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Self-Adjuvanting Therapeutic Peptide-Based Vaccine Induce $CD8^+$ Cytotoxic T Lymphocyte Responses in a Murine Human Papillomavirus Tumor Model

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Abstract: Vaccine candidates for the treatment of human papillomavirus (HPV)-associated cancers are aimed to activate T-cells and induce development of cytotoxic anti-tumor specific responses. Peptide epitopes derived from HPV-16 E7 oncogenic protein have been identified as promising antigens for vaccine development. However, peptide-based antigens alone elicit poor cytotoxic T lymphocyte (CTL) responses and need to be formulated with an adjuvant (immunostimulant) to achieve the desired immune responses. We have reported the ability of polyacrylate 4-arm star-polymer (S4) conjugated with HPV-16 $E7_{44-57}$ ($8Q_{min}$) epitope to reduce and eradicate TC-1 tumor in the mouse model. Herein, we have studied the mechanism of induction of immune responses by this polymer-peptide conjugate and found prompt uptake of conjugate by antigen presenting cells, stimulating stronger $CD8^+$ rather than $CD4^+$ or NK cell responses

Keywords: peptide subunit vaccine, human papillomavirus, therapeutic anticancer vaccine, star-polymer, polyacrylate, self-adjuvanting, cytotoxic T lymphocyte (CTL) responses.

INTRODUCTION

Human papillomavirus (HPV) is major cause of all cervical cancers with HPV type 16 (HPV-16) infection causing the majority of HPV-related cancers [1]. While prophylactic HPV vaccines have already been developed and commercialized, they only protect the host against viral infection but have no therapeutic properties to eradicate established HPV infection. Thus, there is strong demand for development of therapeutic vaccines to eradicate HPV and control cervical cancer mortality. Such vaccines can provide a safe alternative to classical chemotherapy as they use the natural immune system of our body to create a potent immune response against tumor cells.

Therapeutic HPV vaccine candidates are aimed to elicit cytotoxic T-cell responses and mainly focus on targeting HPV E7 oncogenic protein, as E7 protein is required for tumorigenesis and maintenance of tumor growth [1]. The whole oncoprotein cannot be used in vaccine formulation as it can induce genomic instability in normal human cells. However, peptide-based vaccines use only minimal non-oncogenic epitopes necessary to stimulate immune responses. In general, peptides alone are non-immunogenic and the addition of an external adjuvant (immunostimulant) to induce the desired immune responses was required in all reported therapeutic peptide-based HPV vaccine approaches [1]. To date, only a limited number of adjuvants have been approved for human use and they are often toxic or their efficacy is low [2]. Thus, the development of new adjuvants or self-adjuvanting delivery systems with potent immunomodulatory properties and without adverse toxicity is of major importance in the field of anti-cancer therapy [3, 4]. A large number of experimental adjuvants and self-adjuvanting systems are reported to induce humoral immune responses; however, triggering of cellular immunity against cancer cells is a particular challenge [5].

Previously, we have demonstrated that the polyacrylate dendrimers conjugated to multiple copies of minimal B-cell epitope was able to induce humoral immunity without the use of an additional immunostimulant [6-8]. We also reported that star polyacrylate-based vaccine delivery platform had the ability to reduce and eradicate tumor cells that produce the E7 oncoprotein in the mouse model. The lead vaccine candidate called **S4-8Q$_{min}$** possessing **8Q$_{min}$** ($E7_{44-57}$, QAEPDRAHYNIVTF) epitope was able to shrink and eradicate E7-expressing TC-1 tumors after a single-dose immunization in mice [9]. It has been proven that polymer-peptide conjugates can induce humoral immunity but the mode of the anti-cancer action of self-adjuvanting polymer-peptide conjugates has not been studied yet [10]. In this work, we have studied the mechanism of induction of immune responses by

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these polymer-peptide conjugates. It was found that the conjugates were promptly taken up by antigen presenting cells including macrophages and dendritic cells (DCs). In addition, we had proven that $CD8^+$ cytotoxic T lymphocytes (CTLs) played a major role in anti-tumor immunity upon administration of polymer-peptide conjugates.

