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Characterisation of macaque testicular leucocyte populations and T-lymphocyte immunity

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ABSTRACT

The rodent testis is well established as a site of immune privilege where both innate and acquired immune responses are suppressed. Immune cells and responses within human or non-human primate testes, by contrast, are poorly characterised. This study used multi-colour flow cytometry to characterise the leukocytes in testicular cells isolated from 12 young adult pigtail macaques (*Macaca nemestrina*) by collagenase dispersal, and to measure the cytokine responses of macaque testicular T-lymphocytes to mitogens. B-lymphocytes and granulocytes were present in very low numbers (0.24% and 3.3% of leukocytes respectively), indicating minimal blood contamination. A median of 30.8% of the recovered testicular leukocytes were CD3+ lymphocytes, with CD4 and CD8 T-lymphocyte proportions similar to those in the blood. The proportion of naïve T-lymphocytes in the testis was low, with significantly higher frequencies of central memory cells, compared with the blood. A median of 42.7% of the testicular leukocytes were CD163+ macrophages, while 4.5% were $CD14^{+}CD163^{-}$ monocyte-like macrophages. Small populations of myeloid and plasmacytoid dendritic cells, NK cells and NKT cells were also detected. Following mitogen stimulation, 19.7% of blood T-lymphocytes produced IFNγ and/or TNF, whereas significantly fewer (4.4%) of the testicular T-lymphocytes responded to stimulation. Our results characterise the immune cells within the adult macaque testis and identify a suppression of T-lymphocyte responses. This study provides a baseline to examine the immunology of the primate testis and suggests that testicular immune privilege could also be present in primates.

1. Introduction

The rodent testis is well established as a region of immune privilege, where both innate and acquired immune responses are suppressed. This may serve to protect the immunogenic germ cells from acquired immune responses and from the deleterious effects of inflammation (Meinhardt and Hedger, 2011). Currently, there are few studies that have addressed the presence or absence of immune privilege in the testes of humans or non-human primates. Impeding this area of research is the relatively poor characterisation of the leucocyte populations of the primate testis.

The testicular leukocytes reside in the testicular interstitium, and share this compartment with the Leydig cells, connective tissue cells such as fibroblasts and pericytes, and vascular endothelial cells. Leukocytes are never found in the seminiferous tubules under normal conditions (Hedger, 1997). In all species studied to date, the majority of testicular leukocytes are macrophages (Hedger, 2002). The

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macrophage population of the rat testis is the best characterised and consists primarily of resident macrophages that are $CD163^+$. It also includes monocyte-like macrophages that lack CD163, but express other macrophage markers such as CD68 (i.e. $CD68^+CD163^-$) (Wang et al., 1994; Winnall and Hedger, 2013). $CD68^+$ macrophages have been detected in the human and macaque testis (Pöllänen and Niemi, 1987; Frungieri et al., 2002; Shehu-Xhilaga et al., 2007), but the macrophage populations of these species are otherwise poorly characterised. However, data from the human testis suggest that both $CD163^+$ and $CD163^-$ macrophages are present (Frungieri et al., 2002). These testicular macrophages play roles in the response to infection, the regulation of spermatogenesis and steroidogenesis, and are proposed to contribute to immunosuppression in the testis (Hales, 2002; Hedger, 2002; Winnall and Hedger, 2013).

In rat and human testes, T-lymphocytes make up an estimated 10–20% of the leukocytes (El-Demiry et al., 1985; Pöllänen and Niemi, 1987) with $CD8^+$ T-lymphocytes present as the predominant population (Ritchie et al., 1984; Pöllänen and Niemi, 1987; Wang et al., 1994; Tompkins et al., 1998; Jacobo et al., 2009). Smaller populations of dendritic cells, mast cells, eosinophils and natural killer (NK) cells have also been detected in the testes of rodents and some other species (Derrick et al., 1993; Itoh et al., 1995; Anton et al., 1998; Fijak et al., 2005; Fijak and Meinhardt, 2006; Rival et al., 2006), but these remain largely uncharacterised in the primate testis. Neutrophils and B-lymphocytes, which are commonly found in large numbers in the circulation, have not been detected in rodent testes under normal, homeostatic conditions (Hedger, 1997; Tompkins et al., 1998).

The mechanisms behind immune privilege are incompletely defined, but studies in rodents have proposed that anatomical sequestration of germ cells by the Sertoli cell tight junctions, potential immunosuppressive properties of steroid hormones, unique MHC composition inside seminiferous tubules of rodents and humans, as well as the actions of testicular macrophages, contribute to tolerance in the male gonads (Pöllänen and Niemi, 1987; Fiszer et al., 1997; Hutter and Dohr, 1998; Liva and Voskuhl, 2001; Cheng and Mruk, 2002; Hedger and Hales, 2006; Winnall et al., 2011). At least in rodents, the responses of the T-lymphocyte population of the testis to foreign antigens are believed to be inhibited or altered, leading to a prolonged tolerance (Head and Billingham, 1985; Dai et al., 2005; Nasr et al., 2005; Winnall et al., 2011). Local production of immunosuppressive factors such as IL-10, TGFβ, activin A and the presence of immunosuppressive lyso-glycerophosphocholines, which regulate T-lymphocyte activation and survival, are suspected to promote this diminished response of testicular T-lymphocytes (Pollanen et al., 1993; Foulds et al., 2008; Winnall et al., 2011; Bistoni et al., 2012; Hedger and Winnall, 2012). The response of primate testis T-lymphocytes to activation has not been characterised. The present study aims to characterise the leukocytes present in the macaque testis and their response to activation by mitogens.

Table 1
Antibodies.

Antigen[a]	Fluorophore	Clone	Titre	Catalogue number[b]
CD3	Alexa fluor 488	SP34.2	1/200	557705
CD3	Pacific blue	SP34.2	1/100	558124
CD3	APC-Cy7	SP34.2	1/100	557757
CD4	Alexa fluor 700	L200	1/100	560836
CD8	PerCP	SK1	1/70	347314
CD8	APC-H7	SK1	1/100	641400
CD8	PE-Cy7	SK1	1/1000	335787
CD8	APC	SK1	1/800	340584
CD11c	BV711	3.9	1/20	301629[c]
CD14	PE-Cy7	M5E2	1/66	557742
CD14	APC-H7	MPϕ9	1/100	560270
CD16	APC-H7	3G8	1/40	560195
CD19	PE	HIB19	1/20	555413
CD20	PE-Cy7	L27	1/67	335793
CD25	Alexa fluor 488	BC96	1/50	53-0259-42[d]
CD28	PerCP-Cy5.5	L293	1/25	337181
CD45	V450	D058-1283	1/100	561291
CD49d	–	L25	N/A	340976
CD66	PE	B6.2	1/200	551478
CD68	FITC	Y1/82A	1/50	11-0689-41[d]
CD95	FITC	DX2	1/12.5	556640
CD123	APC	7G3	1/125	560087
CD159a (NKG2A)	APC	Z199	1/66	A60797
CD161	BV605	HP-3G10	1/40	339915[c]
CD163	PE	GHI/61	1/25	560933
Lin1	FITC	[e]	1/40	340546
HLA-DR	PerCP-Cy5.5	L243	1/20	552764
Vα7.2	PE	3C10	1/40	351705
TNF	PE-Cy7	mAb11	1/100	557647
IFNγ	APC	B27	1/800	554702

