IGKV3 Proteins as Candidate "Off-the-Shelf" Vaccines for Kappa-Light Chain–Restricted B-Cell Non-Hodgkin Lymphomas

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Abstract

**Purpose:** An increasing set of B-cell non-Hodgkin lymphomas (B-NHL) show a biased usage of IGKV3-20 and IGKV3-15 immunoglobulin genes, a feature that could be exploited for the development of ready-to-use, broadly applicable cancer vaccines.

**Experimental Design:** The immunogenic properties of clonal IGKV3-20 and IGKV3-15 proteins were analyzed with particular focus on their ability to elicit cross-reactive responses against molecularly related IGKV proteins expressed by different B-cell lymphoproliferative disorders.

**Results:** IGK+ lymphoma patients show humoral and T-cell responses to IGKV3-20 and IGKV3-15 proteins and IGKV3-specific cytotoxic T lymphocytes (CTL) can be easily induced ex vivo. IGKV3-20–specific CTLs cross-react against different IGKV3 proteins, an effect mediated by the presence of 21 shared, sometimes promiscuous, T-cell epitopes, presented by common HLA class I allele products, thus assuring a broad HLA coverage of IGKV3-based vaccines. Many natural epitope variants are carried by IGK light chains expressed by a broad spectrum of B-NHLs and we show that IGKV3-20–specific CTLs cross-react also against several of these variant epitopes. Both humoral and CTL-specific responses were induced by KLH-conjugated IGKV3-20 protein in HLA-A2-transgenic mice and coinjection of IGKV3-20–specific CTLs with IGKV3-20+ or IGKV3-15+ lymphoma cells into SCID mice totally prevented tumor growth, thus confirming the ability of these effectors to mediate efficient and cross-reactive cytotoxic responses also in vivo.

**Conclusions:** These results provide the rationale to exploit IGKV3 proteins as "off-the-shelf" vaccines for a large fraction of lymphoma patients. *Clin Cancer Res; 18(15); 4080–91. ©2012 AACR.*

Introduction

Therapeutic vaccines targeting B-cell non-Hodgkin lymphoma (NHL) idiotype (Id) represent a promising approach to improve the clinical control of these malig-
Cancer vaccines for B-cell lymphomas targeting idiotypic proteins constitute a promising therapeutic strategy. Nevertheless, the individualized production of idiotypic vaccines is still complex, costly, and poorly applicable on a large scale. Moreover, the different manufacturing protocols currently used to produce idiotypic vaccines make it difficult to compare the results of different clinical trials. These limitations may be overcome by the exploitation of the immunogenic properties of distinct clonal immunoglobulin light chains, such as IGKV3-15 and IGKV3-20, which are broadly expressed by different B-cell lymphoproliferations. The ability of IGKV3-based recombinant vaccines to elicit cross-reactive responses against molecularly related light chains provides the rationale to activate a phase I/II study aimed at assessing the safety and efficacy of these vaccines. Achievement of these goals would make available a ready-to-use recombinant vaccine for improved consolidation therapy of low-grade B-cell lymphomas.

### Translational Relevance

Cancer vaccines for B-cell lymphomas targeting idiotypic proteins constitute a promising therapeutic strategy. Nevertheless, the individualized production of idiotypic vaccines is still complex, costly, and poorly applicable on a large scale. Moreover, the different manufacturing protocols currently used to produce idiotypic vaccines make it difficult to compare the results of different clinical trials. These limitations may be overcome by the exploitation of the immunogenic properties of distinct clonal immunoglobulin light chains, such as IGKV3-15 and IGKV3-20, which are broadly expressed by different B-cell lymphoproliferations. The ability of IGKV3-based recombinant vaccines to elicit cross-reactive responses against molecularly related light chains provides the rationale to activate a phase I/II study aimed at assessing the safety and efficacy of these vaccines. Achievement of these goals would make available a ready-to-use recombinant vaccine for improved consolidation therapy of low-grade B-cell lymphomas.

### Materials and Methods

#### Patients and healthy donors

Peripheral blood was collected from healthy donors and 13 patients with HCV-NHL (Supplementary Table S1). All participants provided signed informed consent, and the study was approved by local IRB. HLA typing was conducted by standard high-resolution typing.

