K-ras peptide mimotope induces a humoral immune response against G12V K-ras antigen in BALB/c mice

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Abstract. Background. *KRAS* mutations are highly prevalent in pancreatic, lung, and colorectal carcinomas with G12V point substitution being one of the most prevalent mutations. While developments of peptide vaccines for *KRAS*(+) cancers are usually associated with poor immunogenicity, coupling mutant K-ras vaccines with universal CD4⁺ carrier molecules may enhance its outcome. Additionally, recent immunotherapeutic advances also suggest the possibility of inducing mucosal immunity against cancers using *Lactococcus lactis* as a live gastrointestinal delivery vehicle. **Methods.** A region of wild-type K-ras peptide was previously modified with a V7D substitution flanking the G12V mutation, generating a K-ras peptide (termed 68-V) with improved predicted antigenicity. This peptide was fused with a diphtheria toxoid sequence, and cloned into pNZ8048 vector within *Lactococcus lactis* NZ9000. BALB/c mice were then immunized orally, and then subjected to T/B cells immunophenotyping, as well as IgG and IgA detection. **Results.** Modified 68-V K-ras peptide and controls were successfully cloned and detection of His-tagged proteins expressed following induction by nisin was observed. Populations of CD3⁺CD19⁺ immune cells increased following immunization of 68-V, while K-ras specific-IgG and -IgA sera titers were elevated compared to wild-type and G12V K-ras controls. **Conclusion.** 68-V K-ras mimotope was shown to induce humoral-mediated immunity, highlighting the ability of an additional mutation flanking the G12V *KRAS* mutation to induce B cell activation and production of K-ras specific antibodies, while diphtheria toxoid was unable to stimulate an enhanced response when fused to 68-V. Nevertheless, these findings showed that further assessments are required to understand the role of K-ras specific antibodies within a *KRAS*(+) environment.

Keywords: Cancer, diphtheria toxoid, *KRAS*, *Lactococcus lactis*, mimotope, peptide vaccine

INTRODUCTION

KRAS, *HRAS* and *NRAS* each encodes for small GTPases that makes up the Ras superfamily, which plays a role as molecular switches in the regulation between inactive GDP-bound and

active GTP-bound states involved in normal cell signal transduction cascades (Lu *et al.*, 2016; Haigis, 2017). Found in approximately 30% of human cancers, mutations predominantly at

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codons 12 and/or 13 within the oncogenic *KRAS* are frequently found in non-small cell lung carcinomas (NSCLCs), colorectal cancers (CRCs), and pancreatic ductal adenocarcinomas (PDACs) (Prior, Lewis & Mattos, 2012; Herreros-Villanueva *et al.*, 2014; Stephen *et al.*, 2014; McCormick, 2015), with missense mutations in Ras proteins caused by single point mutations disrupting GDP and GTP binding balance (Lu *et al.*, 2016; Haigis, 2017). Altered K-ras proteins are able to reduce the hydrolysis of GTP into GDP or increase the rate of GTP loading, resulting in continuous binding of GTP to K-ras proteins, an active switch that amplifies pro-tumorigenic signaling downstream of the Ras-Raf-MAPK pathway (Lu *et al.*, 2016; Haigis, 2017; Jinesh *et al.*, 2018; McCormick, 2018). Although *KRAS* oncogene is one of the most commonly found aberrations in cancers, with reports indicating the role of exon 2 *KRAS* mutations as an important predictive biomarker for the lack of response to anti-EGFR monoclonal antibodies such as panitumumab and cetuximab in metastatic CRCs (Benvenuti *et al.*, 2007; Banck & Grothey, 2009), minimal advances in *KRAS*-specific treatments with a successful clinical response have been reported.

While it has since been a major challenge in designing effective therapies for *KRAS*(+) cancers, peptide vaccine development has been widely studied and the vaccine design incorporates synthetic peptides derived from epitopes of tumor-specific or tumor-associated antigens (TSAs or TAAs). Aside from being relatively easy and inexpensive to produce, as well as having minimal toxicity towards normal cells (Bei & Scardino, 2010), short peptides of 8 to 10 monomers usually stimulate tumor-specific cytotoxic T lymphocyte (CTL) responses, while long peptides of up to 16 monomers trigger helper T cell responses, albeit being insufficient to support progression free survival (Slingluff, 2011; Hirayama & Nishimura, 2016; Oryazún & Kobe, 2016). Mimotopes, which are modified mimics of natural epitopes, have been hypothesized to potentially trigger enhanced cellular or humoral responses, being capable of stimulating the secretion of response-specific cytokines, stimulating production of antibodies, having superior MHC affinity and potentially stimulate the development of immunological memory

