JLB

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Simian immunodeficiency virus infection and immune responses in the pig-tailed macaque testis

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RECEIVED SEPTEMBER 15, 2014; REVISED NOVEMBER 17, 2014; ACCEPTED DECEMBER 17, 2014. DOI: 10.1189/jlb.4A0914-438R

ABSTRACT

The testis is a site of immune privilege in rodents, and there is evidence that T cell responses are also suppressed in the primate testis. Local immunosuppression is a potential mechanism for HIV persistence in tissue reservoirs that few studies have examined. The response of the pig-tailed macaque testis to SIV_{mac239} infection was characterized to test this possibility. Testes were surgically removed during early-chronic (10 wk) and late-chronic (24–30 wk) SIV infection in 4 animals and compared with those from 7 uninfected animals. SIV infection caused only minor disruption to the seminiferous epithelium without marked evidence of inflammation or consistent changes in total intratesticular leukocyte numbers. Infection also led to an increase in the relative proportion of testicular effector memory $CD8^+$ T cell numbers and a corresponding reduction in central memory $CD4^+$ T cells. A decrease in the relative proportion of resident-type $CD163^+$ macrophages and DCs was also observed. SIV-specific $CD8^+$ T cells were detectable in the testis, 10–11 wk after infection by staining with SIV Gag-specific or Tat-specific MHC-I tetramers. However, testicular $CD8^+$ T cells from the infected animals had suppressed cytokine responses to mitogen activation. These results support the possibility that local immunosuppression in the testis may be restricting the ability of T cells to respond to SIV or HIV infection. Local immunosuppression in the testis may be an underexplored mechanism allowing HIV persistence.
***J. Leukoc. Biol.* 97: 599–609; 2015.**

Introduction

The persistence of HIV, despite ART, which clears virus from the plasma, is a major obstacle to curing this disease. HIV persists as quiescent, integrated DNA, mostly within memory $CD4^+$ T cells [1]. Productively infected cells can also persist in anatomic sanctuaries, such as the CNS [2]. This is presumed to be a result of poor penetration of antiretroviral drugs into the brain. Another potential mechanism for HIV persistence in such regions is immune privilege [3], defined as the local suppression of immune responses that occurs at specific sites. This possibility, however, is currently underexplored. The testis is a tissue where local immunosuppression occurs [4] and is infected by HIV in humans and SIV in macaques [5, 6]. Herein, we examine the immune responses to SIV infection in the macaque testis.

Despite localized immunosuppression, the testis harbors a considerable population of leukocytes. Testicular leukocytes in the primate consist mostly of central and effector memory $CD4^+$ and $CD8^+$ T cells, as well as resident-type $CD163^+$ testicular macrophages and minor populations of monocytes, DCs, and NK cells [7]. The central memory $CD4^+$ T cells, macrophages, and DCs are all potential targets of HIV. There are 2 anatomic compartments in the testis: the seminiferous tubules, in which spermatogenesis occurs, and the interstitium, between the tubules, where the leukocytes reside. Between these anatomic compartments lies the blood-testis barrier, comprised of tight junctions between the epithelial Sertoli cells that constitute the epithelium of the tubules and Sertoli cell expression of multidrug-resistant proteins, such as permeability glycoprotein, which are thought to restrict the access of drugs to the tubules [8, 9].

Historically, the testis has been thought of as an anatomic sanctuary for HIV as a result of the presence of the blood-testis barrier and the reported persistence of HIV in the semen despite antiretrovirals [10–12]. This proposal, however, has never been formally validated or refuted. It is now recognized from vasectomy studies that the testis contributes little to the virus present in seminal plasma [13] and that the blood-testis barrier does not prevent antiretroviral drugs from accessing the infected leukocytes present in the testis [14, 15]. HIV persistence in the testis during ART has not been definitively investigated. SIV

Abbreviations: APC = allophycocyanin, ART = antiretroviral therapy, DC = dendritic cell, Gag = glycosaminoglycan, HEK = human embryonic kidney, IQR = interquartile range, PAS = periodic acid-Schiff, qRT-PCR = quantitative (real-time) RT-PCR, VL = viral load

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DNA, however, has been shown to persist in the macaque testis after 26 wk of combined ART, at higher levels than the CNS, kidney, liver, heart, and bladder but at lower levels than the lymphoid tissue [16]. Regardless of whether the testis is an anatomic sanctuary for HIV, this study addresses the potential for a region of immunosuppression to inhibit responses to the virus.

HIV infection of the human testis is poorly characterized during acute and early infection. Testicular pathology is best described by use of autopsy samples from patients who died of AIDS. Dalton and Harcourt-Webster [17] observed interstitial fibrosis and an arrest of spermatogenesis in testes from such autopsies. Pudney and Anderson [18] reported azospermia, hyalinization of seminiferous tubule boundaries, and significant infiltration of lymphocytes, particularly $CD4^+$ T cells, into the interstitium. SIV infection of macaque testes has been studied in pig-tailed [6] and cynomolgus macaques [5]. SIV infects testicular T cells, macrophages, and possibly DCs, leading to a viral burden of ~100 copies/20 μg testis tissue [6]. SIV infected the testes of cynomolgus macaques during the acute phase, persisted through later phases, and correlated with blood viremia [5].

The testis is known to be a region of immune privilege in rodents, where innate and acquired immune responses are suppressed [4]. Testicular immunosuppression is proposed to protect the immunogenic germ cells from acquired immune responses, as well as minimize inflammation in the testis that could impact spermatogenesis and fertility. Immunosuppression in the rodent testis is thought to be mediated by local factors, such as IL-10 [19], TGF-β [20], IL-12p35 [21], immunosuppressive lysoglycerophosphocholines [22], and other immunosuppressive factors produced by the Sertoli cells [23, 24]. Testicular immunosuppression is best characterized in rodents, where bacterial and viral infections have been studied. Innate immune responses in LPS-treated rats were considerably attenuated in the testis, with hallmark inflammatory cytokines IL-1β and TNF responding poorly to LPS stimulation [25]. These studies are supported by in vitro experiments showing that testicular macrophages have alternatively activated responses, whereby very few proinflammatory cytokines are produced, but IL-10 production is constitutive [19]. The testicular interstitial fluid is capable of inhibiting the T cell-proliferative response to mitogens [22], demonstrating that acquired immune responses can also be inhibited by local factors. Indeed, memory $CD8^+$ T cells homed to testis grafts in a pancreatic islet transplantation mouse model but underwent apoptosis, thereby delaying graft rejection, compared with similar grafts to the kidney graft [26]. Suppression of innate and acquired immune responses by local factors present in the testis may allow persistence of infections, such as HIV in this region.

Although well characterized in rodents, the presence of immune privilege in humans and nonhuman primates has not been established conclusively. Our recent studies in pig-tailed macaques have demonstrated that cytokine responses of testicular T cells to mitogen stimulation are significantly lower than blood T cell responses, consistent with a local suppression of acquired immune responses [7]. The current study makes use of the pig-tailed macaque model of SIV infection to assess the effects of this virus on the testis, as well as the relative proportions and immune responses of testicular leukocytes.

