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Seleção de motivos semelhantes à Papilomavírus, a partir de bibliotecas de "*phage display*", que apresentem potencial aplicação translacional

Tese apresentada a Faculdade de Medicina da Universidade de São Paulo para obtenção do titulo de Doutor em Ciências
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Orientador: Profa. Dr. Luisa Lina Villa

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DECLARAÇÃO

Em adendo ao Certificado 079/2012/CEUA, datado de 24.07.12, e por solicitação da Profa. Dra. **Ana Paula Lepique**, responsável pela linha de Pesquisa, autorizo a inclusão do aluno **Lanre Precieux Sulamain** ao Projeto de Pesquisa "*Potencial terapêutico de peptídeos contra tumores associados ao Papilomavírus humano*", uma vez que se trata de utilização da mesma espécie animal e de métodos experimentais similares ao Projeto.

São Paulo, 21 de outubro de 2013.

Prof. Dr. Wothan Tavares de Lima
Coordenador da CEUA
ICB/USP

EXPEDIÇÃO
DATA 24/10/13
REL. N.º 3961

DEDICATION

To Barbie, Lulu, Omoloye and ‘Kanmbi.

APPRECIATION

Giving thanks to God for where He brought me from, how far he has brought me without fail, and where He is taking me to.

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This journey has been made possible by ALL OF YOU!

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LIST OF ABBREVIATIONS

ACK	Ammonium-Chloride-Potassium lysis buffer
ADXS11	Live attenuated *Listeria monocytogenes* (*Lm*) HPV-16-E7 vaccine
ANOVA	Analysis of variance
BD	Becton-Dickinson
BLAST	Basic Local Alignment Search Tool
BRASIL	Biopanning and Rapid Analysis of Selective Interactive Ligands
BSA	Bovine serum albumin
$CaCl_2$	Calcium Chloride
CCAAT	CYTOSINE-ADENOSINE-THYMIDINE /Enhancer Binding Protein
CCL20	Chemokine (C-C motif) ligand 20
CD	Cluster of differentiation
CDC25A	Cell Division Cycle 25A
CDK	Cyclin Dependent Kinase
CDSR	Cochrane Database of Systematic Reviews
CIN	Cervical intraepithelial neoplasia
CpG-ODN	Cytosine-Guanine oligodinucleotide
CTL	Cytotoxic T Lymphocytes
DNA	Deoxyribonucleic Acid
FUP	Follow up
GM-1	Monosialotetrahexosylganglioside
GST	Glutathione S-transferase

HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER-2	Human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
HLA	Human Leukocyte Antigen
HPV	Human papillomavirus
HR-HPV	High risk Human papillomavirus
Hspg	Heparan Sulfate Proteoglycans
IARC	International Agency for Research on Cancer
IL	Interleukin
LAL	Limulus Amebocyte Lysate
LCR	Long control region
LCs	Langerhans Cells
LPS	Lipopolysaccharide
LRW	LAL Reconstitution Water
MHC	Major Histocompatibility Complex
MTH	Mouse Tonic Hanks solution
NaCl	Sodium Chloride
NCI	National Cancer Institute
NK	Natural Killer cells
NKp30	Natural Killer Cell Cytotoxicity Receptor 30
OC-L	Oxidized tumor cell lysate vaccine against Ovarian Cancer
ORF	Open reading frame

PBS	Phosphate buffered saline
PD-1	Programmed cell death- 1
PEG	Polyethylene glycol
PHA	Phytohaemagglutinin
pRB	Retinoblastoma protein
Raf	Rapidly Accelerated Fibrosarcoma Oncogene
Ras	Rat Sarcoma Viral Oncogene
RT	Room temperature
SDS	Sodium Dodecyl Sulfate
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cells
TU	Transforming Unit
VLPs	Virus-like Particles

RESUMO

Sulaiman LPK. Seleção de motivos semelhantes à Papilomavírus, a partir de bibliotecas de ‘’Phage Display”, que apresentem potencial aplicação translacional

O vínculo entre papilomavírus humano de alto risco e câncer cervical está bem estabelecido. Apesar da existência de vacinas profiláticas contra infecções pelos tipos mais comuns de HPV, para infecções e tumores causados por esses vírus as alternativas terapêuticas são restritas. Encontramos alguns motivos com homologias para proteínas do HPV de alto risco durante o *imunoscreening* de uma biblioteca de phage display com soros de participantes HPV-16-soropositivos da coorte Ludwig-McGill. Após enriquecimento das sequências, os bacteriófagos recombinantes foram purificados e amplificados para uso como imunógenos.Usando uma abordagem profilática, nós vacinamos experimentalmente camundongos imunocompetentes com um dos nossos bacteriófagos recombinantes, usando o bacteriófago sem inserto como controle. Estes camundongos foram então desafiados com células tumorais TC-1 (HPV-16 positivas), tendo-se avaliado as respostas imunes disparadas durante a progressão tumoral. Também usamos uma abordagem terapêutica, aonde os camundongos foram primeiro injetados com as células tumorais e imunizados com o bacteriófago após o estabelecimento do tumor. O crescimento tumoral foi monitorado e os tumores, baço e linfonodos foram avaliados quanto à quantidade e qualidade da resposta imunológica. Os testes de ELISA revelaram que todos os camundongos vacinados responderam à imunização com os diferentes bacteriófagos. O crescimento tumoral foi significativamente reduzido nas imunizações profiláticas e terapêuticas, embora a redução do tumor fosse mínima quando os camundongos foram tratados 9 dias após o enxerto. A redução no crescimento tumoral também se traduziu em uma sobrevivência significativamente maior para os camundongos imunizados. Estudos de infiltração celular não revelaram alterações em diversas sub-populações imunes, mas uma tendência de aumento de linfócitos T citotóxicos foi observada nos camundongos imunizados com PEP1 (bacteriófago contendo inserto). A importância deste aumento de CD8 na redução observada do crescimento tumoral foi confirmada utilizando camundongos CD8-knockout, onde a redução do crescimento tumoral previamente observada foi anulada. Foi observado um aumento de taxa CD8:CD4 nos camundongos imunizados e isto é uma indicação de ambiente tumoral citotóxico. Os ensaios de proliferação celular para testar a especificidade do antígeno dos linfócitos dos camundongos imunizados foram, no entanto, inconclusivos; da mesma forma, não pudemos alterar o padrão observado com o uso de adjuvante CpG. A utilidade da técnica de phage display também foi observada neste trabalho experimental. Trabalhos adicionais para entender o mecanismo de ação desses fagos recombinantes no controle do crescimento de tumores causados por HPV e seu potencial imuno-estimulador são necessários.

ABSTRACT

Sulaiman LPK. Search for Papillomavirus-like motif with Potential Translational Application Selected by Phage Display [thesis] Sao Paulo. ´´Faculdade da Medicina, Universidade de Sao Paulo´´

The link between high-risk human papillomavirus and cervical cancer is well established. Despite the existence of prophylactic vaccines against infections by the most common types of HPV, therapeutic alternatives are limited for infections and tumors caused by these viruses. We found some homology motifs for high-risk HPV proteins during the immune-panning of a phage display library with sera from HPV-16-seropositive participants of the Ludwig-McGill cohort. After enrichment of the sequences, the recombinant bacteriophages were purified and amplified for use as immunogens. Using a prophylactic approach, we vaccinated experimentally immunocompetent mice with one of our recombinant bacteriophages using the insertless bacteriophage as a control. These mice were then challenged with TC-1 tumor cells (HPV-16 positive), and the immune responses triggered during tumor progression were evaluated. We also used a therapeutic approach where mice were first injected with tumor cells and immunized with the bacteriophage after tumor establishment. Tumor growth was monitored and tumors, spleen and lymph nodes were evaluated for the quantity and quality of the immune response. ELISA tests revealed that all vaccinated mice responded to immunization with the different bacteriophages. Tumor growth was significantly reduced in prophylactic and therapeutic immunizations, although tumor reduction was minimal when mice were treated 9 days after TC-1 cells grafting. The reduction in tumor growth also translated into a significantly greater survival for the immunized mice. Cell infiltration studies did not reveal changes in several immune subpopulations, but an upward trend in cytotoxic T lymphocytes was observed in mice immunized with PEP1 (insert-containing bacteriophage). The importance of this increase in CD8 in the observed reduction of tumor growth was confirmed using CD8-knockout mice, where the previously observed reduction of tumor growth was abolished. An increase in CD8:CD4 rate was observed in the immunized mice and this is an indication of a cytotoxic tumor environment. Cell proliferation assays to test the antigen specificity of lymphocytes from immunized mice were, however, inconclusive; likewise, we could not change the pattern observed with the use of CpG adjuvant. The usefulness of the phage display technique was also observed in this experimental work. Additional studies to understand the mechanism of action of these recombinant phages in the control of HPV tumor growth and its immuno-stimulatory potential are warranted.

This thesis is in accordance to the following rules, as at the time of its submission:

Referencias: Associação Brasileira de Normas Técnicas

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Abbreviaturas dos títulos dos periódicos de acordo com List of Journals Indexed in Index Medicus

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1 INTRODUCTION

Cervical cancer is a type of malignancy that occurs in the cells of the cervix, the lower part of the uterus that connects to the vagina. It is the third most commonly diagnosed cancer and the fourth leading cause of cancer death in females worldwide, accounting for 9% (529,800) of the total new cancer cases and 8% (275,100) of the total cancer deaths among females in 2008 (JEMAL et al., 2011) of which more than 80% are from the developing world (TORRE et al 2015). Human Papillomaviruses (HPV) have been associated with the majority of cervical cancer cases. These viruses are classified as low-risk or high-risk types and, although there is a large number of high risk HPV types, HPVs 16 and 18 have been recognized to be most predominant types associated with cervical cancer (WINER et al., 2006), hence, all the available vaccines have been focused on the reduction of the effect of infection by these types of HPV (and others), by raising immunity against them. The results have been very positive in countries where the vaccination programmes have been offered to girls and adolescents in national immunization programmes. Prophylaxis is not effective however for middle-aged and older women who most likely must have been infected before the advent of the vaccines hence periodic cytological screening is undertaken to improve the chances of early detection of the onset of cervical cancer (CHASAN AND MANROW, 2010). New approaches combining this screening with current molecular diagnostics are being proposed to even further improve the chances of early detection. In established cases of cervical cancer however, the treatment options include surgery, radiotherapy and chemotherapy, with the attendant problems. This therefore calls for alternative approaches to the treatment of cervical cancer.

Peptide vaccines for cancer offer the promise of inducing T cells reactive to well-characterized tumor antigens and also enabling assessment of vaccination effect, by monitoring antigen-specific T cell responses. Cancer cells express peptide antigens recognized by $CD8^+$ cytotoxic T lymphocytes, CTL (DARROW et al., 1989). These vaccines offer ease of synthesis, safety and effectiveness at inducing T cell responses although the response rates vary, depending on several factors. Research into possible candidates for this kind of vaccines is therefore of utmost importance.

1.1 HUMAN PAPILLOMAVIRUS

The human papillomavirus is a DNA tumor virus that causes epithelial proliferation at cutaneous and mucosal surfaces. More than 200 different types of the virus exist, including approximately 30 to 40 strains that infect the human genital tract. Of these, there are oncogenic or high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, and 58) that are associated with cervical, vulvar, vaginal, and anal cancers, and non-oncogenic or low-risk types (6, 11, 40, 42, 43, 44, and 54) that are associated with genital warts and low-grade cervical lesions (MUNOZ et al., 2003)

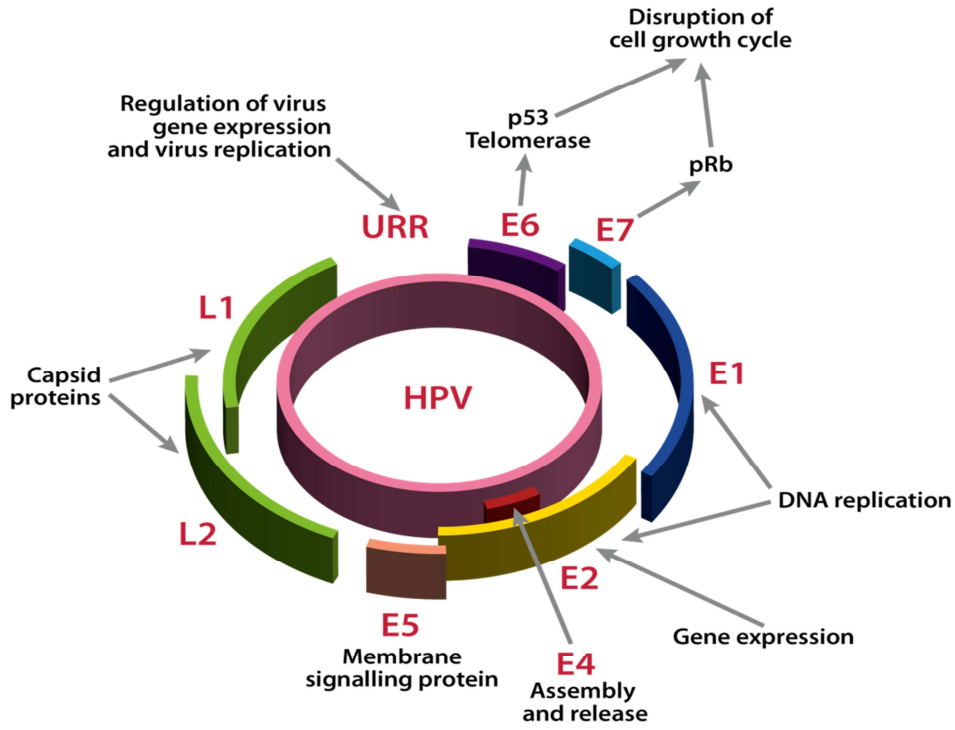

Figure 1: Human papillomavirus genes and their functions (adapted from http://www.genpathdiagnostics.com)

The virus is non-enveloped and different viral subtypes are classified on the basis of their L1 capsid protein into nearly 200 unique types. HPVs can be sub-classified into cutaneous or mucosal types based on their specific tissue tropism [SUZICH et al., 1995) or can be separated into low-risk and high-risk types based on their ability to cause malignant transformation and induce cancer. The high-risk types can cause cancers of

the uterine cervix, anus, vagina, vulva, penis, and head and neck [IARC Monograph, 1995).

The HPV genome encodes approximately 8,000 base pairs of double-stranded DNA. The open reading frames (ORFs) can be divided into three functional parts: the early (E) region that encodes proteins (E1–E7) necessary for viral replication; the late (L) region that encodes the structural proteins (L1–L2) that are required for virion assembly; and a largely non-coding part that is referred to as the long control region (LCR), which contains *cis* elements that are necessary for the replication and transcription of viral DNA (FEHRMANN AND LAIMINS , 2003).

The E1 is one of the most conserved early proteins among different HPV types. It has DNA-binding functions and a binding site in the origin of replication localized in the LCR region. It assembles into a hexameric complex, supported by the E2 protein, and the resultant complex has helicase activity and initiates DNA bidirectional unwinding, constituting a prerequisite for viral DNA replication (WILSON et al., 2002). The E2 proteins derived from the E2 gene are involved in the control of viral transcription, DNA replication, and segregation of viral genomes (KADAJA et al., 2009). The E2 protein can bind to factors on mitotic chromatin and join the virus genome to host cell chromosomes during mitosis (MCPHILLIPS et al., 2006), interact with E1 and stimulate viral DNA replication, favoring the binding of E1 to the origin of replication (CHOW AND BROKER, 1994) or directly represses the expression of early genes as a mechanism to regulate the viral genome copy number. Integration of the HPV genome in the host cell chromosome usually disrupts E2 expression, causing a deregulated expression of early viral genes, including E6 and E7, and this event can favor the transformation of human cells and the transition into a malignant state (ROMANCZUK AND HOWLEY, 1992). The E4 gene is located centrally within the E2 gene. Its main gene product, the E1^E4 gene products generally become detectable at the onset of vegetative viral genome amplification as the late stages of infection begin. E4 contributes to genome amplification success and virus synthesis, with its high level of expression suggesting additional roles in virus release and/or transmission. The precise mechanisms by which E4 contributes to genome amplification-success and capsid protein synthesis are still being studied (DOORBAR, 2013)

The E5 proteins are short transmembrane proteins encoded by many animal and human papillomaviruses. These proteins display transforming activity in cultured cells and animals, and they presumably also play a role in the productive virus life cycle (DIMAIO AND PETTI, 2013). E5 is claimed to have the ability to disrupt antigen presentation by affecting MHC expression, hence protecting infected cells from NK and CTL activity (ASHRAFI et al, 2005). E5 also modulates the immune and inflammatory pathways by interfering in EGF-R activation and transduction signaling such as the mitogen-activated protein kinases (Ras/Raf/MAP kinase) and the phosphoinositide 3-kinase (PI3K/Akt) (VENUTI et al., 2011). E5 binds to calnexin and thus traps the CD1d molecule into the endoplasmic reticulum, reducing CD1d levels at the membrane surface (HORST et al., 2012). Caveolin-1 and GM-1 are upregulated in the plasma membrane, and support viral immune evasion (SUPRYNOWICZ et al., 2008)

Overexpression of the E6 and E7 oncoproteins to affect the cellular regulatory pathways that are targeted by them is a major strategy of high-risk HPV. E6 is a basic protein of approximately 150 amino acids containing two zinc-binding regions capable to associate and degrade many cellular proteins (VANDE POL AND KLINGELHUTZ, 2013), including the pro-apoptotic p53 while E7 is a phospho-protein of approximately 100 amino acids that contains a short motif that mediates the interaction with the retinoblastoma tumor suppressor protein (pRB) and its related proteins p107 and p130 (GARIGLIO et al., 2016).