against targeted TAAs/TSAs, thus potentially preventing cancer occurrence or relapse (Sharav, Wiesmüller & Walden, 2007; Knittelfelder, Riemer & Jensen-Jarolim, 2009; Buhrman & Slansky, 2013). Peptide vaccines reported to induce immunogenicity against Ras antigens include: Targovax's TG01 vaccine targeting mutant Ras combination at codons 12 and 13 that has to be co-administered with GM-CSF, and Globeimmune's GI-4000 vaccine delivered by heat-killed recombinant yeast, which targets three different mutant *KRAS* combinations; both of which have entered Phase I/II clinical trials for PDACs and NSCLCs respectively (Palmer *et al.*, 2017; Chaff *et al.*, 2014; Hartley *et al.*, 2015).

Several studies have reported the incorporation of sequence modifications within tumor antigen-derived peptides to enhance their immunogenicity, among which, includes single amino acid substitution to enhance HLA binding affinity preferably corresponding to MHC class I molecules (Bei & Scardino, 2010; Lipford *et al.*, 1995; Pogue *et al.*, 1995; Fikes, 2004). Such modification has been associated with successful immunogenicity enhancement when assessed *in vivo* (Schreurs *et al.*, 2005; Kumar *et al.*, 2015). Our preliminary study reported on the modification of G12V K-ras peptide via aspartic acid substitution at position 7 within peptide sequence (YKLVVVGAVGVGKSA) from codon 4 to 18 in order to improve the binding of epitopes to MHC class I and II molecules (Ng *et al.*, 2018). Despite highlighting the potency of modified G12V K-ras peptide as an antigenic mimotope *in silico*, the modified peptide was unable to significantly trigger an immune response against G12V K-ras antigens when tested *in vitro*. Several other studies have also reported similar findings and have therefore coupled peptides with carrier molecules such as keyhole limpet hemocyanin (Swaminathan *et al.*, 2014), tetanus toxoid (da Silva Antunes *et al.*, 2018), and diphtheria toxoid (Yano *et al.*, 2015; Diethelm-Okita *et al.*, 2000) to improve overall immunogenicity of peptide-based vaccines.

Another exciting aspect of modern day anti-cancer therapeutic intervention is the use lactic acid bacteria (LAB) as live delivery vehicles of oral vaccines. LAB has the ability to promote both mucosal and systemic immune responses in gut-associated lymphoid tissues (Pellissery & Nair,

2013; Azizpour *et al.*, 2016; Israr *et al.*, 2018). In this study, *Lactococcus lactis* was chosen as the host for the production of mimotopes against KRAS(+) cells as this Gram-positive probiotic bacteria possess advantageous properties such as its generally recognized as safe (GRAS) status, availability of established expression and secretion systems, the absence of inclusion bodies and endotoxins, as well as its non-colonizing nature of the gut. Therefore, this report describes the enhancement and assessment of immune responses triggered by modified G12V K-ras mimotope coupled with CD4⁺ epitope sequence of diphtheria toxoid (DT) following oral administration employing a lactococcal live delivery system. Inbred BALB/c mice were used in the immunological assessments of K-ras mimotopes due to the fact that: (i) mouse biology is highly similar to human biology (Mestas & Hughes, 2004), and (ii) BALB/c is a highly established strain for immunological studies which are significant in the development of drugs, peptides or vaccines (Mills *et al.*, 2000).

MATERIALS AND METHODS

Bacterial strains and plasmid

In this study, *L. lactis* NZ9000 (Universiti Putra Malaysia) host cells harboring pNZ8048 plasmids (Universiti Putra Malaysia) were cultured at 30°C in M17 broth (Merck, Germany) supplemented with 0.5% (w/v) glucose (Chemiz, Malaysia) and 10 µg/ml chloramphenicol (Merck, Germany). Meanwhile, XL-10 GOLD *E. coli* (Stratagene, USA) host cells were cultured in LB broth (Becton, Dickinson and Company, USA) with at 37°C. A supplemental file lists the bacteria strains used, as well as the details of recombinant *L. lactis* clones generated in this study [see Supplemental Table S1].

