



# Immune Modulation by Exosomes in Trichomonas Vaginalis Infection

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**Immune Modulation by Exosomes in *Trichomonas vaginalis* infection**

Tiffany K. Chan

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The Harvard Medical School

in Partial Fulfillment of the Requirements

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Immune Modulation by Exosomes in *Trichomonas vaginalis* infection**Abstract**

Exosomes and their microRNA cargo have been shown to modulate immune gene expression in the context of cancer; however, they are still a largely uncharted territory in the context of reproductive tract immunology. We used a human *in-vitro* model of the most common non-viral infection of the lower female reproductive tract caused by the protozoan parasite *Trichomonas vaginalis* (TV) to begin unveiling the role of exosomes in the host-microbe interactions in this anatomic compartment. We isolated exosomes from monocultures of different TV strains and from vaginal epithelial-TV co-cultures and investigated their effects on the acute immune response by bystander upper reproductive tract endocervical epithelial cells and peripheral blood mononuclear leukocytes (PBMC). It has previously been shown that TV carries endosymbiotic dsRNA *Trichomonas vaginalis* virus (TVV), which amplifies inflammatory responses to TV via TLR3 signaling. Our experimental data showed that the immunoinflammatory effects of exosomes derived from TVV-positive parasites differ from those derived from TVV-negative parasites, including TVV-cured isogenic strains, suggesting a new role of the virus in the host-parasite interactions. The effects of exosomes derived from vaginal-TV co-cultures were also different from those from TV alone, suggesting a contribution of the human vaginal epithelium. Exosomes derived from TVV-uninfected or TVV-cured parasites but not from TVV-infected parasites activated NF- $\kappa$ B and induced selective proinflammatory cytokine expression in bystander endocervical epithelial cells. Similarly, in PBMC, exosomes from TVV-cured but not from the isogenic naturally TVV-infected parasites upregulated a myriad of proinflammatory cytokines. Exosomes from TV-infected vaginal cells suppressed cytokine

upregulation induced via Toll-like receptor signaling and the suppression of proinflammatory cytokines was selectively and specifically induced when the parasites were TVV positive. The TVV exosomes were also capable of abrogating T cell activation by IL-2/PHA as demonstrated by proportion of naïve CD4+CCR7+ cells. These findings suggest that the viral infection of the protozoan parasites affects the exosomal content and function and may serve to suppress human host immunity at the time of establishing the protozoan infection. The differential roles of protozoan and infected host exosomes in underlying inflammatory damage and reproductive outcomes require further studies.

# Table of Contents

Chapter 1: Background .....	1
Epidemiology of Trichomoniasis: .....	1
Biology of Trichomoniasis .....	2
Trichomonas and its own viral infection.....	3
Immunity against Trichomonas vaginalis.....	5
Exosomes: .....	7
MicroRNA: Function, Biogenesis and Role in Immunity .....	9
Unresolved immunology questions .....	11
Chapter 2: Data and Methods.....	13
Short Introduction: .....	13
Glossary of terms: .....	15
Materials .....	16
Table 1: PBMC donors.....	16
Table 2: Immortalized Epithelial Cell lines .....	16
Table 3: Assays .....	17
Table 4: Flow Cytometry Kits and Stains.....	17
I. Exosome Isolation .....	18
II. Exosome Characterization: .....	19
III. Endocervical Experimental Procedures: .....	20
IV. PBMC Experiments: .....	21
<b>Results:</b> .....	23
Brief Discussion:.....	64
<b>Chapter 3: Discussion and Perspectives</b> .....	66
Limitations of the Study:.....	66
Contextualization:.....	688
Future of the field: .....	71
<b>References</b> .....	74

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## Chapter 1: Background Epidemiology of Trichomoniasis:

A common sexually-transmitted infection, trichomoniasis poses a pressing public health concern, due not only to its prevalence and highly disparate distribution down socioeconomic and ethnic lines, but also to its effects on reproductive outcome.

Trichomoniasis presents in the clinic as vaginal discharge, pruritus, odor and irritation in females and urethritis in males(1). While long regarded as simply a nuisance, trichomoniasis has recently been linked to adverse events in reproductive health, before and during pregnancy and for both mother and child. *Trichomonas* infection most commonly occurs in women of child-bearing age, frequently co-presents with bacterial vaginosis (a dysbiotic syndrome of the vaginal microflora) and both of these conditions have been linked to poor reproductive outcomes (2, 3). Although trichomoniasis and other reproductive infections have been treated with metronidazole since the 1960s, the use of this drug has been correlated with adverse pregnancy events and *Trichomonas vaginalis* antibiotic resistance has been increasing in recent years (2). Once infected, patients face increased risk of HIV, high-risk HPV infection and subsequent cervical cancer risk as well as infertility (2, 4). A screening of women in a cancer clinic showed that compared to women without the infection, those with TV had a higher risk of contracting HPV and a significantly higher risk of contracting the most pathogenic strains of the virus (4). During pregnancy, *T. vaginalis* could have adverse effects on fetal development including low birth weight, pre-term birth and vertical transmission from mother to child (2, 5).

Trichomoniasis affects more than 200 million individuals globally and has a higher rate of occurrence than gonorrhea and chlamydia combined, as many as 30% of patients presenting with re-infection upon follow-up (2, 6). TV infection is second-most common STD and second most



common cause of lower genital tract infection world-wide (4). It is already the most common non-viral venereal disease and the number of cases could actually be significantly higher since it is estimated that 80% of cases are asymptomatic (1, 2).

Incidence of trichomoniasis can be as high as 51% in some urban communities as well with a majority of cases presenting in resource-limited settings (2). In urban STI clinics, the prevalence of trichomoniasis is 20% of all cases seen and it comprises 50% of all curable infections worldwide (7). Because trichomoniasis is a recurrent infection, it is a serious burden on a vulnerable demographic (5). Furthermore, prevalence amongst adolescents has been increasing (2).

### **Biology of Trichomoniasis**

Trichomoniasis is a disease caused by the sexually-transmitted urogenital parasite *Trichomonas vaginalis* (TV), which preys on the epithelial cells in the reproductive tract and is best adapted to the vaginal mucosal environment for long term survival (2). Trichomoniasis is often seen in women with bacterial vaginosis (BV) – a condition characterized with a shift in proportion of bacteria in the female reproductive tract in favor of potentially pathogenic anaerobic species (3). BV is one of the most common syndromes in women of child-bearing age who seek primary care. It is contingent on the complex interplay between the host microbiota and the immunoendocrine system. BV increases the susceptibility towards trichomoniasis and together TV and BV exacerbate inflammation and associated risks of poor reproductive outcomes, cancer and other STIs e.g. HIV (2-4).

*T. vaginalis* is a flagellated anaerobe that is remarkably capable of surviving in the acidic and microbially fortified reproductive mucosa for several years (2). *T. vaginalis* was first described in 1836 by French physician-scientist Alfred Donné upon microscopic observations of

cervico-vaginal discharges. It was long thought that *T. vaginalis* was a harmless commensal and was not until 80 years later that it was even linked to vaginitis (2). Although there have been observations of trichomonads in epithelial and sub-epithelial tissues, the parasite is largely extracellular (8). Despite its early discovery, research to understand this parasite and its interactions with other members of the microflora is still an area of emerging research.

*T. vaginalis* is a peculiar protozoan in several respects. It has a large and repetitive genome. It utilizes hydrogenosomes instead of mitochondria to accomplish fermentative carbohydrate metabolism (6). Furthermore, *T. vaginalis* is able to produce enzymes of both prokaryotes and eukaryotes, a specific subset of which allows it to evade immune detection (2).

*T. vaginalis* infection can be divided into four stages: adhesion, contact-dependent cytotoxicity, phagocytosis and intracellular digestion (9). *T. vaginalis* swarms around host cells to attack and can cause cellular injury in under an hour of co-incubation (10). *T. vaginalis* is capable of lysing many different types of cells, including host epithelial cells and lymphocytes as well as other bacteria to establish its pathogenic niche (9, 10). The infection is diagnosed by multiple methods, the oldest and most specific but less sensitive being wet mount under microscope, followed by a rapid antigen test and more recently by nucleic acid amplification test (NAAT).

### **Trichomonas and its own viral infection**

In another layer of immunological complexity, *T. vaginalis* can itself be infected by a virus (Trichomonasvirus, TVV) (5, 7). Many genera of protozoa and fungi carry endosymbiotic viruses (7). These viruses act to chronically but non-cytopathically infect their hosts and can potentially confer evolutionary advantage and aid pathogenicity (7).

Named for its host, Trichomonasvirus is a member of the family *Totiviridae*, icosahedral virions ranging in diameter from 30-40nm (7). The virions are transmitted vertically as *T. vaginalis* undergoes binary fission (7) or also likely after meiosis by mating (11). There are currently four known species of Trichomonasvirus genus (8) and *T. vaginalis* is capable of being stably infected by multiple TVV species concurrently (7). The viral genome consists of a single dsRNA (4300-4900bp) with smaller satellite dsRNA present (~500bp) (9). Although it is unclear how these satellite dsRNA function in TVV, in other members of the *Totiviridae* family this molecular information encodes advantageous toxins that aid host pathogenicity (7). TVV is known to alter the *T. vaginalis* profile of cysteine proteases, a known virulence factor for the species, which are instrumental for the degradation of human immune proteins such as immunoglobulins, complement and adhesion molecules (7).

Trichomonasvirus is sensed by TLR3 which triggers phosphorylation of IRF-3, and a cascade of proinflammatory cytokines and chemokines implicated in pre-term birth and HIV susceptibility (5). Thus, in addition to the *T. vaginalis* itself, the virus exacerbates the inflammatory milieu by upregulating pro-inflammatory pathways, virus stress response genes and chemokines by genital tract epithelial cells (2, 5). The virus also exacerbates the inflammatory response to BV bacteria (5). Because treatment by metronidazole does not improve reproductive outcome, it is believed that metronidazole allows for the release of the virus from inside of *T. vaginalis*, thus increasing exposure, immune activation and inflammatory damage (7). Because Trichomonasvirus activates a different pathway of innate immunity from *T. vaginalis* itself, it also allows the immune response to be diverted from anti-parasitic response to a misguided anti-viral response (to a virus that does not even infect human cells).

## **Immunity against *Trichomonas vaginalis***

The innate immune response plays a key role in sensing and responding to *T. vaginalis*. Epithelial cells, such as those used in this study, constitute an important defense mechanism against the parasite by acting as a physical barrier (1). Epithelial cells sense *T. vaginalis* upon direct contact via galectin-mediated pathways and in the absence of TLR-4 while leukocytes sense the parasite virulence factors via TLR-4 and in both TV can induce the expression of COX-2, TLR 2, 4 and 9 via the MAPK pathway (2). There is also an interesting synergistic effect between inflammatory events in response to *T. vaginalis* and pathogens of bacterial vaginosis (BV) combined. In a study of pregnant women who presented with both types of infection, researchers found higher levels of serum IL-1 $\beta$  and neutrophilic infiltrates in vagina compared to those singly infected with bacterial vaginosis (2). When exposed to *T. vaginalis in vitro*, leukocytes respond by producing IL-8, leukotrienes, reactive nitrogen intermediates and inducible NOS (iNOS) (2). A study conducted on prostate stromal cells also showed that the parasite induced a pro-inflammatory response, upregulating the levels of pro-inflammatory cytokines as well as activating TLR4, ROS and NF- $\kappa$ B expression (8). The dominant inflammatory cells found from vaginal discharge of infected patients are neutrophils. Researchers have shown that TV lysis of neutrophils actually can induce an anti-inflammatory response in macrophages, which limits and leads to the resolution of infection (12). Other studies have shown that the macrophage exposure to *T. vaginalis* suppresses NF- $\kappa$ B induction and is largely anti-inflammatory (13). Research in this Lab has shown that binding of the major surface TV lipophosphoglycan (LPG) to galectin-1 and -3 and the levels of soluble galectins in the extracellular environment can both regulate the inflammatory response by the vaginal epithelial cells (14). Taken as a whole, the pro- or anti-inflammatory nature of the immune response depends largely on the cell type in question and the mucosal context. However,

*T. vaginalis* is unequivocally capable of garnering a robust response from several components of the innate immune system, which is central for clearing the infection.

However, the adaptive immune response to *T. vaginalis* is considerably more complex. In T cells, macrophages, and dendritic cells, exposure to *T. vaginalis* induces the production of immunosuppressive cytokines IL-10, TGF- $\beta$  as well as apoptosis (2). *T. vaginalis* infection challenge experiments in mouse models increased serum levels of IgA, IgG, Th1 cytokines in addition to Nitric Oxide Species in circulation (2). The antibodies were present both in the sera and mucosal sites in the animals (2). Although antibodies specific to *T. vaginalis* are produced upon exposure to the parasite, patients often lack a robust adaptive immune response to the parasite and suffer many recurrent infections (2).

A study conducted by this lab has shown that the Trichomonas lipophosphoglycan (LPG), similarly to LPS found on bacteria, is capable of inducing a robust and specific inflammatory response (2). Because LPG is sufficient for inducing an inflammatory response and does not change structurally over the course of the parasite's development, it provides an interesting and novel target for vaccine development against this elusive parasite (2). Antibodies to LPG have been found in pregnant women who had suffered trichomoniasis. Multiple studies have identified protein immunogens on the TV surface and antibodies against TV proteins have been found in patients with prostate cancer (15). In both cases the antibody presence was merely evidence for exposure to TV that was associated with pathological sequelae e.g. preterm birth or cancer. A study conducted at Yonsei University of Medicine also identified antigenic surface proteins from *T. vaginalis* that are relevant to cytoadherence via membrane fractionation and immunoscreening of a cDNA expression library however their role in immunity and human disease is still to be

established (16). Taken as a sum, these studies indicate that the pathogenic success of *Trichomonas* is not due to an inability to be sensed by the adaptive immune system.

Although there is evidence that the adaptive immune system can recognize *T. vaginalis*, the chronicity and frequency recurrence of the infection suggest evasion of the immune effectors by the parasite. Cysteine proteases produced by *T. vaginalis* are regarded as virulence factors for various strains because they are capable degrading IgG, IgM and IgA, inducing the apoptosis of vaginal epithelial cells and other immune cells and thus attenuating the adaptive response (2). Furthermore, imaging studies have shown that *T. vaginalis* is able to acquire host CD59 (a complement lysis restricting factor) and use it to avoid lysis by the complement system (1, 17). More studies show that surface proteins that *T. vaginalis* shares with other parasites are capable of undergoing a conformational change to avoid detection by immunoglobulins (1). While some *in-vitro* studies on murine models have shown protective immunity *via* vaccination (18). A murine model of Trichomonas infection suggests that the parasite induces CD4+ T-cell infiltration and used this as the basis to argue that a whole-cell TV vaccine conferred protective immunity (19). However, these results have yet to be satisfactorily translated into human (6). Because of this dearth of protective long-lasting adaptive immunity, studies have primarily focused on the innate immune response to *T. vaginalis*.

### **Exosomes:**

Exosomes, extracellular vesicles 50-100nm in diameter, are a mechanism of intercellular communication of emerging interest. Extracellular vesicles contain a specific composition of lipids, mRNA, regulatory miRNA, and functionally-active cytosolic proteins depending on both the source and target cells(20). Exosomes are formed when intraluminal vesicles fuse with the cell membrane and are responsible for transporting a variety of molecular cargo from between cells.

They are an important mechanism for cell-cell communication (20). Exosomes can regulate gene expression and alter fate of their targets depending on their contents. They can cause targets to become activated, differentiated or de-differentiated according to the molecular information received. The generation of extracellular vesicles has been shown both *in vitro* and *in vivo* without affecting target cell viability (21).

Studies suggest that extracellular vesicles can be used as a means of communication between various parasite species, perhaps to promote growth and transmission and manipulate the microenvironment (22). In the context of *Trichomonas* infection specifically, data from Twu et al. found that extracellular vesicles contain strain-specific proteins that affect the ability of TV to adhere to target cells (22, 23). Proteomics of purified preparations from *T. vaginalis* monoculture found a 70% overlap with mammalian exosome markers which suggests that TV is indeed capable of producing exosomes (22).

Definitively identifying exosomes has been a substantial challenge for researchers. Because exosomes constitute a heterogeneous population of extracellular vesicles, size and density alone cannot be used as strict criteria for their isolation and researchers often utilize a combination of various methods. Exosome membranes are highly enriched in tetraspanins, a class of proteins named for their four membrane-spanning domains. Tetraspanins are involved in cell adhesion, motility, invasion or membrane fusion in addition to signaling, protein trafficking (20). Tetraspanins are able to form clusters and interact with a large variety of transmembrane and cytosolic signaling proteins. Several tetraspanins, (CD9, CD63, CD81, CD82, CD151) have a broad tissue distribution and are widely used as exosomal markers (20). CD63 and CD81 expression in particular have been most frequently confirmed on the surfaces of exosomes leading

to the use of them as “classical” markers of exosomes. However, there is still contention in the field about how to best differentiate and confirm populations of extracellular vesicles as exosomes.

Traditional methods of identification, such as flow cytometry, have had limitations in terms of sensitivity. Often, these small vesicles are lost in the “noise” (21). However, emerging technology such as Nanoparticle Tracking Analysis (NTA) has allowed researchers to quantify exosomes with more precision, though it is still recommended to use a combination of various methods to confirm vesicle populations (21, 24). Due to their stability in biofluids, exosomes could have a potentially important role as a diagnostic for various disease states or as a vector for therapeutics (21).

### **MicroRNA: Function, Biogenesis and Role in Immunity**

MicroRNA (miRNA) are short sequences of RNA (~20bp), normally produced by the cell during RNA transcription and are exosomal content of interest for their potential role in post-translational regulation of gene expression. miRNA are transcribed the same way as protein-coding genes via RNA polymerase II and are in fact a normal by-product of transcription. Primary miRNA (pri-miRNA) consisting of several hundred nucleotides in a hairpin-like structure, are first produced within the nucleus and are modified with a 5'-cap and 3'poly-A tail. Pre-miRNA are then exported out of the nucleus and cleaved in the cytosol by Dicer into a double-stranded complex. This duplex then associates with the RNA-induced silencing complex (RISC) and then one strand may be degraded or both strands may remain functional. The miRNA-RISC complex then can associate with mRNA transcripts to mark them for degradation or make them inaccessible for protein translation (25, 26). Perfect base-pairing between mRNA transcript and miRNA is not necessary to silence gene expression, partial sequence complementation is sufficient (25).



MicroRNA were first identified in *C. elegans* but have been reported in a wide variety of organisms from single-cell algae to humans. This suggests that miRNA comprise an evolutionarily ancient and conserved means of modifying gene expression post-transcriptionally (25). MiRNA are implicated to be involved in many processes, from normal development and cell processes to oncogenesis (26). They have also been implicated in the initiation of the immune response, neural development, DNA repair, apoptosis, oxidative stress (25). Furthermore, miRNA are ubiquitously present in body fluids such as blood and urine, each with distinct miRNA profiles, suggesting that they could be a mechanism for extracellular communication (26, 27). MiRNA have been found to be stable in various adverse conditions such as extreme pH, freeze-thaw cycles and are protected from RNase activity (25). MicroRNA expression profiles are dynamic and vary in female patients throughout the course of their cycle (26).

MicroRNA are capable of modulating the activation and function of innate immune cells. It is believed that miRNA are responsible for tolerogenic endometrial dendritic cell phenotype during the periconceptual period (28). It is also possible that microRNA are critical in the initiation of an adaptive immune response (or lack thereof). After depleting miRNA through a Dicer null mutation, the number of CD4<sup>+</sup> and CD8<sup>+</sup> T cells significantly decreased. miRNAs have also been shown to contribute to Treg differentiation and function. Taken as a whole, microRNA may play a key role in establishing the adaptive immune response (28).