MATERIALS AND METHODS

Synthesis of Vaccine Candidate $P(^tBA_{37}\text{-}\equiv)_4\text{-}8Q_{min}$ ($S4\text{-}8Q_{min}$)

Synthesis of polymer-peptide conjugates $S4\text{-}8Q_{min}$ (Scheme **1**) has been described in detail previously [9]. Briefly, the alkyne-functionalized poly(*t*-butyl) acrylate ($P(^tBA)$ 4-arm star-polymer **(S4)** was produced with the help of atom-transfer radical polymerization (ATRP) [11]. The $8Q_{min}$ epitope ended with azide (N_3CH_2CO-QAEPDRAHY-NIVTFCCKCD-NH_2) was synthesized using Fmoc-SPPS and conjugated to the polyacrylate **S4** via 1,3-dipolar copper-catalyzed alkyne–azide cycloaddition (CuAAC) "click" reaction [12] to produce $S4\text{-}8Q_{min}$. Then, the resulting $S4\text{-}8Q_{min}$ conjugate formed particles via self-assembled process in a DMF/water mixture. The dialysis against endotoxin-free water (Millipore, Queensland, Australia) was performed to remove the excess of peptide, residual copper and DMF.

Particle Size Measurement

The particle size and size distribution (span) of the conjugate was measured using the laser diffraction method (Mastersizer 2000, Malvern Instruments, UK) after formulating the conjugate in PBS. The geometrical diameter was presented as volume median diameter (D50%). The size distribution (span) value of the formulation was defined by a standard formula:

$$\text{Span} = (D90\%-D10\%)/D50\%$$

D10%, D50% and D90% are the particle diameters for 10^{th}, 50^{th} and 90^{th} percentile of the undersized particle distribution curve [13]. The size of particle of the conjugate was 12 μm and size distribution was narrow (span < 1.2).

Uptake of Conjugates by Dendritic Cells and Macrophages

An antigen presenting cells uptake assay was performed in a similar manner as described previously [14, 15]. Uptake of $S4\text{-}8Q_{min}$ by DCs and macrophages was investigated using splenocytes marked with CD11c-A660 (eBioscience) and F4/80-APC-Cy7 (BioLegend, Pacific Heights Blvd, San Diego, CA, USA) antibodies for DCs and macrophages, respectively. Spleen was passed through stainless steel mesh to produce single-cell suspensions. Erythrocytes were lysed by erylysis buffer (Sigma-Aldrich) and the remaining cell population was placed in a 6-well plate at a density of 2 x 10^5 cells/well in phenol free IMDM Glutamax medium (Gibco®, Life Technologies), supplemented with 10% FBS, 50 μM 2-mercaptoethanol (Gibco®, Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco®, Life Technologies). The $S4\text{-}8Q_{min}$ was labeled with Dil (1,1' dioctadecyl-3,3,3',3'tetramethylindocarbocyanine perchlorate) (Vybrant®, Life Technologies) by adding 0.5 μl of Dil to 100 μl of $S4\text{-}8Q_{min}$ in PBS and incubating for 30 min (Dil rapidly adsorbs to the $S4\text{-}8Q_{min}$). Dil-labeled $S4\text{-}8Q_{min}$ or Dextran-FITC (positive control) were added to the wells and incubated overnight. The adherent cells were scraped from the plate and incubated with Fc-block for 30 min at 4°C, centrifuged and re-suspended in buffer containing CD11c-A660 and F4/80-APC-Cy7 antibodies for 30 min at 4°C. The cells were then centrifuged and re-suspended in 0.5 mL of FACS buffer (PBS, 0.02% sodium azide, 0.5% BSA) and analyzed using an LSR II flow cytometer (BD Biosciences). The fluorescence intensities of DCs and macrophages treated with PBS were also measured as controls. The actual uptake was calculated as the percentage of cells double positive for $S4\text{-}8Q_{min}$ (Dil) and CD11c (A660), or $S4\text{-}8Q_{min}$ (Dil) and F4/80 (APC-Cy7) markers.

Mice

C57BL/6 (female, 6-8 weeks old) mice were purchased from Animal Resources Centre (Perth, Western Australia). All animal studies were approved by The University of Queensland Animal Ethics Committee (DI/034/11/NHMRC) and performed according to Australia National Health and Medical Research Council (NHMRC) guidelines.

Tumor Cells

TC-1 cells, a mouse C57BL/6 lung epithelial cell line transformed with ras oncogenes, HPV-16 E6 and E7, were kindly provided by TC Wu [16]. TC-1 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% heat inactivated fetal bovine serum (Gibco) and 1% nonessential amino acids (Sigma-Aldrich) at 37 °C/5% CO_2. For injection into mice, TC-1 cells were washed with 1× DPBS, trypsinized, resuspended and viable cells counted (trypan blue exclusion).

