Comparative Medicine
Copyright 2017
by the American Association for Laboratory Animal Science

Vol 67, No 1
February 2017
Pages 67–78

Original Research

Phenotypic and Functional Characterization of Peripheral Blood Lymphocytes from Various Age- and Sex-Specific Groups of Owl Monkeys (*Aotus nancymaae*)

Pramod N Nehete,[1,2,*] Bharti P Nehete,[1] Sriram Chitta,[1] Lawrence E Williams,[1,2] and Christian R Abee[1]

Owl monkeys (*Aotus nancymaae*) are New World NHP that serve an important role in vaccine development and as a model for human disease conditions such as malaria. Despite the past contributions of this animal model, limited information is available about the phenotype and functional properties of peripheral blood lymphocytes in reference to sex and age. Using a panel of human antibodies and a set of standardized human immune assays, we identified and characterized various peripheral blood lymphocyte subsets, evaluated the immune functions of T cells, and analyzed cytokines relative to sex and age in healthy owl monkeys. We noted age- and sex-dependent changes in $CD28^+$ (an essential T cell costimulatory molecule) and $CD95^+$ (an apoptotic surface marker) T cells and various levels of cytokines in the plasma. In immune assays of freshly isolated peripheral blood mononuclear cells, IFNγ and perforin responses were significantly higher in female than in male monkeys and in young adults than in juvenile and geriatric groups, despite similar lymphocyte (particularly T cell) populations in these groups. Our current findings may be useful in exploring *Aotus* monkeys as a model system for the study of aging, susceptibility to infectious diseases, and age-associated differences in vaccine efficacy, and other challenges particular to pediatric and geriatric patients.

Abbreviations: Con A, concanavalin A; ELISPOT, enzyme-linked immunospot; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PHA, phytohemagglutinin; PWM, pokeweed mitogen

Normal aging in humans is associated with a decline in lymphocyte proliferation and cytokine production and in NK cell activity.[5,55] In the present study, we phenotypically and functionally characterized lymphocytes and circulating cytokine levels across a healthy population of owl monkeys (*Aotus nancymaae*) and the age- and sex-dependent variations among study subgroups. *Aotus* spp. are susceptible to malarial infections due to both *Plasmodium falciparum* and *P. vivax*, which produce symptoms similar to those observed in human infections.[51] Consequently, the World Health Organization recommends *Aotus* monkeys as an excellent experimental model for studies of malaria infection.[1,3,16,17,51] Despite the support for this disease model, very little has been published regarding age- and sex-related changes in lymphocyte populations and immune functions in *Aotus* spp., in contrast to the numerous studies conducted in humans, rhesus macaques, and mice.[12,18,22,25,27]

Although human immune reagents and functional assays are often used in research involving NHP, the cross-reactivity of immune reagents, particularly monoclonal antibodies, and the adaptation of immune assays to theses model are species-specific. Although age-associated impairment of the human immune system, including decreased circulating mitogen-specific T cells,[14,54] reduced T cell proliferation, and increased suppressor T-cell activity, have been reported,[26,31,36] the exact cause of aging-related differences in immune dysfunction between male and female NHP has not been systematically studied in detail. Whether age-related immune impairment is influenced by a particular subset of circulating lymphocytes or by the frequency of one or more lymphocyte populations or by the functional activity of these cells is currently unclear.[22,31] Changes in overall immune functionality in elderly men and women reportedly were due to a phenotypic shift from naïve to memory circulating lymphocyte populations.[30] The objective of the current descriptive study was to determine and monitor various immune characteristics including the phenotypes and frequencies of lymphocyte subsets, lymphocyte proliferation, and plasma cytokine levels in male and female owl monkeys of different age groups.

Received: 09 Mar 2016. Revision requested: 12 May 2016. Accepted: 15 Sep 2016.
[1]Department of Veterinary Sciences, MD Anderson Cancer Center, University of Texas, Bastrop, Texas, and [2]Graduate School of Biomedical Sciences, The University of Texas, Houston, Texas
[*]Corresponding author. Email: pnehete@mdanderson.org

Materials and Methods

Care and housing. Healthy owl monkeys (*Aotus nancymaae*) were selected by using a simple random sampling without replacement technique from a population of 117 females and 113

males from the Owl Monkey Breeding and Research Resource at Michale E Keeling Center for Comparative Medicine and Research (MD Anderson Cancer Center, University of Texas, Bastrop, TX). Differences in lymphocytes between male and female monkeys were determined by using an independent group of 30 owl monkeys (15 male and 15 female; age, 2 to 19 y). To investigate the effects of age, animals were classified into juvenile (2 to 4 y), young adult (5 to 8 y), and geriatric (9 to 16 y) subgroups consisting of 10 monkeys per group. All study animals were housed in enclosures that were 4 ft deep × 4 ft wide × 6 ft tall and were kept in their normal social groups, consisting of one male and one female and their offspring (3 y and younger). The lights for this nocturnal species were turned on at 0000 and switched off at 1200, and overhead louvers remained open from 1200 to 1500 to provide a dusky, twilight level of light.

Ethics statement. The animals were housed according to regulations at the AAALAC-accredited facility at Michale E Keeling Center for Comparative Medicine and Research. All animal studies were conducted according to the provisions of the Animal Welfare Act and Animal Welfare Policy and the principles of the *Guide for the Care and Use of Laboratory Animals*.[24] All procedures were approved by the IACUC at MD Anderson (protocol no. 329-RN00).

Diet. Owl monkeys had unrestricted access to New World Primate Diet (no. 5049, Purina, St Louis, MO) and water. In addition, they were fed either a fresh fruit or vegetable daily. Specialty foods, such as seeds, peanuts, raisins, yogurt, cereals, frozen juice cups, and peanut butter, were distributed daily as enrichment. The animals were never food- or water-fasted. In addition, they were given destructible enrichment manipulanda and various travel or perching materials on a rotating basis to promote species-typical behavior.

Blood collection and preparation of PBMC. Blood samples (3 mL) were collected in EDTA-coated tubes from all study animals every 4 to 6 wk; at least 3 samples were collected from each monkey. Before plasma collection by centrifugation, an aliquot (300 to 500 µL) of blood was removed for multicolor flow cytometric assays. Plasma was collected by centrifugation of whole blood and stored at –80 °C until further use to analyze circulating cytokines. PBMC were isolated by Ficoll–Hypaque density-gradient separation, as described previously.[38-40] PBMC were washed twice with RPMI supplemented with 10% heat-inactivated fetal bovine serum and checked for viability by using the trypan blue exclusion method. PBMC preparations showing cell viability of at least 90% were used in functional assays.

Whole-blood staining. Commercially available human monoclonal antibodies (Figure 1) were tested for cross-reactivity with owl monkey mononuclear cells by using flow cytometric analysis. Phenotyping of peripheral blood lymphocytes from owl monkeys was performed as described previously.[38,39,50] Briefly, 100-µL aliquots of EDTA-treated whole blood from each sample were added to individual 12 × 75-mm polystyrene test tubes containing test antibodies (or matched isotype control) and incubated at room temperature in the dark. After 15 min, 1× lysis solution (BD Biosciences, San Jose, CA) was used to remove RBC, and the mononuclear cells were washed twice with PBS and fixed in 400 µL of 2% formaldehyde. Cell signals were acquired on a 4-color flow cytometer (FACSCalibur, BD Biosciences), and for normalization, we acquired 100,000 events for all samples. T cells ($CD3^+$), NK cells ($CD16^+$), and B cells ($CD20^+$) among lymphocytes were identified by gating on a forward scatter by side scatter plot. The $CD3^+$ T-cell population was further analyzed for $CD4^+$, $CD8^+$, $CD4^+CD8^+$, and $CD16^+CD3^+$ (NK T cells) subsets by using Cellquest-pro software (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

Target	Clone	Isotype	Label
CD3	SP34	Mouse IgG3λ	FITC
CD3	SP34-2	Mouse IgG1	APC
CD4	L200	Mouse IgG1	APC
CD4	SK3	Mouse IgG1	PerCP
CD8	RPA-T8	Mouse IgG1	APC
CD8	3B5	Mouse IgG2a	PE
CD16	3G8	Mouse IgG1	PE
CD20	B1-RD1	Mouse IgG2a	PE
CD20	L27	Mouse IgG1	PE
CD27	M-T271	Mouse IgG1	PE
CD28	L293	Mouse IgG1	PerCP–Cy5.5
CD28	CD28.2	Mouse IgG1	APC
CD80	L307.4	Mouse IgG1	PE
CD95	DX2	Mouse IgG1	APC
CCR7	3D12	Rat 1gG2a	AF647
HLA–DR	L243	Mouse IgG2a	PerCP

Figure 1. Human antibodies with cross-reactivity to New World NHP species. All of these antibodies (except clone RPA-T8) are cross-reactive to rhesus macaques (*Macaca mulatta*), squirrel monkeys (*Saimiri boliviensis*), and owl monkey (*Aotus nancymae*); cross-reactivity of RPA-T8 to rhesus macaques has not yet been determined.

