Undergraduate Research Opportunities Program

TITLE: Interleukin-17 Production in CNS by Infiltrating T Cells and Glial Cells in the HIV-1-Infected Brain

INVESTIGATORS: *APPLICANT*: Daniel Joseph Gómez, *MENTOR*: Bruce Shiramizu, MD
SITE(S): John A. Burns School of Medicine (JABSOM); Hawaii Center for AIDS (HICFA), Hawaii HIV Immunobiology and Vaccine Laboratory (HHIVL); Department of Tropical Medicine, Medical Microbiology & Pharmacology (TRMD)

PROBLEM STATEMENT

The prognosis for individuals infected with the human immunodeficiency virus type 1 (HIV) has dramatically improved with the use of combined anti-retroviral therapy (cART) with patients living as long as their non-HIV-infected counterparts. In spite of effective cART, though, neurocognitive complications develop in patients, which can affect their daily lives and daily function. The central nervous system (CNS) is affected in multiple ways during HIV infection. HIV-infected cells cross into the brain through the blood-brain-barrier (BBB) and set up a cascade of events leading to CNS damage, that clinically leads to neurocognitive problems. Because interleukin-17 (IL-17) is capable of disrupting the BBB, the study proposes to characterize expression of IL-17 agonist (IL-17A) in the brain and cerebrospinal fluid (CSF) of patients with HIV-associated neurocognitive disorders (HAND).

OBJECTIVE AND HYPOTHESIS

The objective will be to analyze necropsy brain specimens and CSF samples from individuals with HAND with HIV encephalitis (HIVE) and compare them to samples from HIV-infected individuals with normal cognition (NC).
Hypothesis: IL-17A will be identified in the subcortical structures; specifically, the medium-spiny neurons of the basal ganglia and interneurons in the hippocampus. In addition, astrocytic secretion of the T_H17 cell chemokine (CCL20) and cytokine (IL-17) will be found in CSF samples of HAND patients with HIVE.

BACKGROUND/PRELIMINARY DATA

HIV-1 infection has significant effects on the nervous system; the virus can both infect and affect the brain. The neuropathological changes that result from infection include a wide range of neurological symptoms such as cognitive, behavioral, and motor dysfunction. Collectively, these are the characteristics of HAND[1]. Early in infection, HIV-1 enters the brain through a specific "Trojan horse" mechanism that allows the virus to infiltrate the BBB. This occurs when monocytes/macrophages are attracted to the brain by a chemokine gradient that allows the paracellular transport across the BBB. Once the virus is inside the brain, it can then disseminate infecting endogenous glial cells; microglia and astrocytes are the most predominant[2]. Both neurons and astrocytes have been reported to be undergoing apoptotic death in HAND[3]. It is possible that astrocytes become apoptotic in response to a relatively nonproductive and limited type of HIV-1 infection that occurs in these cells[4]. In 2009, laser capture microdissection shows a substantial proportion of astrocytes in brain tissue from subjects with HAD containing HIV-1 provirus DNA, with or without HIVE[5]. These apoptotic astrocytes have been observed in HIVE, and were associated clinically with progressive worsening of HAND[6]. Neurons that receive support from astrocytes that are apoptotic will lead to neuronal death; in addition, astrocytes connected to other uninfected astrocytes through gap junctions will also die. This may represent subtle and/or early degeneration of neurons and has been related to antemortem HAND[7]. Subcortical structures that are vulnerable include the basal ganglia, composed of both grey matter and neuronal cell bodies. Although HIV-1 does not infect neurons directly, viral proteins released by infected macrophages and microglia leads to neuronal apoptosis and ongoing aberrant neuroinflammation, which contribute to the development of HAND[8]. Substantial loss of a subpopulation of small interneurons that immunostain for parvalbumin occurs in one sector of hippocampus[9]. This study provided rationale for the selection of the interneurons in the hippocampus for

investigation of IL-17A production. Holt, Kraft-Terry, and Chang (2012) have provided both Magnetic Resonance Imaging (MRI) and Magnetic Resonance Spectroscopy (MRS) data supporting basal ganglia volume loss[10]; hence, the selection of the caudate and putamen for inspection.

