VACCINE ADJUVANT APPLICATIONS OF CPG ODN NANORINGS AND DEVELOPMENT OF LEISHMANIA EXTRACELLULAR VESICLE BASED CUTANEOUS LEISHMANIASIS VACCINE

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

VACCINE ADJUVANT APPLICATIONS OF CPG ODN NANORINGS AND DEVELOPMENT OF LEISHMANIA EXTRACELLULAR VESICLE BASED CUTANEOUS LEISHMANIASIS VACCINE

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In our previous studies we converted a conventional K-type CpG ODN with no interferon-alpha stimulating activity into a potent type I interferon inducer by complexing it with the cationic peptide Tat (47-57). These complexes formed well-defined nanorings with potent vaccine adjuvant activity. Herein, we examined the immunostimulatory and vaccine adjuvant activity of K/Tat nanorings in two different mouse tumor models. Results showed that K/Tat nanorings suppressed tumor progression by inducing antigen specific CTL-mediated IFNγ production and CTL-specific EG.7 thymoma killing. However, the nanorings had no beneficial effect in the B16 F10 melanoma model when used as stand-alone immune stimulatory agents. Since the nanorings proved to be of interest when used together with an antigen, in further experiments we explored the optimal vaccination route of nanorings and the model antigen ovalbumin. Results indicated that K/Tat nanorings triggered the highest

antigen specific IgG2c titers and OVA-specific IFNγ production when administered via the intranasal route. The success of K/Tat nanorings in intra nasal administration suggested us the potential use of nanorings as mucosal adjuvants for future trails.

The second half of this thesis analyzed the vaccine potential of extracellular vesicles isolated from *Leishmania*. Leishmaniasis is an infectious disease caused by *Leishmania* protozoa transmitted to mammalian hosts by infected sand flies. The absence of an available licensed vaccine and the cost, toxicity and drug resistance associated with the pentavalent antimonials used for treatment, prompted us to explore a novel strategy of vaccine development based on use of *Leishmania* extracellular vesicles (EVs). For this, antigen-rich EVs secreted from *Leishmania* parasites were purified, characterized and their immune protective vaccine potentials were tested in mice as such or in combination with Th1 type vaccine adjuvants (K-ODN, D-ODN, Nanorings, cGAMP and cGAMP/K-ODN). Herein we show that *Leishmania* extracellular vesicles (EVs) decreased the lesion size when adjuvanted with K/cGAMP and stimulated a Th1 type immune response in *L.tropica* induced cutaneous leishmaniasis model.

Keywords: TLR9, CpG oligodeoxynucleotides, CpG/Tat nanorings, *L.tropica*, cutaneous leishmaniasis, extracellular vesicles, K-cGAMP, vaccine adjuvant.

ÖZ

CPG NANOHALKALARIN AŞI ADJUVANTI UYGULAMALARI VE KÜTENÖZ LEİSHMANİASİSE KARŞI HÜCREDIŞI KESECİKLERE DAYALI AŞI GELİŞTİRİLMESİ

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Önceki çalışmalarımızda, tip I interferon tetiklemeyen K tipi CpG ODN leri, katyonik Tat (47-57) peptidlerle kompleksleştirerek nanometre boyutlarında güçlü tip I IFN indükleyicisi nanohalkalara dönüştürmeyi başarmıştık. Bu çalışmamızda, K/Tat nanohalkaların immün uyarıcı etkileri ve aşı adjuvantı aktivitelerini iki farklı fare tumor modelinde denedik. Elde edilen sonuçlar, K/Tat nanohalkaların antijene özgü sitotoksik T hücrelerinden IFNγ üretimine sebep olduğunu, EG.7 tümör hücrelerinin yok edilmesini sağladığını ve tümör gelişimini engellediğini göstermektedir. Ancak nanohalkaların B16 F10 melanoma modelinde tek başına immün uyarıcı ajanlar olarak kullanıldığında yararlı bir etki göstermediği anlaşılmıştır. Nanohalkalar antijen varlığında etkili olduklarından OVA antijeni ile birlikte kullanıldıklarında en etkin aşı uygulama yolu da araştırılmıştır. Sonuçlar, nanohalkaların intranazal yolla uygulandığında en yüksek antijene özgü IgG2c ve IFNγ üretimine sebep olduğunu

göstermiştir. K/Tat nanohalkaların intranazal uygulamadaki başarısı, bu ajanların mukozal adjuvant olarak kullanılabileceğini ortaya koymaktadır.

Tezin ikinci bölümünde *Leishmania* parazitlerinden izole edilen hücre dışı keseciklerin aşıda kullanılmasına yönelik çalışmalar yapılmıştır. Şark çıbanı olarak da bilinen kütanöz leishmaniasis, *Leishmania* parazitinin sebep olduğu ve memeli konağa enfekte olmuş dişi kum sineklerince taşınan enfeksiyonel bir hastalıktır. Leishmaniasis için şu ana kadar geliştirilmiş koruyucu bir aşı bulunmamaktadır ve tedavi toksik beş değerlikli antimoniallarin kullanımını gerektirmektedir. Bu çalışmamızda, aşı geliştirmede yeni bir strateji olan ve bol miktarda *Leishmania* antijeni içeren *Leishmania* hücre dışı kesecikleri kullanılmıştır. Bunun için *Leishmania* parazitlerinden salgılanan hücre dışı kesecikler toplanmış, karakterize edilmiş ve koruyucu aşı potansiyelleri tek başına ya da güçlü Th1 uyarıcı aşı adjuvanları (K-ODN, D-ODN, Nanohalkalar, cGAMP and cGAMP/K-ODN) ile karıştırılarak denenmiştir. Kütenöz leishmaniasis fare modelinde yapılan çalışmalar sonucunda Leishmania hücre dışı kesecikleri ve K/cGAMP adjuvantı ile aşılanan farelerde *L.tropica* enfeksiyonu sonucu lezyon büyümesinin baskılandığı ve güçlü Th1 tipi immün yantın uyarıldığı belirlenmiştir.

Anahtar Kelimeler: TLR9, CpG oligodeoksinükleotid, CpG/Tat nanohalkalar, *L. torpika*, kütenöz leishmaniasis, hücredışı kesecikler, K-cGAMP, aşı adjuvanı

To my mother

Annem'e

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AFM	Atomic Force Microscopy
AO	Acridine Orange
APC	Antigen presenting cell
BAL	Broncho alveolar lavage
BMDM	Bone marrow derived macrophages
BSA	Bovine Serum Albumin
CBA	Cytometric Bead Array
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
cGAMP	cyclic GMP-AMP
CL	cutaneous leishmaniasis
CpG	unmethylated cytosine-phosphate-guanosine motifs
CXCL	CXC chemokine ligand
DC	Dendritic Cells
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic reticulum
EV	Extracellular vesicles
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal bovine serum
GP63	Glycoprotein 63kDa
HK	Heat killed
HRP	Horse-Radish Peroxidase
id	Intradermal
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

im	Intramuscular
in	Intranasal
IRF	Interferon regulatory factor
MCSF	Macrophage colony stimulating factor
MFI	Mean florescent intensity
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation factor-88
NF-κB	Nuclear factor-kappa B
NK	Natural killer
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate Buffered Saline
PNPP	p-nitrophenyl phosphate
RNA	Ribonucleic acid
SA-AKP	Streptavidin-alkaline phospahatase
sc	subcutaneous
SLA	soluble Leishmania antigen
SV	split vaccine
Th1	T helper type 1
Th2	T helper type 2
TIR	Toll–interleukin 1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF-associated factor
VL	Visceral leishmaniasis

CHAPTER 1

INTRODUCTION

1.1. Therapeutic Potential of CpG ODNs

1.1.1. CpG ODNs

Bacterial DNA contains high frequency of unmethylated cytosine-phosphateguanine dinucleotide motifs (CpG) which are recognized by host Toll-like 9 (TLR9). CpG motifs are rare in mammalian DNA and are mostly methylated (Krieg, 1995). Synthetic oligodeoxynucleotides (ODN) with unmethylated CpG motifs are a family of immunotherapeutics which mimic the immune stimulatory effect of bacterial DNA. CpG ODNs activate plasmacytoid dendritic cells (pDCs) and B cells through TLR9 signaling in humans and induce production of Th1 type cytokines and chemokines that mediate natural killer cell (NK) and T cell activation (Hanagata, 2012).

Multiple factors affect the immunostimulatory activity of CpG ODNs. For example, CpG ODN shorter than 8 bp fail to activate immune cells. Unmodified CpG ODNs with phosphodiester backbone (PO) are digested by serum nucleases and lose their activity rapidly. To enhance nuclease resistance, ODNs with phosphorothioate backbone (PS) are employed. Conversion of CpG to GpC and methylation of cysteines cause loss of activity (Wagner, 2008). Flanking sequences in CpG ODNs are also important. For instance in mice, the optimum stimulatory sequence is PuPuCpGPyPy, whereas in humans this is a PuPyCpGPuPy (Pu: purine; Py: pyrimidine) (Krieg A, 1995; Verthelyi D, 2001).

Based on their sequence, backbone modification and immune stimulatory activity, four different classes of synthetic CpG ODNs have been characterized (Table 1.1; Hanagata, 2012). Class C and Class P were generated in an attempt to mimic the immune stimulatory activity of D-type CpG ODN. Therefore, the next Sections will focus Class A (type D) and Class B (type K) type CpG ODNs (Hanagata, 2012).

Table 1.1. Properties of each class of CpG ODNs (Adapted from Hanagata, 2012).

	Class A (type D)	Class B (type K)	Class C	Class P
ODN structure	Central phosphodiester region containing one or more CpG motifs in a palindrome and 5′ and/or 3′ ends consisting of poly(G) motifs with phosphorothioate backbone	Completely phosphorothioate backbone	One or more 5′ CpG motif(s) and a 3′ palindrome	Two palindromes consisting of phosphorothioate backbone
Examples	ODN2216 (for human) ODN2336 (for human) ODN1585 (for mouse)	ODN2006 (also know as PF-3512676 and CpG7909, for human) ODN1668 (for mouse) ODN1826 (for mouse)	ODN2395 (for human and mouse) ODN M362 (for human and mouse)	ODN21798
Mainly stimulated cell types	pDCs	B cells	pDCs and B cells	pDCs
Actions	Innate immune responses: IFN-α, TNFα, and IL-12 secretion Adaptive immune responses: IL-12 and IP10 secretion	Innate immune responses: IL-6, IL-10, and IL-12 secretion Adaptive immune responses: antibody production; IL-6 and IL-12 secretion	Intermediate between the A and B classes	Potency for IFN-α secretion is higher than that of CpG ODN in class C

Abbreviations: IFNα, interferon-alpha; IL, interleukin; IP10, interferon-gamma-inducible protein of 10 kDa; pDC, plasmacytoid dendritic cell; TNFα, tumor necrosis factor-alpha.

1.1.2. K Type ODNs and D Type ODNs

K type ODNs (also known as Class B) are single stranded linear sequences with multiple CpG motifs and a PS backbone (Vollmer, 2004 ; Hartmann, 2003) (Figure 1.A). In contrast, D type ODNs (also known as Class A) have mixed backbones (PO/PS) and possess guanosine (G) runs on either side of a single CpG motif embedded within a palindromic region (Endres, 2001; Vollmer, 2009)(Figure 1.B).

Both K ODNs and D ODNs are recognized by TLR9 expressing B cells and pDCs in human. K ODNs trigger activation of B cells and production of IL-6 and IgM. They also induce pDC maturation and proinflammatory cytokine production (Marshall, 2003). D ODNs however, are very effective in stimulating IFNα production from pDCs (Gursel, 2002; 2006). D ODNs also stimulate monocytes to mature into CD83+/CD86+ dendritic cells in an IFN-α-dependent manner (Gursel , 2002). This ODN type further induces the expression of co-stimulatory molecules such as CD80, CD86 and HLA-DR on pDCs and B cells albeit, at a much lower extent than K type ODN (Krieg, 2001) K ODNs are more potent in mice in terms of IFNɤ and IP-10 induction while they trigger production of these mediators in low amounts from human PBMCs. In mice, K ODN also stimulate high amounts of IL-6 and IL-12 production and Th1 dominated response generation (Blackwell, 2003). Unlike K ODNs, D ODNs contribute to cytolytic activity of NK cells through induction of IFN-ɤ and IP-10 from PBMC (Vollmer, 2009).

In mouse splenocytes, D-type ODN stimulates moderate levels of IL-6, IL-12 and high levels of IFN-ɤ (Vollmer, 2004).

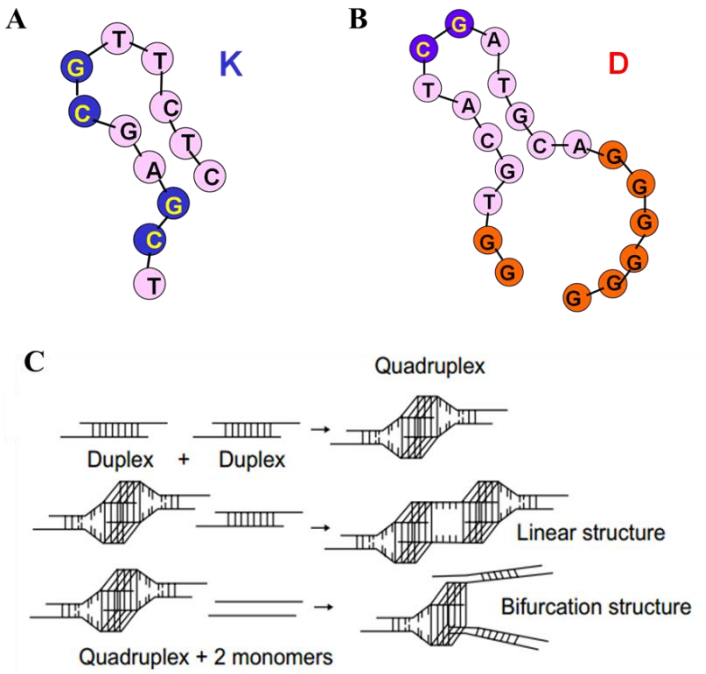

Figure 1.1. Examples of CpG ODN sequences for K-ODN and D-ODN (A and B). G-quadruplex formation and model of higher-order structures formed by D class ODNs (adopted from Hanagata, 2012).

Contrary to K-ODNs, D-ODNs can form higher order structures that can be globular (~50nm size), linear (~100nm size) or two forked (Klein, 2010). As shown in Figure 1.C, palindromic sequences in the center of D-ODN forms duplexes and the four Gs within the poly(G) sequences at the end of these duplexes bond with one another via Hoogsteen base pairing to form a G-tetrad structure. D-ODN is more stable and its cellular uptake is more efficient than K-ODN due to this structural property. Furthermore, Type I IFN inducing ability of D-ODN also depends on this higher order structural feature (Gursel, 2002 and 2006).

1.1.3. Signaling Through TLR9

Similar to other toll like receptors,TLR9, is a single transmembrane protein with an ectodomain containing leucine-rich repeats, a transmembrane domain, and a cytosolic Toll/IL-1 receptor (TIR) domain responsible for transducing signals to downstream adaptors TRIF and MyD88 (Kawai and Akira, 2010). TLR9 is located within the endosomes and recognize CpG motifs in pathogenic DNA. Synthetic K and D ODNs also trigger TL9 signalling in a differential way (Figure 1.2; Gillet et. al., 2008). As D-ODN is internalized by the pDC, it is sequestered into early endosomes for a long period via binding to scavenger receptor CXCL16 (Gursel, 2006). This leads to colocalization of MyD88 and interferon regulatory factor (IRF)-7 with TLR9 and production of IFNα following downstream signaling (Honda et. al., 2005). K-ODN on the other hand, localize to lysosomal vesicles immediately and is recognized by TLR9 in late endosomes, where colocalization with MyD88 and IRF5 induce proinflammatory mediators such as IL-12, IL-6 and TNFα secretion following downstream signaling (Asselin, 2005). The differential TLR9-dependent signaling property of K and D ODNs is due to the higher order structure of D ODN. The complex form of D ODN causes compartmental retention, changing the intracellular distribution of the ODN. Thus, engagement of D ODN and TLR9 happens in a different compartment that recruits different adaptor proteins so that a distinct downstream signaling occurs (Kumagai et. al., 2008).

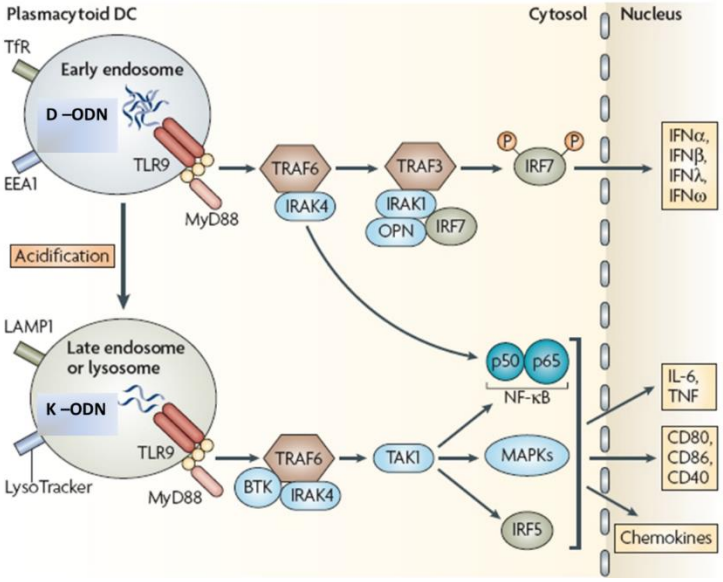

Figure 1.2. Mechanism of differential immune activation induced by D-ODN and K-ODN (Adapted from Gillet et. al., 2008).

1.1.4. K/Tat Nanorings as vaccine adjuvants

K-ODNs have been used in clinical trials as vaccine adjuvants and immunotherapeutic agents whereas D-ODNs could not be progressed into the clinic. The inconsistent G quadruplex structures formed when using D ODNs, preclude them from the clinic, despite their favorable type I IFN inducing activity. Therefore, in our previous study, we developed a simple strategy to convert a conventional K-type ODN into a potent Type I interferon inducer via complexation with a cationic peptide Tat(47–57) (Gungor and Yagci et. al., 2014a). This complexation resulted in formation of stable ring like nanostructures-(nanorings). Nanorings protected K ODN against nucleases, increased their retantion in the early endosomes and induced robust IFNα production from human pDCs (Figure 1.3; Gungor and Yagci et.al, 2014b). We also showed that nanorings boosted T helper 1–mediated immune responses in mice when used together with OVA model antigen in a pDC dependent manner. The detailed mechanism of nanoring action is available in one research and one review article presented in the attachment.

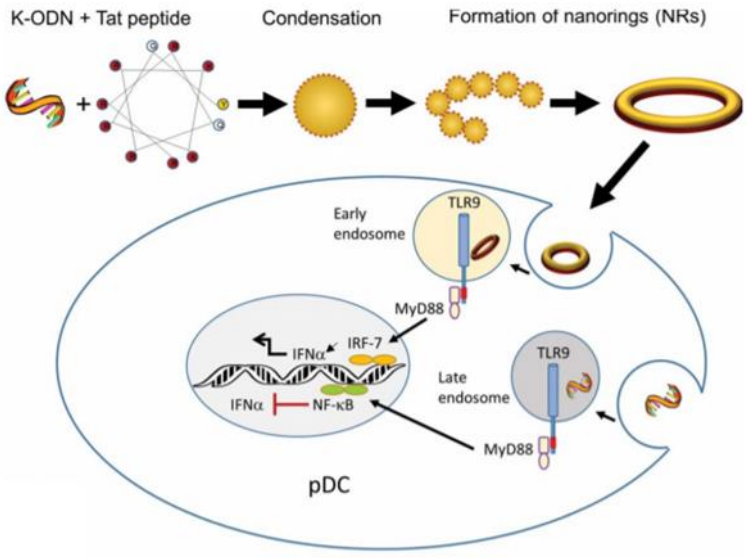

Figure 1.3. Formation of K/Tat nanorings and mechanism of action (adapted from Gungor and Yagci et. al., 2014b).

1.1.5. Combination of K/cGAMP as vaccine adjuvant

1.1.5.1. STING dependent signaling

Stimulator of Interferon Genes (STING) is an adaptor protein required for the signaling cascade initiated by recognition of cytosolic dsDNA. Downstream signaling proceeds through TBK1-IRF3 axis and culminates in Type I IFN production (Sun et al., 2009; Zhong et al., 2008). Internalized pathogen DNA or cytosol-leaked host DNA is recognized by the enzyme cyclic-GMP-AMP synthetase (cGAS) which catalyzes the formation of the second messenger 2'3'-cyclic-GMP-AMP (cGAMP) (from ATP and GTP (Li et. al, 2012; Sun et. al., 2013). Then, cGAMP interacts with STING and initiates Type I IFN (IFN-β) production and NF-κB-mediated pro-inflammatory cytokine production (Cai et. al., 2014). The cGAS/STING dependent signaling pathway is summarized in Figure 1.4 (Barber, 2015).

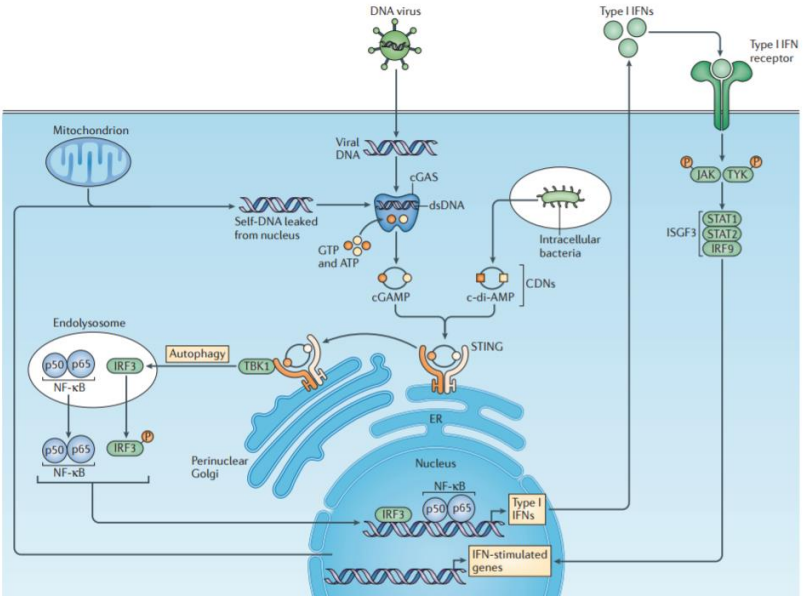

Figure 1.4. STING dependent innate immune signaling (adapted Barber, 2015)

1.1.5.2. Synergistic activity of combined TLR9 and STING agonist administration

Following the discovery of the STING-mediated signaling pathway, synthetic agonists of STING (cyclic dinucleotides and cGAMP) were manufactured and their immune stimulatory properties (Sun et. al, 2013; Karaolis et. al., 2007) and in vivo immunotherapeutic potentials (Ogunniyi et. al, 2008; Hu et. al, 2009) were examined. Results indicated an unexpected Th2 type immune response induction through STING-IRF3 mediated Type I IFN production (Tag et. al., 2013). Meanwhile, Yıldız et. al. in our research group explored the possibility of synergism between c-di-GMP/cGAMP and CpG ODN mediated immune activation. Results showed that combined use of cyclic-di-GMP or cGAMP with CpG ODN augmented cytokine production IFNα/β, IL-6, IP-10, TNF-α) and costimulatory molecule upregulation. In an EG.7 murine thymoma model, immunization of mice with OVA + cyclic-di-GMP and CpG ODN suppressed tumor growth and boosted OVA specific IgG2c antibody production (Yıldız et.al., 2015). At the same time, Temizoz et. al also showed similar synergistic effect of cGAMP and CpG ODN and suggested that
combined use of these ligands served as an advantageous T helper type 1 adjuvant for vaccines and anti-tumor agents (Temizoz et. al., 2015).

1.1.6. Aim of the study I

Unmodified, modified or encapsulated CpG ODNs have been utilized as therapeutic agents for various applications (Figure 1.5; Klinman, 2004). When used as stand-alone immunoprotective agents, CpG ODNs mediate innate immune responses that persist for several weeks and protect the host against a variety of pathogens (Elkins et. al, 1999). In this system, B cells are directly activated by CpG ODNs whereas macrophages and NK cells are indirectly activated via CpG ODN- mediated IFNs and provide protection against bacteria, viruses or extracellular parasites (Zimmerman et. al, 1998). Another broadly used application is coadministration of CpG ODNs as vaccine adjuvants together with various antigens to generate specific immunity (Bode, 2011). Hence CpG ODNs enhance the activity of antigen presenting cells and induce inflammatory cytokine production supporting Th1 development (Eastcott et. al., 2001 and Prince et. al., 2003).

Several preclinical studies have successfully utilized TLR9 antagonists as anti-tumor therapeutic agents (Brignole et. al., 2010; Damiano et. al, 2007; Krieg et. al, 2007). Clinical trials have been conducted to investigate the therapeutic potential of TLR9 antagonists in cancer patients, as single agent treatment, in combination with standard chemotherapies or as adjuvants in cancer vaccines (Melisi et. al, 2014).

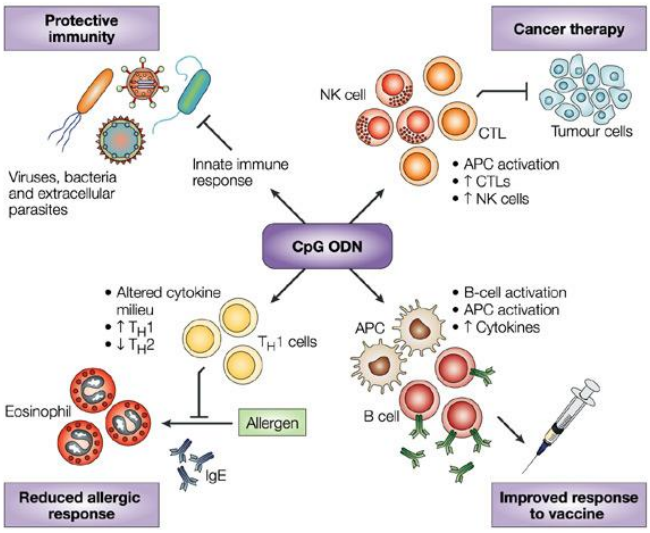

Figure 1.5. Therapeutic applications of CpG ODNs (adapted from Klinman, 2004).

In our previous work, we have described the preparation and characterization of nuclease resistant stable nanorings formed through electrostatic interaction of negatively charged K-ODNs and positively charged Tat peptide(47-57). We showed that the immunostimulatory activity of nanorings reproduced D type ODN activity and induced high amounts of IFNα induction from human pDCs. Nanorings also preserved some K-ODN associated features such as induction of TNFα production. Th1 type immune activation and antigen specific IgG production upon immunization with K/Tat nanorings + OVA were shown to be pDC dependent in mice. K/Tat nanorings were tested as vaccine adjuvants in a low dose commercial vaccine of foot and mouth disease and enhanced the immunogenicity of the vaccine (Gungor and Yagci et. al, 2014). To further asses the therapeutic features of K/Tat nanorings, in the first part of this study, we aimed to investigate the anti-tumor applications of K/Tat nanoring and determine its optimal route of administration as a guide to expand the utility of nanorings as immunotherapeutic agents.

1.2. Leishmaniasis

Leishmaniasis is an infectious disease caused by *Leishmania* protozoa transmitted to mammalian hosts by infected sand flies. The disease is thought to have existed for 50 million years and evidence suggests that ancient civilizations suffered from leishmaniasis (Tuon *et al.*, 2008). The disease was first described in 1756 by Alexander Russel but the relation between the parasite and diseases was confirmed in early 20th century by independent post-mortem examinations of infected patients (Alvar *et al.*, 2006). Today, leishmaniasis is a world-wide health problem affecting 12 million people in more than 80 countries in tropics and subtropics. According to world health organization (WHO), 900,000–1.3 million new cases and 20,000 to 30,000 deaths occur annually due to *Leishmania* infections. The disease is defined within the context of neglected diseases and it affects mostly the poor. Infection is reported to be associated with malnutrition, population displacement, poor housing, a weak immune system and lack of financial resources by WHO. Although leishmaniasis appears to describe only one type of disease, in reality, this is a heterogeneous group of diseases in which the species of *Leishmania* determines the diseases type and pathology. Causative subspecies of leishmaniasis, their geographic locations and reservoirs are displayed in Table 1.2.

Table 1.2. Species of *Leishmania* according to their geographic locations and reservoirs (adapted from Esch et. al., 2013)

Species	Area(s) of endemicity	Predominant reservoir(s)
Leishmania major	Middle East, northwestern China, northwestern India, Pakistan, Africa	Gerbil species, jird, fat sand rat
L. aethiopica	Ethiopia, Kenya, Somalia	Rock hyrax
L. mexicana	Central America, Mexico, TX	Yucatan deer mouse, tree rat, other rodents
L. amazonensis	Brazil	Various forest rodents (grass, pygmy mice)
L. tropica	Mediterranean, Middle East, western Asia, Indian subcontinent	Human, foxes, golden jackals, hyrax, dogs
L. braziliensis	Central, South America	Forest mammals, marsupial species, opossum
L. guyanensis	Guyana, Suriname, northern Amazon basin	Two-toed sloth, forest mammals, marsupial species, opossum
L. peruviana	Peru, Argentinean highlands	Dog?
L. shawi	Brazil	Cebus monkeys, sloths, procyonids
L. lainsoni	Brazil, Bolivia, Peru	Lowland paca, rodents
L. naiffi	Brazil, French Guyana, Ecuador, Peru	Armadillos
L. venezuelensis	Venezuela	Unknown, cat?
L. panamensis	Panama, Costa Rica, Colombia	Sloths, kinkajous, marsupial species, opossum
L. donovani	Indian subcontinent, northern and eastern China, Pakistan, Nepal, eastern Africa, Sudan, Kenya	Human, dogs, goats
L. infantum (syn., *L. chagasi*)	Middle East, Mediterranean basin, northern and northwestern China, northern and sub-Saharan Africa, Central and South America	Dogs, foxes, jackals, wolves

1.2.1. *Leishmania* parasite and life cycle

Unicellular protozoa *Leishmania* are obligate, intracellular parasites belonging to Tryponosomatidae family and Kinetoplastida order. *Leishmania* parasites have a single flagellum and flagellar pocket. They have kinotoplasts, a network of mini and maxi-circle DNAs inside their large mitochondrion (Shapiro, 1995). Life cycle of *Leishmania* include mammalian host and vector sandfly stages as shown in the Figure 1.6. (Esch and Petersen, 2013). The parasite exists in two different morphologically distinct forms depending on its life cycle stage: a motile, spindle shaped form with long flagellum known as a promastigote in the sandfly and a non-motile, round shaped form with shorter flagellum as an amastigote in mammalian host cells (Esch and Petersen, 2013). The lipophosphoglycan (LPG) coat of the parasite can provide advantage in establishment of infection in the host (Spath et. al., 2003).

