Fall 9-29-2014

HIV-1 Transgenic Rats Display Alterations in Immunophenotype and Cellular Responses Associated with Aging

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HIV-1 TRANSGENIC RATS DISPLAY ALTERATIONS IN IMMUNOPHENOTYPE AND CELLULAR RESPONSES ASSOCIATED WITH AGING

By

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Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Molecular Bioscience from the Department of Biological Sciences of Seton Hall University

September, 2014

Acknowledgments

I could not have done this alone. It's hard to find the right words to express my gratitude to all those who supported me through one of the most challenging and exhilarating phases of my life. Without the encouragement I received from the professors and staff at Seton Hall University, along with my family and friends, I would not possess the abilities and knowledge I have today.

I wish to express special thanks to Dr. Sulie L. Chang who mentored and inspired me throughout my research project, spending countless hours discussing my data. I also wish to convey gratitude to my Dissertation Committee for reviewing and guiding my project; and the Institute of NeuroImmune Pharmacology (INIP) staff for animal care and technical assistance.

On a personal note, I want to thank my amazing extended family who were there every step of this journey; my mother Mrs. Rita Bassett who was always willing to listen and give sound advice; my astonishing children Miss Kimberly Abbondanzo and Mr. Peter Abbondanzo who make me so proud everyday and taught me to be a better student; my husband Mr. James Abbondanzo for believing in me and supporting me though this long journey; my resilient sister Mrs. Wend*y* Favorito whose inner strength, after beating cancer,

inspired me to pursue this path; and my siblings Mrs. Diana Carroll and Mr. Brian Bassett, along with their families who coached me along the way. Lastly, I wish to dedicate this to my father and father-in-law, Mr. Peter Bassett and Mr. Vincent Abbondanzo, kind, patient men who were taken from this earth too soon by cancer but taught me to be a better person.

This study was supported, in part, by the following US National Institutes of Health grants to Dr. Sulie L. Chang: RC2 AA019415, R01 DA07058, K02 DA016149, R01 DA026356, and R21 DA036175).

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Abstract

Advances in anti-retroviral therapy over the last two decades have allowed life expectancy in patients infected with the human immunodeficiency virus to approach that of the general population. The process of aging in mammalian species, including rats, results in changes with immune response, immunological phenotypes, and ultimately, increased susceptibility to many infectious diseases. In order to investigate the immunological pathologies associated with chronic HIV-1 disease, particularly in aged individuals, the HIV-1 transgenic (HIV-1Tg) rat model was utilized. Age matched animals were challenged using lipopolysaccharide (LPS) to determine immunological modification of HIV-1Tg animals throughout the aging process. LPS is known to cause imbalances in cytokine and chemokine release, and provides a method to identify alterations of immune response to bacterial infection. In the HIV-1Tg rats, the percentage of T cells decreased with aging, particularly with T cytotoxic cells, whereas T helper cells were increased with age. Neutrophil and monocyte cell numbers increased in HIV-1Tg rats during maturation compared to age-matched control rats. Aging HIV-1Tg rats displayed a significant increase in the pro-inflammatory cytokines, IL-6 and TNF-α in comparison to age-matched controls. Our data indicate that the immunophenotype and immune responses can change during aging in HIV-positive individuals. This information could be important in determining the most beneficial age-dependent therapeutic treatment for HIV patients.

Introduction

Life expectancy of people infected with the human immunodeficiency virus (HIV) is increasing and approximates that of the general population. The current median life expectancy for an HIV patient receiving effective therapy exceeds 70 years of age (High et al., 2012). In the United States, estimates indicate that, by 2015, most of the patients diagnosed with HIV will be 50 years of age and older (Mills et al., 2012; Nath, 2012). A recent publication from the United Kingdom reported that the percentage of HIV-positive individuals who are over 50 years of age has risen from 12% in 2002 to 22% in 2011 due to an increase in length of survival and a surge in the incidence of newly identified older HIV patients (Health Protection Agency, 2012). Furthermore, a majority of the older patients were aware of their HIV status for at least a decade, and were being treated with anti-retroviral therapy since testing positive. However, as the population of HIV-positive patients ages, they often develop other ailments, including cardiovascular disease, cancer, osteoporosis, and liver and renal disease, as well as the neurocognitive deficiencies more commonly associated with aging and chronic inflammation than with HIV infection (Sulkowski and Thomas, 2003; Lucas et al., 2007; Nath et al., 2008; Silverberg et al., 2009; Freiberg et al., 2011; Valcour et al., 2011; Womack et al., 2011).