Immunizations

Prior to immunization, groups of C57BL/6 mice (5 per group) were injected subcutaneously with 2×10^5 TC-1 tumor cells/mouse suspended in 100 μL 1× DPBS in the right flank (day 0), and rested for 3 days to allow for tumor formation. On day 3 after tumor implantation, mice subsequently received a single subcutaneous immunization (100 μL total volume/mouse) at the tail base. This consisted of 100 μg of $S4\text{-}8Q_{min}$ (equivalent of 30 μg of $8Q_{min}$ epitope) in a total volume of 100 μL 1× DPBS (in 6 groups) or 1× DPBS (untreated control group). The size of the tumors was measured every two days using calipers (and palpation) for each mouse. The tumor volume was calculated using the formula [17]:

$$\text{Tumor volume } (cm^3) = \pi \times [\text{largest diameter} \times (\text{perpendicular diameter})^2]/6$$

The mice were euthanized when tumors volume was greater than 1 cm^3 or tumor bleeding was observed.

In vivo Antibody Depletion Experiments

In vivo antibody depletions have been described previously [18]. The antibody depletions were started prior to immunization. Groups of mice (5 groups vaccinated with

S4-8Q$_{min}$, 5 mice per group) were injected intraperitoneally (i.p.) with either 0.5 mg of anti-CD4$^+$ (clone GK 1.5, BioXcell) [19], 0.5 mg of anti-CD8$^+$ (clone 53-6.72, BioXcell) [19], 0.5 mg of anti-NK (PK136, BioXcell) [16] or 0.5 mg of control antibody (rat-IgG and mouse-IgG, BioXcell). Each antibody (100 μL) was administered i.p. on day −1, 0, 1 and once per week throughout the experiment. This antibody treatment resulted in greater than 99% depletion of CD4$^+$ and CD8$^+$ T-cells, and 92% depletion of NK cells as assessed by flow cytometry. Depleted mice were subsequently challenged with TC-1 tumor on day 0 and immunized with vaccine **S4-8Q$_{min}$** or PBS on day 3.