Through E6AP, E6 binds to p53 leading to its ubiquination and consequently to its proteasome-directed degradation. The inactivation of p53 abrogates its binding to target sequences in the genome, maintaining it in the cytoplasm or by effecting the abrogation of the transactivation of p53 responsive genes (KUMAR et al., 2002). The degradation or blocking of the p53 function inhibits apoptotic signaling that would eliminate the HPV infection cell. High-risk HPV E6 has also been shown to interact with two proteins that are part of the innate immune response to viral infection: interferon regulatory factor-3 (IFR-3) and toll-like receptor 9 (TLR9) (HASAN et al., 2007). This is part of an intricate process that HPV have developed to evade the host immune system (BOCCARDO et al., 2010)

The binding of high-risk E7 to pRB disrupts the interaction between pRB and E2F, a family of transcription factors, resulting in the constitutive expression of E2F-responsive genes, such as cyclin A and cyclin E, and promotes premature S phase entry, DNA synthesis, and the progression of cell cycle (ZERFASS et al., 1995). E7 also interacts with the CDK inhibitors (CKI) p21 and p27, efficiently neutralizing their inhibitory effects on CDK2 activities, an important factor for G1 to S phase entry and progression. High-risk E7 has further been shown to increase the levels of the CDC25A phosphatase, which can induce tyrosine dephosphorylation of CDK2, promoting its activation (MOODY AND LAIMINS, 2010). E7 also affects the expression of S phase genes by directly interacting with E2F factors and with histone deacetylases (HDAC): E7-E2F6 interaction prevents repression of gene expression by E2F6, maintaining an S phase environment conductive for viral replication.

The L1 gene corresponds to a sequence of about 1200 base pairs, which encodes a structural protein highly conserved among different HPV types (XU et al., 2006). L1 is the major structural protein of papillomaviruses and its conformation in the virion has largely been elucidated through the use of virus-like particles (VLPs) (HAGENSEE *et al.*, 1993). VLPs are empty capsids that are spontaneously assembled when L1 alone (or L1 plus L2) is overexpressed in heterologous systems such as bacteria, yeast or insect cells. The VLPs are highly immunogenic and contain conformational epitopes that induce the production of neutralizing type-specific antibodies against the virus, which prevent the infection, making it the target of prophylactic vaccines (VILLA et al., 2006).

L2 is the minor capsid protein of papillomaviruses. It is a secondary component of the HPV viral capsid and it is present in a variable number of copies per each capsid, being located on the inner surface in the central cavity below the pentamers of L1, where they are arranged to form the capsid (BUCK et al., 2008). L2 contributes to the binding of virion to the cell receptor, favoring its uptake, transport to the nucleus, and delivery of viral DNA to replication centers. It has also been suggested to be immunogenic and its immunogenicity, unlike L1, may not be type-specific (KARANAM et al., 2009).

1.2 INFECTION AND PATHOGENICITY

Papillomaviruses are highly epitheliotropic and they establish productive infections only within stratified epithelia of the skin, the anogenital tract and the oral cavity. They rely on the early genes E1, E2, E6 and E7 to establish their genome in basal cells. (IARC Monographs, 1995). Micro-injury formation is essential for the virus to access the basement membrane and basal keratinocytes. Heparin-sulfate proteoglycans (HSPGs) on cell membranes have been shown to act as attachment receptors for HPV viruses (JOYCE et al., 1999). The virus first binds to the basal membrane through interactions with HSPGs and then, during the wound healing process, the virus is transferred to basal keratinocytes as they migrate to the wounded area (ROBERTS et al., 2007). The infection with high-risk HPV typically lasts from 12 -18 months and is eventually cleared by the immune system. However, approximately 10% of women fail to clear HPV infections, resulting in a persistent infection (SCHLECHT et al., 2003). Such persistent HPV infection and viral DNA integration into the genome of the cell contributes to increasing the risk of high-grade and malignant lesions because of genomic instability generated. E6 and E7 can induce centrosomal abnormalities and abrogation of cell-cycle checkpoints through the targeting of p53 and pRB family members and this allows retention of cells with chromosomal abnormalities (MÜNGER et al., 2004). This can result in genetic changes that accumulate over an extended period of time leading to cancer development.

1.3 HPV AND CANCER

High-risk types of HPV are identified in nearly all carcinomas of the cervix and the relative risk of cervical cancer associated with infection with high-risk types of HPV is higher than the risk of lung cancer associated with smoking (MUNOZ *et al*. 2003). In a study by DE SAN JOSE et al., (2010), 85% of the 10,575 paraffin-embedded samples of histologically confirmed cases of invasive cancer from 38 countries across all continents taken over a 60 year period, were positive for HPV DNA. There is thus good evidence that HPV infection precedes the development of cervical cancer by a number of decades. Cervical cancer progresses slowly over decades from pre-invasive cervical intra-epithelial neoplasia to invasive cervical cancer (SCHIFFMAN et al., 2016).

Cervical cancer is more prevalent in poor countries for certain reasons including poor health infrastructures, presence of other important diseases like malaria, HIV and tuberculosis, lack of data for proper planning, lack of adequately trained health care workforce among other factors (DENNY, 2005). Alternative strategies for cervical cancer prevention are now incorporating molecular testing in primary screening with the standard Pap smear test as triage to have a better chance at early detection of cervical cancer lesions. Such studies have provided evidence that HPV-based screening is more effective than cytology-based screening (CATARINO *et al*., 2015).

1.4 IMMUNOLOGY OF HPV INFECTION

The immune system uses innate and adaptive immunity to recognize and combat foreign agents that invade the body, but these methods are sometimes ineffective against HPV. HPV has several mechanisms for avoiding the immune system. HPV infects, and multiplies in keratinocytes, which are distant from immune centers and have a naturally short lifespan. The naturally short life cycle of the keratinocyte circumvents the need for the virus to destroy the cell, which would trigger inflammation and immune response. The virus also down-regulates the expression of interferon genes which would otherwise control viral replication. (STANLEY, 2006)

The innate immunity is mediated by epithelial barrier, the complement system, and a variety of cells that phagocytose antigens and present them to other cells or destroy them. Keratinocytes are the main target of HPV and they play an important role in the promotion of an effective immune response as a component of the innate defense mechanism (NESTLE et al., 2009). They can express several toll-like-receptors which recognize pathogen-associated molecular patterns that initiate signaling pathways and result in innate and adaptive immune responses. They may also function as non-professional antigen presenting cells which process the viral antigen for adaptive response (AMADOR-MOLINA et al., 2013). The immunosurveillance of squamous epithelium of the cervix is managed mostly by Langerhans cells (LCs) which are immature dendritic cells. LCs are abundant in the skin and mucosa. They process antigens and present them to the B and T cells, eliciting both innate and adaptive immunity against the virus (MANICKAM et al., 2007). Some studies have however suggested mechanisms by which the HPV confers immunosuppressive phenotype on

dendritic cells through the expression of its immune-regulatory programmed death-1 (PD-1) molecule and its ligand as well as the expression of indoleamine 2,3-dioxygenase (IDO) (MUNN et al., 2004).

Natural Killer (NK) cells represent an important barrier and a key component of the innate immune system. These cells have the capacity to recognize and kill virus-infected and transformed cells through two mechanisms: granule-dependent cytotoxicity; and the apoptosis pathway in the target cells. Nevertheless, tumor cells have developed mechanisms to evade being attacked by NK cells, and viruses such as HPV display intrinsic strategies for preventing infected cells from being eliminated by NK cells (AMADOR-MOLINA *et al.*, 2013). NK cell activity is tightly regulated through a balance between inhibitory and activating receptors. Deregulation of these receptors is common in cancer and HPV infections. NKp30 and NKp46 receptors are found at low levels in NK cells from patients with cervical cancer and precursor lesions, which are correlated with low cytotoxic activity of NK cells (AMADOR-MOLINA et al., 2013).

A combination of all these elements serves to limit the HPV infection as well as initiate the more effective specific response. These roles are influenced by different cytokines and this can be exploited by the virus to affect the direction of the adaptive response.

Adaptive response to HPV infection comprises of cellular and humoral components which collaboratively defeat majority of HPV infections. The cellular aspect involves antigen recognition and uptake by the Langerhans Cells and migration of these cells to the lymphnodes for presentation to naive T cells. The clonally expanded T lymphocytes are the major effector cells and they are helped by chemokines, adhesion molecules and accessory cells such as macrophages to effect HPV clearance. Reduction in Langerhans cells may contribute to impaired immune surveillance (SCOTT et al., 2001). Interleukin-1α and β (IL-1 α, IL-1β) and tissue necrosis factor (TNF) all promote Langerhans cell migration while and interleukin-10 (IL-10) inhibits the migration. Granulocyte-macrophage colony-stimulating factor (GM-CSF) however aids by promoting maturation of Langerhans cells into mature dendritic cells. The cellular response could be Th1 or Th2 regulatory T cell driven depending on the cytokine expression pattern and the dendritic cells secrete cytokines that determine this response. This is the interface between innate and adaptive immunity (KAIKO et al., 2008).

Th1 cells secrete IFN-γ and create a milieu in which key cytotoxic effectors—macrophages, natural killer cells, and principally cytotoxic CD8+ T lymphocytes—are activated, generating cell-mediated immunity while Th2 cells secrete IL-4 and IL-10 which help antigen-primed B lymphocytes differentiate into plasma cells and secrete antibodies, the effector molecules of humoral immune responses (JANEWAY et al., 2001). B cells contribute by elaboration of antibodies. The antibodies against HPV target mainly the L1 capsid protein although weak antibodies directing against E2, E6, E7, and L2 have been described. The vast majority of these antibodies are IgG1 class, a predictable response against viral antigens (VISCIDI et al., 2004). The L1 neutralizing antibodies produced by virus-like particles (VLP) of prophylactic vaccines are produced in much higher levels than those generated in natural infection, and their serum levels remain high in long-term studies.

Regulatory T cells (Tregs) express CD4+CD25+, the signature transcription factor Foxp3 and secrete IL-10 and TGF-β. They recognize self-antigens and function to prevent autoimmunity but they also regulate responses to exogenous antigens. There is increased $Foxp3^{+}$ Treg infiltration following high-risk HPV infection and their presence failure of tumor-suppressive immune response (VAN DER BURG et al., 2007).

1.5 HPV IMMUNE ESCAPE MECHANISMS

As has been said, the HPV has evolved with different mechanisms for escaping the immune system and this contributes to its success as a cancer causing agent. HPV infection has a long incubation period and an effective immune system would normally clear the virus in this period. The HPV does not cause cytolysis or cytopathic effects and therefore avoids stimulating significant immune response (STANLEY AND STERLING 2014). The virus also inhibits interferon synthesis by interfering with the signalling pathway, induces regulatory T cell infiltration and the production of immunosuppressive interleukin-10 and tumor growth factor B. Also, the infected cells express low levels of MHC class 1 thereby causing impaired cytotoxic lymphocyte function. There can thus be an accumulation of ineffective CD4 and CD8 cells in lesions of HPV origin (SONG et al., 2015).

Infection with high-risk HPV decreases the number of Langerhans cells in the transformation zone of the uterine cervix by interaction of E7 with CCAAT/ enhancer-

binding protein β, a transcription factor of chemokine ligand 20 (CCL20) which is a principal factor in migration of Langerhans Cells into the dermis (LE BORGNE et al., 2006). High-risk HPV has also been shown to down-regulate TLR9 expression at the infection site thereby escaping immune-surveillance (HASAN et al., 2007). However, there is up-regulation of TLR4 expression and this correlates with the apoptotic resistance of HPV-infected cells. There is also an accumulation of tumor-associated macrophages (M2) which suppresses anti-tumor response (LEPIQUE et al., 2009). High risk HPV also compromise NK cell activation by reducing the expression levels of NK-activating receptors and by inducing tolerance of the NK cells to HPV infection (JIMENEZ-PEREZ et al., 2012).

The induction of inefficient $CD4^+$ T cells by high risk HPV infection is the factor that promotes lesion progression in immune-compromised patients but the down-regulation of MHC/HLA CLASS 1 is also very important. Other mechanisms involved in the immune evasion tactics include the causing of an imbalance between Th1 and Th2 response and compromised dendritic cell activation, possibly due to the stimulation of programmed death 1 (PD-1)/PD-1 ligand (PD-L1) (CD279/CD274) pathway. These viruses therefore exploit both the innate and the adaptive responses to evade the immune system (SONG et al., 2015)

1.6 HPV CANCER PREVENTION AND TREATMENT

The risk factors to develop cervical cancers include high-risk HPV infection, multiparity, long term use of oral contraceptives, tobacco use, having a weakened immune system, early commencement of sexual activity and having many sexual partners**.** Education on the avoidance of the risk factors would therefore seem to be an important part of a prevention scheme. This will include avoidance of precocious sexual activity, using barrier protection during sexual activity, avoiding the use of tobacco, getting an HPV vaccine, etc. These vaccines provide strong protection against new HPV infections, but they are not effective at treating established HPV infections or disease caused by HPV. Improved and more accessible screening methods for early detection will also go a long way in reducing the cervical cancer burden (VILLA et al., 2011). For decades regular screening with papanicolaou tests have significantly reduced the

incidence rates of cervical cancer in countries with organized screening programmes and high coverage of the population (WHO, 2005). However, we still observe very high rates of cervical cancer in less developed countries where screening programmes are opportunistic or inexistent. The highest morbidity and mortality rates due to cervical cancer are indeed observed in low and middle income countries (OLSON et al., 2016).

There is currently no medical treatment for persistent HPV infections that are not associated with abnormal cell changes. However, genital warts, precancerous changes at the cervix, and cancers resulting from HPV infections can be treated (MIRGHANI et al., 2015). Methods commonly used to treat precancerous cervical changes include cryosurgery, loop electrosurgical excision procedure, surgical conization, and laser vaporization conization. Treatments for other types of precancerous changes caused by HPV (vaginal, vulvar, penile, and anal lesions) and genital warts include topical drugs, excisional surgery, cryosurgery, electrosurgery and laser surgery (BURD, 2003). Most women in developing countries present with advanced disease, often untreatable or suitable only for palliation. Cervical cancer has a range of presentations from asymptomatic screen detected microinvasive disease, to abnormal vaginal bleeding, malodorous vaginal discharge, pelvic pain, or symptoms of more advanced disease such as vesico-vaginal or recto-vaginal fistulae or metastases (CDSR, 2010). Most cases will be treated with primary radiation therapy, either with curative or more commonly, palliative intent. Chemoradiation is an effective treatment for cervical cancer and randomized trials have shown a significantly improved survival with the addition of concomitant chemotherapy. Cisplatinum, 5-Fluorouracil and mitomycin C are the chemotherapeutic drugs usually given (CDSR, 2010). A combination of chemotherapy and radiotherapy improves both overall survival and disease-free survival.

1.7 HPV Vaccines

HPV vaccines are some of the biggest success stories in the battle against cancers of infectious origin. Because over 80% of all HPV associated cancers occur in the cervix, nearly all of the evidence for prophylactic vaccine prevention of incident type-specific HPV infection is in cervical disease (TORRE et al., 2015). There are three licensed

vaccines which contain synthetically manufactured virus like particles (VLPs) of the L1 epitope. Cervarix™ (Glaxo SmithKline) contains HPV 16 and 18 VLPs, Gardasil™ (Merck & Co.) contains HPV 16, 18, 6 and 11 while Gardasil9™ contains VLPs from HPV 16, 18, 31, 33, 45, 52, 58, 6 and 11. In addition, Gardasil and Gardasil9 contain aluminium as adjuvant while Cervarix contains AS04 (a mix of aluminium hydroxide and monophosphoryl lipid A) (POUYANFARD and MULLER, 2017)

Immunogenicity, indicated by antibody titre levels is the primary recognized endpoint in VLP based HPV vaccine studies and titres are dependent on the number of doses and timing (LEHTINEN *et al. 2012*). Studies showed that seropositivity for anti-HPV16 titers after 5 years is high for both Cervarix and Gardasil (GODI et al., 2015) and Gardasil9 induces similar anti-HPV16/18 responses as Gardasil (GUEVARA et al., 2017).

While these vaccines offered the prophylactic protection for which they were intended, they are not known to be effective in established cancer cases. Hence, many researchers are currently exploiting current technologies for the development of therapeutic vaccines that seek to stimulate the immune system to attack the established cancer cells and destroy them.

In contrast to preventive vaccines, therapeutic ones target the oncogenic proteins E6 and E7 which are continuously expressed in the cells throughout the full spectra of HPV infection and disease. Therapeutic vaccines generally seek to generate cell-mediated immunity against HPV lesion in contrast with the humoral immunity which is the target of prophylactic vaccines. Several approaches are being taken and one of them is the use of viral or bacterial live vectors, like *Listeria monocytogenes*, adenovirus, and vaccinia virus, which are highly immunogenic and broadcast the antigens to many APCs for processing, stimulating activation of both CD4+ and CD8+ cells through MHC II and MHC I, respectively (DELIGEOROGLOU et al., 2013). However, despite the ongoing clinical trials to test some of these possibilities, at present there are no available therapeutic vaccines to treat HPV-associated cancers.

1.8 PEPTIDES IN CANCER

Peptides form important parts of various cellular components, biological processes and reactions, in health and in disease. Peptides are therefore of great importance in

cancer research, diagnostics and treatment. The advances in the fields of molecular biology and protein chemistry have increased opportunities for research with peptides. Phage display technology is one such area that has benefited immensely from these advances (AGHEBATI-MALEKI et al., 2016).