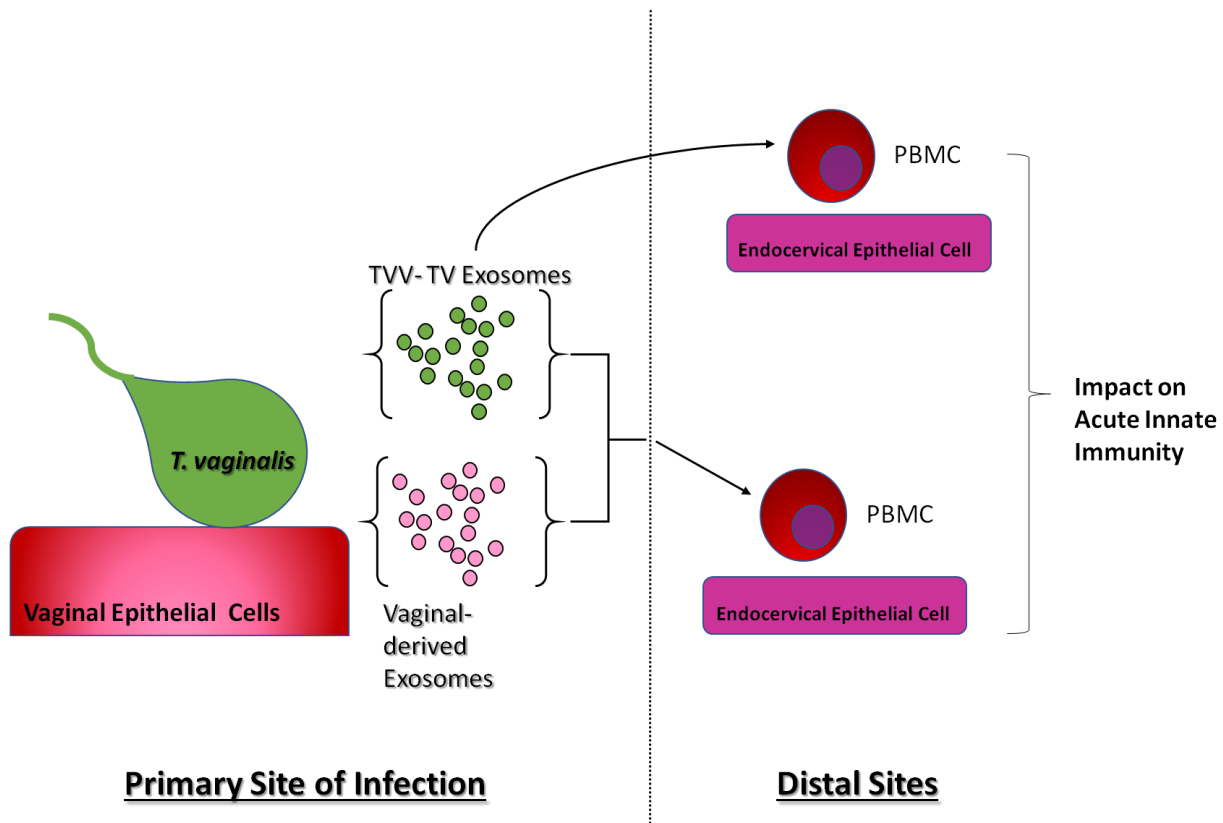
Because miRNAs are capable of dynamically regulating gene expression, they are believed to contribute to various reproductive pathologies. In order to support embryonic implantation and full-term development, the reproductive tract must be tolerogenic before implantation, during the peri-conceptual period and throughout the pregnancy. It is thought that miRNAs play a role in establishing tolerance at conception and play important role in pregnancy, implications for

endometrial receptivity, implantation, placental function and labor (28). MicroRNA have been shown capable of modulating the function of both innate and adaptive immune cells as well as programming immune cells that are critical in the early stages of pregnancy. Sequencing has shown that the expression of specific miRNA(miR-223 and miR-34) are up-regulated in cervical tissue during full-term parturition, suggesting that miRNA can contribute to differentiating normal term vs. pre-term labor. Studies have correlated immune-associated miRNA with miscarriage/pre-eclampsia; suggesting that dysregulated miRNA expression may impact the immune environment during pregnancy (28). Because of the role of miRNA in ovarian function, placental function, uterine receptivity, pregnancy detection, embryonic development, current research suggests that circulating miRNA profiling could hopefully be used as a potential non-invasive diagnostic in the future.

### **Unresolved immunology questions**

Treatment with metronidazole exacerbates inflammatory responses *in-vitro* due to release of TVV (5) and mycoplasma, a member of the disturbed BV microflora frequently carried by the parasite (15). These findings offer an explanation for the failure of metronidazole to prevent poor reproductive outcome in infected pregnant women (5). The elucidation of the full mechanism of the immune response towards *T. vaginalis* and its virus would allow better strategies for prevention of preterm birth and many other risks associated with trichomoniasis. Trichomoniasis is highly recurrent. The lack of effective acquired immunity against *T. vaginalis* has shifted attention toward studying the innate immune response. The evasion of innate and adaptive immunity may be the target of collaboration between TV, TVV and BV species and create a symbiotic network of pathogens. Not much is known of these interactions in the context of mucosal or systemic immune

cells. In this thesis we investigated a proposed mechanism that involves exosomal content dependent on TV and its endosymbiont TVV (Schema 1).



**Schema 1: Proposed hypothesis of the findings of this study. In our model of *T. vaginalis* (TV) infection there are two potential sources of exosomes, one from the *T. vaginalis* itself and the other from infected epithelial cells. In this study we describe differential immune effects of different preparations of exosomes as measured by NF- $\kappa$ B, cytokine, chemokines and immune cell activation markers. We hypothesized that the subset of exosomes derived from axenic TV monocultures (those that lack Trichomonasvirus) will be different in their impact of immunity from those derived from Trichomonasvirus-infected TV. We also hypothesized that exosomes derived from TV-infected vaginal cells will mediate distal immune effects of TV infections e.g. regulate immunity in recipient bystander cells from the upper reproductive tract (uterine endocervical epithelium) and peripheral mononuclear cells in a manner dependent on Trichomonasvirus infection.**

## Chapter 2: Data and Methods

### **Short Introduction:**

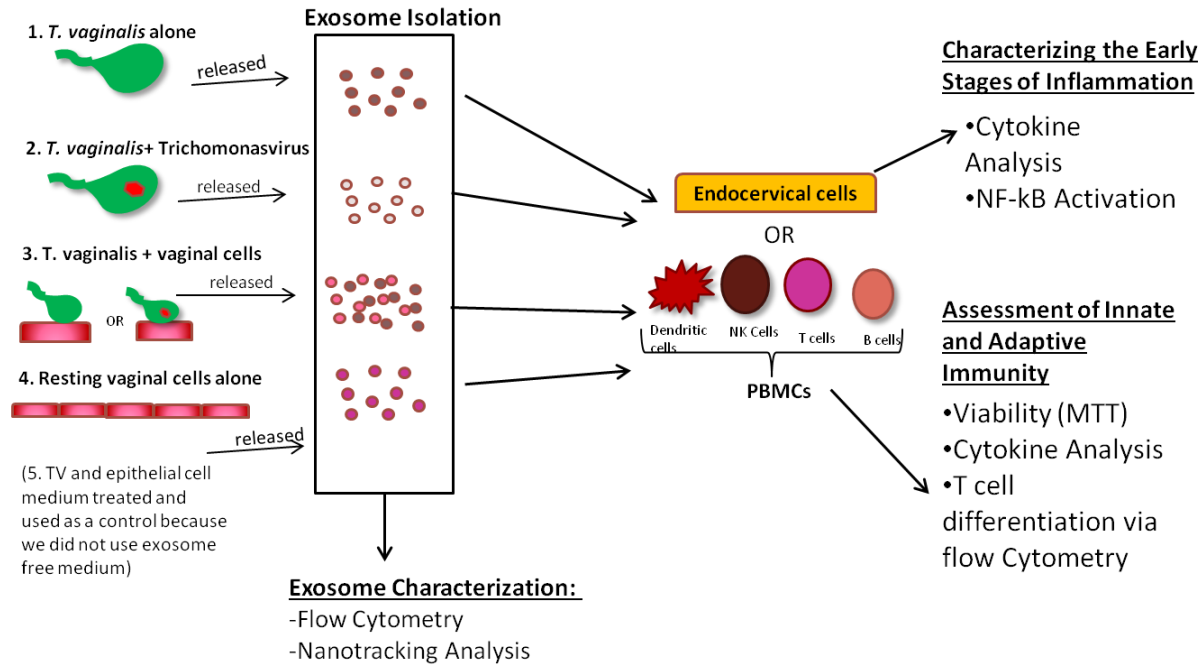
The overall goal of this study was to determine the effect, if any, of exosomes on the innate immune response. We first needed to optimize our protocol for isolating exosomes from cell culture. Although the use of isolation reagent alone yielded usable Nanotracking results (Fichorova lab, unpublished data), after consulting with the company we decided to also use Exosome Spin Columns (Invitrogen) to further purify samples by removing small particles (MW<3000).

Previous experiments have shown that the number of exosomes produced is significantly increased in the TV-epithelial cell infection model compared to other microbes, both pathogenic and symbiotic (Fichorova lab unpublished data). This suggested an important role of exosomes during TV infection but this does not address whether it is the exosomes from TV itself or from infected cells that are being released and influencing bystander cells. To address this, we decided to isolate exosomes both from specific TV strains as well as vaginal epithelial cell infection using the same strains. We also stained isolated epithelial cells and TV and used exosomes isolated from supernatants from the TV-vaginal co-culture to try and determine differences between different treatment conditions via flow cytometry analysis.

We decided to investigate the effect of exosome preparations on two different target cell types: endocervical cells, which represent the upper reproductive tract (the uterine cervix) in close proximity to the infected vaginal cells in vivo, and peripheral blood mononuclear cells, which would be present in circulation. We initially tested various dilutions of the exosomal preparations to determine the highest dilution that maintained the effect in the absence of target cell toxicity and used that exosomal concentration per target cell for all remaining experiments. We investigated both intracellular levels of

gene expression and metabolism (MTT, caspase-3, Luciferase-NFκB) as well as several soluble mediators.

We also have tried to characterize our extracellular vesicle populations by various methods, including proteomics, flow cytometry, fluorescence microscopy and nanotracking.



**Figure 1: Experimental Overview.** Exosomes were isolated from five sources: (1) TVV- or TVV+ strains of *T. vaginalis* (TV) in monocultures, (2) human vaginal epithelial cell co-culture with TVV- or TVV+ TV strains (infection model), (3) human vaginal epithelial cell monocultures (no TV), and (4&5) both TV and epithelial cell culture medium as controls. After isolation, exosomes were characterized using both flow cytometry and nanotracking analysis. Exposure experiments were also conducted on two bystander, non-infected cell types to characterize the immune response to exosomes. Endocervical cells were used to assess early (24h) cytokine response as well as NF-κB activation, using a Luciferase reporter assay. Peripheral Blood Mononuclear cells (PBMCs) were used to assess innate and adaptive immune response parameters. Early (24h) cytokine production was assayed and T cell differentiation was assessed using Flow Cytometry.

## **Glossary of terms:**

### **Media Used:**

AB-: Antibiotic-free keratinocyte serum-free medium (Gibco); epithelial cell medium

D6.0: TV growth medium

### **Strains of *Trichomonas vaginalis* (TV):**

**Trichomonasvirus positive (TVV+):** UR1, 347V+

**Trichomonasvirus negative (TVV-):** PJ, 347V- (TVV cured isogenic derivative of 347v+)

### **Cells:**

VK2/E6E7: vaginal epithelial cells, immortalized with HPV genes E6E7

End/NF- $\kappa$ B: endocervical epithelial cells, immortalized with HPV genes E6E7, stably transfected with a Luciferase reporter gene linked to NF- $\kappa$ B

Ect1/E6E7: ectocervical epithelial cells, immortalized with HPV genes E6E7

### **Adjuvants:**

Poly(I:C): TLR-3 agonist, dsRNA mimic, meant to simulate the presence of virus

IL-2+Phytohaemagglutinin (PHA): blood cell stimulation

### **Cytokines:**

IL: Interleukin

RANTES: CCL5

## **Materials**

**Table 1: PBMC donors**

<b>Donor ID</b>	<b>Demographics</b>	<b>Used in Experiment:</b>
<b>544.011717</b>	Male, Latino  Age: 30yo  Blood Type: O+	1.17.2017
<b>KP49306</b>	Caucasian Female  Age: 26yo  Blood type: A <sup>-</sup>	2.1.2017
<b>KP49695</b>	A.A. Female,  Age: 24yo  Blood type: AB+	3.16.2017

**Table 2: Immortalized Epithelial Cell lines**

<b>Abbreviation</b>	<b>Anatomic location</b>	<b>Function</b>
VK2/E6E7	Human lower reproductive tract: Vaginal epithelial cells immortalized with HPV 16 E6E7	Primary infected Cell
Ect/E6E7	Human upper reproductive tract, Ectocervix uteri: Ectocervical epithelial cells, immortalized with E6E7	Primary infected Cell (fluorescent microscopy only)
End/E6E7	Human upper reproductive tract, Endocervix uteri: Endocervical epithelial cells, immortalized with HPV 16 E6E7	Bystander cell

**Table 3: Assays**

Analyte(s)	Target protein location	Measure of:
IFN- $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, IL-17A, TNF- $\alpha$	Extracellular, soluble mediators	Early inflammatory events (Acute phase cytokines, potent pro-inflammatory cytokines, T helper cell induction)
Luciferase-NF- $\kappa$ B	Intracellular, nuclear	Relative NF- $\kappa$ B activation
MTT Proliferation	Intracellular, mitochondrial	Cell proliferation and metabolism
IL-8/RANTES	Extracellular, soluble mediator	Immune activation; differential profiling between TVV+ and TVV- <i>T. vaginalis</i> strains
Caspase-3	Intracellular, cytosolic	Apoptosis

**Table 4: Flow Cytometry Kits and Stains**

Name	Target Cell	Function
SytoRNA Select Green Fluorescent Protein (Fisher Scientific)	VK2/E6E7	Stains for RNA in target cells
TFL4 (Oncoimmunin)	<i>T. vaginalis</i>	Incorporates into cell membrane of target cells
DuraClone IM T-Cell Subset (Beckman Coulter)	PBMC	Binding to cell surface expression markers as indicators of lymphocyte differentiation (phenotyping)



# I. Exosome Isolation

## Epithelial Cell Culture:

Immortalized vaginal epithelial cell (VK2/E6E7) (29) were cultured in antibiotic-free, keratinocyte serum-free medium (AB-), supplemented with bovine pituitary extract, epidermal growth factor, and calcium chloride as described (29). Cells were trypsinized and grown to confluency in T175 cell culture flasks.

## *T. vaginalis* Culture:

All *T. vaginalis* (TV) strains were cultured in modified Diamond's medium (D6.0) with 10% heat inactivated donor equine serum and iron. (5). TV was harvested in late log phase (24h) by centrifugation (1000xg for 5 min) and re-suspended to a seed density of  $4 \times 10^5$  TV/ml in AB-epithelial cell growth medium.

## Infection Model:

Vaginal epithelial cells were overlaid with 25 ml of suspension of *T. vaginalis* ( $4 \times 10^5$  TV/ml). Cell cultures were incubated under anaerobic conditions on a shaker at 50rpm, 35°C for 24h (5).

## Exosome Isolation:

Exosomes were isolated using the Total Exosome Isolation Reagent (Invitrogen). After 24h of incubation, the reagent was added to cell culture supernatants in a 1:2 ratio. Samples were incubated overnight at 4°C. The following day, samples were centrifuged at 10,000xg for 1 hour at 4°C. Exosome pellets were then re-suspended in either PBS or appropriate media (epithelial or PBMC growth medium) for further purification. Appropriate volume for re-suspension was determined by normalizing to the surface area of epithelial cell growth.

## Purification with Spin columns:

Exosome Spin Columns (Invitrogen) were used to remove small molecules and particulates from exosome samples (MW<3000). Spin columns were rehydrated with 650µl of PBS and centrifuged twice at 760xg for 2min at room temperature. Samples were immediately overlaid 100µl at a time over the gel barrier and spun down at 760xg and collected. Columns were washed with PBS three

times between sample spins to ensure sample purity. Once purified, samples were filtered through 0.22  $\mu\text{m}$  filters and frozen at  $-20^{\circ}\text{C}$ .

## **II. Exosome Characterization:**

### **Exosome Preparation for Nanotracking:**

Exosome preparations were equilibrated to room temperature and diluted in sterile PBS that had been filtered through 0.22  $\mu\text{m}$  vacuum filter. Preparations were diluted 1000x and 50,000x to reach final volumes between 0.5 and 1ml.

### **Nanotracking parameters:**

Exosome preparations were quantified using the ZetaView (Particle Metrix) by translational diffusion size distribution. Particle concentration and size distribution was extrapolated from eleven video scans of samples, two measurements per scan, for a total of 22 data points per treatment.

### **Preparation of Epithelial Cells for Flow Cytometry analysis:**

Vaginal cells were seeded in 6-well flat-bottom plates at a density of  $1.2 \times 10^6$  cells/ml and grown to confluency. Immediately preceding infection, vaginal cells were stained using Syto RNA-select Green Fluorescent Dye (Thermo Fisher) for 30 min, protected from light. Cells were then washed three times with PBS and cultured in KSFMC-AB-.

### **Staining TV:**

*T. vaginalis* was stained using the TFL4 (OncoImmunit). TV was first spun down at 1000xg for 5 min and then washed with PBS. TV was incubated in a 1:3000 dilution of the dye for 1 hour, protected from light. TV was then spun down at 1000xg for 5 min and washed in PBS to remove dye. TV was then adjusted to a concentration of  $8 \times 10^5$  TV/ml in epithelial cell growth medium and overlaid over epithelial cells, for a final concentration of  $4 \times 10^5$  TV/ml upon incubation for 24h.

### **Preparation for Flow Cytometry:**

Cell supernatants were collected and centrifuged at 1000xg for 5 min to remove cells and debris. Supernatants were vacuum-filtered through a 220 nm filter to remove any remaining larger particles. Samples were analyzed using the Becton Dickinson SORP FACS Aria, using polystyrene beads as size controls and a sample of epithelial cell medium to control for medium auto-fluorescence.

#### **Preparation for Fluorescent Microscopy:**

Ectocervical cells (Ect1/E6E7) were first seeded into glass chamber slides. Cells were stained the next day using Syto RNA-select Green Fluorescent dye (Thermo Fisher) following the protocol outlined above in “Staining Epithelial Cells”. Cells were then washed three times with sterile, filtered (0.22 µm) PBS, and cultured in AB- medium. *T. vaginalis* cells were prepared as in “Exosome Isolation: *T. vaginalis* preparation”.

### **III. Endocervical Experimental Procedures:**

#### **Cell Culture:**

Immortalized endocervical epithelial cell (End1/E6E7) (29) were cultured in antibiotic-free, keratinocyte serum-free medium (AB-), supplemented with bovine pituitary extract, epidermal growth factor, and calcium chloride. Cells were seeded into 96-well flat-bottom plates at a density of  $5 \times 10^5$  cells/ml and grown to confluency.

#### **Exosome Exposure:**

Cells were cultured in the presence or absence of Poly(I:C) at a concentration of 10 µg/ml. Exosome preparations were diluted 10x on the plate. Cells were then incubated 24h under anaerobic conditions using an AnaeroPack-Anaero (Mitsubishi) sachet in a sealed chamber on a shaker at 50rpm, 35°C.

#### **NFκB Activation Luciferase Reporter Assay:**

After 24 h of exposure to exosomes and TLR stimulation, supernatants were collected, cells were lysed with GloLysis buffer and luciferase activity was determined using the Bright-Glo Luciferase Assay System by manufacturer’s protocol (Promega, Madison, WI) as described.

### **MSD Assays:**

Concentrations of soluble mediators IL-8 and RANTES were measured in cell culture supernatants simultaneously using an MSD duplex assay, Sector Imager 2400, and Workbench software.

## **IV. PBMC Experiments:**

### **PBMC Isolation:**

PBMCs were isolated from fresh whole blood obtained from donors at the Research Blood Components LLC (Brighton, MA) using density gradient separation. To remove plasma, blood was centrifuged at 3000rpm at 4°C for 20 min. Cells were then re-suspended with sterile PBS and overlaid onto Ficoll Histopaque. Samples were then centrifuged at 400xg for 40min at room temperature. The mononuclear cell layer was then quickly transferred into tubes containing sterile PBS, spun at 400xg for 5 min at room temperature and washed with PBS and re-suspended in RPMI medium with L-Glutamine and 20% heat inactivated Fetal Bovine Serum (FBS). Cells were then counted using an automated cell counter and adjusted to a final working concentration of  $2 \times 10^6$  cells/ml.

### **PBMC Exposure Experiments:**

PBMCs were cultured in the presence or absence of human IL-2 (Roche Diagnostics) and purified Phytohaemagglutinin (PHA), each in a ratio of 1:1000 in growth medium. PBMCs were then cultured in round-bottom 96-well plates. Exosomes were then added to wells at a 10x dilution on plate. Cells were then incubated at 37°C, 5% CO<sub>2</sub>, and cell supernatants were collected at 24h and 48h post-treatment.

