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Research Article

Genetic Immunization with CDR3-Based Fusion Vaccine Confers Protection and Long-Term Tumor-Free Survival in a Mouse Model of Lymphoma

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Therapeutic vaccination against idiotype is a promising strategy for immunotherapy of B-cell malignancies. We have previously shown that CDR3-based DNA immunization can induce immune response against lymphoma and explored this strategy to provide protection in a murine B-cell lymphoma model. Here we performed vaccination employing as immunogen a naked DNA fusion product. The DNA vaccine was generated following fusion of a sequence derived from tetanus toxin fragment C to the $V_HCDR3_{109-116}$ epitope. Induction of tumor-specific immunity as well as ability to inhibit growth of the aggressive 38C13 lymphoma and to prolong survival of vaccinated mice has been tested. We determined that DNA fusion vaccine induced immune response, elicited a strong protective antitumor immunity, and ensured almost complete long-term tumor-free survival of vaccinated mice. Our results show that CDR3-based DNA fusion vaccines hold promise for vaccination against lymphoma.

1. Introduction

Lymphomas represent the fifth most common malignancies. Each year, approximately 55 000 new cases are diagnosed with non-Hodgkin's lymphomas (NHLs) in the United States [1]. Despite current therapeutic strategies including chemotherapy, transplantation, and passive immunotherapy with monoclonal antibodies, many lymphoma patients remain incurable. The recent years have witnessed the development of a variety of promising immunotherapies for treating patients with B-cell NHLs. Vaccine strategies targeting NHLs have largely focused on using the idiotype (Id) of the tumor immunoglobulin (Ig) individually expressed on the surface of malignant B cells as tumor-specific antigen (Ag). After decades of work, some clear evidence of clinical efficacy in phase I/II trials using Id protein vaccines has accumulated, despite results from phase III trials seem disappointing [2, 3]. Furthermore, streamlined production of these patient-specific vaccines is required for eventual clinical application.

Several strategies are being developed to improve these results and include optimization of antigen delivery and presentation as well as enhancement of antitumor T cell function.

DNA vaccines have emerged as a novel lymphoma vaccine formulation for antigen-specific immunotherapy [4]. Such a method is an attractive and effective approach for active therapeutic vaccination since it does not require the production and isolation of a purified protein for each patient, a process that is expensive, laborious, and time-consuming. The protein is endogenously produced and secreted, which may result in more efficient antigen presentation in both classes I and II major histocompatibility complex (MHC) pathways resulting in enhanced anti-Id

immune responses. In addition to their safety, stability, ease of production, DNA vaccines are highly flexible, allowing coexpression of several types of antigens and immunological proteins [5]. Furthermore, the performance of DNA vaccines may be improved by *in vivo* electroporation (EP) as a safe and efficient method of *in vivo* delivery resulting in greatly enhanced DNA uptake, protein expression levels, and degree of local inflammation [6]. DNA vaccination has been applied to therapy of experimental murine lymphomas (for reviews, see Hurvitz and Timmerman [7] and Neelapu *et al*. [8]). DNA vaccines that express either the tumor-derived Id or the tumor variable (V) regions in a single-chain Fv conformation (scFv) have been constructed. However, due to the weak immunogenicity in most cases, their effectiveness depend on carrier proteins or adjuvant proteins linked to the Id structures [9–14].

Idiotypic antigenic determinants lying mainly within the complementary-determining regions (CDRs) 3 have been considered a "hot spot" of particular interest for construction of subunit vaccines [15–18]. Vaccines including only this minimal antigenic domain were proved to induce antibody response [19, 20].

We demonstrated that DNA immunization of outbred mice with different patient-derived V_HCDR3 peptides elicited antibodies able to recognize native antigens on individual patient's tumor cells [20]. Recently, our group has shown the tumor protective effects recruited by CDR3-based DNA vaccines in a poorly immunogenic, highly aggressive murine B-cell lymphoma model. A DNA vaccine containing a V_HCDR3 epitope of the 38C13 B-cell lymphoma [21], administered in combination with the V_LCDR3-encoding plasmid, provided tumor protection and long-term tumor-free survival in 60% of syngeneic mice [22]. In the current study to enhance the potency of this vaccination platform, we used the DNA fusion vaccine design encoding tumor Ags linked to pathogen-derived sequences, aimed to provide $CD4^+$ T cell help. Engagement of $CD4^+$ T helper (T_H) cells has been shown to play a major role in linking and coordinating innate and adaptive immune responses [4, 23]. Many attempts to incorporate exogenous helper antigens into DNA vaccine design to break tolerance to self (tumor) antigens and to improve efficacy by helper T cells have been described [24–28]. Fusion protein of tetanus toxin fragment C (TTFrC) first domain to human CEA-derived peptide provided activating signals required for DNA vaccines against weak Ags [25].