Leishmania parasites are pathogenic for human and animal reservoirs (dogs, foxes, rats and etc.) and are transmitted to mammals via bite of the female sandflies *Phlebotomus* (Old World) or *Lytzomyia* and *Psychodopygus* (New World). Infective promastigotes are injected into human or animal host by sand fly bite during feeding. Simultaneously

chemoattractants in saliva of sandfly are released that helps recruitment of phagocytes such as neutrophils and macrophages to the side of infection (Laufs et. al., 2002). Promastigotes are internalized into host cells via complement and mannose scavenger receptor mediated phagocytosis (Ueno, 2012). Highly motile spindle shaped promastigotes with long flagellum differentiate into oval shaped, immotile amastigote form with short flagellum. Amastigotes are resistant to the acidification of phagolysosomes and replicate by simple division in parasitophorous vacuoles (Lodge and Descoteaux, 2005). Amastigotes continue proliferating till infected cell is lysed and relleased amastigotes proceed to infect other mononuclear phagocytic cells. When a sandfly feeds on an infected host, parasites are transmitted into the sandfly and within the sandfly midgut, amastigotes are transformed back into promastigotes. Following a reproduction stage in sandfly midgut, a high number of promastigotes migrate to the salivary gland of the insect and are converted into metacyclic promastigotes ready to infect new mammalian hosts (Esch and Petersen, 2013).

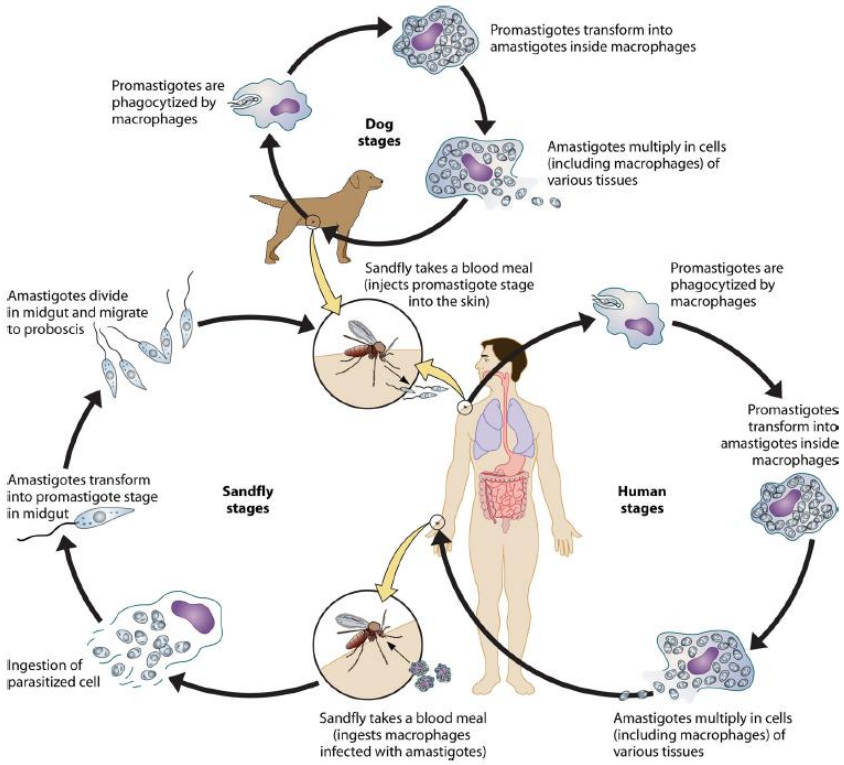

Figure 1.6. The life cycle of Leishmania species (Adapted from Esch and Petersen, 2013).

1.2.2. Types of leishmaniasis

Based on genetic, biological and immunological analyses, more than 30 *Leishmania* sub-species were identified, among which, 20 of them can cause disease in humans (Banuls, 2002). Human *Leishmania* parasites are classified as old world species such as *L. major, L. tropica, L. donovani, L. infantum* and new world species such as *L. mexicana, L. amazonensis, L. brasiliensis* (MacMorris-Alix *et.al.*, 2009). There are three clinical forms of Leishmaniasis: Cutaneous leishmaniasis (CL), Visceral Leishmaniasis (VL) and Mucocutaneous Leishmaniasis (MCL). The clinical presentations of VL, MCL and CL are shown in Fig. 1.7.

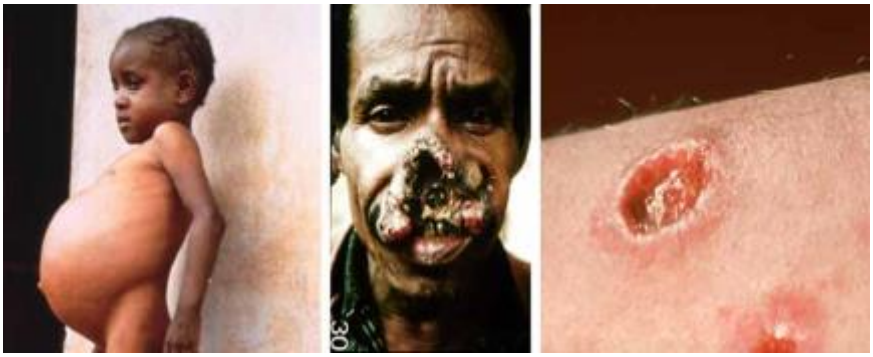

Figure 1.7. Clinical presentations of Leishmaniasis (VL, MCL and CL respectively; adopted from http://web.stanford.edu/class/humbio153/ImmuneEvasion/)

1.2.2.1.Cutaneous Leishmaniasis (CL)

Cutaneous leishmaniasis is the form of the disease restricted to the skin, characterized by formation of skin ulcers due to dermal infection. In the localized form of the disease, ulcers are often self-healing but life-long scarring is frequent. In some instances, the infection may diffuse through lymphatics and cause subcutaneous lesions. CL, the most common type of the leishmaniasis, is reported to have an incidence of 0.7-1.3 million per year. Most of the cases (95%) appear in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria (WHO, 2003). CL causative *Leishmania* species include *L. major, L. tropica, L. mexicana, L. amazonensis, L. braziliensis* and *L. guyanensis.*

1.2.2.2.Visceral Leishmaniasis (VL)

Visceral leishmaniasis is the most fatal form of the disease. In untreated patients, infection metastasizes mostly to the spleen and liver and in some cases to lymph nodes, bone marrow, lungs and rarely to the brain (Kobets and Demant, 2006). In early stages, symptoms are similar to other systemic infections such as fever, chills, fatigue, and weight loss. This is followed by a persistent stage where anemia, diarrhea and hepatosplenomegaly can be seen (Chappuis et. al., 2007). VL is also known as kala-azar (black fever) because of hyperpigmentation and skin darkening (Bryceson, 1996). Secondary infections such as HIV are also observed in VL patients due to suppression of the immune system (Alvar et. al., 2008). Most of the VL incidents (90%) are reported in Bangladesh, Brazil, India, Nepal and Sudan. The number of VL cases is estimated as 0.2 to 0.4 million and 20,000 to 40,000 of them result in death annually (WHO). The Leishmania subspecies that can cause VL are mainly *L. donovani* and *L. infantum* (Banuls et. al., 2011). Post-kala-azar dermal leishmaniasis (PKDL) may develop simultaneously or 6 months to 1 year after cure of the VLs caused by *L.donovani*, especially in Sudan (Zijlstra, 2003).

1.2.2.3. Mucocutaneous Leishmaniasis (MCL)

Mucocutaneous Leishmaniasis (MCL) develops when cutaneous leishmaniasis spreads to the mucosa and form severe lesions due to destruction of mucosal tissues in nose, mouth, throat and adjoining tissues such as nasal septum, lips and palate. In some cases CL and MCL may occur concurrently (David and Craft, 2009). Most of the MCL cases are reported from Brazil, Peru, Bolivia and Ecuador (WHO, 2014). *L. braziliensis, Leishmania amazonensis, L. panamensis, and L. guyanensis* are known causative species of MCL (Ahluwalia *et. al.*, 2004).

1.2.3. Immunology of cutaneous leishmaniasis

1.2.3.1. Immune Response in CL

Several *Leishmania* species can cause cutaneous leishmaniasis and host response differs depending on the characteristics of the infecting *Leishmania* species and the host-evasion mechanisms employed by the parasites (Scott and Novais, 2016).

Infecting promastigotes first encounter tissue resident keratinocytes, Langerhans cells and newly recruited phagocytes, including neutrophils and macrophages soon after transmission to the host (Mougneau et. al., 2011). Keratinocytes immediately secrete inflammatory mediators like IL-12, IL-1β, osteopontin, IL-4, and IL-6 upon invasion by *Leishmania* through the dermal barrier (Ehrchen et. al., 2010). Langerhans are tissue resident dendritic cells which engulf promastigotes and store them in their vacuoles. Langerhans cells are thought to play a role in formation of protective immunity through presentation of *Leishmania* antigens to T cells (Brewig et. al., 2009). Neutrophils are the most abundant cell type infiltrating the site of infection and exert multiple effector functions in cutaneous leishmaniasis. For instance, neutrophils trap/kill extracellular promastigotes via extracellular trap (NET) formation. Promastigotes are also killed after phagocytosis by neutrophils (Guimaraes, 2009; Mougneau et. al., 2011). One week post infection, monocytes migrate to the site of infection and differentiate into mDC in the tissue (Leon et. al.,2007). Signaling of TLRs expressed in mDCs trigger production of potent cytokines such as IL-12, IFN-ɤ and type I IFNs that lead to activation of NK cells & Th1 cells which in turn help establishment of adaptive immunity (Marovic, 2000; Von Stebut, 2003). Macrophages serve as the major reservoir of *Leishmania* parasites since the majority of *Leishmania* replication occurs within macrophages wherein parasite-driven immunosuppressive mechanisms enable evasion from killing (Birnbaum and Craft, 2011). However, macrophages are also the major killer of parasites provided that infected macrophages are first activated by IFN-ɤ and TNF-α. These cytokines stimulate reactive oxygen species (ROS) and nitric oxide (NO) production in infected cells and hence, killing of internalized parasites (Bogdan, 1990).

1.2.3.2.Adaptive immunity in CL

CD4+ T cells have a fundamental role in anti-leishmanial immunity in CL. Following infection, DCs drain to the lymph node and promote development of T helper cells. The type of T helper cell that develops as a result of this interaction determines the outcome of infection. In case of Th1 development, the effectors migrate to the site of

infection and activate macrophages to kill the parasites through secretion of IFN-ɤ (Figure 1.8.A). Therefore, resolution of CL is dependent on induction of Th1 type immunity where IFN-ɤ and TNF-α production activates macrophage microbicidal effector functions. In contrast, development of Th2 immunity results in susceptibility to infection since the cytokines IL-4 and IL-13 produced by Th2 cells support *Leishmania* replication in infected macrophages (Sacks and Noben-Trauth, 2002). Following resolution of the primary infection in CL, development of long-lasting immunity depends on differentiation of long-lived central memory T cells and tissue resident memory T cells (Colpitt, 2009; Zaph, 2004). During the infection resolution process, immune response is down modulated due to IL-10 production that enables persistence of low numbers of parasites at the site of infection (Belkaid et. al., 2001). These remaining parasites support the survival of *Leishmania* specific effector CD4+ T cells which function in rapid clearance of re-infection (Kumar et. al, 2014).

CD8+ cytotoxic T cells play a dual role in parasite killing and pathogenesis (Figure 1.8.B). Whether cytotoxic T cells support parasite clearance or induce pathology depends on the initial parasite load. Uzonna et. al. showed that low dose *Leishmania major* infection induces a transient Th2 response and in the absence of CD8+ T cells, this Th2 response is sustained (Uzonna, 2004). However, in the presence of activated CD8+ T cells that secrete IFNγ, Th2 development is suppressed and ensuing Th1s provide help for pathogen clearance. In contrast, in case of high parasite load, a large numbers of CD8+ T cells accumulate at the site of infection, inflicting tissue damage (Novais, 2013). Cytotoxic CD8+ T cells release molecules such as granzyme, which causes loss of skin integrity, contributing to disease severity in humans (Santos et. al., 2013). How humoral immunity contributes to the outcome of *Leishmania* infection is still unclear. Low levels of neutralizing anti-*Leishmania* antibodies are present in sera of CL patients with active infection. In contrast, high titers of anti-*Leishmania* antibodies were documented in VL patients (Behin, 1989). It is generally believed that protective immunity relies on cellular rather than humoral immunity and in the absence of effective cell mediated immune responses, humoral elements support non-protective responses (Kedziersky and Evans, 2014). IgG antibody-coated amastigotes

can enter into macrophages or DCs through Fc receptor-mediated phagocytosis and the ensuing Fc receptor signaling can induce IL-10 production, supporting infection and enhancing Th2 cell responses (Ronet, 2008 and 2010). Contrary to the negative role of antibodies in leishmaniasis listed above, in the absence of B cells, T cell priming and and T-cell mediated IFN-ɤ production was subobtimal, but this response was recovered when IgG opsonized parasites were used in infection (Woelbing, 2006). Thus the role of B cell and *Leishmania* specific antibodies in CL is still unresolved.

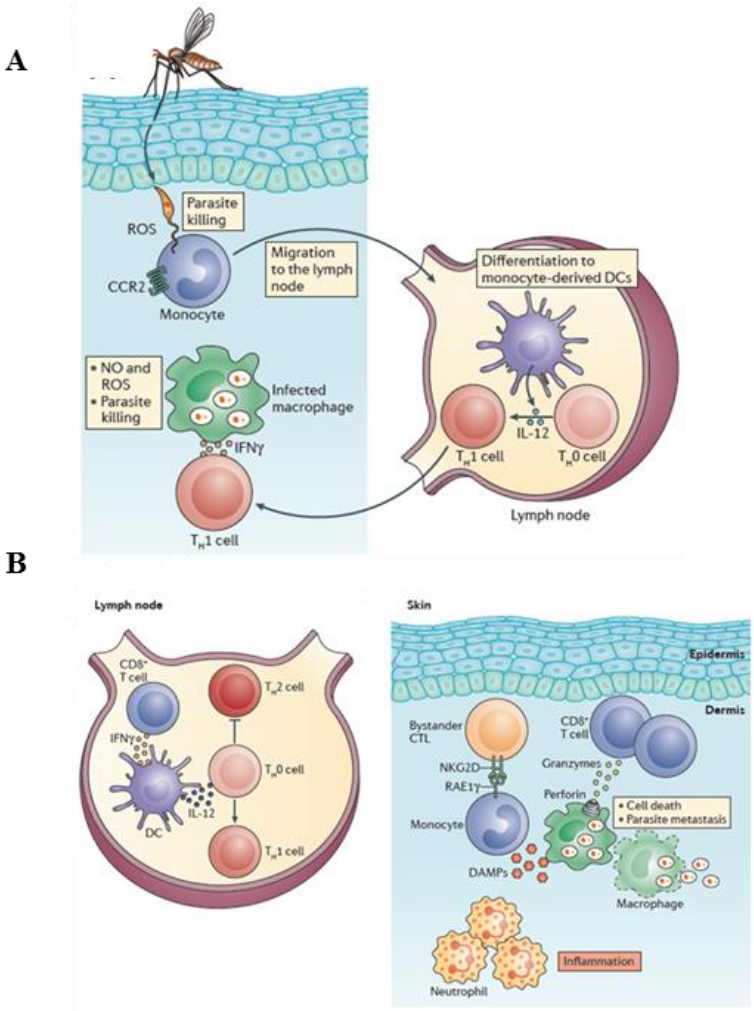

Figure 1.8. Response of immune cells to *Leishmania* parasites in cutaneous leishmaniasis. Involvement of innate cells in Th1 cell polarization and parasite killing (A) Dual role of Cytotoxic T cells in lymph node and skin (B). (Adapted from Scott and Novais 2016).

1.2.4. Diagnosis and Treatment of cutaneous leishmaniasis

Lesions in cutaneous leishmaniasis are similar to lesions observed in leprosy, skin cancers, fungal infections and eczema, requiring parasitological diagnostic tests for correct medical diagnosis (Escobar, 1992). For this, samples from cutaneous lesions are collected through scraping, punch biopsy or needle aspiration and presence of *Leishmania* is examined in Giemsa-stained samples by microscopy. Culture of samples for parasite identification and characterization is doable but is more time consuming and expensive. The Montenegro skin test, a delayed type hypersensitivity test based on positive reactivity to intradermally injected leishmania antigens is also diagnostic in CL (Weigle et. al., 1987). More recently, PCR based assays are routinely employed to detect *Leishmania* DNA in blood or tissue samples but this method is not applicable in economically poor endemic areas due to high cost, lack of technical expertise and poor laboratory infrastructure (Reithinger and Dujardin, 2007).

Lesions in CL are often self-healing and do not require systemic treatment. However, in case of chronic, progressive or mucosal lesions, or in immune suppressed patients, treatment is mandatory (Marke and Makhoul, 2004). Treatment depends on use of pentavalent antimonial drugs, inhibiting glycolysis and metabolic activity. Pentavalent antimonial drugs have serious adverse effects such as renal failure, hepatotoxicity, cardiotoxicity and pancreatitis (Berman, 1997). Other drugs such as amphotericin B, pentomidine isothioate are also used in CL as listed in Table 1.3.

1.2.5. Vaccination against cutaneous leishmaniasis

The cost, toxicity and drug resistance associated with the pentavalent antimonials used for treatment, necessitates the development of an effective preventive vaccine. Vaccination trials in animal models informed researchers about the cellular mechanisms of protection against leishmaniasis. However, to date, an effective and safe vaccine against either cutaneous or visceral leishmaniasis has not been developed (Okwor et al, 2012). The oldest attempt to develop protective immunity is known as "**leishmanization"**, which depends on administration of live virulent parasites to non-immune individuals. This method was successful in generating long-term protective

immunity in endemic regions but also left many with non-healing lesions, immunosuppression and autoimmunity such as psoriasis (Greenblatt, 1980).

Table 1.3. Drugs used in treatment of cutaneous leishmaniasis (adapted from Markle and Makhoul et. al., 2004).

Pentavalent antimony
Meglumine antimoniate (Glucantime) and sodium stibogluconate (Pentostam); cure rate 94 percent; eliminated by kidneys
Dosage: 20 mg per kg per day for 20 days
Stibogluconate supplied as 100 mg Sb per mL light-sensitive solution
Calculated dose (12 to 20 mL for adults) is diluted in 50 mL of 5 percent dextrose in distilled water, infused intravenously over 10 to 15 minutes
Amphotericin B (Fungizone)
Reserved for antimony failures
Dosage: 0.5 to 1.0 mg per kg every other day for up to eight weeks; total dosage is 1.5 to 2 g for the treatment period
Pentamidine isethionate (Pentam 300)
Dosage: 2 mg per kg intramuscularly every other day for seven days
Toxic effects: damage to pancreas, kidney, or bone marrow may be irreversible
May induce diabetes mellitus
Others
Topical paromomycin is effective with *L. major* and *L. mexicana*. It can be combined with antimonials to reduce the number of injections.
Oral antifungals have demonstrated conflicting results, although some good results have been achieved with *L. mexicana*[19] and *L. major*.[18]
Allopurinol (Zyloprim) incorporates into parasite RNA with lethal effect. Studies are conflicting, and it is not recommended, although there is synergistic activity with antimonials.[11-14]
Heat[15-16] and cryotherapy[17] show good results in uncontrolled trials.
Excision is not recommended because of the high risk of local relapse and disfiguration.

The first generation of vaccines attempted to use killed whole parasites in CL endemic regions. This strategy is economically viable, simple and does not involve the risk of lesion development. Although capable of inducing strong Th1 dominated responses, **vaccination with whole killed parasites** failed to induce clinical disease protection in phase III trials in endemic countries (Velez et al.,2000; Armijos et al., 2004). In Venezuella, patients with CL are now treated with three injections of whole killed *L.mexicana* and if this fails, antimony treatment is initiated (Convit et al 2004). To

enhance memory cell formation, whole killed parasites are mixed with adjuvants such as BCG and new adjuvans are investigated in recent studies (Armijos, 2004; Velez, 2005).

Use of live attenuated parasites represents an interesting approach in vaccine development. This strategy depends on generation of replication deficient or genetically modified *Leishmania* parasites with reduced virulence (Daneshvar et al.,2009; Rivier et al.,1993; Elhay et al.,1990 Titus et al.,1995). However, the possibility that the attenuated parasite can regain virulence in the host remains as a major concern.

In **subunit based vaccinnation approach, recombinant *leishmania*** proteins are used as the vaccine antigen. Many *Leishmania* antigens such as GP63, LACK, LmST11 and LeIF have been tested as subunit vaccines alone or in the form of polyproteins in CL (Handman et. al., 1990; Coler et. al., 2007; Melby et. al., 2001). Investigation on the universal protein for split vaccine development is still ongoing and .

DNA Vaccines where direct intramuscular injection of plasmid DNA encoding *Leishmania* specific genes provided either protection (Doroud et. al. 2011), or had no protective activity in mice (Kedzierski et al., 2006). This strategy is still experimental and the fact that DNA vaccines suffer from low immunogenicity in humans than in mice, decreases expectations for this line of work.

1.2.6. Use of CpG ODNs as anti-leishmania vaccine adjuvants

Several studies report on the use of CpG ODNs (explained in detailed in section 1.1.6) as vaccine adjuvants in CL mouse models. For instance, in 2002, Rhee and colleagues tested CpG ODN as a vaccine adjuvant with autoclaved *Leishmania major* (ALM). The authors reported that lesion growth was reduced and infection was controlled in a CD8+ T-cell dependent manner (Rhee et. al., 2002). Mendez et. al. attempted to coinject live *Leishmania major* and CpG ODN with or without Alum to develop long-lasting protection with minimum pathology. Results showed that mice infected with *L. major* plus CpG with or without Alum developed no dermal lesions and were protected against reinfection for up to 6 months. The authors proposed that IFN-ɤ

producing CD4+ T cells were recruited to the infection site, triggering a transient inflammation. Mendez et. al. once more revised this study in 2010, with a follow up article focusing on Th17 cells. Their findings indicated that *L. major* plus CpG vaccination improved the proliferation of Th17 cells which induced neutrophil infiltration to the site of infection. A study by Shargh et al (2012) in which soluble *Leishmania* antigen (SLA) was combined with CpG ODN or liposome encapsulated CpG ODN showed that both free CpG and liposome encapsulated CpG generated protection against cutaneous leishmaniasis. The use of CpG ODNs as vaccine adjuvant was also reported in studies for vaccination against VL (Agallou et. al., 2011 and Ramirez et. al., 2014).

1.2.7. *Leishmania* Extracellular Vesicles

1.2.7.1. Extracellular vesicles

All cells of prokaryotic or eukaryotic origin release membranane vesicles into the extacellular enviroment. These evolutionarily conserved extracellular vesicles (EV) consist of a lipid bilyer and their size differ ranging from 40 to 2000 nm in diameter. Distinguishing EVs according to their size, morphology, density or composition is not possible (Bobrie et al., 2012). However, categorization is based on their biogenesis and there are three broad classes of EVs: 1. Exosomes, 2. Microvesicles and 3. Apoptotic bodies (Bobrie et al., 2012). Exosomes (40-100 nm) originate from early endosomes and are released via fusion of multi vesicular bodies (MVB) with the cell membrane. Microvesicles (50-1000nm) in contrast, are generated by outward budding of the cell membrane (Figure 1.9). Apoptotic blebs are released from cells undergoing apoptosis (Colombo et. al., 2014).

The composition of the microvesicle membrane is more smilar to parent cell membrane when compared to the composition of exosome membranes. However, unlike the parental plasma membrane, phosphatidyserine (PS) is exposed on the surface of exosomes and microvesicles (Devaux et. al., 2008). It is belived that exosome membranes contain lipid rafts, sphingomyelin, cholesterol and ceremide (Record, 2014). EVs also contain high amounts of proteins the content of which varies depending on the EV isolation method and cell type. In general, lipid raft associated

proteins, cytoskeletal proteins, cytosolic proteins, heat shock proteins, plasma membrane proteins and vesicle trafficking related proteins are found in EVs. Those proteins are also used as pan-EV markers (Ostergaard et. al., 2012).

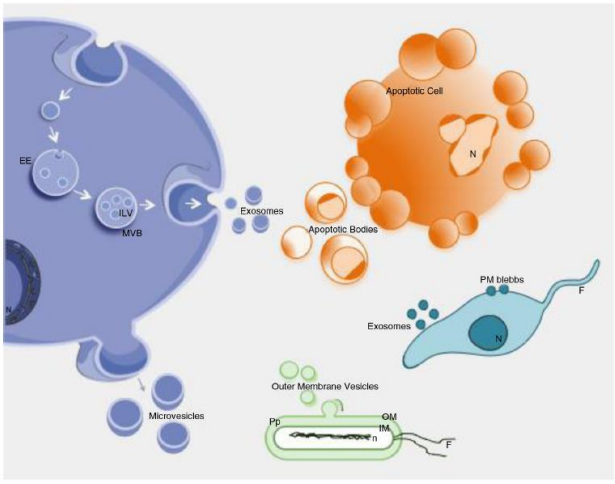

Figure 1.9. Biogenesis and release of EVs (adapted from Yanez-Mo et.al., 2015)

Studies on nucleic acid content of EVs have mainly focused on RNAs. EVs incorporate mRNAs and miRNAs and interestingly, when the cargo is transferred to a target cell, mRNAs can be translated into proteins l (Ratajczak et al., 2006). Recent studies report that EVs contain a large variety of other small noncoding RNA species, such as structural RNAs, tRNA fragments, vault RNA, Y RNAs, and small interfering RNAs (Bellingham et al., 2012; Nolte-'t Hoen et al., 2012). DNA content of EVs are poorly identified. However, evidence suggests that EVs include mitochondrial DNA (mtDNA), single-stranded DNA, double-stranded DNA (dsDNA) and oncogene amplifications (Lee et. al., 2014; Lazaro-Ibanez et. al., 2014). Such EV-associated DNAs and RNAs are proposed as novel tumor-specific biomarkers in cancer detection (Thakur et. al., 2014). Characteristics of different types of EVs are listed in Table 1.4. As multifunctional signaling complexes, EVs play an important role in maintenance of normal physiology but also contribute to pathogenesis of several diseases (Figure 1.10). EVs can either bind to and activate target cell surface receptors directly or deliver their cargo to the cytosol following fusion with the plasma membrane.

Table 1.4. EV types and characteristics (adapted from El Andaloussi et. al., 2013)

Vesicle types	Characteristics			
	Origin	Size	Markers	Contents
Exosomes	Endolysosomal pathway; intra-luminal budding of multivesicular bodies and fusion of multivesicular body with cell membrane	40–120 nm	Tetraspanins (such as TSPAN29 and TSPAN30), ESCRT components, PDCD6IP, TSG101, flotillin, MFGE8	mRNA, microRNA (miRNA) and other non-coding RNAs; cytoplasmic and membrane proteins including receptors and major histocompatibility complex (MHC) molecules
Microvesicles	Cell surface; outward budding of cell membrane	50–1,000 nm	Integrins, selectins, CD40 ligand	mRNA, miRNA, non-coding RNAs, cytoplasmic proteins and membrane proteins, including receptors
Apoptotic bodies	Cell surface; outward blebbing of apoptotic cell membrane	500–2,000 nm	Extensive amounts of phosphatidyl-serine	Nuclear fractions, cell organelles

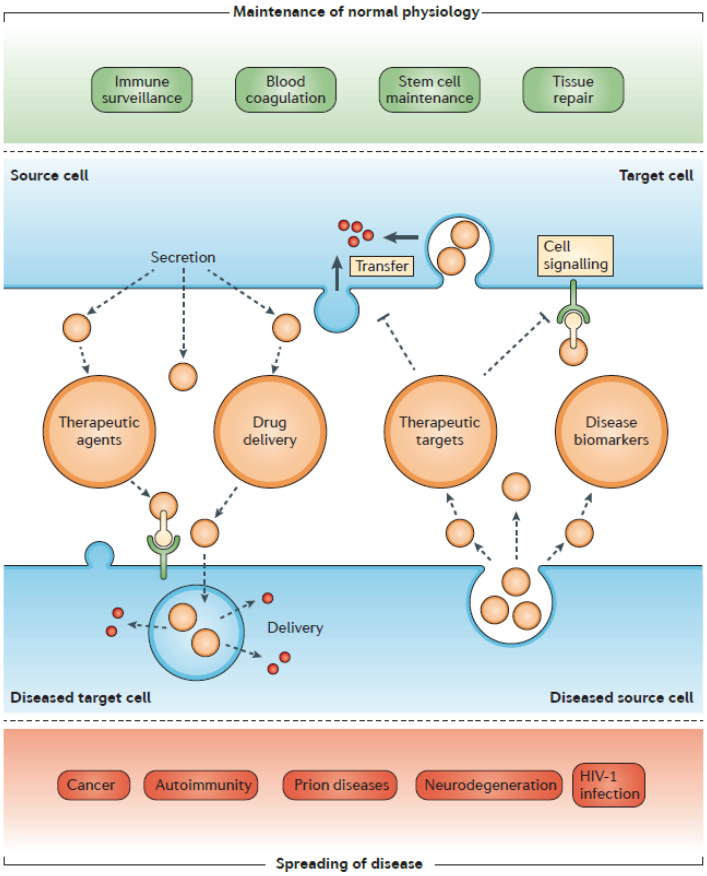

Figure 1.10. Physiological roles of EVs in health and disease (adapted from El Andaloussi et. al., 2013).

1.2.7.2. *Leishmania* Extracellular Vesicles

Similar to all other nucleated cells, *Leishmania* parasites also secrete extracellular vesicles, a process first described by Silverman et.al (Silverman et. al, 2010a and b). Secreted vesicles collected from *Leishmania* were found to be identical to mammalian exosomes in terms of their morphology and density. Of interest, more than 50% of *Leishmania* exosome associated proteins overlapped with the proteome of the mammalian exosomes (Simpson et. al. 2009 and Silverman et al., 2008, 2010a). Several studies analyzing the secretome of different *Leishmania* spp showed that the majority of the secreted proteins lacked N-terminal secretion signal peptide, therefore these could not be secreted via classical protein secretory mechanisms (Lambertz, 2012). In fact, only two of the 151 bone fide secreted proteins from *L. donovani* promastigotes were secreted by a classical mechanisms (Silverman et. al., 2008). Similarly, out of the 42 exoproteome proteins of *L.braziliensis*, just two were thought to be secreted through the classical pathway (Cuervo et al., 2009). For *L.infantum*, 5 of the proteins among the 181 secreted ones were identified as classical secretion candidates (Cuervo, 2010). Several other studies also verified that most of the proteins in the *Leishmania* secretome were secreted through an alternative pathway overlapping with the majority of known exosomal proteins. Collectively, these results indicate that the main secretion system used by *Leishmania* is a vesicle-based secretion system. Table 1.5. presents the shared proteins of *Leishmania* secretome and mammalian extracellular vesicles.

Leishmania EVs were also shown to enclose RNA cargo, mostly of small non-coding RNAs and mature tRNAs in *L. donovani* and *L. braziliensis*. L. braziliensis exosomes also harbored siRNA encoding gene transcripts. Such RNAs could be delivered to host cells and therefore *Leishmania* EVs can potentially modify host cell responses (Lambertz et. al., 2012).

Table 1.5. *Leishmania*-secreted proteins associated with exosome-like vesicles. Microvesicles: BC, B-cell lymphocyte exosome; DC, dendritic cell exosome; AP, adipocyte exosome (adiposome). (Table is adapted from Silverman et. al., 2008).