The process of aging in many mammalian species, including rats, results in changes in immune responses, alterations in immunological phenotypes, and

ultimately increased susceptibility to infectious diseases, cancer, and autoimmune disorders (Dawson and Ross, 1999; Pahlavani and Vargas, 2001; Ropolo et al., 2001; Schmucker et al., 2002). Unraveling the mechanisms underlying the immunophenotypic changes associated with human HIV infection is now possible by utilizing a small animal model, the HIV-1 transgenic (HIV-1Tg) rat (Reid et al., 2001). This animal model possesses human viral genes, including the HIV-1 provirus, but with the deletion of the *gag* and *pol* replication genes. The HIV-1Tg rat exhibits similar clinical manifestations as HIV-positive humans, including wasting, skin lesions, cataracts, neurological and respiratory impairment, and changes in the immune system (Reid et al., 2001; Reid et al., 2004; Chang et al., 2007; Joshi et al., 2008), suggesting that the HIV-1Tg rat model is useful for studying HIV-1-infected patients on highly active anti-retroviral therapy (HAART) (Peng et al., 2010). In the last decade, many reports have demonstrated that HIV-1Tg rat is a valuable model for studying neuroAIDS (Chang et al., 2007; Chang and Connaghan, 2012; Homji et al., 2012). The HIV-1Tg rat is, therefore, an ideal small animal model for studying the immunological alterations and pathology associated with chronic HIV-1 infection during aging.

Viral gene expression has been detected in the lymph nodes, spleen, thymus, and blood of the HIV-1Tg rat, similar to HIV infected humans (Reid et al., 2001; Reid et al., 2004). HIV-specific transcripts (7-, 4-, and 2-kb) were present in spleen, kidney, and thymus, and highest in axillary lymph nodes. Further analysis revealed that the gp120 protein was present in macrophages as

well as B and T cells derived from splenic lysates. In addition, it has been reported that the viral proteins, tat, gp120, nef, and vif, in the spleen of 2-3 mo old HIV-1Tg rats is higher than in 10-11 mo old animals (Peng et al., 2010). Although the HIV-1Tg rat spleen is normal in size, this animal did exhibit a loss of cells in the periarterial lymphatic sheath (PALS), larger marginal zones, follicular hyperplasia, and apoptosis of endothelial cells. However, when immunized with keyhole limpet hemocyanin (KLH) to analyze immune cell function, there was no significant difference in anti-KLH-specific titers and a reduced induration seen in the delayed type hypersensitivity (DTH) response, suggesting that the HIV-1Tg rats have abnormal T helper (Th1) responses but, normal Th2 responses (Reid et al., 2001; Reid et al., 2004).

In older HIV-1Tg rats (12-15 mo old), the $CD4^+$ T cell population displays altered CD28 function, reduced anti-apoptotic Bcl-xL expression, reduced IL-2, and induced apoptosis (Yadav et al., 2006). Mature HIV-1Tg rats also have decreased $CD4^+$ and $CD8^+$ effector and memory cells and an increased number of naïve cells (Reid et al., 2001; Reid et al., 2004). These finding suggest that aging may influence immune function of T cell populations in the HIV-1Tg rat.

In order to explore the effect of aging on immune cell responses, determine the phenotypic changes associated with genotype, and establish immunophenotypic profiles in this small animal model of HIV, a polychromatic flow cytometry approach was employed in this study. Whole blood and spleen from wild-type F344 and HIV-1Tg rats were grouped by age of 2 mo, 5-6 mo, and

18-20 mo. Samples were analyzed for changes in immune cell populations, including B cells, T cells, T helper, T cytotoxic, neutrophil, and monocyte subtypes, classical and non-classical as defined in recent publications (Sengupta 2013; Zhou et al., 2013).

HIV-1Tg rats also display altered pro-inflammatory and anti-inflammatory cytokine and chemokine expression when treated with lipopolysacaride in the endotoxin tolerance (ET) model (Homji et al., 2012). Although the mechanism causing ET appears elusive, evidence suggests that ET produces a downregulation of inflammatory cytokines and an upregulation of anti-inflammatory cytokines (Biswas and Lopez-Collazo, 2009). This led us to investigate whether the immune cell profile and responses are compromised with aging in the HIV-1Tg rat. Using the ET model, animals were rendered tolerant by exposure to two low doses of lipopolysaccharide (LPS) and then challenged with a high dose of LPS as previously described (Chen et al., 2005; Homji et al., 2012). Blood, spleen and lymph nodes of HIV-1Tg rats and age-matched F344 control rats were analyzed for changes in immunophenotype with LPS treatment related to age and genotype. In addition, age-related changes in immune function were determined by examining cytokine and chemokine production in the LPS treated animals.

The results of this study will provide evidence of the effects of aging on immune cellular profile and function in the HIV-positive population compared to non-infected individuals. This information will be helpful in determining the best

treatment for HIV-infected individuals based on multiple factors, including immune cell profiles, cellular responses, and the patient's age.

Materials and Methods

Animals:

All animals were purchased from Harlan Co. (Indianapolis, IN) at approximately 4-8 wks. of age, and maintained in ventilated cages up to 20 mo of age. Male wild-type (F344) and HIV-1 transgenic (HIV-1Tg/F344) rats were grouped according to age. All animal experiments were carried out in Seton Hall University's Animal Care Facility. Animal care and experiments were performed in accordance with the Animal Welfare Act and Public Health Service Policy. Approval was obtained from the Institutional Animal Care and Use Committee prior to the start of experiments. Euthanasia, when necessary, was performed using carbon dioxide asphyxiation and cervical dislocation.

