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Original Research

Comparative Analysis of Cellular Immune Responses in Conventional and SPF Olive Baboons (*Papio anubis*)

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Olive baboons (*P. anubis*) have provided a useful model of human diseases and conditions, including cardiac, respiratory, and infectious diseases; diabetes; and involving genetics, immunology, aging, and xenotransplantation. The development of a immunologically defined SPF baboons has advanced research further, especially for studies involving the immune system and immunosuppression. In this study, we compare normal immunologic changes of PBMC subsets, and their function in age-matched conventional and SPF baboons. Our results revealed that both groups have comparable numbers of different lymphocyte subsets, but phenotypic differences in central and effector memory T-cell subsets are more pronounced in $CD4^+$ T cells. Despite equal proportions of $CD3^+$ T cells among the conventional and SPF baboons, PBMC from the conventional group showed greater proliferative responses to phytohemagglutinin and pokeweed mitogen and higher numbers of IFNγ-producing cells after stimulation with concanavalin A or pokeweed mitogen, whereas plasma levels of the inflammatory cytokine TNFα were significantly higher in SPF baboons. Exposure of PBMC from conventional baboons to various Toll-like (TLR) ligands, including TLR3, TLR4, and TLR8, yielded increased numbers of IFNγ producing cells, whereas PBMC from SPF baboons stimulated with TLR5 or TLR6 ligand had more IFNγ-producing cells. These findings suggest that although lymphocyte subsets share many phenotypic and functional similarities in conventional and SPF baboons, specific differences in the immune function of lymphocytes could differentially influence the quality and quantity of their innate and adaptive immune responses. These differences should be considered in interpreting experimental outcomes, specifically in studies measuring immunologic endpoints.

Abbreviations: Con A, concanavalin A; PHA, phytohemagglutinin; PWM, pokeweed mitogen

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Baboons are a valuable model for the study of human diseases. Compared with other NHP, baboons are large in size, thus allowing researchers to study organ transplantation, medical devices, and collect ample fluid and tissue samples. In addition, baboons share approximately 96% genetic homology with humans, making baboons a more relevant animal model than typical laboratory rodents.[14] Research with baboons has focused on cardiac disease (coronary heart disease, hypertension, and atherosclerosis), respiratory diseases (*Bordetella pertussis* and respiratory syncytial virus), xenotransplantation, reproductive and neonatal physiology, diabetes, genetics, infectious disease, human Ebola virus infection, immunology, and vaccine development.[5,6,15,23,25,26,30,38,42] Baboons also have contributed to the development of adjuvant research to enhance the immunogenicity of vaccines against *Haemophilus* influenza type B and *Neisseria meningitidis* group C polysaccharides.[8,36] Baboons are an excellent model for vaccine development, because their immune system shares similarities with the human immune system given that baboons have the same IgG subclasses (that is, 1 through 4) as humans.[34]

Most baboons are maintained under conventional conditions (that is, nonSPF). Under conventional conditions, baboons harbor a number of adventitious virus infections that typically do not cause disease in immunocompetent animals and are often considered part of the animal's normal flora including a complete complement of herpesviruses and retroviruses. Baboons in SPF colonies have been bred to eliminate many of these agents. The bioexclusion list for SPF animals can vary by institution. The SPF baboons examined in the current study are negative for 21 agents, including multiple viruses (all known herpesviruses, retroviruses, polyomaviruses, paramyxoviruses, and orthopoxviruses), internal parasites (*Trichuris* spp., *Stronglyoides* spp., and *Giardia* spp.), and 2 bacterial species (*Mycobacterium* tuberculosis complex bacteria and *Bordetella* spp.). Retroviruses and herpesviruses are of particular concern in regard to biomedical research due to their ability to influence the immune system and to persist in the host after the initial infection event. Because NHP herpesviruses are closely related to their human virus orthologs, testing of human herpesvirus vaccines in conventional NHP is problematic due to antigenic crossreactivity between human and enzootic simian herpesviruses. In addition,

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the ability of most baboon herpesviruses to infect human cells in vitro raises the risk of zoonotic infection.[4]

These risks highlight the need for SPF baboons in immunologic studies and vaccine development. Studies thus far have primarily used conventional baboons, raising the question of whether cellular immune responses differ between SPF and conventional baboons. In this study, we compared normal immune cell parameters between age-matched conventional (non-SPF) and SPF olive baboons.

Materials and Methods

Ethics statement. This research was conducted at the AAALAC-accredited Michale E Keeling Center for Comparative Medicine and Research (Keeling Center) at the University of Texas MD Anderson Cancer Center (Bastrop, TX). All blood samples were collected as part of routine veterinary medical examination and according to the provisions of the Animal Welfare Act, PHS Animal Welfare Policy, and the principles of the NIH *Guide for the Care and Use of Laboratory Animals*.[10] All procedures were approved by the IACUC at the University of Texas MD Anderson Cancer Center.

Animals, diet, and blood collection. In the present study, we compared the immune responses of conventional and SPF olive baboons (*Papio anubis*), each cohort consisting of 25 females in the 7- to 13-y-old age range that were socially housed in indoor–outdoor runs or Primadomes[32] at our facility. Animals had unlimited access to high-protein NHP chow (no. 5045, LabDiet, St Louis, MO) and water. In addition, they were fed fresh fruits and vegetables daily, as well as regular enrichment items such as forage, seeds, peanuts, raisins, peanut butter, and frozen juice cups. Subjects also were provided with destructible enrichment manipulanda and different travel or perching materials on a rotating basis to promote the occurrence of species-typical behavior. Colony management practices included a comprehensive veterinary program to assess baboon health and psychologic wellbeing along with the daily environmental enrichment opportunities. We used well-established criteria in choosing healthy animals for our study; all were free of illness.[41]

Given that the present study compared the immune responses of SPF and conventional baboons, it was important to define the natural flora of the conventional colony. The conventional baboons had a complete flora of common baboon herpesviruses and of baboon foamy virus, and approximately 50% to 60% of the animals were infected with STLV also. The conventional baboon colony is free of common NHP parasites. The SPF and conventional baboon colonies are geographically separated on our campus and use completely different staff for husbandry and veterinary support. In addition, the SPF baboon colony is surrounded by an 18-foot perimeter fence with badge-only access. Personnel cannot enter the SPF baboon colony on the same day as entering a colony of lower health status and must shower in and wear extensive PPE. Both serologic and PCR testing for an extensive list of common adventitious agents of baboons are performed semiannually to ensure that the animals maintain their SPF status.

SPF baboons are defined[40] as being absent of the following of pathogens: herpesviruses (*Herpesvirus papio* types 1 and 2, simian varicella zoster virus, baboon cytomegalovirus, *Human herpesvirus* type 6, baboon rhinovirus), retroviruses (simian foamy virus, simian retrovirus D, SIV, simian T lymphotropic virus), polyomaviruses (simian virus 40 and SA12 virus), paramyxovirus (morbillivirus, measles), orthopoxvirus (monkeypox virus), arterivirus (Southwest baboon virus 1), internal parasites (*Trichuris trichuria*, whipworms; *Strongyloides* spp., threadworms; *Giardia* spp.), blood parasites (*Babesia* spp.), and bacteria (*Mycobacterium tuberculosis* spp. and *Bordetella* spp.).

