Specific Pathogen-Free Status Alters Immunophenotype in Rhesus Macaques: Implications for the Study of Simian Immunodeficiency Virus

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Abstract

The repertoire of viruses to which research primates are exposed, even in the absence of clinical disease, may contribute to experimental confounding. In this study we examined whether standard specific pathogen-free (SPF) rhesus macaques exposed to a wider spectrum of enzootic viruses and expanded SPF macaques derived to exclude a greater number of viral agents would display alterations in immune activation or immune cell populations. Given the impact of immunophenotype on human immunodeficiency virus (HIV) progression and the importance of the simian immunodeficiency virus (SIV) model for the study of HIV pathogenesis, we elected to additionally examine the impact of SPF status on the capacity of peripheral blood mononuclear cells (PBMCs) to support SIV replication. The expanded SPF group displayed significant immune alterations including increased serum interleukin (IL)-15 and a greater *in vitro* elaboration of GM-CSF, IL1 α , VEGF, IL-10, IL12/23, and MIP-1b. Consistent with reduced viral antigenic exposure in expanded SPF macaques, decreased CD4⁺ and CD8⁺ transitional and effector memory (T_{EM}) cell populations were observed. Expanded SPF PBMC cultures also demonstrated an increased peak (192.61 ng/ml p27) and area under the curve in *in vitro* SIV production (1968.64 ng/ml p27) when compared to standard SPF macaques (99.32 ng/ml p27; $p=0.03$ and 915.17 ng/ml p27; $p=0.03$, respectively). *In vitro* SIV replication did not correlate with CD4⁺ T_{EM} cell counts but was highly correlated with serum IL-15 in the subset of animals examined. Findings suggest that an altered immunophenotype associated with the maintenance of primates under differing levels of bioexclusion has the potential to impact the outcome of SIV studies and models for which the measurement of immunologic endpoints is critical.

Introduction

STUDY DESIGN IN ANIMAL MODELS involves careful consideration of the many factors that influence experimental outcomes including genotype, age, gender, immune status, and aspects of the physical environment. Included among these potential confounders is the presence of naturally occurring viral pathogens. While certain viral agents pose risks to animal health resulting in morbidity and mortality, the more insidious of experimental confounders are those subclinical infections that result in undetected effects on the immune response.

The impact of subclinical viral disease on experimental outcomes is well documented in rodent models and has prompted the establishment of specific pathogen-free (SPF) colonies derived to exclude a defined list of murine viruses, bacteria, and parasites.¹ An increased understanding of the

impact of subclinical or latent infectious disease on experimental outcomes in nonhuman primates (NHP) has similarly prompted the establishment of SPF colonies. The National Council on Research Resources (NCRR) and Office of AIDS Research (OAR) of the National Institutes of Health (NIH) have taken a lead role in facilitating and funding the development of SPF macaque colonies primarily in support of HIV/AIDS research.^{2,3} Agents targeted for elimination include simian T-lymphotropic virus (STLV), simian immunodeficiency virus (SIV), simian type D retrovirus (SRV), and macaque herpesvirus-1 (BV). In addition to excluding the retroviral agents likely to confound experiments utilizing the SIV macaque model, exclusion of BV has the added benefit of reducing zoonotic risks to human handlers. Several facilities have expanded the catalogue of microbial agents targeted for elimination to include animals free of the above mentioned four viruses as well as rhesus rhadinovirus (RRV),

lymphocryptovirus (LCV), simian cytomegalovirus (CMV), simian foamy virus (SFV), simian virus 40 (SV40), and rhesus papillomavirus (RhPV).² These expanded SPF colonies have been utilized for studies examining novel HIV vaccination strategies and the pathophysiology of AIDS-associated opportunistic agents.⁴⁻⁷

Similar to observations in murine models, concomitant immunologic alterations accompany infections with the viral agents targeted for exclusion from SPF colonies. SRV infection results in blunted immune responses associated with pancytopenia, down-regulation of MHC class II expression, reduced mitogen-induced proliferation of peripheral blood mononuclear cells (PBMCs), decreased serum immunoglobulin production, and functional deficits in polymorphonuclear cells.⁸ Experimental infection with RRV in SIV-infected macaques has been shown to result in B cell lymphocytosis, B cell activation, hypergammaglobulinemia, and lymphoid follicular hyperplasia.^{6,9,10} SFV has been documented to alter cell surface markers including increased MHC class I expression and often contaminates primary macrophage/monocytes in cell culture.¹¹ Lastly, simian T-lymphotropic virus 1 (STLV-1) infection commonly remains clinically silent in rhesus macaques although it has been associated with altered cytokine profiles in both transformed cell lines and isolated PBMCs.^{11,12} Exposure to these and other viral agents in the rhesus macaque likely allows for adverse impacts on SIV studies and models that examine immunologic endpoints. *In vivo* investigations of this hypothesis have yielded conflicting results. For instance, experimental STLV-1 coinfection had no impact on SIV viral load or disease course whereas rapid disease progression has been observed with concurrent primary SIV and CMV infection.^{13,14}