PCR amplification of wtKRAS and site-directed mutagenesis of control mimotope

Caco-2 human CRC cell line (Universiti Malaya) harboring wtKRAS was cultured in DMEM (Biowest, France) supplemented with 10% (v/v) FBS (Biowest, France) at 37°C with 5% CO₂. Caco-2 total RNA was harvested using the RNeasy Mini Kit (Qiagen, Germany) and cDNA

was synthesized using Omniscript RT kit (Qiagen, Germany) with poly-dT primers. First 50 codons of wtKRAS (GenBank Accession Number: M54968.1) was amplified from the cDNA prior to PCR-based site-directed mutagenesis (SDM) to generate mimotope control harboring G12V mutation, tagged with a 6x histidine sequence as well as incorporation of restriction sites. All PCR amplifications were carried out using 0.02 U/µl Q5 Hi-Fidelity DNA polymerase (New England Biolabs), 2 mM MgCl₂, 0.2 mM dNTP mix, 0.05 mM primers and 200-1000 ng template. A supplemental file shows details of the primers used in this study [see Supplemental Table S2].

Synthesizing and generating mimotope oligonucleotides

Oligonucleotides encoding peptides 68-V (YKLDVVGAVGVGKSA) (Ng *et al.*, 2018) and DT (QSIALLSSLMVAQAIPLVGEL) (Diethelm-Okita *et al.*, 2000) were synthesized separately in lyophilized pIDT:SMART vectors (Integrated DNA Technologies, USA). Resuspended lyophilized plasmids were transformed into chemically-competent X-10 GOLD *E. coli* host cells via heat shock method, and selected on LB agar (Becton, Dickinson and Company, USA) with 10 µg/mL ampicillin. Oligonucleotide 68-V and DT were amplified from the plasmids extracted using DNA-spin plasmid extraction kit (iNtRON Biotechnology, Korea), and were fused together via splicing by overlap extension (SOE)-PCR.

Cloning recombinant *L. lactis* and expression of mimotopes

pNZ8048 vectors were extracted from *L. lactis* NZ9000 using iNtRON DNA-spin plasmid extraction kit according to manufacturer's protocol. A signal peptide, usp45 (Universiti Putra Malaysia) was first amplified and cloned into pNZ8048 between NcoI and SphI recognition sites. Previously amplified wtKRAS, G12V and 68-V mimotopes were then incorporated into pNZ:usp45 between SphI and SpeI recognition sequences, while 68-V:DT mimotope was added between SphI and HindIII recognition sites (Figure 1A). Recombinant plasmids were then electroporated into *L. lactis* NZ9000 host cells at 2.3 kV at 200 Ω for 5 milliseconds, with minor modifications to the protocol previously

described (Holo & Nes, 1989). Minor modifications were made during preparation of electrocompetent cells whereby M17 media supplemented with 0.5% (w/v) glucose, 2.5% (w/v) glycine and 0.5 M sucrose were used and cells were resuspended in 1/20 culture volume. Transformed samples were selectively grown on M17 agar with 0.5 M sucrose, 0.5% (w/v) glucose, and 10 µg/ml chloramphenicol, with positive transformants confirmed by colony PCR using vector-specific primers before sequencing. All recombinant *L. lactis* were cultured in M17 broth supplemented with 0.5% (w/v) glucose and 10 µg/mL chloramphenicol, and expressed for 27 hours upon induction with 40 ng/mL nisin (Sigma-Aldrich, USA) at $OD_{600} = 0.5$.

Western blotting

Induced cultures were centrifuged at 3,500 g for 10 minutes, with supernatant fractions precipitated using trichloroacetic acid (Subramaniam *et al.*, 2012) to harvest secreted proteins, while intracellular proteins were harvested from pellets following sonication using a Q500 Sonicator (Qsonica LLC, USA) for a total pulse time of 10 minutes with a 10 and 30 seconds on and off pulse cycle. The harvested protein suspensions were separated in 15% (w/v) tricine gel at 120 V for 5 hours, followed by blotting of proteins onto PVDF membrane (Bio-Rad, USA). Blotted membranes were blocked with 1% (w/v) BSA before the detection of His-tagged mimotopes using Nickel-NTA conjugate from HisDetector™ Western blot HRP colorimetric kit (KPL, USA).

Casting recombinant *L. lactis* in edible films

Cultures were induced with 40 ng/mL nisin (Sigma-Aldrich, USA) at $OD_{600} = 0.5$ and expressed for about 4 hours to reach $OD_{600} = 2.8$. Cultures were harvested and casted in films made from 4% (w/v) sodium alginate (Sigma-Aldrich, USA) with 2% (w/v) lycoat (Roquette, France), followed by wrapping a pH-dependent coating layer for every 1 mg of recombinant *L. lactis* film (10^9 cfu). The coating layer was formulated with 0.34 M methacrylic acid-ethyl acrylate copolymer (Sigma-Aldrich, USA), 22.6 mM triethyl citrate (Sigma-Aldrich, USA), 69.2 mM talcum powder (Zulat Pharmacy Pte Ltd, Malaysia) and 62.6 mM titanium (IV) oxide (Sigma-Aldrich, USA)

dissolved in diluent of water, 50% (w/v) isopropanol and 35% (v/v) acetone. The coating solution was sieved and the filtrate was poured into a petri dish and placed into a dryer for 2 hours before used.