MATERIALS AND METHODS

Reagents and antibodies

The antibodies used in this study have been detailed previously [7], with the exception of anti-CD25 PE-Cy7, clone M-A251, used at 1:20 (557741; BD Biosciences, San Jose, CA, USA), and anti-CD16 APC-Cy7, clone 3GB, used at 1:40 (2110090; ICyt, now Sony Biotechnology, Champaign, IL, USA), as well as anti-CD45, used for immunohistochemistry, clone 2B11+PD7/26, used at 1:500 (M0701; Dako, Glostrup, Denmark). All antibodies were mouse monoclonals raised against human antigens, with the exception of mouse anti-nonhuman primate CD45. PE-labeled tetrameric complexes of MHC-I protein *Mane-A1*084*, folded around SIV peptides (either KP9 from gag or KSA10 or KVA10 from tat), as described previously [27], were made in-house and kindly provided by Professor Andrew Brooks, University of Melbourne, Melbourne, VIC, Australia.

Virus production in vitro

Confluent HEK293T cells were transfected with the SIV-producing pKP55-SIV_{mac239} plasmid [28] with the use of FuGENE (Promega, Madison, WI, USA), according to the manufacturer's instructions. After 48 h, media containing virus were recovered from the culture, and contaminating cells were removed by centrifugation. Media (100 μl) were added to 100 μl fresh RPMI/10% FCS containing 1×10^5 CEM-NKR-CCR5 cells and incubated for 48 h at 37°C. CEM-NKR-CCR5 cells were washed extensively after 48 h to remove the original virus and then cultured for a further 5 days. To confirm SIV infection, qRT-PCR of SIV gag showed an exponential increase in virus production up to at least 7 days (data not shown), as described previously [29].

Infection of animals and tissue sampling

Eleven healthy, young-adult, male pig-tailed macaques (*Macaca nemestrina*) were housed in the Australian Animal Health Laboratory (Geelong, VIC, Australia). Animals were sexually mature, with ages ranging from 4 yr and 2 mo to 6 yr and 2 mo. All animals expressed the *Mane-A1*084* allele, which presents SIV Gag epitopes KP9, KSA10, and KVA10, as determined by MHC typing [30]. The Commonwealth Scientific and Industrial Research Organisation Animal Health Animal Ethics Committees approved all studies.

Four animals were infected with SIV_{mac239} by i.v. injection of 2.5×10^9–1×10^{10}-free virus (as measured by qRT-PCR of SIV gag), produced by HEK293T cells, together with 3×10^5 CEM-NKR-CCR5 cells infected with SIV_{mac239}, in a total volume of 500 μl RPMI/10% FCS. A unilateral orchidectomy was performed under ketamine sedation (1 mg/kg i.m.) and with appropriate follow-up analgesia at 10–11 wk after SIV infection. The 2nd testis was removed at autopsy (24–30 wk after SIV infection). Blood samples were taken regularly (1–4 weekly) until autopsy. Infection of animals was confirmed by qRT-PCR of SIV gag in plasma samples, as described previously [29]. Animals were euthanized with pentobarbitone (0.5 ml/kg i.v.) while under ketamine sedation.

Tissue fixing, processing, and staining

After removal of the testis capsule, ~5 mm^3 pieces of testis were fixed in Bouin's fixative for 4 h, followed by 2, 10 min washes in 80% ethanol, followed by 2, 10 min washes in PBS. Based on initial results, formalin fixation was undertaken to enable CD45 immunohistochemistry to be performed. Testis tissue samples from 1 uninfected and 3 infected animals were fixed in formalin for 4 h, followed by 2 washes in PBS. Fixed samples were processed (2 h of 70% ethanol, 1 h of 90% ethanol, 2 h of 100% ethanol, 2 h of new 100% ethanol, 1 h 100% ethanol, 2×2 h xylene, 2 h paraffin, 2×1 h paraffin, and then 30 min paraffin) by use of a Tissue-Tek VIP6 (Sakura, Torrance, CA, USA), followed by sectioning at 5 μm onto SuperFrost Plus slides. Bouin's-fixed sections were de-waxed and then stained with PAS or Masson's trichrome stain. For PAS staining, slides were incubated in 1% periodic acid for 5 min, Schiff's reagent (Australian Biostain, Traralgon, VIC, Australia) for 10 min, counterstained in Mayer's hematoxylin for 5 min, and then 30 s in Scott's tap water. For Masson's trichrome, slides were incubated in Bouin's

fixative for 60 min, washed in water 10 min, 1% Weigerts iron hematoxylin for 2 min, 1% Ponceau/1% acid fuchsin in 1% acetic acid for 5 min, 1% phosphomolybdic acid for 3 min, 2% light green in 1% acetic acid for 5 min, and then finally, acetic acid for 1 min. All slides were dehydrated and mounted by use of distyrene, a plasticizer, and xylene (D.P.X.). Slides were scanned by use of an Aperio system (Leica Biosystems, Nussloch, Germany).

Immunohistochemistry

Immunohistochemistry for CD45 on formalin-fixed sections was performed similar to previous studies [31]. In brief, slides were de-waxed and rehydrated before antigen retrieval of 10 mM sodium citrate, pH 6, with the microwave on defrost for 15 min. Slides were cooled, incubated with 3% H_2O_2 for 10 min, and then blocked by use of serum-free protein block (X0909; Dako) for 10 min, followed by an overnight primary antibody (0.75 μg/ml anti-CD45) at 4°C. The negative control was a matched isotype mouse IgG1 (X0931; Dako) used at the same concentration. Detection used the REAL EnVision peroxidase/diaminobenzidine+ (K5007; Dako) system, according to the manufacturer's instruction, by use of 1 h incubation of secondary antibody. Slides were counterstained with hematoxylin, dehydrated, mounted, and scanned by use of an Aperio system, as above. To quantify the numbers of $CD45^+$ cells/area, images from random areas of sections were recorded by use of the Aperio software. A minimum of 6 images covering >14 mm^2 was used. The number of positive-staining cells in this region was counted, and data presented as the number of cells/mm^2.

Isolation of PBMCs and testicular cells

Interstitial cells were isolated from testes of macaques, as described previously [7]. In brief, after decapsulation, tubules were chopped by use of a scalpel blade and then incubated at 37°C with collagenase and DNase for 1 h. Tubule fragments were allowed to settle, followed by recovery of interstitial cells in the media above the tubules. PBMCs were isolated as described previously [7]. PBMCs and testicular interstitial cells were frozen for long-term storage in liquid nitrogen and thawed, as described previously [7].

Phenotypic analysis of testicular and blood leukocytes

Whole blood (100 μl) or testicular interstitial cells in PBS/0.5% BSA/2 mM EDTA (5×10^6 cells) were incubated with 0.2 μl LIVE/DEAD Aqua (Invitrogen, Carlsbad, CA, USA) for 30 min and then with antibody cocktails for 20 min. Testicular cells were washed in PBS/0.5% BSA/2 mM and fixed in stabilizing fixative (BD Biosciences). Blood samples were incubated in FACS lysing solution (BD Biosciences) for 10 min, followed by 2 washes in PBS/0.5% BSA/2 mM EDTA and fixing with stabilizing fixative. Staining for CD4 T cells, together with the number of leukocytes present in whole blood [counted by use of a CELL-DYN Emerald cell counter (Abbott Diagnostics, Lake Forest, IL, USA)], was used to calculate the absolute $CD4^+$ T cell numbers in **Table 1**. For tetramer staining, cells were first incubated with LIVE/DEAD Aqua stain for 30 min, followed by 1/500 dilution of tetramer in PBS for 40 min and then with antibody cocktail for 30 min. All incubations were performed at room temperature in the dark after mixing by gentle vortex, and centrifugation was performed at 500 g for 5 min. Flow cytometry was performed on an LSR Fortessa or LSRII flow cytometer (BD Biosciences), and data were analyzed by use of FlowJo Version 9.6 (TreeStar, Ashland, OR, USA). Fluorochrome compensation correction was performed by use of the autocompensation function in DIVA 6.1 by use of Calibrite beads (BD Biosciences) for FITC, PE, PerCP, APC, CompBead, or CompBead Plus anti-mouse Ig κ capture beads (BD Biosciences) for other fluorochromes and ArC beads (Invitrogen) for LIVE/DEAD Aqua. Testicular or blood populations were gated according to scatter properties, aggregates and dead cells were excluded, and $CD45^+$ cells were gated to analyze leukocytes.