### **Viability Assay:**

Epithelial cell and PBMC viability was measured using the CellTiter96 MTT proliferation assay, a non-radioactive assay in which MTT (a yellow tetrazole) is reduced to a purple formazan product in metabolically active cells.

### **Lysis for Caspase analysis:**

Cells were centrifuged at 400xg for 10 minutes at room temperature. Working on ice, Tris Lysis buffer containing a protease cocktail (MSD) was added to cells. Plates were incubated while being shaken vigorously at 4°C for 15 min and subsequently collected.

#### **MSD Assays:**

Concentrations of numerous cytokines produced were measured in either cell culture supernatants or lysates simultaneously using an MSD multiplex assay, Sector Imager 2400, and Workbench software.

#### **Preparations of PBMCs for Flow Cytometry:**

PBMCs were isolated from fresh whole blood and incubated for 24h in the presence or absence of IL-2 and PHA (1:1000). After 24h of stimulation, cells were centrifuged at 400xg for 10min at room temperature. Cells were corrected to a concentration of  $4 \times 10^6$  cells/ml in PBMC growth medium. Exosomes were then added to PBMC cultures at a 10x dilution from the stock and incubated overnight in round bottom tubes, on a shaker (50rpm) at 35°C under anaerobic conditions. After stimulating PBMCs with exosomes overnight, samples stained and prepared for flow cytometry using the DuraClone IM T Cell Subset Kit (Beckman Coulter), per manufacturer's protocol. Samples were immediately analyzed without fixation. Samples were run on the CytoFlex LX (Beckman-Coulter).

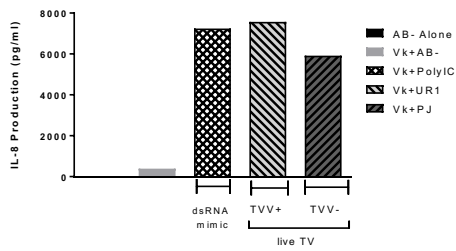
# **Results:**

## **Validation the *in vivo* Infection Model:**

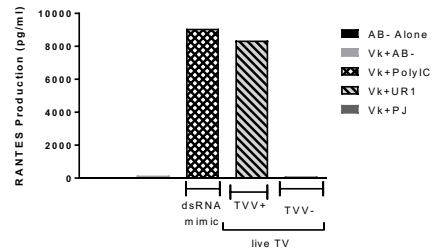
In order to characterize the response of primary infected epithelial cells, supernatants collected prior to exosome isolation were assayed for production of pro-inflammatory cytokines IL-8 and RANTES (Fig 2). Results from two separate isolation experiments are shown from 2.13.17(2A+2B) and 1.10.2017 (2C+2D). In both experiments, vaginal epithelial cells exposed to any TV strains or stimulation by Poly(I:C) produced higher levels of IL-8 compared to those that were not infected (2A+2C). However, only those epithelial cells exposed to TVV+ strains of TV or the dsRNA mimic Poly(I:C) produced high levels of RANTES. Epithelial cells exposed to TVV- strains produced comparable amounts of RANTES compared to uninfected cells. This suggests that the exposure to TVV- strains of TV do not induce viral inflammation via TLR3. These findings are align to those previously described by the lab (5).

**A**

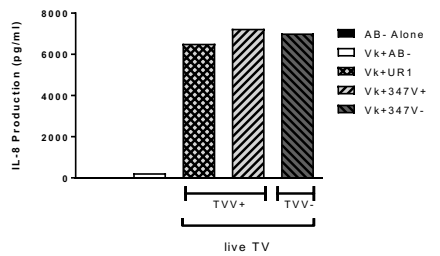
IL-8, Exosome Isolation, 2.13.2017, VK2/E6E7, 24h post treat, pre-isolation

**B**

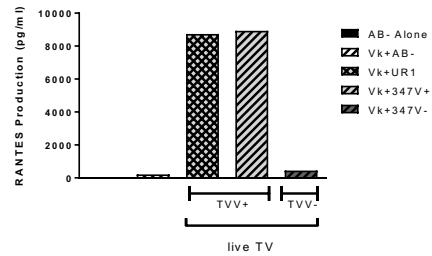
RANTES, Exosome Isolation, 2.13.2017, VK2/E6E7, 24h post treat, pre-isolation

**C**

IL-8, Exosome Isolation, 1.10.2017, VK2/E6E7, 24h post treat, pre-isolation

**D**

RANTES, Exosome Isolation, 1.10.2017, VK2/E6E7, 24h post treat, pre-isolation



**Figure 2: Confirmation of the infection model via cytokine profiling of vaginal epithelial cells. In the presence of TVV+ TV strains or stimulation by dsRNA mimic (Poly(I:C)), there is an increased production of RANTES (CCL5) by vaginal epithelial cells (VK2). Graph results single values from two exosome isolation experiments 2.13.17 (A+B) or 1.10.17 (C+D).**

**A) IL-8 profile of vaginal cells exposed to TVV+ TV, TVV- TV or Poly(I:C) (2.13.17)**

**B) RANTES profile of vaginal cells exposed to TVV+ TV, TVV- TV or Poly(I:C)**

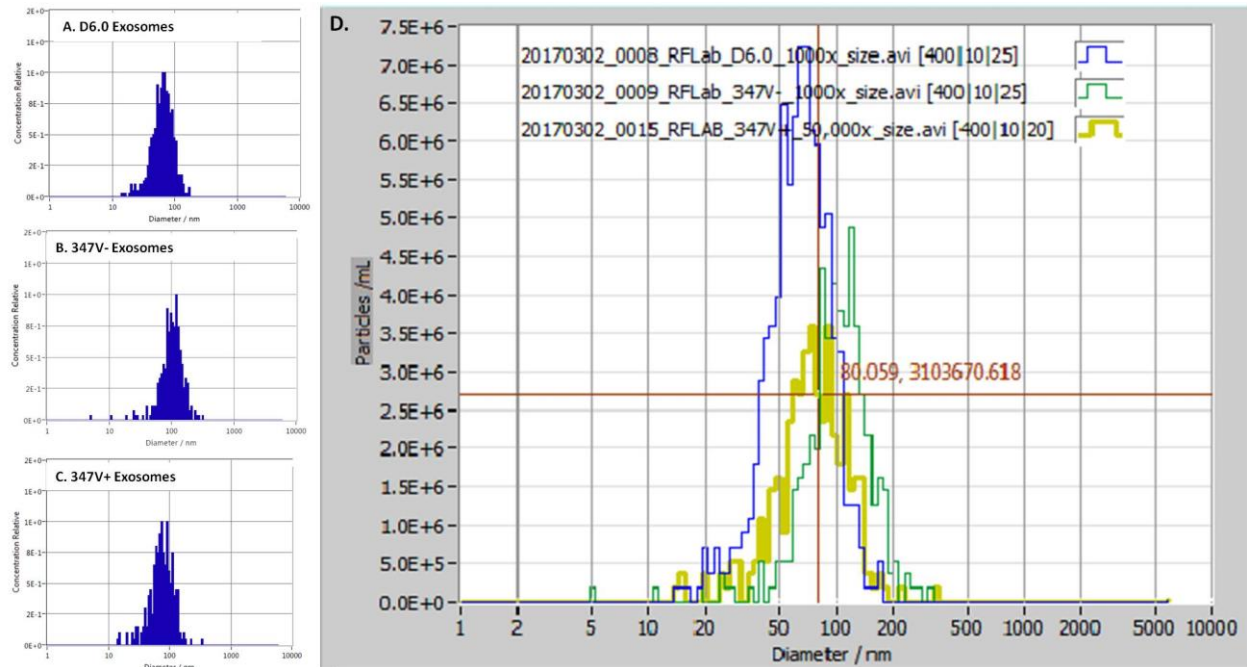
**C) IL-8 profile of vaginal cells exposed to TVV+ TV or TVV- TV (1.10.17)**

**RANTES profile of vaginal cells exposed to TVV+ TV or TVV- TV (1.10.17)**

### **Characterization of Microvesicles Populations:**

In order to characterize the isolated microvesicle populations, exosome preparations from isogenic *T. vaginalis* strains (347V+ and 347V-) and from D6.0 iron-supplemented medium without cells were quantified by size and frequency distribution using the ZetaView (Particle Metrix) for translational diffusion size distribution (Fig 3). Population curves were built from 11 independent sample scans, each with two counts, for a total of 22 data points per sample. Exosome preparations from D6.0 medium yielded an average concentration of  $2.3 \times 10^{10}$  particles/ml with most particles of a diameter of 67.4 nm and an average diameter of 68.3 nm (Fig 3A, Table 5). Exosome preparations from 347V- yielded a concentration of  $1.6 \times 10^{10}$  vesicles/ml with a vesicle population centered around a diameter of 106.9 nm, with an average diameter of 108.9 nm (Fig 3B, Table 5). Exosome preparations from 347V+ yielded a concentration of  $2.3 \times 10^{12}$  particles/ml with a population peak at 76.6 nm and an average diameter of 81.4 nm (Fig 3C, Table 5). A compilation of all microvesicle population distributions prior to dilution corrections is shown in Figure 3D. These averages both agree with values determined previously in the lab (Fichorova lab, unpublished data) and are in line with exosome populations currently defined in the literature (27, 30, 31).





**Figure 3: Nanoparticle Tracking Analysis of TV-derived exosomes. Exosomes from monocultures of (A) D6.0 medium without TV, (B) 347V- , (C) 347V+exosomes and a (D) comparative curve. In the comparative curve, D6.0 microvesicles are shown by the blue curve, 347V- microvesicles are shown in green and 347V+ vesicles are shown in yellow. The comparative curve is prior to dilution corrected. All samples were diluted in filtered sterile Phosphate buffered saline (PBS) and processed using video analysis of 2 data cycles in 11 scanning areas for a total of 22 measurements taken per sample.**

**Table 5: Summary of difference between extracellular vesicle populations after dilution correction.**

Exosome Source	Concentration (particles/ml)	Average Diameter (nm)	Population peak (nm)
D6.0 Iron-supplemented medium, no TV	$2.33 \times 10^{10}$	68.3	67.4
347V-	$1.6 \times 10^{10}$	106.9	108.9
347V+	$2.3 \times 10^{12}$	81.4	76.6

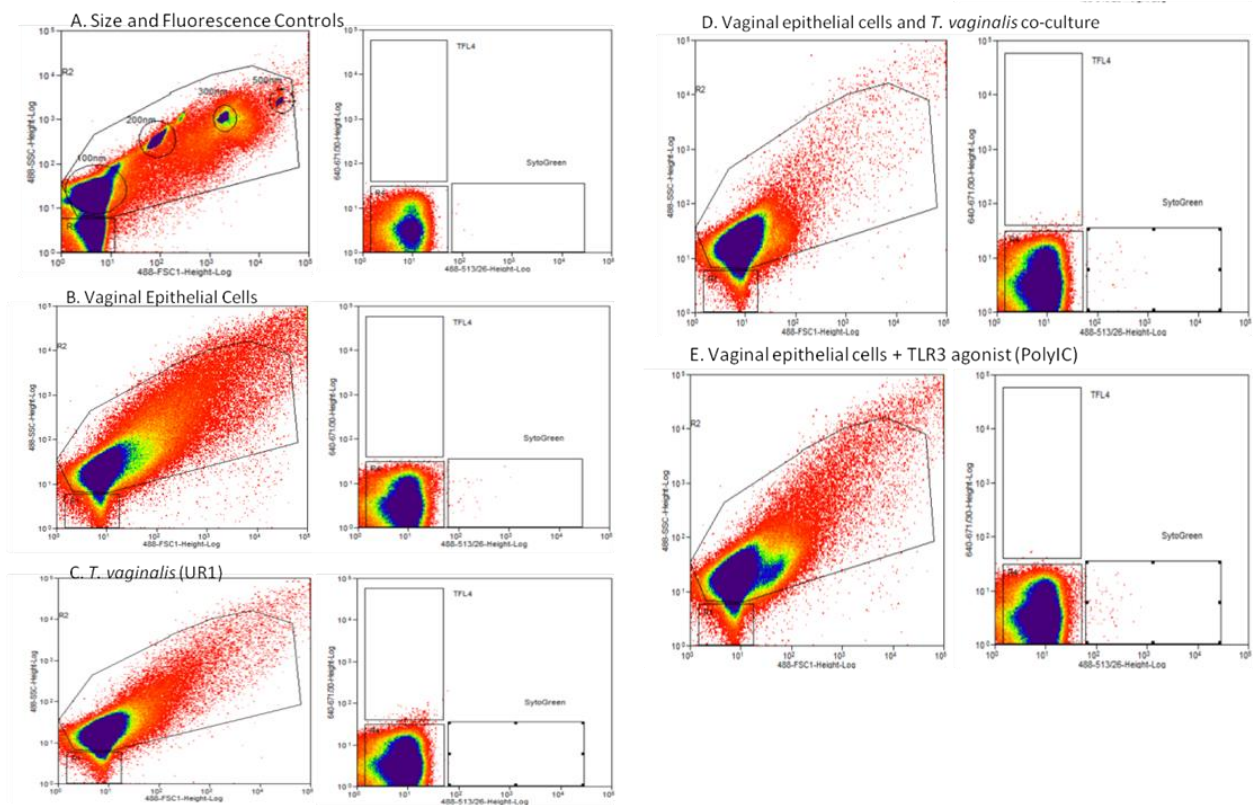
### **Exosome Characterization by Flow Cytometry:**

Exosome preparations from all treatment conditions were also quantified using flow cytometry, using the FACS Aria II (Fig 4). To characterize the predominant source of exosomes in co-culture preparations, two different fluorescent dyes were used in the *in vivo* infection model using live *T. vaginalis*. Vaginal epithelial cells were stained with SytoRNAselect and *T. vaginalis* was stained using TFL4. Polystyrene size controls and medium autofluorescence are shown in Fig 4A. Four experimental conditions were tested, uninfected vaginal cells (4B), *T. vaginalis* monoculture (4C), infected epithelial cells (4D) and epithelial cells stimulated with dsRNA mimic Poly(I:C) (4E). Although both experiments using yielded a relatively low fluorescent staining efficiency when quantified by flow cytometry, significant populations with a diameter around  $10^2$  nm were present in all experimental treatments, indicating the presence of extracellular vesicles of a similar size to exosomes defined in the literature (30, 32). In addition, these experiments showed SytoGreen and TFL4 positive particles in the exosome size range simultaneously present in the vaginal-TV co-cultures suggesting that both the parasite and host cells contribute to the exosome pool in the infection model (Figure 4D) as hypothesized in Schema 1. Vesicle populations previously isolated using this method have been validated via both nanotracking analysis and anti-CD63 gold staining (Fichorova lab, unpublished data). These results indicate that while there is a significant microvesicle population contained in cell supernatants, the staining procedure must still be optimized.

### **Staining of Cells for Fluorescence Microscopy:**

In order to validate the incorporation of the fluorescent dyes into the cell membranes, ectocervical epithelial cells and *T. vaginalis* cells were stained and prepared for fluorescent

microscopy. A representative bright field image is shown in Figure 5. Due to a technical problem that occurred during the handling of microscopic samples, we were unable to obtain high resolution fluorescent images. However, the phase contrast image shown in Figure 5 demonstrates the physiologic density of cells in our model with preserved epithelial morphology suitable for that functional analyses that followed.



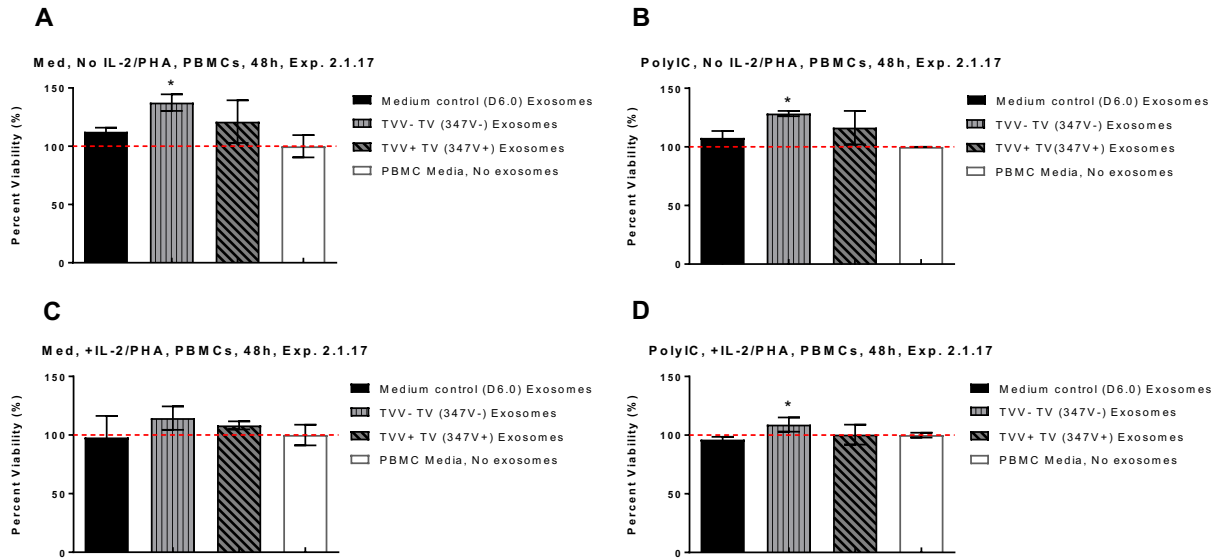
**Figure 4: Flow Cytometry analysis of supernatants containing stained microvesicles. Monocultures of TV and epithelial cells were stained with fluorescent dyes. Epithelial cells were incubated with Syto RNaselect GFP for 30 minutes. TV was incubated with TFL4 for 1 hour. Both samples were then washed three times with PBS before co-culture for 24h. Cell supernatants were collected from monocultures and co-cultures, spun down at 1000xg for 5 minutes to filter out TV and cell debris before vacuum filtration through 220nm filter. Samples were run on the Becton Dickinson SORP FACSaria II, using (A) polystyrene beads to control for size and epithelial cell medium as a control for background fluorescence. Treatments include (B) VK2 cells alone, showing fluorescent microparticles in the SytoGreen spectrum only, (C) TV cells alone, showing only TFL4 positive particles, (D) VK2+TV co-culture showing both SytoGreen and TFL4 positive particles, and (E) VK2 cells stimulated with dsRNA mimic Poly (I:C) showing StoGreen positive particles. Plots represent the results of one experiment of two.**



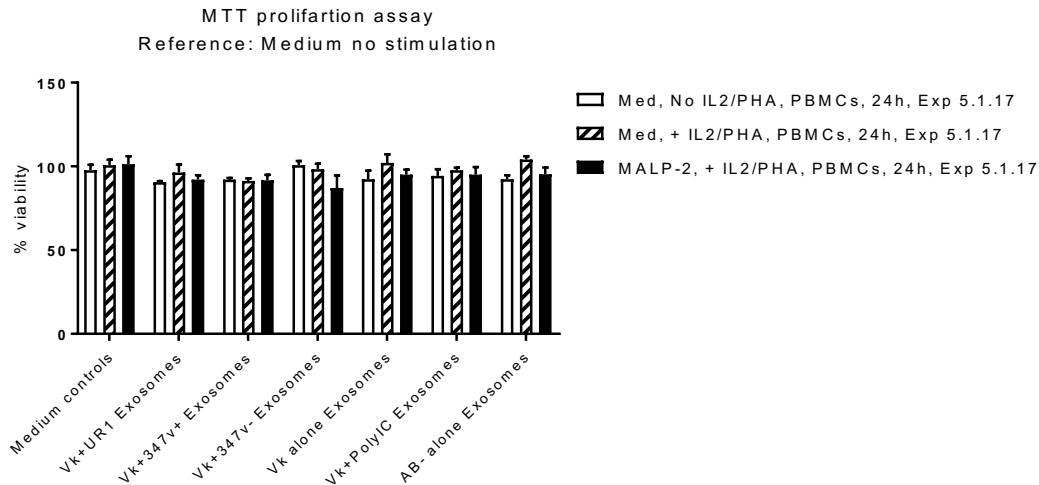
**Figure 5: Fluorescence Microscopy of *in vitro* model of *Trichomonas* infection (ectocervical cells + 347V+), bright field image. Differential morphology is visible: TV is present as smaller adherent cells (blue arrow); ectocervical epithelial cells are flattened, larger and have created a monolayer.**

### **Effect of Exosomes on PBMC viability:**

In order to characterize PBMC proliferative potential and viability, an MTT proliferation assay was conducted 48h post-treatment with exosomes (Fig 6). Four different medium treatments were used: (1) the complete absence of stimulation (6A), (2) the presence of Poly (I:C) but absence of IL-2/PHA (6B), (3) the presence of IL-2/PHA but absence of Poly (I:C) (6C), and (4) the dual presence of both IL-2/PHA and Poly (I:C) (6D). With the exception on one exosome preparation derived from TVV-TV monocultures in one experiment, which showed a slight proliferative effect ( $p < 0.05$ ), there was no significant effect of TV monoculture-derived or TV medium (Diamond 6.0)-derived exosomes compared to PBMC medium control. Furthermore, no treatments with exosomes derived from vaginal-TV co-cultures induced a significant change in PBMC viability/proliferation within the 24h-treatment period (Figure 7). Taken as a sum, this suggests that TV-derived exosomes do not have a cytopathic effect and may in fact actually stimulate cell proliferation.