The interest in peptides by cancer researchers is due to several factors including low toxicity and high specificity of peptides in diagnostic and therapeutic trials, availability of advanced tools for rational peptide design and synthesis, and the possibility of combination with other conventional cancer therapies, thereby reducing the associated (usually dose-related) side effects of those therapies. The other driving force behind this interest in peptides is the chance for creation of cancer vaccines.

Therapeutic peptides are a novel and promising approach for the development of anti-cancer agents (CICERO et al., 2017). They function by targeting the cell cycle, inducing cell death, targeting signal transduction pathways, stimulating specific elements of the immune systems, targeting tumour suppressor proteins or a combination of several of these mechanisms (MARQUS et al., 2017). Existing therapeutic peptides for the treatment of cancer have also been classified into three main groups: (a) antimicrobial/pore forming peptides, (b) cell-permeable peptides and (c) tumour targeting peptides. (BOOHAKER et al., 2012). The development of effective peptide-based cancer vaccines however poses some difficulties, including identifying optimal delivery routes, overcoming the low intrinsic immunogenicity of individual peptides, and the task of combining different types of epitopes to engage the humoral and cellular arms of the adaptive immune response properly, as well as compensating for the poor population coverage of individual T-cell epitopes due to MHC restriction (RECHE et al.,2014).

Apart from stimulating the patient's immune system to target cancer cells, it is also hoped that cancer vaccines can generate long-lasting immunological memory to prevent the recurrence of the cancer. Sipuleucel-T (Provenge®) is one such product that has been licensed for use against prostate cancer. Other products at various phases of trials include PGV001 for solid tumors, NEO-PV-01 for melanoma, lung and bladder cancer; ADXS11 001 for locally advanced cervical cancer; HER-2/neu peptide vaccine for breast and ovarian cancer; OC-L/Montanide ISA 51 VG for Recurrent Ovarian, Fallopian Tube, or Primary Peritoneal Cancer, and several others that are being

investigated (TANYI, 2017). A common feature among these products is the combination with different adjuvants or other products to enhance their efficacy.

1.8.1 Peptide Isolation by Phage Display

Phage display was created by G. Smith in 1985 as a method for presenting polypeptides on the surface of lysogenic filamentous bacteriophages. This method has become one of the most effective ways for producing large amounts of peptides, proteins and antibodies. The phage display technique allows the creation of libraries that contain up to 10^{10} different variants of peptide sequences and could be used for affinity screening of combinatorial peptide libraries to study protein-ligand interactions and to characterize these ligands (HOFFMANN et al., 2010).

E . coli filamentous bacteriophages (f1, fd, M13) are commonly used for phage display. Most antibodies and peptides are displayed at phage proteins pIII and pVIII (CHO et al., 2012; HESS et al., 2012). The major coat protein (pVIII) is a product of gene 8 expression and occurs in nearly 3000 copies, therefore it is used to enhance detection signal when phage displayed antibody associates with antigen. In comparison, minor coat protein (pIII) consists of 406 amino acid residues and occurs at the phage tip in 3 to 5 copies. The vast majority of peptides and folded proteins are displayed as fusions with pIII protein, whereas pVIII, for preserving its functionality, could be coupled only with short (6–7 residues) not containing cysteine peptides (FAGERLUND et al., 2008)

The application of phage display technology now spans several areas of medical research including transfusion medicine, neurological research, Diagnostic and therapeutic agents for autoimmune diseases, tissue homing and anti-angiogenic strategies, molecular imaging and tumor targeting and so on. The technology offers rapid, efficient and relatively inexpensive methods for investigating protein-protein interactions, receptor binding sites, identifying epitopes, mimotopes, functional and accessible sites from antigens. The technology offers high flexibility for exploring the mechanisms of disease and improving diagnostic methods. It is also currently being employed in vaccine development and delivery and this research project is built on the exploration of this last attribute.

2 STUDY BACKGROUND AND OBJECTIVES

As a spin-off of a natural history study of HPV infection and cervical neoplasia conducted in Brazil (the Ludwig-McGill cohort; VILLA and FRANCO, 1989), we screened a 10^9 transforming units of a cyclic CX_7C (C, cysteine; X, any amino acid residue) peptide phage display library for human sera binding ligands by the BRASIL method [GIORDANO *et al*, 2001], to search for peptides identified by immunoglobulins present in the sera of women previously exposed to HPV. The diversity of displayed peptides in this library was approximately 10^8 to 10^9 (VILLA LL, *unpublished data*). Three index sera were used for bio-panning the phage display library and were selected to reflect: (*a*) exposure to different HPV types, measured by HPV DNA detection in cervical smears and HPV-16 serology (*b*) differing disease status. All of the sera were found to be HPV positive by ELISA with HPV 16 VLPs at the first visit of the study and displayed the following characteristics: 1) one index case corresponds to a woman that was consistently HPV DNA positive throughout the first 2 years of follow-up, for both low (6/11 and high-risk (35, 58) HPV types, but she did not develop disease during this period; 2) sera derived from a women whose cervical smears were HPV-negative during the first year of follow-up but had low-grade squamous intraepithelial lesions (LSIL) in smears of subsequent visits; 3) and the third sera corresponded to a woman that turned HPV-16 positive on the fifth visit (18 months of follow-up) after having 4 previous HPV-negative smears;, she then developed a high-grade cervical lesion. Serum collected on this occasion was also subjected to biopanning in independent experiments as summarized below (VILLA LL et al., Unpublished data).

Incubation of the library with sera from HPV-negative women was first performed to clear the library of non-specific binders. The index serum was then diluted in PBS and incubated with 10^9 transducing units (TU) of the phage library. The suspension was centrifuged in a non-miscible oil and the serum bound phages were recovered by infection of *E. coli* K91 as previously described (GIORDANO et al., 2001). The recovered phages were then used for the next round of selection to increase the specificity of the binders. After 3 rounds of selection the PCR amplified DNA corresponding to peptide inserts of hundreds of randomly picked phage clones were sequenced and analyzed according to enrichment and by Clustal W sequence alignment.

Selected motifs were used to search non-redundant protein databanks (National Center for Biotechnology Information [NCBI] BLAST [http://www .ncbi.nlm.nih.gov/BLAST]) and the Papillomavirus Data Bank (VILLA LL, unpublished).

After peptide selection and characterization (BLAST), ELISA was performed with 105 sera (different visits for the first 3 years of follow-up) of 31 women randomly selected from the same study cohort. These included both HPV-positive and negative individuals. A positive control for this test was generated by the injection of the selected recombinant phages into New Zealand rabbits. (VILLA LL et al., Unpublished data)

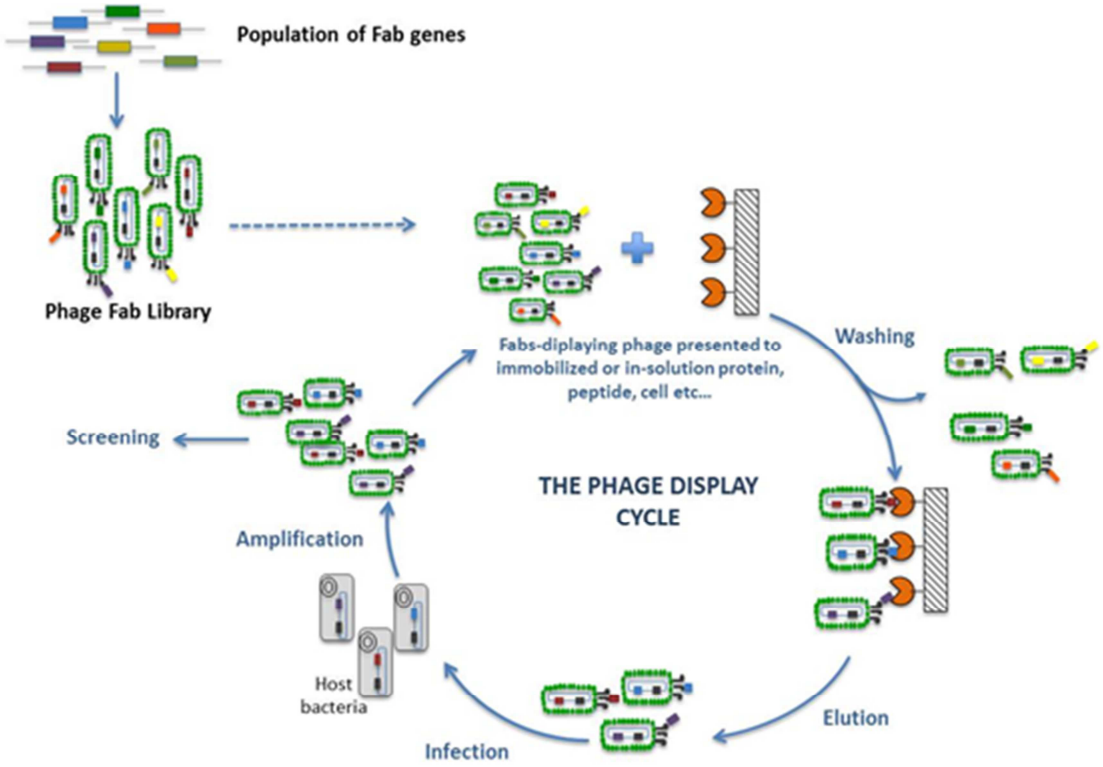

Figure 2. Schematic representation of phage display cycles. Adapted from info@creative-biolabs.com

Analysis of the peptides recovered from phage selection revealed that several amino acid residues were shared among multiple motifs. Searches for each of these peptides in

NCBI BLAST revealed partial matches to motifs contained in both early and late HPV proteins. Specifically, the most often selected peptide motif – MGGSKGP - shared partial homology to the major late protein of HPVs L1. This is a highly conserved protein among different HPV types and therefore not surprisingly we found the motifs in both low and high-risk HPVs. By performing BRASIL with different index sera, we were able to select different recombinant phages with inserts sharing residues with other HPV proteins, namely the early proteins E1 and E2 involved in replication and transcription control of the viral genome. We showed that the index sera could bind specifically to three of the GST-fused peptides, but not to GST only. Also, anti-rabbit antibodies raised against the selected recombinant phages reacted in ELISA with HPV-16 L1 virus-like particles, suggesting that the presented peptides could be mimotopes of the major capsid HPV protein. We tested both HPV-positive and HPV-negative serum samples by ELISA with GST-fused peptides. Most sera showed little or no reactivity with the selected antigens. However, a significantly higher reactivity was observed for some sera, including the index cases, as expected.

We sought to increase immune responses to the selected recombinant phages which could potentially behave as mimotopes of HPV and therefore stimulate the immune to offer protection against HPV-induced tumor growth in an animal model. This was done by the insertion of another sequence - TS-1 – to the recombinant phage. The TS-1 is purported to have the capacity to direct phages containing it to the lymph nodes where anti-tumor adaptive responses are triggered. (TREPEL et al., 2003). The resultant bi-cistronic recombinant phage is thus named PEP1TS1. This construct was done by Dr. Magda Barbu at the MD Anderson Cancer Centre in the laboratory of Dr. Renata Pasqualini.

Immuno-competent isogenic mice *C57Black/6* were immunized prophylactically with the phages (LEPIQUE AP AND VILLA L, unpublished). The immunization protocol consisted of 5 doses, with one week interval between each of them. One week after the last immunization dose, mice were injected subcutaneously with 10^5 TC-1 tumor cells, a mouse cell line containing HPV 16 E6 and E7 and activated *RAS*, a donation from Dr. TC Wu of John Hopkins (Baltimore, US). All mice were sacrificed once any tumor reached 1cm in the larger diameter to avoid animal suffering. From each mouse, tumors, peripheral lymph nodes and spleen were collected for cellular studies while peripheral

blood was collected for cytometric analysis. Cell populations in each of the tumors were incubated with antibodies against phenotype markers CD4, CD8, CD45, CD25 and FoxP3 and analyzed by flow cytometry.

Preliminary data indicated that one of the recombinant phages, PEP1 (containing the sequence corresponding to peptide MGGSKGP), partially inhibits tumor growth when administered prophylactically in mice, as compared to mice immunized with insertless bacteriophage (Figure 3). Addition of the TS-1 peptide to the phage construction (homing to the lymph nodes) does not further modify tumor growth. However, mice immunized with the PEP1 construction displayed significantly less tumor growth, even less than mice immunized with a bacteriophage that contains a construction that combines TS-1 and PEP1.

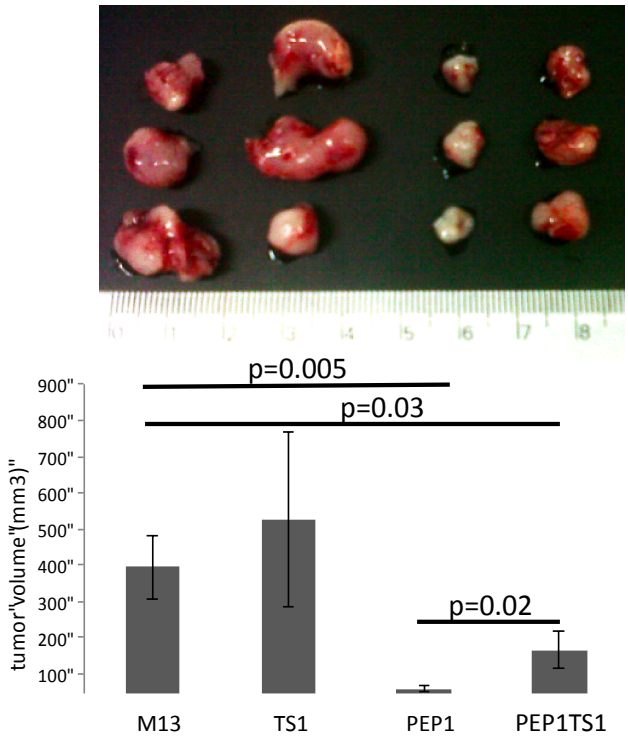

Figure 3: Tumors harvested from mice after prophylactic immunization in the pilot study. The image above shows the tumors harvested and the graph bellow shows the average tumor volume in each experimental group. M13, corresponds to mice immunized with insertless bacteriophage; TS-1, with the construction carrying the TS1 peptide sequence; PEP1, with the construction carrying the MGGSKGP corresponding peptide sequence; PEP1/TS1, with the construction carrying the both peptide sequences. (LEPIQUE A.P *et al.*, unpublished data)

2.1 OBJECTIVES

2.1.1 General Objective

Evaluate the immunologic responses in mice injected with the selected recombinant phages and correlate such responses with HPV-16 tumor growth in a mouse model.

2.1.2 Specific Objectives

- Measure the kinetics of TC-1 induced tumor growth in C57Bl6 mice immunized with recombinant phages in both a prophylactic and therapeutic schemes.
- Characterize the immune responses generated in immunized mice (wild-type and knock-out animals).
- Establish the specificity of the observed response with in-vitro assays.
- Challenge the observed anti-tumor immune responses with the inclusion of adjuvant alongside phage injection.
- Propose the potential mechanism underlying the protective/therapeutic responses triggered by the selected recombinant phages.

3. MATERIALS AND METHODS

3.1 PHAGE CONSTRUCTION

The recombinant phages used in these experiments were obtained at the MD Anderson Cancer Centre in Texas, USA, in the laboratories of Renata Pasqualini and Wadih Arap, by Luisa Villa along 2001-2004. They were selected from a heptameric phage display library constructed on the Fd-tet phage. Thus, the phages in this study are referred to as PEP1, referring to the recombinant phage carrying motif MGGSKGP; TS-1 refers to the recombinant carrying the TS-1 motif; PEP1 TS1 refers to the bi-cistronic recombinant phage containing both the PEP1 and TS1 motifs and M13 refers to the Fd-tet phage without insertion which is used as a control in the experiments.

3.2 *E. coli* K91 CULTURE

From a frozen stock, *E.coli* K91 was streaked onto a minimum medium plate and incubated at 37^0C for 24 hours. Single colonies were picked and vigorously streaked onto an LB Agar plate containing Kanamycin, to form a bacterial carpet. A swipe of about an inch of this carpet is cultured in liquid medium (Terrific broth or LB broth) which is then used to propagate the bacteriophages.

3.3 PHAGE AMPLIFICATION, PURIFICATION AND SEQUENCING

To generate sufficient volume of the recombinant phages for the different experiments, the selected phages were amplified in liquid *E. coli* bacteria culture. 800µL of the bacteria culture in terrific broth is infected with 2µL of the phage stock at room temperature for 30 minutes. The phage-infected culture is then inoculated into 500 millilitres of LB broth containing Kanamycin and Tetracyclin antibiotics and incubated overnight in a shaker-incubator at 37^0C and 200rpm. The grown culture is then processed to harvest the bacteriophages by the processes of PEGylation and centrifugation, as follows.

The culture is decanted into 250ml centrifuge tubes and centrifuged at 8000g for 20minutes. The supernatant is decanted and mixed with 15% Polyethylene glycol/Sodium chloride solution (PEG/NaCl) and incubated on ice for 2 hours and then centrifuged for 30minutes at 8000g. The supernatant is discarded and the pellet is resuspended in 1ml 1xPhosphate buffered saline (PBS). This suspension is shaken at 250RPM and 37^0C for 15minutes and transferred to an eppendorf tube for further

centrifugation at 8000g to remove the pellet of bacterial contaminants. The supernatant is collected, treated with 15% PEG/NaCl for about 30minutes and centrifuged for 40 minutes at 8000g. The observed phage pellet is resuspended with 500 microliters of 1x PBS, incubated and shaken for 15minutes at 37^0C/250RPM for proper resuspension, then centrifuged at 8000g for 15minutes. The clear phage supernatant is then harvested and stored at 4^0C.

