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Original Research

Effects of Chronic Viral Infection on Lymphocyte Populations in Middle-aged Baboons (*Papio anubis*)

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Aging of the immune system is characterized by the loss of naïve T-cells, increased inflammation, and immune function impairment. Chronic infection with cytomegalovirus is thought to play a role in age-related changes in immunity. Therefore, to assess the effect of pathogens such as cytomegalovirus on the immune system, we determined lymphocyte populations and inflammatory markers over a 3-y period in captive, middle-age baboons, with various exposure to pathogens and shedding pressure. Groups included SPF (i.e., pathogen-negative; *n* = 14); large-group, conventionally housed (CONV LG; pathogen-positive; *n* = 14), and small-group, conventionally housed (CONV SM; pathogen-positive; *n* = 7). All baboon groups showed a decrease in CD45RA+ CD28+ (i.e., naive) cells over time during middle age, but the rate of decline appeared faster in CONV LG baboons than in the other groups. In addition, the reduction in CD45RA+ CD28+ cells in the CONV LG baboons coincided with higher IgG levels against baboon cytomegalovirus, increased serum cortisol concentration, and a greater inflammatory phenotype. The results of this project support a role for cytomegalovirus infection in immune system alterations in middle-aged baboons.

Abbreviations: BaCMV, baboon cytomegalovirus; SAA, serum amyloid A

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In 2010, an estimated 8% of the world's population was 65 y or older, and by 2050 the world's aged population is estimated to reach 16%.[29] As people live longer, age-related diseases and conditions place an increased burden on public health and may place tremendous hardship on individual families. Aging is the number one risk factor for numerous diseases, including Alzheimer disease, cardiovascular disease, cancer, and diabetes.[30] Practices that improve wellbeing and allow for extended periods of productivity in the aged population will help lower the costs of long-term care for individual families and to society as a whole.[29] Further complicating matters, many older adults have multiple, concurrent age-related diseases, making specific disease-targeted treatment approaches less efficient. Treatments targeting the deficiencies underlying aging diseases would therefore be preferable. The increased susceptibility to disease in the elderly is due, in part, to a progressive weakening of the immune system, a process known as immunosenescence. Understanding how age affects immunity may offer more broad-reaching therapeutic targets to reduce the cost of long-term care while it helps to improve health and wellbeing among aged populations.

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Aging is associated with an altered immune system that includes loss of naïve T lymphocytes, accumulation of T lymphocytes negative for the costimulatory receptor CD28, altered cytokine expression, and increased inflammatory markers.[7,16,28,39] Evidence indicates that immunosenescence reduces overall health and contributes to a rise of disease in the elderly.[7,8,10] Infection with chronic pathogens has been implicated in the aging of immune function.[32,39] Antigenic stress from chronic viruses, particularly cytomegalovirus, is thought to contribute to age-related changes in the immune system.[1] Cytomegalovirus is a β-herpesvirus that rarely causes symptoms in healthy individuals despite establishing a lifelong infection in its host.[6] Starting in middle age and accumulating over a lifetime, the constant immune response to cytomegalovirus viral antigens is thought to significantly reduce the overall efficiency of the immune system.[13]

Chronic infection with pathogens like cytomegalovirus has been suggested to contribute to immunosenescence by causing decreases in naïve cells and increases in late-differentiated memory lymphocytes.[1,6,32] Naive lymphocytes are an essential defense against infection by new pathogens. Some direct evidence in other species suggests that decreases in the naive T-cell pool could limit the ability of the elderly to respond to a novel immune stimulus appropriately.[36] Naive lymphocytes are activated through a process that involves the costimulatory receptor, CD28.[24] High numbers of lymphocytes negative for CD28 are linked to a reduced response to influenza vaccines and increased inflammatory diseases in the elderly.[35,38,41] Although correlations between chronic viruses and aging of the immune system have been identified in several species, critical questions

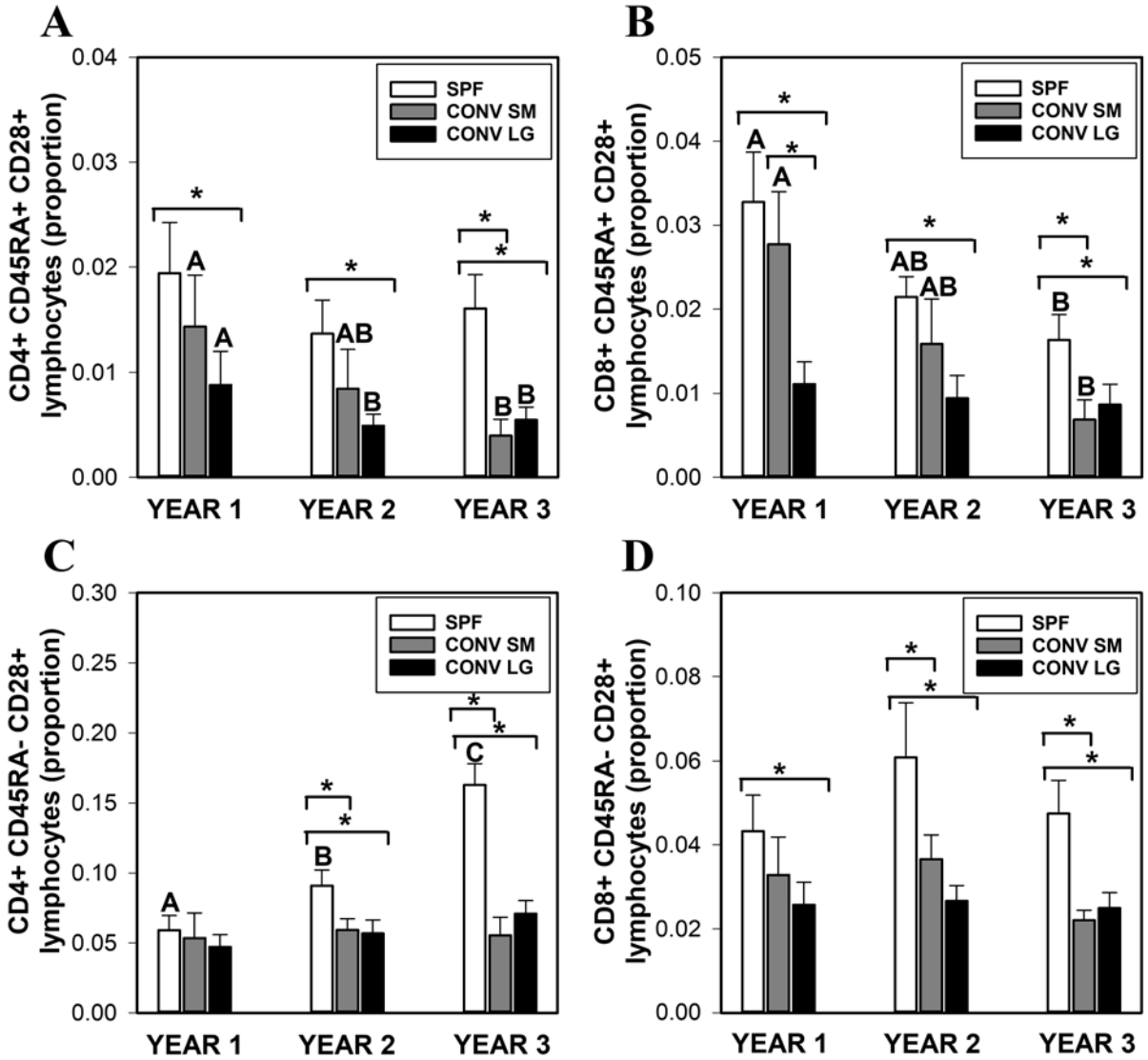

Figure 1. Proportions of CD45RA+ CD28+ and CD45RA– CD28+ lymphocytes (CD3+) in age-matched SPF and conventionally reared, small-group (CONV SM) and large-group (CONV LG) baboons; $n = 14$ matched SPF and CONV LG animals; $n = 7$ CONV SM animals. (A) CD3+ CD4+ CD45RA+ CD28+ lymphocytes. (B) CD3+ CD8+ CD45RA+ CD28+ lymphocytes. (C) CD3+ CD4+ CD45RA– CD28+ lymphocytes. (D) CD3+ CD8+ CD45RA– CD28+ lymphocytes. The mean age of animals was 9.5 ± 0.2, 10.5 ± 0.3, and 11.5 ± 0.3 y in study years 1, 2, and 3, respectively. *, Difference ($P < 0.05$) between groups; uppercase letters denote longitudinal differences ($P < 0.05$) within the same group.

regarding the direct effects of chronic viruses on the immune system changes remain unanswered.