To further investigate the impact of viral exposure history on the immune system, we propose to compare immunologic profiles between cohorts of rhesus macaques housed under differing levels of bioexclusion. To this end, we compare the standard SPF and expanded SPF macaque colonies developed and maintained at the New England Primate Research Center (NEPRC) and hypothesize that those animals with a more limited exposure to viral agents will demonstrate an altered immunophenotype relative to those with a history of exposure to a wider spectrum of viral agents. Based on the observed differences in immunologic profiles between expanded and standard SPF macaques and given the importance of the SIV macaque model for the study of HIV pathogenesis, we also elected to examine the impact of SPF status on SIV replicative capacity using an *in vitro* assay shown to be predictive of *in vivo* SIV disease progression.^{15,16}

Materials and Methods

Animals

All animals were housed at the New England Primate Research Center and maintained in accordance with the "Guide for the Care and Use of Laboratory Animals" of the Institute of Laboratory Animal Resources, National Research Council.¹⁷ The facility is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International and all work was approved by Harvard Medical School's Standing Committee on Animals. Healthy age and gender-matched rhesus macaques of Indian origin were selected from the standard SPF or expanded SPF colony based

on current availability for phlebotomy (not assigned to study and not actively breeding). Standard SPF animals were considered seronegative for BV, STLV-1, SIV, and SRV based on routine quarterly to annual virologic testing for these agents performed on all NEPRC colony animals.² Expanded SPF animals were considered additionally seronegative for RRV, LCV, RhPV, CMV, SFV, and SV40. Serology was performed by the Pathogen Detection Laboratory at the California National Primate Research Center (Davis, CA) to confirm group differences in SPF status. Serology for BV, STLV-1, SIV, SRV, RRV, CMV, SFV, and SV40 was performed using a Multiplex Microbead Immunoassay. Seropositivity for LCV was determined using a cross-reactive Epstein-Barr Virus Viral Capsid Antigen Immunofluorescence Assay. Animals were individually or pair housed in stainless-steel caging equipped with automatic watering systems and maintained *ad libitum* on a commercially available diet in biscuit form (Harlan Teklad, Indianapolis, IN). The expanded SPF colony is maintained in a physically separate and dedicated facility. Animals were sedated with Ketamine HCl (10–15 mg/kg IM, Fort Dodge, Overland Park, KS) and phlebotomy was performed using standard techniques.

Cellular immunophenotyping

EDTA-anticoagulated whole blood was collected from standard and expanded SPF macaques ($n = 12$ per group). Complete blood cell counts were performed with a HEMAVET HV 1700 FS Multispecies Hematology Instrument (Drew Scientific, Oxford, CT). Cellular immunophenotyping was performed using multiple color flow cytometry. All antibodies were obtained from BD Biosciences Pharmingen, San Diego, CA except where indicated. Antibodies used include CD3 (SP34/FITC) or CD3 (SP34/V-500), CD20 (2H7/PerCP), CD8 (RPA-T8/APC-Cy7) or CD8 (RPA-T8/Alexa-700), and CD4 (L200/AmCyan) or CD4 (OKT4/PacBlue, eBioscience Inc., San Diego, CA). CD25 was selected as a cell surface activation marker (M-A251/PE-Cy7; NIH NHP Reagent resource/Beth Israel Deaconess Medical Center, Boston, MA).¹⁸ Memory populations were characterized using antibodies directed against CD95 (DX2/FITC), CD28 (CD28.2/APC), and CCR7 (150503/PE, R&D Systems, Inc., Minneapolis, MN). Memory T cells were defined as CD95⁺. Central memory (T_{CM}; CCR7⁺ CD28⁺), transitional memory (T_{TM}; CCR7⁺ CD28⁺), and effector memory (T_{EM}; CCR7⁺ CD28⁺) were measured through the CD95⁺ memory cell gate. Naïve cells were defined as CD95^{lo} CD28⁺. Briefly, freshly isolated PBMCs were stained in the presence of staining media [phosphate-buffered saline (PBS) containing 1% fetal bovine serum] and fixed in 2% paraformaldehyde. Data were acquired with a FACSCalibur flow cytometer (BD Biosciences Pharmingen) and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR). Appropriate isotype controls were used to establish positive and negative gates. One hundred to two hundred thousand events were collected from a live gate to exclude cellular debris.

Circulating serum cytokine quantification

Detection of cytokines was performed using the 23-plex Milliplex MAP nonhuman primate cytokine kit (Millipore Corp. Billerica, MA). Blood was collected from standard and expanded SPF macaques into serum separator tubes ($n = 19$

per group). Following centrifugation, serum was decanted immediately and frozen at -80°C until assayed. On the day of assay, frozen serum was thawed, mixed by vortexing, and then centrifuged at 10,000 rpm for 5 min to isolate debris prior to use in the assay. Samples were prepared according to manufacturer directions using a 96-well filter membrane microtiter plate and vacuum filtration unit (Millipore Vacuum Manifold, Millipore Corp.). Following the final wash, samples were suspended in 150 μl Luminex Sheath Fluid (Millipore Corp.) and analyzed using the Luminex 200 (Millipore Corp.). All samples were run in duplicate. Acquisition gates were set at 8,000–15,000, sample volume was 100 μl , and 50 events per bead were acquired. Mean fluorescence intensity was analyzed using the Milliplex Analyst software and compared to a standard curve to generate concentration values. Values below the range of the standard curve were set to the lower limit of detection.