Based on such finding, we generated a DNA vaccine consisting of a fusion between a sequence belonging to TTFrC and the V_HCDR3$_{109-116}$ epitope already described [22]. Here we present data on the antitumor efficacy of the CDR3-based DNA fusion vaccine delivered by intramuscular electroporation in a B-cell lymphoma model.

2. Materials and Methods

2.1. Cell Lines. 38C13, a carcinogen-induced B-cell lymphoma in the C3H/HeN murine strain, expresses an IgM/κ surface antigen, is MHC II^- [21], and was cultured in RPMI 1640, 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, and 50 μM β-Mercaptoethanol. This culture medium is referred to as the complete medium throughout this study. 38C13 cell line was used for tumor challenge experiments.

2.2. Construction of DNA Vaccines. Tetanus toxin (TT) fragment-encoding DNA was amplified by PCR from chromosomal DNA of recombinant *Streptococcus gordonii* strain GP1253 (kindly provided by Dr. Pozzi, University of Siena, Italy) [29]. The primers (forward 5′-CCG **CTC GAG** TCA ACA CCA ATT CCA TTT TC-3′ and reverse 5'-CCC **AAG CTT** TGT CCA TCC TTC ATC TGT-3′), containing the restriction sites *Xho*I and *Hind*III (in bold), respectively, were designed to amplify the sequence coding for amino acids 856–1313 of tetanus toxin gene (GenBank Accession No. X04436). The TT fragment spans from aa 856 to aa 1313 (H-chain) and included 9 amino acids of fragment B (aa 856–864).

The amplified fragment was inserted in the cloning vector pUC19, and the resulting recombinant plasmid was named pUC-TT$_{856-1313}$. Sequencing of the cloned TT fragment revealed three-point mutations (already present in the TT-expressing recombinant *S. gordonii* strain), which lead to three amino acids substitutions in the protein sequence: N919D, N998D, and M1240V.

The plasmid pUC-TT$_{856-1313}$ was used as template for the amplification of the partial TT fragment sequence (TT$_{933-1126}$). The fusion vaccines pTT$_{933-1126}$-V_HCDR3 and pTT$_{933-1126}$-V_LCDR3 were assembled by PCR amplification using the TT$_{933}$ forward primer (5′-CTA GCT AGC GCC ACC ATG GTT ATA GTG CAT AAA-3′,*Nhe*I site underlined) in combination with either the TT$_{1126}$$V_H$CDR3 reverse primer (5′-ATAGTTTAGCGGCCGCTTAAATGTAGTC-AAAGTACCCTTCGTATGTATCATATCGTAAAG-3′,*Not*I site underlined) or the TT$_{1126}$ V_LCDR3 reverse primer (5′-ATAGTTTAGCGGCCGCTTATCCAAACGTGTACAG-ATTATCATACTGTAGACATGTATCATATCGTAAAG-3′,*Not*I site underlined). The V_H CDR3 sequence specifies the 8-mer H-$2K^K$ "anchor modified" YEGYFDYI$_{109-116}$ epitope of the murine B-cell lymphoma 38C13 Id, while the V_L sequence expressed the 11-mer peptide starting with the Cysteine88 (i.e., Cys104 in the IMGT unique numbering [30]) and encompassing the CDR3 and the conserved Phenylalanine and Glycine residues of framework (FR)4 [22]. The reverse primer overlapped the TT$_{933-1126}$ carboxyl region and contained an overhang encoding the 38C13 Id peptides, fusing it to TT$_{933-1126}$ C terminus. A DNA fragment encoding the TT$_{933-1126}$ sequence alone was also obtained by means of the TT$_{933}$ forward primer together with TT$_{1126}$ reverse primer (5′-ATAGTTTAGCGGCCGCTTA-TGTATCATATCGTAAAG-3′,*Not*I site underlined). The TT$_{933}$ forward primer also encoded the Kozak consensus sequence and an ATG start codon.

The expression plasmid pRC110-NTS-IL-2 [22] was used as recipient for cloning of the recombinant fragments under the RSV promoter. The resulting PCR products were ligated into pRC110-NTS-IL-2 as *Nhe*I-*Not*I fragments.

All constructs were sequenced, and plasmid DNA was purified for vaccination using a QIAfilter Giga Kit Endotoxin-free (Qiagen S.p.A., Italy).