GeneDB accession number	Protein identification[a]	Microvesicle association
LmjF35.3340	6-Phosphogluconate dehydrogenase, decarboxylating, putative	GLY
LmjF29.0510	Cofilin-like protein	DC
LmjF36.6910	Chaperonin, putative, T-complex protein 1 (theta subunit), putative	AP
LmjF01.0770	Eukaryotic initiation factor 4a, putative	DC
LmjF28.2860	Cytosolic malate dehydrogenase, putative	AP
LmjF24.2060	Transketolase, putative	GLY
LmjF33.2550	Isocitrate dehydrogenase, putative	AP
LmjF28.2770	Heat-shock protein hsp70, putative	BC, DC, AP
LmjF35.3860	T-complex protein 1, eta subunit, putative	AP
LmjF12.0250	Cysteinyl-tRNA synthetase, putative	GLY
LmjF14.1160	Enolase	BC, DC, AP
LmjF36.2030	Chaperonin Hsp60, mitochondrial precursor	AP
LmjF23.1220	T-complex protein 1, gamma subunit, putative	AP
LmjF05.0350	Trypanothione reductase	GLY
LmjF36.2020	Chaperonin Hsp60, mitochondrial precursor	AP
LmjF36.1630	Clathrin heavy chain, putative	BC, AP
LmjF16.0540	Aspartate carbamoyltransferase, putative	GLY
LmjF27.2000	Hypothetical protein, conserved	GLY
LmjF31.1070	Biotin/lipoate protein ligase-like protein	AP
LmjF26.1240	Heat shock protein 70-related protein	BC, DC, AP
LmjF04.0960	Adenylate kinase, putative	GLY
LmjF27.1260	T-complex protein 1, beta subunit, putative	AP
LmjF30.3240	Glutamyl-tRNA synthetase, putative	GLY
LmjF21.0810	Methionyl-tRNA synthetase, putative	GLY
LmjF36.3210	14-3-3 Protein-like protein	DC, AP
LmjF33.2540	Carboxypeptidase, putative, metallo-peptidase	GLY

Since parasites have different characteristics during their life cycle, number of secreted extracellular vesicles, their composition and immunomodulatory role may be influenced by the parasite state. For instance, upon temperature switch from 25ºC to 37ºC, a condition mimicking parasite's entry to the mammalian host, a very quick and dramatic increase in protein and exovesicle release occurs in *L. Mexicana* (Hassani, 2011). Exosomes secreted under infection mimicking conditions (37ºC and acidic pH) possess higher phosphatase activity than exosomes secreted at neutral pH (Silverman, 2010a). Exoproteome analysis by Hassani et. al. also suggests that exosomes secreted at 37ºC have increased enzymatic activity. Exosome associated *Leishmania* virulence factors and their enrichment under acidic pH conditions are shown in Table 1.6.

Table 1.6. Virulence factors carried by *Leishmania* exosomes and their enrichment in acidic pH (Table is adapted from Silverman et. al. 2010a).

Functional class	Gene DB accession no.	Protein identity	Enriched in acidic pH
Immune evasion/suppression	LmjF10.0460	GP63, leishmanolysin	–
	LmjF26.0620	Heat shock protein 10	+
	LmjF26.1240	Heat shock protein 70	–
Intracellular survival	LmjF15.1040	TRYP1, tryparedoxin peroxidase	+
	LmjF11.0350	14-3-3-like protein	+
T cell antigens	LmjF35.2210	Kinetoplastid membrane protein-11	–
	LmjF28.2740	Activated protein kinase c receptor (LACK)	–
	LmjF08.1110	Stress-induced protein sti1	–

Extracellular vesicles released from promastigotes during sand fly bite, just after the infection or from amastigotes after cell lysis, interact with host cells (macrophages, neutrophils and DCs) via surface binding, fusion with plasma membrane or endocytosis (Silverman et. al., 2012). Secreted vesicular components of *Leishmania* were observed within the cytoplasmic compartment of host cells. Exosome based delivery of GP63, EF-1α, HSP70 and 90 into host cells (Nandan et. al., 2002; Gomez et. al., 2009; Silverman et. al., 2010a) could be inhibited by use of reagents blocking vesicle uptake (Keller et. al., 2006). These results indicate that *Leishmania* vesicles can deliver parasite-associated effector molecules directly into host cells.

Immunomodulatory properties of *Leishmania* derived vesicles were first shown by Silverman and collogues (Figure 1.11). Results showed that *L. donovani* vesicles suppressed pro-inflammatory cytokine production (TNFα and IL-12) and HLA-DR expression in parasite infected human monocytes and DCs but induced secretion of IL-10 of s (Silverman et. al. 2010b). Gomez et. al. showed that parasite associated factors (GP63, EF-1α and etc.) delivered by exosomes induced activation of protein-tyrosine phosphatases SHP-1 and PTP1B., targeting vital signaling cascades such as IFN-γ/Jak-STAT1 pathways. Interference with signaling suppresses the IFN-ɤ mediated activation of macrophages, ROS production and hence the anti-leishmanial effect of macrophages (Gomez et. al., 2009). In contrast, PI3K and MAPK signaling pathways are activated by *Leishmania* effector molecules, promoting the production of anti-inflammatory cytokine IL-10 and *Leishmania* survival (Gregory and Olivier, 2005; Nandan and Reiner, 2005).

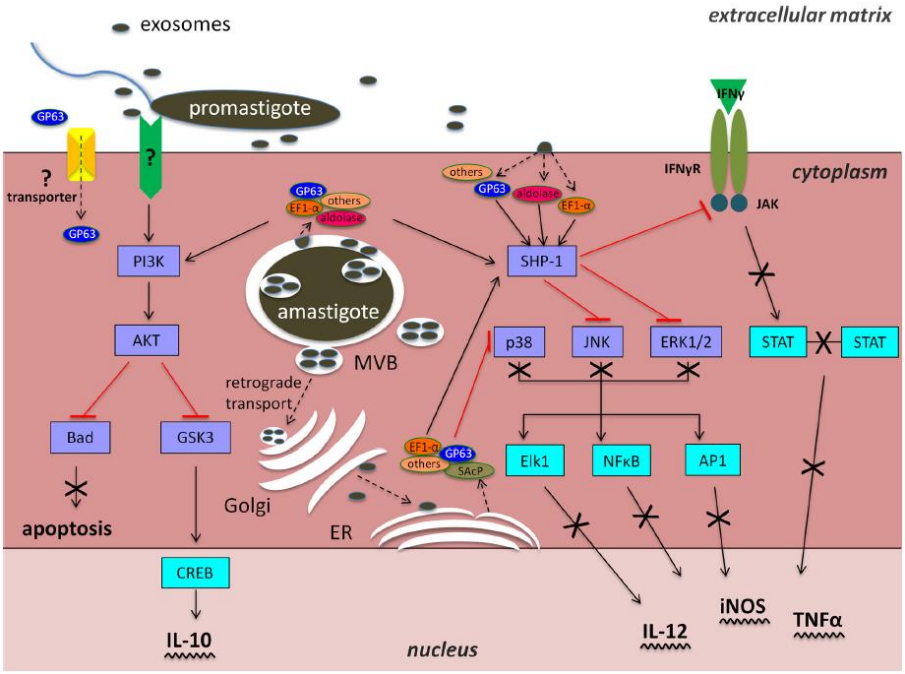

Figure 1.11. *Leishmania* secreted molecules interfere with host signaling pathways. (Adapted from Lambertz et. al.,2012.)

In vivo studies on mouse models for infection showed that parasite loads in both resistant C57B6 and susceptible BALB/c mice were increased i in *L. donovani* or *L. major* exosome pretreated, parasite challenged mice. Increase in parasite burden was associated with enhancement of Th2 type immune response (Silverman et. al., 2010b). *Leishmania* exosomes secreted in parasite mid-gut stage were also shown to be inoculated together with promastigotes during sand fly bite. These mid-gut secreted exosomes were demonstrated to modulate host immune response and exacerbates disease progression of cutaneous leishmaniasis in BALB/c mice. Disease aggravation was linked to induction of IL-17a as well as IL-10 and IL-4 production after co-inoculation of *L.donovani* derived exosomes and parasites (Atayde et. al., 2015).

1.2.8. EV based vaccines

The concept of using extracellular vesicles in vaccines was initially tested in tumor vaccination studies. Dendritic cells were first treated with tumor antigens and DC derived exosomes were shown to activate T cells with anti-tumor effector function (Andre et. al., 2004). Since then, several experimental and clinical EV-based vaccines/immunotherapy agents were tested (Table 1.7). Furthermore, host-derived exosome based vaccines were also tested in a limited number of studies against infectious diseases caused by protozoans. For instance, Aline et.al. reported a cell free EV-based vaccine against *Toxoplasma* (Aline et. al., 2004). Two independent studies by Beauvillain, et al. also claimed that exosome based immunization protected against Toxoplasma infection (Beauvillain, et al. 2007 and 2009). Extracellular vesicles of DC2.4 cell line fed with *T.gondii* triggered a strong *Toxoplasma* specific Th1 type immune response and protection against infection. Another study was conducted to generate anti-malarial vaccine (Martin Jaular et. al., 2011). In that study, exosomes of reticulocytes infected with *Plasmodium yoelii* were shown to include plasmodium specific antigens and vaccination with these *P. yoelii* exosomes plus CpG ODN 1826 combination induced IgG1 and IgG2a production and provided protection in 83% of the animals that were challenged with *P. yoelli*. For leishmaniasis, exosomes secreted from DCs incubated with *L. major* were shown to elicit Th1 mediated protection against cutaneous leishmaniasis in susceptible BALB/c mice (Schnitzer et. al., 2010). EVs are natural antigen transporters between cells, thus use EVs in vaccination has several advantages (Schorey et. al., 2015). For example, proteins are preserved in their stable conformations in EVs; molecular distribution is enhanced by EVs circulating in body fluids; antigen presentation by adhesion molecule expressing EVs are efficient. In contrast, EV based therapies have also some limitations. For instance, reproducibility of EV generation with desirable antigen composition and administration of non-self molecules such as nucleic acids to humans may have unforeseen adverse effects.

Table 1.7. Clinical and preclinical applications of EV based therapies (Table was adapted from György et al. 2015)

Vesicle type and source	Administrative route	Recipient	Therapeutic effect
Preclinical studies: mouse			
Liposomes enriched with *N*-octanoyl-glucosylceramide	i.v.	Tumor-bearing nude mice	Enhanced Dox delivery to tumors via EV versus via liposomes
EVs from mouse MSCs	i.v.	Hypoxia-treated FVB/N mice	Decreased lung inflammation and hypoxia-induced hypertension
EVs from the human ESC-derived MSC line HuES9.E1	i.v.	C57BL6/J mice after myocardial infarction before reperfusion	Reduced myocardial infarct size and inflammation
EVs derived from mouse bone marrow MSCs	s.c. with 4T1 cells	BALB/c mice	Reduced growth and vascularization of tumors
EVs derived from spleen and lymph node cells of TNP-tolerized mice	i.p.	TNP-sensitized C57BL/6 mice	Inhibited contact sensitivity
EVs derived from mouse BMDC overexpressing IL-10, IL-4, or FasL	Footpad	C57BL/6 mice prior to DTH induction	Suppressed DTH
EVs derived from mouse BMDC overexpressing IL-10, IL-4, or FasL	i.v.	Collagen-immunized DBA/1 mice	Delayed collagen-induced arthritis
Bacteroides fragilis OMVs with polysaccharide A	Oral	BALB/c mice prior to colitis induction	Decreased colitis-induced weight loss, colon shrinkage, and colitis
EVs derived from grape juice	Oral	C57BL/6 mice concurrent with colitis induction	Decreased colitis-induced colon shrinkage and mortality
EVs from RAW264.7 cell line pulsed with *Mycobacterium tuberculosis* proteins	i.n. vaccine	C57BL/6 mice	Decreased growth of *M. tuberculosis* in the lung
EVs from a mouse splenic DC cell line, pulsed with *Toxoplasma gondii* antigens	s.c.	Female CBA/J mice prior to mating and *T. gondii* exposure	Fewer *T. gondii* cysts in mother and pup brains, increased pup survival
OMVs derived from *Bordetella pertussis*	i.v.	BALB/c mice prior to *B. pertussis* challenge	Decreased *B. pertussis* counts and increased type 1 antibody production compared to animals given Tdap vaccine before challenge
OMVs derived from *Escherichia coli*	i.p.	C57BL/6 and BALB/c mice prior to *E. coli* challenge	100% survival postchallenge, versus 20% for unvaccinated mice
EVs from BMDCs cultured with OVA peptide and α glucosylceramide	i.v.	Tumor-bearing C57BL/6 mice	Decreased tumor growth and prolonged survival

Table 1.7. ***Continued***

Vesicle type and source	Administrative route	Recipient	Therapeutic effect
EVs from HEK293 expressing let-7a and RGD-targeting peptide	i.v.	Tumor-bearing $RAG^{-/-}$ mice	Decreased tumor growth
EVs from HEK293T expressing CD and UPRT	Intratumoral injection	Tumor-bearing nude mice	Decreased tumor growth; elimination of tumor in two-thirds of treated mice
Dox-loaded EVs from DCs expressing iRGD	i.v.	Tumor-bearing nude mice	Decreased tumor growth compared to free Dox or untargeted EVs
Nanovesicles extruded from RAW264.7 cells in presence of Dox	i.v.	Tumor-bearing BALB/c mice	Decreased tumor growth compared to Dox liposomes
Curcumin-loaded EVs from EL-4 cells	i.p. with LPS	C57BL/6 mice	Increased survival of LPS-induced septic shock
Curcumin-loaded EVs from EL-4 cells	Intranasal	C57BL/6 mice	Decreased EAE clinical score compared to free curcumin
EVs derived from EL-4 cells loaded with Stat 3 inhibitor	Intranasal	Tumor-bearing C57BL/6 mice	Decreased tumor size and prolonged survival compared to free inhibitor
Phase I studies			
EVs from autologous DCs pulsed with melanoma peptide antigens	Intradermal and s.c.	15 patients	Minor inflammation, no major toxicity. One patient: MART-1–T cell response and tumor shrinkage; one patient: minor response (loss of a spinal cord lesion); two patients: stabilization
EVs from autologous DCs pulsed with MAGE peptides	Intradermal and s.c.	9 patients	No major toxicity. One-third of patients: MAGE-specific T cell responses; two patients: increased NK cell lysis
EVs from autologous ascites fluid of colorectal cancer patients	s.c.	37 patients; 13 also received GM-CSF	No major toxicity. Patients receiving EVs and GM-CSF: 80% showed cytotoxic T cell responses to colon cancer peptide CAP-1, with one stabilization and one minor response
OMVs from *Neisseria meningitidis*	i.m. injection	Human patients	MenBVac and MeNZB vaccines are safe and effective

1.2.9. Aim of the study II

In Turkey, more than 50,000 CL cases were reported between 1988 and 2010 (Gurel et. al., 2012). Half of the cases reported in Southeastern Anatolia were caused by *L.tropica,* whereas *L.infantum* prevailed in the Mediterranean region of Turkey (Alvar et. al, 2012). However, more recently, CL cases associated with *L.major* and *L.donovani* were also reported in parient from Adana (Ozbilgin et. al., 2016) and Sanlıurfa (Zeyrek et. al., 2014). Demographic distribution of CL cases in Turkey reported between 2007 and 2009 is shown in Figure 1.12. (Ozbilgin et. al., 2016). Considerable increase in the number of *Leishmania* infections is predicted in the following years due to high amount of *Leishmania* infected refugees emigrating from Syria to Turkey.

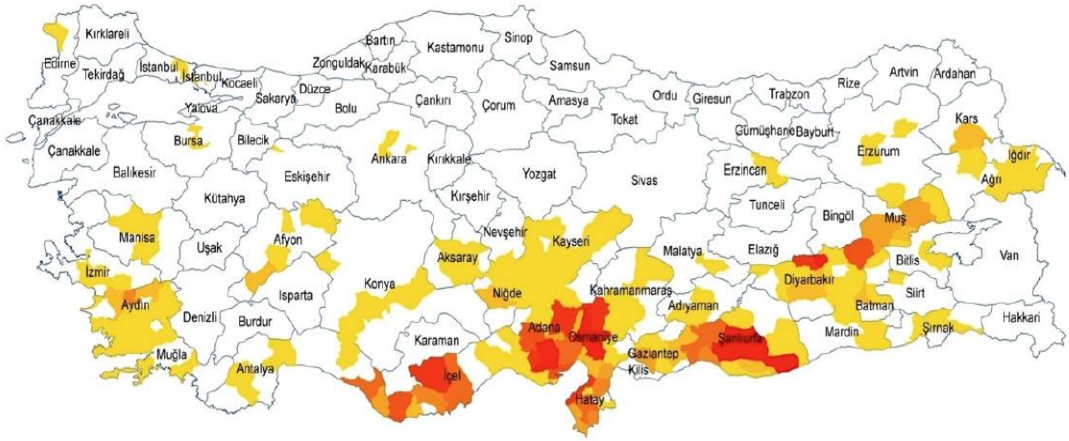

Figure 1.12. CL cases in Turkey between 2007 and 2009 (Adapted from Ozbilgin et. al., 2016).

Leishmania secreted EVs are de facto liposomes composed of *Leishmania* specific antigens. Although EV based vaccination against Leishmaniasis was studied by EVs derived from infected DCs, *Leishmania* derived EVs were not considered as potential vaccine components due to their Th2 inducing properties. Silverman et. al. showed that treatment with CAF01 (a Th1 adjuvant) plus exosomes from HSP100 k/o *L. donovani,* reduced parasite load after challenge with WT *L. donovani* through increased production of *Leishmania*-specific IFN-ɤ, TNF-α, IL-6, IL-10, and IL-17

(Silverman et. al., 2010b). This study suggested that EVs derived from *Leishmania* with low virulence could be good vaccine candidates when combined with strong Th1 adjuvants.

L. tropica, the main causative strain of CL cases in Turkey, is not an aggressive strain and it causes self-healing lesions in the skin. To date, only a handful of studies regarding the mechanism of *L. tropica* infection exists and EVs of *L.tropica* have not been studied before. Therefore, in this study, we aimed to isolate and characterize EVs derived from a patient isolate of *L.tropica* and determined their immunomodulatory properties in vitro. We also intended to develop a *L.tropica* derived EV- based vaccine against cutaneous leishmaniasis. To enhance immunostimulatory activity of EVs, we combined *L.tropica* EVs with CpG ODNs or CpG ODN-based vaccine adjuvants previously developed by our group (Gungor and Yagci et. al., 2014; Yıldız et. al., 2015) as potent Th1 adjuvants.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Reagents for ELISA and Flow Cytometry

Table 2.1. Antibodies and reagents used in cytokine ELISA

Product Name	Brand	Working concentration		SA-AP or HRP 1:1000	TMB or PNPP
		Coating	Detection		
mIFNɤ	Mabtech	1µg/ml	0.5µg/ml	SA-AP	PNPP
mIFNɤ	Biolegend	1/200	1/200	HRP	TMB
mIL12p40	Biolegend	1/200	1/200	HRP	TMB
mIL10	Biolegend	1/200	1/200	HRP	TMB
mIL6	Biolegend	1/200	1/200	HRP	TMB
mTNFα	Biolegend	1/200	1/200	HRP	TMB

Table 2.2. Antibodies and reagents used in IgG ELISA

Product Name	Brand	Working Concentration
mIgG1	Southern Biotech	1/2500 or 1/1000
mIgG2c	Southern Biotech	1/2500 or 1/1000
mIgG2a	Southern Biotech	1/2500 or 1/1000
mIgA	Southern Biotech	1/2500 or 1/1000
mIgE	Southern Biotech	1/2500 or 1/1000

Table 2.3. Antibodies and reagents used in Flow Cytometer

Product Name	Brand	Working concentration
mCD8a-Pe/Cy5	BioLegand	1µg/ml
mIFNγ-Pe	TONBO	1µg/ml
mI-A/I-E -Pe	Biolegend	1µg/ml
mCD69-FITC	Biolegend	1µg/ml
mCD86-AlexaFlour 647	Biolegend	1µg/ml
mTNFα-PE	Biolegend	1µg/ml
leishmania gp63-FITC	Cederlane	1µg/ml
Brefeldin A	Biolegend	10mg/ml

2.1.2. Ligands and Adjuvants

Table 2.4. Immunostimulatory ligands used in vitro stimulations and in vivo treatments

Ligand	Sensor	Brand	Amount	
			in vitro	*in vivo*
K3	TLR 9	GeneDesign	1 µM	25 µg/mice
D35	TLR 9	IDT or GeneDesign	3 µM	25 µg/mice
K23/Tat	TLR 9	IDT/Anaspec	1 µM	15 µg/mice
K3-SPG	TLR 9	IFREC Vaccine Lab	-	15 µg/mice
2’3’cGAMP	STING	Invivogen	10 µg/ml	15 µg/mice

2.1.3. Antigens and peptides

Table 2.5. Proteins and peptides used in immunization and functional assays

Antigen/peptide	Brand name
OVA	Anaspec
SIINFEKL OVA 257–264 class I	Anaspec
Tat	Anaspec or Eurofins
Soluble *Leishmania* Antigen	Home made

2.1.4. Standard Solutions, Buffers, Cell Culture Media Components

RPMI 1640 Medium and Tween20 were purchased from Thermo Fisher Scientific. Na-pyruvate, HEPES, Penicillin/Streptomycin, Non-essential amino acids, and Molecular Biology Grade water were purchased from Hyclone. Fetal Bovine Serum (FBS), Phosphate Buffer Saline (PBS), and ACK Lysis Buffer were purchased from Lonza. Components of various culture media and different buffers are given in detail in Appendix.

2.2. Methods

2.2.1. Methods for Applications of Nanorings

2.2.1.1. Animals

C57BL/6 female, 6-8 weeks old mice were used for the experiments. Mice were housed in Bilkent University, Department of Molecular Biology and Genetics or Osaka University, Immunology Frontier Research Center (IFReC) and Japan National Institute of Biomedical Innovation facilities (NIBIO). All experimental procedures were approved by the animal ethical committee of Bilkent University and Osaka University.

2.2.1.2. Tumor Xenograft models

2.2.1.2.1. E.G7-OVA Tumor Model

2.2.1.2.1.1. Cell culture of E.G7 cell line

E.G7 cells (ATCC, CRL-2113™) were derived from C57BL/6 (H-2 b) mouse lymphoma cell line EL4. They were stably transfected with pAc-neo-OVA including a complete copy of chicken ovalbumin (OVA) mRNA and the neomycin (G-418) resistance gene, thus they synthesize OVA constitutively as a model tumor antigen. Cells were sustained in a RPMI1640 media with 10% regular FBS supplemented with 1 mg/ml G-418 (Hyclone). Cultures were maintained by replenishment of media every 2-3 days.

2.2.1.2.1.2. E.G7 Tumor Inoculation and Treatment

2.5 x 10^6 E.G7 tumor cells in 100 µl PBS per mice were injected subcutaneously into the dorsal flank of five C57BL/6 mice per group. Animals were checked for their tumor development till tumor sizes reached a palpable size (≥50 mm^3). Then, mice were intraperitoneally treated with 25 µg of OVA (Anaspec) plus 10 µg of K-ODN or K/Tat two times at one week intervals. Five days after the first injection (day 0), tumor sizes measurements were initiated with the use of a caliper every other day and tumor volumes were recorded as length x width x height. Mice were sacrificed on day 10 and excised tumors were weighed and photographed. Spleens were removed to assess OVA-specific CD8 T-cell responses (Figure 2.1).

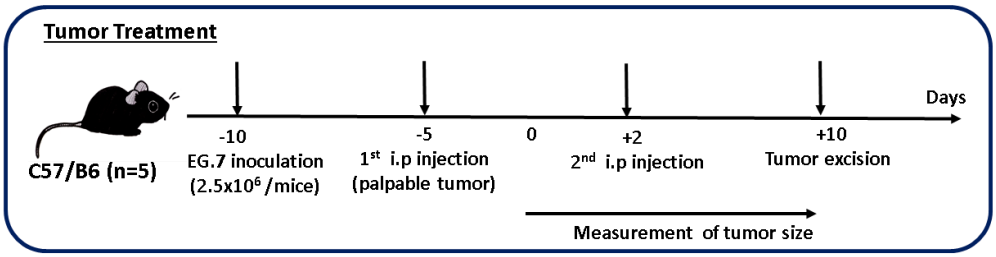

Figure 2.1. Injection schedule for *in vivo* E.G7 tumor inoculation and treatment.

2.2.1.2.1.3 Analysis of OVA specific T cell response from splenocytes

Spleens were surgically removed from tumor inoculated and OVA plus K-ODN or K/Tat treated C57BL/6 mice after cervical dislocation. Single cell splenocytes were prepared by smashing spleens using a sterile syringe plunger in 2% FBS supplemented RPMI 1640 media. As such prepared single cell suspensions were transferred to 15 ml falcon tubes and centrifuged at 300 g for 10 minutes at RT. Supernatants were discarded and red blood cells were lysed by incubation in 2 ml of ACK lysis buffer (Lonza) for 5 minutes. Cells were washed twice in 2% FBS supplemented RPMI 1640 media. $2.5x10^6$/ml cells were stimulated in the absence or presence of SIINFEKL (2 µg/ml) OVA 257–264 class I (Kb)–restricted peptide epitope (Anaspec) in the presence of brefeldin A (10 µg/ml) (Biolegend) for 5 hours. Cells were stained for cell surface CD8 expression by incubation with 1µg/ml anti-mouse CD8a-Pe/Cy5 (Biolegend) for 30 min on ice and washed twice with FACS Buffer (Appendix). Stained cells were fixed in the presence of 100µl/test fixation buffer (4% paraformaldehyde, Medium A from ThermoScientific) for 15 min at RT. Following washing with FACS buffer, cells were incubated in 100µl/test permeabilization buffer, Medium B (Thermo Scientific) containing 1µg/ml anti-mouse IFNγ-PE (Biolegend) for 30 min at RT. Cells were washed again, resuspended in 400 µl PBS and SIINFEKL OVA257-254 specific IFNγ secretion was analyzed from CD8-gated cells using a BD Accuri™ C6 flow cytometer (10000 events were acquired).

2.2.1.2.2. B16 murine melanoma model

2.2.1.2.2.1. Cell Culture of B16 F10 cell line

B16 F10 cells (ATCC, CRL-6475™) were derived from C57BL/6 mouse melanoma skin cells composed of a mixture of spindle-shaped and epithelial-like cells. Cells were sustained in DMEM media supplemented with 10% regular FBS. Cultured cells that reached ~90% confluency were sub-cultured following trypsinization and washing once with PBS (1X).

2.2.1.2.2.2. B16 Tumor Inoculation and treatment

0.5×10^6 B16 F10 tumor cells in 100µl PBS per mice were injected subcutaneously

into the dorsal flank of C57BL/6 mice. Animals were monitored for their tumor development and treated peritumorally with 15 µg of K-ODN (Alpha DNA) (n=7); 10µg K-SpG (n=6); 15µg K/Tat (n=8) and PBS (n=7) every other day starting from the day of palpable tumor (≥50 mm^3) formation. Tumor volumes were recorded for three weeks at two day intervals as width, height and length by a caliper and reported as mm^3 (Figure 2.2).

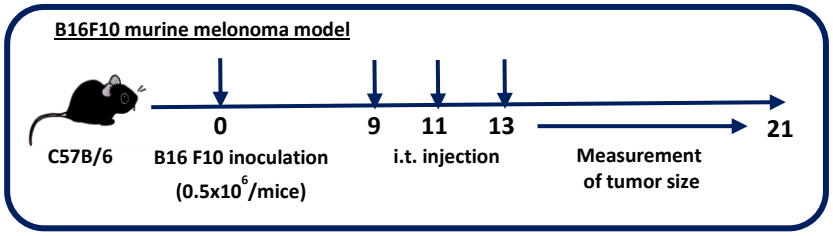

Figure 2.2. Injection schedule for in vivo B16 F10 tumor inoculation and treatment

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2.2.1.3. Determination of the best route of administration of CpG ODN Nanorings

2.2.1.3.1. Immunization against OVA via different route of administration

C57BL/6 mice were anesthetized by subcutaneous injection of 100 µl Ketamine. To determine the best route of administration for CpG ODN nanoring induced vaccine adjuvant activity, five mice/group were treated with 15 µg nanorings with 10 µg OVA as the antigen. Three mice/group were treated with 10 µg OVA alone as the control group. The following injection volumes were used depending on the route of administration: 200 µl intraperitonally (i.p), 100 µl subcutaneously (s.c), 50 µl intamuscularly (i.m), 50 µl intradermally (i.d) or 30 µl intranasally (i.n). All injections were administered on days 0 and 10. For i.n administration, mice were held in the hanging position through their ears and 30 µl of the formulation was applied dropwise into the nostrils (15 µl in each nostril) with the help of a micropipette to allow the mice to inhale the formulation without forming bubbles. Mice were held in this hanging position till their breathing returned to normal. One week after booster injections mice were sacrificed to collect blood, bronchoalveolar lavage (BAL) and spleens for cytokine and immunoglobulin analysis (Figure 2.3).

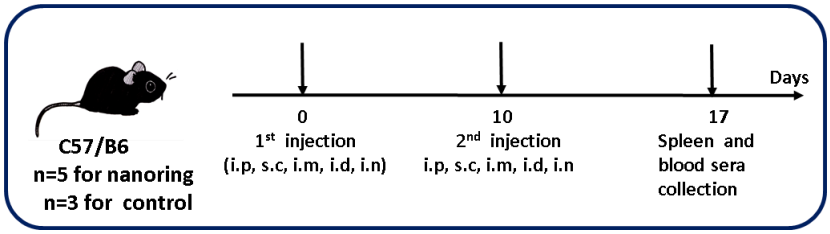


Figure 2.3. Injection schedule to determine the optimal route of administration of the vaccine adjuvant CpG ODN nanorings in mice immunized with OVA.

2.2.1.3.2. anti-OVA antibody detection from sera and BAL

2.2.1.3.2.1. Collection of Blood Samples from the Heart

One week after the last immunization, mice were anesthetized by subcutaneous injection of 100 µl Ketamine. Abdominal cavity of mice were surgically opened and the rib cage was cut to expose both the heart and lungs. Blood was collected from inferior vena cava from the right ventricle of the heart via use of a 26 gauge needle of a 1 ml syringe. Blood was transferred to BD Microtainer® tubes with serum separator additive BD Microgard™ and spun down at 1000 rcf for 10 minutes at RT. Upper phase of the separator gel contained sera and these were collected for further testing in ELISA assays.

2.2.1.3.2.2. Collection of Bronchoalveolar Lavage (BAL)

Mice from i.d, s.c and i.n groups were subjected for BAL collection. One week after last immunization, mice were anesthetized by subcutaneous injection of 100 µl Ketamine. Thoracic cage and neck were disclosed by scissors and tissue from the neck area was dissected to expose the trachea following completion of blood collection from hearts. One inch long piece of a surgical thread was placed underneath each trachea with the help of a forceps. A small incision was made in the trachea, through which a 21 gauge tube was inserted and stabilized by knotting the thread around the tubing. A 1 ml syringe was inserted to the fixed tube and 1 ml cold PBS was injected into lungs. Next, ~1 ml fluid was aspirated from lungs and transferred into microtubes. BAL cells were separated via centrifugation at 300xg for 5 minutes and supernatants were stored at -20ºC to be analyzed by ELISA.