Flow cytometry:

Whole blood was collected at 2, 5-6, 12, and 18-20 mo of age from the rat tail vein into Becton Dickinson (BD) lithium heparin tubes (BD 365965). Twenty microliters of blood was blocked for non-specific staining with 0.25 µg anti-rat CD32 (BD 550271) for 5 min. Fluorescently labeled antibodies were added according to the manufacturer's protocol. Cells were treated with antibodies and labeled as follows: B cells (CD45RA, BD 561624), T cells (CD3, BD 557354), T helper cells (CD4, BD 554839), T cytotoxic cells (CD8a, BD 558824), neutrophils

(RP-1, BD 550002), monocyte population (CD43-AF647, Biolegend 202810; CD172, BD 552298; CD11b, BD 562105), and isotype-matched gating controls. Cells were treated for 30 min at 4°C with fluorescently labeled antibodies, and then centrifuged for 5 min at 300 *xg*. The supernant was removed and the blood was treated with 1X cell lysing solution (BD 349202) for 10 min at room temperature (RT). Cells were centrifuged for 5 min at 300 *xg*, then washed with FACS buffer (PBS, 0.1% BSA, 25 mM HEPES). All samples were re-suspended in FACS buffer, prepared in duplicate, and 30,000 events were acquired on the BD Fortessa. Immune cells populations were gated as follows: B cells ($CD45RA^+/CD3^-$), T cells ($CD3^+/CD45RA^-$), T helper cells ($CD3^+CD4^+$), T cytotoxic cells ($CD3^+CD8^+$), neutrophils ($RP\text{-}1^+$), and classical (mononuclear/$CD172^+/CD43^+$) and non-classical (mononuclear/$CD172^+/CD43^{++}$) monocytes, as described previously (Zhou et al., 2013).

Spleen samples were collected at time of sacrifice from 2 mo, 5-6 mo and 18-20 mo old animals. Spleen cells were isolated by pulverizing the spleen tissue through 100 µm nylon mesh (Fisher, 22363549), washing in FACS buffer, then treating them with red blood cell lysing buffer (Gibco #A10492-01) for 5 min at RT. The cells were washed with FACS buffer, centrifuged, and counted. The spleen cells (10^6 cells/sample) were stained with fluorescently labeled antibodies as described for whole blood.

Lipopolysaccharide (LPS) administration:

Male HIV-1Tg and F344 rats were grouped by age and genotype (N = 5/group): 2, 5-6 and 18-20 mo of age. Treatment with LPS in each group to induce ET was completed as previously established in our laboratory using the HIV-1Tg rat model (Chen et al., 2005; Homji et al., 2012). Briefly, animals were administered IP a non-pyrogenic dose of 250 µg/kg LPS (from *E. Coli* 055:B5, Sigma, L2880) on day 1 at 0h and 10h, respectively and a challenge injection of 5 mg/kg LPS at 24 h. Animals treated with LPS are designated as "LPS treated" in this study. Age- and genotype-matched vehicle control animals were injected with 0.9% saline on the same time schedule as LPS treated rats, and are designated as "Control" in this study.

Cytokine and chemokine analysis:

To measure cytokine levels in the LPS treated animals, the rats were sacrificed 2 h post final LPS treatment (at 26 h). Spleen and lymph nodes (axillary and mesenteric) were isolated and stored at −80°C. Tissue was homogenized in lysis buffer containing 5 mM Tris-HCl (Boston BioProducts, BM-320), 2 mM EDTA (Boston BioProducts, BM-150), 1% Triton X-100 (Sigma, T6878), and 1 vial protease inhibitor cocktail (Sigma P2714) at a ratio of 1:10 (tissue:buffer). Samples were homogenized with a Polytron PT2100 for 30s on ice, centrifuged at 6,800 *rcf* for 5 min at 4° C, and supernants were collected. Total protein concentrations were measured using a micro BCA Protein Assay Kit

(Pierce #23235). Blood was collected into serum separation tubes (BD, 365967), spun down, and the serum was separated. Serum (30 µl) was diluted 1:4 with Diluent 42 (MesoScale Discovery). The levels of ten cytokines and chemokines [interferon (IFN)-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-13, tumor necrosis factor-α (TNF-α)] were determined from 100 µg of spleen., or diluted serum using the rat pro-inflammatory V-plex kit (panel 1, MesoScale Discovery), according to the manufacturer's instructions. Due to limitations of tissue sample, only Il-6 and TNF-α levels were examined in 50μg of lymph node total protein. All samples were run in duplicate, and the mean and standard deviation was calculated.

Western blot analysis:

Tissue was homogenized and total protein concentrations were determined as described above. Protein (40 µg) was added to 2X LDS sample buffer (Invitrogen, NP0007, 4X diluted 1:1 with water) with 5% reducing agent (Invitrogen, NP0004), and heated for 7 min at 95°C. Proteins were resolved on a 4-12% Bis-Tris gel (Novex #NP0322) and transferred onto a nitrocellulose membrane (Novex, 1B3011002) using the Ibot system. Membranes were blocked for 1 h at RT with TBS (Tris-buffered saline, ph 7.4, Boston BioProducts, BM-300) containing 0.05% Tween 20 (Fisher, BP337-500) and 1% bovine serum albumin (BSA, Gemini, 700-100P). Membranes were incubated with primary TNF-α antibody (1:1000, Abcam, ab66579) or IL-6 (1:1000, Abcam, ab6672),

with β-actin (1:1000, cell signaling #8H10D10) as a loading control, followed by secondary antibodies for visualization (1:15,000, goat anti-mouse IgG, Licor 827-08364; donkey anti-rabbit IgG, Licor 926-68073), diluted in blocking buffer (Licor, 927-40000). Proteins were analyzed on the Odyssey infrared imaging system for detection and quantification. The integrated intensity signal of β-actin was used to normalize the signal of TNF-α and IL-6. The percentage change in normalized protein was calculated by comparison to the F344 age-matched vehicle control.