In a separate assay, whole blood (100 µL) was stained with antiCD3 (FITC-labeled; clone SP34-2, BD Biosciences), antiCD4 (PE-labeled; clone L200, BD Biosciences), antiCD28 (PerCP–Cy5.5-labeled; clone CD28.2, BD Biosciences), and antiCD95 (APC-labeled; clone DX2, BD Biosciences) to identify memory T-lymphocyte subsets, according to the same staining protocol described earlier. For acquiring cells and gating on subpopulations during analysis, we used separate compensation (single color), fluorescence-minus-one controls, and isotype controls in each assay.

Enzyme-linked immunoassay for detecting antigen-specific IFNγ- and perforin-producing cells. The population for this immune assay comprised 30 owl monkeys, including both males (n = 15) and females (n = 15) and ranging in age from 2 to 19 y and belonging to juvenile (n = 10), young adult (n = 10), and geriatric (n = 10) groups. To quantify IFNγ- and perforin-producing cells, we followed the standard Enzyme-Linked ImmunoSpot (ELISPOT) protocol.[38-40] Freshly isolated PBMC (viability, 90% or greater; 1×10^5 cells per well) were cultured in the presence of phytohemagglutinin (PHA), concanavalin A (Con A), LPS, or pokeweed mitogen (PWM; all reagents from Sigma [St Louis, MO]; final concentration, 1 µg/mL) to activate cells. Cells were directly stimulated by seeding in triplicate wells of 96-well plates (PVDF-MAIP S 45, Millipore, Bedford, MA) precoated with antiIFNγ or antiperforin primary antibody. After incubation for 30 to 32 h at 37 °C, the cells were removed, the wells were thoroughly washed with 1× PBS, and spots were developed according to the manufacturer's instructions. The quantitative analysis involved counting of the purple-colored spots as cytokine-secreting cells by using the KS-ELISPOT automatic system (Carl Zeiss) by an independent agency (ZellNet Consulting, Fort Lee, NJ). The response was represented as the number of IFNγ or perforin spot-forming cells per 1×10^5 PBMC and was considered positive when the number of spot-forming cells with the test antigen was

at least 5 greater than that of the control wells, where cells were cultured in the absence of test antigen, with medium only.

In vitro mitogen stimulation for analysis of lymphocyte proliferation. For this immune assay, we included 30 owl monkeys, both males (n = 15) and females (n = 15) ranging in age from 2 to 19 y and belonging to juvenile (n = 10), young adult (n = 10), and geriatric (n = 10) groups. The proliferation of PBMC was determined by the standard 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) dye reduction assay.[35,48,57] Briefly, PBMC (10^5/well) were seeded in triplicate wells of 96-well flat-bottomed plates and stimulated for 72 h individually with the mitogens PHA, Con A, PWM, and LPS (final mitogen concentration, 1 µg/mL); culture medium without mitogens served as the negative control. After culture for 72 h at 37 °C in 5% CO_2, each well was loaded with 10 µL of freshly prepared and filtered MTT dye (5 mg/mL in PBS) and incubated for an additional 4 h. The medium was then replaced with 100 µL of acidified 0.04 M HCl–β-isopropanol (Sigma) and left for 30 min at room temperature for color development before being read by using an ELISA plate reader with a 490-nm filter (Victor, PerkinElmer, Shelton, CT). Results were expressed as the optical density after subtraction of the optical density of the medium-only control. Data are reported as the mean of 3 to 6 replicates. The concentration of mitogen, number of PBMC, and incubation time were standardized in our laboratory as optimal for the stimulation of PBMC isolated from healthy owl monkeys.

Multiplex cytokine assays. The concentration of cytokines (IL2, IL4, IL6, IL10, IL12/23[p40], IFNγ, TNFα, and MCP1) in plasma were measured by using an NHP Multiplex Cytokine Kit (Millipore).[39] Briefly, EDTA-preserved plasma samples were centrifuged (900 × g for 10 min), and aliquots were frozen at −80 °C until use. On the day of assay, plasma samples were thawed and precleared by centrifuging at 900 × g for 5 min. The 96-well plates provided in the kit were blocked with assay buffer for 10 min at room temperature and washed, after which 25 µL of standard or control samples were added in appropriate wells. After 25 µL of cytokine-labeled beads was added to each well, the plate was incubated on a shaker overnight at 4 °C. The next day, the plate was washed 2 times with wash buffer, incubated with detection antibody for 1 h, and then incubated with 25 µL of streptavidin–phycoerythrin for 30 min. All incubation and washing steps were performed on a shaker at room temperature. After the plate was washed 2 times with wash buffer, 150 µL of sheath fluid was added to each well, and cytokine concentrations were measured by acquiring bead-associated signals (BioPlex 200 System, BioRad Laboratories, Hercules, CA). Fluorescence data were analyzed with use of Bio-Plex manager 5.0 software (Bio-Rad, Hercules, CA). The minimum detectable concentrations for the various cytokines were calculated by using the Multiplex Analyst immunoassay analysis software (Millipore): IL2, 0.7 pg/mL; IL4, 2.7 pg/mL; IL6, 0.3 pg/mL; IL10, 6.2 pg/mL; IL12(p40), 1.2 pg/mL; IFNγ, 2.2 pg/mL; TNFα, 2.1 pg/mL; and MCP1, 3.1 pg/mL.

Statistical analysis. To determine age-related differences in peripheral lymphocyte populations from owl monkeys, data were compared by using one-way ANOVA followed by Bonferroni correction. The Brown–Forsythe test was used to ensure that the data met the equal variance assumptions of ANOVA. Unpaired 2-tailed t tests were performed for comparisons between male and female owl monkeys after the results of an F test to confirm that the groups had equal variances. A P value of less than 0.05 was considered to be statistically significant. All statistical analyses were performed by using Prism 5.00 software (GraphPad Software, San Diego, CA).

Results

Phenotypic characterization of lymphocytes. Peripheral blood samples from male and female owl monkeys belonging to the 3 target age groups (juvenile, young adult, and geriatric) were analyzed by flow cytometry to identify and enumerate various lymphocyte subsets. Human monoclonal antibodies (Figure 1) were shown to cross-react with rhesus macaques and other NHP; these antibodies were used in the current study after they were being shown to exhibit positive cross-reactivity to owl monkeys. A gating strategy reported earlier[39] was followed for phenotypic analysis of T and B lymphocytes (Figure 2 A). We used a CD16 marker antigen to positively identify NK T lymphocytes and $CD16^+$ NK cells, distinguishing them from lymphocytes in the gated population. Sex- and age-related differences were not observed for lymphocytes that were $CD3^+$, $CD4^+$, $CD8^+$, $CD20^+$, $CD16^+$ NK, or NK T cells (Figure 2 B and C).

To investigate naïve and memory T-cell subsets in the various age and sex groups, we used CD95 and CD28 surface markers, widely used in both humans and rhesus monkeys;[11] we used these surface markers in our study to identify naïve ($CD28^+CD95^-$), central memory ($CD28^+CD95^+$), and effector memory ($CD28^-CD95^+$) subsets of $CD4^+$ and $CD8^+$ T cells by using a gating strategy reported previously[39] (Figure 3 A). The percentages of central memory and effector memory T cells did not differ between male and female owl monkeys (Figure 3 B). However, in all age groups, the $CD8^+$ subpopulations of naïve and central memory T cells were smaller ($P < 0.05$) than those of $CD4^+$ T cells (Figure 3 C).

We also analyzed the percentages of CD28 and CD95 single-positive cells within $CD4^+$ and $CD8^+$ T cells and observed no sex-related changes (Figure 4 A). Among the age groups, however, the geriatric group had a lower ($P < 0.05$) percentage of $CD28^+$ T cells among both $CD4^+$ and $CD8^+$ T cells (Figure 4 B) and a higher ($P < 0.05$) percentage of $CD95^+$ T cells within both $CD4^+$ and $CD8^+$ T-cell populations (Figure 4 C).

ELISPOT assay for detecting mitogen-specific IFNγ- and perforin-producing cells. Owl monkey PBMC were analyzed for IFNγ or perforin production in response to stimulation with PHA, PWM, and LPS by using ELISPOT assays. Compared with males, female owl monkeys had more IFNγ-producing cells in response to stimulation with either PHA or LPS ($P < 0.05$; Figure 5 A) but not PWM. Analysis of IFNγ-producing cells in response to stimulation with PHA, PWM, and LPS among the 3 different age groups revealed significantly ($P < 0.05$) higher numbers of IFNγ-producing cells in adult than in juvenile or geriatric owl monkeys (Figure 5 B).

In the perforin ELISPOT assay, female owl monkeys had more perforin-producing lymphocytes in response to PHA stimulation than did male monkeys, whereas the opposite effect was seen with LPS stimulation (Figure 6 A). Responses to Con A or PWM stimulation did not differ between female and male owl monkeys. In addition, compared with juvenile and geriatric animals, adult owl monkeys demonstrated more ($P < 0.05$) perforin-producing cells in response to stimulation with PHA only; responses to Con A, PWM, or LPS did not differ among the age groups (Figure 6 B).