Cytokine production following in vitro mitogen stimulation

EDTA-anticoagulated whole blood was collected from standard and expanded SPF macaques ($n=6$ per group). PBMCs (2×10^6 per ml/well) were isolated by Ficoll-Hypaque density-gradient centrifugation, suspended in modified RPMI 1640 medium containing 15% fetal bovine serum, and incubated at 37°C . Cells were stimulated with a combination of lipopolysaccharide (LPS; 0.5 $\mu\text{g}/\text{ml}$) and phytohemagglutinin (PHA; 5 $\mu\text{g}/\text{ml}$) or PHA alone (5 $\mu\text{g}/\text{ml}$). Supernatant was harvested 48 h poststimulation and immediately frozen at -80°C . Frozen supernatants were thawed on the day of assay for detection of cytokines using the 23-plex Milliplex MAP nonhuman primate cytokine kit (Millipore Corp.) as described above. Values below the range of the standard curve were set to the lower limit of detection. Values above the range of the standard curve were set to the highest value able to be extrapolated from the standard curve.

Analysis of in vitro SIV viral replication

PBMCs were isolated as above from standard and expanded SPF macaques ($n=8$ per group) and grown in modified RPMI 1640 with 15% fetal bovine serum, penicillin-streptomycin (100 U/ml), L-glutamine (2 mM), HEPES buffer (10 mM), and recombinant human IL-2 (40 U/ml). Cell suspensions containing 1×10^7 PBMCs in 1 ml of RPMI media containing 10% fetal calf serum (FCS) were infected by incubation with 5 ng/ml SIVmac251 for 1 h at 37°C . After infection cells were washed twice in R10 medium, incubated overnight with R15, and then cultured for 3 days with PHA (10 $\mu\text{g}/\text{ml}$). On day 4, cells were washed three times, resuspended, and maintained in culture medium for 21 days at a concentration of 1×10^6 cells/ml. Supernatant was harvested on days 7, 10, 14, 17, and 21. Supernatants were stored at -80°C until analysis. Virus production was quantified using SIV P27 antigen capture (Advanced Bioscience Laboratories, Inc.).

Statistical analysis

Two-tailed Student's *t*-tests were used to assess group differences between standard and expanded SPF macaques. (Sigma Stat 3.5; Systat software Inc., Chicago, IL). *p*-values of less than 0.05 were considered significant. Data are expressed

as mean $\pm$ standard deviation (SD). Linear relationships were examined using scatter plots and correlations determined via calculation of the Pearson correlation coefficient. AUC summary measures were calculated using the trapezoidal rule.

Results

Study population demographics

Age- and sex-matched animals were selected from the standard SPF macaque colony that is seronegative for BV, STLV-1, SIV, and SRV or from the expanded SPF macaque colony that is additionally seronegative for RRV, LCV, RhPV, CMV, SFV, and SV40. To confirm group differences in SPF status, serology was performed on all study animals except for one standard SPF macaque from which banked serum was not available. As expected, all animals from the expanded SPF colony were seronegative for all agents. Seroprevalence data for study animals selected from the standard SPF colony were as follows: RRV seroprevalence of 65.22%, LCV seroprevalence of 100%, SFV seroprevalence of 93.48%, and SV40 seroprevalence of 91.30%. Seroprevalence for CMV in the standard SPF group was lower than expected at 69.57%, however none of the animals were seronegative. The remaining standard SPF animals demonstrated indeterminate results suggesting that CMV seroprevalence may be greater than the observed 69.57%. Of the animals in the standard SPF colony 45.65% were seropositive for all five agents, 32.61% were seropositive for at least four agents, 17.39% were seropositive for at least three agents, and 4.35% were seropositive for at least two agents. Study animals were not matched based on MHC haplotype. Rather, groups were randomly selected to exclude the influence of heritability on assay outcome. Kinship coefficients were determined for each experimental group. Less than 0.5% of possible pairings within each experimental group demonstrated a kinship coefficient value above 0.06 indicating that selected animals represented a largely unrelated population.

Comparison of circulating serum cytokines levels

Circulating cytokines were examined in standard and expanded SPF macaques. Except for a statistically significant increase in IL-15 in expanded (14.10 pg/ml) relative to standard (8.13 pg/ml; $p=0.001$) SPF macaques, there were few appreciable differences in the levels of circulating serum cytokines (Table 1). Of note is the fact that in healthy rhesus macaques a number of cytokines circulate at very low levels with the analytes G-CSF, IL-10, IL-1b, IL4, and IL-5 demonstrating at least 50% of samples below the limit of detection.