After the processes of amplification and purification, the recombinant phage was sequenced to ascertain the integrity of the insert. A colony was used as the template in PCR reaction, using FUSE5 (forward and reverse) primers (CHRISTIANSON, 2007). The resultant product was used as a template in a sequencing reaction with the reverse primer only and the final product was precipitated and sequenced to confirm the insert.

3.4 LIPOPOLISACCHARIDE CONTROL

Lipopolisaccharides (LPS) can contaminate products synthesized in bacteria and because of their biological characteristics; they may be undesirable in certain kinds of experiments. To determine the level of lipo-polisaccharides in the produced phage solutions, Limulus Amebocyte Lysate (LAL) assay was performed. The LAL Kit was purchased from Lonza® Pharma and Biotech and used according to the manufacturer's guide.

3.5 TC-1 CULTURE AND GRAFTING

The TC-1 cell line is a tumor cell line produced from primary lung epithelial cells of C57BL/6 mice co-transformed with HPV-16 E6 and E7 and c-Ha-ras oncogenes LIN et al, 1996). The cells were maintained in culture with RPMI medium 1640 (GIBCO/Invitrogen) containing 10% Fetal Bovine Serum. These cells are used to implant the tumors on the mice which are then treated according to the experimental protocol. The medium is aspirated and the cells are washed with PBS-EDTA for about 30 seconds. Trypsin is then added for about 1minute and the cells are harvested with 6ml of complete medium. The suspension is centrifuged at 1300rpm for 5minutes and the supernatant is discarded. The cells are then resuspended in 5ml of medium and counted to determine the volume required for tumor grafting.

For tumor grafting, 100,000 cells (10^5) are injected subcutaneously on the right flank of each mouse. After counting the harvested cells and calculating the required volume for injection, the cells are washed twice in PBS^{++} (PBS supplemented with 0.5mM $MgCl_2$, 1mM $CaCl_2$) and then resuspended in a final volume of100 µL per mouse. The mouse is restrained and the cells are carefully injected. Tumor is generally palpable from the 6^{th} day of tumor cell injection. All cell manipulations were done under bio-safety cabinet NB1.

3.6 MOUSE LINEAGE

Immuno-competent isogenic mouse strain C57Black/6 and CD8-knock-out mice were housed in the mouse facility at the Department of Immunology, Institute of Biomedical Sciences, Universidade de Sao Paulo and at the mouse facility of the Center for Nuclear Medicine, Instituto de Radiologia da Faculdade de Medicina, Universidade de São Paulo were used in these series of experiments. The mice were all female, between 6-8 weeks old at the start of each experiment and weighed approximately 25 grams each. Mice were housed and handled according to the Brazilian Federal Law and Sao Paulo State Law (LEI Nº 11.794 BR, 2008; ,LEI Nº 11.977 SP, 2005), and our protocols have been approved by the Institution's Ethics Committees for Animal Research.

3.7 ANIMAL INOCULATION

Two routes of inoculation were used in these experiments. The first dose of bacteriophages was $5*10^8$ Transforming Units (TU) per mouse. This is diluted in 500µL of PBS and the injection is given subcutaneously in divided doses to prevent painful swelling. Subsequent doses of $1*10^8$ TU was prepared in 100µL and given intraperitoneally at weekly intervals. All mice manipulations were done at the required biosafety level NB1. The immunization protocol is schematically presented below:

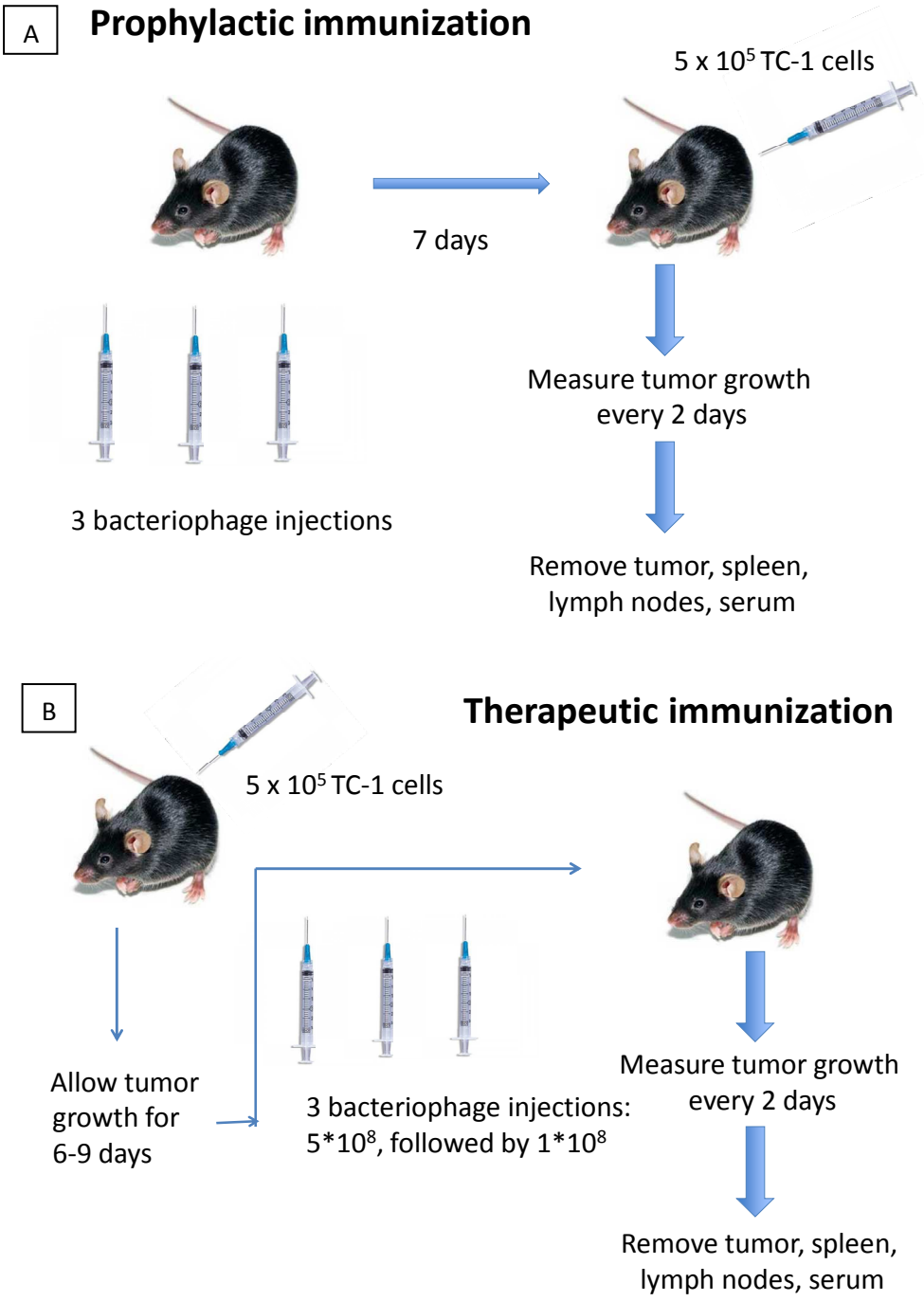

Figure 4: (A) Prophylactic and (B) therapeutic immunization schemes used in the experiments.

3.8 TUMOR MEASUREMENT

Tumor growth is followed carefully and measured by the use of a pachymeter. The mouse is restrained and the tumor diameter is measured in two axes. The volume is calculated by multiplying the square of the shorter diameter (B) with the longer diameter (L). Measurement is done every other day to obtain a growth curve of the tumor. Experiment is generally terminated when any of the tumor diameters reaches 10 millimetres.

3.9 EUTHANASIA AND TISSUE COLLECTION

Sacrificing the mice for tissue collection is done by first anaesthetising the mice with a combination of Xylazine and Ketamine intraperitoneally then placing the mice in a CO_2 chamber for five minutes. The mouse is checked for pain sensation and once there is lack of reflexes, we proceed to collect the required tissues. Blood is collected by intracardiac aspiration while lymph nodes, spleen and the tumor are collected following dissection. The tissues are collected into PBS and placed on ice till processing.

3.10 TISSUE PROCESSING

3.10.1 TUMOR

The tumor is cut in two and one half is fixed in formalin for immunohistochemistry. The other half is macerated with scalpel blade, collected in an eppendorf tube and digested by the addition of 1mg/ml Collagenase I and IV, in Mouse Tonic Hanks solution (MTH - 15 mM HEPES, pH 7.4, 1x Hanks salt solution) complemented with 5% fetal bovine serum and 0.5 U/ml DNAse I. The suspension is shaken at 1300rpm until it gets turbid and then filtered to obtain single cells. The cell suspension is counted in preparation for antibody labelling and flow-cytometry to detect the inflammatory infiltrates.

3.10.2 SPLEEN

The spleen is mechanically strained through a metal filter. The suspension is centrifuged at 1200rpm for 5minutes and the supernatant is discarded. The cell pellet is dislodged and 1ml of hypotonic Ammonium-Chloride-Potassium (ACK) buffer is added for 2 minutes to lyse the cells. Mouse Tonic Hanks' solution (MTH) is added to bring the

volume up to 6ml and then centrifuged for 5 minutes at 1200rpm. The clear pellet is properly resuspended in 2ml MTH and counted in preparation for analysis.

3.10.3 LYMPH NODE

The harvested lymph nodes are macerated on the grainy surface of glass slides and washed into a petri dish. The resulting suspension is filtered through a *'Pasteur filter'* and centrifuged at 1300rpm for 5minutes. The pellet is resuspended in 1ml MTH and counted.

3.10.4 BLOOD

The collected blood is allowed to clot on ice and centrifuged at 3000rpm for 15 minutes to collect serum. Where there is lysis, the collected serum is centrifuged for a further 15minutes at a higher revolution. The serum is stored frozen until ready for antibody evaluation by ELISA.

3.11 ANTIBODY LABELLING

There were two types of antibody labelling depending on the target of interest. For membrane markers like CD45, CD4, CD8, Gr1 and CD11b, the simple protocol for surface labelling was used. For intracellular markers like FoxP3, there is need for a permeabilization process before antibody labelling.

3.11.1 Surface

For surface marker staining, a million cells were aliquoted from each tumor sample and washed with MTH. 50µL of the antibody mix was added to each tube on low speed vortex and the tubes were incubated on ice. After 10 minutes, the tubes were vortexed again and incubated for a further 10 minutes on ice. The cells were then washed with 500µL of MTH and filtered through tip-filters into the FACS tubes. These were kept on ice until ready for acquisition by Flow cytometry.

3.11.2 Internal

When an intracellular marker was evaluated, the cells stained for surface markers were resuspended in 100µL of fixation/permeabilization solution and incubated for 20minutes on ice. The cells were then washed twice with 1ml of 1x BD Perm/Wash buffer and resuspended in 50µL of the intracellular marker antibody for 30 minutes on ice. Subsequently, the cells are washed twice in Perm/Wash buffer and resuspended in

300µL Perm/Wash buffer, then filtered through filter-tips into FACS tube in readiness for acquisition.

3.12 Flow cytometry

Multi-color flow cytometry was used to capture and analyze the different cell populations in the tumor infiltrate and to assess the T-cell clonal expansion/proliferation when unstimulated or stimulated with synthetic peptide equivalent of the sequence inserted in the recombinant phage used for vaccination. This was done at the Laboratório de Citometria, Instituto de Ciências Biomédicas, Universidade de São Paulo. Single color controls were prepared for every antibody/marker used in all the experiments except in tetramer assays which already includes its own control. The basic antibody/fluorophore panels used are shown below, with modifications where necessary.

PANEL A	PANEL B	PANEL C	PANEL D
CD45APC	CD45APC	CD45APC	CD4PerCPCy5.5
CD3PECy7	CD11bFITC	CD11bFITC	CD8APC
CD4FITC	F4/80PECy5	CD4PerCPCy5	CD25PECy7
CD8PE	Gr1PE	CD8PE	FoxP3
CD49B488			

Table 1: Fluorophore-conjugated antibodies panel used in the multicolour Cytometry of the infiltrate populations.

3.12.1 Tumor Infiltrate

The tumor infiltrate study was done with tumors from immunized mice and controls from the different experiments. Tumors were harvested and processed as detailed above. The cells were labeled with surface markers and captured. 300,000 cells were captured per sample.

3.12.3 Lymphocyte Proliferation Assay

Cell Trace assay was performed to see the pattern of T-cell proliferation in response to in-vitro stimulation with the immunizing peptide, phytohaemagglutinin or no stimulation. Single cell suspensions of the spleen and lymph nodes were used for this study. $10*10^6$ cells from the spleen and $6*10^6$ cells from the lymph nodes were aliquoted and washed twice with PBS to remove serum. Cells were resuspended in 500µL of PBS. 500µL of a 20µM solution of Cell Prdiferation Dye eFluor^{TM} 450 at room temperature was added to the cell suspension while vortexing. The mixture was incubated at 37^0Cin the dark for 10 minutes and the labelling was stopped by the addition of cold complete RPMI containing 10% Fetal Bovine Serum. The suspension was allowed to stand on ice for 5 minutes, and then washed 3 times with complete medium. Thereafter, the pellets were resuspended to contain $5*10^5$ of the labeled splenic cells and $3*10^5$ of the lymphoid cells in 100µL respectively and each sample was dispensed into nine wells of a round bottomed culture plate, representing triplicates of the unstimulated, PHA stimulated and peptide stimulated samples. Subsequently, the wells were completed with complete medium containing Penicillin/Streptomycin (1:100), β-Mercaptoethanol, PEP-1 synthetic petide (for the Peptide stimulated group only) and Phytohaemagglutinin (for the PHA stimulated group only). The culture plate was incubated at 37^0C for 5days after which the cells were harvested and labeled with markers for CD4, CD8, CD25 and FoxP3 antibodies as previously described. The samples were subsequently captured by flow cytometry.

3.13 ELISA

Enzyme linked immune-sorbent assay (ELISA) was used to evaluate the humoral response to the treatments/immunization. We checked both response to the immunizing phage and then specific response to the PEP1 sequence.

3.13.1 Phage ELISA

For the evaluation of the antibody response to the phage, the M13 phage was used as the test antigen. Each well of a 96-well ELISA plate (Costar® 3590) was coated with $2*10^7$ Transforming Units (TU) of the phage in PBS and kept overnight at 4^0C. The wells were washed with PBS and blocked with Bovine Serum Albumin for 2 hours after which they were washed again and the test samples added in duplicates. The positive

control (α-FD Bacteriophage Antibody, SIGMA) and negative controls were included. The plate was incubated at room temperature for 2 hours, washed again and the secondary antibody (Anti-mouse antibody conjugated with Horse Radish Peroxidase) was added and the plate further incubated for one hour. The plate is washed; the chromogen (ABTS, SIGMA) is added for 5 minutes and the reaction is stopped by the addition of 1% Sodium Dodecyl Sulfate. The plate is read at 405nm on an iMark® ELISA reader.

3.13.2 Peptide ELISA

Our peptides were synthesized by the Chinese Peptide Company®. The lyophilized peptide was diluted in PBS and stored at -20^{0}C until use. The ELISA plate was coated with 2µg peptide per well (in 50µL PBS) and kept overnight at 4^{0}C. The plate was then washed with PBS, blocked with 2.5% BSA and the samples were applied. The plate was incubated for two hours, washed with the washing solution and the secondary antibody was added. After one hour incubation, the plate was washed again, the chromogen was added for five minutes, stopped with 1%SDS and read at 405nm on an iMark® ELISA reader.

3.14. Immunohistochemistry

Unconjugated primary antibodies against CD4 (AB183685, Abcam®), CD8 (AB203035, Abcam®), CD45 (AB10558, Abcam®) and NKG2D (AB203353, Abcam®) were used in these experiments. Tissue sections from tumors were fixed in formalin, embedded in paraffin and cut onto silanized slides. The slides were deparaffinized in xylene and rehydrated in a graded alcohol series. Endogenous peroxidase was blocked Peroxidase block (Abcam®) for 10 min at room temperature (RT). For epitope retrieval, slides were heated in target retrieval solution (10mM Sodium Citrate solution) in a hot water bath at 95^{0}C for 10min and then allowed to cool to RT for 20 minutes. The slides were washed with PBS-T (PBS containing 0.05%Tween) and then incubated with ProteinBlock (Abcam®) for 30 min to block nonspecific protein binding sites. Subsequently, recommended dilution of the required antibody was added and incubated for 30 minutes at RT. After three washes with PBS-T, the peroxidase-conjugated polyclonal secondary antibody (AB 64261, Abcam®) was added for 20 min at RT. This was washed off and color was developed with 3,3'-

diaminobenzidine and hydrogen peroxide ((Abcam®), and slides were subsequently counterstained with 100% hematoxylin, dehydrated, and mounted.

3.15 STATISTICS

All data obtained were carefully organized for analysis, using Microsoft Excel® and SPSS® programs. Survival data were analyzed by Mantel-Cox Log Rank tests and plotted on Kaplan-Meier survival graph. Humoral responses were analyzed by either student T-test or One-way ANOVA where applicable while cellular data were analyzed by One-way ANOVA followed by post-hoc Tukey-Bonferroni Tests. Tumor volume comparisons were also analyzed by T-test or ANOVA depending on the number of groups being compared.