Proliferation. In colorimetric assays using MTT dye, the proliferative responses to Con A and LPS were significantly greater in

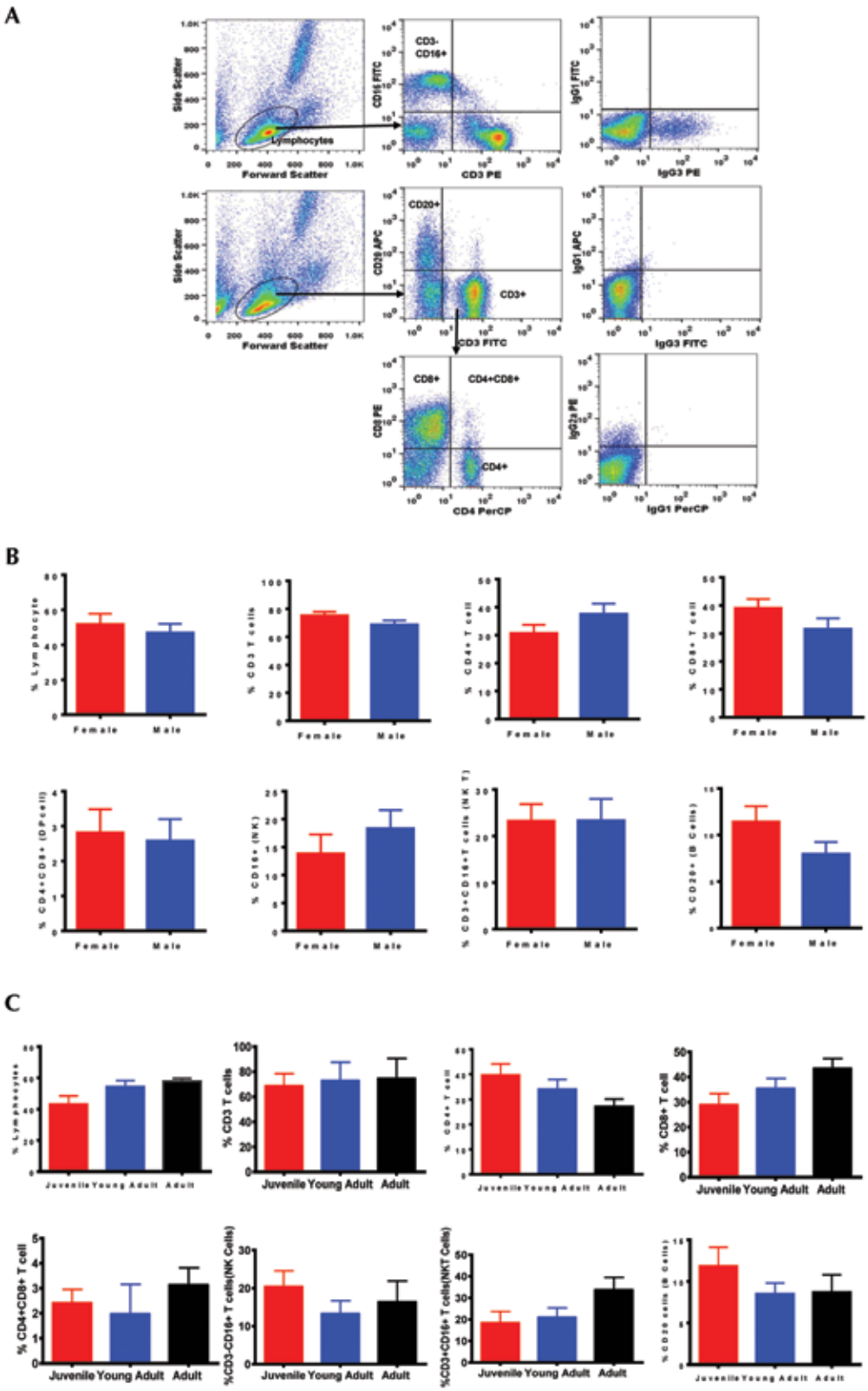

Figure 2. (A) Gating scheme for phenotypic analyses of the various T-cell subsets in the peripheral blood from a representative owl monkey. The lymphocytes were gated based on forward scatter (FSC) compared with side scatter (SSC), and $CD3^+$ T cells, $CD20^+$ B cells, $CD16^+$ NK cells ($CD3^+CD16^+$) were identified by using specific markers. Further analyses of $CD3^+$ cells yielded $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T-cell subpopulations. The specificity of staining for the various markers was ascertained according to the isotype control used for each pair of combination markers, as shown. (B) Sex- and (C) age-dependent differences in owl monkey lymphocyte populations. EDTA-treated whole blood was stained with fluorescently labeled antibodies to

female than in male owl monkeys (Figure 7 A), but no such differences in proliferation were observed for with PHA and PWM. In addition, proliferation in response to PHA, Con A, PWM, or LPS did not differ among the age groups (Figure 7 B).

Circulating peripheral blood levels of Th1, Th2, and proinflammatory cytokines. We analyzed the concentrations of various Th1 (IL2, IL12, IFNγ, TNFα) and Th2 (IL4, IL6, IL10, IL13) cytokines and of the chemokine MCP1 in plasma samples (frozen from freshly collected blood samples) from female and male owl monkeys and from the 3 different age groups. Levels of IL12p40 and IL4 were significantly ($P < 0.05$) higher in male monkeys than in female monkeys; the remaining cytokines showed no such differences (Figure 8 A). In comparing the 3 different age groups, we observed significantly ($P < 0.05$) higher concentrations of Th1 (IL2, IL12, IFNγ, TNFα) and Th2 (IL4, IL6, IL10, IL13) cytokines and MCP1 in the geriatric group than in the juvenile and adult groups (Figure 8 B).

Discussion

One of the most recognized consequences of aging is a decline in immune function. Although aging research has focused on humans and rhesus macaques (*Macaca mulatta*), the findings can be extended to owl monkeys. Research generated by using rhesus monkeys has helped to elucidate and further our understanding of human diseases.[8,9,12,18,22,25,27,52] Age-related diseases such as hypertension, atherosclerosis, infections, cancer, and autoimmune and immune complex pathologies are examples of conditions with comparable pathology between owl monkeys and humans. Some of these diseases are associated with disorders affecting the immune system; we therefore targeted the current study to age-related changes in frequency, phenotype, and function of immune cells. The oldest recorded ages in our colony of owl monkeys are 19 y for a female and 25 y for a male, compared with the 12 to 20 y reported previously.[19] The implication is that the rate of aging in owl monkeys is 3.4 times that of humans and that owl monkeys can be used in research on aging.

Even though owl monkeys represent an important experimental model of human diseases, the normal phenotype and function of their immune system is poorly understood. To our knowledge, the only report describing immune status in owl monkeys concentrates on dendritic cells as a basis for a vaccine against *Plasmodium falciparum* malaria.[13] The goal of our study was to describe the phenotype of immune cells (using cross-reactive human monoclonal antibodies for known immune cell surface markers), in vitro mitogen-activated lymphocyte-secreted cytokines, and circulating cytokines in plasma that might serve as readily accessible markers of aging and early disease severity.

Human immune-cell (CD) markers and commercially available antibodies are currently being used for research involving rhesus macaques, which are being explored as a biologic model in AIDS research and vaccine efficacy studies.[9,33,40,45,52] However, the cross-reactivity of human reagents and functional attributes of immune cells in owl monkeys have not been well characterized. Such information would be valuable in exploring New World owl monkeys as a model for immune-based infectious diseases, vaccine development, and age-related inflammatory diseases.

In the present study, with use of multicolor flow cytometry testing, we assessed a panel of monoclonal antibodies for cross-reactivity against specific subsets of owl monkey lymphoid cells. Considering the many physiologic and genetic characteristics that are similar between owl monkeys and humans as well as owl monkeys' susceptibility to a wide range of infections typically associated with humans (for example, malaria),[1,3,16,17,51] the level of cross-reactivity of the human monoclonal antibodies with the *A. nancymaae* cell markers was not unexpected. Further studies with other species of owl monkeys (*A. azarae* and *A. vociferans*) will extend our knowledge of immune cross-reactivity in general.