To better determine the role of chronic viruses in age-related changes in the immune system, appropriate animal models are necessary. The genetics and physiology of NHP are similar to those in humans and, therefore, these species have distinct advantages over other animal models. In addition, NHP carry viruses that are closely related to viruses naturally infecting humans. However, little research examining the general effects of age on immunocompetence in NHP is available. Previous studies by our laboratory found that olive baboons (*Papio anubis*) show age-associated alterations in the immune system.[26,46] In conventionally raised baboons 6 to 26 y old, we discovered that T cells aged in a manner like what has been described in people.[46] In addition, aged baboons were found to exhibit a proinflammatory state.[26] Although the cited study[26] supports the idea that aging causes impairment of cell-mediated immunity and a proinflammatory phenotype, the cause of these changes was not addressed. Therefore, in the current study, we sought to test the hypothesis that baboons with the most exposure to chronic pathogens, particularly baboon cytomegalovirus (BaCMV), will show the most profound changes in the immune system, whereas animals lacking exposure to chronic pathogens will have better preservation of immune function over time.

Materials and Methods

Animals and sample collection. Given that interventions to prevent age-related changes to immune function likely will be most efficacious before advanced dysfunction or cell loss has occurred, this study focused on changes in middle-aged rather than geriatric animals. Animals were 8 to 13 y old at study initiation and were followed for 3 y (corresponding to a human age of approximately 24 to 39 y). The mean age of the 3 groups was 9.5 ± 0.2, 10.5 ± 0.3, and 11.5 ± 0.3 y in study years 1, 2, and 3, respectively. Both conventionally reared and SPF baboons were

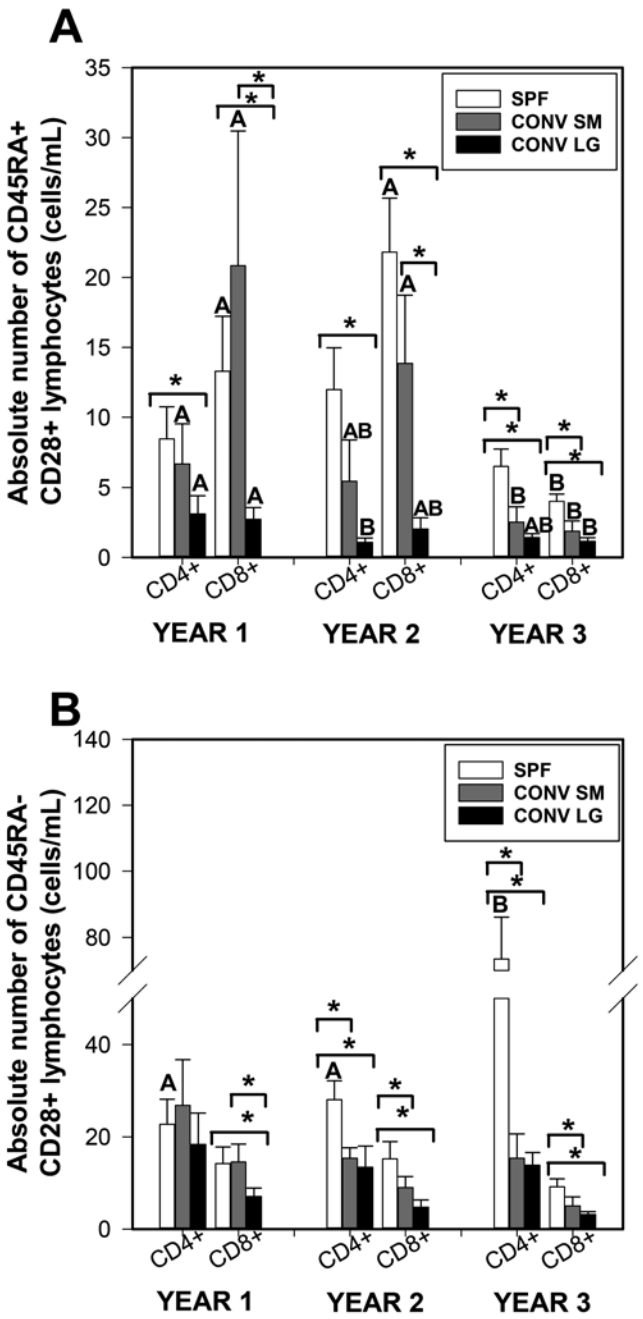

Figure 2. Absolute numbers of (A) CD45RA+ CD28+ and (B) CD45RA– CD28+ lymphocytes (CD3+) in age-matched SPF, CONV SM, and CONV LG baboons; n = 14 matched SPF and CONV LG group animals, n = 7 CONV SM animals. The mean age of animals was 9.5 ± 0.2, 10.5 ± 0.3, and 11.5 ± 0.3 y in study years 1, 2, and 3, respectively. *, Difference ($P < 0.05$) between groups; uppercase letters denote longitudinal differences ($P < 0.05$) within the same group.

used in this study. The SPF baboon colony is free of many of the common viruses that naturally infection baboons, including BaCMV, *Herpesvirus papio 1* (homologous to Epstein–Barr virus in humans), *Herpesvirus papio 2* (homologous to human herpes simplex virus), simian varicella virus, simian foamy virus, simian virus 40, as well as 6 other pathogens (see reference 48 for a full list of targeted pathogens). SPF baboons were raised in large multimale troops (approximately 60 total adults) in an indoor facility. Because the oldest baboons in the SPF colony were used for this study, they were all originally hand-reared as the colony founders. Conventionally raised baboons were raised in large multimale troops (60 to 80 adults total) housed in outdoor corrals with attached indoor housing (CONV LG group) or in small groups (2 or 3 adults total) in an indoor facility (CONV SM group). Both SPF and CONV LG baboons were breeding colonies with mature adults, infants, and juveniles. Each breeding group within a colony contained 4 to 8 mature adults consisting of approximately 1 male per 3 to 7 females. The CONV SM animals were not housed in the CONV LG facilities for reasons including behavioral and health concerns. In addition, 25% of the conventional baboons were nursery-reared for various reasons. All baboons were housed at the National Baboon Research Resource, Department of Comparative Medicine, University of Oklahoma Health Sciences Center and were cared for by 3 clinical veterinarians. All animal procedures were approved by the University of Oklahoma's IACUC. The research was performed in accordance with the guidelines of the Animal Welfare Act and Animal Welfare Regulations[2,3] and with the *Guide for the Care and Use of Laboratory Animals*.[21]

To assess the effect of viral load on the immune system over time, SPF and CONV large-group baboons were matched for age and social status (n = 14 per group). Although our laboratory's previous study did not find differences between males and females in this baboon population, socially high-ranking baboons were found to exhibit a greater age-related decrease in CD3+ lymphocytes positive for CD45RA when compared with subordinate animals.[46] Thus, social status was included in the study design. In addition, 7 CONV SM baboons of comparable age were included. Sample size was chosen based on a power analysis and sample size calculation, and only baboons with consistent housing and husbandry conditions throughout the course of the study were selected to participate in the study. SPF and CONV large-group baboons housed in large hierarchical troops were exposed to similar social stressors and other potentially confounding factors with the foremost exception of pathogen exposure. Similar to CONV LG baboons, CONV SM baboons were exposed to usual pathogens but were housed in different environmental conditions (such as lower animal density, less social pressures, controlled environmental temperature, light exposure, and humidity). The smaller groups contained a similar ratio of originally subordinate to higher social–status animals as in the other 2 groups. All animal housing was equipped with enrichment items for climbing, exercise, and play. Baboons were fed a diet of commercial monkey chow (Purina, St Louis, MO), fresh fruits, and vegetables, with water provided ad libitum.