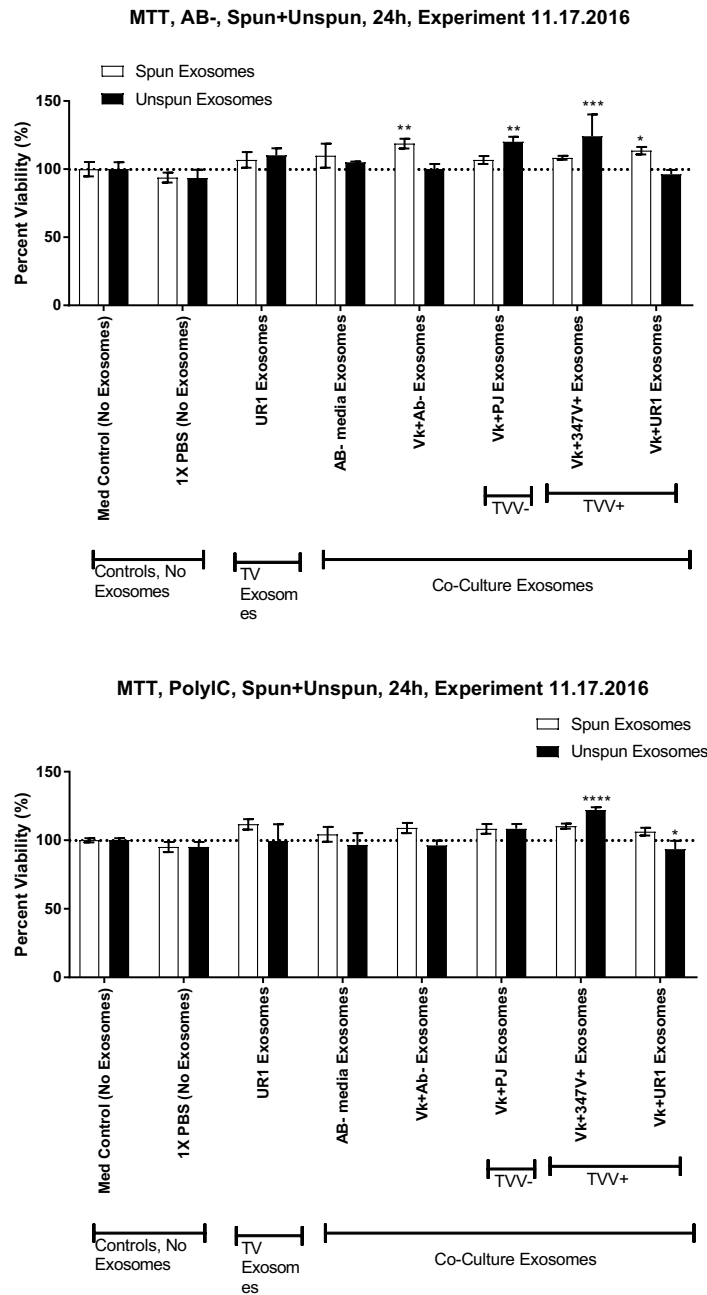
**Figure 6: PBMC Viability in response to treatment with exosomes derived from TV monocultures.** Viability was measured at 48h post-treatment using an MTT Proliferation Assay. Treatments include (A) PBMCs in the absence of stimulation, (B) PBMCs in the presence of stimulation by Poly(I:C), (C) PBMCs in the presence of stimulation by IL-2/PHA and (D), PBMCs in the presence of dual stimulation of IL-2/PHA and Poly(I:C). Results analyzed using a one-way ANOVA,  $p < .05$ , Dunnett post hoc. (\*) denote treatments that differ significantly from PBMC Media control (no exosomes), by  $p < .05$ . Results are graphed as triplicates from one experiment of two.



**Figure 7: PBMC Viability in response to treatment with exosomes derived from TV-vaginal co-cultures.** Viability was measured at 24h post-treatment using an MTT Proliferation Assay. Treatments include (1) PBMCs in the absence of stimulation, (2) PBMCs in the presence of stimulation by MALP, and (3) PBMCs in the presence of stimulation by IL-2/PHA. Results analyzed using a one-way ANOVA,  $p < .05$ , Dunnett post hoc. Results are graphed as triplicates from one experiment of two.

### **Epithelial cell viability:**

In order to characterize epithelial cell viability in response to exosome exposure, an MTT proliferative assay was conducted on vaginal epithelial cells 24h after treatment with both TV-derived and co-culture exosomes (Fig 8). Cells were incubated in the (A) absence or (B) presence of Poly(I:C). “Spun” further purified using exosome spin columns (Invitrogen) as opposed to their “Unspun” counterparts. In the absence of TLR stimulation, cells exposed to spun exosomes from vaginal cell monoculture, as well as several TV-co-culture exosome had higher percent viability than the medium control wells (no exosomes) as analyzed by a two-way ANOVA,  $p < .05$ , Dunnett post-hoc. In the presence of TLR stimulation by Poly(I:C), only unspun exosomes derived from co-culture of vaginal cells and 347V+ increased cell viability significantly compared to medium control wells (two-way ANOVA,  $p < .05$ , Dunnett post-hoc). Interestingly, unspun exosomes derived from co-culture of vaginal cells and TVV+ strain UR1 did significantly decrease cell viability compared to medium control wells (two-way ANOVA,  $p < .05$ , Dunnett post-hoc). However, this was the only exosome source that did adversely affect cell viability and these exosomes were not used in further experiments.



**Figure 8: Epithelial cell viability in response to exosomes derived from TV monocultures and vaginal-TV co-cultures. Viability was measured 24h post treatment using an MTT Viability assay. Treatments include cells co-cultured with exosomes in the (A) Absence or (B) Presence of stimulation by Poly (I:C). “Spun” exosomes were further purified using Exosome Spin Columns (Invitrogen). “Unspun” exosomes did not undergo further purification. Treatments are shown as triplicates from one experiment of two. Results were analyzed using a two-way ANOVA,  $p < .05$ , Dunnett post-hoc, as compared to the Medium Control (No Exosomes). (\*) indicate results significant  $p < .05$ , (\*\*) indicate  $p < .01$ , (\*\*\*)  $p < .001$ , (\*\*\*\*)  $p < .0001$ .**



**T. vaginalis exosomes modulate NF-κB expression in by-stander upper reproductive tract epithelial cells:**

In order to quantify NF-κB activation, a luciferase assay was run on endocervical cells (End/NF-κB) 24h post-treatment (Fig 9).

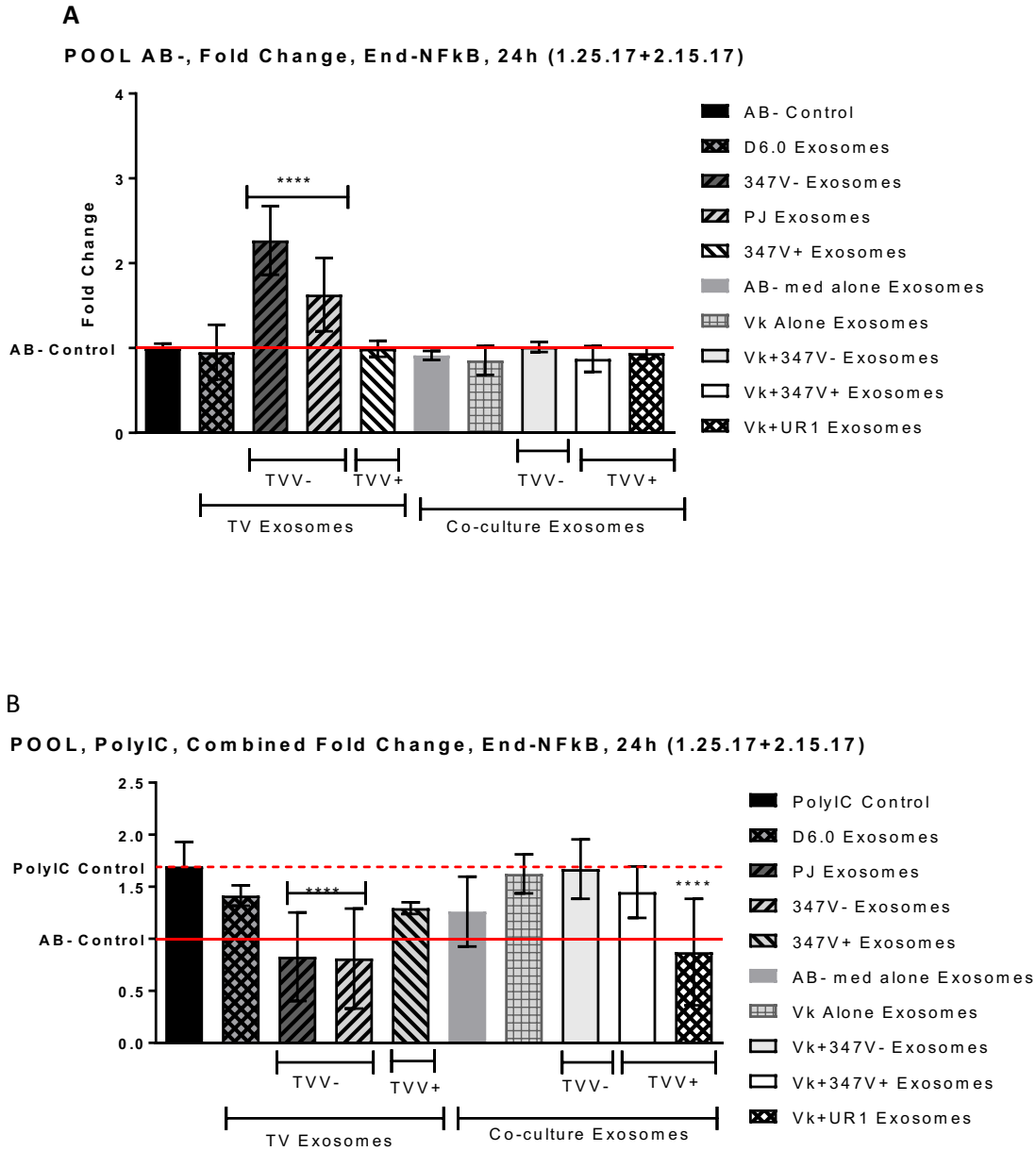
When added to resting cells (Fig. 9A), both PJ and 347V- exosomes significantly increased NF-κB expression ( $p < .05$ , one-way ANOVA, Dunnett post-hoc test). None of the other exosome treatments differed significantly from the medium control.

In cells co-stimulated with the TLR3 agonist Poly (I:C) (Fig. 9B), the TVV- TV monoculture (PJ and 347V) exosomes and the Vκ-UR1 co-culture exosomes, significantly decreased NF-κB activation ( $p < .05$ , one-way ANOVA, Dunnett post-hoc test). The remaining exosome preparations did not cause a statistically significant activation of NF-κB. The decreased activation in the presence of TV-UR1 exosomes may be due to the cytopathic effect of this one preparation. However, the TVV-TV and the remaining TVV+ exosome preparations did not cause any significant effect on cell viability and therefore their effects on NF-κB activation appears to be toxicity-independent. The Poly (I:C) control yielded significantly higher NF-κB expression than the AB- control (over 1.5 fold increase, demonstrated by the dotted line in Fig. 9B), indicating the success of the cell stimulation.

These results indicate that exosomes from TVV- but not TVV+ strains of *T. vaginalis* induce NF-κB activation in resting bystander (non-infected) epithelial endocervical cells, which are anatomically in close proximity to the primary vaginal site of infection. When comparing isogenic strains, the strain infected with TVV virus (347V+) did not increase NF-κB activation

whereas its non-infected counterpart (347V-) did significantly increase NF- $\kappa$ B activation, suggesting a potential immunoprotective anti-inflammatory role of Trichomonasvirus infection.

In cells primed with poly (I:C) stimulation however, this pattern reverses. In the presence of poly (I:C) (synthetically mimicking the effect of TVV), the same exosomes that were previously pro-inflammatory now seemed to counter-balance the poly (I:C) effect suggesting that the parasite may use the exosome mechanism to specifically suppress the NF- $\kappa$ B activation to its own symbiotic virus thus aiding its task to evade host immunity.



**Figure 9: NFkB activation in response to exosomes. Activation of NFkB was measured in endocervical cells using a Luciferase reporter assay 24h post-treatment with exosomes in the (A) Absence or (B) Presence of stimulation via Poly(I:C). Results are shown as triplicate values from two experiments. Treatments were analyzed using a one-way ANOVA,  $p < .05$ , Dunnett post-hoc, as compared to the Medium control and Poly(I:C) control respectively. (\*) indicate results significant  $p < .05$ , (\*\*) indicate  $p < .01$ , (\*\*\*)  $p < .001$ , (\*\*\*\*)  $p < .0001$ .**

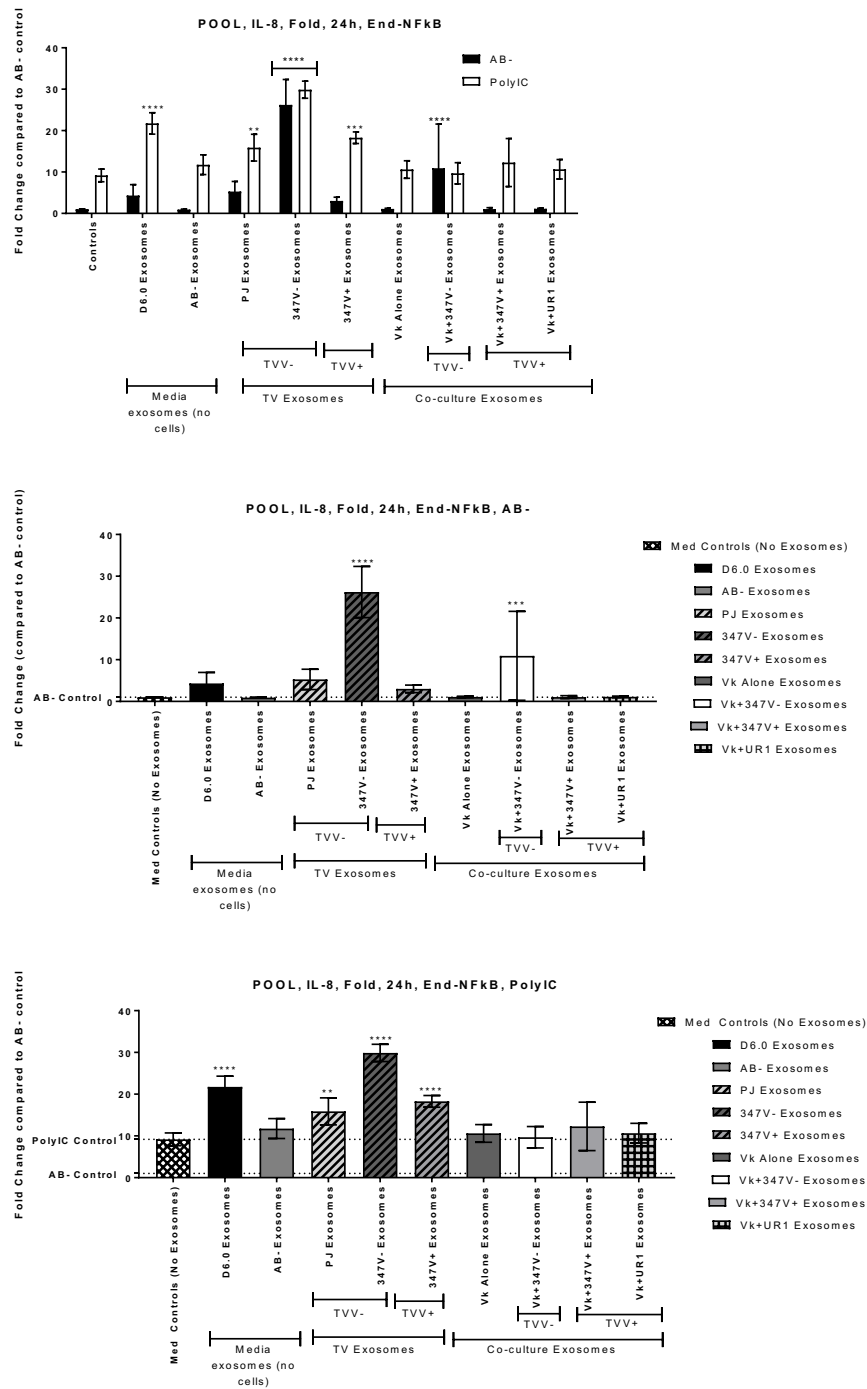
### **T. vaginalis exosomes alter endocervical cytokine profile:**

In order to assess immune response of endocervical epithelial cells to exosomes, levels of proinflammatory cytokines were assayed in cell supernatants. Levels of IL-8 production (Fig. 10) and RANTES (Fig. 11) were assayed from supernatants collected 24h after treatment with exosomes. Results are shown as fold-change from average medium control pooled from two independent experiments. As expected from prior research in this lab, the endocervical epithelial cells responded to poly (I:C) with vigorous upregulation of IL-8 (10-fold, Fig. 10 ) and RANTES (30-fold, Fig. 11) production compared to resting medium controls.

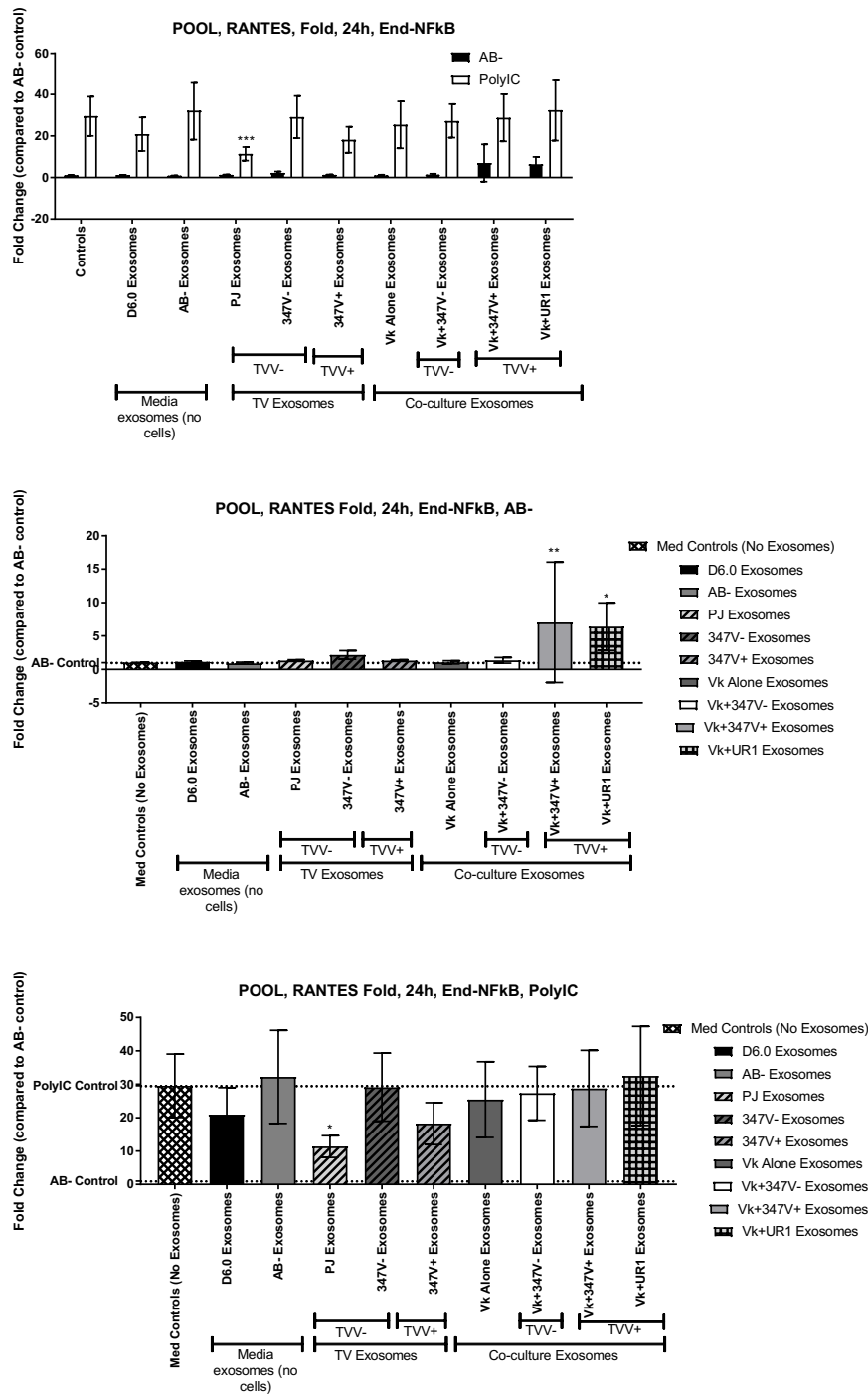
In the absence of poly (I:C), the only exosome preparation that significantly and consistently increased IL-8 but not RANTES was the one isolated from the TVV cured isogenic TV strain 347v- (one-way ANOVA,  $p < 0.05$ , Dunnett post-hoc). Similarly, although more variably, the vaginal-347v- co-culture exosomes induced IL-8 but not RANTES. In the presence of poly (I:C) all preparations of TV monocultures induced IL-8 but so did the TV culture medium control (Fig. 10) suggesting a non-specific effect of possibly vesicle components of the serum-supplemented medium in this case. These effects were not observed with RANTES where the effects of exosomes were inconsistent between experiments (Fig. 11).