2.3. Mice, DNA Vaccination, and Tumor Challenge Protocols. Male C3H/HeN (H-2K^K) mice, 8- to 9-week old, were obtained from Charles River Italia S.p.A. (Calco, Italy) and maintained in the Animal Facility at the "Sacro Cuore" Catholic University of Rome, Italy. All animal experiments, including anaesthetic procedures, were conducted in accordance with protocols approved by the Italian Ministry of Health. For protective experiments, on day 0 anesthetized mice (ketamine-Domitor mixture; pTTV$_H$CDR3 group, $n = 7$; pTT group, $n = 6$) were vaccinated with a total of 50 μg DNA plasmid in 150 mM phosphate saline buffer into two sites of posterior muscle legs and received booster injection 3 weeks later. Both vaccinations were followed by electroporation with BTX ECM 830 Pulse Generator (Harvard Apparatus, MA, USA) at 175 V/cm, 10 ms square-wave pulses, 1 Hz. Muscles were pretreated with bovine hyaluronidase as reported elsewhere [31]. Unimmunized (naïve) mice ($n = 6$) received a mock vaccination by injection with phosphate saline buffer. Serum samples were collected by tail bleeding 3 weeks after priming and 2 weeks after boosting injections. All groups were challenged 2 weeks after the booster vaccination by intraperitoneal injection of 2×10^2 38C13 tumor cells.

In the therapeutic setting, on day 0 C3H/HeN mice were challenged i.p. with a lethal dose of 38C13 (2×10^2) tumor cells. DNA electrotransfer was performed 4 days after challenge and repeated 11 days later, with a total of 80 μg DNA plasmids pTTV$_H$CDR3/pTTV$_L$CDR3 or with 50 μg of pTT (6 mice/experimental group). Unimmunized mice ($n = 5$) received a mock vaccination. EP settings were the same used in the prophylactic experiments.

Clinical evidence of tumor and mouse survival were monitored and compared between groups. Animals were checked for visible abdominal tumors and tumor development was monitored daily by abdominal palpation. Animals were checked daily thereafter for mortality.

2.4. Peptide Synthesis. The native peptides NH$_2$-DPNYY-DGSYEGYFDYWAQGTTL-COOH (IgM 38C13V$_{H\,101–122}$) and NH$_2$ -MHTAVYYCAKGAQGASLGKAYFFDCWGQG-TQVTVSS-COOH (V$_H$ CDR3-PA; [20]) were synthesized by Primm (Primm S.r.l., Italy) and dissolved in the suggested buffer prior to use.

2.5. Anti-Idiotype Antibody Detection by ELISA. ELISA plates were coated with 50 μg/mL of V$_{H\,101–122}$ peptide or V$_H$ CDR3-PA as irrelevant peptide and incubated o.n. at 4°C. Plates were quenched at r.t. for 2 hours with 3% BSA. Mice sera, diluted 1 : 100 in PBS 1X/0,1% BSA/0,05% Tween 20, were added and incubated for 2 hours at r.t. Reactive antibodies were detected with sheep antimouse IgG HRP-conjugated (1 : 5000 diluted, Amersham Biosciences, Italy). Plates were then developed by adding ABTS substrate (Sigma-Aldrich S.r.l., Italy). Absorbance was read at 405 nm using ELISA microplate reader. All measurements of antibody levels in individual animals were determined in triplicate.

2.6. Ex Vivo Intracellular IFN-γ Assay. Mice (3 animals/experimental group) were culled 1 week after booster DNA injection and spleens were removed. Spleens were perfused with 10 mL RPMI 1640 culture medium, cell suspension were passed through 100 μm nylon cell strainer (BD Falcon, BD Biosciences Europe, Belgium) to remove large cells aggregates, and then centrifuged at 1,000 rpm for 10 minutes. Cells were resuspended in 1 mL medium, counted, centrifuged a second time and then resuspended in 90% FBS/10%DMSO and cryopreserved until assessment.

To assess priming of CD8$^+$ T cells, splenocytes harvested from groups of immunized mice were processed for detection on intracellular IFN-γ. Cells (2×10^6/well) were incubated for 5 hours at 37°C in 24-well plates in 2 mL complete medium supplemented with 2 mM sodium pyruvate, 1% nonessential amino acids (1% of 100x stock). Splenocytes were stimulated with 100 μg/mL V$_{H\,101–122}$ in the presence of 1 μL/mL cell culture of Golgi Plug (BD Biosciences Europe, Belgium). Following incubation, stimulated cells were washed twice and Fc receptors were blocked by incubation with rat antimouse CD16/CD32 (Fc Block; BD Biosciences Pharmingen, CA, USA) for 30 minutes. Samples were processed to label surface CD8 (PerCP antimouse CD8a—clone 53–6.7) and subsequently fixed and permeabilized. Cells were stained with Alexa Fluor 488 antimouse IFN-γ (clone XMG1.2) for intracellular labelling and analyzed by FACSCalibur using Cell Quest Pro software (BD, CA, USA). Data collection was gated on live spleen lymphocytes by forward and side angle scatter, utilised to exclude dead cells, debris, nonlymphoid cell, and cell aggregates. Values indicated in the FACS plots refer to double positive cells (CD8$^+$ IFN-γ^+) as percentage of total lymphocytes population. Statistical markers were set using unlabelled cells as reference. Typically, less then 0.08% positive cells were detected beyond the statistical marker in the above negative controls. Fluorochromes-conjugated Abs were purchased from Biolegend, CA, USA.