Astrocytes have receptors for IL-17, IL-17RA, and IL-17RC that work through both NF-ϰB and MAPK pathways[11]. Hence, astrocytes are the cellular CNS target of IL-17. The synergy of both IL-17 and IL-6/sIL-6R ensure further production of IL-6 and the production of chemokine CCL20. CCL20 is a potent chemoattractant for T_H17 cells that are known to bear its receptor CCR6[12]. The chemokine CCL20 secreted by astrocytes invites infiltrating T_H17 cells into the CNS. HIV-1 infection can lead to microglial secretion of pro-inflammatory cytokines for an astrocytic response. The astrocyte will call for T_H17 cells with chemokine CCL20; in addition, it will amplify production of IL-17 and IL-6. This feedback loop will create a microenvironment, which infiltrating $CD4^+$ lymphocytes differentiate into pro-inflammatory T_H17 T cells. Thus, IL-17A production in the CNS can result in dendritic simplification and axonal damage of pyramidal neurons, selective loss of interneurons, BBB damage, and astrogliosis, which characterize the degenerative process in HIV-1 infection of the central nervous system.

METHODS

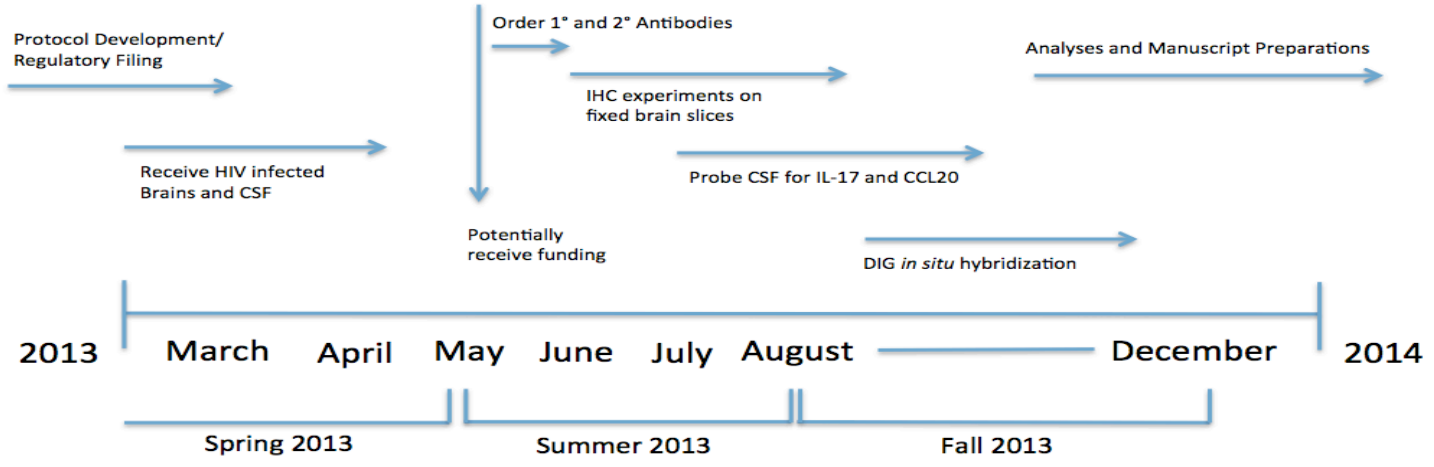
Using molecular biology techniques, the proposed experiments will compare the presence of IL-17A producing cells in necropsy brain sections and cerebrospinal fluid (CSF) from patients with HAD versus NC by characterizing mRNA and protein levels.

Brain Tissue and CSF samples: Unstained necropsy brain sections on microscope slides and CSF will be requested from the National NeuroAIDS Tissue Consortium (nntc.org). Brain sections will be fixed and prepared on slides for analysis will be sent to Hawaii Center for AIDS, Hawaii HIV Immunobiology and Vaccine Laboratory. The regions of the brain that will be requested are the basal ganglia and hippocampus. The CSF will be assessed for IL-17 and CCL20. Two 96-well ELISA kits can screen 40 samples each in duplicate for IL-17 and CCL20.

Immunohistochemistry Methods: Analysis of the data will be collected and processed by confocal microscope, camera, and computer to resolve the locality of the fluorochromes (example: see **Supplement 3—Figure 1**). Double-immunofluorescence Primary Antibodies: CD3+ IL-17+ (T_H17 cells); $CD4^+$ $IL\text{-}17^+$ vs. $CD8^+$ $IL\text{-}17^+$ (CD4/$CD8^+$ T_H17 cells); $GFAP^+$ $IL\text{-}17^+$ (astrocytes); $CAII^+$ $IL\text{-}17^+$ (oligodendrocytes); $CD14^+$ $IL\text{-}17^+$ (macrophages/microglia).