Experimental animals

A total of twenty female 4 weeks old BALB/c mice were obtained from Animal Resource Unit, Universiti Putra Malaysia, and housed at the Laboratory of Vaccines and Immunotherapeutics (LIVES) at Comparative Medicine and Technology Unit (COMeT). Meanwhile, all husbandry and care of animals were carried out in accordance to: (i) the ARRIVE guidelines under Institutional Animal Care and Use Committee ethics approval (UPM IACUC approval number: UPM/IACUC/AUP-R038/2017), and (ii) Animal Welfare Act 2015 (Act 772), Malaysia. BALB/c mice were randomly housed in groups of four inside plastic cages with access to food and water ad libitum, while corn cobs were used as bedding materials. Housing facility was well-ventilated and regulated at 25°C, followed by acclimatization of mice to the housing facility for two weeks. Mice were monitored daily for health status and mice weighed (mean $\pm$ standard deviation) 19.64 ± 1.16 grams at the start of the experiments, while any adverse events were not observed. Groups of BALB/c mice ($n=4$) were each randomly subjected to PBS and different recombinant *L. lactis* actively expressing K-ras mimotopes. Supplemental Table S3 includes the completed ARRIVE guidelines checklist.

Oral immunization in BALB/c mice

Groups of four female 6-8 weeks old immunocompetent BALB/c mice were orally immunized twice with 1 mg of respective recombinant *L. lactis* (pNZ:usp45:wtKRAS, pNZ:usp45:G12V, pNZ:usp45:68-V, and pNZ:usp45:68-V:DT) films and 1× PBS, pH 7.4 (negative control). All films were fed with a Torpac's size M dosing syringe, while PBS was fed using 1.0 ml syringe with 22-gauge $\times$ 1.0 inch feeding needle. Primary immunization was performed on day 1, with two following booster feedings administered at one-week intervals (days 7 and 14).

Isolation of blood, sera and lower GI wash samples

Aesthetic procedures were performed prior to all sample collections. Administration was performed intraperitoneally with 20.0 μL of ketamine/xylazine cocktail (93.33 mg/kg ketamine and 1.33 mg/kg xylazine) per 20.0 g of mice body weight, using a 30-gauge needle. The anesthesia cocktail gave a numbing onset of more than 5 minutes, which can be verified by toe pinch. All sites of injections were cleaned with 70% (v/v) ethanol prior to any invasive procedures. Mice were anaesthetized with ketamine/xylazil cocktail (Vetoquinol, UK/Indian Immunologicals Limited, India), followed by whole blood collection: prior to the primary immunization (day 0) by retro-orbital bleeding, and post-euthanization (day 22) by cardiac puncture. Retro-orbital bleeding was performed by gently pressing down with the thumb and forefinger in the occipital area to pull the skin backwards, and slowly inserting a non-heparinized micro-hematocrit capillary tube medially through the conjunctiva on the inner side of the ocular cavity, whereby blood rushed into the tube was collected (Shimizu, 2004). Meanwhile, cardiac puncture was performed using 1 mL syringe and a 22 gauge needle, whereby needle was pushed vertically below the sternum into the heart for maximum amount of blood collection. Sera were also centrifuged separately at 3,000 g for 15 minutes before storing at -20°C . Mice were then euthanized physically by cervical dislocation before the mice recovered from anesthetic conditions and this choice of euthanasia was chosen for immediate painless death after performing cardiac puncture. Cervical dislocation was performed by placing thumb and forefinger behind the base of the anesthetized mouse's skull and pulling back sharply on the tail at a 30° angle, which damaged the spinal cord and thereby increase the efficacy of the technique as reported by (Carbone *et al.*, 2012). Lower GI tracts were removed and intestinal washes were collected by flushing mice intestines with 3 mL of 1X PBS, pH 7.4 with 1 mM PMSF. After solution was allowed to stand for about 10 minutes, intestinal washes were centrifuged at 3,000 g, at 4°C for 10 minutes to remove solid particles and stools.