#### Production of recombinant Id proteins

Sequences from 1 IGKV3-15 and 2 IGKV3-20 rearranged genes were PCR amplified from NHLs using family-specific primers (21). IGKV3-specific fragments were subcloned into pFLAG-MAc and transformed into JM109 strain *Escherichia coli* (21). The expressed proteins were purified by affinity column and their sequence verified by fingerprinting analysis of trypsin-digested extracts (22). Endotoxin level was <1.0 EU/mg of protein (LAL method).

#### Cell lines

Lymphoblastoid cell lines (LCL) were generated by *in vitro* transformation of B cells with the B.95.8 Epstein–Barr virus isolate. The SH9 LCL was established from peripheral blood mononucleated cells (PBMC) of a patient with Hodgkin lymphoma. The DG75 Burkitt’s lymphoma cell line was obtained from DSMZ and confirmed by fingerprinting (Power Plex 1.2; Promega) in April 2011. Cell lines were cultured in RPMI-1640, containing 10% heat-inactivated FBS (Gibco), 2 mmol/L glutamine, 100 µg/mL streptomycin, and 100 IU/mL penicillin (Sigma-Aldrich) and maintained at 37°C in a 5% CO2 atmosphere.

#### Generation and characterization of IGKV3-specific CTLs

Generation of IGKV3- and peptide-specific CTLs was conducted as described (23, 24). After 3 stimulations with protein- or peptide-loaded DCs, CTL cultures were characterized with the following FITC-, PE-, ECD-, PE7-celled antibodies used in a 5-color combination: anti-CD3 (UCHT1, IgG1), anti-CD45RA (2H4, IgG1), anti-CD4 (13B8.2, IgG1), anti-CD8 (SFCI21ThyD3, IgG1; Beckman Coulter), anti-CCR7 (3D12, Rat, IgG1), BD Biosciences PharMingen), anti-CD11a (H111, IgG1), anti-CD44 (IM7, Rat IgG2b), anti-CD49d (9F10, IgG1, k) and anti-CD62L (DREG56, IgG1, k; ebioscience).  
Mouse FITC- and PE-IgG1 (679.1Mc; Beckman Coulter) and Rat PE-IgG2a (eBRa; ebioscience) were used as isotypic controls. Cytofluorimetric analysis was conducted with a Cytomics FC500 and the data analyzed with CXP software (Beckman Coulter). To generate peptide-specific CTL microcultures, dendritic cells (DC) were differentiated from peripheral blood monocytes of HLA-A*0201– and loaded overnight with 25 µg/mL of each of 10 different IGKV3–20–derived peptides selected for HLA-A*0201 binding. The HLA-A*0201–restricted cyt1 D1–derived peptide (LLNDVRRA) and the Flu-matrix58–66 peptide served as negative and positive controls, respectively. For each peptide, 30 replicate microcultures were set-up and fed with IL-
2 (60 IU/ml) and IL-7 (10 IU/ml) at day 2. All cultures were restimulated at days 7 and 14 with autologous peptide-loaded and irradiated (7 Gy) PBMs (1 × 10⁵ cells/well) and at day 21 analyzed for cytokotoxicity by a calcein-AM release assay (23). All tests were conducted in triplicate. HLA class I w6/32 mAb (10 µg/ml; Abcam) or HLA-A*0201–specific cr11.351 mAb (10 µg/ml; 25) were used to assess HLA restriction, and the anti-Epstein–Barr Virus Nuclear Antigen 1 (EBNA1) mAb (1:50; Millipore) was used as irrelevant antibody. Fluorescence intensity was measured with a SpectraFluorPlus fluorimeter (Tecan).

IFN-γ ELISpot assays
IFN-γ-ELISPOT assay was conducted with a commercial kit (Endogen) according to manufacturer’s instructions and using as stimulators (×10⁵ cells/well) autologous monocytes pulsed with either 10 µg/ml of Id protein or 20 µg/ml peptide, and immunomagnetically purified CD8⁺ T lymphocytes as responders (10⁵ cells/well). Plates were evaluated by a computer-assisted ELISPOT reader (Eli expert. A.EL.VIS). The number of spots in negative controls never exceeded 5 spots/well. Responses were considered significant if >5 IFN-γ-producing cells were detected.

