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IGHV1-69 as a promising candidate for the development of a shared immunotherapy to B-cell lymphomas

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to my father

a mio padre

"Bastava a trasformare le situazioni
la tua visione più ampia e generosa dei fatti"
(Bepi Muraro)

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ABSTRACT

B-cell Non-Hodgkin Lymphomas (B-NHL) are a heterogeneous group of cancers, broadly diffused worldwide and often relapsing after standard treatment and rituximab. Therapeutic vaccines targeting B-NHL idiotype (Id) represent a promising approach to maintain the complete response induced by standard treatments. However, customized idiotypic vaccination still remains a non-approved, experimental therapeutic option, mostly due to the personalized use and penalized by the lack of reliable clinical or biological markers of patient eligibility and responsiveness. Nevertheless, the molecular characterization of different lymphoid tumor histotypes revealed a set of stereotyped immunoglobulins among distinct B-cell lymphoma types. On this ground, we focused our attention on the IGHV1-69 protein, frequently expressed in HCV-associated lymphomas, chronic lymphocytic leukaemia, and auto-immunity related lymphoproliferations, and we characterized the *ex vivo* immunogenicity of this protein.

Seventy IGHV1-69 sequences obtained from patients affected by different B-NHLs or pre-malignant lymphoproliferations were compared to design an optimized sequence characterized by the highest degree of similarity among studied cancers, and thus CDR3 hypervariable region free. Within this "immunogenically" optimized sequence, we identified 13 potential HLA class-I cytotoxic T lymphocyte (CTL) epitopes and synthesized the corresponding pentamers (Pent). We assessed by flow cytometry the presence and extent of epitope-specific T-cell responses in peripheral blood of patients with IGHV1-69$^+$ B-cell lymphoproliferative disorders and healthy donors, and validated these data in IFN-γ ELISPOT (Enzyme-linked immunosorbent spot) assays. Finally, we boosted *in vitro* IGHV1-69-specific responses by stimulating peripheral blood lymphocytes (PBL) from donors and patients with different protocols for the generation of epitope-specific CTL cultures.

Interestingly, the IGHV1-69 Pent$^+$ population observed in patients' samples was generally larger than in donors, supporting the existence of spontaneous memory T-cell responses against IGHV1-69, at least for some HLA-restrictions. Surprisingly, in patients' samples, IGHV1-69-recognizing T cells displayed higher IFN-γ release in ELISPOT assays compared to viral-specific T cells. Moreover, we obtained peptide-specific CTL lines, which showed a weak but specific lysis against peptide-pulsed targets, especially when derived from patients' PBLs. In addition, we were able to generate IGHV1-69-epitope specific CTL clones from healthy donors CD8$^+$ T cells, employing synthetic artificial APC, developed to elicit and expand low-avidity tumor-directed human CTL lines. Finally, IGHV1-69-induced CTL lines showed specific lysis also towards an IGHV1-69 naturally expressing cell line, suggesting that IGHV1-69 memory T-cell responses could be boosted for therapeutic purposes.

These results show that IGHV1-69 constitutes a potential target for the development of a subset-specific Id vaccine. Furthermore, multimer (tetramers and pentamers) and ELISPOT immune-monitoring may partially overcome the main limitations of current Id-targeting vaccinations and further improve their clinical efficacy.

Abstract (Italian)

I Linfomi Non-Hodgkin a cellule-B (B-NHL) rappresentano un gruppo eterogeneo di patologie, ampiamente diffuse nel mondo e caratterizzate da frequenti ricadute in seguito a trattamenti standard o terapia con rituximab. Vaccini terapeutici che hanno come bersaglio l'Idiotipo (Id) dei B-NHL, costituiscono un approccio promettente nel mantenere la risposta completa indotta con trattamenti standard. Tuttavia, i vaccini idiotipici personalizzati

rappresentano ancora un approccio terapeutico sperimentale e non approvato su larga scala, principalmente perché paziente-specifici e perché privi di marcatori attendibili per l'identificazione di pazienti elegibili e di risposta alla terapia. Ciononostante, la caratterizzazione molecolare di differenti istotipi di tumori di origine linfoide, ha rivelato la presenza di una serie di immunoglobuline stereotipate anche fra linfomi di diverso tipo. Su questi presupposti, abbiamo focalizzato la nostra attenzione sulla proteina IGHV1-69, frequentemente espressa in linfomi associati all'infezione da HCV, nella leucemia linfatica cronica (CLL) e in linfoproliferazioni associate ad auto-immunità, e abbiamo valutato *in vitro* l'immunogenicità di questa proteina.

Inizialmente, abbiamo confrontato 70 sequenze relative alla proteina IGHV1-69 e ottenute da pazienti affetti da differenti B-NHLs o linfoproliferazioni pre-maligne, allo scopo di ideare una sequenza ottimizzata, caratterizzata dal maggior grado di similarità fra i tumori presi in esame, e pertanto priva della regione ipervariabile CDR3. All'interno di questa nuova sequenza, abbiamo identificato 13 epitopi potenzialmente riconoscibili da linfociti T citotossici (CTLs) nell'ambito di 7 alleli HLA di classe I, e abbiamo sintetizzato i corrispondenti pentameri (Pent). Tramite citofluorimetria a flusso abbiamo quindi valutato la presenza di risposte T-cellulari specifiche per gli epitopi derivati da IGHV1-69, in campioni di sangue periferico ottenuti da pazienti affetti da linfoproliferazioni esprimenti IGHV1-69 e da donatori sani. Abbiamo inoltre validato questi dati tramite saggi ELISPOT (Enzyme-linked immunosorbent spot) per l'identificazione del rilascio di IFN-γ. Infine abbiamo stimolato *in vitro* le risposte specifiche per IGHV1-69, inducendo i linfociti del sangue periferico (PBLs) di donatori sani e pazienti, attraverso diversi protocolli per la generazione di colture CTL epitopo-specifiche.

E' stato interessante osservare come nei campioni ottenuti da paziente la popolazione di linfociti T $CD8^+$ positiva ai pentameri specifici per gli epitopi di IGHV1-69 sia risultata generalmente più numerosa della corrispondente popolazione osservata in donatori sani. Questo dato supporta l'esistenza di risposte memoria T cellulari nei confronti di IGHV1-69, almeno in alcune restrizioni HLA. Inoltre, nei campioni ottenuti da paziente le cellule T specifiche per gli epitopi di IGHV1-69 hanno rivelato in saggi ELISPOT un maggior rilascio di IFN-γ rispetto ai linfociti T specifici per epitopi virali. In aggiunta, stimolando parallelamente PBLs di donatori sani e pazienti, abbiamo ottenuto linee CTL peptide-specifiche, in grado di riconoscere debolmente, ma in modo specifico target caricati con il peptide d'interesse, soprattutto quando derivate da PBLs di pazienti. Inoltre, tramite l'utilizzo di *antigen-presenting cells* artificiali, prodotte allo scopo di indurre ed espandere linee CTL caratterizzare da bassa affinità nei confronti di antigeni tumorali, abbiamo anche generato cloni CTL specifici per un epitopo di IGHV1-69 a partire da linfociti T $CD8^+$ di donatori. Infine, si è evidenziato che colture CTLs indotte in seguito a stimolo con epitopi derivanti da IGHV1-69, sono in grado di riconoscere in modo specifico una linea cellulare naturalmente esprimente IGHV1-69, suggerendo in questo modo che le risposte memoria T-cellulari specifiche per IGHV1-69, possano essere stimolate ed espanse a scopi terapeutici.

Questi risultati dimostrano che IGHV1-69 costituisce un target potenziale per lo sviluppo di un vaccino Id applicabile su un sottogruppo di linfomi a cellule B. Inoltre, l'immunomonitoraggio tramite marcatura con multimeri (tetrameri o pentameri) e saggi ELISPOT potrebbe eludere almeno parzialmente i principali limiti degli attuali vaccini idiotipici, al fine di incrementare ulteriormente la loro efficacia clinica.

INTRODUCTION

The challenge of anti-tumor vaccination

In the multidisciplinary treatment of cancer patients, therapeutic vaccines represent one of the most fascinating and promising strategies. Compared to standard modalities (chemo- and radiotherapy, surgery, adaptive immunotherapy), anti-tumor vaccination might have the potentiality to persist a lifetime and to induce an effective anti-tumor immune response able to cure the established tumor and keep it under constant control, thus prolonging survival [1].

In contrast to prophylactic vaccines against pathogens, where the target antigens are foreign to the immune system, the majority of cancer vaccine (CV) targets are close to self antigens and therefore generally less immunogenic. However, in the last decades, the identification of tumor-associated antigens (TAA) from human malignancies and the discovery that tumors can be recognised by the immune system, stimulated a broad array of immunological strategies for the successful generation of CVs [2]. Vaccines can be developed against a specific target, such as peptide- or protein-based approaches, or they can be less well defined as whole-tumor cell lysates. In this context, several molecular and cell biology platforms have been produced including engineered DNA plasmids or recombinant viral vectors, and dendritic cell-based vaccines, in order to stimulate B cells, T cells, or professional antigen presenting cells (APCs) (Figure 1). These reagents are expected to exert antitumor effects by engaging the host immune response, and have great potential for circumventing the intrinsic drug resistance that limits standard cancer management [2].

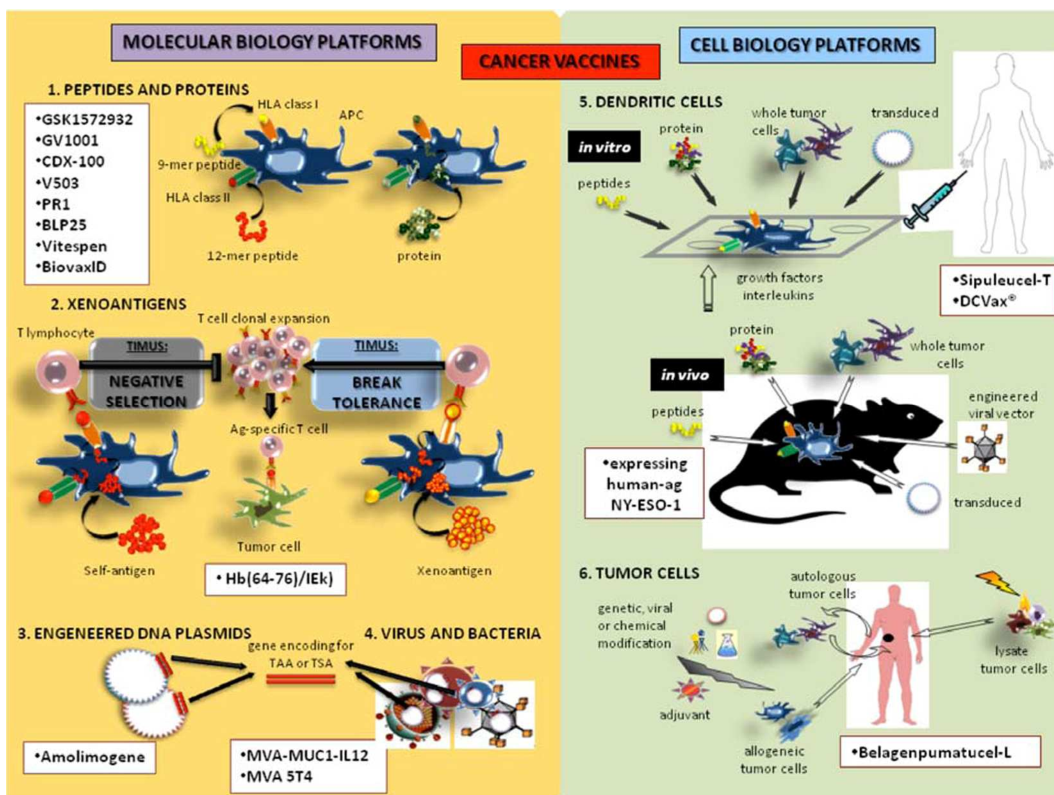


Figure 1. Molecular and cell biology-based cancer vaccine platforms. Examples of current vaccinations are reported in each platform [2].

The quest for the appropriate target

The main intent of therapeutic CVs is to "train" the patient's own immune system to specifically recognize and eliminate tumor cells. To this issue, the target selection becomes pivotal. The potential target for the immune response can be either an antigen expressed only on tumor cells (Tumor-Specific Antigen, TSA), or an antigen present mostly on tumor cells but also shared by some normal cells (TAA). TSAs would be the ideal target for immunotherapy because of their specificity and their frequent involvement in tumorigenesis and cancer progression. However, most of the mutations that identify TSAs are unique to each tumor, and thus required the development of personalized treatments. Conversely, TAAs are shared among tumors with the same histology and also of different origin, but they are limited by their weak immunogenicity due to the tolerance for self antigens acquired during the development of the immune system. Therefore, the most promising TAAs should be broadly expressed among different tumor types, and play a central role in the oncogenic process, to overcome immune escape by mutation or loss of antigen by tumor cells [1].

The validity of a vaccine target depends also on several issues, as the ability of tumor cells to present it in the context of MHC for T- and B-cell recognition, the level of expression, the relative specificity for tumor in comparison to normal tissue, and on the degree of tolerance to the given TAA [3]. The spectrum of current and potential therapeutic CV targets includes different classes of TAA: oncoproteins characterized by somatic point mutations that helps to drive the neoplastic process, as for example mutant RAS in colon and pancreatic cancer, or overexpressed oncoproteins such ad HER2/neu and the carboxy terminus of MUC-1; oncofetal antigens like the carcinoembryonic antigen (CEA) and underglycosylated MUC-1, and cancer-testis antigens such as melanoma-associated antigens (MAGE); "tissue-lineage" proteins, overexpressed in tumors as the melanoma-associated antigens glycoprotein 100 (gp100) and the tyrosinase, and normally expressed in a non-vital organ, like prostate-specific antigen (PSA); molecules associated with cancer "stem cells" and/or the epithelial–mesenchymal transition (EMT) process; and viral proteins [3]. Nevertheless, most of these antigens derive from self-proteins, which may induce immune tolerance. On the other hand, the mutation or modification of self-antigens entails the development of immunogenic TAA. This aspect represents a challenge for the appropriate design of vaccines that have to overcome the immune tolerance in order to elicit specific antitumor immunity without autoimmune side effects. The induction of this specific immune response may cause the selection and expansion of tumor variants, which lack the target tumor antigen and become resistant to the vaccine-induced immune response. Otherwise, such tumor variants may induce a beneficial effect, broadening the immune response against newly expressed antigens not present in the original vaccine in a process defined as "epitope spreading" [4].

The strategy to identify suitable TAAs is relatively easier in hematological disease if compared to solid tumors. Lymphomas originated from B cells, in fact, are characterized by the expression of the immunoglobulin Idiotype (Id), which characterizes the B-cell receptor (BCR) of every single B lymphocyte, and is maintained by the tumor clone during the tumorigenesis process. For these reasons, sequence analysis for the identification of cancer-related mutations can be focused on the immunoglobulin Id, which can be used for the development of a patient-specific vaccine [4].

Selection of patients and clinical trial response endpoints

Recent studies revealed two major issues in the optimal design of clinical trials testing the efficacy of CVs: the selection of a suitable patient population and the choice of the most appropriate clinical trial endpoints [3]. Generally, phase I and some phase II clinical trials are performed on patients with advanced disease and after administration of multiple chemotherapy regimens, thus with severe immune-impairment [2]. In heavy tumor-burden patients, the ability of vaccines to generate anti-tumor immune response is strongly limited by numerous tumor-produced immunosuppressive factors (eg, transforming growth factor-β and vascular endothelial growth factor) and immunoregulatory mechanisms (eg, regulatory T cells [Tregs] and myeloid derived suppressor cells) [5]. Consequently, patients with large tumor burdens would be even less likely to respond to vaccines than to chemotherapy [3].

Conversely, there are several examples of greater vaccine efficacy in patients with low-grade or indolent disease, and in individuals who have received fewer regimens of chemotherapy with a longer time since their last chemotherapy [3]. Thus, the main limitations observed in the first clinical trials can be overcome by testing CVs in the setting of minimal residual disease or in patients with complete remission after primary treatment [2].

Moreover, the optimal clinical trial setting for testing a novel therapeutic CV formulation may not fit the traditional model developed for chemotherapy drug trials, and one may need to select different endpoints such as molecular remission rate and time to progression, rather than response rates [6]. Immune responses often take time to develop and can be potentially enhanced by continued booster vaccination, thus vaccines have the potentiality to apply antitumor activity for a long time, resulting in a slower tumor growth rate. This process may continue for months or years and through subsequent therapies, leading to improved overall survival (OS), but maybe with low rate of objective response [3].

Therefore, early clinical trials with vaccines may have been terminated prematurely when tumor progression occurred, often before a sufficient number of vaccine boosts could have been administered. This phenomenon has led to the quest of a new design for CV trials and to the identification of more suitable immune response criteria [3].

Advantages and drawbacks of current cancer vaccines

The therapeutic approach of CVs has several advantages if compared with conventional anti-cancer treatments, especially if toxicity is considered. Differently to chemotherapy, therapeutic vaccination has the potential ability to circumvent drug cross-resistance and to induce a persistent antitumor effect due to immunologic memory, thus reducing side effects. Moreover, the development of therapies specifically targeting TAAs has the advantage to direct the immune response mainly against tumor cells and only few other normal tissues [7-9].

Unfortunately, despite the promising results coming from pre-clinical models, the first clinical data about CVs were quite disappointing. Several reasons may explain the limited clinical success of anti-tumor vaccination, including the trial design, the specific vaccination approach and host-related factors [2]. One of the main limitations is due to the impaired interplay between tumor and host immune system, which involves the selection of highly aggressive transformed cells that no longer express cancer-specific molecules, the induction of immune tolerance mechanisms (for example the down-regulation of the antigen-processing machinery, the production of immunosuppressive cytokines, the lack of co-stimulatory molecules) and the recruitment of tolerizing dendritic cells and Tregs, which negatively contrast the immune response [2].

One of the main drawbacks of CVs is also the functional dissociation between systemic and local immune responses. This is mainly linked to the lack of reliable immunologic surrogate

markers of clinical response. Antibody production has been reported to correlate with clinical outcome only in some cases [7,8], while findings regarding the diagnostic significance of T-cell responses are conflicting. Some papers claimed indeed that no currently available *in vitro* assays can accurately mirror the *in vivo* antitumor activity of T-cells [2], whilst others were able to show a clear correlation between clinical outcome and tumor-specific T-cell responses in the peripheral blood [10-13].

Presently, CVs can probably have an important role as adjuvant to traditional therapies in the management of minimal residual disease, in particular for patients who are at high risk to relapse [2]. In this context, a recent randomized phase III study reported by Schuster et al [14] suggests that it would be possible to identify a subgroup of patients with follicular lymphoma in first remission after standard treatment, who may benefit from receiving an anti-Id vaccination [15].

The current management for B-cell Non-Hodgkin Lymphomas

Non-Hodgkin Lymphomas: a heterogeneous group of cancers

Lymphomas are a heterogeneous group of solid tumors of the immune system. Hodgkin's lymphomas account about 10% of lymphomas, while the remaining 90% are referred to as Non-Hodgkin's lymphomas (NHLs). NHLs have a wide range of histological appearances and clinical features at presentation, 85-90% arise from B lymphocytes, the remainder originates from T cells or natural killer (NK) cells. This diverse group of malignancies usually develops in the lymph nodes, but can occur in almost any tissue, and ranges from the more indolent follicular lymphoma, to the more aggressive diffuse large B-cell and Burkitt's lymphomas. Several different classification systems have been proposed to group these malignancies according to their histological characteristics [16]. The firt Rappaport (1966) and Working Formulation (WF, 1982) classifications were based on histological characteristics, with the Kiel system (1988) the T/B-cell origin was introduced in the classification parameters, and finally the REAL (Real European American Classification of Lymphoid Neoplasm, 1994) and the most recent WHO classification (2008) takes account also phenotypic, clinical and genetic features[1]. The following list shows the WHO classification of mature B-cell NHL (B-NHL) in force since 2008 [17]:

- Chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma
- B-cell prolymphocytic leukemia
- Splenic marginal zone lymphoma (SMZL)
- Hairy cell leukemia
- Splenic lymphoma/leukemia, unclassifiable
  - Splenic diffuse red pulp small B-cell lymphoma
  - Hairy cell leukemia-variant
- Lymphoplasmacytic lymphoma
  - Waldenström macroglobulinemia
- Heavy chain diseases
  - Alpha heavy chain disease
  - Gamma heavy chain disease
  - Mu heavy chain disease
- Plasma cell myeloma
- Solitary plasmacytoma of bone
- Extraosseous plasmacytoma
- Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)

[1] http://www.cancer-amcc.org/FICHIERS/files/Principles%20of%20WHO%20Classification%20for%20Lymphomas.pdf

Nodal marginal zone B-cell lymphoma (MZL)
    Pediatric type nodal MZL
Follicular lymphoma (FL)
    Pediatric type follicular lymphoma
Primary cutaneous follicle center lymphoma
Mantle cell lymphoma (MCL)
Diffuse large B-cell lymphoma (DLBCL), not otherwise specified
    T cell/histiocyte rich large B-cell lymphoma
    DLBCL associated with chronic inflammation
    Epstein-Barr virus (EBV)+ DLBCL of the elderly
Lymphomatoid granulomatosis
Primary mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
Primary cutaneous DLBCL, leg type
ALK+ large B-cell lymphoma
Plasmablastic lymphoma
Primary effusion lymphoma
Large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease
Burkitt lymphoma
B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma
B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma

Since the various types of B-cell lymphoma can have different clinical behaviors, and thus require diverse treatment strategies, another classification (based on the previous REAL system) was proposed for lymphoproliferative disorders according to whether they belong to the indolent (slow growing) or aggressive subtype. This system includes also T- and NK-cell lymphomas, as well as Hodgkin's diseases [18][2]:

Indolent lymphoma/leukemia

A. Follicular lymphoma (follicular small cleaved cell [grade 1], follicular mixed small cleaved and large cell [grade 2], diffuse small cleaved cell)
B. Chronic lymphocytic leukemia/small lymphocytic lymphoma
C. Lymphoplasmacytic lymphoma (Waldenstrom's macroglobulinemia)
D. Extranodal marginal zone B-cell lymphoma (MALT lymphoma)
E. Nodal marginal zone B-cell lymphoma (monocytoid B-cell lymphoma)
F. Splenic marginal zone lymphoma (splenic lymphoma with villous lymphocytes)
G. Hairy cell leukemia
H. Mycosis fungoides/Sezary syndrome
I. T-cell granular lymphocytic leukemia
J. Primary cutaneous anaplastic large cell lymphoma/lymphomatoid papulosis (CD30+)
K. Nodular lymphocyte predominant Hodgkin's lymphoma

Aggressive lymphoma/leukemia

A. Diffuse large cell lymphoma (includes diffuse mixed cell, diffuse large cell, immunoblastic, T-cell rich large B-cell lymphoma) Distinguish:
    1. Mediastinal large B-cell lymphoma
    2. Follicular large cell lymphoma (grade 3)
    3. Anaplastic large cell lymphoma (CD30+)
    4. Extranodal NK/T-cell lymphoma, nasal type
    5. Lymphomatoid granulomatosis (angiocentric pulmonary B-cell lymphoma)
    6. Angioimmunoblastic T-cell lymphoma
    7. Peripheral T-cell lymphoma, unspecified
    8. Subcutaneous panniculitis-like T-cell lymphoma
    9. Hepatosplenic T-cell lymphoma

[2] (http://www.nhlcyberfamily.org/classification.htm)

10. Enteropathy-type T-cell lymphoma
11. Intravascular large B-cell lymphoma

B. Burkitt lymphoma/Burkitt cell leukaemia/Burkitt-like lymphoma
C. Precursor B- or T-cell lymphoblastic lymphoma/leukaemia
D. Primary CNS lymphoma
E. Adult T-cell leukaemia/lymphoma (HTLV 1+)
F. Mantle cell lymphoma
G. Polymorphic post-transplantation lymphoproliferative disorder (PTLD)
H. AIDS-related lymphoma
I. True histiocytic lymphoma
J. Primary effusion lymphoma
K. Aggressive NK-cell leukemia/blastic NK-cell lymphoma
L. B- or T-cell prolymphocytic leukemia

The annual incidence of B-NHL is about 12 cases in 100,000 inhabitants in Western Europe [19]. In 2007, around 65,540 people were diagnosed with NHL in the USA, and about 20,210 people died of the disease in 2008 [20]. NHL is the fifth most frequently diagnosed cancer in the UK, and more than two-thirds of patients are 60 years and older. The male age-standardized incidence per 100,000 men (17.7) is higher than the female incidence (12.8), while the frequency of specific subtypes of lymphoma varies substantially by geographic region. For example, adult T-cell lymphoma associated with infection by human T-cell lymphotropic virus type 1 (HTLV-1) is much more frequent in east Asia than in other regions, as is nasal NK-cell or T-cell lymphoma associated with Epstein-Barr virus infection, whereas follicular lymphomas are more frequent in western Europe and North America. Diffuse large B-cell lymphoma, by contrast, is common worldwide [16]. The annual mortality from B-NHL ranges around 5 in 100,000 inhabitants, but the individual probability of survival is highly determined by the histological subtype, age, clinical stage and additional risk factors [19].

Several causes have been linked with the etiology of B-NHLs, the most established risk factor is immunosuppression. Indeed, an increased risk to develop high-grade NHL is observed in patients with human immunodeficiency virus (HIV), organ-transplant recipients, patients who have had high-dose chemotherapy with stem-cell transplantation, and those with inherited immunodeficiency syndromes or autoimmune disease. Different infectious agents have been associated with lymphoma development, either by inhibition of the immune function, or by induction of a chronic inflammatory response [16,18]. The Epstein-Barr virus (EBV) for example plays a role in Burkitt's lymphoma etiology [21], another member of the herpes-virus family, human herpes virus 8 (HHV8), is implicated in the pathogenesis of primary effusion lymphomas [22], while Helicobacter pylori is a risk factor for gastric MALT lymphomas [23], hepatitis C virus (HCV) has been associated with splenic marginal zone lymphoma [24], Borrelia burgdorferi has been found in cutaneous MALT [25], and Chlamydophila psittaci infection is involved in ocular adnexal lymphomagenesis [26]. Other than pathogen-derived antigens that cause chronic infections, certain autoantigens can also stimulate reactive B cells. This concept is also supported by the observation that several autoimmune diseases, such as Sjogren's syndrome, rheumatoid arthritis and autoimmune lymphoproliferative syndrome are commonly associated with an increased risk of lymphoma [18]. Moreover, studies in different lymphoma types (B-CLL, follicular lymphoma, MALT lymphomas) indicated that lymphoma cells are able to recognize autoantigens or foreign antigens, and that stimulation by antigen binding contributes to survival and proliferation of lymphoma cells [18]. Recently, the characterization of several subgroups of B-CLL revealed the expression of strikingly similar VH and VL gene-rearrangement sequences among members of the same group [27-31], reflecting the selection and activation of these cases by a restricted set of antigenic epitopes [18].

Reciprocal translocation involving one of the immunoglobulin gene and a proto-oncogene are a hallmark of many types of B-cell lymphomas. This translocation leads to a deregulated, constitutive expression of the oncogene. Somatic hypermutation and class-switch recombination may be the molecular processes mainly involved in chromosomal translocation. Both of them occur mainly in the germinal center (GC), and probably it is for this reason that most of B-cell lymphomas derive from GC B-cells or their descendents (Figure 2). Moreover, these processes do not occur in the DNA of T-cells, which could also partly explain why B cells are more prone to undergo malignant transformation than T cells [18].