DIG In Situ *Hybridization: In situ* hybridization will be used to investigate the presence of IL-17 mRNA in T lymphocytes, astrocytes, and oligodendrocytres. The DIG (digoxigenin) RNA Labeling Kit will be used to incorporate DIG-labeled dUTPs which will then be conjugated by an anti-digoxigenin alkaline phosphatase to Fab fragments to detect IL-17A mRNA. Once the chromogenic reaction is complete, the samples will be visualized for analysis by fluorescence microscopy. Detailed lab protocols available upon request.

TIMELINE



BIOGRAPHICAL SKETCH

After graduating high school in 2007, I prepared myself to enter undergraduate training at Chicago's DePaul University *The Theatre School* with the goal to be a professional Lighting Designer. Our freshman class consisted of five different B.F.A. Lighting Design majors; after application and interview process, I was selected and awarded a talent scholarship to attend their selective private university. It was during this education that I realized my potential could be applied to any field. I was concerned of the employment opportunities as a Lighting Designer and paying ~$40,000 for tuition. After great thought, I decided I would need to choose a goal that would keep myself employed, occupied, and challenged. My conclusion: I wanted to become a doctor that could make a contribution to humanity and the medical community. I began my pursuit to become a doctor in a California Community College – *Ohlone College*, Fremont, CA. There I took the required courses to transfer into a four-year university. I focused on communication courses, as I knew the medical field requires excellent communication.

Matriculating into the B.S. Molecular Cell Biology program at UH Mānoa in *Fall 2010* has fast-tracked my education and career. It has provided me the freedom to take focused electives (*TRMD607, CMB606*) while still requiring a rigorous core (*BIOL407/8, MCB461, etc*); both have been the foundation of my knowledge of cellular and molecular biology. After taking all of these courses in Fall 2012, my focus has been narrowed to studies of the CNS disease HAND. The study of this disease has led me to Dr. Bruce Shiramizu, both physician and private investigator at the Hawaii Center for AIDS; he has allowed me the opportunity to work in his lab on currents projects. I have learned phlebotomy and how to isolate the PBMCs from whole blood plasma with strong cell viability. This was important for processing HAND patients' blood samples at HICFA and Leahi Hospital. Also, I participated in *in vitro* multiple cell cultures during BBB transmigration experiments *(feeding, passaging, transmigration, and flow cytometry participation)*.

The proposed project will further direct me towards understanding pathological situations involving a disturbance in the homeostatic molecular interactions between multiple cell-types in the CNS. I gravitated towards the nervous system for many reasons: arguably, our human evolution has been driven by nervous systems, everything we experience in this world leads to activation of neuronal networks, and with 100 billion neurons it is the most complex system known. The complexity makes the CNS an attractive choice of study. The ultimate goal is to become a neurologist who can diagnose CNS diseases in underrepresented minority populations and provide treatment to either cure or alleviate symptoms. In addition, I would like to continue research in a CNS disease to provide current data in clinical and laboratory settings, a true "bed-to-benchside" experience. I believe clinical research is important and should not be abandoned while becoming a physician. This is why as an undergraduate I would like to begin publishing manuscripts on CNS diseases particularly in the area of neuroinflammation as it pertains to neurodegeneration.

APPLICANT'S ROLE: My role in this project is to gather data stated in the literature about HIVE pathology and fuse the current knowledge of T_H17 cell CNS infiltration in MS with the neuropathology of HAND. Also, production of protocol by applicant with the molecular probes used to address hypothesis is provided. With the guidance of Dr. Bruce Shiramizu and Department of Tropical Medicine, Medical Microbiology & Pharmacology, I have gathered the resources to complete these experiments. Dr. Vivek Nerurkar has approved use of the *RCMI Molecular Pathology Core* in his laboratory and graduate assistant Mukesh Kumar has agreed to supervise in double-immunofluorescence with confocal microscopy analysis (see **Supplement Letter of Support**). Mukesh has extensive experience in this technique (see **Supplement 3—Figure 1)**. It is the applicant's role to follow through with these experiments and analyze the data given. With knowledge gained from experiment, the importance of IL-17A and the neuropathology of HAND will be discussed in the format of a publishable journal article. Also, if any presentation is needed for UROP conference, I will be able to present the findings.