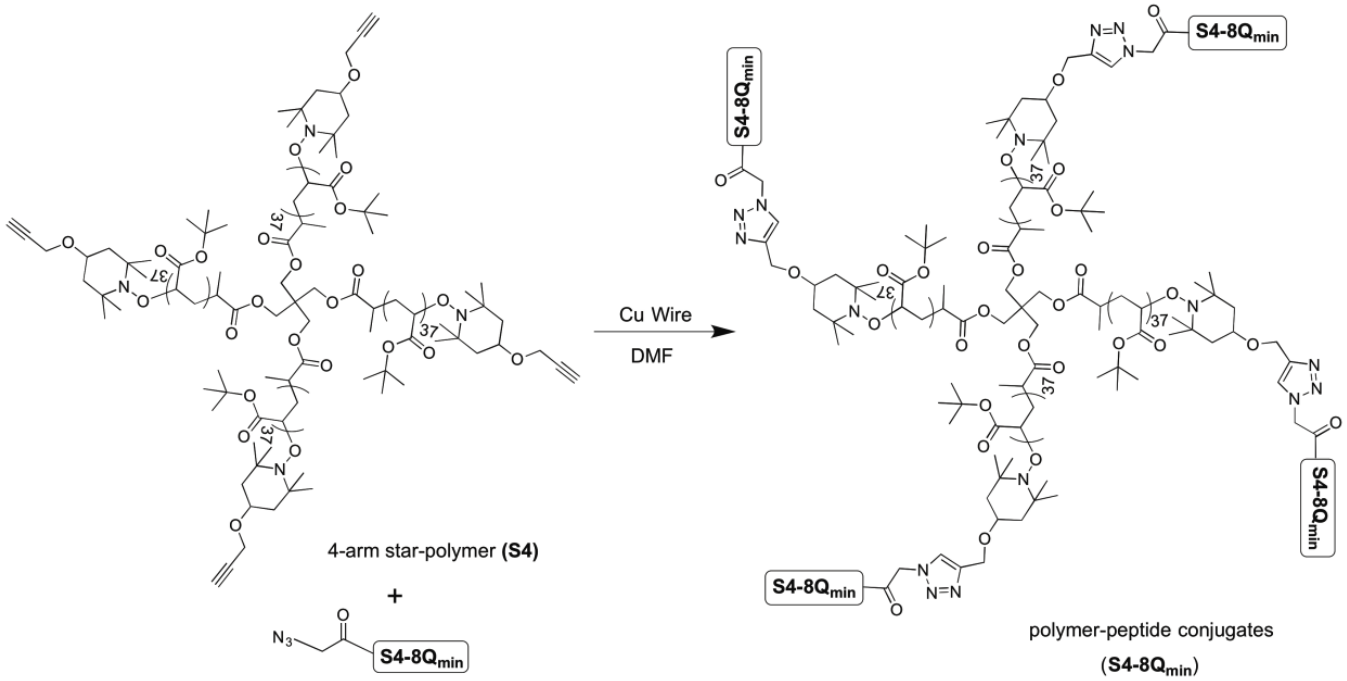
Statistical Analysis

GraphPad Prism 6 software was used for data analysis. Results of tumor volumes among all treatments were evaluated by ANOVA test. Kaplan-Meier survival curves was used for the analysis of tumor challenge experiments. Statistical significance was considered when $p < 0.05$.

RESULTS AND DISCUSSION

We designed and synthesized polymer-peptide chimeras consisting of a polyacrylate core and multiple copies of **8Q$_{min}$** (QAEPDRAHYNIVTF; E7$_{44-57}$). The conjugate, **S4-8Q$_{min}$**, was synthesized by copper-catalyzed alkyne–azide 1,3-dipolar cycloaddition (CuAAC) **"**click**"** reaction of polymer **S4** with the peptide epitope **8Q$_{min}$** in the presence of copper wires (Scheme **1**), then self-assembled into particles. Elemental analysis was used to confirm formation of the product [7] as the conjugate contained higher nitrogen/ carbon ratio (N/C = 0.085) in comparison to that of **S4** (N/C = 0.004). The exact substitution of polymer core with the peptide epitopes was calculated based on the comparison of theoretical and observed N/C ratios. The efficacy of conjugation was high (88% substitution). The **S4-8Q$_{min}$** was self-assembled into microparticles with an average diameter of 12 μm and narrow size distribution (span < 1.2). The **S4-8Q$_{min}$** was built with a hydrophobic polymer core and a hydrophilic peptide epitope. It was demonstrated that the balance between hydrophilic and hydrophobic properties of individual segments of such amphiphiles was responsible for the size of formed particles and the less polar peptide epitopes attached to the polymer core produced larger particles [20]. The production process was highly reproducible in terms of both substitution ratio and size.

The HPV-16 E7 protein epitope **8Q$_{min}$** (QAEPDRAHYNIVTF; E7$_{44-57}$) contained a CTL epitope (CD8$^+$ CTL) and a T-helper cell (CD4$^+$) epitope (Fig. **1**) [21]. This combination made **8Q$_{min}$** a promising candidate to stimulate immune responses against tumor cells. As peptides themselves are very poor immunogens, an additional adjuvant and/or delivery system is essential to induce strong immune responses. Dendrimers are synthetic polymers with well-defined structure which may have similar properties to that of biomolecules and antigens [22]. The antigens can be conjugated to the periphery of dednrimer "arms" to creat multifunctional bio-macromolecule. We have selected polyacrylate as the most promising candidate for vaccine delivery as polyacrylic acid and its esters are easy to synthesize, have very limited toxicity and were reported to possess adjuvanting properties when mixed with an inactivated virus [23, 24]. Here, the **8Q$_{min}$** epitope was applied as an antigen, while **S4** acted as a delivery platform. We have reported previously that polyacrylate polymer conjugated to peptide epitopes were capable to shrink and eradicate TC-1 tumor expressing E7 protein in the mouse model [9]. However, biological evaluation has not yet been performed to demonstrate immunological properties of this construct.



Scheme (1). Synthesis of polymer-peptide conjugates **S4-8Q$_{min}$**.

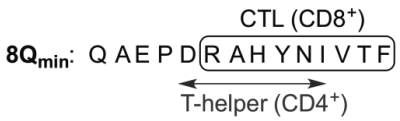


Fig. (1). The sequence of the **8Q$_{min}$** and incorporated CTL ($CD8^+$) and the T-helper cell ($CD4^+$) epitopes.

