-I Reactive BCRs

To test whether IgG1-format neurabin-I BAR-bodies bind to lymphoma cells expressing neurabin-I reactive BCRs, U2932 cells (**Figure 5A**) and U2932 cells transfected with a neurabin-I reactive BCR (**Figure 5B**) were stained with 10 μg/ml IgG1-format neurabin-I BAR-body. Selective binding of the IgG1-format neurabin-I BAR-bodies to lymphoma cells with neurabin-I reactive BCRs was observed (compare right-side histograms of **Figures 5A, B**). IgG1-format BAR-bodies incorporating LRPAP1, an irrelevant BCR antigen of mantle cell lymphomas (25), served as control (left histograms of **Figures 5A, B**).

IgG1-Format Neurabin-I BAR-Bodies Confer Selective, ADCC-Mediated, Cytotoxic Effects to Lymphoma Cells

We tested if IgG1-format neurabin-I BAR-bodies induce PBMC-mediated specific cytotoxicity against lymphoma cells expressing a BCR with neurabin-I reactivity. No construct, IgG1-format BAR-bodies incorporating LRPAP1 (25) or U2932 cells expressing native BCRs were used as controls.

IgG1-format neurabin-I BAR-bodies were tested at different concentrations (1.25–10 μg/ml). After incubation with

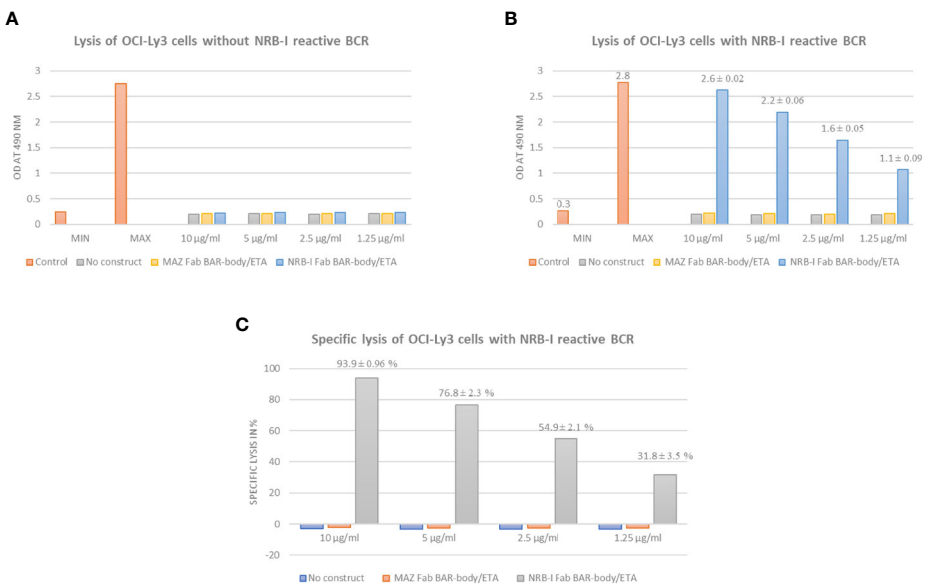

FIGURE 3 | Heavy-chain-only Fab-format neurabin-I BAR-body induced specific cytotoxicity as measured by LDH release. MAZ/ETA, an irrelevant antigen (BCR target of the leukemic cells of a CLL patient) linked to pseudomonas exotoxin A, was used as control. All experiments were performed in triplicate. **(A)** Heavy-chain-only Fab-format neurabin-I BAR-body incubation with OCI-ly3 DLBCL cells at different concentrations (1.25–10 μg/ml) results in no LDH release indicating no unspecific Fab-format BAR-body induced cytotoxicity. **(B)** Heavy-chain-only Fab-format neurabin-I BAR-body at different concentrations (1.25–10 μg/ml) confers cytotoxicity to OCI-ly3 DLBCL cells transfected with neurabin-I reactive BCRs. Maximum LDH release is reached at approximately 10 μg/ml. **(C)** Fab-format neurabin-I BAR-bodies mediate specific lysis (calculated as described in the material and methods section) in a dose dependent manner, starting with approximately 30% at 1.25 μg/ml and reaching >90% at 10 μg/ml.

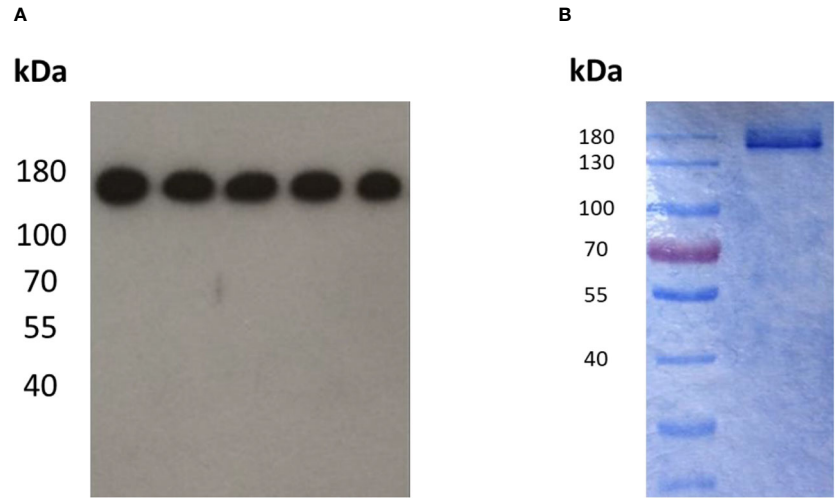

FIGURE 4 | Detection of the IgG1-format neurabin-I BAR-body in Western Blot analysis using anti-Flag antibodies **(A)** and in Coomassie Blue staining **(B).** Both analyses show the estimated molecular weight of the IgG1-format BAR-body of approximately 150 kD comparable to normal IgG antibodies.

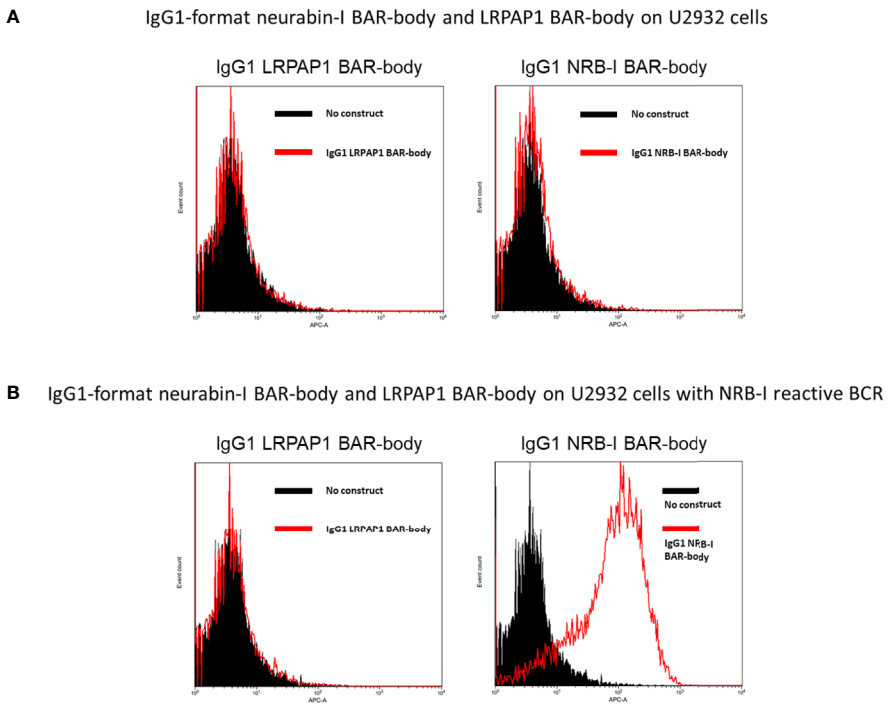

FIGURE 5 | U2932 cells **(A)** and U2932 cells transfected with a neurabin-I reactive BCR **(B)** were stained with 10 µg/ml IgG1-format neurabin-I BAR-body. Mouse anti-Flag antibody followed by APC-conjugated anti-mouse antibody were used for detection. IgG1-format BAR-bodies incorporating LRPAP1, an irrelevant BCR antigen of mantle cell lymphomas, served as controls (left histograms of **A, B**).

unmanipulated U2932 DLBCL cells and PBMCs (E:T ratio of 10:1) no LDH release was observed indicating no unspecific IgG1-format BAR-body-induced cytotoxicity (**Figure 6A**). When applying IgG1-format neurabin-I BAR-body to U2932 DLBCL cells transfected with neurabin-I reactive BCRs and PBMCs (E:T ratio of 10:1), dose-dependent PBMC-mediated cytotoxicity was observed (**Figure 6B**).

Specific lysis is mediated in a dose-dependent manner, starting from 4% at 1.25 µg/ml and going up to 58% at 10 µg/ml (**Figure 6C**) compared to the triton control.

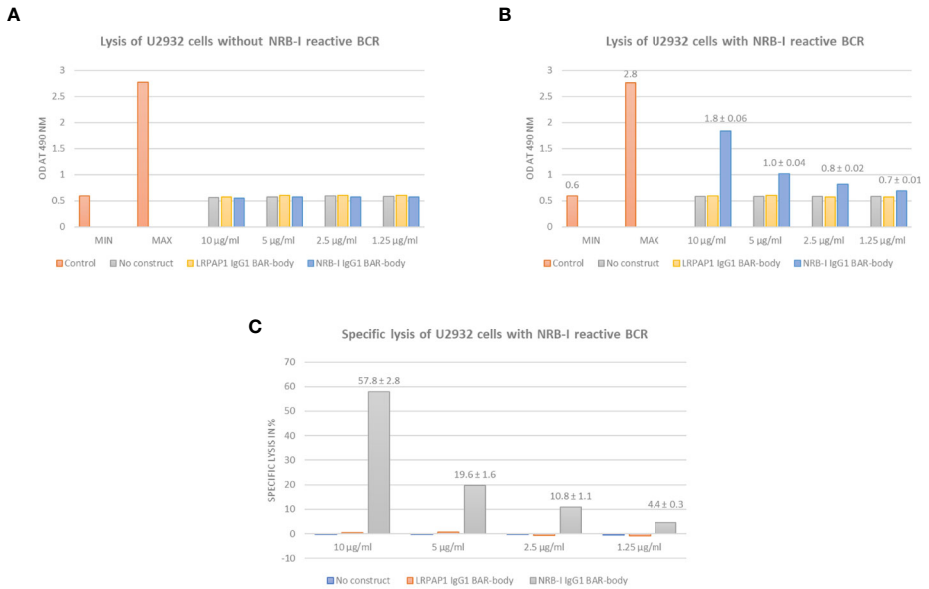

FIGURE 6 | IgG1-format neurabin-I BAR-body induced PBMC-mediated specific cytotoxicity as measured by LDH release. IgG1-format BAR-bodies incorporating LRPAP1, an irrelevant BCR antigen of mantle cell lymphomas, were used as controls. All experiments were performed in triplicate. **(A)** IgG1-format neurabin-I BAR-body at different concentrations (1.25–10 µg/ml) incubated with U2932 DLBCL cells and PBMCs (E:T ratio of 10:1) results in no LDH release indicating no unspecific IgG1-format BAR-body induced cytotoxicity. **(B)** IgG1-format neurabin-I BAR-body (concentrations 1.25–10 µg/ml) confers dose-dependent and PBMC-mediated cytotoxicity to U2932 DLBCL cells transfected with neurabin-I reactive BCRs at an effector to target ratio of 10:1.**(C)** IgG1-format neurabin-I BAR-bodies confer specific lysis to U2932 cells transfected to express a neurabin-I reactive BCR (PBMC-mediated ADCC). LDH release is mediated in a dose-dependent manner, starting from 4% at 1.25 µg/ml going up to 58% at 10 µg/ml.

DISCUSSION

We developed a molecule resembling an IgG1 antibody, that contains the BCR-binding epitope of the common PCNSL antigens SAMD14 and neurabin-I instead of variable regions to target BCRs with specificity for neurabin-I. This molecule, termed IgG1-format neurabin-I BAR-body, showed binding capacity only to lymphoma cells expressing a BCR with neurabin-I reactivity and exerted cytotoxic effects exclusively on lymphoma cells expressing a BCR of this specificity. As shown recently, more than 50% of all PCNSLs express a BCR with reactivity against neurabin-I (12) rendering more than half of all PCNSLs theoretically susceptible to neurabin-I BAR-body therapy.

The BCR of B-cell lymphoma cells is considered to be an ideal target for therapeutic approaches since it has a unique variable region (also called idiotype) distinguishing it from BCRs of normal B-cells and it is expressed abundantly on lymphoma cells. Different strategies have been developed to exploit the distinctiveness of the BCR of B-cell lymphomas. Anti-idiotype antibodies were the first therapeutics to be developed and tested with moderate success. They were either collected from animals immunized with the BCR of a patient´s lymphoma (33–36) or produced by patient vaccination with processed B-cell receptors of lymphoma cells (37, 38). More recently, Ronald Levy et al. developed a treatment approach termed peptibody, where small peptides are selected using a phage display library to develop anti-idiotype peptides that are affixed to an IgG Fc protein (39). The major drawback of anti-idiotype therapies developed so far is that they have to be produced individually on a patient by patient basis. Therapies incorporating a common B-cell receptor antigen that is shared as the antigenic target of a large percentage of a given B-cell lymphoma entity, like the neurabin-I BAR-body in the treatment of CNS lymphomas, would overcome such problems.

The findings of Thurner et al. (12) differ from those published by Montesinos-Rongen, who describes the BCRs of PCNSLs as mostly polyreactive with some BCRs recognizing galectin-3 and other antigens expressed in the CNS (13, 40). Further studies investigating the reactivity of more recombinant PCNSL-derived BCRs using an identical methodological approach will have to resolve these conflicting results. Nevertheless, it is noteworthy that our treatment approach to integrate BCR antigens into antibody formats would be applicable to all antigens identified.

The described neurabin-I BAR-body mimics the molecular structure of an IgG1 antibody with the variable regions exchanged for the shared PCNSL BCR epitope of SAMD14 and neurabin-I. The PCNSL binding epitope of neurabin-I comprises 26 amino acids (aa 1226–1251) which is much shorter than immunoglobulin variable regions, normally consisting of 110–130 aa (12, 41). To match the size of immunoglobulin variable regions in the neurabin-I BAR-body, we elongated the PCNSL-binding epitope to 120 amino acids with adjacent neurabin-I amino acids creating the BAR region. At the start it was unknown how the addition of amino acids to the BCR binding epitope would influence its binding properties. Therefore, we used a heavy-chain-only Fab-format to test different BAR region formations, containing the PCNSL-binding epitope either at its beginning (aa 1204–1324 of

neurabin-I), its middle (aa 1168–1285 of neurabin-I), or its end (aa 1131–1250 of neurabin-I). The amino acid sequence numeration refers to isoform 3 of neurabin-I. These three neurabin-I BAR regions, carrying the common epitope responsible for binding to the BCR of PCNSL at different positions, were produced prokaryotically as heavy-chain-only Fab-format BAR-bodies. These initial exploratory experiments were performed due to lack of information on how the integration of the neurabin-I epitope into an antibody format would influence its binding properties to neurabin-I specific BCRs. When the 26 aa comprising neurabin-I epitope is flanked by non-epitope regions to form a 120 aa neurabin-I BAR region (aa 1168–1285 of neurabin-I) its binding properties to PCNSL BCRs are preserved. The causes for the lack of binding capacity of the other two Fab-format BAR-body variations to PCNSL BCRs with neurabin-I reactivity are not clear. Accordingly, the eukaryotically produced, full-length IgG1 neurabin-I BAR-body incorporating the appropriate BAR region was also able to bind to DLBCL cells, that were genetically engineered to express a patient-derived BCR with neurabin-I reactivity. *In-vitro* LDH-release assays showed the ability of the Fab-format and IgG1-format neurabin-I BAR-bodies to selectively kill DLBCL cells expressing corresponding BCRs.

The glycosylation status of produced BAR-bodies has not been assessed. Mass spectrometry analysis might be able to determine the glycosylation status of integrated neurabin-I epitopes. But while it would be interesting to determine hyper N-glycosylation of the described BAR-bodies, we think it has no bearing on our experiments. In previous work it could be shown that PCNSL-derived BCRs reacted with both the normally glycosylated and the hyper N-glycosylated SAMD14/neurabin-I isoforms (12). The isoform-specific immune reaction may be mediated by CD4+ T cells, which stimulate non-isoform-specific neurabin-I reactive B cells.

It remains to be clarified whether the IgG format is suitable for the further development of BAR-integrating therapeutics. The concentrations that are needed for the reported neurabin-I BAR-body to reach approximately 50% specific lysis are higher than the concentrations that have been reported for rituximab (42). This difference may be attributed to different factors. First, the PCNSL-derived BCR was transfected to be expressed by DLBCL cell lines which also express their natural BCR possibly resulting in a reduced expression of the PCNSL BCR on cells. Secondly, the BAR-body construct targets the variable region of the BCR with its presumed cognate antigen. This BCR-antigen interaction has not been quantified yet, for example by determining the affinity of neurabin-I to the BCR by plasmon resonance imaging. A low affinity may be sufficient to contribute to lymphoma development but may also necessitate high concentrations of BAR-bodies incorporating the cognate BCR antigen.

Regarding the size of full-length IgG antibodies, it is thought to result in an impaired ability to pass the blood-brain barrier and therefore leading to reduced CNS antibody concentrations. Rubenstein et al reported rituximab concentrations of as low as 0.1% of serum antibody concentrations (43). The role of the IgG antibody rituximab in the first-line treatment of PCNSL has been controversial, but evidence from the IELSG trials indicate clinical efficacy (17) and there have been reports of complete remissions after i.v. rituximab administration in the relapse setting (44). Also, intrathecal application of BAR-bodies is a conceivable route of administration (45). We therefore think that the IgG format is appropriate for the treatment of PCNSLs but as long as there is no *in-vivo* data, this notion remains speculative.

Taken together, our study justifies further development of BAR-bodies as promising tools for the treatment of CNS lymphomas. Nevertheless, major challenges might arise that range from the identification of more PCNSL antigens over the penetration of the blood-brain barrier to escape mechanisms of malignant B-cells. When the B-cell receptor is targeted, these problems could result from genetic alterations, common to malignant B cells like somatic hypermutation, to reduce the affinity to a B-cell receptor antigen and therapeutic BAR approaches. Thus, *in-vivo* studies are needed to further investigate the efficacy and potential drawbacks of the IgG1-format neurabin-I BAR-body in the treatment of CNS lymphoma.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MB and LT wrote the manuscript. MB, LT, FN, K-DP, and MP designed and supervised the experiments. LG, CM, MK, ER, and NF performed the experiments. MB and LT are responsible for data analysis. SS, KC, DK-M and MH revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by a grant from Wilhelm-Sander-Stiftung (a charity foundation in Munich, Germany).

ACKNOWLEDGMENTS

We dedicate this work to MP, the intellectual father of the BAR approach. We are grateful to the entire team of the José-Carreras-Centre for Immuno- and Gene Therapy and the Department of Internal Medicine I of Saarland University Medical School for their continuous logistic and intellectual support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.580364/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
published: 18 November 2020
doi: 10.3389/fimmu.2020.594556

Immune Dysfunctions and Immune-Based Therapeutic Interventions in Chronic Lymphocytic Leukemia

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OPEN ACCESS

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: 13 August 2020
Accepted: 14 October 2020
Published: 18 November 2020

Citation:
Griggio V, Perutelli F, Salvetti C, Boccellato E, Boccadoro M, Vitale C and Coscia M (2020) Immune Dysfunctions and Immune-Based Therapeutic Interventions in Chronic Lymphocytic Leukemia. Front. Immunol. 11:594556. doi: 10.3389/fimmu.2020.594556

Chronic lymphocytic leukemia (CLL) is a B-cell malignancy characterized by a wide range of tumor-induced alterations, which affect both the innate and adaptive arms of the immune response, and accumulate during disease progression. In recent years, the development of targeted therapies, such as the B-cell receptor signaling inhibitors and the Bcl-2 protein inhibitor venetoclax, has dramatically changed the treatment landscape of CLL. Despite their remarkable anti-tumor activity, targeted agents have some limitations, which include the development of drug resistance mechanisms and the inferior efficacy observed in high-risk patients. Therefore, additional treatments are necessary to obtain deeper responses and overcome drug resistance. Allogeneic hematopoietic stem cell transplantation (HSCT), which exploits immune-mediated graft-*versus*-leukemia effect to eradicate tumor cells, currently represents the only potentially curative therapeutic option for CLL patients. However, due to its potential toxicities, HSCT can be offered only to a restricted number of younger and fit patients. The growing understanding of the complex interplay between tumor cells and the immune system, which is responsible for immune escape mechanisms and tumor progression, has paved the way for the development of novel immune-based strategies. Despite promising preclinical observations, results from pilot clinical studies exploring the safety and efficacy of novel immune-based therapies have been sometimes suboptimal in terms of long-term tumor control. Therefore, further advances to improve their efficacy are needed. In this context, possible approaches include an earlier timing of immunotherapy within the treatment sequencing, as well as the possibility to improve the efficacy of immunotherapeutic agents by administering them in combination with other anti-tumor drugs. In this review, we will provide a comprehensive overview of main immune defects affecting patients with CLL, also describing the complex networks leading to immune evasion and tumor progression. From the therapeutic standpoint, we will go through the evolution of immune-based therapeutic approaches over time, including i) agents with broad immunomodulatory effects, such as immunomodulatory drugs, ii) currently approved and next-generation monoclonal antibodies, and iii) immunotherapeutic strategies aiming at activating or administering

immune effector cells specifically targeting leukemic cells (e.g. bi-or tri-specific antibodies, tumor vaccines, chimeric antigen receptor T cells, and checkpoint inhibitors).

Keywords: chronic lymphocytic leukemia, immune dysfunction, immunotherapy, immunomodulation, targeted therapy, cellular therapy, chimeric antigen receptor T cells

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a lymphoproliferative disease characterized by the clonal accumulation of mature B lymphocytes in the peripheral blood, bone marrow and secondary lymphoid organs (1). A hallmark of CLL is the variable clinical course, which reflects the biological heterogeneity of tumor cells. The lack of somatic mutations on immunoglobulin heavy chain variable (IGHV) genes, and/or the presence of chromosomal aberrations and genetic lesions identify patients with more aggressive forms of the disease [as reviewed in (1, 2)]. Besides intrinsic features of the malignant clone, profound defects of the immune system and the ability of leukemic cells to circumvent immune recognition and elimination are leading causes of tumor progression. In CLL, tumor cells and cellular components of the microenvironment are reciprocally interconnected and co-evolve, shaping each other during the course of the disease [as reviewed in (3–5)].

Some immunological alterations [e.g. T-cell and natutal killer (NK)-cell expansion, and reduction of circulating normal B cells] are also associated with monoclonal B-cell lymphocytosis (MBL), a premalignant condition that precedes CLL (6, 7). However, most of the immune surveillance dysfunctions accumulate during disease evolution, most likely constributing to the transition from MBL to CLL [as reviewed in (8, 9)]. From the clinical standpoint, these immunologic dysregulations are responsible for the increased susceptibility to infections and secondary malignancies, the occurrence of autoimmune phenomena and the failure to control disease progression (10, 11) [and as reviewed in (5, 12–17)].

In patients with CLL carrying favorable prognostic factors (i.e. mutated IGHV genes), the chemoimmunotherapy combination regimen consisting of fludarabine, cyclophosphamide, and rituximab (FCR) allows the achievement of undetectable minimal residual disease and long-term remissions (18). More recently, combinations including multiple targeted drugs with a different mechanism of action, such as BTK and Bcl-2 inhibitors with or without anti-CD20 monoclonal antibodies (mAb), have shown very promising results in terms of depth and durability of response, although data are not yet mature (19–21).

Nevertheless, to date, the only therapeutic approach with a consolidated, long-term potentially curative effect is the allogeneic hematopoietic stem cell transplantation (HSCT). HSCT, which is still considered a valuable treatment option for younger and fit patients with high-risk CLL (i.e. relapsed/refractory patients with poor prognostic features), exploits a T-cell mediated graft-*versus*-leukemia reaction, thus supporting the evidence that a competent immune system can be effective in controlling and eradicating the tumor. Due to the advanced median age and the high frequency of comorbidities, HSCT can be reserved only to a restricted number of patients with CLL [as reviewed in (22)]. However, other approaches exploiting immunological mechanisms, such as adoptive chimeric antigen receptor (CAR) T-cell therapy, possibly in combination with drugs showing immunomodulatory properties (e.g. lenalidomide and targeted drugs), have shown promising preclinical and/or clinical results.

In this review, we will comprehensively describe immune alterations occurring in CLL, and we will go through the evolution of immune-based therapeutic approaches over time, also addressing most recent advances in the field of immunotherapy.

IMMUNE ESCAPE IN CLL

During the clinical course, CLL cells induce a progressive impairment of the immune system leading to a state of clinically manifest immune suppression, which is in part responsible for the lack of disease control [as reviewed in (3, 4)]. The association between immune deficiency and tumor progression has been widely explored in CLL. Studies report that immune defects characterizing patients since diagnosis frequently exacerbate in advanced CLL stages, and that a wide range of quantitative and qualitative alterations affect both the innate and the adaptive arms of the immune response, have been reported (as summarized in **Figure 1**) [as reviewed in (5, 23, 24)]. Defects in main players of innate immunity, which include cell populations of lymphoid (i.e. NK cells, NKT cells, and γδ T cells) and myeloid [i.e. dendritic cells (DC) and macrophages] lineage, contribute to the ineffective triggering and maintenance of T-cell responses, as well as to their suboptimal cytotoxic activity. In the context of the adaptive immune response, several aberrations of the T-cell compartment, ranging from phenotypical changes to functional impairment, have been described [as reviewed in (24–26)]. Besides cellular components, significant alterations of the humoral response also contribute to the tumor immune escape in CLL (5). Elucidating the immune cell dysfunctions and identifying the mechanisms underlying immune suppression are crucial steps to attempt an immune system reactivation, and develop effective and novel immune-based treatment strategies.

Phenotypic and Functional Alterations of the NK- and NKT-Cell Compartments

Most studies report that NK-cell number is increased in the peripheral blood of CLL patients and associates with better prognosis (27–34). NK cells represent an appealing lymphocyte subset to be exploited in the context of immunotherapy, especially for their non-major histocompatibility complex

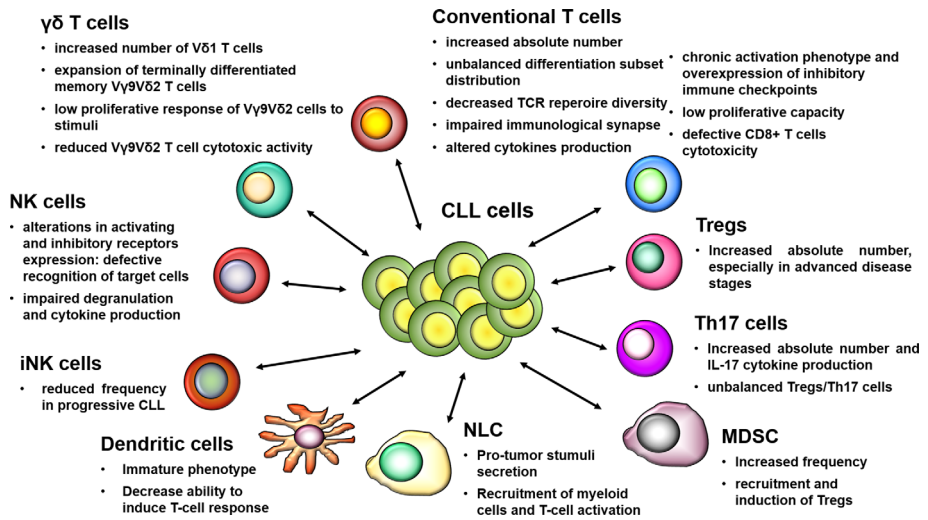

FIGURE 1 | Schematic overview of main defects affecting immune cell populations in CLL. The dysregulation of the immune response in CLL includes phenotypic alterations and functional impairments, which are present since the early stages and exacerbate during the course of the disease, thus promoting immune tolerance and tumor progression. The mutual interactions between leukemic cells and cellular elements of the immune system contribute to the establishment of a permissive or even supportive microenvironment that favors tumor progression, thus playing a key role in immune escape mechanisms.

(MHC)-restricted cytotoxicity (35). However, it has been demonstrated that autologous NK cells are not able to effectively eliminate CLL cells, because of both NK-cell intrinsic defects and immune escape mechanisms employed by tumor cells (36–41). The effector function of NK cells rely on combined signaling *via* a variety of activating and inhibitory receptors whose ligands are either expressed on the surface of target cells or secreted in soluble forms. Several studies focused their attention on the balance between inhibitory and activating receptors signals in CLL, and their role in regulating the final NK-cell-mediated anti-tumor response. The inhibitory receptors NKG2A and the killer-cell immunoglobulin-like receptors (KIRs), through the binding with their respective ligands HLA-E and HLA-A on tumor cells, suppress cytokine secretion and hamper direct cytotoxicity of NK cells against target cells (31, 42). The expression of NKG2A is similar on NK cells from CLL patients and healthy donors, whereas its ligand HLA-E is overexpressed on the surface of leukemic cells (41–44). It has been reported that plasma levels of soluble HLA-E (sHLA-E) are higher in advanced-stage CLL patients and associate to shorter treatment free survival. In addition, sHLA-E secreted by tumor cells *in vitro* inhibits cell degranulation and IFN-γ production by NK cells, thus determining their functional impairment (44). Similarly, plasma samples from CLL patients were reported to contain increased levels of soluble HLA-G, the ligand of the inhibitory receptor (KIR)2DL4, and to be capable of dampening both the viability and cytotoxic function of NK cells from healthy donors *in vitro* (45). HLA-G is also bound with high affinity by the Ig-like transcript 2 (ILT2) inhibitory receptor, which is overexpressed on NK cells from CLL patients (43). As an additional inhibitory mechanism, in line with data on conventional T cells, the immune checkpoint Tim-3 was found to be aberrantly expressed on the NK-cell compartment (28). Concerning activating receptors, the reduced expression of NKG2D, DNAM-1 and natural cytotoxicity receptors (NCRs) reported on NK cells of CLL patients compared to healthy individuals, is paralleled by a defective cytotoxic activity, degranulation and direct killing of target cells (28, 31, 32, 41, 46, 47). Of note, CLL cells have decreased surface level of NKG2D and NCRs ligands, which are also shed as soluble molecules (i.e. sMIC-A, sMIC-B, and sULBP2), thus contributing to a hindered recognition of tumor cells by NK cells (48–50). Notably, NK-cell dysfunctions are not permanent and can be reversed by proper stimulation with cytokines (i.e. IL-2, IL-15, IL-27) (41, 51, 52).

Despite the abnormalities reported so far, NK cells retain their ability to efficiently induce antibody-dependent cellular cytotoxicity (ADCC), through the binding of CD16 (FcγRIIIA) to the Fc-regions of antibody-antigen complexes located on the surface of tumor cells (31, 34, 41, 46, 53). In CLL, ADCC has a pivotal therapeutic role because several treatment strategies include anti-CD20 mAb, whose activity rely on this process. Due to their preserved ADCC function and the reversibility of other CLL-related dysfunctions, NK cells are therefore an attractive source for cellular immunotherapy in this disease.

Within innate immunity, another cell player with a potential anti-tumor role are type I NKT cells, also called invariant NKT (iNKT) cells. iNKT cells have the ability to activate and expand in response to antigens presented by CD1d (54–57). In CLL, little information regarding NKT cells and, specifically, iNKT cells is currently available, and mainly supports their contribution to CLL immune surveillance (58, 59). Interestingly, iNKT-cell frequency is significantly lower in patients with progressive disease than in patients with stable disease, and has shown to be an independent predictor of disease progression (60). Concerning the leukemic counterpart, a reduced expression of

CD1d has been described on CLL cells compared to normal B cells from healthy donors (58, 59, 61, 62). From the functional standpoint, CLL cells have a limited ability to present glycolipid antigens to iNKT cells and to induce their expansion and functional activation (58, 63, 64). Of note, this reduced capacity of leukemic cells to stimulate iNKT cells can be effectively reversed by retinoic acid, which upregulates the expression of CD1d on CLL cells and enhances iNKT-mediated cytotoxicity against tumor targets loaded with α-galactosylceramide (59).

γδ T-Cell Alterations

Among various lymphocyte subsets being considered for cellular immunotherapy of cancer are γδ T cells (both Vδ1- and Vδ2-expressing T cells), which have the ability to mediate responses through the activation of cytotoxic mechanisms against tumor cells in a MHC-unrestricted manner (35). Patients with CLL have increased numbers of circulating Vδ1 T cells which are able to produce TNF-α and INF-γ. Moreover, Vδ1 T cells are able to kill leukemic cells expressing the MIC-A and ULBP3 surface molecules, which are involved in the activation of γδ T-cell effector functions through the engagement of the NKG2D receptor (65). Interestingly, preclinical studies reported that Vδ1 T cells can be properly stimulated to express NCRs that act as costimulatory molecules and improve their cytotoxic activity against CLL cells both *in vitro* and in a xenograft mouse model (66, 67). In healthy subjects, the main subset of circulating γδ T cells is represented by Vγ9Vδ2 T cells, which consist of cytotoxic T lymphocytes with a putative potent anti-tumor activity, triggered by the MHC-independent recognition of non-peptidic phosphoantigens, such as intermediate metabolites of the mevalonate metabolic pathway and aminobisphosphonates [as reviewed in (68)]. We have previously demonstrated that in CLL the Vγ9Vδ2 T cell-compartment is characterized by an unbalanced differentiation subset distribution, with a prominent expansion of effector memory and terminally differentiated effector memory cell subsets, determining a low *in vitro* proliferative response and predicting for a more aggressive clinical course (69). In addition, de Weerdt *et al.* reported that Vγ9Vδ2 T cells from CLL patients are less effective in inducing tumor cell death, due to a dysfunction in effector cytokine production and degranulation. Interestingly, the observations that an altered phenotype is also inducible in healthy Vγ9Vδ2 T cells co-cultured with CLL cells, and that in CLL patients the functional impairment of Vγ9Vδ2 T cells is associated with higher leukocytes counts, indicate a leukemia-induced mechanism of immune suppression (70).

DC Defects

DC are specialized antigen presenting cells (APC) with a crucial role in the initiation and regulation of innate and adaptive immune responses, and whose functional modulation is under investigation with the aim of improving cancer immunotherapy. In CLL, DC show an immature phenotype (lack of CD80 and CD83 expression), and have an altered capacity to stimulate T-cell proliferation, to drive T-cell differentiation toward T helper (Th)1 response and to release IL-12 (71, 72). In addition, Orsini *et al.* reported that CLL cells are able to modify the maturation and function of healthy donor-derived DC through the secretion of IL-6 (71). Recent data demonstrated that the molecular mechanism underlying DC abnormalities in CLL is a disruption of the IL-4R/STAT6 pathway due to enhanced levels of the suppressor of cytokine signaling 5 (SOCS5), a negative regulator that inhibits STAT6 activation and leads to a defective DC differentiation (72). Notably, despite these observed alterations, different studies and clinical trials showed that DC from CLL patients can be properly manipulated and effectively exploited in the context of vaccination approaches (73–77).

Phenotypic and Functional T-Cell Alterations

T lymphocytes have a fundamental role in tumor immune-surveillance. In the context of adaptive immune response, CD4+ Th cells are the main actors in antigen recognition, activation of humoral response, cytokine production, and coordination of CD8+ cytotoxic T lymphocyte response [as reviewed in (25, 26)]. Overall, circulating CD4+ and CD8+ T lymphocytes are increased in patients with CLL (78–80). The expansion of CD8+ T cells is prominent and results in a drop of the CD4:CD8 ratio, which characterizes CLL patients since the early phases of the disease (79, 81). A number of studies attributed a prognostic value to the CD4:CD8 ratio, whose inversion has been associated with advanced disease, and has shown to predict a shorter time to first treatment and overall survival (82–84). Concerning Th subset distribution, most reports agree on the accumulation of Th1 T cells in the peripheral blood of CLL patients compared to healthy controls, whereas data on Th2 T cells are still controversial (85–88). From the functional standpoint, a recent article by Roessner *et al.* has investigated the pro- or anti-tumoral effect of Th1 T cells on CLL development, showing that the accumulation of Th1 T cells observed in human CLL and in a mice with CLL-like disease has no impact on disease progression (87). In terms of T-cell differentiation subsets distribution, a reduction in naïve T cells and an accumulation of effector T cells and highly differentiated memory T cells were observed in CLL patients (82, 85, 89, 90). Several evidences suggest that T lymphocytes in CLL patients are subjected to chronic antigenic stimulation, which shapes their phenotype and functional activity. Indeed, T cells show an increased surface expression of CD57, CD69, and HLA-DR, which are typical markers of activated cells (91, 92). Phenotypic and functional properties of T cells from patients with CLL resemble those of exhausted T cells, which are typically observed during chronic infections (93). Although cytomegalovirus (CMV) infection has shown to induce T-cell expansion and modulate the distribution of differentiation subsets, the exhaustion observed in T cells from CLL patients resulted to be independent from CMV serostatus (82, 93–95). The progressive skew of the T-cell receptor (TCR) repertoire occurring during disease progression, may suggest a tumor-related antigen-mediated selection (96, 97). In line with this observation, an oligoclonal CD8+ effector T-cell population, that expands along with CLL progression and controls disease development, was observed in both CLL patients and mice bearing a CLL-like disease (90). Consistently, through a cell-to-cell-mediated mechanism, leukemic cells induce in CD4+ and

CD8+ T cells purified from CLL patients several changes in the expression of genes involved in CD4+ T-cell differentiation, cytoskeleton formation, and vesicle trafficking, and in CD8+ T-cell cytotoxicity (98). These evidences confirm the contribution of the leukemic counterpart in shaping a pro-tumor microenvironment. The aberrant gene expression profile has an impact on the T-cell functions, mainly in terms of immunological synapse formation with APC, proliferation, migration, and cytotoxic activity (99–102). In particular, a key regulatory mechanism of immune evasion in CLL is the impaired killing of target cells by cytotoxic T lymphocytes, which is associated with the formation of dysfunctional non-lytic immune synapses and to a non-polarized release of lytic granules (101). Lastly, also metabolic features, such as mitochondrial respiration, membrane potential and levels of reactive oxygen species, have an impact on T-cell fitness, and demonstrate to be particularly relevant for CAR T-cell expansion and persistence (103).

The aberrant expression of immune checkpoint molecules, which regulate T-cell activation and function, is a hallmark of an impairment in immune surveillance. The engagement of checkpoint receptors by their ligands leads to the inhibition of T-cell proliferation and cytokine production, thus suppressing immune responses. In CLL, both CD4+ and CD8+ T cells show an increased expression of several inhibitory checkpoints, such as CTLA-4, PD-1, LAG3, Tim-3, TIGIT, CD160, and CD244 (89, 93, 104–110). Of note, this abnormal expression of immune checkpoint receptors on T lymphocytes is paralleled by an increased expression of their corresponding inhibitory ligands, such as PD-L1/PD-L2, CD200, galectin-9, and CD276, on leukemic cells (89, 99, 110, 111). CTLA-4 and PD-1 are the more extensively studied immune checkpoints in CLL. T cells from CLL patients have a higher expression of both the intracellular and surface forms of CTLA-4 compared to healthy controls (85, 112). In addition, the upregulation of PD-1 was observed in CD4+ and CD8+ T cells from patients with CLL and was reported to associate with adverse prognosis (82, 85, 89, 113). PD-1 expression is further increased in T cells from the lymphnode compared to the peripheral blood compartment (90, 114). Interestingly, the double positivity for PD-1 and Tim-3 identifies a T-cell subset with a particularly pronounced impairment in the effector functions (108). Despite their features of impairment, some functional aspects of T cells from the peripheral blood of CLL patients, such as cytokine production, were initially reported to be preserved or even enhanced in comparison to healthy individuals (93). By contrast, CD8+ T cells from secondary lymphoid organs, which are continuously exposed to leukemic cells, express higher levels of PD-1 and are functionally defective (90, 114).

A better understanding of the mechanisms leading to this tumor-induced dysfunction of CD8+ T cells will be important for the development of effective T-cell-based immunotherapeutic strategies for the treatment of CLL patients.

Features of Immunosuppressive Cells and of the Tolerogenic Milieu in CLL

Multiple signals emanated by tumor cells shape the tumor supportive functions of different cellular elements of the tumor microenvironment, including stromal cells, T cells, and myeloid-derived cells. In addition, extrinsic features of the tumor niche—such as hypoxia—contribute to this tolerogenic milieu, leading to the engagement of cell subsets endowed with immune suppressive properties, such as T regulatory cells (Tregs) and myeloid-derived suppressor cells (MDSC) (115).

Several studies agree with the demonstration that circulating Tregs count is increased in CLL patients with respect to healthy controls, while data on Tregs frequency among total CD4+ T cells are not consistent (116–119). Higher Tregs number associates with increased tumor load, advanced stages of disease, disease progression, and poor prognosis (116, 117, 120, 121). Interestingly, Jak *et al.* reported that the expansion of Tregs in CLL can be mediated by CD27-CD70 interactions in the lymphnode and by an impaired sensitivity to apoptosis linked to a Bcl-2 overexpression, rather than being the consequence of chronic antigenic stimulation (122). In parallel to the higher number, an increased production of IL-10 and TGF-β1, and an overexpression of the immunosuppressive molecule CTLA-4 characterize Tregs from CLL patients compared to controls (104, 121, 123). From the functional standpoint, available information indicates that the suppressive activity of Tregs is preserved in patients with CLL (117). Indeed, Tregs i) proliferate in response to TCR stimulation, ii) display CD107a surface expression as a marker of cytotoxic activity, and iii) are able to inhibit T-cell proliferation (120, 124, 125). In addition, a recent study has demonstrated that in CLL patients Tregs switch toward an effector-like T-cell phenotype, which is associated with an altered cytokine profile and transcriptional program of immune genes (126).

Tregs are strictly interconnected with the Th17-cell population, a subset of pro-inflammatory cells involved in the development and evolution of tissue inflammation and autoimmune diseases, and with a dichotomous role in cancer [as reviewed in (127)]. Patients with CLL have an increased frequency and absolute number of Th17 cells, and higher IL-17A and IL-17F serum levels compared to healthy controls. Of note, the Th17-cell number is associated with the presence of favorable prognostic factors, an early stage of the disease and a longer overall survival (85, 88, 128–133). Although these evidences suggest that Th17 cells may have a protective function, their role within the CLL microenvironment is not yet fully understood. Indeed, a recent study by Zhu *at el.* reported that the stimulation of CLL cells and bone marrow mesenchymal stem cells with IL-17 induces the generation of IL-6, a pro-survival cytokine for tumor cells. Consistently, results obtained by *in vivo* experiments have demonstrated that IL-17 treatment favors CLL cells engraftment in a xenograft model through an IL-6-mediated mechanism (134). Lastly, a rise in the Th17-cell count leading to an unbalanced Tregs/Th17 ratio has been found in a subset of patients who undergo autoimmune complications, suggesting a possible contribution of this cell population to the pathogenesis of CLL-related autoimmune cytopenias [as reviewed in (128)].

Another player in the tolerogenic milieu of CLL is represented by MDSC, which accumulate in the peripheral blood of CLL

patients. MDSC are defined as CD14+/HLA-DR^{low} cells and are endowed with potent immunosuppressive activity, limiting the T-cell-mediated anti-tumor responses and the effectiveness of immune therapeutic approaches [as reviewed in (135, 136)]. The frequency of MDSC associates with tumor progression and poor prognosis in CLL (137–140). Different studies provided the evidence of a complex network of interactions occurring between tumor cells, T-cell compartments, and MDSC. CLL cells enhance indoleamine 2,3-dioxygenase (IDO) gene expression in MDSC, which, in turn, suppresses *in vitro* T-cell activation and induces suppressive Tregs (138). On the other hand, MDSC depletion results in a control of CLL development and restores T-cell differentiation subsets skewing in the Eμ-TCL1 mouse model, thus confirming the role of MDSC in mediating CLL-related immune dysfunctions (141). An additional mechanism by which CLL cells maintain a supportive microenvironment is the secretion of exosomes inducing PD-L1 and inflammatory cytokine expression in monocytes, thus triggering their reprogramming toward MDSC (142, 143).

Among cells of myeloid origin, nurse like cells (NLC) are known to generate *in vitro* from monocytes and to contribute to CLL cells protection from spontaneous and drug-induced apoptosis. The *in vivo* counterpart of NLC is represented by tumor-associated M2-polarized macrophages, typically residing in the lymphnode and bone marrow of CLL patients. NLC actively shape the microenvironment through the secretion of several cytokines and chemokines: on one hand, NLC attract tumor cells and sustain their survival and, on the other, they promote the recruitment of accessory myeloid cells and stimulate T-cell activation and proliferation (136, 144).

Humoral Immune Response

Up to date, there are only few data regarding the antibody-mediated responses and healthy B-cell compartment in CLL, and most of them focus on immunoglobulin (Ig) deficiencies. Hypogammaglobulinemia is a very common feature and is associated with an increased risk of infections, which largely contributes to morbidity and mortality of CLL patients. At diagnosis, up to 60% of patients has decreased levels of serum Ig, with IgG, IgA, and IgM as the most affected Ig classes (145). It is well established that hypogammaglobulinemia severity correlates with advanced disease stage and worsens during progression, also correlating with shorter time to first treatment (146–148). The mechanism causing hypogammaglobulinemia is not completely clear, but it could be related to a CLL-mediated inhibition of polyclonal antibodies production and to a reduction in the number of healthy B cells (149, 150).

Another consequence of the altered humoral immunity is the occurrence of autoimmune complications. Episodes of autoimmune cytopenia are frequently observed in CLL patients and are attributable to high affinity polyclonal IgG auto-antibodies which are produced by the non-leukemic B cells and target membrane antigens expressed on red blood cells, platelets, or granulocytes [as reviewed in (17, 151, 152)]. In addition, we previously reported that polyclonal antibodies directed to recurrent antigens expressed by tumor cells can be frequently found in the serum of CLL patients, especially those with progressive disease. However, these antibodies are inefficient in triggering ADCC and complement-derived cytotoxicity (CDC) (153). Alterations in the classical components of the complement cascade are reported in almost 40% of CLL patients, and contribute to the compromised CDC and to their increased susceptibility to infections (154, 155). Of note, complement defects may also impair the clinical efficiency of anti-CD20 mAb, which at least partially rely on CDC for their activity.

IMMUNOMODULATION AND IMMUNOTHERAPY IN CLL

The development of new targeted drugs has dramatically changed the treatment landscape of CLL. Despite their remarkable anti-tumor activity, agents like the B-cell receptor (BCR) signaling inhibitors and the Bcl-2 protein inhibitor venetoclax have some limitations, which include the development of drug resistance mechanisms and the less striking efficacy observed in patients carrying biological high-risk features [as reviewed in (156)]. The observation that allogeneic HSCT, which exploits immune-mediated graft-*versus*-leukemia effects to eradicate tumor cells, is the only treatment option with a long-term curative potential in CLL indicates that the immune system harbors the potential for curing the disease. Therefore, treatment strategies aimed at activating or exploiting effector arms of the autologous immune system to target tumor cells have been and currently are a focus of active investigations in CLL (as summarized in **Figure 2**). Here, we will give an overview of immune-based strategies that are currently used or explored in patients with CLL, thereby also providing an outlook on possible future therapeutic interventions.

Agents With Broad Immunomodulatory Effects

In addition to their direct anti-tumor effect, new targeted drugs (i.e. BTK inhibitors, PI3K inhibitors, and the Bcl-2 inhibitor venetoclax) have demonstrated the ability to modulate non-neoplastic immune cell populations. PI3Kδ inhibition by idelalisib affects effector T-cell differentiation and functionality, and induces a disruption of Tregs suppressor activity, thus breaking immune tolerance both in CLL mice model and in patients (121, 124, 157). These evidences could explain the typical immune-mediated adverse events of the drug, mainly consisting in hepatotoxicity, enterocolitis, skin rash, and pneumonitis (157–159). Notably, idelalisib has also shown to positively impact the generation of T cells modified to express a CAR, when added to the culture during the manufacturing process, by inducing an enrichment of less-differentiated naïve-like T cells, a decrease in the expression of exhaustion markers, and a normalization of the CD4:CD8 ratio (160). These data suggest the possibility of exploiting the immunomodulatory

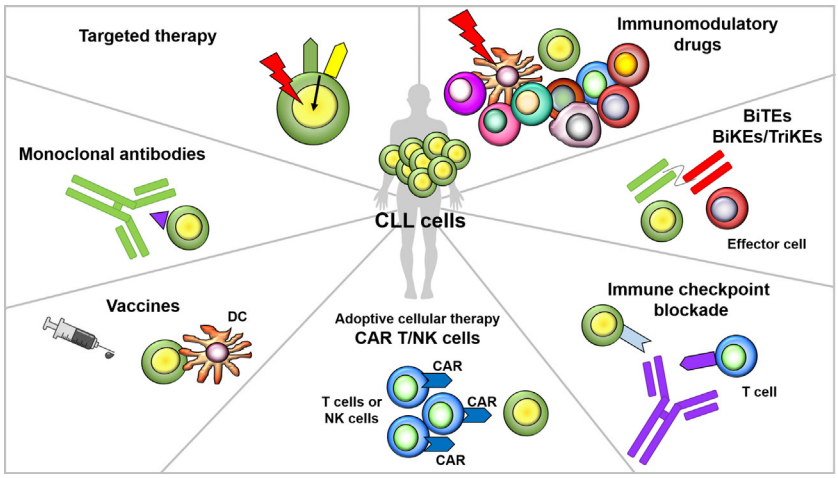

FIGURE 2 | Overview of immunomodulatory agents and immune-based strategies in CLL. Several immunotherapeutic strategies are under evaluation in CLL. Targeted drugs, in addition to a direct anti-tumor activity, can exert off-tumor immunomodulatory effects on T cells and other immune elements. Immunomodulatory drugs (IMiDs) exert their activity through a broad immunomodulation and pleiotropic effects on multiple elements of the immune system (e.g, T, B, NK cells and DC). Monoclonal antibodies act through the recognition of a specific antigen expressed on the surface of tumor cells, which leads to the triggering of cytotoxic responses. Bi- and tri-specific killer cell engagers contain two or three antigen-recognition domains, and are designed to concomitantly target a tumor cell antigen and a molecule expressed on the surface of effectors cells, with the aim of directing immune cell activity toward malignant cells. Cancer vaccines aim at inducing tumor-specific T-cell immunity in an antigen-dependent fashion, thus eliciting immunological memory and long-term protection against cancer relapse. CAR T cells are autologous T lymphocytes engineered to express a chimeric receptor, which recognizes a tumor surface antigen; upon antigen recognition and co-stimulatory domains activation, a cytotoxic response is triggered, leading to tumor cells killing. Immune checkpoint inhibitors target the interactions between co-inhibitory receptors and their ligands thus avoiding the transmission of inhibitory signals that render T cells functionally exhausted.

effects of idelalisib during the preparation of adoptive immunotherapies, thus avoiding the adverse effects determined by drug administration to patients.

Immune changes exerted by ibrutinib have been partially characterized in CLL. Most studies report a normalization of T-cell population counts during treatment with ibrutinib, with the exception of the paper by Long *et al.*, which shows an increase in CD4+ and CD8+ T-cell numbers. Moreover, an increase in TCR diversity repertoire and a decrease in the expression of T-cell exhaustion markers and immune checkpoints have been observed in CLL patients receiving ibrutinib (161–169). From the functional standpoint, preclinical data did not demonstrate a beneficial effect of ibrutinib on T cells (170). However ibrutinib-treated patients showed increased response rates to CAR T cells, thus supporting the potential benefit of combination strategies (171, 172). The immunomodulatory activity of ibrutinib is at least partially attributable to its off-target effects on tyrosine kinases other than BTK, such as the IL-2 inducible kinase (ITK), which is mainly expressed by T cells (173). However, at this stage, it is not completely elucidated whether ibrutinib immunomodulation is also connected to the reduction of tumor burden and to the suppression of tumor-microenvironment interactions, occurring during patients treatment. In line with this latter hypothesis is the observation that also second-generation BTK inhibitors (i.e. acalabrutinib and zanubrutinib), despite their higher selectivity and reduced off-target effects, still show some immunomodulatory properties (165, 174). Consistently, immune changes occurring during venetoclax treatment may be at least partially connected to a clinical response to therapy, driving the attenuation of leukemia-induced immune alterations. In particular, venetoclax has demonstrated to i) normalize B, T, and NK-cell count, ii) decrese the percentage of tumor-supportive T-cell subsets, iii) reduce the frequency of PD-1+ CD8+ T cells, and iv) impair NK-cell activation in CLL patients (114). Recent data also showed that venetoclax increases the anti-tumor effects of T-cell-based therapy against B-cell lines *in vitro* and in solid tumor-bearing mice model (175, 176).

Immunomodulatory drugs (IMiDs) have also been—and currently are—under evaluation for their anti-tumor effects in CLL. In the past 15 years, several clinical trials enrolling CLL patients have demonstrated the clinical efficacy of lenalidomide (**Table 1**), but have also highlighted its unique toxicity profile and a rate of adverse events that suggests caution in incorporating this agent in treatment strategies [as reviewed in (195)]. One aspect of lenalidomide that has been considered worth of investigation consists in its pleiotropic effects on the immune system. In fact, in spite of the absence of a direct *in vitro* cytotoxicity toward CLL cells, lenalidomide is able to favor the immune recognition of leukemic cells—through the upregulation of surface ligands and receptors—and to induce an indirect anti-tumor activity through the immunostimulation of DC as well as T, B, and NK cells. CLL patients treated with lenalidomide have a restored T-cell function, especially in terms of Th1-type cytokine release (IL-2, IFN-γ, TNF-α, IL-21), and formation of functional immune synapses (99, 155). In addition,

TABLE 1 | Clinical trials evaluating the efficacy and toxicity of lenalidomide, used as a single agent or in combination regimens, for the treatment of patients with CLL.

Drug regimen	Setting	Efficacy	Toxicities	References
Lenalidomide single agent days 1–21 of 28-day cycles	Phase II 45 patients R/R CLL	ORR 47% 9% CR 1-year PFS 81%	G≥3 neutropenia 70% G≥3 thrombocytopenia 45% G≥3 anemia 18% G≥3 infections 5% G≥3 febrile neutropenia 15% Fatigue 73% Tumor flare reaction 58% TLS 5%	Chanan-Khan *et al.* (177)
Lenalidomide single agent continuously	Phase II 44 patients R/R CLL	ORR 32% 7% CR 14-month OS 73%	G≥3 neutropenia 41% G≥3 thrombocytopenia 15% G≥3 anemia 3% G≥3 infections 6%	Ferrajoli *et al.* (178) NCT00267059
Lenalidomide single agent days 1–21 of 28-day cycles	Phase II 25 patients Previously untreated CLL	ORR 56% 0 CR 2-year OS 92% PFS 89%	G≥3 neutropenia 72% G≥3 thrombocytopenia 28% Tumor flare reaction 88% Fatigue 72% Infections 60% Rash 64%	Chen *et al.* (179)
Lenalidomide single agent continuously	Phase II 60 elderly patients Previously untreated CLL/SLL	ORR 65% 10% CR 2-year PFS 60% 2-year OS 88%	G≥3 neutropenia 73% G≥3 thrombocytopenia 34% G≥3 anemia 15% G≥3 infections 10% Tumor flare reaction 27%	Badoux *et al.* (180) NCT00535873
Lenalidomide single agent continuously (different starting doses)	Phase II 103 patients R/R CLL	ORR 40% 8% CR; median PFS 9.7 months Median OS 33 months	G≥3 neutropenia 77% G≥3 thrombocytopenia 48% G≥3 anemia 4% G≥3 infections 41% Embolism 6% TLS 3%	Wendtner *et al.* (181) NCT00419250
Lenalidomide single agent continuously	Phase III 225 elderly patients Previously untreated CLL	ORR 55% 3% CR Median PFS 30.8 months	G≥3 neutropenia 49% G≥3 thrombocytopenia 25% G≥3 anemia 7% G≥3 infections 9% Tumor flare reaction 52% Rash 50%	Chanan-Khan *et al.* (182) NCT00910910
Lenalidomide and rituximab	Phase II 59 patients R/R CLL	ORR 66% 12% CR Median TTF 17.4 months 3-year OS 71%	G≥3 neutropenia 73% G≥3 thrombocytopenia 34% G≥3 anemia 15% G≥3 infections 15% Neutropenic fever 10%	Badoux *et al.* (183) NCT00759603
Lenalidomide and rituximab	Phase II 69 patients Previously untreated CLL	ORR 87% 14% CR Median PFS: 19–20 months	G≥3 neutropenia 58% Tumor flare 75% Fatigue 74% Transaminitis 65% Rash 61%	James *et al.* (184) NCT00628238
Lenalidomide and rituximab	Phase II 61 previously untreated + 59 R/R CLL	Previously untreated: ORR 73%; 35% CR; median PFS 50 months R/R: ORR 64%; 28% CR; median PFS 28 months	G≥3 neutropenia 53% G≥3 thrombocytopenia 23% G≥3 anemia 13% G≥3 infections 27% G≥3 cardiovascular 10% Second primary tumors 5%	Strati *et al.* (185) NCT01446133
Lenalidomide and ofatumumab	Phase II 21 patients R/R CLL	ORR 48% 0 CR Median OS 21.5 months	G≥3 neutropenia 47% G≥3 thrombocytopenia 71% Fatigue 52% Tumor flare reaction 43%	Costa *et al.* (186) NCT01123356

(Continued)

TABLE 1 | Continued

Drug regimen	Setting	Efficacy	Toxicities	References
Lenalidomide and ofatumumab	Phase II 34 patients R/R CLL	ORR 71% 18% CR Median PFS 16 months 5-year OS 53%	G≥3 neutropenia 82% G≥3 thrombocytopenia 18% G≥3 anemia 6% G≥3 infections 60% Venous thromboembolysm 9%	Vitale *et al.* (187) NCT01002755
Lenalidomide, rituximab and fludarabine	Phase I 9 patients Previously untreated CLL	ORR 56% 11% CR	78% of patients stopped therapy because of toxicity (44% hematological toxicity)	Brown *et al.* (188)
Lenalidomide, rituximab and bendamustine	Phase I-II 22 patients Previously untreated and R/R CLL	ORR 50% 0 CR Median PFS 8 months	95% of patients had at least one G≥3 adverse event G≥3 neutropenia 71% G≥3 thrombocytopenia 19% G≥3 anemia 5% G≥3 infections 47%	Maurer *et al.* (189) NCT01558167
Lenalidomide, fludarabine and cyclophosphamide	Phase I-II 42 patients R/R CLL	ORR 62% 22% CR Median PFS 19 months Median OS 45 months	G≥3 neutropenia 65% G≥3 thrombocytopenia 17% G≥3 infections 7%	Mauro *et al.* (190) NCT00727415
Lenalidomide, rituximab and ibrutinib	Phase I 12 patients R/R CLL	ORR 67% 8% CR 1-year PFS 78%	G≥3 neutropenia 67% Diarrhea 58% Myalgia/arthralgia 58% Rash 42% G≥3 infections 25%	Ujjani *et al.* (191) NCT02200848
Lenalidomide, rituximab and chlorambucil, followed by lenalidomide consolidation	Phase I-II 63 elderly or unfit patients Previously untreated CLL	ORR after induction 83% (0 CR) ORR after consolidation 93% (14% CR) 2-year PFS 58%	During induction: G≥3 neutropenia 73% G≥3 thrombocytopenia 15% Rash 11% G≥3 infections 9%	Kater *et al.* (192) EudraCT 2010-022294-34
Lenalidomide, rituximab and bendamustine	Phase I 13 patients Previously untreated CLL	ORR 87% 39% CR	G≥3 neutropenia 52% G≥3 anemia 26% G≥3 thrombocytopenia 22% G≥3 febrile neutropenia 13% G≥3 rash 26%	Soumerai *et al.* (193) NCT01400685
Lenalidomide and dexamethasone	Phase II 31 patients Previously untreated CLL	ORR 74% 10% CR Median PFS 27 months	G≥3 neutropenia 61% Febrile neutropenia 19% Rash 65% Diarrhea 61% Edema 55%	Chen *et al.* (194) NCT01133743

CLL, chronic lymphocytic leukemia; CR, complete response; G≥3, grade ≥3; ORR, overall response rate; OS, overall survival; PFS, progression free survival; R/R, relapsed or refractory; SLL, small lymphocytic lymphoma; TLS, tumor lysis syndrome; TTF, time to treatment failure.

lenalidomide induces wide-range immunomodulatory effects on different immune cell compartments, among which i) a decrease in Tregs count, ii) an increase in Th17 cell number, iii) an upregulation of the NKG2D activating receptor on NK cells, and iv) a rise in Ig production by normal polyclonal B cells. Besides the immune system re-education, treatment with lenalidomide also modulates interactions of CLL cells with NLC and stromal cells within the tumor microenvironment (155) [and reviewed in (195, 196)].

Compounds showing the same potent immunomodulatory effects as lenalidomide but characterized by a more manageable safety profile could represent an interesting therapeutic option for patients with CLL. Avadomide (CC-122) is a novel orally available pleiotropic pathway modulator, which has demonstrated enhanced anti-proliferative activities compared to lenalidomide, as well as the ability of eliciting anti-tumor T cell-mediated immune responses when combined with checkpoint inhibitors in preclinical models of CLL (197, 198). Based on these observations, a phase I/II clinical trial is currently evaluating the combination of avadomide plus ibrutinib or avadomide plus obinutuzumab in patients with CLL (NCT02406742).

Monoclonal Antibodies

The targeting of tumor surface antigens with mAb has been the first form of immunotherapy broadly applied in the treatment of CLL. CD20, a transmembrane protein typically expressed on the B-cell surface, represents an ideal target for antibody-based therapy, and its targeting currently provides the best clinical results obtained with mAb therapy in CLL. Rituximab, which was approved by Food and Drug Administration (FDA) in 1998,

is a chimeric mouse-human anti-CD20 mAb. The anti-tumor activity of rituximab is exerted through CDC and ADCC mechanisms, but is limited by the low-level of expression of the CD20 antigen on the surface of CLL cells, by the presence of circulating shed CD20 antigen interfering with the binding of the mAb to leukemic cells and by the selection of antigen loss variants in rituximab-treated patients (199–201). Based on its poor efficacy when used as a single agent, rituximab is mainly used in combination with other drugs for the treatment of CLL patients (202). Rituximab has demonstrated to improve the efficacy of chemotherapy agents, and the combinations FCR and bendamustine plus rituximab (BR) have been validated in phase III randomized clinical trials as the standard therapy for previously untreated, fit patients without *TP53* abnormalities (203–205). However, their use in the frontline setting has been recently challenged by studies comparing chemoimmunotherapy with targeted drugs, used alone or in combinations with rituximab, especially for the treatment of high-risk patients carrying unmutated IGHV and/or del(11q) (206, 207).

Next-generation anti-CD20 mAb with enhanced cytotoxic functions have later been developed. Ofatumumab is an example of type I (or rituximab-like) mAb, that mainly induces tumor cell lysis through CDC and ADCC [as reviewed in (208, 209)]. Obinutuzumab is a type II anti-CD20 mAb (i.e. inducing cytotoxicity mainly through programmed cell death and ADCC) characterized by an engineered structure that allows an increased recruitment of effector cells, a potentiated ADCC and a more efficient NK cell degranulation (210). Ofatumumab and obinutuzumab have shown efficacy in phase II and/or phase III clinical trials when used in combination with conventional chemotherapy (211, 212), BCR inhibitors (213–215), or venetoclax (216). More recently, ublituximab, another type I anti-CD20 mAb, has demonstrated clinical efficacy in CLL patients when administered alone or in combination with chemotherapy or targeted agents (217–219).

Other targets have been or currently are under evaluation for mAb-based therapeutic strategies in CLL. Among them, CD19 is particularly interesting, being a pan-B lymphocyte surface receptor, which is not expressed by hematopoietic stem cells and other immune cells, except for follicular DC [as reviewed in (220)]. CD19 is ubiquitously expressed on CLL cells and currently, results for two different anti-CD19 mAb evaluated in phase I studies are available. Tafasitamab is an anti-CD19 mAb with an engineered Fc region to enhance CD16 binding affinity, whereas inebilizumab is an affinity-optimized anti-CD19 mAb that enacts malignant clone elimination *via* ADCC. Both drugs have demonstrated safety and preliminary efficacy in previously treated CLL patients (221, 222). Tafasitamab containing regimens are currently under evaluation in two different phase II clinical trials enrolling patients with CLL (tafasitamab in combination with lenalidomide in the NCT02005289 trial and in combination with idelalisib or venetoclax in the NCT02639910 trial).