The present investigation revealed no age- or sex-dependent differences in peripheral blood lymphocytes representing the $CD3^+$, $CD4^+$, $CD8^+$, $CD4^+CD8^+$, $CD16^+$ (NK), or $CD3^+CD16^+$ (NKT) T-cell subsets in healthy owl monkeys. These findings are similar to those from studies of aging in humans and various NHP species.[15,18,29,42,53]

We investigated the effect of age on naive and memory T-cell pools by comparing groups of juvenile, young adult, and geriatric owl monkeys containing both male and female animals. We noted no significant differences in the central or effector memory subsets of $CD4^+$ or $CD8^+$ T cells either between male and female animals or among the age groups. These results are different from previously published reports on normal aging, which showed that the slow turnover and long lifespans of naive T cells are preserved.[56] However, other reports support our data and suggest that thymus output gradually declines and ultimately becomes unable to replace naive T cells lost from the periphery and to maintain the breadth of the T-cell repertoire.[28,37]

The findings from our analysis of the effects of the costimulatory molecule CD28 and apoptotic marker CD95 on $CD4^+$ and $CD8^+$ T cells are similar to published reports showing a gradual decline in these markers as animals progress from juvenile to young adult and then to geriatric status.[7,46] This observed change in the loss of CD28 expression and decrease in $CD28^+CD8^+$ T cells is not uncommon and has been reported in humans aging studies.[4]

In aging populations of humans and animals, decreased expression of the essential costimulatory molecule CD28 is a key biomarker, and the decreasing expression of this molecule with age might be an underlying cause for the compromised capacity of these cells to respond to activation signals. Overall, we found that the pattern of age-related T-cell changes differed remarkably between $CD4^+$ and $CD8^+$ naïve and central-memory subsets but not the effector-memory subset. These results highlight fundamental differences in the biology of these 2 T-cell lineages as underlying causes for the changes observed during immune senescence and (possibly) immune exhaustion.[25]

Studies of the biologic activity of T lymphocytes in response to immune activation are often based on in vitro models using polyclonal activators such as antiCD3 antibodies, pharmacologic agents such as phorbol esters, and mitogens such as PHA, Con A, PWM, and LPS. The activation of T lymphocytes results in the expression of cytokine receptors, production and secretion of cytokines, upregulation of cell surface activation markers, and cellular proliferation. In this study, we evaluated the most commonly used polyclonal activators by using target populations of PBMC isolated from normal age- and sex-specific groups of owl mon-

CD3, CD4, CD8, CD16, and CD20 and then analyzed for T-cell subpopulations in (B) female ($n = 9$) and male ($n = 9$) owl monkeys and in (C) the 3 age groups (juvenile, young adults, and geriatric; $n = 9$ per group) of these monkeys. Data are given as the percentage of the total lymphocyte population; error bars, 1 SD. $P < 0.05$ was considered statistically significant.

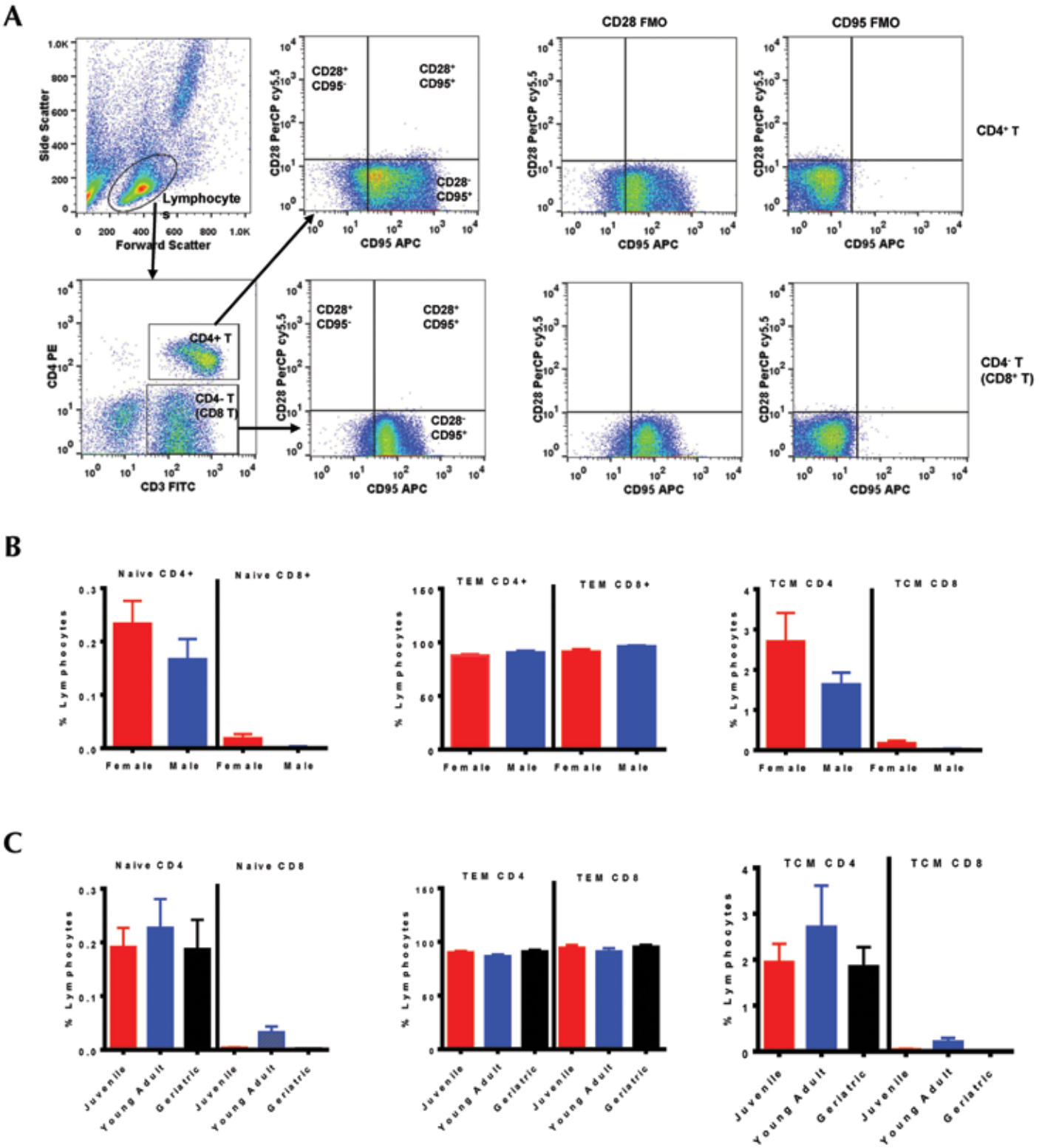

Figure 3. (A) Gating scheme for the analyses of the memory T-cell subsets in the peripheral blood from a representative owl monkey. $CD3^+$, $CD4^+$, and $CD8^+$ cells were identified as described in the legend to Figure 2; naive ($CD28^+CD95^-$), central memory ($CD28^+CD95^+$), and effector memory ($CD28^-CD95^+$) subsets of $CD4^+$ and $CD8^+$ T cells were identified by using antibodies to CD28 and CD95. The specificity of staining for the various markers was ascertained on the basis of the fluorescence-minus-one (FMO) controls shown and as described in the Materials and Methods. (B and C) Differences in memory T-cell subset populations (B) between male and female owl monkeys and (C) among the 3 different age groups. The results shown are the average of 9 monkeys in each group; $P < 0.05$ was considered statistically significant.