Clinical and laboratory assessment of study animals. Animals were observed twice daily by the veterinary staff as part of the comprehensive veterinary care program. Animals were sedated for biannual physical examinations and as needed to treat illness or injury. Blood samples were collected from a peripheral vein and analyzed for CBC (Advia 120, Siemens, Tarrytown, NY) and serum chemistry (model AU680, Beckman Coulter, Brea, CA) profiles. The absolute number of lymphocytes, obtained from hematologic analysis, was used in converting the frequency of the lymphocyte population obtained from FACS analysis, to get the absolute number in each lymphocyte subset population.

Study groups, collection of samples, and PBMC preparation. Blood samples (10 mL) were collected on multiple days between 0900 and 1100 from 50 baboons (25 each conventional and SPF) through venipuncture of the femoral vein into EDTA anticoagulant tubes after the animals had been anesthetized with ketamine (10 mg/kg IM; Vedco, Saint Joseph, MO). Blood sampling volumes were approved by the IACUC and clinical veterinarian, and the baboons appeared healthy throughout the study. Blood samples were processed at the Keeling Center within 2 to 4 h of collection. Plasma was separated by centrifugation and stored at –80 °C until further use. PBMC were isolated by Ficoll–Hypaque density-gradient separation as described previously.[20,22] Erythrocytes were removed by osmotic lysis in ACK lysing buffer (Life Technologies, Grand Island, NY), and the remaining nucleated cells were washed twice in RPMI supplemented with 10% FBS (Atlanta Biologic, Flowery Branch, GA) and used for immune assays.

Flow cytometry. A series of commercially available human monoclonal antibodies that crossreact with NHP mononuclear cells were used in flow cytometric analyses, as described previously.[19,20] Briefly, for each sample, 100 µL of whole blood was added to 12×75-mm polystyrene test tubes (Falcon, Lincoln Park, NJ) each containing one panel of monoclonal antibodies: CD3 PerCP (clone SP-34), CD8 PE (clone SK1), CD16 FITC (clone 3G8), and CD20 APC (clone L27; all from BD Biosciences, San Diego, CA). Tubes were then incubated for 15 min at room temperature in the dark. Red blood cells were lysed with 1× FACS lysing solution (Becton Dickinson, San Diego, CA), diluted according to the manufacturer's instructions. The samples were washed thoroughly in 1× PBS by centrifugation; cell sediments were then suspended in 1% paraformaldehyde buffer (300 µL), and cell were acquired on a 4-color flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA). All samples acquired in this study were compensated by using the single-color stained cells. Lymphocytes that were gated on forward scatter compared with side scatter dot plots were used to analyze $CD3^+$, $CD4^+(CD3^+CD8^-)$, and $CD8^+$ ($CD3^+CD8^+$) T-cell and $CD20^+$ B-cell lymphocyte subsets (FlowJo, Tree Star, Ashland, OR).

For NK and NKT cell analysis, a separate tube containing 100 µL of blood was used for staining with the combination of antibodies to CD3 (PerCP, clone SP-34), CD8 (PE, clone SK1), and CD16 (FITC, clone 3G8; BD Pharmingen, San Jose, CA), as described earlier. The stained cells were acquired through flow cytometry (FACSCalibur, BD Biosciences) and analyzed (FlowJo, Tree Star). Baboon NK cells are identified as $CD3^-CD16^+$ cells and subdivided according to CD8 expression.

For the analyses of T-cell memory subsets, 100 µL of EDTA-preserved whole blood was stained with antiCD3 (FITC, clone SP34-2, BD Biosciences), antiCD4 (PE, clone L200, BD Biosciences), antiCD28 (PerCpCy5.5, BD Biosciences), and antiCD95

(APC, clone DX2, BD Biosciences) and processed as mentioned earlier. Both compensation controls and fluorescence-minus-one controls were used. Results were acquired on a FACSCaliber flow cytometer (BD Biosciences) and analyzed (FlowJo, Tree Star). Naive T cells were identified by intermediate to high expression of CD28 and a lack of CD95; memory T cells acquire surface expression of CD95 and can further be divided into $CD95^+CD28^+$ central memory T cells and $CD95^+CD28^-$ effector memory T cells, hypothesized to be terminally differentiated.

In vitro stimulation with mitogen. PBMC freshly prepared from whole blood collected in an EDTA tube were more than 90% viable, as determined by the trypan blue exclusion method; for each immunoassay, we used 10^5 cells per well. The proliferation of PBMC was determined through the standard MTT dye reduction assay, as previously described.[16,33] Briefly, aliquots of PBMC (10^5/well) were seeded in triplicate into the wells of 96-well, U-bottom plates and individually stimulated for 48 h with phytohemagglutinin (PHA), concanavalin A (Con A), LPS, and pokeweed mitogen (PWM; all from Sigma, St Louis, MO), each at a final concentration of 5 µg/mL. The culture medium without mitogen served as the negative control. After culture for 48 h at 37 °C in 5% CO_2, 175 µL of medium was replaced with 15 µL of freshly prepared MTT dye (5 mg/mL in PBS). After 4 h of incubation, medium was then replaced with 100 µL of 0.04 N acidified isopropanol (Sigma). After 30 min of incubation at room temperature for color development, the plate was read at 490 nm on an ELISA plate reader (Victor, PerkinElmer, Shelton, CT). Results are expressed as optical density after subtraction of the medium-only control. Reported values are the mean of 3 replicates. The optimal concentration of mitogen, number of PBMC, and incubation time were standardized previously in our laboratory by using PBMC isolated from healthy animals.

ELISpot assay for detecting antigen-specific IFNγ-producing cells. Isolated PBMC were stimulated individually with Con A, PHA, and PWM (final concentration, 2 µg/mL) to determine the numbers of IFNγ-producing cells through ELISpot assays (Monkey IFNγ ELISpot^{Pro} ALP, Millipore, Bedford, MA) using the methodology reported earlier.[17,18] Briefly, aliquots of PBMC (10^5/well) were seeded in duplicate into wells of 96-well plates (polyvinylidene difluoride–backed plates, MAIP S 45, Millipore) precoated with the primary antibody to IFNγ and incubated with PHA, Con A, and PWM. After incubation for 24 h at 37 °C, the cells were removed, and the wells were thoroughly washed with PBS and developed according to protocol provided by the manufacturer. Purple spots representing individual cells secreting IFNγ were counted by an independent agency (Zellnet Consulting, Fort Lee, NJ) using the KS-ELISpot automatic system (Carl Zeiss, Thornwood, NY), and the data are shown as number of IFNγ spot-forming cells (SFC) for 10^5 input PBMC. Responses were considered positive when the number of SFC after exposure to the test antigen was at least 5 SFC and was at least 5 SFC above the background control values from cells cultured in medium only.