Supplemental Information: Budget and Budget Justification

UROP BUDGET

[*Double-immunofluorescence*, in situ *hybridization, CSF Biomarkers*]

Experiments

Item #	Description	Manufacture	Price
Double-immunofluorescence to localize IL-17+ cells			
1	mouse anti-CD3	DAKO	$554.40
2	mouse anti-IL-17	R & D Systems	$335.00
3	rat anti-CD3	Serotec	$93.00
4	rat anti-CD4	Serotec	$98.00
5	rat anti-CD8	Serotec	$53.00
6	rabbit anti-GFAP	DAKO	$262.65
7	sheep anti-CAII	Serotec	$404.00
8	rat anti-CD14	Serotec	$294
9	Hoechst 33342	Invitrogen	$62.00
10	TSA - Alexa Fluor 488 anti-mouse Ig	Invitrogen	$457
***In situ* Hybridization to detect IL-17 mRNA expression in HAND Brains**			
11	DIG RNA Labeling Kit (SP6/T7)	Roche Diagnostics	$541.00
12	Anti-Digoxigenin-Rhodamine, Fab fragments from sheep	Roche Diagnostics	$215.00
13	NBT/BCIP Stock Solution	Roche Diagnostics	$111.00
Probing CSF for IL-17 and CCL20			
14	96-well ELISA kit to screen 40 CSF samples in duplicate for IL-17	N/A	$400
15	96-well ELISA kit to screen 40 CSF samples in duplicate for CCL20	N/A	$400
		Total:	$4,280.05

Amount Requested from Undergraduate Opportunities Research Program:

$4,300.00

BUDGET JUSTIFICATION

The estimated budget for my project is $4,300. I am requesting support of $4,300.00 from the University of Hawaii Undergraduate Research Opportunities Program (UROP) to fund supplies mandatory for immunohistochemical analyses with confocal microscopy, *in situ* hybridization, and to probe for HAND CSF biomarkers.

MRI studies stated above show brain atrophy in the caudate nucleus (affecting executive function and verbal learning) and the putamen (affecting processing speed and fine motor skills)[10]. Also, the interneurons of the

hippocampus will be examined. The justification for this region begins with Masliah et al. findings on selective neuronal vulnerability in HIV Encephalitis. There was a trend toward decreased density of neurons in the hippocampus, which only reached significance in the CA3 layer where there was a 50-90% decrease in these neurons[9]. This decrease was closely correlated with the severity of HIV encephalitis. Double-label immunocytochemical analysis confirmed neuritic damage to interneurons. These results suggest that HIV encephalitis differentially involves specific subpopulations of neurons. Since direct HIV infection of neuronal cells was not detected, damage to these cells of the hippocampus and fibers may be indirectly mediated by cytokines released by HIV-infected microglia or astrocytes. Given the data above, I selected both the basal ganglia and hippocampus for inspection of IL-17 protein production.

In the double-immunofluorescence experiment, multiple antibodies are needed to recognize specific targets inside brain tissues. Item **1** recognizes T cells that bear the T-cell receptor containing CD3. Item **2** is required to recognize the suspected pro-inflammatory mediator IL-17. Items **3-5** are for T cell subset analysis ($CD4^+$ or $CD8^+$). Items **6-8** recognize specific endogenous glial cells (astrocytes, oligodendrocytes, and microglia). This are cells involved in the "cross-talk" that occurs in the neurovasacular unit (NVU) where neuroinflammation is propagated. Item **9** is a nuclear stain that will stain the nuclei of all cells. To amplify fluorescence signals, item **10** can be used. Tyramide Signal Amplification (TSA™) is an enzyme mediated detection method that utilizes the catalytic activity of horseradish peroxidase (HRP) to generate high-density labeling of a target protein or nucleic acid sequence *in situ*.