2.7. Statistical Analysis. Data from ELISA assay were analysed by unpaired, two-tailed t-test. Survival analyses were performed using the Kaplan-Meier method and the log-rank test. Tumor-bearing animal proportions and intracellular cytokine staining proportions were compared by X^2 analysis (MedCalc Software, Mariakerke, Belgium).

3. Results

3.1. DNA Vaccines and Experimental Design. In this study a DNA fusion vaccine containing pathogen-derived sequence as an immunoenhancer element was generated. The H-2K^K MHC class I binding motif-guided "Epitope prediction" (SYFPEITHI database, http://www.syfpeithi.de [32]) was applied to a TT fragment that spans from aa 856 to aa 1313 (GenBank Acc. no. X04436). An amino acids region was

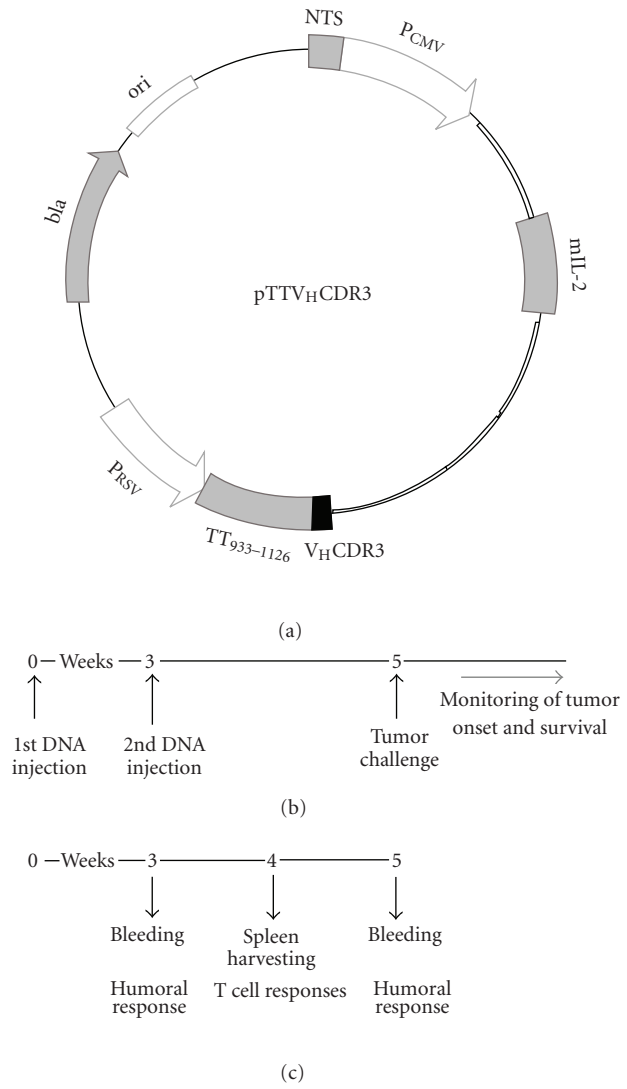

Figure 1: DNA fusion vaccine schematic structure (a). Experimental design of protective (b) and immune responses (c) studies is showed. C3H/HeN mice were immunized twice at 3-week intervals by intramuscular injection followed by electroporation. Unimmunized (naïve) mice received a mock vaccination by injection with phosphate saline buffer. Two weeks after boosting mice were injected i.p. with 2×10^2 38C13 cells.

selected (TTFrC$_{933-1126}$) which overlaps some of CD4$^+$ T-cell epitopes (the TT$_{947-967}$ epitope, the TT$_{1084-1099}$ epitope, TT$_{1058-1077}$ epitope) present on the microbial toxin sequence [33–35]. Furthermore, this TTFrC portion should lack of potentially competing epitopes as regards V_HCDR3$_{109-116}$ epitope, avoiding phenomenon of immunodominance [36].

To construct the DNA vaccine, the amplified fragment TT$_{933-1126}$$V_H$CDR3$_{109-116}$was generated after PCR reactions, as described in Section 2. This fragment was cloned into pRC110-NTS backbone vector [22], and the recombinant plasmid designed as pTTV$_H$CDR3, as reported in Figure 1(a). Additionally our plasmid encodes murine IL-2 as cytokine adjuvant. Likewise, the recombinant plasmid pTTV$_L$CDR3 was obtained by cloning the amplified fragment TT$_{933-1126}$$V_L$CDR3$_{88-98}$ in the same backbone vector. A plasmid encoding the TT$_{933-1126}$ sequence alone in the same backbone vector was also obtained and named pTT.