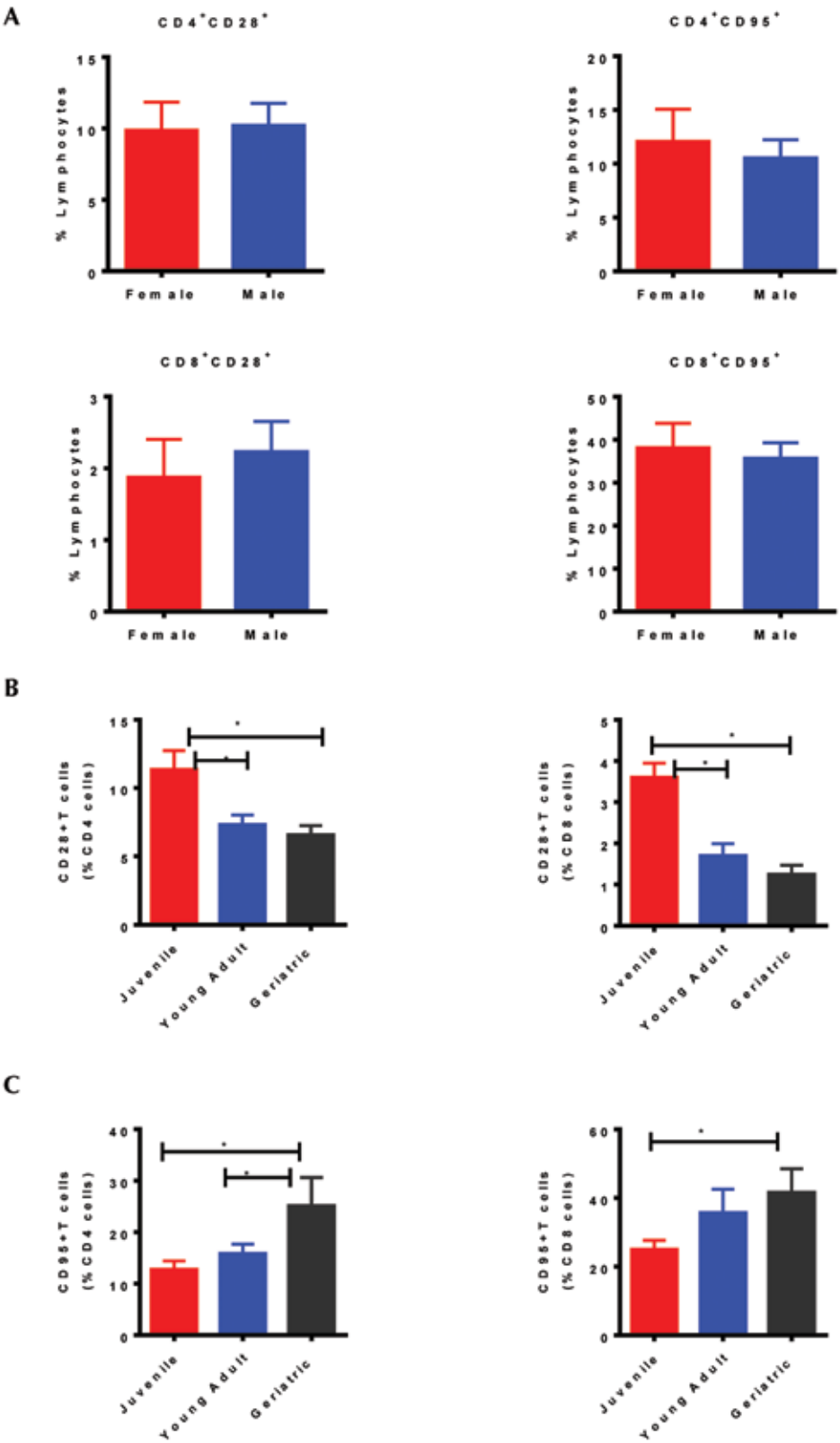

Figure 4. Expression of costimulatory (CD28) and apoptotic (CD95) markers on T-cell subsets in (A) male and female owl monkeys and (B and C) in the 3 different age groups. Percentages of (B) $CD28^+$ and (C) $CD95^+$ populations within peripheral $CD4^+$ (upper panel) and $CD8^+$ (lower panel) T cells were compared among the 3 different age groups. The results are shown as the average of 9 monkeys in each group; $P < 0.05$ was considered statistically significant.

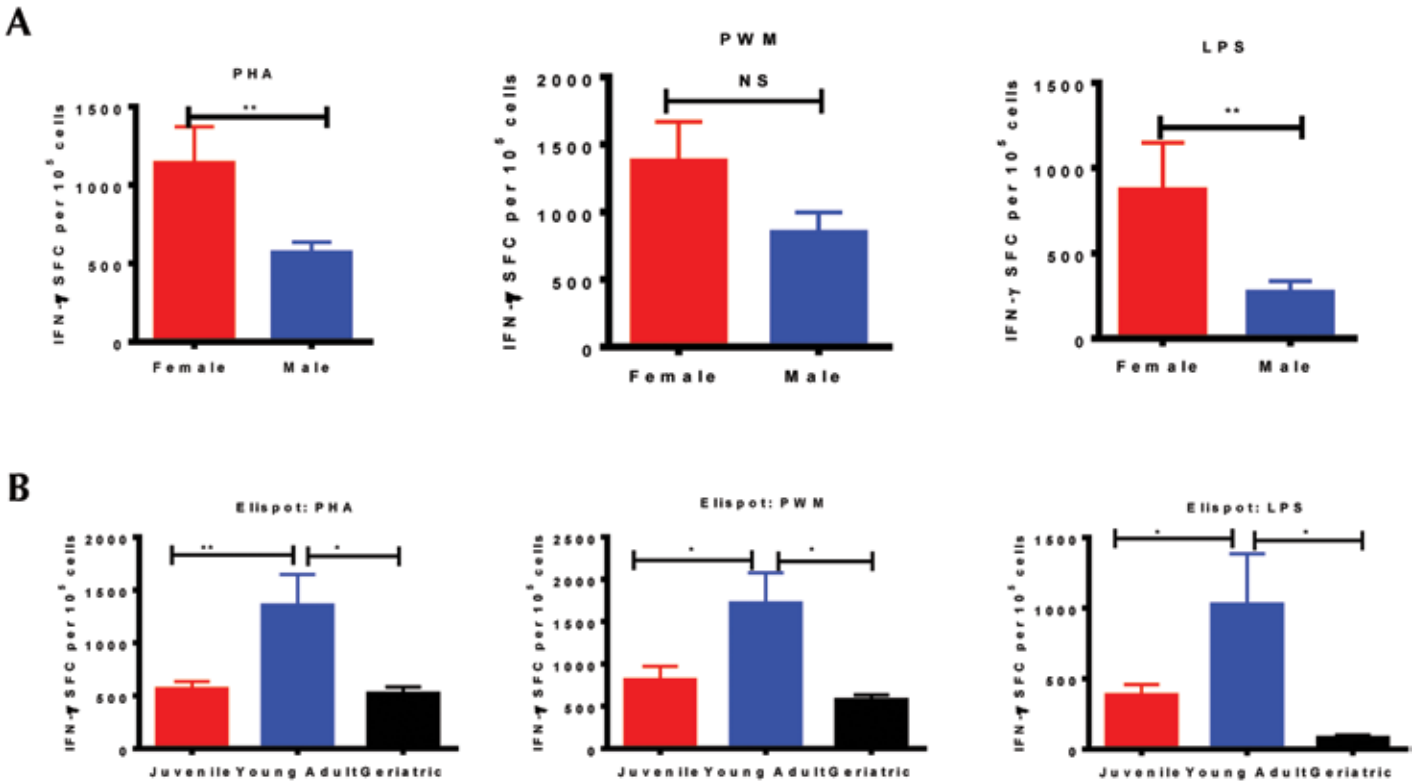

Figure 5. IFNγ ELISPOT response to mitogens. Triplicate wells microtiter plates precoated with antiIFNγ antibody were seeded with PBMC from (A) female (n = 15) and male (n = 15) owl monkeys or (B) the 3 different age groups; the cells were stimulated with each of the indicated mitogens, washed, and then stained with biotinylated secondary antiIFNγ antibody. Data are presented as the number (mean ± 1 SD) of spot-forming cells (SFC) in each of the mitogen-stimulated wells (normalized to the background control); $P < 0.05$ was considered statistically significant.

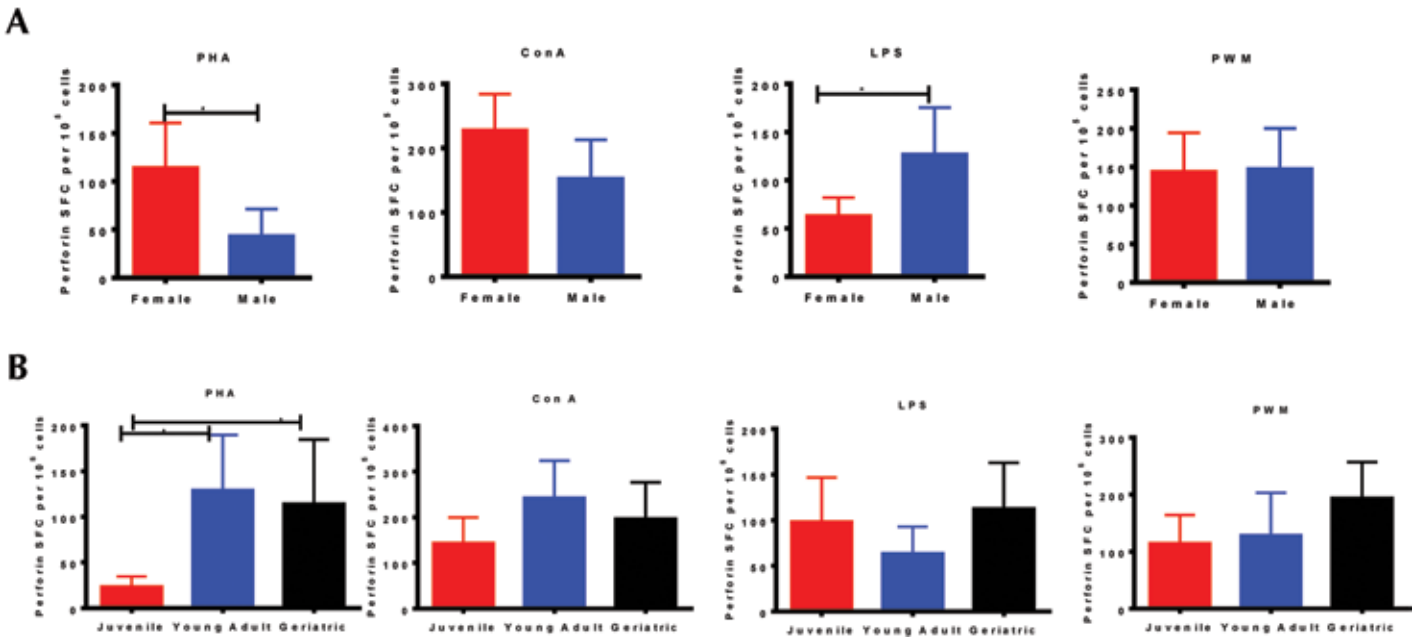

Figure 6. Perforin ELISPOT response to mitogens: Triplicate wells microtiter plates precoated with antiperforin antibody were seeded with PBMC from (A) female (n = 15) and male (n = 15) owl monkeys or (B) the 3 different age groups; the cells were stimulated with each of the indicated mitogens, washed, and then stained with biotinylated secondary antiperforin antibody. Data are presented as the number (mean ± 1 SD) of spot-forming cells (SFC) in each of the mitogen-stimulated wells (normalized to the background control); $P < 0.05$ was considered statistically significant.