Detection of humoral responses to IGKV3-20 in lymphoma patients
The analysis was carried out using a chemiluminescent ELISA test. Briefly, 96-well microtiter plates were coated overnight with either recombinant IGKV3-20 protein or Tetanus Toxoid (TT, Calbiochem) antigens (2 µg/ml). Plates were washed, and horseradish peroxidase-conjugated goat antihuman IgG (Sigma-Aldrich) was added. After incubation for 1 hour at RT, plates were washed, and horseradish peroxidase-conjugated goat anti-mouse antibody (Charles River Laboratories; 0.02 µg/ml in PBS-0.05% Tween). Finally, 100 µl per well of substrate solution (prepared using SIGMA-Fast OPD tablets) were added for 5 minutes at RT in the dark. Reaction was stopped with 50 µl/well of HCl 3N (Carlo Erba), and plates analyzed at 490 nm using a VictorX4 instrument (Perkin-Elmer). Data were used to generate a titration curve. The same sera used in ELISA test were analyzed for their ability to stain DG75 cells (prepared using SIGMA-Fast OPD tablets) were added for 1 hour at RT with secondary HRP-conjugated goat antimouse antibody (Charles River Laboratories; 0.02 µg/ml in PBS-0.05% Tween). Finally, 100 µl per well of substrate solution (prepared using SIGMA-Fast OPD tablets) were added for 5 minutes at RT in the dark. Reaction was stopped with 50 µl/well of HCl 3N (Carlo Erba), and plates analyzed at 490 nm using a VictorX4 instrument (Perkin-Elmer). Data were used to generate a titration curve. The same sera used in ELISA test were analyzed for their ability to stain DG75 cells (prepared using SIGMA-Fast OPD tablets) were added for 5 minutes at RT in the dark.

T-cell epitope mapping and characterization
Identification of IGKV3-20–derived epitopes was carried out with the iTopia Epitope Discovery System (Beckman Coulter; ref. 26). Briefly, 100 9-mer peptides overlapping by 8 residues spanning the entire length of the protein (108 aa) were screened for their ability to bind 8 HLA class I alleles (A*0101, A*0201, A*0301, A*1101, A*2402, B*0702, B*0801, and B*1501) under optimal conditions (27). The percentage of binding was assessed using a fluorimeter and peptides which bound MHC molecules >20% compared with the reference positive control were selected for analysis of affinity, expressed as ED50 (the concentration at which the peptide is bound to the MHC at 50% maximum binding efficiency). Peptides were also analyzed for off-rate (complex stability) where T½ represents the time taken for a reduction from optimal binding of 50% after a shift to dissociating conditions (27).

IGKV3-20–specific humoral and T-cell responses in animal models
Six- to 8-week-old female SCID or HHD mice were used according to the national guidelines (28). HHD mice were immunized by subcutaneous (s.c.) injections of KLH-conjugated IGKV3-20 (100 µg) emulsified in complete Freund adjuvant (Sigma-Aldrich). Subsequent injections were conducted at day 14, 21, 30, and 45 with 100 µg of KLH-conjugated IGKV3-20 emulsified in incomplete Freund adjuvant. HHD sera were analyzed at day 0 and 30 by ELISA. Ten µg/ml of nonconjugated IGKV3-20 were coated onto a 96-well Maxisorp NUNC-immunoplate (Thermo Scientific) in 0.05M sodium carbonate/bicarbonate buffer pH 9.6 (Sigma-Aldrich), overnight at 4°C. After incubation for 1 hour at RT with 100 µl per well of PBS-10% FBS, plates were incubated for 1 hour at RT with sequential dilutions of mouse sera in PBS-10% FBS (from 1:50 to 1:6400). Negative control was PBS-10% FBS alone and sera at day 0 (1:50 in PBS-10% FBS). Then, plates were incubated for 1 hour at RT with secondary HRP-conjugated goat antihuman antibody (Charles River Laboratories; 0.02 µg/ml in PBS-0.05% Tween). Finally, 100 µl per well of substrate solution (prepared using SIGMA-Fast OPD tablets) were added for 5 minutes at RT in the dark. Reaction was stopped with 50 µl/well of HCl 3N (Carlo Erba), and plates analyzed at 490 nm using a VictorX4 instrument (Perkin-Elmer). Data were used to generate a titration curve. The same sera used in ELISA test were analyzed for their ability to stain DG75 cells in flow cytometry.