Multiplex cytokine assays. The concentrations of IFNγ, IL2, IL6, IL10, IL12 (p40), and TNFα in plasma were measured by using a NHP Multiplex Cytokine Kit (Millipore) as described previously.[19] Briefly, EDTA-preserved plasma samples were centrifuged ($1200 \times g$ for 10 min), and aliquots were frozen at −80 °C until used. On the day of assay, plasma samples were thawed and precleared by centrifuging at $1200 \times g$ for 5 min. The 96-well plates provided in the kit were blocked with assay buffer for 10 min at room temperature and washed, and 25 µL of standard, control, or sample was added to appropriate wells. After 25 µL of beads was added to each well, the plate was incubated on a shaker overnight at 4 °C. The next day, the plate was washed twice 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 twice with wash buffer, 150 µL of sheath fluid was added to each well, and cytokines were measured by acquiring beads on the BioPlex 200 system (BioRad, Hercules, CA). Fluorescence data were analyzed by using Bio-Plex Manager 5.0 software (BioRad). The minimal detectable concentration was calculated by using Multiplex Analyst software (Millipore). The minimal detectable concentrations (in pg/mL) for the various cytokines were: IFNγ, 2.2; IL2, 0.7; IL6, 0.3; IL10, 6.2; IL12(p40), 1.2; and TNFα, 2.1.

Ex vivo induction of cytokines by TLR ligands. PBMC obtained after centrifugation of blood by using a density gradient were washed with PBS. Aliquots of 1×10^5 cells were incubated at 37 °C in a 5% CO_2 atmosphere for 24 h with or without TLR ligand (1 µg/mL; TLR1 Pam3CSK4, TLR3 Poly I:C, TLR4 LPS, TLR5 Flagellin, TLR6 FSL1, RLR8 ssRNA40/LyoVec; all from Invivogen [San Diego, CA]) in a 96-well plate for IFNγ ELISpot assay; the ELISpot plate was developed as described earlier.

Statistical analysis. For statistical analysis, samples were grouped according to conventional and SPF animals, and unpaired 2-tailed *t* test analyses were performed. Results are expressed as mean ± 1 SD and *P* value, when statistically significant. An F test for equal variances was done to ensure that the groups had equal variances before the *t* tests were run. *P* values less than 0.05 were considered statistically significant. All statistical analyses were conducted by using Prism 6.00 (GraphPad Software, San Diego, CA).

Results

Conventional baboons harbor a number of adventitious infectious agents common to NHP species, including many of the pathogens on the SPF bioexclusion list provided in the Methods section. Given that these agents have been eliminated from our SPF baboon colony, we assessed whether their absence resulted in changes to the CBC count or blood chemistry. We examined the various CBC and blood chemistry values and found no significant differences between conventional and SPF baboons, as has been reported previously.[40]

Expression of major lymphocyte subsets in the peripheral blood of conventional and SPF baboons. To enumerate various lymphocyte subsets in peripheral blood, samples collected from conventional and SPF baboons were analyzed by flow cytometry. To determine the absolute numbers of various T and B cells subsets in blood, we used the gating strategy shown in Figure 1. We observed no significant differences in the absolute numbers of $CD3^+$ (T cells), $CD4^+$ (helper T cells), $CD8^+$ (suppressor T cells), $CD4^+CD8^+$ (double-positive T cells), or $CD20^+$ (B cells) between conventional and SPF baboons (Figure 2).

NK cells are an important component of the innate immune response, which has fundamental roles in the defense against various cytopathic viruses, primarily herpesviruses.[3] We observed no significant differences in NK and NKT populations overall between conventional and SPF baboons. However, $CD8^+$ NKT cells were significantly more numerous in SPF than conventional baboons ($t = 2.09$, df = 33, $P < 0.05$, $\eta^2 = 0.12$; Figure 3).

Phenotypic analysis of memory markers in conventional and SPF baboons. To investigate naive and memory T-cell subsets in conventional and SPF baboons, we used surface expression of CD95 and CD28, most consistently used in both human and rhesus monkeys.[7,21] We used the flow cytometric gating strategy

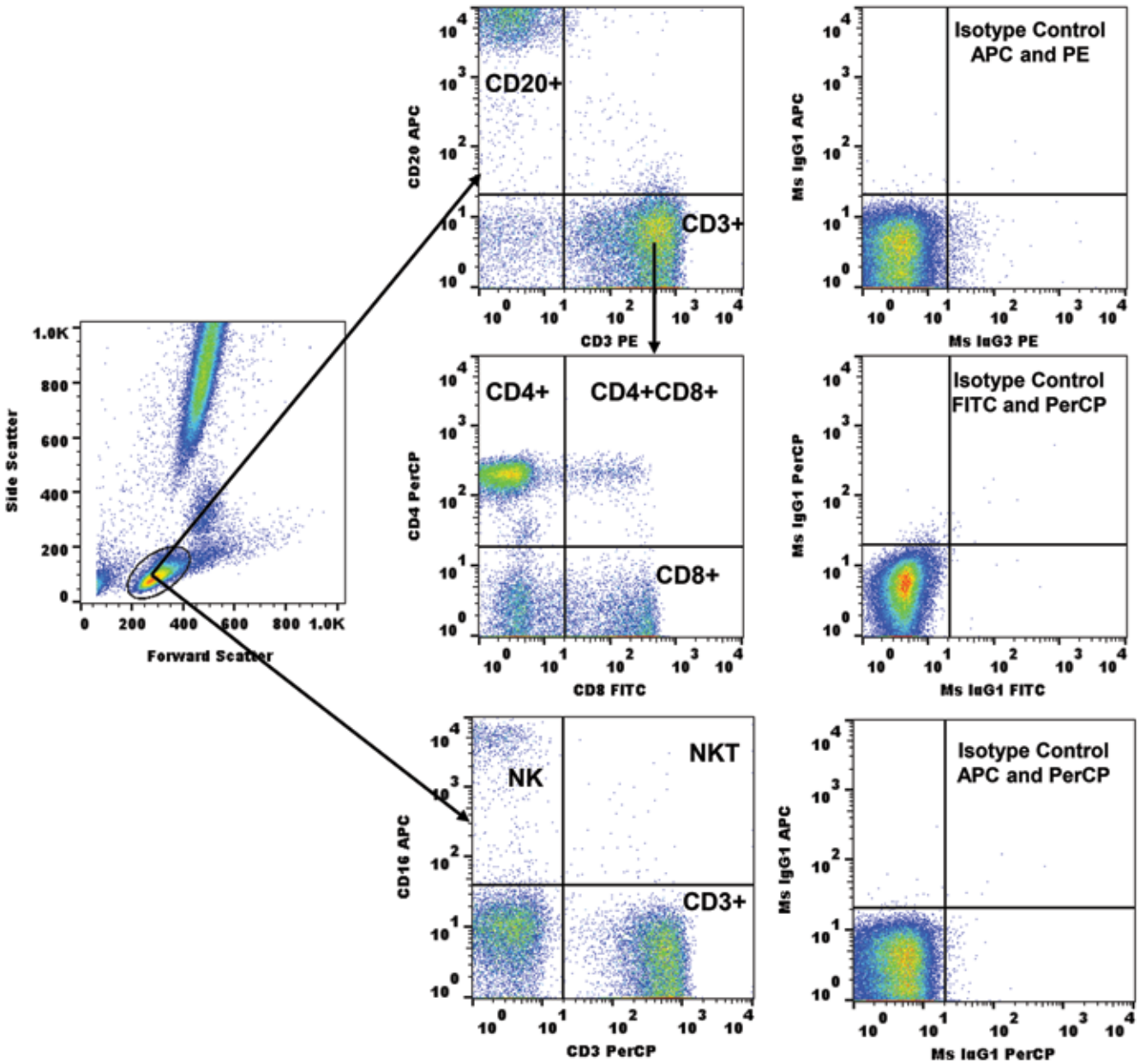

Figure 1. Gating scheme for phenotypic analyses of the various cell markers in peripheral blood from a representative baboon. The lymphocytes and monocytes were first gated according to forward scatter versus side scatter, and then $CD3^+$, $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cells; $CD20^+$ B cells; and $CD16^+$ NK and NKT cells were positively identified. The specificity of staining for the various markers was ascertained according to the isotype control antibody staining used for each pair of combination markers, as shown.