Antigen presenting cells (APCs) play a crucial role in triggering an immune response. They continuously sample and process the antigens and present them to $CD8^+$and $CD4^+$ T-cells via MHC-I and MHC-II molecules, respectively [25]. Antigens in particle form are expected to be efficiently taken up by APCs [26]. It has also been demonstrated that only the particles, which were efficiently taken up by DCs, induced a strong antigen-specific $CD8^+$ T-cell response [27]. Thus, in the present study, we investigated the *in vitro* uptake of conjugate **S4-8Q$_{min}$** by DCs and macrophages by incubating labeled **S4-8Q$_{min}$** with DCs and macrophages derived from splenocytes. The resulting cell-associated fluorescence (Dil labeled **S4-8Q$_{min}$** and CD11c-A660 or F4/8- APC-Cy7) was measured by flow cytometry. Approximately 25% of DCs and 15% of macrophages showed uptake of **S4-8Q$_{min}$** (Fig. **2**). Interestingly, despite their relatively large size the particles were more efficiently taken up by DCs than macrophages (although the opposite trend was expected [28]); however, this difference was not statistically significant.

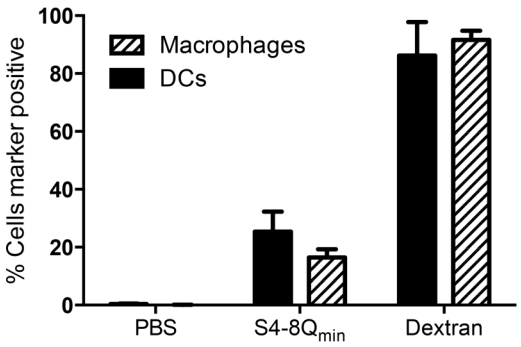


Fig. (2). Uptake of **S4-8Q$_{min}$** by mice splenocyte-derived DCs and macrophages. The experiments were performed in triplicate, and data are presented as a percentage of cells double positive for **S4-8Q$_{min}$** (Dil) and CD11c (A660) or F4/80 (APC-Cy7). CD11c (A660) or F4/80 (APC-Cy7) were markers characteristic of DCs and macrophages, respectively. Bars represent the mean ± S.D. of three experiments.

To determine the ability of the vaccine candidate (without the presence of any adjuvant) to induce a lymphocytic response, *in vivo* antibody depletion experiments were performed [18]. Five groups of mice were injected with anti-$CD4^+$, anti-$CD8^+$, anti-NK, control rat-IgG and control mouse-IgG antibodies. Then, tumor cells were injected, and the vaccine candidate was administrated in a single dose 3 days later. Treatments with PBS and **S4-8Q$_{min}$** without depleting antibodies served as the untreated and treated controls, respectively. All mice immunized with PBS, **S4-8Q$_{min}$**/anti-$CD8^+$ and **S4-8Q$_{min}$**/anti-$CD4^+$ were sacrificed due to tumor burden by day 29, 27 and 29, respectively (Fig. **3a**). In contrast, the survival rate of the mice immunized with **S4-8Q$_{min}$**, **S4-8Q$_{min}$**/rat-IgG, **S4-8Q$_{min}$**/mouse-IgG and **S4-8Q$_{min}$**/anti-NK was 40%, 40%, 60% and 20%, respectively. Among the mice groups treated with **S4-8Q$_{min}$**, the survival rate was statistically significantly lower only in the $CD8^+$-depleted group when compared with mice treated with **S4-8Q$_{min}$** only ($p<0.01$) (Fig. **3a**). Tumor-bearing mice treated with **S4-8Q$_{min}$**, **S4-8Q$_{min}$**/rat-IgG, **S4-8Q$_{min}$**/mouse-IgG, **S4-8Q$_{min}$**/anti-$CD4^+$ and **S4-8Q$_{min}$**/anti-NK showed slower tumor growth than those treated with **S4-8Q$_{min}$**/anti-$CD8^+$ ($p<0.05$) and PBS (Fig. **3b**). Mice treated with the conjugate but depleted of $CD8^+$ T-cells showed fast tumor growth in a manner similar to the untreated control group (PBS). These data suggested that **S4-8Q$_{min}$** anti-tumor potency was related to tumor-associated antigen-specific $CD8^+$ T-cell responses. Administration of the conjugate might also result in stimulation of $CD4^+$ T-cells as all mice in the group injected with anti-$CD4^+$ antibodies were moribund within 30 days (however, the difference in survival rate was not significant when compared to the positive control). In previous studies on peptide-based HPV vaccine candidates, which targeted the E7 oncogene, similar T-cell responses were reported but the use of external adjuvant was essential [29, 30].