Taken as a whole, these results support the hypothesis that exosome control on production of inflammatory cytokines is dependent on the viral status of the *T. vaginalis* strain in question. Exosomes derived from TVV- strain 347V- consistently yielded higher production of IL-8 but not RANTES compared to its isogenic TVV+ counterpart. Because there is no significant difference in the RANTES production by the endocervical bystander cells, it is unlikely that viral contamination of samples is a driving factor in target cell responses. Since RANTES is specific

for anti-viral epithelial response as previously shown by Fichorova's Lab (5), its lack of upregulation by 347v- in contrast to IL-8 supports our hypothesis that once TVV infected the TV strains develop mechanisms to specifically suppress anti-viral response in order to aid the virus in its immune evasion mission.



**Figure 10: Endocervical IL-8 response to exosome treatment.** Cytokine levels were assayed in cell supernatants collected 24h post-treatment with exosomes. Results are shown as triplicate values from two experiments (A) compiled from both medium treatments, cells in the (B) absence and (C) presence of Poly(I:C) stimulation. Results were analyzed using a two-way ANOVA (A only) and one-way ANOVA (B+C);  $p < .05$ , Dunnett post-hoc, as compared to the Medium control (No Exosomes). (\*) indicate results significant  $p < .05$ , (\*\*) indicate  $p < .01$ , (\*\*\*)  $p < .001$ , (\*\*\*\*)  $p < .0001$ .

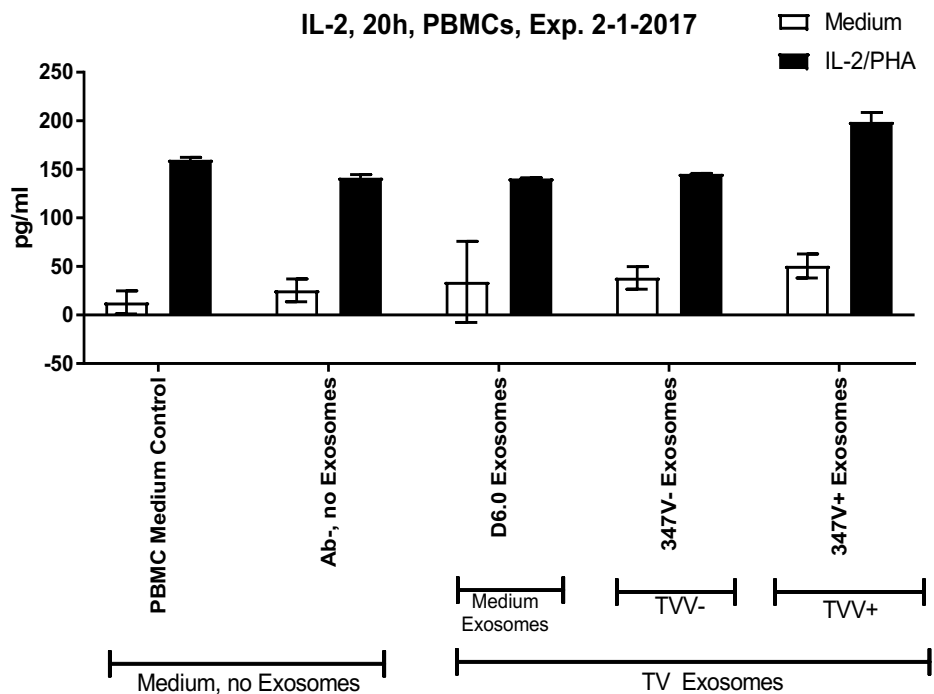


**Figure 11: Endocervical cytokine response (RANTES) in response to exosome treatment. Cytokine levels were assayed in cell supernatants collected 24h post-treatment with exosomes. Results are shown as triplicate values from two experiments (A) compiled from both medium treatments, cells in the (B) absence and (C) presence of Poly(I:C) stimulation. Results were analyzed using a two-way ANOVA (A only) and one-way ANOVA (B+C);  $p < .05$ , Dunnett post-hoc, as compared to the Medium control (No Exosomes). (\*) indicate results significant  $p < .05$ , (\*\*) indicate  $p < .01$ , (\*\*\*)  $p < .001$ , (\*\*\*\*)  $p < .0001$ .**

**Effect of *T. vaginalis* monoculture exosomes on PBMC cytokine profiles:**

To determine the effect of *T. vaginalis* monoculture exosomes on peripheral blood mononuclear cells, cells were treated with exosomes in the presence or absence of IL-2/PHA, and the presence or absence of poly(I:C), as well as in the absence of both types of stimuli and cell supernatants were collected to assay levels of cytokines and chemokines (Fig 12-16).

Although there was no significant difference by exosome treatment, measurements of IL-2 production do indicate higher levels of cytokines in well where exogenous IL-2/PHA was added as stimulation (Fig 12).



**Figure 12: PBMC IL-2 response to exosomes. Cytokine levels were assayed in cell supernatants collected 20h post-treatment with exosomes, in the absence of IL-2/PHA or in the presence of by IL-2/PHA that followed 24h pre-stimulation by IL-2/PHA. Results are shown as duplicate values from one of two experiments. Treatments were analyzed using a two-way ANOVA,  $p < .05$ , Dunnett post-hoc, as compared to the PBMC Medium control (No Exosomes). (\*) indicate results significant  $p < .05$ , (\*\*) indicate  $p < .01$ , (\*\*\*)  $p < .001$ , (\*\*\*\*)  $p < .0001$ .**



In order to assess the acute phase response, we considered the production of IL-1 $\beta$  and TNF- $\alpha$ . Both cytokines were specifically and significantly upregulated in cells exposed to exosomes from the TVV- TV strains regardless of IL2/PHA stimulation (exemplified by 347v- in Fig. 13) (two-way ANOVA,  $p < .05$ , Dunnett post-hoc). No other exosome treatments differed significantly when compared to PBMC medium control (no exosomes).

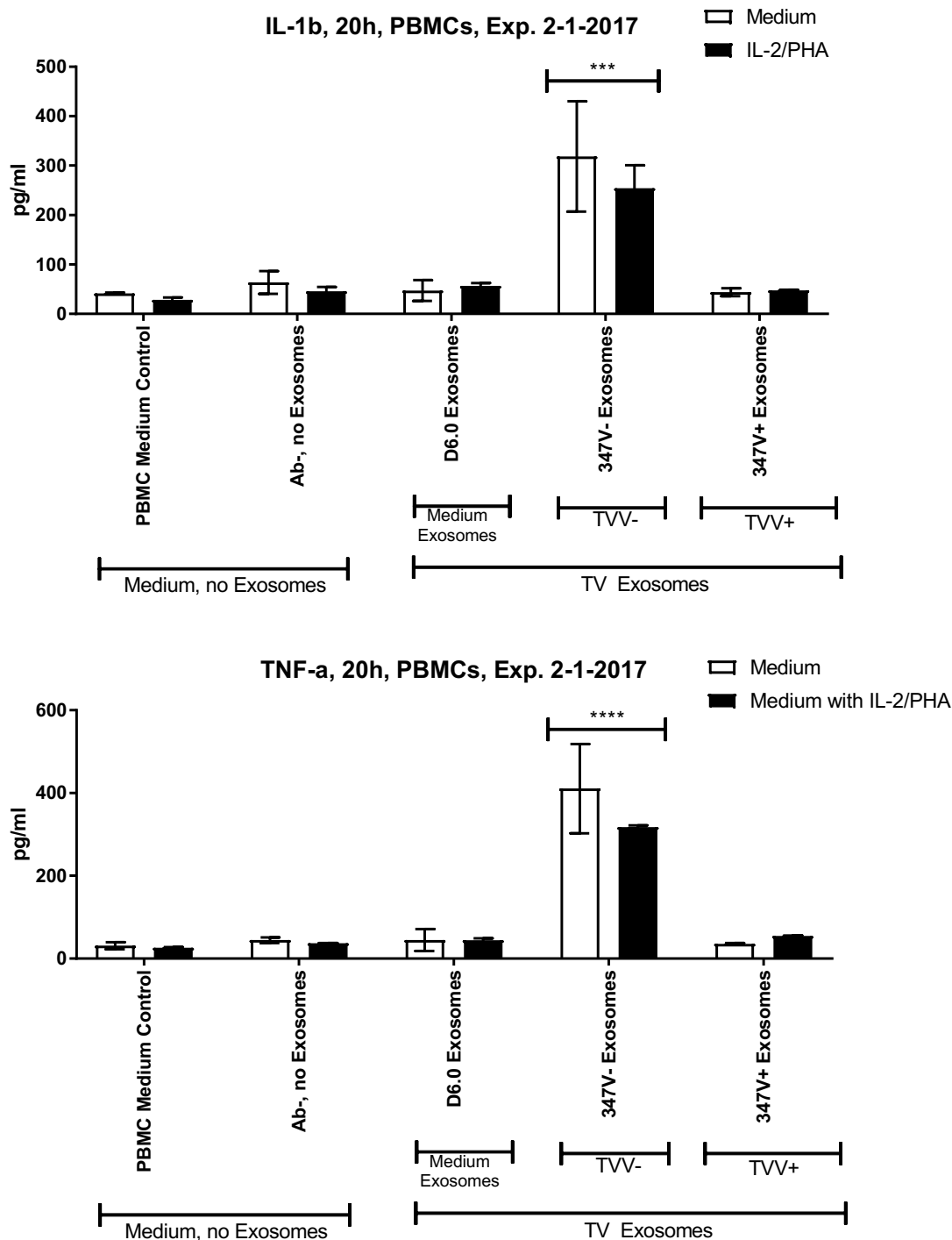
Similarly and consistently with the effects of TV monoculture exosomes on endocervical cells, IL-8, which is a chemokine downstream from NF-kB and IL-1 $\beta$  and TNF $\alpha$  signaling, was upregulated in the PBMC cells by 347v- but not 347v+ exosomes (Fig. 14A,  $p < 0.001$ , two-way ANOVA,  $p < .05$ , Dunnett post-hoc). Again, consistently with the results obtained with endocervical cells, the TV exosomes failed to upregulate RANTES (Fig. 14B).

When measuring immune-regulatory cytokines produced in early stages of inflammation, IL-4 (Fig. 15A) and IL-6 (Fig. 15B) were significantly upregulated by IL-2/PHA and regardless of IL-2/PHA stimulation, also by exosomes derived from the TVV- TV strain (347 v-). IL-4 and IL-6 were upregulated to a much lower extent by exosomes derived from serum-supplemented medium (D 6.0) and the isogenic TVV+ TV (347v+), the latter showing some modest activity in IL-2/PHA stimulated cells only (two-way ANOVA,  $p < .05$ , Dunnett post-hoc).

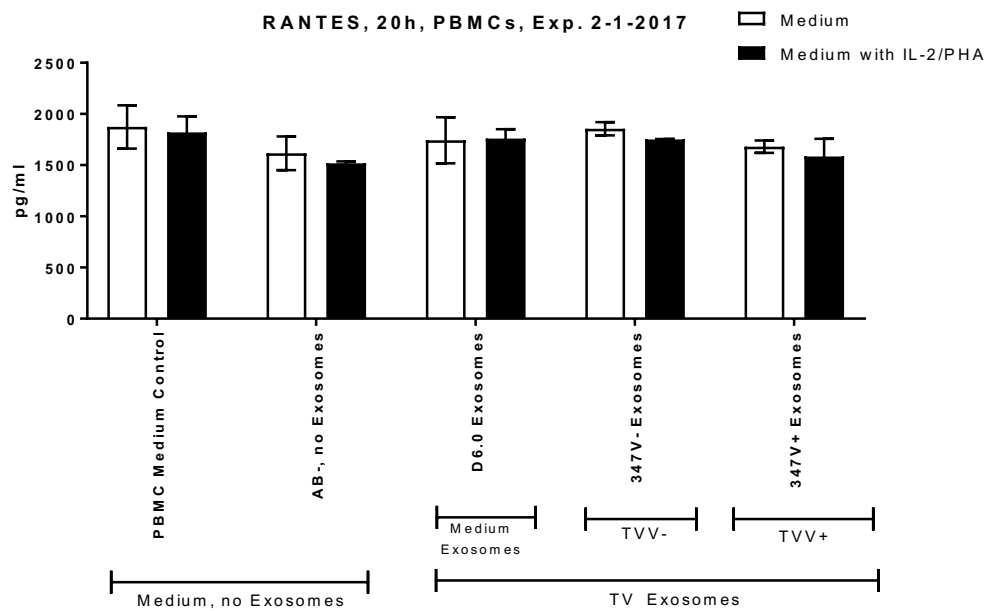
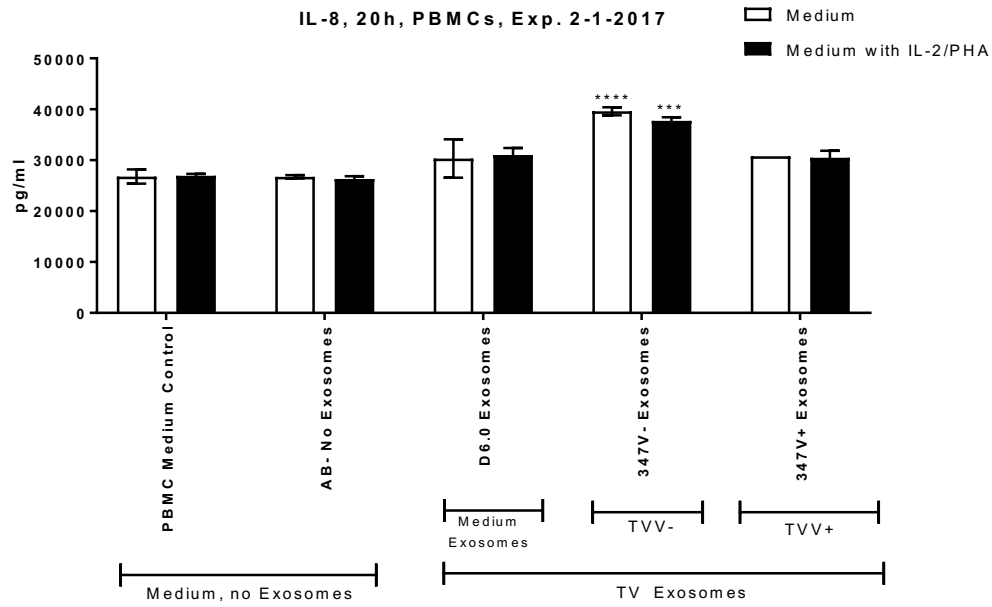
In order to assess the potential effect of exosomes on adaptive immunity, we considered the production of two cytokines characteristic of T helper cell subsets: IL-10 and IL-17A (Fig 16). The patterns were similar to those obtained for IL-4 and IL-6. Both cytokines were upregulated by cells exposed to TVV- exosomes (347v-) compared to medium control (no exosomes), regardless of IL-2/PHA stimulation (two-way ANOVA,  $p < 0.05$ , Dunnett post-hoc). The TVV+TV exosomes (347 v+) upregulated both cytokine only in cells stimulated with IL-2/PHA. The exosomes derived

from serum-supplemented medium (D6.0) also more than two-fold weaker upregulating effect, similar to that of the 347v+ exosomes, and also in IL-2/PHA stimulated cells only, suggesting that the effect of the 347v+ was not specific for TV.

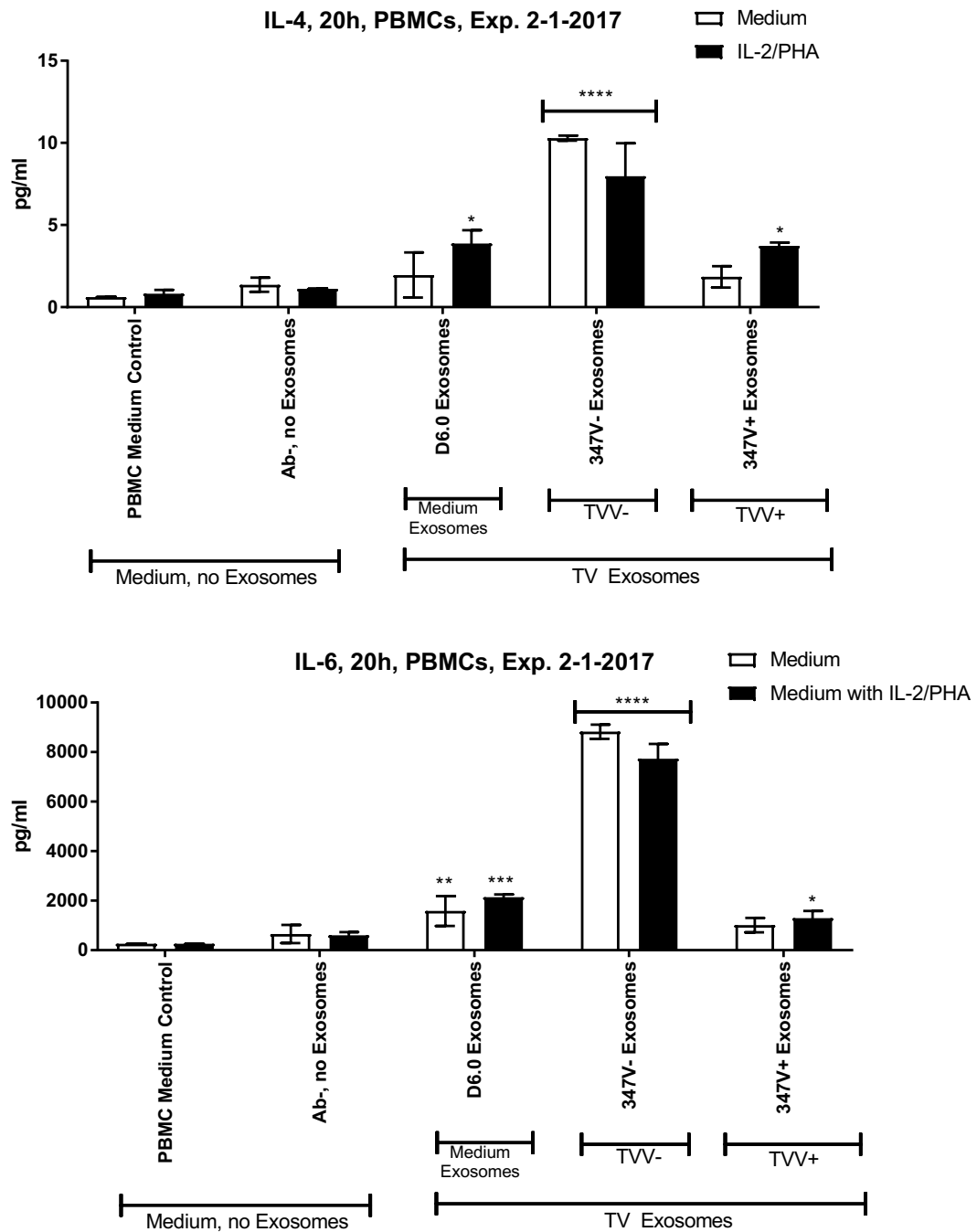
Taken as a whole, these results indicate a selective cytokine upregulating effect of exosomes derived from the TVV-cured TV strain which was absent in the isogenic TVV+ counterpart, supporting our hypothesis of the role of TVV in exosome-regulated immunity. More-specifically, the results suggest an immunosuppressive effect of TVV since the exosomes from TVV infected parasites largely mimicked the medium controls.