An additional target currently under investigation for CLL immunotherapy is CD37. CD37 is broadly and selectively expressed on tumor cells from B-cell malignancies, including CLL cells, where it is involved in various biological processes such as cell adhesion, proliferation, survival, and trafficking [as reviewed in (223)]. Otlertuzumab is an anti-CD37 fusion protein obtained from a chimeric protein (SMIP-016) and engineered to exhibit the full binding activity of an anti-CD37 mAb at one-third of the regular antibody size. The mechanism of action of otlertuzumab consists in the triggering of a direct pro-apoptotic effect and in the induction of ADCC, while sparing the activation of the complement system (224). Single-agent otlertuzumab has demonstrated a modest activity and an acceptable safety profile in a phase I study enrolling treatment-naïve and pre-treated CLL patients (225). In a following phase II trial, the same molecule in combination with bendamustine significantly increased the response rate and prolonged the progression free survival over single agent bendamustine in patients with relapsed or refractory CLL (226). An additional anti-CD37 mAb is BI 836826, which has been Fc-engineered to improve ADCC activity. In a phase I study it has shown an acceptable tolerability and a notable efficacy, being especially active in CLL patients with del(17p) and/or *TP53* mutations (227). BI 836826 is currently under evaluation for its safety and tolerability when given in combination with ibrutinib to patients with relapsed/refractory CLL (NCT02759016).

The ideal targets for a successful anti-tumor immunotherapy are tumor associated antigens (TAA), which are molecules with a unique or highly preferential expression on malignant cells and with a crucial role for the growth and survival of the tumor. In this context, the receptor tyrosine kinase-like orphan receptor 1 (ROR1) can be considered a putative TAA for CLL, being a cancer stem cell antigen almost exclusively expressed on tumor cells and involved in the biology and aggressiveness of the disease (228). Several immune-based strategies targeting ROR1 are currently being investigated, including mAb. Cirmtuzumab, an anti-ROR1 mAb, has demonstrated to be well tolerated and effective at inhibiting ROR1 signaling in a phase I study enrolling patients with progressive, refractory, and relapsed CLL (229), and is currently under evaluation for its safety and efficacy when given in combination with ibrutinib in patients with B-cell lymphoid malignancies in a phase Ib/II protocol (NCT03088878).

Bi-Specific Antibodies and Bi- and Tri-Specific Killer Cell Engagers

Bi-specific antibodies (bsAb) combine specificities of two antibodies simultaneously, addressing different antigens or epitopes on the cell surface, and include a large family of molecules with different formats. Among bsAb, bi-specific T cells engagers (BiTEs) and bi- or tri- specific killer engagers (BiKE or TriKE) are dual or triple targeting antibodies, which act by simultaneously binding tumor antigens and effector cells (T cells or NK cells), thus leading to the creation of a new immunological synapse and the triggering of cytotoxic responses. Of note, TriKEs recognize two different antigens on tumor targets allowing the binding of cancer cells even when one antigen is lost, and thus avoiding the occurrence of escape variants [as reviewed in (230–232)].

Blinatumomab, a CD19/CD3 bsAb designed in the BiTE format, was the first bsAb studied in setting of CLL patients.

In B-cell acute lymphoblastic leukemia (B-ALL), where it is currently approved for the treatment of patients with a relapsed/refractory disease or not achieving an undetectable minimal residual disease, blinatumomab has shown a good anti-leukemic activity associated with a low treatment-related mortality (233). Blinatumomab has demonstrated to effectively kill CLL cells *in vitro*, through the induction of proliferation, cytokine production and granzyme B secretion in autologous T cells. As demonstrated by Wong *et al.*, the formation of immunological synapses between T cells and CLL cells induced *in vitro* by blinatumomab indicates that this CD19/CD3 BiTE is able to overcome the T-cell dysfunction frequently observed in CLL patients (234). In preclinical studies, blinatumomab induced cytotoxicity against tumor cells at very low T-cell:tumor cell ratios, in samples from both treatment-naïve and treated patients, and also in the presence of pro-survival signals (234). A phase I study has demonstrated the feasibility of blinatumomab in relapsed/refractory B-cell non-Hodgkin lymphomas, including small lymphocytic lymphoma (235), and a clinical trial is currently testing the association between lenalidomide and blinatumomab in the same clinical setting (NCT02568553). However, specific data on the tolerability and efficacy of blinatumomab in the CLL patient population are not available.

The main limitation to the efficacy of BiTE constructs is their short half-life that requires these drugs to be administered continuously. To overcome structural limitations of BiTEs, and specifically their poor stability, next-generation bsAb with a more favorable pharmacokinetic profile are currently under investigation. To this aim, a new bsAb platform represented by dual affinity re-targeting (DART) molecules was developed. The MGD011 CD3xCD19 DART (also known as JNJ-64052781) has demonstrated a good *in vitro* efficacy in killing CLL cells by recruiting CLL-derived T cells against the tumor. MGD011 has shown the ability to induce activation and proliferation of T cells from CLL patients, and to promote a partial restore of their immunological disfunctions. Interestingly, MGD011 is also able to kill venetoclax-resistant CLL cells, through a mechanisms that is independent from Bcl-2-mediated apoptosis (236, 237).

Another recently-developed CD19/CD3 bsAb, designed in the single-chain Fv-Fc format (CD19/CD3-scFv-Fc), has shown to induce a particularly rapid killing of CLL cells isolated from ibrutinib-treated patients, including those with acquired ibrutinib-resistance (238).

Although not fully understood, this increased cytotoxicity seems at least in part attributable to an improved performance of T cells isolated from patients treated with ibrutinib. Consistently, a BiTE targeting ROR1 has shown an enhanced cytotoxic activity against primary leukemic cells when used in the presence of T cells isolated from ibrutinib-treated CLL patients (239). Therefore, all these data highlight the importance of the reversal of CLL-related T-cell impairment to improve the BiTE activity.

Another strategy to bypass the T-cell impairment is to exploit engagers designed to target the activity of effector cells of the innate immune system. To date, in CLL, BiKEs and TriKEs engaging NK cells have been exclusively studied in the preclinical setting, but available data encourage their potential translation to the clinic. The therapeutic potential of a CD16/CD19 BiKE and a CD16/CD19/CD22 TriKE has already been demonstrated in a preclinical study showing their ability to trigger NK cell functions in terms of cytokine and chemokine production, secretion of lytic granules and induction of tumor cell death (240). TriKEs recognizing the NKG2D receptor ligand ULBP2 (ULBP2/aCD19/aCD19 and ULBP2/aCD19/aCD33 TriKEs) have also been used to activate NK cells and showed a superior *in vitro* and *in vivo* anti-tumor activity against CLL compared to the bi-specific counterparts (201).

Tumor Vaccines

The ultimate goal of a cancer vaccine is the activation and expansion of cytotoxic T lymphocytes against tumor targets, thus promoting the elimination of the tumor and inducing a long-term protection against possible relapses. In CLL, the main obstacles for the production of effective tumor vaccines are the difficulty in the selection of an ideal TAA, which should be specific for the tumor but broadly expressed in the patients population, and the presence of immune dysfunctions limiting the triggering of effective and peristent anti-tumor responses.

To overcome the difficulties connected to the identification of an optimal TAA, vaccine formulations consisting of autologous whole tumor cells genetically modified to express cytokines or costimulatory molecules have been tested. The manipulation of autologous CLL cells to express a functional ligand for the CD40 molecule (i.e. CD40L or CD154) has shown to upregulate costimulatory factors on leukemic cells and to induce the generation of cytotoxic T lymphocytes capable of specifically recognizing parental non-modified leukemic cells *in vitro* (241). Based on these preclinical results, Wierda *et al.* have designed a phase I study to explore the clinical efficacy of a cancer vaccine consisting of autologous CLL cells genetically modified to express a human form of the CD40L molecule. Results from this study demonstrated that this vaccine formulation is well tolerated, has biological and clinical activity, and may enhance the susceptibility of CLL cells with del(17p) to subsequent chemoimmunotherapy (242). Interestingly, the preclinical use of genetically modified CD40L-expressing CLL cells in combination with IL-2- or OX40L-expressing CLL cells has shown to produce an even more pronounced T-cell activation and trigger therapeutically significant leukemia-specific immune responses (243, 244). Despite the encouraging preliminary results, all the tested tumor cell-based vaccine formulations failed to produce reproducible clinical effects, mainly due to the existence of immune escape mechanisms and deep CLL-driven defects in the immune system. One approach aiming at restoring the immune competence of CLL patients is the use of checkpoint-blockade inhibitors to "release" the immune system to target cancer cells. In this context, the use of a tumor vaccine consisting of irradiated autologous tumor cells coated with an antibody targeting the CD200 immunoregulatory molecule has shown to be effective in a xenogenic model of CLL (245).

An alternative strategy to induce effective anti-tumor responses is the use of vaccine formulations exploiting the antigen presentation ability of DC. The feasibility and safety of a vaccine consisting of DC loaded with apoptotic bodies derived from autologous CLL cells has already been demonstrated in an early-phase clinical trial (74). The lack of meaningful clinical effects, in this as in most other CLL vaccination trials, illustrates the need to identify more potent immune adjuvants for CLL. To this aim, lenalidomide, administered in combination with a DC vaccine, has shown its ability to elicit tumor-specific T-cells responses, although in the presence of relevant autoimmune compications that suggest caution in further exploring this drug as an immune adjuvant in CLL (75).

Cellular Immunotherapy and CAR T Cells

Adoptive cellular immunotherapy is an alternative approach to exploit the immune system to fight tumors, and consists in the isolation and expansion of effector cells that are then transferred to patients.

NK cells and γδ T cells are particularly appealing candidates for cellular immunotherapy, thanks to their peculiar ability of recognizing and targeting tumor cells in an MHC unrestricted manner, which favors the induction of effective allogeneic and autologous anti-tumor responses [as reviewed in (246–248)]. In the setting of CLL, Almeida *et al.* designed a protocol for the clinical grade expansion and the preclinical testing of cytotoxic Vδ1+ T cells, named Delta One T (DOT) cells. DOT cells express NCRs, which synergize with the TCR to mediate leukemic cell targeting *in vitro*, and inhibit tumor growth in xenograft models of CLL (67).

Immune effector cells may also be genetically engineered with the aim of improving and specifically directing their killing properties against the tumor. CAR T cells have been under development for more than 30 years (249), and recently entered the therapeutic armamentarium for lymphoproliferative diseases [as reviewed in (250–252)]. Anti-CD19 CAR T cells are currently approved by FDA and European Medicine Agency for the treatment of patients with aggressive B-cell lymphomas or B-ALL. In other hematological malignancies, including CLL, several challenges still need to be overcome for successful application of CAR T-cell therapies, including identifying alternative or additional target antigens and reversing repressive tumor microenvironments that hamper CAR T-cell function.

Novel CAR constructs that target antigens other than CD19 (e.g. CD20, CD22, ROR1) or that concomitantly target more than one antigen (e.g. CD19 and CD20, CD19 and CD22) are currently under evaluation in early phase clinical studies. Clinical trials evaluating CAR-based cellular therapies in patients with CLL are listed in **Tables 2** and **3**.

In spite of a safety profile not dissimilar to that observed in the setting of other lymphoproliferative diseases, which includes cytokine release syndrome (CRS) and CAR T cell-related encephalopathy syndrome (CRES) as major complications, to date, the efficacy results of CAR T cells in CLL have been relatively discouraging, mainly due to T-cell alterations paralleling disease evolution and hampering effective anti-tumor functions of autologous CAR T cells. Fraietta *et al.* demonstrated that the composition of the cellular product and the intrinsic T-cell functional fitness may have an impact on the therapeutic efficacy of CAR T cells in CLL. They showed that the ability of CAR T cells to expand during the manufacturing process is a predictor of response and correlates with *in vivo* proliferation, which is in turn responsible for a sustained anti-tumor activity. In addition to that, they observed that CAR T cells derived from patients who respond to the treatment are enriched in memory-cell lymphocytes, with enhancement of IL-6/STAT3 signals and STAT3-related cytokine production, whereas CAR T cells from patients who do not respond upregulate genetic programs involved in effector differentiation, glycolysis, exhaustion, and apoptosis (275).

A possible strategy to improve the clinical benefit of CAR T-cell therapy is the co-administration of targeted anti-tumor agents selected for their ability to exert immunomodulatory properties, with the aim of overcoming tumor-induced immune dysfunctions. Based on preclinical studies showing that ibrutinib could improve the anti-tumor efficacy of CAR T cells, a phase I clinical trial was conducted showing the safety and feasibility of ibrutinib administered in combination with anti-CD19 CAR T cells in relapsed and refractory CLL patients (171). In line with these results, recent preclinical data show that also the novel BTK inhibitor acalabrutinib can improve the *in vitro* and *in vivo* anti-tumor functions of CD19-directed CAR T cells (276).

Besides T cells, NK cells could represent a valid cellular carrier for CAR constructs. CAR NK cells have the advantage to be activated not only by the CAR target antigen, but also by NCRs, thus adding ADCC-mediated mechanisms to the CAR-mediated cell lysis. Due to the dysregulation of patient-derived NK cells, most studies addressing the efficacy of NK cells-based adoptive immunotherapy consisted in the transfer of *ex vivo* expanded allogeneic NK cells derived from healthy donors' peripheral blood, umbilical cord blood, or cell lines (277, 278). Allogeneic NK cells can be safely used as effector cells since they do not require a full HLA-matching and they do not induce graft-*versus*-host disease, while harboring strong graft-*versus*-leukemia effects (279, 280). Cord blood-derived anti-CD19 CAR NK cells showed good activity towards CLL cells *in vitro*, and preliminary evidences from a clinical trial have already demonstrated the safety and efficacy of this approach in patients with CD19-positive tumors, including CLL (274, 281). Thanks to these reasons, CAR NK cells can be produced in allogeneic settings and easily used as an "off-the-shelf" treatment (26).

Immune Checkpoint Inhibitors

The targeting of immune checkpoint molecules with the aim of reactivating the T-cell immune responses against tumor cells is an appealing therapeutic strategy. In the context of solid tumors, the blockade of immune checkpoint receptors or their cognate ligands by mAb has brought significant benefits for patients [as reviewed in (282)]. In CLL, encouraging preclinical results have been obtained in the studies that evaluated PD-1/PD-L1 axis disruption (283, 284). Specifically, *in vivo* treatment with an

TABLE 2 | Clinical trials evaluating the efficacy and toxicity of CAR T- and CAR NK-cell treatment in CLL patients.

Drug regimen	Setting	Efficacy	Toxicities	References
Autologous anti-CD19 CAR T cells	3 patients R/R CLL	ORR 100% 67% CR	CRS 100% CRES 0	Kalos *et al.* (253) NCT01029366
Autologous anti-CD19 CAR T cells	14 patients R/R CLL	ORR 57%28% CR	CRS 64% CRES 36%	Porter *et al.* (254) NCT01029366
Autologous anti-CD19 CAR T cells + aldesleukin 720000 UI/kg every 8 hours	4 patients R/R CLL	ORR 75% 25% CR	CRS 100% CRES 0	Kochenderfer *et al.* (255) NCT00924326
Autologous anti-CD19 CAR T cells	5 patients (1 Richter syndrome) R/R CLL	ORR 100% 60% CR	CRS 60% CRES 20%	Kochenderfer *et al.* (256) NCT00924326
Allogeneic anti-CD19 CAR T cells	4 patients (2 Richter syndrome) R/R CLL after allogeneic HSCT	ORR 25% 25% PR	CRS ND CRES ND	Cruz *et al.* (257) NCT00840853
Autologous anti-CD19 CAR T cells + ibrutinib for ≥1 year	3 patients R/R CLL	ORR 100% 33% CR	CRS ND CRES ND	Fraietta *et al.* (258) NCT01747486 NCT01105247 NCT01217749
Autologous anti-κ light chain CAR T cells	2 patients R/R CLL	ORR 0	CRS ND CRES ND	Ramos *et al.* (259) NCT00881920
Allogeneic anti-CD19 CAR T cells	5 patients R/R CLL	ORR 40% 20% CR	CRS 80% CRES 0	Brudno *et al.* (260)
Autologous anti-CD19 CAR T cells	13 patients R/R CLL post ibrutinib	ORR 83% 50% CR	CRS ND CRES ND	Turtle *et al.* (261) NCT01865617
Autologous anti-CD19 CAR T cells	24 patients (5 Richter syndrome) R/R CLL	ORR 67% 17% CR	CRS 83% CRES 33%	Turtle *et al.* (262) NCT01865617
Autologous anti-CD19 CAR T cells	8 patients PR CLL after first-line treatment with pentostatin, cyclophosphamide and rituximab	ORR 25% 25% CR	CRS 50% CRES 0	Geyer *et al.* (263) NCT01416974
Autologous anti-CD19 CAR T cells	19 patients R/R CLL to 6 month-therapy with ibrutinib	ORR 71% 43% CR	CRS 95% CRES 26%	Gill *et al.* (172) NCT02640209
Autologous anti-CD19 CAR T cells	2 patients R/R CLL	ORR 50% 50% CR	CRS 0 CRES 0	Enblad *et al.* (264) NCT02132624
Autologous anti-CD19 CAR T cells	1 patient R/R CLL	ORR 0 100% SD	CRS ND CRES ND	Ramos *et al.* (265) NCT01853631
Autologous anti-CD19 CAR T cells	10 patients R/R CLL	ORR 75% 50% CR	CRS 80% CRES 30%	Siddiqi *et al.* (266) NCT03331198
Autologous anti-CD19 CAR T cells	22 patients R/R CLL after 2 or 3 lines of therapy including ibrutinib	ORR 82% 45% CR	CRS 9% CRES 23%	Siddiqi *et al.* (267) NCT03331198
Autologous anti-CD19 CAR T cells + ibrutinib 420 mg daily for 2 weeks (days −50 to −36)	1 patient R/R CLL	ORR 100% 100% CR	CRS 100% CRES 0	Delgado *et al.* (268) NCT03144583
Autologous anti-CD19 CAR T cells	8 patients R/R CLL	ORR 12% 12% PR 37% SD	CRS 100% CRES 0	Brentjens *et al.* (269) NCT00466531
Autologous anti-CD19 CAR T cells (5 patients received ibrutinib at the time of T-cell collection and/or CAR T-cell administration)	16 patients R/R CLL	ORR 37% 12% CR MRD – 6% CR MRD +	CRS 100% CRES 37%	Geyer *et al.* (270) NCT00466531
Autologous anti-CD19 CAR T cells	2 patients R/R CLL	ND	CRS ND CRES 0	Schubert *et al.* (271) NCT036765041

(Continued)

TABLE 2 | Continued

Drug regimen	Setting	Efficacy	Toxicities	References
Anti-CD19 CAR T cells	13 patients (3 Richter syndrome) R/R CLL	42% CR	CRS 85% CRES 85%	Batlevi *et al.* (272)[1] NCT030851731
Anti-CD19/CD20 CAR T cells	2 patients R/R CLL	ORR 82% 54% CR	CRS 54% CRES 27%	Shah *et al.* (273)[1] NCT03019055
Autologous anti-CD19 CAR T cells + ibrutinib from at least 2 weeks prior to leukapheresis until at least 3 months after CAR T infusion *vs* autologous anti-CD19 CAR T cells alone	19 patients *vs* 30 (4 Richter syndrome)	ORR 83% 22% CR 72% MRD – assessed by cytometry 61% MRD - assessed by *IGH* sequencing *vs* ORR 56% 67% MRD – assesed by cytometry 60% MRD - assesed by *IGH* sequencing	CRS 74% *vs* 95% CRES 26% *vs* 42%	Gauthier *et al.* (150) NCT01865617
Umbilical cord blood derived anti-CD19 CAR NK	5 patients (1 Richter) R/R CLL	3/5 ORR 3 CR	CRS 0 CRES 0	Liu *et al.* (274) NCT03056339

CLL, chronic lymphocytic leukemia; CR, complete remission; CRES, CAR T cell-related encephalopathy syndrome; CRS, cytokine release syndrome; HSCT, hematopoietic stem cell transplantation; MRD, minimal residual disease; ND, no data; ORR, overall response rate; PR, partial response; R/R relapsed or refractory disease; SD, stable disease.
[1]Data for CLL patients only non-available.

TABLE 3 | Ongoing clinical trials evaluating CAR T-cell treatment in CLL patients.

NCT number	Drug regimen	Setting
NCT03881774	Cord blood derived CAR T cells	R/R CLL after autologous CAR T cells therapy or who fail to preparation for autologous CAR T cells
NCT04271410	Anti-CD19 CAR T cells	R/R CLL
NCT04156243	Anti-CD19 CAR T cells	R/R CLL
NCT04014894	Anti-CD19 CAR T cells	R/R CLL
NCT04271800	Anti-CD19 CAR T cells	R/R CLL
NCT03685786	Anti-CD19 CAR T cells + autologous HSCT	MRD+ CLL
NCT03960840	Autologous anti-CD19 CAR T cells	SD or PR CLL after 6-month therapy with ibrutinib or R/R CLL
NCT02963038	Autologous anti-CD19 CAR T cells	R/R CLL
NCT03110640	Autologous anti-CD19 CAR T cells	R/R CLL
NCT03302403	Autologous anti-CD19 CAR T cells	R/R CLL
NCT03579888	Autologous anti-CD19 CAR T cells	R/R CLL who have undergone allogeneic HSCT
NCT02153580	Autologous anti-CD19 CAR T cells	R/R CLL
NCT03050190	Autologous anti-CD19 CAR T cells	R/R CLL
NCT03624036	Autologous anti-CD19 CAR T cells	R/R CLL
NCT03853616	Autologous anti-CD19 CAR T cells	R/R CLL
NCT03383952	Autologous anti-CD19 CAR T cells	R/R CLL
NCT03191773	Autologous anti-CD19 CAR T cells	R/R CLL
NCT03166878	Allogeneic anti-CD19 CAR T cells	R/R CLL
NCT03277729	Autologous anti-CD20 CAR T cells	R/R CLL
NCT00621452	Autologous anti-CD20 CAR T cells	R/R CLL

(Continued)

TABLE 3 | Continued

NCT number	Drug regimen	Setting
NCT00012207	Autologous anti-CD20 CAR T cells	R/R CLL
NCT01735604	Autologous anti-CD20 CAR T cells	R/R CLL
NCT04030195	Allogeneic anti-CD20 CAR T cells	R/R CLL
NCT02794961	Autologous anti-CD22 CAR T cells	R/R CLL
NCT02194374	Autologous anti-ROR1 CAR T cells	R/R or untreated CLL with del17p and not eligible for allogeneic HSCT
NCT02706392	Autologous anti-ROR1 CAR T cells	R/R CLL
NCT04156178	Anti-CD19/CD20 CAR T cells	R/R CLL
NCT04260945	Anti-CD19/CD20 CAR T cells	R/R CLL
NCT04007029	Autologous anti-CD19/CD20 CAR T cells	R/R CLL
NCT03097770	Autologous or allogenic anti-CD19/CD20 CAR T cells	R/R CLL
NCT03398967	Allogeneic anti-CD19/CD20 or anti-CD19/CD22 CAR T cells	R/R CLL
NCT04029038	Autologous anti-CD19/CD22 CAR T cells	R/R CLL
NCT03185494	Autologous or allogenic anti-CD19/CD22 CAR T cells	R/R CLL
NCT03125577	Autologous anti-CD19 and anti-CD20/CD22/CD30/CD38/CD70/CD123 CAR T cells	R/R CLL

CLL, chronic lymphocytic leukemia; HSCT, hematopoietic stem cell transplantation, MRD, minimal residual disease; PR partial response; R/R relapsed or refractory; SD, stable disease.

anti-PD-L1 antibody prevented the development of CLL in the Eμ-TCL1 mice model, also normalizing T cells and myeloid cell populations, and restoring T-cell functions (283). In the same murine model, it has been demonstrated that antibodies targeting PD-L1, but not PD-1, enhance the anti-tumor activity of ibrutinib treatment (170, 284). Notably, Wierz *et al.* have shown, in the Eμ-TCL1 mice model, that the antibody-based dual targeting of PD-1 and LAG3—but not the single targeting of PD-1—effectively limits the tumor development and restores different immune cell populations (284). These data support the concept that the simultaneous inhibition of different immune checkpoint molecules may represent an interesting therapeutic approach, which is worth to be explored.

Unfortunately, so far, disappointing results emerged from clinical trials evaluating immune checkpoint inhibitors in CLL, indicating that these compounds used as single agents are not sufficient to control the disease (**Table 4**). By contrast, interesting preliminary results in terms of response rate have been obtained when checkpoint inhibitors were administered, alone or in association with ibrutinib, to CLL patients developing a Richter's transformation to diffuse large B-cell lymphoma, and particularly to those patients showing a higher expression of PD-L1 and PD-1 in the tumor microenvironment (285, 289). These data encourage further studies exploring the efficacy of checkpoint inhibitors in this setting, which still represents a significant unmet clinical need.

CONCLUSIONS

It is well recognized that, in addition to the direct targeting of malignant cells, the disruption of the immune-tolerant microenvironment and the repair of immune system's defects are necessary steps for disease control. With the aim of harnessing immune responses against tumor cells, during the years, different types of immune-based strategies have been developed and evaluated in CLL. Despite promising preclinical observations, results from pilot clinical studies have been often suboptimal in terms of long-term tumor control, mainly because they were obtained in patients with advanced-stage disease and who had been already heavily pre-treated. In CLL, several observations demonstrate that the tumor negatively affects the host immune system, which progressively accumulates dysfunctions contributing to disease progression. Therefore, in disease like CLL, characterized by a long-acting evolution and the accumulation of immunologic dysfunction, one possibility to improve the efficacy of immunotherapy could be its earlier positioning in the treatment sequencing. By doing this, the development of immune defects could be prevented and subsequent therapies could act in concert with the patient's immune system against the tumor. However, the application of immunotherapy earlier in the course of the disease has to take into account the potential toxicities and the meaningful costs, and should be considered a valuable option only for patients with a high-risk disease and poor prognosis, who benefit less from currently available therapies.

An alternative strategy to improve patients' outcome is the identification of optimal combination treatments targeting both the CLL and the immune system, in order to reshape the functionality of the latter and properly address its reaction toward the tumor. Ibrutinib, and to some extent also next-generation BTK inhibitors and venetoclax, have shown to improve the host T-cell functions. Therefore, these targeted drugs, as well as other agents with more broad

TABLE 4 | Clinical trials evaluating the efficacy and toxicity of immune checkpoint inhibitors, used as single agents or in combination regimens, for the treatment of patients with CLL.

Drug regimen	Setting	Efficacy	Toxicities	References
Pembrolizumab every 3 weeks for up to 2 years	Phase II: 25 patients (9 Richter syndrome) R/R CLL	ORR 16% 1% CR Median PFS 3 months Median OS 10.7 months	G≥3 neutropenia 20% G≥3 thrombocytopenia 20% G≥3 anemia 20% G≥3 dyspnea 8% G≥3 hypoxia 8% G≥3 fatigue 8% G≥3 febrile neutropenia 8% G≥3 fever 4% G≥3 maculopapular rash 4% G3 lung infection 12% G3 hepatic toxicity 8% G5 sepsis 4%	Ding *et al.* (285) NCT02332980
Nivolumab 3 mg/kg IV every 2 weeks and ibrutinib 420 mg/day	Phase II 8 patients (4 Richter sydrome) R/R CLL *vs* 3 patients PR CLL to 9-month ibrutinib therapy	ORR 75% 50% PR *vs* ORR 100% 100% PR	No G≥3 AEs	Jain *et al.* (286) NCT02420912
Pidilizumab	Phase I 3 patients R/R CLL	ORR 0 67% SD	No G≥3 AEs	Berger *et al.* (287)
Samalizumab once every 28 days until progression or toxicity	Phase I 23 patients R/R CLL	ORR 4% 4PR	G≥3 hematological AEs 12% G≥3 reduced visual acuity 4% G≥3 muscular weakness 4% G≥3 RSV infection 4% G≥3 rash 4%	Mahadevan *et al.* (288)[1] NCT00648739
Ibrutinib (420 mg or 560 mg) and nivolumab	Phase I-IIa 56 patients (20 Richter syndrome)	ORR 97%	G≥3 neutropenia 28% G≥3 anemia 23% G≥3 rash 8% G≥3 increased ALT 2% Serious AEs: anemia 4% and pneumonia 4%	Younes *et al.* (289)[1] NCT02329847
For CLL: induction with umbralisib and ublituximab, consolidation with pembrolizumab, umbralisib and ublituximab, maintenance with umbralisib until progression or unacceptable AE. For Richter syndrome: umbralisib, ublituximab and pembrolizumab	Phase I/II 14 patients (5 Richter syndrome) R/R CLL	CLL: ORR 89% Richter syndrome: ORR 50%, 50% CR PFS 71% (median follow-up 15 months)	G≥3 neutropenia 43% G≥3 ALT/AST increase 21% G≥3 hypophosphatemia 21% G≥3 pneumonitis 7%	Mato *et al.* (290)
Nivolumab *vs* pembrolizumab and ibrutinib in 3 patients and venetoclax in 1 patient	7 *vs* 3 patients R/R CLL (Richter syndrome)	ORR 10%	ND	Rogers *et al.* (291)
Ipilimumab and lenalidomide	Phase II 2 patients R/R CLL to allogeneic HSCT	ORR 0	G≥3 neutropenia 40% G2 anemia 40% G2 thrombocytopenia 20% Flare of GVHD 10% G2 nausea 10% G2 headache 10% G2 diarrhea 10%% G2 elevated transaminase 10% G2 hypertension 10% G2 hypothyroidism 10%	Khouri *et al.* (292)[1] NCT01919619
Nivolumab single agent until progression or unacceptable toxicity, with planned deescalation based on toxicity	Phase I 1 patient R/R CLL to allogeneic HSCT	ORR 32% 1-year PFS 23%, 1-year OS 56%	G≥3 thrombocytopenia 14% G≥3 neutropenia 14% G≥3 anemia 11% G≥3 febrile neutropenia 11% G≥3 fatigue 7.1%	Davids *et al.* (293)[1] NCT01822509

(Continued)

TABLE 4 | Continued

Drug regimen	Setting	Efficacy	Toxicities	References
			G≥3 rash 3.6% G≥3 transaminitis 7.1% G≥3 fever 3.6% G≥3 lipase elevation 14% G≥3 pneumonitis 3.6% G≥3 abdominal pain 3.6% G≥3 nausea 3.6% G≥3 arthralgia 3.6% G≥3 bilirubin elevation 11% G 5 acute GVHD (liver and gut) 7.1% G 5 APS complicated by thrombotic CVA 3.6% G 5 sepsis with ARDS 3.6%	
Umbralisib and pembrolizumab	Phase I R/R CLL	ND	ND	NCT03283137
Pembrolizumab	Phase II R/R CLL with Richter Syrndrome	ND	ND	NCT02576990
Ublituximab and umbralisib in combination with targeted immunotherapy	Phase I R/R CLL or Richter syndrome	ND	ND	NCT02535286
Ibrutinib, fludarabine and pembrolizumab	Phase II high-risk or R/R CLL/SLL	ND	ND	NCT03204188
Atezolizumab, obinutuzumab and venetoclax	Phase II CLL, SLL, R/R Richter syndrome	ND	ND	NCT02846623
Durvalumab monotherapy and in combination (with lenalidomide and rituximab; with ibrutinib; with bendamustine and rituximab)	Phase I-II lymphoma or CLL	ND	ND	NCT02733042
Anti-LAG-3 (BMS-986016) single agent and in combination with nivolumab	Phase I/IIa R/R CLL	ND	ND	NCT02061761

AE, adverse events; APS, anti-phospholipid antibody syndrome; ARDS, acute respiratory distress syndrome; CLL, chronic lymphocytic leukemia; CR, complete response; CVA, cerebral vascular accident; G≥3, grade ≥3; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; ND, no data; ORR, overall response rate; OS, overall survival; PFS, progression free survival; PR, partial response; R/R, relapsed or refractory; SD, stable disease; SLL, small lymphocytic lymphoma.
[1]Data for CLL patients only non-available.

immunomodulatory properties—such as IMiDs and checkpoint inhibitors—are currently under evaluation for their ability to potentiate the efficacy of other immunotherapeutic strategies. In this context, combination trials of ibrutinib and T-cell directed immunotherapies, such as anti-CD19 CAR T cells, have already provided promising results and support the potential of this approach.

AUTHOR CONTRIBUTIONS

VG and FP reviewed the literature and wrote the manuscript. VG and FP equally contributed to this work. CS and EB contributed to literature review. MB contributed to manuscript revision. CV and MC designed the review and revised the manuscript. CV and MC equally contributed to this work. All authors contributed to the article and approved the submitted version.

FUNDING

This research received no external funding. VG is a recipient of a fellowship from the "Associazione Damiano per l'Ematologia".

ACKNOWLEDGMENTS

The authors would like to thank for the support Fondazione EMN Italy Onlus, Torino, Italy. MC would like to express her gratitude for the support given by Fondazione Cassa di Risparmio di Torino (CRT) while contributing to this collection of articles.

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Conflict of Interest: MB has received honoraria from Sanofi, Celgene, Amgen, Janssen, Novartis, Bristol-Myers Squibb, and AbbVie; has served on the advisory boards for Janssen and GSK; has received research funding from Sanofi, Celgene, Amgen, Janssen, Novartis, Bristol-Myers Squibb, and Mundipharma. CV has received consultancy fees from Janssen. MC received honoraria from Janssen, Gilead, Abbvie, Shire and research support from Janssen and Karyopharm Therapeutics.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AK declared a past co-authorship with several of the authors CV and MC to the handling editor.

frontiers in Immunology

REVIEW
published: 14 December 2020
doi: 10.3389/fimmu.2020.594841

Microenvironment Remodeling and Subsequent Clinical Implications in Diffuse Large B-Cell Histologic Variant of Richter Syndrome

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: 14 August 2020
Accepted: 03 November 2020
Published: 14 December 2020

Citation:
Augé H, Notarantonio A-B, Morizot R, Quinquenel A, Fornecker L-M, Hergalant S, Feugier P and Broséus J (2020) Microenvironment Remodeling and Subsequent Clinical Implications in Diffuse Large B-Cell Histologic Variant of Richter Syndrome. Front. Immunol. 11:594841. doi: 10.3389/fimmu.2020.594841

Introduction: Richter Syndrome (RS) is defined as the development of an aggressive lymphoma in the context of Chronic Lymphocytic Leukemia (CLL), with a Diffuse Large B-Cell Lymphoma (DLBCL) histology in 95% cases. RS genomic landscape shares only a few features with *de novo* DLBCLs and is marked by a wide spectrum of cytogenetic abnormalities. Little is known about RS microenvironment. Therapeutic options and efficacy are limited, leading to a 12 months median overall survival. The new targeted treatments usually effective in CLL fail to obtain long-term remissions in RS.

Methods: We reviewed available PubMed literature about RS genomics, PD-1/PD-L1 (Programmed Death 1/Programmed Death Ligand 1) pathway triggering and subsequent new therapeutic options.

Results: Data from about 207 patients from four landmark papers were compiled to build an overview of RS genomic lesions and point mutations. A number of these abnormalities may be involved in tumor microenvironment reshaping. T lymphocyte exhaustion through PD-L1 overexpression by tumor cells and subsequent PD-1/PD-L1 pathway triggering is frequently reported in solid cancers. This immune checkpoint inhibitor is also described in B lymphoid malignancies, particularly CLL: PD-1 expression is reported in a subset of prolymphocytes from the CLL lymph node proliferation centers. However, there is only few data about PD-1/PD-L1 pathway in RS. In RS, PD-1 expression is a hallmark of recently described « Regulatory B-cells », which interact with tumor microenvironment by producing inhibiting cytokines such as TGF-β and IL-10, impairing T lymphocytes

anti-tumoral function. Based upon the discovery of high PD-1 expression on tumoral B lymphocyte from RS, immune checkpoint blockade therapies such as anti-PD-1 antibodies have been tested on small RS cohorts and provided heterogeneous but encouraging results.

Conclusion: RS genetic landscape and immune evasion mechanisms are being progressively unraveled. New protocols using targeted treatments such as checkpoint inhibitors as single agents or in combination with immunochemotherapy are currently being evaluated.

Keywords: Richter syndrome, chronic lymphocytic leukemia, diffuse large B-cell lymphoma, genomics, microenvironment, immune checkpoint, immune checkpoint inhibitor

INTRODUCTION

Chronic Lymphocytic Leukemia (CLL) is the most frequent leukemia in Western countries (1). Although considered to be an indolent B-cell neoplasm, CLL actually represents a wide spectrum of diseases from a clinical, biological and prognostic point of view, ranging from non-progressive or poorly progressive to aggressive courses (2). CLL prognosis was first assessed using clinical classifications (3, 4) to which cytogenetic and molecular data were later added. CLLs are distributed among 2 major molecular subtypes that differ in their degree of somatic hypermutations in the *IGHV* (*Immunoglobulin Heavy chain Variable domain*) gene. The *IGHV* unmutated CLLs (U-CLLs), share more than 98% homology with germline sequence and are associated with a worse prognosis than the *IGHV* mutated CLLs (M-CLLs) (5, 6). The combinatorial diversity of VDJ segments at the origin of rearrangements of the *IGHV* gene continuously generates a vast repertoire of B lymphocytes, all different, characterized by a single B-Cell receptor (BCR). A third of the CLLs have been shown to have a stereotypic BCR, meaning that a significant part of B lymphocytes express a restricted immunoglobulin gene repertoire leading to the expression of highly similar BCRs, at a higher rate than statistically expected, indicating a non-random distribution, probably due to chronic antigenic stimulation (7). Certain stereotypic BCR are associated with a poor prognosis (8). Fluorescence In Situ Hybridization (FISH), allows identification of the main CLL-associated cytogenetic abnormalities. About 80% of CLLs are associated with at least one of the four most frequent anomalies: deletion 13q (del 13q), deletion 11q (del 11q), deletion 17p (del 17p), and trisomy 12, encompassing miRNA 15a/16-1 (del 13q), *ATM* and *BIRC3* (del 11q), or *TP53* (del 17p). These abnormalities define different prognostic subgroups (9). The advent of Single Nucleotide Polymorphism (SNP) array allowed the discovery of smaller and less frequent Copy Number Variations (CNV) (10, 11). Next generation sequencing techniques made it possible to precisely define the CLL mutational landscape. This appears to be highly heterogeneous regarding pathway deregulation mechanisms, with a broad spectrum of mutations affecting: i) response to DNA damage and cell cycle control (*TP53, ATM, POT1, ATRX*), ii) RNA maturation and export (*SF3B1, XPO1, RPS15, DDX3X, ZNF292, MED12, NXF1*), iii) NOTCH pathway (*NOTCH1, FBXW7*), iv) BCR pathway (*EGR2, KLHL6, BCOR, IRF4, IKZF3, ITKB, CARD11*), v) chromatin remodeling (*CHD2, BAZ2A, SETD2, ASXL1, ZMYM3, HIST1H1E, ARID1A*), vi) NFKB pathway (*BIRC3, MYD88, TRAF3, NFKB1E*), vii) inflammatory response (*SAMHD1, RIPK1*), viii) early B-cell development (*IKZF3, PAX5*), ix) MAPK-ERK pathway (*MAPK, MAP2K1, ERK, BRAF, KRAS*), and x) MYC-associated signaling (*MGA, PTPN11*). The distribution and frequencies of these abnormalities are different among U-CLLs and M-CLLs (11–15).

Richter Syndrome (RS) is defined as the transformation of CLL or Small Lymphocytic Lymphoma (SLL) into a more aggressive histology (16). Two histopathological variants are described: Diffuse Large B-Cell Lymphoma (DLBCL, for 90%–95% of cases) and Hodgkin Lymphoma (HL, for 5-10% cases). Here we will focus on the DLBCL subtype. In more than 90% of cases, RS presents the immunohistochemical profile of non-Germinal Center-like DLBCL (17–22). RS incidence is very variable, ranging from 2% to 9% of unselected CLLs to more than 20% in the case of refractory and/or 17p deleted CLLs. Cumulative impact is estimated at 2.1% at 5 years and 4.8% at 10 years, representing a transformation risk of 0.5% per year. Median time between CLL diagnosis and RS transformation is 23 months (7, 19, 20, 22). Certain abnormality combinations systematically lead to the CLL transformation into RS, like the co-occurrence of an activating mutation of *NOTCH1*, trisomy 12,

Abbreviations: APC, Antigen Presenting Cell; BCL2, B-Cell Lymphoma 2; BCR, B-Cell Receptor; Bregs, Regulatory B lymphocytes; BTK, Bruton Tyrosine Kinase; CLL, Chronic Lymphocytic Leukemia; CTLA-4, Cytotoxic T Lymphocyte-Associated protein 4; CNV, Copy Number Variation; CR, Complete Response; DC, Dendritic Cells; DLBCL, Diffuse Large B-Cell Lymphoma; FDA, Food and Drug Administration; FL, Follicular Lymphoma; HL, Hodgkin Lymphoma; ICPIs, Immune Checkpoint Inhibitors; IHC, Immunohistochemistry; IFNγ, Interferon Gamma; IGHV, Immunoglobulin Heavy chain Variable domain; IL-2, Interleukine 2; IL-4, Interleukine 4; IL-10, Interleukine 10; M-CLL, Mutated-CLL; MHC, Major Histocompatibility Complex; OFAR, Oxiplatin, Fludarabine, Aracytine, Rituximab; OS, Overall Survival; PD-1, Programmed Death 1; PD-L1, Programmed Death Ligand 1; PD-L2, Programmed Death Ligand 2; PFS, Progression-free Survival; PR, Partial Response; R-CHOP, Rituximab, Cyclophosphamide, Hydroxydaunorubicin, Oncovin, Prednisone; R/R, Relapsed or Refractory; RS, Richter Syndrome; SCT, Stem Cell Transplantation; SLL, Small Lymphocytic Lymphoma; TCR, T-Cell Receptor; TGF-β, Transforming Growth Factor Beta; TL, T Lymphocyte/T-Cell; TIL, Tumor-infiltrating Lymphocytes; TNFα, Tumor Necrosis Factor Alpha; Tregs, Regulatory T Lymphocytes; U-CLL, Unmutated CLL.

and an $IGHV_{4\text{-}39}$ stereotypic BCR. Combination of an *SF3B1* mutation and a stereotypical BCR of the $IGHV_{3\text{-}21}$ type leads to an increased risk of transformation into RS (23). Influence of CLL treatment on the risk of RS transformation is not established as half RS cases occur in the context of an untreated CLL. CLL treatment is associated with a RS risk of 1% per year (5% at 5 years, 15.2% at 10 years) (19). In a large retrospective study, a combination of purine analogs with alkylating agents increased the transformation risk to 1.5% per year. But the use of these different molecules in monotherapy (purine analog, alkylating agents or immunotherapy) does not increase RS risk (22). The impact of conventional chemotherapy on the risk of developing RS remains controversial because this risk is equivalent with Fludarabine alone, Chlorambucil alone or a combination of the two treatments (24). Similarly, after a median follow-up period of 3.5 years, the RS risk was equivalent between Chlorambucil alone, Fludarabine alone and the combination of Fludarabine + Cyclophosphamide (25). Recently, CLL management has been considerably modified with the availability of new BCR (26, 27) and B-cell Lymphoma 2 (BCL2) inhibitors (28). Unfortunately, these drugs do not preserve from RS, accounting for 30 to 50% of CLL progression cases. Transformation is now the principal obstacle for long-term control of the disease and remains a crucial unmet medical need (29, 30).

RICHTER SYNDROME MOLECULAR EVENTS

Exome sequencing shows that RS mutational landscape shares only a few common features with DLBCLs, except for *CARD11*, *MYD88*, *CDKN2A/B*, and *MYC* alterations (23, 31, 32). The genomic complexity of RS is intermediate between that of CLL and *de novo* DLBCL (32). Surprisingly, 64.7% of RS harbors an unmutated *IGHV* sequence, all *de novo* DLBCLs having a mutated *IGHV* profile. This is in line with the fact that U-CLL have a four-time higher RS transformation risk than M-CLL (33). RS exhibits an *IGHV* hypervariable CDR3 region identical to that of the initial CLL in 80-90% cases, proving a clonal relationship between the two stages (7). These clonally related RS have a median survival of 14.2 months. In contrast, the 10 to 20% clonally unrelated RS have a median survival comparable to *de novo* DLBCLs (62.5 months) and are considered by most authors as independent neoplasms (20, 21). Clonal relationship is therefore the most significant prognostic factor. Half RS harbor a stereotypic BCR (20), with an overrepresentation of $IGHV_{4\text{-}39}$ in RS, suggesting a relationship with disease development.

TP53 disruptions (partial or total deletions of the gene, loss of function mutations) are highly frequent at RS stage, with a prevalence of up to 34.4%–60% of cases in documented large cohorts (33). In most cases, *TP53* disruptions are acquired at RS transformation (20). In a large cohort of 131 RS patients, 45 (34.4%) had del (17p) or *TP53* mutation (34). The high proportion of these abnormalities at RS stage could reflect a selective advantage and the conferred chemoresistance. TP53 pathway is also disrupted through other abnormalities affecting related effectors such as *MDM2, MDM4, ATM, BCL2, CREBBP*, and *PRDM1* or *TP53* promoter hypermethylation (35).

NOTCH1 mutations located in exon 34 are identified in up to 30%–40% of RS cases (36). Dominant positive variants devoid of the PEST degradation domain lead to a NOTCH1 protein with extended lifespan and a constitutive pathway activation, constantly triggering the transcription of many genes involved in cell proliferation and therefore uncontrolled cell growth. Trisomy 12 is present in 30% of RS and is frequently associated with *NOTCH1* mutations. Other RS recurrent abnormalities lead to NOTCH pathway deregulation, such as *NCOR1* deletions, *SPEN* mutations, *NLK* deletions, and *MYCN* amplifications.

MYC abnormalities, whether translocations or amplifications, affect 26.5%–35% of RS and are acquired at RS stage in 75% of cases. *MYC* and *TP53* abnormalities are described together in 50% of RS cases (20, 32, 34). An SNP study on a group of 13 RS, 8 of which were clonally related to the initial CLL, identified deletions of miR 17-92, a microRNA cluster regulating *MYC* expression. These anomalies are also acquired at RS stage (31). Last, other MYC pathway effectors are affected in RS, including deletions of negative regulator *MGA*, and mutations of *PIM1* and *PAX5*.

The study of CNVs by Comparative Genomic Hybridization array identified 9p21 deletions in 30% of RS cases. These are systematically acquired at transformation and encompass *CDKN2A/B* genes, encoding distinct negative regulators of cell cycle progression through inhibition of cyclin-dependent kinases 4 and 6 and cyclin D. Silencing through gene promoter hypermethylation is also described (35). These anomalies are bi-allelic in 30% of cases. This study confirmed the large proportion of linear evolutions of the CLL clone and the acquisition of an average of 22 new anomalies between the CLL and RS stage (36).

In the current molecular model, the evolution of the CLL clone into RS is associated with deregulation of cell proliferation, apoptosis and the cell cycle progression, mainly due to abnormalities of *TP53*, *NOTCH1*, *MYC*, and *CDKN2A/B* (37). Half RS cases are associated with the newly acquired *TP53* inactivation, *MYC* activation or *CDKN2A/B* deletion. In 30% of cases, the transformation is associated with trisomy 12 and a mutation of *NOTCH1*.These anomalies are mutually exclusive with *TP53* or *CDKN2A/B* disruptions. The remaining 20% RS are related to other genetic abnormalities (32, 36, 38).

To get a deeper understanding about RS genomic characteristics, we compiled the available data regarding DNA mutations from articles aiming at expanding knowledge about RS genomic features by exploring previously unknown genomic alterations on unselected cases. Richter cohorts documented with only previously known CLL-associated genomic alterations were not retrieved. This led us to select 4 landmark papers, gathering a total of 207 RS (20, 31, 32, 36) and retained: i) the CNVs described in at least 5% cases (with at least five positive cases and series of 50 samples minimal) and ii) SNPs described in at least 5% cases (with at least two positive cases and series of 10 samples minimal). This led to a list of 100 abnormalities affecting 95 different genes, which were annotated functionally with a compilation of gene ontologies (http://geneontology.org/) (39)

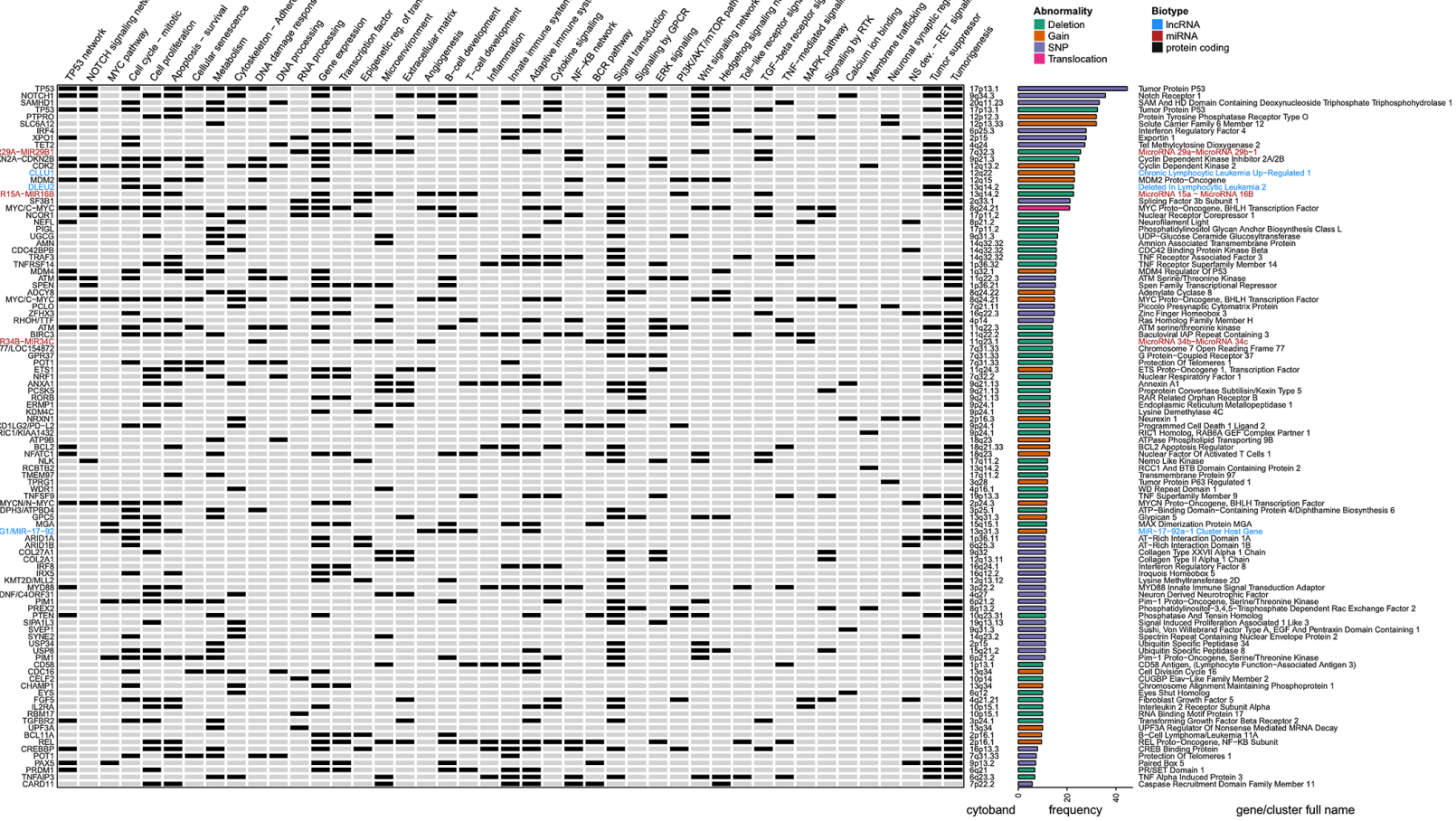

FIGURE 1 | Genomic landscape of Richter syndrome. Genes recurrently mutated and related cellular functions altered in RS as a manually curated heat map of available data from 4 landmark papers, gathering a total of 207 RS patients. From left to right (for each gene): gene name, functional annotations and related pathways (according various databases and literature), cytogenetic band, nature of the reported abnormality (deletion, gain, translocation, or single nucleotide variant) and full gene name written according to a color-code indicating biotype (black: protein coding gene; red: miRNA and blue: lncRNA). Selection thresholds: 5% occurrence in cohorts of at least 50 samples for CNVs and 10 samples for SNPs. BCR, B-cell Receptor; CNV, Copy Number Variation; GPCR, G Protein-Coupled Receptor; lncRNA, Long Non-Coding RNA; MAPK, Mitogen-Activated Protein Kinase; miRNA, MicroRNA; NS, Nervous System; RS, Richter Syndrome; RTK, Receptor Tyrosine Kinase; SNP, Single Nucleotide Polymorphism; TGF, Transforming Growth Factor; TNF, Tumor Necrosis Factor.

and enriched pathways according to Reactome (https://reactome.org/) (40) and retrieved with BioMart (https://www.ensembl.org/biomart) (41). We also added the corresponding literature from OMIM (https://omim.org/) (42), and extensive lists of transcription factors obtained from a recent review on the topic (43), together with tumor suppressor candidates from the Tumor Suppressor Database (https://bioinfo.uth.edu/TSGene/) (44). All these were manually curated and used to complete a detailed heat map of RS genomic abnormalities (**Figure 1**).

According to this compilation, a wide range of cellular functions are deregulated in RS, including cell cycle regulation, cell proliferation, cell survival, senescence, DNA and RNA processing, epigenetic regulation of transcription, nuclear export, signal transduction, ion transport, cytoskeleton, cell adhesion, and migration, as well as numerous essential signaling pathways, implicating TP53, NOTCH, MYC, BCR, NF-KB, JAK-STAT, Toll-like receptors, MAPK, TNF, TGF, Insulin like growth factor, Wnt, Ras, ERK, and PI3K-Akt-mTOR. However, RS mutational landscape does not only affect cell cycle-related functions. Indeed, the alterations described may also disturb the microenvironment, B-cell development, and T-cell expansion/activation, promoting tumor progression by immune response reprogramming.

PTPRO (protein tyrosine phosphatase receptor type O) has been described as highly expressed in the microenvironment of breast cancer, associated with increased tumor growth, angiogenesis, and metastatic spreading (45). It also plays a role in T-cell-mediated anti-tumor immunity, with involvement in regulation of effector T-cells/regulatory T-cells ratio in the tumor microenvironment (46). Besides their known tumor suppressive properties in CLL, miR-29 family members are also regulators of the adaptive immune system, since they regulate helper T-cell development and interferon gamma (IFNγ) secretion by Type 1 helper T-cells (47). Another micro-RNA cluster, miR-15a/16 does not harbor functions restricted to tumor suppressive properties, since it also regulates T-cell expansion and differentiation (48), B cell proliferation (49), contributes to the balance between T-cell activation and T-cell anergy (50), regulates PD-1 (Programmed Death 1) expression and IFNγ excretion by tumor-infiltrating CD8+ T-cells (51), and indirectly governs regulatory T-cell development (52). TGF-β (Transforming Growth Factor β) receptor deregulation through TGF-β receptor 2 abnormalities (53), decreased expression of surface HLA class II molecules due to CD58 locus deletions (54) and impairment of Tumor-Associated Macrophages related to *XPO1* and *NRF1* abnormalities (55, 56) may also represent another player in immune evasion in RS.

XPO1-blocking drug Selinexor has been shown to slow down tumor growth in murine primary central nervous system lymphoma by shifting TAM polarization from PD-1 expressing M2-like macrophages toward PD-1 low-expressing M1 macrophages, providing evidence for the role of microenvironment remodeling in lymphoma (56). The central role of immune checkpoint hijacking through PD-1/PD-L1/PD-L2 pathway deregulation in RS is supported by abnormalities of PD-L1 (Programmed Death Ligand 1) expression-regulating miR-34 cluster (57) and high proportions of alterations of *PDCD1LG2*, encoding PD-L2 (Programmed Death Ligand 2).

ACTIVATION AND LIMITATION OF THE ADAPTIVE IMMUNE RESPONSE: IMMUNE CHECKPOINT DEREGULATION THROUGH PD-1/PD-L1/PD-L2 PATHWAY HIJACKING IN ONCOLOGY

Three signals are required for T-cell/T lymphocyte (TL) activation: i) specific recognition by the T-cell receptor (TCR) of an antigen processed by professional antigen presenting cells (APCs), ii) co-stimulation signals, either through the binding of TL co-stimulatory receptor CD28 with its ligands, CD80 and CD86 expressed on APCs, or the binding of CD40 (receptor) to CD40L (ligand), and iii) TL cytokine production (IL-2 in particular) and expression of their specific receptors, leading to autocrine activation, clonal expansion, TL differentiation, and cytotoxic activity of antigen-specific TL. Co-stimulation is essential for TL activation, since antigen recognition by the TCR without co-stimulation signal leads to an anergy state and/or tolerance to this antigen (**Figure 2**) (58) Three to 5 days after activation, TLs physiologically express co-inhibitory receptors (immune checkpoint inhibitors; ICPIs) on their surface, such as PD-1, CTLA-4 (Cytotoxic T Lymphocyte-Associated protein 4), LAG-3 (Lymphocyte-Activation Gene 3), and TIM-3 (T-cell immunoglobulin and mucin containing protein-3), which bind to their respective ligand, leading to the regulation of the immune response. Once the antigen is eliminated, expression of these checkpoint inhibitors decreases to normal levels (**Figure 2**) (59, 60). The most explored immune checkpoint inhibitors to date are CTLA-4 and PD-1, both members of the B7 receptor family.

TL-specific surface marker CTLA-4 is a CD28 homologue expressed 48 hours after TL activation, with a greater affinity for CD80/86 (61). In lymph node, it acts as a central negative regulator on the surface of naive TLs (CD4+ FOXP3- CD8+), where it competes with CD28 and interacts with CD80/86, enabling the minute regulation of TL activation level, thus limiting early immune response. CTLA-4 is also expressed on CD4+ FOXP3+ regulatory T lymphocytes (Tregs) (62).

PD-1 is a transmembrane protein receptor that functions as a key negative regulator of cellular immunity, orchestrating the delicate balance between immune defense and the protection of healthy tissues from persistent inflammation and autoimmunity through various signaling pathways (59–61, 63–65). PD-1 is expressed on TL, but also on natural killer cells, pro-B cells, macrophages, monocytes, Dendritic Cells (DC), and Innate Lymphoid Cells (66), and has two ligands: Programmed Death Ligand 1 (PD-L1; CD274; B7-H1) and Programmed Death Ligand 2 (PD-L2; CD273; B7-DC). PD-L1 is more widely expressed than PD-L2 (67), notably on non-hematopoietic cells (including epithelial cells, vascular endothelial cells and stromal cells) and is induced by pro-inflammatory cytokines (including type I and type II interferons, TNFα and vascular endothelial growth factor). PD-L2 is mainly expressed on DC and macrophages and is induced by many of the same cytokines as PD-L1, plus IL-4 and granulocyte-macrophage colony-

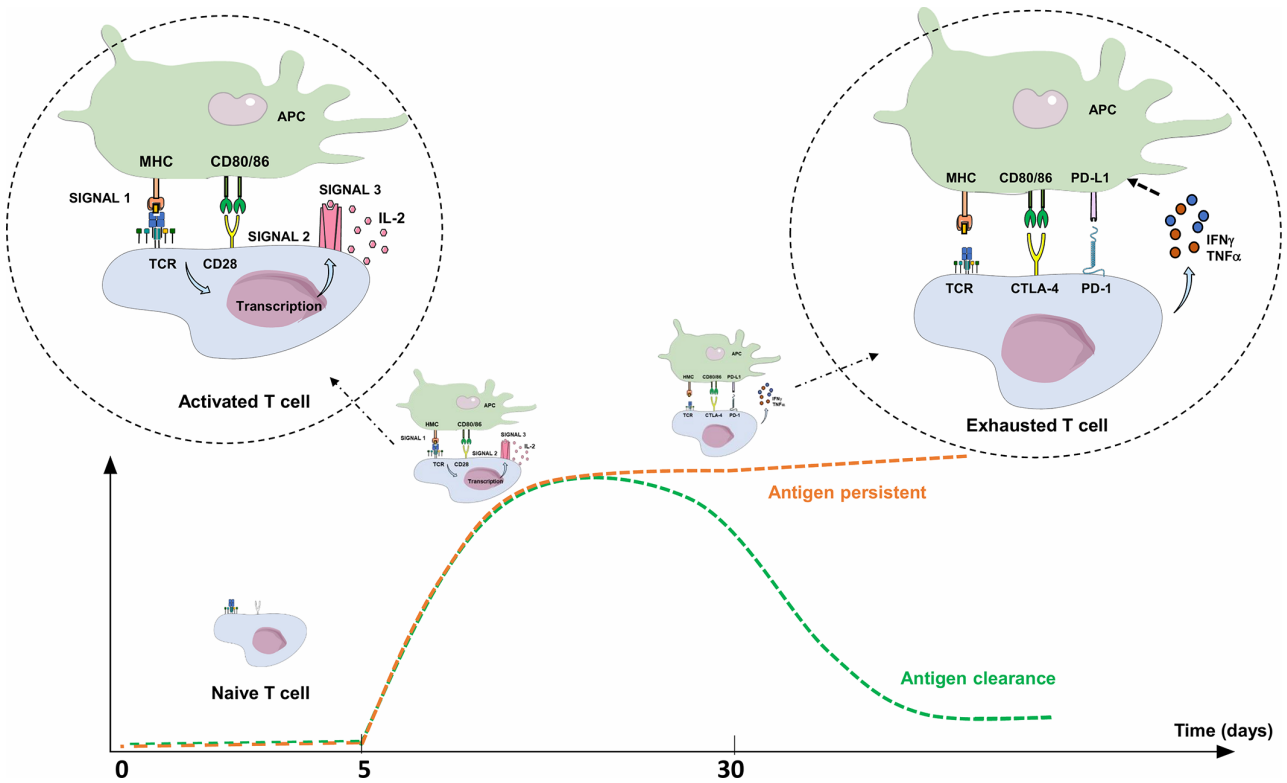

FIGURE 2 | Activation and limitation of the adaptive immune response: physiological concepts. To activate a naive TL during adaptive immune response, APC must present an antigen through MHC to the TCR (signal 1), but also provide an essential co-stimulation signal (signal 2). These co-stimulation molecules, whose expression are induced during innate immune response, are mainly CD80/CD86 (expressed on APC), and CD28 (expressed on T lymphocyte). The activated TL can then proliferate, differentiate into an effector TL, leave the lymph node and move into peripheral tissues to the inflammatory site. Without this co-stimulation, the TL becomes anergic. To limit the immune response to a pathogenic antigen, both central and peripheral immune control checkpoints exist. Three to 5 days after activation, TLs physiologically express co-inhibitory receptors (immune checkpoint inhibitors; ICPIs) on their surface, such as CTLA-4 and PD-1. CTLA-4 is expressed by naive TLs residing in the lymph nodes, competes with CD28 and interacts with CD80/86: this regulates the amplitude of TL activation and limits the initial immune response. PD-1 is expressed on effector T lymphocytes and interacts with its ligands (PD-L1/PD-L2). PD-1 expression occurs within 24 h after T-cell activation and decreases with antigen clearance. Unlike CTLA-4, its role is to limit, through various signaling pathways, the immune response in peripheral tissues during the active phase of inflammation by decreasing TL activity (exhaustion). TL exhaustion is a progressive process consisting in an effector TL dysfunctional state upon repeated or prolonged stimulation by an antigen, happening in a context of chronic inflammation (chronic infection or cancer) with persistent TL stimulation. The expression of PD-L1 is induced by pro-inflammatory cytokines such as IFNγ or TNFα through a negative feedback loop, thus avoiding collateral damage on tissues. APC, Antigen Presenting Cell; CTLA-4, Cytotoxic T lymphocyte-associated Antigen 4; IFNγ: Interferon Gamma; IL-2, Interleukine 2; MHC, Major Histocompatibility Complex; PD-1, Programmed Death 1; PD-L1, Programmed Death Ligand 1; TCR, T-cell Receptor; TNFα, Tumor Necrosis Factor Alpha.

stimulating factor (59, 60, 64, 68). PD-1 expression occurs within 24 h after TL activation and decreases with antigen clearance. The binding of PD-L1 or PD-L2 results in TCR signaling downregulation. This inhibition induces a decrease in IL-2 and IFNγ production by activated TL, leading to a decrease in the downstream stimulation cascade, and a functional alteration of the CD4+ and CD8+ TLs, which in turn reduces their capacity to produce IL-2 and IFNγ, to acquire cytotoxic abilities and to proliferate (68, 69).