Serial blood samples were collected from animals over a 3-y period during routine colony health checks. Three years of aging in baboons is approximately equal to 9 to 10 y of aging in people. Venous blood was collected in the morning under anesthesia (ketamine, 10 mg/kg IM) and, when possible, buccal samples for salivary viral DNA analysis were obtained by using a sterile dental swab. Blood was collected into vacuum phlebotomy tubes containing EDTA as an anticoagulant (for samples used in flow cytometry) or clotted prior to centrifugation at 500 × g for 15 min (for serum collection). Samples used for flow cytometry were prepared on the day of collection. We retained a portion of WBC from each sample for later DNA analysis. Serum and WBC samples were transported to the laboratory on ice and frozen at –80 °C until analysis. Pregnant animals were not anesthetized, and no animals were euthanized for the study.

Assessment of lymphocyte populations by flow cytometry. A CBC and blood differential analyses were performed via an

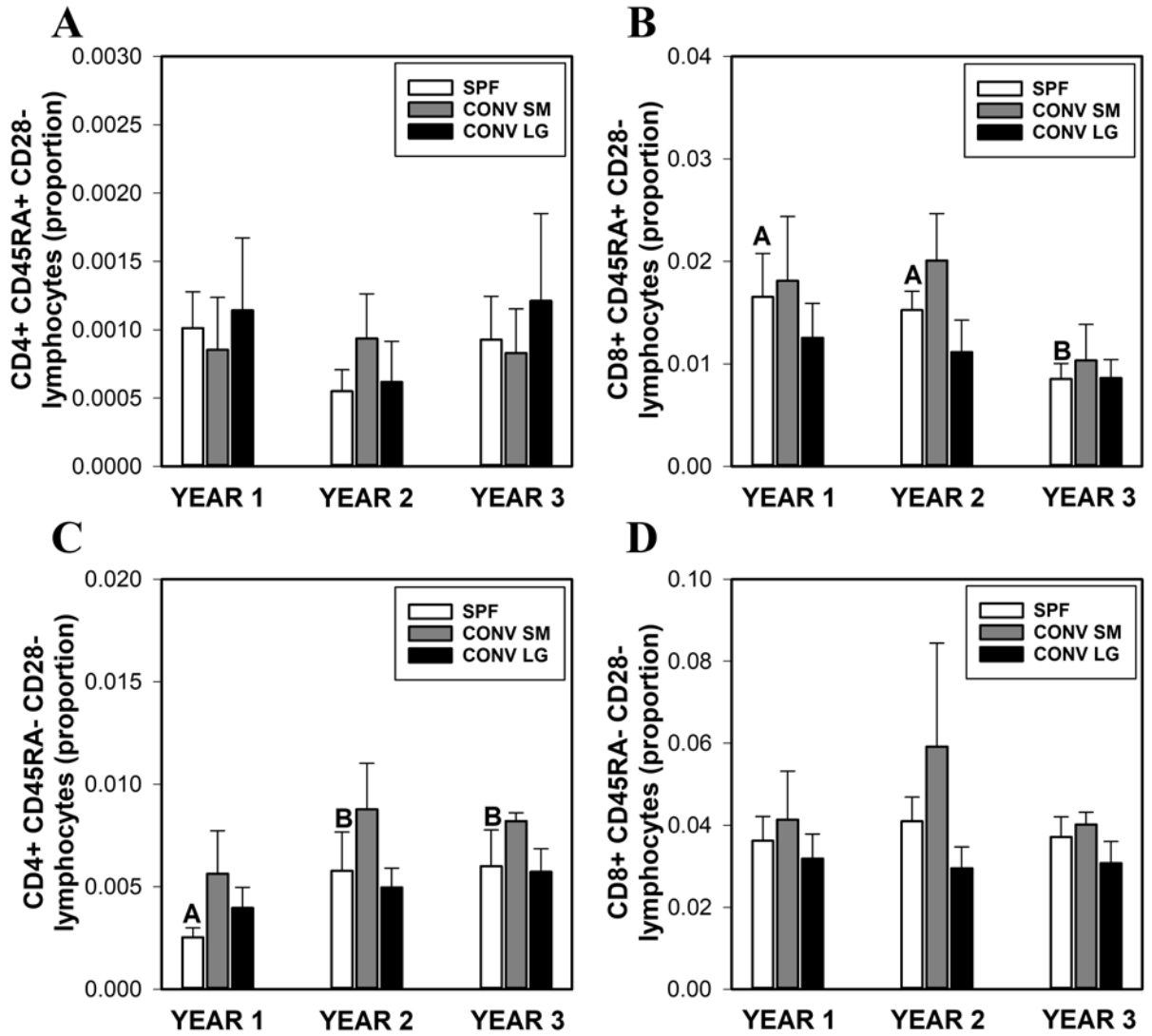

Figure 3. Proportions of CD45RA+ CD28– and CD45RA– CD28- lymphocytes (CD3+) in age-matched SPF, CONV SM, and CONV LG baboons; $n = 14$ matched SPF and CONV LG group animals, $n = 7$ CONV SM animals. (A) CD3+ CD4+ CD45RA+ CD28– lymphocytes. (B) CD3+ CD8+ CD45RA+ CD28– lymphocytes. (C) CD3+ CD4+ CD45RA– CD28– lymphocytes. (D) CD3+ CD8+ CD45RA– CD28– lymphocytes. The mean age of animals was 9.5 ± 0.2, 10.5 ± 0.3, and 11.5 ± 0.3 y in study years 1, 2, and 3, respectively. *, Difference ($P < 0.05$) between groups; uppercase letters denote longitudinal differences ($P < 0.05$) within the same group.

automated hematology analyzer (Vet ABC, Scil Animal Care, Gurnee, IL) and Wright–Geimsa-stained blood smears were used to determine total cell numbers. Relative lymphocyte subset percentages were assessed by flow cytometry, and absolute numbers then were calculated for each sample as described previously.[46] Briefly, fresh whole blood (100 µL) was stained with the appropriate antibodies for 20 min in the dark at room temperature. Cells were labeled with antiCD3–APC (clone SP34-2, Becton Dickinson, San Jose, CA), CD4–FITC (clone L200, Becton Dickinson), CD8–PE-Cy7 (clone RPA-T8, Becton Dickinson), CD28-PE (clone CD28.2, Becton Dickinson), and CD45RA–APC (clone MEM56, Invitrogen, Life Technologies, Carlsbad, CA) individually or in combination. Samples were treated with erythrocyte lysis buffer, incubated for 10 min in the dark at room temperature, and centrifuged at 600 × *g* for 5 min. Pelleted cells were collected and washed twice by using PBS containing 0.2% BSA, fixed with cold BD Cytofix (Becton Dickinson), and resuspended in 500 µL cold PBS containing 0.2% BSA and 0.1% NaN_3. Samples were stored on ice or at 4 °C until analysis on a FACSCalibur flow cytometer (Becton Dickinson). Unstained cells, isotype controls, and single-stained controls were used to define the position of negative cells and to set spectral compensation, and lymphocyte subsets were then determined by using CD3 gating. The same experimental conditions, flow cytometer, and instrument settings, controls, and compensation matrix were used to minimize variation between different analyses. Data are expressed as proportions of positive or negative lymphocytes. Samples were analyzed for lymphocyte populations of CD3+ (total), CD4+ (helper), CD8+ (cytotoxic), and CD3+ CD4+/CD8+ CD45RA+ CD28+ (as an estimate of naive cells), CD45RA– CD28+ (as an estimate of central or effector 1 memory cells), CD45RA+ CD28– (as an estimate of effector or TEMRA cells), and CD45RA– CD28– (as an estimate of late memory - cells) as described elsewhere.[1,23] To assess quality control and possible assay changes over time, the proportion of CD45RA+ CD28+ cells was examined in SPF and CONV LG animals of the same age, collected at different years. Although variation between individual animals occurred, when enough samples were available for statistical analysis, no differences were observed