Comparison of hematologic parameters and cellular immunophenotypes

Cellular immunophenotyping was performed for both standard and expanded SPF macaques to identify the impact of SPF status on immune cell populations. There were no appreciable differences in white blood cell populations, erythrocyte counts, or platelet parameters when comparing standard to expanded SPF macaques (Table 2). Similarly, CD3⁺, CD4⁺, and CD8⁺ lymphocyte subsets were comparable between groups. Expression of the cellular activation marker CD25 on CD3⁺, CD4⁺, or CD8⁺ lymphocyte subsets

TABLE 1. CIRCULATING SERUM CYTOKINE LEVELS FROM STANDARD AND EXPANDED SPECIFIC PATHOGEN-FREE MACAQUES

Analyte (pg/ml)	Expanded SPF (mean $\pm$ SD)	Standard SPF (mean $\pm$ SD)	p-value ^a	Samples below range of standard curve (%)
G-CSF	0.30 $\pm$ 0.00	0.32 $\pm$ 0.06	0.13	87
GM-CSF	4.96 $\pm$ 6.87	10.79 $\pm$ 27.04	0.37	0
IFN- γ	5.59 $\pm$ 15.21	14.21 $\pm$ 30.46	0.28	34
IL-10	0.55 $\pm$ 1.67	0.30 $\pm$ 0.41	0.54	82
IL-12/23	60.38 $\pm$ 58.94	94.29 $\pm$ 132.81	0.32	0
IL-13	2.26 $\pm$ 4.49	1.31 $\pm$ 2.42	0.43	10
IL-15	14.10 $\pm$ 5.23	8.13 $\pm$ 2.83	0.001	0
IL-17	0.80 $\pm$ 1.10	0.63 $\pm$ 0.65	0.56	63
IL-18	38.70 $\pm$ 17.98	38.34 $\pm$ 37.99	0.97	5
IL-1b	0.20 $\pm$ 0.77	0.08 $\pm$ 0.25	0.53	90
IL-1ra	4.14 $\pm$ 6.88	7.23 $\pm$ 11.50	0.32	13
IL-2	122.10 $\pm$ 27.74	132.34 $\pm$ 70.42	0.56	0
IL-4	0.51 $\pm$ 1.39	1.04 $\pm$ 3.70	0.56	95
IL-5	0.44 $\pm$ 0.72	0.63 $\pm$ 0.77	0.42	60
IL-6	3.47 $\pm$ 5.31	2.24 $\pm$ 2.53	0.37	37
IL-8	3970.51 $\pm$ 2375.51	3545.82 $\pm$ 1951.66	0.55	0
MCP-1	480.82 $\pm$ 242.40	375.30 $\pm$ 131.83	0.11	0
MIP1a	11.80 $\pm$ 22.15	5.43 $\pm$ 14.20	0.30	47
MIP1b	2.64 $\pm$ 5.76	2.89 $\pm$ 4.80	0.89	42
sCD40L	1793.54 $\pm$ 935.86	1620.88 $\pm$ 661.64	0.52	0
TGF- α	31.29 $\pm$ 11.09	30.88 $\pm$ 14.55	0.92	0
TNF- α	43.03 $\pm$ 131.35	23.97 $\pm$ 82.70	0.60	32
VEGF	121.93 $\pm$ 59.75	133.63 $\pm$ 60.93	0.55	0

^ap-value determined using two-tailed Student's *t* test of significance.

G-CSF, granulocyte colony-stimulating factor; IL, interleukin; MIP1b, macrophage inflammatory protein 1b; MIP1a, macrophage inflammatory protein 1a; TNF- α , tumor necrosis factor alpha; IFN- γ , interferon gamma; sCD40L, soluble cluster of differentiation 40 ligand; MCP-1, monocyte chemoattractant protein; TGF- α , transforming growth factor alpha; GM-CSF, granulocyte macrophage colony-stimulating factor; VEGF, vascular endothelial growth factor.

TABLE 2. COMPARISON OF HEMATOLOGIC AND IMMUNOPHENOTYPING PARAMETERS BETWEEN EXPANDED AND STANDARD SPECIFIC PATHOGEN-FREE MACAQUES