[a] All were raised to human antigens except non-human primate CD45.
[b] All were from BD Biosciences except where indicated.
[c] Purchased from BioLegend.
[d] Purchased from eBioscience.
[e] Lin1 FITC comprises CD3, CD14, CD16, CD19, CD20 and CD56, which are clones SK7, 3G8, SJ325C1, L27, MϕP9, and NCAM16.2, respectively.

2. Materials and methods

2.1. Animals

Testes were sourced from 12 healthy young adult male pigtail macaques (*Macaca nemestrina*) that were involved in an unrelated study. These animals were uninfected controls and no procedures were performed that could have affected the testes. Animals were euthanised using ketamine sedation (1 mg/kg) and pentobarbitone (0.5 ml/kg) and orchidectomy was performed immediately at autopsy. Concurrent blood samples were collected from animals in heparinised collection tubes. Animals were housed in the Australian Animal Health Laboratory and the CSIRO Animal Health animal ethics committees approved all studies.

2.2. Reagents and antibodies

All antibodies and other reagents were purchased from BD Biosciences (San Jose, CA, USA) and raised against human antigens unless otherwise indicated. Antibodies are described in Table 1 and were all mouse monoclonals raised against human antigens, with the exception of

anti-non-human primate CD45. Mouse CD1d-tetramer PE loaded with an α-galactosylceramide analogue was made in-house (peripheral blood isolated from macaques was stained with CD1d tetramer loaded with an α-GalCer analogue, PBS-44, [kindly provided by Professor Paul Savage, Brigham Young University]). A LIVE/DEAD Fixable Aqua Dead Cell Stain Kit was purchased from Invitrogen (Carlsbad, CA, USA).

2.3. Isolation of testicular cells

Testes were decapsulated and major blood vessels removed manually. Approximately half of each testis, distal to the more vascular rete testis, was recovered. Testicular tissue was roughly chopped using scissors (until tubules were approximate 5-mm pieces) and incubated in a total of 10 ml PBS with 0.07 mg/ml collagenase (Worthington Biochemicals) and 1.4 μg/ml DNase (Roche) at 37 °C for 60 min. After collagenase digestion, 40 ml PBS/0.5% BSA/2 mM EDTA was added, tissue was briefly mixed by two inversions and tubules were allowed to settle for 2 min before removal of the PBS containing suspended cells. The cell preparation contained few aggregates. Cells were pelleted and resuspended in PBS/0.5% BSA/2 mM EDTA for antibody incubations or activation with mitogens. Cell counts were performed using a CELL DYN Emerald cell counter (Abbott Diagnostics, Lake Forest, IL, USA). Light microscopy observations indicated considerable contamination of the testicular interstitial cell preparations with germ cells, mostly spermatids released from broken seminiferous tubules, at approximately 20% of the total. No cells with the characteristic morphology of Sertoli cells could be seen using light microscopy. To confirm that collagenase treatment did not affect the leucocyte surface antigen expression we tested macaque PBMC samples with and without collagenase treatment. Flow cytometry analysis demonstrated that the surface expression of the major antigens used in this study was not significantly changed by collagenase treatment.

2.4. Freezing and thawing testicular cells and PBMC

Fresh blood, stored in heparin-coated vials was carefully layered over 95% Ficoll-Paque PLUS™ (GE Healthcare, Buckinghamshire, England)/5% water (v/v) and centrifuged at 1000 × *g* for 25 min at room temperature. The interface was recovered using a transfer pipette, washed with RPMI media and centrifuged at 500 × *g* for 5 min at room temperature. Cell counts were performed using a CELL DYN Emerald cell counter. For freezing, testicular cell-enriched preparations or PBMC were pelleted and resuspended in a solution of 90% FCS (Bovogen Biologicals, VIC, Australia)/10% (v/v) DMSO (Sigma–Aldrich, St. Louis, MO, USA) at a concentration of 5×10^6 cells/ml and frozen in a "Mr Frosty" freezing container (ThermoFisher Scientific, Waltham, MA, USA) at −80 °C for 12 h before long-term storage in liquid nitrogen. Frozen testicular cells or PBMC were rapidly thawed in a 37 °C water-bath then incubated for 10 min in 3 ml RPMI/10%FCS 66 U/ml DNase in a 37 °C water-bath, followed by washing in PBS. Thawed cells were used for the dendritic cell staining only; freshly isolated blood and testicular interstitial cells were used for all other experiments.

2.5. Phenotypic analysis of testicular and blood leukocytes

Whole blood (100 μl/samples), thawed PBMC (5×10^6 cells in 100 μl, for dendritic cell staining only) or testicular interstitial cells in PBS/0.5% BSA/2 mM EDTA (5×10^6 cells in 200 μl) were incubated with 0.5 μl LIVE/DEAD Aqua to stain dead cells for 30 min, then antibody cocktails were added for a 20-min incubation. Testicular cells were washed in PBS/0.5% BSA/2 mM and fixed in Stabilising Fixative (BD). Blood samples were further incubated 1× FACS™ Lysing Solution (BD) for 10 min to lyse red blood cells, followed by washing in PBS/0.5% BSA/2 mM EDTA and fixing with Stabilising Fixative. All incubations were performed at room temperature in the dark after mixing by gentle vortex and centrifugation was at 500 × *g* for 5 min.