IGKV3-20–specific CTLs were generated from HHD mice showing humoral responses capable of staining DG75 cells cytofluorimetrically. Candidate mice were sacrificed, their splenocytes isolated, and cultured (25). Spleenocytes isolated and cultured (25) were added to the wells in triplicate and incubated for 2 hours at room temperature (RT). Plates were washed, and horseradish peroxidase-conjugated goat anti-human IgG (Sigma-Aldrich) was added. After incubation for 1 hour at RT, plates were washed, developed using chemiluminescence substrate LiteAblot (CELBIO), and read with a GENios Elisa-reader (Tecan).

Results
Ex vivo immunogenicity of IGKV3-20 and IGKV3-15 recombinant proteins
The IGKV3-15 and IGKV3-20 proteins are expressed by a sizeable fraction of B-cell malignancies (Table 1). In particular, IGKV3-20 is used 2 to 3 times more frequently than IGKV3-15. Two representative IGKV3-15 and IGKV3-20 recombinant proteins were used to induce CTL cultures by stimulating PBMCs of healthy donors with protein-pulsed
autologous DC. Specific CTL lines were easily obtained from 14 donors with either IGKV3-20 (n = 12) or IGKV3-15 (n = 4) proteins (Fig. 1A and Supplementary Table S2). Overall, almost 30% of the cells in the cultures (29.5 ± 5.9%; mean ± SD; n = 10) were CD8+ T cells, most of which (>90%) were effector memory (CCR7–/CD45RA–) or terminally-differentiated (CCR7–/CD45RA+) T cells (Supplementary Table S3). Notably, these cultures were also composed of a significant proportion of CD4+ T cells (62.76 ± 6.20%; n = 10). All CTL cultures generated with IGKV3-20 or IGKV3-15 showed class I–restricted cytotoxic activity against autologous LCLs pulsed with the immunizing protein (Fig. 1A).

Considering the derivation from antigen-experienced B cells of most lymphomas expressing IGKV3-20 or IGKV3-15, these proteins may have undergone the process of somatic hypermutation and may thus show a certain degree of inter-patient variation in the amino acid sequence. This does not apparently constitute a major limitation because CTLs induced by a prototypic IGKV3-20 light chain (VK3-20α) were able to specifically kill autologous targets loaded with idiotypic VK proteins of the same subfamily (VK3-15, Figs. 1B and C) derived from unrelated NHL. These results were obtained in 4 donors with different HLA class I haplotypes (Fig. 1B), thus showing that the CTL responses induced by a “prototypic” IGKV3-20 protein may have a potential immunotherapeutic value also in unrelated patients carrying lymphomas showing the same VK restriction.

IGKV3 proteins induce cross-reactive CTL responses

The IGKV3-15 and IGKV3-20 proteins show a sequence homology >80% (Fig. 1D), a biochemical basis for the induction of cross-reactive responses. To address this issue directly, we verified whether IGKV3-15–specific CTLs could kill autologous targets presenting IGKV3-20, and vice versa. As shown in Fig. 1E, IGKV3-15– and IGKV3-20–specific cultures obtained from 3 donors significantly killed, in a class I–restricted fashion, also mismatched targets. Intriguingly, IGKV3-20–induced CTLs killed IGKV3-15–loaded targets with an efficiency comparable to that of matched IGKV3-15–CTLs; only in a single case, IGKV3-20–CTLs showed about a 2-fold higher killing activity against IGKV3-15 targets than matched CTLs (Fig. 1E).

Sequencing of IGKV genes expressed by a large panel of B-cell lines allowed the identification of the DG75 BL cell line (HLA-A*0201/6601, B*4101/5001) naturally expressing IGKV3-20, and the SH9 LCL (HLA-A*0201/0101, B*0702/0801) expressing the IGKV3-15 protein. Notably, both these cell lines were killed in an antigen-specific and HLA-A*02–restricted manner by IGKV3-20–specific CTLs obtained from donors single-matched for HLA-A*0201 with target cell lines (Fig. 2). These findings indicate that IGKV3-20 and IGKV3-15 are naturally processed in lymphoid cells and produce “cross-reactive” epitopes.