Table 1: Reactivity of mouse sera against a CDR3 control (irrelevant) peptide as assessed by ELISA.

Mice groups	Vaccine formulation	Absorbance (nm)[1]	$P^2_{\text{A versus B}}$
A	pTTV$_H$CDR3	0.159 ± 0.161	*NS*
B	pTT	0.049 ± 0.208	

[1] Control values belonging to unimmunized mice were subtracted.
[2] Unpaired, two-tailed *t*-test.
NS: not significant.

Plasmid DNA vaccination was performed using the RSV promoter driving TT$_{933-1126}$$V_H$CDR3$_{109-116}$ expression plasmid (pTTV$_H$CDR3), while pTT was used as control vaccine. Naïve mice received a mock vaccination by injection with phosphate saline buffer. Our DNA vaccination protocol consists of two DNA injections both associated with electroporation [22]. Experimental design of protective and immunological studies is showed in Figures 1(b) and 1(c), respectively.

3.2. Antibody Response Analysis. The levels of antibody response specific to DNA fusion vaccine were evaluated in mice following intramuscular immunization. Humoral immune response elicited after pTTV$_H$CDR3 or pTT immunizations was assayed by ELISA for V_H peptide (D_{101}–L_{122}) reactive antibodies.

We wondered whether the immunization regimen might influence the immune outcome. Individual blood samples were collected from mice (pTTV$_H$CDR3 group, $n = 7$; control groups, $n = 6$) 3 weeks after DNA priming and 2 weeks after boosting injections. The response profile for each vaccine group has been depicted in Figure 2. ELISA test failed to detect antibody titers when performed with mice sera collected after priming as well as analyses of individual sera within the pTTV$_H$CDR3 group revealed no noticeable differences compared to unimmunized and pTT control groups (Figure 2(a)).

Two weeks after boosting, mice immunized with pTTV$_H$CDR3 DNA vaccine showed sera positive for antibodies directed against the V_H peptide (D_{101}–L_{122}) (Figure 2(b)). Compared with pTT control vaccine and unimmunized groups, the pTTV$_H$CDR3 vaccine group antibody level was statistically significant ($P = .0045$ and $P = .0014$, resp.).

The lack of antibody response after priming suggests that boosting is critical for antibody induction. Our data essentially confirmed that immunization schedule was critical for this Ag system [37].

Reactivity of mouse sera against a CDR3 irrelevant peptide (V_H CDR3-PA [22]) generated no significant response (Table 1).

3.3. Induction of IFN-γ Producing CD8$^+$ T Cells by Fusion DNA Vaccine. To investigate whether our vaccination strategy could induce positive CD8$^+$ T cell responses to V_HCDR3

