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T-lymphocyte homeostasis and function in infant baboons: implications for transplantation

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Summary

Laboratory mice are born lymphopenic and demonstrate lymphopenia-induced proliferation that generates memory T cells, yet they are prone to immunologic tolerance. Here we tested whether these fundamental immunologic observations apply to higher animals by studying the immune system of infant baboons. Using flow cytometry of the peripheral blood cells, it was found that baboons are born relatively lymphopenic and subsequently expand their initially naïve T cell pool with increasing numbers of memory T cells. After transplantation of an artery patch allograft or xenograft, non-immunosuppressed recipients readily mounted an immune response against donor-type antigens, as evidenced by mixed lymphocyte reaction. Immunosuppression with anti-thymocyte globulin (ATG), anti-CD154 mAb, and mycophenolate mofetil prevented T cell-mediated rejection. After lymphocyte depletion with ATG, homeostatic T cell proliferation was observed. In conclusion, the baboon proved a suitable model to investigate the infant immune system. In this study, neonatal lymphopenia and expansion of the memory T cell population were observed but, unlike mice, there were no indications that infant baboons are prone to T cell tolerance. The expansion of memory T cells during the neonatal period or after induction therapy may actually form an obstacle to tapering immunosuppressive therapy, or ultimately achieving immunologic tolerance.

Introduction

Laboratory mice are born lymphopenic and demonstrate lymphopenia-induced proliferation (LIP), which generates memory T cells during the first few weeks of life [1]. During the neonatal period, their T cell immune system exhibits remarkable plasticity and, although it is not believed to be immunodeficient, it is prone to tolerance induction after transplantation (Tx) or adoptive transfer of splenocytes under certain conditions (reviewed in Adkins *et al.* [2]). Unlike rodent species, the knowledge available in humans indicates that human neonates are born with an almost entirely functional immune system, a difference believed to be influenced by the duration of gestation [3]. However, also for humans there are several indications that the neonatal immune system still has immature features. For example, (i) the immune system of neonates is still antigen-inexperienced and is constituted by naïve T-lymphocytes [4,5]. (ii) In the unfortunate event that an infant needs an organ transplant, the Tx of a heart across the ABO-blood group barrier is relatively uncomplicated [6–9], while in adults this may lead to graft loss from hyperacute rejection [10]. Moreover, when an ABO-incompatible (AB-I) donor organ is introduced before the development of anti-AB antibodies (Abs), B cell tolerance to the incompatible blood group

antigen(s) can develop [11]. (iii) Pediatric or adult recipients of umbilical cord blood cells experience less graft-versus-host disease than recipients of bone marrow Tx from an adult donor [12,13].

To our knowledge, few studies have closely followed the development of the human infant immune system with advancing age [14,15], possibly because of ethical concerns of involving infants in research protocols. Alternatively, nonhuman primate (NHP) models can be of value for investigation of the developing immune system [16].

The aim of this study was to verify two fundamental immunologic principles observed in mice, i.e., neonatal LIP of T cells and the possibility of inducing T cell tolerance to transplant antigens, in a NHP model using infant baboons. We found that baboon T-lymphocytes significantly increase in number after birth with transient appearance of memory T cells. After alloTx or xenoTx of an artery patch graft [from AB-I baboon, or wild-type (WT) pig donor, respectively] at the age of 3 months, the T cell immune system appeared functional and, in the absence of immunosuppressive therapy (IS), indications for the allowance of immunologic tolerance were not observed.

Materials and methods

Sources of animals and blood samples

Healthy young infant baboons (*Papio anubis*) (Group 1, Table 1) were housed at the specific pathogen-free facility of the University of Oklahoma Health Sciences Center (UOHSC) [17]. Blood was drawn from six baboons during the first week of life, and at 1, 2, 4, and 6 months of age, stored in tubes containing ethylenediamine tetraacetic acid (EDTA), and shipped at 4 °C to the University of Pittsburgh (UPitt) for analysis on the following day. The infants remained healthy and untreated throughout the period of study. Blood samples from healthy, untreated, young baboons aged 1 year ($n = 6$) and 2–3 years ($n = 6$) were drawn and shipped under the same conditions.

In addition, five infant baboons (Group 2) were housed at UPitt from the age of 2 months for immunologic and Tx studies. Blood was drawn and analyzed before and at multiple time-points after Tx of an artery patch graft (see below).

All animal care procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985), and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Transplantation of baboon or pig artery graft

At the age of 3 months, five baboons (Group 2) were transplanted with a length of donor baboon or donor pig carotid artery, as an immunologic model for pediatric heart Tx. Donor artery grafts were obtained either from adult baboons (UOHSC) that were AB-I with the recipient, or from WT pigs (Large White/Landrace) of blood type O (Country View Farms, Schellsburg, PA, USA). Under full anesthesia, and after laparotomy and partial heparinization (100 IU/kg), the recipient aorta was clamped distally to the renal arteries and at the bifurcation, incised longitudinally, and a 1.0 × 0.5 cm patch of donor (baboon or pig) artery was sutured into the aortic wall as a full-thickness onlay graft. The clamps were removed and, after assuring hemostasis, the abdomen was closed.

Two baboons (Group 2A) received either an AB-I or WT pig graft, but no IS; these baboons were followed for immunologic studies for 6 weeks until euthanasia for pathologic studies. Three baboons (Group 2B) received an AB-I ($n = 2$) or a WT pig ($n = 1$) graft, and were immunosuppressed throughout follow-up after Tx (Table 1). IS consisted of induction with ATG, and was maintained using an anti-CD154 mAb and mycophenolate mofetil (MMF) (Table 2). Group 2B baboons were followed for 15 weeks after Tx.