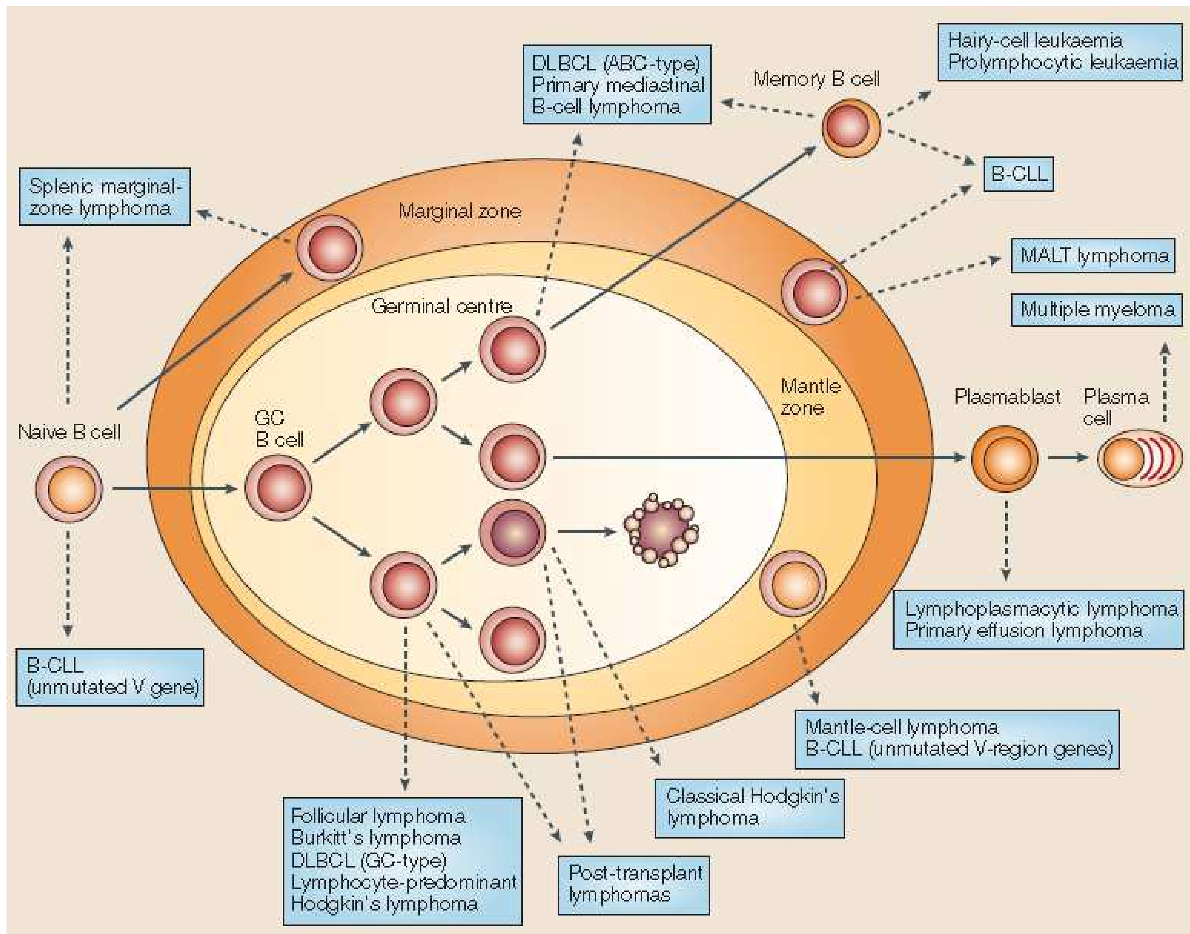


Figure 2. Cellular origin of B-cell lymphomas [18].

It is well known that BCR supplies important survival signals to normal B cells and recent observations support this BCR critical role also in lymphoma B cells. Treatment of patients with follicular lymphoma with anti-Id antibodies did not result in the emergence of BCR-negative lymphoma variants [32], demonstrating the pivotal role of BCR in cancer cell survival [18]. Moreover, immunoglobulin loss variants have rarely been described and only in certain B-NHL, such as follicular lymphoma [6].

The most widely used prognostic model for patients with B-NHL is the International Prognostic Index (IPI), which delineate four risk groups considering independent clinical features such as age (≤60 years vs>60 years), lactate dehydrogenase concentration (normal vs. abnormal), Eastern Cooperative Oncology Group performance status (<2 vs ≥2), Ann Arbor stage (I/II vs. III/IV), and number of extranodal sites implicated (≤one vs. >one). The groups are identified with the number of unfavorable clinical features involved: low risk (zero to one clinical feature), low-intermediate risk (two features), high-intermediate risk (three features),

and high risk (four to five features) [16]. The IPI together with the WHO classification and the aggressive subtypes are all relevant for the choice of the most suitable therapeutic strategy.

Therapeutic approaches

Standard treatments

Over the years, several randomized trials compared the efficacy of different combinations of standard chemo- and radio-therapy in distinct lymphoma histotypes [16]. The purine analogues cladribine and, particularly, fludarabine have activity against small B-cell lymphocytic lymphoma and chronic lymphocytic leukaemia [33]. Bendamustine is a native first-line treatment option for patients for whom fludarabine containing regimens are not appropriate.

Conversely, FL is not usually curable with conventional treatment. The exception is the few patients with limited-stage disease, who can be cured with radiotherapy, a few patients with exceptional responses to initial chemotherapy regimens, and some patients after autologous or allogeneic haemopoietic stem-cell transplantation as second-line therapy. Other treatment regimens include oral chlorambucil, fludarabine, or bendamustine; or combination chemotherapies with cyclophosphamide, vincristine, and prednisolone; or anthracycline-containing regimens such as CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) [34].

Several studies have assessed the role of high-dose chemotherapy and autologous stem-cell transplantation in patients with mantle-cell lymphoma. For the rare, young patients with an HLA-matched donor, allogeneic haemopoietic stem-cell transplantation can be curative [35].

Surgery has proved as the therapeutic choice for localized mucosa-associated lymphoid tissue lymphomas at all sites, and in particular for a rare subtype, splenic marginal-zone lymphoma, which involves the spleen, blood, and bone marrow.

In localized diffuse large B-cell lymphoma (25% of patients) findings from trials comparing radiation alone with radiation plus combination chemotherapy with cyclophosphamide, vincristine, and prednisolone or CHOP, showed an improvement in OS for dual modality therapy, which became the standard treatment. However, disseminated disease is the most common presentation of diffuse large B-cell lymphoma, and about a third of patients with this form of the disease were cured with chemotherapy alone, most by the CHOP regimen [36].

For highly aggressive forms as Burkitt's lymphoma, principles of chemotherapy delivery include maintenance of high dose intensity and the use of alternating non cross-resistant regimens to prevent the emergence of drug resistance [16].

Nevertheless, conventional chemotherapy yields high remission rates in lymphoma by eliminating actively dividing cells, meanwhile a quiescent but lethal subpopulation (i.e., cancer stem cells) may be responsible for disease relapse and its incurability with standard therapy [5].

The introduction of monoclonal antibodies

In the management of lymphoma, especially for the indolent forms, new therapeutic modalities that target tumor cells more specifically and with less toxicity are needed [37]. In this context, the concept of immunotherapy is appealing. Among malignant diseases, lymphoma is ideally suited to immunotherapy. Indeed, B-cell lymphomas express either class I and II MHC molecules, which enables them to present their own tumor associated antigens to both CD8 and CD4 T cells [5].

The first developed antibody-based therapy for B-cell lymphoma tested anti-Id monoclonal antibodies (mAbs). Although these antibodies were then abandoned because they need to be tailored for each patient, they provided evidence that antibody-based therapy can

be safe and highly effective [38], paving the way for subsequent development of the anti-CD20 mAb rituximab [37]. Rituximab (Rituxan, Mabthera) is a chimeric anti-CD20 antibody derived from the mouse mAb 2B8, targeting CD20 antigens, following replacement of the heavy and light chain constant regions with the corresponding regions of a human IgG1 mAb. It depletes both normal and malignant B cells [39]. Four major mechanisms have been proposed for the action of rituximab: antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis, complement-dependent cytotoxicity (CDC), direct induction of apoptosis, and "vaccination effect" resulting from cross-priming. These mechanisms have been demonstrated *in vitro* and in mouse models, and clinical observations support their activity in patients [37].

As a single agent or in combination with chemotherapy, rituximab was able to improve the overall response rate (ORR), the duration of response and OS in several lymphoma subgroups [37]. Findings from one large trial showed an improvement in OS in patients with previously untreated CLL given fludarabine, cyclophosphamide, and rituximab compared with those given chemotherapy alone [16]. Furthermore, since phase III randomised controlled trials have shown improved OS with the addition of this agent to chemotherapy, current guidelines for the treatment of FL recommend that rituximab is administered in combination with standard chemotherapy in previously untreated stage III–IV FL, and at first relapse, and it is recommended as a monotherapy for stage III–IV chemoresistant FL, or at second relapse after chemotherapy [16,39,40]. Studies assessing the addition of rituximab to standard chemotherapy in mantle cell lymphoma have shown significant improvements in complete response rates and time-to-treatment failure, but disappointingly not in OS [16]. Data from the Surveillence, Epidemiology, and End Results (SEER) registry comparing survival in all patients with Diffuse Large B-Cell Lymphoma (DLBCL) from 1973–2004 show that survival has substantially improved since the advent of rituximab [16]. For this reason, rituximab has been approved for previously untreated DLBCL patients in combination with CHOP chemotherapy and with salvage chemotherapy regimens in relapsed/refractory patients [39]. Moreover, several studies are assessing the role of rituximab as part of high-dose regimens (HDT) pre-autologous stem cell transplantation (ASCT) in young high-risk patients with DLBCL achieving a complete remission after a complete course of chemotherapy [39].

Some new generation mAbs are currently undergoing clinical investigation. In particular, humanized anti-CD20 mAbs have been produced with the aim to enhance rituximab mechanism of action and reduce resistance phenomenon. Among them, one of the most promising candidates is the fully human type I Ofatunumab. The monotherapy with this mAb seems to be ineffective in rituximab-refractory FL; however, studies of ofatumumab combined with CHOP chemotherapy in patients with FL are ongoing [37]. The mAb appears to be more effective in CLL than in FL, as demonstrated by the FDA approval and conditional approval in Europe for the treatment of refractory CLL [16]. Some other examples of new-generation mAbs include Epratuzumab, a humanized IgG1 anti-CD22 antibody, which induced objective response in relapsed FL and DLBCL; Galiximab, a primatised anti-CD80 (IgG1λ) mAb able to enhance rituximab antitumor activity in previously untreated NLH patients, with a response reported in 70% of patients; Alemtuzumab, a humanized monoclonal antibody against CD52, indicated for the treatment of patients with B-CLL refractory to fludarabine, and for advanced-stage mycosis fungoides/Sezary syndrome; Apolizumab, a humanized anti-HLA-DR antibody, used in combination with rituximab in relapsed B-CLL [39] (Figure 3).

Finally, to further exploit the ability of mAbs to reach the tumor site, radioimmunotherapy has been developed linking a radioisotope to the antibody. This approach permits the targeting of the radioactive isotopes to cancer tissues and is especially interesting as it allows for killing neighboring cancer cells that either are inaccessible to the antibody or

express insufficient antigen for the antibody to bind in adequate quantities. Two anti-CD20 radioimmunoconjugates are approved for use in patients with relapsed or refractory follicular or low-grade lymphoma: Yttrium-90-labelled ibritumomab tiuxetan (zevalin), and iodine-131-labelled tositumomab (bexxar) [16,37,39] (Figure 3).

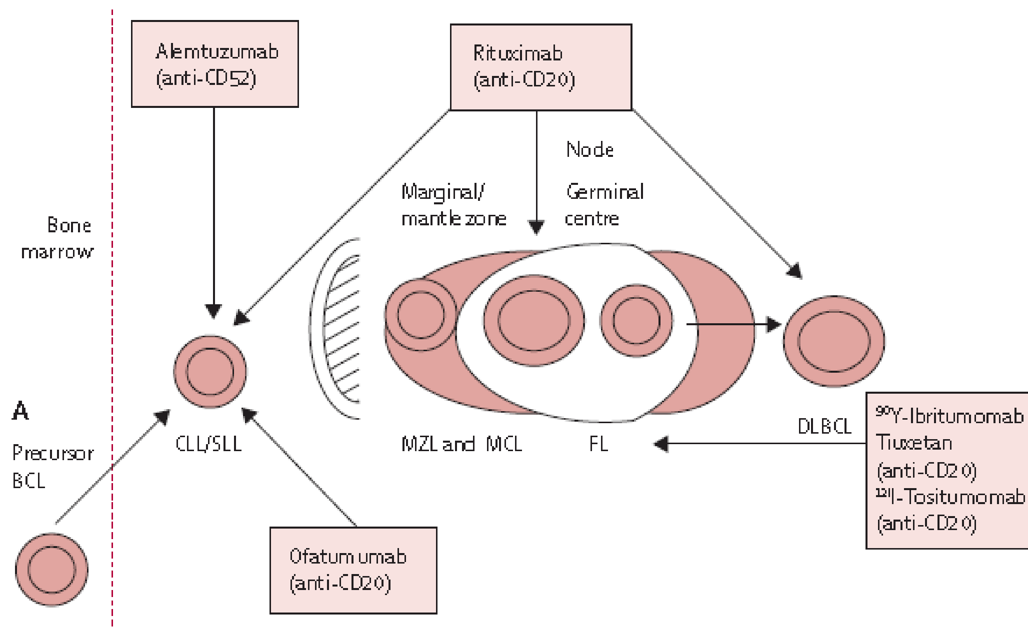


Figure 3. Monoclonal antibodies (and therapeutic targets) employed in the current management of B-NHL (modified by [16]).

Treatment of recurrences and relapses

Efforts to improve rituximab and other mAbs efficacy induced a general enhancement of remission and OS in B-NHL patients, but eventually patients still relapse from residual tumor cells and die of the disease [37]. Indeed, patients usually respond well to the first-line of rituximab and chemotherapy induction, but typically experience repeated relapses and shortening of the time from treatment to treatment [41]. Current guidelines recommend the use of rituximab as a maintenance therapy only in relapsed or refractory FL responding to induction therapy with chemotherapy with or without rituximab [39]. A highly statistically significant progression-free survival benefit is observed during rituximab maintenance. This advantage is not translated to a statistically significant overall survival benefit after first induction treatment in patients with follicular lymphoma. This may be because a longer follow-up is required to demonstrate a statistically significant difference in survival of patients in first remission whose estimated survival is in the range of decades, as opposed to few years in patients in second or third remission [41].

However, most of these patients are not cured and experience relapse after a median of 4 years [41]. Moreover, rituximab maintenance treatment was associated with a higher rate of grade 3 or 4 adverse events and with a higher rate of infections compared with the no maintenance [41].

Rituximab tolerability and mechanisms of resistance

Adverse events were reported in 84% of patients receiving rituximab, during therapy or within the first 30 days following treatment. The most common adverse effects were infusion-related reactions and lymphopenia. Serious adverse effects included severe infusion related reactions, tumor lysis syndrome, mucocutaneous reactions, hypersensitivity reactions, cardiac arrhythmias, angina, and renal failure. One possible haematological adverse event is the

reduction in peripheral B lymphocyte counts, which can last for up to 6 months with a recovery period of 9 to 12 months [39].

Despite being the most successful anti-cancer mAb to date, the effectiveness of rituximab is ultimately limited due to the development of resistance with relapse of the disease. About 30% of patients with aggressive B-NHL develop primary resistance mechanisms, whereas a fraction of indolent B-NHL acquires rituximab resistance only after relapsing [19]. It is currently unclear, whether such secondary resistance to rituximab is based on similar mechanisms as primary unresponsiveness; however, it was demonstrated that rituximab resistance is mediated by both tumor and host factors. Lymphoma cells are able to increase the expression of complement regulatory proteins that impair CDC and ADCC, and to overexpress anti-apoptotic proteins or down regulate pro-apoptotic proteins [37]. The CD20 molecule itself can be involved in resistance to rituximab, by down-modulation, structural changes or loss of its expression. Reduction or loss of CD20 cell surface expression following rituximab treatment has been reported in some patients with B-CLL, and seems to depend on epigenetic mechanisms involving the CD20 promoter or CD20-regulating transcription factors [19]. The immune recognition of CD20-bound rituximab can also depend on the patients' genetic background. Specific FcR or complement component polymorphisms correlate with reduced efficacy of rituximab-based immunotherapy. A well-defined example is the FcR polymorphism FcRIIIa-V158F, which is associated with impaired ADCC in FL, even if it failed to predict the response to rituximab in CLL [19,37,42]. Host-related resistance may be mediated also by exhaustion of complement components or effector cells [37].

To overcome these limitations and the onset of rituximab resistance, many combination therapies have been proposed to modulate intracellular signal transduction, to inhibit proliferation, and to induce apoptosis. For example, combining rituximab with inhibitors of specific pathways involved in CD20 down-modulation, such as Temsirolimus, or with anti-proliferative agents able to sensitize lymphoma cells to rituximab, like Bortezomib, or with immunostimulatory molecules, which can boost ADCC, as GM-CSF or IL-2 [19,37]. Disappointingly, response rates to these combination treatment were not significantly different from those obtained with rituximab alone [37].

One of the main advantages of passive immunotherapy employing mAbs, is the relative independence by the patient's immune system—which may be dysfunctional because of tumor burden or prior therapies—to initiate a response. However, as the targeted lymphoma cells mutate, down-regulate, or otherwise alter the targeted antigen, the main disadvantage is the development of resistance to therapy [5].

<u>*Cell-based therapy*</u>

Besides mAb, as described above, the efficacy of cell-based therapies in B-NHL has been repeatedly proved by different clinical trials, testing allogeneic transplantation, donor-lymphocyte infusion (DLI), and therapies using *ex vivo* expanded T and NK cells, such as lymphokine-activated killer cells, cytokine-induced killer cells, or CD3/CD28-expanded cells. The therapeutic index of such therapies might be improved by specifically targeting lymphoma antigens. Early attempts employed T cells with a natural T-cell receptor selected *ex vivo* to target EBV antigens expressed by distinct lymphoma histotypes [43]. However, a major limitation of adoptive T-cell transfer is the potential to select escape tumor variants, because these therapies are inherently restricted to a single or a few antigen targets. Still, T cells have several advantages as compared with mAbs, including their capacity to migrate to sites of lymphoma, to proliferate, and to take advantage of the lymphodepleting effects of standard cytotoxic therapies [5].

Active immunotherapy for low-grade B-cell lymphomas

In addition to the aforementioned therapeutic choices, many efforts have been done to develop non-toxic approaches as consolidation therapy for different types of B-NHL, after the induction of a first remission with traditional, mAb- or cell-therapy. Agents used for consolidation therapy should ideally have a different mechanism of action compared to induction therapy, because residual tumor cells can be resistant to these approaches. Moreover, they should be safe, improve the duration of remission, and they should not preclude the efficacy of future therapies [44]. In this context, an alternative approach is to vaccinate the patient with a pool of potential target antigens and to allow the immune system to select the most effective BCRs and T-cell receptors (TCRs) [5]. So, the immune system can select novel target overlooked by the guided process of passive immunotherapy and develop a polyclonal response directed against multiple epitopes, hence limiting tumor escape that gives rise to relapse [5,37]. On the other hand, the disadvantage of active immunotherapy is its reliance on the patient's immune system, which may be dysfunctional after the former cycles of chemotherapy [37]. Therefore, the most suitable candidates for active immunotherapy approaches are patients affected by indolent FL in first remission. The indolent nature of FL allows the patients' immune system to recover from the immunesuppression induced by previous therapies. In addition, FL is regarded as particularly immune responsive based on high response rates to mAb treatment, occasional responsiveness to interferon-α and IL-2, and correlation of survival time with gene expression profile of tumor infiltrating immune cells, and especially with the expression of genes encoding T-cell markers [37,45].

Active immunotherapy trials so far consist largely on vaccines that use the immunoglobulin Id as a tumor-specific antigen. A major obstacle in production of Id vaccines derives from their patient-specific nature that requires the generation of a custom-made product. However, Id may be actually used as either protein or DNA in therapeutic vaccines [37]. Id vaccination does not induce immunosuppression, through its mechanism of action it should bypass the development of resistance to rituximab, and does not interfere with possible future therapies. Thus, Id vaccines are ideal candidate as consolidation therapy, and may prolong remission duration without risks associated with currently available consolidation therapies [44]. Nevertheless, unlike passive immunotherapy, regulatory approval has not yet been granted to any regimen of active immunotherapy for lymphoma, despite evidence of biological and clinical efficacy as well as clinical benefit of Id vaccination in lymphoma patients [37]. Further studies and clinical trials are therefore required to consolidate the potentiality of this promising therapeutic approach, in particular as maintenance therapy.

The idiotype as a target for tumor vaccination

Idiotype features

Unlike most other proteins, which, from an immunological standpoint, can function only as antigens, antibodies can function both as antigen-recognizing and -binding molecules and as antigens by virtue of several types of epitopes found on their heavy and light chains [46]. Each B lymphocyte expresses on its surface an immunoglobulin (Ig) molecule, capable of recognizing and binding to a unique epitope. This unique molecular structure is based on the variable regions of the heavy and light chains, and the hypervariable region of the antibody is called the Idiotype (Id) [15] (Figure 4). The Ig sequences differ in 3 short sections within the variable regions (V) of the heavy (H) and light (L) chains, called hypervariable regions or Complementarity-Determining Regions (CDRs). The assembly of the 3 VH-CDRs with the same

regions in the VL develops in a three-dimensional structure responsible for the antigen binding (the BCR). Antigen recognition depends specially by the CDR3 region, which is the closest to the C-terminal sequence and the most variable one. The extreme variability of the BCR structures allows the development of immune responses against a broad number of foreign antigens. Sequence variability arises from the differential usage of several genes, from the genetic recombination, and from the somatic hypermutation. The Ig chains are defined by 3 distinct gene loci, which respectively encode for the light chain κ, the light chain λ, and the heavy chains. The rearrangement, which concerns in particular genes encoding for the variable regions, entails random nucleotide insertions or deletions in the junction points among the V, D, and J segments, and thus results in genes with different sequence and length. Accordingly, 1 x 10^8 to 1 x 10^{12} possible recombinations may potentially encode different BCR structures.

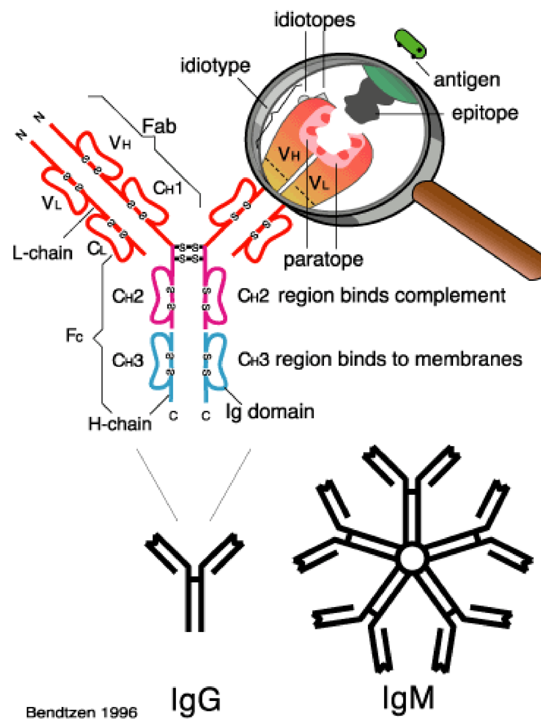


Figure 4. Immunoglobulin structure and idiotype features[3].

Since B-cell lymphomas are clonal in nature, arising from a single B-cell, the Ig rearrangement is preserved by the neoplastic clone, and the consequent Id results differently expressed from the Id of their non-malignant counterparts. Therefore, the Id is regarded as a tumor-specific antigen that can be targeted by immunotherapy [37], provided that the tumor cells express it on their cell membrane either intact as it is in its regular function as B-cell receptor and/or in the form of idiotopes associated with the HLA molecules for epitope presentation [46]. Idiotopes are hence defined as a peculiar variety of antigenic determinants or epitopes exclusively found on the immunoglobulin's heavy and light chain variable regions.

The variable region of the tumor immunoglobulin is different from patient to patient, thus the Id is considered patient-specific and is not a universal tumor antigen. Therefore, the use of idiotype as a therapeutic lymphoma vaccine would require the generation of a custom-made product for each patient [6].

[3] http://www.inflammation.dk/iir/05ig/_igx.htm

Successes and failures of current idiotypic vaccination for B-cell Non-Hodgkin Lymphomas

Compared to the passive anti-Id mAb therapy, abandoned relatively soon due to the onset of Id variants not targetable by the Id-specific Ab, anti-Id vaccination is able to induce a more complete immune activation generating a polyclonal Id-specific humoral and cellular immune response. Since the contribution of both humoral and cellular immunity to reach a survival benefit is still under investigation, it should be highly desirable to elicit both of them [46]. Furthermore, to enhance the immunogenicity of the native Id, based on preclinical and early clinical studies, the Id protein has been coupled to the powerful immunogenic keyhole limpet hemocyanin (KLH) with the addition of granulocyte–macrophage colony-stimulating factor (GM-CSF), which facilitates the induction of tumor-specific $CD8^+$ T-cells [37]. Id-KLH coupled formulation is usually administrated subcutaneously on day 1 of the therapeutic schedule together with a dose of GM-CSF, which is subsequently delivered over the following 3 days. GM-CSF dose and vaccination plan may vary from trial to trial [46]. The antigen is uptaken in the skin by APCs, thus inducing their activation and migration to lymph nodes, where T cell priming takes place.

Two phase I/II clinical trials performed with this vaccine formulation in FL patients in first chemotherapy-induced complete remission, showed the generation of an Id-specific immune response, the clearance of residual tumor cells detected by PCR, and an enhancement of progression-free survival [47,48] (Figure 5). In a phase II study performed in a second chemotherapy-induced complete response, the Id-KLH plus GM-CSF formulation induced a specific immune response in the majority of patients (80%) with FL, and a highly significant increase in disease-free survival [49]. Moreover, in a phase II trial in patients with measurable disease, Id-vaccination alone was able to induce tumor regression and durable clinical response [50] (Figure 5).

Formulation	Induction Therapy	No. of patients	Histology	Anti-Id/tumor immune responses (%)		Clinical response (%)	Molecular response (%)	Ref
				Ab	T-cell			
Phase I/II idiotype vaccine trials after non-rituximab-based chemotherapy								
Id-KLH+SAF	Various	41	FL	41	17	2/20 (10)	n/a	Kwak, *et al* 1992 Hsu, *et al* 1997
Id-KLH+GM-CSF	PACE	20	FL	75	95	n/a	8/11 (73)	Bendandi, *et al* 1999
Id-DC/Id-KLH-DC	None/CVP/CHOP	35	FL	26	49	10/28 (36)	Reported in 1 patient	Hsu, *et al* 1996 Timmerman, *et al* 2002a
Plasmid DNA	CVP	12	FL	0	8	1/12 (8)	n/a	Timmerman, *et al* 2002b
Id-KLH+SAF	Various	9	FL	89	n/a	2/9 (22)	3/5 (60)	Barrios, *et al* 2002
Liposomal Id/IL-2	PACE	10	FL	40	100	Reported in 1 patient	n/a	Neelapu, *et al* 2004
Id-KLH+GM-CSF	CHOP-like	25	FL	52	72	n/a	3/10 (30)	Inoges, *et al* 2006
Phase I/II idiotype vaccine trials after rituximab-based chemotherapy								
Id-KLH+GM-CSF	DA-EPOCH-R	26	MCL	30	87	n/a	n/a	Neelapu, *et al* 2005b
Id-KLH+GM-CSF	Rituximab	89	FL	13	80	47% → 63%[a]	n/a	Koc, *et al* 2005
Phase I/II idiotype vaccine trials as single agent								
Id-KLH+GM-CSF	None	32	FL	20	67	4/31 (13)	n/a	Redfern, *et al* 2006
Id(Fab)-MF59+GM-CSF	None	18	FL	29	47	2/18 (11)	n/a	Bertinetti, *et al* 2006

Abbreviations: SAF: syntex adjuvant formulation; FL: follicular lymphoma; n/a: not assessable; PACE: prednisone, doxorubicin, cyclophosphamide, etoposide; DC: dendritic cell; CVP: cyclophosphamide, vincristine, prednisone; CHOP: cyclophosphamide, doxorubicin, vincristine, prednisone; DA-EPOCH-R: dose-adjusted etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin, rituximab; MCL: mantle cell lymphoma.