In a chronic inflammatory context, TL may enter a dysfunctional state called *lymphocyte exhaustion*, a progressive process consisting in an effector-TL function loss upon repeated activations, coinciding with increasing expression levels of ICPIs and particularly PD-1, considered as the leading inhibitory regulator of TL function. TL are ineffective at eradicating pathogens or tumors, so there is a real interest in reversing the depletion phenomenon (70).

Oncogenic processes begin with an acute inflammatory response, with tumor infiltration by non-specific innate immune cells and increased production of pro-inflammatory cytokines, followed by an activating cascade specific of adaptive immune cells (71, 72). In the microenvironment of solid cancers, the co-stimulatory molecules regulating TL activation are not necessarily overexpressed. The inhibitory molecules that regulate TL functions (and notably the PD-1/PD-L1 axis) are generally overexpressed in tumor cells or in the microenvironment cells such as Tumor-infiltrating Lymphocytes (TIL) and Tregs in melanoma, breast, prostate, ovary, hepatocellular carcinoma

and small cell lung carcinoma (65, 73). PD-1 overexpression in TILs affects the prognosis of several solid cancers (74), and the increased PD-1 expression among tumor-infiltrating CD4+ TLs reflects a usually high level of PD-1 expression on Tregs, which may represent a large proportion of intra-tumor CD4+ TLs (59). In a melanoma mouse model consisting in B16.SIY melanoma cells subcutaneously transplanted into immunocompetent 6-week old wild-type C57BL/6 mice, TL from the tumor microenvironment express very high levels of PD-L1 and indoleamine-2,3-dioxygenase, both induced by IFNγ production by CD8+ TLs (75). This adaptive tumor resistance mechanism uses large quantities of IFNγ from the tumor microenvironment (76, 77): such a negative feedback loop induces PD-L1 expression on the tumor cell surface, which in turn suppresses PD-1+ TLs activity (59, 63, 78). In many cancers, upregulation of PD-L1 appears to be correlated with poorer outcomes (**Figure 3**) (73). PD-L1 is also involved in the maintenance and induction of tumor-associated Tregs by inhibiting the Akt/mTOR signaling cascade, which promotes differentiation and switch from naive CD4+ TL to induced CD4+ CD25+ FOXP3+ Tregs (59, 62, 68, 79). The function of these Tregs is to attenuate the response of effector TLs and PD-1 (59, 78). By overexpressing PD-L1, tumor cells inhibit anti-tumor immune responses in the tumor microenvironment (59).

Blocking the PD-1/PD-L1 signaling pathway can therefore induce a targeted anti-tumor response (73). The presumed mechanism for blocking PD-1/PD-L1 in cancer is that it unleashes the anti-tumor TL response at the tumor site. To date, many monoclonal antibodies targeting PD-1 or its ligand has been approved by the US Food and Drug Administration (FDA) and are available with marketing authorizations in oncology (59, 63, 78, 80, 81).

IMMUNE ESCAPE THROUGH PD-1/PD-L1/PD-L2 PATHWAY IN B-CELL MALIGNANCIES

The model used in oncology is not applicable here because the B lymphocyte tumor cell is also an APC (82, 83). This is because the tumoral microenvironment is usually the lymph node, where immune cells practically reside. In this environment, B lymphocyte interaction is different in each lymphoma subtype.

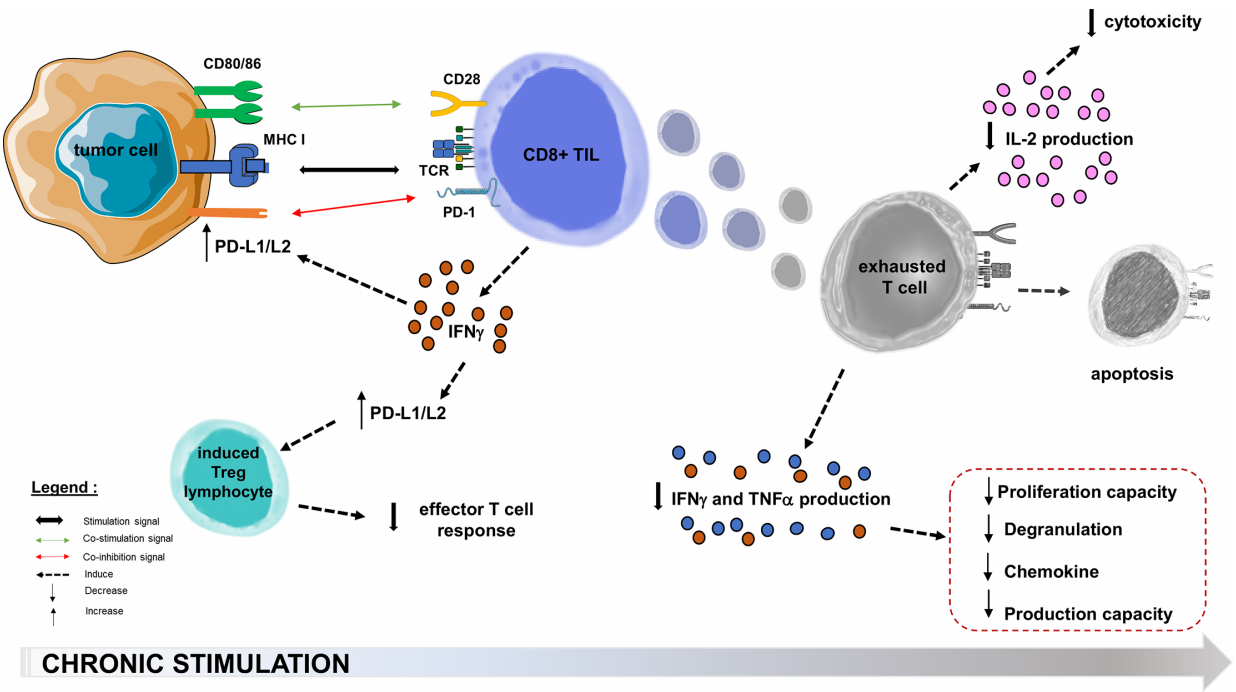

FIGURE 3 | PD-1/PD-L1/PD-L2 pathway hijacking in oncology. Immune checkpoints are exploited by solid tumors to evade or suppress the immune system. The PD-1/PD-L1 co-inhibition axis, within the tumor microenvironment, will allow many PD-L1 tumor cells to escape the immune system through multiple mechanisms. One of these is the decrease of effector functions of cytotoxic TLs, directed against the tumor antigen by inducing their functional depletion (exhaustion), reducing their cytokines (IL-2) production ability, their high proliferation capacity, their cytotoxic activity and consequently the resistance to tumor cell lysis. The second step is the functional alterations in the production of TNFα, IFNγ, β–chemokines, and degranulation. In the most terminal stages of depletion, these cells may enter apoptosis, probably as a consequence of over-stimulation. PD-L1 role is also to maintain and induce tumor-associated regulatory T-cells (induced Tregs), by promoting their switching from naive CD4+ TLs. Infiltration of their microenvironment by activated TLs producing pro-inflammatory cytokines such as IFNγ enhances PD-L1 upregulation in tumor cells. This feedback loop is thought to be a mechanism of adaptive immune resistance by the tumor. APC, Antigen Presenting Cell; CTLA-4, Cytotoxic T lymphocyte-associated Antigen 4; IFNγ, Interferon Gamma; IL-2, Interleukine 2; MHC, Major Histocompatibility Complex; PD-1, Programmed Death 1; PD-L1, Programmed Death Ligand 1; TCR, T-cell Receptor; TIL, Tumor-infiltrating Lymphocyte; TNFα, Tumor Necrosis Factor Alpha; Treg, Regulatory T lymphocyte.

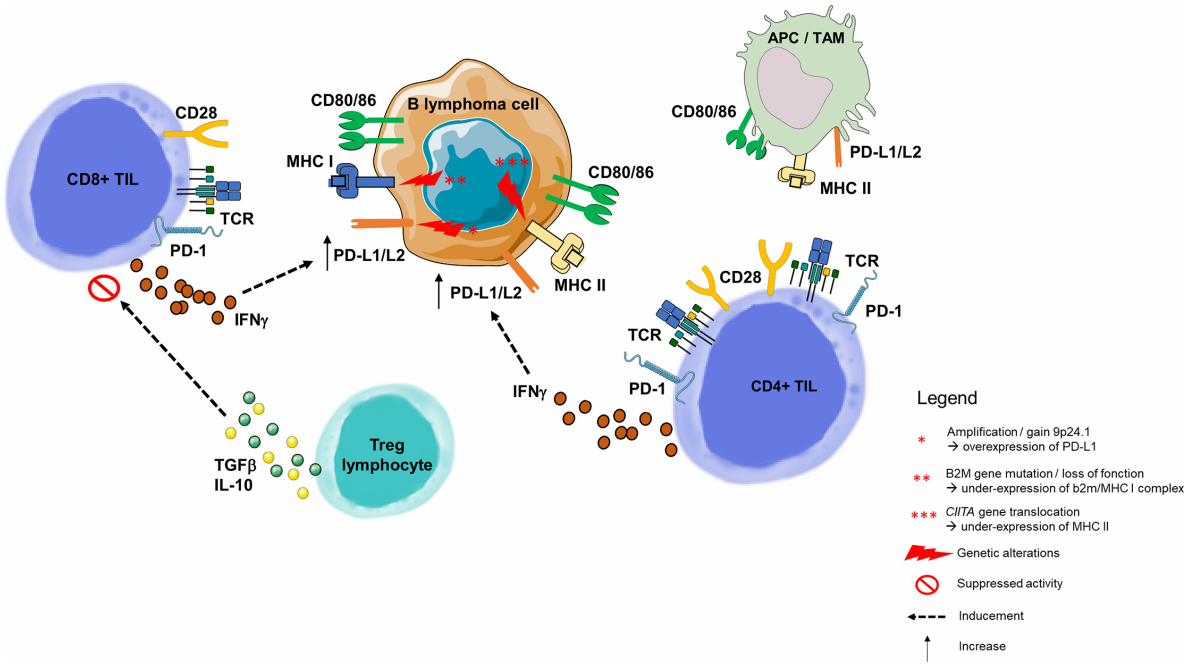

FIGURE 4 | Immune escape through PD-1/PD-L1 pathway in B-cell malignancies. As opposed to solid cancers, the malignant B lymphoma cell is also an immune cell, and its tumor microenvironment contains highly variable numbers of immune cells. B-cell malignancies arise in lymphoid tissues, and more precisely germinal center of lymph nodes (82). Molecular dissection of the malignant B-cell allows to understand the progressive behavior of different B-cell lymphoma subtypes. Among genetic alterations contributing to immune escape is the 9p24.1 copy gain or amplification, described many times in inflammatory lymphomas such as HL or Primary Mediastinal B Lymphoma (84), and correlated with overexpression of PD-L1 and PD-L2 on the surface of malignant cells (85). Two other mechanisms responsible for the malignant B lymphocyte inability to present tumor antigen to the CD8+ or CD4+ TLs are, respectively, loss of function of the gene encoding the β2-microglobulin (MHC I complex dysfunction) (54, 86) and the dysfunction of *CIITA* (encoding MHC II) (87). PD-L1 is highly expressed on tumor-infiltrating macrophages and the surface of tumor cells and APCs in the tumor microenvironment (76). In a HL model, PD-L1+ macrophages were frequently in contact with PD-1 CD4+ TLs, suggesting that macrophages drive CD4+ TL dysfunction *via* PD-1/PD-L1 interactions, and/or by preventing direct access to Hodgkin Reed-Sternberg cells (88). Tumor cells upregulate PD-L1 to dampen cytotoxic TL attack. This upregulation is a consequence of pro-inflammatory cytokine production by tumor infiltrating immune cells: IFNγ is produced by CD4+ and CD8+ TLs and acts as a potent PD-L1 upregulator (76). In a CLL model, Beyer et al. observed a significantly increased expression of TGF-β and IL-10 in Tregs from patients. Both cytokines play an important role for the CD8+ TL inhibitory function of these cells (89). For example, in FL, malignant cells guide differentiation of CD4+ TLs, skewing the population within the tumor towards Tregs. APC, Antigen Presenting Cell; HL, Hodgkin Lymphoma; IFNγ, Interferon Gamma; IL-2, Interleukine 2; IL-10, Interleukine 10; MHC, Major Histocompatibility Complex; PD-1, Programmed Death 1; PD-L1, Programmed Death Ligand 1; PD-L2, Programmed Death Ligand 2; TAM, Tumor Associated Macrophage; TCR, T-cell Receptor; TGF-β, Transforming Growth Factor Beta; TIL, Tumor-infiltrating Lymphocyte; Treg, Regulatory T Lymphocyte.

Malignant B-cells attract non-malignant cells and modulate their plasticity, converting their environment into a supportive niche. It is therefore essential to understand how the dynamic interaction between these B-cells triggers the setting of a supportive niche, and consequently the mechanisms of B-cell immune escape (**Figure 4**) (90).

As in any adaptive immune response, lymphocyte activation requires the three signals described above (58). The B lymphoid tumor cell expresses class II MHC (Major Histocompatibility Complex) and the CD80/86 co-stimulation molecules which are functionally active and allow the tumor lymphocyte to act as an APC (83). An aberrant expression of PD-1 and its ligands PD-L1 and PD-L2 has been detected in many lymphoma subtypes, with a higher frequency for PD-L1. As was the case with solid cancers, PD-L1 reported levels vary highly between studies and within the same lymphoma subtype (58). Potential methodological issues may lead to this variability in PD-1 and PD-L1 expression measurements, and therefore, in the predictive value of these potential biomarkers. In particular, the detection method used (immunohistochemistry/IHC), flow cytometry or RNA sequencing), the specific antibody used for PD-1 or PD-L1 detection, the analyzed cell type, the sample type (bone marrow, blood, lymph node, peripheral organ), and the minimum threshold values to define PD-1 or PD-L1 expression are of consequence (58, 83). Thus, PD-L1 expression appears to be an imperfect predictor of PD-1/PD-L1 pathway inhibition efficacy, although response rates are significantly higher in patients with PD-L1-positive tumors.

Clinico-pathological studies (91–93) have investigated the expression of PD-1, PD-L1, and PD-L2 in a large number of tumor-cells or tumor-microenvironment-cells (403, 899, and 702 biopsies, respectively) in various B-cell neoplasms, whether Hodgkin or non-Hodgkin lymphoma: Burkitt Lymphoma, DLBCL, Follicular Lymphoma (FL), Mantle Cell Lymphoma, Marginal Zone Lymphoma, Primary Mediastinal Lymphoma, and CLL). In these studies, the expression of these tumor markers and

their prognostic values vary in accordance with the lymphoma subtype.

PD-1/PD-L1/PD-L2 IMPAIRMENT IN CLL

In CLL, malignant B-cells interact with neighboring cells in the lymph node, creating a microenvironment that promotes their proliferation and survival by inhibiting apoptosis and protecting them from immune system (94). One of the potential mechanisms responsible for immune escape from cytotoxic TLs in CLL is through PD-1 and both its ligands, PD-L1 and PD-L2. The neoplastic B-cells of the SLL/LLC lymph node weakly express PD-1 in most cases and series (95, 96), but more intensely and predominantly in prolymphocytes and paraimmunoblasts located in the proliferative centers of the lymph node. When expressing PD-1, circulating CLL cells also overexpress PD-L1, allowing tumor escape (94, 96, 97). Brusa et al. found a diffuse expression of PD-L1 in 9/20 samples of nodal B lymphocytes in CLL (96). This PD-L1 overexpression, by CLL B-cells exclusively, was comparable in the lymph node, the circulating blood and the bone marrow (94, 98). It was also significantly greater than that of B lymphocytes in healthy patients. This PD-1/PD-L1 increase is discordant in the literature, as some authors report no (91, 92) or feeble (92, 95) expression of PD-L1/PD-L2 on circulating CLL cells/lymph node SLL (**Table 1A**).

In this neoplastic context, CD4+ and CD8+ TLs circulating or infiltrating the tumor exhibit exhaustion profile since they also express PD-1 (101), either slightly (1/4 cases) (91, 96) or significantly increased (104), and are in close contact with CD23+ CLL cells expressing PD-L1 within the lymph nodes. These CD4+ and CD8+ TLs exhibit the features of chronic activation, with an overexpression of CD69, HLA-DR and CD57 and an under-expression of CD28 and CD62L (104). Exhaustion markers CD244, CD160 and PD-1 (101). These exhausted TLs may be the result of chronic stimulation by low affinity auto-antigens. Due to exhaustion, CD8+ TLs lose their cytotoxicity and become unable to lyse target cells. However, in CLL, and unlike TLs exhausted after chronic stimulation by a high affinity viral antigen, CD8+ TLs keep their ability to produce IFNγ and Tumor necrosis factor alpha (TNFα), with normal IL-2 production potentially protecting CLL cells from apoptosis) (104). There is a specific cytokinic context in CLL proliferative centers where IFNγ production by TL promotes PD-L1 expression on leukemia cells. Conversely, PD-1/PD-L1 interaction triggers a negative feedback loop, with PD-1-mediated significant decrease in IL-4 and IFNγ production by CD4+ and CD8+ TLs, respectively (96). Investigations using human and murine CLL models showed alterations of the immunological synapse between tumor B-cells and CD4+ and CD8+ TLs,

TABLE 1 | PD-1 and PD-L1 expression changes on malignant B-cells and tumor microenvironment TL surfaces, in CLL (**Table 1A**) or RS (**Table 1B**) context.

A (CLL)

Ref.	Number of patients	PD-1 expression				PD-L1 expression			
		Lymph node		Bone Marrow/Peripheral Blood		Lymph Node		Bone Marrow/Peripheral Blood	
		B-CLL	TIL	B-CLL	TIL	B-CLL	TIL	B-CLL	TIL
(95)	13	Yes	Yes	Yes (PB)	UD	No	UD	UD	UD
(96)	117	Yes	Yes	Yes (PB)	Yes (PB)	Yes	UD	Yes (PB)	UD
(94)	68	Yes	Yes	UD	UD	Yes	UD	Yes (PB)	UD
(99)	16	Yes	UD	UD	UD	UD	UD	UD	UD
(100)	39	Yes	Yes	UD	UD	No	UD	UD	UD
(91)	58	No	Yes	UD	UD	UD	UD	UD	UD
(98)	58	Yes	Yes	Yes	UD	Yes	UD	No	UD
(92)	37	UD	UD	UD	UD	No	UD	UD	UD
(97)	112	UD	UD	UD	UD	UD	UD	Only on MNC	UD
(101)	39	UD	UD	UD	Yes	UD	UD	UD	UD
(102)	16 (4 with tumor-invaded tissues)	Yes	Yes	UD	UD	Yes	Yes	UD	UD
(103)	18	UD	UD	UD	UD	No	UD	No	UD

B (Richter Syndrome)

Ref.	Number of patients	PD-1 expression				PD-L1 expression			
		Lymph node		Bone Marrow/Peripheral Blood		Lymph Node		Bone Marrow/Peripheral Blood	
		Richter cells	TIL	Richter cells	TIL	Richter cells	TIL	Richter cells	TIL
(100)	15	Yes	Yes	UD	UD	Yes	No	UD	UD
(99)	17	Yes	UD	UD	UD	Yes	UD	UD	UD
(102)	9 (6 with tumor-invaded tissues)	Yes	Yes	UD	UD	Yes	No	UD	UD
(103)	15 (5 with tumor-invaded tissues)	UD	UD	UD	UD	Yes	No	UD	UD

PD-1, programmed death 1; PD-L1, programmed death ligand 1; B-CLL, CLL malignant B-cells; BM, bone marrow; LN, lymph node; MNC, mononuclear cells; PB, peripheral blood; RS-MO, RS microenvironment; TIL, tumor-infiltrating lymphocytes; UD, undetermined.

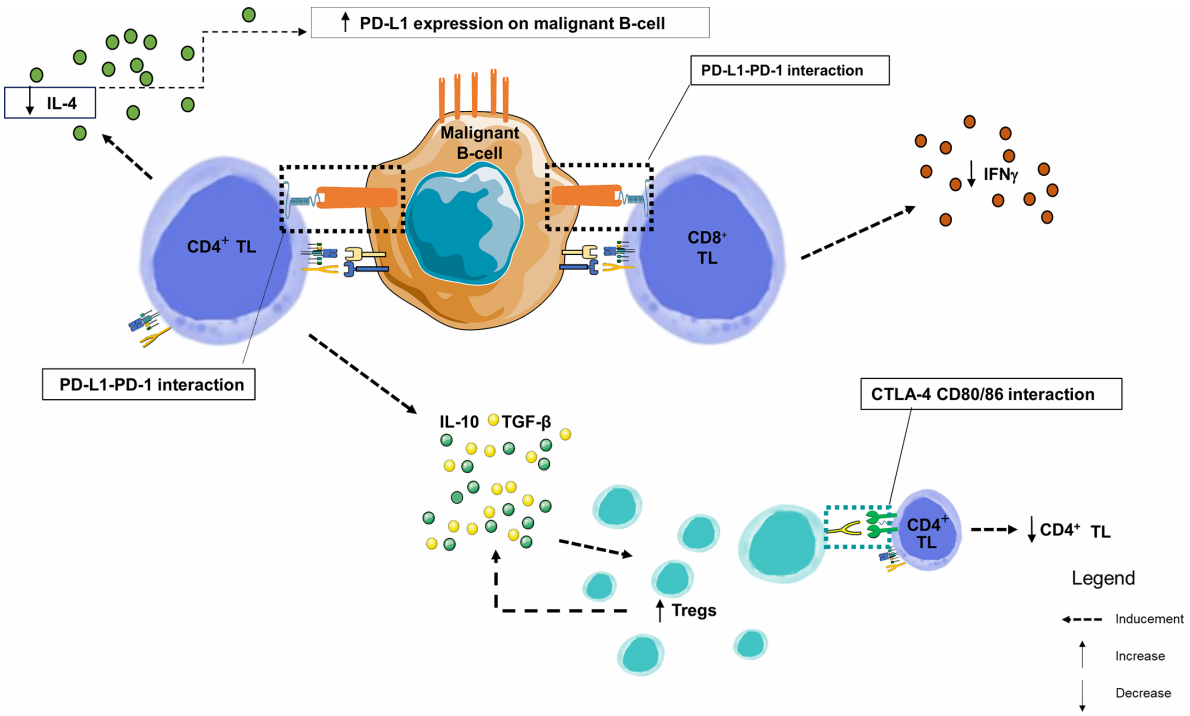

FIGURE 5 | Immune synapse in a CLL node. This figure focuses on the germinal centers of lymph nodes. By combining data from several studies in lymph nodes and peripheral blood, we observe that PD-1 and PD-L1 are heterogeneously expressed markers of B lymphocytes from chronic lymphoid leukemia. Similarly, PD-1 is overexpressed in TLs infiltrating the CLL microenvironment. Within the malignant lymph node proliferation centers, PD-1+ CD4+ TLs are in close contact and interfere with PD-L1+ CLL leukemic B-cells. The number of CD8+ and CD4+ TLs are higher in CLL patients than in healthy donors, with an increase in effector TL number and a decrease in naive TLs. IFNγ production by TLs enhances PD-L1 expression. However, when PD-1 and PD-L1 interact, there is a significant decrease in IL-4 and IFNγ production by CD4+ and CD8+ TLs, respectively, creating a negative feedback loop, and a significant increase in the proportion of CD4+ CD25+ FOXP3 + CTLA-4+ Tregs in CLL patients. TGF-β and IL-10 are overexpressed by the CD4+ TL and Tregs and play an important role in their inhibitory function (106); IFNγ, Interferon Gamma; IL-4, Interleukine 4; IL-10, Interleukine 10; PD-1, Programmed Death 1; PD-L1, Programmed Death Ligand 1; TGF-β, Transforming Growth Factor Beta; TIL, Tumor-infiltrating Lymphocyte; TL, T Lymphocyte; Tregs, Regulatory T Lymphocytes.

primarily due to the disorganization of the TL cytoskeleton through inhibition of TCR components by CLL cells (105). This leads to a decrease in TCR signaling and subsequent proliferation (decrease in production of IL-2 by CD4+ TL) and cytotoxic activity (decrease in cytokines) (**Figure 5**) (92, 101).

To study the CLL microenvironment, particularly the PD-1/PD-L1/PD-L2 axis, and to assess the functional impact of PD-1 expression on the effector function of TLs in CLL, different mouse models have been established (107). In these murine models (which compare an elderly mouse having spontaneously developed CLL to a young one with experimentally induced CLL, and to a healthy aged mouse), CD8+ PD-1+ infiltrating TLs retained their cytotoxicity capabilities but did not maintain a correct immunological synapse. IFNγ production was effectively altered but did not make these TLs exhausted. The ability of CD4+ TLs to switch from a naive phenotype to a memory TL phenotype after meeting the CLL tumor antigen was demonstrated in a similar mouse model, where CD4+ TLs that underwent phenotypic switching were able to protect tumor B-cells from apoptosis *in vitro*, and were associated with a more aggressive disease (106).

In addition to the increased expression of the PD-1/PD-L1 inhibitory receptors, an increase in CD4+ CD25+ FOXP3+ Tregs is observed in CLL, particularly in previously untreated advanced-stage CLL (89). Tregs also induce CTLA-4, another inhibitory receptor. Therefore, CTLA-4 signaling is likely to be another pathway mediating TL dysfunction in CLL.

PD-1/PD-L1/PD-L2 INTERPLAY DEREGULATION BY MALIGNANT B-CELLS PROMOTES IMMUNE ESCAPE IN RS

To date, few data are available on RS regarding the cellular interactions mediating the immunological synapse around the molecular surface marker PD-1 and its ligand PD-L1 (83, 104, 108). Here we will focus on PD-1 and PD-L1 deregulations in the context of RS, both on tumor B-cells and microenvironment cells, and the diagnostic and prognostic impact of the negative bi-directional interaction between these cells.

In a cohort of 80 patients including 39 CLLs, 15 RS, and 26 *de novo* DLBCLs, PD-1 expression in RS is significantly higher than in *de novo* DLBCLs. Only prolymphocytes and paraimmunoblasts

from proliferation centers expressed PD-1 in CLL (100) and even more markedly in "accelerated" CLLs (109). A CLL-Richter clonal relationship, assessed by *IGHV* rearrangement comparison, has been recognized as an adverse prognostic factor in RS (19). In this cohort, He et al. (100). demonstrated that PD-1 expression by large B-cells from RS was highly correlated (90%) with clonally related RS. *IGHV* sequencing is the reference method for assessing CLL-RS clonal relationship (20, 100), but this test is expensive and dependent on the availability of the CLL component at RS diagnosis. It could advantageously be replaced by PD-1 estimation with IHC, which is more accessible for routine practice (101). In contrast, PD-L1 expression was only observed in 1/17 RS samples and 1/26 cases of *de novo* DLBCLs, in the surrounding immune environment consisting of histiocytes and DC.

Evaluation of PD-1 and PD-L1 expression on tumor B-cells from 10 biopsies (6 RS and 4 CLL) available beforehand showed a slight PD-L1 increase in patients with complete response (CR) or partial response (PR) after Pembrolizumab treatment and a tendency to PD-1 overexpression in these same patients *versus* non-responders (102). In tumor-infiltrating CD3+ CD8+ TLs, PD-1 and PD-L1 levels were similar in treatment responders *versus* non-responders. With confocal microscopy, PD-1 expression was observed mainly on tumor B-cells, while PD-L1 expression was observed on histiocytes/monocytes. Of note, FISH analysis did not find amplification or duplication of the 9p24 segment, which is the chromosomal location of PD-L1 and PD-L2. In another study, PD-L1 expression was high (> or equal to 5%) on 3 out of 5 evaluable RS biopsies (103). The study did not specify whether this expression was predominant on lymphoma B-cells or on cells of the microenvironment. PD-1 expression was not measured. These results were confirmed on a 58-patient cohort including 16 CLLs, 17 RS and 25 *de novo* DLBCLs (99), with i) a high correlation between CLL-RS clonal relationship and PD-1 expression (8/9 RS clonally related with matched CLL strongly expressing PD-1) and ii) a difference in PD-1 expression between RS (14/17 positive) and *de novo* DLBCL (2/25 weakly and locally positive). These results highlight the potential role of PD-1 in distinguishing RS from *de novo* DLBCLs or from a clonally unrelated RS. In line with what is described for CLL, PD-1 and PD-L1 expressions are variable within the tumor, either predominant on TIL or on malignant B-cells.

PD-1 positive B-cells in RS share the characteristics of the widely described regulatory B lymphocytes (Bregs) (110, 111), which interact with PD-L1-expressing immune-cells of the tumor stroma and subsequently with inhibitory cytokines (TGF-β and IL-10) from adjacent TL (**Table 1B).** Bregs are described as a B lymphocyte subtype representing less than 10% of total B-cells in a healthy patient but essential for the maintenance of immunotolerance (111). Consistent pre-clinical and clinical studies suggest several distinct Bregs phenotypes involved in autoimmune diseases and cancers (112). Immune suppression mediated by CD19+ CD1d+ CD5+ or CD19+ CD24+ CD3+ Breg subtypes is mediated by IL-10 production, which in turn inhibits Th1 cell activation, Th17 cell differentiation and promotes CD4+ TL conversion into suppressive Tregs. In metastatic hepatocellular carcinoma, a new Breg subtype has been identified within tumor PD-1+ B-cells, presenting a specific phenotype. In the course of hepatocellular carcinoma progression, PD-1 is strongly expressed by tumor-infiltrating B lymphocytes and interacts with PD-L1, expressed by tumor-associated macrophages, leading to IL-10 production by B lymphocytes, inhibition of TL cytotoxic activity and tumor expansion (110). This mechanism could synergistically work with the mechanisms of lymphocyte exhaustion mediated by the interaction of TLs expressing PD-1 with cancer cells expressing PD-L1. This observation is in accordance with similar results in thyroid cancers where tumor-infiltrating PD-1+ B-cells also express PD-L1. However, unlike PD-1+ Bregs, these PD-1+ B-cells do not increase IL-10 production and here, the immunosuppressive effect is mainly mediated by PD-L1 interaction with PD-1+ TLs (113). Several remaining hypothesis need to be explored: a) Bregs expressing IL-10 could also use this PD-1/PD-L1 pathway to neutralize TL activity and b) these Bregs could play an important role in B-cell lymphomas and more particularly in RS.

In B-cell malignancies, tumor B-cells acquire Breg properties through different mechanisms, including the expression of co-inhibitory ligands, such as PD-L1/PD-L2, allowing TL exhaustion, but also the ability to induce FOXP3+ Treg expansion, to recruit myeloid-derived suppressor cells or monocytes/macrophages. Tumor B-cells can also directly express a variety of ligands and suppressive cytokines such as a) TGF-β, which promotes Treg development and inhibits CD4+ TL differentiation into Th1 or Th17, or b) IL-10 which promotes CD4+ CD25+ FOXP3+ Tregs development and CD5+ B-cell expression of Fas-L, leading to cell death *in vitro* (**Figure 6**) (112).

ENSUING THERAPEUTIC OPTIONS IN RS

In B-cell neoplasms, the PD-1/PD-L1 axis has been widely explored. There is a correlation between PD-1 and PD-L1 expression levels and prognosis (58, 83, 91, 93, 108) as well as potential for therapeutic purposes (58, 74, 83, 114). The first indication in which PD-1 and PD-L1 inhibitors have been approved to date is relapsed or refractory classical HL after autologous stem cell transplantation (SCT) and treatment with Brentuximab-Vedotin. Nivolumab was approved by FDA in 2018 (115, 116). In the same indication, Pembrolizumab obtained approval in 2017 (117). Nivolumab is a human monoclonal IgG4 kappa anti-PD-1 antibody. Pembrolizumab is also a humanized monoclonal IgG4 kappa anti-PD-1 antibody, that is devoid of any cytotoxic activity because binding of pembrolizumab to PD-1 does not engage Fc receptors or activate complement. Nivolumab and Pembrolizumab block interactions between PD-1, which is a negative regulator of TL activation, and its ligands PD-L1 and PD-L2 (118). Numerous therapeutic trials concentrate on blocking the PD-1/PD-L1 pathway to modulate anti-cancer immunity are currently ongoing (76).

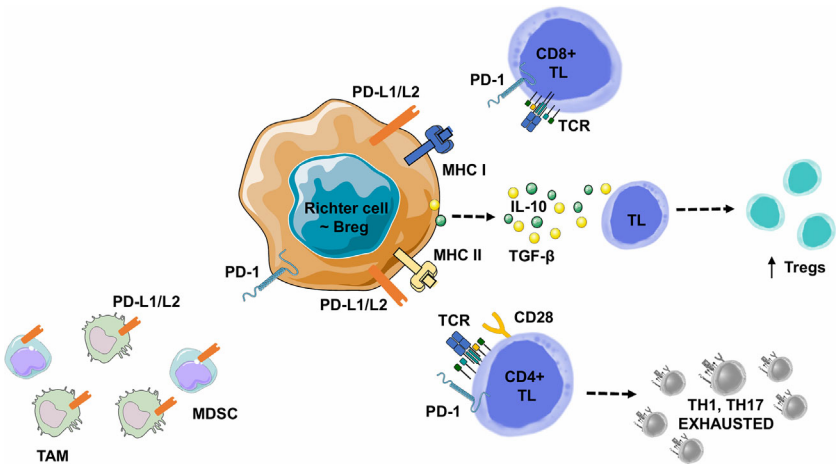

FIGURE 6 | Lymphoma B cell-mediated immune synapse in Richter syndrome. This figure focuses on mechanism of tumoral escape in the germinal center of lymph nodes. PD-1 delivers inhibitory signals to TLs after binding with its ligands PD-L1 or PD-L2, on the surface of malignant B lymphocytes, in the tumor microenvironment. Behdad et al. (99). hypothesize that in RS, tumoral B lymphocytes share characteristics with Bregs: i) expression of co-inhibitory ligands such as PD-L1/PD-L2, allowing TL exhaustion, ii) the ability to induce FOXP3+ Treg expansion, and iii) the ability to recruit Myeloid-Derived Suppressor Cell (MDSC) or monocytes/macrophages (TAMs). Malignant B-cells can also directly express a variety of suppressive ligands and cytokines: i) TGF-β to promote Treg development and inhibit the differentiation of CD4+ TLs into Th1 or Th17 lymphocytes, and ii) IL-10 to promote CD4+ CD25+ FOXP3+ Treg development and Fas-L production (particularly for CD5+ B lymphocytes), leading to cell death *in vitro*. Bregs, Regulatory B Lymphocytes; IL-10, Interleukine 10; IFNγ, Interferon Gamma; MDSC, Myeloid-Derived Suppressor Cell; MHC, Major Histocompatibility Complex; PD-1, Programmed Death 1; PD-L1, Programmed Death Ligand 1; PD-L2, Programmed Death Ligand 2; RS-DLBCL, Richter Syndrome, Diffuse Large B-Cell Lymphoma subtype; TAM, Tumor Associated Macrophage; TCR, T-cell Receptor; TGF-β, Transforming Growth Factor Beta; TL, T Lymphocyte; Tregs, Regulatory T Lymphocytes.

Improved knowledge on tumor microenvironment and particularly on the PD-1/PD-L1 axis in RS context, raises potential therapeutic options with targeted immunotherapies that blocks the interaction between PD-1 and its ligand to restore the activity of the tumor cell/TL immunological synapse. Effectiveness of immune checkpoint blockade therapies, including Pembrolizumab and Nivolumab, are demonstrated and approved for the treatment of many solid cancers (81), and relapsed or refractory HL (115, 116). Clinical trials are ongoing for other hematological malignancies (119).

In RS, three large cohorts of more than one hundred patients showed that median overall survival (OS) of RS patients is short, ranging from 5.9 to 12 months (34, 120, 121). In a large cohort of 103 RS patients, factors associated with RS development were del (17p), elevated thymidine kinase > 10 U/L, and presence of B-symptoms at the first-line treatment of CLL (119). The prognosis of RS patients who did not receive prior CLL therapy is better, with a median OS of 46.3 months versus 7.8 months ($p < 0.001$) (34). RS diagnosis is always an indication to start treatment. There is no randomized study comparing different therapeutic approaches in RS, but different treatment combinations have been individually tested. R-CHOP (Rituximab, Cyclophosphamide, Hydroxydaunorubicin, Oncovin, Prednisone) or R-CHOP-like regimens are widely used as a first-line option, with a response rate of around 67% (7% CR rate) but a progression-free survival (PFS) of only 10 months and a median survival of 15 months in eligible patients (38, 122). More intensive OFAR-type chemotherapy protocols (Oxiplatin, Fludarabine, Aracytine, Rituximab) only resulted in a CR rate of 6.5% and a median survival of 6–8 months (123, 124). These first-line protocols achieved a PR or a CR of short duration in 10%–15% cases and offered a median survival of 12 months (27% survival at 3 years). SCT can improve remission duration, usually short with chemotherapy regimens. Consolidation by an autologous or allogeneic SCT in a small subset of patients selected for their response to chemotherapy makes it possible to obtain a longer survival (75% at 3 years) (19, 125). A European retrospective study has compiled series of patients treated by autologous or allogeneic SCT: at 3 years, relapse-free survival is 27% after allogeneic SCT and 45% after autologous SCT (the non-relapse mortality at 3 years is 26% and 12%, respectively). However, most patients (85%–90%) are unfit or do not achieve an adequate response to be eligible for transplantation (126). Improving response rate to frontline therapy remains critical and new therapeutic approaches, such as Bruton Tyrosine Kinase (BTK) inhibitors yielded encouraging results (127). In this context, immune checkpoint inhibitors could therefore have a great therapeutic interest in RS.

A monocentric phase 2 clinical study tested the humanized anti-PD-1 antibody Pembrolizumab in a small cohort of 25 patients (16 relapsed CLLs and 9 RS). In RS, global response rate was 44%, PFS duration was 5.4 months, and OS was 10.7 months *versus* 3.5 months after classical immunochemotherapy courses. Patients previously treated with Ibrutinib (4/6) were still in clinical response after 11 months. About 20% of hematological adverse events above grade 3 were observed (102). However, none of the 16 CLL patients responded to Pembrolizumab. Notably, and despite a partial RS response, 3 CLLs progressed. Outside clinical trial, 10 patients with active RS and without new

therapeutic options were treated either by Pembrolizumab (n=3) or by Nivolumab (n=7). This "real life" experience showed a time to treatment failure of 1.2 months, with 9/10 patients relapsing (128).

Nivolumab was used in combination with Ibrutinib in a phase II trial on 13 patients, including 5 relapsed or refractory (R/R) CLLs, 5 RS and 3 persistent CLLs after 9 months of Ibrutinib. This study showed PR and CR in 3 and 2 RS, respectively. However, this treatment had minor effects on CLL, with 1 CR and 3 PR in R/R CLLs and none in persistent CLLs (129). The Ibrutinib + Nivolumab association was tested in a second therapeutic trial conducted in 2 steps on 141 patients (103). The first phase (n=14) aimed at evaluating safety and toxicity parameters of the combined Ibrutinib + Nivolumab, administered to patients with high risk R/R CLL/SLL with del(17p) or del(11q), *de novo* DLBCL or FL. The main objective of the second phase (n=127) was to investigate the preliminary activity of this association in 4 patients subgroups: a) 36 high risk R/R CLL/SLL with del (17p) or del (11q); b) 40 FL; c) 45 *de novo* DLBCL and d) 20 RS. None of the 20 RS patients had been previously exposed to Ibrutinib as part of the underlying CLL. In the RS cohort, grade 3-5 adverse events were mainly hematological. 11/141 (8%) patients died, including four from the RS cohort, but none were attributable to Nivolumab-Ibrutinib. In RS, the overall response rate was 65% (13/20), including 10% (2/20) CR and 55% (11/20) PR. One patient had a stable disease and 5 patients (25%) progressed. PFS was 5 months and OS 10.3 months for a median follow-up of 8.7 months; 11 patients either progressed (n=3) or died (n=8). In summary, clinical responses were observed in all cohorts, but the overall response rate was the highest in the RS group. Of note, no CR was observed in the CLL/SLL cohort, but only PR (22/36, 61%). Although the treatment under evaluation was an anti-PD-1, Younes et al. (103) measured PD-L1 expression in five patients (out of 15 available RS samples). PR associated with prolonged OS was observed in the 3/5 patients who had a high (> or equal to 5%) PD-L1 expression, in line with previous results (**Table 2**) (102).

PD-L2 is expressed in a large panel of cancers and is upregulated in various B-cell lymphoma subtypes (130, 131). This may explain PD-1 inhibitors efficacy in the context of PD-L1 negative tumors. In addition, PD-L2 expression in tumor tissues is significantly associated with PFS under Pembrolizumab treatment, regardless of PD-L1 expression (132). On the other hand PD-L2 expression is involved in resistance to anti-PD-L1 monotherapy. In this context, antitumor immunity can be restored either by replacing the anti-PD-L1 therapy by an anti-PD-1 therapy or by combining an anti-PD-L1 with an anti-PD-L2 treatment (133).

Numerous clinical trials are ongoing in RS, combining an anti-PD-1 antibody to other drugs (Jain N et al., NCT02846623 and NCT02420912; Eichhorst B et al., NCT04271956; Danilov A et al., NCT03884998; Ding W et al., NCT02332980; Woyach JA et al., NCT03892044; Acerta Clinical Trials, NCT02362035) or an anti-PD-L1 antibody (Tedeschi A et al., NCT04082897; Herrera AF et al., NCT03321643; Mato AR et al., NCT02535286). Most of these phase II studies are currently evaluating the toxicity and safety of these combinations, with efficacy endpoints as secondary objectives (**Table 3**).

TABLE 2 | Published clinical studies in RS.

Ref	Drugs	Study features	Number of patients	Schedule	CR (%)	ORR (%)	OS (months)	PFS (months)	Grade 3/4 adverse events
(102)	Pembrolizumab	Phase II unicentric clinical trial	25, including: - Relapsed CLL: 16 - RS: 9	200 mg/ 3 weeks	11	44	10.7	5.4	Thrombocytopenia (20%) Anemia (20%) Neutropenia (20%) dyspnea (8%) Hypoxia (8%)
(128)	Pembrolizumab or Nivolumab	Single center experience	10 RS: Nivolumab: 7 Pembrolizumab: 3	Doses according to their US label	10	UD	4.2	1.2	UD
(129)	Nivolumab and Ibrutinib	Phase II clinical trial	13, including: - Relapsed/ refractory CLL: 5 - CLL treated with ibrutinib for ≥ 9 months with persistent disease: 3 - RS: 5	Nivolumab 3mg/kg/15 weeks (cycle 1) Nivolumab 3mg/kg/15 weeks + Ibrutinib 420mg/day (cycle 2)	60	UD	UD	UD	/
(103)	Nivolumab and Ibrutinib	Phase 1/2a open-label multicentric clinical trial	141, including: - CLL/SLL: 36 - de novo DLBCL: 45 - FL: 40 - RS: 20	Nivolumab 3mg/kg / 15weeks + Ibrutinib 420 or 560 mg/day.	10	65	10.3	5	Neutropenia (25%), Anemia (35%), Thrombocytopenia (10%) Pneumoniae (10%) Rash (10%) Dyspnea (10%) hypotension (15%)

CLL, chronic lymphocytic leukemia; CR, complete response; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; SLL, small lymphocytic lymphoma; RS, Richter syndrome; UD, undetermined.

TABLE 3 | Ongoing clinical trials in RS.

Reference	Drugs	Study features	Patients enrollment	Protocol	Primary outcome	Secondary outcome
NCT02846623	Atezolizumab + Obinutuzumab + Venetoclax	Phase II open label clinical trial	65 R/R CLL/SLL and RS	A + O + V for 14 cycles of 28 days vs A+ O + V for 25 cycles of 28 days.	Minimal residual disease negative rate	AEs, best ORR, CRR, duration of response, PFS, OS
NCT04271956	Zanubrutinib + Tislelizumab	Prospective phase II open label multicenter clinical trial	45 RS	T + Z for induction and consolidation (6 cycles each), then maintenance until DP or allo-SCT	ORR after induction according to the Lugano Classification	ORR after induction therapy (IWCLL criteria) ORR after consolidation therapy, PFS, OS, TTNT, duration of response, AEs
NCT03884998	Copanlisib+ Nivolumab	Phase I open label clinical trial	15 RS or Transformed Indolent NHL	C + N for 12 cycles of 28 days in the absence of DP or UT.	Incidence of dose-limiting toxicities & AEs	ORR, duration of treatment, PFS, OS
NCT02332980	Pembrolizumab + Idelalisib or Pembrolizumab + Ibrutinib or Pembrolizumab alone	Phase II open label clinical trial	68 R/R CLL or other low-grade B NHL	P for 12 cycles, or P + I or P + Id for 12-24 cycles in the absence of DP or UT.	Confirmed response	CRR, AEs incidence, ORR, PFS, survival
NCT03892044	Duvelisib and Nivolumab	Phase I open label clinical trial	44 RS or transformed FL	N. + D for 28 days cycles until DP or UT.	Maximal Tolerated Dose	ORR, PFS, OS
NCT04082897	Atezolizumab and Obinituzumab and Venetoclax	Phase II open-labeled, uncontrolled, multicenter clinical trial	28 RS	A + O + V from cycle 1 to 8; A + V from cycle 9 to 18; V only from cycle 19 to 35	ORR	AEs (CTCAE v4), CRR, duration of response, PFS, OS
NCT02420912	Nivolumab and Ibrutinib	Phase II open-labelled non-randomized clinical trial	72 R/R or high-risk untreated CLL, SLL, or RS	For RS: N+ I for 1 or 2-24 cycles if no DP or UT.	CR or CR with incomplete BM recovery	AEs (CTCAE v4), OS, PFS
NCT03321643	Atezolizumab and Rituximab and Oxaliplatin and Gemcitabine	Phase I open-label sign group assignment clinical trial	30 transformed DLBCL (including RS)	Induction: [R + Ox + Gem] + A starting cycle 2. Maintenance: R + A	AEs (CTCAE v5), Maximal Tolerated Dose	CRR, best ORR, biomarker analysis
NCT02362035	Acalabrutinib and Pembrolizumab	Phase Ib/II open label clinical trial	161 B-cell malignancies	UD	AEs	UD
NCT02535286	Ublituximab and Umbralisib and Cosibelimab	Phase I open label clinical trial	20 R/R CLL or RS	Ub followed by maintenance infusions of Um. + Cos	AEs	ORR
NCT03121534	Blinatumomab	Phase II open label clinical trial	10 RS	Induction 8 weeks. If objective response: consolidation 4 weeks	ORR	Toxicity
NCT03931642	R-CHOP and Blinatumomab	Phase II open label clinical trial	35 RS (DLBCL)	2 R-CHOP cycles then Bl if CR and no measurable lesion	CRR	AEs (CTCAE v4), OR, CR
NCT02924402	XmAb13676	Phase I open label clinical trial	66 non B-cell NHL, CLL/SLL/RS.	XmAb13676 administered weekly up to 8 weeks	AEs (CTCAE v4), max tolerated or recommended dose	/

A, atezolizumab (anti-PD-L1); Ac, acalabrutinib (BTK inhibitor); AEs, adverse events; CTCAE, Common Terminology Criteria for Adverse Events; allo-SCT, allogeneic stem cell transplantation; BM, bone marrow; Bl, blinatumomab (anti-CD19 and anti-CD3 bispecific antibody); BTK; Bruton tyrosine kinase; CLL, chronic lymphocytic leukemia; Cop, copanlisib (PI3Kα,δ inhibitor); Cos, cosibelimab (anti-PD-L1); CR, complete response; CRR, complete response rate; D, duvelisib (inhibitor of PI3Kδ and PI3Kγ); DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; DP, disease progression; Gem, gemcitabine; I, ibrutinib (BTK inhibitor); Id, idelalisib (PI3Kδ Inhibitor); IWCLL, International Workshop on Chronic Lymphocytic Leukemia; N, nivolumab (anti-PD-1); NHL, non-Hodgkin lymphoma; O, obinutuzumab (anti-CD20); ORR, overall response rate; OS, overall survival; Ox, oxaliplatin; P, pembrolizumab (anti-PD-1); PFS, progression-free survival; R, rituximab (anti-CD20); R-CHOP, rituximab, cyclophosphamide, hydroxydaunorubicin, oncovin, prednisone; R/R, relapsed or refractory; RS, Richter syndrome; SLL, small lymphocytic lymphoma; T, tislelizumab (anti-PD-1); TTNT, time to next treatment; Ub, ublituximab (anti-CD20); UD, undetermined; Um, umbralisib (anti-PI3Kδ and CK1e); UT, unacceptable toxicity; V, venetoclax (anti-BCL-2); X, XmAb13676 (anti-CD20 and anti-CD3 bispecific antibody); Z, zanubrutinib (BTK inhibitor).

Other immunotherapies have already been successfully tested in hematological diseases such as bispecific CD19-CD3 antibodies (Blinatumomab). Bispecific antibodies transiently induce a synapse between target cancer B-cells and cytotoxic TLs, resulting in TL activation and lysis of the malignant B-cell. This molecule provided a CR at 35 days of treatment in a case report: it involved a 63-year-old man whose RS was refractory to two courses of R-ICE (Rituximab- Ifosfamide-Cisplatine-Etoposide) followed by a course of R-DHAP (Rituximab-Cytarabine-Cisplatine-Dexamethasone) + Ibrutinib. In the aftermath, he was able to benefit from an allogeneic SCT and remained in CR for 130 days of treatment. Of note is the occurrence of grade 3 encephalopathy on day 16, which resolved after a dose reduction (134). A phase II clinical trial evaluating the overall response rate and potential toxicity of Blinatumomab in 10 RS patients is currently underway and results will be available in 2021 (Thompson PA et al., NCT03121534). In the BLINART clinical trial, also currently underway (NCT03931642), Blinatumomab is administered after 2 courses of debulking R-CHOP in 35 RS cases. The hypothesis proposed is the improvement of the CR rate at 8 weeks of treatment. New bispecific antibodies are currently tested. An anti-CD20 combined with an anti-CD3 antibody (XmAb13676) is currently in a phase I study in two groups: patients with non-CLL B-cell malignancies and patients with CLL/SLL/RS (NCT02924402) (**Table 3**, **Figure 7**). Finally, Ipilimumab, an anti-CTLA-4 antibody, has been used in phase I studies in a few relapsed HL cases (135), and in R/R Non-Hodgkin Lymphoma cases (136). There is no ongoing clinical trial or published clinical trial about RS to date.

CONCLUSION

A deeper characterization of RS is ongoing with the advent of novel sequencing and staining technologies, which can lead to a better understanding of this difficult-to-treat entity. Recent data enlighten the role of TL infiltration and immune system in RS specimens. TL exhaustion is driven in part by immune checkpoint deregulation, including high expression levels of checkpoint inhibitory molecules on TLs, such as PD-1, and expression of ligands for these molecules on CLL cells. Recent data showed that PD-1 expression by neoplastic B-cell was weak in both CLL and *de novo* DLBCL and strong in RS. Interestingly, this observation was linked to clinical responses to the PD-1 blocking antibody Pembrolizumab in RS, whereas no clear activity was observed in CLL. Furthermore, high tumor cell mutational burden is emerging as a predictor of improved therapeutic response to these agents. This could increase the neoantigens load at RS, leading to an immune response.

Exploring the expression of the PD-1/PD-L1/PD-L2 axis components in the immune ecosystem of RS at diagnosis is of importance, both for evaluating these potential biomarkers and to initiate a therapy specifically targeting the checkpoint inhibitors.

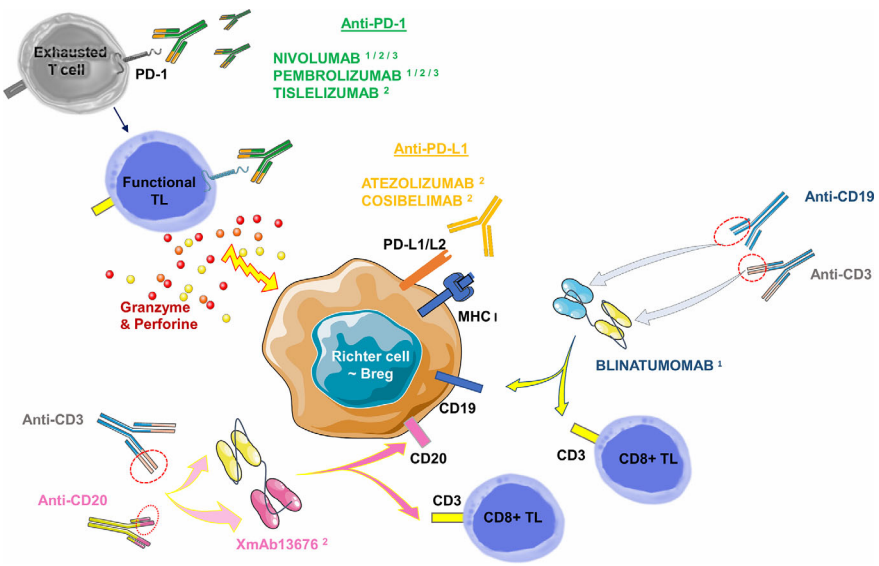

FIGURE 7 | Monoclonal antibodies targeting the PD-1/PD-L1 axis (checkpoint inhibitors) and bispecific antibodies commercially available or in published/ongoing clinical trials in RS. Monoclonal antibodies targeting the PD-1/PD-L1 axis can be divided into two groups: i) against PD-1 receptor and ii) against its ligands PD-L1 or PD-L2. Anti-PD-1 antibodies are mainly represented by Nivolumab: a full human IgG4, and Pembrolizumab: a humanized IgG4. Tislelizumab (BGB-A317) is a new humanized IgG4 anti-PD-1 antibody. Anti-PD-L1 antibodies are mainly represented by Atezolizumab, a humanized antibody. Cosibelimab (TG-1501) is a novel, fully humanized anti-PD-L1 antibody. Bispecific antibodies are designed to direct cytotoxic TLs expressing CD3 towards B-cells expressing CD19 (Blinatumomab) or CD20 (XmAb13676). Breg, Regulatory B Lymphocyte; MHC, Major Histocompatibility Complex; PD-1, Programmed Death 1; PD-L1, Programmed Death Ligand 1; PD-L2, Programmed Death Ligand 2; TL, T Lymphocyte. [1]: commercially approved in other hemopathies (relapsed Hodgkin Lymphoma for Nivolumab and Pembrolizumab, relapsed/refractory Acute B Lymphoid Leukemia for Blinatumomab). [2]: clinical trials published in RS. [3]: clinical trials underway in RS.

These immunotherapies are already approved in relapsed HL and are currently under evaluation in multiple therapeutic trials focusing on B-cell malignancies. Positive results in CLL, DLBCL, FL, and HL make them prone to be included in future therapeutic strategies. Regarding RS, the expression of PD-1 and PD-L1 is variable according to the series, and the data are discordant concerning the location of these membrane proteins. Regarding therapeutic trials, they seem rather encouraging, with better overall response rates than in CLL. PD-1/PD-L1 level measurements using IHC should be systematic at RS diagnosis to tailor the use of these immunotherapies, already widely used in oncology in the context of new clinical trials precisely developed for this indication.

AUTHOR CONTRIBUTIONS

JB and PF supervised the manuscript. JB, HA, and RM selected and reviewed the papers. JB, HA, SH, and ABN made the figures and tables. JB, HA, SH, PF, and ABN wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

MINI REVIEW
published: 08 January 2021
doi: 10.3389/fonc.2020.606540

Toward Therapeutic Targeting of Bone Marrow Leukemic Niche Protective Signals in B-Cell Acute Lymphoblastic Leukemia

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B-cell acute lymphoblastic leukemia (B-ALL) represents the malignant counterpart of bone marrow (BM) differentiating B cells and occurs most frequently in children. While new combinations of chemotherapeutic agents have dramatically improved the prognosis for young patients, disease outcome remains poor after relapse or in adult patients. This is likely due to heterogeneity of B-ALL response to treatment which relies not only on intrinsic properties of leukemic cells, but also on extrinsic protective cues transmitted by the tumor cell microenvironment. Alternatively, leukemic cells have the capacity to shape their microenvironment towards their needs. Most knowledge on the role of protective niches has emerged from the identification of mesenchymal and endothelial cells controlling hematopoietic stem cell self-renewal or B cell differentiation. In this review, we discuss the current knowledge about B-ALL protective niches and the development of therapies targeting the crosstalk between leukemic cells and their microenvironment.

Keywords: B-cell acute lymphoblastic leukemia, bone marrow, leukemic niches, targeted treatments, B cell development

OPEN ACCESS

Edited by:
Jérôme Paggetti, Luxembourg Institute of Health, Luxembourg

Reviewed by:
Rima Haddad, Université Paris-Saclay, France
Simon Mendez-Ferrer, University of Cambridge, United Kingdom

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Specialty section:
This article was submitted to Hematologic Malignancies, a section of the journal Frontiers in Oncology

Received: *15 September 2020*
Accepted: *23 November 2020*
Published: *08 January 2021*

Citation:
Delahaye MC, Salem K-I, Pelletier J, Aurrand-Lions M and Mancini SJC (2021) Toward Therapeutic Targeting of Bone Marrow Leukemic Niche Protective Signals in B-Cell Acute Lymphoblastic Leukemia. Front. Oncol. 10:606540. doi: 10.3389/fonc.2020.606540

INTRODUCTION

B cell Acute Lymphoblastic Leukemia (B-ALL) is characterized by differentiation arrest and malignant transformation of developing bone marrow (BM) B cells. B-ALL is the most common cancer during childhood and is relatively well treated, with a 5-year overall survival reaching 90% (1). The treatment is largely based on chemotherapy and despite the good prognosis, 20% of children relapse with a survival rate of 30% (2). The incidence of B-ALL for adults is lower than for children but concerns a similar number of patients. While adult patient management has also improved and allowed the mortality rate to decrease, prognosis remains poor with 60% of relapse and a median survival of 3 to 6 months following salvage chemotherapies (3–5). Furthermore, there is a high risk to develop long term sequelae related to chemotherapy, including secondary neoplasms, chronic health conditions, or endocrine dysfunctions (6).

Leukemia transformation is driven by a combination of genetic abnormalities including translocations, MLL rearrangements, hyper and hypodiploid karyotypes, gene deletions, point mutations, which lead to aberrant expression of constitutively active or inactive regulatory proteins (7). In addition to cell-autonomous alterations of key regulatory pathways, growing evidences support the influence of non-cell autonomous cues on tumor progression and resistance to therapies that are transmitted by cells

of the BM microenvironment (8). These supportive cellular niches are composed of endothelial, immune, and mesenchymal cells [including C-X-C Motif Chemokine Ligand 12 (CXCL12) abundant reticular (CAR) cells, osteolineage cells, adipocytes] which have the capacity to secrete soluble factors and to establish direct contacts with leukemic cells. Adrenergic nerves were also found to regulate early hematopoiesis as the β2 and β3 adrenergic receptors expressed by the microenvironment are oppositely involved in myeloid and lymphoid skewing of hematopoietic stem cells (HSC) respectively (9, 10). Finally, non-cell autonomous cues related to the hypoxic nature of the BM also play an important role in lymphoid development. However, despite tremendous progress made in the cellular and molecular characterization of normal differentiating B cell niches (11), the knowledge on B-ALL supportive niches remains relatively limited. Therefore, because of the influence of the microenvironment on leukemic growth and chemoresistance, targeting the leukemic niche could represent an attractive therapeutic adjuvant therapy to improve current treatments.

In this review, we present how the BM microenvironment can influence development and survival of normal and leukemic B cells. We also discuss the potential of new innovative therapeutic strategies targeting the crosstalk between leukemic cells and their microenvironment.

NORMAL B CELL NICHES

The organization of the different steps of B lymphopoiesis in the BM is conserved between mouse and human and allows the acquisition of a diverse repertoire of non-autoreactive B cell receptors (BCR). B cell commitment is definitive upon B cell progenitor (pre-pro-B cell) entry in the pro-B cell stage and Pax5 upregulation (12). In mouse and human, pre-pro-B cells are marked by the early expression of pre-BCR and BCR components (CD79a, VpreB) (13, 14). Upon Pax5 expression, pro-B cells upregulate CD19, RAG (recombination activating gene) proteins, and the terminal deoxynucleotidyl transferase (TdT) involved in immunoglobulin heavy chain (IgH) gene recombination. As recombination is a random process forming imprecise junctions between variable gene segments coding for the IgH chain, an initial step of interleukin-7 (IL7)-dependent proliferation increases the odds to express a functional IgH chain and thus increases diversity (15). The IgH chain is then expressed at the pre-B cell stage as part of a pre-BCR, following association with the surrogate light chain (SLC), composed of the invariant λ5 (λ-like in human) and VpreB proteins, and with the CD79a/CD79b signaling complex. Again to increase diversity, Pre-BCR signaling induces clonal expansion of pre-B cells before the initiation of rearrangements of genes coding for the Ig light chain (IgL) in late pre-B cells. Finally, functional IgL chains are associated to the IgH chain at the immature B cell stage to form the BCR. Immature B cells expressing a non-autoreactive BCR can leave the BM to finish their maturation.

Different cellular niches control these differentiation steps through secretion of growth factors and direct cell-cell interactions. Among soluble factors, CXCL12 and IL7 are essential for early B cell development. The main function of CXCL12 is to retain early B cells in the BM, close to their nurturing niches, although a role on lymphopoiesis cannot be excluded as it synergizes with IL7 to induce early B cell proliferation and survival *in vitro* (16–19). In addition to its role as a chemoattractant, CXCL12 induces adhesion of pro-B and pre-B cells by activating the interaction of α4β1-integrin (VLA-4) to VCAM-1 through a focal adhesion kinase (FAK)-dependent pathway (20, 21). VLA-4 expression was indeed shown to be crucial for early B cell development in VLA4 deficient mice (22). Importantly, the decreased expression of CXCR4, receptor for CXCL12, favors immature B cells egress to the periphery through downregulation of VCAM-1-mediated adhesion (23).

In mouse, IL7 is responsible for pre-pro-B and early pro-B cell proliferation (24). In human, as B cells were still found in the peripheral blood of patients presenting defects in IL7 receptor (IL7R) signaling, it was first proposed that IL7 was not required for B cell development (25). However, recent results have demonstrated that IL7 induces human B cell development (26–28). This discrepancy is probably due to the presence of IL7-independent B cells originating from fetal life in patients compromised for IL7R signaling, similarly to what was observed for $IL7^{-/-}$ mice (29).

BM niches for early differentiating B cells have been identified recently (**Figure 1**). The presence of CXCL12 expressing stromal cells associated to BM sinusoids was demonstrated using mice with a knock-in of Green Fluorescent Protein (GFP) under the control of the Cxcl12 promoter (30, 31). Pre-pro-B cells were found in contact with these peri-sinusoidal stromal (PSS) cells (**Figure 1**) (30). Later on, PSS cells were found to co-express IL7 and to support the development of pro-B cells (32–34). Importantly, human pro-B cells identified based on TdT expression were also found to be located close to similar peri-vascular stromal cells expressing both CXCL12 and IL7 (34). The specific interactions of pro-B cells with their supportive niche were further deciphered through the analysis of the trans-interactome between the partner cells and led to the identification of the ligand-receptor pair *Plxdc1/Nid1* (34). Plxdc1 is an adhesion molecule regulated by Pax5 (35) and specifically expressed by pro-B cells as compared to other B cell subsets, while Nidogen-1 is part of the extra-cellular matrix secreted by PSS cells. The analysis of $Nid1^{-/-}$ mice showed that this interaction was important for the retention in the supportive niche and consequently for the IL7-dependent proliferation of early pro-B cells. This result suggests that CXCL12/CXCR4 dependent chemo-attraction and VLA-4/VCAM-1-dependent firm adhesion of pro-B cells to PSS cells depend on initial Plxdc1/Nidogen-1 interaction.