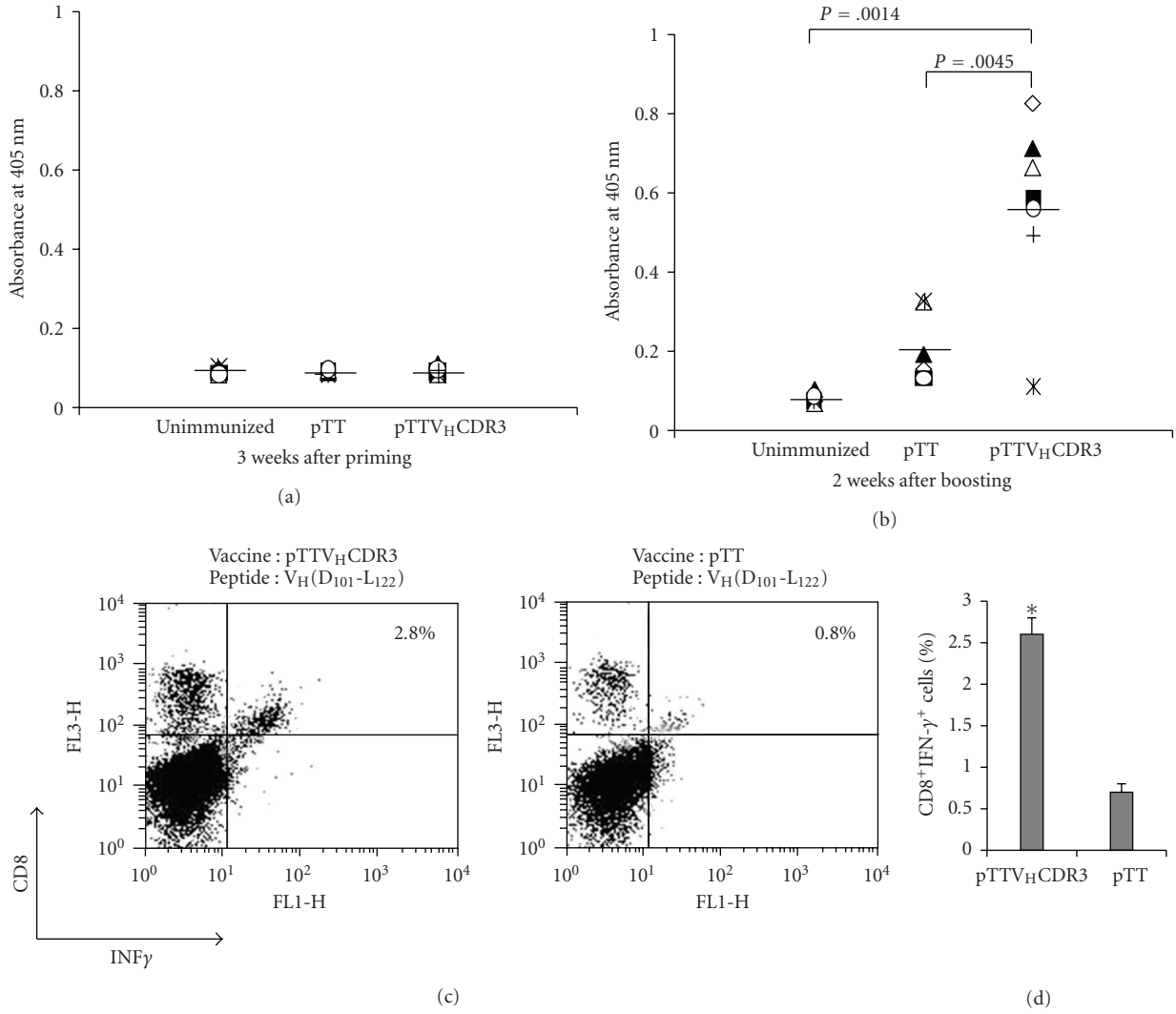

FIGURE 2: Immune responses elicited after pTTV$_H$CDR3 and pTT immunizations. Humoral immunity was assayed by ELISA for mice sera V$_H$ peptide (D$_{101}$-L-$_{122}$) reactive antibodies 3 weeks after priming (a) and 2 weeks after boosting. (b) Unimmunized mice represent the control group. Each marker indicates a value from a single mouse; group means are represented by a horizontal bar. Experimental groups (pTTV$_H$CDR3 group, $n = 7$; control groups, $n = 6$) were compared by unpaired, two-tailed t-test. (c) The frequency of IFN-γ-positive CD8$^+$ T cells was assessed *ex vivo* by intracellular cytokine staining. Splenic lymphocytes were harvested 1 week after booster injection, stimulated with V$_H$ peptide (D$_{101}$-L-$_{122}$), and assayed for IFN-γ production on gated T lymphocytes. Representative flow cytometric plots from pooled mice (3 animals/experimental groups) splenocytes are shown. Numbers in FACS plots refer to CD8$^+$ IFN-γ^+ cells as a percentage of the total T cells population. (d) Data were pooled from two identical independent experiments to indicate the mean percentage of CD8$^+$ T cells producing IFN-γ in response to V$_H$ peptide. An X^2 test for the comparison of the two proportions, expressed as a percentage, was performed. Error bars: SEM. $^{(*)}$denotes a statistically significant value ($P < .0001$).

epitope, C3H/HeN mice ($n = 3$) were vaccinated with the same DNA dose and regimen. Splenic lymphocytes were harvested 1 week after booster injection and processed for their ability to induce V$_H$ peptide (D$_{101}$–L$_{122}$) positive IFN-γ-producing T cells responses. Flow cytometry analyses in Figure 2 showed the percentage of CD8$^+$ T cells producing IFN-γ. Splenocytes isolated from pTTV$_H$CDR3 vaccinated mice generated a significantly higher frequency of IFN-γ-positive CD8$^+$ T cell precursors compared to mice vaccinated with the pTT control vaccine ($P < .0001$). A graphical representation of the number of V$_H$ peptide(D$_{101}$–L$_{122}$)-positive CD8$^+$ T cells is depicted in Figures 2(c) and 2(d). Thus, our data suggest that vaccination with pTTV$_H$CDR3 induces priming of CD8$^+$ T lymphocytes.

3.4. Prophylactic and Therapeutic Experiments. To address the protective tumor immunity of pTTV$_H$CDR3 DNA vaccine, we performed prophylactic vaccination experiments. The details of the immunization protocol and the tumor challenge are described in Figure 1(b). The immunization regimen was previously developed for another CDR3-based vaccine formulation and proved to be efficacious [22].