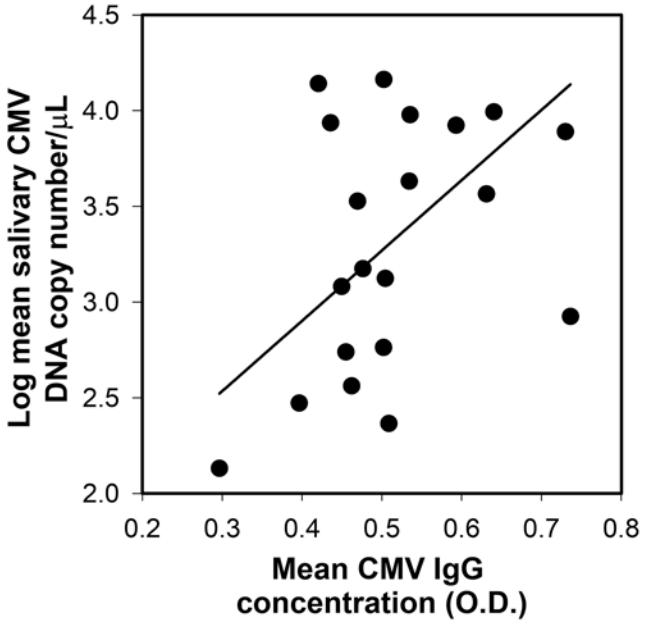

Figure 4. Correlation of salivary BaCMV DNA copy number with antiBaCMV IgG levels in conventionally reared (pathogen-positive) baboon groups; $n = 20$, $r = 0.57$, $P = 0.01$.

in animals of the same age collected at different years (data not shown).

DNA extraction and BaCMV detection. DNA was extracted from buccal swab samples by using DNAzol (Invitrogen, Carlsbad, CA), whereas DNA was extracted from WBC by using QIAamp DNA Blood Mini Kits (Qiagen, Hilden, Germany) according to the manufacturers' directions. DNA concentration was assessed by using a Qubit fluorometer and the Qubit dsDNA BR assay kit (Life Technologies, Carlsbad, CA). BaCMV copy number in saliva was determined through TaqMan real-time PCR by using a standard curve method. In addition, BaCMV DNA in WBC was assessed by using a nested PCR method; both techniques have been described previously and validated by our laboratory.[47]

Stimulation of cytokines in PBMC. At the completion of the study (year 3), immune function was estimated through stimulation of PBMC. PBMCs were freshly prepared from whole blood of matched SPF and CONV LG baboons ($n = 6$ animals per group) by using sodium heparin CPT vacuum phlebotomy tubes (Becton Dickinson Biosciences, San Jose, CA) according to the manufacturer's directions. Cells were plated at a density of 1×10^6 cells/mL in RPMI supplemented with 10% FBS, 2 mM glutamine, and 100 U/mL penicillin–streptomycin. Cells were incubated at 37 °C, 5% CO_2 overnight, and then treated with 20 ng PMA and 1 µm ionomycin (Sigma, St Louis, MO), 1 µg/mL of LPS (055:B5; Sigma), or an equal volume of cell media (no stimulation) for 4 h. Media was collected and frozen at −80 °C for later use in cytokine analyses.

ELISA for serum amyloid A (SAA), ceruloplasmin, cortisol, IL6, IL10, IFNγ, BaCMV, and other chronic viruses. Inflammatory state was assessed by measuring the concentrations of proinflammatory biomarkers SAA, ceruloplasmin, and IL6 as well as the antiinflammatory biomarkers IL10 and serum cortisol. IL6 and IL10 were measured by using an NHP-specific commercial ELISA (U-Cytech, Utrecht, Netherlands) as previously reported.[26,27,46] The acute-phase protein SAA was determined by using a multispecies Phase SAA ELISA kit (Tridelta Development, Maynooth, Ireland), and cortisol was measured by a human-specific ELISA (Neogen, Lexington, KY) validated for use in baboon sera.[46] Standard SAA concentrations for the multispecies kit have not been published for baboons and, therefore, SAA results are expressed as OD_{450}. The concentration of ceruloplasmin in baboon sera was determined by using a human-specific ceruloplasmin ELISA kit (Molecular Innovations, Novi, MI). Assay validations for ceruloplasmin for use in baboon serum were determined by parallelism between diluted pooled baboon samples to a standard curve, percent recovery and linearity of serial diluted baboon samples spiked with known concentrations. Serum antibody levels against common baboon viruses including BaCMV, *Herpesvirus papio 1*, *Herpesvirus papio 1*, simian varicella virus, simian foamy virus, and simian virus 40 (previously shown to have a prevalence greater than 70% in adult baboons at the facility[48]) were measured by using ELISA, as previously described.[33,46,48] IL6, IL10, and IFNγ response to stimulation of PBMC was assessed in cell media by using NHP-specific commercial ELISA (U-Cytech), described earlier. ELISA were completed on all samples at the end of the study. For each assay, the interassay coefficients of variance were below 15%, and the intraassay coefficients of variance were below 10%.

Statistical analyses. Data were tested for normality and homogeneity of variance prior to statistical analyses and were log-transformed when needed. When no suitable transformation was found, data were analyzed by nonparametric methods. Paired *t*-tests were used to determine differences between matched pairs, whereas *t*-tests were used to determine differences between the means of 2 groups. For nonparametric data, a Wilcoxon signed-rank test was used for matched pairs, whereas a Mann–Whitney *U* test was used to compare 2 independent groups. Multiple group comparisons were assessed by using ANOVA, followed by a Tukey HSD (parametric) or Steel–Dwass (nonparametric) test. Longitudinal data were compared by using repeated-measures ANOVA, with Tukey HSD testing for multiple comparisons. Correlations of longitudinal data were determined by repeated-measures correlation with a weighted correlation coefficient, as described by others.[5] Statistical significance was defined as a P value of less than 0.05. JMP 7 Statistical Discovery (SAS, Cary, NC) was used for all statistical analyses.

Results

Changes in lymphocyte populations among baboon groups. Over the 3-y collection period, all middle-aged baboon groups showed a decrease in CD3+ CD45RA+ CD28+ (naive) cells without regard to viral or housing status. Although SPF baboons showed no longitudinal changes in the proportion of CD3+ CD4+ CD45RA+ CD28+ cells, CONV SM and CONV LG baboons showed decreases of 68% and 44%, respectively, over time ($P \leq 0.05$; Figure 1 A). In addition, between-group comparisons revealed 66% fewer ($P \leq 0.05$) CD3+ CD4+ CD45RA+ CD28+ cells in CONV LG than in SPF baboons (Figure 1 A). Furthermore, in year 1, CONV SM animals exhibited a level of CD4+ naive cells that was intermediate between the 2 other groups. By the third year, when the animals had reached a mean age of 11.5 y, the proportion of CD4+ CD45RA+ CD28+ cells in CONV SM was comparable to that of CONV LG ($P > 0.05$; Figure 1 A).