Parameter	Expanded SPF (mean $\pm$ SD)	Standard SPF (mean $\pm$ SD)	p-value ^a
Leukocyte count (cells/ μ l)	7103.33 $\pm$ 2825.71	8710.00 $\pm$ 2846.21	0.18
Lymphocyte count (cells/ μ l)	2126.67 $\pm$ 693.97	2511.67 $\pm$ 984.86	0.28
Neutrophil count (cells/ μ l)	4051.25 $\pm$ 2584.86	5510.52 $\pm$ 2099.01	0.15
Monocyte count (cells/ μ l)	251.18 $\pm$ 199.17	337.67 $\pm$ 283.82	0.40
Hematocrit (%)	33.79 $\pm$ 3.44	33.95 $\pm$ 2.59	0.90
Red blood cell count (10^6 cells/ μ l)	5.59 $\pm$ 0.55	5.84 $\pm$ 0.47	0.24
Platelet count (10^3 cells/ μ l)	348.08 $\pm$ 88.20	378.20 $\pm$ 61.03	0.37
CD3 ⁺ cell count (cells/ μ l)	1250.92 $\pm$ 479.65	1291.29 $\pm$ 566.93	0.85
CD3 ⁺ CD4 ⁺ cell count (cells/ μ l)	548.88 $\pm$ 181.40	608.69 $\pm$ 268.42	0.53
CD3 ⁺ CD8 ⁺ cell count (cells/ μ l)	649.72 $\pm$ 318.78	712.02 $\pm$ 340.78	0.65
CD3 ⁻ CD20 ⁺ cell count (cells/ μ l)	504.76 $\pm$ 245.09	689.60 $\pm$ 410.36	0.19
CD25 expression (% of CD3 ⁺ cells)	3.43 $\pm$ 1.46	2.86 $\pm$ 1.13	0.30
CD25 expression (% of CD3 ⁺ CD4 ⁺ cells)	1.46 $\pm$ 1.04	1.09 $\pm$ 0.97	0.37
CD25 expression (% of CD3 ⁺ CD8 ⁺ cells)	5.76 $\pm$ 2.82	4.42 $\pm$ 1.51	0.16
CD25 expression (% of CD3 ⁻ CD20 ⁺ cells)	3.11 $\pm$ 0.96	4.31 $\pm$ 1.36	0.02
CD4 ⁺ memory T cells (cells/ μ l)	370.38 $\pm$ 163.47	572.40 $\pm$ 237.19	0.02
Central memory cells (cells/ μ l)	345.45 $\pm$ 150.72	459.46 $\pm$ 200.90	0.13
Transitional memory cells (cells/ μ l)	21.88 $\pm$ 13.17	65.84 $\pm$ 39.98	0.003
Effector memory cells (cells/ μ l)	2.77 $\pm$ 4.63	45.03 $\pm$ 33.37	0.001
CD4 ⁺ naive T cells (cells/ μ l)	457.32 $\pm$ 282.47	363.15 $\pm$ 149.39	0.32
CD8 ⁺ memory T cells (cells/ μ l)	188.80 $\pm$ 73.31	569.87 $\pm$ 348.90	0.003
Central memory cells (cells/ μ l)	114.62 $\pm$ 50.14	175.58 $\pm$ 110.37	0.10
Transitional memory cells (cells/ μ l)	19.51 $\pm$ 15.31	60.72 $\pm$ 35.84	0.002
Effector memory cells (cells/ μ l)	36.57 $\pm$ 25.92	320.27 $\pm$ 307.63	0.009
CD8 ⁺ naive T cells (cells/ μ l)	180.72 $\pm$ 89.84	185.27 $\pm$ 114.46	0.91

^ap-value determined using two-tailed Student's *t* test of significance.

also did not vary between groups. The one parameter that demonstrated a measurable difference was the percentage of CD25⁺ cells among the population of CD3⁺/CD20⁺ B cells. Standard SPF macaques (4.31%) demonstrated a modest but statistically significant increase in the percentage of CD25⁺ B cells when compared to expanded SPF macaques (3.11%; $p=0.02$).

Because of the observed differences in circulating IL-15 and the role this cytokine plays in memory T cell homeostasis, the frequency of naive and memory populations of CD4⁺ and CD8⁺ T cells was examined (Table 2). Standard SPF macaques demonstrated a significant increase in CD4⁺ memory cell counts (572.40 cells/ μ l) compared to expanded SPF macaques (370.38 cells/ μ l, $p=0.02$). This difference was primarily associated with alterations in the T_{TM} (increased 3-fold in the standard SPF group, $p=0.003$) and T_{EM} populations (increased 16-fold in the standard SPF group, $p=0.001$). A similar trend in CD8⁺ memory cell counts was observed with standard SPF macaques demonstrating significant increases (569.87 cells/ μ l) relative to expanded SPF macaques (188.80 cells/ μ l, $p=0.003$). Again, the difference was associated with significant alterations in the T_{TM} (increased 3-fold in standard SPF macaques, $p=0.002$) and T_{EM} (increased over 8-fold in standard SPF macaques, $p=0.009$) populations. SPF status was not associated with significant differences in naive or T_{CM} cell populations.

Comparison of cytokine production following *in vitro* mitogen stimulation

As a measure of responsiveness to immune stimulation, freshly isolated PBMCs harvested from standard and expanded SPF macaques were cultured in the presence of PHA and LPS and assayed for the production and release of cytokines to the culture media. Most interestingly, PBMCs harvested from expanded SPF macaques demonstrated an increased elaboration of a number of cytokines when compared to standard SPF macaques (Table 3). Cells cultured for 48 h demonstrated increased production of GM-CSF (51.74 vs. 20.41 pg/ml; $p=0.03$), IL-10 (16.99 vs. 12.57 pg/ml; $p=0.03$), IL-12/23 (128.16 vs. 78.19 pg/ml; $p=0.05$), IL-1ra (2196.12 vs. 748.32 pg/ml; $p=0.004$), MIP-1b (346.17 vs. 113.82 pg/ml; $p=0.02$), and VEGF (324.73 vs. 148.53 pg/ml; $p=0.006$) in expanded relative to standard SPF macaques, respectively. There was a nonstatistically significant trend toward decreased production of G-CSF (588.64 vs. 1383.40 pg/ml; $p=0.12$) and MIP-1a (458.54 vs. 946.85 pg/ml; $p=0.08$) in expanded relative to standard SPF macaques. IL-15 production did not differ by SPF status. It is possible that mitogen stimulation of *in vitro* PBMC cultures is of a greater magnitude or does not closely replicate the degree of immune stimulation experienced *in vivo*.