Statistical analysis:

Flow cytometry analysis was performed using DIVA 6.1.3, FlowJo V10 and Graph Pad Prism 5 software. All data are presented as the mean ± SD. Statistical differences among the groups were assessed by a one-way ANOVA, and post hoc multiple comparisons were performed using the Bonferroni multiple comparisons test. The significance level was set at $p < 0.05$.

Results

Immunophenotypic analysis of untreated peripheral blood cells in aging rats

Immune cell populations from whole blood were gated as illustrated in Fig 1. $CD3^+$ T cell lymphocytes (Fig. 2A) were significantly increased in 2 mo old HIV-1Tg rats compared to age-matched F344 animals. However, T cell percentages decreased in both groups during aging, but to a greater extent in 18 mo old HIV-1Tg rats. The percentage of T helper cells ($CD3^+/CD4^+$) from the HIV-1Tg rats was higher throughout the aging process, with a significant increase in 5-6 and 12 mo old animals (Fig. 2B). There was a significantly lower percentage of T cytotoxic cells ($CD3^+/CD8^+$) in the 6, 12, and 18 mo old HIV-1Tg rats compared to the age-matched control rats (Fig. 2C).

Flow cytometry analysis revealed that the percentage of B cells in both the HIV-1Tg and wild-type F344 rats was higher in the 2 mo old rats and decreases with aging (Fig. 2D).

The percentage of neutrophils was less than 10% in both the HIV-1Tg and F344 groups at 2, 5-6 and 12 mo of age. However, the percentage of neutrophils increased significantly in the 18-20 mo old HIV-1Tg rats compared to the age-

Figure 1. Flow cytometry gating strategy for HIV-1Tg and F344 rat blood.

Mononuclear, lymphocytes, and granulocytes were gated on FSC versus SSC. Cell surface antibodies were used to identify B and T cells, T cytotoxic cells, T helper cells, neutrophils, monocytes, and monocyte subtypes (classical and non-classical).

Figure 2. Analysis of cell populations in whole blood from HIV-1Tg and F344 rats.

Flow cytometry analysis of untreated HIV-1Tg immune cell populations (red line) and untreated F344 age-matched rats (blue line). (A) T cells (CD3+); (B) T helper cells (CD3+/CD4+); (C) T cytotoxic cells (CD3+/CD8+); (D) B cells (CD45RA+); (E) neutrophils; (F) monocytes; (G) classical monocytes (CD43+); (H) non-classical monocytes (CD 43++). N=5 rats/group. *p ¸ 0.05**p ¸ 0.01, ***p ¸ 0.001, ****p ¸ 0.0001.

matched controls (Fig. 2E). There was a significantly lower percentage of monocytes (Fig. 2F) in the 2, 5-6, and 12 mo old HIV-1Tg rats compared to the F344 age-matched control animals, and which remained slightly lower throughout the aging process. In the rat, CD43+ classical monocytes are similar to human CD14+CD16-, and CD43++ non-classical monocytes act like human CD14+CD16+cells.The percentage of classical monocytes in the HIV-1Tg rats declined at 12 mo of age compared to the control animals (Fig. 2G), whereas there was a slight but not significant increase in the percentage of non-classical monocytes in the HIV-1Tg rats at 12 mo of age (Fig. 2H).

Immunophenotypic analysis of peripheral blood cells in LPS treated aging rats

As a model molecule, LPS is commonly used to examine inflammatory response and immunophenotypic changes in animal models. Flow cytometry analysis of whole blood showed that the percentage of $CD3^+$ T cell lymphocytes was significantly higher in both untreated and LPS-treated 2 and 5-6 mo old HIV-1Tg rats compared to age-matched F344 rats (Fig. 3A). However, T cell percentages decreased in 18 mo old HIV-1Tg and F344 rats. The percentage of T helper cells ($CD3^+/CD4^+$) in the LPS-treated HIV-1Tg rats was significantly higher throughout the aging process compared to the F344 rats (Fig. 3B). Conversely, the percentage of T cytotoxic cells ($CD3^+/CD8^+$) was significantly lower in the LPS treated 5-6 and 18-20 mo old HIV-1Tg rats compared to the

LPS treated F344 animals (Fig. 3C). The percentage of B cells was not significantly different in the age matched HIV-1Tg and F344 control or LPS treated groups (Fig.3D).