2.6. Intracellular cytokine staining assay

IFNγ and TNF production by blood and testicular cells was measured by an intracellular cytokine-staining assay similar to previously described assays (De Rose et al., 2007) Peripheral whole blood (100 μl) or 100 μl of 5×10^6 testicular interstitial cells in RMPI/10% FCS were mixed with anti-CD28 and anti-CD49d at 1 μg/μl each, brefeldin A (10 μg/μl) and stimulated with either PBS (unstimulated controls) or a mix of Staphylococcus Enterotoxin-B (1 μg/μl, Sigma–Aldrich), phytohaemagglutinin (100 μg/μl, Sigma–Aldrich) and phorbol myristate acetate (30 μg/μl, Sigma–Aldrich). Cell activation was performed at 37 °C for 6 h in the same media, followed by storage at 4 °C overnight. Cells were then incubated with 0.5 μl LIVE-DEAD Aqua dead cell stain 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 while blood was treated with 1× FACs™ Lysing Solution (BD) for 10 min followed by washing in PBS/0.5% BSA/2 mM EDTA. All cells were permeabilised using 1× FACs™ Permeabilising Solution (BD) for 10 min, washed in PBS/0.5% BSA/2 mM EDTA, then incubated with anti-IFNγ APC and TNF PE-Cy7 for 60 min. Cells were washed then fixed in Stabilising Fixative (BD). All incubations were performed at room temperature in the dark after mixing by gentle vortex. PBS controls were performed for every sample.

2.7. Flow cytometry

Acquisition was performed on an LSR Fortessa or LSRII flow cytometer (BD) according to the manufacturer's instructions and data were analysed using FlowJo Version 9.6 (TreeStar, Ashland, OR, USA). Fluorochrome compensation correction was performed using the auto-compensation function in DIVA 6.1, using Calibrate™ beads (BD) for FITC, PE, PerCP APC, CompBead PLUS anti-mouse Ig κ capture beads (BD) for other fluorochromes and ArC beads (Invitrogen) for LIVE/DEAD Aqua. Testicular or

blood populations were gated according to scatter properties or CD45 expression, and aggregates and dead cells were excluded. Specific populations were gated within testicular interstitial cells compared with blood by calculating the median percentage of the population among all live $CD45^+$ cells (aggregates excluded). Alternatively, in Figs. 2 and 5, medians of specific T-lymphocyte populations were calculated as a percentage of live $CD45^+CD3^+$ cells, and in Fig. 3C, as a percentage of live $CD45^+CD163^+$ cells.

2.8. Statistical analyses

Two testicular cell isolation experiments were performed on different days with 6 animals in each. These samples were analysed by staining with different antibody cocktails, making the sample size $n = 6$ animals for each experiment, with the exception of dendritic cell staining on thawed samples, done at $n = 3$ (statistical analyses were not used for these). Statistical analyses were performed using SPSS version 18 software (IBM, Armonk, NY, USA). Wilcoxon Ranked Sign Tests were used to compare testis and animal-matched blood data in Figs. 2C, D and 5C. A p value ≤ 0.05 was considered to indicate a significant difference.

3. Results

3.1. Macaque testicular interstitial-enriched cell preparations contain low numbers of contaminating blood B-lymphocytes and granulocytes

To characterise the macaque testicular leucocyte population, testicular interstitial cell-enriched preparations were isolated by collagenase digestion of fresh testicular tissue and phenotyped using antibodies. Leukocytes, gated as $CD45^+$, were present at a median of 4.44% (interquartile range (IQR) 2.05%) of the testicular cell preparation, once aggregates and dead cells were excluded (Fig. 1A). There were very few $CD20^+$ B-lymphocytes present in the interstitial cell preparations (0.235% IQR 0.105% of $CD45^+$ leukocytes) compared with the animal-matched blood samples (8.28% IQR 4.02%; Fig. 1B and C). From these data, it was calculated that an average of $\leq$3.7% of the testicular leukocytes were likely to be contaminating blood cells from the testicular blood vessels.

3.2. T-lymphocyte populations in the macaque testicular interstitium

Testicular T-lymphocytes were distinguished from the other testicular interstitial cells according to $CD45^+$ and $CD3^+$ co-expression (Fig. 2A). The proportions of $CD4^+$ and $CD8^+$ T-lymphocytes in the testis were not significantly different from those in blood (Fig. 2A, B, middle panels, and C). Expression of CD28 and CD95 wad used to differentiating populations of naïve, central memory and effector memory T-lymphocytes similar to previous studies (Pitcher et al., 2002). There were significantly fewer naïve T-lymphocytes in the testes (2.2% IQR 2.29% of $CD45^+CD3^+$ leukocytes) than the blood (27.8% IQR 16.7%, Wilcoxon Ranked Sign Test, $p = 0.028$, Fig. 2A, B (right side panels) and D). While effector memory T-lymphocytes were present at similar levels, the central memory proportion was larger in the testis (74.5% IQR 13.9% of $CD45^+CD3^+$ leukocytes) than the blood (41.3% IQR 15.4%, Wilcoxon Ranked Sign Test, $p = 0.046$). A median of 1.08% (range 0.62–1.14%) of the testicular T-lymphocytes expressed the activation marker CD25 compared with 2.57% (range 2.05–4.57%) in the blood samples (data not shown). Also detected was a small population of leukocytes (3.28%, IQR 2.07%) displaying the characteristic large side scatter properties of granulocytes, consistent with low levels of blood contamination (Fig. 2A). This population would likely contain neutrophils from the blood, but may also contain eosinophils, as found in other species (Anton et al., 1998).

3.3. Mononuclear phagocyte and dendritic cell populations in the macaque testicular interstitium

CD3 and CD163 staining of testicular leukocytes indicated that a median of 30.8% (IQR 11.3%) of testicular $CD45^+$ leukocytes were $CD3^+$ T-lymphocytes, 42.7% (IQR 16.75%) were $CD163^+$ resident macrophages and the remainder were mostly a $CD3^-CD163^-$ population (26.7% IQR 5.73%) that could include NK cells, dendritic cells and monocytes (Fig. 3A and B). In rats, the mononuclear phagocyte population of the testis has been characterised based on expression of either or both CD163 and CD68 (Wang et al., 1994; Winnall and Hedger, 2013). CD14 was used to further characterise the mononuclear phagocytes, as the available anti-human CD68 antibody did not cross-react with macaque cells. Two populations could be discerned from the testicular cell preparation: $CD14^+CD163^+$ cells, which were most likely resident testicular macrophages, and $CD14^+CD163^-$, which may be monocytes or monocyte-like macrophages (Fig. 3A). The $CD163^-$ population was calculated to be a median of 4.5% (IQR 5%) of testicular $CD45^+$ leukocytes. Similar proportions of these populations were found in each of 6 animals (Fig. 3A and C).

Small populations of dendritic cells were detected in the testis, identified as lymphocyte- and monocyte-lineage negative (Lin^-), $HLA\text{-}DR^+$ and either $CD11c^+$ (myeloid) or $CD123^+$ (plasmacytoid; Fig. 3D). Overall, dendritic cells were calculated to be a median of 0.22% (range 0.18–0.42%) of the recovered $CD45^+$ testicular leukocytes, whereas 0.09% (range 0.07–0.11%) of macaque blood $CD45^+$ leukocytes were classified as dendritic cells using the same gating strategy (Fig. 3D). Factoring in the low level of blood contamination in the testes sample ($\leq$3.7%), >98% of the dendritic cells identified in the testicular cell preparations were predicted to be of testicular origin.