IGKV3-specific humoral and CD8+ T-cell responses in lymphoma patients

The presence of memory T-cell responses specific for IGKV3-20 and IGKV3-15 proteins was investigated in 13 patients with IGK+ HCV-NHLs. A 48-hour ELISPOT assay disclosed low-to-moderate amounts of IFN-γ–producing T cells in the majority of patients (3–98 SFC/100,000 CD8+ T cells), whereas all donors (n = 4) showed low-level responses (<7 SFC/100,000 CD8+ T cells; Fig. 3A). Moreover, specific humoral responses to IGKV3-20 protein were also observed in most of the patients from whom serum was available (n = 11; Supplementary Fig. S1).

IGKV3-20 CTL epitope mapping and validation

Using the iTopia assay (26), we identified 21 IGKV3-20 peptides able to bind at least 1 of 8 MHC allele products. Interestingly, although most peptides (n = 18) bound only one allele, the P5, P28, and P97 peptides bound 2 to 3 different alleles (Supplementary Table S4). All peptides were low-to-medium binders, with ED50 ranging from 110 to 255 μmol/L (Supplementary Table S4) and half-times varying from 18 to 135 minutes, similarly to other Id-derived peptides (30).

The ability of HLA-A*0201–binder peptides to elicit specific T-cell responses was assessed by IFN-γ-ELISPOT with purified CD8+ T cells obtained from 3 IGK+ HCV-NHL patients and 2 healthy donors. IFN-γ–producing T cells were found in the majority of patients with lymphoma, with higher responses for most epitopes as compared with healthy donors (15–20 vs. 5–10 SFC/100,000 CD8+ T cells).

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

<table>
<thead>
<tr>
<th>B-cell malignancy</th>
<th>IGKV3-15</th>
<th>IGKV3-20</th>
<th>IGKV3-15 or IGKV3-20</th>
<th>IGKV3 family</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV-related NHL</td>
<td>3/21 (14.3)</td>
<td>12/21 (57.1)</td>
<td>15/21 (71.4)</td>
<td>16/21 (76.2)</td>
</tr>
<tr>
<td>Splenic marginal-zone lymphomas</td>
<td>1/31 (3.2)</td>
<td>3/31 (9.7)</td>
<td>4/31 (12.9)</td>
<td>5/31 (16.1)</td>
</tr>
<tr>
<td>Sjogren syndrome–associated lymphoproliferations</td>
<td>2/7 (28.6)</td>
<td>4/7 (57.1)</td>
<td>6/7 (85.7)</td>
<td>6/7 (85.7)</td>
</tr>
<tr>
<td>MALT lymphomas</td>
<td>2/18 (11.1)</td>
<td>7/18 (38.9)</td>
<td>9/18 (50)</td>
<td>9/18 (50)</td>
</tr>
<tr>
<td>Follicular lymphomas</td>
<td>—</td>
<td>3/47 (6.4)</td>
<td>—</td>
<td>6/47 (12.8)</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>4/71 (5.6)</td>
<td>7/71 (9.9)</td>
<td>11/71 (15.5)</td>
<td>11/71 (15.5)</td>
</tr>
<tr>
<td>Chronic lymphocytic leukemias</td>
<td>28/614 (4.6)</td>
<td>72/614 (11.7)</td>
<td>100/614 (16.3)</td>
<td>139/614 (22.6)</td>
</tr>
</tbody>
</table>
In addition, epitope-specific CTLs were successfully induced \textit{ex vivo} from 6 different donors using each of 10 A*0201-restricted, IGKV3-20-derived peptides (Fig. 3C). Notably, CTLs induced with P20 and P33 peptides were able to kill lymphoma cells expressing either IGKV3-20 (DG75) or IGKV3-15 (SH9) in a HLA-A2–restricted fashion (Fig. 3D).

**Cross-reactivity of CTL responses elicited by IGKV3-20–derived epitopes**

The ability of IGKV3-20–specific CTLs to "cross kill" targets presenting or naturally expressing IGKV3-15 prompted us to verify whether the IGKV3-20–derived epitopes are also shared by other IGK proteins from the IGKV3 as well as from unrelated IGKV families, to better define the
extent of lymphoid tumors potentially treatable with IGKV3-20–based vaccines. Sequences of T-cell epitopes were then aligned against all germ-line IGKV sequences retrieved from the ImMunoGeneTics information system (31). As a conservative rule, only peptides showing only one amino acid change compared with the IGKV3-20 epitopes were considered. The analysis showed that most of the IGKV3-20 epitopes are conserved in several light chains of the IGKV3 family (Supplementary Table S5). Interestingly, 2 of these epitopes (P32 and P33) are shared also by several other IGKV families extensively used among B-cell tumors (Supplementary Table S5). Moreover, the HLA-A alleles restricting these peptides (A*1101 and A*2402 for P32 and A*0201 for P33) would ensure a broad population coverage.