Intracellular cytokine staining assay

IFN-γ and TNF production by blood and testicular cells was measured by an intracellular cytokine-staining assay similar to assays described previously [32]. Blood (100 μl) or 100 μl 5×10^6 testicular interstitial cells in RPMI/10% FCS were mixed with anti-CD28 and anti-CD49d at 1 μg/ml each, brefeldin A (10 μg/ml), and stimulated with PBS (controls) or a mix of PMA (2 μg/ml; Sigma-Aldrich, St. Louis, MO, USA), PHA (15 μg/ml; Sigma-Aldrich), and *Staphylococcus* Enterotoxin-B (1 μg/ml; Sigma-Aldrich). Cells were incubated at 37°C for 6 h, followed by storage at 4°C overnight. LIVE-DEAD Aqua (0.5 μl) was added for 30 min, followed by anti-CD3 APC-Cy7, CD4 AF700, CD8 PerCP, and CD45 V450 for 20 min. Testicular cells were fixed by incubation in 1% formaldehyde/PBS. Blood was treated with FACS lysing solution for 10 min, followed by washing in PBS/0.5% BSA/2 mM EDTA. All cells were permeabilized by use of FACS permeabilizing solution (BD Biosciences) for 10 min, washed in PBS/0.5% BSA/2 mM EDTA, and then incubated with anti-IFN-γ APC and TNF PE-Cy7 for 60 min. Cells were washed and then fixed in stabilizing fixative. All incubations were performed at room temperature in the dark after mixing by gentle vortex. Analysis by flow cytometry was performed as described above.

TABLE 1. VL, blood CD4 counts, and $CD45^+$ counts in the testes of uninfected and SIV-infected, pig-tailed macaques

Animal	Time-point	log VL[a]	CD4 count[b]	$CD45^+$ cells[c]
1024	–	NA[d]	ND[e]	ND
26300	–	NA	ND	ND
26783	–	NA	ND	ND
B3099	–	NA	ND	ND
C3752	–	NA	ND	ND
C3763	–	NA	ND	ND
35414	–	NA	ND	18.5
19340	Week 0	<3.2[f]	2398	NA
	Week 10	4.7	673	14.5
	Week 24	5.6	132	17.4
36142	Week 0	<3.2	1983	NA
	Week 11	5.6	130	7.1
	Week 28	5.6	45	10.2
5878	Week 0	<3.2	2320	NA
	Week 10	3.5	1224	19.1
	Week 27	<3.2	840	16.6
C3767	Week 0	<3.2	ND	NA
	Week 11	5.3	196	ND
	Week 30	5.8	238	ND

[a]VL in $\log_{10}$ copies of SIV gag/ml plasma. [b]The absolute number of $CD45^+CD3^+CD4^+$ cells/mm^3 whole blood. [c]Number of $CD45^+$ cells/mm^2, as detected by immunohistochemistry (see Fig. 2). [d]NA, Not applicable, as there was no VL in uninfected animals. [e]ND, Not done. [f]Minimum detection level in this assay: 3.2 copies/ml.

Statistical analyses

Descriptive statistics are quoted in the text as median and IQR or range, where the group has 3 or less data points. Statistical tests were not performed for these data as a result of the low sample size of the infected group ($n = 3$ or 4) and the mix of paired and unpaired data in each graph. The ability to undertake statistical analysis were complicated further by the presence of a treated animal that rapidly controlled the virus after infection.

RESULTS

SIV infection disrupts macaque seminiferous tubules

Testes were recovered from 4 pig-tailed macaques infected with SIV_{mac239} at 2 time-points: early chronic (10–11 wk) and late chronic (24–30 wk). Each animal had a peak of plasma VL within 2 wk of SIV infection (Table 1, and data not shown). One animal (Number 5878) controlled the virus, with very low plasma

VL at wk 10 and no detectable VL at 27 wk. Infected animals exhibited a drop in absolute CD4 T cell numbers in the blood, which was less apparent in the animal that controlled the virus (Table 1). Testes from 7 uninfected pig-tailed macaques were used for comparison, as well as matched blood samples taken at the same time-points from uninfected and SIV-infected macaques.

SIV infection caused only minor disruptions to the seminiferous epithelium, even after 24–30 wk of infection (**Fig. 1** and Table 1). In the infected animals, there were many regions where spermatogenesis looked normal, similar to the uninfected testes (Fig. 1A and B). However, discrete regions could be found where seminiferous tubules appeared abnormal, with disordered spermatogenesis, increased cellular vacuolation, reduction in epithelial thickness and cellularity, and the volume of the tubule lumen (Fig. 1B and D). Conspicuously, there was no obvious progression of this damage between 10 and 11 wk and 24 and 30 wk. Affected tubule profiles were also seen in the SIV-controlling animal, despite very low or no SIV plasma VL at the time. No tubules with this specific phenotype could been found in any of the testis sections from uninfected animals. Damage to tissues through chronic inflammation can result in fibrosis, detected in the testis after damage as a result of vasectomy or cryptorchidism (undescended testes) [33, 34]. To detect fibrosis, collagen fibers were stained by use of Masson's trichrome in the testes of SIV-infected and uninfected animals (Fig. 1E–H). Each tubule was girt by collagen fibers, and lobes of tubules were separated by thicker regions of collagen fibers. There was no marked increase in collagen in the SIV-infected testes around damaged tubules themselves or even in areas where several damaged tubules occurred (Fig. 1G and H).