3.4. Minor lymphocyte populations in the macaque testicular interstitium

Natural killer cells were detected in the $CD3^-$ population of testicular leukocytes by their surface expression of NKG2A, which marks 97% of NK cells in macaques (Mavilio et al., 2005). NK cells were a median of 5.27% of the testicular $CD45^+$ leukocytes compared with 12.6% of blood leukocytes (Fig. 4A and B). Whereas only 1.98% of the NK cells in the blood were $CD8^-$, 35.3% of the

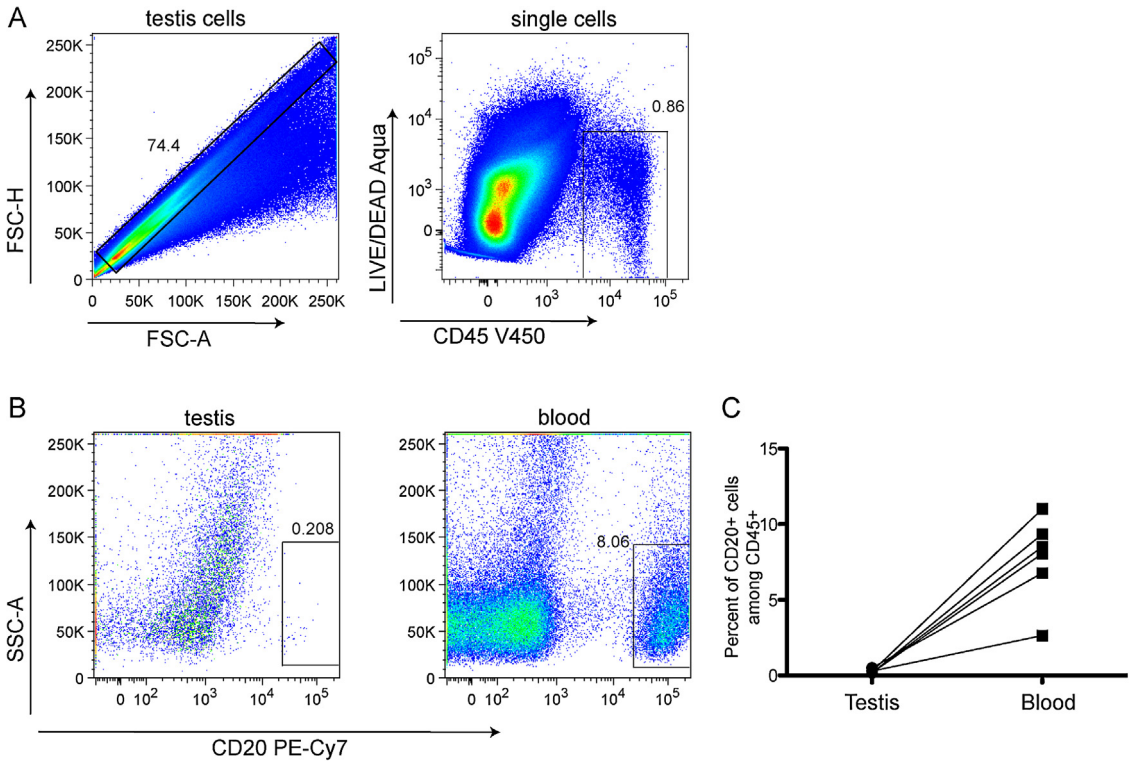

Fig. 1. Macaque testicular interstitial cell preparations contain low numbers of B-lymphocytes. (A) Representative flow cytometry plots showing exclusion of aggregates (left panel) then gating to select for viable CD45$^+$ leukocytes (right panel) among testicular interstitial cells. (B) Representative comparison of CD20$^+$ B-lymphocyte proportions among CD45$^+$ viable cells in testes and blood ($n = 6$). (C) Proportions of B-lymphocytes among CD45$^+$ cells in testis and blood ($n = 6$). FSC-A and FSC-H refer to area (A) and height (H) of the forward scatter (FSC) pulse produced by each cell. Lines indicate animal-matched samples; numbers in gates show the percentage of the gated population within the parental population.

testicular NK cells were CD8$^-$ (Fig. 4B). Natural killer-T (NKT) cells were also detected in the testis by gating on CD1d-tetramer$^+$CD3$^+$ cells with low side-scatter (Fig. 4A and C). The testis NKT population was calculated to be 0.10% (IQR 0.24%) of CD45$^+$ leukocytes compared with a median 0.02% (range 0.01–0.28) in the blood. Co-expression of CD161 and Vα7.2 on CD3$^+$ lymphocytes was used to detect mucosal associated invariant T (MAIT) cells in the blood. There was no convincing population of MAIT cells in the testes of six animals, as very few testicular leukocytes fell into the equivalent gate (Fig. 4A and D).

3.5. T-lymphocytes from the macaque testicular interstitium were not readily activated by mitogens and superantigens in vitro

Activation of T-lymphocytes in fresh testicular interstitial cell-enriched preparations and in the blood was compared using a flow cytometry-based intracellular cytokine-staining assay. Cells were stimulated with a mixture of Staphylococcus enterotoxin-B (SEB), phytohaemagglutinin (PHA) and phorbol-myristate acetate (PMA) in vitro to promote strong responses. Whereas a high proportion of viable blood CD45$^+$CD3$^+$ T-lymphocytes (19.7% IQR 9.16%) responded to stimulation by producing IFNγ and/or TNF, a significantly lower proportion of T-lymphocytes from the testis responded (4.44% IQR 2.05%, Wilcoxon Ranked Sign Test, $p = 0.028$).

4. Discussion

Our results show that the CD45$^+$ leucocyte population recovered from the collagenase-digested macaque testis consists of an average 42.7% resident macrophages, 30.8% CD3$^+$ T-lymphocytes, 4.5% CD14$^+$CD163$^-$ mononuclear phagocytes, 0.29% dendritic cells, 5.27% NK cells and 0.10% NKT, 3.5% granulocytes and 0.207% B-lymphocytes. The low numbers of B-lymphocytes, which are not normally present in the testis parenchyma, indicate that contamination from testicular blood vessels was minimal ($\leq$3.7%). The granulocyte population present in the testis may contain eosinophils, as found in hare and swine testes (Anton et al., 1998). The proportion of testicular T-lymphocytes responding to mitogen and super-antigen stimulation was significantly lower than in the blood (Fig. 5C), suggesting that the suppression of T cell responses may be occurring in the primate testis, as reported in the mouse testis (Dai et al., 2005; Nasr et al., 2005; Fijak and Meinhardt, 2006).