Table 1. Experimental groups.

Group	Experiment				
1	Nontransplanted, no IS	6 Baboons aged 0–6 months 6 Baboons aged 1 year 6 Baboons aged 2–3 years			
		Baboon #	Blood group	Graft type	Age at Tx (days)
2A	Transplanted, no IS	7707	B	A	95
		7607	B	Pig	102
2B	Transplanted, IS	7507	B	A	98
		5008	A	B	107
		5508	B	Pig	98

IS, immunosuppression; Tx, transplantation.

Table 2. Immunosuppressive and supportive therapy in Group 2 baboons.

Induction therapy	Dose	Duration
Thymoglobulin	2.0–2.5 mg/kg i.v,	Days −3 and −1
Methylprednisolone	5 mg/kg i.v.	Before each dose of ATG and on day 0. The dose was then reduced by 1 mg/kg/day, and discontinued on day 5
Maintenance therapy		
Anti-CD154 mAb	20–25 mg/kg i.v.	Days −1, 0, 4, 7, 10, 14, then every 5–7 days
Mycophenolate mofetil	20–150 mg/kg/day p.o. divided in 2 doses	Begun on day 2 (to maintain a blood trough level of 3–6 μg/ml)
Supportive Therapy		
Cefazolin	25 mg/kg bid i.v	For 3 days after surgery
Famotidine	0.25 mg/kg bid i.v.	From day 3
Ganciclovir	5 mg/kg i.v.	From day 4 until 1 month post-Tx
Ketorolac	0.5 mg/kg i.v	Before every dose of anti-CD154 mAb
Buprenorphine	0.01 mg/kg bid i.v.	For 3 days after surgery

ATG, anti-thymocyte globulin; Tx, transplantation.

Flow cytometry studies

The following fluorochrome-conjugated mAbs were used in flow cytometry experiments: anti-CD3 Pacific Blue (clone: SP34-2), anti-CD4 PE-Cy7 (SK3), anti-CD8 APC-Cy7 (RPA-T8), anti-CD20 FITC (2H7), anti-CD25 APC-Cy7 (M-A251), anti-CD45RA PE-Cy5 (5H9), anti-CD62L PE (SK11), anti-CD127 PE (hIL-7R-M21) (all from BD Biosciences, San Jose, CA, USA), and anti-FoxP3 APC (PCH101) (eBioscience, San Diego, CA, USA).

Whole blood was incubated with the conjugated Abs or corresponding isotype controls, after which red blood cells were lysed using PharmLyse (BD Biosciences). Intracellular staining for FoxP3 to identify regulatory T cells (T_{Reg}) was performed according to the manufacturer's protocol (eBioscience). Cells were analyzed with a LSRII multicolor flow cytometer (BD Biosciences). Data were analyzed using FACSDIVA 6.0 software (BD Biosciences). Table 3 shows how different lymphocyte subsets were identified. Absolute cell numbers were calculated based on white blood cell counts obtained from our institution's hematology laboratory.

Mixed lymphocyte reaction

Mixed lymphocyte reaction (MLR) measured by ^{3}H-thymidine incorporation was carried out as previously described [18]. Briefly, as stimulator cells, peripheral blood mononuclear cells (PBMC) were isolated (i) from buffy coats of 150 ml of blood from an unrelated adult baboon (cryopreserved in aliquots to provide stimulator cells for each experiment) or (ii) from freshly obtained blood from Large White/Landrace WT pigs. As responder cells, PBMC obtained from buffy coats of fresh infant baboon blood were isolated. In the MLR, responder cells (0.4×10^6 cells/well) were stimulated with irradiated adult baboon or WT pig PBMC at a 1:1 ratio. All responder–stimulator combinations were set up in quadruplicate and were incubated for 5 days. Ten microliters of ^{3}H-thymidine labeling medium (1 μCi/well; New England Nuclear, Boston, MA, USA) were added to each well during the last 18 h of incubation. The cells were harvested on glass-fiber filter mats with a cell harvester, and were analyzed by beta-scintillation counting on a liquid scintillation counter (PerkinElmer, Waltham, MA, USA). The mean

Table 3. Monoclonal antibodies used to define lymphocyte subsets by flow cytometric analysis.

	Fluorochrome-conjugated monoclonal antibodies						
Lymphocyte subset	Pacific blue	PE-Cy7	APC-Cy7	FITC	PE-Cy5	PE	APC
T and B cells	$CD3^+$	$CD4^+$	$CD8^+$	$CD20^+$			
Naïve T cells (T_N)	$CD3^+$	$CD4^+$	$CD8^+$		$CD45RA^{hi}$	$CD62L^{hi}$	
Total memory T cells (T_{TotMem})	$CD3^+$	$CD4^+$	$CD8^+$		$CD45RA^{lo}$		
Effector memory T cells (T_{EM})	$CD3^+$	$CD4^+$	$CD8^+$		$CD45RA^{lo}$	$CD62L^{lo}$	
Central memory T cells (T_{CM})	$CD3^+$	$CD4^+$	$CD8^+$		$CD45RA^{lo}$	$CD62L^{hi}$	
Terminally differentiated effector memory T cells (T_{EMRA})	$CD3^+$	$CD4^+$	$CD8^+$		$CD45RA^{hi}$	$CD62L^{lo}$	
Regulatory T cells (T_{Reg})	$CD3^+$	$CD4^+$	$CD25^{hi}$			$CD127^-$	$FoxP3^+$

results of quadruplicate tests were expressed as counts per million and stimulation index (average counts of anti-baboon or anti-pig response divided by anti-self response).