Upon differentiation towards the pre-B cell stage, cells were shown to be located in close vicinity to Galectin-1 (GAL1) expressing mesenchymal cells which are away from the sinusoids and IL7-expressing PSS cells (32). This result is consistent with the fact that pro-B cells need high IL7 concentrations to be maintained and proliferate, while a low dose favors pre-B cell differentiation and expansion (36). GAL1 is a ligand for the pre-BCR identified in both human and mouse which binds the λ5 subunit through direct protein-protein interactions (37, 38). GAL1 is also a lectin that binds specifically β-galactosides and particularly glycosylated chains of the VLA-4, VLA-5, and LFA-1 integrins expressed by pre-B cells (39). Efficient differentiation of pre-B cells depends on specific

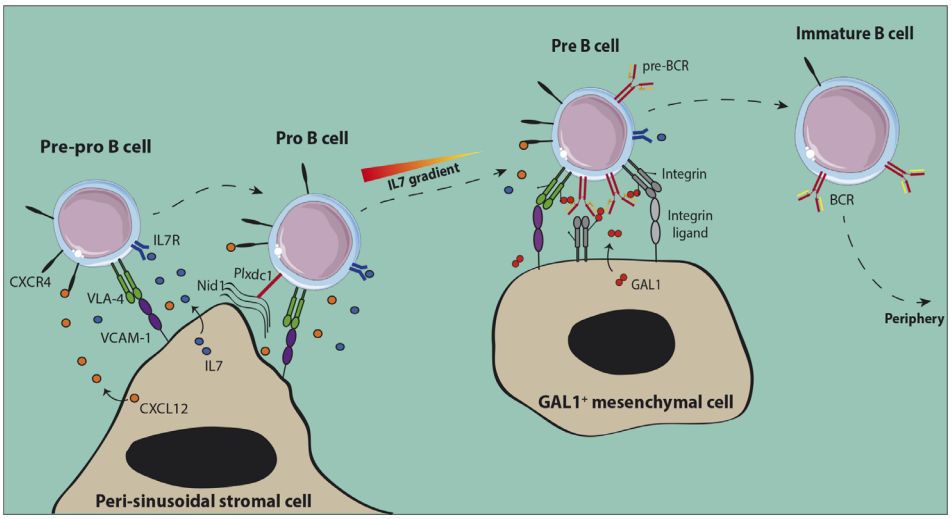

FIGURE 1 | Normal early B cell niches in the bone marrow. Both pre-pro-B and pro-B cells are in contact with peri-sinusoidal stromal cells which express high levels of CXCL12 and IL-7. Upon expression of the pre-BCR, pre-B cells move to IL-7^{low} regions close to Galectin-1 (GAL1) expressing stromal cells. GAL1-dependent cross-linking of the pre-BCR induces proliferation of pre-B cells before the initiation of IgL rearrangements. After expression of the BCR, non-autoreactive immature B cells leave the BM to achieve their maturation in the periphery.

contacts with stromal cells, involving tri-partite interactions between pre-BCR, GAL1, and integrins, and then binding of integrins with their respective ligands expressed at the surface of stromal cells (37–39).

The BM is a highly hypoxic organ with a local oxygen tension (pO_2) ranging from 1.3% away from the endosteum to 1.8% close to it (40). Hypoxia induces a cellular response through the stabilization of the hypoxia-inducible factor (HIF)-1α and -2α and then activation of downstream effectors (41). It has been shown for human lymphoid-primed multipotent progenitors (LMPPs) that *in vitro* cultures in hypoxic versus normoxic conditions stabilize HIF-1α and HIF-2α and favor a lymphoid gene expression program (42). In mouse and human, hypoxia-dependent genes are highly expressed from pre-pro-B to pre-B cells and decreased in immature B cells (43). The B cell specific deletion of the *Vhl* (von Hippel-Lindau) gene, a E3 ubiquitin ligase which drives HIF proteins to degradation in presence of oxygen, leads to a constitutive activation of HIF proteins and a severe decrease in peripheral B cells. This phenotype was found to be HIF-1α-dependent and linked to a decrease in IgH repertoire diversity from the pro-B cell stage, a block at the immature B cell stage and a lower BCR editing. Dysregulation of HIF-1α in immature B cells leads to a decreased BCR and CD19 expression and to a higher cell death related to an increased expression of the pro-apoptotic protein BIM. Consequently, HIF-1α and more generally hypoxia contributes to the normal development of B cells.

LEUKEMIC B CELL NICHES

For a long time, it has been considered that tumorigenesis was a cell-autonomous process. However, it has been observed 50 years ago in the case of hematopoietic cell transplantation that some patients could develop donor cell leukemia although the donor was presumably healthy and did not develop leukemia in the following months either (44). These observations were in accordance with the seed and soil theory which stands that similarly to plants, malignant cells ("seeds") likely need a favorable or permissive microenvironment ("soil") to grow (45).

B-ALL in co-culture with stromal cells were indeed found to be protected from apoptosis, confirming that genetic alterations are not sufficient for the maintenance of leukemic cells (46). Stromal cells were further shown to protect leukemic cells from chemotherapeutic drugs *in vitro* (47). More recent studies have highlighted *in vivo* the presence of dormant cells, called leukemia initiating cells (LIC), protected by the microenvironment, which are resistant to therapy and involved in relapse (48, 49). These cells, which were identified following transplantation of primary B-ALL to immunodeficient mice, are similar to leukemic cells isolated from patients with minimal residual disease (MRD). Furthermore, they present gene expression signatures of quiescent cells and most particularly of hematopoietic stem cells (HSC). These LIC are latent sub-clones, in a dormant state, which may be rare and present at the time of diagnosis or may be the result of sub-clonal evolution (48). Their quiescence is probably induced by interactions with the microenvironment as they start proliferating and become sensitive to chemotherapy in *in vitro* cultures, similarly to bulk leukemic cells (49). The influence of stromal cells was confirmed in patient derived xenograft (PDX) mice. Upon transplantation of B-ALL cells, the invasion of BM by blast cells led to progressive damages in the vascular structure and a loss in N-Cadherin$^+$ cells likely composed of stromal and osteoblastic cells (50, 51). Interestingly, following Ara-C treatment, resistant leukemic cells were found associated to stromal cells phenotypically similar to CD51$^+$LepR$^+$NG2$^-$ PSS cells (34). Furthermore, specific expression of the chemoattractant CCL3 by the resistant leukemic cells favored migration of the

CCR1$^+$ protective stromal cells in their vicinity (**Figure 2**). These results confirm the existence of protective leukemic niches and that leukemic cells actively shape their own supportive niches under the pressure of chemotherapeutic treatments.

Stromal cells have the capacity to directly inhibit the effect of therapeutic agents used for B-ALL treatment. B-ALL strongly relies on extracellular sources of asparagine (Asn) related to their low expression level of Asn synthetase (ASNS) (52). Therapeutic treatments therefore take advantage of L-asparaginase to deprive leukemic cells from this crucial amino acid. Stromal cells were shown to express 20 times higher levels of ASNS than B-ALL and to be an important source of Asn probably at the origin of L-asparaginase treatment resistance (53). Interestingly, it has been observed that B-ALL in co-culture with stromal cells produce insulin-like growth factor (IGF) binding protein 7 (IGFBP7) associated with L-asparaginase resistance (54). In this study, the authors found that IGFBP7 enhanced ASNS expression and Asn secretion by stromal cells in an insulin/IGF dependent manner (**Figure 2**).

B-ALL subtypes still possess features of their normal differentiating B cell counterparts and their survival and development therefore partly rely on similar signals transmitted by stromal cell niches. B-ALL express VLA-4 and the importance of the VLA-4/VCAM-1 pair in adhesion to stromal cells was first shown *in vitro* using blocking antibodies (47, 55). Furthermore, VCAM-1 over-expression in stromal cell lines enhanced B-ALL resistance to chemotherapeutic drugs *in vitro* while VLA-4 negative variants of the B-ALL cell line Nalm6 had a decreased capacity to engraft in immunodeficient mice (56, 57). Finally, the outcome was poorer in MRD$^+$ childhood B-ALL with a high expression of VLA-4 (58). Integrin mediated adhesion involves the reciprocal activation of the phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway. In B-ALL, AKT activation is responsible for the inhibition of chemotherapy-induced apoptosis and was shown to be sustained by stromal cells *in vitro* (59). In addition, the PI3K inhibitors wortmannin or LY294002 impaired the protective effect of stromal cells (56, 59). Activation of AKT was associated with a poor prognosis and to chemoresistance in childhood B-ALL (60). Finally, VLA-4/VCAM-1 interaction also induces the activation of nuclear factor (NF)-κB in both leukemic cells and stromal cells *in vitro* (61). Importantly, inhibition of the NF-kB signaling pathway in stromal cells affects their chemoprotective function *in vitro* and *in vivo*,

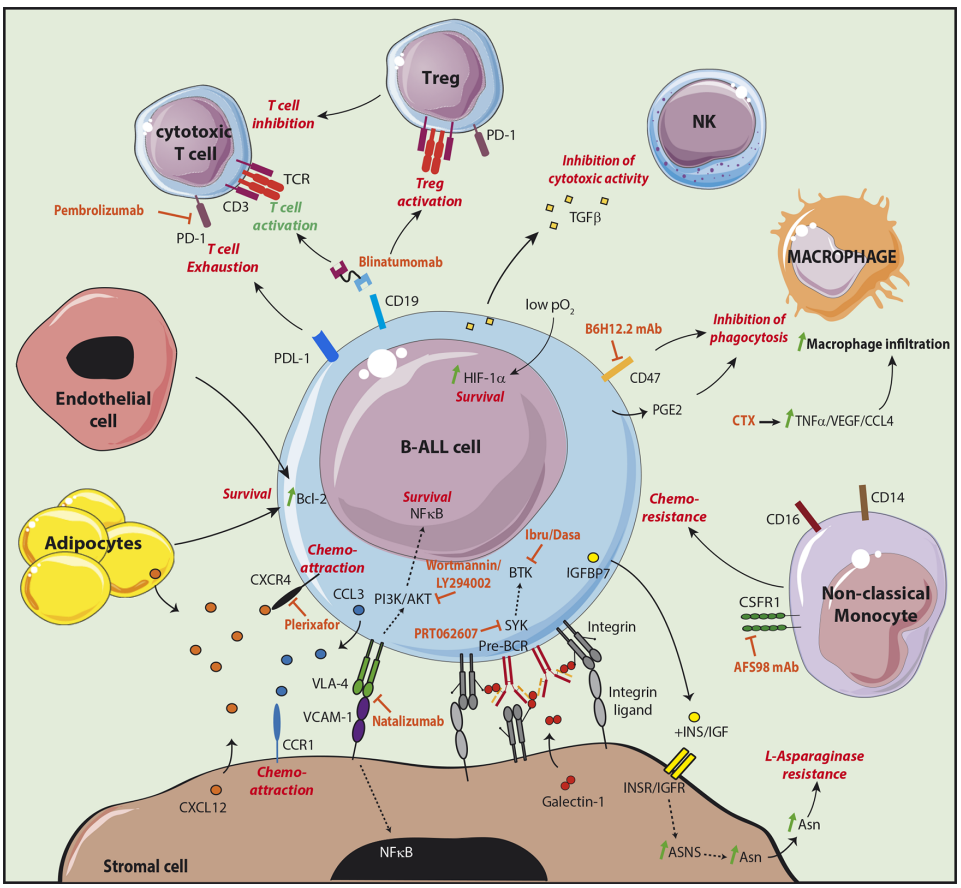

FIGURE 2 | Targeting the leukemic B cell niche. The different molecular interactions of B-ALL cells with cells supporting their survival and development are shown. The supportive signals transmitted by the different partners are shown in red. The treatments targeting the supportive niche are shown in orange. The CD19/CD3 bispecific T-cell engager blinatumomab allows the redirection and the activation of cytotoxic T cells. However, an increased Treg activation was observed in non-responder patients, as well as an increase of the checkpoint molecule PD-1 on T cells and of its ligand PDL-1 on leukemic cells.

indicating that the reciprocal activation of leukemic cells and stromal cells by integrins is a key event in chemoresistance mechanisms.

Similarly to their normal counterparts, B-ALL integrin β1-dependent adhesion to stromal cells was shown to be increased through the activation of the CXCL12/CXCR4 signaling pathway (62). However, the influence of CXCL12 as a pro-survival factor per se remains controversial (63, 64). The chemoattractive function of CXCL12 on B-ALL cell lines and patient samples was first demonstrated with transmigration assays *in vitro* (65). Furthermore, homing to the BM of immunodeficient mice was decreased after desensitization of CXCR4 with an *in vitro* exposure to CXCL12 but also after pre-treatment with pertussis toxin—inhibitor of the chemokine receptor Gαi-mediated signaling—or with the CXCR4 inhibitor AMD3100 (62, 66). As expected, Nalm6 cells injected to immunodeficient mice home to BM peri-sinusoidal regions in the vicinity of the CXCL12-expressing PSS cells (66). Importantly, in childhood B-ALL, higher levels of CXCR4 were correlated to an increased extramedullary organ infiltration and to a poor outcome. However, it is not clear whether high CXCR4 is a prognosis factor for extramedullary relapse (67, 68). In adult, overall survival was correlated to phosphorylated CXCR4 but not to CXCR4 expression, indicating that activated CXCR4 may be a better prognosis factor (69). Larger cohorts will be needed to confirm these results.

The pre-BCR is expressed by a B-ALL subset equivalent to pre-B cells which is identified as being intracellular Igμ positive ($ic\mu^+$) (70). Importantly, phosphorylation of key tyrosine kinases downstream of the pre-BCR in $ic\mu^+$ patient samples is increased and treatment with an anti IgH antibody effectively stimulates pre-BCR signaling (71). In addition, similarly to normal mouse pre-B cells, co-culture of the human pre-BCR^+ cell line Nalm6 with stromal cells induced a GAL1-dependent clustering of the pre-BCR at the leukemic/stromal cell synapse and activation of the pre-BCR (37–39). Stromal cells are therefore likely to give a proliferative advantage to pre-BCR^+ leukemic cells through direct contacts and production of GAL1.

In addition to stromal cells, adipocytes were also shown to play protective roles in B-ALL. CXCL12 produced by adipocytes induces the migration of leukemic cells into adipose tissue (72, 73). Interestingly, co-cultures of B-ALL cell lines with the 3T3-L1 cell line differentiated in adipocytes or with adipose tissue induce a resistance to chemotherapeutic treatments (72, 74). Furthermore, while the overall survival of obese C57Bl/6 mice transplanted with the murine leukemic 8093 cell line was similar to control mice, their survival upon Vincristine treatment was decreased (74). The 8083 cell line treated *in vitro* with Vincristine was found to upregulate the anti-apoptotic proteins Pim2 and Bcl2 when co-cultured in presence of the 3T3-L1 fibroblasts differentiated in adipocytes as compared to undifferentiated cells. It has been further demonstrated in the case of human T-ALL transplanted to immunodeficient mice, that leukemic cells are more quiescent and metabolically less active in the BM of adipocyte-rich tail vertebrae or in gonadal adipose tissue than in the BM of adipocyte-poor femur or thoracic vertebrae (75). Adipocyte-dependent molecular cues controlling leukemic cell survival and quiescence remain to be identified. Although these different results point to a role of adipose tissue and adipocytes in the resistance to treatments, the correlation between obesity and outcome remains controversial and needs to be confirmed through the analysis of large cohorts of patients (76).

Finally, the cross-talk between endothelial cells and B-ALL has been poorly studied. B-ALL patients show an increased microvascularization in the BM associated to an increase in most of the cases of the pro-angiogenic factor basic fibroblast growth factor (bFGF) but not of vascular endothelial growth factor (VEGF) (77, 78). The importance of angiogenesis in B-ALL prognosis is still controversial and has been described in details elsewhere (79). It has been however shown *in vitro* that endothelial cells protect B-ALL blasts from apoptosis through over-expression of the anti-apoptotic protein Bcl-2 (78). Further investigations will be needed to confirm this result and identify the molecular interactions taking place. Vascular remodeling is a feature of leukemia progression. It has been shown in a murine model of BCR-ABL1 B-ALL that during leukemia progression, there is an increase in blood vessel density and therefore a higher oxygen supply (80). However, because of high O_2 consumption by leukemic blasts and despite an important extracellular O_2 supply, intracellular hypoxia is elevated. In advanced disease stages, the O_2 supply becomes limited and the extracellular space highly hypoxic. As a consequence of hypoxia, HIF-1α was found to be stabilized in B-ALL and high HIF-1α levels were related to a poor outcome and resistance to chemotherapy through a decrease in pro-apoptotic and an increase in anti-apoptotic proteins (81, 82). Interestingly, co-culture of B-ALL cells with the MS-5 mesenchymal cell line further enhanced HIF-1α protein levels through AKT phosphorylation in hypoxic conditions, and through ERK phosphorylation independently of hypoxia (81).

IMMUNE CELLS IN THE LEUKEMIC NICHE

Tumor progression relies on the capacity of malignant cells to evade the immune system. In the BM, immune protection is supported by regulatory T cells (Tregs), which were shown to protect allogeneic HSC from rejection (83). Furthermore, Treg depletion in mouse leads to a differentiation arrest at the pre-proB stage (84). Tregs were found to control B cell differentiation through the regulation of IL7 production by PSS cells. Therefore, BM can be considered as an immune-privileged site. In the case of B-ALL, the proportion of $CD4^+CD25^+$ Tregs was found to be slightly but significantly increased for B-ALL patients at diagnosis in two independent studies (85, 86). Furthermore, Tregs from B-ALL (and T-ALL) patients secreted lower levels of the T cell stimulating cytokine IL-2 *in vitro*, and higher levels of the immunosuppressive IL-10 and TGFβ, as compared to healthy individuals (85). In contradiction with these results, another study showed a decrease in $CD4^+CD25^+$ Tregs (87). However, by refining the immunophenotyping the authors found a strong increase in the proportion of functional $FoxP3^+$ and $IL\text{-}10^+$ Tregs. Furthermore, Tregs from B-ALL patients secreted higher levels of IL-10 and TGFβ, and inhibited T cell

proliferation *in vitro* more strongly than Tregs from healthy individuals. Altogether these results suggest that B-ALL may escape immune-surveillance by activating Tregs.

Natural Killer (NK) cells are cells of the innate immune system which have the capacity to kill their target cell and are involved in tumor immune surveillance (88). Accordingly, B-ALL patients with a higher proportion of NK cells in the BM respond better to treatment (89). However, the frequency of $CD56^+CD3^-$ NK cells is decreased for most patients as compared to healthy individuals (90, 91). Interestingly, expression of the activating receptor NKp46 was decreased while the inhibitory receptor NKG2A was increased (91). Furthermore, NK cells from patients had a decreased capacity to degranulate, to secrete IFNγ and to induce the lysis of target cells compared to NK cells from healthy controls. Leukemic cells were further shown to directly inhibit NK cell cytotoxic activity and thus to drive immune evasion partly through the secretion of TGFβ and the activation of the Smad2/3 signaling pathway in NK cells.

B-ALL also has the capacity to evade the immune system by inhibiting macrophage phagocytosis. CD47, an inhibitor of phagocytosis by macrophages (92), is overexpressed by a subset of B-ALL, and was found to be an indicator of poor survival and associated with a high risk of refractory disease (93). Furthermore, it has been demonstrated in a humanized mouse model of leukemia/lymphoma that leukemic cells expressing a shRNA specific for the prostaglandin synthetase 3 gene (PTGES3) were more sensitive to antibody-dependent cell-mediated cytotoxicity (ADCC) (94). This result is in agreement with the fact that PTGES3 is involved in the catalysis of prostaglandin E2, an inhibitor of macrophage phagocytosis (95).

Finally, a recent study performed an in depth analysis of the immune microenvironment of B-ALL patient samples by single cell RNAseq (96). Although the myeloid compartment was strongly diminished in the BM of B-ALL patients at diagnosis compared to healthy individuals, the frequency of $CD14^+CD16^+$ non-classical monocytes was increased at the expense of $CD14^+CD16^-$ classical monocytes. Importantly, the frequency of this population strongly decreased upon remission but re-emerged at relapse. Furthermore, the authors found that a high monocyte count in both adult and childhood B-ALL was associated with a lower overall survival (OS) and relapse free survival (RFS). Finally, in a murine model of BCR-$ABL1^+$ B-ALL, mice transplanted with leukemic cells were treated with an antibody specific for CSF1R, highly expressed by non-classical monocytes (antibody AFS98), with Nilotinib, a BCR-ABL1 tyrosine kinase inhibitor (TKI) or a combination of both. The antibody-induced depletion of the non-classical monocyte subset had no effect on survival, however, the antibody improved the responsiveness of mice to Nilotinib. This result suggests that the non-classical monocyte subpopulation may promote leukemic blast survival and protect them from treatment.

TREATMENTS TARGETING THE LEUKEMIC NICHE

Novel therapeutics, have been introduced in the standard treatment regimens based on multiagent chemotherapies. TKIs which target dysregulated signaling pathways have given promising results with improved overall survival (97). Immune cells can also be addressed to leukemic cells through the use of monoclonal antibodies targeting surface receptors. As an example, Blinatumomab is a CD3-CD19 bispecific T-cell engaging antibody able to activate T-cells without the need for additional costimulatory signals. Blinatumomab is FDA approved for the treatment of MRD^+ or relapsed/refractory B-ALL (98, 99). However, part of the patients does not respond to Blinatumomab treatment. Exhaustion markers including PD-1 and CTLA-4 were upregulated by T cells following treatment, together with an upregulation of their respective ligands PDL-1 and CD86 at the surface of leukemic cells (100, 101). The *in vitro* Blinatumomab-mediated T cell response was inhibited when patient T cells as compared to healthy donor T cells were incubated with ALL cells. However, this block was relieved by using an anti-PD-1 alone or together with an anti CTLA-4. Blinatumomab non responders were also found to have an increased proportion of Tregs which impair T cell response in a contact-dependent manner (102). Clinical trials are currently underway to determine the relevance of combining Blinatumomab with checkpoint inhibitors like the anti PD-1 Pembrolizumab (103).

Immunotherapies have moved a step forward with the advance on chimeric antigen receptor (CAR) T-cell treatments. CAR-T cells are amplified from autologous T cells from the patient. They are engineered to express a single-chain variable fragment (scFv) of an antibody specific for a tumor cell marker (e.g. anti CD19 antibody), fused to the CD3ζ stimulatory domain and to an additional costimulatory domain in the case of second-generation therapies. CAR-T cells have been approved in 2017 by the FDA for the management of refractory or second/later relapsed ALL (104).

Mechanisms involved in the evasion from innate immune response can also be targeted. CD47, shown to be overexpressed by B-ALL, inhibits phagocytosis by macrophages. Treatment with the anti CD47 neutralizing antibody B6H12.2 relieved the block on phagocytosis *in vitro* and impaired leukemia engraftment *in vivo* (**Figure 2**) (93). In a humanized mouse model of leukemia, resistance to the anti CD52 antibody Alemtuzumab used to target $CD52^+$ lymphoid leukemia by ADCC was observed in the BM but not in periphery (94). This resistance was linked to a decrease in macrophage numbers at the time of BM invasion by leukemic cells. Importantly, most mice treated with a combination of Alemtuzumab and the alkylating agent cyclophosphamide (CTX) survived for at least 6 months while all mice under monotherapies died in about 2 months. CTX was found to specifically induce production by the BM microenvironment of TNFα, VEGF, and CCL4, at the origin of an increased penetrance of macrophages in the BM as well as the activation of their phagocytic properties. Altogether these results clearly demonstrated that reactivation of the immune microenvironment of the BM can improve the therapeutic outcome.

As leukemic niches have the capacity to give pro-survival and quiescence signals through direct interactions, it has been postulated that targeting the crosstalk between non immune cells

of the BM microenvironment and leukemic cells could improve current therapies. A high expression of VLA-4 in a cohort of MRD^+ patient is of bad prognosis (58). In a PDX model of B-ALL, the use of Natalizumab, an anti-VLA-4 monoclonal antibody, in combination with a multi-agent chemotherapy led to a complete remission of all animals for at least 4 months after treatment, while mice with the chemotherapy alone died less than 2 months after treatment (58). The anti VLA-4 alone marginally or did not improve survival (57, 58) therefore supporting that the loss of adhesion of leukemic cells with their niche sensitizes them to chemotherapy.

CXCR4 inhibitors have also been tested in B-ALL. The T140 and AMD3100 (Plerixafor) inhibitors impaired *in vitro* migration of the Nalm6 cell line and patient samples towards a CXCL12 gradient and into stromal layers (105). Furthermore, the loss of contact with stromal cells induced by the inhibitors increased the proliferative status of leukemic cells and their sensitivity to chemotherapeutic drugs. Treatment of mice engrafted with B-ALL from patients with plerixafor induced mobilization of leukemic cells in the circulation with a decreased tumor burden in the spleen but not in the BM (106, 107). Nevertheless, treatment of PDX mice with a combination of Plerixafor and the antimetabolite Ara-C led to a significant decrease in the tumor burden of the spleen as well as the BM compared to the Ara-C monotherapy (107).

Finally, pre-BCR signaling has been shown to be effective in $c\mu^+$ B-ALL and to rely on contacts with stromal cells (37, 39, 71). As a consequence, *in vitro* treatment of patient samples with TKIs specific for kinases involved in pre-BCR signaling (Ibrutinib and Dasatinib: BTK inhibitors; PRT062607: Syk inhibitor) induces a strong death at low half maximal inhibitory concentration (IC50) (71). Furthermore, survival of mice xenografted with pre-BCR^+ B-ALL is increased upon treatment with Ibrutinib or Dasatinib, and a synergistic effect of Ibrutinib was observed *in vitro* in combination with either Dexamethasone or Vincristine (71, 108).

CONCLUSION

B-ALL mutational status has been extensively studied; however, the crosstalk of B-ALL with their immune and non-immune microenvironment remains poorly characterized despite their known function in the control of residual disease and relapse. Chemotherapy toxicity and secondary effects are also a heavy burden for survivors, and there would be a strong benefit to develop adjuvant targeted therapies. Tremendous progress has been made in the outcome of patients by reactivating the immune tumor microenvironment through the development of immunotherapies. Nevertheless resistance and relapse in B-ALL still concerns an important number of patients, particularly among elderly. One of the main challenges remains to understand how the interactions between partner cells are orchestrated into the leukemic microenvironment in order to find which of these interactions could be inhibited to lead to the collapse of the leukemic cell ecosystem. Mouse models have been essential in understanding the organization of the BM non-immune microenvironment and its importance in normal and pathological hematopoietic progenitor maintenance and development. Thanks to advances in single cell transcriptomics and high resolution microscopy, the spatial organization of the BM starts to be resolved (109). However, the translation to human BM organization is more difficult due to the paucity of BM samples. PDX models are valuable for the analysis of the non-immune microenvironment, but the simultaneous contribution of immune cells cannot be evaluated. In addition, one has to consider that B-ALL are composed of different subtypes with diverse mutational landscapes. The composition of their supportive microenvironment and their dependence towards it may therefore be strongly dependent on their intrinsic properties. Therefore, future studies may consist in the establishment of experimental immunocompetent models in which new adjuvant therapies may be tested. This will require to better understand the natural history of microenvironment shaping during leukemic development starting from LIC in order to identify key steps in B-ALL ecosystem establishment. No doubts that mathematical modeling and new technological breakthroughs such as single cell technology will lead to the identification of cellular cross-talks constituting the Achilles heel for the development of efficient innovative therapies.

AUTHOR CONTRIBUTIONS

SM and MA-L conceptualized and finalized the manuscript. MD and K-IS provided the content and prepared the figures. JP provided the content. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the Ligue Nationale contre le Cancer (EL2020). MD is supported by the Ligue Nationale Contre le Cancer (IP/SC-16060) and K-IS by the Algerian "Ministère de l'enseignement supérieur et de la recherche scientifique" (MESRS). JP was the recipient of a conjoint PhD grant from the Région PACA/ Inserm/Innate Pharma and from la Ligue Nationale contre le Cancer (#TDCU19117).

ACKNOWLEDGMENTS

We are grateful to all the members of the lab and to the GDR 3697 Micronit for fruitful discussions. **Figures 1** and **2** were created from Servier Medical Art by Servier, licensed under a Creative Common Attribution 3.0 Unported License.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 20 January 2021
doi: 10.3389/fimmu.2020.612244

T Cells in Chronic Lymphocytic Leukemia: A Two-Edged Sword

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Chronic lymphocytic leukemia (CLL) is a malignancy of mature, antigen-experienced B lymphocytes. Despite great progress recently achieved in the management of CLL, the disease remains incurable, underscoring the need for further investigation into the underlying pathophysiology. Microenvironmental crosstalk has an established role in CLL pathogenesis and progression. Indeed, the malignant CLL cells are strongly dependent on interactions with other immune and non-immune cell populations that shape a highly orchestrated network, the tumor microenvironment (TME). The composition of the TME, as well as the bidirectional interactions between the malignant clone and the microenvironmental elements have been linked to disease heterogeneity. Mounting evidence implicates T cells present in the TME in the natural history of the CLL as well as in the establishment of certain CLL hallmarks e.g. tumor evasion and immune suppression. CLL is characterized by restrictions in the T cell receptor gene repertoire, T cell oligoclonal expansions, as well as shared T cell receptor clonotypes amongst patients, strongly alluding to selection by restricted antigenic elements of as yet undisclosed identity. Further, the T cells in CLL exhibit a distinctive phenotype with features of "exhaustion" likely as a result of chronic antigenic stimulation. This might be relevant to the fact that, despite increased numbers of oligoclonal T cells in the periphery, these cells are incapable of mounting effective anti-tumor immune responses, a feature perhaps also linked with the elevated numbers of T regulatory subpopulations. Alterations of T cell gene expression profile are associated with defects in both the cytoskeleton and immune synapse formation, and are generally induced by direct contact with the malignant clone. That said, these abnormalities appear to be reversible, which is why therapies targeting the T cell compartment represent a reasonable therapeutic option in CLL. Indeed, novel strategies, including CAR T cell immunotherapy, immune checkpoint blockade and immunomodulation, have come to the spotlight in an attempt to restore the functionality of T cells and enhance targeted cytotoxic activity against the malignant clone.

Keywords: chronic lymphocytic leukemia, T lymphocytes, anti-tumor immunity, tumor microenvironment, T cell-based therapies

OPEN ACCESS

Edited by:
Jérôme Paggetti, Luxembourg Institute of Health, Luxembourg

Reviewed by:
Alan G. Ramsay, King's College London, United Kingdom
Silvia Deaglio, University of Turin, Italy

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *30 September 2020*
Accepted: *08 December 2020*
Published: *20 January 2021*

Citation:
Vlachonikola E, Stamatopoulos K and Chatzidimitriou A (2021) T Cells in Chronic Lymphocytic Leukemia: A Two-Edged Sword. Front. Immunol. 11:612244. doi: 10.3389/fimmu.2020.612244

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is an age-related malignancy characterized by the accumulation of monoclonal mature B cells expressing CD5, CD19 and CD23 on their surface (1). Numerous studies support that the clonotypic B cell receptor immunoglobulin (BcR IG) is critically implicated in disease pathophysiology (2). Indeed, the intensity of intracellular signaling downstream of the BcR in CLL cells is associated with cell proliferation and disease severity (3), whereas restrictions in the gene repertoire of the clonotypic BcR IG strongly highlight the role of antigenic triggering in disease pathogenesis (4, 5).

Different lines of evidence suggest that interactions with two main categories of microenvironmental components i.e. soluble factors and other extracellular elements as well as bystander cells are implicated in shaping a supportive tumor microenvironment (TME) in CLL (6–8). The former category includes various extracellular molecules (e.g.) cytokines, chemokines, antigens but also, in a broader sense, products of metabolic processes. Indeed, although cellular metabolism in CLL remains largely unexplored, increasing evidence implicates oxidative stress in the natural history of CLL by showing e.g. that the accumulation of reactive oxygen species (ROS) may hold prognostic significance (9, 10).

Turning to bystander cells, different subpopulations of T cells as well as natural killer (NK) cells accumulate *in vivo* along with mesenchymal stromal cells (MSC) and nurse-like cells (NLCs), forming a complex network that favors clonal expansion and proliferation of the malignant clone (11–13). Ongoing crosstalk of CLL malignant cells with these other cell populations in the TME affects the function of both parties. On the one hand, this leads to immunosuppression, a hallmark of CLL associated with increased susceptibility to infections, autoimmune manifestations, and a higher incidence of secondary malignancies (14). On the other hand, external triggers support the survival and proliferation of the neoplastic cells (15); this was first made evident when it was found that CLL cells undergo apoptosis in suspension cultures, which can be partially rescued by co-cultures with stromal cells or NLC (11).

T cells are major contributors to adaptive immunity, actively engaged in defense against pathogens and tumor cells through a great variety of accessory and effector functions. Upon encounter with a specific antigen, T cells are activated and eventually differentiate into various distinct subpopulations, acquiring either cytotoxic or helper properties. Pathogen clearance, mediated by cytotoxic T cells or through the activation of other cell types induced by cytokines secreted from T helper cells, is followed by the apoptosis of the effector T cells as a homeostatic mechanism that restores the immune system at the pre-activation state. Simultaneously, a small fraction of antigen-specific memory T cells are resting in the body, ready to generate an immediate and effective secondary response (16, 17). This homeostatic balance is perturbed in CLL, where, similar to various solid or hematological malignancies, T cells exhibit a number of phenotypic and functional defects undermining their normal immune responses (18). Moreover, T cells appear to have an active involvement in CLL development and evolution, as supported by experimental evidence that the transfer of autologous activated T cells in NOD/Shi-scid, γcnull (NSG) mice is a prerequisite for successful engraftment of CLL cells in murine models (19, 20). Interestingly, the post-transfer outgrowth of functionally competent Th1 T cells seen in NSG mice highlights the suppressive and inhibitory TME in CLL patients, particularly considering reports that these T cells can regain their functionality and promote B cell diversification and differentiation (18). It has been proposed that this phenomenon may reflect selection for Th1 cells *in vivo*, release from various inhibitory mechanisms operating within the human host or a special effect of the distinct NSG microenvironment. Arguably, similar mechanisms may also act in CLL patients, however, strong correlations remain elusive (20), highlighting the need for caution when attempting extrapolations based on findings deriving from model systems.

In CLL, as in other cancers, genomic instability leads to various alterations that serve as a source of cognate antigens, sufficient for the induction of tumor-reactive T cell responses (21–24). Although tumor-/antigen-specific T cells have been identified in CLL patients, these are incapable of effectively eliminating neoplastic cells due to several intertwined immunosuppressive mechanisms that establish T cell dysfunction (25, 26), thus, favoring escape from immune surveillance (27). Evidence suggests that this tolerogenic TME is actively induced by the CLL cells themselves through the secretion of cytokines and chemokines that affect cellular functions in the bystander cells (15). As a matter of fact, a recent study demonstrated that shared peptides from the highly conserved BcR IG of patients belonging to CLL stereotyped subsets 1 and 2 could generate antigen-specific T cells upon presentation in HLA-restricted manner. Furthermore, that study reported T cell specific reactivity against CLL cells, while also showing that immunization of Eμ-TCL1 mice with BcR IG-derived peptides led to anti-leukemic T cell responses, implying that targeted T cell immunotherapy against the clonotypic BcR IG represents an appealing treatment option (28). Along similar lines, the identification of the unique set of neo-epitopes of each patient could lead to the isolation of leukemia-specific T cells, that can be used as a novel treatment approach (29).

Taken together, deciphering the cross-talk mechanisms between CLL cells and bystander T cells as well as the implicated signaling pathways is reasonably anticipated to offer insights into disease pathophysiology, and, potentially, a great opportunity for designing microenvironment-directed treatment approaches.

T CELL SUBPOPULATION IMBALANCES IN CLL

Alterations in the T cell composition have been extensively described in CLL, however the exact impact of these changes on disease development and progression remains controversial. The first described T cell abnormality in a CLL-specific context concerned the finding of increased numbers of T cells in the

periphery, accounting mostly for $CD8^+$ T cell expansion that leads to inversion of the normal $CD4^+/CD8^+$ cell ratio (30). Of note, these $CD8^+$ T cells are severely compromised as to their capacity to exert cytotoxicity (31). Regarding the potential clinical relevance of these findings, it has been reported that elevated numbers of $CD8^+$ cells are associated with disease progression as well as shorter time to first treatment and progression-free survival, perhaps partly due to co-expression of the inhibitory receptor of programmed death-1 (PD-1) (32). However, under different conditions oligoclonal expansion of $CD8^+$ effector cells in the Eμ-TCL1 mouse model of CLL has been associated with disease control, whereas conversely, ablation of $CD4^+$ T cells did not affect disease progression (33).

Expansions of different $CD4^+$ T cell subpopulations that exert either pro-tumoral activity or immunosuppression have been reported in CLL. Early studies have shown that IFN-γ secreting Th1 cells can provide trophic signal for CLL cells inhibiting programmed cell death and supporting survival (34). On the other hand, T cell-mediated immune responses have been linked with disease control in cases of autologous CLL regression, highlighting the multi-dimensional T cell activity into the CLL TME (35). Further, expansion of a novel $CD4^+$ T subpopulation characterized by the expression of the TIGIT (T cell immunoreceptor with Ig and ITIM domains) inhibitory receptor has been recently observed in patients at advanced stages of CLL; these cells were shown to support CLL survival in *in vitro* experiments (36). Finally, $CD4^+PD\text{-}1^+HLA\text{-}DR^+$ T cells that co-express inhibitory and activation markers have been associated with aggressive disease (37). Altogether, these apparently conflicting findings clearly indicate the need for delving deeper into the distinct subsets and functions of the T cell compartment in CLL.

A well-characterized finding in CLL concerns the elevated numbers of T regulatory cells (Tregs) (30, 38) that are generally known to contribute to cancer progression through dampened antitumor responses and immunosuppression (39, 40). Of note, CLL Tregs are more suppressive than normal Tregs, whereas depletion of these cells led to efficient anti-tumor responses in animal models of CLL (41, 42). Additionally, interleukin 4 (IL-4) secreted from Tregs also induces anti-apoptotic pathways in CLL cells through the overexpression of the anti-apoptotic protein BCL2, which is therapeutically targeted by venetoclax, an effective agent for the treatment of CLL (43–46). Hence, efforts to inhibit Tregs through targeting the FoxP3 transcription factor, that is critical for their function (47, 48), or other interacting molecules in the downstream pathway could be clinically useful, at least in principle, similar to what has been proposed for other cancers (49, 50).

Th17 cells represent another T cell subpopulation with a critical role in immune homeostasis, actively participating in inflammatory processes. Imbalances in Th17 populations have been linked with autoimmune disorders, however their role in cancer remains to be fully elucidated (51). In CLL, Th17 cells are increased compared to healthy individuals; however, increased Th17 cells in cases at early disease stages have been reported to be associated with a favorable clinical outcome, conceivably through controlling the expansion of Tregs (52, 53). Altogether, the aforementioned evidence suggests that the balance between Tregs and Th17 cells may impact on the clinical outcome through as yet unknown mechanisms (14, 51).

FUNCTIONAL IMPAIRMENT OF T CELLS IN CLL: CAUSES, FUNCTIONAL CONSEQUENCES, AND CLINICAL IMPLICATIONS

T cell activation starts when an antigen-presenting cell (APC) introduces an epitope bound on an HLA molecule to a specific TR. A second signal from the CD28 receptor, constitutively expressed on T cells, enhances cell proliferation, generation of cytotoxic lymphocytes, and cytokine production (54). In CLL, *in vitro* T cell activation through CD28 followed by infusion of the activated autologous T cells to the patients was shown to lead to competent anti-leukemic effects and decreased TR clonality. Based on this evidence, *ex vivo* T cell activation and expansion was proposed as a valid treatment option in CLL, however failed to gain ground (55).

Effective activation of naïve T cells through normal interactions with APCs requires the formation of the immune synapse, a specialized contact area between T cells and APCs (56). Impaired immune synapse formation has been documented as a hallmark of CLL and shown to be mechanistically linked to alterations in the expression of genes associated with actin polymerization, cytoskeletal organization and vesicle trafficking (57). Of note, immune synapse defects appear to be contact-dependent, as shown in both *ex vivo* studies of primary patient samples and *in vivo* studies of animal models of CLL (58, 59).

Such effects may dampen effective antitumor responses, through a complex network of inhibitory signals and immunosuppressive interactions amongst CLL cells and T cells into TME. Importantly, signals delivered through the CTLA-4 and PD-1 receptors modulate T cell activation status through reduction of TR signaling and cytolytic functions. These inhibitory molecules are overexpressed in T cells of patients with CLL, while their ligands are also overexpressed by the malignant cells, hence putting the brakes on anti-tumor activity (60). Specifically, CLL cells express inhibitory surface molecules from B7 and TNF-receptor families, namely CD200, CD274 (PD-L1), CD276 and CD270 which are implicated in synapse formation defects in both autologous and allogeneic T cells, suggesting a novel evasion mechanism (61).

On these grounds, research into these receptors and ligands could pave the way to novel treatment modalities for CLL, whereby T cell-driven immune responses could be re-invigorated through targeting the interactions between inhibitory receptors and their ligands. However, despite encouraging pre-clinical results, the clinical application of antibodies against these receptors (immune checkpoint inhibitors [ICI]) has been frustratingly disappointing in CLL,

with only minimal effectiveness when administered as monotherapy (62, 63). Evidently, more research is warranted in order to define the precise role of ICIs in the management of CLL and also better understand the mechanisms underlying the emergence of severe adverse events reported for these agents (64, 65).

In CLL, immune synapse defects were found to be reversible by the administration of lenalidomide, an immunomodulatory drug (IMiD) that appears to reshape the expression of cytokines and orchestrate cell-mediated responses, mobilizing the T cell compartment (66, 67). In addition, lenalidomide treatment invigorates T cell motility and migration through activation of integrin lymphocyte function–associated antigen-1 (LFA-1), also affected by direct contact with CLL cells (68). Relevant to mention, clinical studies of lenalidomide as monotherapy or consolidation therapy after chemoimmunotherapy have reported restored immune synapse formation and downstream signaling and well as improved quality of clinical responses (69, 70).

T cells in CLL display features of exhaustion, a functional state initially described in the setting of chronic infections but later also recognized in various cancer types (25, 71–75). In such conditions, exhaustion has been postulated to arise as a result of persistent antigenic stimulation, leading to gradual loss of T cell effector functions (76–78). Similar to other contexts, exhausted T cells in CLL are characterized by low proliferative rates, reduced cytotoxicity, altered cytokine production and, as already mentioned, expression of multiple inhibitory receptors on their surfaces, including CTLA-4, PD-1 and LAG-3 (25, 79, 80).

The functional analogies between T cells in individuals exposed to persistent antigenic stimulation due to a chronic infection and individuals affected by CLL allows arguing that antigenic pressure is key to shaping the T cell repertoire and functionality also in CLL. That notwithstanding, the exact nature of the cognate antigens/epitopes implicated in T cell selection in CLL is yet to be defined. However, the most prominent (classes of) candidates concern: (i) the same antigens that are implicated in the selection of the CLL progenitors or the malignant cells themselves; (ii) tumor-derived epitopes from aberrant proteins expressed by the malignant cells e.g. due to a pathogenic mutation or other genomic aberration; and (iii) the clonotypic IG, perhaps the most abundant clone-specific molecule (81–84) (**Figure 1**).

ANTIGEN SELECTION SHAPES THE T CELL COMPARTMENT IN CLL: IMMUNOGENETIC EVIDENCE

The clonotypic BcR IG is critically implicated in the natural history of CLL, a fact amply supported by the therapeutic efficacy of agents interfering with BcR IG signaling, which have changed dramatically the therapeutic landscape of CLL (85–87). This clinical evidence complements immunogenetic evidence that the BcR IG is a key driver in disease ontogeny and evolution. In more detail, the BcR IG gene repertoire is characterized by remarkable restrictions (88), culminating in BcR IG stereotypy where, at

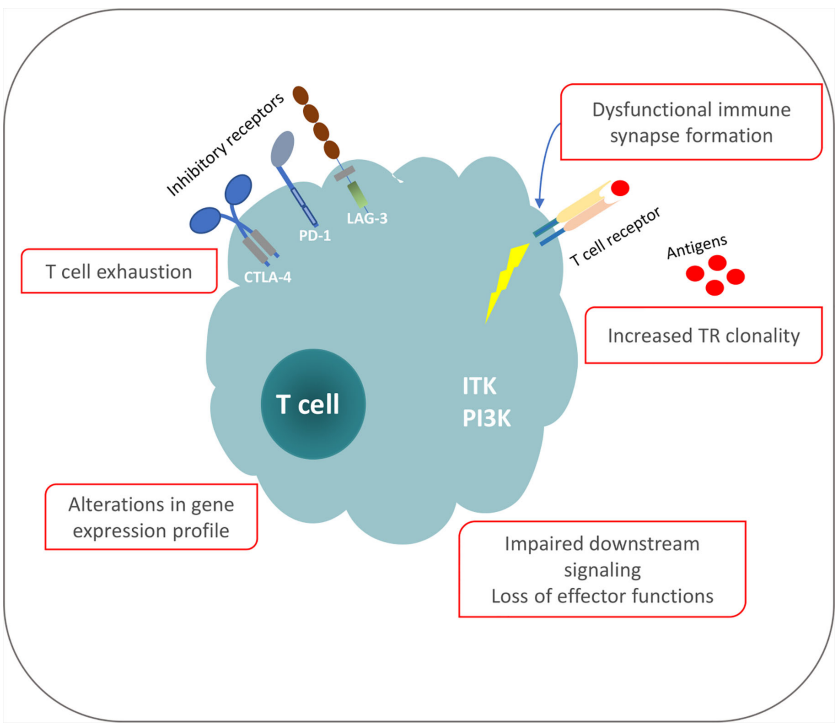

FIGURE 1 | Phenotypic and functional defects of T cells in CLL.

clear odds with serendipity, different clones share (quasi) identical IG (4, 5, 89). Moreover, the molecular characteristics of the clonotypic BcR IG have established prognostic and predictive significance since patients with a significant imprint of somatic hypermutation (SHM) display indolent disease, in contrast to those with few or no SHM who generally follow a more aggressive clinical course (90, 91). Taken together, these features emphasize the links between BcR IG structure and clonal behavior, while also highlighting the importance of antigen selection in CLL pathophysiology (92, 93).

Considering the importance of extracellular cues for CLL cell survival and proliferation, as well the T cell compartment defects in CLL patients, the interest in the TR gene repertoire of T cells in CLL and the implicated antigens is hardly surprising. Immunophenotypic studies in the 1990s described T cell clonal expansions in CLL patients, prompting speculations that these cells could be possibly related to the host immune response against CLL-derived antigens, or emerged as a result of a longitudinal interplay between CLL and T cells in the proliferative centers (94). Moreover, analyses of the TR beta (TRB) chain gene rearrangements by Southern blotting offered the first molecular evidence for skewed TRBV gene repertoire and oligoclonality in both $CD4^+$ and $CD8^+$ T cells of CLL patients compared to healthy aged-matched controls (95–97). Additionally, stimulation of isolated T cells from CLL patients with autologous malignant cells resulted in monoclonal TR expansions, prompting speculations that CLL patients could bear a pool of T cells that specifically recognize leukemia-associated antigens (95, 98). Similar findings of decreased clonal diversity were also reported in Eμ-TCL1 mice supporting the notion of CLL-dependent antigen-driven selection pressure on the TR gene repertoire (99).

These initial studies were followed by significantly more extended examinations of the TR gene repertoire by our group, first through subcloning of TRBV-TRBD-TRBJ gene rearrangements followed by Sanger sequencing (100) and, more recently, by high-throughput approaches using next generation sequencing (NGS) (101, 102). Summarizing our findings: (i) the TRBV gene repertoire is skewed; (ii) oligoclonal T cell expansions feature prominently, particularly amongst cytotoxic T cells; (iii) such expansions persist and may even increase during the disease course; and, (iv) identical or highly similar TR clonotypes were shared between different patients, especially those belonging to the same stereotyped subset, and these appeared to be CLL-biased as they were not present in other contexts. Altogether, these findings allow arguing that antigen selection has a key role in shaping the TR repertoire in CLL, while also favoring the intriguing hypothesis that the relevant antigens are most likely CLL-related (101). However, the exact mechanism that underlies T cell clonality and retains these expanded populations during the disease course remains elusive.

In order to further understand T cell compartment dynamics in CLL, we extended our studies to longitudinal investigation (pre/post-treatment) of patients treated with chemoimmunotherapy with the fludarabine-cyclophosphamide-rituximab (FCR) regimen or the BcR signaling inhibitors (BcRi) ibrutinib (IB) and idelalisib (the latter in combination with rituximab, R-ID). We found that T-cell clonality significantly increased at (i) 3 months in the FCR and R-ID treatment groups, and (ii) over deepening clinical response in the R-ID group, with a similar trend detected in the IB group. Perhaps more importantly, in contrast to FCR that induced T-cell repertoire reconstitution, BcRi retained pretreatment clones. Extensive comparisons of the CLL dataset against external TR sequence databases showed little similarity with other entities, but instead revealed major TR clonotypes shared exclusively by patients with CLL, supporting selection by conserved CLL-associated antigens. In addition, we assessed the functional impact of these treatments on T cells and found that (i) R-ID upregulated the expression of activation markers in effector memory T cells, and (ii) both BcRi improved antitumor T-cell immune synapse formation, in marked contrast to FCR (102). Here, it is worth mentioning the contradictory findings of an another NGS-based longitudinal study of the TR gene repertoire in IB-treated patients, where this therapy resulted in TR repertoire diversification (103). However, major differences in the experimental and analytical procedures render these two studies incomparable, highlighting the need for further harmonization of the NGS protocols and bioinformatics workflows. A limitation inherent to both studies concerns the fact that total $CD3^+$ T cells rather than particular T cell subpopulations were investigated, which could lead to misinterpretation due to a "dilution effect" by bulk T cell analysis. Finally, robust conclusions regarding the links, if any, between T cell dynamics overtime and clinical outcome are still not possible: hence studies on larger, well-characterized cohorts remain of paramount importance.

Considering mounting evidence that the TR gene repertoire in CLL is antigen selected, identifying the cognate antigens is an obvious line of research, not least because it would assist in designing effective antigen-specific immunotherapy. Studies of HLA-presented antigenome and computational models for tumor-associated antigen prediction proposed the existence of disease specific antigens, deriving from CLL-related genomic aberrations, that were identified exclusively on neoplastic cells of CLL patients (104–106). Additionally, the broad representation of BcR IG-derived epitopes in patients across different disease (Binet) stages and through various treatments suggest them as potential candidates for peptide vaccines and objectives of neoantigen-targeting immunotherapies. However, immune tolerance mechanisms can turn the BcR IG-derived peptides, a potentially significant pool of cognate neo-antigens in B cell lymphomas (107, 108), into ineffective targets for T cells. This phenomenon, known as immunoediting, has been described in different contexts (including cancer and viral infections) and can neutralize an immunogenic phenotype favoring cells to survive and proliferate (27, 109).

TREATMENT OPTIONS IN CLL AND IMPLICATION OF THE T CELL COMPARTMENT

T cells in the CLL TME exhibit a number of phenotypic and functional defects, acquired through persistent crosstalk with the

neoplastic cells. The T cell compartment aberrations appear to be CLL-specific and induced by the CLL cells, leading to functional exhaustion, reduced activation and proliferation of effector subpopulations and decreased cytotoxic responses. Thus, CLL cells have the ability to amend alter T cell functions in order to evade immune-surveillance, proliferate and further expand overtime.

Despite the severe T cell deficiencies identified in CLL patients and models, studies demonstrate that subsets of these defective T cells can restore their functionality and mount anti-tumor responses under particular therapeutic interventions (**Figure 2**). Moreover, the identification of tumor-derived antigens implicated in T cell clonal expansions that may harbor anti-tumor properties can pave the way for therapeutic peptide vaccination strategies and adoptive T-cell transfer protocols in order to augment efficacy and guide the specificities of anticancer immune responses. Consequently, deep understanding of tumor-derived T cell dysfunction is highly significant for the development of new therapeutic modalities to restore anti-tumor immunity and result in tumor control.

In recent years, the advent of BCRi has led to a major paradigm change in the treatment of CLL. Due to structural and functional similarities of kinases between T and B cells, cross-reactivity of these agents has been described, highlighting an additional immunomodulatory mechanism of action in TME. In more detail, besides inhibiting the Bruton's tyrosine kinase (BTK) that is implicated in signal transduction of the activated BcR in CLL cells (110), ibrutinib targets also the T-cell associated kinase ITK, modulating T cell immunity. This is made manifest by effective immune synapse formation, shift towards Th1 polarization, and expansion of functionally competent specific-T clones, possibly contributing in deepening clinical response (102, 111, 112). In addition, ibrutinib-induced down-regulation of the PD-1/PD-L1 axis and inhibition of the STAT3 pathway modulates the immunosuppressive TME: on these grounds, combination protocols with lenalidomide or checkpoint inhibitors hold promises for reactivation of anti-tumor immunity (113–115). Similar effects have been demonstrated by treatment with idelalisib that also targets other PI3K isoforms in T cells, enhancing anti-tumor functions through modulation of Tregs (74). This is unsurprising considering that intracellular signaling that leads to suppression of T cell effector functions is extensively dependent on pathways implicating an isoform of PI3Kδ that constitutes an excellent target for idelalisib. At a first glance, this effect might be considered as an alternative mechanism to reduce immunosuppression and invigorate T cell responses; however, the described severe autoimmune adverse effects cannot be overlooked (116). Effects on the CLL TME are not restricted to BCRi though, since treatment with the BCL2 inhibitor venetoclax has been associated with reduced numbers of PD-1$^+$ T cells and expression of inflammatory cytokines, suggesting a mechanism of immune recovery (117). Collectively, thorough investigation of the possible immunomodulatory effects of novel agents in CLL is of great relevance for future development of new combination therapeutic strategies.

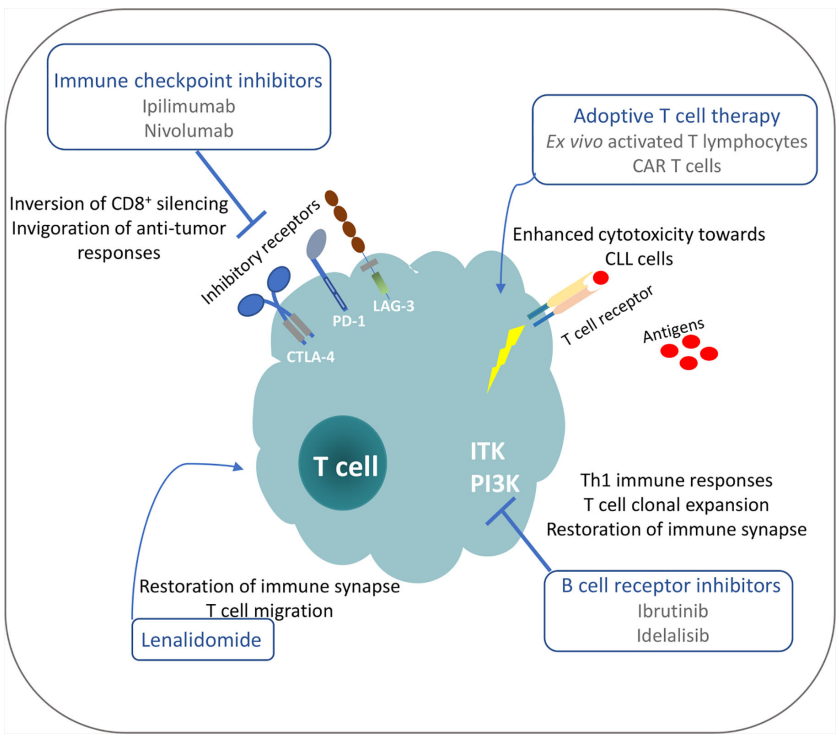

FIGURE 2 | Possible mechanisms for restoration of T cell functionality in CLL under different treatment manipulations.

As already mentioned, interactions between inhibitory receptors and their ligands, including the PD-1/PD-L1 axis, enable CLL cells to evade T cell immunosurveillance, thus proposing a new area for therapeutic targeting. Preclinical studies in a murine CLL model showed that immune checkpoint inhibition of PD-1/PD-L1 led to prevention of immune dysfunction and leukemia development, hence offering a rationale for the clinical testing of these regiments (58). Despite the promising results of PD-1 blockade in clinical trials for metastatic melanoma (MM), non-small cell lung cancer (NSCLC) and renal cell cancer (RCC) (118), anti–PD-1 monotherapy benefits only a small fraction of CLL patients who developed Richter syndrome (64). Nevertheless, combination therapies with regimens targeting different inhibitory pathways may offer better outcomes (71, 119).

Adoptive T cell therapy is an evolving field that shows promises in recent trials. The protocol is based on the infusion of genetically modified T lymphocytes with a particular specificity for antigen recognition on the malignant cells. In CLL, the expression of the CD19 surface molecule is used as a selective B cell target and autologous cytotoxic T cells are modified *ex vivo* to express a chimeric antigen receptor (CAR) with affinity to CD19 of CLL cells. The engagement of a CAR-T cell with a $CD19^+$ B cell leads to T cell activation in a MHC-independent manner, redirecting cytotoxic effects towards CLL cells (120). Encouraging results from adoptive T cell therapy in B acute lymphoblastic leukemia have paved the way for trials in CLL: initial results were deemed positive, but the overall efficiency of the approach was limited in patients with bulky lymph node involvement (121). Attempts to induce the anti-tumor properties and the efficiency of the CAR-T cells are based on, amongst others, modifications for constitutive expression of the CD40 ligand that lead to proliferation and Th1 immune responses, whereas also combination with PD-1 blockade has been used in order to bypass CAR-T exhaustion (121, 122).

A major challenge for effective CAR-T cell designs is to enhance and guide specificity towards the malignant cells. Thus, identification and in-depth characterization of T cell clones bearing anti-tumor properties emerge as highly relevant. In parallel, detailed studies on the HLA-lingadome highlight tumor-derived antigens that are exclusively expressed on the leukemic cells and represent possible effective targets for adoptive T cell therapy (107, 123). Additionally, the identification of anti-CLL T clones and the respective disease-specific cognate antigens offers a rationale for peptide vaccination strategies in order to induce anti-tumor responses, but also for target recognition for T-cell based immunotherapy. Indeed, spectrometric studies on the naturally presented HLA ligands in CLL identified a pool of antigens exclusively expressed in CLL patients, providing information regarding the immunogenicity of the malignant cells while also offering new treatment targets (107). However, clinical studies targeting neoepitopes expressed in the malignant cells demonstrate enhanced rejection of tumor cells, underling the safety profile of these approaches (124, 125).

CONCLUDING REMARKS

CLL cells have the ability to transform the effector functions of the bystander T cells in the TME, thus rendering them a source of trophic signals for the survival and proliferation of the malignant clone. Moreover, alterations in the T cell transcriptome combined with functional exhaustion undermine cytotoxic responses against tumor cells, establishing tumor evasion and escape of CLL cells from immunosurveillance. That said, despite the observed defects in the T cell compartment, several studies have identified subsets of T cells bearing anti-tumor properties that can be unleashed under the proper stimulation. This binary, contradicting role of T lymphocytes in CLL TME supports the analogy with a two-edged sword. Based on the observations discussed in the current review, the mobilization of exhausted T cells and the invigoration of anti-tumor pathways could be translated to clinical efficiency, tumor control and durable remissions. However, in order for this objective to be met, detailed characterization of the implicated mechanisms at the molecular and cellular level is urgently warranted.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version. EV wrote the manuscript. KS and AC edited the text and gave final approval.

FUNDING

This work is supported in part by the ERANET Transcan-2, Project Acronym: Novel, and the Hellenic Precision Medicine Network in Oncology.

ACKNOWLEDGMENTS

The authors wish to acknowledge the work of past and current group members, particularly Drs Anna Vardi, Fotis Psomopoulos, Kostas Pasentsis, and Niki Stavroyianni.

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Conflict of Interest: KS and AC have received unrestricted grant support from Jannsen Pharmaceutica and Abbvie.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 20 January 2021
doi: 10.3389/fimmu.2020.595818

Phosphoinositide 3-Kinase Signaling in the Tumor Microenvironment: What Do We Need to Consider When Treating Chronic Lymphocytic Leukemia With PI3K Inhibitors?

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Phosphoinositide 3-kinases (PI3Ks) and their downstream proteins constitute a signaling pathway that is involved in both normal cell growth and malignant transformation of cells. Under physiological conditions, PI3K signaling regulates various cellular functions such as apoptosis, survival, proliferation, and growth, depending on the extracellular signals. A deterioration of these extracellular signals caused by mutational damage in oncogenes or growth factor receptors may result in hyperactivation of this signaling cascade, which is recognized as a hallmark of cancer. Although higher activation of PI3K pathway is common in many types of cancer, it has been therapeutically targeted for the first time in chronic lymphocytic leukemia (CLL), demonstrating its significance in B-cell receptor (BCR) signaling and malignant B-cell expansion. The biological activity of the PI3K pathway is not only limited to cancer cells but is also crucial for many components of the tumor microenvironment, as PI3K signaling regulates cytokine responses, and ensures the development and function of immune cells. Therefore, the success or failure of the PI3K inhibition is strongly related to microenvironmental stimuli. In this review, we outline the impacts of PI3K inhibition on the tumor microenvironment with a specific focus on CLL. Acknowledging the effects of PI3K inhibitor-based therapies on the tumor microenvironment in CLL can serve as a rationale for improved drug development, explain treatment-associated adverse events, and suggest novel combinatory treatment strategies in CLL.

Keywords: phosphoinositide 3-kinase (PI3K), chronic lymphocytic leukemia, tumor microenvironment, idelalisib, phosphoinositide 3-kinase (PI3K) inhibition

OPEN ACCESS

Edited by:
Dominik Wolf,
Innsbruck Medical University, Austria

Reviewed by:
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *17 August 2020*
Accepted: *07 December 2020*
Published: *20 January 2021*

Citation:
Aydin E, Faehling S, Saleh M, Llaó Cid L, Seiffert M and Roessner PM (2021) Phosphoinositide 3-Kinase Signaling in the Tumor Microenvironment: What Do We Need to Consider When Treating Chronic Lymphocytic Leukemia With PI3K Inhibitors? Front. Immunol. 11:595818. doi: 10.3389/fimmu.2020.595818

PHOSPHOINOSITIDE 3-KINASE SIGNALING PATHWAY

The phosphoinositide 3-kinase (PI3K) family mediates nutrient sensing and metabolic control to prevent cell growth and proliferation in conditions of energy or nutrient deficiency. Several roles are ascribed to PI3K signaling both in normal cell function and cancer. The four best-characterized pathways that activate PI3K are cytokine/chemokine receptors, receptor tyrosine kinases (RTK), B-cell receptor (BCR), and G-protein coupled receptors (GPCR) (**Figure 1**). Stimulation of these receptors causes the autophosphorylation of tyrosine residues on immunoreceptor tyrosine-based activation motifs (ITAMs) that are important mediators of signal transduction in immune cells, leading to the activation of a PI3K p110 catalytic isoform.

PI3Ks phosphorylate the 3-position hydroxy group of the phosphatidylinositol ring. The resulting products are the secondary messengers phosphatidylinositol$_{(4,5)}$P2 (PIP2) and phosphatidylinositol$_{(3,4,5)}$P3 (PIP3), which trigger important physiological changes in cells through AKT/Protein Kinase B (PKB) phosphorylation and downstream kinases. One of those, mammalian target of rapamycin complex 1 (mTORC1), which is a central mediator of the metabolic control, combines signals from nutrients and PI3K to induce cell growth and proliferation. Major components of this pathway are outlined in **Figure 1**.

When a ligand binds to RTK, two RTK monomers dimerize, which results in the activation of intracellular tyrosine kinase domain and auto phosphorylation followed by indirect PI3K activation *via* adapter molecules such as the insulin receptor substrate (IRS). Alternatively, BCR-dependent activation of PI3Ks is mediated by Src and Syk family of receptor-associated tyrosine kinases. First, Src-family proteins phosphorylate the tyrosine residues of ITAMs that reside on the cytoplasmic part of the signal transducing subunits of the BCR-associated Ig-α and Ig-β. Phosphorylated ITAMs serve as binding sites for Src-homology 2 (SH2) domain-containing proteins such as B-cell PI3K adaptor protein (BCAP) and CD19 (**Figure 2**). With the help of these proteins, PI3Ks are recruited to the BCR signalosome (2, 3). Next, the regulatory subunit interacts with the intracellular section of the activated receptor *via* its SH2 domain and this event leads to the activation of catalytic p110 isoform which triggers a lipid membrane-associated cascade of phosphorylations (PIP2 to PIP3). The PI3K pathway is mediated mainly by PIP3, which is a secondary messenger that acts as a docking site. In the PI3K-AKT pathway, PIP3 can bind to both downstream effector proteins phosphoinositide dependent kinase (PDK1) and AKT. AKT modification activates the mTORC1 by direct phosphorylation, which results in synthesis of growth, proliferation-, and survival-related proteins (1).