In this work, we have clearly demonstrated that the polymer-peptide conjugate could serve as a self-adjuvanting delivery system and the conjugate was promptly taken up by APCs and stimulated $CD8^+$ T-cells to produce a therapeutic effect against tumor cells. The self-adjuvanting properties of the conjugates can be related to their ability to form particles. [28, 31]. It has been demonstrated that antigens in particle form are expected to be more efficiently taken up by APCs [26]. In addition, large particles are able to induce strong immune responses through the depot effect (i.e., retention and slow release of an antigen at the injection site) [32]. Along the same line, activation of cellular immunity is often enhanced by depot-forming adjuvants, which elongate time of antigen presentation to the immune cells [31]. Thus, it might be assumed that microparticles, presented here, have formed a depot and have the ability to induce cellular immunity following uptake by peripheral APCs. Though, the induction of immunity as a consequence of disaggregation of these self-assembled particles and slow release of the conjugate in the form of single molecules or smaller particles cannot be ruled out.

CONCLUSION

In conclusion, we synthesized and evaluated a delivery system for anti-cancer peptide-based vaccines. This delivery system, based on a polyacrylate polymer conjugated with HPV-16 E7 protein-derived peptide epitope, induced potent therapeutic immune responses against a tumor without the use of additional adjuvant. Until now, the ability of self-assembled polymer-peptide conjugates to induce $CD8^+$ CTL immunity has not been proven. In this work, we have shown that the polymer-peptide conjugate **S4-8Q$_{min}$** was efficiently taken up by APCs, that is, DCs and macrophages, and $CD8^+$ CTLs have played a major role in the anti-tumor immunity induced by **S4-8Q$_{min}$**. Thus, we have developed the first self-adjuvanting therapeutic peptide vaccine delivery system against cervical cancer. We believe that this strategy can be

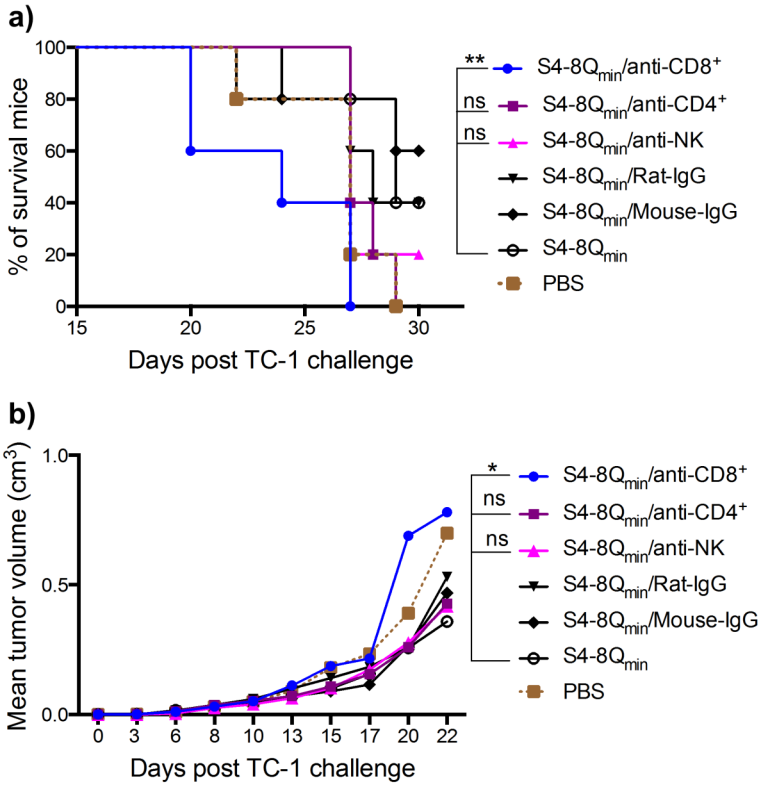


Fig. (3). Effect of lymphocyte subset depletions on the vaccine candidate **S4-8Q$_{min}$**. (**a**) Kaplan-Meier survival curve (30 days), and (**b**) mean tumor volume (cm^3). Tumor volume is plotted until the first mouse from any group has reached the endpoint (day 22). Five groups of C57BL/6 mice (5 per group) were injected anti-CD4$^+$, anti-CD8$^+$, anti-NK and control antibodies (day-1, 0, 1 and weekly), respectively. All groups of mice were challenged with 2 x 10^5 TC-1 cells/mouse (day 0), and immunized (**S4-8Q$_{min}$** or PBS) on day 3.

used to design other therapeutics that will be able to trigger CTL responses against targeted diseases.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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PATIENT CONSENT

Declared none.

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