TABLE 3. CYTOKINE PRODUCTION IN 48-h MITOGEN-STIMULATED PERIPHERAL BLOOD MONONUCLEAR CELL CULTURES FROM STANDARD AND EXPANDED SPECIFIC PATHOGEN-FREE MACAQUES

Analyte (pg/ml)	Expanded SPF (mean $\pm$ SD)	Standard SPF (mean $\pm$ SD)	p-value ^a
G-CSF	588.64 $\pm$ 757.18	1383.40 $\pm$ 878.29	0.12
GM-CSF	51.74 $\pm$ 26.72	20.41 $\pm$ 16.51	0.03
IFN- γ	46.44 $\pm$ 40.77	98.76 $\pm$ 143.61	0.41
IL-10	16.99 $\pm$ 2.52	12.57 $\pm$ 3.62	0.03
IL-12/23	128.16 $\pm$ 48.26	78.19 $\pm$ 27.32	0.05
IL-13	11.44 $\pm$ 2.10	11.35 $\pm$ 4.35	0.97
IL-15	3.49 $\pm$ 0.71	2.82 $\pm$ 0.99	0.21
IL-17	7.17 $\pm$ 2.77	4.72 $\pm$ 2.01	0.11
IL-18	32.55 $\pm$ 3.00	30.52 $\pm$ 1.49	0.17
IL-1b	1327.85 $\pm$ 1303.43	976.77 $\pm$ 1314.70	0.65
IL-1ra	2196.12 $\pm$ 879.33	748.32 $\pm$ 415.00	0.004
IL-2	80.71 $\pm$ 6.59	80.16 $\pm$ 5.94	0.88
IL-4	0.70 $\pm$ 0.33	0.78 $\pm$ 0.27	0.68
IL-5	0.64 $\pm$ 0.46	0.64 $\pm$ 0.21	0.98
IL-6	5855.06 $\pm$ 3568.59	4607.05 $\pm$ 3956.13	0.58
IL-8 ^b	7365.17 $\pm$ 2059.82	7420.43 $\pm$ 2025.07	0.96
MCP-1	2976.62 $\pm$ 1569.75	2848.06 $\pm$ 1134.36	0.87
MIP1a	458.54 $\pm$ 97.08	946.85 $\pm$ 545.04	0.08
MIP1b	346.17 $\pm$ 209.59	113.82 $\pm$ 43.33	0.02
sCD40L	68.07 $\pm$ 4.60	102.38 $\pm$ 80.44	0.32
TGF- α	0.43 $\pm$ 0.41	0.25 $\pm$ 0.21	0.38
TNF- α	338.10 $\pm$ 234.32	411.15 $\pm$ 377.12	0.70
VEGF	324.73 $\pm$ 121.06	148.53 $\pm$ 34.30	0.006

^ap-value determined using two-tailed Student's *t* test of significance.

^bData presented for all analytes except IL-8 represent stimulation of cells with lipopolysaccharide (LPS; 0.5 μ g/ml) and phytohemagglutinin (PHA; 5 μ g/ml). IL-8 data represent stimulation with PHA alone (5 μ g/ml).

G-CSF, granulocyte colony-stimulating factor; IL, interleukin; MIP1b, macrophage inflammatory protein 1b; MIP1a, macrophage inflammatory protein 1a; TNF- α , tumor necrosis factor alpha; IFN- γ , interferon gamma; sCD40L, soluble cluster of differentiation 40 ligand; MCP-1, monocyte chemoattractant protein; TGF- α , transforming growth factor alpha; GM-CSF, granulocyte macrophage colony-stimulating factor; VEGF, vascular endothelial growth factor.

Analysis of *in vitro* SIV viral replication

Because $CD4^+$ T_{EM} cells represent the main target population during acute SIV infection and because of the potential impact of immune alterations on HIV/SIV progression, the capacity of cultured PBMCs from standard and expanded SPF macaques to support virus growth was investigated. Purified PBMCs from standard ($n=8$) and expanded ($n=8$) SPF macaques were infected with SIVmac251, cultured for a 21-day period, and assayed for virus production via p27 antigen capture. Cells isolated from expanded SPF macaques demonstrated a significantly increased peak *in vitro* virus production (occurring on either day 10 or 14 based on virus production within each individual culture) when compared to standard SPF macaques (192.61 ng/ml p27 vs. 99.32 ng/ml p27; $p=0.03$). In addition, expanded SPF macaques demonstrated a significantly increased area under the curve in *in vitro* virus production when compared to standard SPF macaques (1968.64 ng/ml p27 vs. 915.17 ng/ml p27; $p=0.03$). Mean p27 antigen production in supernatant over the duration of cell culture for standard and expanded SPF macaques is depicted in Fig. 1A. Examination of a subset of animals ($n=11$) revealed no significant correlation between measures of *in vitro* virus production and $CD4^+$ T_{EM} cell counts. In contrast, a significant correlation between IL-15 and both area under the curve *in vitro* virus production ($r=0.86$, $p=0.001$) and peak *in vitro* virus production ($r=0.70$, $p=0.02$) was observed in a subset of animals ($n=10$) (Fig. 1B and C).