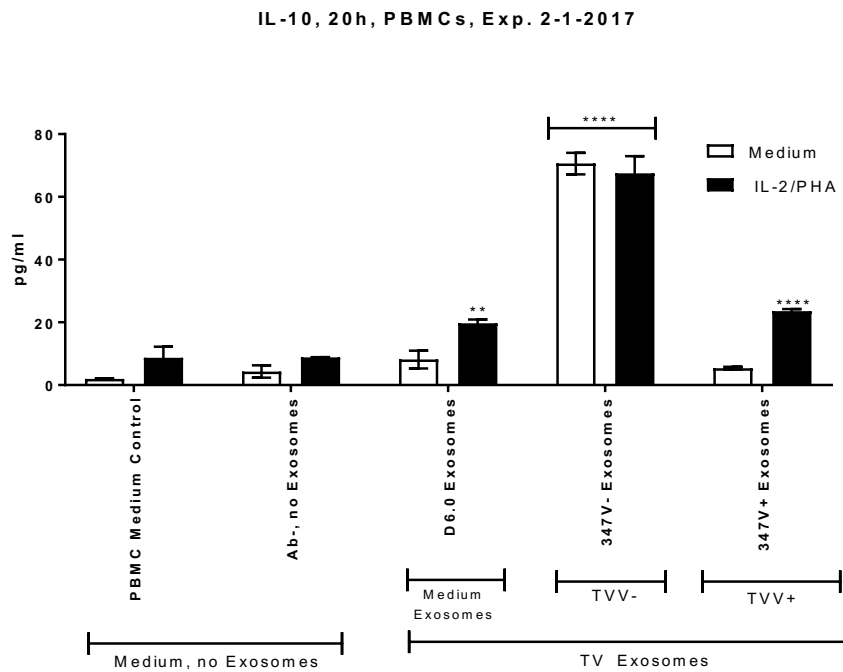
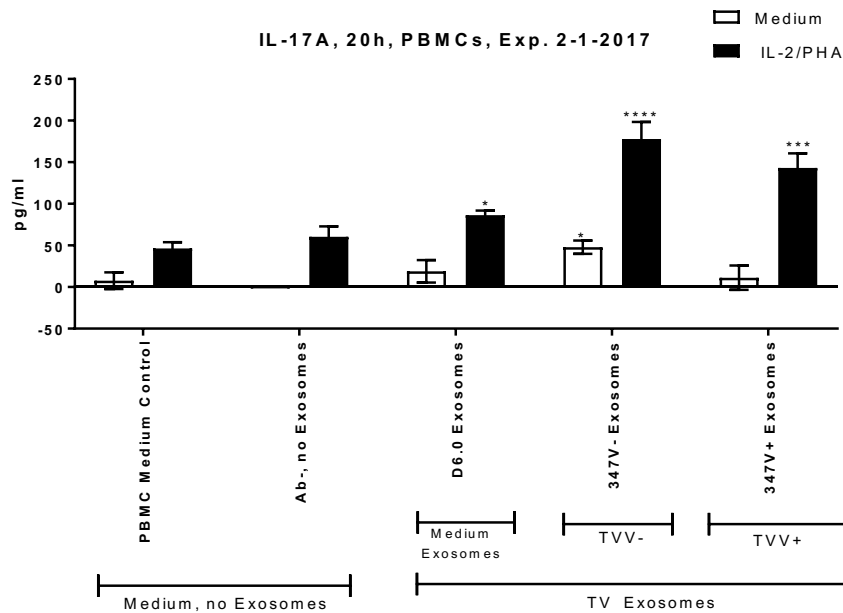
**Figure 13: Acute phase cytokine response to exosomes. (A) IL-1  $\beta$  and (B) TNF- $\alpha$  levels were assayed in cell supernatants collected 20h post-treatment with exosomes, in the absence of IL-2/PHA or in the presence of by IL-2/PHA that followed 24h pre-stimulation by IL-2/PHA. Results are shown as duplicate values from one of two experiments. Treatments were analyzed using a two-way ANOVA,  $p < .05$ , Dunnett post-hoc, as compared to the PBMC Medium control (No Exosomes). (\*) indicate results significant  $p < .05$ , (\*\*) indicate  $p < .01$ , (\*\*\*)  $p < .001$ , (\*\*\*\*)  $p < .0001$ .**



**Figure 14: IL-8 (A) and RANTES (B) response to exosome treatment. Cytokine levels were assayed in cell supernatants collected 20h post-treatment with exosomes, in the absence of IL-2/PHA or in the presence of by IL-2/PHA that followed 24h pre-stimulation by IL-2/PHA. Results are shown as duplicate values from one of two experiments. Treatments were analyzed using a two-way ANOVA,  $p < .05$ , Dunnett post-hoc, as compared to the PBMC Medium control (No Exosomes). (\*) indicate results significant  $p < .05$ , (\*\*) indicate  $p < .01$ , (\*\*\*)  $p < .001$ , (\*\*\*\*)  $p < .0001$ .**



**Figure 15: Pro-inflammatory cytokine response to exosomes. (A) IL-4 and (B) IL-6 levels were assayed in cell supernatants collected 20h post-treatment with exosomes, in the absence of IL-2/PHA or in the presence of by IL-2/PHA that followed 24h pre-stimulation by IL-2/PHA. Results are shown as duplicate values from one of two experiments. Treatments were analyzed using a two-way ANOVA,  $p < .05$ , Dunnett post-hoc, as compared to the PBMC Medium control (No Exosomes). (\*) indicate results significant  $p < .05$ , (\*\*) indicate  $p < .01$ , (\*\*\*)  $p < .001$ , (\*\*\*\*)  $p < .0001$ .**



**Fig 16: PBMC Fig #: PBMC production of characteristic T-helper cell cytokines. (A) IL-17A and (B) IL-10 levels were assayed in cell supernatants collected 20h post-treatment with exosomes, in the absence of IL-2/PHA or in the presence of by IL-2/PHA that followed 24h pre-stimulation by IL-2/PHA. Results are shown as duplicate values from one of two experiments. Treatments were analyzed using a two-way ANOVA,  $p < .05$ , Dunnett post-hoc, as compared to the PBMC Medium control (No Exosomes). (\*) indicate results significant  $p < .05$ , (\*\*) indicate  $p < .01$ , (\*\*\*)  $p < .001$ , (\*\*\*\*)  $p < .0001$ .**

### **Effect of vaginal-*T. vaginalis* co-culture exosomes on PBMC cytokine profiles:**

To determine the effect of vaginal-*T. vaginalis* co-culture exosomes on peripheral blood mononuclear cells, cells were treated with exosomes in the absence or presence of IL-2/PHA, and the presence or absence of MALP-2, as well as a standard TLR-2/TLR6 ligand known to upregulate pro-inflammatory pathways in PBMCs. In these experiments the PBMCs were not pre-stimulated but rather exposed directly to all stimuli for 24h and cytokines assessed in cell culture supernatants in parallel with flow cytometry. The most dramatic effect was seen for all cytokines and chemokines tested in the presence of MALP-2 stimulation (Fig 17).

As expected MALP-2 significantly upregulated all measured cytokines and chemokines in resting PBMCs. Interestingly, the exosomes from the exosomes derived from vaginal cells infected with TVV+ strains (347v+ strain featured in Fig. 17) were efficient in abrogating all cytokines (IL-12p70, IL-1 $\beta$ , TNF $\alpha$ , IL-17A, IL-2, IL-6, IL-4 and IL-10), and the chemokines (IL-8 and MCP-1) responses to MALP-2 in comparison to exosomes derived from uninfected vaginal epithelial cells (“Vk alone exosomes”).

Compared to non-exosome treated MALP-2 stimulated vaginal cells, the Vk alone exosomes had no effect on their own with two exceptions: 1) they selectively upregulated IL-6, and 2) they selectively suppressed MALP-2 induced MCP-1 (P<0.001); however, the exosomes generated from 347v+ infected vaginal cells suppressed MCP-1 even further compared to Vk alone exosomes (P<0.001). The effects of the 347v- exosomes were closely mimicked by exosomes produced in response to poly (I:C) in the cases of IL-12p70, IL-17A, IL-2, IL-6, IL-4, IL-10 and IL-8, implicating the TLR3 signaling pathway in regulating the vaginal exosome content. The exosomes derived from the vaginal cells infected with the TVV-cured TV did not suppress

proinflammatory cytokines and chemokines but suppressed predominantly T cell immune-regulatory cytokines e.g. IL-2, IL-6, IL-10, and IL-17A. On the other hand, the most powerful proinflammatory mediators IL-1 $\beta$ , TNF $\alpha$  and IL-8 were suppressed exclusively by exosomes released in the context of the TVV+TV vaginal infection suggesting a synergistic effect of TV and TVV.

Taken together, these data support our hypothesis that TVV infection has a significant impact on the exosome content released in the context of the human vaginal infection. More specifically, these data suggest that the TVV infection supports an immunosuppressive phenotype and that TV and TVV act in concert to suppress leukocyte immune response via exosome content.



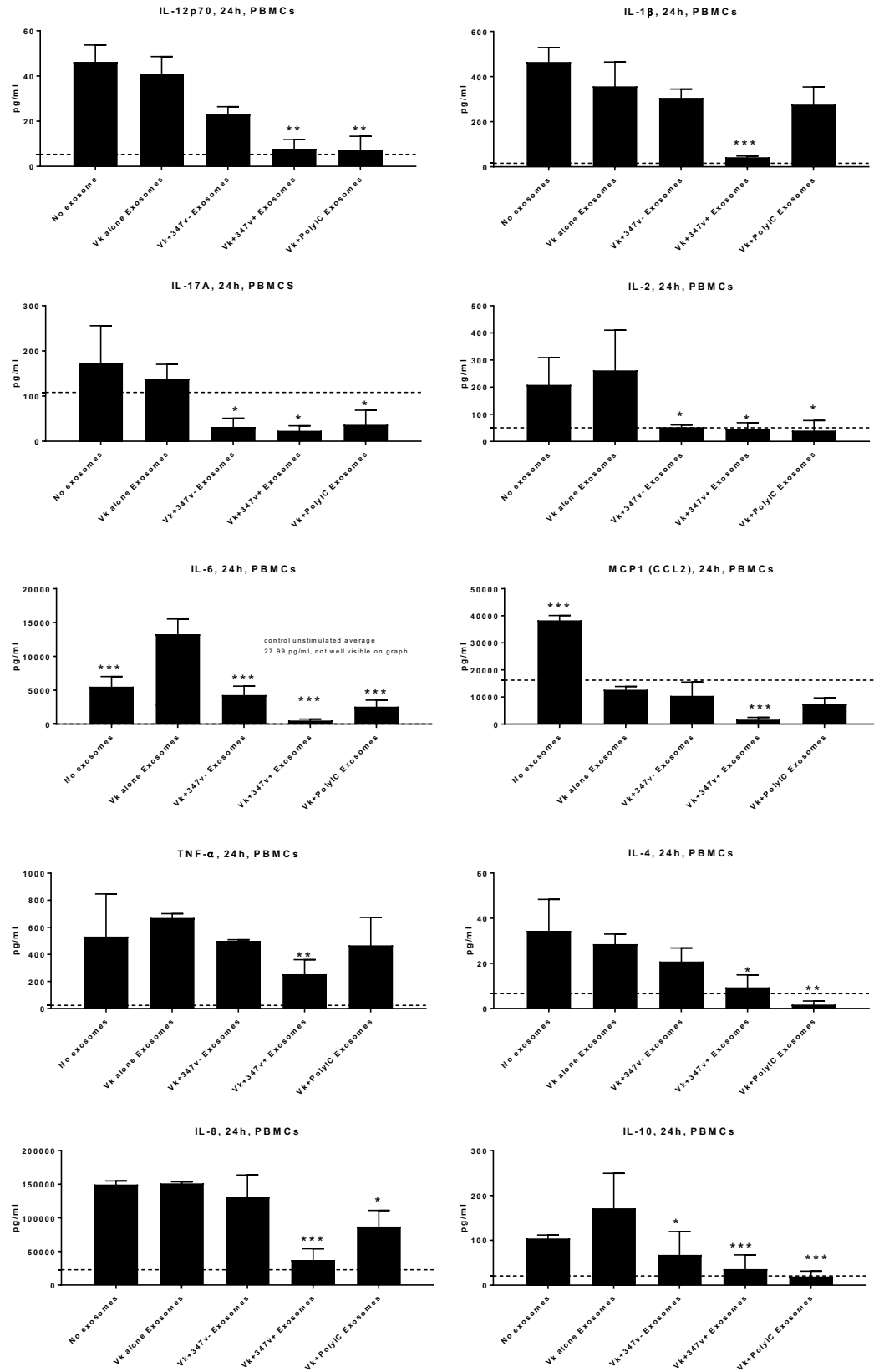


Figure 17. Acute phase cytokine response to exosomes derived from vaginal-TV co-cultures in the presence of MALP-2. Results are triplicate measurements from one in two experiments, analyzed by ANOVA, Dunnet's pot-hoc.

## Effect of *T. vaginalis* Exosomes and live TV on Lymphocyte differentiation:

In order to determine the effect of both live TV and TV-derived exosomes on lymphocyte differentiation, peripheral blood mononuclear cells were incubated in the presence or absence of stimulation, TV and TV derived exosomes. A prototype gating schematic provided by the company is shown in Figure 18.

### SAMPLE ANALYSIS (Example)

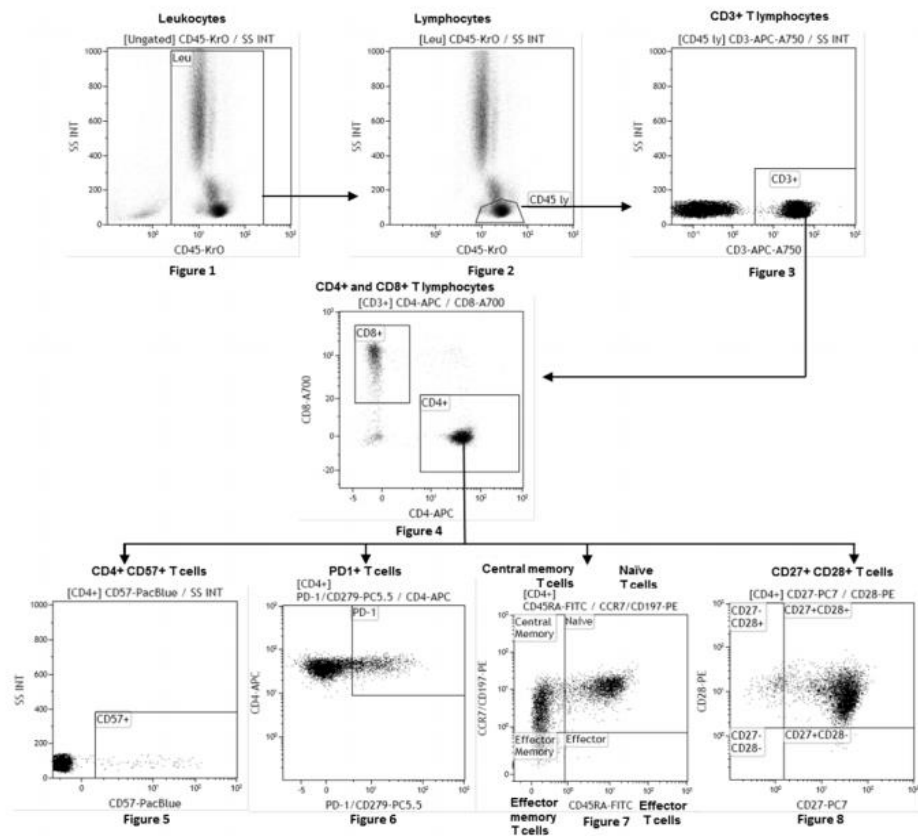


Figure1-8: Gating protocol followed for sample analysis (example)

Figure 18: Gating schematic used in cell Flow Cytometry Analysis. This was included in the manufacturer's instructions of the Duraclone IM T cell Subsets Kit (Beckman Coulter). The first gate is set on CD45+ cells to isolate the Leukocyte population. Then, a gate is placed on the CD3+ population, which defines the T-cell population. Gates are then set to denote CD4+ and CD8+ populations. Using the CD4+ population, a variety of different phenotypes can be determined, such as exhaustion state (PD1+ cells) or formation of memory (CCR7+ or CD45RA). Analysis was conducted on Beckman-Coulter CytoFlex LX.

In order to assess the effect of IL-2/PHA stimulation, we compared three major subsets of cells between the cells in the absence (Fig.19A B), or presence (Fig. 19C, D) of IL-2/PHA stimulation. In unstimulated cells, CD3<sup>+</sup> lymphocytes comprised 75% of the total population of cells (19A) and of the CD3<sup>+</sup> population, 16.96% were CD8<sup>+</sup> cytotoxic cells and 67.42% were CD4<sup>+</sup> cells (19B). In comparison, cells stimulated with IL-2/PHA have a CD3<sup>+</sup> population of 73.68%, which is comparable to the unstimulated cells (19C). At 16.52% CD8<sup>+</sup> cells, this subset is also comparable between the two treatments (19B+D). However, the CD4<sup>+</sup> population of the IL-2/PHA stimulated cells only comprised 2.87% of the CD3<sup>+</sup> population, a substantial decrease from 67.42% in the unstimulated cells. The effect of IL-2/PHA can also be observed in the proportion of naïve versus memory T cells. In unstimulated PBMCs, the predominant population of cells is CCR7<sup>+</sup>CD45RA<sup>+</sup>, which traditionally characterizes naïve T cells (19A). In comparison, in response to IL-2/PHA stimulation, the naïve T cell population decreased within 48h to 8.33% (20B). Taken as a sum, these results indicate that stimulation of PBMCs by IL-2/PHA could have a potentially adverse effect on the relative proportion of CD4<sup>+</sup> cells detectable by flow cytometry but also on the relative percentage of the naïve T cell population.

We then examined the effect of TV infection on PBMC differentiation. Figure 21 shows the cell populations of (A) unstimulated PBMCs, (B) PBMCs exposed to live 347V<sup>-</sup> (the cured TVV<sup>-</sup> strain) and (C) live 347V<sup>+</sup> (the naturally infected TVV<sup>+</sup> strain). Compared to the unstimulated control cells, cells infected with live TV both had lower proportions of CD3<sup>+</sup> cells and somewhat mimicked the effects of IL2/PHA stimulation by demonstrating lower proportions of CD3<sup>+</sup>CD4<sup>+</sup> cells. PBMCs infected with 347V<sup>-</sup> had 18.49% CD3<sup>+</sup> cells and PBMCs infected with 347V<sup>+</sup> had 48.27% CD3<sup>+</sup> cell, both of which are lower than 75.38% in the unstimulated controls. This suggests a potential role of TV infection to deplete the CD3<sup>+</sup> population. Compared

to unstimulated controls, infection by both strains of TV skewed the proportion of CD8+ to CD4+ cells. In the unstimulated control the percentage of CD8+ cells (out of all CD3+ cells) is 16.96% compared to a CD4+ percentage of 67.42% (21A, bottom). In cells treated with 347V-, CD4+ cells comprised 29.90% of CD3+ cells the CD8+ cells were 25.43%. In cells treated with 347V+, CD4+ cells comprised 20.82% of CD3+ cells and CD8+ cells 9.67%. In both treatment conditions with TV, CD4+ cells comprised a smaller relative proportion of the CD3+ lymphocytes.