4 RESULTS

4.1 PHAGE AMPLIFICATION, PCR AND SEQUENCING

Following the isolation of the phages with the different peptide-sequence inserts at the MD Anderson Laboratory in Texas, I cultured the phages in bacteria to expand the volume sufficiently for all the subsequent experiments. The new batch was sequenced after polymerase chain reaction to ensure that there was no contamination during the culture process. As expected, the amplicons gave around 300bp band on 1.2% agarose gel electrophoresis

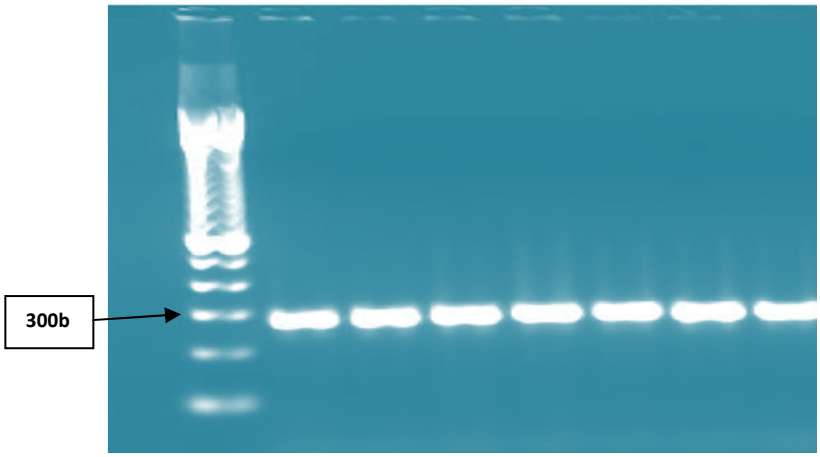

Figure 5: Agar Gel Electrophoresis of the PCR-Amplified recombinant phages, using the primers Fuse5 forward and reverse. The insert band can be seen at around 300bp marker.

The sequencing reaction was also done utilising the FUSE5 Reverse primer. Both the original phage stock and the expanded stock used in this work were sequenced to ensure the integrity of the insert. The insert sequence was confirmed to be the same as the isolated peptide.

4.2 Phage Titration

The new phage stock was titrated on LB Agar plates (containing Tetracycline and Kanamycin) to determine the volume needed for the treatment doses.

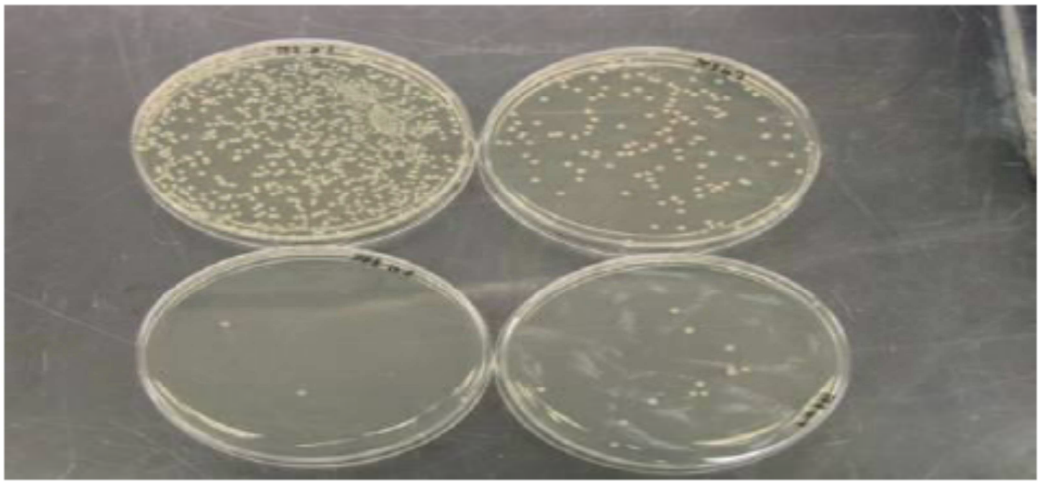

Figure 6: Phage titration on LB/Kanamycin/Tetracyclin agar plates.

4. 3 Lipopolysaccharide Evaluation By Limulus Amebocyte Assay

To determine the level of lipo-polisaccharides in the produced phage solutions, Limulus Amebocyte Lysate (LAL) assay was performed. Each phage stock was diluted ten times and twenty times as advised by the LAL protocol before testing. Each standard solution and sample was run in triplicates and the mean and endotoxin concentrations were calculated according to the manufacturer's guide.

	TRIPLICATE READING			MEAN	CONC (EU/mL)
SAMPLE	1	2	3		y=1,9571x+0,1046
PEP1	3,045	3,229	3,266	3,18	**1,519033**
PEP1TS	3,039	3,126	3,218	3,13	**1,493485**
TS1	3,01	2,978	2,997	2,995	**1,424506**
M13	2,888	2,998	2,876	2,921	**1,386695**

Table 2: LAL Assay for assessment of endotoxin levels in the phage solutions.

FDA recommended maximum LPS in injectable materials is 5EU/Kg. If average mouse weight is 25g, this translates to 0.125EU/mouse For M13 with the highest LPS content of 0.01438576 EU/microlitre, this will translate to 8.7microlitres of phage prep. However, with a maximum dosage of 5 * 10ê8 and minimum phage titre of 4*10ê8, there is no situation that will warrant the injection of this volume of phage prep. In summary therefore, all the phage preps contain well below the levels of LPS allowed in injectable substances, according to the US FDA recommendation.

4.4 Prophylactic immunization of mice with PEP1 recombinant bacteriophages generates humoral response against the phage antigen as well as against the inserted epitope.

As a proof of effective delivery of the phage immunogens, we performed ELISA with sera recovered from immunized mice using the M13 phage and the synthetic PEP1 peptide as antigen. All immunized mice showed high production of IgG against the phage antigen but only those immunized with PEP1 phage (in prophylactic and therapeutic protocols) and PEP1TS1 (in prophylactic protocol only) had detectable immunoglobulins against the inserted sequence, as expected.

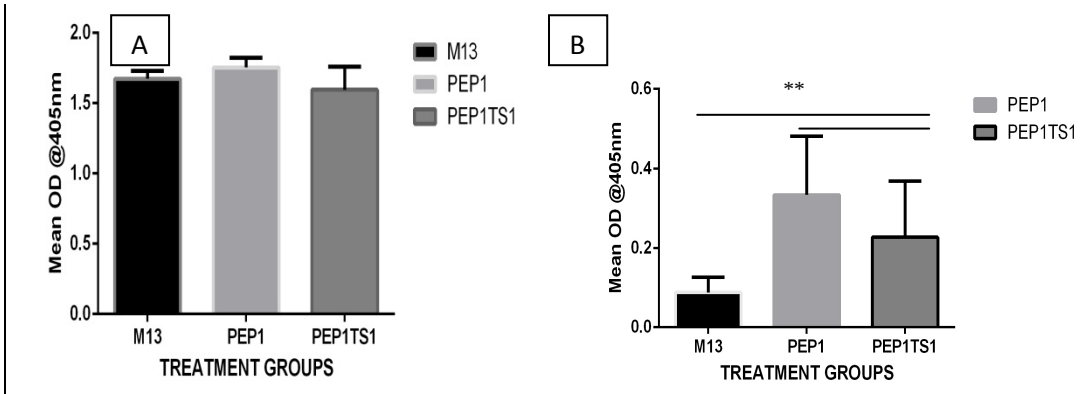

Figure 7: Humoral response against the M13 phage and the peptide insert in prophylactically immunized mice. The M13 phage was used as the antigen in A while the synthetic peptide was used as the antigen in B. Data presented represents mean titre with standard deviation. Statistical analysis was done using One way ANOVA, followed by Bonferroni test. **P<0.01

All the immunized mice showed appreciable antibody production against the M13 phage. This is proof that the immunization was effectively done. The specific response against the inserted peptide was however limited to the groups immunized with PEP1 and PEP1TS1, as expected.

4.5 Prophylactic administration of the recombinant phage Pep1 can stimulate immune responses which reduce TC-1 tumor growth but additional insertion of TS-1 did not improve tumor reduction.

To verify if these recombinant bacteriophages can generate an immune response against an HPV-positive tumor cell line in a mouse model when administered prophylactically, we immunized three groups of mice with M13, PEP1 and PEP1TS1 recombinant bacteriophages, starting with a dose of $5*10^8$ transforming units (TU) and two booster doses of $1*10^8$ TU, with each immunization given one week apart. We then injected TC-1 tumor cells as described under methodology and monitored tumor growth in the different groups of animals (Figure.8).

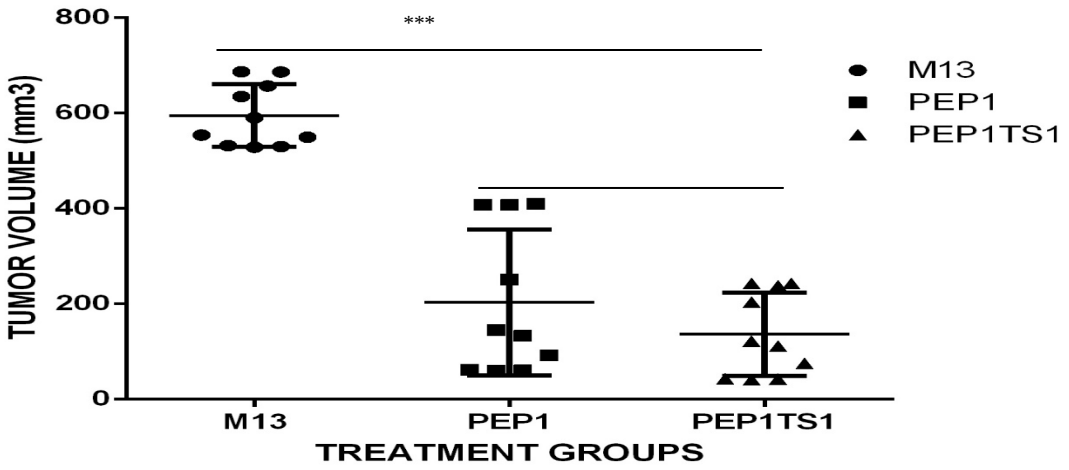

Figure 8. Tumor volume of mice immunized with PEP1, PEP1TS1 and M13 recombinant bacteriophages. The experiment was terminated when tumors in any group get to the preset limit of 10millimeter in any diagonal measurement. This graph represents the results of two independent experiments. Data was analyzed by One way ANOVA followed by Bonferroni test. ***P<0.001

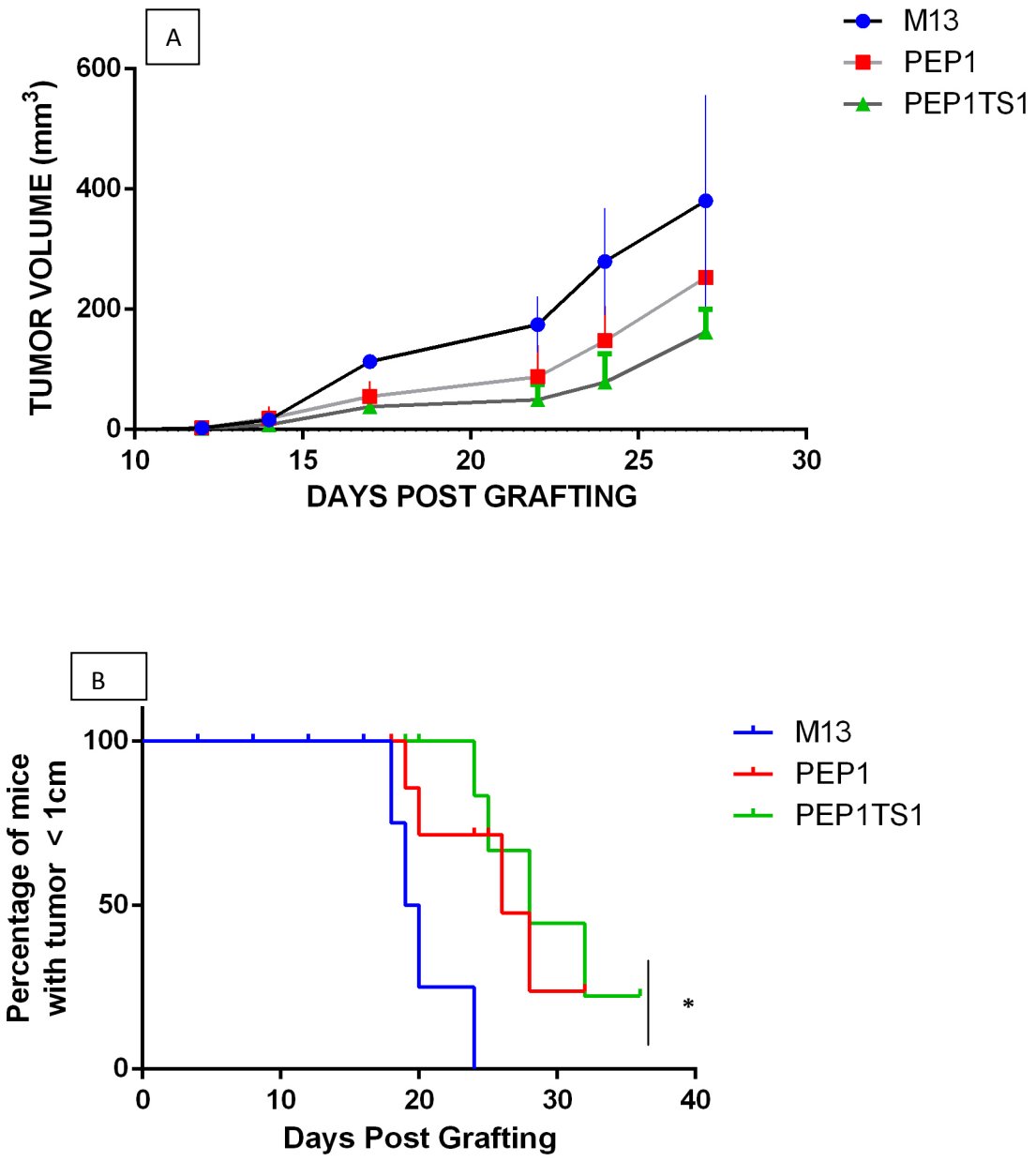

Figure 9: A. Tumor growth curve of TC-1 cells injected in the left flank of prophylactically immunized BL57/B6 mice. Three treatment-groups containing three mice each were monitored over time to obtain data for the tumor growth pattern in the individual subjects. **B**. **The percentage of mice with tumors less than 1cm** in this same experiment was plotted against time in a Kaplan Meier graph. Mice were censored when tumor reached 1cm in either diameter. Statistical analysis was done using Mantel-Cox log rank test. *P<0.05 (*Endpoint was predetermined as when tumor reaches 1cm in either diameter*)

Tumor measurements were plotted against time (days post graft) to generate the kinetic profile for the tumor growth while the final tumor volumes were compared for evaluation of the tumor growth reduction effect of the immunizations (figure 9). The groups immunized with PEP1 and PEP1TS1 had comparatively slower tumor growth rate and the reduction in final tumor volume was statistically significant ($p<0.001$). There was however no statistically significant difference between the PEP1 immunized and the PEP1TS1 immunized mice (figure 9A). The survival analysis of the mice was plotted in a Kaplan Meier curve (figure 9B).

4.6. Prophylactic administration of the recombinant phages affects immune cellular responses and promotes a cytotoxic tumor environment.

In order to understand the role of cellular immune effectors in the observed tumor reduction in the treated mice, we evaluated the relative percentages of tumor infiltrating leucocytes between the experimental groups, quantitatively measuring the presence of CD45, CD4, CD8, NK, F4/80 and CD11bGr1 positive cells in the digested tumor tissues. Figure 10 shows the level of expression of these cells in the treatment groups.

There was no statistically significant difference in the level of expression of these immune cell populations between the treatment groups. However, we observed a trend of higher cytotoxic CD8 cell stimulation in the groups immunized with PEP1 and PEP1TS1 recombinant bacteriophages (figure 10E).