Cell surface staining for detection of T and B cell populations

Procedures on cell surface immunofluorescent staining of whole blood was referred according to BioLegend's protocol (BioLegend, 2018). Every 100 μL of anti-coagulated whole blood sample was pre-incubated on ice with 1 μg of purified anti-mouse CD16/32 (BioLegend®, USA) to block Fc receptors prior to staining. Cell surface staining was performed on mice whole blood using: 0.125 μg of Brilliant Violet 421-conjugated anti-mouse CD3, 0.125 μg of APC/Fire 750-conjugated anti-mouse CD4, 0.25 μg of Brilliant Violet 510-conjugated anti-mouse CD8a, 0.0625 μg of APC-conjugated anti-mouse CD19, 0.15 μg of Brilliant Violet 605-conjugated anti-mouse CD25, and 0.125 μg of PerCP/Cy5.5-conjugated anti-mouse CD335 antibodies (BioLegend®, USA), for every 100.0 μL of whole blood. After staining using fluorochrome-conjugated antibodies, red blood cells (RBCs) were lysed with 1X RBC lysis buffer (0.155 M ammonium chloride, 0.012 M sodium hydrogen carbonate and 0.1 mM EDTA, adjusted to pH 7.3). Every stained sample was suspended in 400 μL of Cell Staining Buffer (BioLegend®, USA) containing 0.125 $\mu\text{g}/\text{mL}$ propidium iodide staining solution (BD Pharmingen™, USA), followed by sample acquisition using CytoFLEX S flow cytometer (Beckman Coulter, USA). Data were reported as mean percentage $\pm$ standard deviation of three biological replicates, and Student's paired and unpaired T-test were also performed to measure the significant differences.

Assaying for mimotope-specific IgG and IgA responses

Cross-reactivity test was validated prior to indirect ELISA to detect mimotope-specific IgG and IgA. Assessments were first performed by coating 100 μL of each mimotope (5 $\mu\text{g}/\text{mL}$) diluted in 1X PBS, pH 7.4 into MaxiBinding microtiter plates (SPL Life Sciences, Korea). Plates were incubated overnight at 4°C and blocked with 2% (w/v) skim milk. After diluting each mouse serum in the blocking buffer at 1:10, the diluted sera and undiluted intestinal washes were added into respective wells for 2-hour incubation. Mimotope-bound IgGs or IgAs were then captured using HRP-labeled goat anti-mouse IgG (Santa Cruz Biotechnology, USA) or HRP-labeled

goat anti-mouse IgA (Invitrogen, USA), which were diluted at 1:12,500 and 1:4,000 respectively. Detection was performed using TMB substrate solution (Elabscience, USA) and stopped by the addition of 2 N H₂SO₄ before measuring absorbance at 450 nm using FLUOstar® Omega (BMG LABTECH GmbH, Germany). All samples and controls were assayed in duplicates, and all normalized mean data were expressed as net absorbance by subtracting mean absorbance of non-coated wells and no sample controls. IgG and IgA concentrations were calculated by interpolation from a standard curve and expressed as mean normalized IgG or IgA concentration $\pm$ standard deviation. Differences between pre-immunization and post-euthanization were analyzed using Student's paired t-test, while independent samples T-tests were also performed between mice groups, with a p -value ≤ 0.05 (*) threshold representing statistically significant differences.

RESULTS

***L. lactis* NZ9000 as a host for expression and extracellular secretion of mutated K-ras mimotopes**

Figure 1B shows colony PCR screening of positive *L. lactis* NZ9000 transformants using pNZ8048-specific primers flanking the insert. Colony PCR amplicons of transformed pNZ:usp45:wtKR4S, pNZ:usp45:G12V, pNZ:usp45:68-V and pNZ:usp45:68-V:DT showed positive band sizes of 437 bp, 437 bp, 335 bp and 401 bp respectively, while 198 bp bands represent amplicons of vector without the desired insert (data not shown). Undesired mutations were absent in the sequencing analysis of positive transformants (data not shown), indicating that the sequences are in frame and would not affect subsequent downstream expression of recombinant *L. lactis*. Owing to the relatively low molecular weight of target peptides, tricine gels were used to provide better resolution during the separation and detection. K-ras mimotopes were successfully expressed and were confirmed by His-tag detection via Western blotting, with

usp45:wtKR4S and usp45:G12V mimotope controls having sizes of 9.7 kDa, while usp45:68-V and usp45:68-V:DT mimotopes were 5.7 kDa and 9.0 kDa respectively (Figure 1C). Western blot of both intracellular and extracellular protein fractions, with nisin concentration at 40 ng/mL being optimal for maximal expression of His-tagged mimotopes after 6 hours of induction are also shown (Figure 1C).