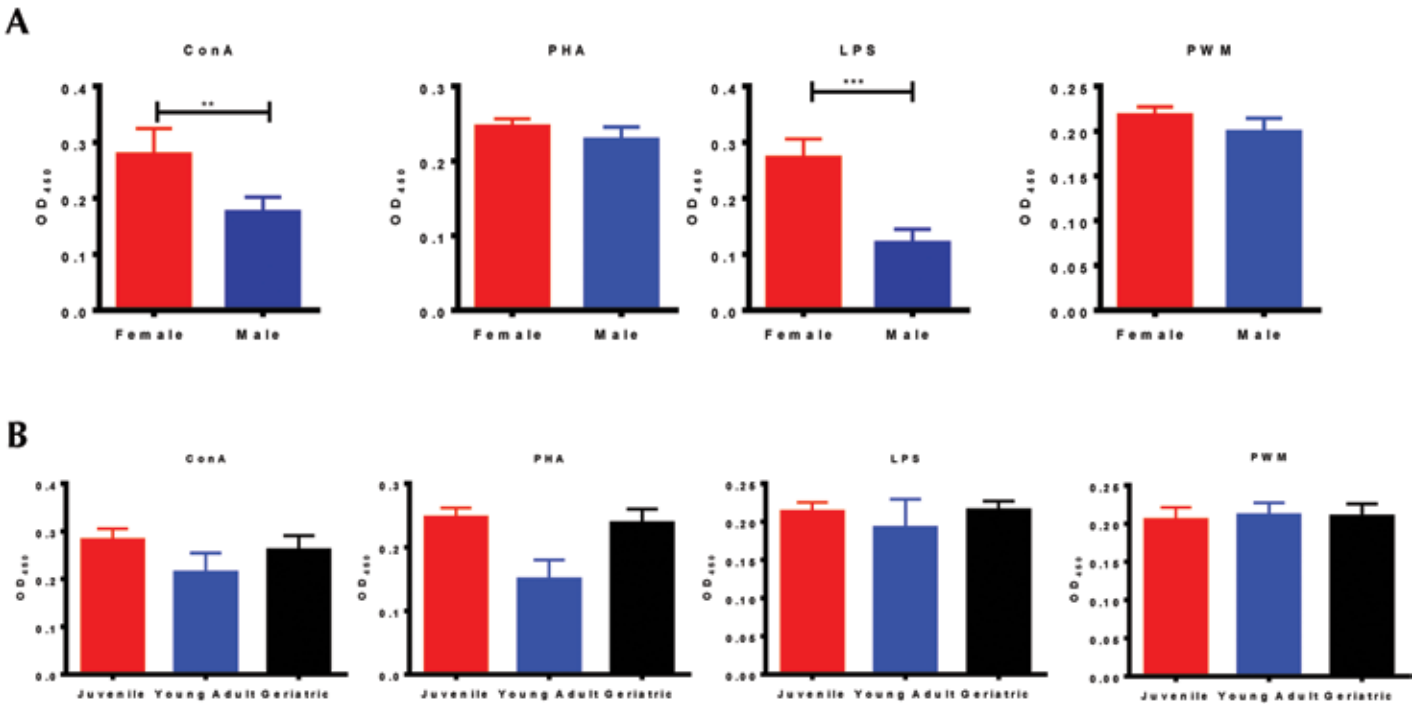

Figure 7. Proliferative response of PBMC to mitogens. PBMC isolated from (A) female (n = 15) and male (n = 15) owl monkeys and (B) the 3 different age groups (n = 10 per group) were used for determining the lymphoproliferative response to the indicated mitogens by using the standard MTT dye reduction assay. The proliferation responses are expressed as optical density (OD; mean ± 1 SD) after subtraction of that for the medium-only control subtraction; $P < 0.05$ was considered statistically significant.

keys; our findings can be applied to in vitro functionality studies involving immunomodulation agents. The age-related decline in antigen-specific cell functions in humans and animals are believed to be associated with increased susceptibility to infections, failure to respond to vaccines, and chronic inflammation.[2,6,36,58] This age-related decline in T-cell function is due to involution of the thymus gland, a decrease in thymic hormone levels and thymic proliferative capacity, impaired expression of IL2, and an increase in incompetent memory lymphocytes.[34]

Our results demonstrated moderate mitogenic activity of Con A in female owl monkeys compared with males but no differences among age groups. These results are similar to those from previous studies in cynomolgus and owl monkeys, which demonstrated moderate proliferative responses to PHA and Con A.[44,54] In the present investigation, we also observed significantly reduced IFNγ- and perforin-producing cell responses to PHA, PWM, and LPS in the juvenile and geriatric owl monkeys, compared with the responses of the young adult animals.

We believe that our study is the first to assess IFNγ and perforin production in response to mitogens in owl monkeys. The homology of the immune system between *Aotus* spp. and humans has led to the supposition that human reagents will cross-react with *Aotus* PBMC.[10,21,41] It is important to consider that the immune response data we present here represent those of stimulated total PBMC; responses specific to T and B lymphocytes as well as $CD4^+$ and $CD8^+$ subsets of T cells will have to be assessed independent of each other to understand the activity of each lymphocyte subtype and which significant changes, if any, are specific to cell type.

The current study used a multiplexed, microsphere-based technology to simultaneously quantitate and compare multiple cytokines in the plasma of owl monkeys. The simultaneous measurement of multiple cytokines in a single biologic sample is critical for determining the appropriate application of these methods in deciphering the role of cytokines in pathology of disease and to evaluate candidate drugs.[47] Squirrel monkey cytokine gene sequences share 91.4% to 98.1% homology with those of humans and other NHP species.[20] We determined the associations between aging and circulating levels of cytokine in the plasma and observed increased levels of Th1 (IL2, IL12, IFNγ, TNFα) and Th2 (IL4, IL6, IL10, IL13) markers in the geriatric population of owl monkeys. Similar results were observed for 71 elderly participants in the Framingham Heart Study (mean age, 79 y) and in 21 young healthy volunteers (mean age, 39 y).[49] Similarly, a study in healthy blood donors[32] demonstrated that aging was associated with an increase in Th2 cells and MCP1. In another study,[43] age and sex were associated with significant alterations in bone metabolism and with estradiol or testosterone deficiency in the production of cytokines. We observed increased MCP1 levels in geriatric owl monkeys; a similar observation was reported from a study of an aging human population, which demonstrated an increased MCP1 concentration and the appearance of atherosclerosis among patients with coronary artery disease or cerebrovascular accidents.[23]

Using ELISPOT assays, we noted significantly more IFNγ- and perforin-producing cells in response to a variety of mitogens in female and young adult monkeys. We also observed increased concentrations of plasma Th1 and Th2 circulating cytokines in a geriatric group of owl monkeys by using multiplex cytokine analysis. In the geriatric monkeys, the data indicate decreased mitogen-mediated IFNγ by ELISPOT assay but increased levels of same cytokine in plasma. These higher levels of cytokines in plasma are likely produced by immune cells in either the lymph