To assess whether the cross-reactivity of IGKV3-20–specific CTLs against IGKV3-15–loaded targets was mediated by the recognition of identical or variant epitopes, 30 independent CTL microcultures, for each of 13 different peptide/donor conditions, were generated either using P20, P32, P33, P46, P47, and P48 peptides. Each peptide-specific culture was tested using both the native and the corresponding IGKV3-15–derived variant peptide epitope as target. Part of peptide-specific microcultures were able to efficiently recognize and kill T2 cells loaded not only with the same native peptide, but also with the variant epitope peptides (Supplementary Fig. S2A and S2B). More interestingly, few cultures showed cell lysis selectively to variant peptides, although no cytotoxicity against the native epitope was observed (Supplementary Fig. S2). Thus, IGKV3-20–specific CTLs recognize both similar and variant epitopes in IGKV3-15, further emphasizing the cross-reactivity of IGKV3-induced responses.

We also analyzed the ability of 2 representative IGKV3-20–derived A*0201 peptides to generate CTLs able to cross-react against 9 epitope variants (2 for P20 and 7 for P33) differing only for 1 amino acid and carried by IGK chains expressed by different B-cell tumors (Supplementary Table S5). Four of these “variant” peptides had a single amino acid substitution at the MHC anchor sites. As shown in Fig. 4A, T-cell lines generated from A*0201 donors with the P20 and P33 peptides specifically recognized and efficiently killed autologous LCLs pulsed with all the epitope variants as well as IGKV3-15+ and IGKV3-20+ lymphoma cell lines (Fig. 4B). Notably, in some instance, the killing of LCLs pulsed with the variant peptides was even higher than that induced by the “native” peptides (Fig. 4A and B). The extent of cytotoxic responses against variant peptides was retained at comparable levels also at low effector:target ratios (1:1) and when LCLs were pulsed with peptide concentrations as low as 1 μg/ml (not shown), thus assuring about the specificity of recognition. All the variant peptides investigated showed a similar or, more frequently, lower predicted binding affinity to A*0201 as compared with the “native” epitopes (Supplementary Table S5). These findings suggest that the IGKV3-20 protein carries natural heteroclytic versions of several CTL epitopes in the IGK proteins broadly expressed among lymphoid malignancies.

Humoral and cellular responses to IGKV3-20 in mice

Immunization of HHD mice with KLH-conjugated IGKV3-20 yielded high titers of specific antibodies (Fig. 5A). Sera were also tested for the capacity of staining cytofluorimetrically the IGKV3-20+ DG75 cells. After a first round of 3 immunizations, DG75 cells were not recognized and additional immunizations were required to obtain a detectable staining signal (Fig. 5B). Interestingly, although ELISA Ig titer remained almost constant (not shown), indicating that antibodies to IGKV3-20 had already reached a plateau, repeated immunizations boosted detectable levels of Ig capable of recognizing naturally folded, public epitopes physiologically present on the membrane-mounded protein.

Immunization-induced CTL generation was also exploited in the HHD mouse model, which allows the presentation of epitopes in the HLA-A*0201 context, and hence the generation of mouse CTLs potentially able to recognize human A*0201+ target cells. Splenocytes from IGKV3-20–immunized mice were restimulated in vitro with irradiated DG75 cells and tested 4 days later for lytic activity. As shown in Fig. 5C, these effectors showed a strong IGKV3-20–specific CTL activity. To assess whether the in vitro ability of CTL lines to target and lyse IGKV3+ cells could find an in vivo counterpart in the capacity of restraining tumor growth, SCID mice were injected in one flank with DG75 tumor cells admixed with human IGKV3-20-specific CTLs (ratio 1:1), although the opposite flank was inoculated with tumor cells alone. Control DG75 tumors developed in 100% of injected animals 8 to 9 days after inoculation, and reached the allowed maximal tumor volume in about 15 days. Conversely, at the site where DG75 cells and IGKV3-20–specific CTLs were coinjected, no growth, nor formation of tumor masses occurred over the entire observation period, thus
showing the tumor growth inhibitory activity of IGKV3-20–specific CTLs ($P < 0.001$, $n = 3$; Fig. 5D). Similar findings were observed with the IGKV3-15–SH9 cell line, thus confirming the cross-reactivity of IGKV3-20 CTL responses also in vivo ($P = 0.001$, $n = 3$; Fig. 5E).