The percentage of neutrophils remained low in the control blood of both the HIV-1Tg and F344 rats, but increased in response to LPS in both the HIV-1Tg and F344 rats in all age groups (Fig. 3E). There was no significant difference in the percentage of monocytes in the control and LPS treated HIV-1Tg compared to the age and treatment matched F344 group. However, the percentage of classical monocytes was significantly lower in 18-20 mo control HIV-1Tg rats compared to the age-matched control F344 animals (Fig. 3G). Conversely, the percentage of non-classical monocytes was significantly increased in 18-20 mo control HIV-1Tg rats compared to age matched control F344 animals. (Fig. 3H).

Immunophenotypic analysis of immune cells from the spleens of LPS treated aging rats

The distribution of immune cells in the spleens of HIV-1Tg rats, with and without an LPS treatment, was compared to age-matched F344 rats using flow cytometry analysis. The percentage of $CD3^+$ T cell lymphocytes was significantly increased in 5-6 mo old LPS-treated HIV-1Tg rats compared to the F344

Figure 3. Analysis of cell populations in whole blood from aging HIV-1Tg and F344 rats, with and without LPS treatment. Flow cytometry analysis of HIV-1Tg cell populations (red bars) and F344 age-matched control rats (blue bars). Solid bars indicate control samples and bars with striped lines indicate samples that have been treated with LPS. (A) T cells; (B) T helper cells; (C) T cytotoxic cells; (D) B cells; (E) neutrophils; (F) monocytes; (G) classical monocytes; (H) non-classical monocytes. N=5 rats/group. *p ¸ 0.05, **p ¸ 0.01, ***p ¸ 0.001, ****p ¸ 0.0001.

animals, and significantly decreased in 18-20 months old HIV-1Tg rats (Fig. 4A). The percentage of T helper cells ($CD3^+/CD4^+$) was significantly higher in the 2 mo old control HIV-1Tg rats compared to the age-matched F344 animals (Fig. 4B). There was a significantly lower percentage of T cytotoxic cells ($CD3^+/CD8^+$) in the 18-20 mo old LPS treated HIV-1Tg rats compared to age matched LPS treated F344 rats (Fig. 4C).

The percentage of B cells was significantly higher in the 18-20 mo old LPS-treated HIV-1Tg rats compared to age matched LPS treated F344 rats (Fig. 4D). The percentage of neutrophils in the spleen was significantly decreased in the 2 mo old LPS-treated HIV-1Tg rats compared to the age-matched F344 control animals (Fig. 4E) There was a significantly lower percentage of monocytes in the 18-20 mo old LPS-treated HIV-1Tg rats compared to LPS treated age matched F344 rats (Fig. 4F). However, the percentage of classical monocytes (Fig.4G) or non-classical monocytes (Fig 4H) was not significantly different when comparing age and treatment matched HIV-1Tg and F344 rats.

Figure 4. Immune cells in the spleens of aging HIV-1Tg and F344 rats, with and without LPS treatment. Flow cytometry analysis of immune cells from the spleens of HIV-1Tg rats (red bars) and F344 age-matched control rats (blue bars). Solid bars indicate control samples and bars with striped lines indicate samples that have been treated with LPS. (A) T cells; (B) T helper cells; (C) T cytotoxic cells; (D) B cells; (E) neutrophils; (F) monocytes; (G) non-classical monocytes; (H) classical monocytes. N=5 rats/group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Cytokines and chemokines in the serum, spleen, and lymph nodes of LPS-treated aging rats

The protein levels of IFN-γ, IL-1β,IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-13, and TNF-α in the serum, spleen, and lymph nodes of HIV-1Tg and F344 age-matched rats treated with LPS or saline (control) were determined using an electrochemiluminescent assay. There was no difference in the levels of IL-2, INF-γ, IL-4, and IL-13 in the serum of the LPS- and saline-treated HIV-1Tg and F344 rats (Fig. 5A-D). Although not significant, there was a slight decrease in the IL-5 levels associated with increased age in both the untreated F344 and HIV-1Tg rats; however, IL-5 was elevated in all the LPS-treated groups (Fig. 5E). There was no difference in IL-10 levels in any age group in either the control or LPS-treated F344 rats, and no difference in IL-10 in the 2 mo old control and LPS-treated HIV-1Tg rats. However, there was an increase, although not significant, with IL-10 in the 5-6 mo LPS-treated HIV-1Tg rats as well as the control and LPS-treated18-20 mo old HIV-1Tg rats compared to the treated and age-matched F344 rats (Fig. 5F). IL-1β in both the control HIV-1Tg and F344 rats was below detection levels for this assay (<3 pg/ml). The IL-1β levels in both the LPS-treated F344 and HIV-1Tg rats declined with increased age (Fig. 5G). KC/GRO levels were low in both untreated HIV-1Tg and F344 rats; however, KC/GRO levels increased significantly in 5-6 and 18-20 mo old HIV-1Tg rats treated with LPS compared to age matched LPS treated F344 rats (Fig. 5H). Both IL-6 and TNF-α level were minimal at all ages in both the control HIV-

Figure 5. Cytokines and chemokines in the serum of aging HIV-1Tg and F344 rats, with and without LPS treatment. Cytokine and chemokine levels of serum from HIV-1Tg rats (red bars) compared to F344 age-matched controls (blue bars). Solid bars indicate control (saline) treated samples and bars with striped lines indicate samples treated with LPS. (A) IL-2; (B) IFN-γ; (C) IL-4; (D) IL-13; (E) IL-5; (F) IL-10; (G) IL-1β; (H) KC/GRO; (I) IL-6 and (J) TNF-α. N=5 rats/group. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

1Tg and F344 animals. There was a significant increase in both IL-6 and TNF-α level in the serum of LPS-treated HIV-1Tg rats at 18-20 mo of age compared to the age-matched control and LPS-treated F344 rats (Figs. 5I and 5J).