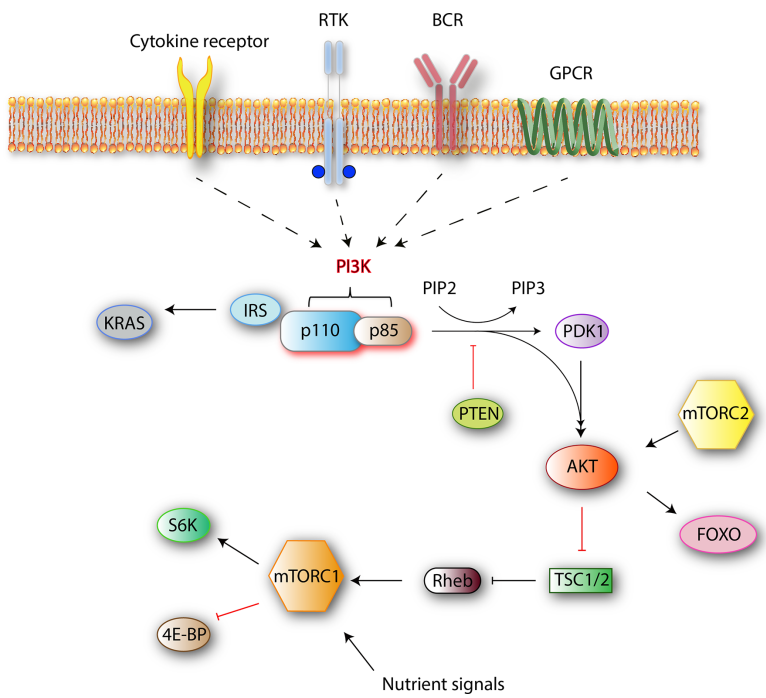

FIGURE 1 | Class I PI3K signaling pathway simplified. Class I PI3Ks reside as dimers in the cells. After receiving an external stimulus, the regulatory p85 subunit interacts with the intracellular section of the activated receptor. The activation of a catalytic p110 isoform triggers a lipid membrane-associated cascade of phosphorylations (PIP2 to PIP3). The PI3K pathway is mediated mainly by PIP3, which is a secondary messenger that acts as a docking site. In the PI3K-AKT pathway, PIP3 can bind to both downstream effector proteins PDK1 and AKT. AKT modification activates the mammalian target of rapamycin complex 1 (mTORC1) by direct phosphorylation, which results in synthesis of growth-, proliferation-, and survival-related proteins (1).

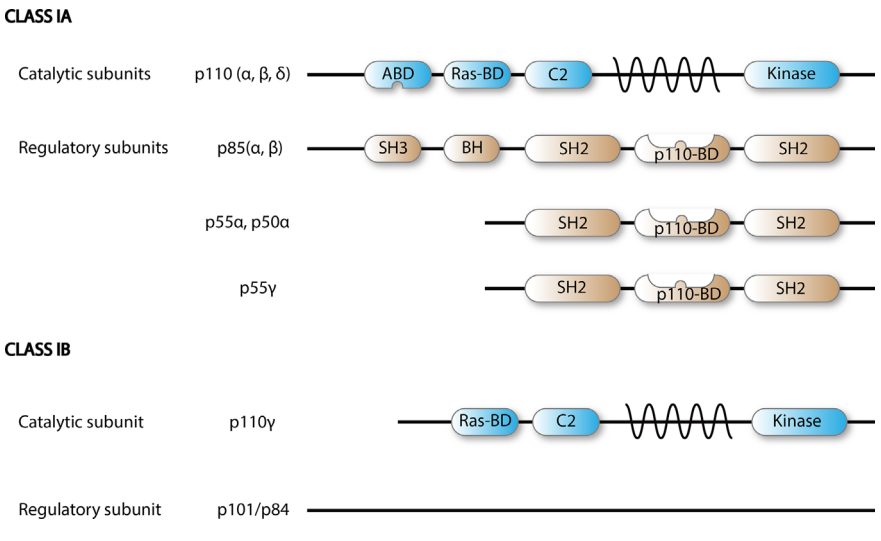

FIGURE 2 | Scheme of PI3K class I isoforms. Functional PI3K is built upon dimerization of a catalytic p110 and a regulatory subunit.

Isoforms of Phosphoinositide 3-Kinases

There are eight different isoforms of PI3Ks that are grouped into three classes (class I, II, and III) based on their primary structure and regulation. Class I PI3Ks, which this review is focused on, consist of four catalytic isoforms, namely p110α, -β, -γ, and -δ. Class IA PI3Ks are dimers of a p110 catalytic protein and a p85 or p55 regulatory adapter subunit as detailed in **Figure 2** (4). Each regulatory subunit can associate with any of the three catalytic class IA isoforms: p110α, p110β, and p110δ (5).

The expression of distinct catalytic isoforms is cell type specific. P110α and -β are expressed in all cell types. In mice, homozygous knockouts of p110α and p110β are embryonic lethal, emphasizing their physiological importance (6–8). In contrast, p110δ expression is mainly confined to leukocytes (4). Genetically modified mice expressing catalytically inactive PI3Kδ (PI3Kδ^{KI}), manifest impaired B-cell, NK cell, and T-cell function (9, 10).

Class IB PI3Ks, which are built by a p110γ catalytic and a p101 regulatory subunit, are selectively expressed by leukocytes (4, 11) and their activation is mediated by GPCRs (4). Knockdown of p110γ in mice causes altered migration and recruitment of myeloid cell populations (12–14), which is in concordance with other reports indicating that the PI3Kγ isoform is expressed mostly in the myeloid cell lineage (15–17).

PHOSPHOINOSITIDE 3-KINASE SIGNALING IN CANCER AND CHRONIC LYMPHOCYTIC LEUKEMIA

Phosphoinositide 3-Kinase Signaling in Cancer

One of the best-characterized hallmarks of cancer cells is their ability to sustain chronic proliferation (18). While healthy cells can strictly regulate the production and release of growth-promoting signals, cancer cells often fail to do so and malignant transformation occurs as a result of mutations in one or more components of these signaling pathways (18). There are several pathways that influence each other through multifaceted interactions and contribute to tumor development. Some of the major signaling pathways whose components are likely to be cancer drivers include proteins like growth factor receptor tyrosine kinases (e.g., EGFR), lipid kinases (e.g., PI3K), small GTPases (e.g., RAS), oncogenes and tumor suppressors (e.g., MYC, P53), serine/threonine kinases (e.g., RAF, AKT), and cytoplasmic tyrosine kinases (e.g., SRC, ABL) (19–24). Among these, involvement of the PI3K signaling pathway in cancer has been revealed first when PI3K signaling was found to be hyperactive in phosphatase and tensin homologue (PTEN)-deficient tumors (25). Independent of stimulating receptors, PI3Ks can be activated directly *via* oncogenes like *RAS* (26) and their activity is negatively regulated by the tumor suppressor PTEN, that acts as a PI3K phosphatase (**Figure 1**) (27).

Altered PI3K/AKT signaling has been associated with many types of cancer (28). Chronic lymphocytic leukemia (CLL), breast, ovarian, liver, lung cancers, and glioblastomas are well-studied examples in which altered PI3K activity was observed (29–32). Genetic alterations such as amplifications, gain of copy numbers and increased gene expression have been observed for all class I PI3Ks in many different cancer entities (26). P110α (*PIK3CA*) is affected most frequently and a direct oncogenic potential was attributed to it (33), as the signaling of mutant p110α is active and less dependent on external stimuli (34).

Phosphoinositide 3-Kinase Signaling in Chronic Lymphocytic Leukemia Cells

Compared to the mutation rates in other cancer entities, activating mutations of PI3K are scarce in CLL (35, 36). Genomic analysis revealed an amplification of *PIK3CA* in 3.5%

of CLL patients, which is perhaps surprising when all the significant pharmaceutical effort to inhibit the PI3K pathway in this malignancy is considered (37). However, PI3K signaling is constitutively active in CLL cells, even in the absence of genetic drivers (38, 39). There are three well-studied pathways that are capable of activating and maintaining PI3K signaling in CLL cells; BCR, RTKs and cytokine/chemokine receptors (40–42). Although the stimulation of these pathways initiates different cascades of downstream events, they converge at a final point, that is, generation of a domain that can bind to the SH2 region of the regulatory p85 subunit. If the SH2 domain remains occupied, p85 fails to bind a p110 catalytic isoform and inactivate it, which results in constitutively active PI3K (43). The importance of these three pathways varies for different cell types, however, BCR is shown to be the dominant contributor to PI3K activity in B-cell malignancies. Autonomous events or continuous BCR activation by autoantigens have been reasoned for tonic BCR stimulation in CLL (44, 45).

In conclusion, the highly active PI3K signaling pathway in CLL serves as an ideal therapeutic target to inhibit CLL cell proliferation and survival directly.

PHOSPHOINOSITIDE 3-KINASE SIGNALING IN THE TUMOR MICROENVIRONMENT

Although CLL is a genetically heterogeneous disease, it is widely accepted that CLL cells are highly dependent on supporting stimuli of bystander cells in their microenvironment for their survival, proliferation, homing to lymphoid tissues as well as chemo-resistance. Different types of stromal cells, myeloid, NK and T-cells were shown to have dual functions in the tumor microenvironment (TME) of CLL both in patient samples and more recently the Eμ-TCL1 mouse model as reviewed extensively by us and others (46, 47).

In the last 20 years, many mouse models were generated in which different components of the PI3Ks were knocked out (6, 48–51). The results of the studies using these mice need careful interpretation as the alteration of one isoform's expression can impact on those of other isoforms. As the different catalytic and regulatory subunits have the ability to interact in various combinations (4), the outcome of the expression variations in these knockout models might be far from reflecting the true functions of these proteins. For instance, an increased expression of p85β in addition to decreased expression of the three p110 isoforms (α, β, δ) has been shown in NK cells from p85$\alpha^{-/-}$ mice (5, 52). In another study where p110δ was knocked out, levels of p85α were decreased, p110β and -γ were increased but p110α remained unchanged in NK cells (53). Methodologies involving the generation of point mutations that completely inactivate the catalytic function of isoforms without altering the normal expression levels may provide more reliable conclusions compared to the knockout models that alter the expression of all isoforms, as the former may mimic the normal physiological state better and make it easier to assign functions directly.

Phosphoinositide 3-Kinase Signaling in Non-Immune Components of the Tumor Microenvironment

In the TME, complex interactions between cancer associated fibroblasts (CAFs), immune cells, signaling molecules, extracellular matrix (ECM), vasculature-associated cells, and malignant cells take place (54). Cancer cells are known to orchestrate the host tissue to facilitate tumor progression and regulate the recruitment of immune cells to the TME. PI3K signaling is engaged in almost all these components, including the stromal compartment. As discussed above, CLL cells require strong support from stromal cells for their proliferation and survival (55). Similarly, Bertrand et al. have shown that stromal cells protect the B-cell acute lymphoblastic leukemia (B-ALL) cells from apoptosis *via* PI3K and MEK pathway dependent mechanisms and co-inhibition of these pathways can synergistically break this apoptotic resistance (56).

The ability of solid tumors to grow depends strictly on oxygen and nutrient delivery through newly developing blood vessels. Accumulating evidence indicates that angiogenesis is also important in hematologic malignancies, including CLL (57). Neovascularization is a cooperative event involving endothelial cells, myeloid cells, and the tumor cells that secrete angiogenic factors such as vascular endothelial growth factor (VEGF). Inhibitors of different PI3K isoforms were shown to generate different vascular responses as the dominant PI3K isoform diverges among these cells (58). Up to date, most of the research that investigates the effect of PI3K inhibition on tumor vascular function was performed with high doses of pan-PI3K inhibitors and these studies are pointing towards a vascular dysfunction which is associated with decreased blood flow and vessel density (59–61). As the anti-angiogenic effect of PI3K inhibition is outcompeted by anti-VEGF therapies (62, 63), PI3K inhibition has not been considered specifically for targeting tumor neoangiogenesis. However, recent preclinical studies show that low doses of PI3K inhibition contribute to vessel normalization which might enable more efficient delivery of chemotherapy drugs and immune infiltration to the tumor sites (64, 65). Overall, these studies support that targeting PI3Ks in tumor and endothelial cells selectively hold the potential to synergize with conventional therapies.

CAFs constitute one of the most abundant but poorly defined cell populations in the TME. A commonly held view is that CAFs promote tumorigenesis *via* remodeling of ECM components, facilitating metastasis, promoting vascularization, and secreting cytokines and growth factors (66). This view is supported also for CLL, where leukemic cells are able to induce the transformation of stromal cells to a CAF-like phenotype by an exosome-mediated fashion (67). In this study, it has been shown that the CLL-derived exosomes are actively taken up by stromal cells both *in vitro* and *in vivo*. Upon uptake, stromal cells gained pro-tumoral characteristics, evident by enhanced proliferation, migration, angiogenesis, and secretion of inflammatory cytokines *via* mechanisms involving activation of AKT and NF-κB. These events are known to be partly involving PI3K signaling (68–70). Thus, it is expected that PI3K inhibition might dampen the pro-tumoral activity of CAFs.

Phosphoinositide 3-Kinase Signaling Pathway in Immune Cells

All immune cells express receptors that can initiate PI3K signaling, but the components and wiring of the pathway are different in several aspects compared to other cells (1). The major difference is the expression of the catalytic isoform. P110δ and p110γ comprise the dominantly expressed isoforms while p110α and p110β are rarely engaged in immune cells (71). In non-immune cells, a higher degree of PI3K activation usually correlates with an increased downstream function such as apoptosis, survival, proliferation, and growth. However, in immune cells, an increased activation of the PI3K pathway does not necessarily mean a stronger immune reaction. Depending on the cell type, the pathway has the potential to augment or alleviate the response to pathogens as well as tumors. The network of the PI3K pathway downstream of AKT seems to be different for lymphoid cells subsets. MTORC1 phosphorylates a wide range of downstream proteins when stimulated *via* PI3K/AKT and RAS/ERK pathways but it also requires signals delivered through nutrient-sensing pathways to ensure that cells do not intent to proliferate under stress conditions like nutrient deprivation (72, 73). In lymphocytes, mTORC1 was shown to be more dependent on nutrient inputs than on PI3K/AKT activity (74–77). The two best-characterized mTORC1 substrates in mammals are p70 ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (78). 4E-BP/Eukaryotic translation initiation factor 4E (eIF4E) arm is known to be responsible for regulation of growth and proliferation in lymphocytes (79), whereas in other cell types, S6Ks are the main mediators of cell growth (80). It has also been hypothesized that S6Ks might be involved in lymphocyte differentiation, but to our knowledge this has not been validated yet (81–83) (**Figure 1**). Overall, considering these immune cell-specific features might be useful while estimating the overall impact of PI3K-mTOR inhibition on the TME.

Phosphoinositide 3-Kinase Signaling in Myeloid Cells

Innate immune cells such as monocytes, neutrophils, DCs, mast cells, and macrophages are the key components that mediate the initial phase of acute inflammation *via* cytokine/chemokine secretion, extravasation, migration, and phagocytosis. Each of these events requires class I PI3K activation (84). Upon detection of a stimulus, tissue resident macrophages immediately start producing cytokines and chemokines to attract neutrophils and macrophages to the site of infection, which requires p110δ expression (84). These chemoattractants are known to activate neutrophil p110γ through GPCR (14). After reaching the site of inflammation, macrophages and neutrophils phagocyte and lyse the pathogens with nicotinamide adenine dinucleotide phosphate oxidase (NOX)-derived ROS. It has been shown that p110β and p110δ are involved in the regulation of phagocytosis (85–87). In line with this, p110β-deficient mice fail to mediate Fc gamma receptor (FcγR)-dependent activation of neutrophils and produce ROS (88). In addition to initiating ROS production, maintenance of NOX activity for ROS production also requires the activation of p110δ (89).

All class I PI3K catalytic unit isoforms are expressed in myeloid cells. However, current literature suggests that p110δ and p110γ are the critical isoforms for mediating their immune functions. Deficiency of these two isoforms has been reported to induce neutrophilia in mice (90), while inhibition of only one of them did not have a striking effect. Although the p110γ isoform is expressed in many hematopoietic cells, highest level of expression was detected in cells of the myeloid lineage and the inhibition of p110γ resulted in dampened inflammatory responses (14, 91). Of note, neither neutrophil nor macrophage development was impaired in $p110\gamma^{-/-}$ mice. However, chemoattractant-stimulated neutrophils failed to produce PIP3 and implement respiratory burst (14). Motility and migration mechanisms have also been noted to be defective, both in neutrophils and macrophages (14). Similarly, the migration of antigen-loaded DCs from the tissue to draining lymph nodes was impaired demonstrating the non-redundant functions of p110γ (92).

Effect of PI3K Inhibition in Myeloid Cells in Cancer

PI3K signaling mediates an immunosuppressive phenotype in myeloid cells to prevent excessive innate immunity in chronic infections and inflammation (89, 93–95). This phenomenon supported the idea to pharmacologically target the PI3K p110γ isoform with specific inhibitors for the treatment of inflammatory diseases but the possibility of dampening the host defense has slowed down the development of this idea.

However, the pro-inflammatory potential of PI3K inhibitors might provide new opportunities in cancer treatment. In many cancer entities, pro-inflammatory cytokine expression is correlated with better prognosis. High expression levels of p110γ that induces immunosuppression in TAMs, were reported in several studies (15, 96, 97). Selective inhibition of PI3Kγ in mouse models of melanoma and breast cancer revealed a downregulation of M2 markers (*Tgfb1*, *Arg1*, *Ido1*) and an upregulation of M1 markers (*Il12a* and *Nos2*) (98), demonstrating the influence of this isoform on the phenotype and polarization of TAMs. Interestingly, reduced tumor growth as well as enhanced anti-tumoral T-cell responses have been noted upon genetic or pharmacological p110γ inhibition (15, 96, 98). Of note, cancer cells of most solid tumors do not express p110γ and anti-tumoral effects of p110γ inhibition might be attributed to TAMs and the TME.

Recently, the immune microenvironment has gained attention in cancer therapy. Although the approval of checkpoint inhibitors has been a significant breakthrough in immunotherapy, multiple resistance mechanisms exist, and the presence of tumor infiltrating myeloid cells are among these. Following encouraging pre-clinical results, a phase 1 trial combining immune checkpoint blockade and inhibition of p110γ has been initiated, but results are pending (NCT02637531) (98).

Inactivation of p110δ isoforms mostly interferes with the functions of adaptive immune cells but this isoform has also been implicated in controlling the pro-tumorigenic effect of myeloid cells in cancer (99, 100). In one study, histological analysis of patient breast tumors demonstrated a correlation between the

disease progression and levels of PI3Kδ in tumor cells. In addition to the tumor cells, $CD68^+$ macrophages also manifested strong PI3Kδ staining (101). Treatment with a PI3Kδ-specific inhibitor, IC87114, reduced the volume of human breast tumors in xenograft models when administered intratumorally or orally (101). Intratumoral treatment only slowed down tumor growth, as a small but significant proliferation of tumor cells was still detectable (101). However, oral administration of the inhibitor totally stopped tumor growth and reduced macrophage infiltration (101), suggesting that PI3Kδ inhibition in these cells might be a contributing factor to the anti-tumoral effect. To further evaluate the impact of macrophage PI3Kδ inhibition on tumor growth, the authors adoptively transferred either PI3Kδ-knockout (KO) or wildtype (WT) macrophages to tumor bearing NOD/SCID-gamma null (NSG) mice (101). Tumor growth was significantly reduced in the mice that received PI3Kδ-KO macrophages compared to the ones that received WT macrophages (101). In addition, PI3Kδ-KO macrophages were less competent in infiltrating the tumors compared to WT macrophages (101), demonstrating that inhibition of macrophage PI3Kδ might be sufficient to reduce the infiltration of macrophages to tumors and thereby impact on tumor growth. In the final set of experiments, the researchers inoculated PI3Kδ expressing tumors to macrophage-deficient NSG mice and PI3Kδ-deficient tumors to Balb/c nude mice with functional and PI3Kδ-proficient macrophages. While treatment with the PI3Kδ inhibitor IC87114 reduced tumor growth in both groups, complete abrogation was only seen in mice with PI3Kδ-expressing tumors and WT macrophages (101). Overall, these results suggest that targeting PI3Kδ both in tumor cells and TAMs is likely to be a promising strategy for treatment of breast cancer.

The exact function of PI3K isoforms in DCs is unclear. To study the impact of PI3K inhibition in DCs, Marshall et al. have employed different mouse tumor models and reported that either broad or β-, δ-, or γ-isoform-specific inhibition reduced levels of IL-10 and TGFβ but enhanced IL-12 expression (99). This caused a less immunosuppressive TME and facilitated the priming of Th1 T-cells *in vivo*. An important finding of this study is that selective inhibition of PI3Kβ and -δ isoforms reduced IL-10 and TGFβ expression in toll-like receptor (TLR)-activated DCs pulsed with dead tumor cells *in vitro*. Adoptive transfer of pulsed DCs into tumor-bearing mice in combination with pan-PI3K inhibition resulted in a significant increase in anti-tumoral T-cell response, decrease in tumor growth and prolonged overall survival, demonstrating the potential of PI3K inhibitors for the development of tumor DC-based vaccines.

Effect of Phosphoinositide 3-Kinase Inhibition on Myeloid Cells in Chronic Lymphocytic Leukemia

Twenty years ago, Burger et al. discovered that peripheral blood monocytes differentiate in co-culture with CLL cells to adherent cells that support the survival of CLL cells *in vitro* (102). These cells were named as nurse-like cells (NLCs), and only later on, it was understood that they resembled tumor-associated macrophages (TAMs) in solid tumors (103, 104). NLCs exhibit an M2-like phenotype and besides delivering survival signals, they were suggested to have immunosuppressive functions (55, 105).

Research investigating the effect of PI3K inhibition on myeloid cells in CLL is scarce. Thus far, efforts have been dedicated to identifying the effect of clinically approved PI3K inhibitors in modulating the effect of type I anti-CD20 monoclonal antibody (e.g., rituximab and ofatumumab) treatment. More specifically, the role of PI3K in regulating antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent phagocytosis (ADP) responses has been investigated. Da Roit et al. described an effect of the PI3Kδ inhibitor idelalisib on inhibiting activation of polymorphonuclear neutrophils (PMN) and phagocytosis of anti-CD20 opsonized CLL cells by macrophages (106). Based on these observations, Enya Chen et al. investigated the role of PI3K-p110 in modulating antibody-mediated responses by macrophages in CLL (107). The authors demonstrated that class I PI3K p110 isoforms α, β, γ, and δ are expressed in nurse-like cells (NLCs) of both anti-CD20 antibody-sensitive and -resistant CLL patients (107). The effect of PI3K inhibition on ADCC and ADP was found to be FcγR-mediated and independent of downstream spleen tyrosine kinase (SYK) and Bruton's tyrosine kinase (BTK) signaling. Inhibition of p110δ, both pharmacologically and *via* siRNA knockdown, decreased ADCC and ADP in CLL-derived NLCs (107), suggesting that this catalytic isoform is essential for CLL-derived NLC as well as macrophage response to therapeutic antibodies.

In a recent study, idelalisib has been shown to prevent AKT-dependent phosphorylation of the $p47^{phox}$ subunit of NOX2, resulting in suppressed rituximab-induced ROS formation in human monocytes (108). In co-cultures of monocytes and NK cells, idelalisib rescued NK cells from ROS-induced toxicity and thus improved NK cell ADCC against primary CLL cells and 221 B-lymphoblastoid cells (108).

In addition to its role in regulating ADCC, the PI3K pathway is also involved in migration and homing of CLL cells which circulate between peripheral blood and secondary lymphoid organs (109). Trafficking of CLL cells is regulated by stromal cells and NLCs in the lymph nodes that secrete chemokines including C-X-C motif chemokine ligand (CXCL)12, CXCL13, C-C motif ligand (CCL)19, and CCL21 (110). Upon the activation of chemokine receptors, PI3K signaling directs the CLL cells towards stromal cells that provide tumor supportive stimuli (111). As expected, inhibition of the PI3K pathway *in vitro* with PI3Kδ-specific idelalisib was shown to result in impaired homing of CLL cells to stromal cells, due to their reduced response to CXCL12 and CXCL13 (109). These findings partly explain the rapid lymph node shrinkage in CLL patients treated with PI3Kδ inhibitor idelalisib (112).

Overall, current research highlights the importance of PI3K activity in immune responses to therapeutic antibodies and suggests that both enhancing and inhibiting this pathway might improve these responses (**Figure 3**). Nevertheless, further *in vitro* and *in vivo* studies are needed to unravel the

diverse and complex effects of PI3K inhibition in the myeloid compartment of CLL.

Phosphoinositide 3-Kinase Signaling in Natural Killer Cells

Information about how NK cells are affected by PI3K inhibition is relatively sparse and derived mainly from knockouts of PI3K isoforms in mice. NK cells are critical mediators of innate immunity that do not require prior sensitization or antigenic stimulation. Upon activation by tumor cells, they can exert their cytolytic functions *via* granzyme (Gzm)- or receptor-mediated fashion (113, 114). They are also producers of an array of cytokines including interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα), which act on other immune cells to enhance the immune response. There are several studies providing evidence that PI3K isoforms control signaling checkpoints in cytokine expression and secretion in mouse NK cells (115). PI3Ks are involved in the signaling downstream of different NK cell activation receptors such as NKG2D, CD16, and CD28. In addition, PI3K–mTOR pathway is indispensable for efficient NK cell activity, specifically for cytokine responses in IL-15 primed NK cells (116). PI3K inhibitors were also shown to have an impact on NK cell granule polarization. Upon tumor contact, NK cells start to mobilize perforins and GzmB towards the tumor cells (117). Zhong et al. have shown that pre-treatment of NK cells with pan-PI3K inhibitors (wortmannin or LY294002) effectively stopped this intracellular polarization of cytotoxic granules (118). A complementary study has also shown that mice lacking the regulatory p85α subunit and its alternatively spliced variants (p55α/p50α) had impaired lineage commitment leading to reduced NK cell numbers in the bone marrow and liver. In addition to genetic inhibition, broad pharmacological PI3K inhibition with wortmannin also reduced the cytotoxicity against EL-4 lymphoma cells and cytokine/chemokine generation was significantly compromised (119). Other studies that support these findings exist, in which treatment with different PI3K inhibitors (wortmannin, LY294002, IPI-145) quenched mouse and human NK cell cytotoxicity against tumor cells (120–122) while inhibition of a single p110 isoform did not have a profound effect. This phenomenon brings up the question whether different p110 isoforms can compensate for/replace each other in NK cells. Kim et al. have investigated the relative contribution of p110δ and -γ isoforms on NK cell anti-tumor cytotoxicity and provided an answer to this question to some extent (123). They reported a reduction in NK cell numbers and production of the cytokines IFNγ, TNFα, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in p110δ-deficient, but not p110γ-deficient NK cells. When both p110δ and p110γ isoforms were inactivated by a combination of genetic and biochemical approaches, NK cell cytotoxicity was attenuated, which was not observed upon inhibition of a single isoform (123). Therefore, it is likely that cooperation of isoforms can compensate for the maintenance of cytotoxic function in NK cells. However, the overall results suggest that p110δ is the dominant PI3K isoform for cytokine secretion by NK cells. In line with this study, Saudemont et al., have also proposed that P110δ is the dominantly expressed and preferentially activated isoform in NK cells (124).

Using a murine model of catalytically inactive p110δ ($PI3K\delta^{KI}$), Guo et al. have shown that p110δ plays a crucial

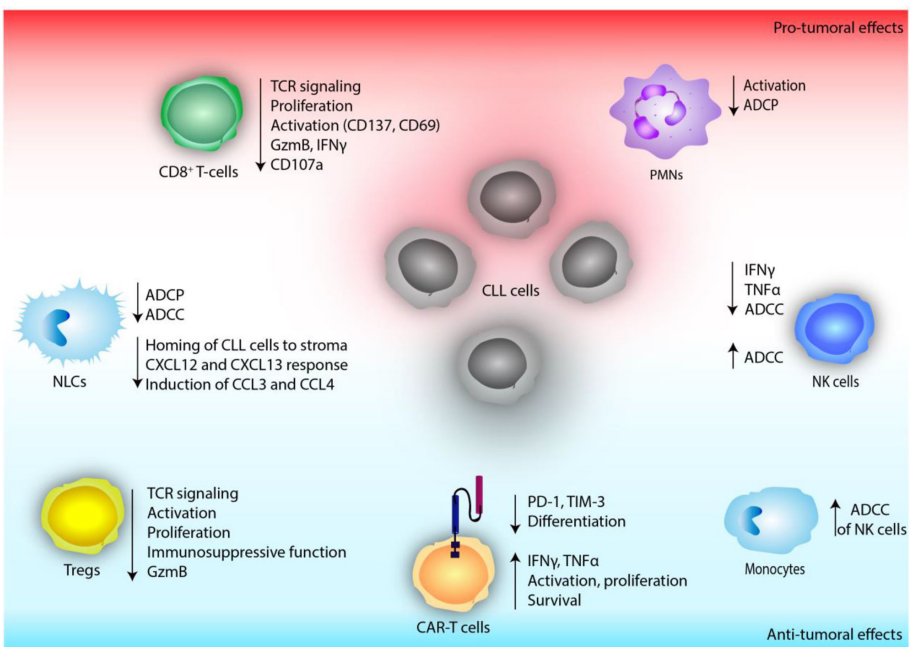

FIGURE 3 | The effect of defective PI3K signaling on immune cells of the tumor microenvironment in chronic lymphocytic leukemia (CLL).

role in NK cell terminal maturation and cytokine/chemokine generation (10). Moreover, the PI3Kδ^{KI} mice had reduced NK cell numbers and maturation. NK-mediated cytotoxicity was slightly impaired against EL-4, YAC-1, and RMA-S tumor cells *in vitro* and *in vivo*. However, cytokine and chemokine generation by NK cells was severely affected accompanied by a reduced c-Jun N-terminal kinase (JNK) 1/2 phosphorylation in response to NKG2D-mediated activation. The study provides evidence for the role of PI3Kδ in downstream JNK activation that regulates NK cell cytokine generation (10).

Another study using the same mouse model showed that the NK cells derived from PI3Kδ^{KI} mice showed comparable cytotoxicity to WT NK cells, suggesting that p110δ is not indispensable to kill RMA-S lymphoma cells *in vitro* (115). On the contrary, mice lacking a functional p110δ failed to eliminate *i.p.* administered syngeneic tumor cells. The authors hypothesized that the failure might be specific to the intraperitoneal model they have used, or it may possibly indicate a defect of NK cell migration to the tumor site (115). Two years later, the same group has tested whether the p110δ isoform was essential for NK cell trafficking. Using genetically modified mice, they observed that p110δ was indispensable for chemotaxis to sphingosine-1-phosphate (S1P) and CXCL10, NK cell distribution throughout lymphoid and non-lymphoid tissues and for extravasation to tumors (124).

Effect of Phosphoinositide 3-Kinase Inhibition on Natural Killer Cells in Chronic Lymphocytic Leukemia

NK cells are not able to kill CLL cells directly, however they contribute substantially to ADCC (125, 126). Rituximab, a chimeric monoclonal antibody that targets CD20, was approved in 2010 and served as standard therapy in combination with chemotherapy for the treatment of CLL. Fc region of the antibody communicates with the NK cell surface Fc-γ-receptor CD16 (FcγRIIIA) (127). Fc receptor ligation leads to PI3K activation in NK cells, triggering release of cytotoxic granules and ADCC (123, 128). Earlier *in vitro* studies using idelalisib have not reported any significant cytotoxicity towards NK cells (38). Interestingly, pre-treatment of NK cells with idelalisib inhibited their production of inflammatory cytokines, such as TNFα and IFNγ but did not alter their ADCC against CLL cells (38) (**Figure 3**).

Considering that the ADCC of NK cells is mediated through PI3K activation, it might be expected that inhibition of PI3Kδ would limit the clinical efficacy of rituximab. For clinical use, idelalisib is only approved in the combination with αCD20 antibodies and this treatment is efficacious (129). To investigate whether idelalisib dampens NK cell mediated ADCC, a direct comparison of idelalisib *versus* idelalisib + rituximab would be necessary.

Phosphoinositide 3-Kinase Signaling in T-Cells

PI3K signaling is involved in several T-cell specific functions spanning from their activation to apoptosis. The activity of PI3K is regulated by the co-stimulatory receptors CD28 (4, 9) and inducible T-cell co-stimulator (ICOS) (4). Activation of these receptors results in subsequent activation of PI3Kδ, the dominantly expressed PI3K isoform, which leads to proliferation and differentiation into regulatory, effector, and memory T-cell phenotypes (4, 9). In addition, PI3Ks regulate survival and apoptosis by increase of B-cell lymphoma-extra large (BCL-XL) expression or IL-2 secretion in response to T-cell receptor (TCR) activation (4). These functions of T-cells involve calcium flux, in which PI3Ks are also involved (4). Resting T-cells sustain a low level of Ca^{2+}. Upon TCR activation, the cells experience an influx of Ca^{2+} (130). It is reported that PI3Ks are involved in the regulation of calcium signaling *via* IL-2-inducible T-cell kinase (ITK), indicating the significance of the crosstalk between these pathways (4).

Effect of Phosphoinositide 3-Kinase δ Inhibition on T-Cells

PI3Kδ is crucial for the function of T-cells, as reduced activity of PI3Kδ has been shown to diminish TCR signaling of murine (9, 131–134) as well as human T-cells (135–137) *in vitro*, including all major CD4$^+$ and CD8$^+$ T-cell subsets. Subsequently, reduced proliferation of murine (9, 132, 133, 135, 137, 138) as well as human T-cells (134–137) has been observed upon abrogated PI3Kδ signaling, without induction of apoptosis (133). Of note, some combinatory approaches targeting the TCR as well as the co-stimulatory receptor CD28 or supplying IL-2 have been shown to overcome the negative effects of PI3Kδ inhibition on T-cell activation (9, 133, 135).

T-cells differentiate after the activation of their TCR into antigen-experienced effector and memory cells (139, 140). During this process, surface marker expression as well as transcriptional programs change, which for example enables effector and memory T-cells to home to distinct organs. Memory T-cells are equipped to provide fast cytotoxic functions and proliferation upon secondary recall infections (140, 141). In line with reduced TCR-activation, impaired PI3Kδ signaling has been shown to result in a higher expression of CD62L (138, 142, 143), CD127 (143) as well as CCR7 (9, 143), and therefore, less terminally differentiated T-cells. Long-term culture of T-cells using repeated TCR stimulations in the presence of PI3Kδ inhibitors resulted in higher proliferation rates and IL-2 secretion (138), likely caused by initial blockade of T-cell activation and differentiation. The typical memory T-cell markers such as CD62L or CCR7 are important mediators of migration and homing. In contrast to the higher expression of these markers in PI3Kδ-defective T-cells, transfer of WT, or PI3Kδ^{KI} T-cells into syngeneic hosts did result in comparable homing to lymph nodes (133), suggesting that the expression of naïve/memory markers does not affect the migration of these cells.

Following the clonal expansion of T-cells, particularly their production of effector molecules is essential to control pathogens as well as cancer. After *in vitro* or *in vivo* stimulation of PI3Kδ-defective T-cells, a lower cytokine production of IFNγ, IL-2, IL-17, and TNFα (135) has been observed compared to WT cells. This was confirmed using an antigen-specific stimulation of

T-cells of WT or PI3Kδ^{KI} mice, in which a reduced expansion as well as IFNγ production of mutant T-cells was noted (133).

In conclusion, PI3Kδ is essential for TCR signaling and subsequent activation, proliferation, and cytokine production of T-cells. However, it is likely that the negative effects of PI3Kδ inhibition in T-cells can be partially overcome by applying strong or combinatory stimuli.

Effect of Phosphoinositide 3-Kinase δ Inhibition on CD4$^+$ T-Cells

CD4$^+$ helper T-cells (Th cells) are involved in multiple immune functions, such as control of immune reactions by Tregs as well as providing help in the clearance of pathogens by Th cells (46).

IFNγ, IL-4 and IL-17 are the key cytokines of Th1-, Th2-, and Th17-mediated responses, respectively (46). Following *in vitro* stimulation in the presence of PI3Kδ inhibitors, a reduced production of IFNγ (133, 135), IL-4 (133), and IL-17 have been noted (135). Of interest, memory CD4$^+$ T-cells were more susceptible to abrogated PI3Kδ signaling than their naïve counterparts (135). These data highlight that PI3Kδ inhibition affects the function of Th cell subsets and suggest that antigen-experienced T-cell subsets might be more dependent on PI3Kδ signaling than naïve T-cells.

Tregs are crucial in maintaining orchestrated immune reactions by paracrine secretion of IL-10 or direct, contact-mediated inhibition of effector responses (46). In line with the reduced proliferation of this cell type upon PI3Kδ inhibition *in vitro* (136, 137), lower frequencies of Tregs in lymphoid organs and blood of p110δ^{KI} mice have been observed (100, 144), and shown to be most likely due to an increased rate of apoptosis (137). In line with reduced TCR activation upon PI3Kδ inhibition, lower expression levels of FOXP3, CD25, ICOS, and PD-1 have been noted in human Tregs *in vitro* (136), indicating a reduced activation state and immunosuppressive potential of this cell type. Accordingly, PI3Kδ-inhibited murine (144) as well as human Tregs (136) were reported to be less efficient in suppressing effector T-cell proliferation *in vitro*, and lower concentrations of the Treg-specific cytokine IL-10 but higher concentrations of the effector cytokines IL-2 and TNFα have been observed in the culture supernatants upon PI3Kδ inhibition (136). Interestingly, the negative effect of PI3Kδ inhibition could be overcome upon co-culture with antigen-presenting cells (APCs) (144), which could provide strong co-stimulatory signals to compensate for the inhibitory effects of abrogated PI3Kδ signaling. Comparing the sensitivity of Tregs and effector T-cell populations to PI3Kδ inhibition, *in vitro* assays showed that Tregs seem to be more sensitive (134, 136, 137). In sum, the functions of helper T-cells as well as Tregs are dependent on PI3Kδ signaling. Moreover, it is likely that Tregs are more sensitive to PI3Kδ inhibition than effector T-cells, which could unleash immunosuppressive bonds from effector cells and enhance their effector function.

Effect of Phosphoinositide 3-Kinase δ Inhibition on CD8$^+$ T-Cells

CD8$^+$ T-cells are important for the control of pathogens as well as anti-tumoral immunity (46). In addition to the previously mentioned reduced *in vitro* proliferation of T-cells, the numbers of pathogen-specific CD8$^+$ T-cells was also reduced in PI3Kδ-deficient mouse models of infections (131, 132). Therefore, a thorough analysis of CD8$^+$ T-cell function upon PI3Kδ inhibition is essential, as enhanced effector functions could compensate for the reduced expansion of these cells and result in similar immunological properties.

The expression of early activation markers is often used to analyze TCR signaling activity and the activation status of T-cells. Upon TCR stimulation reduced expression of the activation markers CD25 (132), CD69 (132, 134, 143), killer cell lectin-like receptor subfamily G member 1 (KLRG1) (143) as well as PD-1 (143) have been noted in murine, PI3Kδ-inhibited CD8$^+$ T-cells compared to untreated cells. Similarly, abrogated PI3Kδ signaling resulted in a reduced expression of CD69 (134, 135) as well as T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) (143), a marker of activation-induced dysfunction, on human CD8$^+$ T-cells after TCR engagement in comparison to PI3Kδ-proficient T-cells, suggesting that not only numbers, but also activation of CD8$^+$ T-cells could be altered by PI3Kδ inhibitors.

Once activated, CD8$^+$ T-cells secrete granzymes and cytokines such as IFNγ or TNFα, which is essential for their immune control of pathogens and cancer (46). *In vitro* analysis of CD8$^+$ T-cell function after antigen-specific or antibody-mediated TCR stimulation revealed lower expression of the effector molecules IFNγ (131, 135), TNFα (132), GzmB (100, 131), GzmA (100, 142), perforin (100), and IL-2 (135) upon PI3Kδ inhibition.

In summary, PI3Kδ is essential for the activation and cytotoxic function of CD8$^+$ T-cells, which could in turn result in a reduced immune surveillance and anti-tumoral immunity in individuals that are treated with PI3Kδ inhibitors.

Targeting Phosphoinositide 3-Kinase δ in T-Cells in Cancer

The function of PI3Kδ in T-cells has extensively been investigated in many murine cancer models. According to previously mentioned results, global PI3Kδ abrogation in mice caused a reduced frequency and absolute number of Tregs at tumor sites (137, 142, 145) and in draining lymph nodes (100). Of interest, reduced frequencies of Tregs have already been found in spleens of mice as early as 3 days after a single dose of idelalisib (137), suggesting a very fast reaction to PI3Kδ inhibition. Concomitantly with reduced Treg numbers, improved tumor control has been noted in various tumor models using either pharmacological or genetic inhibition of PI3Kδ (100, 142, 145). Utilizing FoxP3^{Cre} x δ$^{flox/flox}$ mice, in which PI3Kδ-deficiency is specific to Tregs, a reduced tumor growth and improved survival was noted in comparison to systemic PI3Kδ-deficient or PI3Kδ-proficient mice (100). The improved tumor control in the presence of PI3Kδ-deficient Tregs was further confirmed by transfer of WT or PI3Kδ^{KI} Tregs into PI3Kδ^{KI}-mice (100). This data highlight that inhibition of Treg-PI3Kδ reduces the accumulation and immunosuppressive function of Tregs which results in an enhanced anti-tumoral immunity in mouse models of cancer.

Supported by reduced Treg functions upon PI3Kδ inhibition, enhanced tumor-infiltration, and cytotoxic function of $CD8^+$ T-cells were expected. Accordingly, systemic PI3Kδ inhibition resulted in an increased ratio of $CD8^+$ T-cells to Tregs and increased numbers of $CD8^+$ tumor-infiltrating lymphocytes (TIL) in mouse models of carcinoma (100, 145). Further, transfer of WT but not $PI3K\delta^{KI}$ Tregs into $PI3K\delta^{KI}$ mice resulted in reduced infiltration of $CD8^+$ TILs (100). These data strongly support the hypothesis that reduced Treg function, caused by defective PI3Kδ signaling, increases TIL infiltration and enhances anti-tumoral immunity.

Seminal work by Lim et al. investigated several tumor models expressing the ovalbumin (OVA) antigen, namely EL-4 lymphoma, MC-38 colon, and LLC lung carcinoma (142). For EL-4 and MC-38 cancer models, tumor growth was dependent on Treg-PI3Kδ, as depletion of Tregs using $FoxP3^{DTR}$ mice, as well as Treg-specific, genetic PI3Kδ deficiency resulted in a reduced tumor burden (142). In line, transfer of WT or $PI3K\delta^{KI}$, tumor-specific $CD8^+$ T-cells into EL-4 tumor-bearing mice did not impact on the disease progression (100). This data suggest that $CD8^+$ T-cells are not sufficient to mediate disease control in the EL4 model while targeting PI3Kδ in Tregs has a high potential.

In contrast, growth of LLC- and MC-38 tumors was neither altered by depletion of Tregs, nor using a systemic $PI3K\delta^{KI}$ model (142), suggesting that tumor control in these models relies at least partially on functional PI3Kδ in other immune cells, likely $CD8^+$ T-cells. In line, LLC tumor cells were most susceptible to $CD8^+$ T-cell mediated killing *in vitro*, followed by MC-38 and EL-4 tumors (142). This anti-tumoral immunity was abolished if $CD8^+$ T-cells harbored a $PI3K\delta^{KI}$ mutation (142). Similarly, *ex vivo* analysis of $CD8^+$ TILs of LLC-OVA or MC-38 tumors revealed a reduced expression of the cytotoxic molecules GzmA and GzmB if PI3Kδ-signaling was abrogated (142).

In conclusion, the effects of PI3Kδ inhibition on T-cells and subsequently on tumor control are highly contextual. In tumors that are controlled by an immunosuppressive, Treg-dependent microenvironment, PI3Kδ inhibition can succumb the pro-tumoral TME and result in slowed tumor progression. On the contrary, in tumors that are controlled by $CD8^+$ T-cells, PI3Kδ inhibition reduces the tumor infiltration, activity as well as tumor control of this cell type. Of note, even though PI3Kδ deficiency does not have a significant impact on tumors that are resistant to $CD8^+$ T-cell mediated killing, it is likely that abolished $CD8^+$ T-cell function could cause a reduced response to pathogens and therefore, limit the success of combining immunotherapeutic treatment approaches and PI3Kδ inhibitors.

Effects of Phosphoinositide 3-Kinase δ Inhibition on T-Cells in Chronic Lymphocytic Leukemia

T-cells, their subsets and function are profoundly altered in CLL and known to be of pathological relevance, as recently reviewed by us (46), emphasizing the need to evaluate the effect of PI3K inhibitors on these cells.

Similar as in the above-mentioned tumor models, PI3Kδ inhibition by idelalisib treatment of CLL patient-derived T-cells *in vitro* also resulted in reduced proliferation, expression of activation markers (CD69, CD25) (146), and cytokine production, including IL-2, TNFα, and IFNγ (146). Using Boyden-chamber migration assays, a reduced migration of idelalisib pre-treated T-cells obtained from CLL patients has been observed (146). This data highlight that idelalisib pre-treatment reduces the function of CLL patient-derived T-cells, emphasizing the need for more detailed investigations.

Effect of Phosphoinositide 3-Kinase δ Inhibition on Tregs in Chronic Lymphocytic Leukemia

Tregs have repeatedly been reported to be more abundant in CLL patients than healthy individuals, as recently reviewed by us (46). As expected, PI3Kδ inhibition decreased TCR signaling of Tregs, which were isolated from TCL1-leukemia bearing mice, in a dose-dependent manner (134). Accordingly, reduced proliferation and numbers of Tregs have been noted *in vivo* using either pharmacological PI3Kδ inhibition in the TCL1 AT model of CLL, in which leukemic splenocytes of Eμ-TCL1 mice were transplanted into syngeneic WT mice (134) or $PI3K\delta^{KI}$ x Eμ-TCL1 mice (147). Functionally, PI3Kδ inhibition reduced the expression of activation markers and their immunosuppressive function, as a reduced secretion of GzmB was noted (134). These findings indicate that preclinical PI3Kδ inhibition in CLL results not only in defects of Treg proliferation but also induces their reduced activation and immunosuppressive phenotype.

Until now, reports analyzing Tregs in samples of CLL patients undergoing idelalisib treatment are scarce. Comparison of blood samples before idelalisib treatment initiation and during the treatment revealed a reduced frequency of circulating Tregs (148) as well as a reduced fraction of $KI\text{-}67^+$, proliferating Tregs, in comparison to pre-treatment samples (136), implicating that idelalisib treatment of CLL patients likely reduces accumulation and proliferation of Tregs (**Figure 3**). Although effects of reduced CLL burden cannot be excluded to contribute to these observations, these results are concordant with the findings of other pre-clinical models of different cancer entities.

Effect of Phosphoinositide 3-Kinase δ Inhibition on $CD8^+$ T-Cells in Chronic Lymphocytic Leukemia

In CLL, $CD8^+$ T-cells have been proposed to contribute to the control of disease progression (46, 149), thus, their function has to be maintained during treatment. As suggested by other models, PI3Kδ inhibition *ex vivo* decreased TCR signaling of $CD8^+$ T-cells, which were isolated from TCL1-leukemia bearing mice, in a dose-dependent manner (134). And a reduced proliferation of $CD8^+$ T-cells has been observed after PI3Kδ inhibitor treatment *in vivo* in the preclinical TCL1 AT model of CLL (134) as well as in another model, in which OVA-expressing TCL1 leukemic cells were transplanted into $PI3K\delta^{KI}$ mice (147). Concomitantly with their reduced TCR signaling activity and proliferation upon defective PI3Kδ signaling, a reduced expression of the activation markers CD137 and CD69 and an enrichment of naïve $CD8^+$ T-cells have been noted in the TCL1 AT model (134). Functionally, PI3Kδ inhibition caused a reduced effector molecule production of $CD8^+$ T-cells, as less

IFNγ, GzmB, and CD107a were detected, even if focusing the analysis on the antigen-experienced effector population (134). In conclusion, preclinical PI3Kδ inhibition in the TCL1 AT mouse model of CLL reduces the proliferation, expression of activation markers, differentiation, as well as effector function of $CD8^+$ T-cells, which likely contributes to a diminished CLL-control by this cell type.

In contrast to these results, Dong et al. performed a transplantation study of TCL1 leukemic cells into $PI3K\delta^{KI}$ mice, which resulted in reduced TCL1 leukemia growth in comparison to WT recipient mice (147). Of importance, tumor cell transplantation in mice with genetic differences between the donor and recipient has been shown to cause tumor rejection in the TCL1 mouse model of CLL (150) as well as a model of multiple myeloma (151), which seems a likely explanation for the lower TCL1 tumor burden in $PI3K\delta^{KI}$ *versus* WT mice. Re-challenge of these $PI3K\delta^{KI}$ mice, which previously rejected TCL1-leukemia, with tumor cells resulted again in tumor rejection and expansion of $CD44^+$ effector/memory $CD4^+$ as well as $CD8^+$ T-cells (147). Rather than providing evidence on the role of PI3Kδ for $CD8^+$ T-cell function in CLL, these results suggest that $PI3K\delta^{KI}$ mice are still proficient in a recall, memory T-cell response. Moreover, graft-rejection of TCL1-leukemia is based on a strong T-cell stimulus which potentially overcomes inhibitory effects of abrogated PI3Kδ signaling.

Most evidence about the impact of PI3Kδ inhibition on $CD8^+$ T-cells in CLL patients is derived from preclinical studies. In the clinical study by Chellappa et al. analysis of blood samples of idelalisib-treated CLL patient showed a reduced frequency of KI-67 expressing, proliferating $CD8^+$ effector T-cells during idelalisib treatment for two out of three patients (136), suggesting that PI3Kδ inhibition might not be beneficial for $CD8^+$ T-cell mediated tumor control as well as their clearance of pathogens. But based on the very small patient cohort in this study and recent findings showing that $CD8^+$ T-cells derived from lymphoid organs of CLL patients are more active than their blood-derived counterparts (149, 152), the provided analysis of blood T-cells during idelalisib-treatment allows only for very limited conclusions.

In summary, although there is evidence suggesting that pre-clinical as well as clinical PI3Kδ inhibition in CLL diminishes $CD8^+$ T-cell function (**Figure 3**), further studies investigating patient samples are needed to fully elucidate the effects of inhibited PI3Kδ signaling on $CD8^+$ T-cells in CLL.

Impact of Phosphoinositide 3-Kinase δ Inhibition on Adoptive Cell Transfers and Chimeric Antigen Receptor T-Cell Therapy in Chronic Lymphocytic Leukemia

Adoptive cell transfer-based therapies such as chimeric antigen receptor (CAR) T-cell therapy, are new approaches to specifically target tumor cells that are currently also investigated in CLL, as recently reviewed by us (46). So far, the effect of PI3Kδ inhibition on T-cells during the generation of the infusion T-cell product on anti-tumor activity has been investigated in different cancer entities, including CLL. Transplantation of PI3Kδ inhibitor pre-treated $CD8^+$ T-cells into B16 melanoma-bearing WT mice, resulted in a better tumor control (138, 143), which could be further enhanced by vaccination with the tumor antigen (138). Similarly to beforehand mentioned, *in vitro* PI3Kδ inhibition results in a less differentiated phenotype of T-cells (143) and in increased proliferation rates of T-cells after long-term cultivation (138). Accordingly, the frequency of PI3Kδ inhibitor pre-treated T-cells within the tumor site trended to be higher in comparison to vehicle-treated controls (143). Of interest, transfer of naïve T-cells similarly reduced the tumor size as seen for PI3Kδ inhibitor pre-treated $CD8^+$ T-cells (143). This data suggest that PI3Kδ inhibition during the generation of the T-cell product results in their reduced differentiation, activation, and subsequently higher proliferation *in vivo* as well as a better tumor control.

In comparison to other B-cell malignancies, the remission rates of CD19 CAR T-cell therapies in CLL have been disappointing (46). Therefore, different efforts were taken to improve treatment efficacy. Among them, pre-treatment of CAR T-cells with ibrutinib (153) or idelalisib (154) have been explored. PI3Kδ inhibition during CAR T-cell production of patient derived T-cells resulted in lower proliferation rates of $CD4^+$ T-cells, while proliferation of $CD8^+$ T-cells was enhanced (154). Of note, in healthy donor-derived T-cells, no difference in the proliferation between PI3Kδ inhibited and control cells was observed (154). Phenotypically, an expansion of naïve and effector T-cells, accompanied by a reduction of memory cell populations (143, 154), and lower levels of PD-1 and TIM-3 expression of idelalisib-treated compared to untreated CAR T-cells of CLL patients were seen (154). This lower state of pre-activation resulted in an increased TNFα and IFNγ production *ex vivo* (154). And chromium release assays showed that cytotoxicity of idelalisib-treated CAR T-cells was not altered *in vitro* compared to untreated CAR T-cells (154). This data suggest that priming of CAR T-cells by idelalisib causes a less differentiated and activated phenotype of the T-cell product, which could result in an enhanced anti-tumoral activity *in vivo*. Investigating their function *in vivo*, two independent groups showed an enhanced tumor control and prolonged survival of PI3Kδ inhibitor pre-treated CAR T-cells in xenograft mouse models of CLL (143, 154) (**Figure 3**).

In conclusion, pre-treatment of cells for adoptive transfer therapies, such as CAR T-cells, with PI3Kδ inhibitors results in a less differentiated and activated cell product accompanied by an enhanced *in vivo* efficiency in xenograft models. Further studies are needed to assess the long-term persistence and tumor control of PI3Kδ inhibitor-primed CAR T-cells in an immunocompetent TME. Ultimately, a confirmation of these results in patients is still pending.

Effect of Phosphoinositide 3-Kinase α and β Inhibition on T-Cells

PI3Kδ is the most studied isoform in T-cells. To investigate the role of other PI3K subtypes in $CD4^+$ as well as $CD8^+$ T-cells, initially pan-class IA PI3K inhibitors were utilized. These studies showed a similar effect as p110δ isoform specific inhibition, such as reduced TCR signaling, proliferation, and higher expression of

naïve/memory T-cell markers (137, 138). Intriguingly, neither the specific inhibition of PI3Kα nor -β did result in an altered TCR signaling, proliferation (137, 138), or affected the phenotype or cytokine production of $CD8^+$ T-cells *in vitro* (138). This data suggest that neither PI3Kα nor -β alone are indispensable for T-cell function. To elucidate whether PI3Kα or -β inhibition have additive effects to PI3Kδ inhibition on T-cells, combinations of Class IA inhibitors and genetically inactive isoforms have been investigated. Treatment of PI3Kδ-deficient cells with PI3Kα or -β inhibitors further reduced TCR signaling as well as proliferation of $CD4^+$ T-cells *in vitro* (137, 145) and *in vivo* (145). Concomitantly, higher frequencies of $CD8^+$ T-cells with lower PD-1 expression at the tumor site have been noted upon PI3Kα and -δ inhibition (145). Of importance, all results were comparable to single δ-specific inhibition (145), and differences in T-cell phenotype as well as function have not been observed upon combination of PI3Kα and -β inhibition (137).

These results highlight that PI3Kδ is indispensable for T-cell activation and proliferation and its loss of function cannot be compensated by class IA PI3K isoforms. Dual inhibition of PI3Kα and -δ likely does not have additive effects, although a direct head to head comparison is missing.

Effect of Phosphoinositide 3-Kinase γ Inhibition on T-Cells

PI3Kγ inhibition is mostly targeting myeloid cells, as detailed above. Interestingly, an increased frequency of T-cells in tumor-bearing animals with either pharmacological or genetical PI3Kγ inhibition has been noted (96). To exclude bystander effects *via* the myeloid cell compartment, which is affected by dysfunctional PI3Kγ, myeloid cells of LLC-tumor bearing mice were depleted using clodronate liposomes and mice were treated with PI3Kγ inhibitors. In comparison to vehicle-treated animals, PI3Kγ inhibition did not result in altered T-cell numbers suggesting that it does not affect T-cell function markedly (96). In line, genetical or antibody-mediated depletion of $CD8^+$ T-cells in $PI3K\gamma^{-/-}$ mice caused a reduced tumor control but no alterations in proliferation or production of IFNγ or GzmB of PI3Kγ-deficient T-cells isolated from either naïve or tumor bearing mice have been observed *ex vivo* (96). In conclusion, PI3Kγ inhibition is likely not affecting T-cell function directly. Nevertheless, the effects of PI3Kγ inhibition on other components of the TME, such as myeloid cells, can enhance the recruitment and anti-tumoral of T-cells.

RESISTANCE TO PHOSPHOINOSITIDE 3-KINASE INHIBITION IN CANCER AND CHRONIC LYMPHOCYTIC LEUKEMIA

Although both isoform-specific and pan-PI3K inhibitors show promising clinical efficacy in certain human cancers, there are several intrinsic and acquired resistance mechanisms that challenge their use. The PI3K/AKT pathway involves numerous feedback loops and converges with other signaling pathways at several points. This crosstalk mechanism enables the tumors to adapt by directing the signaling to an alternative path and thereby influencing the therapeutic outcomes (155, 156). *RAS* oncogene that activates both RAF-MAPK and PI3K signaling is one of the striking examples. The two pathways converge at early points after the growth factor receptor stimulation and interestingly, they evolved as antagonists (156). It has been shown that inhibition of the PI3K pathway can cause over-activation of the RAF-MAPK pathway, which induces tumor growth and quenches the efficacy of PI3K inhibition (156). Dual inhibition strategies have strong potential to overcome this dilemma but likely to result in high toxicities and a narrow therapeutic window.

Presence of a genetic alteration that leads to over-activation of the targeted kinase usually predicts the success of the inhibition therapy, as proven in different malignancies (28). However, cancer cells are genetically complex and mutations downstream of the targeted node might also influence the cellular responses to inhibition resulting in reduced sensitivity. In line, activating mutations of the NOTCH pathway induced resistance to PI3K inhibition in breast cancer (157).

Besides pathway-related mutations, so-called "gatekeeper" mutations that deteriorate the sensitivity of tumor cells to the inhibitor can be acquired during the therapy. A common feature of these "gatekeeper" mutations is their presence in the kinase domain of the targeted protein kinase which hinders its binding to the inhibitor. Similar mutations in PI3K are likely to exist. However, studies analyzing the resistance mutations are scarce. Zunder et al. have detected a potential hotspot mutation in p110α that confers resistance by inhibiting the potency of several PI3K inhibitors 5- to 30-fold (158). To our knowledge, other PI3K isoforms have not been analyzed but these results might serve as a start point for the development of new generation, isoform selective PI3K inhibitors since the hotspot is conserved in the entire PI3K family (158). The nature of PI3K inhibition itself is likely to provide a supportive environment for new mutations to occur, as PI3K inhibition *in vitro* is not cytotoxic but mostly cytostatic for cancer cells (58, 159, 160). Upon inhibition, it has been shown that cells enter a dormant state and can survive with subtle amount of PI3K activity for a significant time (161) during which the accumulation of new mutations might be facilitated.

In CLL, treatment failure of idelalisib caused by *de-novo* mutations seems to be a rather rare event but disease progression is observed (162). Overall response rates (ORR) are in general relatively high in phase III trials of relapsed/refractory CLL patients with 83.6% for idelalisib/rituximab (median progression-free survival (PFS) 19.4 months) (129), and 70.0% for idelalisib/bendamustine + rituximab (median PFS 20.8 months) (163), but results of long-term follow-ups are still pending. Nevertheless, activating mutations of the BRAF-MAPK pathway have been observed in 10 non-responders to idelalisib (N=7) or voxtalisib (N=3), which targets the PI3K/mTOR pathway. Recurrent mutations were identified in *BRAF* (N=5), *MAP2K1* (N=2), and *KRAS* (N=2), but no information is provided whether these mutations resulted in resistance to idelalisib or voxtalisib (164). To investigate acquired resistance to idelalisib, samples of 13 CLL patients that initially responded to idelalisib treatment and developed progressive disease were analyzed by whole exome

sequencing across three phase III trials (165). Intriguingly, neither recurrent, nor "gatekeeper" mutations or genomic alterations of any other related signaling pathways were identified that could confer resistance to idelalisib treatment. Similarly, in a preclinical mouse model, long-term PI3Kδ inhibition and serial tumor transplantations caused resistance to the treatment, but no recurrent mutations were identified by whole exome sequencing (166). Intriguingly, expression of insulin-like growth factor 1 receptor (IGF1R) was upregulated due to enhanced activity of forkhead box protein O1 (FOXO1) and glycogen synthase kinase 3 beta (GSK3β) which resulted in pronounced MAPK signaling pathway activity (166).

Despite all the challenges and resistance mechanisms, pharmacological targeting of the PI3K pathway is an efficient strategy. It is highly likely that long-term follow up data of CLL patients will provide more information about resistance mechanisms and combination strategies to achieve a durable effect with minimum toxicity.

ON-TARGET SIDE EFFECTS OF PHOSPHOINOSITIDE 3-KINASE INHIBITION IN CANCER AND CHRONIC LYMPHOCYTIC LEUKEMIA

As PI3Ks are involved in many different cellular functions and crucial signaling pathways, off-target effects need to be considered when treating patients, which has been extensively reviewed (167–169). In brief, inhibition of the α-isoform with alpelisib in breast cancer frequently caused hyperglycemia, rash, diarrhea or stomatitis (167). In CLL, PI3Kδ inhibition using idelalisib caused immune-cell mediated adverse events like pneumonitis, neutropenia, diarrhea, colitis or transaminitis, and an infiltration of T-cells to inflamed tissues has been observed. These adverse events were more frequent when combining idelalisib with rituximab-bendamustine or ofatumumab (129, 148, 168, 169). Recently, Kienle and Stilgenbauer raised optimism that i) the development of more specific PI3Kδ inhibitors like umbralisib, ii) the adaptation of the scheduling of PI3K inhibitors (e.g., intermittent dosing), and iii) combinatory treatment approaches could result in a more favorable toxicity profile and deep treatment responses with PI3K inhibitors (169).

CONCLUSION

Initial success of idelalisib, the first approved PI3K-inhibitor for CLL, paved the development of a plethora of PI3K inhibitors, as recently reviewed by Kienle and Stilgenbauer (169). However already in 2016, concerns arose that clinical PI3Kδ inhibition can be associated with an increased frequency of immune-related adverse events (AE), such as colitis, pneumonitis, neutropenia as well as elevated hepatic transaminases (129, 168). This highlights the need for a detailed review of the expression as well as function of PI3Ks in components of the TME of CLL, which could serve as potential explanation for the observed AE during idelalisib treatment.

PI3Ks are involved in crucial signaling cascades of both, the non-immune and immune compartment of the TME in CLL. Some PI3K isoforms are expressed by all cell types whereas others are specific for distinct leukocyte subsets. The use of selective inhibitors gives the opportunity to enhance anti-tumoral immune responses of some cell types. An example is the effect of PI3Kγ inhibition on myeloid cells. Therefore, the challenge of subtype specific PI3K inhibition is not to dampen the function of other immune cells that are essential for tumor or pathogen control.

PI3Kδ-selective inhibition and its effect on the immune environment has so far been studied most in CLL. Apart its great success in controlling the CLL progression (129, 163, 170), adverse events, such as immune cell-mediated liver toxicities are a drawback. Reduced frequencies of circulating Tregs accompanied by an enhanced $CD8^+$ T-cell infiltration to liver tissues have been reasoned to be involved in those cases (148). For Tregs, accumulating pre-clinical as well as clinical evidence supports that PI3Kδ inhibition reduces this population in numbers and dampens their immunosuppressive function. In contrast, the effect of PI3kδ inhibition on $CD8^+$ T-cells is still under debate. On the one hand, diminished Treg-mediated immunosuppression was suggested to unleash $CD8^+$ T-cell function, but on the other hand direct, negative effects of PI3Kδ inhibition on the function of this cell type have been observed. The latter could be causative for compromised clearance of pathogens, especially viruses, as well as a reduced anti-tumor control.

Neutrophils are important for the clearance of extracellular pathogens such as bacteria and fungi. Therefore, neutropenia, an AE observed in idelalisib-treated CLL patients, could be reasoned for higher rates of infections such as pneumonia. *In vitro* treatment of neutrophils with PI3Kδ inhibitors hindered their activation and their ADCC, supporting that inhibition of PI3Kδ in neutrophils could be involved in reduced immune function of idelalisib-treated CLL patients.

Today, little is known about the effect of other PI3K isoforms on the function of the TME in CLL. In contrast, many clinical trials investigating novel PI3Kδ inhibitors, dual inhibitors of PI3Kδ and -γ, as well as pan-Class IA inhibitors have been initiated (169). Therefore, thorough investigations of the TME in these trials are essential to elucidate the role of PI3K class I isoforms on the function of distinct cell types and to reassure that PI3K inhibitors can serve as a highly active, safe, and tolerable treatment option in CLL.