shown in Figure 4 to count the various memory and naïve subsets of $CD4^+$ and $CD8^+$ T cells. In addition, naïve, effector, and memory subsets of $CD4^+$ and $CD8^+$ T cells were enumerated by using costimulatory (CD28) and proapoptotic (CD95) markers of expression by using a previously reported gating strategy.[21] Between the conventional and SPF baboons, within the $CD4^+$ T cell subsets, conventional baboons had significantly higher numbers of $CD4^+$ central and effector memory T cells than SPF groups ($t = 2.09$, df = 33, $P < 0.05$, $\eta^2 = 0.12$). In $CD8^+$ T-cell subsets, conventional baboons had significantly higher numbers of $CD8^+$ effector memory T cells than SPF baboons ($t = 2.09$, df = 33, $P < 0.05$, $\eta^2 = 0.12$; Figure 5). However, no significant differences between conventional and SPF baboons were observed for naïve $CD4^+$ and $CD8^+$ T cells or for central memory subsets of $CD8^+$ T cells (Figure 5).

Proliferative responses. Because we did not find significant differences in the expression of T and B cells, we investigated a functional hallmark of proliferation of PBMC samples from the conventional and SPF baboons. We measured proliferation as the reduction of tetrazolium salts by using MTT dye, and optical density was expressed as percentage viability (Figure 6). Absorbance values that are lower than that of the control cells indicate a reduction in the rate of cell proliferation; conversely, a higher absorbance indicates an increase in cell proliferation. The proliferative responses to PHA (t = 3.55, df = 38, $P < 0.0001$, $\eta^2 = 0.24$) and PWM ($t = 2.91$, df = 38, $P < 0.001$ $\eta^2 = 0.19$) were significantly greater in conventional than SPF baboons (Figure 6), but the response to Con A was greater ($t = 9.11$, df = 38, $P < 0.05$, $\eta^2 = 0.69$) in SPF baboons compared with conventional baboons (Figure 6).

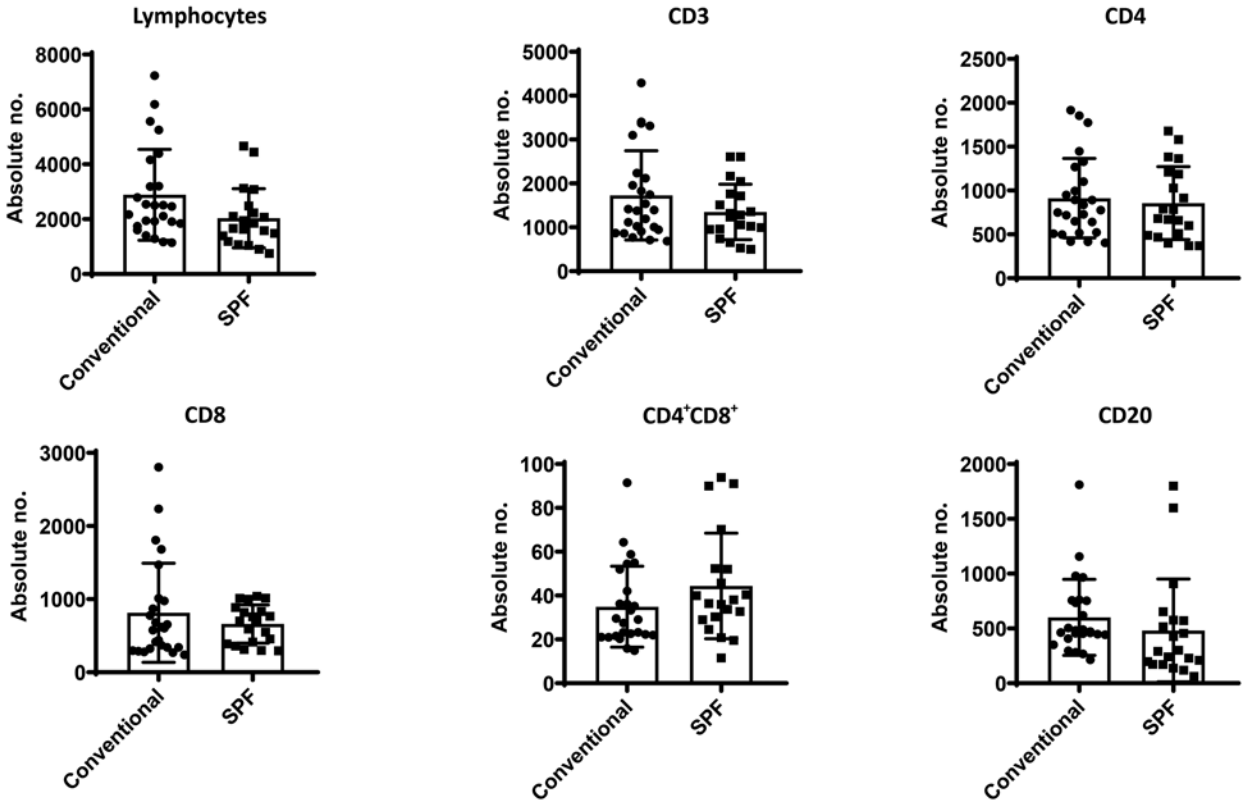

Figure 2. Absolute numbers of total lymphocytes, $CD3^+$ T cells, subsets $CD4^+$ T cells, $CD8^+$ T cells, $CD4^+CD8^+$, and $CD20^+$ B cells. Phenotypic analyses of lymphocytes in conventional and SPF baboons. Aliquots of EDTA-treated whole blood were stained with fluorescently labeled antibodies to the $CD3^+$, $CD4^+$, $CD8^+$, $CD4^+CD8^+$, and $CD20^+$ lymphocytes and analyzed for T-cell subpopulations in conventional and SPF baboons.

ELISpot assay for detecting mitogen-specific IFNγ-producing cells in conventional and SPF baboons. In addition, we used ELISpot measured functional activity as IFNγ production by PBMC in response to stimulation with PHA, PWM, and LPS. Conventional baboons showed significantly more IFNγ-producing cells in response to stimulation with Con A (t = 3.88,df = 42, $P < 0.05$,$\eta^2 = 0.26$), PWM (t = 4.23,df = 38, $P < 0.05$, $\eta^2 = 0.32$), and LPS (t = 4.14,df = 42, $P < 0.05$, $\eta^2 = 0.29$) than SPF baboons. The response to stimulation with PHA was greater in conventional baboons than SPF baboons (Figure 7).

Cytokine levels in plasma. Using a bead array kit, we analyzed plasma samples from conventional and SPF baboons for various Th1 (IFNγ, IL2, TNFα) and Th2 (IL6, IL10, IL12[p40]) cytokines and noted significantly higher levels of TNFα (t = 2.89, df = 45, $P < 0.05$, $\eta^2 = 0.16$) in SPF compared with conventional baboons. However, the remaining cytokines evaluated showed no differences in concentration between SPF and conventional baboons (Figure 8).