Using *in situ* hybridization, I will be able to detect even low levels of IL-17A mRNA expression in cerebral cell types. Using the DIG RNA Labeling Kit (**11**), I will be able to synthesize an IL-17A riboprobe that is "anti-sense" to IL-17A mRNA. That is, hybridization will occur in anti-parallel fashion. On the riboprobe are digoxigenin (DIG)-labeled-dUTPs; these are recognized by item **12.** The anti-DIG is covalently attached to an alkaline phosphatase (AP). Alkaline phosphatase produces many fluorescence molecules with a colorimetric substrate (**13**). This amplification is needed because IL-17A mRNA levels may be in low concentration.

Neuroimaging has demonstrated the ventricles in the HAND brain to be expanded[10]. The choroid plexus line the ventricles of the brain, and this is where CSF is produced daily. The circulation of CSF is aided by the pulsations of the choroid plexus and by the motion of the cilia of ependymal cells. CSF is absorbed across the arachnoid villi into the venous circulation and a significant amount probably also drains into lymphatic vessels around the cranial cavity and spinal canal. Thus, the CNS, CSF, peripheral blood, and lymphatic vessels are all connected; hence, HIV-1 infection resulting in a systemic infection. The CSF is actually produced by the glial cells (ependymal cells) of the brain. Because of the tight relationship with the CNS, I chose to probe the CSF for anomalies that reveal a pro-inflammatory environment that is tied to the involvement of T_H17 cells.

To find CNS biomarkers in HAND, I will need two ELISA kits (**14, 15**). These ELISAs will sandwich both suspected cytokines (IL-17, CCL20) in the CSF samples. Finding these T_H17 cell cytokines/chemokines will further support feedback loops that involved T_H17 cell recruitment (CCL20) and differentiation (IL-17). Elevated levels of plasma LPS are found in HAND patients[14]; LPS can stimulate microglia (through TLR4/CD14) to influence astrocytic secretion of both IL-17 and CCL20.

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Mukesh Kumar's GFAP$^+$ Staining on Fixed Brain Sections

From Mukesh Kumar's PhD defense:
Diabetes as a Risk Factor for West Nile Virus-Associated Encephalitis
Kumar M., Nerurkar VK – Unpublished Data.

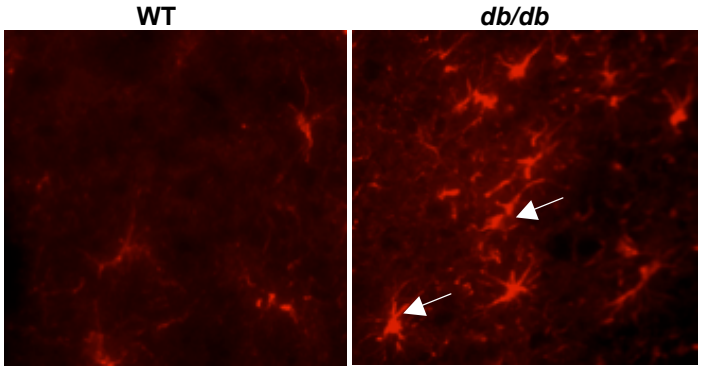

Figure 1: GFAP Staining. PFA-fixed brain sections from West Nile virus (WNV)-infected wild-type (WT) and diabetic *db/db* mice at day 8 after infection were stained for GFAP (DAKO, 1:100 dilution). White arrows identify activated astrocytes. Immunoreactivity of GFAP was higher in WNV-infected *db/db* mice. Bars, 20 μm.

As shown, through immunohistochemistry protocols have been set in place in Dr. Nerurkar's laboratory. Mukesh Kumar has much experience in immunostaining and confocal microscopy analysis. Daniel Gómez will begin training by Mukesh Kumar in Spring 2013.

Dear UROP Committee Members:

I am delighted to assist and train Mr. Daniel Gómez to conduct immunohistochemistry experiments outlined in his research proposal. In Fall 2012, Daniel was enrolled in my Advances in Neurovirology class (TRMD607). Daniel is a bright undergraduate student and I am confident that if funded he will successfully complete the aims outlined in the grant proposal.

Our laboratory is fully equipped to conduct immunohistochemistry related experiments, and we have trained students and staff who routinely conduct the protocols described in the grant proposal. Daniel's research site is next to our laboratory on the third floor of JABSOM BioSciences Building and his mentor Dr. Bruce Shiramizu is a faculty in our department.

I enthusiastically support Daniel's project submitted to UROP.

Please call me if you need additional information or clarification.

Aloha.

Vivek R. Nerurkar

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