Exposure to recombinant L. lactis expressing 68-V stimulates B cell production

Provoking either antigen-specific cell-mediated or humoral immune response requires stimulation from effector helper T cells (CD3⁺CD4⁺), and immunization with recombinant *L. lactis* expressing K-ras mimotopes were unable to elevate a significant CD3⁺CD4⁺ response (Figure 2A). This was presumably due to late sampling of sera at 7-days post-second booster, resulting in the inability to capture the changes in T helper population. Assessing CD3⁺CD8⁺ will determine a cell-mediated immune response, and Figure 2B shows that there was no stimulation of CTLs in mice peripheral blood. While a fluctuation in CD3⁺CD19⁺ response was detected in mice peripheral blood immunized with recombinant *L. lactis* expressing 68-V and 68-V:DT mimotopes compared to wild-type K-ras and G12V controls, this elevation was found to be statistically insignificant (Figure 2C). Nevertheless, such minute elevations still suggest the possible potential of 68-V K-ras mimotope to induce a humoral immunity, while coupling 68-V with DT failed to significantly improve stimulation of CD4⁺ response, presumably due to epitope masking of 68-V by the more prominent and antigenic DT epitope. Meanwhile, Figure 2D suggest that CD3⁺CD19⁺CD335⁺ (NK cell) populations were triggered by wild-type and G12V K-ras controls instead of 68-V and 68-V:DT K-ras mimotopes. As NK cells are part of the innate system that induces non-specific immunity against invading pathogens (Aziz & Bonavida, 2016), the variations in NK cell population may be triggered by the presence of *L. lactis*, which constitutes the K-ras live delivery system.

Figure 1. Recombinant *L. lactis* expressing 68-V and 68-V:DT mimotopes and their corresponding wtKRAS and G12V KRAS controls. (A) Graphical map of pNZ:usp45:wtKRAS, pNZ:usp45:G12V, pNZ:usp45:68-V, and pNZ:usp45:68-V:DT. (B) Positive transformants of pNZ:usp45:wtKRAS, pNZ:usp45:G12V, pNZ:usp45:68-V, and pNZ:usp45:68-V:DT. (C) His-tag detection of expressed usp45:wtKRAS, usp45:G12V, usp45:68-V, and usp45:68-V:DT mimotopes. Colony PCR was performed using pNZ8048-specific primers flanking the inserts, while western blotting was performed using Ni-NTA conjugates. M, marker; In, intracellular protein; Ex, extracellular protein; usp45, an extracellular secretion signal peptide; DT, diphtheria toxin.

Figure 2. Immunophenotyping profile of mice whole blood after oral immunization of recombinant *L. lactis* expressing K-ras controls (wtKRAS, G12V) and K-ras mimotopes (68-V, 68-V:DT). Charts display change (Δ) in percentage of immune cell populations: (A) helper T cells / CD3⁺CD4⁺, (B) cytotoxic T lymphocytes / CD3⁺CD8a⁺, (C) B cells / CD3⁻CD19⁺, and (D) NK cells / CD3⁻CD19⁻CD335⁺. Negative control group comprises of mice fed with PBS. Mice whole blood samples were harvested at day 0 (pre-immunization) and day 22 (post-euthanasia), represented by grey and black bars respectively. All data are presented as change (Δ) in percentage (%) of immune cell populations between pre-immunized and post-euthanized mice whole blood. Data were reported in relative mean values $\pm$ standard deviation and data of p -value ≤ 0.05 (*) is denoted as statistically significant.

Probiotic bacteria have been reported to enhance innate immunity involving NK cell activity, though there is no evidence on the effect of acquired immunity that follows (Calder, 2013). According to these data [see Supplemental Data S1], immunophenotyping of mice whole blood further confirmed the inability of 68-V K-Ras mimotope to stimulate a CTL response.

pNZ:usp45:68-V elicits G12V K-ras specific IgG- and IgA-mediated response in BALB/c mice sera

Mutated K-ras specific IgG and IgA levels in mice sera between pre-immunization (day 0) and post-euthanasia (day 22) indicated that pNZ:usp45:68-V was able to elevate both antigen-specific IgG-mediated (p -value ≤ 0.05) and mucosal immune responses (p -value ≥ 0.05).

(Figure 3A and 3C). On the other hand, pNZ:usp45:68-V:DT failed to elicit significant IgG or IgA responses, while low to undetectable levels of IgG and IgA were detected in mice sera immunized with recombinant *L. lactis* expressing G12V mimotope control (p -value ≥ 0.05). In addition to that, there was little to no detectable IgG response at either time for negative control groups (PBS and wtKR4S). Assessment of cross-reactivity in Figure 3B shows that pNZ:usp45:68-V sera samples were highly reactive across all K-

ras antigens compared to other immunized sera counterparts. On the other hand, cross-reactivities were not detected between mimotopes and IgA across all mice intestinal wash samples (Figure 3D). These cross-reactivity tests suggest the specificity of IgG antibodies raised by 68-V mimotope to target G12V KR4S(+) tumor cells with a cross reaction against wtKR4S is not desirable. Supplemental Data S2 includes the data obtained from the ELISAs performed.