[a] Clinical response rate improved from 47% after rituximab therapy to 63% after vaccination.

Figure 5. Summary of immunological and clinical outcomes in Id vaccine trials in B-cell NHL [6].

Id proteins were generated through hybridoma techniques as in the Bendandi et al [47] clinical trial, or by recombinant production, through PCR amplification of the tumor-specific variable region Ig sequences and cloning into expression vectors carrying the desired isotype backbone [48]. The MyVax® phase I/II clinical trial sponsored by the Genitope Corporation (Fremont, CA) demonstrated that the recombinant Id was well-tolerated, and that

immunogenicity was comparable to that of hybridoma-derived Id. Moreover, induced serum anti-Id antibodies recognized native tumor Id structure as well as recombinant Id, and could specifically bind to patient's tumor cells [48]. This technology has the advantage of a reduced time to obtain the Id-formulation, and it does not need a surgical biopsy, as adequate tumor cells can be obtained by core needle biopsy, bone marrow aspiration or sampling of involved peripheral blood. The phase III clinical trial employing this vaccine formulation corroborated the capability of this technique to obtain Id protein in more than 99% of cases in which tumor cells express surface Ig [48].

Nevertheless, this double-blind, randomized phase III study, as well as the clinical trial sponsored by Favrille Inc, found no statistically significant differences in PFS between the Id–KLH plus GM-CSF vaccine and a control vaccine containing KLH plus GM-CSF, although the vaccine was generally safe [51][4]. An interesting correlation between clinical and immune response was highlighted in the Genitope study, showing an increased progression-free survival in patients developing an anti-Id immune response, compared to patients who did not mounted such a response (40 versus 16 months). This observation suggests that there can be a group of patients, who may benefit from the Id vaccination [37]. In addition to these clinical trials, another phase III study was performed by NCI/Biovest International, and demonstrated an improved disease free survival (p=0.045) in Id-vaccinated patients if compared to the control arm [14] (Figure 6). This trial was criticized because it did not reach the sample size and the statistical power (p<0.01) originally planned [37,46].

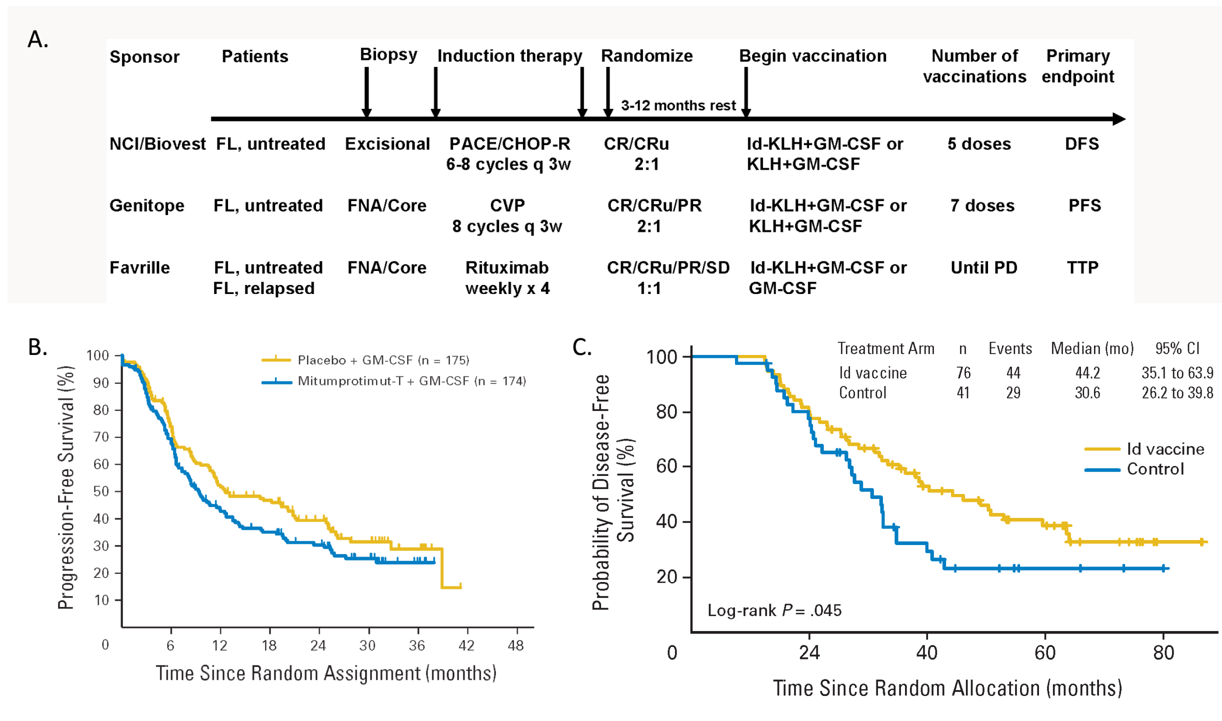


Figure 6. Phase III idiotype vaccine clinical trials. **A.** Scheme summarizing similarities and differences between the three trials [6]. NCI – National Cancer Institute; q 3w – every 3 weeks; FNA – fine needle aspiration; PD – progressive disease; DFS – disease-free survival; PFS – progression-free survival; TTP – time to progression. **B.** Time to progression of intent-to-treat population in Favrille study [51]. **C.** Disease-free survival for randomly assigned patients who received at least one dose of Id vaccine (n=76) or control (n=41) in the NCI/Biovest study [14].

[4] Levy,R., Robertson, M.J., Ganjoo,K., Leonard,J., Vose,J., andDenney,D.(2008). Results of a phase 3 trial evaluating safety and efficacy of specific immunotherapy, recombinant idiotype (Id) conjugated to KLH (Id-KLH) with GM-CSF, compared to non-specific immunotherapy, KLH with GM-CSF, in patients with follicular non-Hodgkin's lymphoma. American Association for Cancer Research Meeting Abstract, San Diego, LB-204.

There are several factors, which differentiated the 3 studies and can be responsible for the different clinical outcome observed. First patients' eligibility criteria: in Biovest study only patients in complete remission were recruited, conversely Genitope and Favrille also included patients who achieved only partial response or even stable disease after chemotherapy [3]. The persistence of significant numbers of malignant cells may make the immune environment unfavorable for the development of an adequate immune response to the vaccine [15]. In addition, the pre-vaccine therapy was different in the 3 trials, with the Favrille trial being the only one employing rituximab instead of chemotherapy as debulking agent [51]. Moreover, while Genitope and Favrille trials used recombinant Id and exclusively with the IgG isotype, Biovest vaccination was performed with hybridoma products, which could express either IgG or IgM isotypes [37]. Interestingly, the latter trial revealed that vaccination with IgM-Id but not with IgG-Id significantly prolonged disease free survival compared with isotype-matched controls, suggesting that the isotype of the constant region may influence the immunogenicity of variable region epitopes [14]. Preclinical studies investigated and beard this hypothesis showing that Ids switching to IgG became tolerogenic, whereas Ids of their IgM progenitors were highly immunogenic [52,53]. These findings suggest that the determination of the Id isotype may facilitate early identification of patients who are more likely to benefit from a vaccine approach [15], and that the Id produced by hybridoma technique may resemble more closely the native Ig on the tumor cell surface, compared with the recombinant DNA–derived Id proteins [14,44].

Notwithstanding, some observations have to be done about the real failure of Id vaccination. The lack of a statistical significance in progression-free survival between patients receiving vaccination and those receiving the control may be due to factors other than vaccination efficacy [46]. Thus, consideration should be given not only to the clinical benefit, which is the capacity to influence disease endpoints, but also to biological and clinical efficacy, which indicate respectively the vaccine capacity to elicit an immune response and to induce *in vivo* anti-tumor effects [46]. Moreover, considering that the current standard of care for FL includes the use of rituximab, a careful investigation should be done on the effects of B-cell depletion on vaccination T-cell priming [37]. Indeed, while some mouse models suggest that B-cells inhibit the induction of T-cell dependent immunity, skewing the T-helper response towards a T helper 2 profile, others prompt that B lymphocytes are necessary for T-cell priming [6].

Increasing antigen immunogenicity, delivery and presentation are other major issues of Id-vaccination. The co-administration of the Id-vaccines with immunostimulatory adjuvants as CpG may enhance the immunogenicity, activating antigen presenting cells, B cells, and NK cells by engaging TLR9 [37]. These reagents were employed also in a recent phase I study of *in situ* vaccination, which consists of low-dose irradiation to a single lymphoma site, followed by intratumoral injection of CpG at the same site. The first results were encouraging and showed that the *in situ* vaccination was able to induce tumor-reactive $CD8^+$ T cells [54]. In addition, incorporation of Id into cytokine-carrying liposomes has been proposed as a tool to enhance Id delivery [37]. Dendritic cells (DCs) loaded *in vitro* with antigen are also attractive vehicles for therapeutic cancer vaccines [37]. In this respect, Di Nicola *et al*., in a pilot study recruiting 18 patients with measurable indolent NHL who had relapsed after at least one chemoradiotherapy regimen, used unfractionated whole tumor cell preparations as the source of antigens. DCs were loaded with heat-shocked apoptotic and necrotic tumor cells. The vaccination was well tolerated without autoimmune reactions, achieved significant objective clinical responses, with enhanced antitumor activity in 6 patients (3 objective radiographic complete responses and 3 partial responses), and was associated with significant immune modulation [55]. The authors

selected the whole tumor as a source of antigen with the intent to promote activation of immune responses not only to tumor-specific Ig heavy chain-encoded epitopes, but also to the wide array of recently identified shared lymphoma-associated antigens recognized by T cells in NHL patients [55]. On these grounds, the identification of universally expressed lymphoma-specific antigens will be useful in the future to develop vaccine formulations that can be used in all lymphoma patients, being therefore easier and less costly to produce [6].

Idiotype characterization of different lymphoproliferations

Recent evidence suggests that the limitations due to the individualized production of Id-vaccines may be overcome. In fact, distinct set of stereotyped Ig have been identified in several subsets of B-NHL, suggesting that a much higher frequency of Id sharing exists among patients than appreciated previously [56]. In this context, sequencing of the immunoglobulin variable regions genes in 21 HCV-associated NHLs revealed a highly restricted use of gene segments, with all variable regions of the VH chain using exclusively the VH1, VH3 and VH4 family and the majority of VK chains expressing the kv325 (V_K3-20) and the kv328 (V_K3-15) genes [57]. The same VH family members were observed in 13 patients affected by Sjögren's Syndrome cell lymphoproliferative disorders, which also preferentially expressed a VK3 family gene [58].

A biased usage of particular VH segments has been reported also in MALT lymphomas. Gastric MALT inflammation-dependent tumors frequently used V_H3-23 and V_H3-30 fragments, which have been closely associated also with autoimmune diseases. Indeed, they have been frequently used in thymic MALT lymphoma, a tumor closely associated with autoimmune disease [59]; while salivary MALT lymphomas B-cells mainly express V_H1-69 [60].

Some papers revealed also the shared use of a specific Ig rearrangement in different lymphoma histotypes, as for example the expression of V_H4-34 frequently found in diffuse large-cell lymphoma, primary central nervous system lymphoma, B-chronic lymphocytic leukemia (CLL), and autoimmune disorders, and expressed in about 5% of healthy adult B lymphocytes [61].

In CLL, the mutational status of the immunoglobulin genes expressed in tumor B-cells can be used to segregate patients into 2 subsets that have significantly different rates of disease progression: the unmutated VH genes are associated with a greater tendency for disease progression and shorter survival than the mutated ones. Within the unmutated group several studies revealed that the Ig repertoire shows a reduce variability compared with that of adult blood B cells, with a frequent use of V_H1-69, V_H3-07, V_H3-21, and V_H4-34, and a skewed D3-3 and J6 gene usage with restricted reading frames (RFs) in particularly linked to the V_H1-69 expression [30,31]. Some of these IGHV genes have also been implicated with prognosis: the V_H1-69 was associated with a poor prognosis, V_H3-48 and V_H3-53 were predominantly found in female patients without adverse prognostic cytogenetics, and V_H3-72 was associated with highly stable disease [62].

Some recent papers aimed to assign the stereotyped BCRs observed in CLL patients to defined clusters identified by shared HCDR3 motifs. These analysis, carried out on very broad cohorts of patients, revealed an over-representation of certain IGHV genes among clustered cases, especially V_H3-21 and V_H1-69, and an under-presentation of others, as for example V_H3-7, V_H3-23, V_H3-30 [27-29]. Interestingly, Darzentas *et al*. observed that just six IGHV genes (V_H1-69, V_H1-3, V_H1-2, V_H3-21, V_H4-34 and V_H4-39) accounted for almost 80% of cases belonging to high-level clusters with stereotyped BCR structures [29].

The restricted use of specific gene segments in many different lymphomas is unlikely to occur by chance only, thus leading to the speculation of a possible antigen component involved in the neoplastic transformation of CLL and other B-NHLs [30,57]. Moreover, the limited

repertoire of the Ig expressed by different B-NHLs could be potentially exploited for the development of tailored treatment strategies applicable to each major stereotyped subset [27,31].

Thus, the identification of T-cell epitopes shared by multiple patients, may overcome one of the major limitations of current Id-vaccination, which is the requirement of individualized manufacturing [63]. In 2000, Trojan *et al.* described the generation of autologous T cells specific for framework-derived peptides from patients with CLL [64]. This observation may indicate that there is neither a defect in the T-cell repertoire against such self peptides, nor T-cell tolerance, or simply that *ex vivo* presentation of such peptides in the context of professional antigen-presenting cells might have been capable of overcoming any *in vivo* tolerance [64]. In this field, other 2 more recent papers demonstrated the induction of epitope-specific T cells stimulating healthy donors PBMCs with Ig-derived peptides shared by different families of both heavy [65] and light immunoglobulin chains [63]. The immunogenic epitopes were selected for their shared expression in several lymphoma patients-derived sequences, and were localized in both the complementarity determining regions and the framework regions [63,65]. This strategy may be useful to selectively expand Id-specific T cells *ex vivo* in order to generate "educated" DLI [63], or even better to develop a shared vaccine [27]. However, for the latter hypothesis, it will be important to determine whether Ig light chain–specific cytotoxic T lymphocytes (CTLs) preexist in the blood of patients with B-cell tumors and whether such precursors can be expanded by vaccination or adoptive transfer [63].

IGHV1-69 as a promising candidate for a shared immunotherapy

The identification of a common antigen expressed on B-cell malignancies, and preferentially not present on normal B cells, could offer a potential for the development of an off-the-shelf vaccine for lymphoma, which avoid the expensive, laborious and time-consuming production of patient-specific therapies [66]. To prevent the induction of autoimmunity, tumor-associated antigens should be uniquely expressed or overexpressed in lymphoma cells compared with normal B-cells [63].

In this respect, a recent paper from Weng *et al*., selected the T-cell leukemia/lymphoma 1 (TCL1) oncoprotein as an aberrantly expressed antigen in many B-cell malignancies, including FL, CLL, MCL, DLBCL, and SMZL, and identified $TCL1_{71\text{-}78}$ as the minimal epitope that binds to HLA-A*0201. They were able to generate peptide-specific CTLs lines from HLA-A*0201 healthy donors and patients, and these CTLs can efficiently kill lymphoma cell lines and primary human lymphoma cells [63]. These results suggested that TCL1 protein is naturally processed and presented on the surface of primary lymphoma cells for recognition by CTLs in an HLA-restricted manner. Moreover, since recognition by antigen-specific CTLs needs only few MHC-peptide complexes on the cell surface, also low-expressing lymphoma cells can be specifically killed. Furthermore, lysis of TCL1-expressing tumor cells by TCL1-specific CTL may potentially induce epitope spreading and broaden the immune response against tumor cells with or without TCL1 expression [67].

These data pave the way to identify and characterize shared antigens among B-cell lymphomas in a broader MHC background, in order to develop a vaccine therapy for wide lymphoma patients' subsets.

Lessons from IGKV3-20

In this respect, we recently characterized the immunogenic properties of IGKV3-20 and IGKV3-15 proteins [56], highly expressed, as mentioned above, in HCV-associated lymphomas autoimmunity-associated lymphoproliferations, DLBCL, MZL, FL, and CLL (Figure 7) [30,58,68-73]. We found IGKV3-20 and IGKV3-15-specific T-cell and humoral responses in NHL patients, and proved that these 2 proteins are immunogenic *ex vivo*. The 2 targets were able to elicit specific CTLs that cross-react against related IGVK proteins expressed by different lymphomas. These results suggest that the interpatient variation in the IGKV3-20 or IGKV3-15 amino acid sequence does not constitute a major limitation in inducing cross-reactive responses. Actually, CTLs induced with a "prototypic" IGKV3-20 protein were able to specifically kill autologous targets loaded with idiotypic VK proteins of the same subfamily derived from unrelated NHL. Moreover, when generated from the PBMCs of HLA-A*0201 donors, IGKV3-20-stimulated-CTLs specifically recognized also 2 different lymphoma cell lines respectively expressing IGKV3-20 and IGK3-15 in a HLA-A*0201 restricted manner (Figure 7). These findings demonstrate that the 2 proteins are naturally processed in lymphoid cells and confirm the ability to induce cross-reactive responses against proteins characterized by a sequence homology of >80%, as IGKV3-20 and IGKV3-15. In addition, the identification of MHC-class I restricted epitopes within IGKV3-20 protein and the generation of epitope-specific CTLs able to recognize also similar and variant epitope in IGKV3-15, demonstrated that IGKV3-20 protein carries natural heteroclytic versions of several CTL epitopes in the IGK proteins broadly expressed among lymphoid malignancies. Finally, through the immunization of mice with KLH-conjugated IGKV3-20, we demonstrated also the *in vivo* induction of humoral and T-cell responses against IGKV3-20, as well as the cross-reactivity of IGKV3-20 CTL responses [56]. These results may also rule out the lack of specificity against these Id proteins in the TCR repertoire due to central tolerance, and/or the occurrence of peripheral tolerance [56].

A.

Table 1. Distribution of IGKV3-15 and IGKV3-20 proteins among B-cell malignancies

B-cell malignancy	IGKV3-15 N (%)	IGKV3-20 N (%)	IGKV3-15 or IGKV3-20 N (%)	IGKV3 family N (%)
HCV-related NHL	3/21 (14.3)	12/21 (57.1)	15/21 (71.4)	16/21 (76.2)
Diffuse large B-cell lymphoma	4/20 (20)	1/20 (5)	5/20 (25)	7/20 (35)
Splenic marginal-zone lymphomas	1/31 (3.2)	3/31 (9.7)	4/31 (12.9)	5/31 (16.1)
Sjögren syndrome–associated lymphoproliferations	2/7 (28.6)	4/7 (57.1)	6/7 (85.7)	6/7 (85.7)
MALT lymphomas	2/18 (11.1)	7/18 (38.9)	9/18 (50)	9/18 (50)
Follicular lymphomas	—	3/47 (6.4)	—	6/47 (12.8)
Multiple myeloma	4/71 (5.6)	7/71 (9.9)	11/71 (15.5)	11/71 (15.5)
Chronic lymphocytic leukemias	28/614 (4.6)	72/614 (11.7)	100/614 (16.3)	139/614 (22.6)

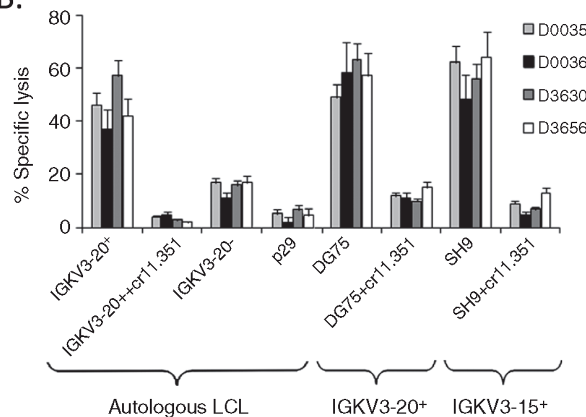


Figure 7. Characterization of IGKV3-15 and IGKV3-20 expression and immunogenicity features. **A.** Summary table of IGKV3-15, IGKV3-20, and in general IGKV3 proteins expression among B-cell lymphoproliferations. **B.** Results of cytotoxic assays performed with IGKV3-20-induced CTLs. DG75 (IGKV3-20^{+}) and SH9 (IGKV3-15^{+}) cells were efficiently killed in a HLA-A*0201–restricted manner (cr11.351 mAb) by IGKV3-20–specific CTLs obtained from donors single-matched for HLA-A*0201 with target cells. Effector:Target ratio - 20:1. IGKV3^{-} LCLs and BARF1 p29 pulsed LCL were used as negative controls ($P < 0.02$, Student t test) [56].

Previous papers supported the IGKV3-20 eligibility as a promising target for a shared immunotherapy to B-NHL. In particular, a multiparametric investigation of markers of immune response induced in human monocyte-derived dendritic cells, demonstrated an immune activation and responsiveness after IGKV3-20 stimulation, with a prevalence of Th2-cytokines production. Moreover, HCV-seropositivity did not impair the immune activation induced by IGKV3-20, thus confirming the potential utility of this protein also in HCV-associated lymphomas [74]. Such results have a prospective application in the development of idiotype based on the IGKV3-20 protein, suggesting that a specific Th1-driving adjuvant formulation should be adopted to elicit therapeutically relevant immune responses [75].

In addition, a method to optimize Id vaccines was developed expressing IGKV3-20 in mature DC with the use of recombinant lentivirus. The protein was fused to the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen (EBNA) 1, which act as a portable inhibitor of proteasomal degradation, in order to improve antigen stability. The manipulation of the antigen processing machinery allowed reshaping the Id-specific immune response. Indeed, this strategy induced effective responses to subdominant epitopes that could bypass the tolerance to self antigens expressed in malignant cells [76].

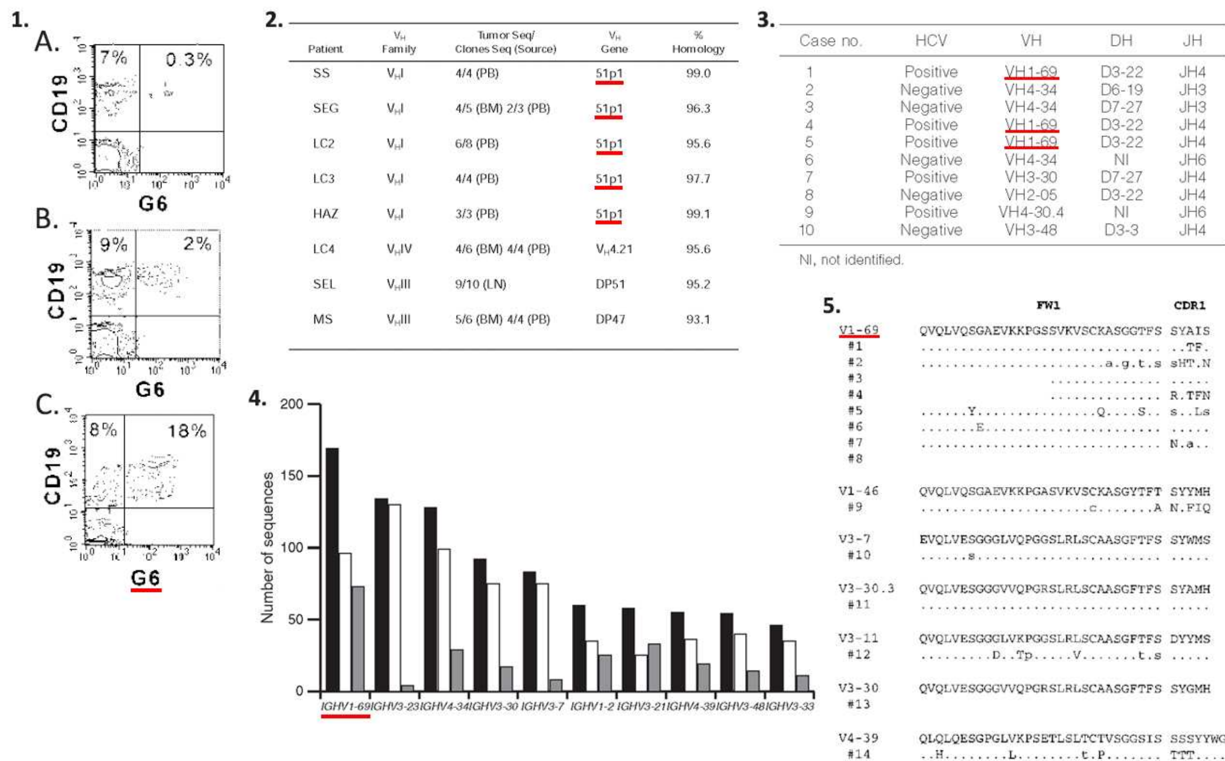
Shared IGHV1-69 expression among different lymphoproliferative diseases

On the bases of the previous considerations, other Id proteins, expressed by different B-NHLs, could be investigated as targets of a shared immunotherapy. Among the various stereotyped immunoglobulins found in B-NHL, IGHV1-69 could constitute a good candidate for the development of a therapeutic vaccination because of its wide expression, identified also in pre-malignant lymphoproliferations (Figure 8). Indeed, in type II mixed cryoglobulinemia (MC), HCV-infection drives a non-malignant expansion of monoclonal B-cells, which produce a polyreactive natural antibody of the IgM class commonly encoded by the V_H1-69 variable gene [77]. This V_H gene, together with the $V_L$kv325, was found highly overrepresented also in HCV-associated immunocytoma, suggesting that HCV-associated immunocytoma most likely represents the malignant counterpart of type II MC [71].

Moreover, sequencing of the clonal V_H genes found in lymphoepithelial (myoepithelial) sialadenitis, a reactive infiltrate that frequently characterizes Sjögren's syndrome, revealed the use of V_H1-69 gene in 61% of cases [60]. Also in this case, this non-malignant clonal B-cell expansion can evolve to salivary gland MALT lymphomas, which in turn express the IGHV1-69 protein on lymphoma B-cells [60].

Furthermore, the V_H1-69 gene is preferentially used also in nodal-marginal zone B-cell lymphoma, indicating a highly biased and non-random use of the V_H segments in this subtype of tumors [61]. Sequence analysis of the rearranged V_H segments in these tumors revealed a substantial deviation from the germline V_H1-69 sequence with a degree of mutations very similar among the different cases of lymphoma [61].

The V_H1-69 immunoglobulin segment is expressed in the restricted repertoire of fetal liver B lymphocytes [78,79], and a productive V_H1-69 is present in about 1.6% of normal B lymphocytes in adults [80]. However, the expression of this gene results much more prevalent in lymphoma B-cells, and in particular it characterizes 13% of all CLL and 25% to 30% of un-mutated CLL [31,81]. Again the sequence analysis performed in a large cohort of CLL patients revealed that V_H1-69 sequence in normal B cells did not reflect the corresponding sequence in CLL [81].