Spleen samples showed similar levels of IL-2, INF-γ, IL-4, and IL-5 in age and treatment matched HIV-1Tg and F344 rats during aging (Figs. 6A, B, C, and E). IL-13 increased in response to LPS in both the F344 and HIV-1Tg rats (Fig. 6D). The increase in IL-10 in response to LPS was similar in the 2 and 5-6 mo old F344 rats, but had declined at 18-20 mo of age (Fig. 6F). IL-10 in the HIV-1Tg rats was increased in response to LPS at all ages, and was significantly higher at 18-20 mo of age compared to age-matched F344 animals (Fig. 6F). IL-1β levels were comparable in the spleen of control HIV-1Tg and F344 rats, and increased in response to LPS in both groups. However, at 5-6 mo of age, the level of IL-1β was significantly lower in the LPS-treated HIV-1Tg rats compared to the treated age-matched F344 rats (Fig. 6G). KC/GRO concentrations were similar in control HIV-1Tg and F344 rats, and increased in response to LPS in both groups. The LPS-induced levels of KC/GRO appeared to decrease with age in the F344 rats, whereas KC/GRO remained significantly higher at 18-20 mo of age in the HIV-1Tg animals (Fig. 6H). IL-6 and TNF-α were at basal levels in the spleens of the control HIV-1Tg and F344 rats. However, both IL-6 and TNF-α increased significantly in response to LPS in the spleens of the 18-20 mo old HIV-1Tg rats compared to age and treatment

Figure 6. Cytokines and chemokines in the spleens of aging HIV-1Tg and F344 rats, with and without LPS treatment. Cytokine and chemokine levels of spleen protein from HIV-1Tg rats (red bars) compared to F344 age-matched controls (blue bars). Solid bars indicate control (saline) treated samples and bars with striped lines indicate samples treated with LPS. (A) IL-2; (B) IFN-γ; (C) IL-4; (D) IL-13; (E) IL-5; (F) IL-10; (G) IL-1β; (H) KC/GRO; (I) IL-6 and (J) TNF-α. N=5 rats/group. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

matched F344 rats (Figs. 6I and 6J).

Low levels of IL-6 and TNF-α were found in the lymph nodes in control HIV-1Tg and F344 rats as well as in 2 and 5-6 mo old LPS-treated HIV-1Tg and F344 animals (Figs. 7A and B). There were significantly higher levels of IL-6 and TNF-α in the lymph nodes of LPS treated 18-20 mo old HIV-1Tg rats compared to age-matched LPS treated F344 rats.

IL-6 and TNF-α protein analysis

The IL-6 protein signal was comparable in both the control treated 2 and 5-6 mo old HIV-1Tg and F344 rats; however, there was a slight increase in the IL-6 signal in the spleens of the control 18-20 mo old HIV-1Tg rats compared to the-age matched control F344 rats (Fig. 8A). IL-6 protein increased with age in the LPS-treated HIV-1Tg rats, with a significant increase seen in the 18-20 mo old HIV-1Tg rats compared to the age-matched LPS-treated F344 rats (Fig. 8B). There was a significant increase in TNF-α levels in the spleens of the 18-20 mo old HIV-1Tg control rats (Fig. 8C), and no significant difference in TNF-α levels in the 2 mo and 5-6 mo control HIV-1Tg, and 2 mo, 5-6 mo and 18-20 mo control F344 animals. There was no difference in the TNF-α protein levels following LPS treatment in the 2 mo old HIV-1Tg and F344 rats, but a slight decrease in both groups at 5-6 mo of age. There was a significant increase in TNF-α level in both the LPS treated HIV-1Tg and F344 rats at 18-20 mo of age compared to the age-matched F344 controls (Fig. 8D).

Figure 7. IL-6 and TNF-α in lymph nodes of aging HIV-1Tg and F344 rats, with and without LPS treatment.

Pro-inflammatory cytokines, (A) IL-6 and (B) TNF-α, were examined in the lymph nodes of HIV-1Tg (red bars) and age-matched F344 control rats (blue bars), with and without LPS treatment. Solid bars indicate control samples and bars with striped lines indicate samples treated with LPS. N=5 rats/group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 8. IL-6 and TNF-α in the spleens of aging HIV-1Tg and F344 rat spleen, with and without LPS treatment.