Figure 3. Mice sera and intestinal washes were assayed for IgG and IgA levels respectively after oral immunization of recombinant *L. lactis* expressing K-ras controls (wtKRAS, G12V) and K-ras mimotopes (68-V, 68-V:DT). Charts displayed change (Δ) in concentrations of: (A) IgG levels for all samples (B) IgG cross-reactivity test, (C) IgA levels for all samples, and (D) IgA cross-reactivity test. Mice whole blood samples were harvested at day 0 (pre-immunization) and day 22 (post-euthanasia), while mice group fed with PBS was used as negative control. All data are presented as change in IgG or IgA concentrations (ng/mL) between pre-immunized and post-euthanized mice sera. Data were reported in mean values $\pm$ standard deviation and data of p -value ≤ 0.05 (*) is denoted as statistically significant when compared against PBS and G12V control groups.

DISCUSSION

While there were studies which have reported on immunized mice with engineered *L. lactis* expressing: E7 antigen against HPV-16-induced cervical cancers (Bermúdez-Humarán *et al.*, 2015), and carcinoembryonic antigen against colon cancer in mice (Zhang *et al.*, 2016), there was yet any attempt on engineering *L. lactis* to target *KRAS*(+) cancers. With an established safety profile in the food industry, *L. lactis* is widely used in production of dairy products and its use has been extended into biomedical applications in the recent years (Bahey-El-Din & Gahan, 2011; Song *et al.*, 2017). Besides low levels of native exoproteins makes *L. lactis* a more desired strain compared to *E. coli* for expression and oral delivery of heterologous proteins (Bahey-El-Din & Gahan, 2011; Azizpour *et al.*, 2017). Additionally, the non-invasive, non-pathogenic, non-colonising and non-commensal nature of *L. lactis* will not cause high risk in triggering immunotolerance or many unwanted side effects when used in long term (Bahey-El-Din and Gahan 2011; Sasan *et al.*, 2011), while the ability of *L. lactis* to extracellularly secrete recombinant proteins will ease all the downstream processing steps (Jørgensen *et al.*, 2013). In this study, the modified mutant G12V K-ras mimotope was successfully fused with DT and inserted at the cloning site downstream of *PnisA* promoter and *usp45* signal peptide within the *L. lactis* cloning and expression vector. Expressions of K-ras mimotopes from positive transformants selectively resistant to chloramphenicol were also detected when induced with addition of sub-inhibitory amounts of nisin. Nisin is able to trigger transcription of *PnisA* promoter within the vector to express the K-ras mimotope, and is then secreted extracellularly with the help of a signal peptide, which is an added advantage when recombinant *L. lactis* is used as an oral live vaccine host (Bahey-El-Din & Gahan, 2011).

Even though *L. lactis* is able to induce high expression levels of proteins and only produce a single extracellular housekeeping protease (Poquet *et al.*, 2000), its gastrointestinal survival rate is strain-dependent, thus oral immunization becomes a challenge as this affects its ability to deliver expressed proteins into the GI tract for

significant induction of host mucosal and systemic immune responses. *L. lactis* subsp. *cremoris* is known to have poor tolerance towards low gastric pH and intestinal bile salts (Nomura *et al.*, 1999; Kimoto *et al.*, 2003), therefore approaches via encapsulation (Divya & Nampoothiri, 2015) or packaging in enteric-coated mini-capsules (Lei *et al.*, 2010) have been established to protect *L. lactis* against sensitive gastrointestinal environment. Enteric-coated polymer was used in this study to protect recombinant *L. lactis* from low pH degradation and upon exposure to neutral pH environment at the lower GI tract, the enteric-coated polymer breaks down and exposes recombinant *L. lactis* casted on alginate film (Dea-Ayuela *et al.*, 2006). The mucoadhesive properties of the alginate film will allow recombinant *L. lactis* to adhere to the mucosal surface and trigger host immunity towards *KRAS*(+) antigens.

Efficacy of a peptide vaccine in triggering an immune response in host either via a cell-mediated or humoral response relies on its animal *in vivo* assessment before advancing into clinical trials. In this study, the use of inbred BALB/c mice offers consistency in terms of results compared to outbred models due to consistent genetic background and lesser phenotypic variability (Vaickus *et al.*, 2010); and since sex gender was not crucial in this study, female mice were used over male mice owing to their less aggressive nature. Mice and humans were known to be somewhat similar in terms of immune system, genetics and components (Mestas & Hughes, 2004), thus the choice of animals is especially important as findings using immunocompetent BALB/c mice will highly reflect to human biology. The immunological *in vitro* data (Ng *et al.*, 2018) has shortlisted only the potential mutant G12V K-ras vaccine candidate, thus vastly reducing the number of mice groups needed in this study and this approach is in line with “UPM Policy and Code of Practice for the Care and Use of Animal for Scientific Purposes”.