2.2.1.3.3. Determination of OVA specific antibody response by ELISA

2.2.1.3.3.1. Anti-OVA IgG ELISA

OVA specific IgG1 and IgG2c antibodies induced in immunized mice were detected by ELISA. Briefly, 96-well PolySorp plates (F96 Nunc-Immunoplate, NUNC, Germany) were coated with 50 µl of 10 µg/ml OVA antigen in PBS at 4 ◦C overnight. Plates were washed five times by a microplate washer (The ImmunoWash™ 1574, BioRad). Wells were blocked with 200 µl assay buffer (1% BSA in PBS) for 1ht at RT. After washing, 5000 X and 500 X diluted mouse serum or broncho alveolar lavage (BAL) from each mice was added into wells of the first rows and serially diluted 2-fold thereafter for IgG1 and IgG2c, respectively. After 2hr incubation at RT and washing, goat anti-mouse IgG1/HRP or IgG2a/HRP (Southern Biotech) were 1:2500 diluted in assay buffer and were added to plates (50 µl/well). Following 1hr incubation at RT and a final wash, 50 µl/well TMB substrate was added (Pierce, Thermo Fisher Scientific) followed by addition of 50 µl/well stop solution (Pierce, Thermo Fisher Scientific). Development of yellow color was measured at OD 450 nm using an ELISA reader (Thermo Scientific) and OVA specific IgG1 and IgG2c titers were calculated and graphed.

2.2.1.3.3.2. Anti-OVA IgE ELISA

OVA specific IgE antibodies induced in immunized mice were detected by ELISA. 96-well PolySorp plates (F96 Nunc-Immunoplate, NUNC, Germany) were coated with 100 µl of 2 µg/ml anti-mouse IgE antibody (Table 2.2) in PBS and incubated overnight at RT. Plates were washed three times by a microplate washer (The ImmunoWash™ 1574, BioRad). Wells were blocked with 200 µl assay buffer (1% BSA in PBS) for 1h at RT. After washing, 5X diluted mouse serum and recombinant IgE standarts (532, 266, 133, 66.5, 33.3, 0 ng/ml) were added (100 µl/well). After 24hr incubation at RT and washing, goat anti-mouse IgE/HRP (Southern Biotech) was 1:100 diluted in assay buffer and distributed to wells (100 µl/well). Following 1hr incubation at RT and a final wash, 100 µl/well TMB substrate was added (Pierce, Thermo Fisher Scientific) followed by addition of 100 µl/well stop solution (Pierce, Thermo Fisher Scientific).

Development of yellow color was measured at OD 450 nm using an ELISA reader (Thermo Scientific) and concentrations of OVA specific IgE antibodies were calculated with respect to the standard curve and calculated values were reported as pg/ml.

2.2.1.3.3.3. Anti-OVA IgA ELISA

OVA specific IgA antibodies induced in immunized mice were detected by ELISA. 96-well PolySorp plates (F96 Nunc-Immunoplate, NUNC, Germany) were coated with 50 µl of 10 µg/ml OVA antigen in bicarbonate buffer (pH=9.5) and incubated overnight at 4 ◦C. Wells were blocked with 200 µl assay buffer (1% BSA in PBS) for 1ht at RT. Plates were washed four times by a microplate washer (The ImmunoWash^{TM} 1574, BioRad). Mouse broncho alveolar lavage (BAL) from each animal was added into wells. After 2 h incubation at RT and washing, goat anti-mouse IgA/HRP (Southern Biotech) were 1:2500 diluted in assay buffer and were distributed to wells (50 µl/well). Following 1 h incubation at RT and a final wash, 50 µl/well TMB substrate was added (Pierce, Thermo Fisher Scientific) followed by addition of 50 µl/well stop solution (Pierce, Thermo Fisher Scientific). Development of yellow color was measured at OD 450 nm using an ELISA reader (Thermo Scientific).

2.2.1.3.4. Determination of OVA specific cytokine production by ELISA

2.2.1.3.4.1. Incubation of OVA Pulsed Splenocytes

Spleens were surgically removed from immunized or naive C57BL/6 mice after cervical dislocation and gentleMACS Dissociator (Miltenyi Biotech) was used in order to prepare single cell splenocytes according to the recommended protocol by the manufacturer. For this, spleens were transferred to C Tubes (Miltenyi Biotech) in 3 ml media. Tubes were attached upside down onto the sleeve of the gentleMACS Dissociator and gentleMACS Program m_spleen_01 was run. Cell suspensions were filtered through 0.22 micron cell strainers, transferred to 15ml falcon tubes and centrifuged at 300 g for 10 minutes at RT. Supernatants were discarded and red blood cells were lysed by incubation in 2 ml of ACK lysis buffer (Lonza) for 5 minutes. Cells that were washed in 2% FBS supplemented RPMI1640 media (twice) were counted.

Concentration of splenocytes were adjusted to 10^7 cells/ml and 100 µl cell suspensions from each mouse were layered into 6 wells of a 96 well round bottom tissue culture plate. Splenocytes were untreated or treated with OVA (10 µg/ml) or OVA peptide (10 µg/ml) that is specific for MHC class I (OVA 257) in duplicate. Following 48 h incubation of treated and untreated control wells, cells were removed by centrifugation at 300xg for 10 min and supernatant was stored at -20 ˚C until further use for cytokine detection.

2.2.1.3.4.2. Detection of Antigen Specific IFNγ Production by ELISA

96-well PolySorp plates (F96 Nunc-Immunoplate, NUNC, Germany) were coated with 1/200 diluted mouse recombinant IFNγ (50 µl/well) (Biolegend) and plates were incubated at 4°C overnight. Following blocking in 200 µl blocking buffer for 2 h at room temperature, plates were washed three times by a microplate washer (The ImmunoWash™ 1574, BioRad). 50 µl of supernatants were added into the wells and incubated for 2-3 hours at room temperature or overnight at 4°C. After washing, 50 µl of 1:200 diluted biotinylated-secondary IFNγ antibody (Biolegend) was added into wells and plates were incubated 2 hours at room temperature. Plates were washed as before and 50 µl of 1:1000 diluted streptavidin-HRP (Biolegend) was added to each well for 1hr at room temperature. Plates were washed and incubated with 50 µl TMB substrate (Pierce, Thermo Fisher Scientific). Color development was followed and 50 µl stop solution (Pierce, Thermo Fisher Scientific) was added to stop the reaction. Development of yellow color was measured at OD 450 nm using an ELISA reader (Thermo Scientific).

2.2.2. Methods for EV based *Leishmania* Vaccine

2.2.2.1. Parasite Culture

2.2.2.1.1. Parasites

Leishmania tropica parasites were isolated from Turkish patients with cutaneous leishmaniasis, in Department of Parasitology, Celal Bayar University and were kindly provided by Prof. Dr. Ahmet Ozbilgin. Detection of *Leishmania* species in biopsies from patients and identification of the strain of parasites were carried out via real time

PCR assay and sequencing techniques by Seray Ozensoy Toz, in Department of Parasitology, Ege University.

2.2.2.1.2. Culture of *Leishmania* Promastigotes

Parasites isolated from patients were sustained in parasite growth medium (RPMI 1640 supplemented with 20% heat inactivated FBS; Appendix). Parasites were incubated in upright airtight tissue culture flasks in a non-CO_2 incubator (Nuve) set at 25 ˚C and passaged every 2-3 days. Dilution factor for each passage was decided according to the growth curve. Passage number of promastigotes was kept under 5-6 due to the fact that they lose their infectivity after serial passaging.

2.2.2.1.3. Counting of *Leishmania* Parasites

Parasites were counted after fixation using a hemocytometer and/or flow cytometer. 20 µL of parasites from culture was mixed with 20 µL of 4% paraformaldehyde. After 5 min, the volume was completed with PBS to 200 µl. 10 µL of fixed parasites was applied to a hemocytometer and left for 5 min to allow the cells to settle down. Parasites in 4 big squares were counted and parasite count per ml was calculated by Average number of promastigotes in 4 squares x 10^5 x dilution factor. Alternatively, parasite numbers in 20 µL of fixed parasite solution was counted (N) by flow cytometery (BD Accuri C6, USA) and parasite count per ml was calculated using the formula N x 500 x dilution factor.

2.2.2.1.4. Assessment of Growth Kinetics of *L. tropica* patient isolate

Culture was initiated using 10^5 *Leishmania* cells/ml in 10 ml parasite medium. Parasite number was counted once a day until cells stopped growing. Mid log, late log and stationary phases of promastigote life cycle were defined in accordance with the growth curve for further experiments.

2.2.2.2. Isolation of $EV_{L.tropica}$ and Preparation of SLA and HK Parasites

2.2.2.2.1. Isolation of Extracellular Vesicles from *L.tropica* ($EV_{L.tropica}$)

30 ml stationary phase promastigotes (~2.5x10^6/ml) were centrifuged at 1500xg for 15 min and pellet was dissolved in 1X PBS. Following two washing steps with 1X PBS, parasites were transferred into 30 ml of exosome collection medium (RPMI-1640

supplemented with exosome-depleted FBS (20%); pH 5.5) (Appendix), and incubated at 37˚C for 24 hours in a CO_2 incubator (Oasis CO_2 Incubator) Parasite EVs were then purified by differential centrifugation method (Figure 2.4) adopted from mammalian EV purification protocol (Cristodero, 2008). Briefly, parasite cultures were centrifuged at 1500xg for 15 min (Nuve, NF 800R) and the supernatants were transferred to ultracentrifuge tubes (Hitachi Preparative ultracentrifuge) CP100WX) and centrifuged at 15,000xg for 30 min at 4˚C to remove cell debris and large vesicles such as apoptotic blebs. Supernatants were again collected and filtered through 0.2 µm filter and re-centrifuged at 100,000xg for 60 min at 4˚C. Extracellular vesicle (EV) containing pellets were dissolved in 1X PBS following two washing steps. The size of EVs are too small to be quantified by flow cytometer, therefore EV quantitation was based on protein content determination. The protein contents of EVs were measured at A280 nm using nanodrop (BioDrop DUO).

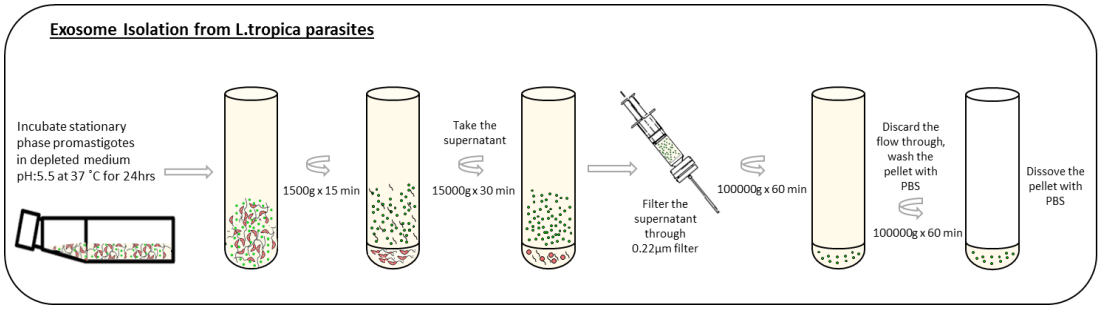


Figure 2.4. Isolation protocol of extracellular vesicles from parasite culture.

2.2.2.2.2. Preparation of Heat Killed (HK) Parasites

After the step in which supernatants were transferred to ultracentrifugation tubes in EV isolation protocol (section 2.2.2.1), the remaining parasite pellet was washed 2 times with 1X PBS and re-suspended pellet was incubated at 70˚C for 60 min in a heat block (Biosan TDB-120) Heat killed parasites were centrifuged at 2500g for 20 min and the pellet was resuspended in 1X PBS. HK parasite concentrations were based on the original parasite numbers present in the EV collection medium. HK parasites served as as a positive control in future experiments.

2.2.2.2.3. Preparation of Soluble *Leishmania* Antigen (SLA)

Stationary phase *L. Tropica* parasites (~2.5x10^6ml) were washed in 1X PBS twice by centrifugation at 1,500xg for 10 min at 4˚C. The pellet was dissolved in lysis buffer (1 ml buffer per 10^9 parasites; Appendix). Following three rapid freeze thaw cycles, three pulses of sonication (20 sec; 40W) was applied. Supernatant was collected after centrifugation at 5,000xg for 20 min at 4˚C. Protein amount was determined using Micro BCA™ Protein Assay Kit (Pierce) according to the manufacturer's protocol.

2.2.2.3. Characterization of $EV_{L.tropica}$

2.2.2.3.1. Quantification of $EV_{L.tropica}$ by CFSE Staining

Stationary phase *L. Tropica* parasites (~2.5x10^6ml) were washed in 1X PBS twice by centrifugation at 1,500xg for 10 min. Cell pellet was resuspended with 1 ml 5µM Carboxyfluorescein succinimidyl ester (CFSE, ThermoFisher Scientific) in 1X PBS rapidly. After incubation for 10 min at 37 ˚C, 9 ml RPMI with 10% FBS was added immediately and parasites were washed with 1X PBS twice. Stained parasites were placed into exosome collection medium (RPMI-1640 supplemented with exosome-depleted FBS (20%); pH 5.5) and incubated at 37˚C for 24 hours in an incubator. Parasite derived CFSE labeled EVs were isolated as described in section 2.2.2.1. CFSE labelled exosomes were analyzed by a flow cytometer (BD Accuri C6).

2.2.2.3.2. Morphological and Size Analysis of $EV_{L.tropica}$ by Atomic Force Microscopy (AFM)

The morphology and size of EVs were determined by Atomic Force Microscopy (AFM) (NanoMagnetics Instruments, SHPM using the NMI Image Analyzer 1.5 software). EVs were diluted 100X in Dnase/Rnase free H_2O and 5 µl was deposited on silicon wafers. Samples were air dried at room temperature. Images were obtained using a Multi75Al model cantilever from Budget Sensors in non-contact mode. Cantilever's resonance frequency and force constant were 75 kHz and 3 N/m, respectively. Scan rate was kept at 0.73-0.79 Hz. The scanned area sizes were in 10X10 mm^2, 3X3 mm^2 and 2X2 mm^2 range. Images were analyzed using NMI Image Analyzer software.

2.2.2.3.3. Analysis of gp63 Content of EV*L.tropica* by Flow Cytometer

Extracellular vesicles (200µl) were incubated with 1 µg/ml anti-*leishmania* gp63-FITC antibody for 1h at RT. Unincorporated dye was removed from labeled exosomes using exosome spin columns (MW3000) (Invitrogen) according to the manufacturer's protocol. Briefly, dried gel in the spin columns were hydrated by 650µl PBS for 15 min at RT. Spin columns were placed in collection tubes and spun at 750 g for 2 min at RT to remove the excess PBS. Spin columns were placed into elution tubes and labelled and unlabeled EV suspensions were directly applied to the center of the gels without disturbing the gel surface. Columns were centrifuged at 750 g for 2 min at RT and eluted EVs were analyzed on a NovoCyte flow cytometer (ACEA Biosciences). 20 µl of EVs were acquired and FSC-SSC gating was adjusted using unlabeled EVs. gp63 positivity of labeled EVs was determined using the FL-1 channel in comparison to unlabeled EVs.

2.2.2.3.4. Analysis of EV*L.tropica* Associated Nucleic Acids by Flow Cytometer

Extracellular vesicles (200µl) were incubated with 20 µM acridine orange dye for 10min at RT. To digest intact vesicles, 100µl of stained EVs was treated with 100 µl %10 SDS. Unstained, acridine orange stained and SDS treated EVs were acquired (20µl) on a flow cytometer (BD Accuri C6). Single stranded nucleic acids were assessed in FL3 (red) channel and double stranded nucleic acids were assessed in FL1 (green) channel. Remaining stained and SDS treated EV suspensions were loaded onto a %1 agarose gel and subjected to electrophoresis at 70V for 1h. Acridine orange signal was detected on an electrophoresis and imaging system (Cleaver Scientific, runVIEW).

2.2.2.4. Maintenance of Animals

Adult male or female BALB/c (8-12 weeks old) mice were used for all *in vitro* stimulation and *in vivo* immunization experiments. The animals were kept in the laboratory animal facility of the Department of Molecular Biology and Genetics at Bilkent University under controlled ambient conditions (22 ˚C ±2) regulated with 12 hour light and 12 hour dark cycles. Animals were provided with unlimited access of

food and water. Our experimental procedures have been approved by the animal ethical committee of Bilkent University and METU (Bil-AEC).

2.2.2.5. Cell Culture

2.2.2.5.1. Generation of Bone Marrow Derived Macrophages (BMDM)

Female BALB/c mice were sacrificed via cervical dislocation and back legs were removed, skin and muscles were dissected to expose the femur and tibiae bones. Bones were sterilized in 70% EtOH for 5 mins. Both ends (epiphysis) of each bone was cut with a scissor and bone marrows were flushed out with 2 ml RPMI 1640 supplemented with 2% FBS using a 25G attached to a 5 ml syringe., Marrow plugs were minced using a sterile plunger and cells were washed in RPMI medium at 300x g for 10 min. . Cell pellets were treated with 1 ml ACK lysis buffer (5 min incubation) and washed 2 times. Cells were resuspended, counted and plated as $1.5x10^5$ bone marrow cells/well in RPMI 1640 supplemented with 20% FBS in in 48 well tissue culture plates. For macrophage differentiation, mouse recombinant M-CSF (20 ng/ml) (TONBO) was added into culture plates on days 0 and 3. After 7 days, untouched attached cells were stimulated with various ligands and/or EVs.

2.2.2.5.2. Preparation of Single Cell Suspension from Spleen

Female BALB/c mice were sacrificed via cervical dislocation and spleens were removed and placed into a 35cm petri dishes containing 3 ml of complete RPMI supplemented with 2% FBS. Single cell suspensions were obtained by mashing the spleens with the back of a sterile syringe plunger using circular movements under sterile cell culture conditions. The cells were washed 2 times in complete RPMI by centrifugation at 300x g for 10 minutes. After the final washing step, cells were counted and resuspended in complete RPMI supplemented with 10% FBS.

2.2.2.5.3. Cell Counting

At the end of the washing steps, c, supernatants were aspirated and pellets were resuspended in 1 ml RPMI 1640 supplemented with 10% FBS media. 20 µl of samples

were transferred into 10 mL isotonic solution. Remaining RBCs were lysed by adding 3 drops of ZAP-OGLOBIN II Lytic Reagent (Beckman Coulter, USA). Number of events (N) in 20µl was counted on a flow cytometer (BD Accuri C6, USA), by gating on live cells and excluding dead cells and debris. Cell number per ml was calculated by N x 5000 (Dilution Factor) x 500.

2.2.2.6. Determination of Immunostimulatory Properties of EV*L.tropica*

2.2.2.6.1. In Vitro Stimulation of Cells with EV*L.tropica*

For assessment of Immunostimulatory properties of EVs, 400,000 cells/well splenocytes, 200,000 cells/well BMDM in 48 well cell culture plates were stimulated with 3 different EV concentrations (0.2, 1, 5, 10 µg/ml) or HK parasites (1:5, 1:10, 1:20 cell to HK ratio) as such or in combination with various nucleic acid sensor ligands (Table 2.4) in 10% FBS supplemented complete RPMI. After 24h incubation at 37ºC in a CO_2 incubator, cell culture supernatants were collected and cytokine levels were measured via ELISA (2.2.6.2). Upregulation of activation markers were determined from cells stained for specific cell surface molecules (2.2.6.3). For intracellular cytokine staining, 500,000 splenocytes/well were stimulated in round bottom 96 well cell culture plates for 6h at 37ºC in a CO_2 incubator in the presence of 10 µg/ml brefeldin A.

2.2.2.6.2. Enzyme Linked-ImmunoSorbent Assay (ELISA) for Cytokine Detection

Following 24h incubation period, plates were spun at 300xg for 5 min and supernatants were collected and stored at -20ºC until use. 96 well ELISA Immuno plates (SPL) were coated with monoclonal mouse antibodies (50µl/well) at a concentration described in Table 2.1 and plates were gently tapped to ensure uniform distribution. After incubation at RT for 4h or at 4 ºC overnight, coating medium was decanted and wells were blocked with 200µl blocking buffer (Appendix) for 2h at RT. Plates were rinsed with ELISA wash buffer (Appendix) 4 times with 2 minute incubation intervals and dried by tapping. Supernatants (50µl/well) were added into the wells and plates were incubated for 2h at RT or overnight at 4 ºC. Following washing step, 50µl of

biotinylated capture antibody in T-cell buffer (Appendix) was added into wells according to concentrations given in Table 2.1) and incubated 2h at RT or overnight at 4 ºC. After washing step, 50µl of 1:1000 diluted streptavidin-alkaline phosphatase solution (SA-AP) prepared in T-cell buffer at least 2h prior to use or 1:1000 diluted streptavidin-HRP solution in T-cell buffer was added into wells for 1h at room temperature (Table 2.1). Plates were washed and to develop the SA-AP containing plates, 50 µl of PNPP solution was added after a PNPP tablet was dissolved in 4 ml ddH2O and 1 ml PNPP buffer. Color development was followed at 405 nm over time using an ELISA reader, Multiskan FC Microplate Photometer (Thermo Scientific). To develop streptavidin-HRP conjugate containing plates, washed plates were incubated with 50 µl TMB substrate (Pierce, Thermo Fisher Scientific). Color development was followed and 50 µl stop solution (Pierce, Thermo Fisher Scientific) was added to stop the reaction. Development of yellow color was measured at OD 450 nm using an ELISA reader.

2.2.2.6.3. Fluorescence Activated Cell Sorting (FACS)

2.2.2.6.3.1. Fixation of Cells

Following incubation period, cells were centrifuged at 300 x g for 5 minutes and supernatants were removed. Pellets were resuspended in PBS and transferred to round bottom 96 well plates. Plates were spun down at 300 x g for 5 minutes and cell pellets were fixed in the presence of 100 µl/test fixation buffer (4% paraformaldehyde, Medium A from ThermoScientific) for 15 min at RT. Fixed cells were washed twice using 200 µl PBS-BSA-Azide (FACS Buffer; Appendix). Supernatants were aspirated following centrifugation and pellets were resuspended in FACS buffer for following procedures.

2.2.2.6.3.2. Cell Surface Marker Staining

Fixed or live cells in round bottom 96 well plate were centrifuged and pellets were resuspended in 100µl FACS buffer containing flourochrome conjugated antibodies against various cell surface markers as stated in the Table 2.3 and incubated for 30 min in dark at RT (for fixed cells) or at 4ºC (for live cells). At the end of incubation period,

cells were washed twice with FACS Buffer and resuspended in 200 µl PBS. Upregulation of cell surface markers were analyzed on a NovoCyte Flow Cytometer (ACEA Biosciences).

2.2.2.6.3.3. Intracellular Cytokine Staining

To assess intracellular TNFα production, cytokine secretion from cells was prevented by using 10µg/ml Brefeldin A (Biolegend) during 5 hours of stimulation period. Cells were fixed and washed as described in the previous section (2.2.6.3.1). Cell pellets were mixed with anti-mouse TNFα-PE (1µg/ml) in 100µl permabilization medium (Medium B from ThermoScientific) and incubated for 30 min in dark at RT. Cells were washed twice in FACS Buffer and resuspend in 200µl PBS. Intracellular TNFα was analyzed on a NovoCyte Flow Cytometer (ACEA Biosciences).

2.2.2.7. In vitro T cell Proliferation Assay

2.2.2.7.1. T Cell Isolation via Magnetic Separation

Splenocytes of four BALB/c mice were prepared as described previously (2.2.5.3) and stained with 10µM CFSE dye (2.2.3.1). Splenocytes of four spleen were pooled and cell concentration was determined for magnetic separation after washing. MACS mouse naïve $CD4^+$ T cell isolation kit was used according to the manufacturer's protocol (Miltenyi Biotech). Briefly, cells were centrifuged, the pellet was dissolved in 400µl MACS Buffer (Appendix) and 100µl of biotin antibody cocktail per 10^8 cells was added to the cell suspension to label non-T cell populations. After incubation for 15 minutes on ice, 300 µl of MACS Buffer and 200 µl of anti-biotin micro beads per 10^8 cells were added and cells were further incubated on ice for 15 minutes. Following washing with 10 ml MACS Buffer twice, pellet was resuspended in 500 µl MACS Buffer. Cell suspension was then applied onto a pre-hydrated LS column attached to a magnet and the column was washed with 3 ml MACS Buffer three times. Unlabeled T cells enriched in the effluent was collected into a collection tube and spun down at 300 x g for 5 minutes. Pellet was resuspended in 800 µl MACS Buffer. $CD62L^+$ micro beads per 10^8 cells were added and incubated for 15 min on ice. Following washing, cells were resuspended in 400µl MACS Buffer and applied onto

a pre-hydrated MS column attached to a magnet. The column was washed three times with 500µl MACS Buffer. Then, the column was removed from the magnetic field and placed into a collection tube. Magnetically labeled $CD4^+$ $CD62L^+$ T cells were flushed out by adding 1ml MACS Buffer onto column and pushing the plunger into the column firmly. This protocol enabled us to obtain naive T cells to be used in proliferation assay.

2.2.2.7.2. CFSE Proliferation Assay

Round bottom 96 well cell culture plates were coated with 2µg/ml anti mouse CD3 antibody (TONBO) (200µl/well) and incubated for 1h at 37ºC. Plate was washed three times with PBS and dried in laminar flow hood until use. Isolated $CD4^+$ $CD62L^+$ T cells were counted and cell number was adjusted to $4x10^6$ cells/ml. 100µl of cell suspension was distributed into wells in the presence of 1.5 µg/ml anti-mouse CD28. T cells were treated with different doses of $EV_{L.tropica}$ (1, 5 and 10 µg/ml) in 100µl for 72h at 37ºC in a CO_2 incubator. CFSE labelled cells were analyzed by flow cytometer (BD Accuri C6, USA) in the FL1 channel.

2.2.2.8. Immunization Studies

2.2.2.8.1. *L. tropica* Footpad Infection Model for Cutaneous Leishmaniasis

2.2.2.8.1.1. Operation Schedule for Immunization and Challenge Experiments

In order to determine the most effective adjuvant to be used in combination with $EV_{L.tropica}$ as a protective vaccine against cutaneous leishmaniasis, the immunoprotective activity of vaccine formulations were examined in *L. tropica* footpad infection model (Figure 2.5). 6-8 week old female BALB/c mice (5 mice/group) were immunized 2 times (intraperitoneal (ip), day 0 and 15) using parasite derived EVs (20 µg/mouse) or heat killed (HK) parasites ($5x10^6$ /mouse) as such or in combination with Th1 type adjuvants: K-ODN (25µg), D-ODN (25µg), cGAMP (15µg), K/TAT nanorings or K+cGAMP (including 15µg K-ODN). Blood was collected from tail veins 2 weeks after each immunization. Sera were prepared by centrifugation at 8000 rpm for 5 min and stored at −20 ˚C until use. Two weeks after booster injection, left footpads were infected with *L. tropica* parasites. For parasite

challenge, stationary phase *L. tropica* promastigotes were centrifuged at 1500xg for 10 min and washed 2 times with 1X PBS. Promastigote pellet was resuspended in 1X PBS, counted and parasite concentration was adjusted to $200x10^6$ parasites/ml. Left footpads were inoculated with $10x10^6$ live promastigotes (50µl/mouse) using a 26 gauge needle.

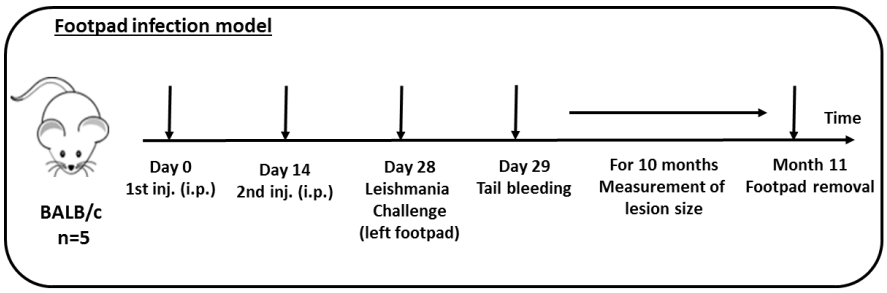


Figure 2.5. Schedule of immunization against cutaneous leishmaniasis, parasite challenge and monitoring of lesion growth

2.2.2.8.1.2. Monitoring of Lesion Growth

Lesion sizes were monitored after inoculation with *L. tropica* promastigotes for the duration of 10 months. Lesions started to develop 4 months post-challenge. From then on, lesion sizes were measured from footpads as height and width using a digital caliper on a monthly basis. Lesion sizes were calculated as size differences between the infected left and uninfected right footpads.

2.2.2.8.1.3. SLA Specific IgG ELISA

Antigen specific IgG1 and IgG2a antibodies produced in immunized mice were detected by ELISA. Immulon 1B plates (Thermo Labsystems, USA) were coated with 10 µg/ml soluble *leishmania* antigen (SLA) in bicarbonate buffer (50ul/well) and incubated overnight at 4˚C. Blocking was carried out in 200 µl blocking buffer (Appendix) for 2 h at room temperature. Plates were washed with ELISA wash buffer 5 times with 5 minute incubation intervals and then rinsed with ddH2O and dried. Following washing, 100X and 200X diluted mouse serum from each animal was added into wells of the first row and serially diluted 2-fold thereafter for IgG1 and

IgG2a ELISA, respectively. After overnight incubation at 4˚C and washing, goat anti-mouse IgG1/AP or IgG2a/AP (Southern Biotech) were 1:2000 diluted in T-cell buffer and were distributed to plates (50ul/well). Following 2 h incubation and a final wash, PNPP substrate was added (Perbio Pierce, USA) and formation of yellow color was followed at OD 405 nm using an ELISA reader (Thermo Scientific).

2.2.2.8.2. Immunization to determine antigen Specific Th1/Th2/Th17 Cytokine Levels

2.2.2.8.2.1. Operation Schedule for Immunization

8 week old male BALB/c mice (5 mice/group) were immunized 2 times (subcutaneous (sc), day 0 and 10) using parasite derived EVs (20 μg/mouse) or heat killed (HK) parasites ($5x10^6$ /mouse) as such or in combination with Th1 type adjuvants: K-ODN (25μg), cGAMP (15μg) and K+cGAMP (including 15μg K-ODN). Seven days after booster injection, mice were sacrificed and spleens were removed (Figure 2.6).

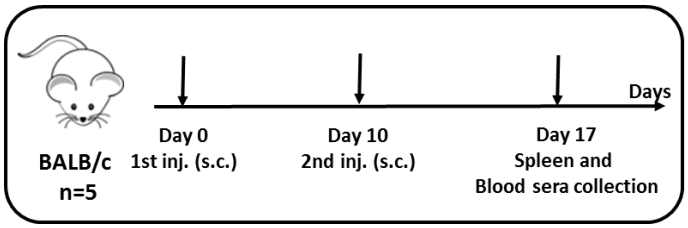


Figure 2.6. Schedule of immunization against cutaneous leishmaniasis for T cell response assessment.