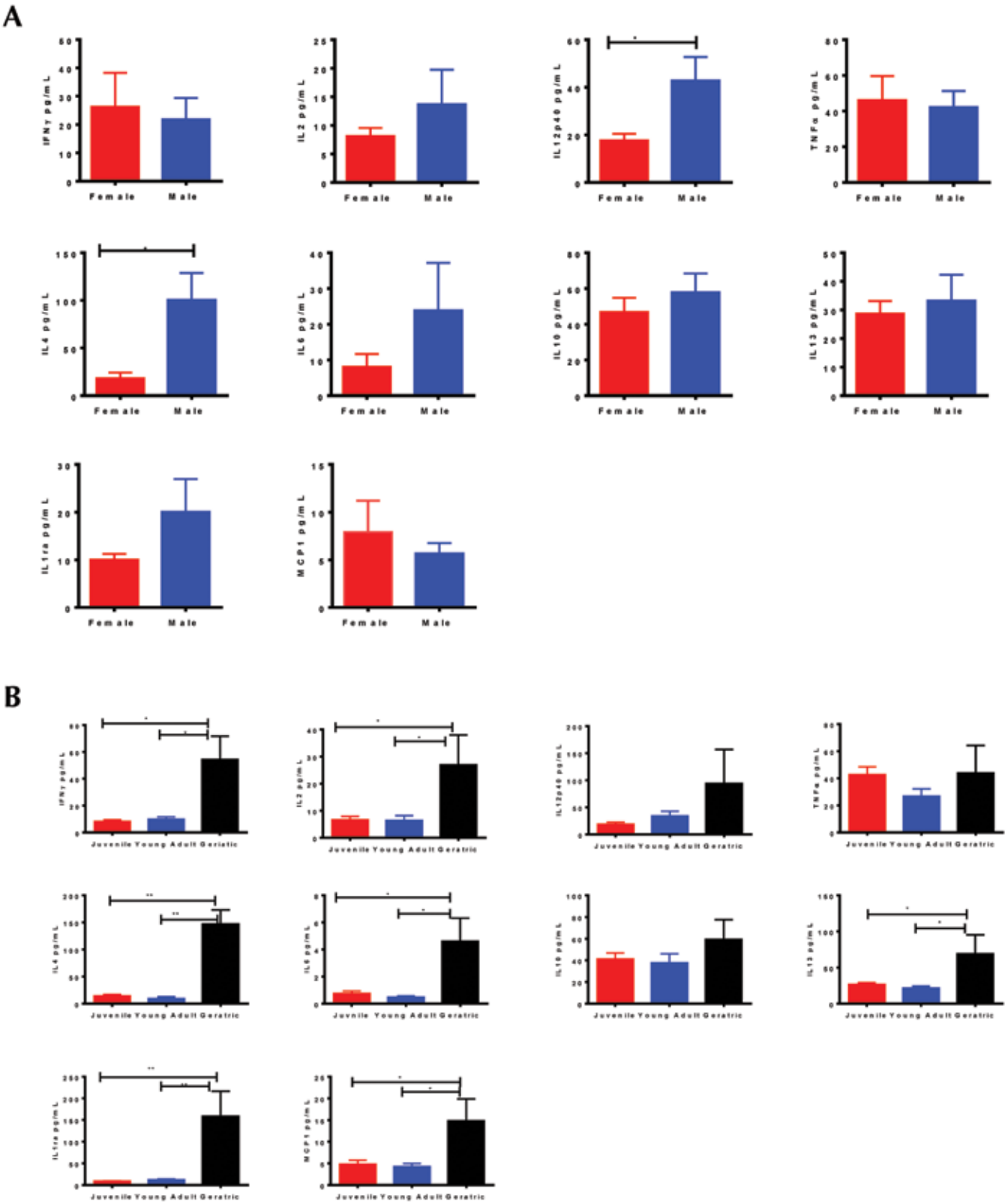

Figure 8. Cytokine bead array analyses of plasma samples. Duplicate samples of plasma from (A) female (n = 15) and male (n = 15) owl monkeys and (B) the 3 different age groups were incubated with cytokine-coupled beads, washed, and then stained with biotinylated detection antibody. The data are reported as concentrations (pg/mL; mean ± 1 SD); $P < 0.05$ was considered statistically significant.

nodes, spleen, or central organs and possibly by macrophages as well. However, the decreased IFNγ production in the geriatric group reflects decreases in $CD4^+$ and $CD8^+$ T-cell function. We believe our study is the first to compare cytokine and chemokine profiles among juvenile, young adult, and geriatric owl monkeys.

Overall, our results suggest that, in general, age-associated phenotypic and functional features of lymphocyte subsets are similar between humans and owl monkeys; furthermore, sex-specific differences exist in the immune function of lymphocytes between young and old animals that might potentially affect experimental outcomes. This concern is especially true for immunologic studies. It is important to recognize that the small size of these monkeys permits the use of decreased doses of potential vaccine candidates and of therapeutic agents during efficacy studies.

In conclusion, our current study provides basic information showing that the owl monkey immune system can be explored by using reagents and assays that have been standardized for human and rhesus immune cells. Such extended studies help to illustrate that owl monkeys are a useful animal model with which to study human diseases. Moreover, any differences specific to *A. nancymaae* relative to other NHP and humans may help researchers adjust the immune parameters during efficacy studies of potential therapeutic agents or vaccine candidates.

Acknowledgments

We thank George Tustin for providing blood samples for this study. This study is partly supported by the Cattleman for Cancer Research (PN) and the Owl Monkey Breeding and Research Resource (CRA) at the Michale E Keeling Center for Comparative Medicine and Research at the MD Anderson Cancer Center.

References

1. **Caldwell CW, Mrema JE, Taylor HM, Green TJ.** 1987. Reference values for peripheral blood lymphocytes from *Aotus lemurinus* ssp. *griseimembra* (owl monkey). J Med Primatol **16:**261–271.
2. **Castle SC.** 2000. Clinical relevance of age-related immune dysfunction. Clin Infect Dis **31:**578–585.
3. **Collins WE**. 1994. The owl monkey as a model for malaria. p 217–244. In: Baer JF, Weller RE, Kakoma I, editors. *Aotus*: the owl monkey. New York (NY): Elsevier.
4. **Czesnikiewicz-Guzik M, Lee WW, Cui D, Hiruma Y, Lamar DL, Yang ZZ, Ouslander JG, Weyand CM, Goronzy JJ.** 2008. T cell subset-specific susceptibility to aging. Clin Immunol **127:**107–118.
5. **De Martinis M, Modesti M, Ginaldi L.** 2004. Phenotypic and functional changes of circulating monocytes and polymorphonuclear leucocytes from elderly persons. Immunol Cell Biol **82:**415–420.
6. **De Paoli P, Battistin S, Santini GF.** 1988. Age-related changes in human lymphocyte subsets: progressive reduction of the CD4 CD45R (suppressor inducer) population. Clin Immunol Immunopathol **48:**290–296.
7. **Dennett NS, Barcia RN, McLeod JD.** 2002. Age associated decline in CD25 and CD28 expression correlate with an increased susceptibility to CD95-mediated apoptosis in T cells. Exp Gerontol **37:**271–283.
8. **Desrosiers RC.** 1997. The value of specific pathogen-free rhesus monkey breeding colonies for AIDS research. AIDS Res Hum Retroviruses **13:**5–6.
9. **Desrosiers RC, Sasseville VG, Czajak SC, Zhang X, Mansfield KG, Kaur A, Johnson RP, Lackner AA, Jung JU.** 1997. A herpesvirus of rhesus monkeys related to the human Kaposi's sarcoma-associated herpesvirus. J Virol **71:**9764–9769.
10. **Diaz D, Naegeli M, Rodriguez R, Nino-Vasquez JJ, Moreno A, Patarroyo ME, Pluschke G, Daubenberger CA.** 2000. Sequence and diversity of MHC DQA and DQB genes of the owl monkey *Aotus nancymaae*. Immunogenetics **51:**528–537.
11. **Fagnoni FF, Vescovini R, Mazzola M, Bologna G, Nigro E, Lavagetto G, Franceschi C, Passeri M, Sansoni P.** 1996. Expansion of cytotoxic $CD8^+$ $CD28^-$ T cells in healthy ageing people, including centenarians. Immunology **88:**501–507.
12. **Frasca D, Riley RL, Blomberg BB.** 2005. Humoral immune response and B-cell functions including immunoglobulin class switch are downregulated in aged mice and humans. Semin Immunol **17:**378–384.
13. **Gabriela D, Carlos P-L, Clara S, Elkin PM.** 2005. Phenotypical and functional characterization of nonhuman primate *Aotus* spp. dendritic cells and their use as a tool for characterizing immune response to protein antigens. Vaccine **23:**3386–3395.
14. **Goronzy JJ Jr, Weyand CM.** 2005. T-cell development and receptor diversity during aging. Curr Opin Immunol **17:**468–475.
15. **Gruver AL, Hudson LL, Sempowski GD.** 2007. Immunosenescence of ageing. J Pathol **211:**144–156.
16. **Gysin J**. 1998. Animal models: primates. p 419–441. In: Sherman IW, editor. Malaria: parasite biology, pathogenesis, and protection. Washington (DC): American Society for Microbiology.
17. **Gysin J, Aikawa M, Tourneur N, Tegoshi T.** 1992. Experimental *Plasmodium falciparum* cerebral malaria in the squirrel monkey *Saimiri sciureus*. Exp Parasitol **75:**390–398.
18. **Haynes L, Eaton SM, Burns EM, Randall TD, Swain SL.** 2005. Newly generated CD4 T cells in aged animals do not exhibit age-related defects in response to antigen. J Exp Med **201:**845–851.
19. **Hearn JP, Dixson AF.** 1984. Assessment of comfort and wellbeing in New World primates.p 206–216. In: **The Universities Federation for Animal Welfare (UFAW)**. Standards in Laboratory Animal Management Potters Bar (United Kingdom): The Universities Federation for Animal Welfare (UFAW).
20. **Heraud JM, Lavergne A, Kazanji M.** 2002. Molecular cloning, characterization, and quantification of squirrel monkey (*Saimiri sciureus*) Th1 and Th2 cytokines. Immunogenetics **54:**20–29.
21. **Hernández EC, Suárez CF, Méndez JA, Echeverry SJ, Murillo LA, Patarroyo ME.** 2002. Identification, cloning, and sequencing of different cytokine genes in 4 species of owl monkey. Immunogenetics **54:**645–653.
22. **Hong MS, Dan JM, Choi JY, Kang I.** 2004. Age-associated changes in the frequency of naive, memory and effector $CD8^+$ T cells. Mech Ageing Dev **125:**615–618.
23. **Inadera H, Egashira K, Takemoto M, Ouchi Y, Matsushima K**. 1999. Increase in circulating levels of monocyte chemoattractant protein 1 with aging. J Interferon Cytokine Res **19:**1179–1182.
24. **Institute for Laboratory Animal Research**. 2011. Guide for the care and use of laboratory animals, 8th ed. Washington (DC): National Academies Press.
25. **Janković V, Messaoudi I, Nikolich-Žugich J.** 2003. Phenotypic and functional T-cell aging in rhesus macaques (*Macaca mulatta*): differential behavior of CD4 and CD8 subsets. Blood **102:**3244–3251.
26. **Jiang J, Gross D, Elbaum P, Murasko D.** 2007. Aging affects initiation and continuation of T-cell proliferation. Mech Ageing Dev **128:**332–339.
27. **Kang I, Hong MS, Nolasco H, Park SH, Dan JM, Choi JY, Craft J.** 2004. Age-associated change in the frequency of memory $CD4^+$ T cells impairs long-term $CD4^+$ T-cell responses to influenza vaccine. J Immunol **173:**673–681.
28. **Kohler S, Wagner U, Pierer M, Kimmig S, Oppmann B, Möwes B, Jülke K, Romagnani C, Thiel A.** 2005. Post-thymic in vivo proliferation of naive $CD4^+$ T cells constrains the TCR repertoire in healthy human adults. Eur J Immunol **35:**1987–1994.
29. **Kovacs EJ, Palmer JL, Fortin CF, Fülöp T Jr, Goldstein DR, Linton PJ.** 2009. Aging and innate immunity in the mouse: impact of intrinsic and extrinsic factors. Trends Immunol **30:**319–324.
30. **Kovaiou RD, Weiskirchner I, Keller M, Pfister G, Cioca DP, Grubeck-Loebenstein B.** 2005. Age-related differences in phenotype