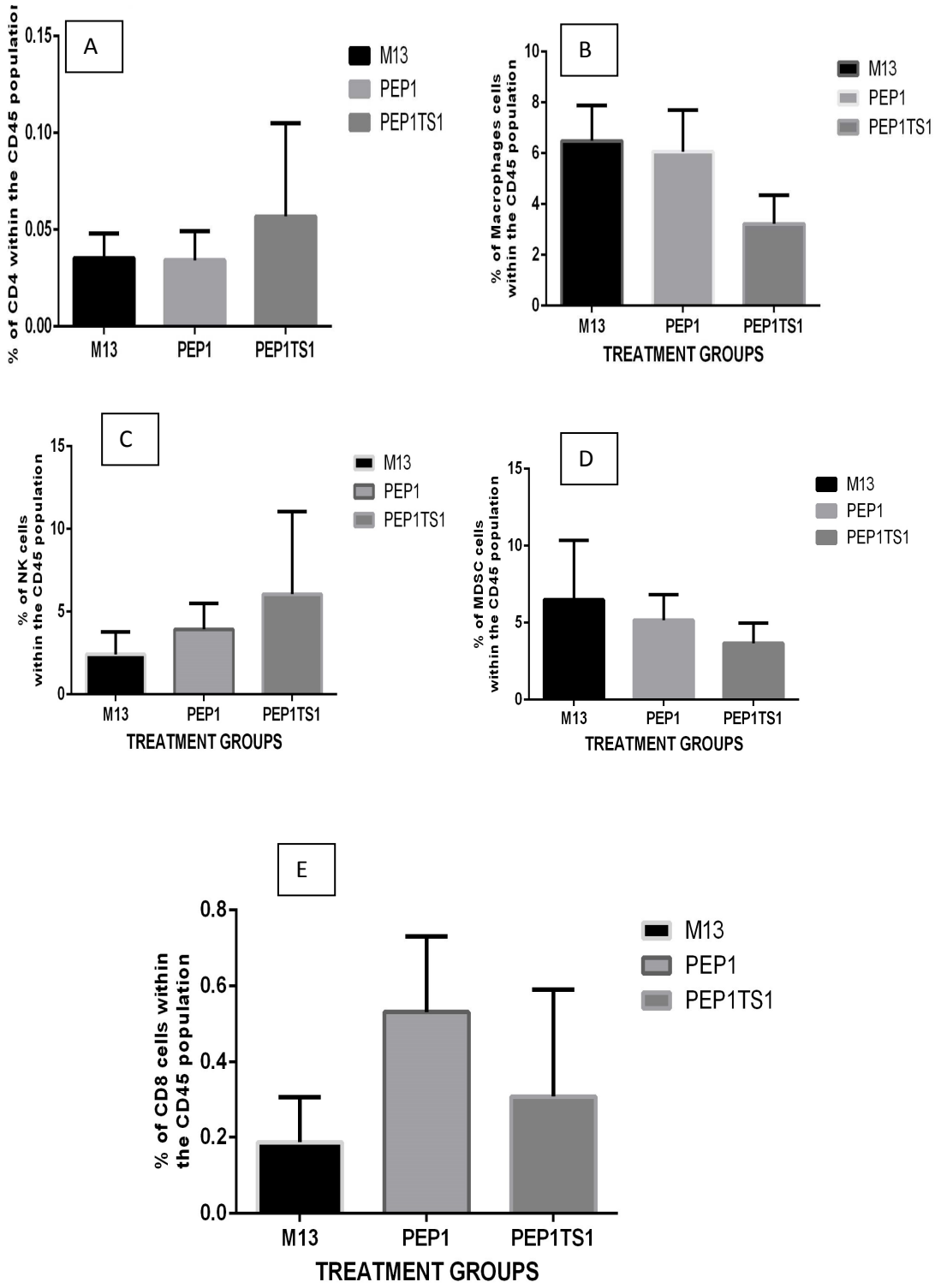

Figure 10: Quantitative evaluation of the different immune cell infiltrates within the tumor tissues from prophylactically immunized mice. (A) helper CD4T cells, (B) Macrophages, (C) Natural Killer (NK)cells, (D) Myeloid derived suppressor cells and (E) cytotoxic CD8 T cells are all expressed as a percentage of the Leucocyte population (CD45+). The graphs represent the result of the experiment with the most consistent pattern.

4.7 The observed immune response against TC-1 tumor in prophylactically immunized mice is based on CD8 T cells and this response is lost in CD8-Knockout mice

We hypothesised that the observed slight increase in the CD8 cell numbers in the prophylactically immunized mice could be a contributing factor for the associated tumor reduction in the PEP1 and PEP1TS1 immunized groups. In order to investigate this, we immunized CD8 knockout and wild-type B57C/BL6 (B6) mice with the recombinant phages and then grafted the mice with TC-1 cells as previously done. The graphs below represent the obtained results (figure 11).

The results of the experiment performed in CD8-knock-out mice confirmed the suggested role of the cytotoxic CD8 cells in the tumor reduction mechanism seen is the previous experiments: the only group with a statistically significant tumor reduction was the immunocompetent mice group treated with PEP1 recombinant phages whereas the CD8 knockout group treated with the same PEP1 phages had a pattern of tumor growth similar to the mice immunized with the insertless phage (figure 11 A and B).

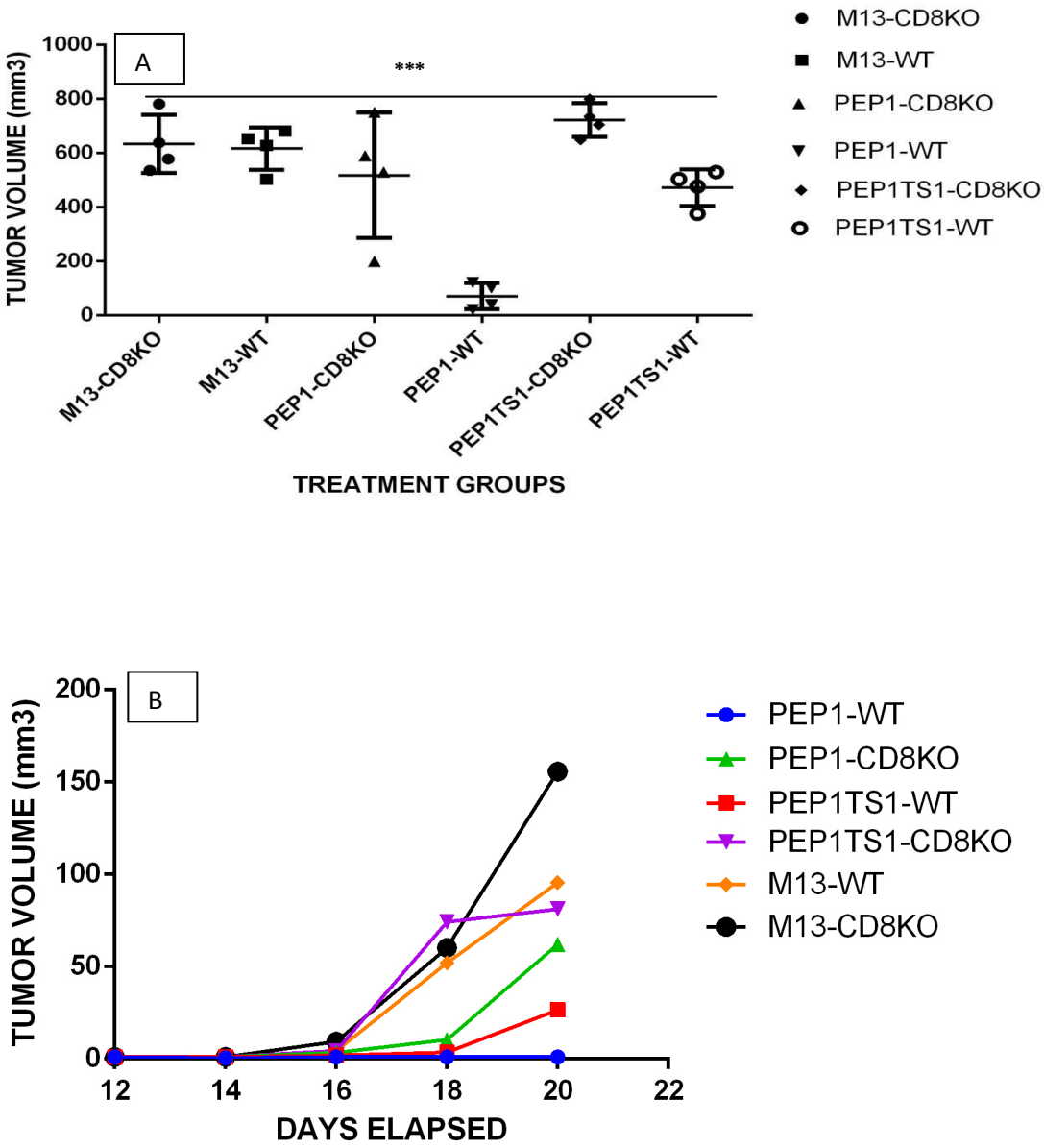

Figure 11. Tumor volumes (A) and tumor kinetics (B) of CD8 knockout mice and immuno-competent mice immunized with recombinant phages and grafted with TC-1 tumor cells. Each treatment group had 4 mice. The different groups represented in the graphs are CD8 knockout mice treated with M13 recombinant bacteriophages (M13CD8KO), immunocompetent mice treated with M13 recombinant bacteriophages (M13WT), CD8 knockout mice treated with PEP1 recombinant bacteriophages (PEP1CD8KO), immunocompetent mice treated with PEP1 recombinant bacteriophages (PEP1WT), (e) CD8 knockout mice treated with PEP1TS1 recombinant bacteriophages (PEP1TS1CD8KO) and immunocompetent mice treated with PEP1TS1 recombinant bacteriophages (PEP1TS1WT). The immuno-competent group treated with PEP1 recombinant phage had statistically significant reduction in tumor growth when compared with the other groups and with the CD8 knockout treated with PEP1.Statistical analysis was done by One-way ANOVA test followed by Bonferroni. ***P<0.001

Finally, we plotted a survival curve for the mice in the different groups over the course of the treatment to see if the observed tumor reduction translated into a significantly longer survival for the mice. The result is presented in Figure 12.

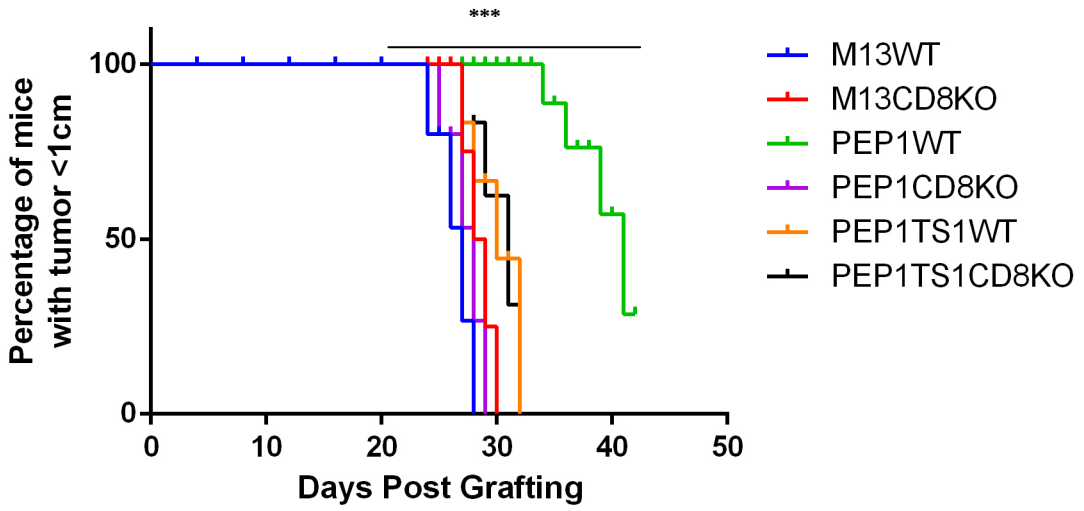

Figure 12: Percentage of mice with tumors less than 1cm in was plotted in a Kaplan Meier graph. Each mouse in the different groups was euthanized when the tumor reached 1cm in either diameter. (*Endpoint was predetermined as when tumor reaches 1cm in either diameter*)

Death was determined as the experimental end-point in this evaluation since the mice can not be allowed to bear the tumor indefinitely, by ethical rules. The last mouse in the group of immunocompetent mice treated with rcombinant PEP1 bacteriophage suvived for 41 days before the experiment was terminated. Statistical analysis was done by Mantel Cox log rank test. ***P<0.001

4.8 Therapeutic administration of the recombinant phage PEP1 can stimulate immune responses which reduce TC-1 tumor growth

Following the observation that prophylactic immunization of mice with our recombinant phages stimulates an immune response which reduces TC-1 tumor growth, we wished to find out if the same effect can be obtained when mice are immunized in a therapeutic manner, i.e. after the tumor has been established. We injected the TC-1 tumor cells as described above and after a 6-day wait, commenced immunization with recombinant phages M13 and PEP1. Treatment for two other groups was commenced after 9-days post tumor grafting. The experiment was terminated when tumor in any of the groups grew up to 10mm diameter.

Figure 13 shows the differences in levels of antibodies in mice that received the different bacteriophages post TC1 cells grafting (see M&M for immunization scheme). We can observe a positive response to immunization, as expected (figure 13).

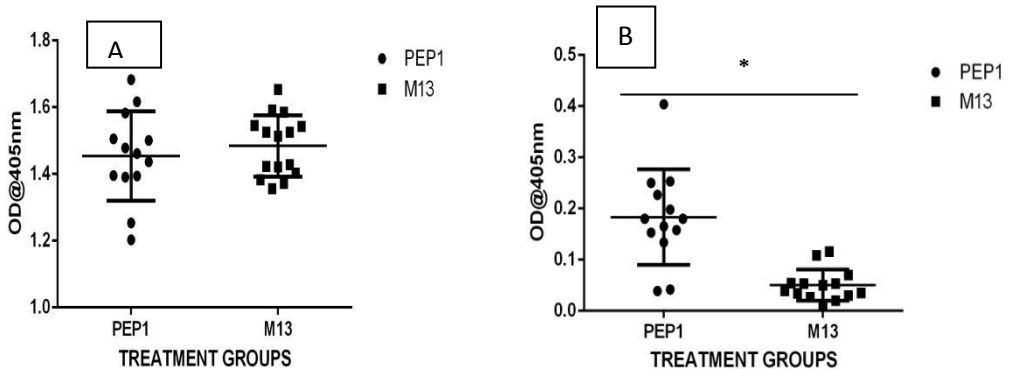

Figure 13: Humoral response against the M13 phage and the peptide insert in therapeutically immunized mice. The M13 phage was used as the antigen in A while the synthetic peptide was used as the antigen in B. The graph represents samples from three independent experiments. Data analysis was done by t-test.*P<0.05

There was no difference between the M13 and PEP1 group treated on the 9th day post grafting. However, there was a significant difference between the two groups treated 6 days post grafting (figure 14)

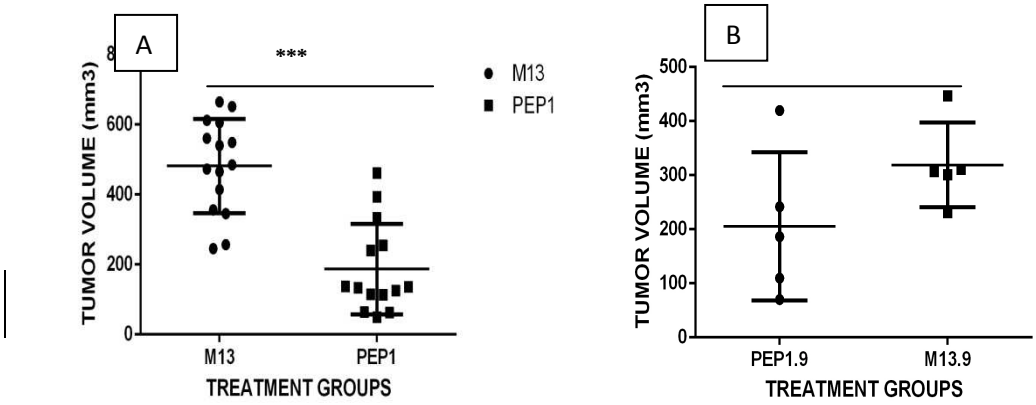

Figure 14: Final tumor volumes from therapeutically immunized mice. Mice treated with M13 insertless bacteriophage and recombinant PEP1 phage on day 6 (A) and day 9 (B) post grafting TC-1 cells. Graph A represents data from three independent experiments while B is from a single experiment. Data was analyzed by t-test. ***P<0.001

4.9 Therapeutic administration of the recombinant phages affects immune cellular responses and promotes a cytotoxic tumor environment.

The percentage of immune cellular infiltrates relative to total leucocytes in the recovered tumors from the therapeutic experiment was also measured and the results are shown in the graphs below (figure 15).

There was no statistically significant difference in the percentage of immune cell infiltrate between the two groups. Likewise, the CD4 and CD8 subsets showed no statistically significant difference between the groups. However, the ratio of CD8 to CD4 was slightly higher in the groups treated with the recombinant bacteriophage PEP1. This points towards a cytotoxic TH1 response and may be responsible for the observed reduction in tumor growth in this group. There is need however for further studies to have a clearer understanding of this tumor environment.