2.2.2.8.2.2. Incubation of SLA Pulsed Splenocytes

Single cell suspensions from whole spleen were prepared as previously described in section 2.2.4.1.4. Concentration of splenocytes was adjusted to 10^7 cells/ml and duplicate of 100μl cell suspensions from each mouse were layered in 96 well round bottom tissue plates SLA was prepared according to the protocol described in section 2.2.2.3. Concentration of SLA solution was adjusted to 100μg/ml and 100μl was added to one well of each mouse whereas paired wells were left untreated as negative controls. Following 48 h incubation, splenocytes were removed by centrifugation at

300xg for 10 min and supernatants were stored at -20 ˚C until use for cytokine detection.

2.2.2.8.2.3. Detection of Th1/Th2/Th17 Cytokine Levels in Splenocyte Culture Supernatant by Cytometric Bead Array

In vitro SLA specific cytokine responses of splenocytes from immunized mice were assessed via Cytometric Bead Array (CBA) using the Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, USA) according to the manufacturer's protocol. This kit contains seven bead populations with distinct fluorescence intensities. These populations are mixed together to form the bead array, which is resolved in a red channel of a flow cytometer (Figure 2.7). Beads are coated with capture antibodies specific for IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17A, and IL-10 proteins, that enables detection of multiple cytokine proteins simultaneously in samples. Briefly, lyophilized standards were reconstituted and two fold serially diluted (20-625 pg/ml). For each assay sample to be analysed, 10µl aliquote of each mouse Th1/Th2/Th17 capture bead were pooled in to a tube and 50µl of the mixture per well was added in round bottom 96 well plate. Then, 50µl standards and unknown samples were mixed with 50µl PE labelled mouse Th1/Th2/Th17 detection reagent in wells containing capture beads. After 2 h incubation at room temperature, 100 µl wash buffer was added, centrifuged at 200x g for 5 min and supernatants were discarded. Washing was repeated with 200 µl wash buffer and final pellet was resuspended in 200 µl. Samples were acquired on an Accuri C6 Flow Cytometer (BD Biosciences, USA) by using a template as instructed in the manual and results were analyzed using FCAP array software.

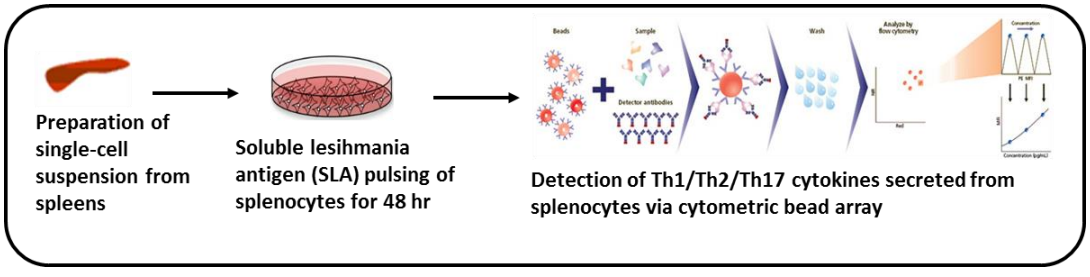


Figure 2.7. Production of Th1/Th2/Th17 cytokines from immunized mice splenocytes in response to SLA treatment.

2.3. Statistical Analysis

For statistical analysis, Mann Whitney U-test was used to compare untreated vs. treated groups and Kruskal Wallis was used to test the difference among groups. For indicated experiments, t-test was used after normality and equality of variances were verified by Shapiro Wilk Test and F test. For all comparisons, 95% confidence intervals were used and P values of <0.05 were considered significant. All analyses were done with the GraphPad Prism 6.1 (GraphPad Software Inc.) or R Studio software.

CHAPTER 3

RESULTS & DISCUSSION

3.1. Therapeutic applications of CpG ODN nanorings

3.1.1. Use of CpG ODN Nanorings in Tumor Therapy

The antitumor activity of K/Tat nanorings was examined in two different mouse tumor models for therapy. In the first EG.7 thymoma model, the nanorings were employed as a vaccine adjuvant to enhance tumor-antigen (OVA) specific adaptive immunity. In the second tumor model in which the B16 F10 mouse melanoma cells do not express a known tumor specific antigen, nanorings were administered as a stand-alone immunostimulatory/immunotherapeutic agent. Both tumors were subcutaneously inoculated in the dorsal skin region of C57BL/6 mice. Since the EG.7 model incorporated therapeutic vaccination with the model tumor antigen OVA+ vaccine adjuvant (K ODN versus CpG nanorings), it was expected that tumor specific immunity would depend on the successful generation of OVA-specific cytotoxic T cell responses. In contrast, in the B16 tumor melanoma model, an immune stimulatory agent (K-ODN, CpG ODN nanorings or K ODN complexed with a fungus derived polysaccharide SPG) was administered in the absence of any tumor antigens and it was expected that tumor rejection would depend on enhancement of natural killer cell (NKs)-mediated killing. The following Sections, summarizes the results of these two studies.

3.1.1.1. Anti-tumor vaccine adjuvant activity of CpG ODN nanorings in OVA expressing EG.7 tumor model

The antitumor effect of vaccination with nanoring and tumor antigen OVA was examined in a therapeutic tumor vaccination model. C57BL/6 mice were inoculated with OVA-expressing EG-7 tumor cells. Mice were then treated with OVA+K-ODN or OVA+K/Tat (nanoring) by intraperitoneal injection when tumors became of palpable size (≥50 mm^3), followed by a booster injection one week after the first immunization. Tumor growth was monitored for 10 days and volumes were recorded. Results showed that tumor growth was significantly reduced in OVA + K/Tat treated groups in comparison to OVA + K-ODN–treated groups ($p = 0.0005, 0.0004, 0.0003, 0.0024$, and 0.0001 on days 2, 4, 6, 8, and 10, respectively) (Figure 3.1.A). At the end of 10 days, mice were sacrificed, tumors were excised and weighed. The average of tumor weight in OVA+K ODN treated group was 4-fold more than the average of tumor weights in OVA+K/Tat treated group ($p= 0.0001$) (Figure 3.1.B). The tumor sizes were significantly larger in OVA+K ODN treated mice as presented in Figure 3.1.C. These results suggest that K/Tat nanorings are more effective than the K-ODN as a vaccine adjuvant to trigger an immune response culminating in delayed tumor growth.

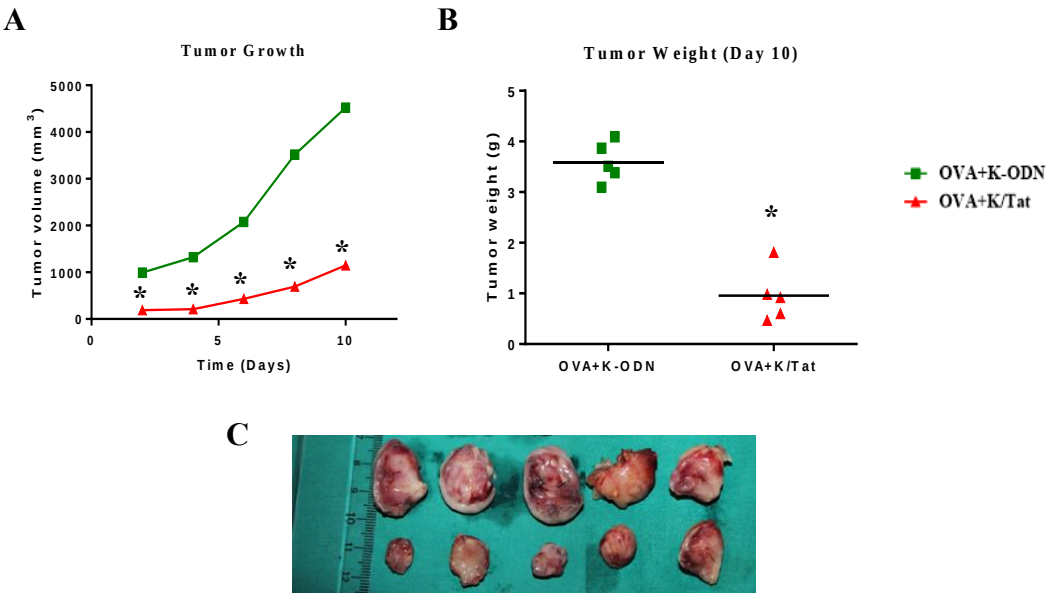


Figure 3.1. Subcutaneous EG.7 thymoma tumor progression following therapeutic vaccination with OVA+K-ODN or OVA+K/Tat.

OVA expressing EG.7 cells (2.5x106) were inoculated subcutaneously in C57B/6 mice. 25µg of OVA + 10 µg of K-ODN or 25µg of OVA + 10 µg of K/Tat nanorings were intraperitoneally injected twice (1 week apart) when tumors became palpable (≥50 mm3). Tumor volumes were recorded (length x width x height) throughout 10 days (A). Tumors were weighed after surgical removal (B) and photographed (C). Statistical comparison between groups was based on two-tailed unpaired Student's t test (n =5). * indicates $p<0.005$.

Tumor specific cytotoxic T cells (CTLs) are key effector cells, mediating recognition and killing of cancer cells (Wakita, 2006). Therefore, use of immune stimulatory agents to enhance the number and functionality of CTLs constitute a major goal of anti-tumor therapeutics. To assess CTL induction by OVA+K-ODN and OVA+K/Tat nanorings in the EG.7 thymoma model, splenocytes of tumor bearing immunized mice were incubated with brefeldin A in the absence or presence of SIINFEKL OVA peptide (MHC Class I associated CTL epiptope) for 5hrs. Cells were stained by CD8-Pe/Cy5 and IFNγ-PE for flow cytometeric analysis. Increase in total CTL numbers were represented as % CD8+ lymphocytes (Figure 3.2.A) whereas antigen specific IFNγ production from CD8+ CTLs was reported as % producers and fold-induction in number of producers responding to antigenic re-stimulation (Figure 3.2.B, dot plots and fold-induction bar graph, respectively). It was shown that K/Tat adjuvanted treatment stimulated an evident expansion in the number of CD8+ T cells ($p = 0.0001$, Figure 3.2.A). Stimulation of antigen specific IFNγ production from this T cell population ($p=0.0017$, Figure 3.2.B) was 4-fold higher when compared to K-ODN adjuvanted treatment. These results demonstrated that OVA+K/Tat adjuvanted vaccine was more efficient in triggering tumor specific cytotoxic T cell response when compared to immunization with OVA+K-ODN.

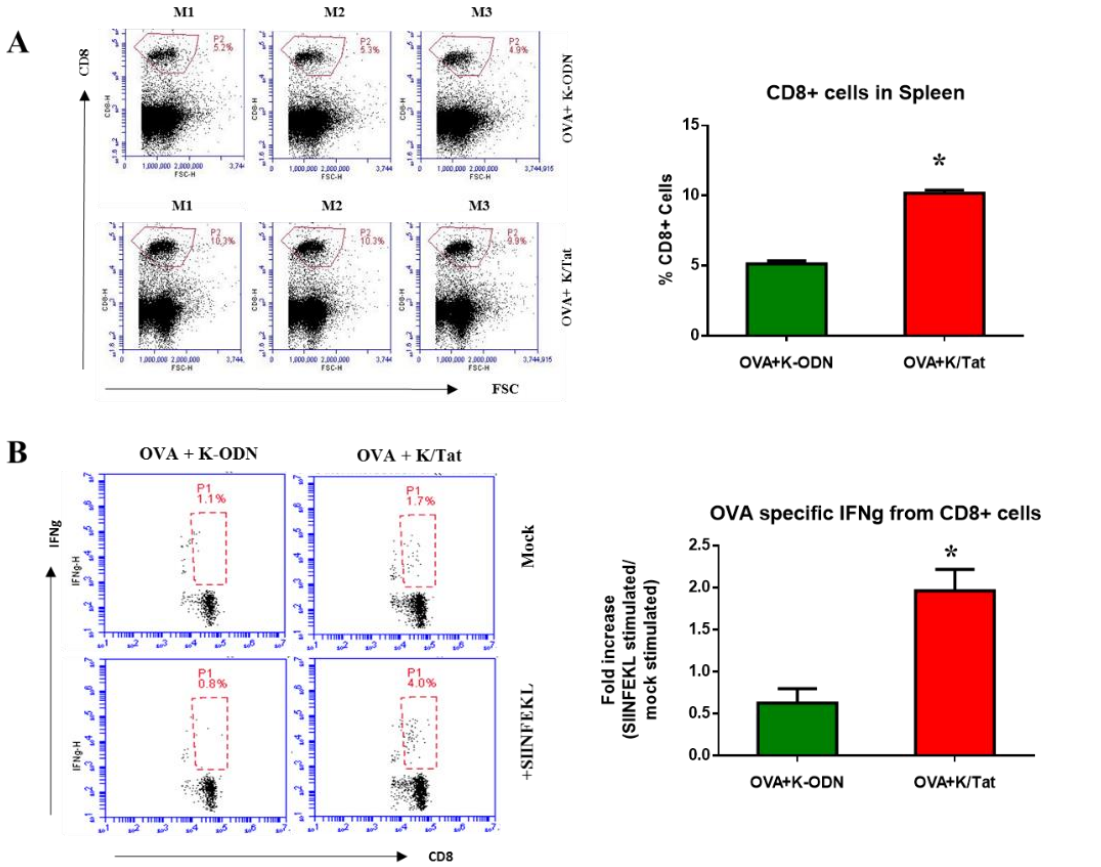


Figure 3.2. Cytotoxic T cell expansion and antigen specific IFNγ production in spleen cells isolated from immunized mice.

Splenocytes (2.5x106/ml) were incubated with or without SIINFEKL peptide (2 µg/ml) for 5 hours in the presence of brefeldin A (10 µg/ml). Cells were stained for cell surface CD8 expression (A), and intracellular IFNγ production from gated CD8+ cells was determined by flow cytometry (B). Statistical comparison between groups was based on two-tailed unpaired Student's t test (n =3). * indicates $p<0.005$.

3.1.1.2. Stand alone antit-umor immunotherapeutic activity of CpG ODN Nanorings in the B16 melanoma tumor model

CpG ODN nanorings have been tested as stand-alone immunotherapeutic agents that can potentially activate NK cells. Peri-tumoral administration of nanorings would cause destruction of the tumor provided that the nanorings can directly activate NK cells. To test this possibility, C57BL/6 mice were first challenged with B16 F10 melanoma cells. Animals were monitored for tumor development and following the

day of palpable tumor (≥50 mm^3) formation, mice were treated peri-tumorally with K-ODN, K/Tat or K-SPG using a one day-on and one day-off treatment strategy. In this model, we have also included another type of K CpG ODN based immunotherapeutic agent (K-SPG), a formulation generated by complexation of a modified K type CpG ODN with the β-glucan schizophyllan (SPG). This TLR9/dectin-1 targeting immune stimulatory agent was previously shown to possess potent vaccine adjuvant activity (Kobiyama, 2014) and was included here to assess its activity in parallel to K/Tat nanorings. Tumor volumes were recorded every other day for the duration of three weeks and graphed as mm^3. Kruskal Wallis tests revealed no significant difference amoung treatment groups and control in terms of tumor size (Fig. 3.3.A. p= 0.99 and Figure 3.3.B. p=0.12). Thus we concluded that K/Tat nanorings, K-ODN and K-SPG peritumoral treatments had no beneficial effects as stand alone anti-cancer agents in B16 F10 melanoma tumor model.

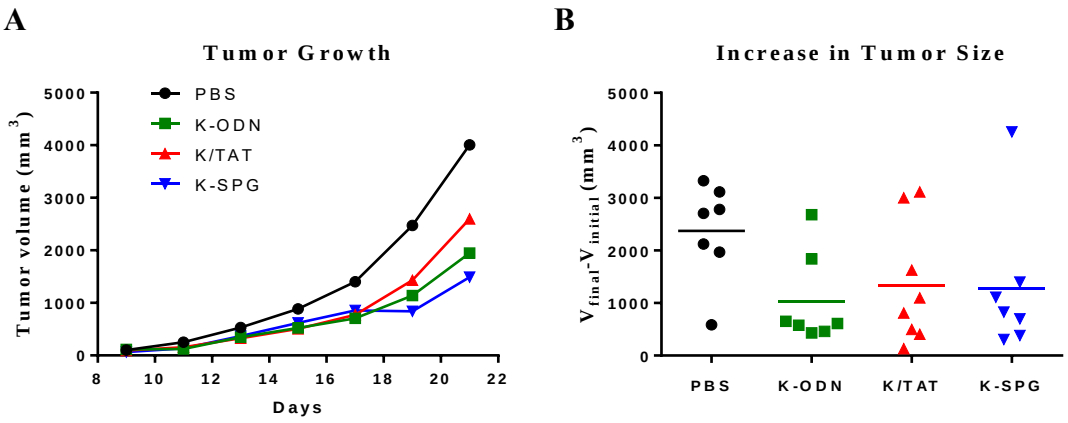


Figure 3.3. Subcutaneous B16 F10 mouse melanoma tumor progression following treatment with stand alone immunotherapeutic agents.

B16 F10 tumor cells (0.5x106) were injected subcutaneously into C57BL/6 mice. After tumors reached a palpable size (≥50 mm3), 15 µg of K-ODN (n=7); 10µg K-SpG (n=6); 15µg K/Tat (n=8) and PBS (n=7) were administered peritumorally using a one day-on and one day-off treatment protocol. Tumor volumes were recorded every other day for three weeks (A). Increase in tumor size was calculated by subtracting final tumor volumes from initial volumes of tumors (B). Multiple comperison between groups was based on Kruskal Wallis Test.

Collectively, antitumor effects of OVA+K/Tat nanorings was investigated in two therapeutic tumor models. The results of experiments imply that K/Tat nanorings are more effective than K-ODN in terms of hindering tumor progression by inducing antigen specific CTL-mediated IFNγ production and CTL-specific EG.7 thymoma killing. However, K/Tat nanorings were not effective in B16 F10 melanoma tumor model in which tumor elimination is dependent on antigen independent NK cell activity. In conclusion, K/Tat nanoring may be a powerful candidate as anti-tumor vaccine agents in antigen-dependent tumor treatment in which mainly cytotoxic T cells play a major protective role.

3.1.2. Determination of the best route of administration of Nanorings

In our previous experiences with K/Tat nanorings, we have verified many times that nanorings are potent Th1 immune response stimulators when injected intraperitonally (a systemic administration route used in mice). The route of administration can impact the outcome of immunization. Therefore, to determine the best route of administration that can trigger optimal antigen-specific immune activation with minimal adverse effects, mice were treated with 15 μg CpG ODN nanorings together with 10 μg model antigen OVA (n=5) intraperitonally (i.p), subcutaneously (s.c), intamuscularly (i.m), intradermally (i.d) or intranasally (i.n) twice on days 0 and 10 in comparison to control mice treated similarly with 10 μg OVA alone (n=3). To investigate the induced antigen-specific immune responses, in mice immunized via alternate routes of administration, serum OVA specific IgG1 and IgG2c levels were assessed (day 17). Antigen specific IFNγ induction, a hallmark of Th1 mediated immunity, was determined following re-stimulation of splenocytes from immunized mice with OVA specific MHC Class I peptide or intact OVA antigen for 48 hours (day 17). Moreover, to determine whether or not the vaccine would trigger any unwanted allergic adverse events, OVA specific IgE production (negative indicator of vaccine safety) was also quantified in sera collected from mice in all groups (Nagao, 2016).

Sera of immunized mice were collected from hearts on day 17 and examined for OVA specific IgG1, IgG2c and IgE antibody production. Results were reported as anti-OVA antibody titers (Figure 3.4). K/Tat nanoring adjuvanted immunization induced significant levels of OVA specific IgG1 antibody production through the s.c and i.n route ($p= 0.036$ for s.c and i.n) (Figure 3.4.A), whereas OVA specific IgG2c antibody levels were increased significantly through i.p , s.c, i.d and i.n routes ($p=0.036$ for i.p , s.c , i.d and i.n) (Figure 3.4.B). Comparison of titers revealed that intranasal administration induced the strongest OVA-specific IgG antibody responses. Specifically, significance testing among adjuvanted groups demonstrated that the amount of OVA specific IgG1 antibody titers generated via the i.n route was significantly higher than i.p , i.m , i.d routes ($p=0.008$). Similarly, the i.n route induced OVA specific IgG2c titers that was several magnitute higher than those triggered via i.p, s.c , i.m and i.d routes ($p=0.008$). Of note, IgG2c is an important indicator of Th1 type response induction and elevated levels of anti-OVA IgG2c observed in all groups except the i.m route was an important validation for us to show that the nanorings were potent agents supporting Th1 mediated immunity.

To test the safety of the adjuvant, anti-OVA IgE levels in serum was also tested (Figure 3.4.C). There was no significant difference in serum IgE titers between OVA antigen and K/Tat adjuvanted OVA treatment among all tested administration routes. This result indicate that vaccination with antigen plus K/Tat nanorings did not induce an unwanted allergic response in mice, providing evidence for the safety of this vaccination strategy.

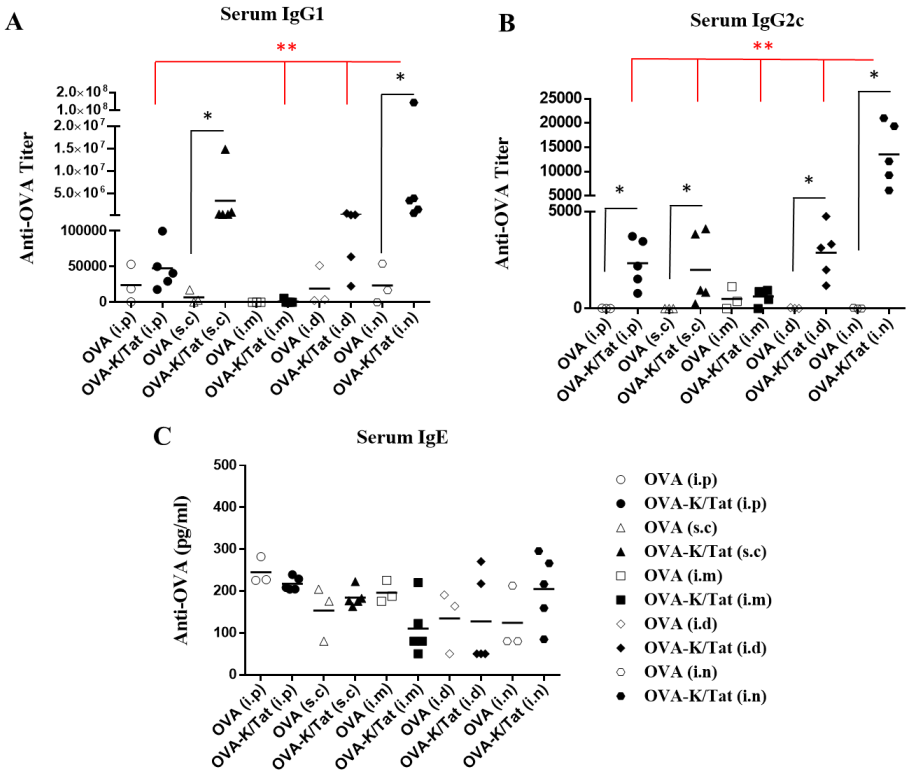


Figure 3.4. OVA specific antibody titers in sera of mice immunized via different routes. Mice were treated with 15 µg nanorings plus 10 µg OVA antigen (n=5) or with 10 µg OVA (n=3) as the control group intraperitonally (i.p), subcutaneously (s.c), intamuscularly (i.m), intradermally (i.d) or intranasally (i.n) twice on days 0 and 10. Mice were sacrificed on day 17 and blood was collected from hearts for investigation of OVA specific IgG1 (A), IgG2c (B) and IgE (C) antibody titers. Comparison of titers between OVA and OVA-K/Tat and for different routes was based on Mann Whitney Test * indicates $p<0.05$; ** indicates $p<0.001$.

To further analyze the best route of administration of nanorings as Th1 inducing vaccine adjuvants, splenocytes of immunized mice were stimulated with OVA (10 µg/ml) or MHC class I specific OVA peptide (10 µg/ml) for 48hr and antigen specific IFNγ was determined. Results showed that re-stimulation with MHC class I peptide or OVA induced significant levels of IFNγ from splenocytes of mice immunized via the i.p and i.n routes as compared to OVA-PBS (Figure 3.5A, P=0.036 for both i.p and i.n route). This response was highly antigen specific since IFNγ secretion was only observed in samples stimulated with MHC class I peptide or OVA and not in those

without antigenic stimulation (indicated by Med for medium alone treated groups; p=0.008 for both i.n and i.p for no antigen versus antigen re-stimulated groups). Subcutaneous (s.c) or intramascular (i.m) administration of OVA+K/Tat nanorings failed to induce detectable levels of IFNγ in response to peptide and antigen treatment. With intradermal administration (i.d),, there was only a modest MHC class I peptide-induced IFNγ production which was found to be significant when compared to control mice or OVA treated and OVA-K/Tat immunized mice (Figure 3.5.A, p= 0.015 and p=0.031, respectively).

When we tested the significance of intact OVA antigen specific IFNγ production among all immunized groups, i.n route was found to be the most potent inducer of this cytokine when compared to i.d, s.c and i.m routes (p=0.008). The second most effective group was the i.p immunized mice and antigen-specific IFNγ production was found to be significantly higher than s.c, i.d or i.m immunizations (p=0.008; Fig 3.5.B). MHC Class I molecules are expressed on all nucleated cells and play a crucial role in presentation of intracellular antigens to cytotoxic T cells. If splenocytes from immunized mice are re-stimulated with a known MHC Class I binding peptide derived from OVA (i.e, the SIINFEKL peptide used here), the peptide can directly bind to MHC Class I molecules without the need for antigen processing, and the measured IFNγ production would be an indicator of solely antigen-specific CTL activity. Therefore, we tested IFNγ production in response to class I peptide re-stimulation, and found that i.n administration of OVA+K/Tat nanorings resulted in the most potent CTL-dependent cytokine production compared to i.p (p=0.016), i.d, s.c and i.m (p=0.008) routes. The second most successful group (i.p route) was superior to s.c and i.m immunized groups (p=0.008; Figure 3.5.B, blue line indicates test results for i.p; red line indicates results for i.n).

Our previous study demonstrated that K/Tat nanorings had Th1 inducing vaccine adjuvant activity when administered intraperitoneally (Gungor and Yagci, 2016). However, whether or not this proved to be the most effective route of administration remained unknown. Results of our current research addressed this issue and demonstrated that K/Tat nanorings triggered the highest antigen specific IgG2c titers

and OVA-specific IFNγ production when administered via the intranasal route. Intraperitoneal injection proved to be second most effective route of administration.

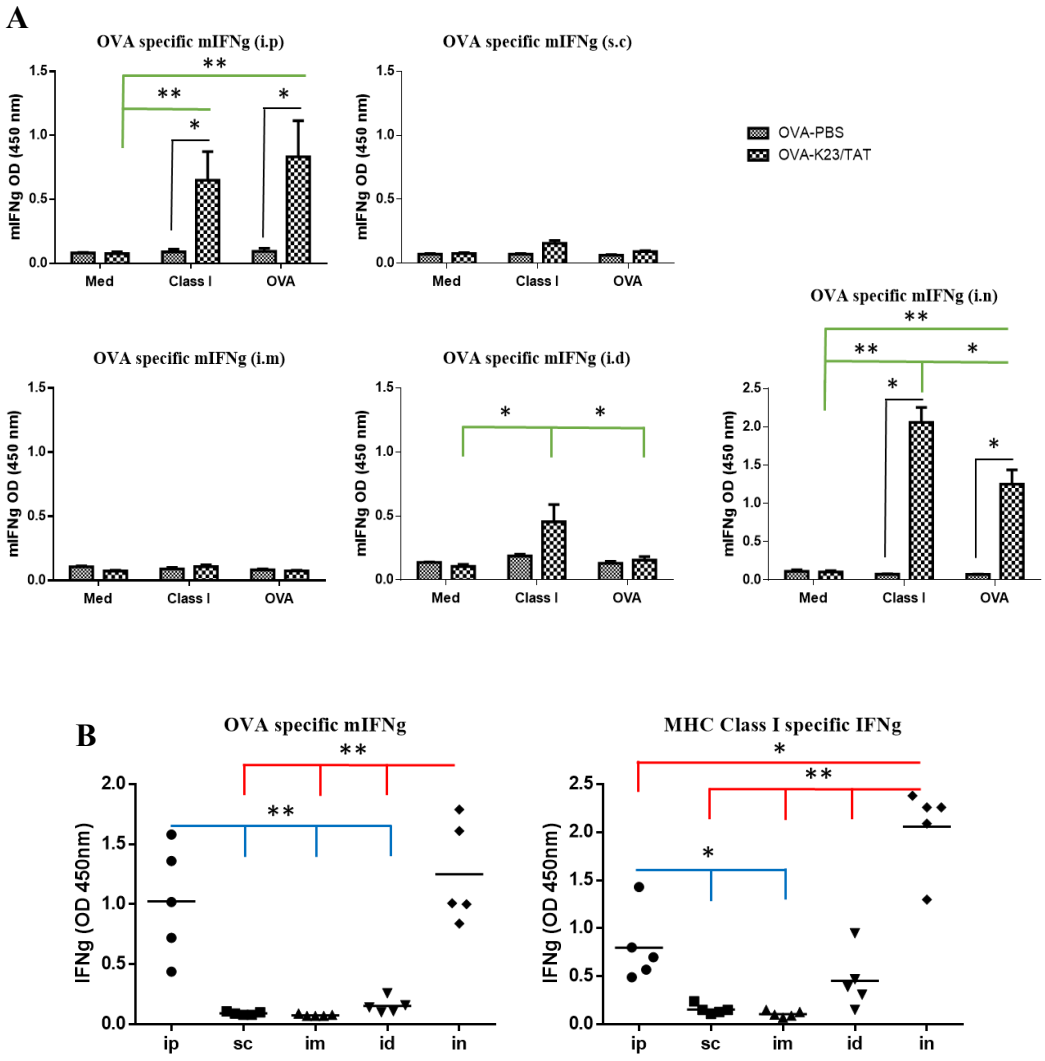


Figure 3.5. Antigen specific IFNγ production from splenocytes of mice vaccinated through different routes. Splenocytes from immunized mice were either untreated (Med for medium alone) or re-stimulated with OVA (10 µg/ml) or OVA peptide (10 µg/ml) specific for MHC class I (OVA 257). Following 48hr incubation, secreted IFNγ levels were determined from culture supernatants by ELISA. IFNγ production (A) per route i.p, s.c, i.d, i.m and i.n (B) or per re-stimulation with OVA antigen and MHC classI peptide. Comparisons between treatments relied on Mann Whitney Test. * indicates $p<0.05$; ** indicates $p<0.001$. Blue line indicates test results for i.p; red line indicates results for i.n comparisons.

Since potent adjuvant activity observed via the intranasal route would suggest that the nanorings could be effective mucosal adjuvants, we decided to assess immunological parameters associated with mucosal immunity. For this purpose, the bronchoalveolar lavage (BAL) of immunized mice from groups that can potentially generate mucosal immunity (i.d, s.c and i.n) was collected and OVA-specific IgA and IgG2c titer were determined (Figure 3.6).