Both SPF and CONV SM baboons exhibited a decrease (range, 50% to 75%) in the proportion of CD3+ CD8+ CD45RA+ CD28+ cells over time ($P \leq 0.05$; Figure 1 B). Although CONV LG baboons did not show longitudinal changes in this cell type, intergroup comparisons revealed that the proportion of CD8+ naive cells in CONV LG baboons was lower than that of SPF animals

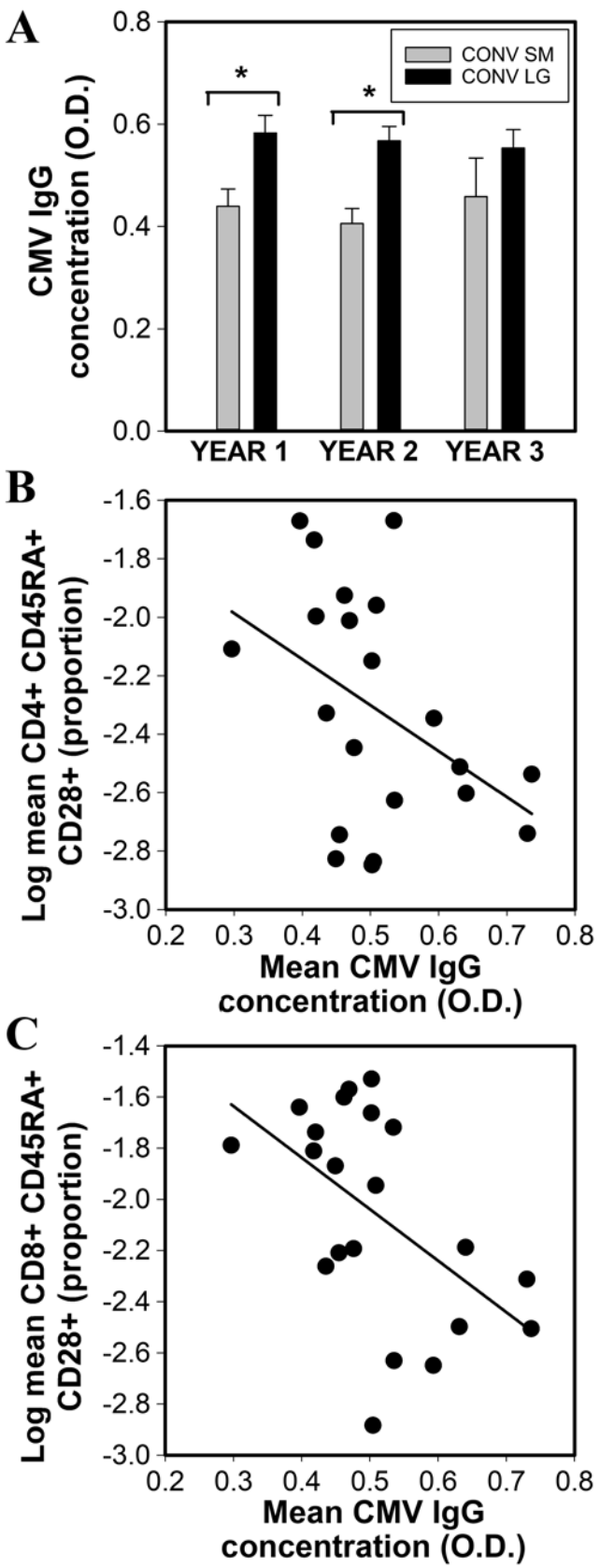

Figure 5. (A) AntiBaCMV IgG concentration in conventionally reared (pathogen-positive) baboon groups; n = 14 matched SPF and CONV LG animals, and n = 7 CONV SM animals. *, Difference ($P < 0.05$) between groups. (B) The relationship of CD3+ CD4+ CD45RA+ CD28+ lymphocytes to BaCMV IgG level in conventionally reared (pathogen-positive) baboon groups; n = 21, r = –0.4, P = 0.05. (C) Correlation of CD3+ CD8+ CD45RA+ CD28+ lymphocytes with antiBaCMV IgG concentration in pathogen-positive, conventionally reared baboons; n = 21, r = –0.5, P = 0.01.

(ranging from 66% to 47%) throughout the collection period. In contrast, the proportion of CD3+ CD8+ CD45RA+ CD28+ lymphocytes in CONV SM baboons initially was comparable to that of the SPF baboons. However, by year 3, this population of lymphocytes had dropped to less than 53% of that of the SPF baboons ($P \leq 0.05$; Figure 1 B). Similar patterns were evident in the absolute number of helper and cytotoxic CD45RA+ CD28+ lymphocytes (Figure 2 A).

The proportion of CD3+ CD4+ CD45RA– CD28+ memory cells increased ($P \leq 0.05$) in SPF animals during middle age but did not increase in either CONV baboon group (Figure 1 C). Likewise, the proportion of CD3+ CD8+ CD45RA– CD28+ cells was as much as 54% greater in the SPF group compared with CONV baboons ($P \leq 0.05$; Figure 1 D). The absolute numbers of helper and cytotoxic CD45RA– CD28+ memory lymphocytes showed a similar pattern (Figure 2 B). Conversely, no differences were found between groups for CD45RA+ CD28– (effector or TEMRA) or for CD45RA– CD28– (late memory) cell populations (Figure 3 A through D). Although some changes in these cell types were observed over time in SPF baboons (CD8+ CD45RA+ CD28– decreased; CD4+ CD45RA– CD28- increased), no changes were found in the CONV groups during middle age (Figure 3 A through D).

BaCMV DNA copy number and viral antibodies in serum. To determine whether antibody concentration paralleled viral shedding in saliva, BaCMV antibody concentration was compared with salivary and WBC BaCMV copy number in CONV baboons. Levels of BaCMV IgG in serum were positively correlated with BaCMV DNA copy numbers in saliva (Figure 4; n = 20, r = 0.57, P = 0.01). Although cytomegalovirus is reported to be maintained in monocytes in humans,[37] only one baboon sample tested positive for BaCMV DNA in WBC. This sample was from a dominant male baboon in CONV LG, and subsequent WBC samples from the same animal did not test positive for BaCMV DNA. To examine potential exposure differences between CONV SM and CONV LG baboons, antibody levels for BaCMV and other viruses were compared between groups. In years 1 and 2, antiBaCMV IgG levels in CONV SM baboons were approximately 26% lower ($P \leq 0.05$) than in CONV LG baboons (Figure 5 A). In contrast, in year 3, no difference was detected in BaCMV antibody levels between the 2 CONV baboon groups. In addition, CONV SM baboons exhibited lower IgG levels to simian varicella virus and *Herpesvirus papio 2* throughout the 3-y study period, compared with CONV LG animals ($P \leq 0.05$; Figure 6). No differences between the 2 CONV groups were observed in IgG levels for other viruses (Figure 6).

The relationship between the proportion of lymphocytes and viral antibody levels was also assessed using repeated measures correlation. BaCMV IgG levels were negatively correlated with CD3+ CD4+ CD45RA+ CD28+ cells (Figure 5 B; n = 21, r = –0.4, P = 0.05). In addition, high levels of serum IgG against BaCMV IgG (Figure 5 C; n = 21, r = –0.5, P = 0.01) and simian varicella virus (Figure 7; n = 21, r = –0.5, P = 0.05) were negatively correlated with reduced relative amounts of CD3+ CD8+ CD45RA+ CD28+ cells. Serum concentrations of other viral antibodies were not correlated with the proportion of CD45RA+ CD28+ lymphocytes, and no correlations were found among viral antibodies and the other lymphocyte populations examined.