Ex vivo stimulation of cytokines by TLR ligands. Because TLR are a class of proteins that play key roles in the innate immune system and recognize various microbial and viral molecules known as pathogen-associated molecular patterns,[12] we also investigated the effect of TLR in conventional and SPF baboons. In IFNγ ELISpot plates, we incubated PBMC with ultrapurified TLR ligands (TLR 1, 3 through 5, 6, and 8). Compared with conventional animals, SPF baboons showed significantly increased responses to stimulation by using TLR5 (t =1.034, df = 7, $P <$ 0.335, $\eta^2 = 0.13$) and TLR6 (t =2.11, df = 7, $P < 0.072$, η^2 =0.38) and significant decreases in response to TLR4 (t = 2.87, df = 7, $P < 0.0002$, $\eta^2 = 0.54$), TLR3, and TLR8 (for both: t = 2.87, df = 7, $P < 0.05$, $\eta^2 = 0.54$) in SPF baboons (Figure 9).

Discussion

The high level of genetic homology between baboons and humans, their outbred nature, and their large size has made baboons a valuable animal model. Although their research contributions are vast, we have focused on their immune system, which shares many similarities to the human immune system. We specifically focused on potential differences in immune systems between conventional and SPF baboons. Differences in immunity are likely to exist between conventional baboons—which carry an array of endogenous viruses, parasites, and bacteria—and SPF baboons, which are devoid of many of the pathogens found in conventional baboons. The preservation of a functional and diverse T-cell population is a dynamic process controlled by exposure to antigen and cytokines. An analysis of baboon T cells has demonstrated that $CD4^+$ and $CD8^+$ T cells can be subdivided into naïve, central memory, and effector memory T cells, as reported for rhesus macaques, by using CD28 and CD95 as the primary cell surface markers.[11,19] We found that conventional and SPF baboons have equivalent absolute numbers of helper and cytotoxic T cells. Our analysis of memory markers revealed that the expression of $CD4^+$ effector memory, $CD4^+$ central memory, and $CD8^+$ effector memory T cells is significantly greater in conventional compared with SPF baboons. This finding parallels those in infant, adolescent, and adult macaques in conventional compared with SPF colonies.[27,29] In these previous studies, the observed differences in specific lymphocyte subsets likely contributed to the distinct cytokine responses after mitogenic T-cell stimulation. Researchers noted that the conventional adolescent macaques produced higher levels of inflammatory cytokines compared with their age-matched SPF adolescent macaques.[27,29]

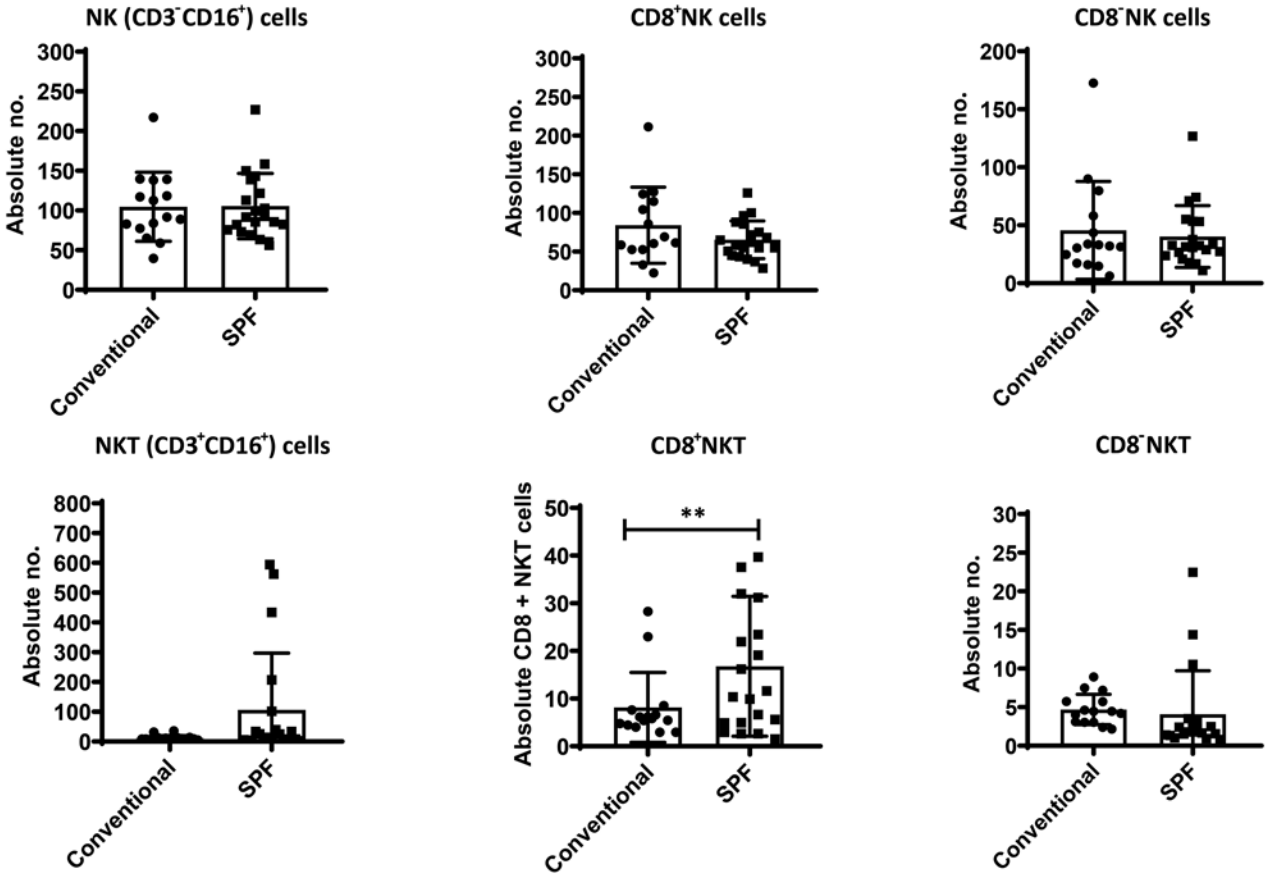

Figure 3. Analysis of CD16⁺ and its subsets are shown. NK (CD3⁻CD16⁺) cells, CD8+ NK cells, CD8⁻NK cells, NK T (CD3⁺CD16⁺) cells, CD8⁺ NKT cells and CD8⁻NKT cells subsets are shown. Values on the *y*-axis are absolute numbers of lymphocytes. Data are compared by using the Student *t* test and are shown as mean ± SEM (n = 20–25). Differences were considered significant at **, ($P < 0.05$).