In contrast to plasma, the predominant immunoglobulin class in mucosal environments (such as lungs) is IgA, which has a vital role in neutralization of toxins and pathogens (Macpherson, 2008). Therefore, OVA specific IgA was also examined in addition to OVA specific IgG2c from bronchoalveolar lavage (BAL). Results showed that i.d, s.c and i.n administration of OVA+K/Tat nanorings significantly boosted the levels of OVA specific IgA antibodies (Figure 3.6.A; p=0.0005, 0.018 and $2.7x10^{-5}$, respectively) and IgG2c antibodies (Figure 3.6.B; p= 0.037, 0.014 and $3.9x10^{-6}$, respectively) in the BAL when compared to OVA vaccinated controls. Intranasal administration was the most effective method of administration in terms of OVA specific IgA and IgG2c production in the lung.

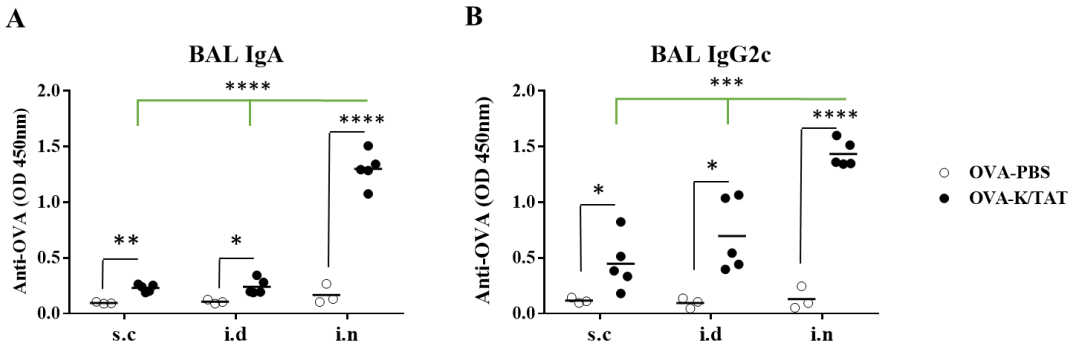


Figure 3.6. OVA specific antibody titers in BAL of mice immunized via different routes. BAL was collected from lungs one week after the last immunization for investigation of OVA specific IgA (A), IgG2c (B) antibody levels. Comparison of antibody levels between OVA and OVA+K/Tat administered via different routes was based on two sample t-test. Normality of data from each treatment were verified by Shapiro-Wilk normality test and equality of variances were tested by F test. Comparisons of groups with unequal variances were subjected to Welch two sample t-test. Multiple comparisons among groups were tested via one-way ANOVA* indicates $p<0.05$; ** indicates $p<0.01$, *** indicates $p<0.001$ and **** indicates $p<0.0001$.

In summary, results of experiments to determine the best route of administration for K/Tat nanorings as vaccine adjuvants showed that the intranasal route was the most effective vaccination strategy followed by the intraperitoneal route among the tested routes of intraperitoneal (i.p), intradermal (i.d), subcutaneous (s.c), intramuscular (i.m) and intranasal (i.n). Antigen specific IgG1 and IgG2c antibody induction in blood serum as well as OVA and class I peptide specific IFNγ production from splenocytes were significantly higher in animals vaccinated through the intranasal and intraperitoneal routes. Furthermore, antigen specific IgA and IgG2c antibody levels were significantly elevated in the mucosa in animals immunized via the intranasal route. Collectively, these findings indicate that CpG ODN nanorings could also be of interest as novel adjuvants for mucosal immunizations.

3.2. EV based vaccine development against Cutaneous Leishmaniasis

3.2.1. Assessment of Growth Kinetics of *L. tropica* patient isolate

Extracellular vesicles (EVs) and soluble *Leishmania* antigen (SLA) were isolated from stationary phase promastigotes. Challenge of immunized mice also depended on late stationary phase metacyclic promastigote production. To determine the day of entry into stationary phase, *L.tropica* growth kinetics of cultures were determined. For this, parasites were counted daily for 8 days and number of parasites per ml culture medium was graphed using log10 scale (Figure 3.7). Experiments demonstrated that promastigotes reached stationary phase on 6th day of culture and the parasite concentration was ~25x10^6 parasites/ml.

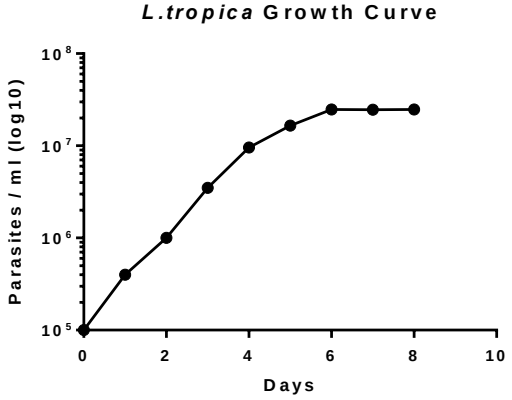


Figure 3.7. Growth Curve of L.tropica promastigotes in culture. Growth of parasites was monitored by counting the number of parasites growing at 26˚C for 8 days.

3.2.2. Characterization of EV*L.tropica*

Extracellular Vesicles (EVs) were purified from *L.tropica* culture supernatants as described in the section 2.2.2.1. The obtained pellets were analyzed by a flow cytometer after staining and labelling with specific markers (such as GP63). For further characterization by size and morphological evaluation, EVs were investigated by Atomic Force Microscopy (AFM).

3.2.2.1. Quantification of EV*L.tropica* by CFSE Staining

The size of the extracellular vesicles range between 30 nm to 1µm. Conventional flow cytometers are suitable for analysis of relatively large EVs (>200nm) because of

inadequate noise/particle discrimination for smaller particle range. Thus, to enhance single vesicle based flow cytometric detection, flourescently labelled EVs were analysed. For this, EVs were isolated from Carboxyfluorescein succinimidyl ester (CFSE) labelled parasites. CFSE is a flourescent dye which covalently couples, via its succinimidyl group, to intracellular molecules, thereby stably labeling cells. CFSE labelled EVs secreted from labelled parasites enabled quantification of EVs in the FL1 channel on a BD Accuri C6 flow cytometer.

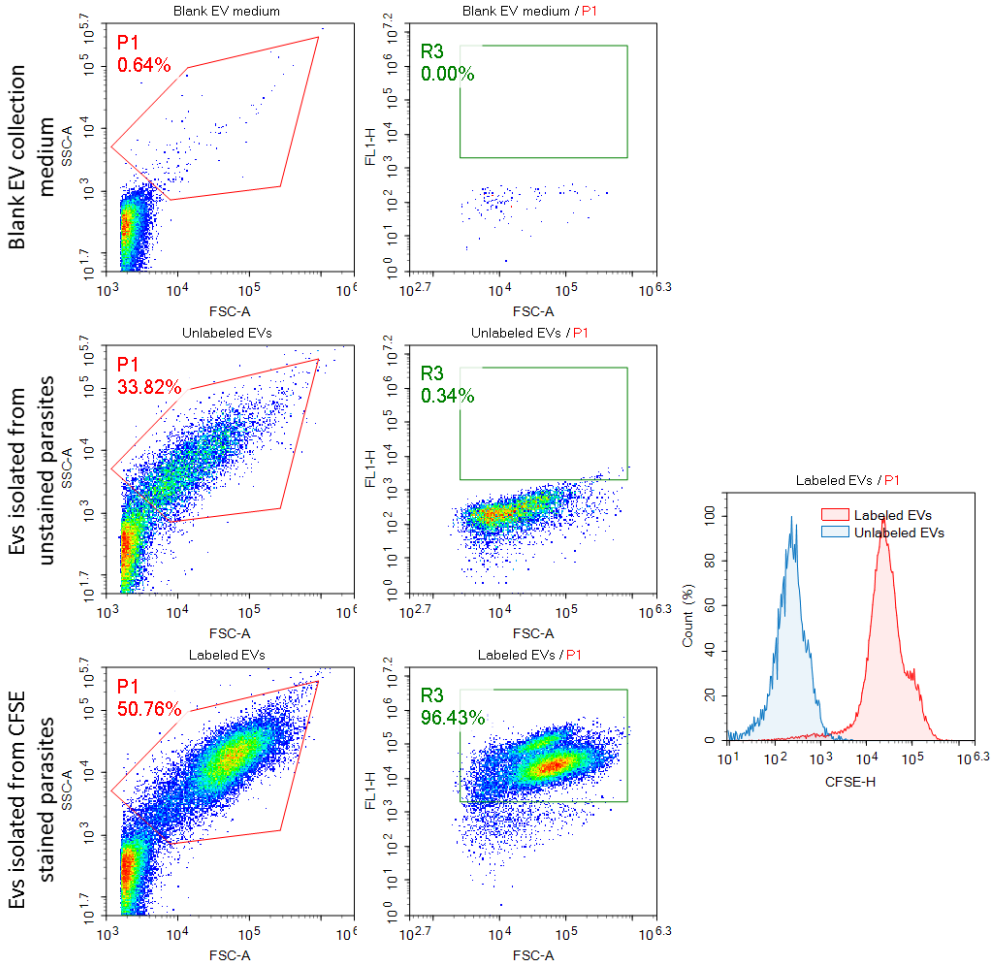


Figure 3.8. Exosome quantification by flow cytometer after purification of exosomes from CFSE pre-stained L. tropica parasites. Blank EV collection medium (devoid of parasites or EVs) served as the negative control in establishing EV unrelated noise. EVs isolated from unstained parasites were used to determine FSC and SSC gate. EVs isolated from CFSE stained parasites were analyzed in FL1 channel. CFSE labeling enabled us to quantification of EVs by flow cytometry.

To analyze small vesicles, 20µl of samples were acquired using a FSC threshold of 7500 in which small particles in the medium was also detected. To eliminate signals associated with noise, a gating strategy based on exclusion of particles from blank EV collection medium but inclusion of those associated with EVs was used and defined as the P1 gate (Figure 3.8). Then, intensity of CFSE signal was analyzed in FL1 channel from P1 gated samples. In EVs isolated from CFSE stained parasites, almost all of the P1 gated particles were CFSE positive (96.4%), verifying the success of the EV isolation protocol from supernatants of the parasite culture.

3.2.2.2. Analysis of GP63 content of EVs by Flow Cytometer

To further characterize *Leishmania* purified extracellular vesicles, use of *Leishmania* EV-specific markers would be desirable. However, in contrast to mammalian EVs that characteristically express specific markers such as Alix and CD63, as of now, there are no established *Leishmania* EV specific markers available for use, except for glycoprotein 63 (GP63), a zinc-dependent metalloprotease, which is a major surface antigen of *Leishmania* promastigotes (Olivier, 2012). It was previously reported that *Leishmania* secreted vesicles contain GP63 antigen (Isnard, 2012). Therefore, GP63 content of isolated *Leishmania* EVs were also analyzed. Anti *Leishmania* GP63-FITC antibody labelled and unlabeled EV samples were acquired (FSC threshold 5000) and analyzed on a NovoCyte flow cytometer (Figure 3.9). EVs were gated in FSC versus SSC plot by exclusion of the background signal of particles present in blank medium (Figure 3.9.A). GP63 positive population among gated EVs were determined by comparing the signal of unlabeled and labeled EVs in FL1 channel (MFI for unlabeled and labeled EVs were 2847 and 54965, respectively). Results showed that 88.4% of the EVs were positive for the *Leishmania* specific marker GP63 (Figure 3.9.B), demonstrating the vaccine potential of EVs in terms of their rich antigen content.

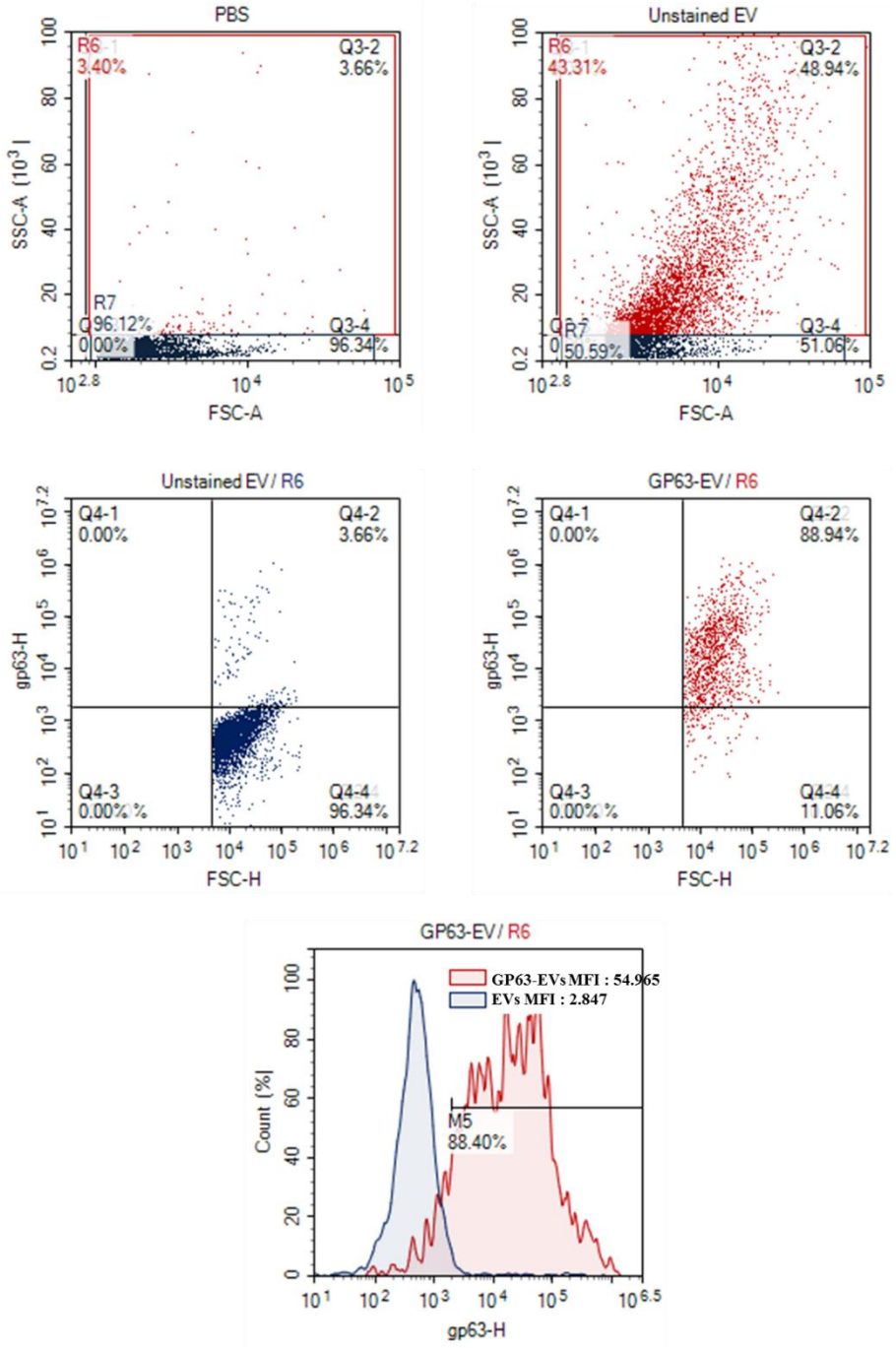


Figure 3.9. Analysis of Leishmania specific GP63 marker on EVs. 100µl of EVs were stained with 1µg/ml FITC conjugated anti-leishmania GP63 antibody for 1 hr at RT. 20µl of EV suspension was analyzed by flow cytometer. Gating on EVs was achieved by excluding signals associated with blank medium FSC-SSC plot (A) and GP63 positive EVs (88.4%) were detected from gated samples in the FL-1 channel (B).

3.2.2.3. Analysis of EV*L.tropica* by Atomic Force Microscopy

Morphology and dry state sizes of parasite derived EVs were investigated with Atomic Force Microscopy (AFM). 10µl of 100X diluted EVs was deposited onto silicon wafers and topographic images were obtained in non-contact mode. Scanning was initiated using a surface area of 10x10 mm^2 and selected parts were successively analyzed on a smaller scales of 3x3 and 2x2 mm^2 for more detailed investigation. AFM micrographs revealed the presence of spherical shaped EVs with various sizes (Figure 3.10.A). Isolated EVs were a mixture of small vesicles with ~100nm in diameter and ~100nm in height (exosomes) and bigger vesicles with ~300nm in diameter and ~150nm in height (microvesicles) (Figure 3.10.B and 3.10.C).

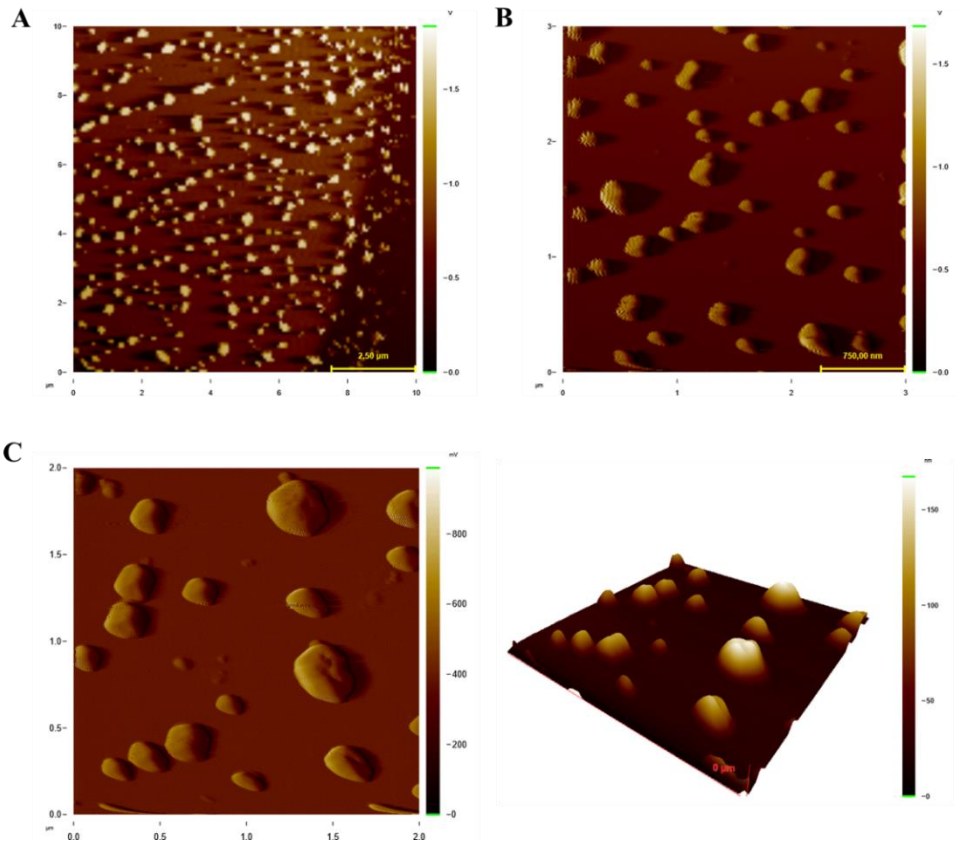


Figure 3.10. Imaging of isolated extracellular vesicles by atomic force microscopy. Scanned area is (A) 10x10 mm^2, (B) 3x3 mm^2 and (C) 2x2 mm^2. Isolated exosomes were spherical in morphology and variable in size from 100nm-350nm in diameter and 50-150nm in height.

3.2.2.4. Analysis of $EV_{L.tropica}$ Associated Nucleic Acids by Flow Cytometer

Acridine orange (AO) is a fluorescent, cell-permeant nucleic acid dye. It is excited at 488 nm and emits green fluorescence when bound to double stranded nucleic acids (emission: 525nm) and red fluorescence when bound to single stranded nucleic acids (emission: 650nm). Thus, AO was used to distinguish double stranded (ds) and single stranded (ss) nucleic acid content of $EV_{L.tropica}$ on a flow cytometer before and after disintegration of EVs by SDS treatment (Figure 3.11.A).

As expected, EV associated ss nucleic acids (47.9 %) and ds nucleic acids (39.7 %) signals were substantially reduced following SDS lysis (9.7 % and 11.4 % respectively), accounting for the vesicular structure of EVs that is destroyed post-detergent lysis (Figure 3.11.B). When AO labeled EVs were electrophoresed on an agarose gel, vesicles were moved against the current towards – pole but SDS treated vesicles lost integrity and released nucleic acids moved towards to + pole (Figure 3.11.C). This indicated that EVs were negatively charged particles associated with nucleic acids that were released when vesicle integrity was disrupted.

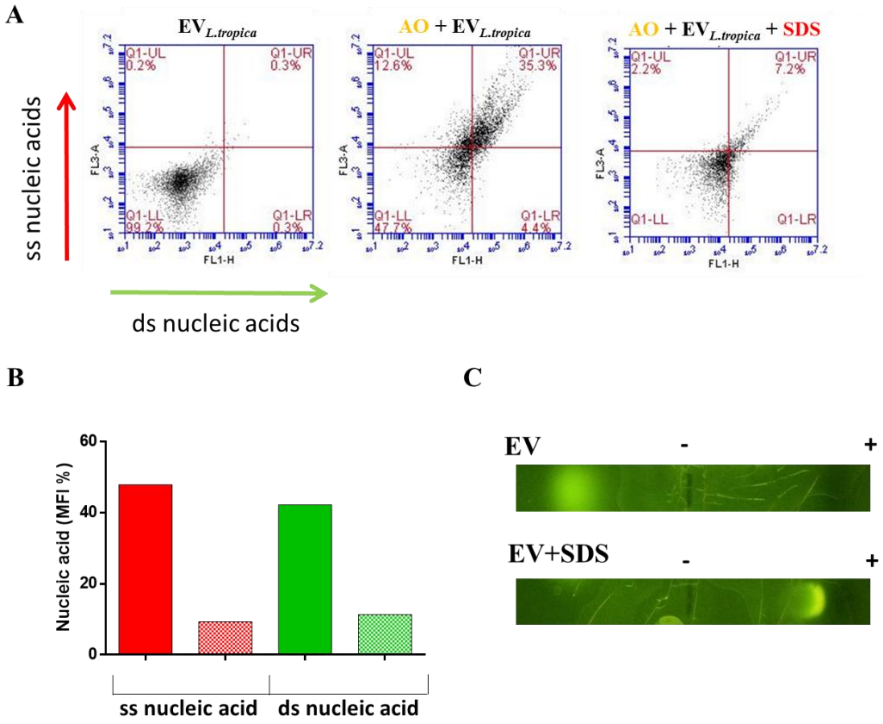


Figure 3.11. Analysis of EV associated nucleic acids. EVs were stained with 20μM AO for 10 min at RT. Intact EVs or those treated with an equal volume of 10% SDS were analyzed by flow cytometry for ds nucleic acid (green channel) and ss nucleic acid (red channel) contents (A). Both ss and ds nucleic acid signals were sharply reduced after SDS treatment (B). Intact EVs moved towards the negative pole during electrophoresis initiated from the middle of a 1% agarose gel, whereas nucleic acids moved towards the + pole only after loss of vesicular integrity following SDS treatment.

3.3. Determination of Immunomodulatory Properties of EV*L.tropica*

Immunomodulatory properties of EVs secreted from *Leishmania* parasite was investigated and EVs were thought to be immunosuppressive predominantly. It was reported that EVs induced Th2 polarization in mice and had a role in disease exacerbation (Silverman, 2010). However combination of EVs with strong immunostimulatory ligands such as CpG ODNs were not studied before. To test the modulatory effect of EVs when combined with either various types of CpG ODNs or complexes and combinations of CpG ODNs, upregulation of activation markers and changes in cytokine production upon stimulation in splenocytes and in bone marrow derived macrophages (BMDM) were studied.

3.3.1. Determination of Immunomodulatory Properties of EV*L.tropica*

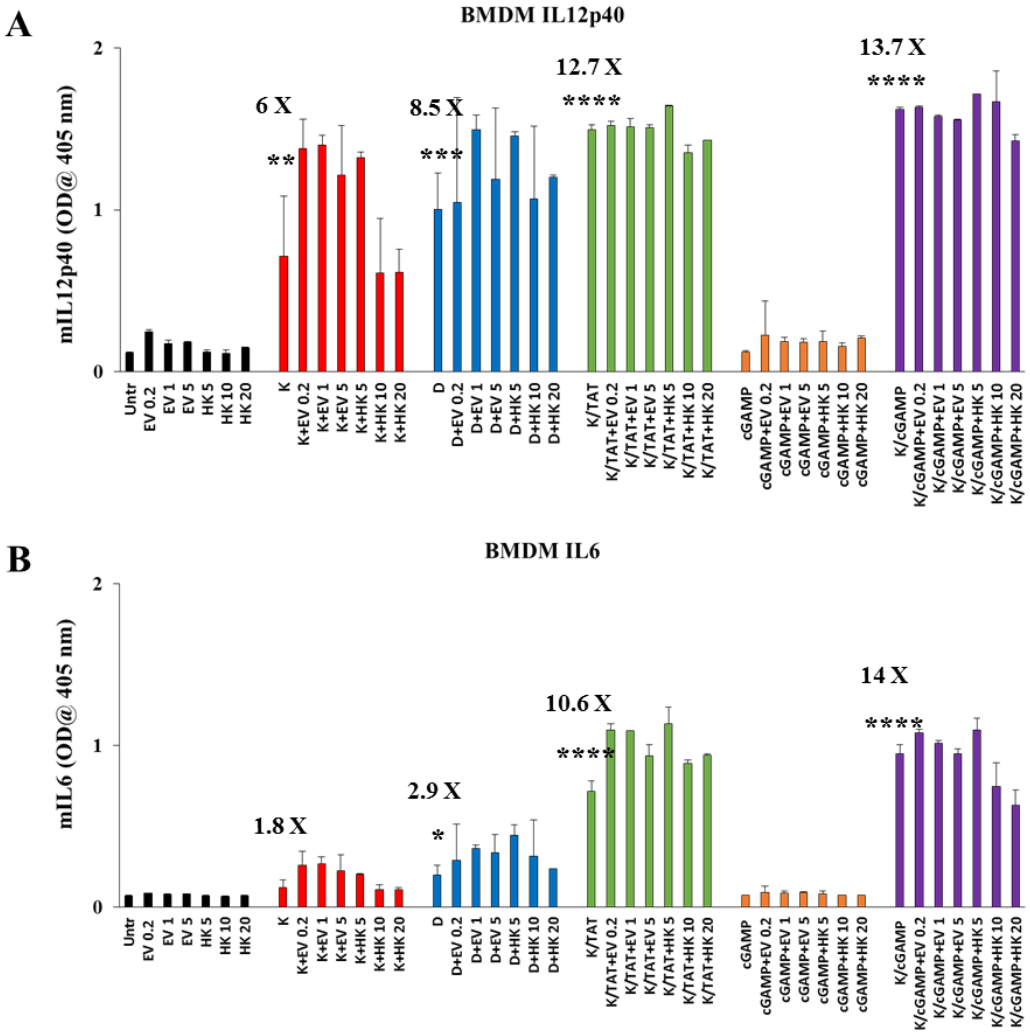
Published results suggest that *Leishmania* EVs may have immunomodulatory properties (Atayde, 2015). For example, one study demonstrated that EVs induced Th2 polarization in mice and had a role in disease exacerbation (Silverman et. al., 2011). Since our aim was to use the EVs as a source of parasite antigens, should these vesicles possessed powerful immunomodulatory properties overriding the immunostimulatory effects of the intended vaccine adjuvants, vaccination using EVs would have been counterproductive. Whether *Leishmania* EVs are capable of interfering with the immunostimulatory activity of adjuvants such as CpG ODNs have never been studies before. Therefore, to test the immunomodulatory effect of EVs, we stimulated bone marrow derived macrophages (BMDM) or splenocytes with CpG ODNs, nanorings, the cyclic dinucleotide cGAMP or its combination with K type CpG

ODN in the absence or presence of EVs and assessed cytokine production or activation marker upregulation.

3.3.1.1. Assessment of immunomodulatory effects of EVs in BMDM

Macrophages constitute the major cell type prone to *Leishmania* infection. *Leishmania* promastigotes differentiate into amastigotes in macrophage acidic endosomes and are known to modulate macrophage responses to proliferate inside macrophage vesicles (Liu, 2012). To study the immunomodulatory effects of EVs on macrophages, bone marrow derived macrophages (BMDM) were stimulated with EVs (0.2, 1, 5, μg/ml) and HK parasites (1:5, 1:10, 1:20 cell to parasite ratio) as such or in combination with K-ODN, D-ODN, K-ODN nanorings, cGAMP or K+cGAMP and pro-inflammatory cytokines produced by macrophages were determined (Figure 3.12). Stimulated groups were compared with untreated controls and the effect of EVs and HK parasites used in combination with the ligands were compared with the results induced by ligands only. IL-12 is an important cytokine, stimulating IFNɤ production which in turn prevents lesion growth in cutaneous leishmaniasis. Among the tested ligands, IL-12 p40 production from BMDM was significantly increased in K-ODN ($p= 0.004$); D-ODN ($p=0.0001$); K/Tat nanorings ($p<0.0001$) and K/cGAMP ($p<0.0001$) stimulated samples (Figure 3.12.A). EVs did not interfere with IL-12 p40 induction. In contrast, HK parasites downmodulated K/Tat induced IL12p40 production ($p=0.012$ for K/Tat+HK1:5; and $p=0.013$ for K/Tat+HK 1:10 versusK/Tat comparisons). Levels of IL-6 were elevated in samples stimulated with D-ODN ($p=0.019$); K/Tat nanorings ($p<0.0001$) and K/cGAMP ($p<0.0001$) (Figure 3.12.B). K/cGAMP induced IL-6 secretion was inhibited by HK (1:20) ($p=0.025$), whereas EVs did not induce or suppress IL-6 production. TNFα levels, a cytokine that activates macrophages to kill intracellular parasites (Liew, 1989), was upregulated following stimulation with K-ODN, D-ODN, K-ODN nanorings and K+cGAMP ($p=0.03$, 0.0005, 0.0002 and 0.0008 respectively) (Figure 3.12.C). EVs and HK did not alter ligand-induced TNFα production. IL-10, a regulatory cytokine which limit the exacerbation of Th1 related inflammation (Kanne and Mosser, 2001), was produced in response to K/Tat nanorings and K-cGAMP ($p<0.0001$) and EVs or HK parasites had no effect on IL-10 production.

In summary, experiments on BMDM showed that K/Tat nanorings and K-cGAMP were the most potent immunostimulatory ligands, upregulating IL12p40, IL-6 and TNFα production 13,11,11-fold and 14,14,5-fold, respectively. These agents also elevated IL-10 secretion 11-fold, an expected regulatory mechanism observed with potent immunostimulatory agents to minimize collateral damage caused by exaggerated inflammation. Experiments on BMDM demonstrated that EVs did not alter the pattern of cytokines secreted in ligand stimulated samples, suggesting that their reported immunomodulatory activity remains too weak to override/interfere with the activity of the tested ligands.