and function of $CD4^+$ T cells are due to a phenotypic shift from naive to memory effector $CD4^+$ T cells. Int Immunol **17:**1359–1366.
31. **Linton PJ, Dorshkind K.** 2004. Age-related changes in lymphocyte development and function. Nat Immunol **5:**133–139.
32. **Mansfield AS, Nevala WK, Dronca RS, Leontovich AA, Shuster L, Markovic SN.** 2012. Normal ageing is associated with an increase in Th2 cells, MCP1 (CCL1), and RANTES (CCL5), with differences in sCD40L and PDGF-AA between sexes. Clin Exp Immunol **170:**186–193.
33. **Mercier GT, Nehete PN, Passeri MF, Nehete BN, Weaver EA, Templeton NS, Schluns K, Buchl SS, Sastry KJ, Barry MA.** 2007. Oral immunization of rhesus macaques with adenoviral HIV vaccines using enteric-coated capsules. Vaccine **25:**8687–8701.
34. **Miller RA.** 1995. Cellular and biochemical changes in the aging mouse immune system. Nutr Rev **53:**S8–S17.
35. **Mosmann T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods **65:**55–63.
36. **Murasko DM, Weiner P, Kaye D.** 1987. Decline in mitogen-induced proliferation of lymphocytes with increasing age. Clin Exp Immunol **70:**440–448.
37. **Naylor K, Li G, Vallejo AN, Lee WW, Koetz K, Bryl E, Witkowski J, Fulbright J, Weyand CM, Goronzy JJ.** 2005. The influence of age on T-cell generation and TCR diversity. J Immunol **174:**7446–7452.
38. **Nehete PN, Gambhira R, Nehete BP, Sastry KJ.** 2003. Dendritic cells enhance detection of antigen-specific cellular immune responses by lymphocytes from rhesus macaques immunized with an HIV envelope peptide cocktail vaccine. J Med Primatol **32:**67–73.
39. **Nehete PN, Hanley PW, Nehete BP, Yang G, Ruiz JC, Williams L, Abee CR, Sastry KJ.** 2013. Phenotypic and functional characterization of lymphocytes from different age groups of Bolivian squirrel monkeys (*Saimiri boliviensis boliviensis*). PLoS One **8:**e79836.
40. **Nehete PN, Nehete BP, Manuri P, Hill L, Palmer JL, Sastry KJ.** 2005. Protection by dendritic cells-based HIV synthetic peptide cocktail vaccine: preclinical studies in the SHIV–rhesus model. Vaccine **23:**2154–2159.
41. **Nino-Vasquez JJ, Vogel D, Rodriguez R, Moreno A, Patarroyo ME, Pluschke G, Daubenberger CA.** 2000. Sequence and diversity of DRB genes of *Aotus nancymaae*, a primate model for human malaria parasites. Immunogenetics **51:**219–230.
42. **Panda A, Arjona A, Sapey E, Bai F, Fikrig E, Montgomery RR, Lord JM, Shaw AC.** 2009. Human innate immunosenescence: causes and consequences for immunity in old age. Trends Immunol **30:**325–333.
43. **Pietschmann P, Gollob E, Brosch S, Hahn P, Kudlacek S, Willheim M, Woloszczuk W, Peterlik M, Tragl KH.** 2003. The effect of age and gender on cytokine production by human peripheral blood mononuclear cells and markers of bone metabolism. Exp Gerontol **38:**1119–1127.
44. **Pinzón-Charry A, Vernot JP, Rodríguez R, Patarroyo ME.** 2003. Proliferative response of peripheral blood lymphocytes to mitogens in the owl monkey *Aotus nancymae*. J Med Primatol **32:**31–38.
45. **Pitcher CJ, Quittner C, Peterson DM, Connors M, Koup RA, Maino VC, Picker LJ.** 1999. HIV1-specific $CD4^+$ T cells are detectable in most individuals with active HIV1 infection but decline with prolonged viral suppression. Nat Med **5:**518–525.
46. **Potestio M, Pawelec G, Di Lorenzo G, Candore G, D'Anna C, Gervasi F, Lio D, Tranchida G, Caruso C, Romano GC.** 1999. Age-related changes in the expression of CD95 (APO1/FAS) on blood lymphocytes. J Exp Gerontol **34:**659–673.
47. **Prabhakar U, Eirikis E, Miller BE, Davis HM.** 2005. Multiplexed cytokine sandwich immunoassays: clinical applications. Methods Mol Med **114:**223–232.
48. **Roehm NW, Rodgers GH, Hatfield SM, Glasebrook AL.** 1991. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. J Immunol Methods **142:**257–265.
49. **Roubenoff R, Harris TB, Abad LW, Wilson PWF, Dallal GE, Dinarello CA.** 1998. Monocyte cytokine production in an elderly population: effect of age and inflammation. J Gerontol A Biol Sci Med Sci **53:**M20–M26.
50. **Schapiro SJ, Nehete PN, Perlman JE, Sastry KJ.** 2000. A comparison of cell-mediated immune responses in rhesus macaques housed singly, in pairs, or in groups. Appl Anim Behav Sci **68:**67–84.
51. **Schmidt LH, Esslinger JH.** 1981. Courses of infections with *Plasmodium falciparum* in owl monkeys displaying a microfilaremia. Am J Trop Med Hyg **30:**5–11.
52. **Sestak K, McNeal MM, Choi A, Cole MJ, Ramesh G, Alvarez X, Aye PP, Bohm RP, Mohamadzadeh M, Ward RL.** 2004. Defining T-cell–mediated immune responses in rotavirus-infected juvenile rhesus macaques. J Virol **78:**10258–10264.
53. **Shaw AC, Joshi S, Greenwood H, Panda A, Lord JM.** 2010. Aging of the innate immune system. Curr Opin Immunol **22:**507–513.
54. **Tatsumi M, Fujiwara T, Hayami M.** 1982. Blastogenic response of peripheral blood lymphocytes to mitogens in cynomolgus monkeys (*Macaca fascicularis*). Am J Primatol **3:**111–119.
55. **Tollerud DJ, Clark JW, Brown LM, Neuland CY, Pankiw-Trost LK, Blattner WA, Hoover RN.** 1989. The influence of age, race, and gender on peripheral blood mononuclear-cell subsets in healthy nonsmokers. J Clin Immunol **9:**214–222.
56. **Wallace DL, Zhang Y, Ghattas H, Worth A, Irvine A, Bennett AR, Griffin GE, Beverley PCL, Tough DF, Macallan DC.** 2004. Direct measurement of T-cell subset kinetics in vivo in elderly men and women. J Immunol **173:**1787–1794.
57. **Weichert H, Blechschmidt I, Schröder S, Ambrosius H.** 1991. The MTT assay as a rapid test for cell proliferation and cell killing: application to human peripheral blood lymphocytes (PBL). Allerg Immunol (Leipz) **37:**139–144.
58. **Yan J, Greer J, Hull R, O'Sullivan J, Henderson R, Read S, McCombe P.** 2010. The effect of ageing on human lymphocyte subsets: comparison of males and females. Immun Ageing **7:**4.