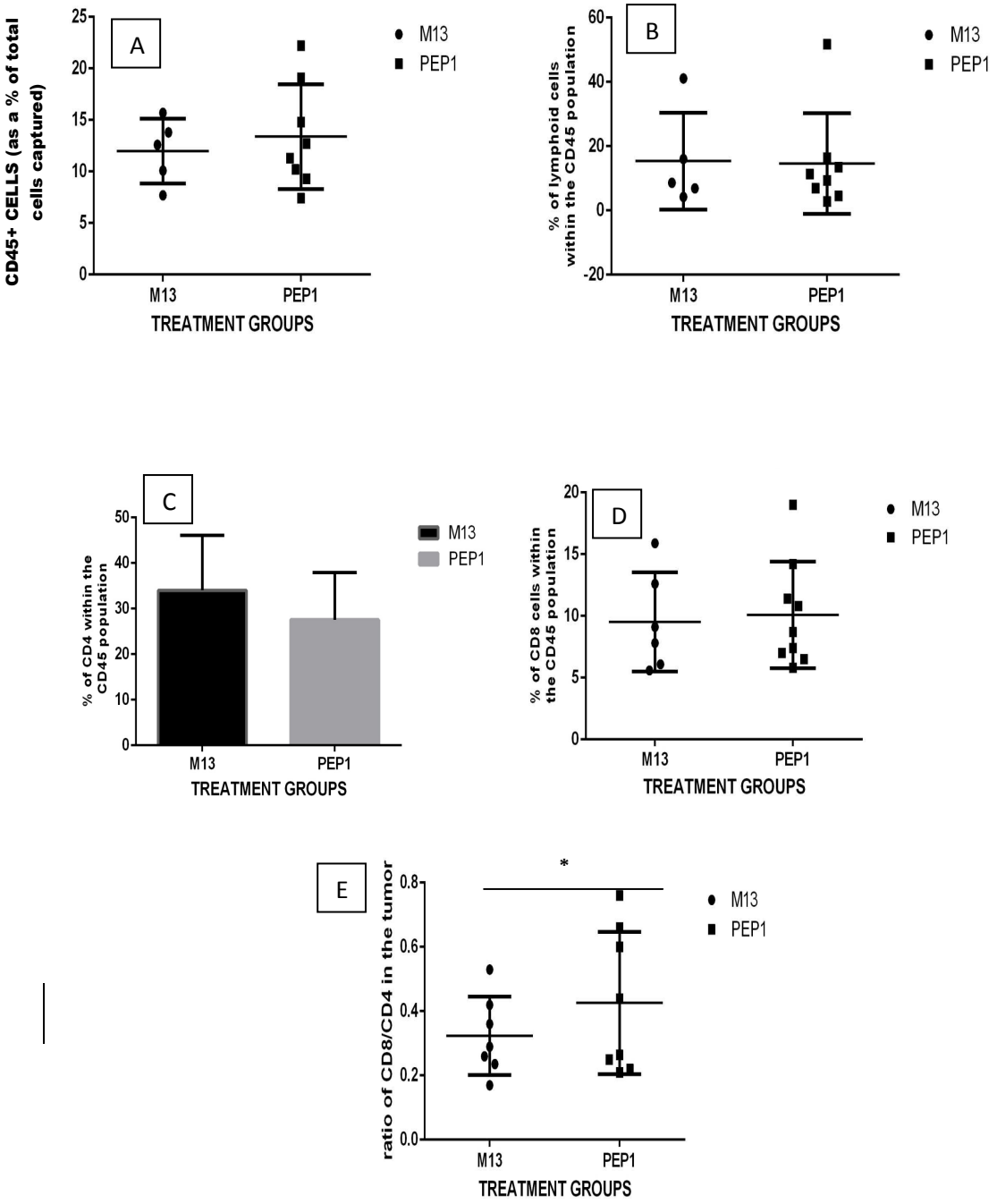

Fig 15: Tumor infiltrating leucocytes profile from mice grafted with TC-1 tumor and treated with recombinant bacteriophages on day 6 post-grafting. Total Leukocyte count (A) from the mice treated with PEP1 and M13 bacteriophages. Lymphoid infiltrates (B) are expressed as a percentage of total leukocytes while CD4 (C) and CD8 (D) are expressed as a percentage of lymphoid cells. The ratio of CD8 to CD4 in the two groups is shown in E. Data was analyzed by t-test. *P<0.05

4.10 The T lymphocytes primed in-vivo by treatment of mice with the recombinant bacteriophage PEP1 did not appear to be antigen specific and did not proliferate significantly in response to stimulation by the synthetic equivalent of the insert, in vitro

Lymphocytes can be stimulated specifically by a previously encountered antigen and non-specifically by certain agents. We cultured cells from the lymph-nodes and spleens of mice immunized with PEP1 and M13 bacteriophages and stimulated them with the synthetic peptide insert of PEP1 and phytohaemagglutinin. The proliferation of the different samples is shown in Figure 16.

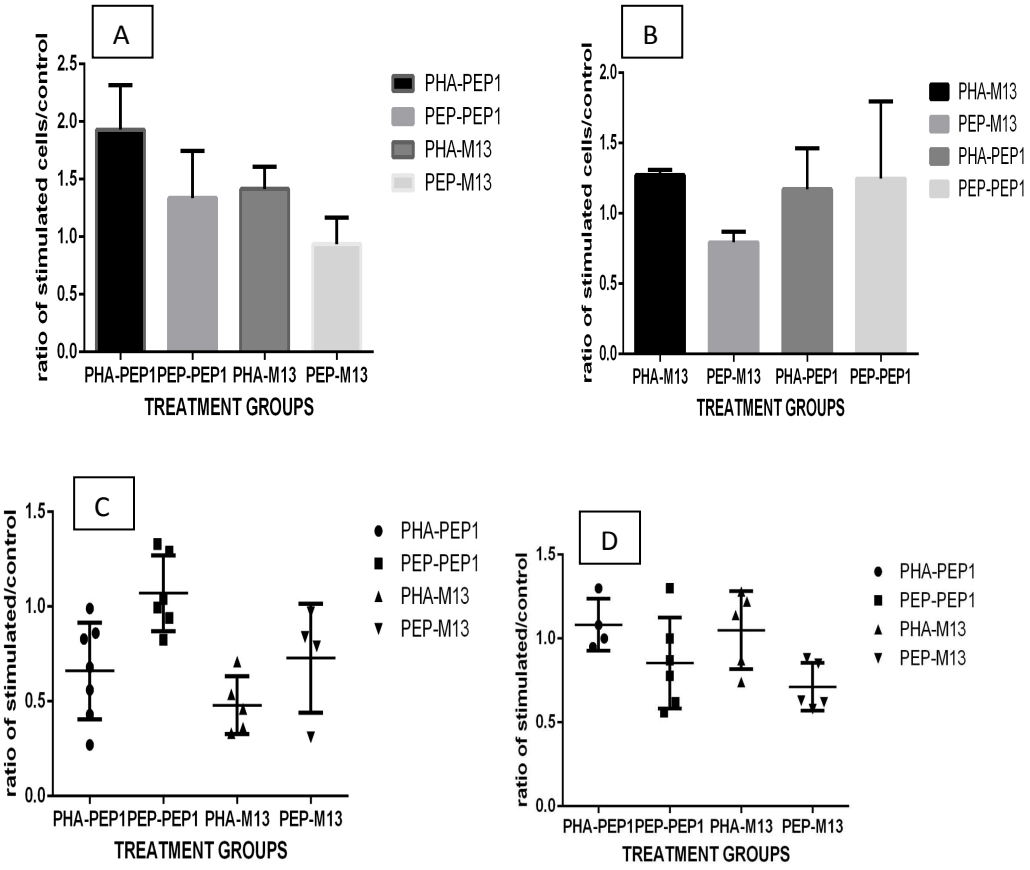

Figure 16. Lymphocyte proliferation profiles for lymphnodes and spleen from the immunized mice. (A) CD8 T cells from the lymphnodes of PEP1 immunized and M13 immunized mice, invitro stimulated with phytohaemagglutinin (PHA) or synthetic peptide insert. (B) CD4 T cells from the lymphnodes of PEP1 immunized and M13 immunized mice, invitro stimulated with PHA or synthetic peptide insert. (C) CD8 T cells from the spleen of PEP1 immunized and M13 immunized mice, invitro stimulated with PHA or synthetic peptide insert. (D) CD4 T cells from the spleen of PEP1 immunized and M13 immunized mice, invitro stimulated with PHA or synthetic peptide insert. There was no statistically significant difference between any of the groups.

The concept of the proliferation experiment is to show that the T cells can specifically recognize the antigen they were primed with in-vivo. However, in these experiments, we could not confirm the specificity of these lymphocytes as there was no significant difference between the groups.

4.11 **Immuno-histochemistry suggested higher infiltration of immune cells in tumors from mice immunized with PEP1-phage, in comparison with control mice**

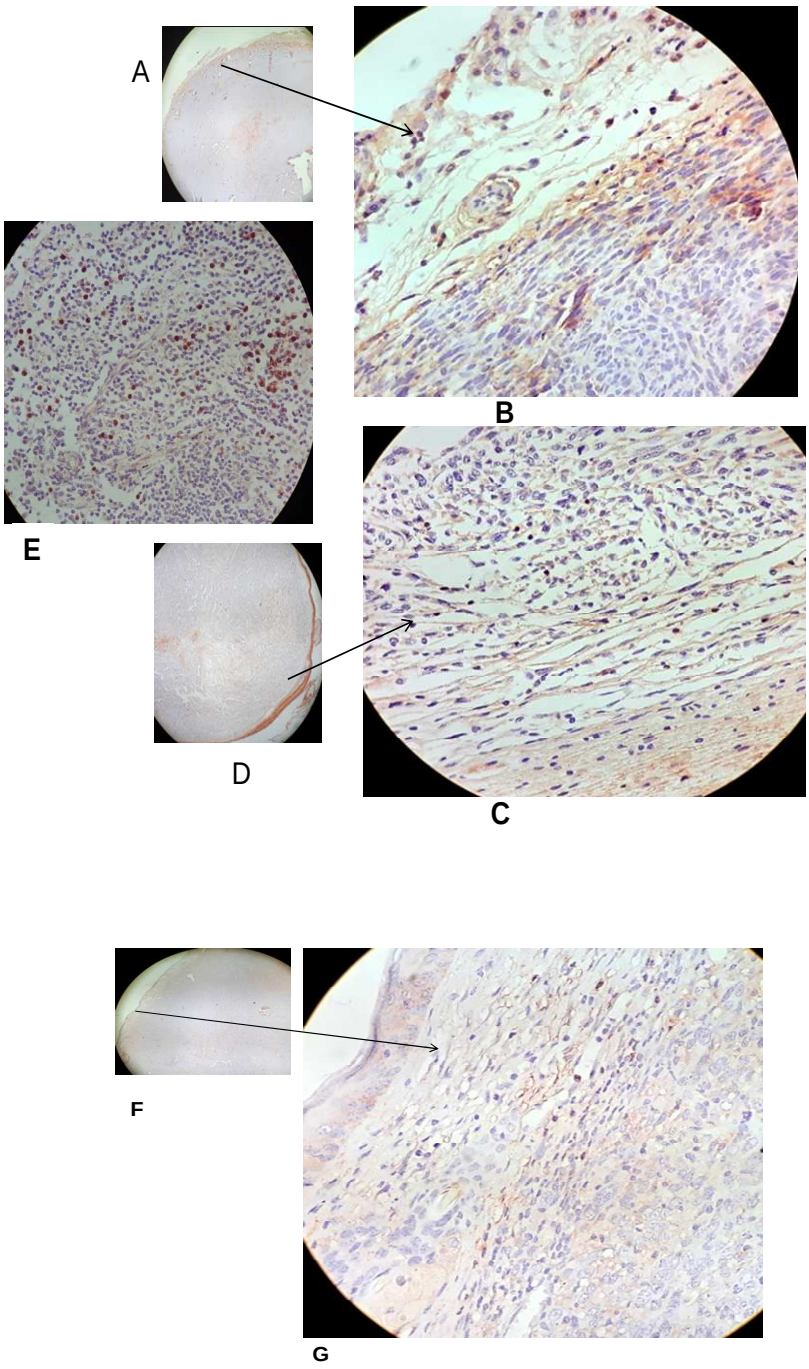

Figure 17: Immuno-histochemical staining of CD8 cells on tumor tissues. TC-1 tumor tissues from therapeutically PEP1 immunized mice (A and B; B==400X magnification), prophylactically PEP1 immunized mice (D and C; C = 400X magnification) and M13 immunized mice (F and G; G = 400X magnification). Positive Control tissue from spleen is labelled E.

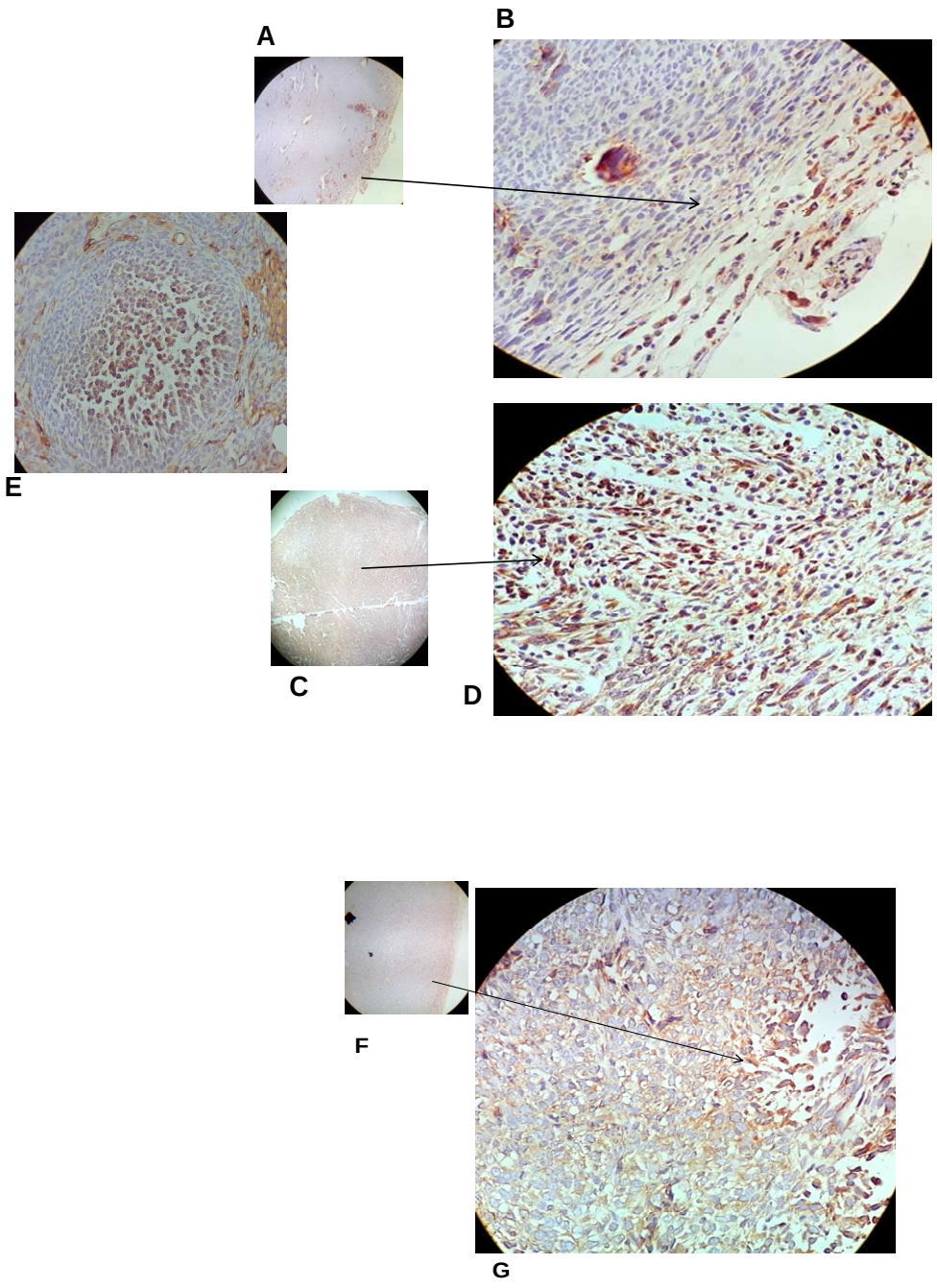

Figure 18: Immuno-histochemical staining of CD45 infiltrates in tumor tissues. TC-1 tumor tissues from therapeutically PEP1 immunized mice (A and B; B= 400X magnification), prophylactically PEP1 immunized mice (C and D; D = 400X magnification) and M13 immunized mice (F and G; G = 400X magnification). Positive Control tissue from Thymus is labelled E.

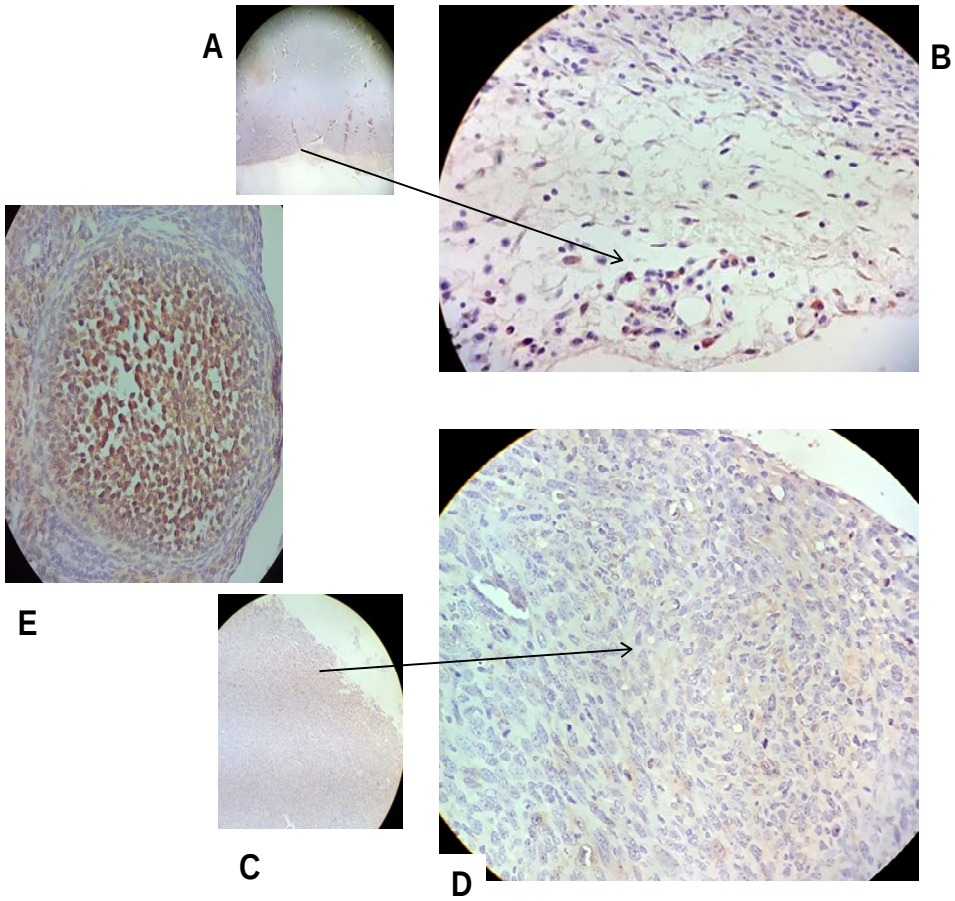

Figure 19: Immuno-histochemical staining of Natural Killer infiltrates in tumor tissues. TC-1 tumor tissues from therapeutically PEP1 immunized mice (A and B; B= 400X magnification) and M13 immunized mice (C and D; D = 400X magnification). Positive Control tissue from Thymus is labelled E.