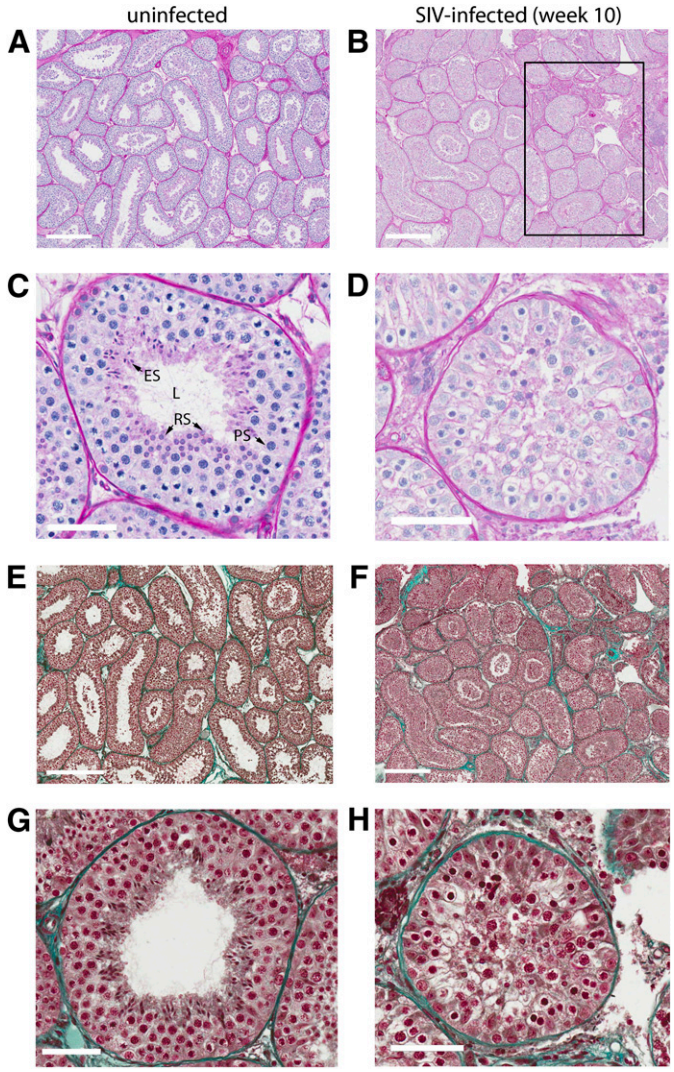

Figure 1. Histology of uninfected and SIV-infected macaque testes. Bouin's-fixed testes were sectioned and stained with PAS (A–D) or Masson's trichrome (E–H). PAS-stained sections show seminiferous epithelium morphology of uninfected (A and C) and SIV-infected animals at wk 10 (B and D). (B) A region is shown (in the square) in which abnormal tubules are observed, showing disordered spermatogenesis and reduced tubule and lumen diameter. (C) ES, Elongated spermatids; RS, round spermatids; PS, pachytene spermatocytes; L, lumen. Bars represent 300 μm (A and B) and 60 μm (C and D). (D) An example of a tubule with disordered spermatogenesis and cellular vacuolization. Green staining in Masson's trichrome-stained sections shows collagen fibers in testes from uninfected (E and G) and SIV-infected animals (wk 10; F and H). Bars represent 200 μm (E and F) and 60 μm (G and H).

Testicular leukocyte numbers after SIV infection

Immunohistochemistry for CD45 confirmed that leukocytes were present in the testes of uninfected and SIV-infected animals (**Fig. 2**). Leukocytes were only found in the interstitial region and never inside tubules (Fig. 2A–D). Staining on only 1 uninfected animal could be performed; this animal had 18.5 $CD45^+$ leukocytes/mm^2 testis section (Table 1). This limitation means that we cannot estimate the normal range of $CD45^+$ cells/mm^2 testis section. The infected animals had $CD45^+$ cells ranging from 7.1 to 19.1 cells/mm^2 testis section (Table 1). With this limited data set, we have not observed an obvious reduction in $CD45^+$ cell number. These data are consistent with the absence of inflammatory leukocytic infiltrates in the testis sections of the infected animals.

Testicular T cell phenotype changes with SIV infection

SIV infection of pig-tailed macaques is known to result in a gradual loss of $CD4^+$ T cells in the peripheral blood, but a more dramatic, early loss of $CD4^+$ T cells in lymphatic tissues [35]. The fate of $CD4^+$ T cells from within testicular interstitial cells has not been reported previously. We found that SIV infection led to a dramatic reduction in the relative proportion of $CD4^+$ T cells from the testis within 10–11 wk of infection (**Fig. 3A** and **B**). $CD4^+$ T cells fell from a median of 40.7% (IQR 12.5%) of recovered $CD3^+$ cells in uninfected animals to 5.2% (IQR 20.5%) during early chronic infection and 9.6% (IQR 28.7%) during late chronic infection. $CD4^+$ T cells were also reduced slightly in the blood of infected animals. The SIV-controlling animal was not as severely affected as the others (Fig. 3B, triangles). Accordingly, the proportion of recovered testicular $CD3^+$ T cells that was $CD8^+$ increased considerably with SIV infection (Fig. 3A and C). $CD8^+$ T cells increased from a median of 40.3% (IQR 16.5%) of leukocytes in uninfected animals to 75.8% (IQR 20.4%) during early chronic infection and 76.7% (IQR 15.1%) during late chronic SIV infection.

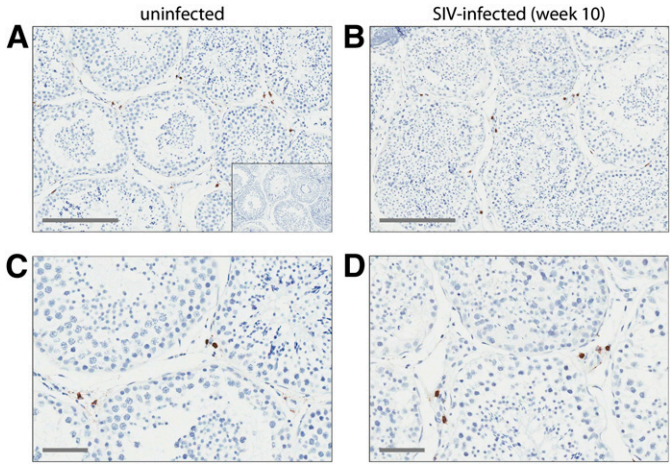

Figure 2. CD45 immunohistochemistry of uninfected and SIV-infected macaque testes. Formalin-fixed testes were sectioned and immunohistochemistry performed to detect CD45, followed by hematoxylin counterstain of testes from uninfected (A and C) and SIV-infected animals (wk 10; B and D). Positive cells can be seen stained brown in the interstitial regions only. Bars represent 200 μm (A and B) and 60 μm (C and D). An isotype-matched, negative control is shown (A, inset).

The phenotyping of macaque T cells by use of CD28 and CD95 surface expression allows classification as naïve, central memory or effector memory T cells [36]. After SIV infection, $CD4^+$ T cell phenotypes in the testis were more likely to be naïve or effector memory, with a loss of central memory $CD4^+$ T cells, as would be expected during SIV infection (Fig. 3D and E). $CD4^+$ T cells were likewise altered in the blood (Fig. 3F). These CD4 T cell data are limited though, as in some samples, there were a relatively low numbers of CD4 T cells in the infected animals. The data points (Fig 3E) do cluster tightly within groups, indicating that the biologic replicates were similar, regardless of the number of cells analyzed. $CD8^+$ T cells in the testis were skewed toward effector memory phenotypes after infection and away from central memory, with the proportion of naïve cells unchanged (Fig. 3D and G). Similar but less pronounced changes were seen in the blood $CD8^+$ T cell memory phenotypes (Fig. 3H).

The changes in $CD8^+$ and $CD4^+$ T cells described above represent changes to the relative proportions of $CD3^+$ cells with these phenotypes; however, the effect on the absolute numbers of $CD4^+$ and $CD8^+$ T cells in the testis cannot be established by these data alone. Flow cytometric analyses showed that the proportion of leukocytes expressing $CD3^+$ increased in the testis samples during early and late chronic SIV infection (**Fig. 4A** and **B**). Uninfected animal testes had a median of 35.4% (IQR 11.4%) leukocytes expressing CD3, whereas 10–11 wk SIV-infected animals had 54.2% (IQR 21.8%), and 24–30 wk infected animals had 53.8% (IQR 7.5%) $CD3^+$ T cells among leukocytes. SIV infection did not lead to such a marked increase in $CD3^+$ cells in the blood (Fig. 4C). Given that the CD45 immunohistochemistry suggested minimal substantial changes in overall number of testicular leukocytes after infection, the flow cytometry data suggest a loss of $CD4^+$ T cell numbers and an increase in the absolute numbers of $CD3^+$ and $CD8^+$ T cells in the SIV-infected testis.