Patient	V_H Family	Tumor Seq/ Clones Seq (Source)	V_H Gene	% Homology
SS	V_HI	4/4 (PB)	51p1	99.0
SEG	V_HI	4/5 (BM) 2/3 (PB)	51p1	96.3
LC2	V_HI	6/8 (PB)	51p1	95.6
LC3	V_HI	4/4 (PB)	51p1	97.7
HAZ	V_HI	3/3 (PB)	51p1	99.1
LC4	V_HIV	4/6 (BM) 4/4 (PB)	V_H4.21	95.6
SEL	V_HIII	9/10 (LN)	DP51	95.2
MS	V_HIII	5/6 (BM) 4/4 (PB)	DP47	93.1

Case no.	HCV	VH	DH	JH
1	Positive	VH1-69	D3-22	JH4
2	Negative	VH4-34	D6-19	JH3
3	Negative	VH4-34	D7-27	JH3
4	Positive	VH1-69	D3-22	JH4
5	Positive	VH1-69	D3-22	JH4
6	Negative	VH4-34	NI	JH6
7	Positive	VH3-30	D7-27	JH4
8	Negative	VH2-05	D3-22	JH4
9	Positive	VH4-30.4	NI	JH6
10	Negative	VH3-48	D3-3	JH4

NI, not identified.

Figure 8. IGHV1-69 documented expression in different B-cell lymphoproliferations. **1.** Flow cytometric analysis of B cells expressing the G6 (IGHV1-69) Idiotype in HCV$^+$ type-II cryoglobulinemia (cryo-II). **A.** Healthy subject. **B.** Cryo-II patient with mild blood invasion by G6$^+$ cells. **C.** Cryo-II patient with more pronounced blood invasion by G6$^+$ cells. Extracted and modified from [77]. **2.** Distribution of V_H genes in HCV-associated immunocytomas. 51p1 gene encodes for IGHV1-69 protein. Extracted and modified from [71]. **3.** VH, D, and JH usage and HCV infection in Nodal Marginal Zone B-Cell Lymphoma. Extracted and modified from [61]. **4.** Usage of immunoglobulin heavy-chain variable (IGHV) subgroups and IGHV genes and chance to belong to clusters of homologous heavy chain complementarity determining region-3 (HCDR3) in Chronic Lymphocytic Leukaemia. Expression of the ten most frequently used IGHV genes. For each IGHV gene, histograms reported the total number of IG rearrangements (closed histograms), the total number of IG rearrangements not included in clusters of homologous HCDR3 (open histograms) and the total number of IG rearrangements included in clusters of homologous HCDR3 (dotted histograms). Extracted and modified from [28]. **5.** Deduced amino acid sequences of the consensus lymphoma VH gene segments in salivary gland MALT lymphomas. The sequences are compared to the amino acid sequences of the germline V_H gene segments showing the greatest homology where identity is indicated by a dot. The positions of mutations in the nucleotide sequences that would not result in a change in amino acid sequence are indicated by small case letters. Extracted and modified from [60].

Could the shared expression of IGHV1-69 imply a chronic antigenic stimulation?

The frequent occurrence of stereotyped receptors in different lymphoma patients raises the question on the nature of the molecules they can effectively recognize. In this regard, the almost identical CDR3 sequence of the IGHV1-69 protein expressed in HCV chronic infection suggests that a same or similar HCV antigen epitope may be responsible for the B-cell selection by a direct antigenic stimulation [61]. This hypothesis is also sustained by the observation that hybridomas derived from the peripheral B cells of HCV-infected patients and selected by reactivity with the viral E2 glycoprotein, used highly restricted V_H genes, with a prevalence of V_H1-69 [69]. Taken together, these studies strongly support the concept that the growth and survival of monoclonal B cells in HCV-associated lymphoproliferative disorders depend strictly on the continued stimulation by HCV [77].

The frequent usage of the V_H1-69 gene in autoimmunity-associated lymphoproliferations suggests a similar chronic stimulation due to auto-antigens, as for

example the rheumatoid factor in salivary gland MALT lymphomas. Indeed, the expression of IGHV1-69 is frequently observed in antibodies with rheumatoid activity in patients with salivary gland lymphomas and/or Sjögren's syndrome [60]. Conversely, gastric MALT lymphomas do not usually express IGHV1-69, thus suggesting that pathogenesis of MALT lymphomas may have features that are specific for certain sites or locations [60].

A mechanism involving autoreactive antibodies, or bacterial antigens, or antigenic epitopes present on viable and apoptotic T cells, was proposed also in the pathogenesis of CLL [62]. Even if the trigger of this disease is still not known, the stereotyped immunoglobulin repertoire in CLL suggests that its development and evolution is not a stochastic event, but may be favoured by an antigen that drives the cell of origin for at least some subsets of CLL [29]. Several IGHV1-69 expressing IgM obtained from CLL cases showed reactivity against apoptotic cells, while others recognized polysaccharide derived from *Streptococcus pneumoniae* [81]. Moreover, a typical characteristic of CLL is the presence of a sizeable amount of normal activated $CD3^+CD4^+CD40L^+$ T cells, which themselves are a sign of chronic inflammation [82]. In addition, CLL cells have the phenotypic profile of B cells activated by antigen interaction and display a gene expression profile that also recalls that of antigen-experienced B lymphocytes [82]. In particular, B-CLL-derived mAbs characterized by the un-mutated rearrangements involving IGHV1-69 were shown to selectively bind a molecule of 250 kDa, identified as non-muscle myosin heavy chain IIA, a major molecular motor of normal cells [83]. It is interesting to note that this molecule transmigrates to the cell surface of cells undergoing programmed cell death, thus sustaining the hypothesis of an autoreactivity against apoptotic cells [82]. Interestingly, autoantibodies against non-muscle myosin have been reported to occur also in the setting of HCV infection [84].

The use of IGHV1-69 was recently documented in recombinant antibodies against different viruses as HIV, SARS and influenza [85-88]. In this context, a recent paper describes the ability of CLL recombinant antibodies encoded by IGHV1-69 to react with pUL32, a phosphoprotein of cytomegalovirus (CMV). CMV has been linked to CLL by several lines of evidence: rates of CMV seroprevalence, frequency, and magnitude of CMV reactivation, and risk for CLL increase in parallel with increasing age [89]. In addition, an expansion of $perforin^+CD4^+$ T cells was observed in the peripheral blood of CLL patients, and it was strongly associated with CMV seropositivity [90]. These $perforin^+CD4^+$ T population was enriched for MHC class II-restricted, CMV-specific cells, indicating a direct role for CMV in promoting their expansion, and characterized by a $IL4^+$ and $IL10^+$ phenotype, thus driving a Th2 response. Hence, it is possible that anti-CMV $CD4^+$ T cells could support further B-CLL expansion [90]. Finally, a more pronounced expansion of CMV-specific $CD8^+$ T cells was observed in CLL patients than in age-matched healthy adults, suggesting that an antigenic determinant of CMV could be involved as a specific antigen or superantigen in BCR-mediated stimulation of CLL cells [89]. It is interesting to observe that the IGHV1-69 expressed on the majority of normal B-cells are distinct from those expressed on leukemic cells, and that there is only a little subset of healthy B-cells expressing 51p1-encoded IGHV similar to those found in CLL. This suggests that, conceivably, a subset of such normal B cells may be selected to undergo transformation by virtue of their expression of surface Ig with some distinctive binding activity to specific antigens, as for example the pUL32 of CMV [89].

Immune monitoring for cancer immunotherapy

One of the main challenges of tumor vaccination is the identification of suitable biomarkers of response to the therapy, which could have a reliable correlation with a meaningful clinical endpoint. In this respect, the measurement of T-cell mediated immune responses has been widely investigated in order to validate shared protocols for the immune monitoring during cancer immunotherapy [91,92].

The most common biomarker used in cancer vaccine protocols has been the immune response of patients to TAA pre- and post-vaccination. Some trials have analysed also the antibody response to TAA, or variations in the percentage of Treg, myeloid derived suppressor cells, NK and DCs, often evaluating the ratio of effector to regulatory cells. In addition, many studies have used analysis of multiple serum cytokines and chemokines [3,93-98].

The study from Di Nicola *et al.,* for example, documented significant changes in Treg, NK and anti-tumor T-cell frequencies, and humoral responses in responding patients compared with non-responding ones. These observations indicate that the clinical responses observed after tumor-loaded DC-based vaccination may be the result of an active modulatory effect on different components of the immune system [55]. Moreover, the responding patients carrying the HLA-A*0201 allele showed after vaccination an increase of IFN-γ and IL-4 releasing lymphocytes against peptides derived from the tumor specific IgH sequence. Notably, these responses were found up to 17 months after vaccination, suggesting a long lasting effect, if compared to other Id-vaccinations [55].

In this context, Id-specific T-cell lines were successfully generated from post-vaccine PBMCs of FL patients who had been actively immunized with the unique Id protein expressed by their tumors, which induced $CD4^+$ and $CD8^+$ T cell responses and molecular remissions [99]. The identification of such immunodominant epitopes in each Id protein may serve to validate suitable candidates for the development of reagents for immune monitoring in vaccinated patients.

Many assays has been developed and improved in the last decades to perform immune monitoring. The multiparameter approaches used to characterized TAA-responses in vaccinated patients mainly comprise enzyme-linked immunosorbent spot (ELISPOT) to quantify the release of relevant cytokines (IFN-γ, IL2, IL-4 and IL-5) by TAA-stimulated T cells, flow cytometry identification of TAA-specific T cells through peptide-multimer complexes able to bind to the TCR on T cell surface, and intracellular staining to determine the production of multiple cytokines after *in vitro* stimulation of T lymphocytes with TAA. The latter two techniques can be combined in order to obtain a more complete profile of TAA-specific immune responses [100].

The enumeration of anti-tumor T cells through fluorochrome labeled multimers conjugate of MHC molecules with a tumor antigen peptide has the main advantage to detect rare T cell populations, due to the high sensitivity of these reagents [101-104]. Drawbacks are mainly linked to the necessity to know exactly which peptide to use and which HLA background characterized each patient [5]. The lack of information about the functional state of T cells identified with this technique could instead be bypassed by the combination of tetramer staining with surface markers of T cell memory status or activation [105-108], or with the measurement of T cell proliferation in response to antigen re-exposure *in vitro* [5].

In addition, combined with this approach, intracellular staining can simultaneously assess cell subtype and cytokine production and allow measurement of multiple cytokines within a single cell [5,109,110]. New evidence suggest that the quality of T cell responses, and especially the presence of multifunctional T cells, correlates with the disease outcome to various infections and with the induction of an effective anti-viral immune response after vaccination [111]. Accordingly, the incorporation of such T-cell assays in the design of future Id

vaccines could increase the sensitivity to identify relevant immunologic correlates of tumor regression or/and improved survival [48].

AIM OF THE STUDY

Idiotypic vaccinations were considered a promising therapeutic approach for the consolidation of compete remission in B-NHL patients after standard treatments and monoclonal antibodies. However, recent evidence coming from phase III clinical trials revealed the inability of 2 out of 3 studies to reach a significant increase in PFS, thus reducing confidence and trust placed in this kind of treatment. In our opinion, a better design and definition of the vaccine target and the clinical endpoints may increase the potentiality of Id vaccination, which can be regarded as an integrative adjuvant approach during the maintenance therapy for NHL patients in first remission.

To this end, the identification of a common Id, shared by different B-cell lymphomas/leukemias, may overcome the patient-specificity of current Id vaccination and facilitate the identification of eligibility and responsiveness markers. We therefore identified IGHV1-69 as a promising candidate for the development of a shared immunotherapy for B-cell lymphoproliferations, due to the common expression of this protein in HCV-associated NHLs, autoimmunity-related lymphomas, and CLL [28,61,71,81]. Moreover, the presence of this Ig rearrangement in pre-malignant lymphoproliferations, as for example in type II mixed cryoglobulinemia, suggests a potential application of this protein also for preventative purposes [58,60,77].

On these grounds, the aim of this study was to characterize the *ex vivo* immunogenicity of IGHV1-69, and to select the most immunogenic epitopes in order to develop antigen-specific reagents for the identification of eligible patients and for the immune-monitoring of anti-tumor responses. In particular, we investigated the ability of this protein to induce epitope-specific T-cell responses in lymphoma patients, and its efficiency in boosting these epitope-specific T-cells *in vitro*. Finally, we also evaluated if CTLs induced with epitopes derived from a selected IGHV1-69 sequence, are able to cross-react against a cell line expressing a variant IGHV1-69 sequence. Such evidence could provide a strong premise for the development of recombinant Id vaccines, potentially able to elicit tumor-specific immune responses in a quite broad number of patients, affected by lymphoproliferative disorders expressing molecularly related Id.

MATERIALS AND METHODS

Patients and Healthy Donors

Peripheral blood samples from 23 patients with an IGHV1-69^{+} B-cell lymphoproliferation were collected for this study. Six samples were kindly provided by Prof. Salvatore De Vita and Dr. Martina Fabris (Clinic of Rheumatology, DSMB, University of Udine, Italy), 10 by Dr. Valter Gattei (Clinical and Experimental Onco-Hematology Unit, Centro di Riferimento Oncologico, I.R.C.C.S., Aviano, PN, Italy), 6 by Dr. Massimo Di Nicola ("C. Gandini" Medical Oncology, Bone Marrow Transplantation Unit; Fondazione I.R.C.C.S., Istituto Nazionale per lo Studio e la Cura dei Tumori, National Cancer Institute, Milan, Italy), and 1 was collected at the Centro di Riferimento Oncologico, National Cancer Institute (I.R.C.C.S., Aviano, PN, Italy). IGHV1-69 expression was determined by sequencing of IGHV amplicons derived from either reverse-transcribed total RNA or genomic DNA [28,61]. Buffy coats from 51 healthy donors were also collected and included in this study.

Peripheral blood mononuclear cells (PBMCs) were freshly isolated from heparinised blood of patients or from buffy coats of healthy donors by Ficoll-Hypaque gradient (Lymphoprep, Fresenius Kabi Norge Halden, Norway) using standard procedures, and viably frozen in fetal bovine serum (Gibco, Grand Island, NY) with 10% Dimethyl sulfoxide (DMSO), at -180°C until use.

Patient's and healthy donor's HLA-class I genotyping was performed by PCR sequencing based typing with primers specific for both locus A and B [112].

Cell lines and culture conditions

The following human cell lines were used in this study: the transporter associated with antigen-processing-deficient T2 cells transfected with the HLA-A*0201 gene (T2-A2); the lymphoid C1R cell line, characterized by a low expression of its endogenous HLA class I antigens and transfected to express the HLA-A*0301 allele (C1R-A3; kindly provided by Prof Andrea Anichini, Department of Experimental Oncology, IRCCS Foundation, National Institute of Tumors, Milan, Italy); the lymphoblastoid cell line (LCL) PG B.95.8 (HLA-A*02; -A*03; -B*35; -B*39) derived from the immortalization of peripheral B cells of a CLL patient through the standard EBV isolate B.95.8 (kindly provided by Dr. Javier Avila-Carino, Karolinska Institutet, Stockholm); the MHC-negative erythroleukemia K562 cell line; EBV-transformed LCLs generated *in vitro* by transformation of HLA-A*2402 or HLA-B*3501-expressing B cells using the standard EBV isolate B.95.8; and the EBV-transformed B-cell line LG2-EBV (HLA-A*24; -A*32; -B*35; -B*44; kindly provided by Dr Pierre van der Bruggen Ludwig Institute for Cancer Research, Brussels, Belgium).

All cell lines were cultured in RPMI-1640 (Gibco), containing 2 mM L-glutamine, 10% fetal bovine serum (Gibco), 100 µg/ml streptomycin and 100 IU/ml penicillin (Sigma), with the exception of LG2-EBV cell line, which was cultured in complete IMDM ([Lonza, Verviers, Belgium]) supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 µg/ml streptomycin and 100 IU/ml penicillin). C1R-A3 cell line was kept in culture with the selective hygromycin (220µg/ml; Sigma) in order to express the HLA-A*0301 allele.

Bioinformatics tools, T-cell epitope mapping and peptide synthesis

IGHV1-69 aminoacid (aa) sequences derived from the lymphoma cells of 55 CLL patients and 15 patients with HCV-related lymphoproliferations were kindly provided respectively by Dr Valter Gattei (Clinical and Experimental Onco-Hematology Unit, Centro di Riferimento Oncologico, I.R.C.C.S., Aviano, PN, Italy) and Dr Gianluca Gaidano (Division of Hematology, Department of Clinical and Experimental Medicine, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy), and by Dr Valli De Re (Experimental and Clinical Pharmacology, Centro di Riferimento Oncologico, National Cancer Institute, Aviano, Italy). Sequence comparison and alignment were performed through the bioinformatic MultAlin sequence alignment software (http://multalin.toulouse.inra.fr/multalin/) [113], generating a unique IGHV1-69 optimized sequence.

Within a IGHV1-69 germline sequence derived from a patient affected by a HCV-associated lymphoma and within the optimized IGHV1-69 sequence, 2 libraries of respectively 112 and 87 overlapping 9-mers with 1 amino acid offset, spanning the entire sequence (PEPscreen, ProImmune, Oxford), were screened in HLA-binding assay. The binding affinity of each peptide to the HLA-A*0101, -A*0201, -A*0301, -A*2402, -B*0702, -B*0801, and -B*3501 alleles, was evaluated through the REVEAL binding assay (ProImmune). This novel *in vitro* technology screens peptide libraries for their ability to stabilize the MHC complex. The approach measures the 'on-rate' and 'off-rate' of each peptide for the interested HLA allele investigating its binding kinetics. In general, good T cell epitopes tend to have rapid on-rates and slow off-rates [114]. The assay assigns then to each peptide a score relative to a known T cell epitope that has a borderline affinity to the allele of interest and identifies the most immunogenic peptides. Finally, the peptide-MHC complexes with the highest binding affinity are selected for the synthesis of MHC class I-peptide pentamer complexes (ProVE^{TM} pentamers; ProImmune) for epitope validation.

The probability of proteasomal processing was assessed for the first and the last aa residues of each predicted epitope, with NETchop, a predictor of proteasomal processing based upon a neural network (http://www.cbs.dtu.dk/services/NetChop/) [115]. The MHC affinity of IGHV1-69 epitopes was investigated through 3 different bioinformatics software: Syfpeithi (www.syfpeithi.de) [116], Bimas (www-bimas.cit.nih.gov/molbio/hla_bind/) [117], and NetMHC (www.cbs.dtu.dk/services/NetMHC/) [118].

The following virus-derived peptides were used as positive controls: HLA-A*0201-restricted Flu matrix 1 M1$_{58-66}$ peptide (GILGFVFTL), -A*0301-restricted EBV EBNA3A-RLR$_{603-611}$ (RLRAEAQYK), -A*2402-restricted CMV pp65-QYD$_{341-349}$ (QYDPVAALF), and –B*3501 CMV pp65-IPS$_{123-131}$ (IPSINVHHY). The HLA-A*0201-restricted MelanA/MART1$_{26-35}$ peptide analogue ELAGIGILTV was employed as TAA positive control in the generation of peptide-specific cytotoxic T lymphocytes. Selected IGHV1-69-derived peptides (ASG$_{22-30}$, RSE$_{85-93}$, QLV$_{3-11}$, GTA$_{54-62}$, EWM$_{44-52}$, EVK$_{8-16}$, IPI$_{50-58}$, TAY$_{76-84}$) together with the positive controls were synthesized by fluorenylmethoxycarbonil synthesis (Primm, Milan, Italy), purity (>95%) was determined by reverse-phase high-performance liquid chromatography and verified by mass spectral MALDI-TOF analysis. Peptides were dissolved in DMSO at a concentration of 5 mg/ml and stored at -80°C until use. Work stocks for each peptide were prepared in phosphate-buffered saline (PBS; Biomerieux, Marcy l'Etoile, France) at a final concentration of 500µg/ml and stored frozen. IGHV1-69 (QLV$_{3-11}$, EWM$_{44-52}$, IPI$_{50-58}$), viral (Flu M1-GIL$_{58-66}$, EBV BRLF1-YVL$_{109-117}$, EBV EBNA3A-RLR$_{603-611}$, EBV BRLF1-TYP$_{198-206}$, CMV pp65-QYD$_{341-349}$, CMV pp65-IPS$_{123-131}$), and control (HIV Pol-PMD$_{908-916}$, human NNMT-QLL$_{70-79}$, human NNMT-YYM$_{203-211}$, human DDX-TPV$_{688-696}$) peptides were kindly synthesized by Prof Stefan Stefanović group (Department of Immunology,

University of Tübingen, Tübingen, Germany), dissolved in DMSO at a concentration of 1 mg/ml with distilled water and 10% DMSO, and stored at -80°C until use.

Monomers refolding and Multimers production

MHC class-I/peptide monomers were refolded and biotinylated using standards protocols [103,119]. Specifically, HLA-heavy chain inclusion bodies (kindly provided by Dr Cécile Gouttefangeas, Department of Immunology, University of Tübingen, Tübingen, Germany), diluted in Urea buffer (8M Urea [UCB], 10mM Tris pH 8 [Sigma], 100mM NaH_2PO_4 [Merk], 0.1 mM EDTA [Roth], 0.1 mM DTT) 20mg/ml, together with MHC-light chain inclusion bodies were supplemented with injection buffer (3M guanidine HCl [Fluka], 10mM NaAcetate [Sigma], 10mM EDTA [Sigma], pH4.2). Both were added to 250ml of refolding buffer (L-Arginine base [Fluka], HEPES [Roth], EDTA [Sigma] in ddH_2O, pH 7.76), supplemented with reduced glutathione, oxidised glutathione, PMSF (phenylmethanesulfonylfluoride, Sigma), and 2.5-7.5mg of the selected peptide diluted 10mg/ml in DMSO. The reaction was then incubated overnight at 10°C while gently shaking. The 2° day heavy chain was added twice every 12h.

At day 3 the reaction was filtered through 0.22µm vacuum filter and concentrated to 25ml at 60psi in an Assemble Amicon stirred cell (Fisher Scientific). Retentate was taken apart at 4°C till day 5, while MHC heavy chain and MHC light chain were added together with PMSF to the permeate. Heavy chain was appended other 2 times every 12 hours.

At day 6 the reaction was filtered again and concentrated till 25ml, added to the concentrate of day 3 and spinned at 3200g for 10 min. The obtained supernatant was further concentrated using the Amicon Ultra-15 devise (Millipore) until obtaining 5ml of retentate. After spinning the retentate at 3200g for 10 min, the supernatant was used to do a size exclusion chromatography (Fast protein liquid chromatography, FPLC) on a S75 column using TBS (20mM Tris [Sigma] pH 8.0, 150mM NaCl [Merck], 0.5% Sodium azide [Sigma]) as buffer. Five ml fractions were stored through the FPLC and the UV absorption at 280nm was recorded. Fractions correspondent to the monomer peak were collected and immediately supplemented with PMSF, Leupeptin (100µg/ml, Roche) and Pepstatin (70µg/ml, Roche). The reaction was then gradually concentrated with the Amicon Ultra-15 devise till having 5 ml of retentate. Afterwards, the collected monomer was biotinylated adding Tris (80mM) pH 8, $MgCl_2$ (5mM), ATP (5mM), BirA enzyme (Affinity) and D-Biotin (0.6mM; Sigma), and incubated 16h at 27°C.

Finally, the biotinylated monomer was centrifuged at 3200 g for 5 min and employed in another FPLC separation on the S75 column as described above. The biotinylated monomer peak was collected and immediately supplemented with PMSF, leupeptin and pepstatin, together with sodium azide 0.1% and EDTA 2mM. The reaction was further concentrated with the Amicon Ultra-15 devise till about 500µl. Protein concentration was evaluated through Bradford assay and adjusted to 2.0mg/ml with permeate. Monomer aliquots were stored at -80°C until use.

Peptide sequence in the refolded monomer was verified with mass spectral MALDI-TOF analysis (kindly performed by Claudia Falkenburger, Department of Immunology, University of Tübingen, Tübingen, Germany).

Monomers were tetramerized adding the appropriate volume of Streptavidin-Phycoerythrin (PE; 159µg for 100µg monomer; Invitrogen, Molecular Probes) or Streptavidin-Allophycocyanin (APC; 87µg for 100µg monomer; Invitrogen, Molecular Probes) in 10 times every 30 min. To store labelled-tetramers, a freezing solution (containing 16% glycerol) was added to the tetramer, and aliquots were kept at -80°C till use.

In addition to the tetramers, PE-labelled Pro5® Pentamers were synthesized by ProImmune for selected epitopes (HLA-A*0201 $QLV_{3\text{-}11}$, HLA-A*2402 $EWM_{44\text{-}52}$, and HLA-B*3501 $IPI_{50\text{-}58}$), after epitope validation studies done with unlabelled ProVE™ Pentamers. PE-labelled HLA-A*0201-MelanA/$MART1_{26\text{-}35}$, -A*0201-Flu M1-$GIL_{58\text{-}66}$, -A*2402-CMV pp65-$QYD_{341\text{-}349}$, and –B*3501 CMV pp65-$IPS_{123\text{-}131}$ pentamers were also purchased from ProImmune.

Multimer and intracellular staining in flow cytometry

IGHV1-69-specific ProVE™ and Pro5® pentamers were tested *ex vivo* on patients' and healthy donors' PBMCs. Peptide-specific CTLs cultures were analysed with both, IGHV1-69-specific tetramers and pentamers. The following fluorescent-conjugated monoclonal antibodies were combined with multimers in order to better characterized epitope-specific T cells: α-CD4 phycoerythrin-cyanine5 (PeCy5; mouse IgG1, 13B8.2), α-CD8 phycoerythrin-cyanine7 (PeCy7; mouse IgG1, SFCI2IThy2D3), α-CD19 fluorescein isothiocyanate (FITC, mouse IgG1 k, J3-119), and α-CD45RA ECD (PE-Texas Red, mouse IgG1, 2H4LDH11LDB9) all from Beckman Coulter (Fullerton, CA, USA); α-CD4 FITC (mouse IgG1, RPA-T4), α-CD4 APC-Cy7 (mouse IgG1, RPA-T4), α-CD8 Peridinin chlorophyll (PerCP, mouse IgG1, SK1), α-CD8-APC (mouse IgG1, RPA-T8), α-CD19-FITC (mouse IgG1, HIB19), α-CD107a FITC (mouse IgG1, H4A3), α-CD197 PE (CCR7, rat IgG2a k, 3D12), α-IFNγ PeCy7 (mouse IgG1, 4S.B3), α-IL2 APC (rat IgG2a, MQ1-17H12) purchased from BD Biosciences (Becton Dickinson, Franklin Lakes, NJ, USA); α-TNFα PacificBlue (mouse IgG1, MAb11) from BioLegend (San Diego, CA, USA); α-CD8-FITC (OKT8) was kindly provided by Dr Cécile Gouttefangeas (Department of Immunology, University of Tübingen, Tübingen, Germany), together with PE- and APC-labelled Flu (HLA-A*0201-restricted, $M1_{58\text{-}66}$-specific), EBV (HLA-A*0201-restricted, $BRLF1_{109\text{-}117}$-specific) and CMV (HLA-A*2402-restricted, $pp65_{341\text{-}349}$-specific) specific tetramers. Properly labelled isotypic antibodies were used as negative controls.

Pentamer staining was performed under manufacture's instructions, with a variation in the reagent volume and the staining time. Briefly, pentamer vial was centrifuged at 14,000g for 5 min, to avoid the collection of protein aggregates. Then, 15 µl of pentamer were added to the cells (1-2x10^6 PBMCs, or 5x10^5 CTLs) in 50µl of wash buffer (PBS) for 15 min at room temperature. Finally, without washing, optimal amounts (1µg/1x10^6 cells) of surface antibodies were added to the sample for 30 min at 4°C. All antibodies were used in an appropriate volume of 10% rabbit serum (Dako, Glosdrup, Denmark) and PBS to reduce unspecific signal. After 2 washes in PBS, cells were then re-suspended in a fixing solution (1% Paraformaldehyd in PBS) and read at the flow cytometer. Cytofluorimetric analysis was performed with a FACSCalibur (Becton Dickinson) and a Cytomics FC500 (Beckman Coulter, Fullerton, CA) and at least 1 x 10^6 events were acquired. Obtained data were analyzed with CXP software (Beckman Coulter, Fullerton, CA, USA) and WinMDI 2.9 software (Windows Multiple Document Interafce for Flow Cytometry, Microsoft).