5. DISCUSSION

The use of phage display technology for the isolation of peptide motifs has gained traction in the last decade and important contributions are being made in various fields of biomedicine from this line of research. This research work constitutes one of such additions as we were able to isolate peptide motifs that seem to have immunogenic capacity against an HPV-induced animal tumor model.

The immune system is very important in the neutralization of HPV infections; hence many infections do not progress to the formation of warts and cancers. Cells infected with some viruses are known to release type-1 interferons which inhibit viral replication in host cells and activate natural killer (NK) cells that can kill cells carrying viruses. Other elements of the innate immunity are also stimulated as a first line of defence and then the adaptive immunity takes over with the production of antibodies and the activation of cytotoxic cells (STANLEY, 2009). This natural flow of defence mechanism is what vaccination seeks to stimulate and the performance reports of the currently available vaccines are impressive. In the case of established cervical tumors however, there is no proven advantage of these conventional vaccines either towards tumor regression or arrest, hence the search for alternative options (HILDESHEIM et al, 2007; MARKOWITZ et al, 2007). Peptide vaccines have become generally popular by reasons of their ease of application and near complete lack of adverse effects. The discovery and selection of these important peptides are being pursued with different techniques and many of them offer varying advantages.

In these series of experiments, we used our recombinant phages which are inserted with the nucleotide sequences of our phage display selected peptide to immunize mice in both prophylactic and therapeutic fashion. Bacteriophages have been recognized as strong immunogens (OCHS et al, 1971) and this technique has also been used by other researchers (WU et al, 2002; GHAEMI et al, 2010). Cytolytic responses have also previously been observed against HIV-1 by displaying viral peptide epitopes within the phage capsid (DE BERARDINIS *et al*., 2000).

We observed consistent reduction in TC-1 tumor growth in mice prophylactically immunized with our PEP1 phage in comparison with an insertless phage. A bi-cistronic recombinant phage containing our PEP1 sequence and an additional sequence (TS1) did

not improve the tumor reduction observed with the PEP1 phage. Therapeutic administration of phage PEP1 in mouse bearing established TC-1 tumors also produced significant reduction in tumor growth. This is similar to the findings of GHAEMI et al. (2010) who used Lambda phage recombinants to immunize tumor-grafted mice. WU et al. (2002) have also demonstrated both prophylactic and therapeutic immunity generated by immunization with recombinant phages, against a mastocytoma tumor model. Although cytometry revealed that there was no statistically significant change in the immune cell infiltration into tumors, we observed a trend of higher percentages of cytotoxic T cells in the PEP1 treated groups. To assess the actual contribution of these CD8 cells to the antitumor immunity, we performed a CD-8 knockout experiment which resulted in a total loss of the observed tumor reduction in the CD8-knockout mice. This is a pointer to the fact that the observed tumor reduction was based on the higher infiltration of these cytotoxic cells into the tumors in the treated groups. We also tried to verify the epitope specificity of the T lymphocytes recovered from these experiments lymphocyte proliferation assay. The results of the T-cell proliferation assay showed only slight increase in the proliferation of cells recovered from the spleen and lymph nodes of mice that were immunized with our recombinant phages and stimulated invitro with the synthetic form of our isolated peptide. The specificity of the generated immune cells was therefore in doubt. This is contrary to the finding of NONN *et al.* (2003) which reported the induction of specific T cells to HPV16 E7 or HPV18 E7 by in vitro stimulation with recombinant protein-pulsed dendritic cells. In natural infections, antigen presenting cells capture HPV proteins, digest them into peptides and then translocate to the lymph nodes where HPV peptides are exposed on the cell surface to MHC class 2 antigens. Naive CD4 cells then differentiate into Th1 which induce cell mediated immunity or Th2 which induce antibody production (SASAGAWA et al, 2012). This understanding informed our inclusion of the TS-1 molecule in one of our constructs. The TS-1 molecule preferentially targets the lymphatic system (TREPEL et al; 2003) and we hoped the inclusion of this molecule could improve the immunogenicity of our recombinant phages. From the prophylactic experiments, it was observed that the inclusion of this molecule did not improve the immunogenicity of our phages; hence there was no significant tumor reduction in the mice that were immunized with the phage constructs that included the TS-1 molecule. This construct was thus excluded from subsequent experiments.

Cytotoxic lymphocytes are able to kill CIN or cancer cells expressing HPV antigens. higher numbers of these lymphocytes have been reported in patients with CIN compared with normal individuals (NIMAKO et al, 1997); are associated with favourable prognosis in ovarian cancer (SATO et al, 2005) and breast cancer (MAHMOUD et al, 2011) . The TC-1 tumor cells express HPV 16 E6 and E7. The presence of cytotoxic CD8 cells in the tumors from mice that were immunized with the recombinant phage PEP1 therefore seems to suggest the immunization was able to slightly increase the infiltration of cytotoxic lymphocytes and this was responsible for the observed tumor reduction. The abolition of this tumor reduction in the CD8 knockout mice seems to also confirm this hypothesis.

We were interested in how this immuno protection would fare in a therapeutic approach, where the tumor is already established before the commencement of treatment. We also thought an effect like this would be of greater significance because of the problems associated with conventional cancer treatment. Mice that were treated 9 days after tumor grafting had tumor growth comparable to the control group while there was a significant difference between the treated group and the control group in mice treated 6days after tumor grafting ($P< 0.001$). Cancer immunotherapies are often effective for a limited time due to escape mechanisms that include down-modulation of the tumor antigen, decrease in MHC class I expression, induction of 'clonal anergy' of tumor-specific T cells, and alterations in the antigen presentation pathway. (SINGH and PATTERSON, 2007; TING-KOH et al, 2006). While there is a lot of variation in the rate of T cell infiltration in different tumors, probably due to the interplay between tumor and the immune system, it has been speculated that some tumors progress faster and reach the "escape" phase early (HADRUP et al, 2013). The immune system may be able to kill tumor cells in the early stages of development but as the tumors grow, they may acquire genetic and phenotypic characteristics that enable them escape the generated antitumor response (VILLUNGER AND STRASSER, 1999). Breaking this tolerance induction and sufficiently raising the immune response to completely clear the tumor before this escape point is reached should therefore be a target of effective immunotherapy.

While we did not find a statistically significant difference in the tumor cellular infiltrates in the therapeutic experiments, the difference observed in the CD8/CD4 ratio

is significant ($P<0.05$) and a good pointer to a cytotoxic tumor environment. Cytotoxic CD8 cells are the major effector cells in cancers and an immunotherapeutic intervention will seek to increase the activation and infiltration of these cells into the tumor. However, the generation and maintenance of an effective and durable immune response is dependent on both the CD8 and CD4 populations (DING AND ZHOU, 2012)

Our immunization protocols, both prophylactic and therapeutic, provoked antibody production to the phage antigen as well as to the inserted HPV mimotope sequence. The phage platform is an effective carrier for small epitopes as thus demonstrated. The specific antibody to the inserted sequence may have a contributory role in the tumor reduction. This will need to be further investigated.

One of the militating factors to cancer vaccines is the use of limited epitopes which may offer the tumor an escape route by devoting the immune system to this epitope while the tumor escapes through other pathways (SUHRBIER, 1997, ELLIOTT et al., 1999). In these experiments, we used a recombinant phage carrying one of the sequences we isolated by phage display. This may be responsible for the lack of protection in the non-responders. A future approach may be to combine several of our isolated sequences to provide multiple epitopes for stronger immune stimulation.

Other problems that limit the efficacy of some cancer vaccines include advanced disease stage and aggressive tumors. The TC-1 tumor cells are quite aggressive. In our therapeutic protocols, we observed that the tumors are more difficult to control by immunization once they reach the immune escape point, as was evident in the tumor control in mice treated 6 days post graft than in those treated 9-days post graft, in this TC-1 model. This highlights again, the importance of early detection to the success of immunotherapy. One suggestion is to use immunotherapy as post-operative, treatment (SCHIRRMACHER et al, 2014) or in combination therapy with chemotherapy or radiotherapy (WARGO et al, 2015). This seems to be the current trend as the approaches are complementary and generally offer greater safety. A trial of this type may also be an option for the use of our selected motifs in future experiments.

The results obtained from our immuno-histochemical investigation showed greater infiltration of inflammatory cells into tumor tissues in PEP1 immunized mice compared to mice immunized with the insertless phage. This largely corroborates the

flowcytometry results. Even though it’s not a quantitative technique, the result offer5s an in-situ view of the tumor infiltration patterns of the immune cells in the different subjects.

CONCLUSION

Phage display remains a good tool for the isolation of important ligands which may be biologically important in disease processes. We isolated a peptide motif from the sera of HPV infected women and this motif was proven to be effective in prophylactic immunization of immunecompetent mice with HPV-induced tumors (TC-1 model) The immunity thus generated was able to control TC-1 tumor growth in immunized mice.

The generation of cytotoxic lymphocytes is very important for the control of TC-1 tumors and immunization of mice with our recombinant phage PEP1 appears to be capable of generating these cytotoxic lymphocytes. The absence of these cytotoxic lymphocytes nullified the tumor growth reduction that was visible in immunocompetent mice.

However, therapeutic administration of the recombinant phage PEP1 did not sufficiently stimulate the immune system to control TC-1 tumor growth. Length of time between the grafting of tumors and the commencement of immunization also appears to be important as the tumors in mice treated 6 days post tumor grafting seemed to develop slightly more slowly than in the mice treated later.

Further studies on the mechanisms of action of this peptide will be needed, to elucidate the cytokine profiles, explore better on the specificity of immune cells generated and propose other approaches to improve the immunotherapy benefits that this peptide might have in the control of HPV-associated tumors.

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FICHA DO ALUNO

5155 - 8743424/1 - Lanre Precieux Kabir Sulaiman

Email:	lanreprecieux@usp.br
Data de Nascimento:	28/05/1976
Cédula de Identidade:	RNE - V920410-S - SP
Local de Nascimento:	Nigéria
Nacionalidade:	Nigeriana
Graduação:	Doctor of Veterinary Medicine - University of Ibadan - Nigéria - 2002
Mestrado:	Master of Veterinary Science in Avian Medicine (1) - University of Ibadan - Nigéria - 2009

Curso:	Doutorado
Programa:	Oncologia
Data de Matrícula:	25/06/2013
Início da Contagem de Prazo:	25/06/2013
Data Limite para o Depósito:	23/10/2017
Orientador:	Prof(a). Dr(a). Luisa Lina Villa - 25/06/2013 até 16/11/2017. Email: l.villa@hc.fm.usp.br
Proficiência em Línguas:	Inglês, Aprovado em 26/06/2013 Português, Aprovado em 28/01/2016
Prorrogação(ões):	120 dias Período de 25/06/2017 até 23/10/2017
Data de Aprovação no Exame de Qualificação:	Aprovado em 27/01/2016
Data do Depósito do Trabalho:	23/10/2017
Título do Trabalho:	"Seleção de motivos semelhantes à Papilomavírus, a partir de bibliotecas de "*phage display*", que apresentem potencial aplicação translacional"
Data Máxima para Aprovação da Banca:	22/12/2017
Data de Aprovação da Banca:	24/10/2017
Data Máxima para Defesa:	22/01/2018
Data da Defesa:	16/11/2017
Resultado da Defesa:	Aprovado
Histórico de Ocorrências:	Primeira Matrícula em 25/06/2013 Prorrogação em 11/04/2017

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 5473 em vigor de 18/09/2008 até 19/04/2013).

Última ocorrência: Matrícula de Acompanhamento em 17/07/2017

Impresso em: 06/12/2017 17:14:15

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Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
MCM5912-1/1	Fatores Associados ao Desenvolvimento do Câncer em Pacientes Jovens	07/04/2014	27/04/2014	60	4	83	A	N	Concluída
MCM5907-1/2	Terapia Gênica do Câncer: Tecnologia e Aplicação	08/09/2014	02/11/2014	120	8	100	A	N	Concluída
MCM5851-4/2	Adesão Celular e Câncer	08/10/2014	09/12/2014	90	6	100	A	N	Concluída
MPT5760-4/5	Metodologia de Ensino I: Preparação Pedagógica	14/08/2015	15/10/2015	90	6	87	A	N	Concluída
MCM5918-1/1	Mecanismos de Ação de Agentes Imunomoduladores. "Mechanisms of Action of Immunomodulatory Agents"	21/09/2015	27/09/2015	30	2	100	A	N	Concluída
MIP5722-5/5	Pesquisa Bibliográfica Automatizada em Bases de Dados de Medicina Clínica e Especializada em Doenças Infecciosas e Parasitárias	04/11/2015	24/11/2015	45	3	100	A	N	Concluída
MCM5919-1/1	Imunoterapia para Doenças Neoplásicas. "Cancer Immunotherapy"	16/11/2015	22/11/2015	30	2	100	A	N	Concluída
MCM5921-1/1	Imunoterapia para Doenças Inflamatórias Crônicas. "Immunotherapy of Chronic Inflammation"	07/03/2016	13/03/2016	30	0	-	-	N	Matrícula cancelada
MCM5920-1/1	Desenvolvimento de Vacinas em Doenças Infecciosas. "Vaccine Development in Infectious Diseases" (2)	09/05/2016	15/05/2016	30	2	100	A	N	Concluída

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	6	8	33
Estágios:			
Total:	6	8	33

Créditos Atribuídos à Tese: 176

Observações:

1) Título de mestre obtido no(a) University of Ibadan. Reconhecimento da equivalência do título aprovada pela Câmara Curricular do Conselho de Pós-Graduação, em Sessão de 08/10/2014..

2) Disciplina(s) cursada(s) voluntariamente pelo(a) candidato(a) após ter cumprido as exigências regulamentares.

Conceito a partir de 02/01/1997:

A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.

Um(1) crédito equivale a 15 horas de atividade programada.

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Comissão julgadora da tese de doutorado:

NUSP	Nome	Vínculo	Função
797570	Luisa Lina Villa	FM - USP	Presidente
52433	Maria Lucia Zaidan Dagli	FMVZ - USP	
919280	Luciana Nogueira de Sousa Andrade	USP - Externo	
1811650	Ricardo José Giordano	IQ - USP	
1953645	Roger Chammas	FM - USP	

Última ocorrência: Matrícula de Acompanhamento em 17/07/2017
Impresso em: 06/12/2017 17:14:15

MEDICINA
USP

Julgamento da Tese de Doutorado

ALUNO(A): **Lanre Precieux Kabir Sulaiman**

Programa de Pós-Graduação em **ONCOLOGIA**

Relatório da Comissão Julgadora:

Aos **16/11/2017**, atendendo ao disposto no Regimento da Pós-Graduação da Universidade de São Paulo, o(a) candidato(a) **Lanre Precieux Kabir Sulaiman** ao título de **Doutor em Ciências**, apresentou sua **Tese de Doutorado** com o título: *"Seleção de motivos semelhantes à Papilomavírus, a partir de bibliotecas de "phage display", que apresentem potencial aplicação translacional"*

Esta Comissão, indicada pela Comissão de Pós-Graduação, é de parecer que:

O candidato apresentou seus resultados de forma clara, com ênfase na apresentação oral. Respondeu adequadamente às questões formuladas pela banca examinadora, demonstrando maturidade científica compatível com seu momento de formação.

Sugestões da banca examinadora devem ser incorporadas à versão final da tese.

De maneira geral, o aluno atingiu os requisitos necessários para obtenção do título de Doutor em Ciências pelo Programa de Oncologia da Universidade de São Paulo.

De acordo com o disposto no Regimento da Pós-Graduação da Universidade de São Paulo, os Examinadores consideraram o(a) candidato(a): **Lanre Precieux Kabir Sulaiman**

São Paulo, **16/11/2017**

COMISSÃO EXAMINADORA:

1 . (X) APR ( ) REP _____
Luisa Lina Villa - Presidente

2 . (X) APR ( ) REP _____
Luciana Nogueira de Sousa Andrade – Titular

3 . (X) APR ( ) REP _____
Maria Lucia Zaidan Dagli – Titular

4 . (X) APR ( ) REP _____
Ricardo José Giordano – Titular

5 . (X) APR ( ) REP _____
Roger Chammas - Titular

6 . ( ) APR ( ) REP _____
Bryan Eric Strauss – Suplente

7 . ( ) APR ( ) REP _____
Luis Carlos de Souza Ferreira – Suplente

8 . ( ) APR ( ) REP _____
Maria Aparecida Azevedo Koike Folgueira – Suplente

9 . ( ) APR ( ) REP _____
Maria Del Pilar Estevez Diz – Suplente

10 . ( ) APR ( ) REP _____
Maricy Tacla - Suplente