SIV infection changes the macrophage, granulocyte, NK, and DC populations of the macaque testis

Given the marked changes observed in T cell subsets within the testes, we asked whether other leukocyte subsets were also perturbed, reflective of a broader immune dysregulation in the SIV-infected macaque testes. The proportion of recovered $CD45^+$ cells in the uninfected testis that were $CD3^-CD20^+$ B cells was a median of 0.364% (IQR 0.18%), which was very low compared with matched blood samples (median 15.5%, IQR 2.8%). As B cells do not enter the testis under normal conditions, these cells presumably represent cells present within the blood vessels of the testis. The proportion of testicular B cells among leukocytes remained low with infection, with a median of 0.4% (IQR 0.6%) at the 10–11 wk time-point and 0.9% (IQR 0.5%) during late chronic infection. Very low B cell numbers in the testis samples indicate that blood contamination of the testicular interstitial cell populations is minimal.

The proportion of recovered leukocytes that expressed the "resident" macrophage marker CD163 decreased in the testis, from a median of 43.5% (IQR 17.1%) in uninfected animals to 24.8% (IQR 14.8%) by 10–11 wks of infection and 15.2% (IQR 12.6%) by 24–30 wks of infection (Fig. 4A and B). The SIV-controlling animal (Fig. 4, triangles) was not as severely affected as the other animals at the chronic time-point. The proportion of recovered leukocytes negative for CD3 and CD163 was ~25% and was not affected by SIV infection. Curiously, CD163 surface expression was found in the blood of 1 animal at the latest time-point (Fig. 4C). DCs were a very small proportion of testicular leukocytes, with a median of 0.7% (IQR 1.2%) of recovered leukocytes in the uninfected testis. During early chronic infection. this fell to a median of 0.1% (range 0.06–0.45%) and rose to a median 0.3% (IQR 0.07%) by late chronic infection. In contrast, the proportion of DCs in the blood did not undergo any notable change with infection.

The granulocyte population of the testis was detected by gating on $CD3^-$ cells with high side-scatter (Fig. 4D). Very few granulocytes were present in uninfected testes (median 3.4%, IQR 2.1% of testicular leukocytes). As granulocytes do not enter the testis under normal conditions, these cells presumably represent granulocytes present within the blood vessels of the testis. Infection had no effect on this population during the early chronic phase (median 4.7%, IQR 3.2%) but led to a small increase in the proportion of granulocytes among recovered testicular leukocytes at the late chronic stage (median of 8.1%, IQR 15.47%). NK cells in the testes were detected as $CD159^+$ ($NKG2a^+$) among the $CD3^-CD163^-$ population. The proportion of NK cells among testicular leukocytes in uninfected testes was a median of 5.3% (IQR 9.9%) compared with blood (median 12.2%, IQR 10.4%). Testis NK cell levels increased with infection, with a median of 18.3% (range 15.7–18.4%) at the early time-point and 13.6% (IQR 8.9%) during late chronic infection, with similar changes seen in the blood.

SIV-specific $CD8^+$ T cells in the testis during early SIV infection

SIV-specific T cell responses may assist in the local control of SIV infection but have not been studied previously in the

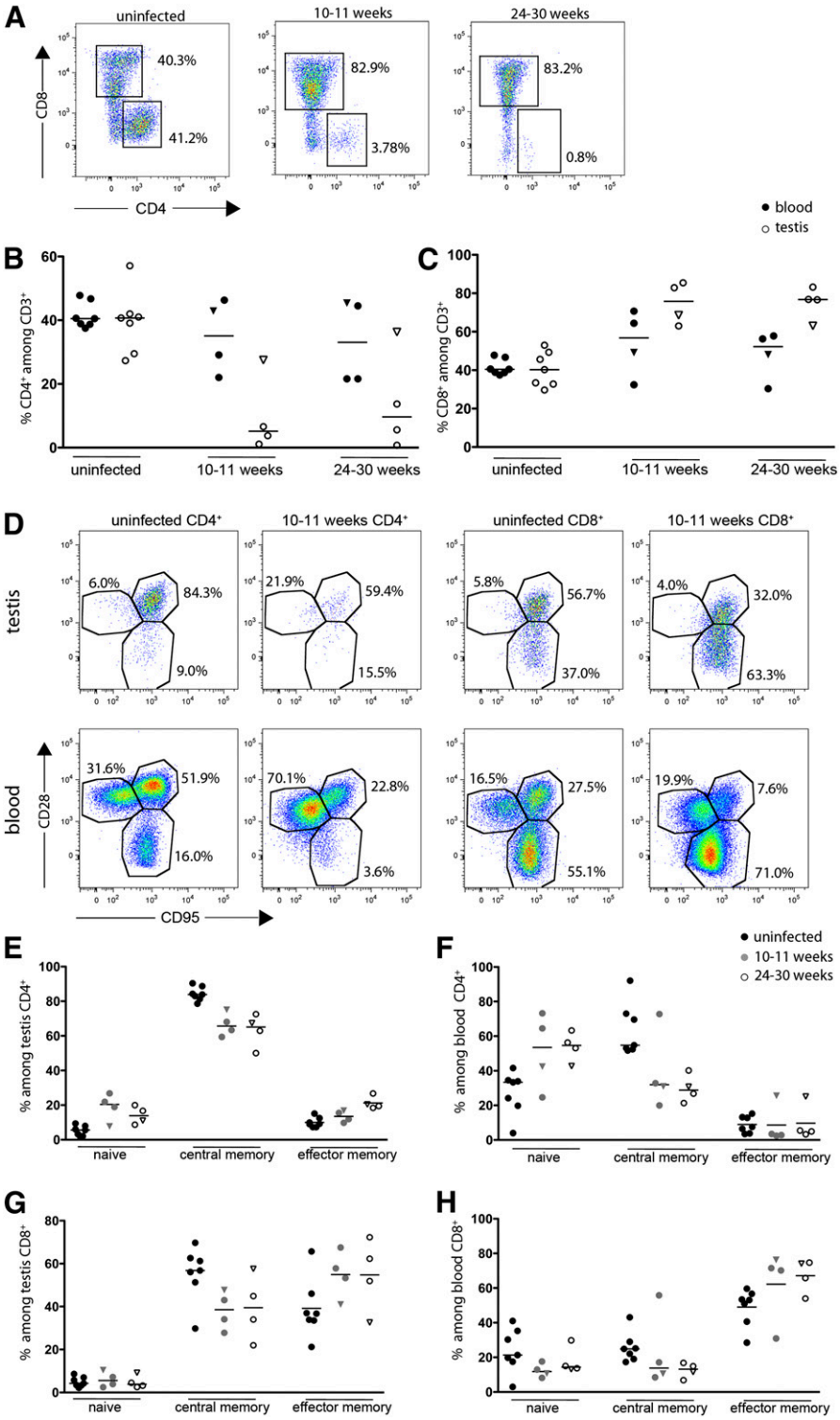

Figure 3. T Cell phenotypes in the testes and blood. Flow cytometry was used to determine the proportion of $CD3^+$ leukocytes that was $CD4^+$ T cells or $CD8^+$ T cells. Cells were gated for live, single, $CD45^+$, and $CD3^+$ before the plots shown. (A) Examples of CD4 and CD8 staining for uninfected, 10–11 wk and 24–30 wk infection timepoints for testicular leukocytes. Proportion of $CD45^+CD3^+$ cells from blood or testicular interstitial cell preparations that were $CD4^+$ T cells (B) or $CD8^+$ T cells (C) is shown for all animals. (D) Examples of gating on $CD4^+$ T cells (left 2 rows) or $CD8^+$ T cells (right 2 rows) for memory populations by use of CD28 and CD95 expression, as described previously [36]. Cells that were $CD28^+CD95^-$ were gated as naïve, $CD28^+CD95^+$ as central memory, and $CD28^-CD95^+$ as effector memory (D). $CD4^+$ T cell memory populations in the testis (E) and blood (F) and $CD8^+$ T cell memory populations in the testis (G) and blood (H) are shown for all animals. (E) It should be noted that some data points had relatively low cell numbers as a result of the low numbers of CD4 T cells present in the testes of infected animals. Horizontal lines on the graphs denote median values. Triangles on graphs denote the SIV-controlling animal.