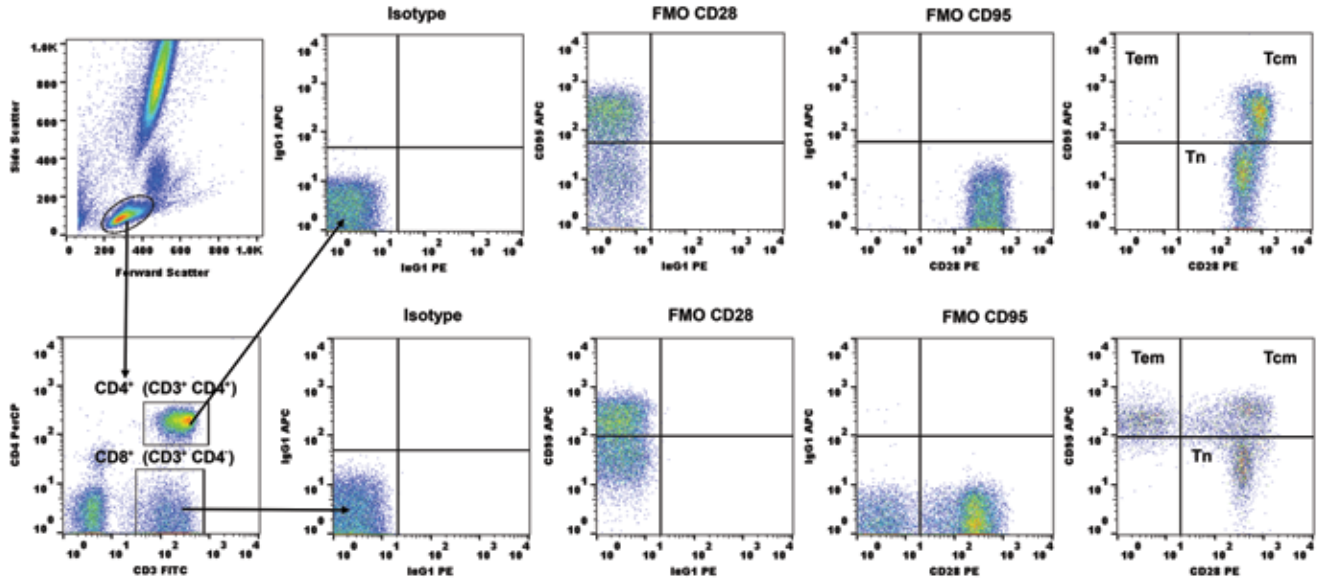

Figure 4. Gating scheme for naïve and memory T-cell markers in peripheral blood from a representative baboon. The lymphocytes were first gated according to forward scatter versus side scatter. T cells were then positively identified by CD3 expression followed by the detection of the CD4⁺CD8⁻ (CD4⁺ T cells) and CD4⁻CD8⁺ (CD8⁺ T cells) populations within the CD3⁺ T cells. On the basis of CD28 and CD95 expression, CD4⁺ and CD8⁺ T cells were further differentiated into naive (CD28⁺CD95⁻), central memory (CD28⁺CD95⁺), and effector memory (CD28⁻CD95⁺) T-cell subsets. The specificity of staining for the different markers was ascertained according to fluorescence-minus-one controls as shown.

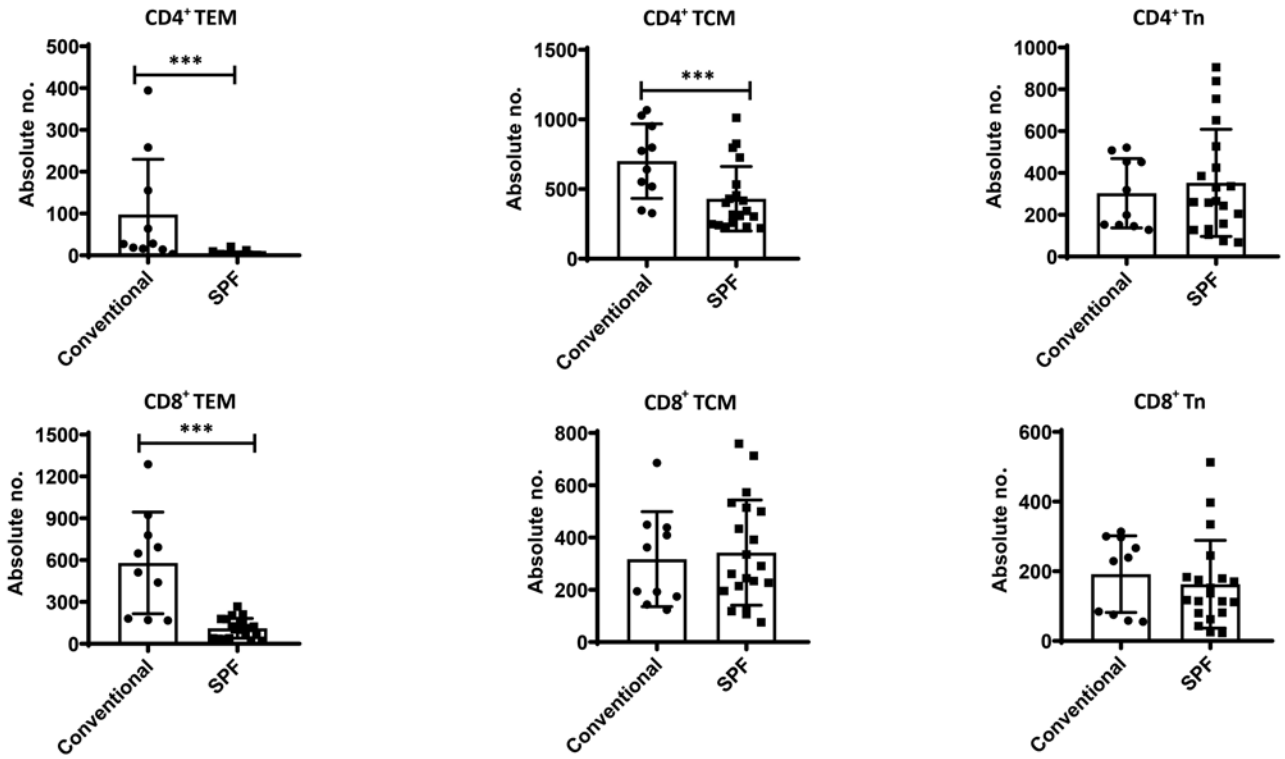

Figure 5. Analyses of memory T-cell subpopulations. Blood samples from conventional and SPF baboons were stained and analyzed for T-cell subpopulations by flow cytometry. Absolute numbers of CD4 + effector memory (TEM; $CD28^{-}CD95^{+}$), central memory (TCM; $CD28^{+}CD95^{+}$), naïve (Tn; $CD28^{+}CD95^{-}$)and CD8 + effector memory (TEM; $CD28^{-}CD95^{+}$), central memory (TCM; $CD28^{+}CD95^{+}$), naïve (Tn; $CD28^{+}CD95^{-}$)were compared between conventional and SPF baboons. The results shown are an average of 10 baboons in each group. Data are compared by using the Student *t* test and are presented as mean ± SEM ($n = 20$). Differences of $P < 0.05$ were considered statistically significant.