Graft histology

At necropsy, lengths of aorta, including the graft, were fixed in 10% formalin and embedded in paraffin. Four-micrometer (4 μm) sections were cut and stained with hematoxylin and eosin for light microscopy.

Statistical analyses

Continuous variables are expressed as mean ± standard error of the mean, and compared using the (paired) Student *t*-test, linear regression, and repeated measures ANOVA for changes over time, as appropriate. Repeated measures ANOVA was followed by pairwise comparisons of peak values with values at birth. *P*-values < 0.05 were considered statistically significant. All analyses were performed with GRAPHPAD PRISM 4 for Macintosh (GRAPHPAD Software, La Jolla, CA, USA).

Results

Early lymphocyte development in healthy infant baboons

In Group 1 baboons aged 0–30 months, a linear decrease in the percentage of $CD4^+$ T-lymphocytes ($CD4^+$ cells) was observed (Fig. 1a). This decrease was complemented by a steady increase in the proportion of $CD8^+$ T-lymphocytes ($CD8^+$ cells) (Fig. 1a). During the first months of life, an increase in the absolute number of lymphocytes occurred. Numbers of $CD4^+$ cells were 2.8 ± 0.8 times higher at the age of 4 months compared with numbers at birth (repeated measures ANOVA, $P < 0.0001$, Fig. 1b). Numbers of $CD8^+$ cells were 6.6 ± 1.4 times higher at the age of 4 months compared with numbers at birth (repeated measures ANOVA, $P < 0.0001$). Because of the changing proportions over time, the fold-increase in $CD8^+$ cells was greater than of $CD4^+$ cells (*t*-test, $P = 0.034$). After 4 months, a decline in $CD4^+$ cells and stabilization of the numbers of $CD8^+$ cells was observed. No distinctive pattern in the development of $CD20^+$ B lymphocytes (B cells) could be discerned (Fig. 1c and d).

After observing the above trends in lymphocyte development early in life, we were interested in characterizing the phenotype of different T-lymphocyte subpopulations (Fig. 2a). In the first week of life, the majority of lymphocytes (62.1 ± 7.1% of $CD4^+$, and 58.5 ± 7.9% of $CD8^+$) possessed the phenotypic characteristics of naïve T cells (T_N), which stain positive for CD45RA and CD62L (Fig. 2b and e). The absolute numbers of $CD4^+$ and $CD8^+$ T_N increased during the first 4 months of life (Fig. 2c and f). However, as a percentage of total $CD4^+$ and $CD8^+$ cells, a decline in T_N occurred during the first 2 months (repeated measures ANOVA, $P = 0.015$ for $CD4^+$, $P = 0.003$ for $CD8^+$, Fig. 2b and e). Simultaneously, an increase in the percentages of effector memory T cells (T_{EM}) and central memory T cells (T_{CM}) was observed [in Fig. 2 presented separately as well as combined as total memory T cells (T_{TotMem})]. The changes were most pronounced in the $CD8^+$ population. When compared for differences in kinetics, the appearance of T_{EM} occurred earlier than the appearance of T_{CM} (Fig. 2 e). Figure 2d and g show the development of T_N and

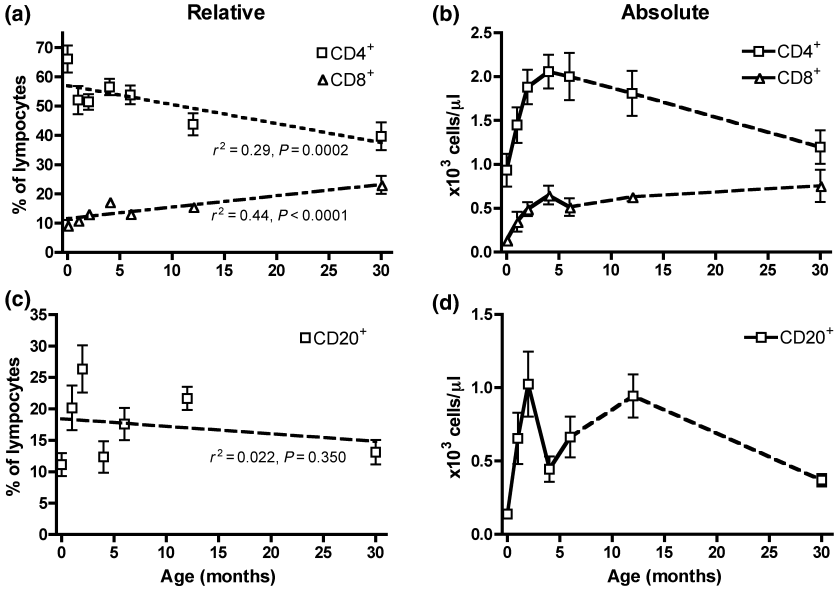

Figure 1 Changes in $CD4^+$ and $CD8^+$ T cells, and $CD20^+$ B cells in healthy infant baboons (Group 1). Left panels show proportions of total lymphocytes (a, c), right panels show absolute numbers, which increased three- to sevenfold during the first 4 months of life (b, d). Dashed lines beyond 6 months indicate that at 12 months and 30 months different subsets of baboons were investigated.