testes. All animals in this study expressed the *Mane-A1*084* MHC-I allele, which presents a known SIV Gag epitope (KP9) and two Tat epitopes (KSA10 and KVA10) [30]. SIV-specific cells were detected in the testis by use of a labeled tetramer of the SIV peptide and this MHC-I. **Figure 5** shows detection of SIV-specific $CD8^+$ T cells in the testes and blood of infected animals, i.e cells that have TCRs that bind the SIV peptide/MHC-I tetramer. SIV-specific cells that bound the SIV peptides KP9 and KSA10 were present in the testis during early chronic infection, with numbers reduced by late chronic infection, whereas KVA10-specific $CD8^+$ T cells were not detectable. The proportion of $CD8^+$ cells that was SIV specific for these peptides in the testis was

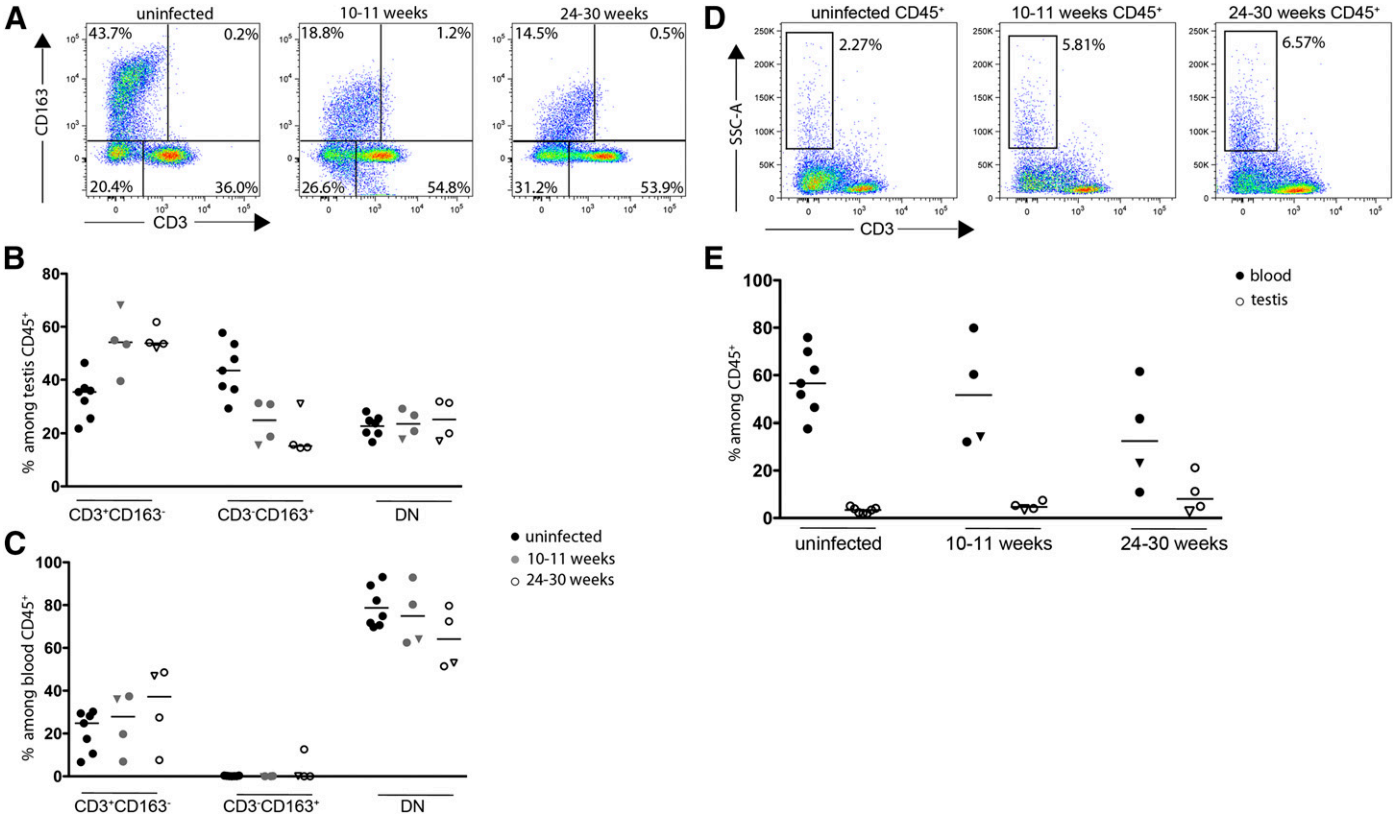

Figure 4. Leukocyte populations in the testis and blood. Flow cytometry was used to determine the proportion of leukocytes that were T cells, macrophages, and granulocytes in the blood and testicular interstitial preparations. Cells were gated for live, single, $CD45^+$ cells before the plots shown. Examples of CD163 (resident macrophage marker) and CD3 (T cell marker) detection for uninfected, 10–11 wk and 24–30 wk infection time-points show gating of these populations (A). Proportion of $CD45^+$ cells that were T cells ($CD3^+CD163^-$), resident macrophages ($CD3^-CD163^+$), or neither [double negative (DN)] is shown for the testis (B) and blood (C) of all animals. (D) Examples of gating for granulocytes as $CD3^-$ cells with high side-scatter [(SSC)-area (A)] from the $CD45^+$ population in the testicular interstitial cells, as described previously [7]. The proportion of $CD45^+$ cells that were granulocytes for all animals is presented for blood and testis (E). Horizontal lines on the graphs denote medians. Triangles on graphs denote the SIV-controlling animal.

similar, and in some cases, higher than in the blood at the same time-points (Fig. 5B and C). These results show that $CD8^+$ T cells are found in the infected testis at similar levels to the blood, peaking 10–11 wks after infection.

$CD8^+$ T cell cytokine responses

Our previous study in macaques demonstrated that significantly less testicular T cells produced cytokines after mitogen stimulation than did blood T cells [7]. An intracellular cytokine-staining assay was used in the present study to detect IFN-γ and TNF production by T cells in uninfected and SIV-infected animals after mitogen stimulation (**Fig. 6**). The proportion of $CD4^+$ T cells (median 1.9%, IQR 6.1%) and $CD8^+$ T cells (median 5.8%, IQR 4.4%) producing cytokines (IFN-γ, TNF, or both) after stimulation in SIV-uninfected testes was distinctly lower in the testis than the blood (13.0%, IQR 9.8% for $CD4^+$ T cells and 13.5%, IQR 3.1% for $CD8^+$ T cells; Fig. 6A–C). SIV infection reduced the proportion of $CD4^+$ T cells responding to stimulation in the blood, and levels remained low in the testis (Fig. 6B). In the blood and testis, the proportion of responding $CD8^+$ T cells was reduced by infection, but the effect on the testis was much more profound. Only 2.2% (range 1.5–3.2%) testicular $CD8^+$ T cells produced cytokines upon stimulation by 10–11 wk of infection compared with 8.0% (range 7.4–17.0%) in the blood. Furthermore, almost no $CD8^+$ T cells or $CD4^+$ T cells from the 24–30 wk infected animals could be activated (Fig.6A–C), with the exception of the SIV-controlling animal, which was not as strongly affected by infection in this assay. These results demonstrate that considerably less testicular T cells were capable of effective immune responses than T cells from the blood and that SIV infection reduced T cell responses further, essentially ablating them by chronic infection.