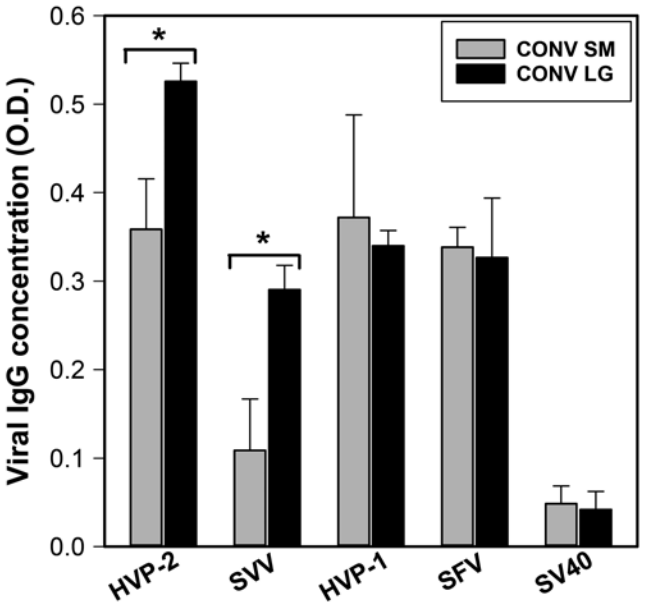

Figure 6. Antiviral IgG concentration against *Herpesvirus papio 2* (HVP2), simian varicella virus (SVV), *Herpesvirus papio 1* (HVP1), simian foamy virus (SFV), and simian virus 40 (SV40) from a representative year in pathogen-positive, conventionally reared baboon groups; $n = 14$ matched SPF and CONV LG animals; $n = 7$ CONV SM animals; *, $P < 0.05$. No longitudinal changes were observed within the same group, and differences between groups were consistent during the 3-year collection period.

Serum concentrations of cortisol, SAA, ceruloplasmin, IL6, and IL10. No measurable changes were observed over time in serum cortisol or inflammation markers within each baboon group. However, marked differences were observed when groups were compared (Figure 8). Serum cortisol concentration was 40% lower in CONV SM baboons compared with SPF and CONV LG baboons ($P \leq 0.05$; Figure 8 A). Although baboons reared in small groups initially had lower ($P \leq 0.05$) SAA and ceruloplasmin concentrations, this difference was no longer evident by year 3 (Figure 8 B and C).

In addition, CONV small-group baboons had serum IL6 concentration 75% below ($P \leq 0.05$) that of the other 2 groups throughout the collection period (data from a representative year are shown in Figure 8 D). No difference was found among groups in the serum IL10 concentration (results not shown) and, consequently, the ratio of IL6:IL10 was lower ($P \leq 0.05$) in CONV SM baboons (Figure 8 D).

Cytokine response to stimulation in PBMC. At the end of the study, stimulation of PBMC was used to estimate immune function in a subset of paired SPF and CONV, pathogen-positive baboons. T-cell function was assessed through IFNγ release after stimulation with PMA–ionomycin, whereas release of IL6 and IL10 was assessed after stimulation with LPS as a positive control. SPF baboons exhibited a 223% greater ($P \leq 0.05$) release of IFNγ in response to PMA–ionomycin, when compared with the CONV baboon group (Figure 9 A). The IL10 response was not different between groups (Figure 9 B), but the IL6 response to LPS was 124% greater ($P \leq 0.05$) in SPF baboons as compared with CONV baboons (Figure 9 C).

Discussion

Cytomegalovirus is thought to accelerate age-related changes in the immune system. However, determining a causal relationship between chronic cytomegalovirus infection and rate

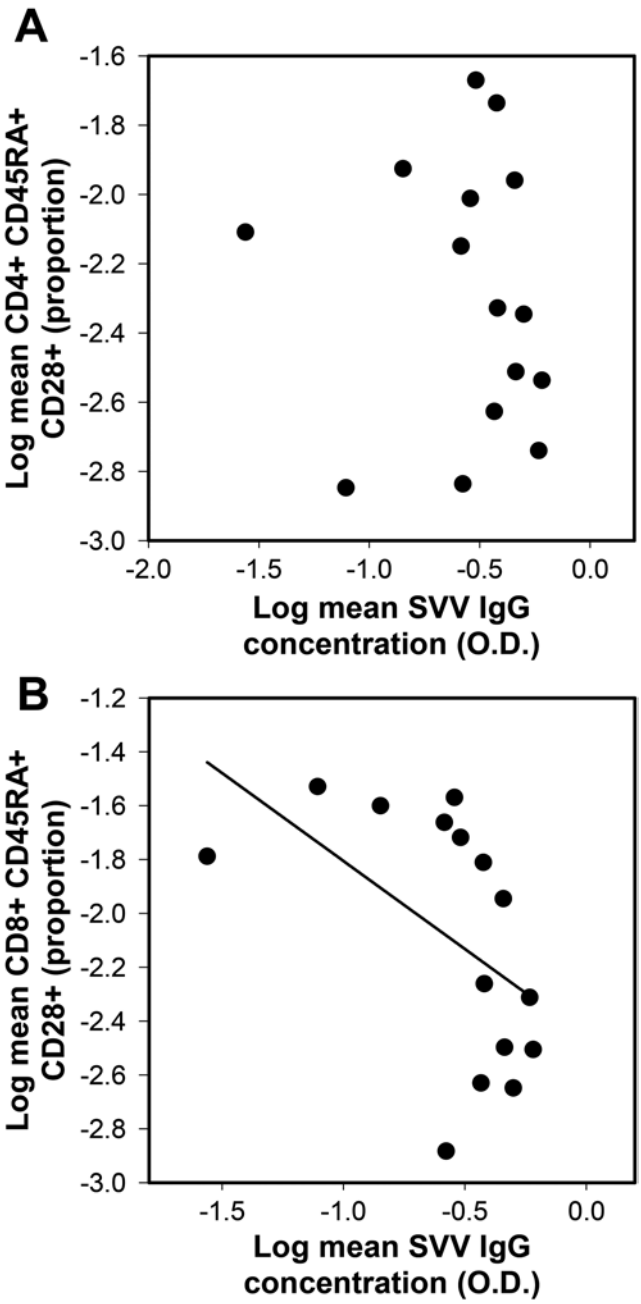

Figure 7. Relationship of (A) CD3+ CD4+ CD45RA+ CD28+ and (B) CD3+ CD8+ CD45RA+ CD28+ lymphocytes to antiSVV IgG levels in conventionally reared (pathogen-positive) baboons; $n = 21$. CD8+ CD45RA+ CD28+ lymphocytes were negatively correlated ($r = –0.5$, $P = 0.05$) with antiSVV IgG levels.

of change in immunity during aging in people is difficult. In contrast to humans, animals provide a greater supply of naive subjects and more controlled study parameters. Baboons are a highly relevant model for studying the complex process of immunosenescence; however, almost all adult wild and conventionally raised captive baboons are infected with BaCMV. Most conventionally reared baboons test positive for BaCMV by 3 y of age, and infection profiles for other viruses are similar to those for the homologous human viruses.[33,48] SPF baboon colonies provide a valuable resource of pathogen-free controls to examine how chronic viruses can influence the immune system. Our investigation focused on the period when aging-associated changes have begun but the development of age-associated