The testicular interstitium of macaques contained similar ratios of CD4$^+$ and CD8$^+$ T-lymphocytes to that found in the blood (Fig. 2A and C). It should be noted that young adult macaque blood contains approximately equal proportions of CD4$^+$ and CD8$^+$ T-lymphocytes, unlike human

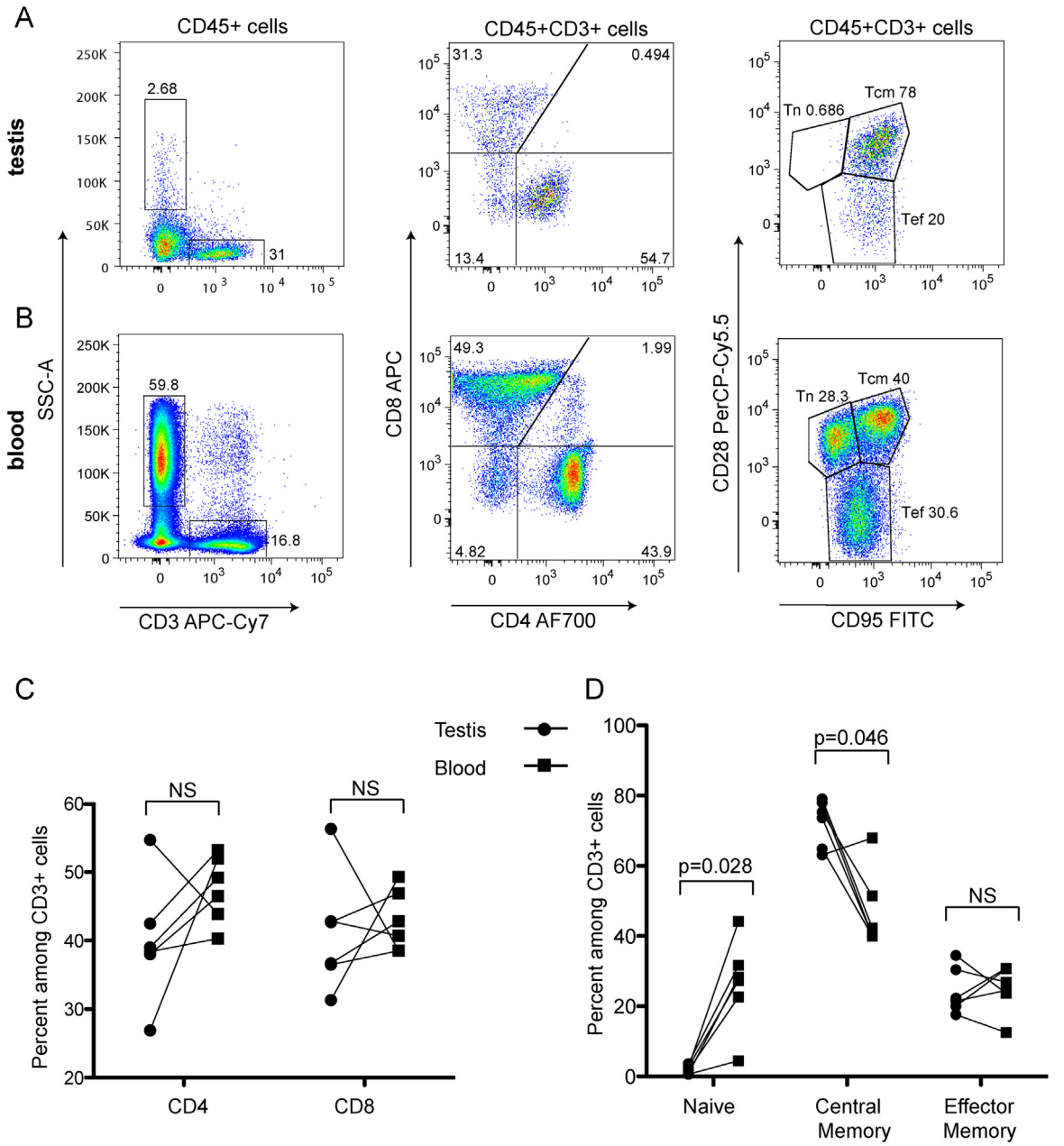

Fig. 2. Comparison of T-lymphocyte populations in the testicular interstitium (A) and the blood (B). Representative flow cytometry plots showing viable $CD45^+CD3^+$ T-lymphocytes and cells with high side-scatter that were assumed to be granulocytes (left panels), $CD4^+$ and $CD8^+$ T cells (middle panels) and naïve (Tn), central memory (Tcm) and effector memory (Tef) T-lymphocytes (right panels). (C) Proportions of $CD4^+$ and $CD8^+$ cells amongst $CD45^+CD3^+$ cells in testes and blood ($n = 6$). (D) Proportions of naïve, central and effector memory cells among $CD3^+CD45^+$ cells for all six tested animals comparing testis with blood. Statistics were performed using the Wilcoxon Ranked Sign Tests, $n = 6$ animals. Lines indicate animal-matched samples; numbers in gates show the percentage of the gated population within the parental population.

blood where $CD4^+$ T-lymphocytes predominate (Terao et al., 1988). The ratios of $CD4^+$ and $CD8^+$ cells contrasts to the previous studies in the rat testis, showing an increase in the proportion of $CD8^+$ and subsequent decrease in $CD4^+$ T-lymphocytes compared with the blood (Tompkins et al., 1998; Jacobo et al., 2009), but are consistent with one previous human study where testicular T-lymphocytes had approximately equal ratios of $CD4^+$ and $CD8^+$ cells (Ritchie et al., 1984). Together these results indicate that the ratio of $CD4^+$ to $CD8^+$ cells in the testes of primates is similar to the blood whereas in rodent testes $CD8^+$ cells dominate. The T-lymphocyte population of the macaque testis had a significantly reduced proportion of naïve cells ($CD28^+CD95^-$) and an increase in central memory T-lymphocytes compared with the blood (Fig. 2A and D). These findings are consistent with a study in the rat