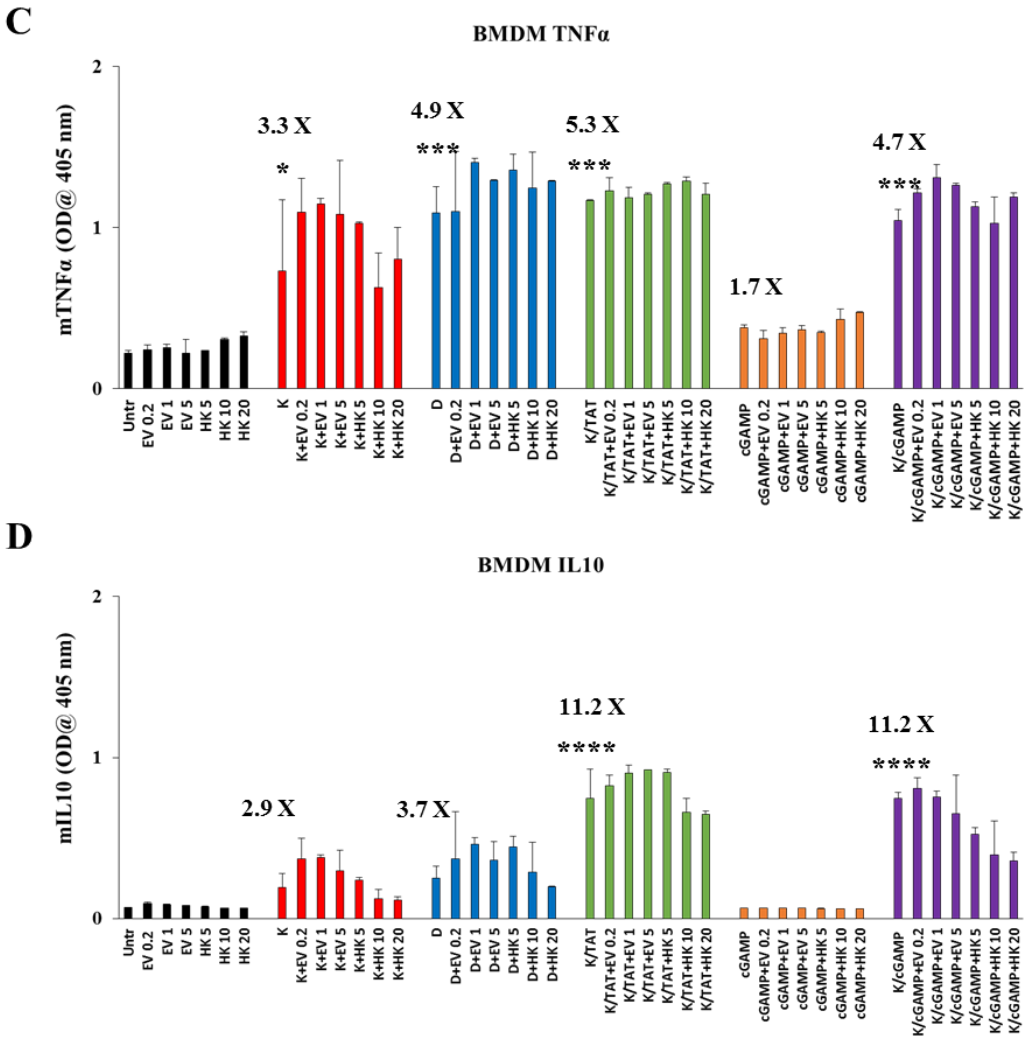
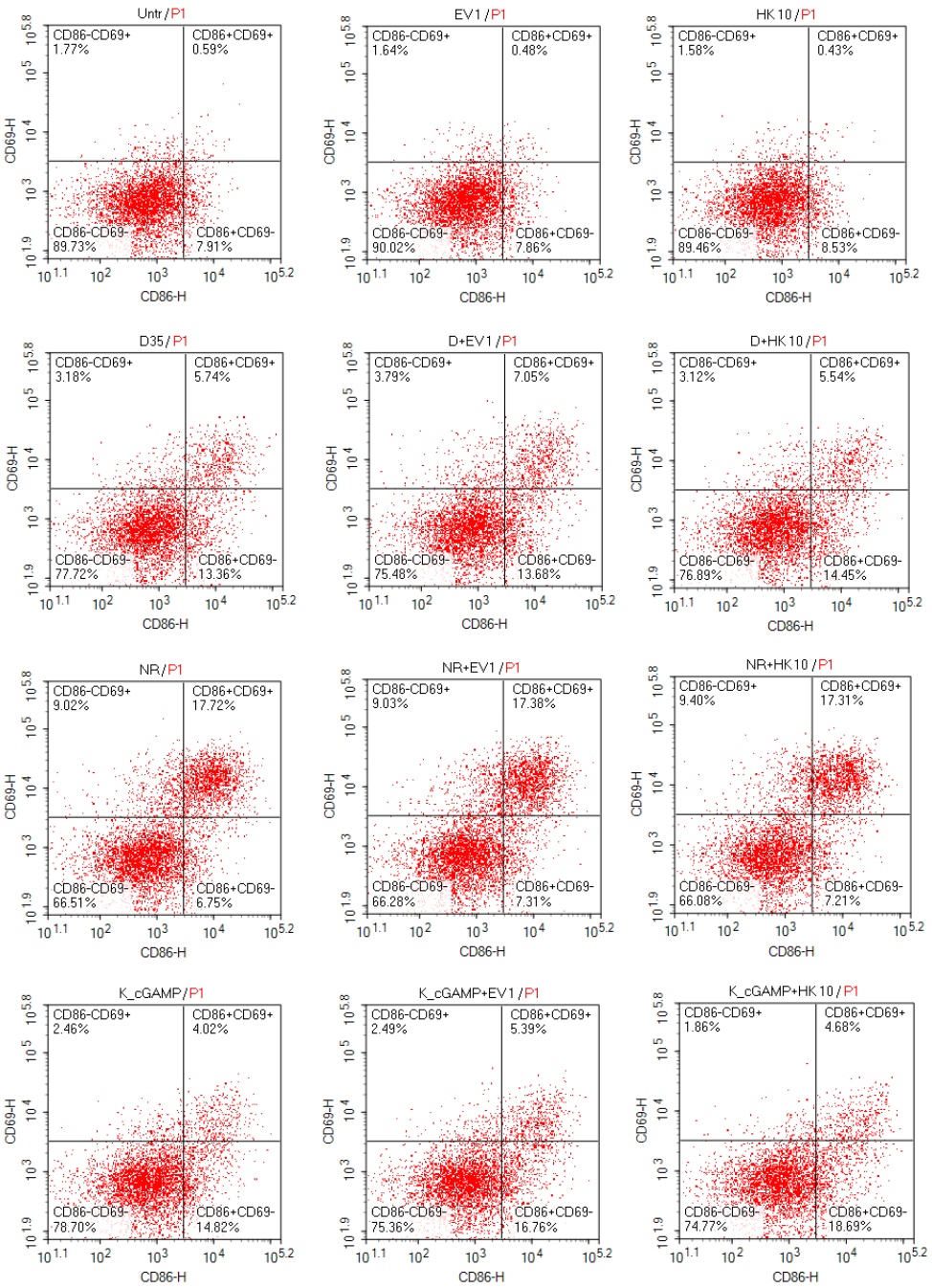


Figure 3.12. Stimulation of BMDM by EV*L.tropica* and ligands. BMDM were stimulated with EVs (0.2, 1, 5 µg/ml) or HK parasites (1:5; 1:10; 1:20 cell to parasite ratio) as such or in combination with K-ODN, D-ODN, K/Tat nanorings, cGAMP, K-cGAMP for 24 hr. Cytokine production IL-12p40 (A), IL-6 (B) , TNFα (C) and IL-10 (D) were detected from culture supernatant by ELISA. Significance among treated groups was determined by one-way ANOVA, Holm Sidak's multiple comperisons test. * indicates $p<0.05$; ** indicates $p<0.01$, *** indicates $p<0.001$ and **** indicates $p<0.0001$.

3.3.1.2. Assessment of immunomodulatory effects of EVs in splenocytes

Possible immunomodulatory effect of EVs and their combinations with various forms of CpG ODNs or cyclic dinucleotides were also tested in splenocytes through monitoring of activation (CD69) and costimulatory molecule (CD86) upregulation in MHC Class II positive antigen presenting cells. Briefly, splenocytes were stained for MHC Class II, C86 and CD69 after 24 h stimulation and analyzed by flow cytometer. MHC Class II positive antigen presenting cells were gated and upregulation of CD86/CD69 was assessed. Representative dot plots for untreated EV (1μg/ml), HK (1:10), D-ODN, K/Tat nanoring, K-cGAMP treated groups are displayed in Figure 3.1.3.A. Percent of CD69/CD86 double positive cells are displayed for all tested groups in Figure 3.13.B. Results indicate that K/Tat treatment caused a 34-fold induction in CD69/CD86 expression whereas D-ODN and K-cGAMP stimulated only 12 and 8-fold increase, respectively. No significant change in CD69/CD86 expression was observed in samples stimulated with EVs or HK parasites alone and these did not alter the activity of other ligands (Figure 3.13.B).

A


Untr / P1
CD86-CD69+ 1.77%
CD86+CD69+ 0.59%
CD86-CD69- 89.73%
CD86+CD69- 7.91%
EV1 / P1
CD86-CD69+ 1.64%
CD86+CD69+ 0.48%
CD86-CD69- 90.02%
CD86+CD69- 7.86%
HK 10 / P1
CD86-CD69+ 1.58%
CD86+CD69+ 0.43%
CD86-CD69- 89.46%
CD86+CD69- 8.53%
D35 / P1
CD86-CD69+ 3.18%
CD86+CD69+ 5.74%
CD86-CD69- 77.72%
CD86+CD69- 13.36%
D+EV1 / P1
CD86-CD69+ 3.79%
CD86+CD69+ 7.05%
CD86-CD69- 75.48%
CD86+CD69- 13.68%
D+HK 10 / P1
CD86-CD69+ 3.12%
CD86+CD69+ 5.54%
CD86-CD69- 76.89%
CD86+CD69- 14.45%
NR / P1
CD86-CD69+ 9.02%
CD86+CD69+ 17.72%
CD86-CD69- 66.51%
CD86+CD69- 6.75%
NR+EV1 / P1
CD86-CD69+ 9.03%
CD86+CD69+ 17.38%
CD86-CD69- 66.28%
CD86+CD69- 7.31%
NR+HK 10 / P1
CD86-CD69+ 9.40%
CD86+CD69+ 17.31%
CD86-CD69- 66.08%
CD86+CD69- 7.21%
K_cGAMP / P1
CD86-CD69+ 2.46%
CD86+CD69+ 4.02%
CD86-CD69- 78.70%
CD86+CD69- 14.82%
K_cGAMP+EV1 / P1
CD86-CD69+ 2.49%
CD86+CD69+ 5.39%
CD86-CD69- 75.36%
CD86+CD69- 16.76%
K_cGAMP+HK 10 / P1
CD86-CD69+ 1.86%
CD86+CD69+ 4.68%
CD86-CD69- 74.77%
CD86+CD69- 18.69%
CD69-H
CD86-H

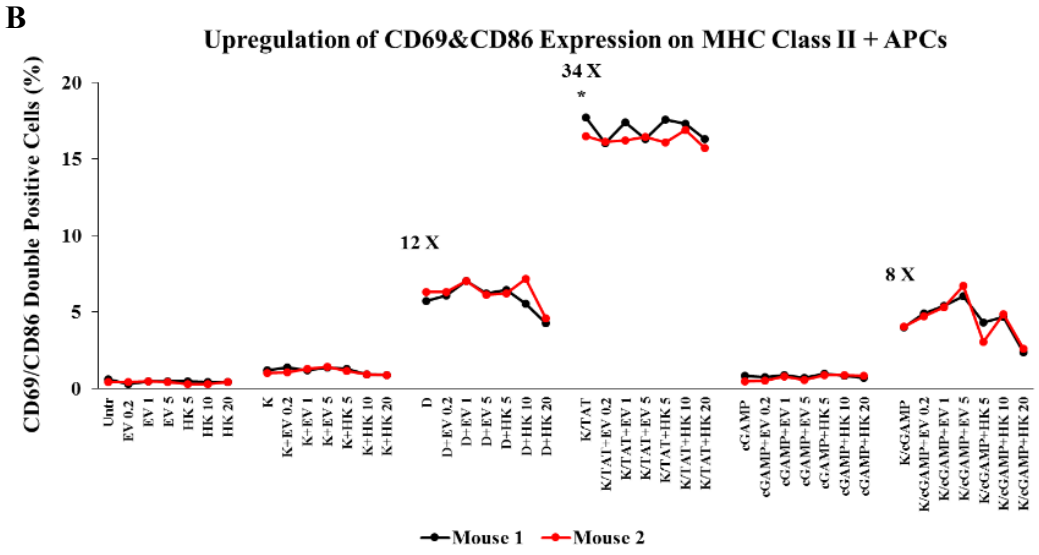
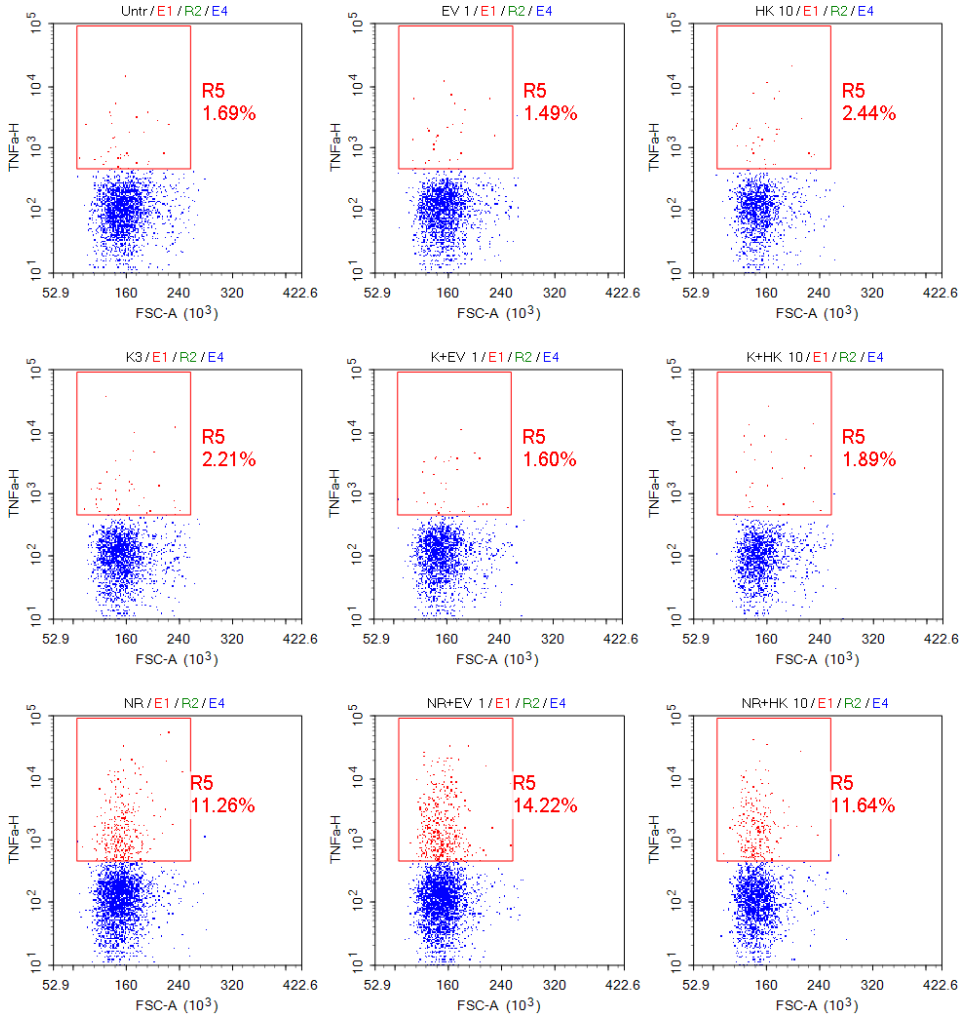


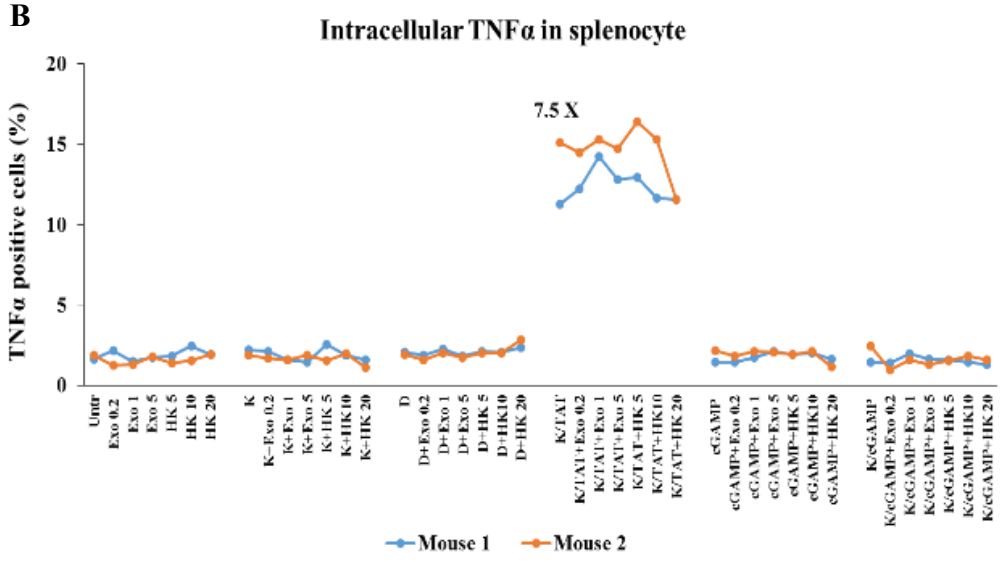
Figure 3.13. Upregulation of activation markers in response to EVL.tropica and ligands. Splenocytes of two Balb/c mice were stimulated with EVs (0.2, 1, 5 µg/ml) and HK parasites (1:5; 1:10; 1:20 cell to parasite ratio) as such or in combination with K-ODN, D-ODN, K/Tat nanorings, cGAMP, K-cGAMP for 24 hr. Upregulation of CD69 and CD86 markers on MHC Class II positive APCs were determined by flow cytometry after cell surface staining. Representative dot plots of selected groups (A). Percent of CD69/CD86double positive cells in MHC Class II gated APCs (B). Multiple comparisons among groups were tested via Kruskal Wallis Test. * indicates $p<0.05$.

To further investigate any immunomodulatory effect of EVs when combined with various ligands, splenocyte induced TNFα and IL-12 production was analyzed by intracellular cytokine staining (Figure 3.14.A-B) and ELISA (Figure 3.14.C), respectively. Results showed that only the K/Tat nanorings induced measurable TNFα production (7.5-fold increase with respect to untreated) from splenocytes. There was no suppression of TNFα induced by K/Tat nanorings in the presence of EVs or HK parasites. IL-12 p40 cytokine levels in supernatant of treated splenocytes showed that D-ODN and K/Tat induced 7-fold and ~13-fold more IL-12 p40 than unstimulated controls. Other treatments (K-ODN, cGAMP and K/cGAMP) stimulated either no IL-12 response or induced very low levels of this cytokine (Figure 3.14.C). EVs and HK parasites did not interfere with cytokine production.

A


Untr / E1 / R2 / E4
R5
1.69%
EV 1 / E1 / R2 / E4
R5
1.49%
HK 10 / E1 / R2 / E4
R5
2.44%
K3 / E1 / R2 / E4
R5
2.21%
K+EV 1 / E1 / R2 / E4
R5
1.60%
K+HK 10 / E1 / R2 / E4
R5
1.89%
NR / E1 / R2 / E4
R5
11.26%
NR+EV 1 / E1 / R2 / E4
R5
14.22%
NR+HK 10 / E1 / R2 / E4
R5
11.64%
TNFa-H
FSC-A (10^3)
52.9
160
240
320
422.6

B


Intracellular TNFα in splenocyte
TNFα positive cells (%)
7.5 X
Mouse 1
Mouse 2

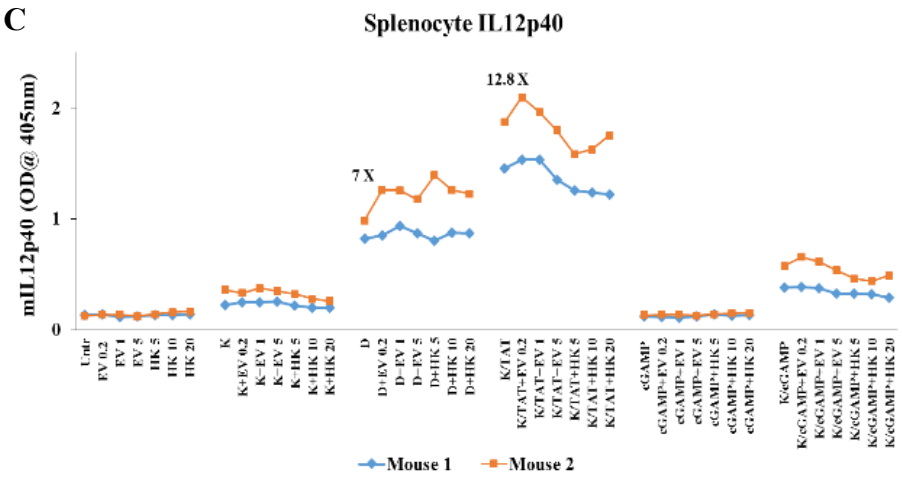


Figure 3.14. TNFα and IL12-p40 production from splenocytes in response to EV$_{L.tropica}$ and ligands. Splenocytes of two BLAB/c mice were stimulated with EVs (0.2, 1, 5 µg/ml) and HK parasites (1:5; 1:10; 1:20 cell to parasite ratio) as such or in combination with K-ODN, D-ODN, K/Tat nanorings, cGAMP, K-cGAMP. TNFα levels were determined by intracellular cytokine staining and flow cytometry following 5h incubation. Representative dot plots of selected groups (A). Percent of TNFα positive cells (B). IL-12p 40 cytokine production upon 24h stimulation was determined from culture supernatant by ELISA. Multiple comparisons among groups were tested via Kruskal Wallis Test. * indicates $p<0.05$.

3.3.2. Assessment of immunomodulatory effects of EVs in T cell proliferation

To assess whether or not EV$_{L.tropica}$ by itself may affect T cell proliferation, an in vitro T cell proliferation assay was performed. For this purpose, $CD4^+$ $CD62L^+$ naive T cells were sorted from mouse spleen by magnetic beads (Trickett and Kwan, 2003) and stained with CFSE dye (Quah, 2007). To provide the signals for T cell proliferation, wells of a 96 well plate were coated with anti-CD3 and anti-CD28 was supplemented as the costimulatory initiator. Cells were then incubated for 3 days in the absence or presence of EV$_{L.tropica}$ (1, 5 and 10µg/ml). Division of T cells were defined by CFSE dye dilution and percentages in dividing subfractions were analyzed by flow cytomery. CFSE signal intensities in the FL1 channel showed that T cells were divided four times during this incubation period (Figure 3.15). EV$_{L.tropica}$ had no effect on T cell proliferation kinetics, suggesting that EVs have no direct immunomodulatory effect on T cells.

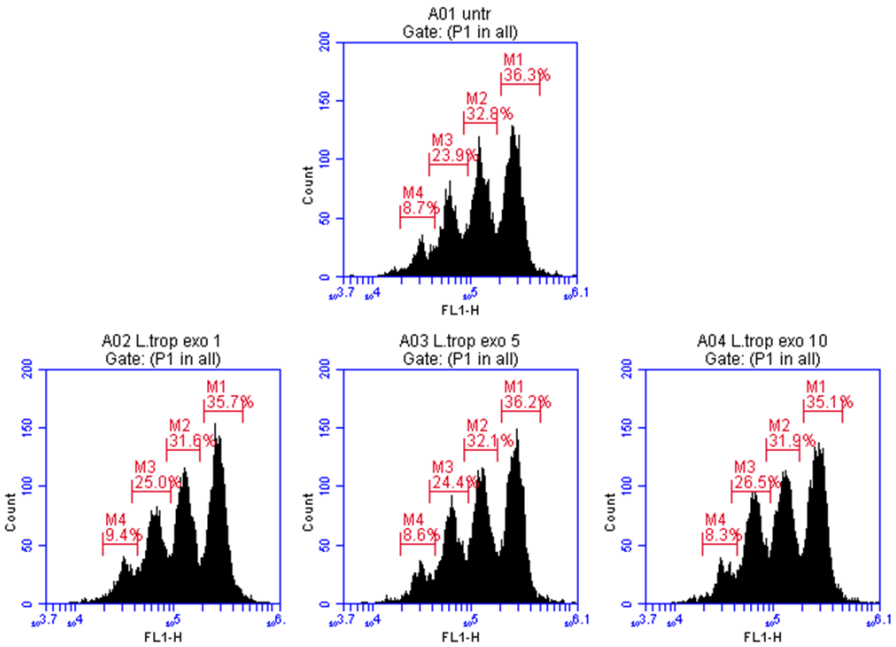


Figure 3.15. Effect of $EV_{L.tropica}$ on T cell proliferation. CFSE labelled $CD4^+$ $CD62L^+$ T cells ($4x10^6$ cells/ml) were treated with different doses of $EV_{L.tropica}$ (1, 5 and 10 µg/ml) on anti-CD3 pre-coated plates (2µg/ml) supplemented with 1.5 µg/ml of anti-CD28 for 72h at 37ºC. CFSE dye dilution was determined on a BD Accuri flow cytometer on the FL1 channel.

3.4. Immunization Studies

Published evidence indicates that *Leishmania* EVs are enriched in parasite antigens, an important attribute making these vesicles suitable for vaccine applications (Schorey et. al., 2015). In our study, we also aimed to determine the most effective adjuvant to be used in combination with $EV_{L.tropica}$ as a protective vaccine against cutaneous leishmaniasis (CL). For this purpose, immunizations and live parasite challenge studies were performed in *L.tropica* susceptible BALB/c mice using a footpad infection model for CL. Susceptibility to *Leishmania* infection in BALB/c mice stems from the development of a Th2 type immune response, supporting lesion development. In contrast, C57BL/c mice are resistant to *Leishmania* infection since in this strain *Leishmania* specific immunity is Th1 dominated, culminating in spontaneous healing of parasite induced lesions (Von Stebut and Udey, 2004). Based on this information

and the results of several published studies emphasizing the role of a Th1 dominated response in establishment of anti-*leishmanial* immunity, we aimed to generate *Leishmania*-specific Th1 immune cell development by combining potent Th1 inducing vaccine adjuvants together with parasite derived EVs in BALB/c mice. To evaluate whether our strategy would be of interest for further vaccine development, the following parameters were monitored:

1. Cutaneous lesions were monitored in naive and immunized mice following live parasite challenge 2. Humoral indicators of a Th1-dominated immune response was assessed through measurement of antigen specific IgG2a production as opposed to Th2-dependent IgG1 secretion

3. Cellular indicators of antigen-specific Th1/Th2/Th17 development was monitored through assessment of T-helper subset specific cytokines from naive and immunized mice splenocytes after antigen re-challenge.

3.4.1. Preliminary *L. tropica* Footpad Infection Model for Cutaneous Leishmaniasis

The immunoprotective activity of the vaccine formulations were tested in *L.tropica* challenged mice. For this, mice were first immunized twice (i.p) with parasite derived EVs as such or in combination with Th1 inducing adjuvants: K-ODN, D-ODN, K/TAT nanorings, cGAMP, or K+cGAMP. Two weeks after booster injection, left footpads of mice were inoculated with $10x10^6$ live *L. tropica* promastigotes and lesion development was monitored for the next 10 months. Footpad swelling was apparent 4 months post-infection and from then on, lesion sizes were measured by a digital caliper at indicated time intervals. Figure 3.16 shows individual lesion sizes measured from mice in each group 4, 5 and 10 months after challenge. As expected, footpad swelling was significant in infected- versus uninfected mice throughout the course of infection ($p= 0.036$ for 4^{th} month; 0.01 for 5^{th} month; 0.0017 for 10^{th} month of infection). Comparison of footpad swelling of infected unimmunized versus infected immunized groups.at 4 months revealed that D type CpG ODN delayed primary lesion development significantly ($p=0.0195$) (Figure 3.16.A). 5 months post infection,

footpad swelling in unimmunized mice were prominent, whereas lesion development in EV, D type CpG and cGAMP immunized mice were more limited when compared to unimmunized mice ($p= 0.01$, $p=0.016$ and $p=0.0397$ respectively; Figure 3.16.B). We observed that after 5.5 months, all groups entered into a temporary remission period. Such a process of temporary remission followed by re-appearance of lesions was also reported in several other cutaneous leishmaniasis models (Gangneus et.al., 2007). In our preliminary in vivo model, apparent footpad swelling was re-observed 7 months post-infection that reached maximal levels by 10 months (Figure 3.16.C). Representative photographs demonstrating the amount of footpad swelling in unimmunized (PBS) and immunized groups 10 months post-infection are also displayed in Figure 3.16.D. At this later stage, EV, D type CpG and K/cGAMP groups had significantly smaller lesions ($p=0.0089$, $p=0.0131$ and $p=0.0255$) than the unimmunized controls. Interestingly, although cGAMP adjuvanted group appeared to control lesion development significantly at earlier time points (4 and 5 months), this group failed to show a protective effect by 10 months. These results suggest that EV, D type CpG and K/cGAMP adjuvanted groups might be more successful in inducing Leishmania-specific memory cell formation. Furthermore, our results also indicate that EVs can be of value when used as a carrier of *Leishmanial* antigens and would be protective as a vaccine despite the fact that their presence during active infection (i.e when released by *Leishmania* parasites at the infection site) might have disease accelerating properties (Silverman et. al., 2012). This experiment was our first attempt to establish an in vivo infection model of cutaneous leishmaniasis and we have encountered several hurdles to be rectified in future studies. One important factor was that we tried to establish this model from an *L.Tropica* human isolate that was not adapted to mice and therefore lesions started developing 4 months (a very late response) after challenge. Based on this data, we are now adapting *L.tropica* to mice and will repeat these experiments using parasites that are more virulent and hence would represent a better model to evaluate vaccination efficiencies.