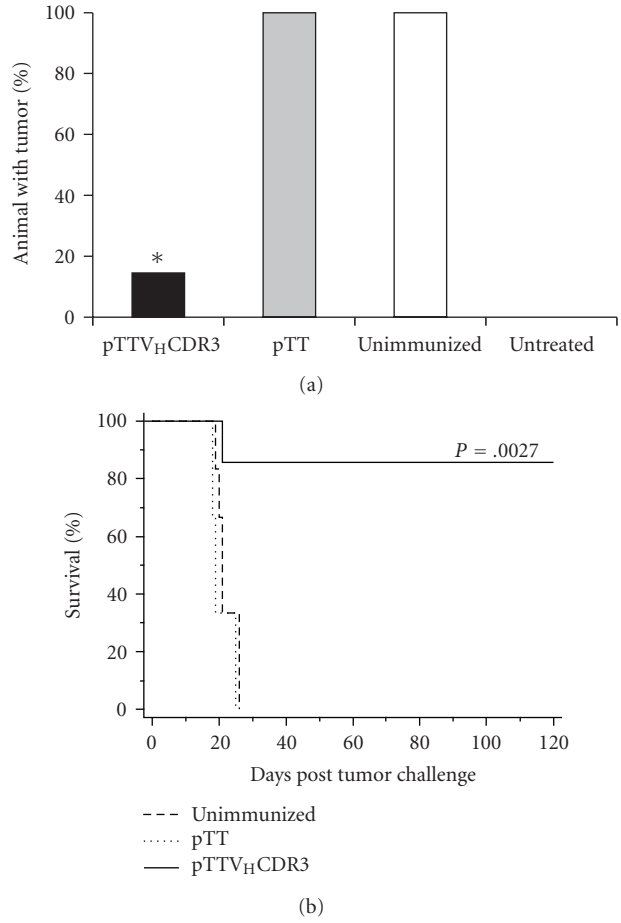

Figure 3: *In vivo* antitumor effects generated by immunization with pTTV$_H$CDR3 vaccine. (a) Tumor resistance. $^{(*)}$ denotes a statistically significant value ($P < .001$) by X^2 analysis when comparing the pTTV$_H$CDR3 group ($n = 7$) to all other groups ($n = 6$). (b) Representative long-term tumor-free survival. Survival analyses were performed using the Kaplan-Meier method and the log-rank test ($P = .0027$).

Two weeks after the last DNA electrotransfer, animals (pTTV$_H$CDR3 group, $n = 7$; control groups, $n = 6$) were challenged intraperitoneally (i.p.) with a lethal dose of 38C13 tumor B-cells. The development of i.p. lymphoma was monitored for each mouse and the protective efficacy of fusion vaccine was evaluated in terms of survival of mice over the next 120 days. Immunization with the pTTV$_H$CDR3 DNA significantly impacted tumor growth and ensured long-term tumor-free survival of about 85% of treated mice ($P = .0027$) (Figures 3(a) and 3(b)). Cohorts vaccinated with the pTT control vaccine or phosphate buffer showed poor lymphoma resistance, with all mice showing median survival time of 19 days.

The potent prophylactic antitumor effect prompted us to assess the therapeutic vaccination against established 38C13 tumor. Therefore, based on our previous data [22] and recent findings (manuscript in preparation), we evaluated the combined effect of V$_H$CDR3 and V$_L$CDR3 peptides fused to TT$_{933-1126}$ FrC portion in a therapeutic setting. Four days after challenging C3H/HeN mice (6 mice/experimental group) with a tumorigenic dose of 38C13 cells, DNA electrotransfer with pTTV$_H$CDR3/pTTV$_L$CDR3 or with pTT was performed and repeated 11 days later. Even though the timing of tumor onset was similar for the plasmids injected mice and the control mice, at days 18–22 postchallenge all untreated and pTT control mice succumbed. DNA vaccination with pTTV$_H$CDR3/pTTV$_L$CDR3 resulted in a trend toward a prolongation of life span through day 35 posttumor challenge, although the delay in death rate was not statically significant (Table 2).

4. Discussion

We have previously developed a DNA-based vaccine containing the 8-mer H-2K^K "anchor modified" YEGYFDYI$_{109-116}$ epitope of V$_H$CDR3 sequence of the murine 38C13 B-cell lymphoma. The V$_H$CDR3 epitope has been shown to be protective in combination with the V$_L$CDR3 peptide in a murine tumor protection experiment [22].

In the current study, we aim to gain insights into the enhancement of the effectiveness of the V$_H$CDR3-based DNA vaccine in terms of specific immune responses and tumor protection in mice.

Induction of potent immune responses against self-tumor antigens is not an easy task. Fusion of the antigen with foreign universal T$_H$ epitopes (such as tetanus toxoid epitopes) has been shown to brake the tolerance to self-antigen and render a weak tumor antigen more immunogenic.