DISCUSSION

These experiments have characterized the effects of SIV infection on the macaque testis and provided evidence for a localized suppression of $CD8^+$ T cell responses in this region. SIV infection reduced the numbers of central memory $CD4^+$ T cells, macrophages, and DCs in the testis and led to an increase in effector memory $CD8^+$ T cells. Although SIV-specific $CD8^+$ T cells were present in the infected testes at similar proportions

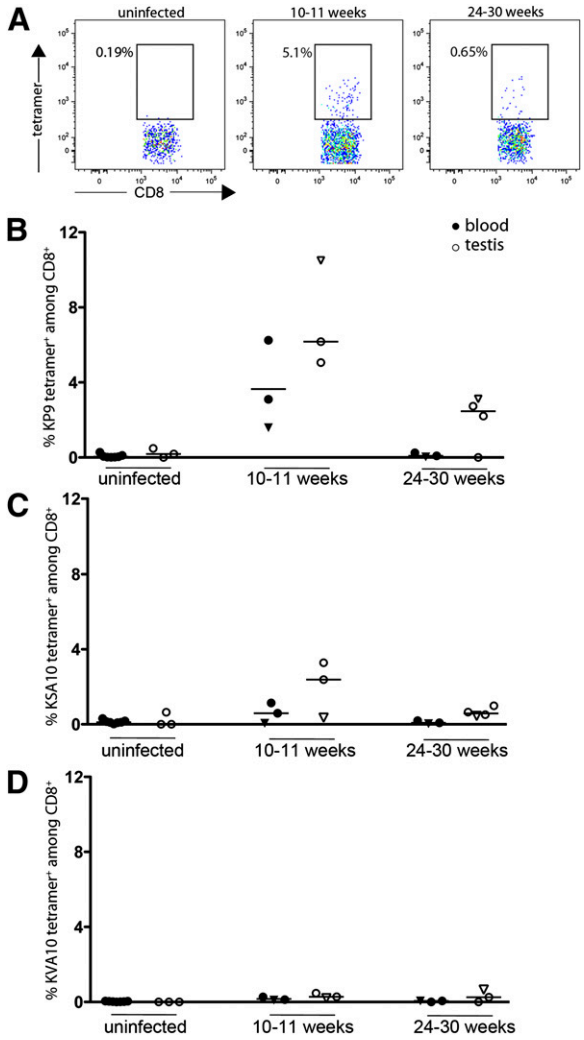

Figure 5. SIV-specific CD8$^+$ T cells in the testis and blood. SIV-specific cells were detected in the testis by use of a PE-labeled tetramer of SIV peptides (KP9 from gag; KSA10 and KVA10 from tat) and *Mane-A1*084* MHC-I protein. Blood and testicular interstitial cells were gated for live, single, CD45$^+$, CD3$^+$, CD8$^+$ cells before the plots shown. (A) Examples of KP9 tetramer staining of testicular interstitial CD8$^+$ T cells from uninfected, 10–11 wk and 24–30 wk infection time-points. Shown is the proportion of SIV-specific (tetramer-positive) cells among CD8$^+$ T cells in the testis and blood in all animals for the SIV peptides KP9 (B), KSA10 (C) and KVA10 (D). Horizontal lines on the graphs denote medians. Triangles on graphs denote the SIV-controlling animal.

to those found in the blood, the capacity of CD8$^+$ T cells from the testis to be activated was reduced severely with SIV infection. These data imply that local suppression of immune responses in a region of immune privilege may be reducing the ability of CD8$^+$ T cells to respond to SIV or HIV.

In this study, the small sample size ($n = 4$) for many comparisons unfortunately limits the conclusions that we can make and make it impossible to use valid statistical tests. Many differences in cell phenotypes, cytokine production, and SIV-peptide-tetramer staining were large, obvious, and reproducible; however, more subtle differences may have gone unnoticed as a result of the small sample sizes. Furthermore, the memory phenotyping of testicular CD4$^+$ T cell subsets (Fig 3D and E) was compromised by some samples having low numbers of cells, as a result of the reduction in CD4$^+$ T cell numbers with SIV infection.

Damage to the seminiferous tubules may have occurred as a result of early inflammatory responses to the infection or of prolonged effects of chronic infection. All 4 infected animals had high peak SIV viremia early in infection, although 1 of the animals in this study controlled viremia, with very low levels at wk 10 of infection and no viremia by the late time-point. For the

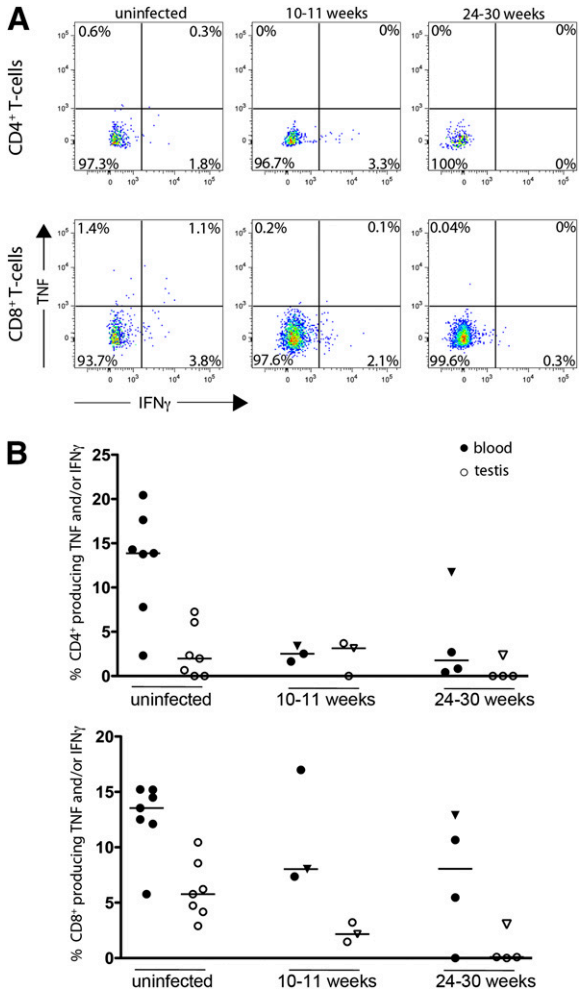

Figure 6. Cytokine production by testicular and blood T cells after mitogen stimulation. Blood or testicular cells from uninfected or SIV-infected animals were stimulated with 2 μg/ml PMA, 15 mg/ml PHA, and 1 mg/ml *Staphylococcus* Enterotoxin-B for 6 h. T cells producing TNF, IFN-γ, or both cytokines were detected by use of an intracellular cytokine staining assay. (A) Examples of gating on testicular interstitial cells expressing TNF, IFN-γ, or both cytokines after stimulation. Before these plots, cells were gated on live, single CD45$^+$ and CD3$^+$ cells, as well as CD4$^+$ (upper) or CD8$^+$ (lower). The proportion of CD4$^+$ cells (B) and CD8$^+$ cells (C) producing TNF, IFN-γ, or both cytokines was calculated for all animals by use of the percentage of cells that were producing either or both cytokines. Horizontal lines on the graphs denote medians. Triangles on graphs denote the SIV-controlling animal.