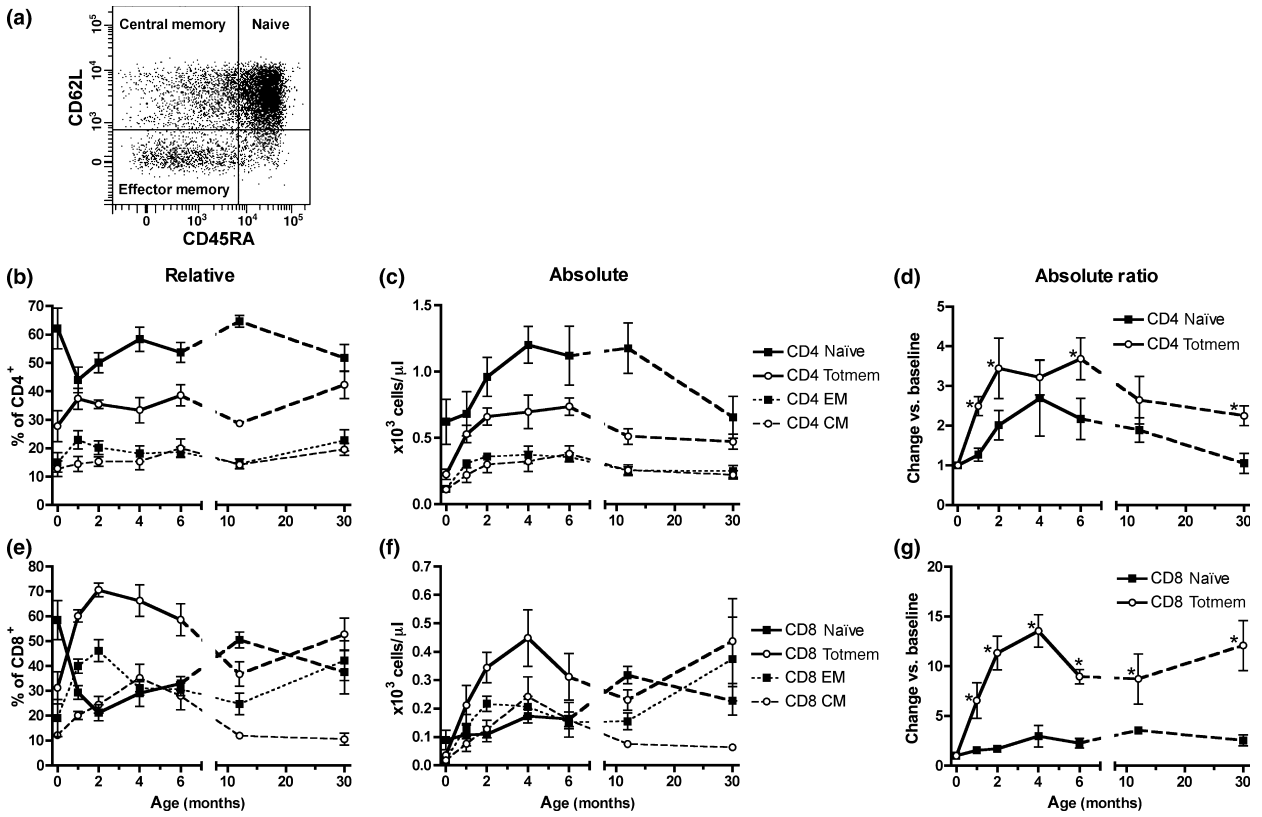

Figure 2 Lymphopenia-induced proliferation of memory T cells in healthy infant baboons (Group 1). (a) Dot plot of $CD4^+$ cells stained for CD45RA and CD62L to distinguish naïve and memory T cells. Proportions (b, e) and absolute numbers (c, f) of naïve and memory subpopulations among $CD4^+$ and $CD8^+$ cells. Absolute numbers expressed as a ratio of numbers measured <1 week after birth (d, g) show that expansion of T_{TotMem} was more significant than of T_N, indicating the occurrence of LIP. $^*P < 0.05$ vs. Naïve. (TotMem, total memory T cells; CM, central memory T cells; EM, effector memory T cells). Dashed lines beyond 6 months indicate that at 12 months and 30 months different subsets of baboons were investigated.

T_{TotMem} as a ratio of their respective baseline numbers at birth (set as 1.0), indicating that the expansion of T_{TotMem} was more significant than of T_N (paired *t*-test, $P < 0.05$ for most time-points). Beyond 2 months of age, the proportions of T_N and T_{TotMem} reversed towards proportions more comparable with those at birth. The percentages and numbers of terminally differentiated effector memory T cells (T_{EMRA}) remained relatively stable (not shown).

The relative and absolute numbers of $CD25^{hi}FoxP3^+$ $CD127^-$ T_{Reg} peaked at the age of 4 months (repeated measures ANOVA, both $P < 0.0001$, Fig. 3), at the same time-point as the highest measured total numbers of $CD4^+$ cells.

The dynamic rearrangements of T_N and T_{TotMem} did not seem to affect the functionality of the cellular immune system to mount an immune response, as investigated by MLR. The responses of infant baboon PBMC after stimulation with irradiated baboon or WT pig PBMC did not vary with age ($P > 0.05$ for each), (Fig. 4).

Changes in lymphocyte subpopulations after artery patch Tx

Group 2A: Tx in absence of IS

After Tx of an artery patch graft in two untreated infant baboons, the gradual decline in percentage of $CD4^+$ and increase of $CD8^+$ cells did not appear different from those in healthy untreated and nontransplanted baboons of comparable age (Fig. 5a). The presence of an AB-I or WT pig graft therefore did not seem to influence this evolution.

Three weeks post-Tx, a slight and transient, and nonsignificant, shift to increased proportions of memory T cells (T_{TotMem}, T_{EM}, T_{CM}) was seen for $CD4^+$ as well as $CD8^+$ cells (repeated measures ANOVA, $P = 0.374$ for $CD4^+$, Fig. 6a, and $P = 0.243$ for $CD8^+$, Fig. 6e), without major changes in absolute numbers (Fig. 6b and f).