**Discussion**

In this study, we have extensively characterized the immunogenic properties of the homologous IGKV3-20 and IGKV3-15 proteins, which can be considered as prototypic “off-the-shelf” vaccines. In fact, these proteins are...
collectively expressed by a large fraction of HCV-related NHL (>70%) and by a sizeable proportion of HCV-unrelated lymphoid malignancies (Table 1). Both IGKV3 proteins were shown to easily elicit HLA class I–restricted specific CTL responses ex vivo from different donors, thus excluding the existence of “holes” in the TCR repertoire because of central tolerance. These proteins are also naturally immunogenic in vivo, as shown by the detection of specific humoral and CD8+ T-cell responses in patients with lymphoma, ruling out also the occurrence of peripheral tolerance/ignorance. Notably, the presence of a significant proportion of CD4+ T lymphocytes within the cultures generated with IGKV3-20 and IGKV3-15 is of relevance in the light of the critical role of CD4+ T-cell responses in mediating the vaccine effect.

An ideal “shared” IGKV3 vaccine should elicit CTL responses effective against unrelated lymphomas expressing IGKV3 proteins. This is a relevant issue if we consider that most of NHLs potentially treatable with these vaccines derive from antigen-experienced B cells, whose Id proteins may thus carry somatic mutations possibly affecting immune recognition. Our findings indicate that CTLs induced with a prototypic IGKV3-20 may be effective also against IGKV3-20 proteins derived from different NHLs, supporting the potential immunotherapeutic value of this vaccine for unrelated patients with IGKV3-20+ lymphomas. Furthermore, we also show that CTLs induced by IGKV3-20 or IGKV3-15 elicit mutually cross-reactive responses, being able to efficiently kill target cells presenting or naturally expressing the mismatched IGKV3 protein. Considering the relatively frequent usage of IGKV3-20 and IGKV3-15 among lymphoid malignancies, these findings support a broad applicability of these vaccines. The higher prevalence of IGKV3-20 expression and its slightly higher efficiency in eliciting “cross-reactive” CTL responses indicate that IGKV3-20 is the preferred candidate for vaccination.

The immunogenicity of IGKV3-20 is supported by the identification of 21 different CTL epitopes presented by the vaccine.
common HLA class I alleles. All these peptides were low-to-medium binders, consistently with their derivation from a self-protein and with the features of other Id-derived epitopes (30). Memory CD8+ T-cell responses to the majority of HLA-A*0201–restricted IGKV3-20 epitopes were detected in patients with HCV-NHL, indicating that these epitopes are immunogenic in vivo. Moreover, we also showed that CTLs specific for these epitopes can be generated from different A*0201+ donors. Notably, these effectors were able to kill in a HLA-A2–restricted fashion lymphoma cells expressing either IGKV3-20 or IGKV3-15, showing that these shared epitopes are efficiently generated and presented by tumor cells.