Discussion

The repertoire of viruses to which research primates are exposed, even in the absence of clinical disease, may contribute to experimental confounding, particularly when measuring immunologic endpoints. In this study we compared two populations of rhesus macaques, those with a limited exposure to viral agents (expanded SPF macaques) and those exposed to a wider spectrum of viral agents (standard SPF macaques). Surprisingly, the proinflammatory cytokine IL-15 circulated at greater levels in the serum of macaques from the expanded SPF group. This group also demonstrated decreased $CD4^+$ and $CD8^+$ memory T cell counts including significant reductions of both T_{TM} and T_{EM} cell populations. Cultured PBMCs isolated from expanded SPF macaques demonstrated a greater capacity to support SIV replication compared to cultures of PBMCs from standard SPF macaques and, although SIV replication did not correlate with $CD4^+$ T_{EM} cell counts, *in vitro* virus production was highly correlated with serum IL-15 levels in the subset of animals examined.

Following antigen exposure, T_{EM} cells differentiate from T_{CM} cells to gain cytotoxic potential, polarized cytokine production, and the ability to home to effector sites.^{19,20} It is conceivable that the degree of antigenic stimulation associated with SPF status contributes to the observed difference in T_{EM} cell counts. This is consistent with the observation by Pitcher *et al.* who ascribed the attainment of memory cell

FIG. 1. (A) Comparison of *in vitro* virus production between expanded and standard specific pathogen-free (SPF) macaques demonstrating increased simian immunodeficiency virus (SIV) replicative capacity in expanded SPF animals. Error bars depict standard error of the mean. (B) Correlation between serum interleukin (IL)-15 and area under the curve in *in vitro* virus production. (C) Correlation between serum IL-15 and peak *in vitro* virus production.

frequencies in juvenile macaques comparable to those of adult humans to an early and more frequent exposure to infectious organisms.²⁰ It was counter to our expectations that both lower T_{EM} cell counts and increased serum IL-15 were observed within the expanded SPF group. Constitutive production of IL-15 allows for homeostatic proliferation and differentiation of T_{EM} cells independent of T cell receptor-antigen interaction.^{21–23} Administration of recombinant IL-15 (rIL-15) to rhesus macaques has been shown to induce proliferation of $CD4^+$ and $CD8^+$ T_{TM} and T_{EM} cells.^{21,22} However, the doses administered were greater than physiologic levels and repeated administration resulted in refractoriness to IL-15 signaling.²² It is possible that the degree of antigen exposure associated with SPF status rather than the amount of circulating IL-15 plays an equal or greater role in the expansion of the T_{EM} pool. Unmeasured cytokines, such as IL-7, may also impact the magnitude of this cell population. Lastly, we did not examine T_{EM} populations in lymphoid or effector sites. T_{EM} cells rapidly migrate to lymph nodes, lung, liver, and gut raising the possibility that circulating T_{EM} cell counts are not reflective of cell frequencies in extravascular sites.²²

Members of the $CD4^+$ $CCR5^+$ T_{EM} population are the initial cellular targets of acute SIV infection. In this study, SIV replication in PBMC cultures was not correlated with circulating T_{EM} cell numbers and but instead was highly correlated with serum IL-15 levels. *In vivo* studies support these findings and have shown that expansion of the T_{EM} target cell pool in rIL-15-treated macaques was not associated with increased SIV plasma viral loads.^{21,22} Others have also shown that cocubation of infected PBMCs with IL-15 results in increased HIV replication and that increased susceptibility of IL-15-treated $CD4^+$ T cell cultures to SIV infection was associated with an IL-15 dependent increase in CD4 expression.^{24,25} Although the utility of IL-15 has been explored in the SIV model as an immunomodulatory therapeutic and vaccine adjuvant,^{26–29} a recent report suggests that treatment with IL-15 may augment $CD4^+$ T cell proliferation and activation resulting in a 3-log increase in viral set point and accelerated onset to simian AIDS.³⁰ Our findings suggest that the endogenous level of circulating serum IL-15 associated with SPF status may serve as a host factor with the capacity to modulate SIV pathogenesis.

The enhancement of cytokine production following mitogen stimulation of cultured PBMCs from expanded SPF macaques suggests an increased responsiveness to immune stimulation in this group. Cytokines elaborated at greater levels included IL-10, GM-CSF, IL-12, MIP-1b, IL-1ra and VEGF. A number of these are reported to play critical roles in SIV/HIV pathogenesis. For instance, genetic polymorphisms resulting in decreased IL-10 production have been linked to increased HIV susceptibility and accelerated $CD4^+$ T cell loss.^{31,32} IL-10 production also contributes to the anti-inflammatory cytokine profile in African green monkeys with apathogenic SIV infection.³³ Suppressive effects of IL-10 on HIV replication *in vitro* have also been reported.^{34,35} While these findings would suggest that increased IL-10 elaborated in expanded SPF macaque PBMC cultures would not contribute to the observed increased *in vitro* SIV replication, there have also been numerous contradictory reports indicating an IL-10 synergizes with other cytokines to enhance HIV production.^{36–38} Similar contradictory reports exist in the litera-

ture for GM-CSF and IL-12, illustrating the importance of cell type, culture conditions, and cytokine milieu.