After Tx without IS, no change in T_{Reg} as percentage of $CD4^+$ cells was detected (Fig. 7a). However, although we

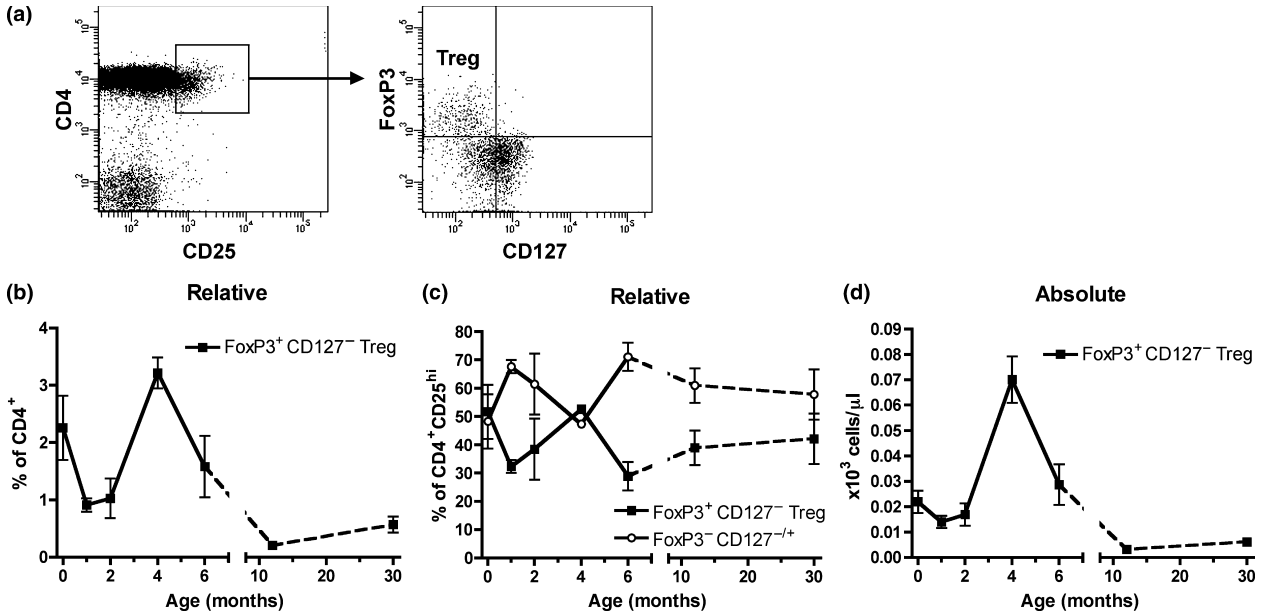

Figure 3 Regulatory T cells in healthy infant baboons (Group 1). (a) Identification of regulatory T cells among $CD4^+CD25^{hi}$ T cells. Proportions among $CD4^+$ (b) and $CD4^+CD25^{hi}$ (c) cells, and absolute numbers (d). (Treg, regulatory T cells). Dashed lines beyond 6 months indicate that at 12 months and 30 months different subsets of baboons were investigated.

were not able to provide statistical evidence because of the small number of baboons, within the $CD4^+CD25^{hi}$ population greater numbers of cells expressed FoxP3 (Fig. 7b), resulting in a peak of absolute numbers of T_{Reg} 4 weeks after Tx (Fig. 7c).

Although the changes in T_N and T_{TotMem} were small, during follow-up donor type-specific immunosensitization had readily occurred. This was evidenced by an increased response in the donor type-specific MLR (Fig. 4a–d), the appearance of Abs against graft antigens in recipient serum (anti-A Abs in AB-I Tx and anti-Gal Abs in WT xenoTx, respectively, Dons EM *et al.* manuscript submitted), and fibrosis and lymphocyte infiltration of the graft on histological examination (Fig. 4e).

Group 2B: Tx in presence of IS

Induction therapy with ATG resulted in depletion of >95% of $CD4^+$ cells, and >90% of $CD8^+$ cells in three infant baboons (Fig. 5b and d). $CD4^+$ cells were maintained at low levels with anti-CD154 mAb and MMF, as has been previously described in older animals [19] (Fig. 5a and b). $CD8^+$ cells recovered during the 3.5 months follow-up to levels comparable with those measured at baseline (Fig. 5c and d). The increased proportion of B cells within the lymphocyte gate (Fig. 5e) was a consequence of depletion of T cells, as no significant change in absolute numbers of B cells was noted (Fig. 5f).

During follow-up, pronounced changes in relative numbers of T_N, T_{TotMem}, T_{EM}, and T_{CM} were seen. $CD4^+$ T_N fell from 55.8 ± 6.6% at baseline to 14.8 ± 4.9% at 6 weeks post-Tx (repeated measures ANOVA, $P = 0.013$, Fig. 6c). During this period, $CD4^+$ T_{TotMem} had increased from 42.0 ± 6.6% to 82.2 ± 6.7% (repeated measures ANOVA, $P = 0.044$). At the age of 4 months, a major shift from T_N to T_{TotMem} as part of the natural development of the immune system would no longer be expected (compare with Fig. 2b), and is therefore likely to be caused by lymphocyte depletion and repopulation phenomena. Although absolute numbers of $CD4^+$ cells were still low, the altered proportions of T_N and T_{TotMem} resulted in the earlier repopulation of T_{TotMem} than T_N (Fig. 6d). Starting 2 months after Tx (at age 5 months), the proportions largely reversed to those measured pre-Tx (Fig. 6c).