Engagement of CD4$^+$ T$_H$ cells has been shown to play a major role in linking and coordinating innate and adaptive immune responses [4, 23].

Thus, a DNA fusion vaccine was generated following fusion of a sequence derived from TTFrC to the V$_H$CDR3$_{109-116}$ epitope to help immune responses against the tumor antigen. Vaccine efficacy was assayed in a highly aggressive and weakly immunogenic murine model of B-cell lymphoma.

We demonstrated that the fusion DNA vaccine pTTV$_H$CDR3 was able to induce detectable levels of antibodies against the peptide encompassing the V$_H$CDR3 sequence. Humoral immune response could not be achieved by first plasmid electrotransfer suggesting that boosting is critical for antibody induction for this antigen system.

Furthermore, plasmid-driven TTV$_H$CDR3 immunization resulted in the induction of significantly higher frequency of IFN-γ-producing CD8$^+$ T cell precursors as compared to control group.

Prophylactic vaccination with pTTV$_H$CDR3 DNA vaccine through the intramuscular route in combination with electroporation strongly affected the onset of highly aggressive 38C13 B-cell lymphoma. Inhibition of lymphoma growth led to significant and long-lasting protection from tumor in syngeneic mice with about 85% surviving, compared to naïve animals or those given the pTT control vaccine. This study demonstrates that fusion of exogenous protein to tumor-specific epitope converted an ineffective

Table 2: Therapeutic vaccination induces life span prolongation.

Mice group	Vaccine formulation	Survival % (n./tot)			Log-rank *P*-values versus	
		Day 15	Day 22	Day 28	Group C	Group B
A	$pTTV_HCDR3/pTTV_LCDR3$	100 (6/6)	50 (3/6)	50 (3/6)	0.083	0.115
B	pTT	100 (6/6)	0 (0/6)		0.408	—
C	Unimmunized	80 (4/5)	0 (0/5)		—	—

vaccine, namely, pV_H [22], into one with considerable activity.

The potent prophylactic antitumor effect prompted us to assess the tumor immunity in a therapeutic setting, which is more clinically relevant. Preliminary data obtained by using this DNA platform strategy provide proof of principle for the treatment of already established tumor in our model. Further enhancement of the potency of CDR3-based DNA vaccines is necessary in a therapeutic scenario; experiments testing new combinations of other crucial cytokines are under evaluation.

Attempts to identify the mechanism of Id-induced antitumor immunity to malignant B-cells have yielded variable results. Despite results from early clinical trials with Id vaccines suggest that both humoral and cellular immune responses may be independently associated with tumor regression and improved progression-free survival [38–42], the relative importance of the antibody and cell mediated immune response is still uncertain. Experiments are currently ongoing to explore the relative role of cellular versus humoral immunity for vaccine efficacy in our system.

The functional insertion of microbial sequence within the DNA vaccine was aimed to stimulate $CD4^+$ T cell help that is critical for inducing and maintaining an effective CTL response [23, 43]. Deeper analyses are needed to explore the role, if any, of V_HCDR3 peptide-specific $CD8^+$ T cells precursors in the generation of immune responses via $CD4^+$ T cell-mediated mechanisms. The involvement of $CD4^+$ T helper lymphocytes at the effector phase of anti-tumor responses is coherent with T_H cell-dependent "DCs licensing" [44] required for optimal vaccine efficacy, in the absence of MHC class II molecules expression by tumor cells [28, 45]. Licensed DCs presenting peptides from both TTFrC portion and tumor antigen can be able to activate the large repertoire of anti-TTFrC $CD4^+$ T cells. Hence, by ligand-receptor pairs interactions, "DCs licensing" mechanism supports priming and boosting of the weak tumor-derived peptides response [4].

Lastly, employing electrotransfer for the delivery of a DNA vaccine should improve the availability of the antigen, since EP increases and prolongs protein expression level and also results in recruitments of infiltrating inflammatory cells to the administration site [6].

5. Conclusions

This study demonstrates that fusion of exogenous protein to tumor-specific epitope converted an ineffective vaccine into one with considerable activity. Immune responses recruited by CDR3-based DNA fusion vaccine involve anti-Id antibody production and suggest the possible contribution of $CD8^+$ T lymphocytes.

A vaccination protocol consisting of a naked DNA priming and boosting is attractive by virtue of ease and less time-consuming production. Furthermore, safety is also achieved since adverse immune response can be avoided (i.e., immunity generated against some viral vectors).

The CDR3-based DNA fusion vaccines strategy may prove to be a highly useful approach against B-cell lymphoma. Optimal integration of active immunization approaches into standard therapies suggests DNA vaccination as an effective treatment to eradicate minimal residual diseases during clinical remission following standard chemotherapy in lymphoma patients.

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