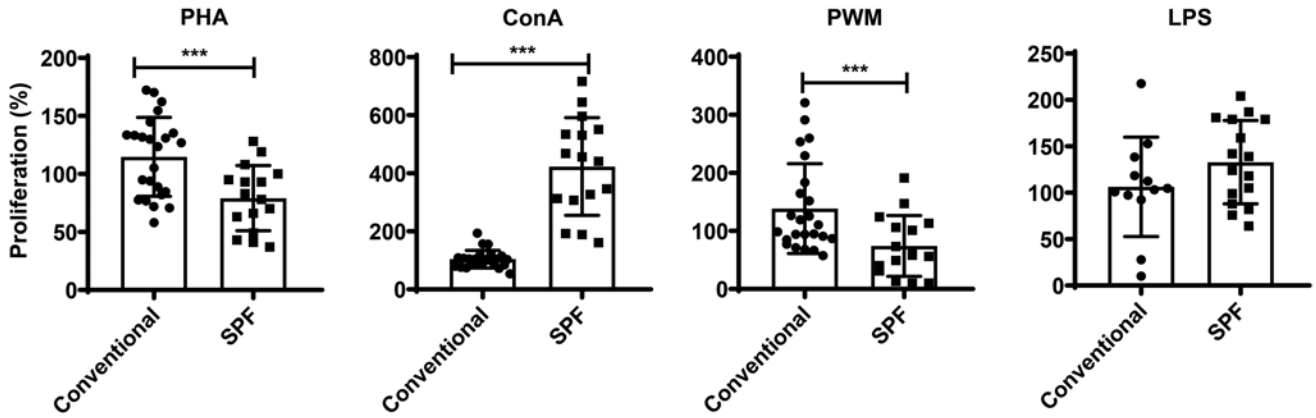

Figure 6. Proliferative responses of PBMC to mitogens in conventional and SPF baboons. We used PBMC that were isolated from blood samples of baboons to determine the proliferative response to various mitogens PHA, ConA, PWM, and LPS by using the standard MTT dye reduction assay. Proliferative responses were measured as optical density (OD) and expressed as percentage viability in excess of the medium-only control. Data are compared by using the Student *t* test and are presented as mean ±SEM ($n = 38$). Differences of *** $P < 0.05$ were considered statistically significant.

NK cells are critical to the innate immune system as the first line of defense against many virus-infected cells and tumor cells. In addition, NK cells are important mediators of transplantation rejection reactions, as seen during baboon xenotransplantation studies.[1,2,9] The expression of NK markers is variable in both humans and NHP.[28] The majority (greater than 85%) of circulating NK cells are CD16 single-positive; other NK subsets population, including CD56 single-positive and CD16–CD56 double-positive or double-negative cells, represent only a small fraction of the overall cell population.[28] In the current study, we identified baboon NK cells as $CD3^{-}CD16^{+}$ cells, as we reported previously regarding the blood of healthy rhesus macaques.[19] In NHP, NK cells have also been characterized as CD8α+$NKG2A^{+}CD14^{-}$[31] and $CD4^{-}CD8^{-}$[35] cells. In humans, 85% to 90% of NK cells are $CD3^{-}CD56^{+}$ and can be subdivided according to the expression of CD16.[13] One major difference between human and NHP NK cells are that baboon and macaque NK cells—but not human NK cells—express high levels of CD8α.[13,39]

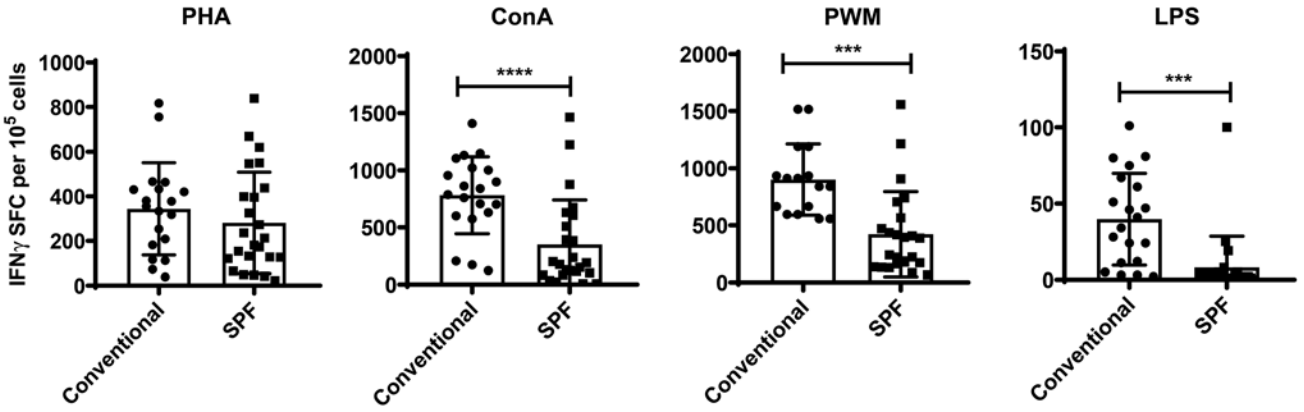

Figure 7. IFNγ-secreting cells among mitogen-stimulated PBMC from conventional and SPF baboons. PBMC were analyzed through ELISpot assays by staining for IFNγ cells that were stimulated with mitogens PHA, ConA, PWM and LPS. The total number of spot-forming cells (SFC) in each of the stimulated wells was counted and adjusted to that in control medium as background. Data are compared by using the Student *t* test and are presented as mean ± SEM (n = 24). Differences were considered significant at ****, $P < 0.001$, ***, $P < 0.05$.