Tetramers were employed as follow: cells were washed twice with FACS-Washing buffer (PBS with 0.01% sodium azide, 2mM EDTA and 2% FCS) and stained with 5µg/ml or 2.5µg/ml of the correspondent tetramer. Tetramer mix was prepared in staining buffer (PBS with 50% FCS, 2mM EDTA and 0.02% sodium azide) and centrifuged at 14,000g for 5min at 4°C to eliminate possible aggregates. Fifty µl of this solution were added to the cells, which were then incubated for 15 min at room temperature. Without washing, an appropriate volume of surface antibodies was then added to the cells in 20µl of FACS washing buffer, together with the viability control AquaLive/Dead® Reagent (LifeTechnologies), and incubated 20min at 4°C. Cells were hence washed and re-suspended in FACS-Washing buffer for the analysis or permeabilized

with Cytoperm/Cytofix Reagent (Becton Dickinson) for 20min at 4°C. After a further washing step in Permwash buffer (PBS with 0.02% sodium azide, 0.5% BSA [Sigma], and 0.1% Saponin [Sigma]), permeabilized cells were stained for intracellular marker detection in 50µl of Permwash buffer, and incubated 20min at 4°C. Finally, samples were washed twice in Permwash buffer and re-suspended in FACS-Washing buffer for the analysis. Cytofluorimetric analysis was performed with a BD FACS Canto II flow cytometer (Becton Dickinson) equipped with the DIVA software, and all cells were acquired for each sample. Data analysis was carried out with FlowJo software (Tree Star, Ashland, OR).

IFN-γ ELISPOT assays

The interferon (IFN)-γ release enzyme-linked immunosorbent spot (ELISPOT) assay was performed using a commercial kit (Human IFN gamma ELISPOT; Thermo scientific, Rockford, IL, USA), according to manufacturer's instructions. The assay was carried out employing purified effector T cells, obtained by immunomagnetic enrichment protocols using the human T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) under manufacture's instructions, and collecting the non-T cell fraction (mainly B cells and monocytes), which was used as a source of antigen presenting cells (APCs). Ten thousand APCs were seeded in each well of the ELISPOT plate, loaded with 10 µg/ml of 9-mer peptide in complete RPMI medium, and incubated for two hours at 37°C with 5% CO_2. Purified effectors were then added to peptide-loaded APCs (50,000 cells/well) at a 5:1 effector:target ratio. APCs stimulated with viral peptides and unrelated peptides (derived from simian virus 40) were used as positive and negative controls, respectively. Finally, some wells were stimulated with phytohaemagglutinin (PHA) to value T-cell functionality. Cells were seeded onto ELISPOT capture plates in triplicates and incubated for 24 hours at 37°C with 5% CO_2. All plates were evaluated by a computer-assisted ELISPOT reader (Eli.Expert, A.EL.VIS GmbH, Hannover, Germany). Responses were considered significant if a minimum of 5 IFN-γ producing cells were detected in the wells, and data were indicated as IFN-γ Spot Forming Cells (SFC).

Generation and characterization of peptide-specific CTLs

Several CTLs generation protocols were tested in this study in order to improve the yield of peptide-stimulated T cells and to select the most appropriate scheme to induce epitope-specific T cells also from lymphoma patients' PBMCs samples.

Stimulation with autologous monocytes-derived dendritic cells

Healthy donors' purified PBMCs were re-suspended in serum-free medium and incubated at 37°C to allow for plastic-adherent step. After 1 h and 30 min, non-adherent peripheral blood lymphocytes (PBLs) were removed and vitally cryopreserved, while immature dendritic cells (DC) were obtained by culture adherent monocytes in complete RPMI 1640 supplemented with 10% of heat-inactivated healthy donors' serum, recombinant human GM-CSF (50ng/ml; R&D Systems, Abingdon, UK) and IL-4 (25ng/ml, R&D Systems, Abingdon, UK). Cells were substituted with 50% of fresh medium and cytokines concentrations were re-established on day 3 and day 6. On day 6, DC maturation was induced with lipopolysaccharide (LPS; 1.5µg/ml) from *Salmonella Typhimurium* (Sigma). After over-night incubation at 37°C, 10^6 mature DC/ml were seeded in 48-wells plates, pulsed with IGHV1-69- or viral-derived peptides (25µg/ml), and then incubated for 2 hours at 37°C in 5% CO_2. Peptide-pulsed DC were re-suspended in complete medium, supplemented with recombinant human IL-7 (rhIL7; 20ng/ml,

R&D Systems) and co-cultured in 48-wells plates with 10^6/ml autologous PBLs, cryopreserved on day 1, at a 20:1 effector:target ratio. Recombinant human IL-2 (rhIL2; 3ng/ml; R&D Systems) was added on day 3.

The CTLs cultures were re-stimulated twice every 7 days with freshly prepared peptide-pulsed-DC added at a ratio of 20:1 CTLs:DCs. Other 2 more re-stimulations were performed using autologous PBMCs pulsed with 100µg/ml peptide for 2h, γ–irradiated with 30 Gray (Gy), and added at a ratio of 10:1 (CTLs:PBMCs) to the CTLs culture. Alternatively, T2-A2 or heterologous HLA-matched LCLs were used as stimulators. One million cells/ml were pulsed with 20µg/ml of the correspondent peptide for 2h, then γ-irradiated with 60 Gy (T2-A2) or 30 Gy (LCLs), and finally added to the CTLs at a ratio of 10:1 CTLs:stimulators. RhIL7 (20ng/ml) was added at each stimulation, while medium was supplemented with rhIL2 (3ng/ml final concentration) 3 days after the stimulation, and then every 3 days.

CTLs cultures were characterized in flow cytometry for their memory phenotype before each re-stimulation and their specificity was evaluated 1 week after the last re-stimulation in a cytotoxic assay, or with pentamer staining after further 5 days in medium without cytokines and 2h in ice, to promote TCR expression.

Stimulation with peptide-loaded HLA-matched cell lines

An alternative protocol was used to compare the ability to induce epitope-specific CTLs in healthy donors' and patients' PBMCs [101,120]. T2-A2, C1R-A3 or HLA-matched LCLs were stimulated with different concentrations of IGHV1-69-derived peptides (0.1µg/ml, 1µg/ml, 10µg/ml) in complete medium, for 2h at 37°C and 5% CO_2, and then γ-irradiated with 30Gy. Empty targets and targets stimulated with HLA-matched viral-derived peptides (5µg/ml) were used respectively as negative and positive induction controls. Healthy donors' and patients' PBLs, obtained after monocytes depletion with plastic-adherent step, were cultivated in medium with 10% heat-inactivated human serum, seeded 10^6/ml in 48-wells plates, and stimulators were added at a 4:1 (effectors:stimulators) ratio. At day 3, and every 3 days, cultures were supplemented with rhIL-2 (3ng/ml). Weekly re-stimulations were performed with peptide-pulsed cell lines as at day 1, stimulating CTLs cultures with a ratio of 2:1 CTLs:stimulators. After the 3° and the 4° stimulations, CTLs cultures were characterized for their memory phenotype in flow cytometry, and tested for their specificity in cytotoxic assays and with pentamer staining.

Stimulation with artificial antigen presenting cells

Finally, we tested a protocol employing synthetic artificial APC (aAPC) developed to elicit and expand also low-avidity tumor-directed human CTL lines [119]. PBMCs were freshly isolated from a healthy donor's leukapheresis, by Ficoll-Hypaque gradient using standard procedures. $CD8^+$ T cells were purified using CD8 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), staining up to 250×10^6 cells with 300µl beads in 1200µl MACS buffer (PBS, 0.5% BSA, 2mM EDTA) and collecting the fraction linked to the MACS column (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD8^+$ purified T cells were maintained overnight in IMDM medium with 10% human serum (c.c. pro, Oberdorla, Germany) and 50 µM β–Mercaptoethanol (Roth, Karlsruhe, Germany), supplemented with 2.5ng/ml IL7 (Promokine, Heidelberg, Germany) and 10U/ml IL2 (Proleukin, Novartis, Basel, Switzerland), at 37°C and 7.5% CO_2.

Streptavidin Coated Microspheres (BangsLaboratories, Inc., USA) were loaded with the biotinylated anti-human CD28 antibody 9.3 [121] (kindly provided by Claudia Falkenburger, Department of Immunology, University of Tübingen, Tübingen, Germany), and the biotinylated IGHV1-69 correspondent monomer, the HLA-A*0201 $DDX_{168-176}$ (used as negative control), or

the HLA-A*0201 BRLF1$_{109-117}$ (used as positive control; both controls were kindly provided by Dr Cécile Gouttefangeas, Department of Immunology, University of Tübingen, Tübingen, Germany) monomers. aAPC were arranged accounting 200,000 beads, 150ng CD28 biotinylated mAb, and 50ng monomer, for 1 stimulation of each well. Each monomer was tested in 24 different wells and cells were stimulated 3 times.

After the overnight incubation, purified CD8$^+$ T cells were harvested, counted, and seeded 10^6 cells/well in 100µl T-cell complete medium, together with CD28/MHC loaded beads in the presence of IL12 (final concentration in the culture 5ng/ml; Promokine, Heidelberg, Germany), and incubated at 37°C with 7.5% CO_2. At day 3 medium was changed and supplemented with IL2 (40U/ml).

After 3 stimulations, the specificity of each well was evaluated in flow cytometry through tetramer staining (2.5µl/ml tetramer), blocking possible streptavidin-free binding sites with a previous incubation of 30 min at 4°C with 10µg/ml streptavidin (New England BioLabs GmbH, Frankfurt, Germany).

Tetramer-positive cells isolation and cloning

Tetramer-positive wells were re-stimulated once with aAPCs and after 10 days collected, stained with 2.5µg/ml of the correspondent PE-labeled tetramer and isolated with anti-PE microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) under manufacture's instructions. The positive fraction was eluted twice from the MACS column, while the negative fraction was kept in culture with 2.6U/ml IL2. Tetramer-positive purified cells were cultivated parallel 100,000cells /well, 10cells/well, and 5cells/well in 96-wells plates, in T-cell medium with IL2 (150UI/ml), IL7 (5ng/ml), and IL15 (5ng/ml; Promokine, Heidelberg, Germany). Every 2 weeks cells were stimulated with $1.5x10^5$/well freshly isolated PBMCs from 3 different healthy donors and γ-irradiated with 60Gy, and $0.15x10^5$/well LG2-EBV γ-irradiated with 200Gy. The bulk culture (100,000cells/well) was supplemented with 0.1µg/ml of the correspondent peptide, while the clones (10cells/well and 5cells/well) were stimulated with 1µg/ml PHA. The specificity of bulk and clones were finally analyzed with tetramer and intracellular staining after peptide stimulation.

Cytotoxicity assays

Cytotoxic activity of peptide-specific CTLs was evaluated in a Calcein–AM release assay using peptide-pulsed T2-A2, C1R-A3, or heterologous HLA-A*2402- or HLA-B*3501-expressing LCLs as targets. Target cells ($1x10^6$) were resuspended in 0.5 ml RPMI with 10% human serum, and pulsed with 20 µg/ml of corresponding peptide for 2 hours at 37°C and 5% CO_2. After washing, cells were diluted $1x10^6$/ml in Hanks Balanced Salt Solution without phenol red (HBSS), supplemented with 10 % FCS, labelled with 8 µM of Calcein-AM (Calbiochem, San Diego, CA) and incubated 1 h and 30 min at 37°C and 5% CO_2. Empty targets, the IGHV1-69$^+$ PG B.95.8, and the NK-sensitive erythroleukemia K562 cell lines were also included in the cytotoxic assays, staining them with Calcein-AM as the peptide-pulsed targets. Labeled cells were washed three times, counted, and seeded in 96-wells plate at a concentration of $5x10^3$ cells/50 µl/well. One half of so prepared target cells were incubated with 1µg/$1x10^6$ cells anti-MHC class-I W6/32 antibody (Abcam, Cambridge, MA) 30 min at room temperature, washed and re-suspended $5x10^3$ cells/50 µl/well, to assess the HLA-class I restriction of CTL responses. Unlabeled K562 were eventually added in each well ($5x10^3$ cells/50 µl/well) to reduce un-specific cytotoxicity signal. CTLs were added at 20:1, 10:1, 5:1, and 2.5:1 effector:target ratio. All tests were performed in triplicate. To obtain total calcein-releasing cells, targets were incubated with 100µl/well of lysis buffer (25 mM sodium borate, 0.1% Triton-X100 in HBSS, pH 9.0). While spontaneous release was determined by seeding target cells and adding 100µl/well

HBSS. Plates were centrifuged and incubated 4 h at 37°C and 5% CO_2 in a final volume of 200µl/well. Following incubation, the content of each well was mixed, plates were centrifuged and 100µl of the supernatant was transferred to a 96-well black culture plate. Fluorescence intensity was measured by reading the plates from the top using a SpectraFluorPlus (Tecan, Austria). Excitation and emission filters were 485 and 535 nm, respectively and gain was set at 70. The percentage of lysis was calculated as follows:

$$\text{Lysis \%} = \frac{\text{Experimental release - Spontaneous release}}{\text{Total release - Spontaneous release}} \times 100$$

Statistical analysis

Data obtained from multiple independent experiments were expressed as mean and standard deviation for immunophenotypic analysis of peptide-specific PBMCs and CTLs cultures. Percentage of specific lysis in cytotoxic assays was indicated as mean of triplicates obtained in the same experiment. The Student's t test for two tailed distributions was used for the statistical analysis of *ex vivo* pentamer staining and IFN-γ ELISPOT assays to compare data obtained from patients' samples with results achieved on healthy donors' cells. Data were considered to be statistically significant when $P \leq 0.05$ (two-sided).

RESULTS

Outline of the study

The main purpose of this study was the investigation of IGHV1-69 eligibility as a promising candidate for the development of an immunotherapeutic approach designed for a subset of patients affected by lymphoproliferations sharing similar Id proteins.

On these assumptions, we first compared IGHV1-69 sequences derived from B-NHL patients in order to design an optimized sequence with the highest degree of similarity among the selected variants. Within this sequence, we identified the most immunogenic epitopes in the 7 HLA class-I restrictions most frequent in the Caucasian population [122], and synthesized the correspondent PE-labeled pentamers and tetramers.

Since the immunogenicity of a TAA depends also on its ability to induce specific host immune responses, T-cell memory responses against IGHV1-69 were investigated in IGHV1-69$^+$ B-NHL patients and in individuals affected by an IGHV1-69$^+$ lymphoproliferation with the newly synthesized multimers. IGHV1-69-specific T-cell responses were then characterized for their memory phenotype in flow cytometry and for their ability to secrete IFN-γ in ELISPOT assays.

Finally, considering that a good candidate for an anti-tumor vaccination should be able to boost the specific immune responses naturally developed, CD8$^+$ T-cells specific for IGHV1-69-derived epitopes were expanded *in vitro* from both healthy donors' and patients' PBMCs using several protocols for the *in vitro* boosting of epitope-specific T-cell responses. The specificity of the CTLs lines obtained with these approaches was tested in flow cytometry with epitope-specific multimers, and in cytotoxicity assays.

Lastly, we also investigated the ability of IGHV1-69 to induce cross-reactive responses against targets expressing molecularly related Id, in order to validate the eligibility of IGHV1-69 as a target for a subset-specific immunotherapy. For this purpose, we included as target in cytotoxicity assays a cell line derived from the lymphoma B-cells of an IGHV1-69$^+$ CLL patient, and sharing the same HLA alleles with the selected CTLs lines.

The thorough characterization of IGHV1-69 immunogenicity will provide a solid rational background for further *in vivo* studies aimed at verifying whether the optimized IGHV1-69 can function as a good candidate for a shared immunotherapy for a subset of patients carrying different lymphoproliferations expressing not only IGHV1-69, but also molecularly related Ids.

Patients' characteristics

Twenty-three patients carrying an IGHV1-69-expressing B-cell lymphoproliferation were included in this study. We collected samples from patients suffering from different diseases, including pre-malignant lymphoproliferations associated with HCV infection or autoimmunity, HCV- or autoimmunity-related lymphomas, CLL and follicular lymphomas (Table 1). All these disorders were selected on the basis of the shared expression of IGHV1-69 by clonal B cells and relevant HLA class I alleles of the patients.

Patient number	Disease	HLA class I genotyping
#1	Type II Mixed Cryoglobulinemia (HCV+)	-A*2402, -B*3501
#2	Type II Mixed Cryoglobulinemia	-A*0301
#3	Type III Mixed Cryoglobulinemia, Sjögren's Syndrome, Parotid MALT Lymphoma	-A*0201
#4	Type III Mixed Cryoglobulinemia, Sjögren's Syndrome (HCV+)	-A*2402, -B*0801, -B*3501
#5	Type II Mixed Cryoglobulinemia (HCV+)	-A*2402
#6	Mantle Cell Lymphoma (HCV+)	-A*0201, -B*3501
#7	Chronic Lymphocytic Leukaemia	-A*0201, -A*0301
#8	Chronic Lymphocytic Leukaemia	-A*0201, -A*2402
#9	Chronic Lymphocytic Leukaemia	-A*0201, -A*2402
#10	Chronic Lymphocytic Leukaemia	-A*2402, -B*3501
#11	Chronic Lymphocytic Leukaemia	-A*0101, -A*0201
#12	Chronic Lymphocytic Leukaemia	-A*0301
#13	Chronic Lymphocytic Leukaemia	-A*0301, -A*2402
#14	Chronic Lymphocytic Leukaemia	-A*0201, -A*0301, -B*3501
#15	Chronic Lymphocytic Leukaemia	-A*0201, -B*3501
#16	Chronic Lymphocytic Leukaemia	-A*0101, -A*2402, -B*0702, -B*3501
#17	Type II Mixed Cryoglobulinemia (HBV+, HCV+)	-A*2402, -B*0702
#18	Chronic Lymphocytic Leukaemia	-A*0301
#19	Non Germinal Center Diffuse Large B-Cell Lymphoma	-A*0301, -A*2402
#20	Burkitt Lymphoma	-A*0201, -B*3501
#21	Mediastinal Large B-Cell Lymphoma	-A*0201
#22	Follicular G1-2 Lymphoma	-A*0201
#23	Plasmablastic Lymphoma (HHV8+)	-A*0201, -B*3501

Table 1. Patients' clinical-pathological characteristics and HLA class-I genotyping. The presence of infection by HCV (or HBV, or HHV8) is indicated in brackets. MALT: Mucosa-Associated Lymphoid Tissue

Optimization of the IGHV1-69 sequence

One of the main limitations of current Id vaccinations is the individual feature of the vaccine target. In order to bypass the uniqueness of each Ids, we selected a shared Id protein, IGHV1-69, whose expression was documented in several B-NHL and pre-malignant lymphoproliferations [28,58,60,61,71,77,81]. To further improve the immunogenic features of IGHV1-69 as shared antigen, we compared the predicted IGHV1-69 aminoacid sequences derived from 55 CLL patients and from 15 individuals with HCV- or autoimmunity-related lymphoproliferations. The alignment of these 70 aa sequences resulted in a single "consensus sequence", which was deprived of the hypervariable CDR3 region, to achieve the highest degree of similarity among the selected IGHV1-69 variants. For each position we selected the

most represented aminoacid among the collected sequences, and obtained an optimized sequence characterized by a homology degree >80% with the IGHV1-69 reference germ line sequence (derived from a HCV-related lymphoma). Moreover, analysing the germ line sequence with bioinformatics engines for the prediction of MHC affinity (Syfpeithi, Bimas and NetMHC), we identified the most immunogenic epitope candidates in the context of the 7 most common HLA class-I alleles of the Caucasian population (-A*0101, -A*0201, -A*0301, -A*2402, -B*0702, -B*0801, and -B*3501) [122], and we maintained unchanged the corresponding aa residues in the final sequence. Using this approach, we selected the most immunogenic regions, and obtained an IGHV1-69 new variant, optimized for the extent of similarity and predicted immunogenic features (Figure 9).

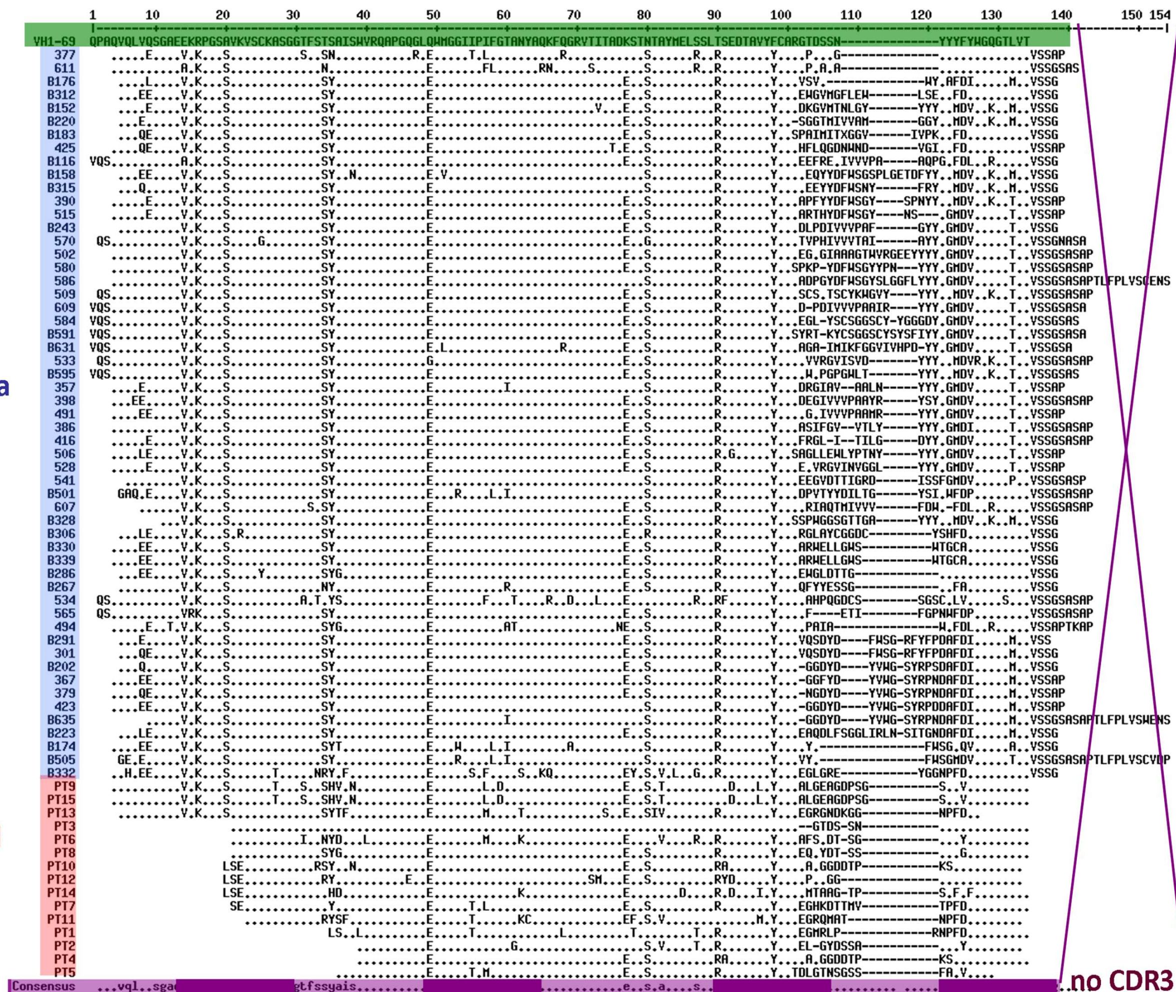


Figure 9. Sequence alignment and design of the IGHV1-69 optimized sequence. IGHV1-69 sequences are compared to the aa sequence of a reference germ line IGHV1-69 segment derived from a HCV-associated lymphoma. The 100% homology on the aa level with the germ line sequence is indicated by a dot, while a change of the aa residue is underlined with capital letters.

Identification and validation of potential CTLs epitopes within IGHV1-69 sequences

Through the REVEAL binding assay (ProImmune) [123,124], we investigated the binding affinity to HLA molecules of 9-aa peptides derived from an IGHV1-69 sequence (obtained from a patient affected by a HCV-associated lymphoma), and the optimized IGHV1-69 sequence. We identified 28 peptides within the patient's sequence and 13 in the optimized one, able to bind 1 of the selected MHC allele products (HLA-A*0101, -A*0201, -A*0301, -A*2402, -B*0702, -B*0801, -B*3501).

Table 2 shows the aa sequence of each predicted epitope, the comparison between the patient's sequence-derived peptides and their corresponding sequence in the optimized IGHV1-69, and *vice versa*.

HLA	IGHV1-69 patient's sequence	IGHV1-69 optimized sequence	Sequence comparison
-A*0101	LTSEDTAVY [89-97]		LRSEDTAVY [86-94] no affinity
	GTDSSNYYY [102-110]		CDR3 region, absent in the optimized IGHV1-69
	DSSNYYYFY [104-112]		CDR3 region, absent in the optimized IGHV1-69
		ASGGTFSSY [24-32]	ASGGTFSTS [27-35] no affinity
		RSEDTAVYY [87-95]	TSEDTAVYF [90-98] no affinity
-A*0201	FSTSAISWV [32-40]		FSSYAISWV [29-37] no affinity
	SLTSEDTAV [88-96]		SLRSEDTAV [85-93] no affinity
		QLVQSGAEV [3-11]	QLVQSGAEE [6-14] no affinity
-A*0301	LVQSGAEEK [7-15]		LVQSGAEVK [4-12] no affinity
	GSAVKVSCK [18-26]		GSSVKVSCK [15-23] no affinity
	KASGGTFST [26-34]		KASGGTFSS [23-31] no affinity
	STSAISWVR [33-41]		SSYAISWVR [30-38] no affinity
	GTANYAQKF [59-67]		GTANYAQKF [56-64] not changed
	GTDSSNYYY [102-110]		CDR3 region, absent in the optimized IGHV1-69
		GTANYAQKF [56-64]	GTANYAQKF [59-67] not changed
-A*2402	TFSTSAISW [31-39]		TFSSYAISW [28-36] no affinity
	QWMGGIIPI [49-57]		EWMGGIIPI [46-54] confirmed affinity
	AYMELSSLT [82-90]		AYMELSSLR [79-87] no affinity
	SSNYYYFYW [105-113]		CDR3 region, absent in the optimized IGHV1-69
		GSSVKVSCK [15-23]	GSAVKVSCK [18-26] no affinity
		GTFSSYAIS [27-35]	GTFSTSAIS [30-38] no affinity
		EWMGGIIPI [46-54]	QWMGGIIPI [49-57] confirmed affinity
-B*0702	QPAQVQLVQ [1-9]		Absent in the optimized IGHV1-69
	EEKRPGSAV [13-21]		EVKKPGSSV [10-18] confirmed affinity
	KRPGSAVKV [15-23]		KKPGSSVKV [12-20] no affinity
	RPGSAVKVS [16-24]		KPGSSVKVS [13-21] no affinity
	SCKASGGTF [24-32]		SCKASGGTF [21-29] not changed
	AISWVRQAP [36-44]		AISWVRQAP [33-41] not changed
	WVRQAPGQG [39-47]		WVRQAPGQG [36-44] not changed
	APGQGLQWM [43-51]		APGQGLEWM [40-48] no affinity
		EVKKPGSSV [10-18]	EEKRPGSAV [13-21] confirmed affinity
		SCKASGGTF [21-29]	SCKASGGTF [24-32] not changed
		AISWVRQAP [33-41]	AISWVRQAP [36-44] not changed
		WVRQAPGQG [36-44]	WVRQAPGQG [39-47] not changed

HLA	IGHV1-69 germline sequence	IGHV1-69 optimized sequence	Sequence comparison
-B*0801	None	None	
-B*3501	QPAQVQLVQ [1-9] IPIFGTANY [55-63] TAYMELSSL [81-89] LTSEDTAVY [89-97] DSSNYYYFY [104-112]		Absent in the optimized IGHV1-69 IPIFGTANY [52-60] not changed TAYMELSSL [78-86] not changed LRSEDTAVY [86-94] no affinity CDR3 region, absent in the optimized IGHV1-69
		IPIFGTANY [52-60] TAYMELSSL [78-86]	IPIFGTANY [55-63] not changed TAYMELSSL [81-89] not changed

Table 2. Potential HLA class-I restricted epitopes predicted through REVEAL binding assay in a patient-derived IGHV1-69 sequence and in the optimized one. [ ] indicate the aa positions of the first and the last residues of the nonamer in the source sequence. The comparison shows the corresponding peptide in the second sequence underling the differences in aa sequence and reporting the binding affinity results.