The changes in $CD8^+$ T_N and T_{TotMem} followed similar patterns to those in $CD4^+$ cells. $CD8^+$ T_N levels fell from 38.9 ± 6.3% at baseline to 21.1 ± 3.6% at 3 weeks post-Tx (repeated measures ANOVA, $P = 0.003$, Fig. 6g). $CD8^+$ T_{TotMem} transiently increased from 58.2 ± 5.8% to 71.3 ± 3.2% (repeated measures ANOVA, $P = 0.027$), which, however, did not greatly influence absolute numbers (Fig. 6h). By 6 weeks post-Tx reversal to proportions comparable with baseline had occurred.

When changes in T_{Reg} after Tx without (Group 2A) and with (Group 2B) IS were compared, opposite

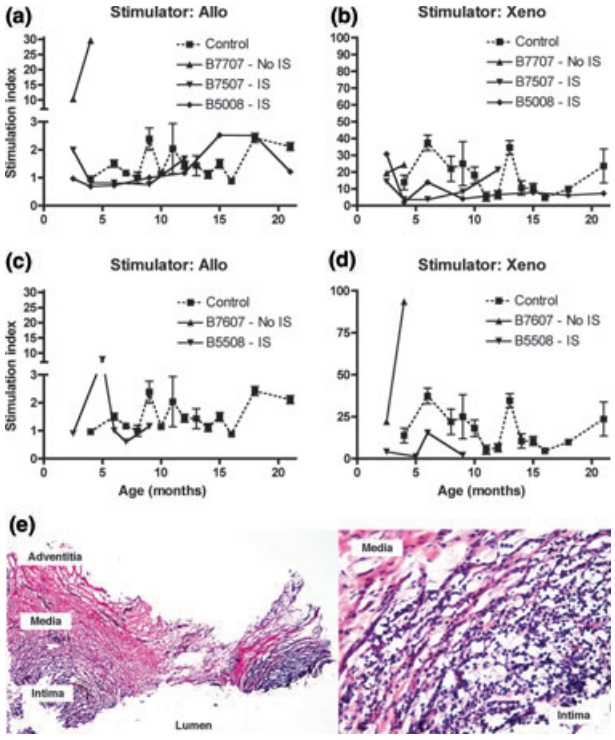

Figure 4 Stimulation indices after mixed lymphocyte reaction (MLR), and histology of artery grafts in Group 2 baboons. Responses in healthy infant baboons (Control) are compared with responses in baboons after AB-I allo-Tx (a, b) and WT pig xeno-Tx (c, d) in the absence (No IS) or presence (IS) of immunosuppressive therapy. Data for B7607 after allo-stimulation are missing because of technical error (c). (e) Graft histology 6 weeks after xenotransplantation without IS (Group 2A). Left panel (magnification 4×) shows loss of architecture. The location of the media in the vessel wall can still be recognized by collagen and smooth muscle (pink), but intense cellular infiltration has caused damage throughout the vessel wall (B7607). Right panel: lymphocytic infiltration of intima and media (magnification 40×, B7607). Clear (transparent) spaces are artifacts because of suboptimal tissue storage. Cellular infiltration was also seen after allo-transplantation (B7707, not shown).

phenomena were observed. While without IS the percentage of T_{Reg} among $CD4^+$ cells did not change (Fig. 7a), after T cell depletion and chronic IS a relative increase in T_{Reg} among $CD4^+$ cells was seen (although nonsignificant, Fig. 7d). However, this led neither to an increase in percentage of $FoxP3^+$ T_{reg} within the $CD4^+CD25^{hi}$ cell population nor to an increase in absolute numbers ($P > 0.05$, Fig. 7e and f). This indicated that FoxP3 expression did not increase among $CD4^+CD25^{hi}$ cells, and that the increase among $CD4^+$ cells was more likely a result of lymphocyte depletion, which relatively spared T_{Reg}.

The administered immunosuppressive protocol (ATG, anti-CD154 mAb, MMF) adequately suppressed the response in the MLR to donor-type stimulation (Fig. 4) and inhibited the induction of anti-donor Ab (not shown) during the course of follow-up.

Discussion

The aims of the studies presented here were to verify if infant NHP (i) undergo LIP to expand their T cell numbers after birth and (ii) are prone to the induction of immunologic tolerance to transplant antigens. Both phenomena have been previously documented in laboratory rodents, but it remained unknown if these fundamental immunological principles are similar in NHP and humans. We observed that infant baboons were relatively lymphopenic at birth. The initial naïve immune status and subsequent LIP of T cells, which generated memory T cells, did not affect the functionality of the immune system when challenged with AB-I baboon or WT pig antigens. IS including lymphocyte depletion was effective in the suppression of cellular rejection, but caused significant shifts in T cell phenotype.

During the first 4 months of life of the baboons, the blood T-lymphocyte pool expanded three- to sevenfold. Numbers of $CD8^+$ cells increased more substantially than the $CD4^+$ subpopulation (which relative numbers actually slightly declined, as previously reported in humans [5,14]). Although early in life the thymus still contributes importantly to T cell homeostasis, in our studies evidenced by increasing T_N, a relatively greater expansion of memory T-lymphocytes was seen. Two plausible mechanisms could have contributed to this observation, being (i) LIP and (ii) exposure to antigens in the environment, likely as a result of intestinal colonization [20], while further antigen exposure was limited in the specific pathogen-free housing environment. The observation that the shift toward increased T_{TotMem} was transient and reversed after approximately 6 months of age suggests a dominant role for LIP.