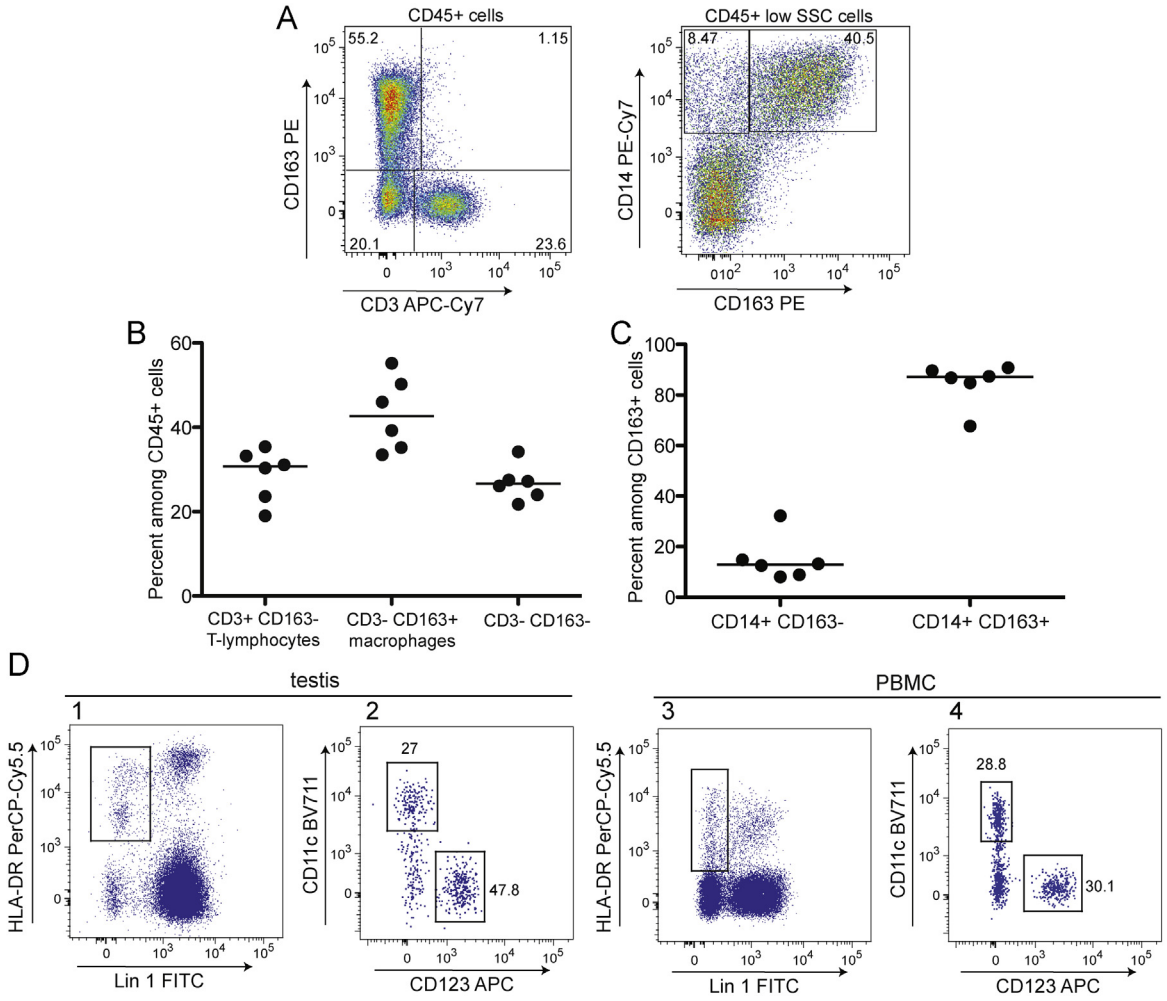

Fig. 3. Mononuclear phagocyte (A)–(C) and dendritic cell (D) populations in the testicular interstitium compared with the blood. (A) Representative flow cytometry plot showing CD163$^+$ macrophages and CD3$^+$ T-lymphocytes gated from CD45$^+$ testicular leukocytes (left panel). Cells with high side scatter (likely granulocytes) were excluded from CD45$^+$ leukocytes to avoid CD14$^+$ neutrophils (not shown), then mononuclear phagocytes were gated as CD14$^+$CD163$^-$ and CD14$^+$CD163$^+$ populations (right panel). (B) Proportions of T-lymphocytes (CD3$^+$) resident macrophages (CD163$^+$) and CD3$^-$CD163$^-$ cells among CD45$^+$ cells for all six animals. (C) Proportions of CD14$^+$CD163$^-$ and CD14$^+$CD163$^-$ cells among CD45$^+$ with low side scatter for the same six animals. (D) Representative flow cytometry plots showing gating of dendritic cell populations (Lin1$^-$HLA-DR$^+$ cells) from live testicular single cells (panel 1) compared with the blood (panel 3). Dendritic cells were classified as plasmacytoid (CD123$^+$) or myeloid (CD11c$^+$) in panels 2 and 4. Only Lin1$^-$HLA-DR$^+$ cells that were either CD123$^+$ or CD11c$^+$ were counted as dendritic cells to calculate the proportion of dendritic cells among leukocytes. Numbers in gates show the percentage of the gated population within the parental population.

showing only 19% of testicular T-lymphocytes as being positive for CD45RA, indicating a naïve phenotype (Tompkins et al., 1998) and being consistent with low proportions of naïve T-lymphocytes found in other peripheral tissues (Weninger et al., 2002).

Regulatory T-lymphocytes (Tregs) play an important role in peripheral tolerance and the prevention of autoimmunity (Chatila, 2009). Since the testicular environment contains high levels of TGFβ (Le Magueresse-Battistoni et al., 1995), which can stimulate Treg production, and low levels of T-lymphocyte activation, at least in rodents, it might be expected that numerous Tregs would be found in this region. In one previous study, approximately 2% of testicular interstitial cells of the rat were CD4$^+$CD25$^+$Foxp3$^+$ Tregs (Jacobo et al., 2009). The present study found that only 1% of testicular lymphocytes were CD25$^+$ in the macaque testis (data not shown), suggesting that levels of CD25$^+$Foxp3$^+$ T cells are likely to be low. Further study of Tregs in the macaque testes is warranted.

Stimulation of macaque testicular T-lymphocytes with a combination of three mitogens, including the superantigen SEB, resulted in very little production of TNF or IFNγ compared with stimulation of blood T-lymphocytes. The exact mechanisms behind this immunosuppression are unknown. We propose that the failure of the T-lymphocytes to be activated in our cultures may be due either to an intrinsically suppressed state as a result of originating in the testis, or alternatively that testicular cells present in the culture (and in vivo) exert an immunosuppressive effect in the assay. The current view of testicular immunosuppression is that local factors affect the responses of T-lymphocytes (Hedger and Hales, 2006).