Immunophenotyping of mice whole blood has confirmed the inability of 68-V K-ras mimotope to stimulate a CTL response, which is a preferable response in cancer vaccine design. Recombinant *L. lactis* expressing 68-V K-ras mimotope was also shown to elevate K-ras specific IgG and IgA levels. Our findings

therefore suggested that a modification within the mutant K-ras peptide via an amino acid substitution flanking the mutation site is able to enhance its B cell epitope binding affinity, thus mediating a humoral response through activation of plasma cells, which subsequently produce immunoglobulins against mutant G12V K-ras antigens. Using *L. lactis* as the vehicle for GI delivery provokes the immune system to secrete mucosal K-ras-specific IgAs acting as a first line of defense, which is advantageous when targeting tumors found along mucosal surfaces (Dechant & Valerius, 2001; Ludvigsson *et al.*, 2015). DT fused to the modified mutant G12V K-ras mimotope, on the other hand, failed to stimulate significant induction of immune responses higher than that of 68-V mimotope alone, and this also suggest that the CD4⁺ epitope sequence of DT was unable to enhance immunity of its conjugated epitope in mice as compared to human subjects in the study reported by (Diethelm-Okita *et al.*, 2000), probably due to the fact that their human subjects include those that were previously boosted by diphtheria vaccination. There is also the possibility of epitope masking being involved, whereby magnitude of an antibody or immune response to the epitope is reduced when epitope was bound to pre-existing antibodies or antibodies already generated during the immune response triggered following immunization. A study by (Zarnitsyna *et al.*, 2015) has previously demonstrated that the epitope masking model of HA antigen results in the reduction of primary immune response, while removal of epitope masking from the model reflects an opposite response. In addition, the unknown effect size in sample size estimation would require a follow up study to include a larger sample size to detect an association and reduce the margin of error between samples which will in turn improve the accuracy of results obtained (Kadam & Bhalerao, 2010).

Majority of peptide vaccines predicted with high binding affinity to MHC molecules were able to stimulate infiltration of CTLs and induce tumor cell killing with the purpose of eradicating antigen specific tumor cells in cancer treatment (Sahin & Türeci, 2018). The 68-V mimotope, although predicted with high binding affinity towards human MHC molecules, it was also previously predicted that it has average binding affinity

towards mouse MHC molecules (Ng *et al.*, 2018). These suggest that 68-V mimotope has poor CTL recognition in mice, which is not a preferable immune response in cancer treatment; however, humoral response induced by 68-V mimotope would shift the direction of a cancer treatment to a B cell-based anti-cancer immunity. Peptide vaccines that induce tumor-specific B cell responses will first initiate Th activation upon presentation of antigen-bound MHC class II. The following B cell signaling produces tumor-specific antibodies that induce passive humoral immunity via antibody-dependent cellular cytotoxicity, antibody dependent cellular phagocytosis or complement-dependent cytotoxicity (Kametani *et al.*, 2015; Kaumaya, 2015; Yuen, Demissie & Pillai, 2016). An example of a study by (Foy *et al.*, 2013) had demonstrated the ability of B cell epitope vaccine to induce EGFR-specific anti-tumor immunity in mice. B cells can also play a role in tumor immunosuppression; therefore, one must distinguish between different B cell subpopulations to understand the efficacy of a peptide vaccine to promote tumor regression (Yuen, Demissie & Pillai, 2016; Li, Yuan & Deng, 2016).

CONCLUSION

Overall, this study has demonstrated that the 68-V K-ras vaccine with an additional mutation (V7D) flanking the native G12V *KRAS* mutation is able to induce activation of B cell signaling and production of anti-K-ras specific B cells. Further tumor regression assessments concerning the efficacy of this mimotope vaccine within a *KRAS*(+) tumor environment is currently underway. Distribution study of the live vaccine can also be performed via imaging to observe the delivery of the labeled live vaccine to the targeted mucosal region of the test subjects, while dosage-response kinetic can be evaluated to determine the efficacy and safety of the live vaccine. Moving forward, immunogenicity of this K-ras vaccine could also be further enhanced by other means including, but not limited to, emulsification, multimerization or fusion with other adjuvants and carrier molecules.

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