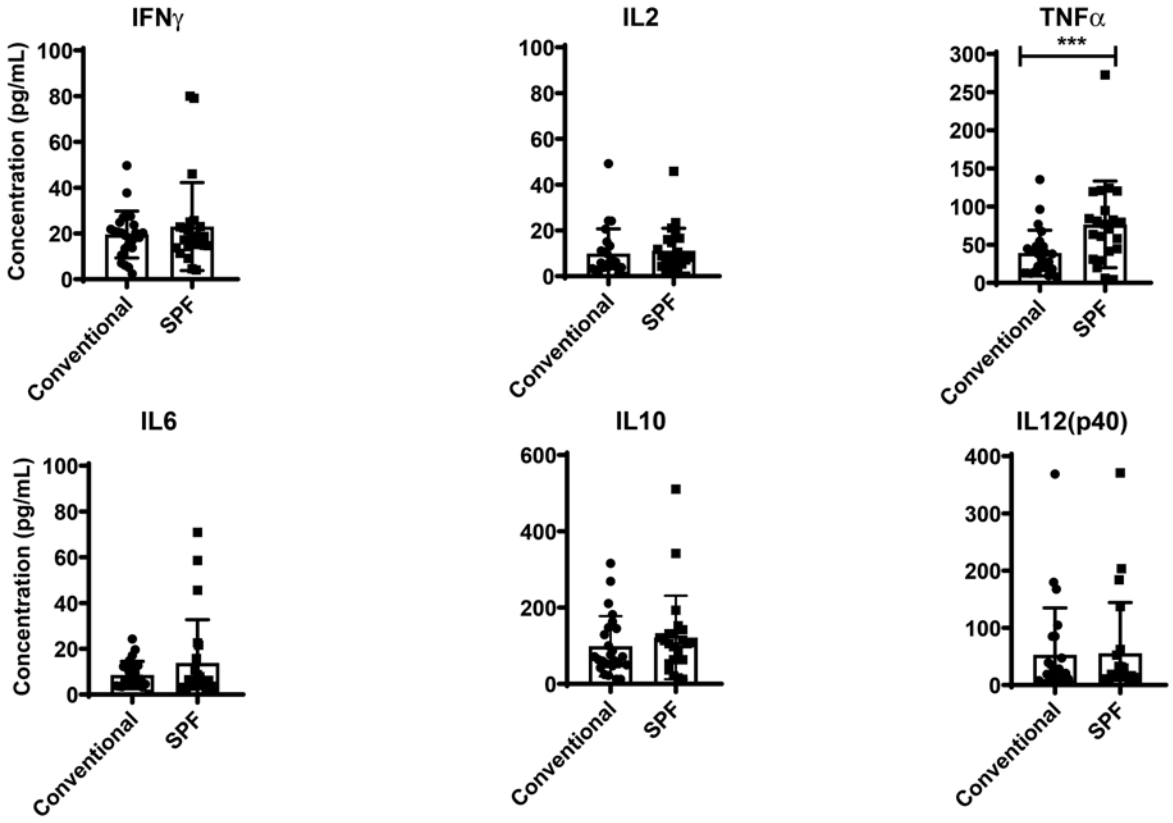

Figure 8. Cytokine bead array analysis of plasma from conventional and SPF baboons. In duplicate wells of 96-well filter plates, 25 µL of plasma was incubated overnight with 25 µL of cytokine-coupled beads 4 °C followed by washing and staining with biotynylated detection antibody. Results for cytokines IFNγ; TNFα; IL2; IL6; IL10; and IL12(p40) are expressed in pg/mL; minimal detectable concentrations were: IFNγ, 2.2; TNFα, 2.1; IL2, 0.7; IL6, 0.3; IL10, 6.2; and IL12(p40), 1.2. Standard deviations did not exceed 15% of the mean value. Data are compared by using the Student *t* test and are presented as mean ±SEM (n = 25). Differences were considered significant at ***, $P < 0.05$.

A previous report has shown that CD8+ NKT-like cells can function as suppressive cells to regulate the immune response.[37] We similarly observed increased numbers of CD8+ NKT cells and a decreased proliferative response to against PHA and PWM in SPF baboons (Figure 6).[1]

To determine whether the phenotypic changes we observed in this study were indicative of altered T-cell function, we measured mitogen-specific T-cell responses in the plasma of conventional and SPF baboons. We found a significant increase in circulating TNFα in SPF compared with conventional baboons, whereas IL2, IL6, IL10, IL12(p40), and IFNγ did not differ between groups. The identification of noticeable differences between conventional and SPF baboons suggests that chronic infections modulate host immune development, as previously reported for SPF and conventional rhesus macaques.[24] TLR function as central mediators of the innate immune response

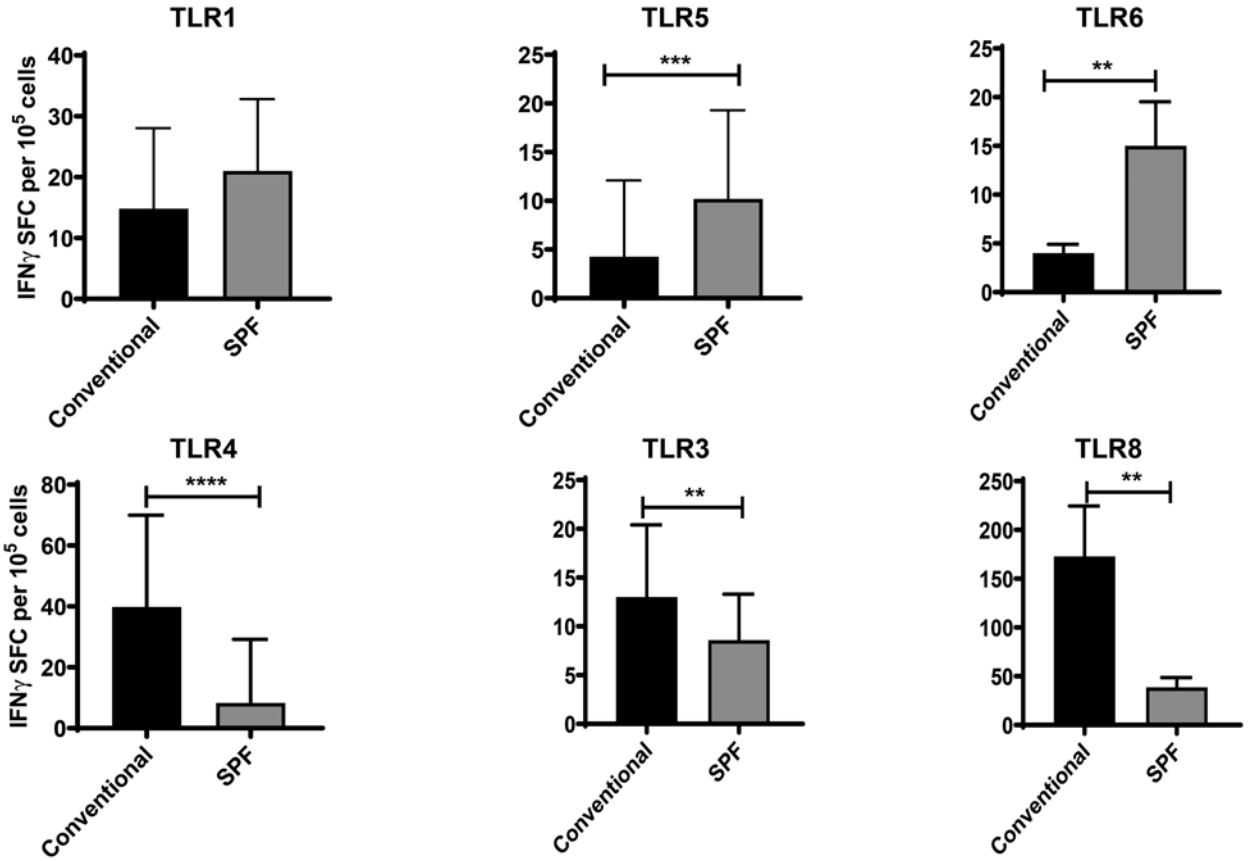

Figure 9. Ex vivo induction of IFNγ production after stimulation by TLR ligand. PBMC isolated from female conventional and SPF baboons were stimulated with TLR ligands TLR1, TLR5, TLR6, TLR4, TLR3 and TLR8 for 24 h, and cells were evaluated in IFNγ ELISpot assays. *P* values were obtained by using the Student *t* test; data are presented as mean ±SEM ($n = 5$). Differences were considered significant at ** $P < 0.05$, ***, $P < 0.005$, ****, $P < 0.0002$.

to diverse pathogens. In the present study, we expected that PBMC isolated from SPF baboons and exposed in vitro to various TLR ligands would have a greater response to specific TLR ligands.

Although the lymphocyte subsets of conventional and SPF baboons share many phenotypic and functional similarities, the findings of this study suggest that specific differences in lymphocyte immune function exist and might alter innate and adaptive immune responses. As we plan immunologic studies going forward, these differences should be considered because they potentially could influence results and outcomes for studies examining various immunologic endpoints.

We experienced 2 limitations during this study: scarcity of crossreactive antibodies recognizing certain baboon antigens, such as NK receptors, and the inclusion of females only. We recognize the study would have had greater value if we had included male baboons.

In conclusion, baboons are invaluable models to advance our understanding of disease pathogenesis, immunity, and vaccine development, due to the animals' genetic similarity to humans and natural susceptibility to a wide variety of human pathogens. Comparative studies of the immune system of conventional and SPF olive baboons enhance our knowledge of the baboon model and accelerate research on the important mechanisms associated with inflammation. This research will help to identify and validate biomarkers and develop novel vaccine strategies.

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