purposes of this study, this animal was considered to be "infected," but each graph was depicted as a triangle to distinguish it from the other 3 that had much higher plasma SIV at both time-points. Interestingly, this "controller" animal shared some characteristics with the other 3 infected animals but not in all analyses. For instance, the controller had a damaged testis tubule phenotype, increase in $CD8^+$ T cell numbers (Fig. 4C), and SIV-specific $CD8^+$ T cells in the testis (Fig. 5B–D), similar to the other infected animals. However, this animal did not have the loss of $CD4^+$ T cells (Fig. 4B) or the severe loss of $CD8^+$ T cell cytokine production upon activation (Fig. 6B) that the other infected animals displayed. These results suggest the testable hypothesis that early viremia is sufficient to affect spermatogenesis and lead to an influx of $CD8^+$ T cells.

The lack of marked total leukocyte infiltration into the testis, minimal influx of neutrophils, modest damage to testicular architecture, and no accumulation of collagen after SIV exposure indicates that SIV infection induces minimal inflammation in the testis. The time-points used in this study (10–11 wks and 24–30 wk), however, may not be optimal for observing acute inflammatory and innate immune responses. Both HIV and SIV lead to innate immune responses in other regions that include release of type 1 IFNs and TNF by DCs, activation of NK cells, and the release of proinflammatory and immunoregulatory factors [37]. These responses may have occurred in the testis at earlier time-points than we studied. A previous macaque study, however, is consistent with the lack of inflammatory damage seen here. Le Tortorec et al. [5] report that acute SIV infection of cynomolgus macaques did not lead to major changes in tubule morphology when examined after 2 wk of infection, despite the presence of SIV in the testis. Together the present results and those of Le Tortorec et al. [5] indicate that SIV does not lead to major inflammatory damage in the testis of macaques; however, this needs to be confirmed by studies specifically examining inflammation and innate immune responses in the testis. Overall, the minimal inflammatory response to SIV in the macaque testis is consistent with immunosuppression and therefore, a degree of immune privilege in the primate testis.

The most notable effect of SIV on testicular leukocyte populations was a dramatic loss of $CD4^+$ T cells, particularly those with a central memory phenotype. In the blood, the loss of $CD4^+$ T cells was not as pronounced at the same time-points. $CD163^+$ macrophages also appeared to be reduced in the testis after infection, as well as DCs. This was not unexpected, as these cells are all potential targets of SIV; however, it is unlikely that all of these cells are infected. Indeed, in a previous study from our group, SIV-infected $CD4^+$ T cells, macrophages, and DCs could be detected among testicular leukocytes, but they were only a small proportion of these cells in the testis [6]. One likely explanation for the dramatic loss of testicular $CD4^+$ T cells is SIV-induced bystander apoptosis [38, 39].

With the use of specific MHC-I tetramers, we observed SIV-specific $CD8^+$ T cells in the testes of infected animals at similar levels to the blood. The presence of these antigen-specific cells does not indicate whether they are activated or capable of being activated to control infection. Our previous study demonstrated that significantly less $CD8^+$ T cells in the testis could produce cytokines upon antigenic stimulation compared with $CD8^+$ T cells in the blood [7]. In this study, we found that SIV infection reduced the ability of all testicular CD8 T cells to become activated and express cytokines. These results indicate that testicular $CD8^+$ T cell responses are generally inhibited during SIV infection. An analysis of the cytokine responses of the SIV-specific $CD8^+$ T cells from the testis will provide more definitive information on SIV-specific immune responses in the testis during future studies. Two important studies of immune responses in the mouse testis are mostly consistent with the present results. During pancreatic islet allografts in the mouse testis, $CD8^+$ T cells homed to the testis but underwent apoptosis instead of activation [26]. Nasr et al. [40] found that the testis attracted less antigen-specific $CD8^+$ T cells and more $CD4^+CD25^+$ regulatory T cells during transplantations compared with other sites. It is possible that regulatory T cells influx into the macaque testis with SIV infection, although experiments that use CD25 staining showed no convincing increase in $CD25^+$ cells in the testis (data not shown). A more detailed study is required to determine whether regulatory T cells are present in the macaque testis at basal levels and after infection.

Whereas the existence of immune privilege in the rodent testis is well characterized, whether immune privilege exists in humans or nonhuman primates has not been fully investigated. Immunosuppression in the rodent testis is predicted to protect germ cells from autoimmune attack, as they are immunogenic [41]. Evidence in favor of immunosuppression in the primate testis includes the findings that 1) germ cells are protected from autoimmune attack, which can lead to infertility [42]; 2) local injury of seminiferous tubules caused by fine-needle biopsies causing tubule injury in humans does not cause orchitis [43]; 3) T cell responses in the macaque are relatively weak [7]; and 4) human germ cells tolerate early HIV infection with little response [44]. There is, however, some evidence against immune privilege in the testis, namely the rejection of thyroid tissue transplants by macaque testis [45] and the rejection of human testis tissue transplanted into the mouse [46]. It is not known whether germ cells are immunogenic in primates; they may be protected from autoimmune attack as a result of thymic, or central, tolerance instead. The present study showing that activation of $CD8^+$ T cell cytokine responses is very low in the SIV-infected testis and absent at the late time-point provides further evidence in favor of local immunosuppression in the primate testis.

The persistence of HIV during antiretroviral treatment is a major barrier to HIV cure. Herein is examined the possibility that regions of local immune suppression, such as sites of immune privilege, might diminish the responses to SIV in the macaque model. Our results indicate that despite an early SIV-induced reduction in $CD4^+$ T cells in the testes, $CD8^+$ T cell responses are substantially inhibited during SIV infection. Exploring the mechanism by which this occurs may allow immunomodulatory approaches to control active and quiescent HIV infection in the testes.

AUTHORSHIP

W.R.W. contributed to the conception and experimental design, acquisition of data, analysis of data, and drafting of the manuscript. S.B.L., S.A., and T.H.A. contributed to the acquisition of data. R.D.R. contributed to the experimental design, acquisition, and analysis of data. M.P.H., J.E.G., and S.J.K. contributed to the experimental design, interpretation of data, and drafting of the manuscript. All authors approved the final version.

ACKNOWLEDGMENTS

This work was supported by Australian National Health and Medical Research Council (NHMRC) fellowships (1013221, 1041832, 1020269, and 1011578), NHMRC Program Grant 1052979, and the Victorian government's Operational Infrastructure Support Program. The authors thank Hyon-Xhi Tan, Shayarana Gooneratne, Marit Kramski, Caroline Fernandez Megan Schepers, and Lisa Reid for technical assistance. The authors also thank the staff of the Biomedical Science Histology Facility (University of Melbourne) and the Royal Melbourne Hospital Tissue Bank for expert tecnical assistance.

DISCLOSURES

The authors have no conflicts of interest to declare.

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KEY WORDS:
$CD4^+$ T cells · $CD8^+$ T cells · immune privilege · HIV · testicular macrophages