We also wanted to assess the effect (if any) of the exosomes from serum-supplemented medium (D6.0 medium) on T cell differentiation, since our TV monocultures exosomes were derived from TV cultures in D6.0 medium. In Figure 22, we compared cells stimulated with IL-2/PHA for 24h and then treated for another 24h in the presence of IL-2/PHA with either medium mock (no exosomes) control or exosomes isolated from D6.0 growth medium without cells. In the medium-mock treated cells, the CD3+ population was 73.68% of the total cell population (22A) compared to D6.0 exosome treated cells which had a CD3+ population of 74.55% (22D). The proportion of CD8+ cells is 16.52% in mock treated cells (22B) compared to 16.42% in those treated with D6.0 exosomes (22E). The proportion of CD4+ cells is 2.87% in mock treated cells (22B) compared to 2.80% in those treated with D6.0 exosomes (22E). The similarities between the treatments were upheld in the proportion of naïve and memory phenotypes (22C+22F). In sum, the D6.0 exosome treated PBMCs appeared to have a similar proportion of phenotypes compared to those cells treated with IL-2/PHA alone (no exosome mock), suggesting that any differences we see in the forthcoming exosome treatments with TV derived exosomes were unlikely due to the effect of the TV growth medium.

Finally, we assessed the effect of TV-derived exosomes on T cell differentiation (Fig 23-25).

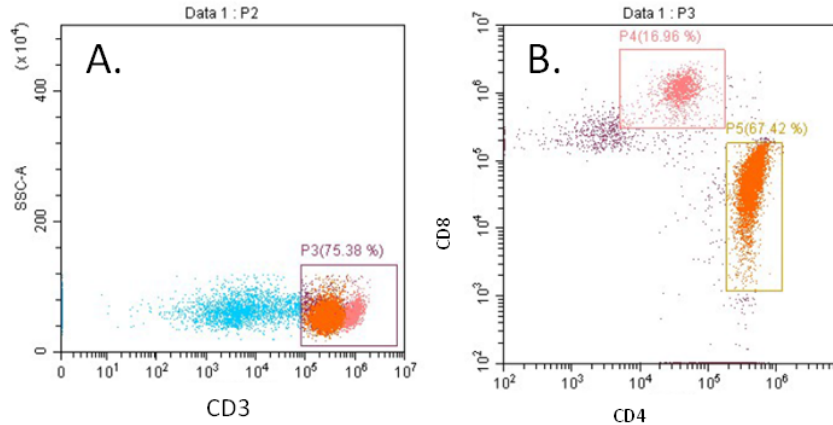
We first examined the proportion of CD3<sup>+</sup> cells among the CD45<sup>+</sup> cells. Compared to cells treated with mock exosome doses (23A), cells treated with exosomes from both 347V<sup>-</sup> (23B) and 347V<sup>+</sup> (23C) produced similar proportions of CD3<sup>+</sup> cells. All treatments stimulated with IL-2/PHA had a similar proportion of CD3<sup>+</sup> cells compared to the unstimulated (no IL-2/PHA, no exosome) control as well (23D).

We then examined the populations of CD4<sup>+</sup> and CD8<sup>+</sup> cells among the CD3<sup>+</sup> cell population (Fig 23). The proportion of CD8<sup>+</sup> cells was consistent amongst all treatments. However, compared to the no exosome control, there was slightly higher percentage of CD4<sup>+</sup> in cells treated with 347V<sup>-</sup> and 347V<sup>+</sup> exosomes, 3.44% and 5.47% respectively, compared to 2.87% (Fig 24B+C). While this increase did not fully recover the 67.42% CD4<sup>+</sup> population seen in unstimulated cells (Fig 24D), it is interesting to see this slight shift in proportions happening after only 24h stimulation.

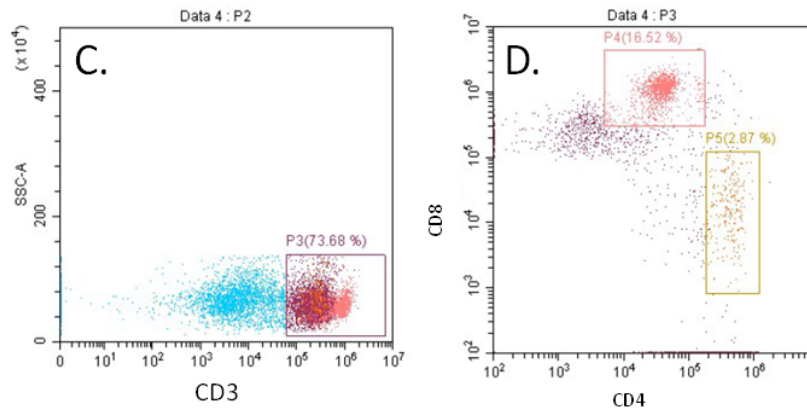
We also compared the differentiation of T cell memory subsets (Fig 25). Compared to the mock-dose (no exosome) control, there were higher percentages of naïve T cells (CD45RA<sup>+</sup>CCR7<sup>+</sup>) in all TV exosome treatments. Cells stimulated with IL-2/PHA only had a naïve T cell population of 8.33% (Fig 25A, upper right quadrant). This number increased to 16.37% in cells treated with 347V<sup>-</sup> exosomes (Fig 25B) and 21.99% in cells treated with 347V<sup>+</sup> exosomes (Fig 25C). While this also does not completely align with unstimulated cells, which have a naïve population of 51.42% (Fig 25C), it is interesting to note that treatment with TV-exosomes is sufficient to double the percentage of naïve T cells, perhaps acting by suppressing the effect of IL-2/PHA stimulation. Although further experiments are needed to validate these results, they support the immunosuppressive cytokine profiles and the potential role of TV-exosomes to oppose T cells differentiation.

Figure 26 graphically summarizes the compilation of effector and memory phenotypes across all treatments as a percentage of the CD3+CD4+ population. In unstimulated cells, naive T cells are the dominant population and this percentage dramatically decreases when cells are stimulated by live TV strains either infected or uninfected by TVV. In the context of live TV encounter, the central T cell population also increases. Although live TV does not infect leukocytes and does not enter the blood circulation, the effect of live TV on PBMC suggests that they similarly affect immune cells in mucosal inflammatory infiltrates and thus these effects should be further studied. In the presence of stimulation by IL-2/PHA the naïve cell population decreases, compared to the unstimulated control. However, in IL-2/PHA stimulated cells treated with TV exosomes, naive T cells appear to be rescued. Taken as a whole, these findings suggest that exosomes could function to mask infection by quelling the activation of naive T cells.

Unstimulated PBMCs

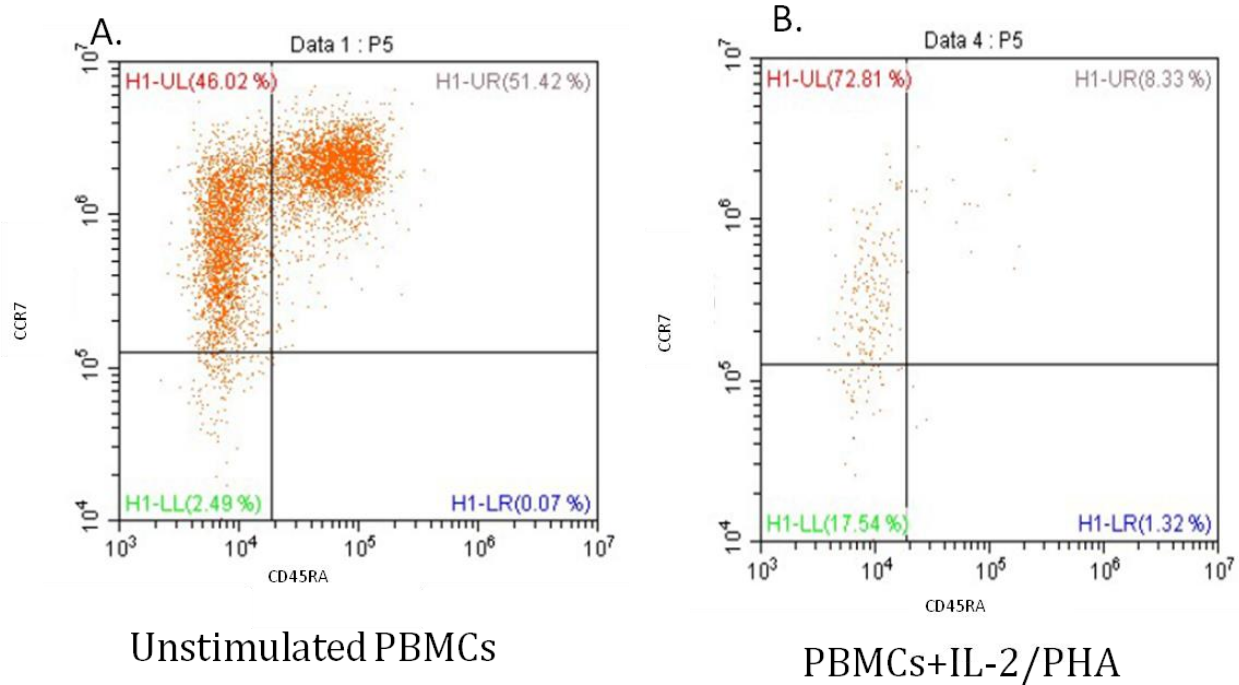


PBMCs+IL-2/PHA

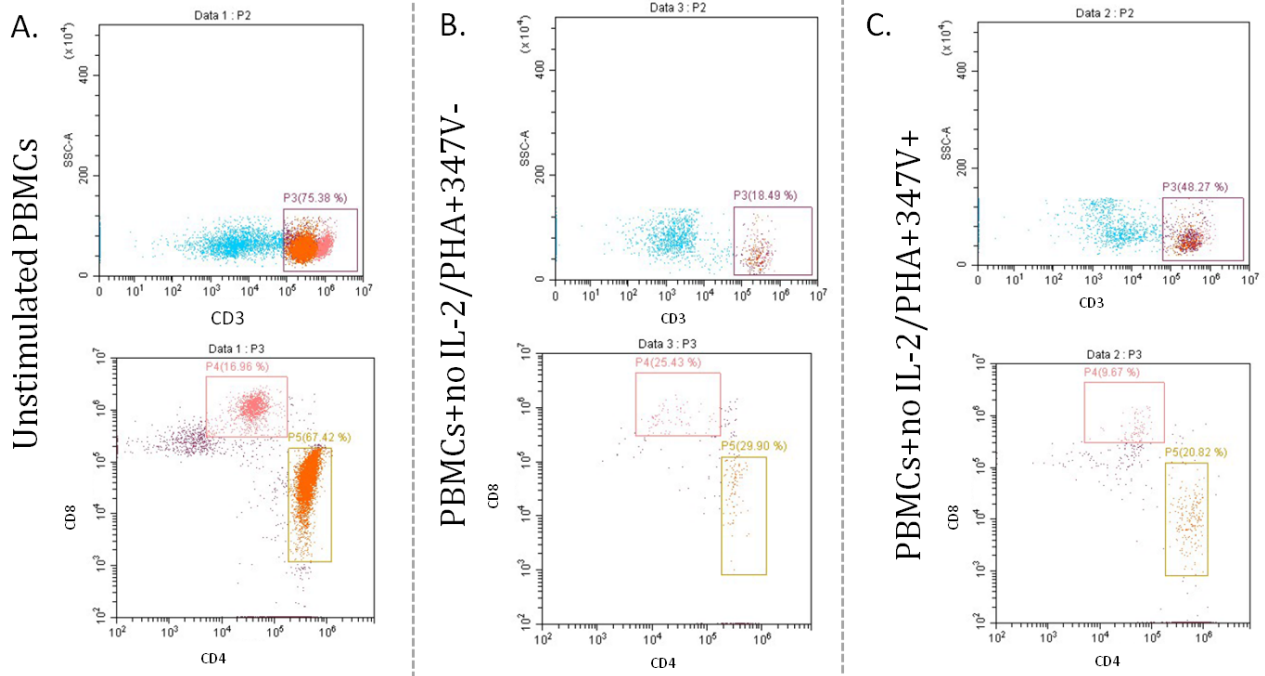


**Figure 19: PBMC Flow Cytometry Effect of IL-2 on CD3+ cells (A+C) and CD4 and CD8 populations (B+D). PBMCs were incubated for ~48h with medium in the absence (A+B) or presence (C+D) of stimulation by IL-2/PHA. After 48h of incubation, cells were stained using Duraclone IM T cell Subset Kit (Beckman-Coulter) and immediately analyzed, without fixation. Cells were run on the CytoFlex LX (Beckman-Coulter) and all samples are normalized to 25,000 events. Plots represent the results of an independent experiment.**



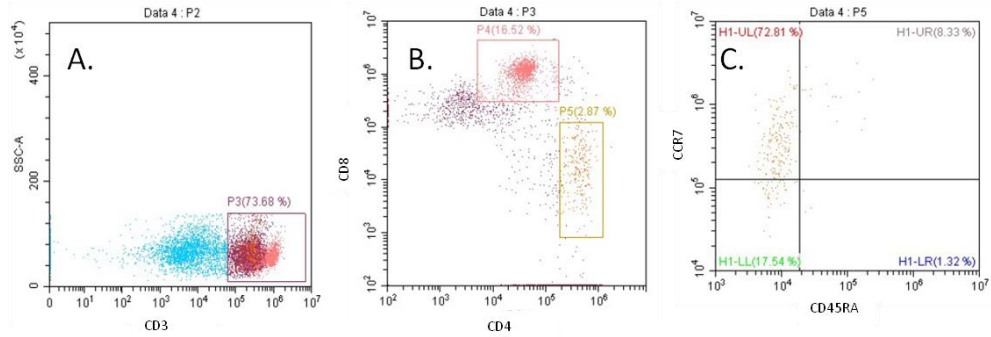


**Figure 20: Effect of IL-2/PHA on memory populations.** Cells were incubated for 48h in the absence (A) or presence (B) of stimulation by IL-2/PHA. After 48h of incubation, cells were stained using Duraclone IM T cell Subset Kit (Beckman-Coulter) and immediately analyzed, without fixation. Cells were run on the CytoFlex LX (Beckman-Coulter) and all samples are normalized to 25,000 events. Plots represent the results of an independent experiment.

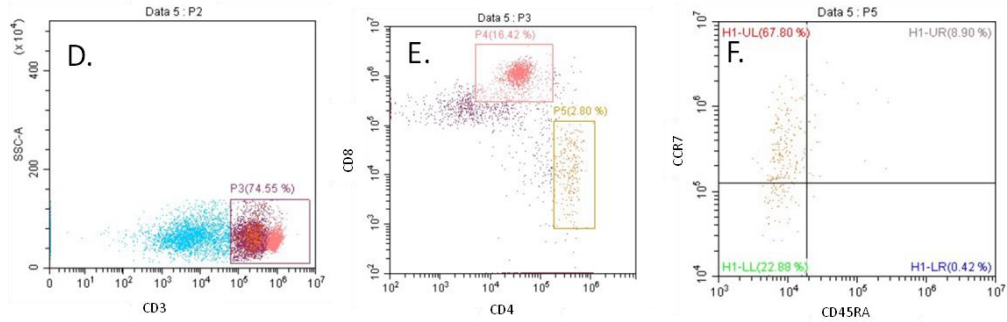


**Figure 21: Effect of live TV (and TVV status) on T-cell populations. Cells were cultured for 24h in the absence of IL-2/PHA and the (A) absence of TV, (B) presence of TVV- strain 347V- or (C) TVV+ strain 347V+. Top plots show populations of CD3+ lymphocytes out of the entire population of cells. The bottom plots represent the population of CD4+ and CD8+ cells from the CD3+ population. After a total of 48h of stimulation, cells were stained using Duraclone IM T cell Subset Kit (Beckman-Coulter) and immediately analyzed, without fixation. Cells were run on the CytoFlex LX (Beckman-Coulter) and all samples are normalized to 25,000 events. Plots represent the results of an independent experiment.**

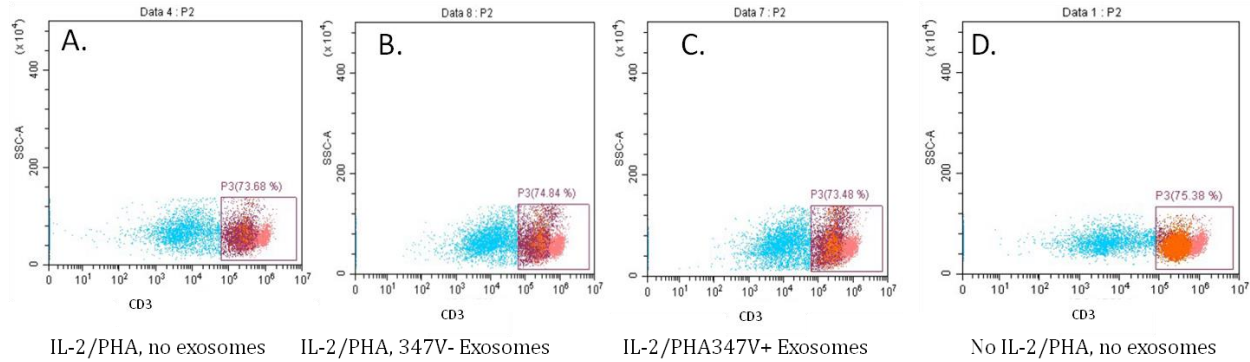
PBMCs+IL-2/PHA+AB-media



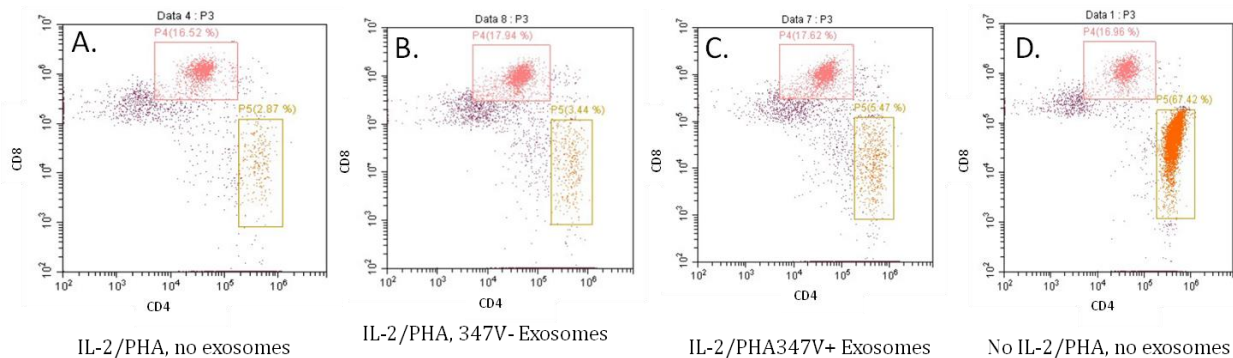
PBMCs+IL-2/PHA+D6.0 exosomes



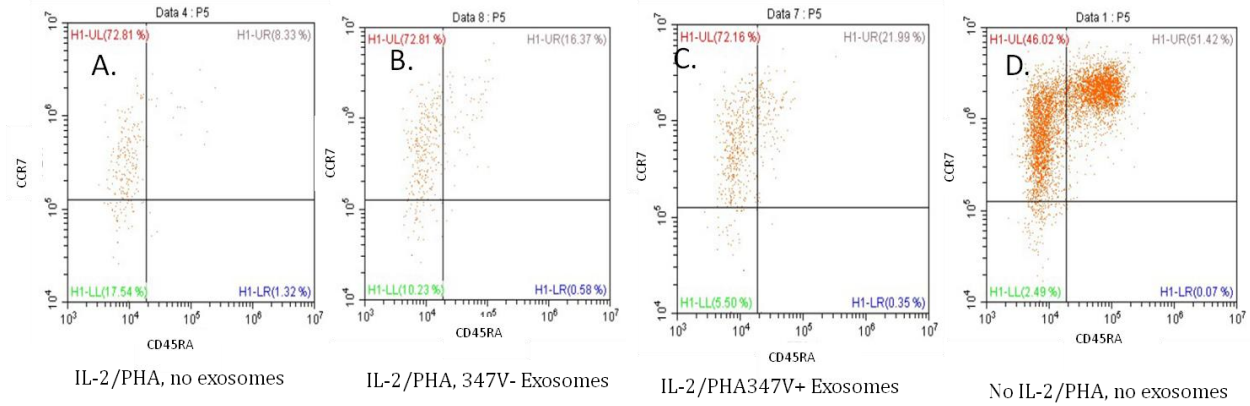
**Figure 22: Effect exosomes derived from serum-supplemented TV medium (D6.0) on PBMC differentiation markers. Cells were stimulated with IL-2/PHA for 48h and 24h with (A+B+C) medium alone (a mock dose, no exosomes) or (D+E+F) exosomes isolated from D6.0 TV growth medium. (A+D) show the population of lymphocytes from all cells isolated (B+E) show populations of CD4+ and CD8+ cells and (C+F) show populations of memory T cells. After a total of 48h of stimulation, cells were stained using Duraclone IM T cell Subset Kit (Beckman-Coulter) and immediately analyzed, without fixation. Cells were run on the CytoFlex LX (Beckman-Coulter) and all samples are normalized to 25,000 events. Plots represent the results of an independent experiment.**