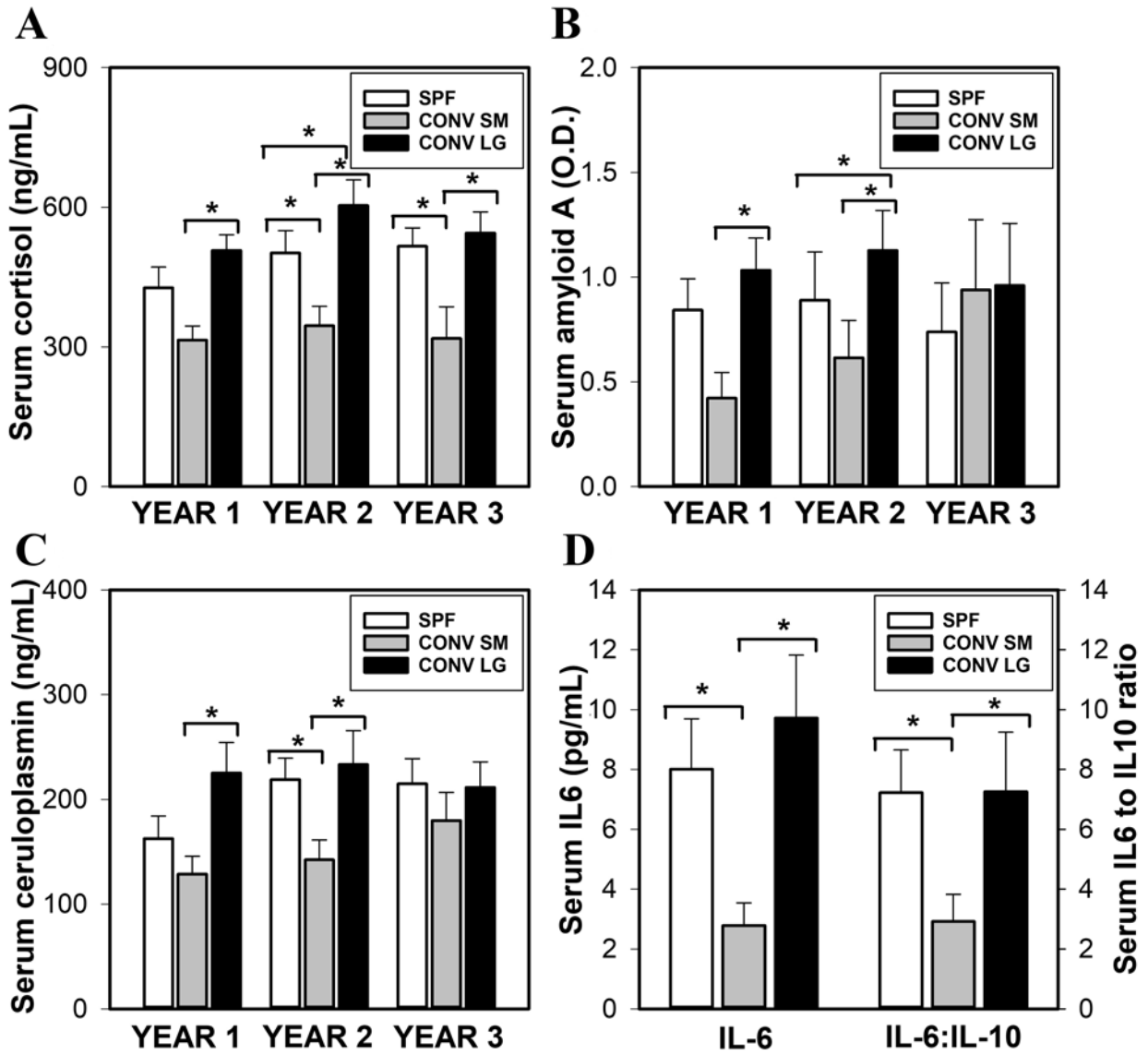

Figure 8. Serum concentration of cortisol, markers of inflammation, and cytokines in age-matched SPF, CONV SM, and CONV LG baboons. (A) Serum concentration of cortisol (ng/mL) in age-matched baboon groups. (B) Serum concentration of serum amyloid A (OD) in age-matched baboon groups. (C) Serum concentration of ceruloplasmin (ng/mL) in age-matched baboon groups. (D) Serum concentration of IL6 and the IL6:IL10 ratio in age-matched baboon groups during a representative year. Differences between groups were consistent during the 3-y collection period; $n = 14$ matched SPF and CONV LG animals, and $n = 7$ CONV SM animals. The mean age of baboons was 9.5 ± 0.2, 10.5 ± 0.3, and 11.5 ± 0.3 y in years 1, 2, and 3, respectively. *, Difference ($P < 0.05$) between groups; data are given as mean ± SEM.

disease has not, thus providing an ideal target for interventions to promoting health-span in people. Although SPF baboons are negative for numerous viruses, including but not limited to BaCMV, cytomegalovirus is the most common chronic virus linked to age-associated changes in the immune system.[39] Consequently, the goal of the current study was to compare the rate of change in immune aging biomarkers over a 3-y period (equivalent to about 10 y in people) in middle-aged baboons with or without chronic BaCMV infection.

The current study revealed that BaCMV DNA is rarely detected in the blood of healthy baboons. Monocytes are a key cell type for active and latent cytomegalovirus infection.[37,44] However only one blood sample from a CONV (pathogen-positive) baboon had measurable levels of BaCMV DNA. Likewise, in healthy humans, cytomegalovirus DNA during latent infection is generally detected only at a very low level in blood.[17,34] In contrast to blood measurements, CONV baboons consistently shed BaCMV in the oral cavity, and salivary BaCMV DNA copy numbers were positively correlated with corresponding antiBaCMV IgG levels in serum. Although no changes in antibodies were observed within groups over the 3-y collection period in the current study, we did observe lower antibody levels for certain pathogens (BaCMV, simian varicella virus, and *Herpesvirus papio 2*) in the baboons housed in small groups compared with the large-group baboons throughout the study. This finding supports the concept that low population density reduces pathogen exposure (that is, social distancing), most likely due to fewer opportunities for direct interaction with house mates shedding BaCMV and other viruses.

The current study also indicated that populations of CD45RA+ CD28+ T cells (an estimate of naive cells) decrease in middle-aged baboons with greater environmental pressures and exposure to chronic viruses. CONV LG baboons had a substantial reduction in their CD45RA+ CD28+ cell populations