Heart Tx is the only life-saving therapy in certain congenital cardiac abnormalities. In neonates, a heart can be successfully transplanted across the ABO-blood group barrier [6–9], likely because the natural Abs against non-self blood groups do not develop until later. Moreover, after Tx the development of Abs against the donor blood group never occurred, while Abs against nondonor, non-self readily appeared. This indicates that B cell tolerance was induced [11], although it should be noted that recipients were thymectomized and remained fully immunosuppressed during follow-up. It is unknown if the T cell compartment contributed to this tolerant state, and whether it would be possible to reduce the (T cell-directed) IS in infant Tx recipients. We therefore established the infant baboon artery patch Tx model to undertake immunological studies.

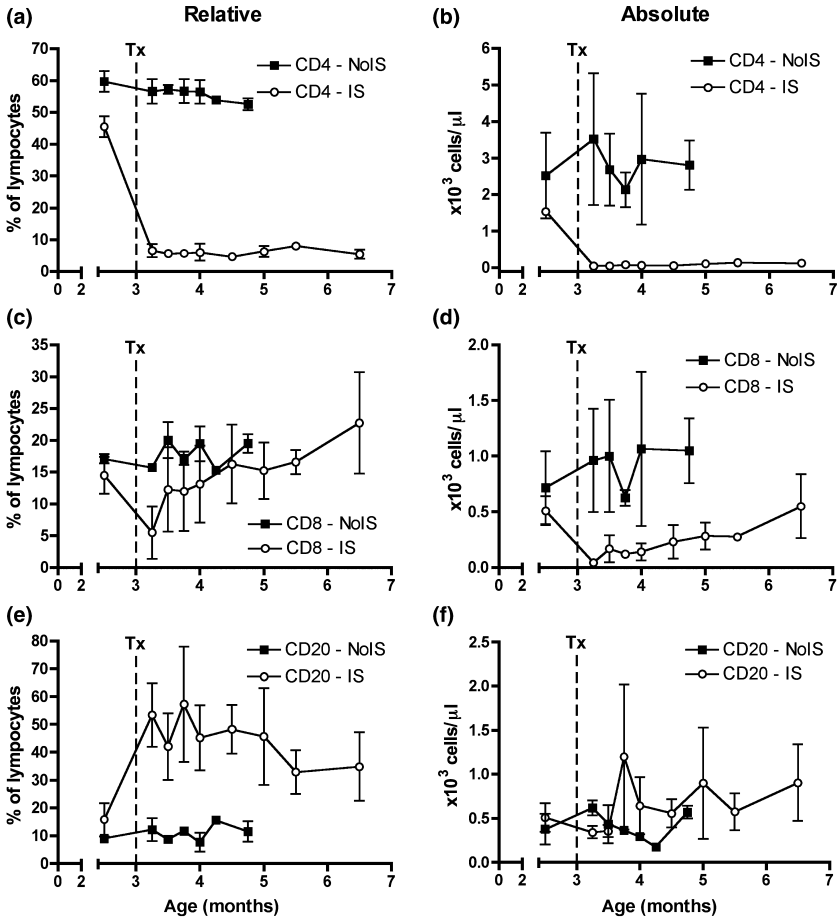

Figure 5 Changes in CD4$^+$ and CD8$^+$ T cells, and CD20$^+$ B cells in infant baboons after artery Tx with and without immunosuppression (Group 2). Left panels show percentages of total CD4$^+$ T cells (a), CD8$^+$ T cells (c), and B cells (e), right panels show corresponding absolute numbers (b, d, f). Data are presented in the absence (No IS – Group 2A) or presence (IS – Group 2B) of immunosuppressive therapy.

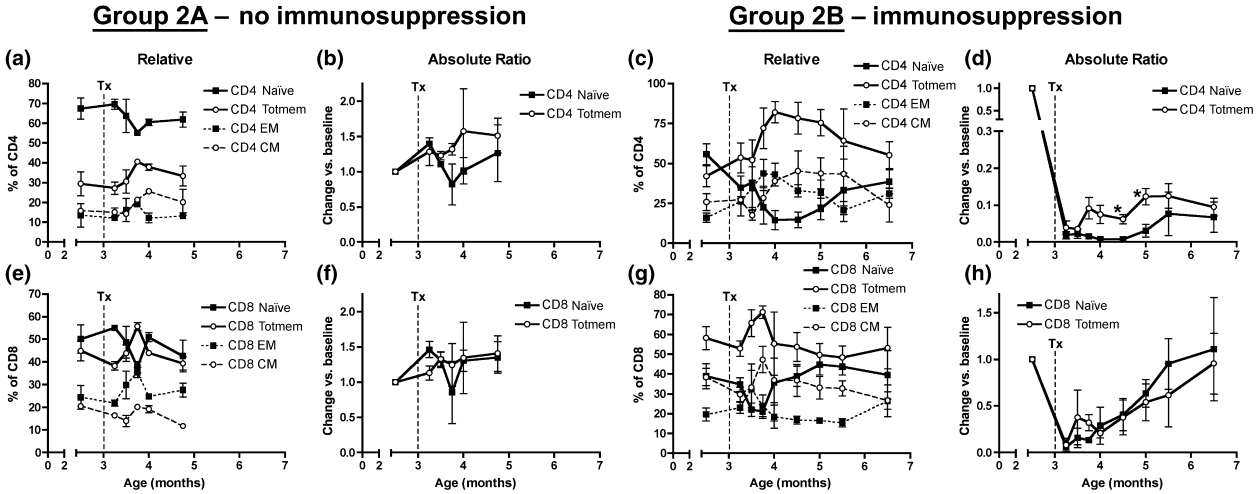

Figure 6 Naïve and memory subpopulations among CD4$^+$ and CD8$^+$ T cells in infant baboons after artery Tx with and without immunosuppression (Group 2). (a, b, e, f) Group 2A – No Immunosuppression. (c, d, g, h) Group 2B – Immunosuppression. Shifts toward a memory phenotype (c, g) and significantly greater expansion of memory T cells (d) indicated homeostatic proliferation after induction therapy with anti-thymocyte globulin. *$P < 0.05$ vs. Naïve. (TotMem, total memory T cells; CM, central memory T cells; EM, effector memory T cells).