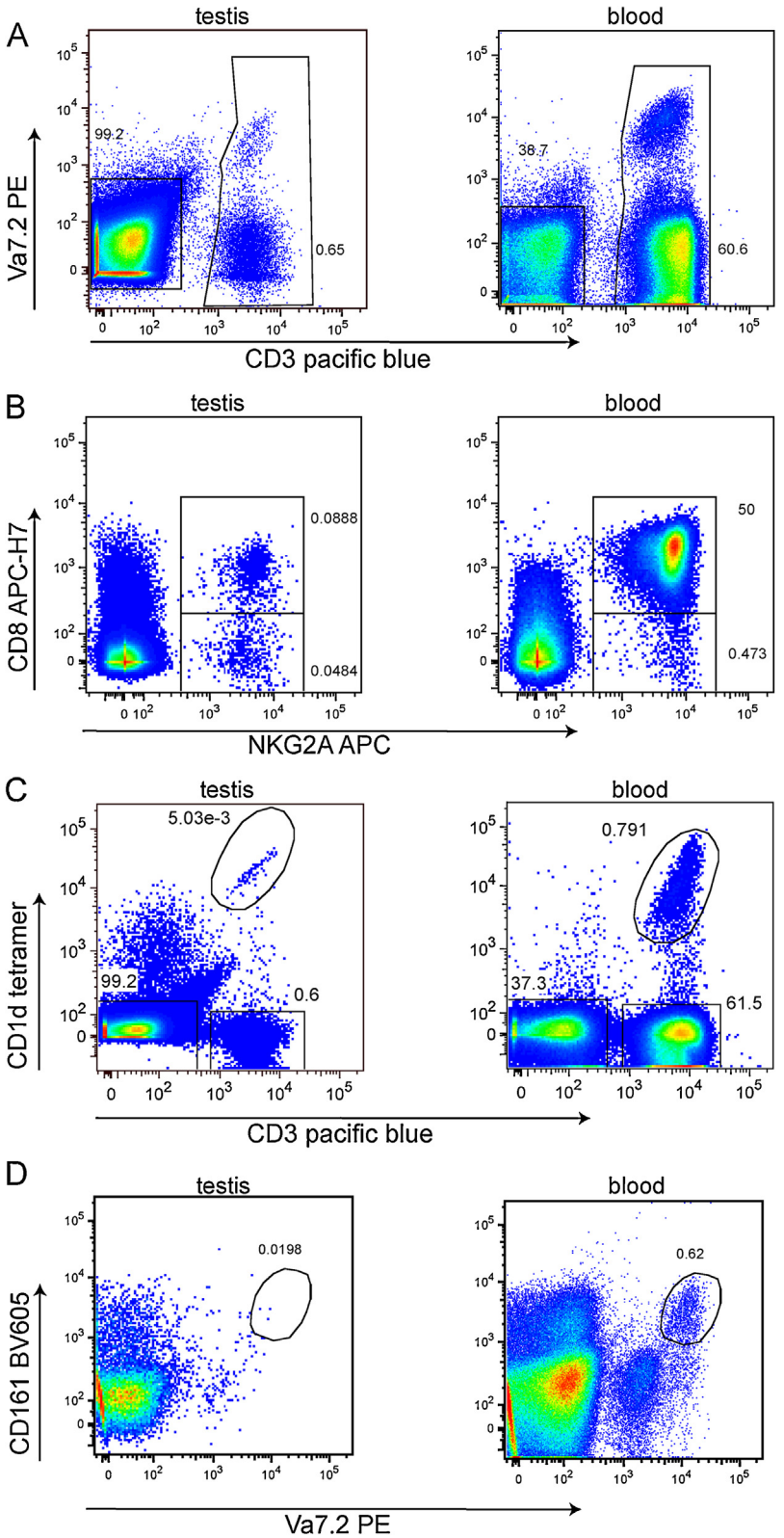

Fig. 4. Minor lymphocyte populations in the testicular interstitium compared with the blood. Lymphocytes/smaller cells were selected based on scatter properties and then aggregates were excluded prior to this gating (not shown). (A) Representative flow cytometry plot showing gating strategy for detecting $CD3^-$ NK and CD3+ NKT cells and MAIT cells among testicular cells (left panels) and blood (right panels). (B) $NKG2A^+$ NK cells were divided into $CD8^+$ and $CD8^-$ NK cells. (C) NKT cells were selected as $CD3^+CD1d$ $tetramer^+$. (D) A $V\alpha7.2^+CD161^+$ MAIT cell population could be found in the blood, but not in the testis. Numbers in gates show the percentage of the gated population within the parental population.