Notably, IGKV3-20 carries 3 promiscuous epitopes able to bind 2 or 3 different HLA class I allele products. Given the relatively well-established class I binding motifs for most alleles (32, 33), binding of epitopes across a broad spectrum of HLA class I alleles has been considered the exception rather than the rule (34, 35). Indeed, several CTL epitopes can be presented in the context of more than 1 HLA class I allele product, although this is largely limited to the same HLA supertype (34–37). The 3 IGKV3-20 promiscuous CTL epitopes show binding properties that extend beyond a single HLA supertype, being in this respect similar to viral epitopes (38). Therefore, a broad HLA coverage of the IGKV3-20 vaccine is assured not only by a relatively high number of CTL epitopes presented by common HLA molecules, but also by the promiscuity of distinct epitopes. Notably, IGKV3-20 promiscuous peptides are able to induce specific effectors with similar cytotoxic activity from donors with unrelated HLA alleles (Supplementary Fig. S2C and S2D), a relevant prerequisite for the induction of comparable CTL responses across different HLA backgrounds.
One of the most intriguing results of this study is the demonstration that 2 IGKV3-20–derived epitopes are fully conserved and shared by various IGKV families broadly expressed among lymphomas/leukemias. Moreover, several "variants" of the IGKV3-20–derived epitopes are carried by clonotypic IGKV proteins expressed by different lymphoid tumors. Notably, IGKV3-20–specific CTLs 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. This is consistent with the lower predicted affinity showed by some of the "variant" peptides, which carry single amino-acid substitutions at the MHC anchor sites. These findings indicate that the IGKV3-20 protein carries "natural" heteroclitic versions of several CTL epitopes shared among various IGKV families broadly expressed among lymphoid malignancies. Available evidence indicates that heteroclitic peptide modifications can increase the immunogenicity of low-binding peptides, thus leading to improved ability to generate CTL responses against primary tumors (39–41). The enhanced immunogenicity of heteroclitic peptides has been elegantly shown also using analog peptides derived from native epitopes of Ig heavy chains (42). Moreover, heteroclitic peptides from various tumor antigens are now widely applied in clinical studies, both as single peptides and included in multiepitope vaccines (43). The demonstration that the IGKV3-20 protein naturally carries multiple CTL epitopes shared among various IGKV families, and that may behave as heteroclitic peptides further supports the applicability and the potential efficacy of this vaccine in a discrete proportion of IGK-expressing leukemias/lymphomas.

We also show that the IGKV3-20 protein is immunogenic in mice, being able to elicit both humoral and CTL-specific responses. Although the relevance of this model may be considered somehow modest because of the foreign nature of the IGKV3-20 protein into a mouse environment, nonetheless HHD mice proved capable of generating IGKV3-20–specific, HLA-A2–restricted CTLs that, in turn, correctly recognized and lysed the relevant human targets, thus supporting the potential role of vaccination to elicit antilymphoma therapeutic T-cell responses. In this regard, coinjection of human IGKV3-20–specific CTLs with IGKV3-20+ or IGKV3-15+ lymphoma cells into SCID mice totally prevented the growth of tumor cells, thus confirming that these effectors mediate efficient and cross-reactive cytotoxic responses also in vivo.

One possible limitation of the use of single-chain, recombinant IGKV3 proteins as "shared" vaccines is related to the loss of the unique B-cell epitopes generated by the association of the variable portions of the heavy and light chains. Although this may prevent the induction of therapeutically relevant anti-Id antibodies (44), single-chain Id proteins may also elicit the generation of antibodies to public determinants conserved in the native Ig. Our results support this possibility, even though repeated immunizations are probably needed to obtain antibodies able to detect the native IGKV3-20 and thus potentially able to mediate clinically relevant responses. Another potential limitation of our "off-the-shelf" vaccine is the possible deletion of a fraction of normal IGKV3+B lymphocytes. Available evidence, however, indicate that it is unlikely that this could constitute a major concern, as phase II and III trials have convincingly shown that Id vaccines including 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.

Our findings are consistent with the recent identification of CTL epitopes in clonal Ig light chains, mainly belonging to different IgV lambda gene families (45). Notably, the single IGKV epitope identified was a somatically mutated version of our IGKV3-20 p48 peptide (Supplementary Table S2). The demonstration that several CTL epitopes are carried by a single Id light chain such as IGKV3-20 supports a role for this protein as "off-the-shelf" vaccine for extended subsets of B-cell tumors. Besides overcoming the need for customized vaccine production, the IGKV3-20 vaccine may have the additional immunological advantages to be a carrier of a set of peptides naturally selected for heteroclitic substitutions associated with nearly optimal immunogenic features. This set of T-cell epitopes potentially covers a high proportion of IGKV-restricted B-cell tumors, as at least 1 IGKV3-20 epitope can be found in several light chains from different IGKV families. Moreover, IGKV3-20 also contains promiscuous epitopes, thus assuring a broad HLA coverage. On the whole, these data provide the rationale to activate a phase I/II study aimed at assessing the safety and efficacy of the IGKV3-20 vaccine for patients with lymphomas expressing molecularly correlated Id proteins. Achievement of these goals would make available a ready-to-use recombinant vaccine for improved consolidation therapy of low-grade B-cell lymphomas.

**Disclosure of Potential Conflicts of Interest**

R. Dolcetti is a consultant/advisory board member of Areta Int. No potential conflicts of interest were disclosed by the other authors.

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