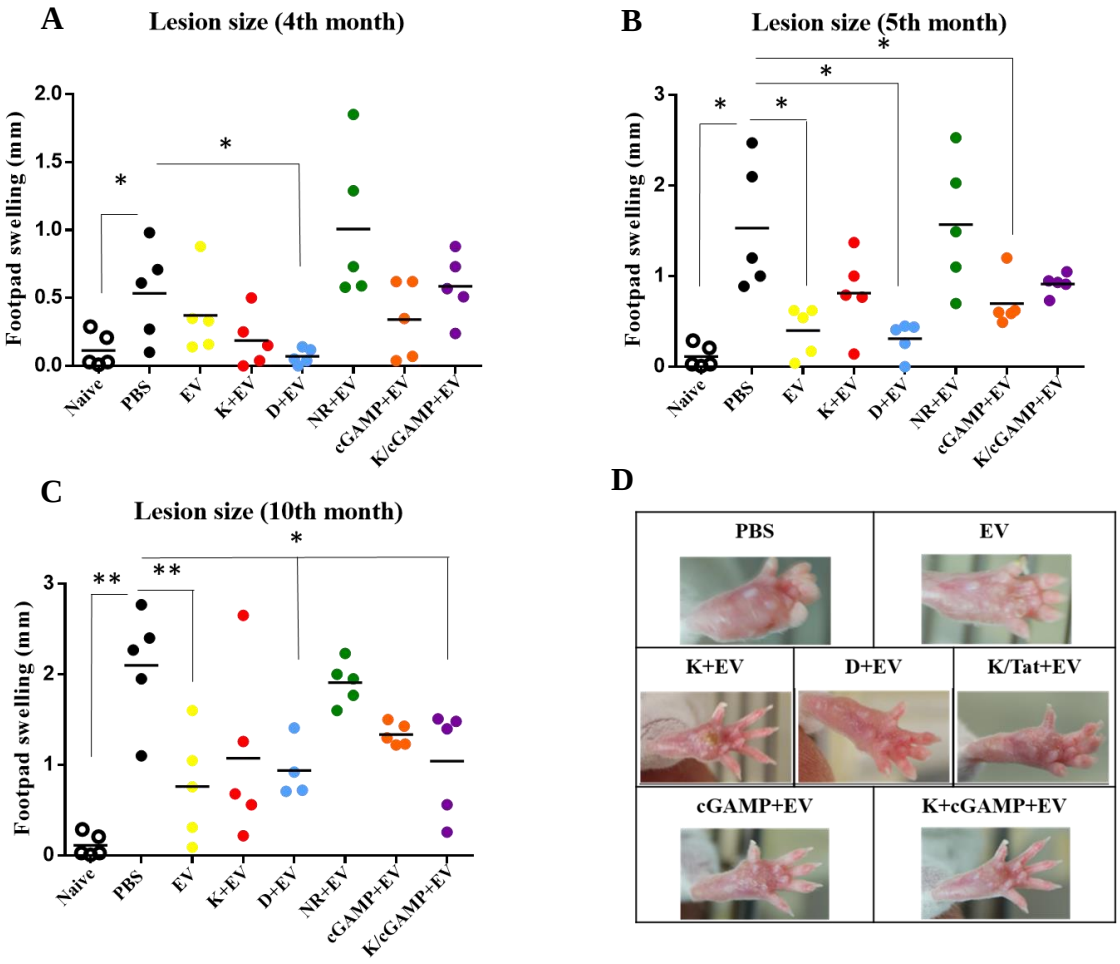


Figure 3.16. Lesion development in uninfected (Naive) or infected footpads of unimmunized (PBS) or immunized mice. Mice were either unimmunized or immunized twice with the indicated groups. Two weeks after booster injection, left footpads were inoculated with $10x10^6$ live stationary phase *L. tropica* promastigotes. Lesion development was monitored for 10 months based on footpad swelling measurement achieved through the use of a digital caliper. Lesion sizes were reported as size difference between the infected left and uninfected right footpads (A,B and C). Statistical comparison of lesion size between PBS and vaccinated groups was based on two sample t-test. Normality of data from each treatment were verified by Kolmogorov-Simirnov test and equality of variances were tested by F test. Comparisons of groups with unequal variances were subjected to Welch two sample t-test. * indicates $p<0.05$; ** indicates $p<0.01$ and *** indicates $p<0.001$.

To determine *Leishmania* specific IgG induced by vaccination, blood was collected from tail veins, IgG1 and IgG2a were assessed from sera by ELISA and results were reported as anti-SLA antibody titers (Figure 3.17). Multiple comparisons of immunized groups with the control group (PBS) revealed that cGAMP+EV immunization induced substantially high levels of SLA- specific IgG1 ($p=0.0001$). K/cGAMP+EV treatment was the next most potent IgG1 inducer ($p=0.0021$). Apart from these two groups, none of the other treatments induced significant IgG1 antibody production (Figure 3.17.A). In contrast, production of SLA-specific IgG2a (a hallmark of Th1 type response) was triggered most effectively by K/cGAMP+EV treatment ($p=0.0013$ for K/cGAMP+EV vs PBS and for K/cGAMP+EV vs EV). cGAMP+EV also induced significant levels of SLA-specific IgG2a ($p=0.0165$ when compared to PBS and EV), albeit at much lower titers than the K/cGAMP group. Immunization with K/Tat+EV resulted in measurable IgG2a production which was not statistically different from PBS (Figure 3.17.B). To better interpret the Th1/Th2 dominated responses, ratio of SLA antibody titers (IgG2a/IgG1) were also calculated (Figure 3.17.C). Results showed that IgG2a/IgG1 ratio was the highest in K/cGAMP (0.29) and K/Tat nanoring (0.23) adjuvanted groups.

Collectively, these findings implicated that among the tested groups K/cGAMP+EV was the most effective formulation for late-stage lesion size control and in inducing *L. tropica* specific antibody production. Although K/Tat nanorings proved to be a potent Th1 inducing adjuvant when combined with OVA (section (3.1.1)), in the *Leishmania* immunization/challenge model, the nanoring adjuvanted EVs triggered rapid footpad swelling and lesion sizes were even bigger than the untreated control. Although speculative, it is possible that negatively charged EVs could destabilize the K/Tat nanoring integrity, forming EV-Tat based large aggregates, which might compromise the immunostimulatory activity of the K/T nanorings and the antigen carrier function of EVs. To eliminate such possible unfavorable interactions, we will test the adjuvanticity of K/Tat nanoring and EV antigen in a “separate component injection model” in future experiments. Among other formulations, K type CpG generated a very poor response which failed to reduce lesion growth and resulted in

no leishmania specific antibody production. D type CpG was another potent Th1 inducer, and D-ODN adjuvanted groups had delayed lesion growth. However, this adjuvant failed to induce *Leishmania*-specific IgG2a. cGAMP triggered high amount of *Leishmania* specific IgG1 and IgG2a production and partially protected lesion formation in early but not the later stages. EVs themselves significantly reduced lesion growth, although they triggered very low levels of parasite-specific antibodies. To be able to better evaluate T-cell specific immunity generated by EVs+adjuvants, we decided to narrow our focus to K/cGAMP adjuvanted group since this formulation was the only one that simultaneously controlled lesion development and triggered significant SLA-specific IgG2a. Therefore, further studies were conducted using the adjuvant K/cGAMP combined with EV and its relevant controls EV, K and cGAMP in the next immunization experiment.

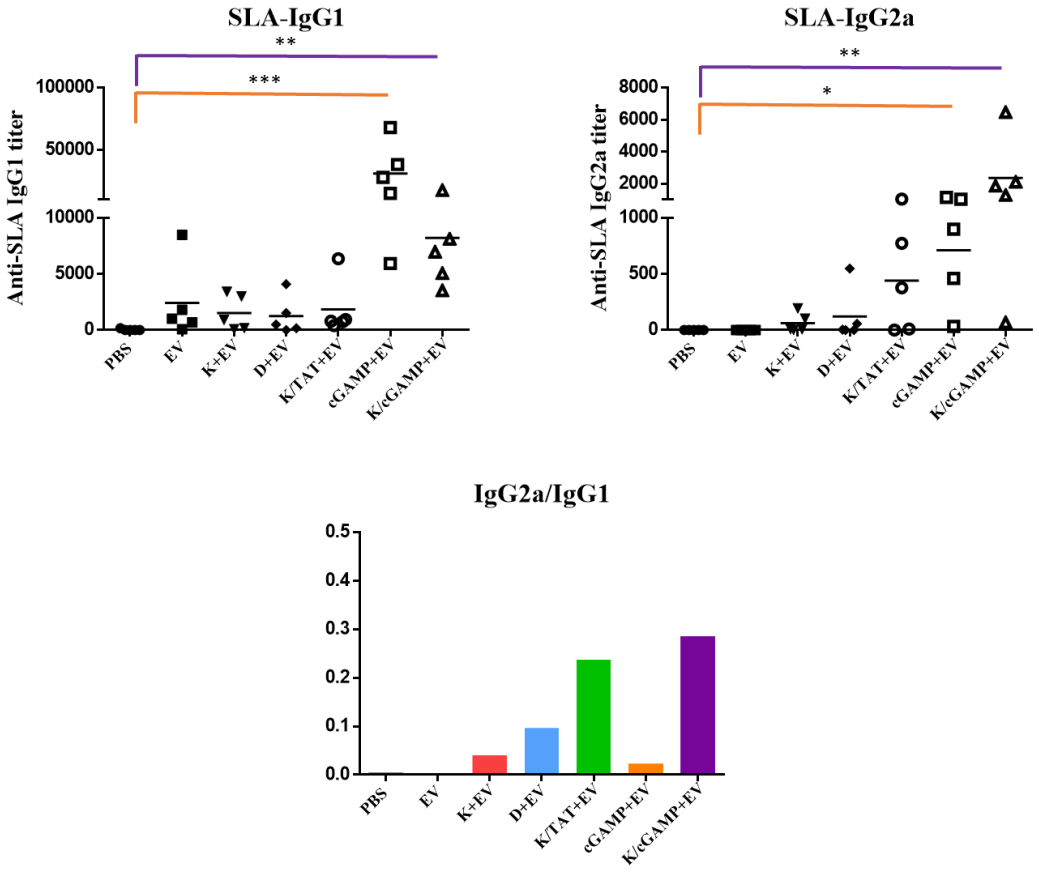


Figure 3.17. Determination of SLA specific antibodies in sera. 6-8 week old female BALB/c mice (5 mice/group) were immunized 2 times (intraperitoneal (ip), day 0 and 15) using parasite derived EVs (20 μg/mouse) as such or in combination with Th1 type adjuvants: K-ODN (25μg), D-ODN (25μg), cGAMP (15μg), K/TAT nanorings or K+cGAMP (including 15μg K-ODN). Blood was collected from tail veins 2 weeks after immunization. Soluble leishmania specific (SLA) IgG1 (A) and IgG2c titers were determined by ELISA. Multiple comparisons among groups were tested via Kruskal-Wallis and Dunn's multiple comparison tests. * indicates $p<0.05$; ** indicates $p<0.01$, *** indicates $p<0.001$. Purple lines indicate test results for K/cGAMP+EV; orange lines indicate results for cGAMP+EV.

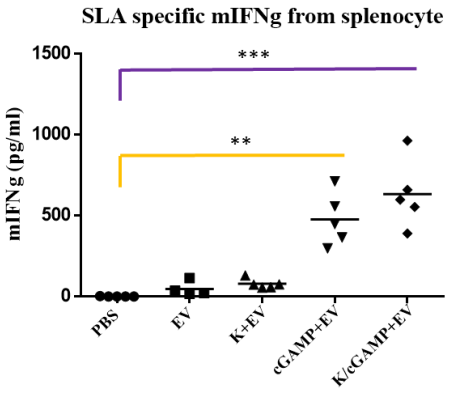
3.4.2. Determination of antigen Specific Th1/Th2/Th17 Cytokine Levels

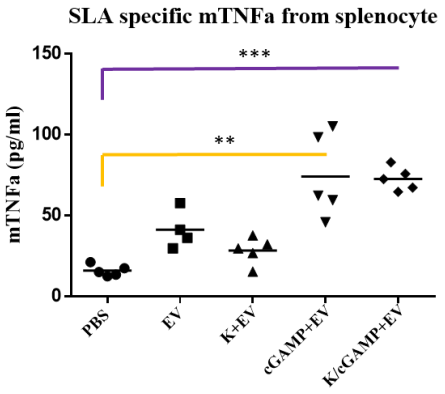
Production of IFNɤ and TNFα by CD4+ Th1 cells was identified as the most efficient mechanism for induction of parasite killing and clearance. In contrast, non-healing lesions are supported by IL-4 and IL-10 secreted from Th2 cells (Shahi et. al., 2013). The role of Th-17 type immune response in leishmaniasis is still a controversial issue. Evidence suggests that IL-17 secreted from Th-17 cells could help parasite clearance but could also contribute to pathology (Katara et. al., 2013). Protective role of Th-17 based immunity is thought to be *Leishmania* strain and disease type specific (Gonzalez-Lombana et. al., 2013).

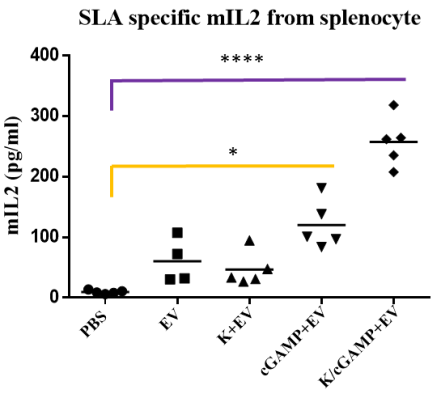
To assess the helper T cell responses induced by immunization, BALB/c mice were immunized two times (day 0 and 10) by EV, K+EV, cGAMP+EV or K/cGAMP+EV. One week later, splenocytes were incubated with SLA for 48 h. Th1/Th2/Th17 associated cytokines IFNɤ, TNFα, IL2, IL4, IL6, IL10 and IL17 were detected from culture supernatant using cytometric bead array by flow cytometry (Figure 3.18). Results showed that the highest level of Th1 cytokines, IFNɤ, and TNFα were produced by splenocytes of K/cGAMP+EV immunized mice in response to SLA stimulation ($p=0.0003$ for IFNɤ and $p=0.0008$ for TNFα). Similarly, splenocytes of cGAMP+EV immunized mice produced a significant amounts of IFNɤ, and TNFα ($p=0.0023$ for both). However, cGAMP+EV immunization also produced Th2 cytokines, IL4, IL10 and IL6 more than the other immunization groups ($p= 0.0004$, $p=0.0003$ and 0.0002 respectively). EV immunization alone induced Th2 cytokines

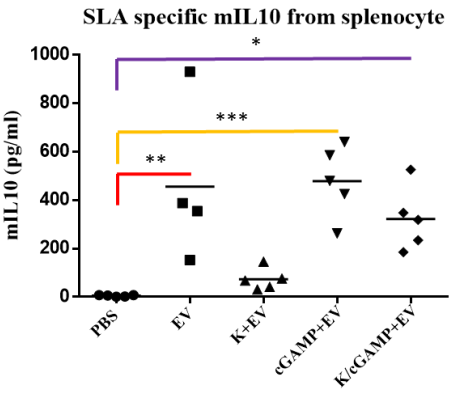
IL4, IL6 (p=0.0178) and IL10 (p=0.009) but no Th1 or Th17 associated cytokines. IL2 is required for survival and proliferation of both Th1 and Th2 cells. Splenocytes of K/cGAMP+EV and cGAMP immunized mice produced IL2 significantly (p= 0.0001 and p=0.0127 respectively). SLA specific IL17A was produced only by splenocytes of K/cGAMP+EV immunized mice (p=0.0037).

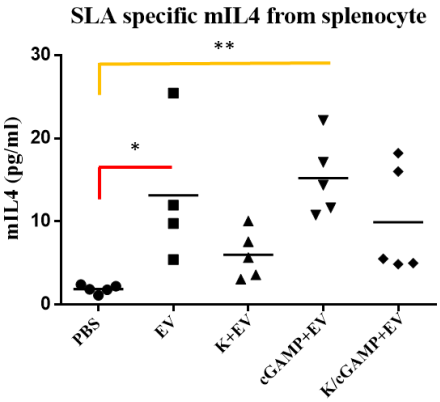
Of note, in the absence of regulatory mechanisms, exacerbation of inflammatory cytokines IL17 and IFNɤ contribute the disease pathology and delay the healing process (Kostka et. al., 2009). IL10 is an important dual function cytokine. In the initial phase of infection it may enhance disease (Belkaid, et. al, 2001), yet under certain circumstances, it may decrease parasite numbers (Kane and Mosser, 2010). In resolution of inflammation, Th1 cells can release IL10 as a regulatory factor (Trinchieri, 2007). Gonzales-Lombana et al reported that in the absence of IL10, IFNɤ and IL17 could lead to immunopathology in cutaneous leishmaniasis (Gonzales-Lombana et. al., 2013). Therefore, based on the aforementioned information and our results, we believe that elevated production of IL10, as well as Th1 and Th17 cytokines in K/cGAMP+EV immunized groups would indicate that K/cGAMP could be a safe adjuvant candidate for vaccination against cutaneous leishmaniasis.

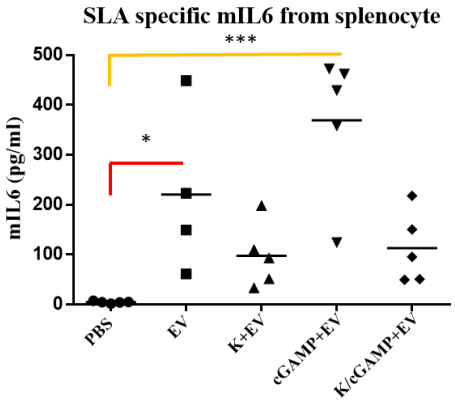

SLA specific mIFNg from splenocyte
mIFNg (pg/ml)
1500
1000
500
0
***
**
PBS
EV
K+EV
cGAMP+EV
K/cGAMP+EV


SLA specific mTNFa from splenocyte
mTNFa (pg/ml)
150
100
50
0
***
**
PBS
EV
K+EV
cGAMP+EV
K/cGAMP+EV


SLA specific mIL2 from splenocyte
mIL2 (pg/ml)
400
300
200
100
0
****
*
PBS
EV
K+EV
cGAMP+EV
K/cGAMP+EV


SLA specific mIL10 from splenocyte
mIL10 (pg/ml)
1000
800
600
400
200
0
*
***
**
PBS
EV
K+EV
cGAMP+EV
K/cGAMP+EV


SLA specific mIL4 from splenocyte
mIL4 (pg/ml)
30
20
10
0
**
*
PBS
EV
K+EV
cGAMP+EV
K/cGAMP+EV


SLA specific mIL6 from splenocyte
mIL6 (pg/ml)
500
400
300
200
100
0
***
*
PBS
EV
K+EV
cGAMP+EV
K/cGAMP+EV

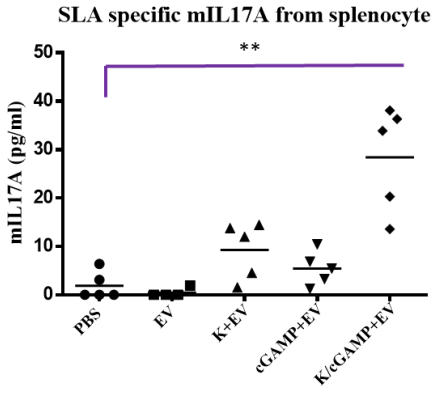

SLA specific mIL17A from splenocyte
mIL17A (pg/ml)
50
40
30
20
10
0
**
PBS
EV
K+EV
cGAMP+EV
K/cGAMP+EV

Figure 3.18. Detection of SLA specific Th1/Th2/Th17 cytokines. BALB/c mice (5 mice/group) were immunized 2 times (subcutaneous (sc), day 0 and 10) using parasite derived EVs (20 μg/mouse) as such or in combination with Th1 type adjuvants: K-ODN (25μg), cGAMP (15μg) and K+cGAMP (including 15μg K-ODN). Seven days after booster injection, splenocytes were incubated with 10 μg/well soluble leishmania antigen (SLA) for 48 h and SLA specific IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17A, and IL-10 responses of splenocytes were assessed via Cytometric Bead Array (CBA) using the Mouse Th1/Th2/Th17 Cytokine Kit. Results were analyzed using FCAP array software. Multiple comparisons among groups were tested via Kruskal Wallis Test. Multiple comparisons among groups were tested via Kruskal-Wallis and Dunn's multiple comparison tests. * indicates $p<0.05$; ** indicates $p<0.01$, *** indicates $p<0.001$. Red lines indicate test results for EV; Purple lines indicate test results for K/cGAMP+EV; orange lines indicate results for cGAMP+EV.

In summary, in our preliminary experiments the immunoprotective activity of vaccine formulations were tested in a footpad infection model of cutaneous leishmaniasis. Results suggested that K/cGAMP could be a promising adjuvant among the tested immune stimulatory agents. In this group, late stage lesion sizes were significantly reduced and IgG2a to IgG1 titers of sera were elevated. Measurement of T cell responses in further studies showed that parasite-specific Th1 and Th17 related cytokines were produced by splenocytes taken from K/cGAMP+EV immunized mice. When used alone, EVs generated Th2 dependent IL4, IL6 and IL10 induction in the absence of IFNγ or IL17. However, use of K/cGAMP together with EVs could shift this balance towards a more favorable Th1 dominated response. Collectively, these preliminary results indicate that in vaccination against cutaneous leishmaniasis, K/cGAMP could be employed as a Th1 inducing adjuvant in combination with EVs. We hope that future experiments will shed more light on the type of immunity required to provide protection against *Leishmania* infections and would pave the way for generating a reliable vaccine for this parasitic disease which afflicts the poorest of the poor and is therefore neglected for a very long time.

CHAPTER 4

CONCLUSIONS & FUTURE PERSPECTIVES

Synthetic CpG ODNs are potent immune stimulatory agents mimicking the natural unmethylated CpG motifs present in pathogen genome that are recognized by TLR9 receptor (Krieg, 1995). K and D type ODNs are two main classes of CpG ODNs Vollmer, 2004; Hartmann, 2003). Altough both are recognized by TLR9, they elicit different downstream signaling cascades and so induce production of different types of immune mediators (Gillet et. al., 2008). K-ODN is linear in structure, and are localized in the late endosomes following their uptake. This ODN class activates B cells, pDCs and trigger production of pro-inflammatory cytokines such as IL-6, IP-10 and TNFα. D-ODN are more complex and form G-quadruplex based higher order structures. The multimerized forms of D-ODNs cause their retention in early endosomes whereby they interact with different signaling partners and uniquely trigger type I IFNs production (Gursel, 2006). Type I IFNs play vital roles in anti-viral and anti-tumor response of immune system. Therefore, D-ODNs possess potentially important therepeutic applications in treatment of viral infections and cancer (Klinman, 2004). However, pharmaceutical production of GMP grade D-ODNs is not possible due to their unpredictable and uncontrollable multimer forming structural species. Therefore, in contrast to K ODNs, use of D-ODN in clinical trials has not been tested. In our previous study, we developed a stable nanoring therapeutic agent by mixing a negatively charged K-ODN with the HIV derived polycationic Tat peptide (47-57). Complexation generated structurally stable nanorings which protected K-ODN from nuclease attack, retained them in early endosome and stimulated type I IFN

production from pDCs, reproducing the immune stimulatory properties of D-ODNs. In vivo investigation of K/Tat nanorings with OVA antigen showed that OVA specific serum IgG2c was increased in a pDC dependent manner. When K/Tat nanorings were administered with a low dose commercial vaccine against foot and mouth disease (FMD), this novel adjuvant enhanced the immunogenicity of the vaccine (Gungor and Yagci et. al., 2014a).

In the first part of this study, we examined the anti-tumor effect of K/Tat nanorings in two different tumor models. In the first model, OVA expressing EG.7 thymoma cells were subcutaneously inoculated into mice, followed by intraperitoneal therapeutic immunization with K/Tat+OVA nanorings. In a second tumor model of B16 melenoma challenge, K/Tat nanorings were administered peritumorally as stand-alone anti-cancer agents. Results showed that K/Tat nanorings suppressed tumor progression by inducing antigen specific CTL-mediated IFNγ production and CTL-specific EG.7 thymoma killing in the first model. However, the nanorings had no significant effect in the B16 F10 melanoma model when used as stand-alone immune stimulatory agents. Since the route of administration differed in these models, we next, we explored the optimal vaccination route of nanorings using the model antigen ovalbumin. For this, K/Tat nanoring + OVA was administered via intraperitoneal, intradermal, subcutaneous, intramuscular or intranasal routes. Sera associated anti- OVA IgG1, IgG2a and IgE titers and bronchoalveolar lavage (BAL) associated anti-OVA IgG2c and IgA titers were determined. Furthermore, splenocytes from immunize mice were pulsed with OVA and antigen specific IFNγ production was assessed. Results showed that K/Tat nanorings triggered the highest antigen specific IgG2c titers and OVA-specific IFNγ production when administered via the intranasal route. Of note, induction of very low IgE antibody titers indicated that K/Tat nanorings did not cause any unwanted allergic responses when used as mucosal adjuvants. The success of K/Tat nanorings in intra nasal administration suggested us the potential use of nanorings as mucosal adjuvants for future trials.

In the second part of the study we aimed to develop a protective vaccine against cutaneous leishmaniasis. *Leishmania* protozoan is an intracellular parasite transmitted to the host through blood-feeding of the vector sandfly (Shapiro, 1995). Parasite clearance in the host depends on the generation of a Th1 type immune response. Specifically, IFNɤ secreted from Th1 cells activate infected macrophages and provide help in killing of intracellular parasites (Sacks and Noben-Trauth, 2002). *Leishmania* spp. have been shown to secrete extracellular vesicles into their environment to deliver *Leishmania*-specific mediators to host cells (Silverman et. al, 2010). The absence of a protective vaccine against cutaneous Leishmaniasis prompted us to investigate a novel strategy of vaccine development based on the use of *Leishmania* extracellular vesicles (EVs) together with Th1 promoting immunostimulatory CpG ODNs or cyclic dinucleotides. Briefly, we isolated *Leishmania* derived EVs from promastigote culture supernatants by differential centrifugation. According to atomic force microscopic analysis, isolated $EV_{L.tropica}$, were determined to be of spherical morhology and their size ranged from 30-250nm in diameter. Nearly 90% of the $EV_{L.tropica}$ were positive for the *Leishmania* GP63 antigen and vesicles contained double- and single stranded-nucleic acids. Previously, extracellular vesicles of some *Leishmania* strains (*L. major, L. donovani*) were reported to induce Th2/Treg dominated immune responses that exacerbated parasite spreading in infected cells (Silverman et. al., 2010). In this study, the immune modulatory effects of $EV_{L.tropica}$ was examined for the first time. Results showed that $EV_{L.tropica}$ had no immune modulatory effects on bone marrow derived macrophages or murine splenocytes when used as such or in combination with immune stimulatory agents. In partcular, $EV_{L.tropica}$ neither enhances nor inhibited the activity of TLR9 and/or STING ligands (K-ODN, D-ODN, Nanorings, cGAMP and cGAMP/K-ODN). Furthermore, $EV_{L.tropica}$ did not alter T-cell proliferation in vitro. These results indicated that *Leishmania tropica* EVs had no detectable immune regulatory effects on their own and therefore could be of value as antigen carriers in vaccine development. Based on these findings, a *L.tropica* footpad infection model for CL was established and the immune protective activity of EVs as such or in combination with Th1 inducing adjuvants: K-ODN, D-ODN, K/TAT nanorings,

cGAMP, or K+cGAMP was tested. Naive or immunized mice were challenged with live parasites and lesion growth was monitored for the duration of 10 months. Extent of *Leishmania* Antigen (SLA) specific IgG1 and IgG2a antibody production was assessed from mice sera. To evaluate the development of adaptive immunity, splenocytes from immunized mice were pulsed with SLA and antigen specific Th1/Th2/Th17/Treg cytokines were determined. Collectively, our results showed that $EV_{L.tropica}$ K/cGAMP combination significantly reduced lesion development and stimulated a Th1/Th17 type immune response. Surprisingly, although $EV_{L.tropica}$ alone failed to augment SLA-specific IFNγ in immunized mice, lesions remained small and did not significantly differ than those observed in EV+K/cGAMP immunized groups. Although we require further experiments to understand the role of B cells and T cells in generating *Leishmania* specific protective immunity, these preliminary results demonstrate the applicability of EVs in vaccination.

This was our first attempt to establish a vivo infection model for *L.tropica* in mice. Future work will focus on improving some of the difficulties we experienced as follows:

1. In this study, *L.tropica* parasites directly isolated from a human patient were used in EV purification and live parasite challenge experiments. However, we noticed that lesion development took a very long time in mice, suggesting that use of murine adapted *L.tropica* might be a better alternative to establish an infection model. For this purpose, we are planning to isolate amastigotes from infected footpads and expand and use these murine adapted parasites in challenge experiments.

2. Measurement of lesion size using a caliper is not precise and often, lesion size may not correlate with parasite load. We are trying to generate parasites expressing green fluorescent protein and luciferase for simultaneous real time monitoring of both the lesion size and the parasite burden using an in vivo imaging system.

3. Recent studies showed that encapsulation of CpG ODNs into extracellular vesicles enhanced their immune stimulatory/immunoprotective activity when compared to

mixture of EV plus CpG ODN. Therefore, immunoprotective activity of CpG ODN and/or cGAMP encapsulating EVs will also be tested in future experiments.

4. K/Tat nanorings triggered a potent Th1 dominated immunity when used with the protein antigen OVA but failed to show a favorable activity when used together with Leishmania EVs. It is possible that the negatively charged EVs could destabilize the K/Tat nanoring integrity, forming EV-Tat based large aggregates, which might compromise the immunostimulatory activity of the K/T nanorings and the antigen carrier function of EVs. To eliminate this possibility, we will test the adjuvanticity of K/Tat nanorings and EVsin a "separate component injection model" in future experiments.

5. This study was restricted to EVs and parasites of *L.tropica,* a non-aggressive *Leishmania* strain. However, *L. major* and *L. infantum* are also circulating among CL patients in Turkey. One of our future perspective is to compare extracellular vesicles of these three strains in terms of their immune modulatory properties and their potential use in development of an anti-leishmania specific vaccine.

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APPENDIX

Recipes for Buffers, Cell Culture Media and Solutions

1. Blocking Buffer (ELISA): For total 500 mL,

-500 mL PBS (1X)

-20 grams of BSA (2%)

-500 µl Tween20 (0.05%)

BSA is dissolved in PBS totally by using a magnetic stirrer (15-20 min mixing). After preparation buffer is stored at -20°C as aliquots of 50 mL.

2. Wash Buffer (ELISA): For total 5 lt,

-500 mL PBS (10X)

-2.5 mL Tween20

-4.5 lt ddH2

3. T –Cell Buffer (ELISA): For total 500 mL,

-475 mL PBS (1X)

-25 mL FBS (5%)

-250 µl Tween20 (2.5%)

4. FACS Buffer: For total of 500 mL,

-500 mL PBS (1X)

-5 g BSA (1%)

-125 mg Na-Azide (0.125%)

5. MACs Buffer For total of 500 mL,

- 5 ml FBS (1%)

- 2 ml EDTA (2mM)

-500 ml PBS

6. Regular RPMI 1640 containing 2%, 5%, 10% FBS: For total 500 mL,

-10 mL, 25 mL or 50 mL FBS (inactivated at 55°C)→ for 2%, 5%, 10% respectively

-5 mL PenStrep (Penicillin/Streptomycin, 50 µg/mL)

-5 mL NaPyruvate (0.11 mg/mL)

- 5 mL HEPES (10 mM)

-5 mL NEAA (non-essential amino acids, diluted 1X from 100X stock)

Final volume is brought to 500 mL by using RPMI-1640 (w/L-Glutamine, w/Phenol Red) from ThermoFisher Scientific, USA.

7. *Leishmania* growth medium 10%, 20% FBS: For total 500 mL,

-50 mL, 100mL FBS (inactivated at 55°C)→ for 10%, 20% respectively

-5 mL PenStrep (Penicillin/Streptomycin, 50 µg/mL)

-10 mL HEPES (20 mM)

Final volume is brought to 500 mL by using RPMI-1640 (w/L-Glutamine, w/Phenol Red) from ThermoFisher Scientific.

8. Exosome collection medium for total 35mL

- 31 ml EV free Leishmania growth medium

- 3.5ml MES Buffer from 250mM (25mM)

- 560µl HCl (1N) --- pH=5.5

9. Lysis Buffer for *Leishmania* Parasite,

- 50 mM Tris-HCl

- 5mM EDTA

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