As expected, sequence variations in the epitope anchor sites modified the binding affinity on the MHC allele [125], therefore, we did not identify the same epitopes in the 2 sequences. However, most (8 out of 13) of the candidates found in the optimized IGHV1-69 were present or confirmed also in the patient’s sequence. Conversely, since the optimized sequence was deprived of the CDR3 region, the potential epitopes (5 out of 23) detected in the CDR3 of the patient’s sequence, can not be considered as broadly usable candidates, because of the extremely variability observed among IGHV1-69 sequences in this region.

We also compared the epitopes derived from the optimized IGHV1-69 sequence with several IGHV1-69 variants and with IGHV proteins belonging to the IGHV1 family. Interestingly, we observed that these epitopes are highly conserved not only in sequences derived from the same Id, but also in molecularly-related Ids (Table 3).

HLA allele	IGHV chain	Peptide sequence	HLA allele	IGHV chain	Peptide sequence
HLA-A*0101	IGHV1-69 optim	ASGGTFSSY	HLA-A*0101	IGHV1-69 optim	RSEDTAVYY
	IGHV1-69*01	ASGGTFSSY		IGHV1-46*02	RSEDTAVYY
	IGHV1-69*02	ASGGTFSSY		IGHV1-46*03	RSEDTAVYY
	IGHV1-69*04	ASGGTFSSY		IGHV1-24*01	RSEDTAVYY
	IGHV1-69*05	ASGGTFSSY		IGHV1-58*01	RSEDTAVYY
	IGHV1-69*06	ASGGTFSSY		IGHV1-58*02	RSEDTAVYY
	IGHV1-69*08	ASGGTFSSY	HLA-A*0201	IGHV1-69 optim	QLVQSGAEV
	IGHV1-69*09	ASGGTFSSY		IGHV1-69*01	QLVQSGAEV
	IGHV1-69*10	ASGGTFSSY		IGHV1-69*02	QLVQSGAEV
	IGHV1-69*11	ASGGTFSSY		IGHV1-69*04	QLVQSGAEV
	IGHV1-69*12	ASGGTFSSY		IGHV1-69*05	QLVQSGAEV
	IGHV1-69*13	ASGGTFSSY		IGHV1-69*06	QLVQSGAEV
	IGHV1-69*07	ASGGTFSSY		IGHV1-69*08	QLVQSGAEV
HLA-A*0101	IGHV1-69 optim	RSEDTAVYY		IGHV1-69*09	QLVQSGAEV
	IGHV1-69*01	RSEDTAVYY		IGHV1-69*10	QLVQSGAEV
	IGHV1-69*02	RSEDTAVYY		IGHV1-69*11	QLVQSGAEV
	IGHV1-69*04	RSEDTAVYY		IGHV1-69*12	QLVQSGAEV
	IGHV1-69*05	RSEDTAVYY		IGHV1-69*13	QLVQSGAEV
	IGHV1-69*06	RSEDTAVYY		IGHV1-18*01	QLVQSGAEV
	IGHV1-69*08	RSEDTAVYY		IGHV1-18*02	QLVQSGAEV
	IGHV1-69*09	RSEDTAVYY		IGHV1-2*01	QLVQSGAEV
	IGHV1-69*10	RSEDTAVYY		IGHV1-2*02	QLVQSGAEV
	IGHV1-69*11	RSEDTAVYY		IGHV1-2*03	QLVQSGAEV
	IGHV1-69*12	RSEDTAVYY		IGHV1-2*04	QLVQSGAEV
	IGHV1-69*13	RSEDTAVYY		IGHV1-3*01	QLVQSGAEV
	IGHV1-46*01	RSEDTAVYY		IGHV1-3*02	QLVQSGAEV

HLA allele	IGHV chain	Peptide sequence	HLA allele	IGHV chain	Peptide sequence
HLA-A*0201	IGHV1-69 optim	QLVQSGAEV	HLA-A*2402	IGHV1-69 optim	GSSVKVSCK
	IGHV1-46*01	QLVQSGAEV		IGHV1-46*02	GASVKVSCK
	IGHV1-46*02	QLVQSGAEV		IGHV1-46*03	GASVKVSCK
	IGHV1-46*03	QLVQSGAEV		IGHV1-58*01	GASVKVSCK
	IGHV1-12*01	QLVQSGAEV		IGHV1-58*02	GASVKVSCK
	IGHV1-12*02	QLVQSGAEV		IGHV1-24*01	GASVKVSCK
	IGHV5-51*01	QLVQSGAEV		IGHV1-68*01	GASVKVSCK
	IGHV5-51*02	QLVQSGAEV		IGHV1-12*01	GASVKVSCK
	IGHV5-51*03	QLVQSGAEV		IGHV1-67*01	GASVKVSCK
	IGHV5-51*04	QLVQSGAEV		IGHV7-4-1*01	GASVKVSCK
	IGHV5-a*01	QLVQSGAEV		IGHV7-4-1*02	GASVKVSCK
	IGHV5-a*02	QLVQSGAEV		IGHV7-81*01	GASVKVSCK
	IGHV5-a*03	QLVQSGAEV		IGHV7-NL1*01	GASVKVSCK
	IGHV5-a*04	QLVQSGAEV		IGHV7-4-1*03	GASVKVSCK
	IGHV1-67*01	QLVQSGA**D**V	HLA-A*2402	IGHV1-69 optim	GTFSSYAIS
HLA-A*0301	IGHV1-69 optim	GTANYAQKF		IGHV1-69*01	GTFSSYAIS
	IGHV1-69*01	GTANYAQKF		IGHV1-69*03	GTFSSYAIS
	IGHV1-69*03	GTANYAQKF		IGHV1-69*04	GTFSSYAIS
	IGHV1-69*05	GTANYAQKF		IGHV1-69*05	GTFSSYAIS
	IGHV1-69*06	GTANYAQKF		IGHV1-69*06	GTFSSYAIS
	IGHV1-69*07	GTANYAQKF		IGHV1-69*07	GTFSSYAIS
	IGHV1-69*10	GTANYAQKF		IGHV1-69*09	GTFSSYAIS
	IGHV1-69*12	GTANYAQKF		IGHV1-69*10	GTFSSYAIS
	IGHV1-69*13	GTANYAQKF		IGHV1-69*11	GTFSSYAIS
	IGHV1-69*01	GTANYAQKF		IGHV1-69*12	GTFSSYAIS
	IGHV1-69*03	GTANYAQKF		IGHV1-69*13	GTFSSYAIS
HLA-A*2402	IGHV1-69 optim	EWMGGIIPI	HLA-B*0702	IGHV1-69 optim	EVKKPGSSV
	IGHV1-69*01	EWMGGIIPI		IGHV1-69*01	EVKKPGSSV
	IGHV1-69*03	EWMGGIIPI		IGHV1-69*02	EVKKPGSSV
	IGHV1-69*05	EWMGGIIPI		IGHV1-69*04	EVKKPGSSV
	IGHV1-69*06	EWMGGIIPI		IGHV1-69*05	EVKKPGSSV
	IGHV1-69*07	EWMGGIIPI		IGHV1-69*06	EVKKPGSSV
	IGHV1-69*10	EWMGGIIPI		IGHV1-69*08	EVKKPGSSV
	IGHV1-69*12	EWMGGIIPI		IGHV1-69*09	EVKKPGSSV
	IGHV1-69*13	EWMGGIIPI		IGHV1-69*10	EVKKPGSSV
HLA-A*2402	IGHV1-69 optim	GSSVKVSCK		IGHV1-69*11	EVKKPGSSV
	IGHV1-69*01	GSSVKVSCK		IGHV1-69*12	EVKKPGSSV
	IGHV1-69*02	GSSVKVSCK		IGHV1-69*13	EVKKPGSSV
	IGHV1-69*03	GSSVKVSCK		IGHV1-45*01	EVKKPGSSV
	IGHV1-69*04	GSSVKVSCK		IGHV1-45*02	EVKKPGSSV
	IGHV1-69*05	GSSVKVSCK		IGHV1-18*01	EVKKPGSSA
	IGHV1-69*06	GSSVKVSCK		IGHV1-18*02	EVKKPGSSA
	IGHV1-69*08	GSSVKVSCK		IGHV1-2*01	EVKKPGSSA
	IGHV1-69*09	GSSVKVSCK		IGHV1-2*02	EVKKPGSSA
	IGHV1-69*10	GSSVKVSCK		IGHV1-2*04	EVKKPGSSA
	IGHV1-69*11	GSSVKVSCK		IGHV1-3*01	EVKKPGSSA
	IGHV1-69*12	GSSVKVSCK		IGHV1-3*02	EVKKPGSSA
	IGHV1-69*13	GSSVKVSCK		IGHV1-46*01	EVKKPGSSA
	IGHV1-45*01	GSSVKVSCK		IGHV1-46*02	EVKKPGSSA
	IGHV1-45*02	GSSVKVSCK		IGHV1-46*03	EVKKPGSSA
	IGHV1-45*03	GSSVKVSCK		IGHV1-12*01	EVKKPGSSA
	IGHV1-18*01	GASVKVSCK		IGHV1-12*02	EVKKPGSSA
	IGHV1-18*02	GASVKVSCK		IGHV1-17*01	EVKKPGSAV
	IGHV1-2*01	GASVKVSCK		IGHV1-17*02	EVKKPGSAV
	IGHV1-2*02	GASVKVSCK		IGHV1-58*01	EVKKPGSST
	IGHV1-2*03	GASVKVSCK		IGHV1-58*02	EVKKPGSST
	IGHV1-2*04	GASVKVSCK	HLA-B*0702	IGHV1-69 optim	SCKASGGTF
	IGHV1-3*01	GASVKVSCK		IGHV1-69*07	SCKASGGTF
	IGHV1-3*02	GASVKVSCK		IGHV1-69*01	SCKASGGTF
	IGHV1-46*01	GASVKVSCK		IGHV1-69*02	SCKASGGTF

HLA allele	IGHV chain	Peptide sequence
HLA-B*0702	IGHV1-69 optim	SCKASGGTF
	IGHV1-69*03	SCKASGGTF
	IGHV1-69*04	SCKASGGTF
	IGHV1-69*05	SCKASGGTF
	IGHV1-69*06	SCKASGGTF
	IGHV1-69*08	SCKASGGTF
	IGHV1-69*09	SCKASGGTF
	IGHV1-69*10	SCKASGGTF
	IGHV1-69*11	SCKASGGTF
	IGHV1-69*12	SCKASGGTF
	IGHV1-69*13	SCKASGGTF
HLA-B*0702	IGHV1-69 optim	AISWVRQAP
	IGHV1-69*07	AISWVRQAP
	IGHV1-69*01	AISWVRQAP
	IGHV1-69*03	AISWVRQAP
	IGHV1-69*04	AISWVRQAP
	IGHV1-69*05	AISWVRQAP
	IGHV1-69*06	AISWVRQAP
	IGHV1-69*09	AISWVRQAP
	IGHV1-69*10	AISWVRQAP
	IGHV1-69*11	AISWVRQAP
	IGHV1-69*12	AISWVRQAP
	IGHV1-69*13	AISWVRQAP
	IGHV1-69*02	TISWVRQAP
	IGHV1-69*08	TISWVRQAP
	IGHV1-18*01	GISWVRQAP
	IGHV1-18*02	GISWVRQAP
HLA-B*0702	IGHV1-69 optim	WVRQAPGQG
	IGHV1-69*07	WVRQAPGQG
	IGHV1-69*01	WVRQAPGQG
	IGHV1-69*02	WVRQAPGQG
	IGHV1-69*03	WVRQAPGQG
	IGHV1-69*04	WVRQAPGQG
	IGHV1-69*05	WVRQAPGQG
	IGHV1-69*06	WVRQAPGQG
	IGHV1-69*08	WVRQAPGQG
	IGHV1-69*09	WVRQAPGQG
	IGHV1-69*10	WVRQAPGQG
	IGHV1-69*11	WVRQAPGQG
	IGHV1-69*12	WVRQAPGQG
	IGHV1-69*13	WVRQAPGQG
	IGHV1-18*01	WVRQAPGQG
	IGHV1-18*02	WVRQAPGQG
	IGHV1-2*01	WVRQAPGQG
	IGHV1-2*02	WVRQAPGQG
	IGHV1-46*01	WVRQAPGQG
	IGHV1-46*02	WVRQAPGQG
	IGHV1-46*03	WVRQAPGQG
	IGHV1-45*01	WVRQAPGQA
HLA-B*0702	IGHV1-69 optim	WVRQAPGQG
	IGHV1-45*02	WVRQAPGQA
	IGHV1-3*01	WVRQAPGQR
	IGHV1-3*02	WVRQAPGQR
	IGHV1-24*01	WVRQAPGKG
	IGHV4-4*01	WVRQAPPQG
	IGHV4-4*02	WVRQAPPQG
	IGHV4-4*03	WVRQAPPQG
	IGHV4-4*04	WVRQAPPQG
	IGHV4-4*05	WVRQAPPQG
HLA-B*3501	IGHV1-69 optim	IPIFGTANY
	IGHV1-69*01	IPIFGTANY
	IGHV1-69*03	IPIFGTANY
	IGHV1-69*05	IPIFGTANY
	IGHV1-69*06	IPIFGTANY
	IGHV1-69*07	IPIFGTANY
	IGHV1-69*12	IPIFGTANY
	IGHV1-69*13	IPIFGTANY
HLA-B*3501	IGHV1-69 optim	TAYMELSSL
	IGHV1-24*01	TAYMELSSL
	IGHV1-3*01	TAYMELSSL
	IGHV1-3*02	TAYMELSSL
	IGHV1-45*01	TAYMELSSL
	IGHV1-45*02	TAYMELSSL
	IGHV1-45*03	TAYMELSSL
	IGHV1-58*01	TAYMELSSL
	IGHV1-58*02	TAYMELSSL
	IGHV1-69*01	TAYMELSSL
	IGHV1-69*02	TAYMELSSL
	IGHV1-69*03	TAYMELSSL
	IGHV1-69*04	TAYMELSSL
	IGHV1-69*05	TAYMELSSL
	IGHV1-69*06	TAYMELSSL
	IGHV1-69*07	TAYMELSSL
	IGHV1-69*08	TAYMELSSL
	IGHV1-69*09	TAYMELSSL
	IGHV1-69*10	TAYMELSSL
	IGHV1-69*11	TAYMELSSL
	IGHV1-69*12	TAYMELSSL
	IGHV1-69*13	TAYMELSSL
	IGHV1-2*01	TAYMELSRL
	IGHV1-2*02	TAYMELSRL
	IGHV1-2*03	TAYMELSRL
	IGHV1-2*04	TAYMELSRL
	IGHV1-46*01	TVYMELSSL
	IGHV1-46*02	TVYMELSSL
	IGHV1-46*03	TVYMELSSL
	IGHV1-14*01	TAYMELSSQ

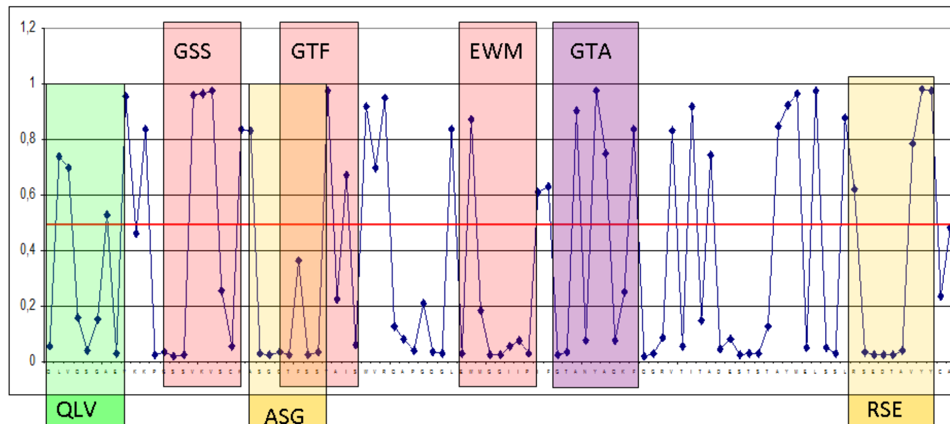
Table 3. Sequence comparison of the optimized IGHV1-69 predicted epitopes with IGHV1-69 variants and sequences derived from Id proteins of the IGHV1 family. Optim is the abbreviation for optimized. Changes in the aa sequences are underlined.

Validation of the predicted epitopes

Bioinformatics analysis of proteasomal cleavage in IGHV1-69 sequences

The IGHV1-69 predicted epitopes were identified through a method based on their binding affinity to the HLA-allele molecules, but this system does not consider the natural processing of these peptides through the proteasomal machinery [115]. To further investigate this aspect, we analyzed the IGHV1-69 optimized sequence (Figure 10) and the germline sequence (data not shown) with the NETchop software and fixed at 0.5 (in order to maintain a good balance between the specificity and the sensitivity of the prediction) the threshold to detect a positive prediction, namely a cleavage of the downstream peptide bond following the aa residue. As shown in Figure 10, almost all (10 out of 13) of the predicted epitopes presented a high probability of proteasomal cleavage in at least one of the terminal aa residues (aa1 or aa9 of the sequence). HLA-A*2402-restricted $GTF_{27\text{-}35}$, and HLA-B*0702-restricted $SCK_{21\text{-}29}$ and $AIS_{33\text{-}41}$ had low probability values in both aa1 and aa9, while HLA-A*0101-restricted epitopes seemed to have a high probability of proteasomal processing (i.e. positive prediction values in both the terminal residues).

A.



B.

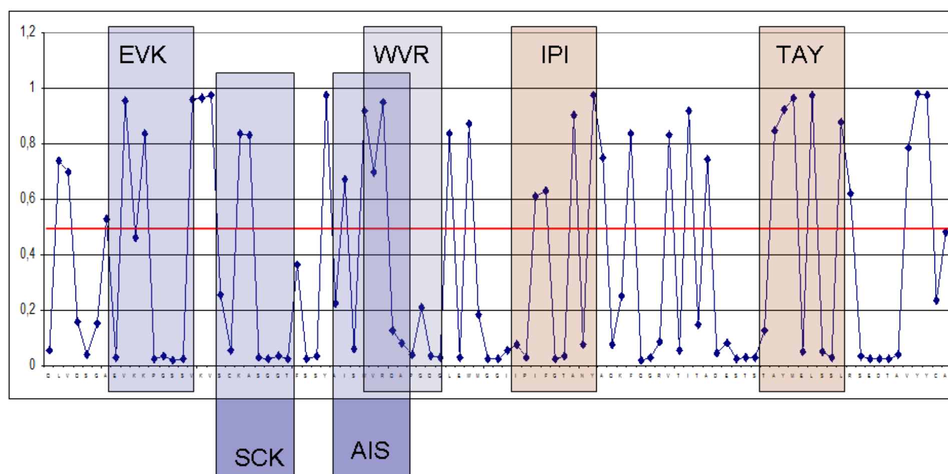


Figure 10. Graphical view of the proteasomal processing probability calculated for the IGHV1-69 optimized sequence and carried out with NETchop. The X axis represents the aa residues, while on Y axis are reported the predictions values. A cut-off of 0.5 was previously fixed to discriminate between positive and negative predictions. Epitopes are indicated with the first 3 aa residues of their sequence and colors characterized every HLA allele. **A.** HLA-A alleles: yellow –A*0101, green –A*0201, violet –A*0301, pink –A*2402. **B.** HLA-B alleles: blue –B*0702, brown –B*3501.

Flow cytometry analysis with ProVE™ Pentamers

To further validate the natural processing of predicted epitopes, we investigated the existence of epitope-specific $CD8^+$ T cells through ProVE™ Pentamers, which were synthesized for each predicted epitope of both the optimized and the patient-derived sequence. We first selected the most frequently expressed HLA alleles among the collected patients and decided to focus our attention on the HLA-A*0201, -A*0301, -A*2402 and -B*3501 specific pentamers. Moreover, to assess the possible background due to un-specific binding, we tested HLA-specific pentamers with PBMCs of HLA mismatched healthy donors (n=4). Figure 11 displays the adopted scheme of flow cytometric analysis, which was performed in the presence of α-CD4, α-CD19 and α-CD8 to define the specific binding of these new reagents.

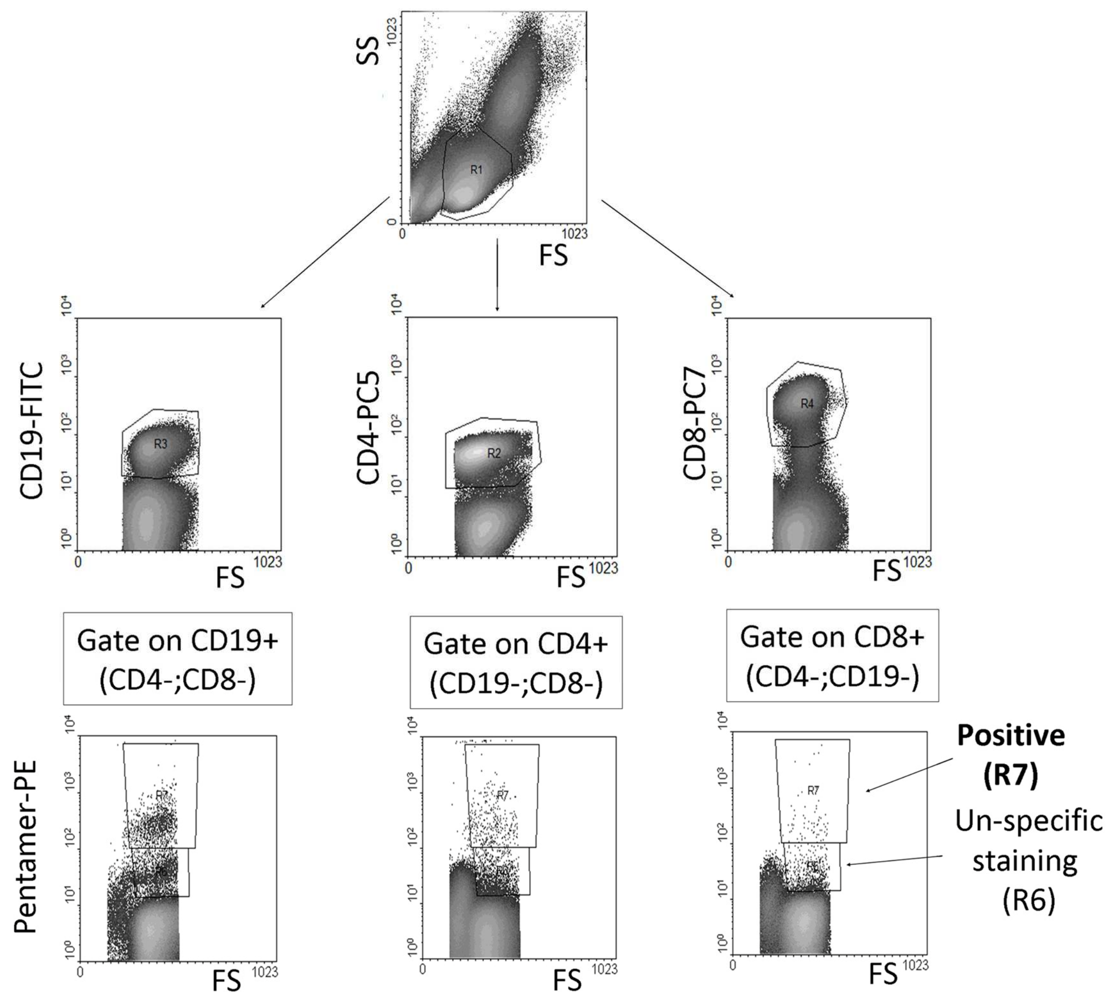


Figure 11. Scheme of flow cytometry analysis performed to validate ProVE™ Pentamers. α–CD19 and α–CD4 antibodies were included to exclude un-specific binding signal. The staining was considered positive when the Mean Fluorescence Intensity (MFI) was >10^2 in $CD8^+CD4^-CD19^-$ cells (R7, calculated excluding $CD19^+$ [R3] and $CD4^+$ [R2] cells).

As shown in Table 4, the background signal in the HLA mismatched donors was generally very low, with the exception of pentamers $SSN_{105\text{-}113}$ (HLA-A*2402) and $DSS_{104\text{-}112}$ (HLA-B*3501) derived from the patient's IGHV1-69, where the observed positivity was mainly due to an un-specific binding to B cells. Interestingly, the specific positive signals were usually higher in samples obtained from patients (n=5) carrying IGHV1-69$^+$ lymphoproliferation, than in HLA-

matched healthy donors (n=6). Table 4 reports representative data obtained for some of the tested ProVE^{TM} pentamers.

		HLA-mismatched donor % of pentamer$^+$ cells on			HLA-matched donor % of pentamer$^+$ cells on			Patient % of pentamer$^+$ cells on		
HLA-allele	Peptide	CD8$^+$	CD4$^+$	CD19$^+$	CD8$^+$	CD4$^+$	CD19$^+$	CD8$^+$	CD4$^+$	CD19$^+$
-A*0201	FST_{32-40}	0.06	0.00	0.00	0.05	0.01	0.10	0.12	0.01	0.06
-A*0301	GSA_{18-26}	0.02	0.00	0.17	0.07	0.01	0.06	0.10	0.00	0.05
-A*2402	EWM_{46-54}	0.03	0.02	0.19	0.02	0.03	0.09	0.28	0.08	0.18
	$SSN_{105-113}$	0.03	0.04	0.32	0.27	0.28	0.75	0.11	0.08	0.18
-B*3501	IPI_{52-60}	0.01	0.00	0.01	0.01	0.02	0.49	0.37	0.09	0.26
	$DSS_{104-112}$	0.14	0.12	0.59	0.03	0.04	0.56	0.84	0.22	1.08

Table 4. Percentages of ProVE^{TM} pentamer-positive cells observed among CD8$^+$, CD4$^+$ and CD19$^+$ lymphocytes in the peripheral blood of patients affected by a IGHV1-69$^+$ lymphoproliferation, HLA-matched healthy donors, and healthy subjects with a different HLA-background.

In view of the noticeable un-specific binding of pentamers obtained from the CDR3 epitopes ($SSN_{105-113}$, $DSS_{104-112}$) identified in the patient's IGHV1-69 sequence, we decided to focus further analyses only on the candidates identified in the IGHV1-69 optimized sequence.