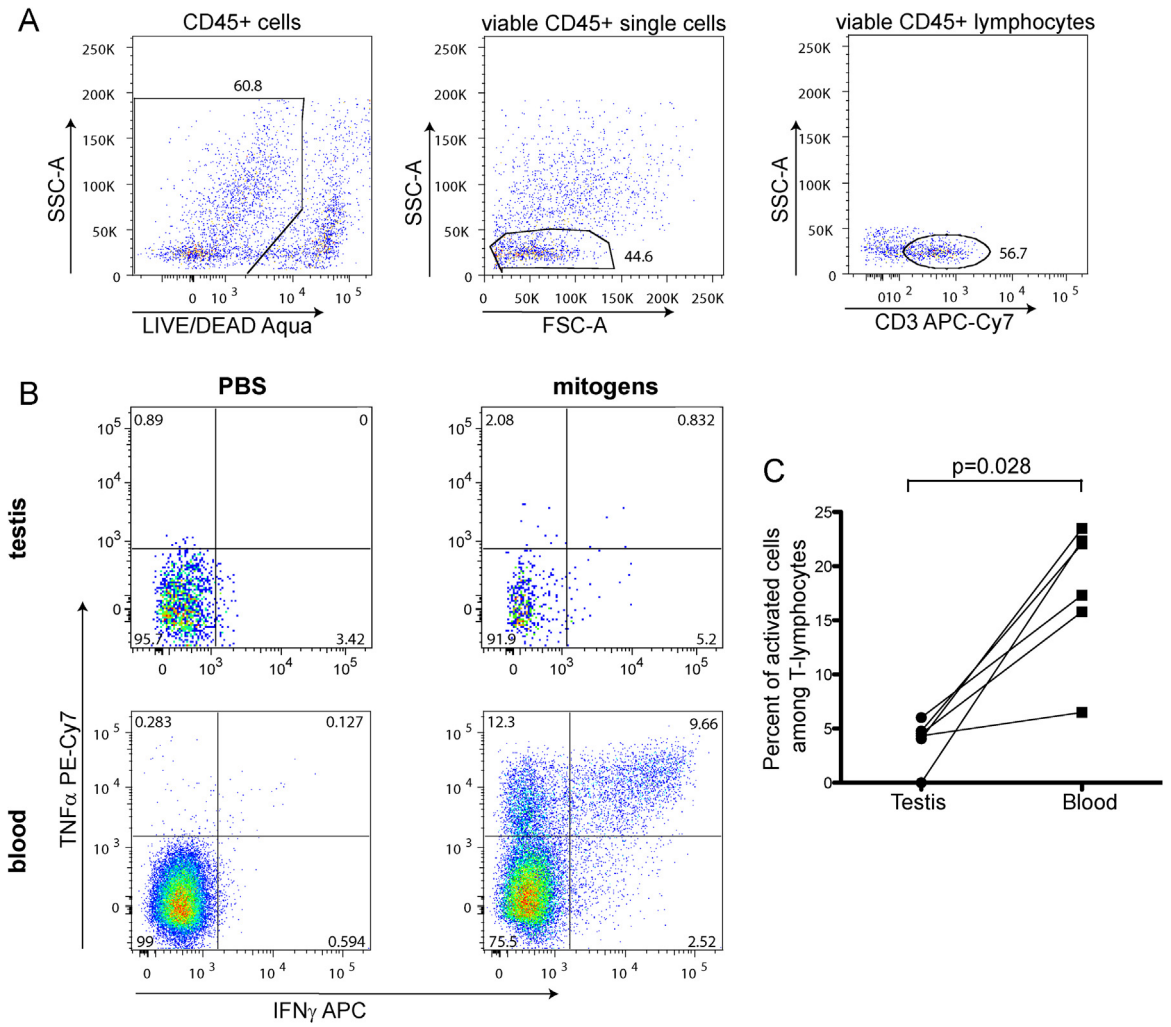

Fig. 5. T-lymphocytes from the macaque testicular interstitium were not readily activated by mitogens in vitro. (A) Representative flow cytometry plots showing gating on viable CD45$^+$ cells (left panel), gating on cells with the low forward and side scatter characteristics of lymphocytes (middle panel) then CD3$^+$ cells (right panel). (B) Representative flow cytometry plots showing cytokine production by CD3$^+$ testicular T-lymphocytes (upper row) compared with blood (lower row). Unstimulated controls (PBS) are shown on the left side compared with mitogen-stimulated samples on the right. (C) Proportions of T-lymphocytes producing IFNγ and/or TNF in all six animals tested. Plots show background-subtracted proportions (as percentage) of responding cells. Statistics were performed using the Wilcoxon Ranked Sign Tests, $n = 6$ animals. SSC-A refers to area (A) of the side scatter (SSC) pulse produced by each cell. Lines indicate animal-matched samples; numbers in gates show the percentage of the gated population within the parental population.

Although factors produced by Sertoli cells can be immunosuppressive (Fallarino et al., 2009; Bistoni et al., 2012), few Sertoli cells were present in our cultures. However ample testicular macrophages and Leydig cells were present that could be affecting T-lymphocyte activation, likely by production of soluble factors that inhibit activation (Born and Wekerle, 1982; Hedger et al., 1990; Winnall et al., 2011). Previous studies showed that T-lymphocytes of the mouse testis entered into apoptosis upon transplantation of pancreatic islet allografts or are more likely to become Tregs (Dai et al., 2005; Nasr et al., 2005). The fate of mitogen-exposed T-lymphocytes in our cultures is unknown and will be interesting to determine in future studies. Importantly, these results are the first to demonstrate a suppression of immune responses in macaque testis cultures and suggest that immune privilege, which has so far only been demonstrated in the rodent testis, may also extend to the primate testis.

The rat testis contains three populations of mononuclear phagocytes: CD68$^-$CD163$^+$ cells, which are considered tissue-resident macrophages, CD68$^+$CD163$^-$, which are predicted to be "newly arrived", monocyte-like macrophages, and a double-positive population that may be in the process of differentiating into resident macrophages (Wang et al., 1994; Winnall et al., 2011). The present study indicates that CD163$^+$ cells are the majority of the recovered testicular leukocytes in the macaque, at an average of 42.7% (Fig. 3A and B). Analysis of CD14 versus CD168 staining revealed two potential populations of mononuclear phagocytes: a predominating CD14$^+$CD163^{hi} population that is likely to be the resident macrophage population, and CD14$^+$CD163$^-$ that is likely to be "newly

arrived" monocyte-like macrophages (Fig. 3A and C). These results confirm that the macaque testis has at least two populations of macrophages that are CD163$^+$ and CD163$^-$ cells, similar to the rat and human testis (Frungieri et al., 2002).

We detected a population of NK cells in the testis at 5.27% of leukocytes (17% of lymphocytes), compared with 12.6% of blood lymphocytes (Fig. 4B). The macaque testicular NK population was similar in proportion to the rat testicular NK population at 25% of lymphocytes (Tompkins et al., 1998). Interestingly, the macaque testicular NK cells were more likely to be CD8$^-$ than blood NK cells, indicating that they are less prone to activation (Srour et al., 1990; Vargas-Inchaustegui et al., 2011). We also detected NKT cells in the testis that were present at 5-fold higher levels than in the blood, indicating that the small amount of blood contamination (3.7%) in our testicular cell preparations could not have been the source of all of these cells. Mucosal associated invariant T (MAIT) cells are unique T cells found in mucosal regions that are the portals of entry for many pathogens (Gold and Lewinsohn, 2011). Small MAIT cell populations were detected in macaque blood samples, but no discernable population could be seen in the testicular interstitial cell samples. These results are not surprising, as the interstitium of the testis is neither a mucosal region nor a common portal of pathogen entry.

Dendritic cells were detected in the testis, with both myeloid and plasmacytoid phenotypes. Myeloid dendritic cells, which are CD11c$^+$, are more commonly found in peripheral tissues (i.e. non-mucosal tissues) and have high spontaneous rates of migration out from the circulation into peripheral tissues (De La Rosa et al., 2003). Plasmacytoid dendritic cells (CD123$^+$), on the other hand, are primarily found in the blood and peripheral lymphoid tissues, with their migration more tightly controlled by specific integrins and chemokines (De La Rosa et al., 2003). Interestingly, the testicular cell preparations contained relatively large proportions of plasmacytoid dendritic cells compared with myeloid, indicating that specific signals, as yet uncharacterised, may be stimulating their migration into the testis.

Our results provide a baseline for future analyses of the immunomodulatory environment of the primate testis. Further, they allow us to probe the effects of local and systemic infections, such as simian immunodeficiency virus, on immune cell populations and immune responses in the primate testis in detail.

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