MIP-1 β production was also increased in mitogen stimulated PBMC from expanded SPF macaques. This chemokine is secreted by a variety of cells and is involved in recruitment of immunocompetent cells. As a ligand for the CCR5 coreceptor, MIP-1 β , like MIP-1 α and RANTES, is an endogenous inhibitor of viral entry and capable of inducing a dose dependent suppression of HIV-1, HIV-2 and SIV.^{39,40} Higher levels of MIP-1 α and MIP-1 β have been reported in asymptomatic HIV positive subjects compared to patients that progressed to AIDS.⁴¹ While this literature would argue against the increase in viral replicative capacity observed in PBMC cultures from expanded SPF macaques, effects of elevated MIP-1 β may be negated by alterations in the remaining cytokines such as the observed trend towards decreased MIP-1 α production in expanded SPF macaque PBMC cultures. It is unlikely that specific cytokines contribute to the variation in viral replicative capacity but rather the overall shift in cytokine environment combined with other factors.

The immunologic and viral milieu established during the acute stages of HIV/SIV infection, in part, defines the pathogenic potential of infection in a given individual.^{42–44} A number of host factors are thought to contribute to these early set points. One such host factor postulated to result in a poor prognosis for HIV-infected populations in developing countries is increased exposure to bacterial, viral, and parasitic agents.^{45,46} In this study we suggest the opposite. Macaques exposed to fewer viral agents via maintenance under an increased level of bioexclusion had increased levels of serum IL-15, greater PBMC stimulability, and an increased capacity to support *in vitro* SIV replication. Although counter to our expectations, this finding may be explained by the “hygiene hypothesis.” This theory suggests that the reduction in bacterial, viral, and parasitic infections associated with antibiotic use, vaccination programs, and improved living conditions in developed countries may explain an increased incidence of allergic and autoimmune disease.^{47,48} Animal models have provided some proof of this hypothesis. Elimination of environmental viruses results in an increased frequency and rapidity of onset of diabetes and an increased incidence and severity of adjuvant induced arthritis in rodent models.^{49,50} Adoptive transfer of lymphocytes from virus-exposed animals or the introduction of specific rodent viruses has been shown to provide a protective effect from the induction of autoimmune disease.^{51–53} In rhesus macaques, animals with reduced pathogen burden as a result of indoor housing display skin pathology consistent with chronic hypersensitivity dermatitis and animals with a prior history of outdoor housing or exposure to lung mites had a reduced incidence of dermatitis.⁵⁴ A mechanistic explanation for the “hygiene hypothesis” is not completely elucidated, although shifts in cytokine profiles, down-regulation of $CD25^+$ T regulatory cells, and antigenic competition have been suggested as contributing factors.⁴⁷

The examination of hematologic parameters and cellular immunophenotypes revealed an increased percentage of $CD25^+$ B lymphocytes in standard SPF macaques. $CD25$ expression indicates a mature phenotype with increased expression in memory B cells and decreased expression in naive and precursor populations.⁵⁵ $CD25^+$ B cells are also described as having an activated phenotype with improved antigen

presentation capabilities and the ability to trigger CD4⁺ T cell proliferation in mixed lymphocyte reaction cultures.⁵⁶ Alternately and depending on the cytokine environment, CD25⁺ B cells may be tolerogenic, participating in inhibition of the T cell proliferation and cytokine elaboration.⁵⁷ Similar to the increase in the memory T cell pool, a more mature B cell phenotype in standard SPF animals may be attributed to an early and recurring exposure to viral pathogens to which expanded SPF animals are not subject. A complete characterization of the B cell phenotype, including an assessment of additional markers of memory populations, in expanded and standard SPF macaques and the potential contribution to alterations in T cell function require further investigation. Such further studies should also utilize additional markers of T cell activation and proliferation such as HLA-DR and Ki67.

There are limitations to this study. Although randomly selected animals were age and gender matched for each experiment, sampling was restricted to those animals not currently assigned to other studies and not currently in active breeding arrangements. Because samples from all animals were not able to be used for all assays, only a limited examination of correlations between *in vitro* SIV replicative capacity, cytokine production, and cellular immunophenotype was able to be performed. These animals were also not MHC haplotyped, although the random selection of unrelated animals likely limits this as a study confound. Lastly, we do not have data on the response of expanded and standard SPF animals to *in vivo* SIV inoculation. Despite these limitations we have been able to demonstrate the profound impact of SPF status on memory T cell populations and the capacity to support *in vitro* SIV replication. Based on the findings presented here, performance of a subsequent study in which SIV inoculation is performed in MHC haplotyped expanded and standard SPF animals subject to a complete battery of baseline phenotyping may further elucidate the contribution of environmental infectious agent exposure to HIV/SIV progression.

In this study we report significant differences in immune cell populations, cytokine production, and the capacity to support *in vitro* SIV virus replication between cohorts of standard and expanded SPF macaques. Although additional experiments are required, findings here suggest that an altered immunophenotype associated with housing of animals under differential levels of bioexclusion has the potential to impact the outcome of studies using the SIV model. This risk of experimental confounding associated with SPF status may extend beyond nonhuman primate models employed in HIV research to other studies for which the measurement of immunologic endpoints is critical, including models of solid organ transplantation, drug discovery safety assessment, and vaccine development. Results of this study suggest that critical consideration be given to virus exposure history when selecting animals for study inclusion and allocating animals to experimental groups.

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Author Disclosure Statement

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