AUTHOR CONTRIBUTIONS

EA reviewed the literature, prepared the figures, wrote and revised the manuscript. SF, MSa, and LC reviewed the literature and prepared a draft of the manuscript. MSe reviewed the literature and revised the final version of the manuscript. PMR reviewed the literature, wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 18 March 2021
doi: 10.3389/fimmu.2021.660085

Heat Shock Proteins in Lymphoma Immunotherapy

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Immunotherapy harnessing the host immune system for tumor destruction revolutionized oncology research and advanced treatment strategies for lymphoma patients. Lymphoma is a heterogeneous group of cancer, where the central roles in pathogenesis play immune evasion and dysregulation of multiple signaling pathways. Immunotherapy-based approaches such as engineered T cells (CAR T), immune checkpoint modulators and NK cell-based therapies are now in the frontline of lymphoma research. Even though emerging immunotherapies showed promising results in treating lymphoma patients, low efficacy and on-target/off-tumor toxicity are of a major concern. To address that issue it is suggested to look into the emerging role of heat shock proteins. Heat shock proteins (HSPs) showed to be highly expressed in lymphoma cells. HSPs are known for their abilities to modulate immune responses and inhibit apoptosis, which made their successful entry into cancer clinical trials. Here, we explore the role of HSPs in Hodgkin and Non-Hodgkin lymphoma and their involvement in CAR T therapy, checkpoint blockade and NK cell- based therapies. Understanding the role of HSPs in lymphoma pathogenesis and the ways how HSPs may enhance anti-tumor responses, may help in the development of more effective, specific and safe immunotherapy.

Keywords: heat shock proteins, lymphoma, CAR T, CAR NK, checkpoint inhibitors

OPEN ACCESS

Edited by:
Jérôme Paggetti, Luxembourg Institute of Health, Luxembourg

Reviewed by:
Gabriele Multhoff, Technical University of Munich, Germany
Carmen Garrido, Institut National de la Santé et de la Recherche Médicale (INSERM), France

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *28 January 2021*
Accepted: *26 February 2021*
Published: *18 March 2021*

Citation:
Albakova Z, Mangasarova Y and Sapozhnikov A (2021) Heat Shock Proteins in Lymphoma Immunotherapy. Front. Immunol. 12:660085. doi: 10.3389/fimmu.2021.660085

INTRODUCTION

Lymphoma is a heterogeneous cancer divided into two major types such as Hodgkin lymphoma (HL) and Non-Hodgkin lymphoma (NHL) (1, 2). In oncology research, management of lymphoma stands out as the choice of treatment is largely based on the results obtained from prospective clinical trials (1). Standard treatment regimen includes chemotherapy and radiation therapy for the treatment of HL and chemotherapy combined with anti-CD20 antibodies for NHL patients, reaching the cure rate of 80-90% (1, 2). Even though the response rate is high, treatment-related toxicity such as induction of second malignancy and cardiotoxicity is of a major concern (1). Following initial treatment, 10-30% of lymphoma patients develop refractory or recurrent (r/r) disease which is treated with high-dose chemotherapy followed by an autologous hematopoietic stem cell transplantation (ASCT) (1, 2). The overall goal of current and emerging treatments for HL and NHL is to cure disease and minimize treatment-related toxicity (1, 2). Current treatments for lymphoma patients are summarized in **Table 1**. Recently approved treatments for r/r HL and NHL subtypes include anti-CD30 antibody-drug conjugate brentuximab vedotin, PD1 inhibitors

(pembrolizumab and nivolumab), Bruton's tyrosine kinase inhibitors (ibrutinib and acalabrutinib), phosphoinositide 3-kinase γ and/or δ inhibitors (idelalisib, copanlisib and duvelisib) and CD19 chimeric antigen receptor (CAR) T cell therapy (tisagenlecleucel and axicabtagene ciloleucel) (**Table 1**) (1, 2, 29).

Heat shock proteins (HSPs) are molecular chaperones highly expressed in various types of cancer. HSPs are classified into several families such as HSP110, HSP90, HSP70, HSP40, chaperonins and HSPB (30). HSPs are largely known for their role in blocking apoptosis which was further translated into development of HSP inhibitors (31–49). Along this line, Kamal and colleagues showed that HSP90 inhibitor 17-allylamino-17-demethoxy-geldanamycin (17-AAG) selectively targets cancer cells (50). In light of the reported, several HSP90 inhibitors such as alvespimycin (NCT01126502), luminespib (NCT01485536), PU-H71 (NCT01581541) and SNX-5422 (NCT02914327) currently are assessed in clinical trials for the treatment of lymphoma patients. Furthermore, HSPs showed to be potent immune system activators through the induction of cytotoxic CD8+T cell response (51–56). Several HSP-based vaccines have been evaluated in clinical trials (57–61). Specifically, the efficacy and safety of HSPPC-96 vaccine, which is an autologous gp96 heat shock protein-peptide complex vaccine, was assessed in patients with indolent non-Hodgkin lymphoma (62). Inspired by the ability of HSPs to induce immune responses, Li and colleagues developed a novel nanovaccine that mimics HSPs, so it can be used to stimulate anti-tumor immune responses (55). Additionally, numerous studies have assessed extracellular HSPs derived from various liquid biopsies (serum, plasma, urine, plasma/serum/urine-derived exosomes) as potential cancer biomarkers [reviewed in (31)] (63–71).

Novel emerging immunotherapy approaches involve CAR T cell therapy, checkpoint inhibitors and NK cell-based therapies that are aimed at improving effectiveness in treating of lymphoma patients. In this review, we focus on the role of HSP family in Hodgkin and Non-Hodgkin lymphoma. Since the mechanism of apoptosis and immune modulation are the key features in lymphoma pathogenesis, it is of particular interest to explore the contribution of HSPs in this process. We explain how the understanding of the cross-talk between tumor microenvironment (TME) and malignant cells and the role of HSPs in this process may help to improve emerging treatments for lymphoma patients.

HSPs IN HODGKIN LYMPHOMA

Hodgkin lymphoma (HL) is a B cell lymphoma divided into classic HL (cHL) which accounts for the majority of the cases and nodular lymphocyte-predominant HL (NLPHL) (1). Histologically, cHL is classified into four types such as nodular sclerosis HL (NSHL), mixed cellularity HL (MCHL), lymphocyte-rich HL (LRHL) and lymphocyte-depleted HL (LDHL) (1).

cHL is characterized by the presence of malignant Hodgkin and Reed-Sternberg (HRS) cells that constitute minor population (~1%) of the tumor mass (72). The majority of infiltrate surrounding HRS cells is represented by different types of non-malignant immune cells such as dendritic cells, macrophages, lymphocytes, mast cells, neutrophils, eosinophils and fibroblasts which form tumor microenvironment (TME) (1, 72). Even though, HRS cells are germinal center (GC)- derived B cells, they resemble immunophenotype that does not associate with any known cells of hematopoietic origin (**Figure 1**) (74). Specifically, HRS cells rarely express typical B-cell lineage markers such as CD19, CD20, CD22, CD79, CD79B and instead express markers of dendritic cells (CD83), myeloid markers (CD15) and T cell markers (CD2, CD3, CD4) (1, 74, 75, 81). Additionally, members of tumor necrosis factor family namely CD30 and CD40 are expressed on HRS cells (75). It is interesting to point out that cHL TME is composed of variable cellularity that is different in each cHL subtype (1). As an example, NSHL is rich in fibroblast-like cells and fibrosis, MCHL is composed of B cells, T cells, neutrophils, histiocytes, plasma cells and mast cells, LRHL is characterized by HRS cells surrounded by mantle zone B cells and histiocytes whereas LDHL predominantly consists of $CD4^{+}$T cells, histiocytes and fibrosis (1).

Nuclear factor- kappa B (NF-kB) is constitutively activated in HRS cells (1, 82). Engagement of CD40, CD30, RANK and BCMA/TACI with their cognate ligands showed to activate NF-kB leading to increased production of IL-6, IL-8, CCL5, IFNγ and IL-13 (75). During activation process, the IKK complex, which is composed of two kinase subunits IKKα, IKKβ and a regulatory subunit IKKγ, phosphorylates IkBα, an inhibitor of NF-kB, allowing NF-kB translocation to the nucleus and activation of genes responsible for B-cell proliferation and survival (73). Notably, treatment with HSP90 inhibitor geldanamycin resulted in inactivation of NF-kB and IKK activity in HRS cell lines (83). HSP90 showed to stabilize subunits of IKK complex (IKKα and IKKβ) and protect them from proteasomal degradation (83).

Another important signaling cascade which is constitutively activated in HRS cells is Janus kinase (JAK)-signal transducer and activator of transcription (STAT) (1). Activation of JAK-STAT pathway leads to hyperphosphorylation of STAT proteins (1). In particular, STAT3, STAT6 and STAT5 showed to be constitutively phosphorylated in cHL cell lines (84–86). Intriguingly, Schoof and coworkers reported that pharmacological blocking of HSP90 inhibited the phosphorylation of STAT1, STAT3, STAT5 and STAT6 in cHL cell lines (84). Overall, HSP90-targeting agents may be a promising strategies in cHL where deregulated NF-kB and JAK-STAT signaling pathways play a major role in cHL pathogenesis.

Phosphatidylinositol 3-kinase (PI3K)-serine/threonine protein kinase (AKT) pathway is also constitutively activated in HRS cells as a result of activation of multiple receptor tyrosine kinases (RTKs) (1, 87). RTKs such as platelet-derived growth factor receptor A (PDGFRA), discoidin domain-containing receptor 2(DDR2),tyrosine kinase receptor A (TRKA) and

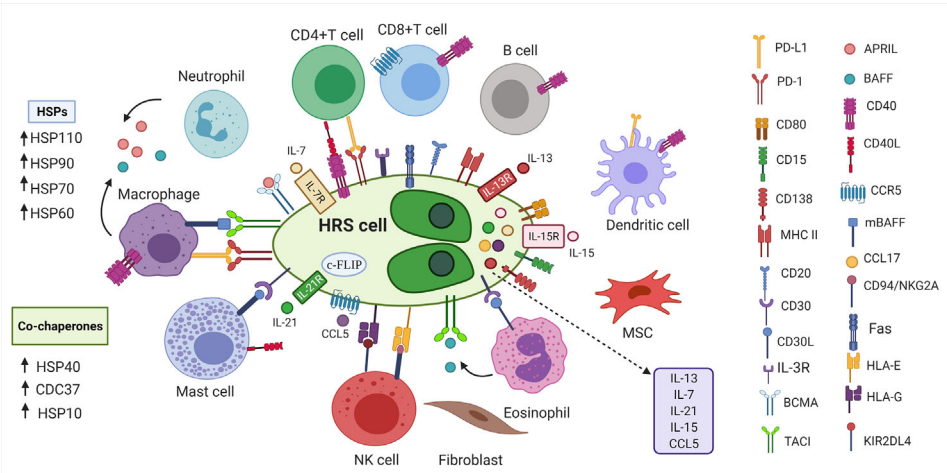

FIGURE 1 | Tumor microenvironment in cHL. HRS cells are surrounded by non-malignant immune and stromal cells. Inflammatory cells secrete cytokines, tumor necrosis family members (CD40L, CD30L) and other molecules (APRIL, BAFF) that bind to the proteins on the surface of HRS cells to promote growth and survival of HRS cells (1, 73). HRS cells express various markers of B cells, T cells, myeloid markers, markers of dendritic cells (74). HRS cells express PD-L1 to escape anti-tumor responses (75). HRS cells express Fas, but avoid FasL-mediated apoptosis by overexpressing c-FLIP (75–77). HRS cells express FasL leading to apoptosis of Fas-expressing NK cells (76, 78). HSP chaperones and their corresponding co-chaperones are highly expressed in HRS cells, which further contribute to immunosuppressive TME (79, 80). HRS, Hodgkin and Reed-Sternberg cells; BCMA, B cell maturation antigen; APRIL, a proliferation-inducing ligand; BAFF, B-cell activating factor;PD-L1, programmed death ligand 1; PD-1, programmed death 1; CD30L, CD30 ligand; CD40L, CD40 ligand; CCL5, CC-chemokine ligand 5; IL-3R, interleukin-3 receptor; TACI, transmembrane activator and calcium-modulator and cyclophilin ligand interactor; HSP, Heat shock protein; MSC, mesenchymal stromal cells; mBAFF, membrane-bound B-cell activating factor; MHC II, Major histocompatibility complex class II; HLA-E/G, Human leukocyte antigen- E/G; KIR2DL4, killer cell immunoglobulin-like receptor family member; c-FLIP, cellular FLICE-inhibitory protein; cHL, classic Hodgkin lymphoma; NK cells, natural killer cells.

TRKB showed to be aberrantly expressed in HRS cells of HL patients, while no expression of these RTKs was observed in normal B cells or B-cell NHL cells (87). Furthermore, aberrant expression of mitogen-activated protein kinase (MAPK)/ERK has been reported in HL (88).In light of the reported, HSP90 inhibitor 17-AAG showed to deplete AKT and inhibit extracellular signal-regulated kinase (ERK) phosphorylation, leading to growth arrest and apoptosis in HL cell lines (89) (88). This was further supported by the finding that HSP90 inhibitor celastrol induced anti-tumor effects in HRS cells by downregulating RAS, ERK1/2 and c-Fos (90). In another experiment, inhibition of HSP90 by geldanamycin induced apoptosis in HRS cells with wild-type *IkBα* in p53-independent manner (91). Taken together, these observations suggest that targeting AKT, NF-kB and MAPK/ERK pathways with HSP90 inhibitors may prove effective in HL treatment.

In addition to unique immunophenotype and multiple deregulated signaling pathways, HRS cells express high level of HSPs. Hsu and colleagues assessed HSP expression of formalin-fixed, paraffin-embedded tissues derived from patients with different cHL subtypes (80). High cytoplasmic expression of HSP90 and HSP60 in HRS cells was found in NSHL, MCHL, LRHL and LDHL (80). By contrast, no cytosolic HSP27 expression was found in HRS cells in LRHL and low expression in LDLH while 20% of patients with NSHL and MCHL showed strong HSP27 expression (80). Later, Santon and co-workers used tissue microarray to analyze immunohistochemical expression of HSPs in HRS cells of cHL patients (79). More than 90 percent of cHL patients in HRS cells showed high cytoplasmic expression of HSP60, HSP10, HSP90, and CDC37, nuclear HSF1 whereas HSP110 showed to be highly expressed in nucleus and cytoplasm of HRS cells (79). Positive cytoplasmic staining of HSP70 and cytoplasmic/nuclear expression of HSP40 was observed in 78% of cHL patients whereas 54% had positive cytoplasmic expression of HSP27 (79). Expression of HSP90 and HSP70 positively correlated with expression of their co-chaperones CDC37 and HSP40, respectively (79). Furthermore, expression of HSP40 positively correlated with p53, caspase 9 and cellular FLICE-inhibitory protein (c-FLIP) whereas HSP70 expression correlated with caspase 3 (79). In another study, high cytoplasmic expression of HSP60 was observed in HRS cells in 100% of NSHL and MCHL cases (92).

HSPs IN NON-HODGKIN LYMPHOMA

Non-Hodgkin lymphoma (NHL) is comprised of B-cell lymphoma, accounting for the majority of the NHL lymphoma subtypes, while other NHLs include T-cell lymphoma and NK-cell lymphoma (93). NHL is classified into indolent (slow-growing) and aggressive (fast-growing) lymphoma. The most common indolent lymphoma is follicular lymphoma (FL), while other slow-growing lymphoma subtypes include marginal zone lymphoma (MZL), chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL) and lymphoplasmacytic lymphoma (94). The most common aggressive NHL subtype is represented by diffuse large B-cell lymphoma (DLBCL), while

other aggressive lymphoma subtypes include mantle cell lymphoma (MCL), Burkitt lymphoma (BL) and primary effusion lymphoma (94).

Lymphoma cells largely depend on microenvironment for their growth and survival (95). Continuous signaling from B cell receptor (BCR), immune and stromal cells are required to support proliferation activity and survival of lymphoma cells. BCR is required for B cell survival and the loss of BCR results in B cell death (95, 96). BCR activation by self-antigens showed to be a driving force in various NHL subtypes (97–99). Moreover, some subtypes of DLBCL carry genetic mutations that activate BCR signaling, including mutation in *CD79B* and *CARD11*, where the latter mutation leads to constitutive activation of NF-kB in activated B cell-like (ABC) subtype of DLBCL (95, 97, 100–102). Notably, Walter and colleagues demonstrated that HSP90 and its client protein spleen tyrosine kinase (SYK) are required for tonic BCR signaling in BL lymphoma, suggesting potential use of HSP90 as potential target for BL lymphoma treatment (103, 104). Additionally, HSP90 showed to stabilize BCR kinases such as Bruton tyrosine kinase (BTK), SYK, LYN and AKT in chronic lymphocytic leukemia cells (105). Recent studies have added more insight into the role of HSP90 in BCR signaling in NHL subtypes. Jacobson and colleagues reported that HSP90 inhibition led to the complete loss of BTK and IKKα and downstream loss of phosphorylated ERK1/2 in mantle cell lymphoma cell lines (106).Moreover, HSP90 inhibitor showed to downregulate BTK in cells expressing BTK C481S mutation, which was found to be associated with resistance to BTK inhibitor ibrutinib in MCL and CLL patients (106–108). Importantly, Cerchietti and colleagues showed that HSP90 interacts with B-cell lymphoma-6 (Bcl-6) which was further supported by the finding that HSP90 inhibitor PU-H71 selectively killed Bcl-6-dependent DLBCL cells (109). Subsequently, Goldstein and co-workers used PU-H71 and tumor-enriched HSP90 (teHSP90) complexes derived from DLBCL cell lines to show that LYN, SYK, BTK and phospholipase C γ 2(PLCγ2) are dependent on teHSP90 (110). Furthermore, treatment with PU-H71 showed to disrupt BCR signaling, calcium influx and NF-kB activity, resulting in cell growth inhibition (110). Additionally, PU-H71 in combination with ibrutinib led to the killing of lymphoma cells, suggesting that combinatorial therapeutic approach may be more effective in NHL patients (110).

In addition to continuous BCR signaling, lymphoma cells require additional signals to survive. Early experiments in establishing NHL cell lines showed that FL cells require signals from T cells for CD40-mediated interaction and IL-4 stimulation for sustained proliferation of lymphoma cells (95, 111–113). Furthermore, in NHL subtypes myeloid cells secrete high level of B cell- activating factor (BAFF) and a proliferation inducing ligand (APRIL) that are critical for survival and differentiation of B cells (73, 95, 114–117). In addition to the signals provided by immune cells, the cross-talk between stromal cells and FL cells plays important role for the growth of FL B cells [reviewed in (118)].

Members of HSP family showed to be highly expressed in NHL subtypes. Valbuena and colleagues reported moderate-to-strong cytoplasmic expression of HSP90 in 100% of cases of BL,61% of FL patients, 59% of DLBCL, 38% of nodal MZL and 33% of cases with SLL/CLL and 30% of lymphoplasmacytic lymphoma (119). Weak cytoplasmic expression of HSP90 was observed in 43% of cases with extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (119). Patients with T-cell lymphoma showed moderate/strong cytoplasmic expression of HSP90 (119). HSP60 also showed to be highly expressed in DLBCL and high-grade FL whereas no HSP60 was detected in low-grade FL (92). NK/T-cell lymphomas showed positive cytoplasmic expression of HSP60 (92).

Recent studies have emphasized the role of HSP110 in aggressive subtypes of B-cell NHLs such as DLBCL and BL (120). Zappasodi and colleagues demonstrated that inactivation of HSP105/HSPH1 leads to downregulation of c-Myc and Bcl-6 (120).Mechanistically, HSP105 showed to interact with c-Myc and Bcl-6 in nucleus in primary human DLBCL and BL cells, suggesting that HSP105 may function as a chaperone for both c-Myc and Bcl-6 (120).Additionally, higher expression of HSP105 was found in DLBCL expressing c-Myc compared to c-Myc low/negative counterparts (120). In light of the reported, Boudesco and co-workers showed that overexpression of HSP110 resulted in upregulation of NF-kB, whereas silencing of HSP110 donwregulated NF-kB, suggesting that there is an interplay between HSP110 and NF-kB (121). Mechanistically, HSP110 showed to stabilize myeloid differentiation factor 88 (MyD88), leading to chronic NF-kB activation in ABC-DLBCL (121). Therefore, targeting HSP110 may be a promising strategy for the treatment of B-cell NHLs.

Phase II clinical trial was conducted to assess the safety and efficacy of HSP90 inhibitor AUY922 in patients with r/r DLBCL and peripheral T-cell lymphoma (PTCL) (92). Overall, 14 patients with DLBCL and 6 with PTCL were enrolled, 1 patient with DLBCL reached complete response (CR) and 1 patient with PTCL achieved partial response (122). Treatment-related adverse effects included fatigue, visual disturbance that was fully reversible and anemia (122). Authors concluded that HSP90 inhibitors may be a good target in some cases, though, combination with chemotherapeutic agents and histone deacetylase (HDAC) inhibitors may be used to improve anti-tumor activity (122). Several studies assessing combination of HSP90 inhibitors with chemotherapeutic drugs such as fludarabine, doxorubicin, cytarabine, melphalan, or HDAC inhibitors demonstrated promising results in hematological malignancies (122–126). Taken together, NHL subtypes have high expression of specific HSP members, however, use of combinatorial approach in NHL patients warrants further investigation.

HSPs AND EMERGING LYMPHOMA IMMUNOTHERAPY

HSPs and CAR T

CAR T therapy involves *ex vivo* expansion and genetic modification of an autologous (self) or allogeneic (donor) T

cells that specifically identify and eliminate cognate target ligand (127, 128). CAR consists of antigen-recognition domain represented by a single-chain variable fragment (scFv), hinge, transmembrane and intracellular signaling domains (127). Majority of CARs contain CD3ζ which is critical for T cell receptor (TCR) signaling (129). Due to low CAR T cell activity and persistence, second generation CARs have been developed that integrated co-stimulatory domains derived from CD28 or 4-1BB into the CAR design (128, 129). Importantly, CAR T cells that contain CD28 domains differentiate into effector memory T cells whereas 4-1BB-domain CAR T cells differentiate into central memory T cells (128, 130).

CAR T showed to be effective in the treatment of B-cell malignancies (129). In 2017 the first CAR T immunotherapy tisagenlecleucel (CD19-specific 4-1BB-CAR) was approved by FDA for the treatment of r/r B-cell acute lymphoblastic leukemia (B-ALL) (128). Later, in 2018, axicabtagene ciloucel (CD19-specific CD28-CAR) was approved for the treatment of r/r DLBCL (93). Nevertheless, the main challenge in CAR T therapy now is to find an antigen universally expressed on tumor cells that can be targeted by CAR T. Since HRS cells almost exclusively express CD30, CD30 was proposed as an attractive target for the CAR T therapy. Up till now only 3 studies assessed the efficacy and safety of CD30 CAR T immunotherapy for the treatment of Hodgkin lymphoma (129). Recently, Ramos and colleagues have conducted two phase I/II clinical trials where autologous CD30 CAR Ts were administered to patients with r/r Hodgkin lymphoma after lymphodepletion with fludarabine in combination with either bendamustine or cyclophosphamide (131). The overall response rate (ORR) for 32 patients was 72%, 19 (59%) of which achieved a complete remission (131). It is encouraging that no neurotoxicity was observed. Cytokine release syndrome (CRS) and skin rash that occurred in 10 and 20 patients, respectively, were found to be associated with cyclophosphamide rather than with bendamustine and both spontaneously resolved (131). In another study, Wang and colleagues designed anti-CD30 CAR and conducted a pilot study in patients with r/r Hodgkin lymphoma (132). After lymphodepletion, patients were infused with anti-CD30 CAR Ts. A total of 9 patients received CD30 CAR T infusion, 3 achieved CR, six experienced CRS from which 4 were low-grade and no neurotoxicity was observed (132). Authors also demonstrated promising results in combination therapy where CD30 CAR T treatment was combined with anti-PD-1 antibody (132). Notably, Watanabe and colleagues showed that CD30 induces expression of HSP90α and HSP90β in cHL by activating heat shock factor 1 (HSF1) (133). Since CD30 and HSP90 are overexpressed in cHL, future studies should explore the effect of anti-CD30 CAR T therapy on HSP90 (133).

The use of CARs against HSP70 was proposed by Smith and colleagues (134). Their invention particularly aims to target membrane-bound form of HSP70, so that specifically HSP70-surface positive tumor cells can be killed (134). Similar to the Smith group, Claffey and co-workers identified heavy chain antibody (HCAb2) that selectively targets HSP90 on malignant cells (135). Based on their findings, authors described an antibody that specifically binds to the cell surface HSP90β isoform (135, 136). Therefore, targeting surface expression of HSPs can be a new promising strategy for the development of more efficient CAR T therapy (**Figure 2**).

Due to on-target/off-tumor toxicity that associated with the use of CAR T therapy, several investigators proposed that the activity of CAR T cells can be controlled by thermal regulation with the use of HSP-based promoters (pHSP) (141, 142). Shapiro and colleagues used genetically engineered circuits pHSP-CAR to induce CAR expression in T cells in response to thermal stimuli (142). Studies assessing the use of pHSP for thermal control of CAR T highlighted that, despite their names, HSP members respond to various cellular stresses such as heat, hypoxia, radiation, heavy metal toxicity and cytokines (142–144). Therefore, the choice of HSP-based promoter should largely depend on the context in which these promoters will be used (142).

Several investigators demonstrated that HSP90 is crucial for the functional activity of T lymphocytes. For example, Bae and coworkers demonstrated that HSP90 inhibition downregulates the expression of CD3, CD4, CD8 as well as CD28, CD40L and αβ receptors and cripples T cell proliferation and interferon-γ (IFN-γ) secretion (145). In another study, pharmacological blocking of HSP90 resulted in decreased expression of Linker

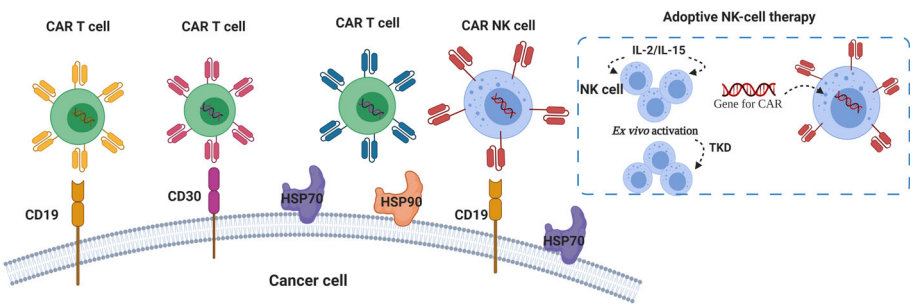

FIGURE 2 | HSPs and CAR T/NK cells in lymphoma immunotherapy. Chimeric Antigens Receptor (CAR) targeting CD19 and CD30 showed promising results in patients with r/r lymphoma (93, 129, 131, 132). Autologous NK cells pre-activated with cytokines (IL-2, IL-15) and 14-mer HSP70-derived peptide (TKD) can be used for *ex vivo* activation of NK cells for the adoptive transfer therapy (137–140). Anti- HSP70 and HSP90 CARs were proposed for specific targeting of membrane-bound forms of HSP70 and HSP90 (134, 136).

TABLE 1 | Current treatments for HL and NHL.

Hodgkin lymphoma		
Lymphoma type	**Standard treatment regimen**	**Refs**
cHL	Chemotherapy + ISRT	(1, 3, 4)
r/r cHL	High-dose chemotherapy +ASCT	(1, 4–7)
NLPHL	Rituximab	(3, 8)
	New agents	
cHL, including r/r cHL	Brentuximab vedotin	(1, 3, 9)
	Nivolumab	(3, 10)
	Pembrolizumab	(3, 11)
Non-Hodgkin lymphoma		
	Standard treatment regimen	
NHL, including r/r NHL	Rituximab+chemotherapy	(2)
	Lenalidomide+Rituximab	(2)
	High-dose chemotherapy +ASCT	(2)
	New agents	
PTCL	Brentuximab vedotin	(12)
CLL/SLL	Ibrutinib+rituximab	(13, 14)
CLL/SLL; MCL	Acalabrutinib	(15–20)
FL and SLL	Idelalisib	(21)
FL	Copanlisib	(22)
r/r CLL/SLL	Duvelisib	(23)
r/r primary mediastinal BCL	Pembrolizumab	(24, 25)
r/r DLBCL	Tisagenlecleucel	(26)
r/r DLBCL	Axicabtagene ciloucel	(27, 28)

ISRT, Involved site radiation therapy; ASCT, autologous haemotopoietic stem cell transplantation; cHL, classic Hodgkin lymphoma; NLPHL, nodular lymphocyte-predominant Hodgkin lymphoma;r/r, Refractory or recurrent disease; PTCL, peripheral T-cell lymphoma; CLL, chronic lymphocytic leukemia (CLL); SLL, small lymphocytic lymphoma; MCL, mantle cell lymphoma.

for activation of T cells (LAT) in activated T cells (146). Therefore, taking into account that HSP90 is important for the T cell function and phenotype, and that CAR T-containing CD28 and CD3 domains may affect intracellular and extracellular HSP90 expression, further studies should address the effect of CAR T on HSP90 expression.

HSPs and NK Cell-Based Immunotherapy

The immunosuppressive TME specifically inhibits functional activity and proliferation of NK cells (76). Several investigators reported deficiency in the number of NK cells in the biopsies of cHL patients (76, 147). Moreover, lower number of circulating NK cells were detected in peripheral blood of cHL patients (76, 148, 149). The main goal of NK-based immunotherapy in HL is to reactivate NK cells (150). Boll and colleagues demonstrated that HSP90 inhibitor called BIIB021 combined with doxorubicin and gemcitabine selectively killed Hodgkin lymphoma cells by inhibiting NF-kB activation (150). Moreover, HSP90 inhibition resulted in upregulation of NKG2D ligands such as MHC class I chain-related A (MICA), MICB and ULBP2 on HL cells, making HL cells susceptible to NK-mediated killing (150). In line with that, Fionda and colleagues demonstrated that HSP90 inhibition upregulates MICA and MICB and leads to increased NK cell degranulation in myeloma cell lines (151).

Contrary to T cells, NK cells do not recognize an antigen in the form of MHC-peptide complex, but rather sense the absence of self-MHC class I on tumor cells. HSP90 and HSP70 chaperones showed to be crucial for antigen presentation by MHC I molecule. Binder and colleagues reported that peptide antigens bound to HSPs, such as HSP90, HSP70, gp96, were presented 100 fold more efficiently by MHC I compared to free peptides (152, 153). Callahan and co-workers demonstrated that HSP90 inhibition disrupts the loading of peptides on MHC I (153). Furthermore, Kunizawa and Shastri showed that TCP-1 ring complex (TRiC/CCT) chaperonin is required for the expression of peptide-loaded MHC I on the cell surface (154). Later the same team found that inhibition of HSP90α or co-chaperone carboxyl terminus of Hsc70-interacting protein (CHIP) reduced presentation of peptide-bound MHC I on the cell surface (155).

Several investigators proposed the use of engineered NK cells as promising strategy for adoptive cell therapy in hematological malignancies (156). NK cells that are used in adoptive transfer can be allogeneic, autologous or immortalized such as NK-92 (156, 157). For research studies, NK cells can be isolated from peripheral blood or differentiated from stem cells, *ex vivo* expanded, activated with cytokines (IL-2, IL-15) and co-cultured with γ-irradiated feeder cells. It is important to point out that NK cells derived from peripheral blood mononuclear cells (PBMC) differ from NK cells differentiated from stem cells (156). For example, NK cells derived from cord blood showed no expression of CD57, high expression of NKG2A, lower expression of killer-immunoglobulin-like receptors (KIRs) and lower secretion of interferon-γ (156, 158). Several studies showed that NK cells can also be activated by HSP70 protein or 14-mer HSP70-derived peptide (TKD) in combination with IL-2 or IL-15 (**Figure 2**) (137–139). Multhoff and colleagues demonstrated that aggressive tumors have high expression of membrane-bound HSP70 (mHSP70) and that radio and/or- chemotherapy further increases surface expression of HSP70 (159–161). Furthermore, they reported that NK cells pre-activated with TKD and IL-2 recognize mHSP70 on tumor cells (137). Translating this to clinical trial, Multhoff et al. showed that four cycles of adoptive transfer of autologous NK cells pre-stimulated with TKD and IL-2 was well-tolerated and resulted in increase in the number of NK cells in peripheral blood of patients with mHSP70-positive non-small cell lung cancer (NSCLC) following radiochemotherapy in phase II clinical trial (140). Earlier, same research team demonstrated that NK cells activated with IL-2 and TKD and combined with anti-PD-1 antibody increased cytolytic activity of NK cells toward cancer cells and delayed tumor growth *in vivo* (162).

NK cells that express tumor-specific CARs showed to be efficiently applied in B cell malignancies (156). Currently, six CAR-NK therapies (CD19-CAR NK, CD22-CAR NK, CD19/CD22, CD7-CAR NK,CD19-t-haNK) are assessed in clinical trials for the treatment of lymphoma patients (156). Liu and colleagues used NK cells from the cord blood (CB) for incorporation of genes for CAR-CD19, IL-15 cytokine and caspase 9 as safety switch (iC9/CAR.19/IL15) to efficiently kill CD19-positive leukemia/lymphoma cells lines (156, 163). Same research group further assessed administration of HLA-mismatched iC9/CAR.19/IL15-transduced CB-NK cells in Phase I/II clinical trial to patients with r/r CD19- positive

NHL and chronic lymphocytic leukemia (CLL) (164). Overall, 11 patients were administrated with anti-CD19-CAR NK cells, 8 patients (73%) achieved clinical response from which 4 patients with lymphoma and 3 patients with CLL had a complete remission (164). Notably, no neurotoxicity, CRS or increase in IL-6 were observed, which are frequently associated with the administration of CAR T therapies (156, 164).

HSPs and Immune Checkpoints

Immunotherapy in the form of checkpoint modulation has advanced cancer research (165). In 2011, the first monoclonal antibody targeting immune checkpoint cytotoxic T-lymphocyte antigen-4 (CTLA-4) called ipilimumab received FDA approval. Later, monoclonal antibodies that target programmed death 1 (PD-1) such as pembrolizumab and nivolumab, and antibodies against PD-L1 such as atezolizumab and durvalumab were developed (165). Novel combinatorial approaches currently emerge, where the use of immune checkpoint modulators in combination with other anti-cancer therapies may further improve clinical response (166).

Taking into account that a major challenge in immunotherapy is a loss of tumor-associated antigens, HSP90 inhibitors were proposed as complementary approach to checkpoint inhibitors for cancer treatment (167). Rao and colleagues reported that inhibition of HSP90 resulted in proteasome-dependent degradation of its client oncoprotein EphA2 and, hence, increased tumor recognition by EphA2-specific CD8+T lymphocytes (168, 169). Haggerty and colleagues demonstrated that HSP90 inhibitors showed to upregulate the expression of tumor antigens such as Melan-A/MART-1, TPR-2, gp100 and enhance T cell recognition in melanoma cell lines (170). HSP90 client protein nucleophosmin-anaplastic lymphoma kinase (NPM/ALK) showed to induce PD-L1 *via* STAT3 activation in T cell lymphoma (171). Notably, administration of anti-PD-L1 antibody in combination with HSP90 inhibitor ganetespib showed significantly higher anti-tumor activity than when treated with anti-PD-L1 alone in syngeneic mouse models of melanoma and colon carcinoma (167). Furthermore, Mbofung and colleagues demonstrated that ganetespib in combination with anti-CTLA4 and anti-PD-1 antibodies improved survival and anti-tumor response in mice bearing MC38/gp100 tumors (172). Authors also showed that combinatorial treatment of HSP90 inhibitors and checkpoint blockade therapy decreased number of T regulatory cells and increased the expression of CXCL9, CXCL10 and IFN-γ (172). Combinatorial treatment also increased the number of CD8+T cells producing granzyme A and granzyme B, suggesting that combination of anti-CTLA4 and HSP90 inhibition enhances cytotoxic activity of CD8+T cells (172).

CD47 is an innate immune checkpoint highly expressed on the surface of tumor cells (93, 173). CD47 forms complex with signal regulatory protein α (SIRPα), which is expressed on phagocytic cells such as monocytes, macrophages and dendritic cells (93). CD47/SIRPα sends 'don't eat me" signals to innate immune cells, thus, inhibiting phagocytosis (174). Chao and colleagues reported that CD47 was overexpressed on samples derived from patients with various NHL subtypes (DLBCL,B-CLL, MCL, FL, MZL, pre-B ALL) and high CD47 expression correlated with poor clinical prognosis in NHL patients (173, 175). Blocking of CD47/SIRPα with anti-CD47 monoclonal antibody resulted in phagocytosis of acute myeloid leukemia cells (173). Combination of anti-CD47 monoclonal antibody (Hu5F9-G4) and rituximab showed promising results in patients with r/r DLBCL and FL in phase I clinical trial (176). Interestingly, Cook et al. demonstrated that inhibition of glucose-regulated protein-78 (GRP78), a member of HSP70 family, downregulated CD47 expression in tumor cells, leading to enhanced macrophage infiltration (177). Moreover, co-expression of CD47 and GRP78 showed to associate with poor survival in breast cancer patients (177). HSP90 also showed to play a role in CD47 regulation as inactivation of Myc, a client protein of HSP90, resulted in reduced expression of CD47 and PD-L1 (178, 179). It is interesting to note that HSP90 inhibitor PU-H71 induced apoptosis and inhibited tumor growth in patient-derived xenograft model of MCL *via* downregulating Myc (179, 180). Therefore, further studies are required to understand the HSP90-MYC-CD47/PD-L1 relationship and the role of GRP78 in CD47 regulation for the development of more specific and effective CD47-based therapies.

DISCUSSION

Lymphoma represents a unique group of cancer derived from major effector cells of an immune system such as B cells, T and NK cells (81, 93). Immunotherapy-based approaches showed encouraging results for patients with HL and NHL, however, severe toxicity and low efficacy profiles restrict the use of immunotherapy in lymphoma patients (2, 81). To overcome these limitations, various therapeutic strategies are developed that include the use of HSPs.

HSPs belong to evolutionally conserved family of chaperones that assist client proteins in folding, trafficking, degradation and showed to be involved in the most stages of cancer development (56, 181–184). High expression of HSPs on the surface correlates with the aggressiveness and resistance to therapy in many types of cancer (185, 186). Furthermore, HSPs are largely known for their critical role in regulating cell death mechanisms and immune responses (187–189).

Lymphoma cells create a complex and unique immune-modulatory tumor microenvironment, where inflammatory and stromal cells provide essential signals for growth, proliferation and survival of tumor cells (75, 95, 118). One of the major hallmark of lymphoma is represented by the deregulated critical signaling pathways including NF-kB, JAK-STAT, BCR signaling, PI3K/AKT, MAPK/ERK and apoptosis signaling pathways (82, 85–88, 190).Noticeably, specific members of HSP families, in particular, HSP90 showed to interfere with all these signaling cascades (83, 89, 103). Based on positive results from preclinical studies, several researchers proposed the use of HSP90 inhibitors for the treatment of lymphoma (122). However, result from phase II clinical trial of HSP90 inhibitor revealed low efficacy, but durable response, and

acceptable toxicity profile in patients with r/r NHL (122). Taking into account critical role of HSP90 in lymphoma pathogenesis, further studies should be performed to assess the role of HSP90 in different subtypes of HL and NHL lymphomas.

For effective development of HSP-based therapy in lymphoma, it is critical to bear in mind that different HSP members reside in different cellular compartments where they perform specific functions (30, 191, 192). For example, mitochondrial HSP90 homolog known as tumor necrosis factor receptor-associated protein 1 (TRAP1) is involved in mitochondrial bioenergetics while endoplasmic reticulum (ER) HSP90 member referred to as glucose-regulated protein 94 (GRP94/gp96/Endoplasmin/HSP90B1) is critical for the unfolded protein response (193–196). It also appears that cell has some form of a balance of HSP distribution across compartments and cancer seems to impair this equilibrium, leading to the translocation of HSPs, which further reflects their functions (197–199). For example, surface expression of GRP94 showed to increase tumor immunogenicity and stabilize plasma membrane HER2 in breast cancer cells (200–202). So whether HSP-based immunotherapy also affects distribution of HSPs in HL and NHL, shifting HSPs from their primary locations is not yet clear and requires further investigation.

It is also important to point out that HSPs in extracellular milieu exist in several forms either secreted or membrane-bound and each form has distinct function (203–208). For example, dying tumor cells secrete HSP70s that serve as damage-associated molecular patters (DAMPs) and showed to elicit strong T cell response which with long-term exposure leads to the induction of immune tolerance and tumor growth (209–212). Conversely, viable tumor cells export HSP70 in exosomes which showed to activate myeloid-derived suppressor cells (MDSCs) and macrophages for the production of IL-6 and TNF-α, respectively (213, 214). Additionally, HSP70 on the surface of tumor cells serves as a recognition structure for NK cells (199, 215). In light of the reported, extracellular HSP70 activates T regulatory cells leading to the downregulation of interferon-γ and TNF-α section and upregulation of IL-10 and transforming growth factor-β (TGF-β) production (216). Along this line, interaction of extracellular HSP70 with antigen-presenting cells resulted in activation of NF-kB, leading to the production of TNF-α, IL-1β, IL-6 and IL-12 (56, 217–220).Figueiredo and colleagues reported that soluble HSP70 alone or in combination with IL-2 resulted in increased production of IFN-γ by T cells (221). Furthermore, stimulation of T cells with HSP70 and IL-2 or IL-7/IL-12/IL-15 resulted in upregulation of Granzyme B in CD4+T cells in target-independent manner, suggesting that extracellular HSP70 can induce target-independent cytotoxicity in T- helper cells (221). In light of the reported, extracellular HSP110 induce pro-inflammatory phenotype in macrophages (222). Along this line, HSP27-positive tumor –derived exosomes enhance immunosuppressive activity of MDSCs whereas soluble HSP27 induces tolerogenic phenotype in macrophages (223, 224). Furthermore, extracellular HSP27 inhibits differentiation of monocytes to DCs (225). Therefore, taking into account immunologic role of extracellular HSPs, it is important to study their functions in lymphoma pathogenesis and further monitor expression of HSPs in different stages and subtypes of HL and NHL.

Despite their extracellular roles in tumor immunology, HSP members have also distinct intracellular immunologic functions. For example, ER HSP90 homolog GRP94 plays important role in immunosuppressive activity of T regulatory cells, in lymphopoiesis of T and B cells, in production of proinflammatory cytokines by tumor-associated macrophages, in the regulation of platelet GPIbα subunit of GPIb-IX-V complex and maturation of dendritic cells (195, 201, 202, 226–231). Along this line, mitochondrial HSP70 homolog GRP75/mtHSP70/mortalin/HSPA9 showed to interact with complement C9 and protect tumor cell from complement-dependent cytotoxicity (232–234).Furthermore, HSPs regulate an important component of innate immune response- the Nod-like receptor protein-3 (NLRP3) inflammasome (235, 236). Therefore, taking into account that central role in lymphoma pathogenesis play immune evasion mechanisms, intracellular immunologic roles of HSPs should also be considered for the development of more effective and safe HSP-based immunotherapy for the HL and NHL treatment.

HSP chaperones also showed to interact with each other. For example, mortalin interacts with HSP60 and HSP90 whereas GRP94 interacts with binding immunoglobulin protein (BiP/GRP78/HSPA5) (234, 237–239). Furthermore, individual homologs may have specific client networks. For example, ER HSP90 member GRP94 has client network that does not overlap with client network of cytosolic HSP90 homologs (195, 240, 241). Conversely, members from different HSP families may have overlapping client network. For example, HSP110 and HSP90 showed to stabilize c-Myc and Bcl-6 (109, 120, 179). Therefore, it is critical to note that blocking HSP member may further affect its co-chaperones, its client network and other HSP chaperones. Therefore, further studies should explore what happens on the level of individual HSP members in different subtypes of HL and NHL lymphoma and whether specific blocking of a particular homolog and, hence, its client and co-chaperone network, will be more advantageous for the lymphoma treatment.

HSP90 showed to affect CD3 and CD28, thus, the effect of HSP90 on CAR- containing CD3 and CD28-derived domains requires further investigation (133). From the other hand, HSP70-derived peptide TKD has been used for *ex vivo* activation of NK cells for adoptive transfer therapy and, since no severe toxicity was observed for NK-based immunotherapy, this strategy may be exploited for the development of lymphoma immunotherapy (140). In light of the reported, two research teams proposed to target membrane-bound HSP70 and HSP90 isoforms on tumors by CARs (134, 136).

Evidently, HSP90 *via* its client network (NPM/ALK and Myc) showed to be involved in the regulation of immune checkpoints such as PD-L1 and CD47 whereas HSP70 ER member GRP78 showed to be co-expressed with CD47 (171, 177–179). Since combination of HSP90 inhibitors with either anti-PD-1, anti-PD-L1 or anti-CTLA-4 antibodies showed anti-tumor effect in mouse models, combinatorial approaches of using HSP90 inhibitors and checkpoint inhibitors or HSP70 inhibitors coupled with anti-CD47 antibodies may further improve anti-tumor response (167, 172).

Another strategy to improve therapy responses involves the use of biomarkers that can predict clinical outcome. Large body of evidence suggests that extracellular HSPs can be used as predictive, prognostic and diagnostic biomarkers of cancer (31, 63, 64, 242–244). Further studies should be performed to assess expression of HSPs in extracellular milieu in their potential to predict clinical response in patients with HL and NHL. Recently, Dunphy and colleagues have conducted phase I clinical trial to test the safety and feasibility of administering ^{124}I-PU-H71 radiologic agent followed by positron emission tomography (PET) to detect HSP90 within epichaperome complex in various types of tumors, thus supporting further development of HSP90-based targeted- therapeutics (245).

Taken together, members of HSP family may be exploited for the development of more efficacious treatment, though, further studies are required to understand the effect of HSP expression in various lymphoma subtypes and their use in the development of T/NK-based immunotherapies and combination approaches. Moreover, the role of HSPs as biomarker to predict clinical outcome in lymphoma patients warrants further investigation.

CONCLUSION

Lymphoma is a heterogeneous group of cancer, derived from immune cells and characterized into two major subtypes such as Hodgkin and Non-Hodgkin lymphoma. HSP members in particular HSP90, HSP60 and HSP70 are highly expressed in most subtypes of HL and NHL lymphoma. Evidently, HSPs play a major role in hallmarks of lymphoma pathogenesis including their involvement in immune evasion and dysregulation of key signaling cascades. Exploiting HSPs in immunotherapy-based approaches and as biomarkers for the lymphoma therapy may prove effective, however, requires further investigation.

AUTHOR CONTRIBUTIONS

ZA: conceptualization and manuscript writing. YM: manuscript editing. AS: Resources. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by RFBR, project number 20-315-90081. YM was supported by the RAKFOND grant 2020-02.

ACKNOWLEDGMENTS

The figures were created with BioRender.com.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 25 March 2021
doi: 10.3389/fonc.2021.626818

Understanding the Immune-Stroma Microenvironment in B Cell Malignancies for Effective Immunotherapy

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OPEN ACCESS

Edited by:
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Reviewed by:
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Eric Eldering, Amsterdam University Medical Center (UMC), Netherlands
Philipp M. Roessner, German Cancer Research Center (DKFZ), Germany

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Specialty section:
This article was submitted to Hematologic Malignancies, a section of the journal Frontiers in Oncology

Received: *06 November 2020*
Accepted: *04 January 2021*
Published: *25 March 2021*

Citation:
Apollonio B, Ioannou N, Papazoglou D and Ramsay AG (2021) Understanding the Immune-Stroma Microenvironment in B Cell Malignancies for Effective Immunotherapy. Front. Oncol. 11:626818. doi: 10.3389/fonc.2021.626818

Cancers, including lymphomas, develop in complex tissue environments where malignant cells actively promote the creation of a pro-tumoral niche that suppresses effective anti-tumor effector T cell responses. Research is revealing that the tumor microenvironment (TME) differs between different types of lymphoma, covering inflamed environments, as exemplified by Hodgkin lymphoma, to non-inflamed TMEs as seen in chronic lymphocytic leukemia (CLL) or diffuse-large B-cell lymphoma (DLBCL). In this review we consider how T cells and interferon-driven inflammatory signaling contribute to the regulation of anti-tumor immune responses, as well as sensitivity to anti-PD-1 immune checkpoint blockade immunotherapy. We discuss tumor intrinsic and extrinsic mechanisms critical to anti-tumor immune responses, as well as sensitivity to immunotherapies, before adding an additional layer of complexity within the TME: the immunoregulatory role of non-hematopoietic stromal cells that co-evolve with tumors. Studying the intricate interactions between the immune-stroma lymphoma TME should help to design next-generation immunotherapies and combination treatment strategies to overcome complex TME-driven immune suppression.

Keywords: immunotherapy, tumor microenviroment, stroma, anti-PD1, lymphoma, interferon, T cells, CAR T

INTRODUCTION

There is a clinical need to identify novel treatments for lymphoid malignancies (1). Effective immunotherapy promotes the killing of cancer cells by cytotoxic T cells. Immune checkpoint blockade has demonstrated that reinvigorating anti-tumor immune activity can induce durable responses across multiple cancer types and serves as an illustrative example of therapeutically targeting the tumor microenvironment (TME) (2). The most promising clinical responses to PD-1 blockade have been seen in classical Hodgkin lymphoma (cHL) and primary mediastinal B cell lymphoma (PMBL) with up to 87% overall response rate (ORR) detected in relapsed/refractory (R/R) cHL (3). However, the efficacy of anti-PD-1 immunotherapy in non-Hodgkin lymphomas (NHLs) including diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) has been more modest (4). Unexpectedly, no activity was seen in a trial of anti-PD-1 therapy for R/R chronic

lymphocytic leukemia (CLL) although PD-L1-PD-1-mediated T cell dysfunction has been described (5–7). This clinical experience suggests that profound immunosuppressive barriers operate within the TME.

The current immuno-oncology era is directing attention towards the relevance of studying the composition and function of the immune TME, together with genomic analysis. In this review, we discuss the current understanding concerning the role of T cells and inflammatory signaling ("inflamed" *versus* "non-inflamed" immune TMEs) in generating both endogenous anti-tumor immune responses, as well as sensitivity to immunotherapy. We describe how cancer immunology has been the subject of intense research in the solid tumor field but has been comparatively ill-defined in the lymphomas. We highlight tumor intrinsic and extrinsic mechanisms of resistance in generating an effective anti-tumor immune response, before introducing an understudied area of lymphoma research—the role of non-hematopoietic stromal cell types in regulating anti-tumor immunity, that could also represent a targetable obstacle that immune cells face in the cancer-immunity cycle.

THE IMMUNE TME AND ANTI-TUMOR IMMUNITY

It is known that the immune system can paradoxically both inhibit and promote tumor development through a dynamic process where complex interactions between malignant cells and the immune system determine the fate of tumors, termed cancer immunoediting, which progresses through three phases: elimination, equilibrium, and escape (8, 9). During the elimination phase, the immune system recognizes and eradicates transformed cells; however, some tumor clones can avoid elimination leading to the equilibrium phase during which tumor growth is kept in check (10). However, chronic inflammation as well as evolutionary pressure from immune cells, can allow tumor subclones to escape immune surveillance, leading to tumor outgrowth and disease (10). Although cancer immunoediting has been defined using murine models, next generation technology is now in a position to shed light on the relevance of these concepts for human patients and clinical observations (11).

The immune composition of the TME is a major determinant of tumor progression through these phases and includes innate (natural killer cells, macrophages, neutrophils, mast cells and dendritic cells), and adaptive (T and B cells) immune cells. The composition of the TME in B cell lymphomas that arise in secondary lymphoid tissues, can vary from resembling normal reactive lymph nodes with germinal centers as seen in FL, to tumor effacement as exemplified by CLL and DLBCL (12). In common with solid cancers, malignant cells actively influence the composition of the TME and educate surrounding immune and stromal cells that can acquire pro-tumorigenic and immunomodulatory activity.

Particular cell types of the innate and the adaptive immune system can function in a tumor-promoting or inhibitory way with neutrophils, M2-polarized tumor-associated macrophages (TAMs), T_H2 $CD4^+$ T cells and T_{Regs} generally considered as pro-tumor cells, whereas, M1-macrophages/TAMs, T_H1 $CD4^+$ T cells and cytotoxic $CD8^+$ T cells are associated with anti-tumor functions (13). Importantly, in a significant proportion of cancer patients, including the lymphomas, there is evidence of an active anti-tumor immune response directed against tumor-specific (neoantigens) (14, 15) or tumor-associated antigens (16). However, although acute inflammatory signals can stimulate adaptive immunity, chronic inflammation can be antagonistic and promote immune suppression. Numerous studies in solid cancer have shown that an activated adaptive immune response involving effective antigen presentation and interferon (IFN) signaling, as well as sufficient numbers of T cells in the TME, is associated with a favorable prognosis (13, 17, 18).

T Cells and Anti-Tumor Immunity

The crucial role of T cell lymphocytes as the main regulators and effectors in anti-tumor immunity has been well established. Seminal studies have revealed the role of immune surveillance and the vital contribution made by adaptive immune cells in suppressing the formation of tumors (19–21). Murine models have shown that tumor killing is mainly mediated by cytotoxic $CD8^+$ T cells in both solid (13) and B cell lymphomas (22). Elegant studies using the transgenic Eμ-TCL1 mouse model of CLL have shown that $CD8^+$ T cells play an important role in controlling disease development in an IFNγ-dependent manner (23). $CD8^+$ T cells, following successful priming, recognize antigens presented by tumor cells on their surface in complexes with HLA class I molecules and kill their targets, primarily *via* the release of cytotoxic molecules such as perforin and granzymes (24, 25). While the anti-tumor role of $CD8^+$ is generally accepted, the role of $CD4^+$ T cells in cancer immunity is controversial. T_H1 $CD4^+$ T cells, *via* the secretion of pro-inflammatory cytokines such as IFNγ and IL-2, can activate antigen presentation and costimulatory function on antigen-presenting cells (APCs), promote the effector differentiation of $CD8^+$ T cells and enhance their migration (26). However, as mentioned above, some subsets of $CD4^+$ cells can exhibit pro-tumor functions. T_H2-polarized $CD4^+$ T cells secrete cytokines that can limit CTL differentiation and proliferation such as IL-10 and IL-4, while $CD4^+$ T_{Regs} ($FOXP3^+$) have significant immunosuppressive functions through various mechanisms and their pro-tumor role has been described in both solid tumors and lymphomas (13, 27–29). In CLL, *in vitro* and xenograft murine models have demonstrated a pro-tumor effect of the $CD4^+$ T cell compartment in patients, with correlations of $CD4^+$ subset counts and clinical outcome supporting this role (28, 30). However, research using Eμ-TCL1-based murine models has suggested a more complex role of $CD4^+$ T cells with some studies showing an anti-tumor function (31), while others demonstrating a dispensable role (23). Such differences likely reflect differences in the assay systems used, and the degree to which they model complex immune TMEs, as well as highlighting the divergent role of T_H1 *versus* T_H2-polarized $CD4^+$ T cell subsets and their plasticity. Interestingly, $CD4^+$ T cells specific for immunoglobulin-derived

neoantigens in mantle cell lymphoma (MCL) were shown to express granzyme B and possess cytolytic function against autologous tumor cells following their engagement and expansion (14, 15), as previously described for granzyme B^+ tumor-reactive $CD4^+$ T cells in solid cancer (32). However, despite their crucial roles in controlling tumor growth, T cells become dysfunctional due to exhaustion in the chronically inflamed TME. T cell exhaustion is characterized by low proliferative capacity and limited effector function, due to chronic tumor antigen exposure (33, 34). This, in combination with a multitude of additional mechanisms covered later in this review, allow some tumors to escape both endogenous and therapy-mediated anti-tumor responses.

The Importance of Type I and II IFNs in the Immune TME

Numerous studies have established the important role of the type II IFN, IFNγ as a key player in host anti-tumor immunity through direct anti-tumor and indirect immunoregulatory actions (19, 35–39). IFNs can suppress tumors directly by triggering pro-apoptotic signaling or inhibiting their proliferation, while increasing MHC expression (40), antigen presentation (41) and promoting tumor immunogenicity, a critical process for effective tumor recognition and elimination by the immune system (19). IFNγ strongly promotes inflammatory responses and has been shown to augment the function of tumor-infiltrating immune cells including $CD4^+$ T_H1 cells, $CD8^+$ T cells, dendritic cells and macrophages, while suppressing T_H2 cells and T_{regs} (20, 39). Despite its pleiotropic effects, it has been demonstrated that IFNγ-expressing T cells play a dominant role in mediating effective anti-tumor activity (42).

Although the majority of studies have used solid tumor models, the importance of IFNγ in anti-tumor immunity has also been shown in B cell malignancy models. Effective lymphoma immune surveillance using murine models was shown to be mediated by tumor-specific $CD4^+$ T cells and associated with proinflammatory cytokines, particularly those that promote a T_H1 phenotype including IFNγ, IL-2, and IL-12 (43). As discussed above, a non-redundant role of IFNγ-expressing $CD8^+$ T cells in suppressing CLL progression was demonstrated using a murine transgenic model (23). Recent work has revealed that a subset of DLBCL harboring a T cell-inflamed TME (discussed later in this review) expressed a number of inflammatory and effector cytokine pathways including IFNγ and TNFα (44). Moreover, a T_H1 cytokine profile including type II IFN T cell responses has been associated with a favorable prognosis and response to traditional chemotherapy, the immunomodulatory drug lenalidomide and anti-PD-1 immunotherapy in NHL patients (44–48).

Type I interferons (including IFNα and IFNβ) can be produced by all nucleated cells and act on both tumor and immune cells (38, 49). Even though some decades-old studies had suggested an anti-tumor activity for IFNα/β, only recently, their role in the elimination of tumor cells and immunoediting has started to be elucidated (50, 51). Several studies have established their direct anti-tumor effect which is mediated through growth inhibition and/or induction of apoptosis by the regulation of the cell cycle (G1 phase arrest) and activation of both intrinsic and extrinsic apoptotic pathways (TRAIL, FAS and FASL among others) (52, 53). Moreover, type I IFNs have been shown to increase both HLA class I and tumor antigen expression and consequently, increase immune recognition and successful generation of an anti-tumor response (54, 55).

In addition to direct effects on tumor cells, there is substantial evidence showing that type I IFNs mainly function through stimulating anti-tumor immune responses that is relevant for both natural and therapy-induced immunity. The autocrine and paracrine circuits within the immune TME that are triggered by type I IFN show similarity with IFNγ signaling but they do not overlap completely (50, 56). Type I IFN deficient dendritic cells (DCs) in murine TME models have been correlated with ineffective T cell priming (57, 58). In common with IFNγ, the ability of type I IFN to promote immune-mediated anti-tumor activity is also through their effects on T cells. More specifically, type I IFNs have been reported to play an important role in T_H1 $CD4^+$ T cell polarization as well as promoting the survival and effector function of $CD8^+$ cytotoxic T cells by increasing granzyme expression (51, 59, 60). In addition, type I IFNs have been shown to negatively regulate the numbers and activity of T_{Regs} (61) and myeloid-derived suppressor cells (MDSCs) (62).

Remarkably, in the context of hematological malignancies, one of the earliest studies indicating a role of type I IFNs in promoting immune-mediated anti-tumor responses was reported in a mouse lymphocytic leukemia model (63). Recent work has demonstrated that avadomide, a cereblon E3 ligase modulator (CELMoD), can induce IFN signaling in DLBCL B cells that triggered tumor apoptosis (64). In addition, the immunomodulatory drug lenalidomide has been shown to induce IFNβ signaling in DLBCL that promotes tumor cell death (65). In contrast, lenalidomide and avadomide have anti-proliferative effects on CLL cells that are likely a direct effect of IFN signaling induction but does not induce direct tumor B cell apoptosis (66, 67). Interestingly, the fusion of IFNα to anti-CD20 antibody induced a superior anti-lymphoma effect than anti-CD20 alone by direct and potent killing of type I IFNα receptor-positive lymphoma cells (68). It was further demonstrated that an important additional mechanism of action of tumor-directed antibodies, in addition to direct anti-tumor effects, was the induction of type I IFN in the TME that activated DC cross-presentation and T cell activation (69). This work highlights the potential of next-generation Ab-based immunotherapy (Ab–IFN fusion) (70) to induce direct anti-tumor effects and reconnect suppressed innate and adaptive immune responses in the TME (69). However, it is worth noting that earlier work in CLL has described conflicting data regarding a pro-survival effect when treating CLL cells with recombinant type I (71–73) or type II IFNs (74), that may be associated with studies using peripheral blood-derived CLL cells, rather than tissue culture or *in vivo* models that mimic activated CLL TME biology (75). Recently, impaired IFN signaling in CLL B cells (loss of IFN regulatory

factor 4, IRF4 using a murine model or its reduced expression in human CLL cells) has been linked to downregulated antigen presentation and co-stimulatory molecules that prevented the generation of activated, exhausted T cell responses and was associated with accelerated disease progression. In addition to this tumor immune evasion mechanism, T cells from treatment naïve CLL patients have been shown to express deregulated IFN type I and II signaling genes compared to healthy age-matched control T cells (67), in keeping with impaired IFN signaling in T cells representing a common immune defect in cancer (76). Together these recent findings provide evidence that reduced IFN signaling in the CLL TME could contribute the development of an immunosuppressive/non-inflamed TME.

Inflamed *Versus* Non-Inflamed Immune TMEs

Analysis of solid cancer tumors including colorectal, melanoma, and ovarian cancer among others has established the prognostic significance of the type, density, and localization (immune contexture) of immune cells that reside within the TME for predicting a patient's overall survival (77–80). The prognostic power of these variables is so powerful that they led to the development of a new scoring system termed the 'immunoscore', which has been internationally validated for colorectal carcinomas (CRC) (81). The immunoscore is based on the quantification and localization of $CD3^+$ and $CD8^+$ lymphocytes within tumors and their invasive margin and evidence so far suggests that this alternative scoring system is a more robust classification system for CRC for predicting survival than the classical tumor-node-metastasis staging system (81, 82). This was the first attempt of cancer classification/stratification through a system which was not tumor-based but rather immune-based, leading to a basic distinction of TMEs as T cell inflamed (or hot) that contain high numbers of infiltrated T cells, *versus* non-inflamed (or cold) phenotypes that are non-infiltrated (3, 18).

Transcriptome profiling studies have revealed additional molecular characteristics of T cell inflamed TMEs including the number of tumor-infiltrating T cells and the expression of IFN-inducible activated T cell biology gene signatures including granzymes, chemokines, PD-L1, as well as tumor mutational burden and neoantigen load (83, 84). In contrast, non-inflamed TMEs are generally characterized by low or absent infiltration of $CD8^+$ cytotoxic T cells, low frequency of neoantigen expression, and a paucity of IFN signaling (18, 85). It should be noted that solid cancer and B cell lymphoma subtype TMEs fall into a spectrum of T cell inflamed to non-inflamed phenotypes reflecting expected heterogeneity, and can include "excluded" phenotypes where understudied stromal cells may present a barrier to infiltrating T cells (18). Applying the concept of inflamed *versus* non-inflamed TME phenotypes to lymphoma is challenging due to two major characteristics of these cancers. First, lymphomas are cancers of immune cells and as a consequence, tumor cells can regulate immunological functions themselves, making the tumor-immune cell interaction significantly more complex than solid tumors. Secondly, the origin and residence of malignant cells in most B cell lymphomas are secondary lymphoid organs that serve as sites of immune cell surveillance. Therefore, the presence of T cell subsets or inflammatory signatures in the TME may also represent active non-tumor immune responses. For example, cytomegalovirus causes a marked expansion of virus-specific T cells in CLL patients (86). However, the role of Epstein–Barr virus (EBV) in the pathogenesis of some lymphomas may also be a source of antigens for tumor T cell recognition (87). Regardless, a classification of lymphomas based on inflamed versus non-inflamed immune landscapes ('immune contexture') and responsiveness to immune checkpoint blockade has been described (3).

In the current era of immunotherapy, the characterization of tumors according to their immune landscape is more relevant than ever (88), as IFNγ-expressing T cell inflamed tumors have shown increased sensitivity to anti-PD-1 therapy with both predictive and prognostic significance (83, 89–91). PD-1 has been shown to be a negative regulator of pre-existing immune responses in tumors, thus blocking the interaction of PD-1 with its ligands (PD-L1 or PD-L2) prevents this inhibitory signaling and allows tumor-specific T cells to remain activated and kill tumor cells. Pre-clinical studies in solid cancer have suggested that anti-PD-1 therapy cannot function in the absence of primed tumor antigen-specific $CD8^+$ cytotoxic T cells which express high levels of PD-1 (exhausted phenotype) (2, 92, 93). However, high-dimensional correlative analysis of circulating immune cells from melanoma patients receiving anti-PD-1 immunotherapy has revealed a more complex role of the T cell compartment during therapy. In particular, higher numbers of $PD\text{-}1^+$, $CTLA\text{-}4^+$, $IL\text{-}17A^+$, $IFN\gamma^+$ activated memory $CD4^+$ and $CD8^+$ T cells including granzyme B^+ $CD4^+$ T cells were detected after therapy and in responding patients (94). Interestingly, the frequency of classical $CD14^+$ $HLA\text{-}DR^+$ monocytes prior to therapy was found to be a strong predictor of response, thought to reflect myeloid expansion triggered by IFNγ produced by activated tumor-specific and infiltrated T cells in patients who are more likely to become responders.