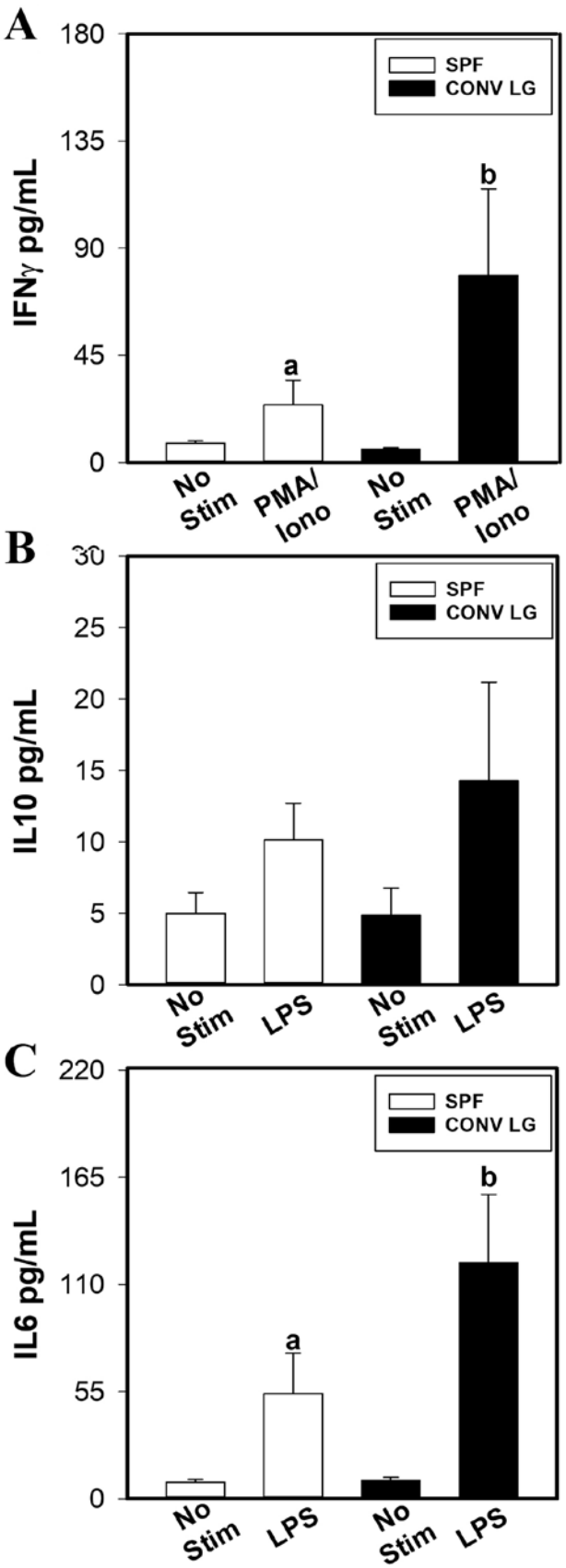

Figure 9. Cytokine release (IL6, IL10, and IFNγ) after PMA–ionomycin or LPS stimulation of PBMC from middle-aged SPF and conventionally housed (pathogen-positive) baboons. (A) IFNγ concentration after no stimulation (No Stim) or stimulation with PMA–ionomycin. (B) IL10 concentration after no stimulation or stimulation with LPS. (C) IL6 concentration after no stimulation or stimulation with LPS. The mean age of baboons was 11.6 y; $n = 6$ paired SPF and CONV LG animals. *, Difference ($P < 0.05$) between groups; data are given as mean ± SEM.

starting in year 1, when the group's mean age was only 10 y, and this reduction was consistent over time. Although the CD3+ CD8+ CD45RA+ CD28+ cell population in SPF baboons also decreased over the 3-y collection period, SPF animals maintained higher levels than did CONV baboons. Similar to cytotoxic cells, fewer CD3+ CD4+ CD45RA+ CD28+ cells also were present in CONV LG baboons in year 1, suggesting that these animals were already undergoing changes in their naive T-helper cell population as well. CONV SM baboons initially showed an intermediate rate of decline of CD45RA+ CD28+ T cells, between those of the SPF and CONV large-group baboons. However, by the time the CONV SM animals had reached an average of 11.5 y of age (year 3), both CD45RA+ CD28+ cell populations had fallen to the level in CONV LG baboons, whereas the SPF group maintained larger populations of both of these cell types.

In mice, available evidence suggests that murine cytomegalovirus infection directly reduces populations of naive lymphocytes.[9] One group found that infecting SPF mice with murine cytomegalovirus caused an irreversible decrease in CD8+ naïve cells.[9] Furthermore, multiple studies in people indicate that cytomegalovirus infection is associated with reduced levels of naive lymphocytes.[1,11,12,15,25,45] Studies in people also have indicated that cytomegalovirus seropositivity is correlated with a reduced CD8+ naive cell population in middle-aged adults and even in people as young as approximately 20 y.[1,40] In the present study, antiBaCMV IgG levels in the CONV baboons were negatively correlated with CD45RA+ CD28+ cells, providing further support that cytomegalovirus exposure can influence naive cell populations. Furthermore, an association between the concentration of antibodies to simian varicella virus and decline of CD3+ CD8+ CD45RA+ CD28+ T cells was observed, although the correlation was not as strong as that with BaCMV.

The current study also revealed that levels of CD45RA- CD28+ were elevated in middle-aged SPF baboons. This finding is consistent with studies in people, where aged individuals negative for cytomegalovirus had a greater proportion of memory lymphocytes expressing CD28 but not CD45RA than did seropositive cytomegalovirus persons.[1,12,45] Furthermore, cytomegalovirus is associated with late-differentiated lymphocytes with an inflammatory profile in people.[31] However, no significant differences in late-differentiated CD45RA+ CD28– or CD45RA– CD28– cells were observed between our pathogen-free and pathogen-positive groups during middle age. When total CD4+ and CD8+ lymphocytes negative for CD28 were examined in a population of baboons, results indicated that a progressive accumulation occurs with age, similar to what has been found in people.[46] Thus, greater changes in these cell types are likely to be present in geriatric baboons.

Concentrations of immunomodulatory hormones and both pro- and antiinflammatory cytokines are likely to be altered in elderly people as compared with younger adults.[22] The progressive chronic low-grade inflammation observed in the aged immune system has been correlated with an increased morbidity and mortality, and multiple studies have shown that higher concentrations of proinflammatory markers, such as IL6, increase mortality risk in the elderly.[14,18,19] Furthermore, oxidative and inflammatory stress as well as stress-related glucocorticoids can affect the rate of change in the immune system over time.[4,20,42,43] Previous studies by our laboratory showed that baboons exhibit

age-related changes in markers of inflammation and stress similar to humans.[26,46] In the present study, results likewise revealed differences in stress and inflammatory markers among middle-aged baboons. However, most differences occurred between baboons housed in small versus large groups, rather than between the pathogen-positive and pathogen-free baboons.

Collectively, these data indicate that all baboons, independent of pathogen status and environmental conditions, show reductions in their CD45RA+ CD28+ lymphocyte populations during middle-age (particularly in CD3+ CD8+ CD45RA+ CD28+ cells). The results also suggest these lymphocyte changes occur more quickly in baboons exposed to chronic pathogens. When conventionally reared baboons are housed in smaller groups, they show a delay in the onset of decline in CD45RA+ CD28+ cell populations as compared with CONV LG baboons. These small-group baboons have lower inflammation and stress markers, suggesting that housing and environment play a role in the rate of immune changes found in middle-aged baboons. In contrast, the examination of markers of inflammation and stress revealed few differences between the 2 baboon groups housed in a similar manner: the SPF and CONV LG baboons. Nevertheless, the naive CD45RA+ CD28+ and memory CD45RA– CD28+ T-cell populations as well as PBMC response to stimulation were more robust in SPF baboons, indicating that during middle age, baboons free of chronic pathogens are able to maintain higher levels of CD45RA± CD28+ lymphocytes and a more responsive immune system despite experiencing similar states of inflammation and stress as the large-group, conventionally housed counterparts. However, whether this difference is a direct consequence of their pathogen-free status could not be determined within the present study.

In conclusion, the results presented here provide further validation of baboons as a model of aging and provide evidence that chronic infection with cytomegalovirus and potentially other chronic pathogens can alter the rate of change in the immune system during middle age. SPF baboons that were housed with similar environmental stressors as large-group, pathogen-positive animals were able to maintain higher populations of naïve and memory lymphocytes and showed a greater response to immune stimuli, despite similar increases in inflammatory and stress markers. We also observed that CONV baboons housed in small groups with less environmental stress exhibited a reduced inflammatory phenotype and a delayed reduction in their CD45RA+ CD28+ T-cell populations as compared with CONV LG animals. Although multiple contributing factors likely influence the rate of age-related changes in the immune system, cytomegalovirus and potentially other chronic infections may accelerate these changes or may work synergistically with other factors to increase the rate of decline in the immune system, leading to an enhanced immune risk profile and a potential greater susceptibility to age-related diseases during old age. Future studies using additional markers to further define lymphocyte populations and functional changes between conventional and SPF baboons as they age would be beneficial. Ultimately, the infection of SPF baboons with a single chronic virus, such as cytomegalovirus, would provide further insight into the specific role of chronic viral exposure in immune aging.

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