Identification and characterization of IGHV1-69 memory T-cell responses

Ex vivo pentamer staining of patients and healthy donor PBMCs

The validated epitope-specific pentamers were then tested *ex vivo* on PBMCs from 14 patients carrying an IGHV1-69$^+$ lymphoproliferation, 15 HLA-matched healthy donors, and 5 healthy individuals with a different HLA background, in the context of HLA-A*0201, -A*2402 and –B*3501 alleles (Figure 12A) (the HLA-A*0301-derived epitope was not evaluable in flow cytometry analyses because of the lack of a corresponding Pro5® pentamer. Experiments testing this epitope were carried out in IFN-γ ELISPOT assays and with the generation of epitope-specific T-cells). Overall, the pentamer$^+$ percentage among CD8$^+$ lymphocytes was significantly higher in patients (median=0.280; min-max 0.030-1.080) if compared with healthy donors expressing the same HLA alleles (median=0.095; min-max 0.015-0.650; $p=0.014$) (Figure 12C). Moreover, both groups displayed higher numbers of pentamer-specific cells than HLA mismatched donors (median=0.009; min-max 0.002-0.060; $p<0.001$ compared to patients; $p=0.002$ compared to HLA matched donors) (Figure 12C), demonstrating the HLA-specificity of custom reagents. In parallel, the analysis with viral-specific pentamers (flu, CMV) revealed similar percentages of pentamer$^+$ CD8$^+$ lymphocytes between patients (n=10; median=0.170; min-max 0.040-1.720) and HLA-matched healthy donors (n=11; median=0.190; min-max 0.040-4.080; $p=0.817$) (Figure 12B). This latter observation underlined and confirmed that in lymphoma patients we observed an exclusive prevalence of pentamer$^+$ T-cells specific for the IGHV1-69 protein-derived epitopes.

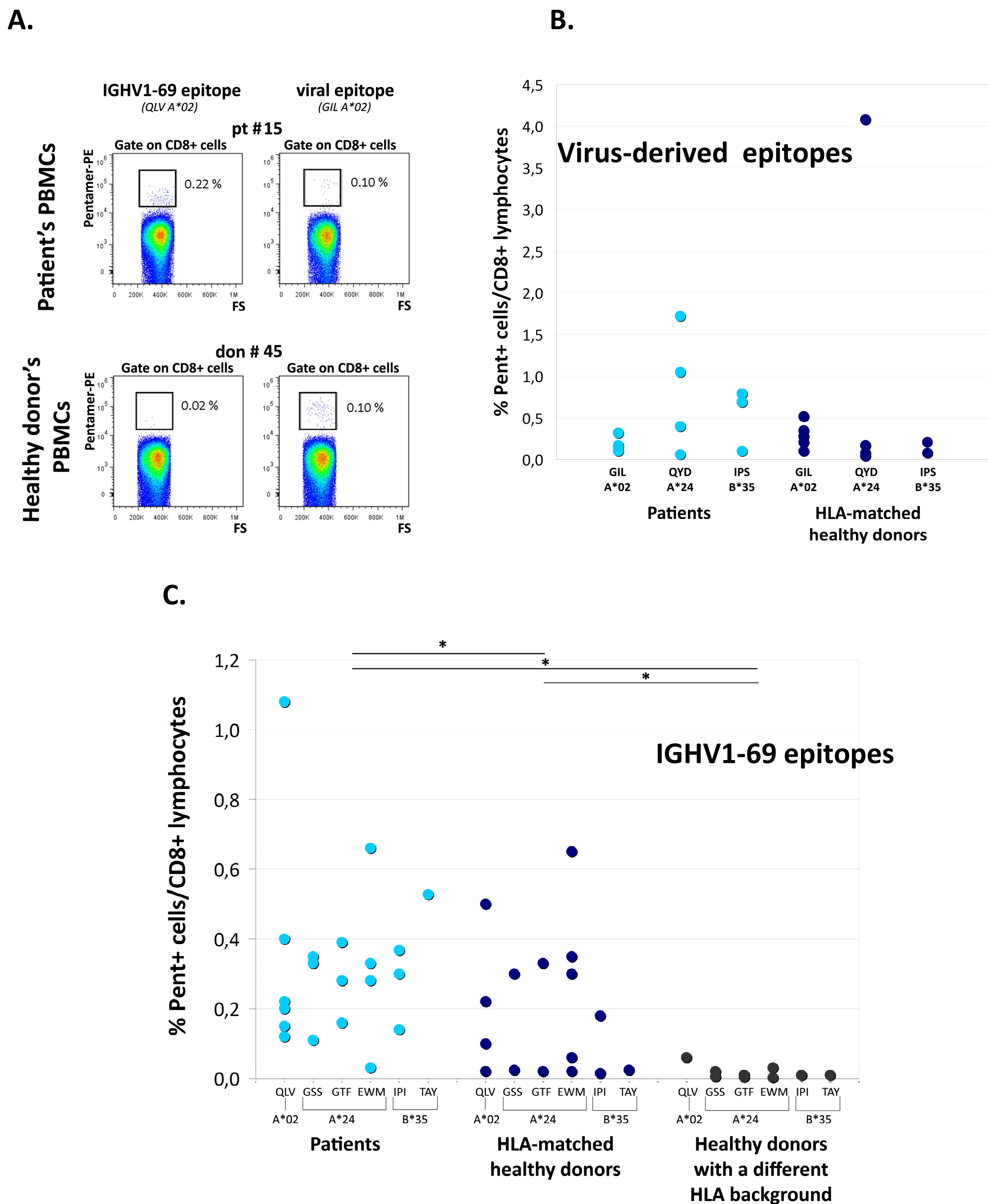


Figure 12. *Ex vivo* IGHV1-69- and viral-specific pentamer staining on patients' and healthy donors' PBMCs. **A.** Examples of flow cytometry dot plots gated on living $CD8^+$ lymphocytes. **B.** Graphical view of the global case study for virus-derived epitopes (patients n=10; healthy donors n=11). Every dot corresponds to the percentage detected in a single tested patient/donor for the specific pentamer. Percentages are calculated among living $CD8^+$ lymphocytes. Virus-derived epitopes are represented in the X axis with the first 3 aa residues: GIL is the HLA-A*0201-restricted flu $M1_{58\text{-}66}$ peptide; QYD the HLA-A*2402-restricted CMV $pp65_{341\text{-}349}$ peptide; IPS the HLA-B*3501-restricted CMV $pp65_{123\text{-}131}$ peptide. **C.** Graphical view of the global case study for IGHV1-69 specific epitopes (patients n=14; HLA-matched healthy donors n=15; HLA-mismatched healthy donors n=5). Epitopes are indicated in the X axis with the first 3 aa residues. Results were considered significant if p<0.05.

Characterization of memory phenotype in IGHV1-69- and virus-specific CD8⁺ T cells

Because of the different contribution of memory T cells subsets in mediating anti-tumor immune responses [105-107], the differentiation stage of pentamer$^+$ CD8$^+$ T cells was investigated through the combined analysis of the chemokine receptor CCR7 and the CD45RA isoform, to distinguish CCR7$^+$CD45RA$^+$ naïve ($T_{naïve}$), CCR7$^+$CD45RA$^-$ central memory (T_{CM}), CCR7$^-$CD45RA$^-$ effector memory (T_{EM}), and CCR7$^-$CD45RA$^+$ terminally differentiated (T_{Temra}) cells [108] (Figure 13A). While healthy donors (n=9) displayed a higher frequency of T_{EM} among virus specific CD8$^+$ cells if compared to IGHV1-69-pentamer$^+$ cells (55% *versus* 31.9%; p=0.03), no differences were highlighted in patients (n=10) pentamer$^+$ cells when comparing the memory subsets between viral- and IGHV1-69-specific cells (T_{EM} 38.5% versus 28.7%) (Figure 13B). Memory subsets in CD8$^+$ T cells were not differently distributed between patients and healthy donors (Figure 13C).

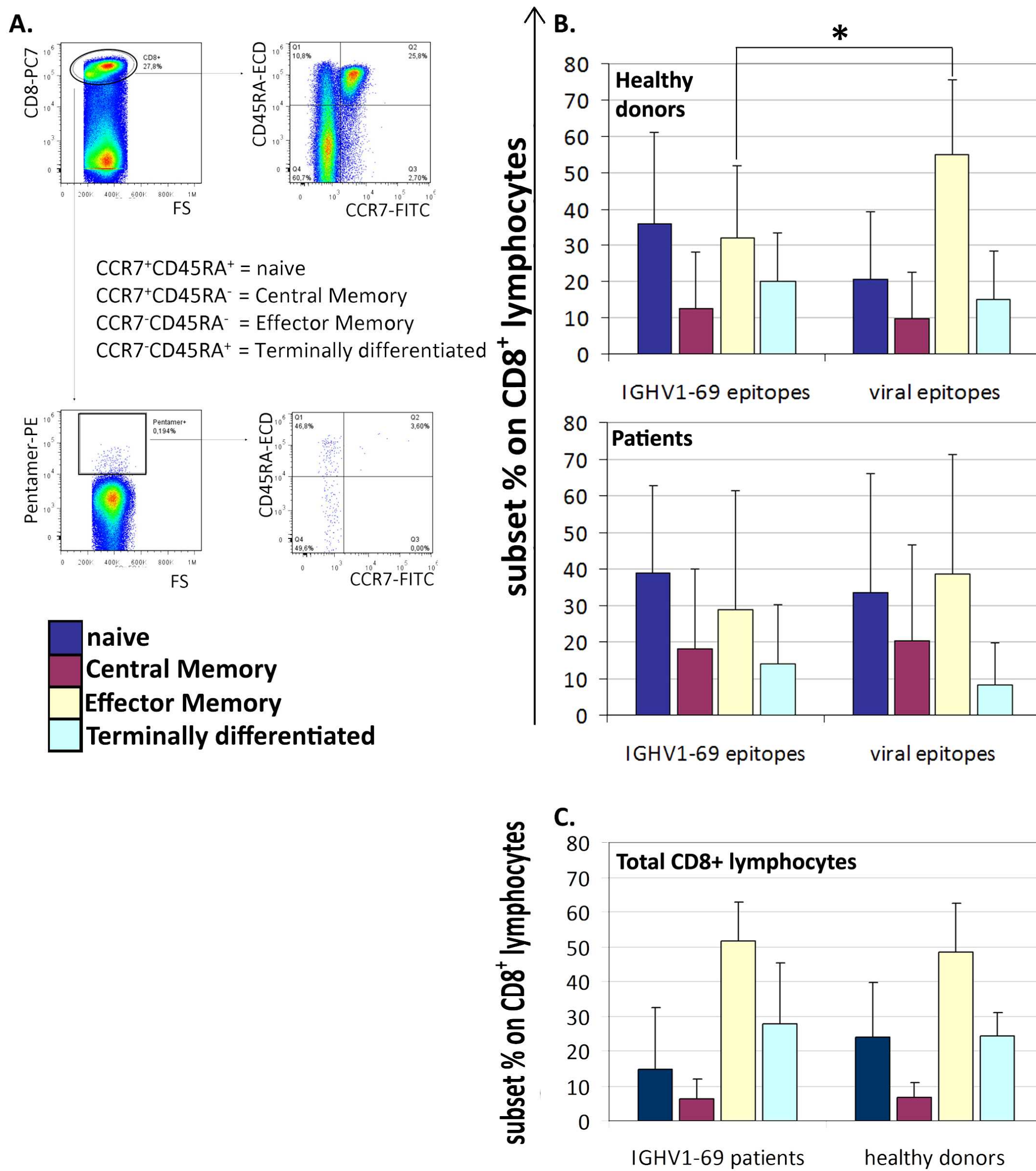


Figure 13. Characterization of the memory phenotype in pentamer$^+$ CD8$^+$ cells. **A.** Example of the flow cytometry gating strategy. **B.** Percentages of memory phenotype subsets in IGHV1-69$^+$ cells and viral$^+$ lymphocytes, measured in both healthy donors and patients. **C.** Percentages of memory phenotype subsets within the global CD8$^+$ population in patients and healthy donors. Results were considered significant if $p<0.05$.

Analysis of cytokine production by IGHV1-69-specific T cells

To further characterize IGHV1-69-specific T cells, we investigated their functional status through the ability to produce cytokines after *in vitro* stimulation. In particular, we measured the production of IFN-γ in ELISPOT assays after stimulation with both IGHV1-69- and virus-derived peptides in the context of HLA-A*0201, -A*0301, -A*2402, and -B*3501. Purified T lymphocytes isolated from patients (n=8) seemed to display a slightly higher number of IFN-γ secreting cells if stimulated with IGHV1-69-derived peptides as compared to viral-peptides stimulation ($median_{ratio\ IGHV1\text{-}69\ epitope/viral\ epitope}$=1.024; 0.399-3.600) (Figure 14). Moreover, the ratio between IGHV1-69-induced and viral-induced IFN-γ responses measured in patients' T cells was significantly higher than the correspondent ratio obtained in healthy donors' (n=8) purified T cells ($median_{ratio\ IGHV1\text{-}69\ epitope/viral\ epitope}$=0.700; 0.200-1.500; p=0.017) (Figure 14). These data were consistent with the higher prevalence of IGHV1-69-specific T cells (Figure 12) and the similar percentages of T_{EM} between IGHV1-69- and viral-specific T cells observed in patients' PBMCs (Figure 13B), if compared to the same analyses performed with healthy donors' cells.

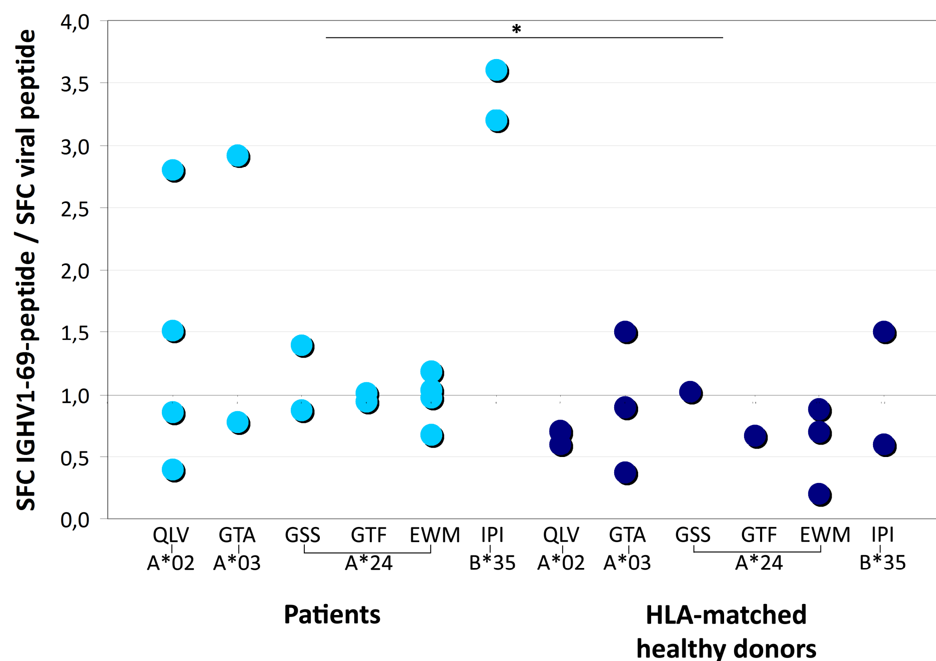


Figure 14. IFN-γ responses induced after *in vitro* stimulation with IGHV1-69- and viral-derived peptides in patients' and healthy donors' PBMCs. Data are represented as the ratio between IGHV1-69- and viral-induced IFN-γ responses. Every dot represents the ratio measured for a single IGHV1-69 and viral peptide in each patient/healthy donor. IFN-γ responses are indicated as number of SFCs (spot forming cells). IGHV1-69 epitopes are represented in the X axis with the first 3 aa residues. Viral epitopes used as controls are: the HLA-A*0201-restricted flu $M1_{58\text{-}66}$ peptide, the HLA-A*0301-restricted EBV $EBNA3A\text{-}RLR_{603\text{-}611}$ peptide, the HLA-A*2402-restricted CMV $pp65_{341\text{-}349}$ peptide, and the HLA-B*3501-restricted CMV $pp65_{123\text{-}131}$ peptide. Results were considered significant if p<0.05.

In vitro boosting of IGHV1-69-specific responses

The *in vitro* immunogenicity of IGHV1-69-derived peptides was evaluated through their ability to induce epitope-specific CTLs from healthy donors' and patients' PBMCs, using different CTL generation protocols.

Low IGHV1-69-specificity induced in healthy donors' PBMCs

IGHV1-69-derived peptides were used to induce CTLs by stimulating healthy donors PBMCs with peptide-pulsed autologous dendritic cells. After 5 stimulations, CTL cultures showed a specific, even if low, lytic activity against HLA-matched target cells loaded with the corresponding peptide, if compared to CTLs stimulated against known immunogenic peptides (MelanA/MART1 ELA_{26-35} for HLA-A*0201; CMV pp65 $QYD_{341-349}$ for HLA-A*2402; CMV pp65 $IPS_{123-131}$ for HLA-B*3501) (Figure 15). We also tested the ability of CTL cultures to recognize a HLA-matched lymphoid B-cell line naturally expressing IGHV1-69. Interestingly, we observed some specific lysis, especially in 1 B*3501 donor, against the IGHV1-69+ PG B.95.8 cell line (HLA-A*0201; HLA-A*0301; HLA-B*3501) (Figure 15).

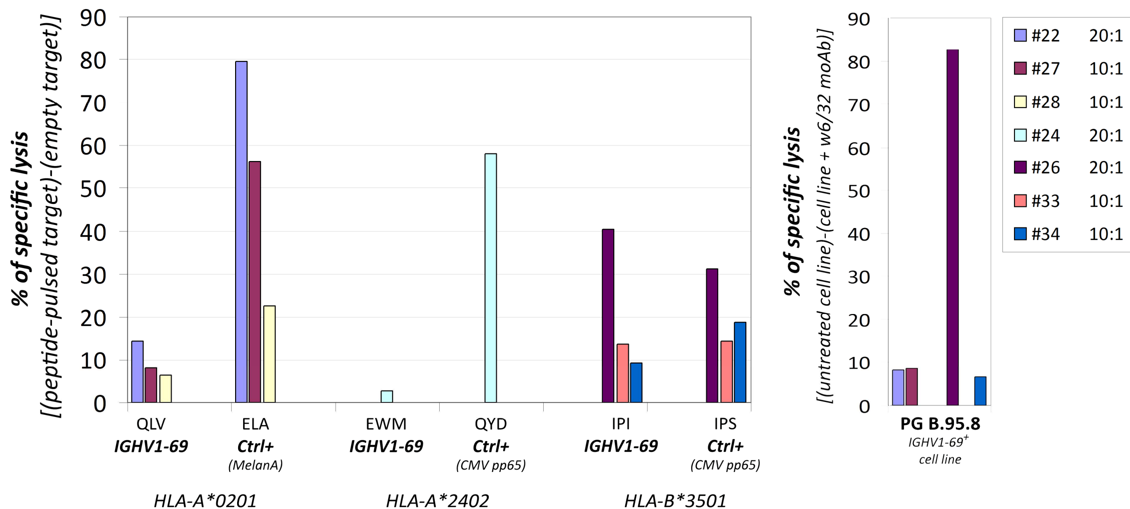


Figure 15. Results of cytotoxicity assays performed with CTLs cultures generated from the PBMCs of 7 different healthy donors. Each color represents a single donor as indicated in the legend, which displays also the selected Effector:Target ratios. Tested peptides are indicated in the X axis with the first 3 aa residues and their source. Specificity was calculated subtracting to the lysis obtained in peptide-pulsed targets, the lysis measured in empty targets. The lysis against the IGHV1-69-expressing PG B.95.8 cell line (HLA-A*0201, -A*0301, -B*3501) was estimated incubating target cells also with the α–HLA class-I antibody w6/32. The difference between the lysis of the untreated- and the antibody-coated-cell line was considered as specific lysis.

However, when compared to CTLs generated against positive controls, the pentamer staining of IGHV1-69-specific cultures was not clearly detectable (Figure 16), possibly due to the low affinity of the IGHV1-69-specific CTLs' TCR to the pentamer, or to the low frequency of IGHV1-69-specific cells in the culture that justify the lower cytotoxicity observed in respect to ELA- or viral-specific CTLs (Figure 15).

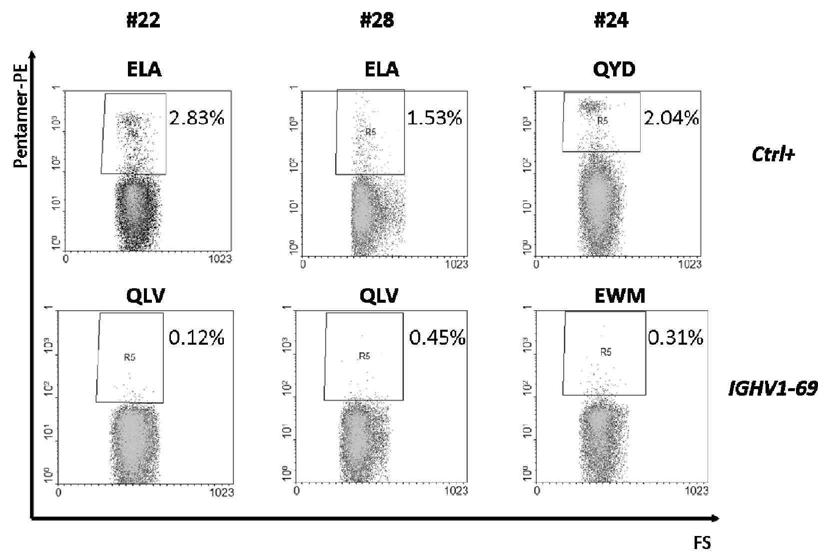


Figure 16. Examples of dot plots achieved with pentamer staining of peptide-specific CTLs cultures obtained from healthy donors PBMCs. Graphs display the $CD4^{-}CD8^{+}CD19^{-}$ population. Pentamer signalling was considered positive when mean fluorescence intensity > 10^2, with the exception of CMV $QYD_{341-349}$-specific pentamer that show a higher background. Peptide-specificity is indicated with the first 3 aa residues on the top of the correspondent dot plot (ELA is the HLA-A*0201-restricted MelanA/$MART1_{26-35}$ peptide; QYD the HLA-A*2402-restricted CMV $pp65_{341-349}$; QLV the HLA-A*0201-restricted IGHV1-69_{3-11} peptide; EWM the HLA-A*2402-restricted IGHV1-69_{44-52} peptide).

IGHV1-69-specific CTLs obtained from patient's PBMCs displayed a higher specificity in cytotoxic assays than healthy donors' derived CTLs cultures

Considering the higher frequency of IGHV1-69-precursors observed in patients' PBMCs (Figure 12C), we thought that it would be interesting to stimulate *ex vivo* patients IGHV1-69-specific cells in order to obtain IGHV1-69 specific CTL cultures. We pulsed in parallel healthy donors' (n=5) and patients' (n=5) PBMCs with HLA-matched cell lines (T2-A2 for HLA-A*0201, C1R-A3 for HLA-A*0301) loaded with different concentrations of IGHV1-69-derived peptides (0.1µg/ml, 1µg/ml, or 10µg/ml) or with a positive control (5µg/ml). We first monitored the cell growth, and interestingly observed a higher rate of growth of patients' cells if stimulated with IGHV1-69 peptides as compared to control stimuli, whereas in donors' PBMCs the grater growth ratio was obtained with virus-derived peptides (Figure 17).

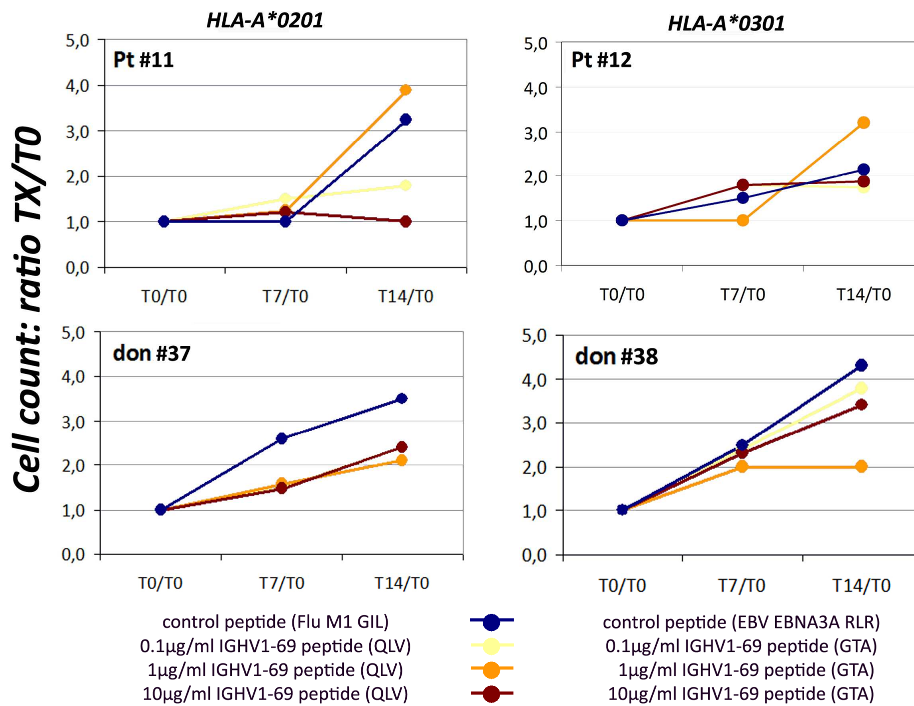


Figure 17. Cell count ratios in 4 exemplary CTLs cultures obtained from PBMCs of 2 patients and 2 healthy donors in the HLA-A*0201 and -A*0301 restrictions. Before each re-stimulation (T7 and T14), the number of cells was determined and divided for the starting amount of cells (T0).

After 3 stimulations, we also characterized the CD8 and CD4 phenotype of CTLs cultures, and their memory differentiation status through a combined analysis of the surface markers CCR7 and CD45RA (Figure 18A) [108]. Within CTLs cultures derived from patients' PBMCs, we interestingly observed an increase in the number of $CD8^+$ T cells when a higher amount of IGHV1-69-peptide was used in the stimulations (between 0.1µg/ml and 10µg/ml, $p=0.05$) (Figure 18B). Moreover, we noticed that CTLs cultures generated against IGHV1-69 or viral peptides displayed a comparable proportion of $CD8^+$ T cells and similar effector memory phenotype, if derived from both patients' and healthy donors' PBMCs (Figure 18B).