Inflamed lymphomas such as cHL tumors have shown increased sensitivity to anti-PD-1 blockade therapy and are characterized by an unusually high immune cell infiltrate, an inflammatory $PD\text{-}L1^+$ TME, a high mutational burden (95) and genetic alterations that facilitate cancer immunoediting escape including aberrant MHC class I molecule expression (96). Intriguingly, the majority of T cells that infiltrate HL tumors are IFNγ-expressing T_H1-polarized $CD4^+$ T cells (96, 97), which is again at odds with the concept that PD-1 inhibition works solely through the reactivation of MHC class I-restricted $CD8^+$ T cells (98), given that cHL is sensitive to this immunotherapy. Indeed, recent immune monitoring studies have shown that circulating cytotoxic granzyme B^+, $PD\text{-}1^+$ $CD4^+$ T cells, as well as $PD\text{-}1^+$ differentiated effector $CD8^+$ T cells are detected in HL patients. Interestingly, only $CD4^+$ T cell receptor (TCR) diversity at baseline and during therapy correlated with responses to anti-PD-1 therapy (99). Additionally, the study identified an IFN-experienced circulating $CD68^+$ $CD4^+$ Granzyme B^+ innate

effector subset in patients who responded to therapy. This correlative data highlights the potential cytotoxic anti-tumor capability of specific $CD4^+$ T cell subsets that is relevant for both solid cancers (94) and lymphomas (99–102). It is also interesting to note that the presence of a baseline T cell inflamed TME has correlated with improved responses to other therapies including adoptive cell therapy in solid cancer models (103), that may have relevance for CAR T therapy in lymphoma (104), and the CELMoD avadomide which induced higher responses in immune-rich DLBCL TMEs (105). However, although a proportion of DLBCL (44) and FL tumors (106, 107) exhibit features of T cell inflamed TMEs, the immune landscape of the NHLs is more heterogenous with most harboring a cold or non-inflamed environment, and are typically resistant to anti-PD-1 therapy. A recent phase 2 study of anti-PD-1 in R/R DLBCL following autologous transplant reported an ORR of 10%, although some patients did show encouraging disease stabilization and durable responses (108). Non-inflamed lymphomas are thought to contain fewer infiltrating immune cells, particularly T cells, that can predict poor survival in DLBCL (109). Sparse infiltration of immune cells is linked to lymphoma intrinsic oncogenic pathways that prevent the recruitment or retention of immune cells in TME lymphoid tissues or downregulate APC capability (110). Interestingly, non-inflamed lymphomas typically lack genetic aberrations that facilitate immune escape (111). We have recently demonstrated that CLL lymph nodes show salient features of non-inflamed tumors including sparse numbers of $CD8^+$ T cells relative to reactive non-malignant tissues and low PD-L1 expression in the TME, as well as profound T cell functional exhaustion (67).

TUMOR INTRINSIC AND EXTRINSIC MECHANISMS THAT INFLUENCE RESPONSE TO IMMUNOTHERAPY

It is now clear that distinct mechanisms may confer sensitivity and resistance to different types of immunotherapy in tumors that show a large degree of heterogeneity in their immune TMEs, falling within the extremes of the inflamed *versus* non-inflamed classification. The key factors that shape the TME include tumor immunogenicity, oncogenic pathways, and genetic alterations that regulate T cell infiltration and function (3, 112). The sculpting of the immune TME is inherently interconnected with the cancer immunoediting process. Available evidence from studies of patients treated with immune checkpoint blockade drugs suggests that immunoediting takes place not only during tumor progression but, at least in some form, also in response to therapy (9). Several factors of immune escape and resistance to immunotherapy (innate or acquired) that have been characterized to date, can be broadly divided in tumor-intrinsic and tumor-extrinsic mechanisms (113, 114). While the bulk of evidence regarding the role of these processes in regulating anti-tumor immunity and response to immunotherapy comes from solid tumor research, recent studies are revealing similar mechanisms operative in the lymphomas.

Tumor Cell-Intrinsic Mechanisms That Shape the Immune TME

Tumor intrinsic mechanisms generally include genetic aberrations that can affect antigen recognition and influence immune function and immune contexture in TMEs including neoantigen load (**Figure 1**). HLA class I and II are required for the display and presentation of tumor-associated antigens and consequently an effective adaptive anti-tumor response. Several studies have reported HLA class I and II molecule loss or downregulation in lymphomas through genetic, epigenetic or transcriptional mechanisms. Intriguingly, acquisition of recurrent genetic alterations in genes encoding antigen presentation machinery appears to be a shared characteristic of T cell inflamed lymphomas such as cHL (115) and a subset of DLBCLs (44). This likely represents a critical cancer immunoediting escape mechanism used by lymphoma cells to evade the effector activity of lymphoma-specific $CD8^+$ and $CD4^+$ T cells. Impaired or loss of expression of HLA class I molecules is frequently detected in HL and DLBCL but has not been reported or is extremely rare in indolent lymphomas such as MCL, marginal zone lymphoma and CLL (16, 115–118). The most common mechanism leading to this altered HLA expression is caused by mutations and deletions in the β2-microglobulin gene, although direct genetic alterations of the HLA I genes have also been reported (115, 117, 119). HLA class II molecule downregulation is also observed in HL and DLBCL and mediated mainly at a transcriptional level through inactivating mutations CIITA, as well as homozygous deletions of chromosome 6p21.3 (116, 136–139).

Another mechanism contributing to immune evasion in lymphoma is the genetic overexpression of PD-L1, that is also a common feature of checkpoint blockade-sensitive T cell inflamed TMEs including cHL and PMBL (3). Upregulated expression of PD-1 ligands has been found to be driven by genetic alterations driving amplification of structural variations (SVs) of the chromosome region 9p24.1 which contains the loci for *PD-L1*, *PD-L2* and *JAK2* (termed the PDJ amplicon) (120–122). PD-L1 expression is less prevalent in DLBCL, but *PD-L1* gene alterations have also been detected in a subset of DLBCLs, particularly the non-germinal center B cell (GCB) subtypes, harboring T cell inflamed phenotypes with high numbers of infiltrating T cells, downregulated HLA expression and upregulation of inflammatory NF-kB, TNFα and IFNγ gene pathways (44, 120, 140). Recurrent SVs in the 3′ UTR of the *PD-L1* gene, which are thought to stabilize PD-L1 transcripts leading to increased protein expression, have also been uniquely described in DLBCL (141).

As mentioned earlier, type I and II IFNs are important mediators of both innate and adaptive anti-tumor immune function and are key players in cancer immunoediting. Importantly, defective IFN signaling in tumor cells has emerged as a major tumor-intrinsic resistance mechanism during immune checkpoint blockade therapy (114, 142, 143). Loss-of-function mutations in IFNγ receptor signaling pathway genes *JAK1* and *JAK2* have been seen in melanoma patients who developed late relapses after initial successful anti-PD-1 therapy

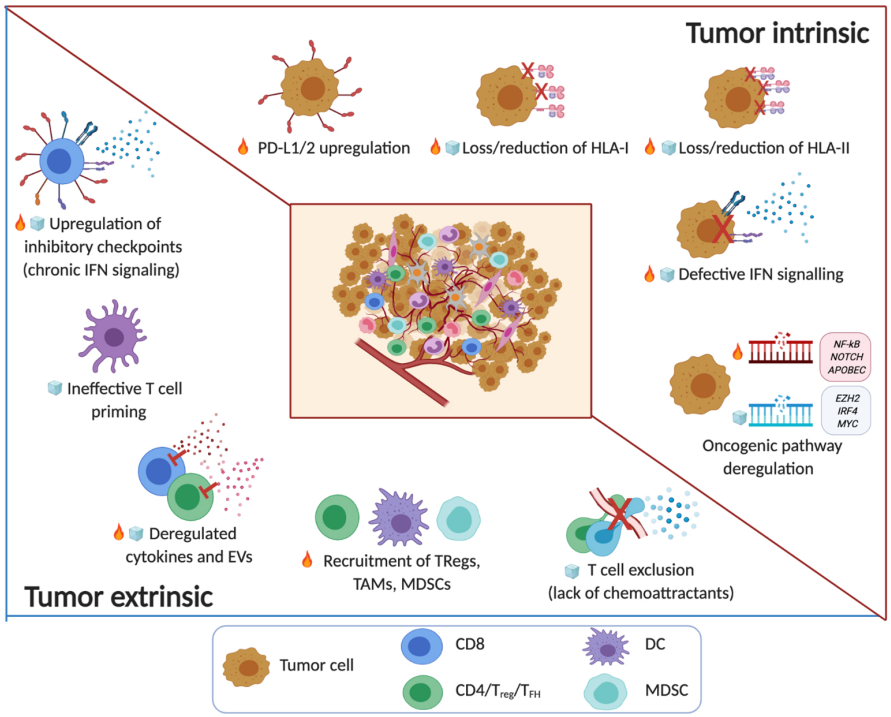

FIGURE 1 | The immune TME in B cell malignancies. Tumor intrinsic and tumor extrinsic mechanisms associated with noninflamed/cold ( ) or inflamed/hot ( ) TMEs in B cell malignancy. The key factors that shape the TME include tumor immunogenicity, oncogenic pathways and genetic alterations that regulate T cell infiltration and function. Note: the timeline or sequence of events during the evolution of the altered pro-tumor, yet immunoprivileged TME is a current topic in the field. Emerging evidence from patients treated with immune checkpoint blockade drugs suggests that cancer immunoediting takes place not only during tumor progression but also in response to therapy (*e.g.* the acquisition of mutations that contribute to 'defective IFN signaling') (8–11). Several factors of immune escape and resistance to immunotherapy (innate or acquired) that have been characterized to date, can be broadly divided in tumor-intrinsic and tumor-extrinsic mechanisms. Tumor intrinsic mechanisms generally include genetic aberrations that can affect antigen recognition ('loss/reduction of HLA-I/-II molecules') (16, 115–119) and influence immune function ('PD-L1/2 upregulation') (120–122) and immune contexture in TMEs ('oncogenic pathway deregulation') including neoantigen load (83, 84). Tumor cell extrinsic factors that regulate anti-tumor immunity, immune evasion or resistance to immunotherapy involve non-tumor cellular and molecular components within the immune TME including 'upregulation of inhibitory immune checkpoints' (linked to chronic IFN signaling) (123–125), the 'recruitment of TAMs, MDSCs, T_{Regs}' and stromal cells, as well as 'deregulated cytokines and EVs' (126–130), 'ineffective T cell priming' and 'T cell exclusion' (131–135). (*Created with Biorender.com*).

(144), as well as patients with primary resistance to this immunotherapy (145). Mutations in IFNγ-related genes were also observed in non-responsive patients following anti-CTLA-4 checkpoint therapy (146). This loss of IFNγ receptor signaling allows the tumor to evade the effects of IFNγ produced by anti-tumor T cells that would reduce tumor antigen presentation capability, decrease the expression of IFN-associated chemoattractants and promote insensitivity to the anti-proliferative and pro-apoptotic effects of IFNγ in tumor cells. Several studies have found evidence of deregulation of tumor-intrinsic IFN signaling in lymphoma but this has not yet been directly linked to immunotherapy resistance. Suppression of type I interferon by STAT3 has been described in non-GCB DLBCL, with inhibition of STAT3 activity using ruxolitinib inducing a synergistic growth inhibition effect when combined with the IFN-inducing immunomodulatory drug lenalidomide (147). Evidence for a CLL cell-intrinsic IFN signaling defect was described earlier in this review with low expression of IRF4 associated with inferior prognosis and associated studies supporting a novel tumor immune evasion mechanism (148). This IFN signaling defect reduced expression of tumor genes required to activate T cells including antigen processing and presentation, that could contribute to resistance to immunotherapies in this disease.

In addition to altering intrinsic tumor cell properties, oncogenic signaling has been found to contribute to the immune contexture of inflamed and non-inflamed TMEs. Genetic aberrations affecting MYC, p53 and NF-kB among other genetic events can dictate the immune landscape (84, 149). Importantly, pathways including WNT-β-catenin and MAPK signaling, as well as those associated with loss of PTEN, have all been implicated in driving intrinsic resistance to immune checkpoint blockade in solid tumors (114). Recent genomic studies in DLBCL have revealed associations between alterations in oncogenes or tumor suppressors including *PTEN*, *EZH2* and *TP53* and reduced expression of genes linked to immune cell activation (150). However, mechanistic data on how oncogenic alterations promote a non-inflamed immune TME or immunotherapy resistance in lymphomas is currently

lacking. Double hit GCB DLBCLs with *MYC*, *BCL2*, and/or *BCL6* gene rearrangements have been shown to contain low number of infiltrating T cells (111). These lymphomas are enriched for *EZH2* activating mutations that have recently been shown to underlie acquired deficiency in MHC I and II expression and low T cell infiltrates in murine lymphoid TMEs (110). Interestingly, *MYC* has been shown to regulate the anti-tumor immune response in murine models of T cell acute lymphoblastic leukemia (ALL) and liver cancer by inducing the expression of CD47 and/or PD-L1 immune checkpoint molecules and recruiting TAMs, while excluding T cells among other mechanisms (151–153). However, it should be noted that the ability of oncogenes like *MYC* to control the recruitment and function of immune cells in tumors can be counteracted by other genetic aberrations as has been seen using the *Eμ-MYC* lymphoma model (149, 154), that highlights the complexity of defining the roles of oncogenic alterations in modulating immune cell landscapes both within and between complex molecular lymphoma subtypes. Conversely, oncogenes and tumor suppression genes can equally foster an inflammatory immune TME. One notable example in solid cancer is NF-kB that controls cell survival and proliferation, but also production of inflammatory cytokines (149). In common, recurrent genetic modifications that lead to NF-kB activation have been described in inflamed lymphomas including cHL, PMBL and a subset of T cell-rich DLBCLs that contain *PD-L1* SVs and downregulated HLA expression (44, 155, 156). In addition, oncogenic NOTCH signaling has been implicated in the regulation of inflammatory DLBCLs that harbor genetic immune escape mechanisms including inactivating *CD70* and *FAS* mutations (157–159). Overall, further studies involving pre-clinical murine models will be required to define the how specific oncogenic signaling pathways contribute to tumor-immune cell interactions and T cell recruitment in lymphoma TMEs.

Several studies have shown that tumor neoantigens can function as targets for anti-tumor T cells and there is a positive correlation between tumor mutational burden and response to immune checkpoint blockade across cancers (160–162). In particular, deficiency in DNA repair mechanisms has been found to be the main driver of genomic instability and has been associated with response to immunotherapy (163). Interestingly, mutations that lead to DNA mismatch repair defects and microsatellite instability, as well as APOBEC mutational signatures have been identified in PD-1 blockade sensitive ''inflamed'' lymphomas such as cHL and PMBL; however, these seem more rare in other B cell malignancies (164–166). On the other hand, low tumor immunogenicity with low frequency of neoantigen generation as is seen in CLL, is linked to reduced intrinsic sensitivity to immune checkpoint blockade (5, 167). However, recent antigen-presentation profiling work is revealing that B cell lymphomas including CLL can present immunoglobulin neoantigens (15), with preferential MHC-II presentation that has implications for promoting the cytolytic T_H1 differentiation of neoantigen-specific $CD4^+$ T cells with immunotherapy, as has been demonstrated in solid cancer (168).

Recent studies have demonstrated that metabolically active tumor cells chronically deprive the TME of essential nutrients that affect T cell effector function and promote the creation of a tolerogenic TME [metabolic reprogramming in the lymphomas has been reviewed elsewhere (169–171)].

Tumor Cell Extrinsic Mechanisms That Shape the Immune TME

Tumor cell extrinsic factors that regulate anti-tumor immunity, immune evasion or resistance to immunotherapy involve non-tumor cellular and molecular components within the immune TME including inhibitory immune checkpoints, TAMs, MDSCs, T_{Regs} and stromal cells (**Figure 1**). Although tumor extrinsic components have been linked to cold or non-inflamed TMEs and response to therapy, it should be noted that their roles in cancer immunology are highly dynamic and context dependent, including the nature and duration of the driving forces implicated. For example, cytokines within immune TMEs including IL-10 (131), IL-6 (132) and TGFβ (133) or extracellular vesicles (EVs) (134, 135) are known to have complex, context-dependent effects on both immune and cancer cells. Although there is evidence that these secreted factors have relevant immunomodulatory activity in B cell malignancies including CLL (172–176) and FL (177), our understanding of the hierarchy and cooperation required between cytokines and chemokines or EVs and their cellular sources for the licensing of immune evasion or the promotion of anti-tumor immune responses is currently ill-defined.

Type I and II IFNs can act as double-edged swords in cancer, promoting both feedforward immune activation responses described earlier, as well as feedback inhibitory mechanisms. Importantly, persistent IFN signaling in cancer, in common with chronic virus infection, can be immunosuppressive by inducing PD-L1, IDO and LAG-3 in the immune TME (123–125). Indeed, prolonged type I and II IFN-driven expression of multiple immune checkpoint ligands, receptors and inhibitory pathways linked to exhaustion including PD-1, LAG-3 and TIM-3, have been shown to be upregulated on T cell subsets during adaptive or acquired resistance to immune checkpoint therapy (125, 178, 179). It is plausible that combination checkpoint inhibition may be able to bypass negative feedback and multiple inhibitory receptors as has been demonstrated using a CLL murine model with dual anti-PD-1 and LAG-3 blockade (180). Interestingly, $CD8^+$ CAR T cells co-expressing PD-1 with either LAG-3 or TIM-3 were associated with poor responses in CLL, whereas patients who had complete and durable remissions were infused with CAR T cells containing lower frequencies of these exhausted phenotypes. Moreover, the clinical effectiveness of CAR T cells in CLL was increased when co-stimulatory receptor $CD27^+$ was expressed on CAR T cells that may reflect a less exhausted and functionally competent T cell phenotype ('intrinsic T cell fitness') (181). This data supports the relevance of multiple inhibitory pathways induced by IFN signaling that could hinder CAR T therapy and promote therapy resistance.

TAMs are an important subset of terminally differentiated myeloid cells that regulate anti-tumor immunity and response to

therapy in many cancers including lymphoma (126, 127). Early work demonstrated that TAMs promoted chemotherapy resistance in cHL and in DLBCL in the pre-rituximab treatment era (182, 183). However, TAMs have been shown to mediate antibody-dependent cellular phagocytosis of rituximab engaged malignant B cells, highlighting therapeutic context. Studies have demonstrated the capability of TAMs to directly suppress T cells through PD-L1 expression, as well as the production of cytokines such as IL-10 and TGF-β or enzymes that can limit effector activity (126). Interestingly, inflamed cHLs harbor high numbers of PD-L1$^+$ TAMs, that presumably represent an active immune evasive strategy elicited within the TME to suppress lymphoma-specific PD-1$^+$ CD4$^+$ T cells (184). In addition, it has been shown that lymphoma cells can upregulate expression of the membrane anti-phagocytic protein CD47 that allows them to escape elimination by TAMs. Combining anti-CD47 blocking antibody with rituximab has elicited synergistic anti-tumor activity in pre-clinical models and early clinical results have been promising (185, 186). These studies highlight the potential to harness anti-lymphoma TAM activity that could be combined with immune checkpoint blockade therapy to re-educate their pro-tumor, immunosuppressive activity and optimize immunotherapy. In addition, MDSCs have also emerged as potentially important regulators of immune responses, through the production of several immunosuppressive factors (ARG1, NO, PGE2) (187), and their presence in solid cancer TMEs correlates with decreased efficacy of immunotherapies including checkpoint blockade and adoptive T cell therapy (128, 129). However, a role in contributing to immunotherapy resistance in B cell malignancy has not been defined to date (188). Notably, in CLL it has been demonstrated that MDSCs, characterized by a CD14$^+$HLA-DR^{lo} phenotype, can be induced by malignant B cells *in vitro* to suppress T cell effector function and promote T_{Reg}-differentiation mediated by upregulation of indoleamine 2,3-dioxygenase (IDO) (189).

T_{Regs} comprise a subtype of CD4$^+$ cells which are mainly defined by the expression of FOXP3 and play an important role in maintaining self-tolerance (190). T_{Regs} suppress effector T cells by secretion of inhibitory cytokines including IL-10 and TGFβ, as well as direct cell contact. Indeed, many murine studies of solid cancer have shown that the depletion of T_{Regs} cells from the TME can enhance or restore anti-tumor immunity (130). Studies in CLL using the TCL1 transgenic model indicated that partial depletion of T_{Regs} numbers did not impact on CLL disease progression but did result in enhanced CD8$^+$ T cell functional capacity (191). Increased numbers of circulating T_{regs} have been found in CLL patients and correlated with disease progression (192, 193), and lymph node tissue was shown to harbor twice the number of these suppressive cells than the peripheral blood compartment (194). Most immune checkpoint molecules including PD-1 and CTLA-4 are expressed on T_{Regs} but the effects of checkpoint inhibitors on this T cell subset and treatment response remain unclear. Intriguingly, studies have suggested that anti-PD-1 immunotherapy might enhance the immunosuppressive function of T_{Regs} (130, 195), whereas anti-CTLA-4 inhibitors might deplete these cells (196). It is possible that T_{Regs} cells may coincide with lymphoma-specific T cells, indicating a potentially immune-responsive tumor. Correlative studies to determine the impact of T_{Regs} on clinical outcomes for lymphoma patients receiving immunotherapies should be informative, as well as the development of targeted treatment approaches to deplete these cells in order to activate anti-tumor immunity (130).

STROMA CELLS AS A KEY TUMOR CELL EXTRINSIC MECHANISM THAT REGULATE IMMUNE RESPONSES IN THE TME

Emerging evidence has now demonstrated that the creation of a "cold" TME requires the coordinated intervention of several other non-immune cell types that co-evolve with the tumor. Cancer-reprogrammed stromal cells [endothelial cells (ECs) and fibroblasts] acquire altered features that promote tumor progression and contribute to immunosuppression (197). This is achieved by a plethora of tightly orchestrated mechanisms influencing both spatial organization and function of immune subsets within the TME.

Evidence That Stromal Cells Regulate Anti-Tumor Immunity in Solid Tumors

Extensive studies of tumor-associated endothelial cells (TECs) in solid cancer are highlighting not only the significance of angiogenesis in disease progression but also the role of tumor-reprogrammed ECs in recruiting and polarizing immune cells in the TME (198). EC-mediated T cell trafficking, a crucial and highly dynamic process, is initiated by the release of chemokines, to attract circulating leukocytes, followed by selectin-dependent leukocyte rolling on the endothelium and integrin-induced trans-endothelial migration (199). Multiple steps in this tightly controlled process are disrupted in tumors.

The tumor vasculature has been found to be less functional with abnormal 'leaky' vessels (200), contributing to the hypoxic state that characterizes the majority of solid tumors. In this hypoxic environment, ECs release nitric oxide (NO) that suppresses the expression of leukocyte adhesion molecules (VCAM-1, ICAM-1) (201). Decreased ICAM-1 expression on TECs has been shown to impair T cell extravasation and has been associated with decreased numbers of tumor-infiltrating lymphocytes (TILs) (202).

Together with ECs, cancer-associated fibroblasts (CAFs), the resident fibroblasts activated in a chronic inflamed TME, have been shown to actively shape the immune infiltrate. The CAF secretome promotes recruitment and polarization of regulatory cells from both the innate and adaptive immune arms (203). Recently, a subpopulation of immunoregulatory CAFs (CAF-S1) has been functionally involved in the attraction, retention and activation of T_{Regs} in the breast cancer TME through the secretion of CXCL12 (204). Moreover, other CAF-derived

factors like Chi3L1, MCP-1 and SDF-1 drive the recruitment of monocytes and their polarization into pro-tumoral, immunosuppressive M2-macrophages in breast and prostate cancers (205–207), while CAF-derived IL-6 has been shown to promote immunosuppressive neutrophils in hepatocellular carcinoma (208). In addition, CAF-driven abnormal extracellular matrix (ECM) deposition and remodeling have been shown to induce the physical trapping of anti-tumor T cells to prevent effective tumor access (209).

Besides their role in mapping the spatial organization of immune cells in the TME, stromal cells can also directly influence or suppress endogenous anti-tumor immune responses through additional mechanisms, including antigen presentation. ECs upregulate both MHC-I and MHC-II in response to inflammatory cytokines (such as INFγ) acting as semi-professional non-hematopoietic APCs (210). As a consequence, ECs can mediate Ag-specific stimulation of effector memory $CD4^+$ and $CD8^+$ T cells (211–215). Interestingly, Motz and colleagues found that FasL expression on the vasculature of human and mouse solid tumors induced specific killing of $CD8^+$ TILs, but not T_{regs}, thus skewing the lymphocyte infiltrate towards a more regulatory phenotype (216). Moreover, ECs can upregulate PD-L1 and PD-L2 molecules inhibiting T cell activation and cytotoxic capacity (217–220).

Along the same lines, CAFs also directly impact the cytolytic activity of anti-tumor effector T cells through a number of different mechanisms. Prostaglandin E2 (PGE2) and NO produced by tumor-associated fibroblasts dampen $CD8^+$ T cells proliferation in pancreatic and breast cancer respectively (221, 222) and CAFs expressing tumor antigens promote $CD8^+$ apoptosis *via* PD-L2 and FasL expression (223). Overall, these intricate studies have shown that stromal cells shape the TME by affecting the recruitment and function of different adaptive and innate immune cells in solid tumors, but accumulating evidence is now revealing that similar mechanisms are operative in B cell malignancies.

The Importance of Stromal Cells in B Cell Malignancies

In lymphoid organs [lymph nodes (LNs), spleen and bone marrow (BM)] resident stromal cells engage in bidirectional interactions with lymphocytes that directly contribute to the shaping and function of the immune system (224, 225). Lymphocytes enter the LN *via* both afferent lymphatics formed by lymphatic endothelial cells (LECs) and peripheral circulation through high endothelial venues (HEVs). Lymphocyte homing to secondary lymphoid organs is coordinated by the expression of integrin and adhesion molecules, as well as chemokine–chemokine receptors on ECs lining HEVs (199). Emerging evidence also shows that LN ECs also acquire distinct immunomodulatory roles such as priming T cells (226), while BM ECs critically influence bone marrow remodeling and hematopoiesis, also contributing to the differentiation of immature B cells (225). Besides ECs, lymphocytes also closely interact with stromal cells of mesenchymal origin: mesenchymal stromal cells (MSCs) in the BM and both fibroblastic reticular cells (FRCs) and follicular dendritic cells (FDCs) in the LNs. MSCs primarily function to create a reticular network in the BM and to produce factors essential to lymphoid lineage development and differentiation. MSCs are also a key component of the regenerative system and they possess the ability to migrate to a damaged tissue, promote its repair and suppress the associated inflammation through a number of immunomodulatory factors (227). In the LN, FRCs and FDCs maintain immune system homeostasis by guiding the correct compartmentalization of immune cells through the display and secretion of chemokines and cytokines. They also actively participate in the control of immune responses by regulating the recruitment and the activation status of myeloid cells and lymphocytes using similar mechanisms to the ones described for MSCs (228). Thus, it is reasonable to hypothesize that stromal cells could similarly participate in shaping the immune TME, with potential contribution to the immunosuppressive state in B cell malignancies.

Several studies have shown that stromal cell architecture is altered in both the BM and LN tissue compartments of patients with different hematological malignancies (229, 230). Increased proliferation of VEGFR-1^+ neovasculature has been observed in aggressive lymphomas (DLBCL and Burkitt's lymphoma), and an elevated numbers of αSMA^+ mesenchymal cells described in indolent NHLs including CLL and small lymphocytic lymphoma (SLL) (231). Despite this general distinction, the stromal landscape of hematological malignancies is far more complex, with evidence that both ECs and fibroblasts cooperate in shaping disease biology and promoting tumor progression. Angiogenesis contributes to CLL development and correlates with disease progression (232–234). In line with this observation, overexpression of vascular endothelial growth factor (VEGF) in tissue biopsies has been shown to be a promising prognostic factor for NHL (235). In aggressive DLBCL, Lenz and colleagues described two different stromal gene signatures (stromal 1 and stromal 2) associated with patient survival following CHOP and R-CHOP treatment. The 'stromal-2' signature comprised of angiogenesis-associated genes (including CD31, VEGF), increased tumor blood-vessel density and correlated with poor prognosis following R-CHOP. On the other hand, the prognostically favorable stromal-1 signature showed enrichment for fibroblasts and ECM-associated genes (236). The importance of fibroblasts in aggressive lymphoma was functionally demonstrated in the Eμ-Myc mouse model, where expanded FRCs created a pro-tumor niche directing the homing and survival of lymphoma cells in the spleen (237). It has also been demonstrated in CLL using the Em-TCL1 murine model, that splenic stromal cells co-evolve with disease, in particular an expansion and reprogramming of splenic fibroblasts that produce the pro-B cell cytokine CXCL13 (238). To complicate the picture further, endothelial and mesenchymal cells are intimately linked to each other as exemplified by PDGF-activated MSCs in CLL that were shown to up-regulate VEGF production and promote the 'angiogenic switch' associated with disease progression (239). The importance of crosstalk in CLL

between tumor cells and stroma cells has been recently reviewed by Dubois et al. (240).

Malignant B Cells Hijack Stromal Cells in the Lymphoma TME

Altered vascular patterns and angiogenesis characterize aggressive and indolent lymphomas (241, 242). Tumor B cells secrete pro-angiogenic factors such as VEGF (243, 244), that promote the activation of endothelial cells and neoangiogenesis. In turn, as highlighted by numerous studies in CLL (245–248) and other hematological malignancies (249, 250), activated ECs critically contribute to malignant B cell survival through factors such as B-cell activating factor (BAFF) (245, 251, 252) (**Figure 2A**).

Moreover, CLL and HL cells secrete EVs that promote the acquisition of a CAF-like phenotype in previously healthy ECs and fibroblasts from different origins (267, 268). Importantly, tumor-derived EVs can also modulate other cellular components of the TME (269), and their role in B-cell malignancies has been recently reviewed (135).

In multiple myeloma (MM), the combination of both soluble (SDF-1α) and membrane-bound factors (integrins) induce stroma activation (ECs and BM-MSCs) (263), while in FL and CLL, tumor-derived TNFα and lymphotoxin (LT) are involved in the remodeling of BM-MSCs and LN-FDCs respectively (253, 254). These tumor-specific factors ultimately converge in the activation of NF-kB-dependent transcriptional programs (253, 263) that promote the secretion of pro-inflammatory soluble mediators involved in the enhancement of cancer survival (255) and in the modulation of TME immune infiltration (**Figure 2A**).

Evidence for the Immunomodulatory Roles of Reprogrammed Stromal Cells in B Cell Malignancies

As described above, tumor-activated stroma has a direct impact on the retention of tumor B cells and could therefore impact the recruitment or exclusion of other lymphocytes *via* similar mechanisms. The upregulation of adhesion molecules on EC surfaces (250) may negatively impact T cell trans-endothelial migration, which is a critical step of T cell homing into the LNs (270). In CLL, $CD4^+$ and $CD8^+$ T cells exhibit impaired integrin lymphocyte function-associated antigen-1 (LFA-1)–driven migration (271). Altered expression of adhesion molecules on TECs, together with T cell motility defects, could therefore block effective T cell trafficking into LN tissues.

Although studies showing a direct impact of activated endothelium on T cell recruitment in hematological malignancies are lacking, recently de Weerdt et al. revealed that CLL LNs contained twice the amount of T_{Regs} and a lower frequency of cytotoxic lymphocytes compared to the peripheral blood compartment (194). Under inflammatory conditions, activated ECs induce the expansion of T_{Regs} (215, 216) and the proliferation of memory $CD4^+$ T cells (272), and it could be speculated that similar mechanisms occur in the CLL TME.

Despite the lack of evidence for ECs, several studies have demonstrated the contribution of mesenchymal cells in the regulation of immune infiltration in B cell malignancies. In a mouse model of aggressive lymphoma, where tumor cells rely less on the TME for survival, the co-injection of MSCs with tumor cells induced a marked increase of immune cells including $CD4^+$ T cells, $CD11b^+$ cells, $CD4^+Foxp3^+$ T_{Regs}, and $CD11b^+Ly6C^+Ly6G^-$ MDSCs (256), demonstrating that the CAF secretome can modulate the recruitment and/or polarization of different immune subpopulations. In another model of aggressive lymphoma (Eμ-Myc), T cell zone resident stromal cells were shown to selectively retain $CD4^+$ T cells in the TME to support tumor progression *via* CD40L signaling (237).

In FL, where tumor cells strongly depend on TME interactions, stromal-derived IL-6 supports the survival of T-follicular helper (T_{FH}) and T-follicular regulatory (T_{FR}) cells (257). T_{FH} cells, that sustain lymphoma through a number of mechanisms (258), were also shown to establish a feedback loop with stromal cells. IL-4, the predominant cytokine produced by T_{FH}, was shown to trigger production of CXCL12 by FRC-like stromal cells, thus supporting FL tumor cell activation and survival (259). Additionally, FL-associated stroma (BM-MSCs) derived CCL2 and IL-8 were shown to promote the recruitment of monocytes/TAMs and neutrophils respectively, using *in vitro* assays (253, 260). Once in contact with FL-MSCs, monocytes/TAMs have been demonstrated to acquire an immunosuppressive phenotype with a reduced capacity to respond to pro-inflammatory stimuli such as LPS, while neutrophils supported the inflammatory stromal phenotype. Also in MM, malignant B cells have been shown to induce MSCs to secrete pro-tumor cytokines, as well thymic stromal lymphopoietin (TSLP) that activated T_H2-type inflammation in the BM TME resulting in tumor progression (261) (**Figure 2B**).

Another important mechanism of how stromal cells contribute to an immunosuppressive TME is through the expression of immune checkpoint molecules. Lymphoma-derived ECs preferentially express TIM-3. Expression levels of TIM-3 in the B-cell lymphoma endothelium has been correlated with poor prognosis and $TIM\text{-}3^+$ ECs suppressed the activation of $CD4^+$ T cells inhibiting T_H1 polarization *in vitro* and *in vivo* (273). In DLBCL, besides its expression on tumor and immune cells described earlier in this review, PD-L1 has also been associated to the non-malignant cellular compartment ("microenvironmental PD-L1"or mPD-L1) (262). Our own work has reported that PD-L1 upregulation on lymphoma-educated stromal cells can dampen TIL cytolytic killing activity against DLBCL tumor cells, highlighting that stromal cells expressing inhibitory ligands can directly modulate anti-tumor immune responses (274).

In addition to expression of immunomodulatory immune checkpoints, both LN and BM-derived stromal cells can adopt a number of other immunosuppressive mechanisms to modulate immune responses (**Figure 2B**). In MM, MSCs exhibit a distinctive gene expression profile compared to healthy MSCs, characterized by the expression of a number of immunoregulatory factors and cytokines including NOS2, IL-10, IL-6 and TGF-β, involved in the generation and activation of MDSCs (263, 264). In FL, MSCs also produce prostaglandin E2 (PGE2) that not only

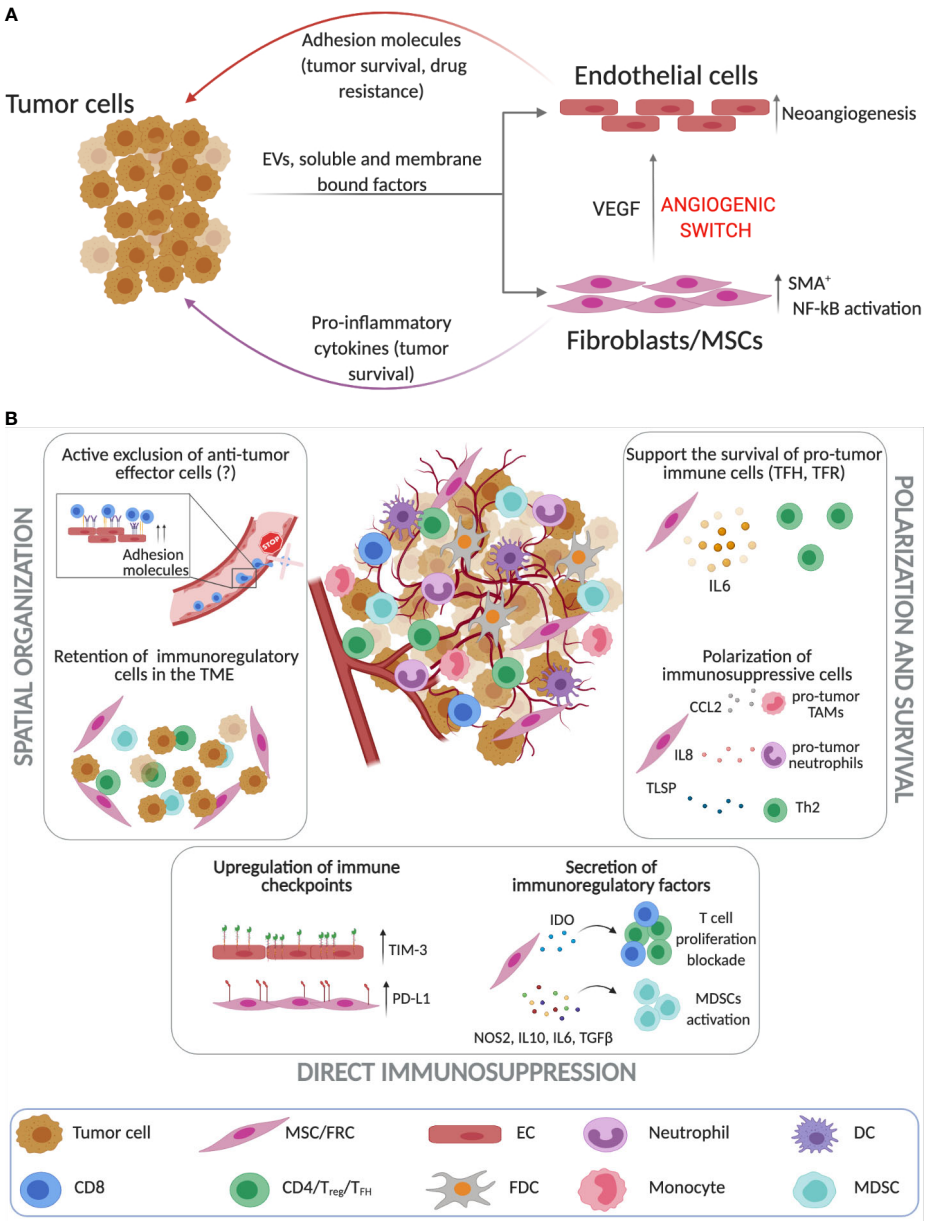

FIGURE 2 | Stroma cells as key players in regulating immune responses in the TME of B cell malignancies. **(A)** Tumor cells and tissue-resident stroma (endothelial cells (ECs) and FRCs [fibroblastic reticular cells)/MSCs (mesenchymal stromal cells)] engage into complex bidirectional interactions that promote cancer progression while simultaneously altering the stroma cell phenotype which can then further contribute to resistance to therapy. Tumor B cells induce neoangiogenesis and the upregulation of adhesion molecules on ECs (243–245, 248, 252). Similarly, lymphoma cells through cell-to-cell contact interactions, secretion of soluble factors and extracellular vesicles (EVs) promote the activation of FRCs and MSCs that contribute to increased tumor survival and neoangiogenesis (135, 253–255). **(B)** Unlike solid tumors the investigation of the immunosuppressive roles of stroma cells in the lymphoma TME is still in its infancy. Stromal cells play a crucial role in spatial organization of the TME as they can retain immunoregulatory cells and possibly actively exclude anti-tumor effector cell populations (237, 253, 256–261). Additionally, lymphoma ECs and FRCs upregulate immune checkpoints such as TIM-3 and PD-L1 (261, 262) and secrete immunoregulatory factors such as IDO and IL-10 that block T cell proliferation, while activating immunosuppressive cells (263–266). FRCs have been also found to drive the survival of pro-tumoral immune subsets such as T_{FH}, T_{H2} and TAMs (257–260). (*Created with Biorender.com).*

promotes neutrophil survival, but can have additional effect on other immune subpopulations (260). In physiological conditions, MSCs and LN-fibroblasts produce IDO to keep activated immune responses in check by inhibiting T cell proliferation, a mechanism also described in solid tumors (265). In FL, MSCs-derived IDO was also shown to repress not only T cell proliferation, but also the pro-lymphoma activity of stromal cells, highlighting the complex effects of cytokines in the TME (266). Clearly, our understanding of the immunomodulatory function of the different stromal cell populations that reside within lymphoma TMEs is still in its

infancy. However, it is becoming clear that different non-hemopoietic cells within lymphoid TMEs can alter the recruitment, polarization and function of immune cells, suggesting that the tumor stroma may also influence immune responses elicited by current immunotherapy.

IMMUNE AND STROMA TARGETED IMMUNOTHERAPY TO ACTIVATE ANTI-LYMPHOMA ACTIVITY

A number of studies have shown that the TME can interfere with clinical response to tumor-targeting therapy *via* different mechanisms. Reduced macrophage infiltration in the BM of ALL mice limits the response to anti-CD20 antibodies (alemtuzumab) (275), while adhesion to BM-derived stromal cells provides protection to CLL cells from rituximab-induced apoptosis (276). On the other side, tumor-targeting drugs can have indirect effects on the TME that may boost clinical responses. In CLL, there is evidence that ibrutinib treatment alleviates T cell exhaustion (277), while chemotherapy-induced cancer cell death promotes the exposure of tumor neoantigens and could re-invigorate anti-tumor immune responses (278). Understanding how the TME is shaped by currently available treatments can help to understand resistance mechanisms and to design combination therapies to boost clinical responses.

The immuno-oncology era has introduced a vast array of drugs designed to engage and promote innate and adaptive immune responses within the TME. Thus, lymphoma therapy is changing dramatically with the introduction of several new therapeutic approaches, including the use of checkpoint inhibitors (3). However, as described here, only a subset of patients achieve long-lasting responses to anti-PD-1 monotherapy even if they harbor inflamed TMEs. This clinical experience, together with the lack of clinical activity of anti-PD-1 in NHL (108) and CLL (5), has highlighted the need to incorporate checkpoint blockade therapies into more powerful combinations to unleash the power of anti-tumor immune cells, with potential therapeutic partners including CELMoDs and immunomodulatory drugs, CAR T cells and bispecific antibodies (**Figure 3**).

As discussed earlier, there is substantial evidence demonstrating that type I and II IFN signaling is required within TMEs to prevent development of an immunosuppressive state (37, 49). Recent work by our group has demonstrated that the CELMoD avadomide can induce type I and II IFN signaling in the T cell compartment that sensitizes CLL to anti-PD-1 or anti-PD-L1 checkpoint blockers (67). Avadomide was shown to trigger a feedforward cascade of reinvigorated T cell responses, as well as IFN-inducible feedback inhibition through upregulation of PD-L1. Patient-derived xenograft tumor models revealed that inducing IFN-driven T cell responses with avadomide could convert non-inflamed CLL tumors into $CD8^+$ T cell-inflamed TMEs that responded to anti-PD-L1/PD-1-based combination therapy. This pre-clinical study provides encouraging proof of concept that inducing inflammatory IFN type I and II signaling in patient T cells can successfully re-shape anti-tumor T cell responses and sensitize CLL to immunotherapy (67).

The re-activation of autologous anti-tumor immune responses has been also demonstrated in a number of different hematological malignancies by using "off the shelf" bispecific T cell engager (BiTE) antibodies. This dual binding of both neoplastic cells and tumor-infiltrating lymphocytes is providing an attractive therapeutic approach for B cell malignancies (279). Blinatumumab, a BiTE with dual specificity for CD3 and CD19, has shown activity in ALL and in different NHL, and has been approved by FDA in 2014 (280). However, due to its short half-life, Blinatumumab requires continuous infusion for weeks, causing patients discomfort. Moreover, the occurrence of different resistance mechanisms described for ALL patients (effector T cell exhaustion/dysfunction and expansion of T_{Regs}), prompted the optimization and testing of alternative BiTE drugs in recent years [reviewed in (288)]. Among them, the T cell bispecific (TCB) antibody drug CD20-TCB (RG6026), with its 2:1 CD20-CD3 binding format, has shown superior potency in T cell activation and increased half-life in pre-clinical settings (281). CD20-TCB can be efficiently combined with the bispecific antibody fusion protein CD19-4-1BBL, that provides co-stimulatory signals to tumor-engaged immune cells (T cells or NK cells) and this combination immunotherapy has been shown to promote intratumoral TILs accumulation and increased anti-tumor efficacy in preclinical models of NHL (282). These pre-clinical results suggest that combination immunotherapy may achieve better clinical responses and overcome immune suppression in the TME. Currently, BiTEs and CD20 TCBs, including RG6026, are being evaluated in B cell malignancy patients with activity detected.

CD19-directed CAR-T cells have shown remarkable efficiency in the treatment of ALL and aggressive NHL (283, 289, 290) and, along the same line, new CD19-CAR-NK cells are showing effective anti-tumor activity with minor side effects (291). Indeed, CAR-T cell therapy has been the first FDA-approved cellular therapy in lymphoma [covered in a recent review (4)]. Despite this clinical success, emerging data is showing that durable responses induced by CAR Ts are seen in only a subset of patients and their anti-tumor activity depends on the persistence of CARs (181). While peripheral blood analysis of treated patients has shown that CAR-T concentrations peak at 14 days post-infusion (292), recent data has demonstrated that only a minimal amount of CAR-T cells infiltrate TME tissue at 5 days post-infusion, and that virtually no CAR-Ts were present in the TME 10 days following infusion (104). These results support the concept that the stroma-rich TME may contain ill-defined immunosuppressive mechanisms that interfere with the effective trafficking and optimal activation of anti-tumor immune cells using current immunotherapies.

Combining immunotherapy with drugs that target stromal cells is an attractive, next-generation treatment strategy. Different approaches can be adopted to target stromal cells in cancer including the direct targeting of fibroblasts/ECs or their associated proteins, activated signaling pathways or secreted factors. The majority of studies to date targeting tumor stroma

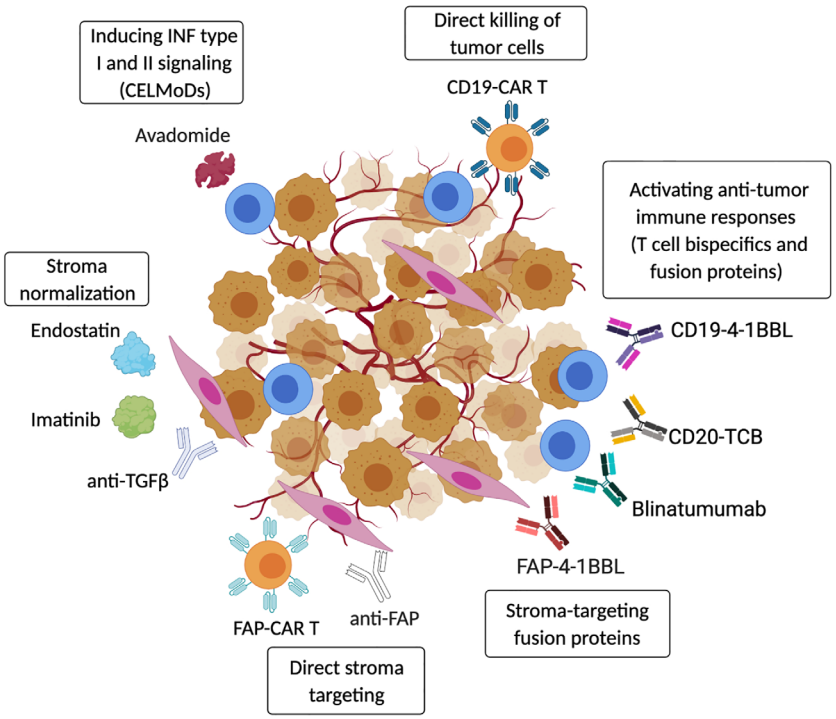

FIGURE 3 | Targeting strategies to activate anti-tumor immunity in the TME. Therapeutic strategies include re-activation of autologous anti-tumor immune responses using CELMoDs (*e.g.* avadomide) (67) or T-cell bispecific antibodies and fusion proteins (CD20-TCB, CD19-4-1BBL, Blinatumumab) (279–282). Anti-tumor immunity can also be induced by transferring CAR-T/NK effector cells that directly target cancer cells (CD19-CAR-T) (283). In addition, cancer-associated stroma can be efficiently targeted to boost anti-tumor immunity, and fibroblasts-specific pharmacological approaches (anti-FAP antibodies or CAR-T) have been shown to induce tumor regression (284). Tumor stroma can also be 'normalized' by blocking or neutralizing cancer-secreted factors that promote stromal cell activation (Endostatin, Imatinib, anti-TGFβ) (285–287). Moreover, the presence of cancer-stroma-specific proteins can be used to activate tissue resident anti-tumor T cells using stroma-specific/T cells co-stimulatory fusion proteins (FAP-4-1BBL) (282). (*Created with Biorender.com*).

have used solid cancer models, establishing a strong rationale for developing therapies for hematological malignancies. A strong case in point is fibroblast activating protein (FAP), a cell-surface serine protease which is expressed at high levels on tumor stroma and has been considered a suitable therapeutic candidate on CAFs for some time. Several FAP-specific pharmacological approaches (vaccines, CAR-T cells) designed to selectively target CAFs, were shown to induce tumor regression in different murine models (284). However, depletion of FAP^+ fibroblasts has been shown to cause profound systemic immune-mediated toxicity, indicating their importance not only in cancer but also in physiological tissue functions (293). This example suggests that caution should be taken in considering direct elimination of stromal cells forming the TME given their critical functions in tissue architecture and immune homeostasis. Therefore, therapies designed to 'normalize' or 're-educate' aberrant stromal cells by targeting or overcoming their pro-tumor, immune evasive pathways could be more clinically relevant (**Figure 3**).

To this direction, stromal cell normalization can be achieved by directly blocking or neutralizing cancer-secreted factors that promote stromal cell activation. To date, in lymphoma this approach has been exploited through the use of endostatin, an endogenous inhibitor of angiogenesis which inhibits matrix metalloproteinases (MMP) activity and blocks VEGF binding to VEGFR-2. Administration of endostatin in lymphoma-bearing mice was shown to delay tumor growth (285). Imatinib, a PDGFRβ inhibitor, has also been shown to disrupt lymphoma angiogenesis by targeting vascular pericytes (286). Moreover, as PDGFR ligation also has a role in promoting fibroblast activation, the use of specific inhibitors can also affect fibroblast differentiation and function in the TME. As described above, TGFβ, TNFα and LT are all master regulators of immunosuppressive fibroblast function in hematological malignancies, and the use of neutralizing antibodies against these molecules could provide an interesting approach for stroma normalization, as already demonstrated in solid tumors (294). Combined blockade of TGFβ and anti-PD-1 in MM has been shown to promote anti-tumor T cell activation and proliferation, indicating that targeting the different immunosuppressive pathways occurring in the TME can reactivate endogenous anti-tumor immune responses and favor tumor clearance (287).

In conclusion, understanding the functional role of stroma and its specific features will help to design new combination immunotherapies to improve clinical responses. An example of effective immune-stroma dual targeting therapy comes from recent work from Claus and colleagues in solid tumors (282),

where the combination of tumor antigen-TCB (CEA-TCB) with a stroma-specific/T cell co-stimulatory fusion protein (FAP-4-1BBL) promoted tumor remission and accumulation of activated $CD8^+$ in the TME (**Figure 3**).

CONCLUSIONS

Immunotherapy has revolutionized cancer treatment, achieving significant responses in patients with B cell malignancies, although sensitivity and resistance remain major challenges. Despite the extreme inflamed *vs* non-inflamed classification of immune TMEs, most B cell malignancies and their subtypes show a high degree of heterogeneity that likely influences responses to immunotherapy. For this reason, it is crucial for further research to fully unravel TME complexity including the major tumor intrinsic and extrinsic drivers of immune composition and spatial organization of immune and stromal cell subsets. Defining how malignant B cells alter both immune and stromal cells, as well as how these reprogrammed cells contribute to the creation of a pro-tumor and immunosuppressive TME will be essential to design next-generation immunotherapies and combination treatment strategies to overcome TME-driven immune suppression and optimize therapy for patients.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Conflict of Interest: AGR has received research funding to Institution from Bristol-Myers Squibb Roche Glycart AG and AstraZeneca.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GLOSSARY

CLL	**chronic lymphocytic leukemia**
SLL	**small lymphocytic lymphoma**
DLBCL	**diffuse large B-cell lymphoma**
cHL	**classical non-Hodgkin lymphoma**
NHL	**non-Hodgkin lymphoma**
MM	**multiple myeloma**
PMBL	**primary mediastinal B-cell lymphoma**
FL	**follicular lymphoma**
MCL	mantle-cell lymphoma
ALL	acute lymphoblastic leukemia
CRC	colorectal carcinomas
EBV	Epstein–Barr virus
GCB	non-germinal center B-cell subtype
R/R	relapsed/refractory
ORR	overall response rate
TME	tumor microenvironment
LN(s)	lymph nodes
BM	bone marrow
TILs	tumor-infiltrating lymphocytes
TAMs	tumor-associated macrophages
APCs	antigen-presenting cells
DCs	dendritic cells
NK(s)	natural killer cells
FDCs	follicular dendritic cells
MDSCs	myeloid-derived suppressor cells
ECs	endothelial cells
TECs	tumor-associated endothelial cells
LECs	lymphatic endothelial cells
BECs	blood endothelial cells
HEVs	high endothelial venules
FRCs	fibroblastic reticular cells
CAFs	cancer-associated fibroblasts
MSCs	mesenchymal stromal cells
PD-1	programmed cell death protein 1
IFN	interferon
HLA	human leukocyte antigen
IL-	Interleukin-
MHC	major histocompatibility complex
TRAIL	TNF-related apoptosis-inducing ligand
CELMoD	cereblon E3 ligase modulator
IRF4	IFN regulatory factor 4
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CAR T cells	chimeric antigen receptor T cells
JAK2	Janus kinase 2
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
STAT3	signal transducer and activator of transcription 3
MAPK	mitogen-activated protein kinase
PTEN	phosphatase and tensin homolog
EZH2	enhancer of zeste homolog 2
TP53	tumor protein P53
BCL2	B-cell lymphoma 2
TGF-β	transforming growth factor beta
EVs	extracellular vesicles
ARG1	arginase 1
IDO	indoleamine-pyrrole 2,3-dioxygenase
LAG-3	lymphocyte activation gene-3
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
FOXP3	forkhead box p3
NO	nitric oxide
ICAM-1	intercellular adhesion molecule 1
VCAM-1	vascular cell adhesion protein 1
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
CCR	(C-C motif) chemokine receptor
SDF-1	stromal cell-derived factor 1
MCP-1	monocyte chemoattractant protein-1
Chi3L1	chitinase 3 like 1
ECM	extracellular matrix
PGE2	prostaglandin E2
VEGF	vascular endothelial growth factor
αSMA	alpha smooth muscle actin
R-)	(rituximab-) cyclophosphamide doxorubicin hydrochloride
CHOP	(hydroxydaunorubicin) vincristine sulfate (Oncovin) and prednisone
PDGF	platelet-derived growth factor
BAFF	B-cell activating factor
LT	lymphotoxin
TSLP	thymic stromal lymphopoietin
NOS2	nitric oxide synthase 2
FAP	fibroblast activating protein

frontiers in Immunology

ORIGINAL RESEARCH
published: 24 May 2021
doi: 10.3389/fimmu.2021.619069

Intrinsic Resistance of Chronic Lymphocytic Leukemia Cells to NK Cell-Mediated Lysis Can Be Overcome *In Vitro* by Pharmacological Inhibition of Cdc42-Induced Actin Cytoskeleton Remodeling

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Natural killer (NK) cells are innate effector lymphocytes with strong antitumor effects against hematologic malignancies such as chronic lymphocytic leukemia (CLL). However, NK cells fail to control CLL progression on the long term. For effective lysis of their targets, NK cells use a specific cell-cell interface, known as the immunological synapse (IS), whose assembly and effector function critically rely on dynamic cytoskeletal changes in NK cells. Here we explored the role of CLL cell actin cytoskeleton during NK cell attack. We found that CLL cells can undergo fast actin cytoskeleton remodeling which is characterized by a NK cell contact-induced accumulation of actin filaments at the IS. Such polarization of the actin cytoskeleton was strongly associated with resistance against NK cell-mediated cytotoxicity and reduced amounts of the cell-death inducing molecule granzyme B in target CLL cells. Selective pharmacological targeting of the key actin regulator Cdc42 abrogated the capacity of CLL cells to reorganize their actin cytoskeleton during NK cell attack, increased levels of transferred granzyme B and restored CLL cell susceptibility to NK cell cytotoxicity. This resistance mechanism was confirmed in primary CLL cells from patients. In addition, pharmacological inhibition of actin dynamics in combination with blocking antibodies increased conjugation frequency and improved CLL cell elimination by

OPEN ACCESS

Edited by:
Benjamin Frey,
University Hospital Erlangen, Germany

Reviewed by:
Salem Chouaib,
Institut Gustave Roussy, France
Alan G. Ramsay,
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *19 October 2020*
Accepted: *23 April 2021*
Published: *24 May 2021*

Citation:
Wurzer H, Filali L, Hoffmann C, Krecke M, Biolato AM, Mastio J, De Wilde S, François JH, Largeot A, Berchem G, Paggetti J, Moussay E and Thomas C (2021) Intrinsic Resistance of Chronic Lymphocytic Leukemia Cells to NK Cell-Mediated Lysis Can Be Overcome In Vitro by Pharmacological Inhibition of Cdc42-Induced Actin Cytoskeleton Remodeling. Front. Immunol. 12:619069. doi: 10.3389/fimmu.2021.619069

NK cells. Together our results highlight the critical role of CLL cell actin cytoskeleton in driving resistance against NK cell cytotoxicity and provide new potential therapeutic point of intervention to target CLL immune escape.

Keywords: actin cytoskeleton, Cdc42, immune evasion, immunological synapse, tumor immunology, natural killer (NK), B cell neoplasms

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most prevalent lymphoproliferative disorder in the United States and Europe and is characterized by the clonal expansion of mature $CD5^+$ $CD23^+$ B cells (1, 2). As first reported in 1999, the mutational status of the immunoglobulin heavy chain variable region genes (*IGHV*) is associated with overall survival and is by now considered one of the most important molecular prognostic factors (3, 4). A higher degree of somatic mutations is considered a good prognostic marker, while patients with a non-mutated IgH_V region show shorter progression-free and overall survivals (5). An additional marker of prognostic and predictive value is the tumor protein 53 gene (*TP53*) status that can be affected by 17p13 deletion ((del(17p)) and/or somatic *TP53* mutations (6, 7). The double knockout of the *TP53* gene renders CLL cells resistant to most chemo-immunotherapies and has also been shown to be involved in resistance against monoclonal antibodies, such as rituximab (8).

A cardinal feature of CLL is an acquired immune system dysregulation and immune response dysfunction of both innate and adaptive immunity that gradually worsens over time even without disease progression (9–19). The immune response of cytotoxic lymphocytes, such as cytotoxic $CD8^+$ T and (natural killer) NK cells, is regulated by molecular interactions occurring in the context of immunological synapses (IS). The formation of a lytic IS between cytotoxic lymphocytes and their target cells is a tightly coordinated process that ensures that only infected or transformed cells are lysed (20–22). Because the IS is the point of convergence for cytolytic effector functions, it is susceptible to immune evasion strategies of cancer (23, 24). Interestingly, it has been shown that immune escape of CLL can be achieved by various means, notably by those interfering with the formation or the function of the lytic IS (25–29). Lytic IS formation of $CD8^+$ T cells and NK cells with CLL cells can be rescued in part by drug treatments, such as lenalidomide or blocking antibodies, or can even be bypassed by infusion of genetically modified cytotoxic lymphocytes which do not rely on MHC-mediated antigen presentation as they form a non-classical IS (30–36). Nevertheless, toxic side effects or acquired resistance against these new therapeutic options have been reported and result in disease progression (37, 38).

NK cells are commonly described to play an important role in the immunosurveillance of hematologic malignancies (23). NK cell effector function is regulated by the balance between inhibitory ligands, mainly canonical and non-canonical MHC-I, and activating ligands presented by the target cell at the IS. Down-modulation of MHC-I is a common feature on cancerous cells and, if accompanied by upregulation of stress-induced activating ligands, leads to activation of NK cells and subsequent target cell lysis (24). Sufficient activating signal results in the release of cytokines, such as IFN-γ and TNF-α, and the formation of a lytic IS that includes directed degranulation of cytotoxic molecules, such as perforin and granzyme B, towards the conjugated target cell (23). CLL immune evasion from NK cells has been described to occur mainly through the upregulation of non-canonical MHC-I isoforms HLA-G and HLA-E (39–41). The NK cell repertoire of an individual can be defined by the simultaneous expression of different receptors and is quite diverse with up to 30'000 different phenotypic populations. Interestingly, the expression of the HLA-G receptor KIR2DL4 is universally found on all NK cells (40, 42, 43). This indicates that overexpression of HLA-G on CLL cells can provide immune evasion from any NK cell subpopulation. Accordingly, monoclonal antibody blockade therapies targeting HLA-E or HLA-G overexpression successfully increased the natural cytotoxicity of NK cells from CLL patients *in vitro* (40, 44). However, commercial HLA-E monoclonal antibodies are not specific and show cross-reactivity with HLA-A/B/C (45) and HLA-G is characterized by the presence of several isoforms and a high intra- and interpatient heterogeneity, making it a difficult target (23). Alternative inhibition of the inhibitory HLA-E receptor NKG2A showed promising results *in vitro* (46) and is currently tested in several clinical trials, however a phase I/II study of Monalizumab in combination with Ibrutinib including CLL patients was terminated in 2018 (NCT02557516).

Even though NK cell expansion in CLL patients has been reported, these NK cells are described to be hyporesponsive due to a downregulation of activating receptors. They also show a reduced degranulation efficiency against malignant B lymphocytes, through both natural or antibody dependent cell cytotoxicity (ADCC) triggered by rituximab (23). The exhausted NK cell phenotype is enhanced in patients with a progressive disease and results in a loss of NK cell cytotoxicity against CLL target cells. However, CLL patients' exhausted NK cells can be replaced by activated NK cells coming from a healthy donor. Such allogenic adoptive cell therapy studies showed that unmutated CLL cells are susceptible targets for activated NK cells (46, 47). As demonstrated for other hematologic malignancies, cytotoxic lymphocytes expressing chimeric antigen receptors (CARs) can efficiently lyse tumor cells, and in an attempt to circumvent toxic side effects of CAR-T cell therapy, anti-CD19 CAR-NK cells have been tried for B cell malignancies (48). While this new therapeutic approach holds promising results in first clinical trials (48), little is known about the CAR IS (49). Although some preliminaries studies suggest

that CAR IS are superior to conventional NK/T cells IS (50, 51), it remains unclear whether these IS can also be affected by resistant subpopulations of tumors that can modulate IS formation or functions.

These new treatment options set the focus on the lytic IS formed between CLL cells and NK cells and the underlying resistance mechanisms that could result in disease progression. Actin cytoskeleton remodeling has recently emerged as an important process underlying evasion of solid tumor cells, such as breast cancer cells, from NK cell cytotoxicity (23, 24, 52–55). However, the role of the actin cytoskeleton in CLL cells during NK cell attack has not been evaluated so far. Here, we show that a subset of CLL cells from four cell lines, but also patient-derived cells respond to NK cell attack by fast polarization of actin filaments at the IS. Live cell imaging and imaging flow cytometry analyses suggest that synaptic actin accumulation protects CLL cells against NK cell-mediated killing by reducing intracellular levels of granzyme B. Remarkably, pharmacological inhibition of an actin regulatory pathway in CLL cells was sufficient to prevent actin cytoskeleton remodeling, promote granzyme B accumulation, and restore high susceptibility to NK cell-mediated cytotoxicity. Similar results were obtained with patient-derived CLL cells that showed reduced resistance to NK cell-mediated cell death after inhibition of actin dynamics. In this context, blocking antibodies targeting HLA-G also demonstrated that release of the inhibitory interaction of HLA-G with its receptor in NK cells improves conjugate formation. Our data support that interfering with actin cytoskeleton remodeling in CLL cells in combination with antibody blockade provides an opportunity to restore a potent NK cell anti-tumor response in aggressive CLL.