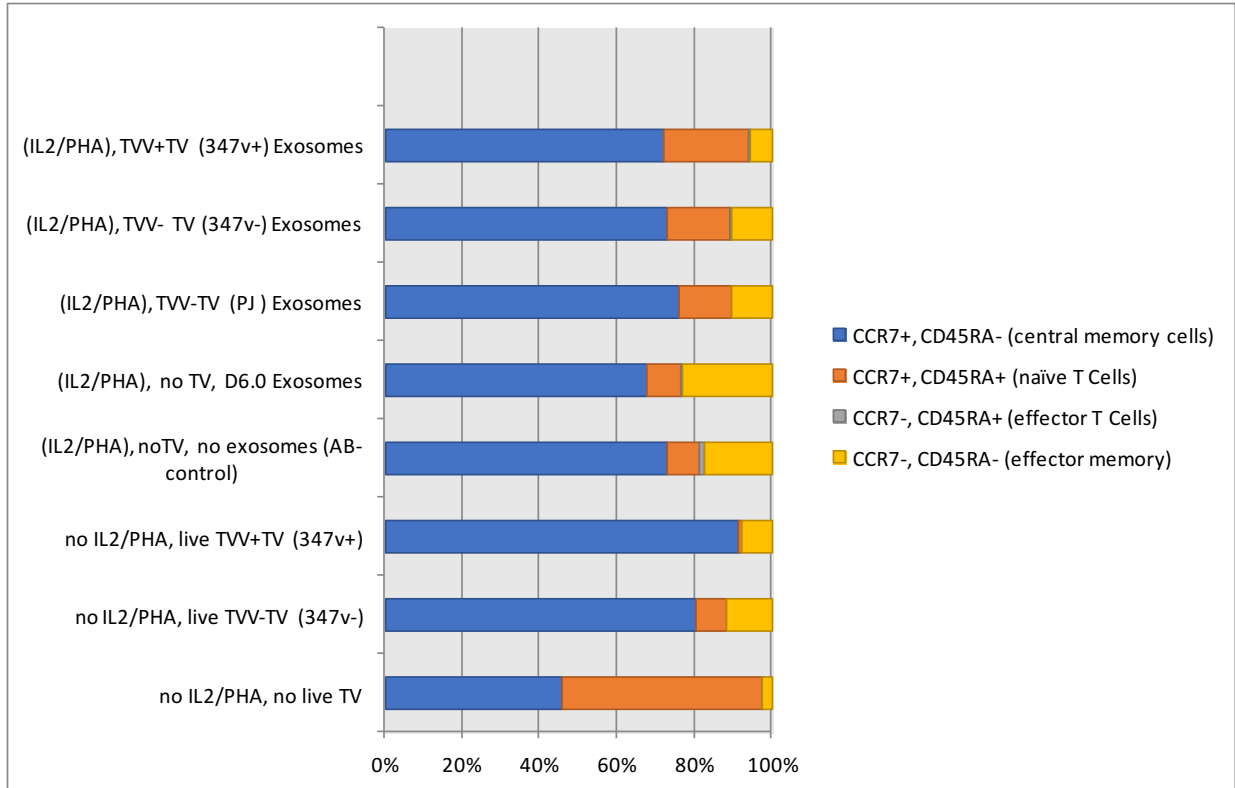
**Figure 23: Effect of TV exosomes on PBMC CD3+ differentiation.** Cells were stimulated with IL-2/PHA for 24h, followed by 24h incubation in IL-2/PHA supplemented medium in the (A) absence of exosomes, (B) presence of exosomes from TVV- strain 347V-, (C) presence of exosomes from TVV+ strain 347V+. (D) Shows PBMCs that were not stimulated with either IL-2/PHA or exosomes. After a total of 48h of stimulation, cells were stained using Duraclone IM T cell Subset Kit (Beckman-Coulter) and immediately analyzed, without fixation. Cells were run on the CytoFlex LX (Beckman-Coulter) and all samples are normalized to 25,000 events. Plots represent the results of an independent experiment.



**Figure 24: Effect of TV exosomes on PBMC CD4 and CD8 T cell populations.** Cells were stimulated with IL-2/PHA for 24h, followed by 24h incubation in IL-2/PHA supplemented medium in the (A) absence of exosomes, (B) presence of exosomes from TVV- strain 347V-, (C) presence of exosomes from TVV+ strain 347V+. (D) Shows PBMCs that were not stimulated with either IL-2/PHA or exosomes. After a total of 48h of stimulation, cells were stained using Duraclone IM T cell Subset Kit (Beckman-Coulter) and immediately analyzed, without fixation. Cells were run on the CytoFlex LX (Beckman-Coulter) and all samples are normalized to 25,000 events. Plots represent the results of an independent experiment.



**Figure 25: Effect of TV exosomes on naïve and effector memory populations. Cells were stimulated with IL-2/PHA for 24h, followed by 24h incubation in IL-2/PHA supplemented medium in the (A) absence of exosomes, (B) presence of exosomes from TVV- strain 347V-, (C) presence of exosomes from TVV+ strain 347V+. (D) Shows PBMCs that were not stimulated with either IL-2/PHA or exosomes. After a total of 48h of stimulation, cells were stained using Duraclone IM T cell Subset Kit (Beckman-Coulter) and immediately analyzed, without fixation. Cells were run on the CytoFlex LX (Beckman-Coulter) and all samples are normalized to 25,000 events. Plots represent the results of an independent experiment.**



**Figure 26: Effect of live TV, and TV-derived exosomes on memory populations. Cells were incubated in the presence or absence of IL-2/PHA stimulation for 48h of which 24h in the presence or absence of exosomes. Results are shown as percentages of the CD3+CD4+ cell population. Graph represent the results of an independent experiment.**

### **Brief Discussion:**

The study was one of the first to perform a comparative functional characterization on exosomes from a variety of sources and their effects on the early inflammatory events in reproductive infections. Furthermore, this study aims to shed the light on differences of TVV infection state on the pathogenic inflammatory potential of different isolates/strains of *Trichomonas vaginalis*.

Our nanotracking analysis and flow cytometry characterization studies of our exosome preparations confirmed the presence of microvesicles in the size range of exosomes previously defined in the literature.

We determined that the exosomes largely do not have an adverse effect on epithelial cell or PBMC viability.

In bystander uninfected upper reproductive tract endocervical epithelial cells, exosomes from TVV- strains of *T. vaginalis*, including a TVV-cured naturally infected strain, had a specific and significant pro-inflammatory effect in resting cells, upregulating the transcription factor NF- $\kappa$ B and selectively the pro-inflammatory chemokine IL-8, but not RANTES. In the presence of the TVV viral mimic (synthetic dsRNA poly(I:C)), the same exosomes that were before pro-inflammatory instead significantly and specifically downregulated NF- $\kappa$ B and IL-8.

In isolated PBMCs, regardless of stimulation by IL-2/PHA, exosomes derived from monocultures of the cured TVV- strain but not its isogenic infected counterpart, significantly upregulated the production of many key proinflammatory cytokines. Acute phase response cytokines IL-1 $\beta$  and TNF- $\alpha$  were specifically upregulated as well as immunoregulatory cytokines IL-6 and IL-4, and even cytokines indicative of a T helper cell response, such as IL-17A and IL-10.

In PBMCs stimulated with a TLR2/6 ligand, exosomes derived from vaginal epithelial cell cultures infected with the TVV positive strain induced a profound immunosuppressive responses, some of which mimicked exosomes from viral mimic (polyI:C) treated vaginal cell, some appeared to be common for TVV infected and TVV-cured TV and some appeared to be a synergistic effect of TV and TVV as they were absent in the viral mimic.

Flow Cytometry conducted on PBMCs also yielded interesting results, showing that TV exosomes have the capacity to counter the stimulating effect of IL-2/PHA treatment. All exosomes derived from TV increased the relative proportion of naive T cells when compared to cells without exosomes, indicating that perhaps exosomes constitute a common mechanism of suppressing the adaptive immune response against the parasite.

Taken as a whole, these findings support our hypotheses and show that exosomes derived from *T. vaginalis* have an undeniable capacity to modulate both the innate and adaptive immune response. When comparing isogenic strains 347V+ and its virus-cured counterpart 347V-, the loss of the virus correlated with an increase in pro-inflammatory potential in terms of early, innate cytokine production. This suggests that Trichomonasvirus plays a potential role in suppressing host immunity and could contribute to chronic infection for the humans and evolutionary advantage for its parasitic host.



## **Chapter 3: Discussion and Perspectives**

### **Limitations of the Study:**

While the findings of this research thesis are promising, they are somewhat inevitably limited in scope.

In our experiments, we were able to isolate peripheral blood mononuclear cells with relatively high yield from fresh whole blood obtained from a commercial source within several hours of collection time. However, we were able to confirm donor demographics from this source only after the experiments were conducted. Thus we had two female donors (one African American and one Caucasian), one male donor and the demographics of the last donor are still to be confirmed. In future experiments, donors should consistently be females of reproductive age to be most relevant to the public health concern at hand. There is evidence to suggest that sex hormones modulate the immune response, with testosterone being largely immunosuppressive and estrogen/progesterone being largely immunoenhancing (33). More specifically, there is evidence to suggest that the sex hormones are capable of skewing the helper-T cell responses. Researchers propose that testosterone suppresses TH2 adaptive immune responses in favor of TH1 responses whereas female sex hormones promote the TH2 response and suppress the TH1 response. By using female donors matched menstrual cycle phase and hormonal contraceptives use in the future, we could account for sex hormones as a confounding factor in our results. In our study the donors were of reproductive age and we have not seen significant differences at baseline or after IL-2/PHA stimulation in our cytokine or flow cytometry profiles.

To investigate the effect of *Trichomonas vaginalis*-derived monoculture and co-culture exosomes, we also studied a model of TV in isolation. While necessary to build an experimental

protocol and testable hypothesis, this is nonetheless simplified system. Under physiological conditions *T. vaginalis* is at the center of the nexus of reproductive tract infections, working in concert to increase colonization of the lower reproductive tract by anaerobic species (34, 35). While trichomoniasis has largely been seen as simply a nuisance, studies show an emerging role in enabling other chronic reproductive infections (34). Furthermore, previous studies indicate that other pathogenic protozoans may be capable to establish their infective niches via extracellular vesicles with evidence that there is communication within each species and potentially between species (36). Studies on TV-derived exosomes in the context of other pathogenic microbes or mixtures of exosomal sources would lead to new insights on the ways that the exosomes contribute to the microenvironment in perhaps a more physiologically relevant way.

In this study, exosomes from TVV- *T. vaginalis* monoculture were effective in upregulating the production of pro-inflammatory cytokines, whereas this effect was abrogated in exosomes derived from TVV infected parasites and TV-epithelial cell co-culture using the same strains. These results could suggest that both TVV and the vaginal epithelial host cells modify the effect or content of TV exosomes. Further content analyses e.g. mRNA and proteomics studies would be needed to confirm or deny these hypotheses and identify which molecular pathways are synergistically targeted by exosomes in the context of TV and TVV infection.

Although it is unlikely that our isolation procedure selected for cell-free virions from TVV+ *T. vaginalis* strains due to the non-lytic nature of the virus and the predominant size exceeding this of TVV, treatment of exosome samples with anti-viral staining antibodies could be used to confirm this finding. Although we have made preliminary attempts to stain both TV and epithelial cells with fluorescent dyes (Syto and TFL4), we had a low staining percentage when considering the microvesicle population as a whole using Flow Cytometry. Further work is needed

to optimize this staining procedure. Specific inhibitors of TVV signaling and other methods will also be applied in our future studies to investigate whether TVV dsRNA is part of the exosome signaling paradigm.

It is also possible that our preparations contain a mixture of different types of extracellular vesicles, as would be expected under physiological conditions (30). Using a combination of rigorous characterization methods would strengthen our argument that there are exosomes present in the extracellular vesicle populations used.

### **Contextualization:**

The interest in *Trichomonas vaginalis* research represents the paradigm shift in recent years as we gain a deeper understanding and appreciation for the far-reaching impact of the parasite. Despite discovering *T. vaginalis* in the early 1900s, our knowledge on the host immune response and implications of the parasite are still evolving. This project adds a layer of complexity to the picture by beginning to address how Trichomonasvirus contributes to the early inflammatory events, with potential insights into reproductive outcome.

Although defining what exactly constitutes “exosomes” is still a topic of much debate (24), extracellular vesicles have been isolated from every prokaryotic and eukaryotic species studied thus far including many human parasites (36). In this study, we were able to isolate extracellular vesicles from *T. vaginalis* strains that were previously characterized by this lab to be of a similar in nature to mammalian exosomes (Fichorova lab, unpublished data). Furthermore, our work is in accordance with the findings of Twu et al, in that the exosomes isolated from TVV- strains were sufficient to induce a pro-inflammatory response in target recipient cells as measured by the production of cytokines such as IL-6 and IL-8 (23, 36). Twu et al observed that TV-derived

exosomes recapitulated the full extent of IL-6 production as exposure to live TV; this is not in accordance with our findings which found the live TV, and especially TVV infected TV to be 100x more potent at producing IL-6 than TV-derived exosomes. By considering the relative proportion of cytokine production induced by exosomes derived from different strains our study expands upon previous findings but elaborating the differences between strains infected with TVV and those that are not. While exosomes derived from TVV- strains yielded an overall pro-inflammatory cytokine profile (23), TVV+ *T. vaginalis* derived exosomes suppressed the pro-inflammatory response attributable to the exosomes from axenic cultures and suppressed inflammatory response triggered by Toll-like receptor pathways. This suggests a potential anti-inflammatory role of the Trichomonasvirus in altering the exosomal content of the parasite. Other researchers have found that in addition to delivering protein and nucleic acids to target cells, TV-derived exosomes are capable of increasing the cytoadherence of the parasites (23). Other researchers have found that other protozoan parasites are capable of secreting extracellular vesicles that are pro-inflammatory, which is in line with some of our findings. Furthermore, recent studies have shown that exosomal content differs by strain and perhaps confer an evolutionary advantage (37). The researcher postulates that the vesicles contribute to establishing an infective niche in the context of the microbiota as a whole (36).

The methods used to characterize and quantify microvesicle populations are still in the early stages of development and optimization. Previous studies have shown that when working with exosome preparations, many dilutions are needed in order to account for a “swarm effect” and losing the vesicle data due to their size and interference with one another (38). This is in-line with our observations to optimize samples for characterization with Nanoparticle Tracking

Analysis, as samples needed to be diluted at least 5000x and one sample being diluted even further to 10,000x before vesicles were within the instrument's reading range.

Although the study of exosomes is still in its nascent stages, exosomes appear to be an important part of the gene regulatory networks particularly in the context of reproductive fitness. Functional studies suggest that exosomes (from many sources) contribute to the regulation of reproductive processes (39). Extracellular vesicles have been shown to be secreted from the embryo, oviduct epithelial cells, endometrium and placenta (40). Due to their ubiquitous nature, it is therefore unsurprising that placental, maternally-derived and embryonically-derived exosomes are all instrumental to the normal development of a healthy embryo.

Several studies have shown that exosomes may constitute a mechanism of communication between the mother and developing embryo via the placenta (40-42). Western blots of maternally-derived exosomes have been shown to contain proteins that play a role in fertilization and early pregnancy (40). A recent study has shown that exosomes derived from maternal macrophages are internalized by the placental tissue in a time and dose-dependent manner. Furthermore, this uptake induced the production of cytokines by the placenta, IL-6, IL-8, IL-10 and IL-12 (41). This study shows that exosomes if taken up by the blood circulation can be used to mediate placental immune responses during pregnancy.

Embryonically derived exosomes also pose an interesting new avenue of research. The number of exosomes fluctuates based on developmental stage and can even be a marker of embryonic quality (32, 40). In a recent study conducted in Genoa, researchers found that there was a significant difference in the exosomes released by term and pre-term infants. Exosomes from mesenchymal stem cells are capable of conducting extramitochondrial aerobic respiration.

However, only exosomes derived from full-term newborns were able to synthesize ATP in this manner and exosomes derived from pre-term newborns lacked this potential for oxidative metabolism (43).

Maternal and paternal exosomes also appear to play a role in regulating the success of implantation and embryonic development as well as longer-term development of disease. Exosomes have also been found in the follicular fluid and perhaps guide fertilization by regulating normal follicle maturation and meiosis resumption (44). This suggests that the maternal exosomes play a role in overall fertility. Furthermore, parental diet has also been shown to be a factor in altering the exosomal content and by extension playing a role in long-term embryonic development (45). A recent review summarizes that embryonic exposure to exosomes derived from male mice fed a high fat diet give rise to offspring with metabolic disorders and altered genetic patterns compared to controls (45). This study using a murine model of diabetes suggests that paternally-derived exosomes might function to determine the metabolism of the developing embryo and has implications for inherited metabolic disorders.

Taken as a sum, these findings suggest that the extracellular vesicle milieu may contribute significantly to the physiologic or pathologic development in the womb. Therefore, it is of interest to study exosomes as a marker of potential pathologic processes as a result of infection or even as a marker of uterine health and receptivity as a whole.

### **Future of the field:**

As stated before, there is still many unknowns in the field of *Trichomonas vaginalis* research. One author has stated that trichomoniasis is one of the CDC's "neglected parasitic infections" especially considering the parasites implications in long-term reproductive health

(insert citation). However, because of this, there are many different experimental factors of interest to investigate regarding *Trichomonas* pathogenicity.

An experimental condition of interest would be to use different levels of iron in the TV media. Previous studies have shown that iron has the capacity to change the proteome of TV hydrogenosomes, their mitochondria-like organelle used for substrate level phosphorylation (46). Since iron-levels change throughout menses, the effects of iron on the exosomal content of TV could lead to new insights about fluctuations in vulnerability to infection throughout the menstrual cycle.

Because exosomes are incredibly stable and ubiquitous in many different types of bodily fluids, it is hoped that exosomes will one day be useful as a diagnostic tool of current disease state or to gauge reproductive potential. Biomarkers from exosomes isolated from clinical samples could perhaps be used as non-invasive means to diagnose patients in the early stages of various parasitic infections not only trichomoniasis. In the course of performing this study, we learned that new plates have been recently developed to quickly scan for various known markers for disease state currently known to be found in exosomes. The scanning procedure has been simplified so that it would be similar to performing an ELISA or any other immunoassay. While these tools are not commercially available or economically sustainable, it is an interesting avenue for technological advances.

It is my hope that insights into the exosomes produced by different strains of *T. vaginalis* may be better used to inform treatment of trichomoniasis and perhaps one day a cost-effective preventative measure. Incidence of trichomoniasis has been on the rise in recent years and those affected are often in resource limited settings. There is evidence to suggest that metronidazole-

resistant strains of *T. vaginalis* have been emerging and as of yet, physicians have no other recourse. Recurrent *Trichomonas* infections do not lead to protection nor shorter infections or less severe symptoms as one would expect of a robust adaptive immune response (47, 48). Furthermore, clearance of the infection does not guarantee that there are no adverse effects on reproductive health. The inflammatory damage wrought by the parasite has implications beyond just the symptoms of inflammation but can affect fertility and even fetal development.

Development of vaccinations using murine and bovine models of trichomoniasis has yielded moderate success in conferring protective immunity. However, these results have not yet led to a viable human vaccine, as clinical trials have been inconclusive about the vaccine's efficacy (47, 48). *Trichomonas vaginalis* has several sophisticated mechanisms of immune evasion (cytoadherence, cysteine proteases to degrade immunoglobulins, sequestration of host protein to avoid detection by complement). Exosomes pose the possibility of perhaps negating several pathways at once (17, 48). Because of its involvement in several pathologic processes due to inflammatory damage, it is imperative that attention be brought to understanding how *Trichomonas* causes the inflammatory damage and the implications therein.



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