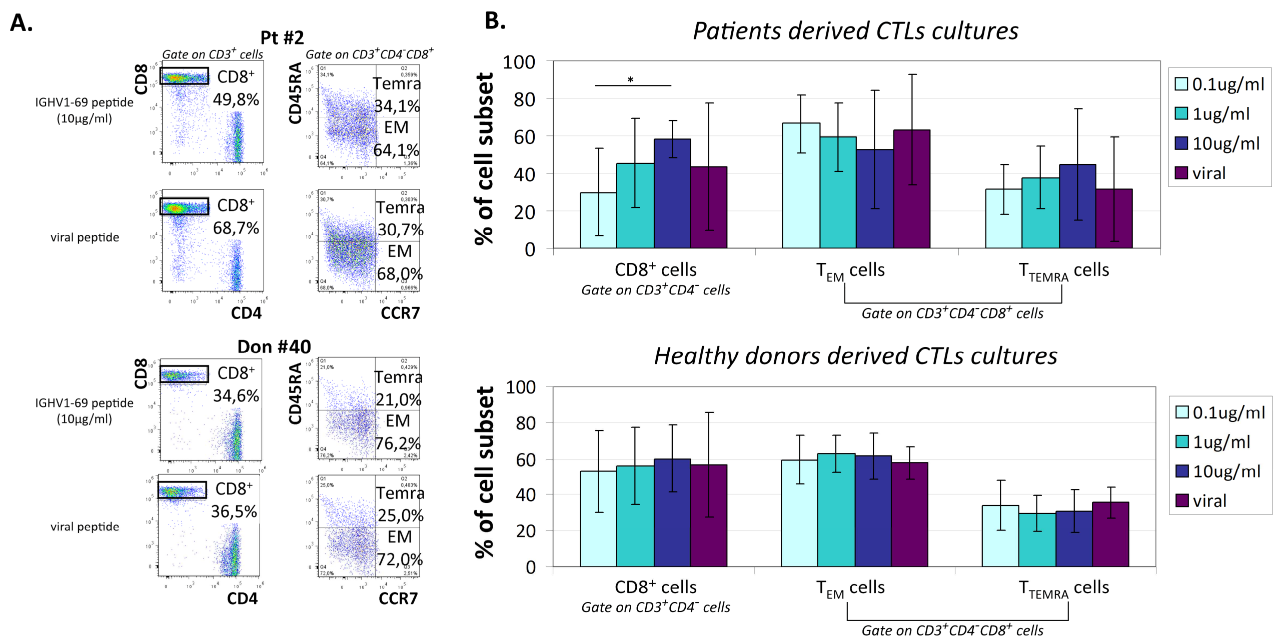


Figure 18. Phenotype characterization of CTL cultures derived from patients' and healthy donors' PBMCs. **A.** Examples of dot plots obtained gating live CD3$^+$ lymphocytes (left column) and CD3$^+$CD4$^-$CD8$^+$ cells (right column) in CTLs cultured obtained from one patient and one healthy donor. **B.** Summary of collected data regarding CTLs cultures generated from 4 patients and 4 healthy donors using 3 different concentration of IGHV1-69 peptide (0.1µg/ml, 1µg/ml, 10µg/ml) or a viral control (HLA-A*0201 restricted Flu M1-GIL$_{58\text{-}66}$, and HLA-A*0301 restricted EBV EBNA3A-RLR$_{603\text{-}611}$). T_{EM} (Effector Memory) cells were identified as CCR7$^-$CD45RA$^-$, T_{TEMRA} (Terminally differentiated) as CCR7$^-$CD45RA$^+$. Results were considered significant when p≤0.05.

IGHV1-69-specific CTLs generated from PBMCs of 2 different patients revealed a detectable cytotoxicity against an HLA-matched cell line (T2-A2 for HLA-A*0201, and C1R-A3 for HLA-A*0301) pulsed with the specific peptide. Interestingly, IGHV1-69-specific cultures generated from 1 patient's cells were able to kill more efficiently peptide-pulsed targets, if compared with the similarly-generated CTLs derived from healthy donor's PBMCs (Figure 19A). Furthermore, in 8 CTLs cultures (4 from healthy donors' and 4 from patients' cells), we also measured a weak, but specific lysis, against the IGHV1-69$^+$ PG B.95.8 cell line, with a more evident recognition by HLA-A*0301-restricted cultures (Figure 19B).

Nevertheless, we were not able to observe a clear epitope-specific signal when testing these CTL cultures in flow cytometry with peptide-specific pentamers (data not shown).

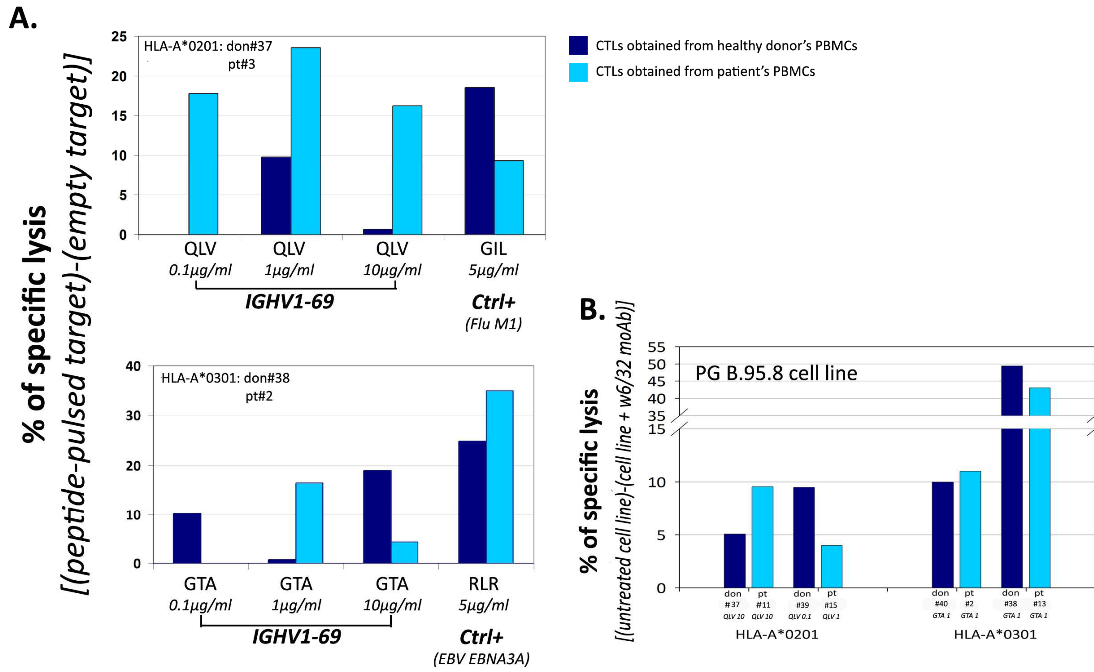


Figure 19. Evaluation of CTLs specificity in cytotoxicity assays. **A.** Results obtained with CTLs cultures generated from the PBMCs of 2 different healthy donors and 2 patients in the HLA-A*0201 and –A*0301 restrictions. Peptide specificity is indicated along the X axis with the first 3 aa residues, the concentration used to load the APC, and the peptide source. Specific lysis was calculated as the subtraction between the value relating to the killing of empty targets, and the result obtained with peptide-pulsed targets. The Effector:Target ratio was 2.5:1. **B.** Lysis against the IGHV1-69-expressing PG B.95.8 cell line (HLA-A*0201, -A*0301, -B*3501). The specificity was estimated incubating target cells with the α–HLA class-I antibody w6/32. The difference between the lysis of the untreated- and the antibody-coated-cell line was considered as specific lysis. Patients' and healthy donors' origin, peptide-specificity, and peptide concentration used to load APC are indicated along the X axis. The Effector:Target ratio was 10:1.

Successful generation of IGHV1-69-epitope-specific CTLs cultures from healthy donor PBMCs using artificial APC

At last, we tested a CTL generation protocol developed to boost low-avidity tumor-directed T-cells [119] based on the use of artificial APC. After 3 stimulations, we successfully obtained IGHV1-69-epitope specific T-cells from $CD8^+$ T cells of 1 healthy donor (#48), in the HLA-B*3501 restriction. These CTL cultures displayed a clear positivity in flow cytometry with a tetramer positive signal varying from 0.26% to 2.69% of $CD8^+$ T cells (Figure 20). Parallel HLA-A*0201 and -A*2402-restricted stimulations did not induce epitope-specific $CD8^+$ T cells in this donor (data not shown).

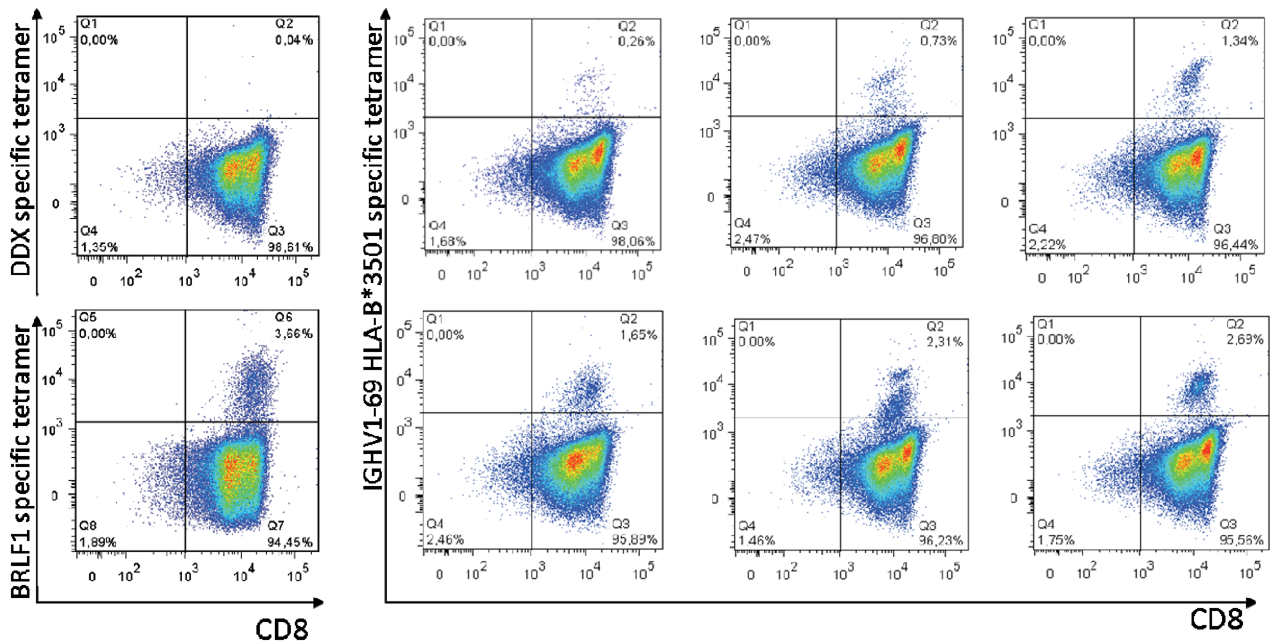


Figure 20. Tetramer staining of CTL cultures obtained with aAPC stimulation of healthy donor $CD8^+$ T cells. Dot plots on the left show the staining of CTLs cultures obtained with the negative control ($DDX_{168-176}$, upper) and the positive control (EBV $BRLF1_{109-117}$, lower) stimulation and stained with the corresponding tetramers and an α-CD8 moAb. The right panel displays exemplary dot plots obtained after the staining of IGHV1-69 IPI_{50-58} stimulated CTLs cultures with IGHV1-69 HLA-B*3501 specific tetramer and an α-CD8 moAb.

We finally isolate through immunomagnetic methods the IGHV1-69 IPI_{50-58} tetramer-positive fraction of the CTLs cultures and clone it. Interestingly, after 1 month, we achieved the first T-cell clones, which resulted clearly positive in tetramer staining (Figure 21), even if, unfortunately, they did not display any cytokine release after peptide stimulation (data not shown). A more careful and deeper analysis of the obtained clones is ongoing.

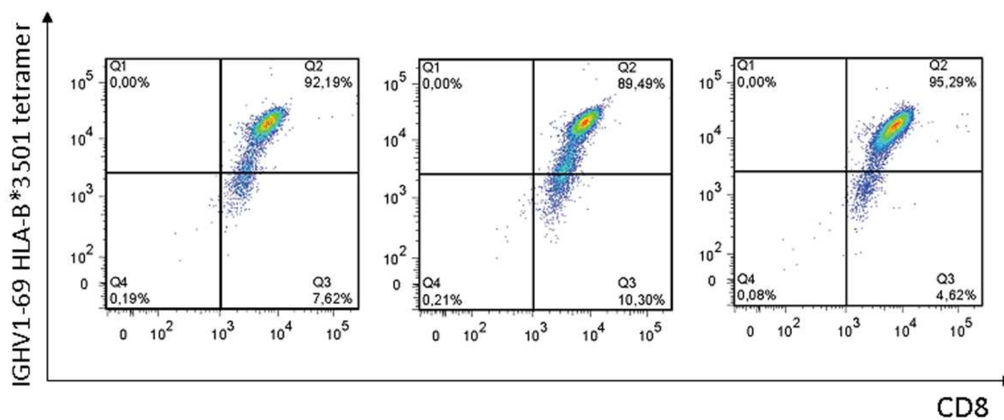


Figure 21. Tetramer staining of CTL clones obtained after immunomagnetic isolation of IGHV1-69-tetramer positive cells and stimulation with feeder cells every 2 weeks. Exemplary dot plots achieved with IGHV1-69 HLA-B*3501 specific tetramer and α-CD8 moAb staining. All the graphs are gated on live lymphocytes.

DISCUSSION

In this study we aimed at investigating the *in vitro* immunogenic features of the Id protein IGHV1-69, in order to establish its potential role as a suitable target for a shared immunotherapy against B-cell lymphomas/leukemias. The extreme heterogeneity of B-NHLs, together with their frequent resistance and relapses after chemo-radiotherapy and monoclonal antibodies, has made it difficult to develop a common therapy able to maintain the complete remission induced with standard treatments. In this field, in the last decades many efforts have been spent to investigate if Idiotype vaccines may represent the most suitable maintenance therapy, due to their high tumor-specificity and their low toxicity. However, despite encouraging results coming from phase I and II studies, 2 out of 3 phase III Id clinical trials failed to achieve the expected benefit in progression-free survival, thus abating the expectations placed in this type of vaccination. Several reasons have been ascribed to the failure of these trials, with particular attention to the study design and the selected endpoints. For example, the recruitment of both patients undergoing a complete response and those with a partial response after standard treatments resulted in a quite various population survey, which could be responsible for the different outcome after Id vaccination. Moreover, the lack of rituximab, currently used in the management of B-NHLs, in the pre-vaccination therapy of 2 clinical trials, did not allow to investigate the effect of B-cells depletion in the vaccination treatment. Finally, the choice of progression free survival as the major endpoint may be constrictive, and other evaluation criteria, as biological and clinical efficacy, should be considered. In our opinion, a more stringent definition of patients' enrolment and the choice of suitable criteria of response to therapy, may overcome the current misses of Id vaccinations and enrich their potentialities. In this respect, the identification of a shared target for Id vaccines may facilitate the development of markers suitable for patient eligibility and response monitoring. Moreover, the selection of a common target could promote the design of an off-the-shelf Id vaccine, thus reappraising the present expensive and time-consuming production of patient-specific Id vaccines.

Available data clearly indicate that IGHV1-69 is characterized by both tumor specificity issues and a shared expression among different B-NHL [28,30,57-61,71,77,81], thus combining the advantages of a reduced toxicity due to limited side effects, and decreased times and costs of production if compared to patient-tailored vaccines. In order to emphasize the properties of shared antigen, we designed an optimized IGHV1-69 sequence, conceived to enhance its similarity degree in respect to several IGHV1-69 sequences, obtained not only from different patients, but also in the context of various malignancies, while preserving or even enhancing immunogenicity. In this respect, the inclusion of a heterogeneous group of patients, affected by B-cell lymphoproliferations with different histological characteristics (Table 1), stresses the broad applicability of a therapeutic approach exploiting the features of IGHV1-69. In addition, the expression of this protein in pre-malignant lymphoproliferations evokes also a potential role of this target in preventive anti-tumor vaccination (Table 1) [58,60,77].

Sequence optimization was performed to further support the shared expression of the same aa variants among patients. To do so, we excluded the hypervariable CDR3 region. In fact, even if this sequence's portion is often a carrier of immunogenic epitopes [126], it is primarily responsible for sequence variability and thus involved in the individualized character of current Id vaccinations. Moreover, most of NHLs considered in this study, derived from antigen-experienced B cells, whose Id proteins may carry somatic mutations, especially in the CDR3 region, that could possibly affect immune recognition [56].

When designing the optimized sequence we also took care of the most immunogenic regions using bioinformatics tools able to predict within a sequence the epitopes characterized by the highest binding affinity to selected MHC molecules [116-118]. The obtained sequence is thus optimized not only in the similarity degree among IGHV1-69 variants (Figure 9), but also in the preservation of immunogenic regions, which could at least in part overcome the low affinity to HLA molecules usually observed for self-antigen-derived epitopes, including Ig-derived peptides [64]. Indeed, one strategy to increase MHC binding and immunogenicity of low-binding peptides is to modify the peptide at the MHC-binding aa residues while leaving the T-cell–recognition residues intact [127]. The resultant “heteroclitic” peptides led to improved induction of CTL responses against melanoma in clinical trials [128], and interestingly also against IGV proteins *in vitro* [129]. Importantly, heteroclitic peptides elicited T-cell responses not only against the altered peptide but also against the native peptide from which they were derived. Moreover, they also intriguingly increased the killing efficacy of primary leukemic cells expressing the correspondent IGV protein and thus presumably the native peptide [129]. These observations suggest that MHC class I–restricted heteroclitic peptides can enlarge the pool of immunogenic peptides in B-cell malignant diseases and enhance the immunogenicity of these peptides [129]. In this context, we recently characterized the ability of IGKV3-20-specific CTLs to efficiently recognize and kill autologous targets presenting different A*0201-restricted IGKV3-20 "variant" epitopes, often with a higher efficiency as compared with that elicited against the "native" epitopes [56]. Accordingly, these data strongly suggest that IGV proteins, in this case IGKV3-20, can be carriers of "natural" heteroclitic versions of several CTL epitopes shared by IGV proteins broadly expressed among lymphoid malignancies [56].

To definitively validate the most promising epitopes within the optimized IGHV1-69 sequence, we did not restrict our self to the *in silico* analysis. We also investigated the ability of all possible IGHV1-69-derived nonamers to stabilize MHC complexes in the context of the 7 most common HLA class-I alleles in the Caucasian population [122]. For this purpose, we used a novel technology able to measure the *in vitro* binding kinetics of T-cell epitopes to MHC molecules (ProImmune REVEAL & ProVE® Rapid Epitope Discovery System) [123,124]. It was interesting to observe that, through the HLA-binding affinity assay, the 13 predicted epitopes in the optimized sequence (Table 2) appear conserved or barely modified in several IGHV1-69 variants, and surprisingly also in other proteins of the IGHV1 family or of molecularly related Id proteins (Table 3). This remark inspires a further extension of IGHV1-69-derived epitopes pertinence, even if a deeper analysis is needed to investigate the effects on IGHV1-69 immunogenicity after heteroclitic peptide modifications.

Nonetheless, the immunogenicity of a potential epitope depends also on the avidity of TCR binding to the peptide-MHC complex [114,125,130], which in turn should be investigated through the survey of epitope-specific T-cell responses in patients affected by lymphoproliferations expressing the relevant target. Presently, the results obtained so far support the existence of spontaneous T-cell memory-responses against IGHV1-69-derived epitopes in lymphoma patients, at least for some HLA restrictions (Figure 12). Therefore, the protein seems to be naturally processed and presented on MHC molecules, as further demonstrated by the ability of epitope-induced CTLs to specifically recognize an IGHV1-69$^+$ cell line (Figures 15 and 19B). Moreover, the *in silico* analysis of proteasomal processing revealed that most of the predicted epitopes are potentially naturally produced (Figure 10), thus supporting the predicted immunogenicity of the identified epitopes [115].

In addition, the ability to elicit HLA class I-restricted specific CTL responses *ex vivo* from different donors and patients (Figure 15, 19, and 20) rules out the possible existence of a central tolerance (against this Id protein) that could entail the lack of IGHV1-69-specific TCR

[131]. In this context, previous evidence supported the ability to generate, from healthy donors' PBMCs, specific CTLs against epitopes shared by different proteins of the IGHV1 family, among whom we identified the IGHV1-69-derived QLV_{3-11} epitope [65]. With our analysis we further enforced this observation, demonstrating the capability of this epitope to induce specific CTLs also from patient's PBMCs (Figure 19A). The common expression of the QLV_{3-11} epitope in different IGHV proteins (Table 3) suggests that IGHV1-69-derived epitopes could potentially induce a cross-reactive response also against other molecularly related Id proteins, expressed in different lymphomas/leukemias. This prospective effect has already been described for IGKV3-20 protein, which was able to elicit cross-reactive responses against IGKV3-20 proteins derived from different NHLs, and interestingly, also towards IGKV3-15, a related Id protein showing a 80% similarity degree with IGKV3-20 [56]. CTLs generated against IGKV3-20 were able to efficiently recognize also IGKV3-20- and IGKV3-15-expressing lymphoma cells, in a HLA-A*0201-restricted manner [56]. On these grounds, the specific recognition that we observed against the IGHV1-69-expressing cell line with QLV_{3-11} stimulated CTL cultures (Figure 15, and figure 19B), may assume a potential cross-reactive response induced by the optimized IGHV1-69 protein, against other IGHV1-69 proteins. The PG B.95.8 cell line in fact, expresses a variant IGHV1-69 sequence, which differ for some aa residues from the optimized sequence (80% homology between the 2 sequences), and could pave the way to a broader analysis of cross-reactive responses against other Id proteins of the IGHV1 family.

To exclude the presence of peripheral tolerance mechanisms against IGHV1-69, we then searched for IGHV1-69 IFN-γ responses in lymphoma patients. Comparing the amount of IFN-γ release after peptide stimulation, we interestingly found that IGHV1-69-derived epitopes induced a higher response than known immunogenic epitopes do, only in lymphoma patients (Figure 14). These data are consistent with the increased number of IGHV1-69-specific T cells observed in lymphoma patients (Figure 12C), and with the similar memory phenotype between IGHV1-69- and virus- specific T cells documented in the same subjects (Figure 13), and further support the *in vivo* immunogenicity of this protein or at least of its derived epitopes. The almost evident higher response against IGHV1-69 epitopes observed *ex vivo* in patients' cells, when compared to effects induced by viral epitopes, was further supported by the ability to stimulate *in vitro* epitope-specific CTLs more efficiently than viral-derived peptides (Figure 16, and figure 18A pt#3). Withal, an increased dose of IGHV1-69 stimulating peptide enhanced the number of $CD8^+$ T cells only within CTL cultures derived from patients' PBMCs (Figure 17), thus underlying the stronger effect of IGHV1-69 stimulation in cells from lymphoma patients. Incidentally, the comparable memory phenotype observed in patients' and healthy donors' CTL cultures (Figure 18), ruled out the possibility of an impairment of patients' cells to differentiate into effector memory and terminally differentiated T cells.

In this study, we mainly focused our attention on Id-derived epitopes, primarily because of the optimized character of IGHV1-69 sequence, which would require a dedicate production of the optimized protein. However, several papers demonstrated that peptides-based vaccines are often able to induce a more effective and specific response than the whole protein, maybe because they can elude the tolerance developed against the original protein [132-135]. Moreover, a recent paper proposed the use of Id-derived immunogenic peptides to selectively expand Id-specific T cells *ex vivo* and generate "educated" DLIs. Such primed T cells might enhance tumor specificity and limit Graft *versus* Host Disease complications of current DLI strategies [63]. For this purpose, a promising alternative is represented by the quite fast generation of epitope-specific T cells through artificial APC [119]. This strategy, in fact, allowed us to obtain an epitope-specific CTL population (Figure 20) in a shorter time if compared to traditional protocols, and to successfully generate a large number of epitope-positive clones

(Figure 21), which could be in turn expanded for DLI intentions, even if we still need to confirm their functionality. Furthermore, this approach does not require the production of autologous dendritic cells, which often represents the limiting step of TAA-specific CTLs generation and expansion.

The early identification of immunogenic epitopes may also simplify the development of markers for patients' eligibility and eventually therapy response. Indeed, multimers (tetramers and pentamers in this study) constitute a quick instrument to identify rare T cell populations, and to monitor their expansion during therapy, investigating their relationship with clinical response. Moreover, multimer staining offers many advantages over other T cell assays. In particular, this method is quantitative and fast, it is the only technique that allows an estimation of the avidity between TCR and peptide-loaded HLA molecules, and cells can be stained with additional markers simultaneously. An important issue of multimer utilization is that specific T cells can be analyzed regardless of their functional status including anergic or non-reactive T cells [92], thus requiring further techniques able to identify only functional T cells. For this reason, besides this method, other two assays are recommended as first-line monitoring tools in clinical vaccination studies: IFN-γ ELISPOT assays and intracellular cytokine staining (ICS) in flow cytometry [136]. IFN-γ ELISPOT owns the lowest detection limit for specific T cell responses at the single cell levels [137], while ICS allows the simultaneous analysis of multiple cytokines and phenotypic markers of T-cell activation, thus identifying multifunctional T cells [109,138]. These 3 assays, strongly suggested for the immune-monitoring of vaccination trials, require the early identification of the target of interest, and thus may benefit from previously identified epitopes.

However, the lack of investigations on the whole IGHV1-69 protein represents a limitation of this study, and a crucial future step to definitively confirm the *in vitro* and *in vivo* immunogenicity of this protein. Another possible hindrance related to our study design may be the selection of a single chain Id protein, which causes the loss of the unique B-cell epitopes generated by the association of the variable portions of the heavy and light chains. Nevertheless, the choice of a single chain protein enlarges the potential targeted malignancies and the number of eligible patients. Moreover, we recently described that in a mouse model repeated immunizations with the single chain Id protein IGKV3-20 boosted detectable levels of Ig capable of recognizing naturally folded, public epitopes physiologically present on the membrane-mounted protein [56]. For this reason, we speculate that the same effect could be obtained also with other single chain Id proteins, as IGHV1-69. Finally, the selection of a self antigen, which is also expressed in a small proportion of normal B cells, may cause the deletion of IGHV1-69^{+} normal B cells [78-80]. Available evidence, however, indicate that Id vaccines, which include both heavy and light chains, are safe and associated with only modest toxic effects, with no evidence of immune compromise related to depletion of part of B-cell repertoire [56].

Hence, the data presented in this study support the *in vitro* immunogenicity of IGHV1-69-derived epitopes, and pave the way to further *in vivo* pre-clinical experiments aimed at definitively confirm the IGHV1-69 ability to induce an immune response, which could be able to restrain tumor growth *in vivo*. Moreover, we intend to design and prepare viral vectors allowing the expression of the optimized IGHV1-69 sequence fused to an enhancer of the immune response, as *mycobacterium tubercolosis* heat shock protein 70, in order to develop an IGHV1-69-targeting DNA vaccination, which could extend the application of this approach.

In conclusion, the results of the present study indicate that IGHV1-69 constitutes an attractive target candidate for the development of a *subset-specific* idiotypic vaccine,

potentially addressed to the 40% of patients affected by an HCV-associated lymphoma, the 13% of CLL patients, and almost the 70% of patients with an autoimmunity-related B-cell lymphoproliferation. Furthermore, multimers and ELISPOT-based immune-assays may be particularly useful to select patients who could be eligible for the IGHV1-69-based vaccination and to identify those showing specific T-cell responses. Accordingly, improved selection of eligible patients and a more precise monitoring of specific immune responses may further improve the clinical efficacy of “shared Id” vaccines in the maintenance of a complete response after standard therapy in B-NHL patients.

APPENDIX

Healthy donor number	HLA class I genotyping	Healthy donor number	HLA class I genotyping	Healthy donor number	HLA class I genotyping
#1	negative for interested alleles	#18	-A*2402	#35	-A*0201
#2	-A*2402	#19	negative for interested alleles	#36	-A*0101, -A*0201
#3	-B*3501	#20	-A*2402	#37	-A*0201
#4	negative for interested alleles	#21	-A*2402	#38	-A*0301, -A*2402
#5	-B*0702	#22	-A*0201	#39	-A*0201
#6	-A*0201	#23	-A*0201	#40	-A*0301
#7	-A*0301	#24	-A*2402	#41	-A*0201, -B*3501
#8	-A*0201	#25	-B*3501	#42	-A*0301, -A*2402
#9	-A*0301	#26	-B*3501	#43	-A*0201, -A*2402
#10	-B*0702	#27	-A*0201	#44	-B*3501
#11	-A*0301, -A*2402	#28	-A*0201	#45	-A*0201
#12	-A*0301	#29	-A*2402	#46	-A*2402
#13	-A*2402	#30	-A*2402	#47	-B*3501
#14	-A*0301	#31	-A*0301	#48	-A*0201, -A*2402, -B*3501
#15	-A*0101, -A*0201	#32	-A*2402	#49	-A*0201
#16	-A*0101	#33	-A*0201, -B*3501	#50	-A*0301, -A*2402
#17	-A*0201, A*0301	#34	-A*0201, -B*3501	#51	-A*0201

Table 5. HLA genotyping of healthy donors collected for the study.

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