METHODS

Cell Lines and Cell Culture Conditions

The CLL cell lines used in this study were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany). Cell lines were authenticated through STR profiling analysis (Microsynth, Switzerland) or purchased directly from DSMZ. HG-3, PGA-1, JVM-3 and MEC-1 cell lines were cultured in RPMI-1640 (ThermoFisher Scientific, cat. # 61870010) supplemented with 10% (v/v) fetal bovine serum (FBS, Life Technologies, cat. #10500-064), 100 U/mL penicillin and 0.1 mg/ml streptomycin (Westburg, cat. #LO DE17-602E). The NK-92MI cell line was kept in RPMI-1640 supplemented with 10% (v/v) FBS, 10% (v/v) horse serum (ATCC, cat. # 30-2040), 100U/ml penicillin and 0.1 mg/mL streptomycin. All cell lines were cultured under humidifying conditions at 37°C and 5% CO_2 and were checked routinely for mycoplasma contamination using the MycoAlert detection kit (Lonza, cat. # LT07-318).

Isolation of Human Primary NK Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy, anonymous donors provided by the Luxembourg Red Cross. Upon receipt, buffy coats were diluted ten times with Ca^{2+}/Mg^{2+} free phosphate buffered saline (PBS) and the low-density PBMC fraction was isolated by centrifugation over a Lymphoprep density gradient (Stemcell Technologies, cat. # 07861). After centrifugation, the PBMC layer was collected, washed several times with Ca^{2+}/Mg^{2+} free PBS and red blood cells were lysed with ACK buffer (ThermoFisher Scientific, cat. # A1049201). Following erythrocytes lysis, cells were washed once with Ca^{2+}/Mg^{2+} free PBS, counted with Trypan blue and cell concentration adjusted for NK cell isolation. NK cells were isolated with the MojoSort human NK cell isolation kit (BioLegend, cat. # 480054) combined with a LS column (Miltenyi Biotec, cat. # 130-042-401). Isolated NK cells were cultured overnight in RPMI 1640 supplemented with 10% FBS, 10 mM HEPES (ThermoFisher Scientific, cat. # 15630056), 100 U/mL penicillin, 0.1 mg/mL streptomycin, 100 U/mL recombinant human interleukin-2 (IL-2; Peprotech, cat. # 200-02) and 10 ng/mL recombinant human IL-15 (IL-2; Peprotech, cat. # 200-15).

Isolation of Human Primary CLL Cells

Peripheral blood samples were collected from anonymous CLL patients. All samples used in this study were obtained after informed consent in accordance with the Declaration of Helsinki and the Comité National d'Ethique de Recherche Luxembourg (CNER No. 201707/02 Version 1.2). CLL was diagnosed according to standard clinical criteria. PBMCs were isolated from fresh blood samples using standard density centrifugation over a Lymphoprep gradient. Isolated cells were washed twice in Ca^{2+}/Mg^{2+} free PBS and suspended in complete medium (RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin). PBMCs were either used immediately or were cryopreserved in FBS with 10% DMSO. After thawing, cells were allowed to recover overnight before being used for further experiments.

Cell Transduction and Cdc42 Inhibition

mEmerald-Lifeact-7 was a gift from Dr. M. Davidson (Addgene plasmid # 54148). For generation of stable cell lines, the mEmerald-Lifeact fragment was subcloned into the viral pCDH-EF1α-MCS-IRES-puro plasmid (System Biosciences, cat. # CD532A-2). Infectious particles were produced using HEK293 cells and used to infect HG-3, PGA-1, JVM-3, and MEC-1cell lines. Transduced cells were selected with puromycin (0.5 µg/ml, Sigma-Aldrich, cat. #P8833).

To inhibit Cdc42 activity in CLL cell lines, the cells were incubated for 1 h with 50 µM of the cell-permeable Cdc42 inhibitor ZCL278 (Sigma Aldrich Merck Calbiochem, cat. # 500503) (56). Cells were washed after treatment and allowed to recover for 1 h or 5 hrs in complete medium before stimulation with human recombinant EGF (0.1µg/mL; PeproTech, cat. # AF-100-15) for 15 min. Inhibition of Cdc42 activity upon stimulation was confirmed using a Cdc42 G-LISA Activation Assay following the manufacturer protocol (Cytoskeleton Inc., cat. # BK127-S).

Cytotoxicity Assay

For cytotoxicity assays, NK cells (effectors) were counted and stained with anti-human CD56-PE/Cy7 (BioLegend, cat. # 318318, clone HCD56). Effector cells were co-cultured with mEmerald-Lifeact$^+$ HG-3, PGA-1, JVM-3 or MEC-1 (target cells) at effector-target (E:T) ratios of 1:1 and 5:1 for 4 hrs at 37°C/5% CO_2. After incubation, the plate was placed on ice in the dark to stop the experiment until acquisition on the flow cytometer. Immediately before acquisition on a CytoFLEX (Beckman Coulter), TO-PRO-3 Iodide (ThermoFisher Scientific, cat. # T3605) was added to the samples (0.05 µM final concentration). Generated data were analyzed with FlowJo v10.6.2. software.

Flow Cytometry

To assess cell death in target cells, mEmerald-Lifeact$^+$ HG-3, PGA-1, JVM-3 or MEC-1 target cells were incubated for 45 min with CD56-PE/Cy7-labeled NK-92MI cells. Cells were washed with cold Annexin V binding buffer (Biolegend, cat. # 422201) twice. Afterwards, cells were resuspended in 100 µl Annexin V binding buffer with 5 µl Alexa Fluor® 647 Annexin V (Biolegend, cat. #640912) and 5 µl propidium iodide staining solution (Sigma-Aldrich, cat. #P4864) per million cells. Cells were incubated for 15 min at RT in the dark, before addition of 400 µl Annexin V binding buffer and analysis by flow cytometry on a CytoFLEX (Beckman Coulter). Generated data were analyzed with FlowJo v10.6.2. software.

Imaging Flow Cytometry

For conjugate formation, NK cells were counted and stained with anti-human CD56-PE/Cy7 (BioLegend, cat. # 318318, clone HCD56), before co-culture with mEmerald-Lifeact$^+$ target cells at an E:T ratio of 3:1 in the presence of Hoechst 33342 (0.5 µg/mL final; Miltenyi Biotec, cat. # 130-111-569). Conjugation was allowed for 40 min at 37°C before fixation with 2% paraformaldehyde (PFA; Agar scientific, cat. # R1026) for 15 min at 37°C, and permeabilization with 0.1% Triton X-100 (Sigma Aldrich, cat. # T9284) for 10 min at room temperature (RT). Prior to intracellular staining, samples were washed twice with PBS and then stained for anti-Granzyme B-APC (BioLegend, cat. # 372204, clone QA16A02).

To analyze apoptosis in target cells, cells were centrifuged after 30 min of co-incubation and stained with Zombie Red (BioLegend, cat. # 423110) in PBS for 10 min at RT. Cells were then washed with cold cell staining buffer (BioLegend, cat. # 420201), resuspended in 100 µl Annexin V binding buffer (BioLegend, cat. # 422201) with 5 µl Alexa Fluor® 647 Annexin V (BioLegend, cat. # 640943) and stained for 15 min at RT in the dark. Cells were then washed with Annexin V binding buffer and fixed in 2% v/v PFA diluted in Annexin V binding buffer. After fixation, cells were washed in Annexin V binding buffer and kept at 4°C in this buffer until acquisition. For acquisition, ImageStream®X Mark II (EMD Millipore) with four built-in lasers (405 nm, 488 nm, 561 nm, 642 nm) and the INSPIRE® software (EMD Millipore) were used. Analysis for AR, including the gating strategy, masks and features, were described previously (52) and are shown in **Supplementary Figures S1C, D**.

For analysis of patient-derived CLL cells, PBMCs were stained for 30 min with 0.2 µM of the cell permeable F-actin probe SiR-actin (Spirochrome AG, cat. #SC001). To inhibit Cdc42 activity, cells were then treated for 1 h with 50 µM of ZCL278 or vehicle control in complete medium. Before co-culture with NK-92MI cells, patient CLL cells were stained with anti-human CD19-FITC (BioLegend, cat. #302256, clone HIB19), anti-human CD5-BV605 (BioLegend, cat. #364019, clone L17F12), and 10 µg/mL anti-human HLA-G (BioLegend, cat. #335902, clone 87G) or control IgG (BioLegend, cat. #400201, clone MOPC-173) for 30 min at 4°C. Cells were allowed to conjugate with NK-92MI for 30 min in the presence of Hoechst 33342 (0.5 µg/mL final concentration), before staining with 0.1X Live-or-Dye NucFix™ Red for 15 min in PBS. Conjugates were washed in PBS containing Ca^{2+}/Mg^{2+} and fixed with 2% PFA for 15 min at 37°C. For analysis of AR and cell death, CLL cells were identified as CD19+/CD5+ cells in conjugation with CD56+ NK-92MI cells.

Confocal Microscopy

For labelling, mEmerald-Lifeact$^+$ target cells were settled on a Poly-L-Lysin (25 µg/mL, Sigma-Aldrich, cat. # P4707) coated µ-slide 8 well (Ibidi, cat. # 80826) for 10 min before fixation with 2% paraformaldehyde (PFA). Cells were permeabilized with 0.1% Triton X-100 and labelled with anti-α-tubulin antibody (Sigma-Aldrich, cat. # T5168, clone B-5-1-2), goat-anti-mouse Alexa Fluor 633 (Invitrogen, cat. # A-21126) and with acti-stain 555 phalloidin (100 nM, Cytoskeleton Inc., cat. # PHDH1) and DAPI (0.2 µg/mL, Sigma-Aldrich). For conjugate formation, NK cells were counted and stained with the CellTracker™ Orange CMRA dye (1 µM, Invitrogen, cat. # C34551), before co-culture with mEmerald-Lifeact target cells at an E:T ratio of 1:1 in the presence of Hoechst 33342 (0.5 µg/mL final). Conjugation was allowed for 40 min at 37°C, then cells were settled on a Poly-L-Lysin coated µ-slide 8 well for 10 min before fixation with 2% PFA. After 2 washings, PBS was replaced by mounting medium (Ibidi, cat. # 50001) before cell imaging. For acquisition, high-resolution pictures were acquired on a Zeiss LSM880 fastAiry confocal microscope, in the Airy mode. A multitrack configuration was used with laser 405 nm, 488 nm, 543 nm, and 633 nm for excitation. A stack of 50 slices with an interval of 0.2 µm was acquired. The fluorescence intensity was measured on the maximum intensity projection picture of the stack in a rectangle of 5 µm width set in the center of the IS, with the macro "GetProfileExample" in the Image J v1.53e software.

For live cell imaging, NK92MI cells were counted and stained with the CellTracker™ Orange CMRA dye (1 µM), before co-culture with mEmerald-Lifeact target cells at an E:T ratio of 1:1 in the presence of SYTOX™ Blue (2 µM, Invitrogen, cat. # S11348). For acquisition, cells were maintained under the microscope at 37°C and 5% CO_2. A single-track configuration was used with excitation at 405 nm, 488 nm, and 543 nm. The pinhole was open to acquire a 2 µm depth slice. A stack of 4 slices with an interval of 2 µm was acquired for 1 h at a rate of one picture every 4 min.

Statistical Analysis

The paired Student's t-test and 2-way ANOVA in Prism 9 (GraphPad) were used to determine the statistical significance of the results obtained. For apoptosis experiments, a Z-score test for two population proportions was used to determine the statistical significance between samples. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

RESULTS

CLL Cell Resistance to NK Cell-Mediated Cytotoxicity Correlates With Actin Cytoskeleton Polarization to the Immunological Synapse

To evaluate actin cytoskeleton organization and dynamics in aggressive forms of CLL, three IGHV mutated cell lines, namely PGA-1, JVM-3 and MEC-1, and one IGHV non-mutated cell line (HG-3) (**Supplemental Figure S1A**) were modified to stably express the mEmerald-tagged actin marker Lifeact (**Figure 1A**) (57). With this approach, labeling of the actin cytoskeleton became obsolete and a spill-over from the NK cell actin cytoskeleton could be avoided. Cytotoxicity assay found MEC-1 as a highly resistant CLL cell line compared to the other three CLL cell lines (**Figure 1B**). After 4 hrs of co-culture with an excess of NK-92MI cells at a 5:1 E:T ratio, MEC-1 cells were lysed with an average rate of 17%. In comparison, HG-3, PGA-1, and JVM-3 cells were significantly more susceptible with average NK cell-specific lysis rates of 54%, 47%, or 44%, respectively. Using confocal microscopy, we found that some NK cell-conjugated CLL cells showed a strong polarization of filamentous actin to the synaptic area (**Figure 1C** and **Supplementary Figure S1B**). We recently reported similar synaptic accumulation of filamentous actin during NK cell attack in breast cancer cells and termed this

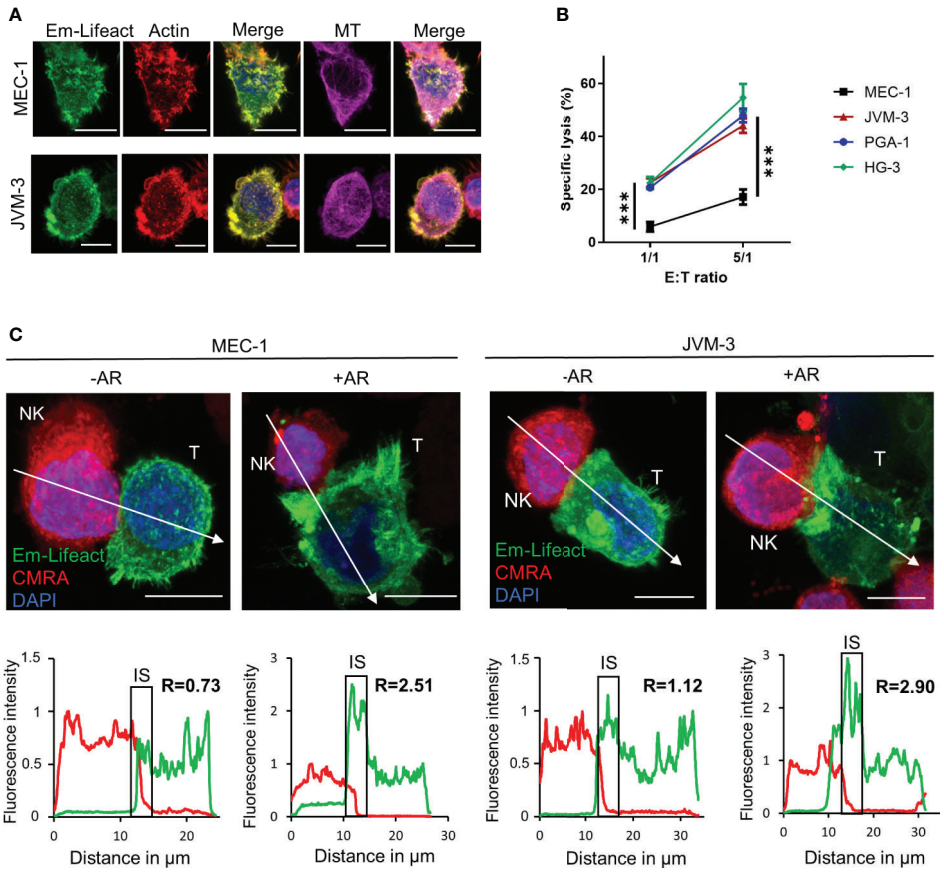

FIGURE 1 | CLL cells have the ability to respond to NK cell attack with an actin response associated to their resistance. **(A)** JVM-3 and MEC-1 cells were transduced to express the actin cytoskeleton marker Emerald-Lifeact (green). Stable cell lines were stained with Acti-stain 555 phalloidin (red) and anti-tubulin antibody (MT, violet). The yellow-green signal shows the co-localization of the two actin cytoskeleton probes. Bars: 10 µm. **(B)** Cytotoxicity assays with four CLL target cell lines and effector NK-92MI cells at 1:1 and 5:1 E:T ratios for 4 hrs. 2-way ANOVA was applied to determine statistical significance; *** denotes $p < 0.0001$. **(C)** Confocal microscopy pictures of MEC-1 (left) and JVM-3 (right) cells (T) in conjugation with NK-92MI cells (NK) with and without an actin response. The charts below show the relative fluorescent intensity of Emerald-Lifeact and CMRA along the trajectories (white arrow). The fluorescence was normalized to 1 at the opposite site of the synapse. The region of the immunological synapse is indicated with "IS". Compared to the opposing end, target cells with an actin response have a more than 2-fold higher fluorescent signal at the IS. Bars: 10µm.

phenomenon "actin response" (AR) (52). Analysis of confocal microscopy images revealed that CLL cells with an AR exhibit a more than 2-fold increase of F-actin at the IS as compared to CLL cells without an AR (**Figure 1C** and **Supplemental Figure S1B**). CLL cells without an AR showed a relatively homogenous distribution of F-actin.

Quantitative analysis of the relative number of NK cell-conjugated CLL cells with and without an AR was conducted using high-throughput imaging flow cytometry. For analysis of conjugates, $5x10^3$ double-positive events were acquired per experiment with the same settings. After quality control, over 1000 conjugates between CLL and NK cells from 3 independent experiments were evaluated for the presence or absence of an AR (**Supplementary Figures S1C, D**). Our data revealed that a majority (about 63%) of highly resistant MEC-1 cells exhibited an AR, while in more susceptible CLL cell lines only a small fraction exhibited this phenotype (**Figure 2A**). HG-3 cells showed the lowest rate of AR with an average of only 14% of conjugated CLL cells forming an AR, while PGA-1 and JVM-3 had an AR frequency of 21 and 23%, respectively. To better characterize and compare the AR in CLL cells, we analyzed both the total F-actin content in AR^- and AR^+ cells, as well as the

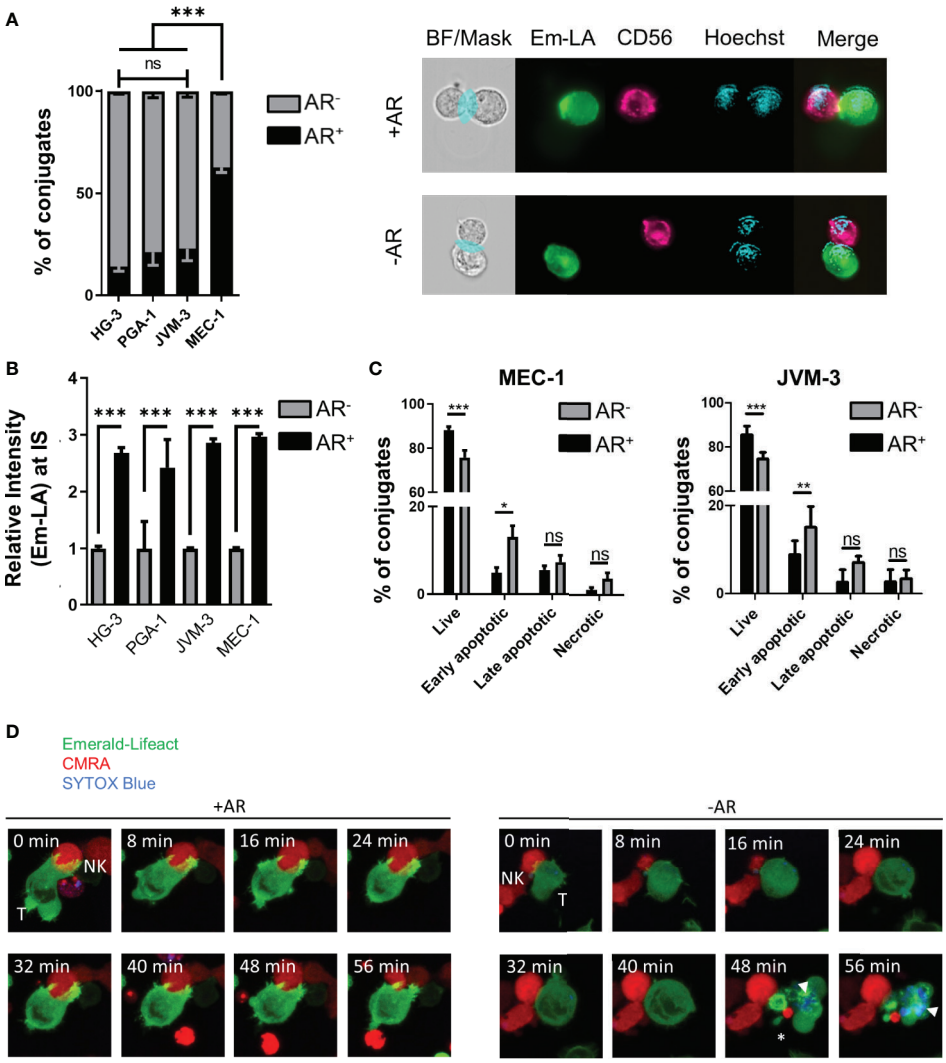

FIGURE 2 | Quantification and functional consequence of the actin response during NK cell attack. **(A)** Quantitative Imagestream analysis of CLL-NK cell conjugates. CLL cells HG-3, PGA-1, JVM-3 and MEC-1 were analyzed for their actin response frequency in conjugates with NK-92MI cells. Percentages of target cells in conjugation with NK-92MI cells with (black, AR^+) and without (grey, AR^-) an actin response. *** denotes $p < 0.0001$ **(B)** Relative intensity of Emerald-Lifeact at the IS in target cells conjugates with NK cells with respect to absence (grey, AR^-) to presence (black, AR^+). Data represents results of 6 different experiments and plots over 2000 conjugates per cell line. Data was normalized to conjugates without an AR. *** denotes $p < 0.0001$ **(C)** Imagestream analysis of target cell death in CLL target cells conjugated with NK cells in the presence (black, AR^+) or absence (grey, AR^-) of an actin response. Target cell death was assessed by Annexin V and propidium iodide staining. * denotes $p<0.05$, ** denotes $p< 0.001$, *** denotes $p < 0.0001$ **(D)** Time lapse imaging of actin dynamics in MEC-1 CLL cells upon NK cell attack. The AR^+ target cell can resist NK cell-induced cell death. Target cells not capable to produce an actin response are effectively lysed as seen by the SYTOX blue staining (white arrow head) and disappearance of normal cellular structures and membrane blebbing (asterisk). ns, non significant.

relative signal intensity of Emerald-Lifeact within the synaptic region of each type of cells. The results show that the total F-actin content is not significantly different in conjugated CLL cells with or without an AR (**Supplementary Figure S2A**). However, in CLL cells with an AR, the relative intensity of F-actin at the IS was increased almost 3-fold (**Figure 2B**), indicating a prominent polarization of the actin cytoskeleton toward NK cells. Additionally, these data suggest the accumulation of F-actin at the IS is compensated by equivalent depolymerization of actin filaments in other parts of the cells, resulting in no net increase of the overall F-actin content in target cells.

To further characterize the link between the AR and resistance to NK cell-mediated death, individual cell-cell conjugates were analyzed using imaging flow cytometry after tumor cells were labeled with the live/dead cell discrimination marker Zombie Red and Annexin V. Early apoptotic cells were characterized as Annexin V^+/Zombie Red^-, late apoptotic cells as Annexin V^+/Zombie Red^+, and Annexin V^-/Zombie Red^+ cells were classified as necrotic for quantitative analysis. In both MEC-1 and JVM-3 cell lines, AR^+ cells showed significantly less signs of apoptosis, especially early apoptosis, than AR^- cells (**Figure 2C**). Similar results were obtained for the other two CLL cell lines HG-3 and PGA-1 (**Supplementary Figure S2B**). Since NK and target cells were allowed to conjugate for only 45 minutes, induction of primarily early apoptosis is within the time frame of normal NK cell cytotoxic activity (58, 59). The protective effect of the AR was similar in all cell lines. Yet, it is important to consider that the size of the AR^+ cell subpopulation greatly differs between the MEC-1 cell line and the HG-3, PGA-1, and JVM-3 cell lines, explaining the difference in their overall susceptibility.

Live cell imaging analysis revealed that the AR in CLL cells is induced immediately after their first physical contact with NK cells and persisted throughout the whole cell-to-cell interaction time (**Figure 2D** left and **Supplementary Movie 1**). In addition, it provides direct evidence that NK cell-conjugated CLL cells that successfully assembled an AR survived the immune cell attack, while those that failed to mount an AR were efficiently lysed, as shown by uptake of SYTOX blue viability dye (**Figure 2D** right, **Supplementary Movie 2** and **Supplementary Figure S2C**).

In conclusion, we identified a subpopulation of cells in four CLL cell lines that responds to NK cell attack with fast polarization of the actin cytoskeleton to the IS (or AR), a process that closely correlates with resistance to NK cell-mediated lysis. Thus, the overall susceptibility of a given cell line can be directly deduced from the relative size of this subpopulation within the cell, with a large subpopulation being predictive of a highly resistant phenotype.

Targeted Inhibition of Actin Remodeling in CLL Cells Restores High Susceptibility to NK Cell-Mediated Killing

The Rho GTPase cell division control protein 42 homolog (CDC42) is a key regulator of actin polymerization and cell polarity (60). It promotes F-actin polymerization in association with the neuronal Wiskott-Aldrich syndrome protein (N-WASP) and the Arp2/3 complex (61). In addition, CDC42 localizes the N-WASp-Arp2/3 complex close to the cell membrane through interaction with phosphatidylinositol (4, 5) bisphosphate (62). In an attempt to inhibit fast actin remodeling in CLL cells during NK cell attack and to confirm the causal relation between the AR and CLL cell-intrinsic resistance to NK cell-mediated lysis, CDC42 was pharmacologically inhibited using the cell-permeable CDC42-specific inhibitor ZCL278 (56). 50 μM of the inhibitor was found to achieve significant and sustained inhibition of CDC42 activity, without inducing significant toxicity (**Supplementary Figure S2D**). To avoid side effects on the actin cytoskeleton of NK cells, CLL cells were pre-treated with ZCL278 and the drug was washed out before co-culture with NK cells for following experimental assays (63).

Inhibition of CDC42 activity in the resistant MEC-1 cell line resulted in potently impaired AR formation with a more than four-fold decrease in the relative number of conjugated cells exhibiting an AR as compared to DMSO-treated control cells (16.9% and 73.3%, respectively) (**Figure 3A**, left). Remarkably, such an effect was paralleled by an almost five-fold increase in tumor cell susceptibility to NK cell-mediated lysis. Indeed, 52.2% of ZCL278-treated MEC-1 cells were lysed at a 5:1 E:T ratio, while only 10.6% of DMSO-treated cells were lysed in the same conditions (**Figure 3B**, left). Thus, inhibition of *de novo* F-actin polymerization and the AR in CLL cells was sufficient to turn the initially highly resistant MEC-1 cell line into a highly susceptible phenotype. Spontaneous cell death did not change in response to ZCL278 treatment (**Supplementary Figure S2E**), indicating indeed increased susceptibility of MEC-1 cells to NK cell-mediated lysis after CDC42 inhibition. Pharmacological inhibition of CDC42 in HG-3, PGA-1, or JVM-3 cells could not further reduce the small subpopulation of CLL cells with an AR in these cell lines (**Figure 3A**, right and **Supplementary Figure S3A**) and had accordingly no effect on their already highly susceptible phenotype (**Figure 3B**, right and **Supplementary Figure S3B**).

Then MEC-1 cells were pre-treated with DMSO or 50 μM ZCL278 prior to 45 min co-culture with NK cells (1:1 E:T ratio), subsequent labelling with AnnexinV and propidium iodide and quantification of target cell killing by standard flow cytometry. Inhibition of the AR using ZCL278 resulted in a significant increase in apoptotic MEC-1 cells, especially early apoptotic cells, compared to DMSO-treated control cells (**Figure 3C**). The percentage of early apoptotic cells increased from 5.8% to 15%, a value that parallels the imaging flow cytometry analysis of cell death in AR^- MEC-1 conjugates with NK cells (**Figure 2C**), indicating restoration of a susceptible phenotype. Longer incubation time points did not change the distribution of the populations significantly, as late apoptotic and necrotic cells were removed during the washing steps (data not shown). Consistent with our previous results and the intrinsically low AR frequency in the other CLL cell lines, ZCL278 treatment did not significantly modify apoptosis in these cell lines (**Figure 3C** and **Supplementary Figure S3C**). Altogether, these results indicate that the AR is mediated by CDC42 dependent actin polymerization and that inhibition of CDC42 activity potently restores CLL cell susceptibility to NK cell-mediated cytotoxicity.

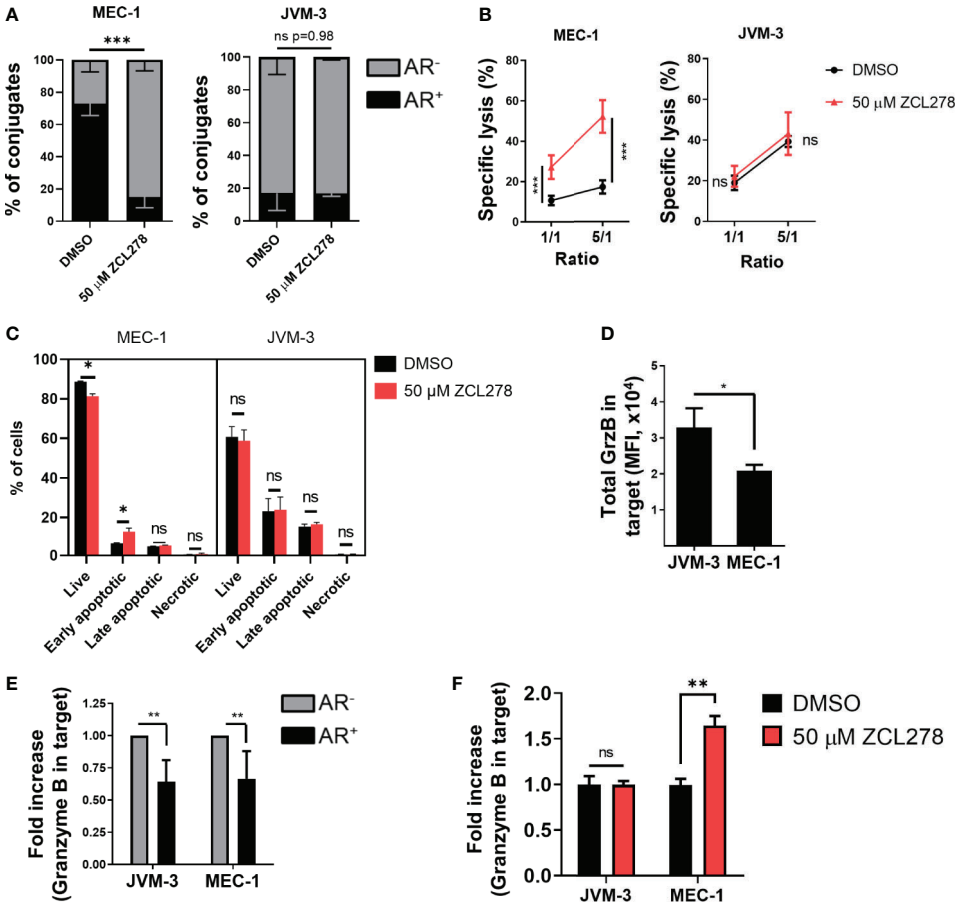

FIGURE 3 | Pharmacological inhibition of Cdc42 increases CLL cell susceptibility to NK cell attack by lowering actin response frequency. **(A)** Quantitative Imagestream analysis of CLL-NK cell conjugates. CLL cells were pre-treated with 50 μM ZCL278 and analyzed for their actin response frequency in conjugates with NK-92MI cells. *** denotes $p < 0.0001$ **(B)** NK cell-mediated cytotoxicity against DMSO- or ZCL278-treated CLL cells. Pre-treated JVM-3 and MEC-1 cells were co-cultured for 4 hrs with NK-92MI cells at E:T ratios of 1:1 and 5:1. Cell death was evaluated by To-Pro-3 staining and adjusted to NK cell-specific lysis. *** denotes $p < 0.0001$ **(C)** Flow cytometry analysis of DMSO- or ZCL278-pretreated CLL target cells after 45 minutes of co-culture with effector NK-92MI cells at a 1:1 E:T ratio. Apoptosis was evaluated by Annexin V and PI staining. * denotes $p < 0.05$, ** denotes $p < 0.001$, *** denotes $p < 0.0001$ **(D)** Imagestream analysis of total granzyme B load in target cells conjugated to NK-92MI cells after 45 minutes of co-culture. * denotes $p < 0.05$ **(E)** Target cells were categorized into AR^+ (black) and AR^- (grey) and granzyme B load in target cells evaluated. Data was normalized to AR^- conjugates. ** denotes $p < 0.001$ **(F)** Imagestream analysis of intracellular granzyme B in target cells after ZCL278-induced Cdc42 inhibition. ** denotes $p < 0.001$. ns, non significant.

Synaptic Actin Remodeling Leads to Reduced Granzyme B Levels in NK Cell-Conjugated CLL Cells

Direct cytotoxicity of NK cells occurs through the release of cytotoxic granules (23, 24). These granules contain among others granzymes and perforin that trigger cancer cell lysis through formation of membrane lesions and induction of caspase-3 and caspase-8 activation. We assessed the levels of one key granzyme, namely granzyme B, transferred into CLL cells. On average, JVM-3 cells in conjugation with NK cells showed a higher intracellular intensity of granzyme B compared to MEC-1 cells (**Figure 3D**), which is consistent with the respective cell line susceptibility to NK cell-mediated lysis and ability to remodel actin cytoskeleton following immune attack. Moreover, in all four cell lines, intracellular levels of granzyme B were considerably reduced (by approximately 35%) in the cell subpopulation exhibiting an AR as compared to the cell subpopulation without an AR, suggesting that the AR leads to reduced amounts of granzyme B transferred to target cells (**Figure 3E** and **Supplementary Figure S3D**).

To assess if inhibiting the AR could restore elevated levels of granzyme B in MEC-1 cells, MEC-1 cells were pre-treated with 50 μM ZCL278 to lower the cell subpopulation with an AR and granzyme B levels were quantified after 45 minutes incubation with effector cells (**Figure 3F** and **Supplementary Figure S3E**). The results show that AR inhibition increased the intracellular granzyme B intensity to levels comparable to JVM-3 cells. Altogether our data provide strong indication that the AR protects CLL cells from cytotoxicity mediated by NK cells through the granzyme B/perforin pathway.

Primary NK Cells Induce the Actin Response and Confirm CLL Cell Line Intrinsic Actin Response Frequency

The NK-92MI cell line used in this study is a CD16$^-$ effector cell lines that additionally lacks major inhibitory receptors such as Killer-cell immunoglobulin-like receptors (KIR) and NKG2A receptors (64, 65). These results in induction of natural cytotoxicity through recognition of activating ligands such as MHC class I homologues MIC-A (MIC-A), MIC-B, and UL-16 binding protein (ULBP) on target cells and interaction of lymphocyte function-associated antigen 1 (LFA-1) with its ligand intercellular adhesion molecule 1 (ICAM-1). According to its "hyperactive" phenotype, the NK cell line kills target cells in an unrestricted manner and with low specificity. Thus, we re-evaluated the AR in CLL cell lines challenged with primary NK cells isolated from healthy donors.

To this end, primary NK cells were isolated from PBMCs using a negative selection kit reaching a purity of >90% (**Supplementary Figure S3F**) and kept in culture overnight with IL-2 and IL-15 for activation. Cytotoxicity of primary NK cells against JVM-3 and MEC-1 target cells was lower compared to lysis rates achieved with the NK-92MI cell line. However, the previously established difference in intrinsic susceptibility between the two target cell lines was confirmed (**Figure 4A**). Indeed, despite inter-donor variability, JVM-3 cells were more effectively lysed by donor-derived NK cells than MEC-1 target cells.

Quantitative analysis of the AR frequency with primary NK cells using imaging flow cytometry resulted in remarkably comparable results as seen with the NK-92MI cell line (**Figure 4B**), with about 25% of AR$^+$ conjugates with JVM-3 cells and 60-71% of conjugated MEC-1 cells showing an AR. Confocal microscopy provided direct evidence that primary NK cell attacks also invoked an AR in some individual CLL cells (**Figure 4C**).

In conclusion, activated donor-derived healthy NK cells induce an AR in CLL cells at a same frequency as the NK-92MI cell line, supporting that the AR is a process intrinsic to the CLL cells and is not dependent on the origin of the effector NK cells, being a cell line or isolated form a healthy donor.

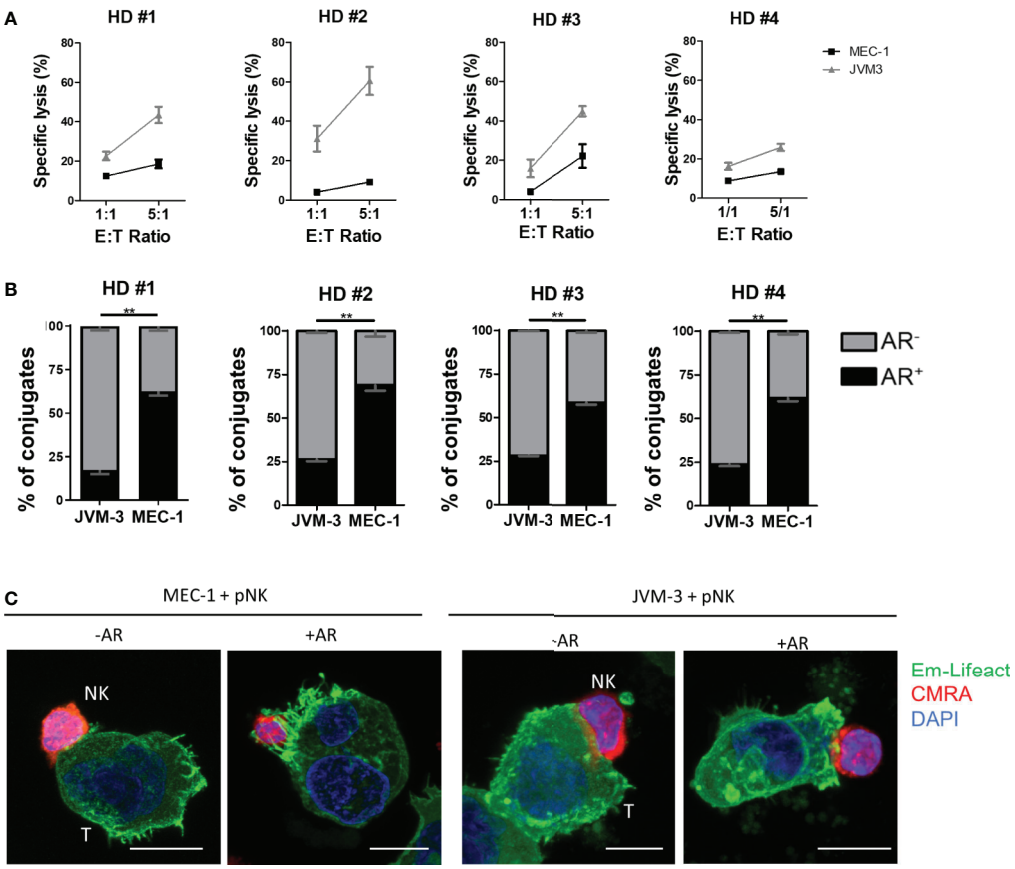

FIGURE 4 | Primary human NK cells can invoke the actin response in MEC-1 and JVM-3 cells. **(A)** Cytotoxicity assay with primary NK cells isolated from four healthy human donors and JVM-3 and MEC-1 CLL target cells at 1:1 and 5:1 E:T ratios. Target cells and primary NK cells were co-cultured for 4 hrs before analysis of NK cell-specific lysis by flow cytometry. **(B)** Quantitative analysis of primary NK-CLL conjugates with (black, AR$^+$) and without (grey, AR$^-$) actin response by imaging flow cytometry. For each donors a minimum of 200 conjugates were analyzed. ** denotes $p < 0.001$ **(C)** Representative confocal microscopy pictures of primary NK cells (CMRA, red) in conjugation with MEC-1 (left) and JVM-3 (right) cells with or without an actin response. Bars: 10 µm.

Inhibition of the Actin Response in Combination With HLA-G Blocking Antibody Restores Patient-Derived CLL Cell Susceptibility to NK Cell-Mediated Killing

To investigate if primary CLL cells mount an AR and if inhibition of the latter improves their susceptibility to NK cell-mediated cytotoxicity, CLL patient samples (n=10) were analyzed in a series of *ex vivo* assays. In these assays, PBMCs were isolated from peripheral blood and their actin cytoskeleton was stained with SiR-actin, a cell-permeable and F-actin specific probe. As illustrated in **Figure 5A**, ARs were observed in primary CLL cells conjugated with NK-92MI cells. These ARs were of slightly lower intensity compared to those seen with CLL cell lines, with a roughly 1.8-fold increase of fluorescence intensity for F-actin at the IS as compared to the opposing cell side. This could be explained by the smaller cell size of primary CLL cells. The AR was then quantified in CLL cells originating from six patients using imaging flow cytometry. Our results revealed a similar and relatively high rate of AR in all primary CLL cell samples with values ranging from 38.4% to 51.2% of analyzed primary CLL-NK-92MI cell conjugates (**Figure 5B**). We noticed that the conjugation rate of primary CLL cells with NK-92MI cells was particularly low (~7% of all $CD5^+/CD19^+$ cells; **Figure 5C**), which can be explained by a high surface expression of HLA-G on *ex vivo* CLL cells (40) and expression of the cognate inhibitory receptor immunoglobulin-like transcript 2 (ILT-2, LILRB1) on NK-92MI cells (66). Interaction of ILT-2 with HLA-G has been reported to negatively impact not only NK cell polarization, but also to interfere with F-actin assembly at the NK cell side of the IS (67) and can thereby prevent conjugate formation between patient-derived CLL and NK-92MI cells (24).

To improve conjugate formation, primary CLL cells were treated with an anti-HLA-G blocking antibody for 1 h prior to co-culture with NK-92MI cells. It is noteworthy that NK-92MI cells do not express the Fc receptor CD16 (64), excluding the risk of ADCC. As anticipated, HLA-G blocking antibody increased the frequency of target CLL cells in conjugation with NK-92MI cells compared to control IgG, indicating a release of the inhibitory ILT-2 signaling and restoration of IS formation (**Figure 6A**). Additionally, we aimed at evaluating the effect of ZCL278 on primary CLL cells. Inhibition of CDC42 activity with 50 µM ZCL278 in combination with blocking antibody was found to only minimally or not to alter the rate of conjugation in comparison to vehicle control (DMSO). Conversely, treatment with anti-HLA-G antibody did not alter the rate of AR in primary CLL cells, while ZCL278 treatment reduced the AR in three out of five patients (**Figure 6B**). Although this remains speculative, the lack of response in CLL cells originating from patient 8 and 9 could be explained by an initially low AR frequency (patient no. 9) or upregulation of alternative, CDC42-independent actin polymerization pathways (patient no. 8).

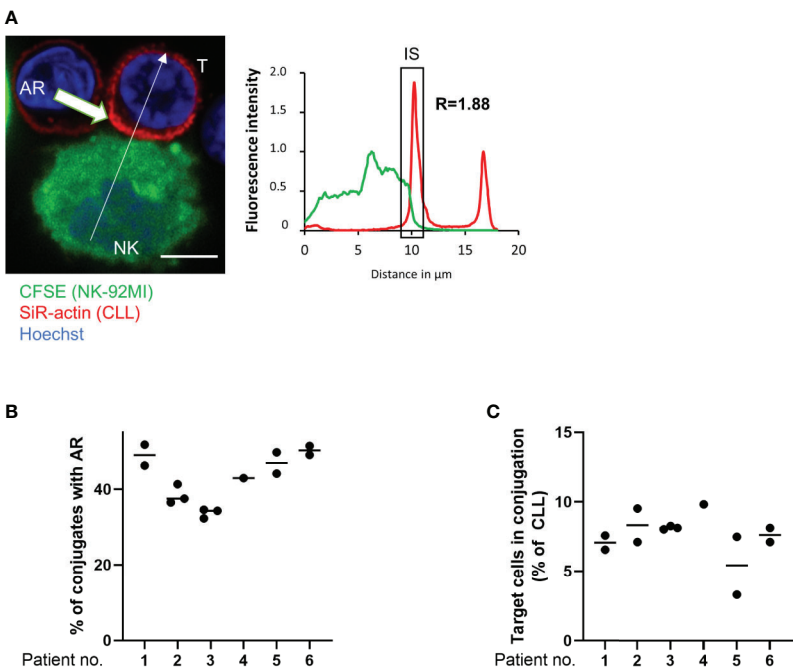

FIGURE 5 | The actin response in patient-derived primary CLL cells. **(A)** Representative confocal microscopy image of an immune synapse between a primary CLL cell (T) and NK-92MI cell (NK). The chart to the right shows the relative fluorescent intensity of SiR-actin along the trajectory (white arrow). The fluorescence was normalized to 1 at the opposite site of the synapse. The region of the immunological synapse is indicated with "IS". Bar: 10 µm **(B)** Quantitative imaging flow cytometry analysis of the AR in primary CLL cells in conjugation with NK-92MI effector cells. CLL cells were identified as $CD5^+/CD19^+$ cells. Actin staining was performed using spirochrome labelling. **(C)** Quantification of primary CLL cells in conjugation with NK-92MI cells after 45 minutes of co-culture as percentage of total target population.

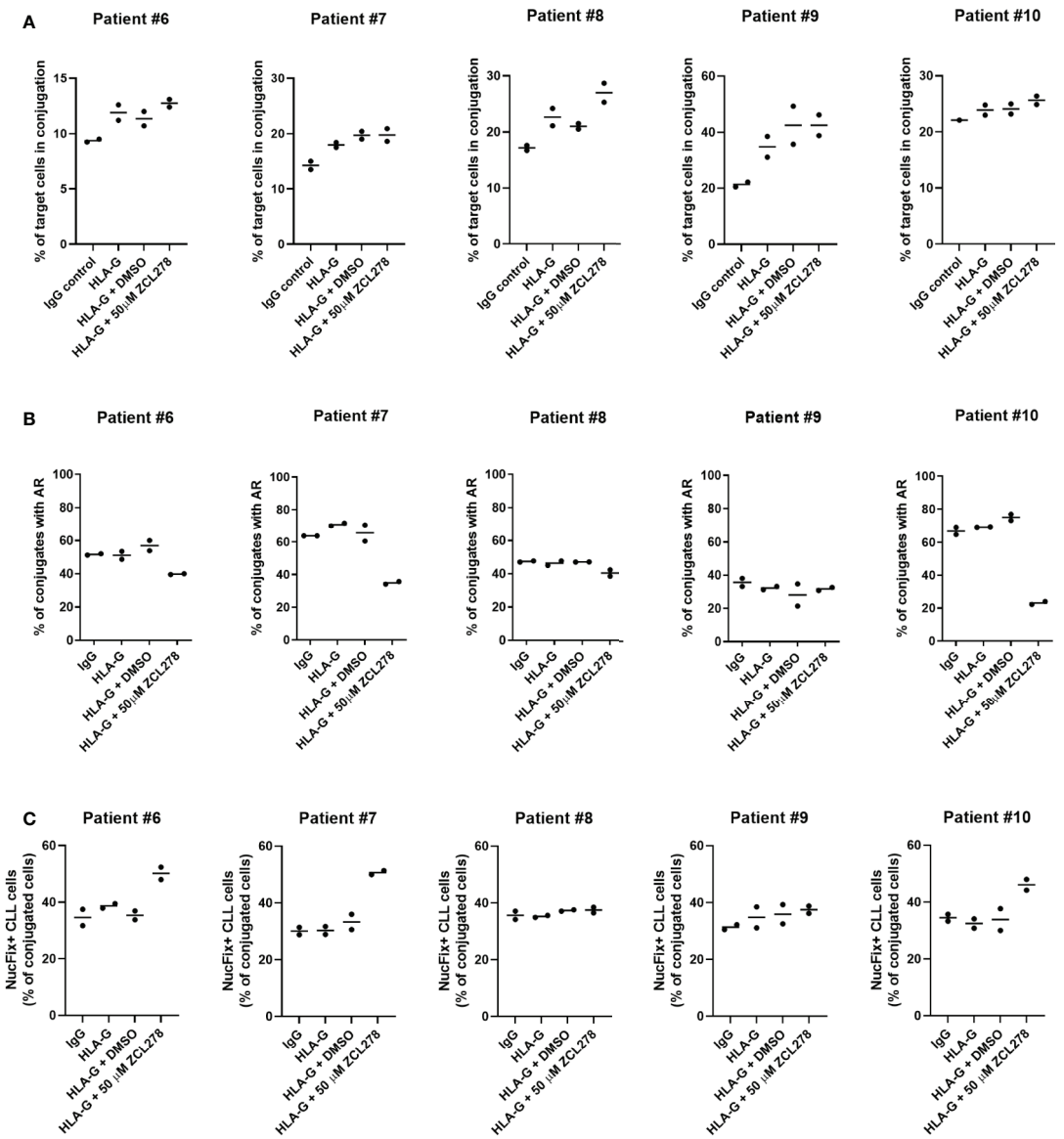

FIGURE 6 | Inhibition of the actin response in combination with anti-HLA-G blocking antibody substantially improves NK cell-mediated killing of CLL cells. **(A)** Conjugation frequency of primary CLL cells as quantified by imaging flow cytometry in the absence or presence of HLA-G blocking antibody. Target cells were incubated with 10 µg/mL control IgG or blocking antibody against HLA-G, and were used either untreated (IgG, HLA-G), DMSO treated, or after incubation with 50 µM ZCL278 for 1 h before conjugation. Effector and target cells were co-cultured at a 3:1 E:T ratio for 45 min at 37°C and fixed with 2 v/v% PFA. Target cells in conjugation are shown as % of total target cells. **(B)** Quantitative analysis of primary CLL-NK cell conjugates with an actin response in the presence of control IgG or anti-HLA-G blocking antibody by imaging flow cytometry. Target cell were either untreated (IgG, HLA-G) or conjugated after treatment with vehicle (DMSO) or 50 µM ZCL278. For each patient, a minimum of 100 conjugates were analyzed. **(C)** Average percentage of Live-or-Dye NucFix™ Red positive primary CLL cells in conjugation with NK-92MI cells in the presence of control IgG or anti-HLA-G blocking antibody. Target cells are either untreated, treated with vehicle control or 50 µM ZCL278.

Finally, apoptosis in primary CLL cells in conjugation with NK-92MI was evaluated with regards to anti-HLA-G antibody treatment and CDC42 inhibition. Samples treated with 50 µM ZCL278 that previously showed no changes in the AR frequency (**Figure 6B**) demonstrated similar levels of apoptosis as compared to IgG control, HLA-G, or HLA-G in combination with vehicle treated samples from the same patient (**Figure 6C**, patient no. 8 and 9). In contrast, with CLL samples that showed a reduction of the AR in response to CDC42 inhibition (**Figure 6B**), an increased percentage of conjugated primary CLL cells showed signs of apoptosis (**Figure 6C**, patient no. 6, 7, and 10). These results extend our analysis with CLL cell lines and indicate that the AR is a frequent process in primary CLL cells (with more than 50% of NK cell-conjugated CLL cells showing an AR for

most patients) and that targeting of this process can substantially increase CLL cell susceptibility to NK cell-mediated cell death.

In conclusion, we report here for the first time AR in patient-derived cancer cells and show that specific targeting of key actin regulators in combination with anti-HLA-G blocking antibody, increases conjugate formation and target cell susceptibility to NK cell-mediated cytotoxicity opening up the possibility of combinational targeting for CLL patients.

DISCUSSION

Despite substantial recent advances in the therapy of CLL, treatment options, especially for patients diagnosed with an aggressive disease, particularly with *TP53* deletion and/or mutation, are limited. Effectors of both, the adaptive and the innate immunity immune systems, show severe signs of dysfunction that allow for successful immune evasion of malignant B cells. This includes inhibition of cytotoxic $CD8^+$ and activated $CD4^+$ T lymphocytes and induction of the immune suppressive M2-like monocyte phenotype instead of pro-inflammatory immune sub-populations. Additionally, CLL induces expansion of regulatory T cells (T_{Reg}), overall resulting in the development of a tolerogenic environment and disease progression (11, 68). While special attention has been paid investigating the interaction between CLL and T lymphocytes, recent studies focused on NK cells as an alternative target of chemo-immunotherapy. NK cells derived from CLL patients were described as hyporesponsive due to a loss of the mature, $CD56^{dim}$ NK cell population, possibly due to activation-induced apoptosis as a result of constant exposure to malignant B cells. This was accompanied by a downregulation of activating receptors such as NKG2D that affects the natural cytotoxicity of NK cells (23). However, upon sufficient activating signal through CD16, NK cell function can still be induced, showing that CLL-derived NK cells of the CMV-associated $NKG2C^+$/$CD16^+$ phenotype are fully functional (69). Total NK cell numbers have repeatedly been reported to be elevated in CLL patients compared to healthy controls, often with an emphasis on CMV-related $NKG2C^+/CD56^{dim}/CD16^+$ NK cells (23, 70, 71). These phenotypes accordingly cannot explain the lack of anti-tumor response or disease progression in CLL.

The NK cell line we used in the present study is negative for CD16 and a common model of natural cytotoxicity of NK cells. Although these NK cells are fully activated and effectively recognize their targets, a subpopulation of CLL cells was still resistant to NK cell-mediated cytotoxicity. This indicates an additional intrinsic resistance of CLL that allows escape from NK cells that can be activated either through activating ligands or possibly even through ADCC. Here we show that the actin cytoskeleton of CLL cells plays a critical role in the intrinsic capacity of these cells to avoid destruction by degranulating immune effector cells.

The fast synaptic actin remodeling we observed in CLL cells attacked by NK cells strongly resembled the "actin response" or AR previously described for breast cancer cells and was strongly associated with resistance to NK cell cytotoxicity. Independent of IGHV mutational status, CLL cell lines showed a resistant subpopulation that was characterized by the AR. While HG-3, PGA-1, and JVM-3 showed similar results in all experimental assays with a high susceptibility to NK cell-mediated lysis and high intracellular granzyme B load, MEC-1 cells demonstrated a high resistance to NK cell-mediated cytotoxicity and decreased uptake of NK cell-derived granzyme B. We attributed these differences to the relative size of the AR^+ subpopulation as we were able to show that *de novo* F-actin polymerization on the cancer side of the IS is strongly associated with survival and resistance during NK cell attack. Pharmacological inhibition of CDC42 activity drastically reduced the size of the AR^+ subpopulation and resulted in an increase of early apoptotic cells and overall cell death in MEC-1 that can be explained by increased amounts of granzyme B transferred into target cells. Although CDC42 is a central actin cytoskeleton regulator, other pathways might be involved in the process of the AR, as suggested by the remaining AR^+ subpopulation in HG-3, PGA-1, JVM-3 and MEC-1 cells that resisted treatment with the CDC42-specific inhibitor ZCL278 (56).

An interesting aspect that could be worth further investigation is the potential role of *TP53* in enabling the AR, as the four cell lines differ in their *TP53* mutational status. HG-3, PGA-1, and JVM-3 cells have all been reported to express wildtype *TP53*, while MEC-1 are identified as a $TP53^{del/mut}$ cell line, expressing a truncated 40kDa version of the p53 protein without transcriptional activity. In CLL patients, mutations of *TP53* or loss of one *TP53* allele are associated with a significant decrease in survival and are predictive for an impaired response to chemo-immunotherapy (72). Additionally *in vitro* experiments attributed expression of wildtype *TP53* or mutational *TP53* and/or loss of *TP53* to differential drug response in several CLL cell lines, including JVM-3 and MEC-1 (73–75). Other studies have shown, that rescue of mutational *TP53* function can restore granzyme B-mediated apoptosis in breast cancer through down-modulation of anti-apoptotic proteins (76). However, in these studies, *TP53* reactivation was achieved in cell lines expressing a missense mutational p53, while in many CLL cases with aberrant *TP53* expression, deletion of the short arm of chromosome 17 (del17p13) results in a complete loss of *TP53*, often in association with *TP53* mutations on the other allele (77). Whether *TP53* status is therefore a critical determinant of the AR frequency in CLL will need to be determined with a larger, better defined patient cohort in the future.

Most importantly, we were able to show that the AR is not an artefact of cell lines but can indeed be found in patient-derived CLL cells. In this context, the inhibitory interaction of surface molecules, such as the non-classical MHC-I isoforms HLA-E and HLA-G, with their corresponding ligands on NK cells might have been underestimated in their significance. Even the NK-92MI cell, known to not express key inhibitory receptors such as KIR or NKG2A, showed a deficiency in conjugate formation with patient-derived CLL cells. This dysfunction could be rescued by antibody blockade targeting HLA-G. However, antibody opsonization of target CLL cells had no impact on the

frequency of the AR in primary CLL cells. In combination with CDC42 inhibition, anti-HLA-G blocking antibody greatly increased NK cell-induced apoptosis in patient-derived CLL cells. We hypothesize that this is a consequence of the relative size of the AR^+ population that could be decreased by pharmacological CDC42 inhibition. This is further supported by the observation that in individual patient samples in which this treatment failed to reduce the size of the AR^+ population, the frequency of CLL cells showing signs of apoptosis was unchanged compared to untreated and DMSO-treated samples. Since NK-92MI cells are $CD16^-$, antibody blockade did not trigger ADCC that could explain the increase in target cell death. It is however worth speculating that NK cells capable of ADCC could be even more effective in inducing apoptosis in CLL cells that underwent dual blocking antibody and CDC42 inhibition therapy.

Yet, actin cytoskeleton targeting drugs, such as cytochalasins or latrunculins, show intolerable toxicity with particularly severe adverse effects on cardiac structure and function and are therefore unfit for clinical trials. Experimental drugs that target cancer-specific F-actin components and confirmation of their efficiency *in vivo* (78, 79) demonstrate however, that targeting of the actin cytoskeleton dynamics is a possibility in our search for new innovative cancer drugs. Importantly, targeting of intrinsic immune escape mechanisms such as the AR can only be effective in combination with other therapies, such as immune checkpoint inhibitor blockade and/or opsonization with tumor-targeting antibodies. Without these therapies, cytotoxic lymphocyte activation, but also IS formation that is fundamental to a functional anti-tumor immune response cannot take place.

Overall, NK cells are emerging as a valuable tool for the control of CLL disease progression and reactivation of their cytotoxic capabilities against cancer cells could potentially improve overall outcome. Selective targeting of intrinsic immune escape mechanisms, such as the here described AR, could provide a new line of therapy for the difficult to treat or relapsing CLL subtypes. It is important to highlight that irrespective of the expression status of poor prognostic markers, such as *TP53* and *IGHV*, all four CLL cell lines, as well as all ten patient-derived CLL samples demonstrated an AR^+ subpopulation that proved to be resistant against pharmacologic inhibition of CDC42 activity. This shows the presence of another signaling pathway allowing cancer cells to maintain resistance against NK cell cytotoxicity. Further studies employing patient cohorts will be needed to address and confirm the clinical importance of the AR in CLL and its therapeutic value. Further it needs to be evaluated whether the hyporesponsive phenotypical state of CLL-patient derived NK cells is revertible or if allogeneic NK cell therapy could benefit from selective targeting of the AR (47), possibly in combination with other immunomodulatory drugs (44, 80, 81).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité National d'Ethique de Recherche Luxembourg (CNER No. 201707/02 Version 1.2). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HW designed and performed most of the experimental work, analyzed results, and wrote the manuscript. LF, CH, MK, and AB performed experiments and analyzed results. JM performed NK cell isolation. HW, LF, and AL performed patient CLL cell isolation. Patient samples were provided by SD, JF, and GB. JP and EM organized CLL sample collection and contributed to supervise the study. CT supervised the study and wrote the final version of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING SUPPORT

Work in CT's group is supported by grants from the National Research Fund (FNR, Luxembourg, ACTIVASION, C19/BM/13579644), La Fondation Cancer (Luxembourg, ACTIMMUNE, FC/2019/02) and Think Pink Lux (Luxembourg). HW is recipient of a PhD fellowship from the National Research Fund (FNR, Luxembourg), Luxembourg (PRIDE15/10675146/CANBIO). AB and MK are recipient of PhD fellowships from Fonds National de la Recherche Scientifique (FNRS, Belgium, Televie #7.4536.19 ACTIPHAGY and Televie #7.4531.20 TRANSACTINg, respectively). AL is supported by a post-doctoral fellowship from the FNRS (Televie #7.4503.19). JP is supported by the National Research Fund (FNR), Luxembourg (INTER/DFG/16/11509946).

ACKNOWLEDGMENTS

We thank Flora Moreau and Odile Lecha for providing Emerald-Lifeact expressing cell lines and help with data collection.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.619069/full#supplementary-material

Supplementary Figure 1 | (A) List of CLL cell line characteristics. **(B)** Confocal microscopy pictures of HG-3 and PGA-1 Emerald-Lifeact cells (T) in conjugation with NK-92MI cells (NK) with and without an actin response. Charts below show the relative fluorescent intensity of Emerald-Lifeact and CMRA along the trajectories (white arrow). The fluorescence was normalized to 1 at the opposite site of the synapse. The immunological synapse is indicated with "IS". Cells with an actin response show a more than 2-fold higher fluorescent signal at the IS. Bars: 10µm.

(C) Analysis of imaging flow cytometry images. First, a scatter plot of aspect ratio vs. area was used to gate for cells and exclude debris and control beads (R1). Second, a gradient RMS (root mean square for image sharpness) histogram was used to gate for cells in focus (Focus). Third, the intensity of PE/Cy7 (CD56) was gated against the intensity of Emerald-Lifeact (EmLA). Double positive events were categorized as conjugates and used for subsequent image analysis. We defined AR^+ CLL cells as target cells with an increased relative intensity of EmLA fluorescence in the IS mask in relation to the total intensity of EmLA in target cells. For determination of granzyme B content in CLL cells, the NK cell part of the immune synapse is excluded from the analysis. **(D)** Immune synapse definition using IDEAS. Cell shape defined by surface labelling (CD56) or EmLA expression was extended by 3px in all dimensions using the dilate function. The overlapping region was defined as the immune synapse mask (IS mask) in NK cell-CLL cell conjugates. Created with BioRender.com

Supplementary Figure 2 | (A) Imaging flow cytometry analysis of total F-actin fluorescent intensity in AR^+ and AR^- HG-3, PGA-1, JVM-3, and MEC-1 cells. Differences between cell lines are the result of differences in transduction efficiency and transgene expression. Analysis was performed using a Wilcoxon paired t-test. **(B)** Apoptosis in HG-3 and PGA-1 conjugates with NK-92MI as evaluated by imaging flow cytometry. Cells were gated based on the absence (AR^-) or presence (AR^+) of an actin response. * denotes to $p < 0.05$, ** denotes to $p < 0.01$ **(C)** Measurement of SYTOX Blue fluorescence intensity over time in the MEC-1 cells with and without an actin response following conjugation with NK cells. Image J software was used to do the quantification of LSM880 acquired images. **(D)** Evaluation of GTP-loaded Cdc42 by G-LISA colorimetric activation assay. MEC-1 cells were pre-treated for 1 h with 50 µM ZCL278 before drug was removed and cells resuspended in fresh complete medium. Cells were allowed to recover for 1 and 5 hrs after drug removal before stimulation with 0.1 µg/mL human recombinant EGF for 15 minutes to measure inducible CDC42 activity. *** denotes to $p < 0.0001$. **(E)** Spontaneous cell death in HG-3, PGA-1, JVM-3, MEC-1 cells treated with either DMSO or 50 µM ZCL278 for 1 h. Drugs were removed and cells allowed to recover for 45 minutes before cell death analysis using Annexin V and propidium iodide.

Supplementary Figure 3 | (A) Imaging flow cytometry analysis of DMSO- or ZCL278-treated HG-3 and PGA-1 with regards to actin response frequency in conjugates. **(B)** Cytotoxicity assays of DMSO- or ZCL278-treated HG-3 and PGA-1 cells with NK-92MI at E:T ratio of 1:1 and 5:1. **(C)** Apoptosis assay of DMSO- or ZCL278-treated HG-3 and PGA-1 cells with NK-92MI effector cells. Cells were co-cultured for 45 minutes before live/dead staining with Annexin V and propidium iodide. **(D)** Quantitative imaging flow cytometry analysis of granzyme B load in HG-3 and PGA-1 CLL cells in conjugation with NK-92MI cells. **(E)** Imaging flow cytometry analysis of granzyme B load in DMSO- or ZCL278-treated HG-3 and PGA-1 CLL cells after 45 minutes of co-culture with NK-92MI cells. **(F)** Flow cytometry gating strategy for buffy coat-derived PBMC before and after negative selection for NK cells.

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