The integrated intensity of TNF-α and IL-6 protein from the spleens of HIV-1Tg (red bars) and F344 age-matched control rats (blue bars), with and without LPS treatment, was measured using Western blot analysis and normalized to β-actin protein. (A) IL-6 after saline treatment; (B) IL-6 after LPS treatment; (C) TNF-α after saline treatment; (D) TNF-α after LPS treatment. N=5 rats/group.*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Discussion

It is well known that aging causes alterations in immune responses, in general, with increased susceptibility to many infectious diseases (Aberg, 2011; Barrett et al., 2012; Cavanagh et al., 2012; Goronzy and Weyand, 2012; Kogut, et al., 2012; Rymkiewicz et al., 2012). Understanding the changes in immune responses in HIV-positive individuals is further complicated when factors such as age and disease progression are taken into consideration. Utilizing the HIV-1Tg rat, we were able to demonstrate that, in this animal model of human HIV-1-positive patients receiving HAART, alterations in immune cell populations and response to an LPS challenge occur with aging.

Flow cytometric analysis of untreated blood revealed that, in the HIV-1Tg rat, there is a decrease in T cells, particularly T cytotoxic cells, but an increase in T helper cells with increased aging. Interestingly, the untreated F344 rat did not display changes in the percentage of T cells within the lymphocyte due to age and showed percentages of 52-55%. This result is similar to a study by Schmucker *et al*. in which they compared young adult (4-6 mo old) and senescent (24-26 mo old) F344 rats finding that the percentage of the T cell populations within the blood were consistent regardless of age (Schmucker et al., 2002). These results suggest that age of HIV-1Tg may play a role in the decrease of T cell populations that are not displayed in the F344 animals.

In healthy humans, $CD14^{+}CD16^{-}$ cells represent 90-95% of the total monocyte population in the blood and are described as “classical”, while the remaining 5-10% are proinflammatory monocytes ($CD14^{+}CD16^{+}$) termed “non-classical” (Strauss-Ayali et al., 2007). In the rat, two subsets of monocytes have been described based on chemokine receptor expression and CD43 amounts. $CD43^{+}$ are similar to human $CD14^{+}CD16^{-}$ classical monocytes, while in the rat $CD43^{++}$ monocytes act like human non-classical $CD14^{+}CD16^{+}$ populations (Grau et al., 2000; Strauss et al., 2007). Monocytes are precursors for macrophage and dendritic cell populations, and have important functions in innate and adaptive immunity throughout inflammation (Tacke and Randolph, 2006). Increases in non-classical monocytes have been associated with infections, including HIV and aging. Non-classical monocytes produce higher amounts of inflammatory cytokines including TNF-α protein, while classical monocytes produce a broad range of cytokines including IL-10 and IL-6 (Wong et al., 2011).

The percentages of monocytes in this study were decreased in the untreated HIV-1Tg rats compared to the F344 animals, but increased with aging. Interestingly, the percentage of classical and non-classical monocytes was similar throughout aging in the HIV-1 and F344 rats. Perhaps the changes exhibited in the monocyte populations and cytokine increases associated with HIV-1 aging may be related to alterations with the macrophage or dendritic populations not detected in these studies. These results, however, demonstrated

that untreated HIV-1Tg rats display alterations in immunophenotype due to aging that are not evident in age-matched F344 rats. Thus, the persistent presence of HIV-1 viral proteins appears to lead to aging dependent immunomodulation.

LPS is a glycolipid component of gram-negative bacteria commonly used to study inflammatory responses and cytokine and chemokine changes (Schletter et al., 1995; Fujihara et al., 2003; Biswas and Lopez-Collazo, 2009). A balance in pro-inflammatory and anti-inflammatory responses is imperative in achieving an appropriate immune response to a stimulus. An imbalance between pro-inflammatory and anti-inflammatory cytokines can cause adverse biological events, such as septic shock (Biswas and Lopez-Collazo, 2009). Repeated exposure to an endotoxin such as LPS can cause endotoxin tolerance (ET), in which there is an increase in anti-inflammatory cytokines and a decrease in pro-inflammatory cytokines (Biswas and Lopez-Collazo, 2009). A recent study reported that ET causes an imbalance in the cytokine/chemokine response in an HIV-1Tg animal (Homji et al., 2012). While innate immunity is deregulated in both HIV infection and ET (Lester et al., 2009), identifying changes in immune responses that are also related to age may provide useful information in treating bacterial and other infections in the HIV patient.

Aging is linked with heightened amounts of circulating cytokines and proinflammatory markers (Michaud et al., 2013). Increased levels of the proinflammatory cytokines IL-6 and TNFα, along with IL-1 and C-reactive protein, have been associated with an elevated risk of morbidity and mortality found in

aging population (Michaud et al., 2013). In humans, a condition referred to as 'inflamm-aging', exhibits an increase in IL-6 and TNF-α which occurs with aging (Franceschi et al., 2000; Wolf et al., 2012). In mature HIV-positive individuals, elevated levels of IL-6 and TNF-α have been reported (Nixon and Landay, 2010). Immunoscenescence or aging of the immune system caused by a prolonged antigen burden could be accelerated in HIV-positive individuals (Franceschi et al., 2007; Vasto et al., 2007; Smith et al., 2013).

IL-6 is secreted by T cells and macrophages and has a role in inflammation and aging whereas TNF-α is mainly secreted by macrophages (Michaud et al., 2013). KC/GRO is produced by macrophages and is involved in neutrophil activation. In our study, there was a significant increase in the percentage of neutrophils in the blood of untreated 18 mo HIV-1Tg rats and increases in monocyte populations with aging. IL-10 inhibits the synthesis of many cytokines that have a role in suppression of Th1 pro-inflammatory responses and phagocytic uptake. The cytokines IL-6 and TNF-α, along with the CXC chemokine, KC/GRO, were significantly elevated in the blood and spleen protein of 18-20 mo old LPS treated HIV-1Tg rats. In addition, there was increased IL-6 and TNF-α protein expression in the lymph nodes. IL-10 levels were increased in the spleen of LPS-treated 18-20 mo old HIV-1Tg rats compared to LPS-treated and age-matched F344 rats. These results further indicate that age may play a role in the alterations of the immune cell responses in HIV-positive individuals.

In HIV-infected patients, anti-retroviral therapies have been successful in controlling viral replication, allowing those individuals to achieve a relatively normal life span (Manfredi, 2004; Manfredi and Calza, 2004; Gebo, 2006). Viral proteins, however, are capable of independently inducing organ dysfunction and affecting host target cells (T cells and macrophages), resulting in the clinical manifestations of AIDS (van Maanen and Sutton, 2003; Joshi et al., 2008; Peng et al., 2010). In addition, Peng, et al (2010) observed that the HIV-1Tg rat died at an earlier age than F344 rats. As an HIV-infected individual matures, immune cell function may be compromised even more. Age may, therefore, be a factor in determining appropriate therapies for both younger and older HIV-infected patients.

These results suggested that the HIV-1Tg rat displayed immune alterations in T cells, neutrophils and monocytes with aging. Thus, in addition to serving as a rodent neuroAIDS model to study the HIV patients given HAART, our current studies suggest that the HIV-1Tg rat could be an ideal model to study aging associated immune alterations of HIV patients.

Future studies following the immunological changes in T cells, neutrophils and monocytes with aging, may focus on investigating specific immune subtypes, not examined in this current report. Using flow cytometry, immunophenotypic analysis of cell populations such as activated and regulatory T cell populations, mature and immature monocytic derived dendritic cells, natural killer (NK), and B cell subtypes (naïve, mature, memory) may exhibit additional changes in the

aging HIV-1Tg rat. Further analysis of cellular response at the protein level may include surveying additional cytokines and chemokines, not currently examined, with western blots and/or electrochemiluminescent assays. These results may unearth a specific cellular subtype which may have an important role in aging of the HIV-1Tg rat.

In addition, it would be interesting to inspect immunophenotype and cellular response in an acute immune challenge model not examined in this report. Aged animals treated with a single dose of LPS, may respond differently compared to the ET model used in this study. *In vitro* treatments of splenocytes or whole blood from aged animals challenged with agents such as LPS or phorbol myristate acetate (PMA), may display different cellular response in the aged HIV-1Tg rat compared to the F344 animals.

Founded on the results established in this report, potential studies may include exploring the immunological effects of HIV and aging within the brain. Cognitive impairment linked to human HIV has been demonstrate in the HIV-1Tg rat (Rao et al, 2011). Rao showed that protein and mRNA in the brains of 7-9 mo old animals displayed increases in cytokines, including TNF-α, along with changes in the microglial and the macrophage marker CD11b. A detailed study examining the protein and mRNA levels of neuroinflammatory markers in the aged (18-20 mo) rat may reveal alterations in the HIV-1Tg rat brain not displayed in F344 rats. In addition, evidence suggests that LPS treatment may increase RNA turnover of IL-6 and TNFα along with corticosterone levels in the rat brain

with ET treatment (Chen et al., 2005). Aged HIV-1 Tg rat brains treated with LPS may be examined at the protein and mRNA level to understand changes occurring in the aged brain. Developmental learning, such as associated with dendritic spine turnover, may be altered in the aging HIV-1Tg rat. Cognitive animal tests, such as the Morris water maze test, may reveal further changes in the aging rat. Perhaps these and other differences with the molecular mechanism underlying the age-related neuronal and immune compromised status many be revealed in the aging HIV-1Tg rats.

Another area to explore with the HIV-1Tg rat involves the cellular aging pathway and structural alterations to telomeres. It is known that shortening of the telemetric sequences and telomeres is associated with increased age (Abrass et al, 2012). Data suggests that human immune cell types, including effector CD8 T cells, display shortened telomeres and lack of telomerase activity in seniors with decreased vaccine response, bone deficiency, neurocognitive regression and cardiovascular illness. Immunohistochemistry experiments focusing on measuring telomeres through the aging process in HIV-1Tg compared to F344 rat may provide evidence that HIV status influences telomere shortening. Exploring the structural attributes of the telomeres in immune cells such as T cell, monocytes and neutrophils in the HIV-1Tg rat may reveal changes associated with increased aging.

Understanding HIV status and relating age related consequences in the HIV-1Tg rat may provide new avenues into treating the aging human population

currently infected with HIV. Treatments specifically targeting alterations in IL-6 and TNFα may prevent or delay senescence in the aging HIV patient. The findings from this study provide evidence pointing to age-related alterations in immune cell function in the HIV-positive population. This information could be important in the development of novel therapeutic treatments for HIV-infected individuals based on multiple factors, including immune cell profile, cellular responses, and age of the patient.

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