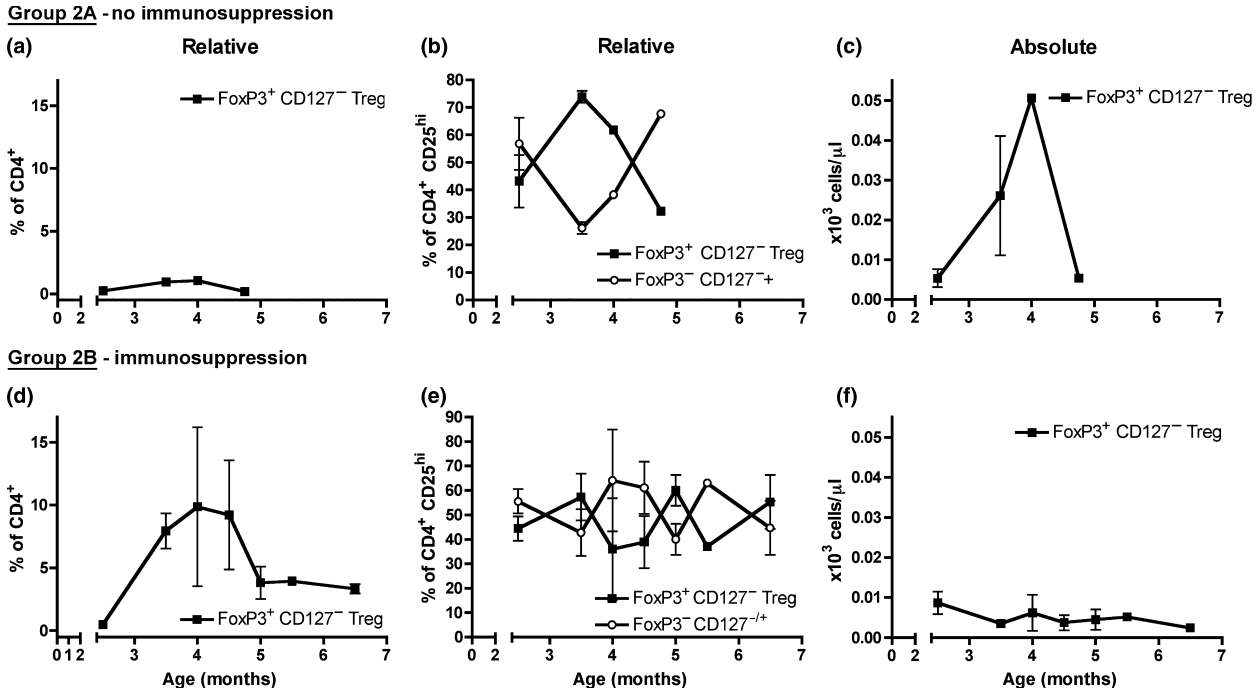

Figure 7 Regulatory T cells in infant baboons after artery Tx (Group 2). Proportions among $CD4^+$ (a, d) and $CD4^+CD25^{hi}$ (b, e) cells, and absolute numbers (c, f) in the absence (No Immunosuppression – Group 2A) or presence (Immunosuppression – Group 2B) of immunosuppressive therapy. (Treg, regulatory T cells).

Because heart Tx in infants is limited by the scarcity of size-matched donor hearts, we also investigated xenoTx, i.e., pig-to-human, which could provide at least a 'bridge' for the patient until a suitable human donor heart becomes available [21]. The observation that natural anti-pig Abs develop in a similar pattern as anti-A or B blood group Abs led us to hypothesize there might be an opportunity for tolerance induction to pig antigens [22].

Regarding T cell immunity, no major phenotypic changes in the T_N and T_{TotMem} compartments occurred after Tx without IS during 6 weeks of follow-up. Donor-type specific sensitization was induced, as proven by an increased response in the MLR and pathologic changes in the graft. These results indicate that the T cell immune system of baboon infants (at 3 months of age), although still antigen-naïve, can readily mount an immune response against donor antigens. The appearance of donor-type specific Abs in recipient serum indicated that B cell tolerance was not achieved in non-immunosuppressed baboons (described in detail by Dons EM *et al.*, manuscript submitted).

The administered IS prevented the induction of anti-donor-type Abs, and reduced the response in the MLR against donor-type stimulators, as well as against third-party stimulators. Signs of selective tolerance induction were therefore not observed, as this is traditionally defined as specific unresponsiveness to a specific foreign (Tx) antigen while maintaining reactivity to other (third-party) antigens [23]. Lymphocyte depletion with ATG caused significant shifts in naïve and memory T cells. Thereafter, peripheral blood was mainly repopulated with memory T-lymphocytes, a phenomenon known as homeostatic repopulation [24,25]. Possible effects of these vigorous phenotypic changes seem to have been overruled by the immunosuppressive regimen with respect to their *in vitro* functional potential in the MLR. However, it should be acknowledged that at the time of the major T_N–T_{TotMem} phenotypic shifts, T cell numbers were too low to harvest sufficient numbers to perform MLR. The intervals between MLR results are thus larger than the time required for phenotypic changes. We also acknowledge that the number of animals in our studies is small and that further conclusions might not be justified.

Homeostatic repopulation of memory T cells after lymphocyte depletion has been observed in patients with organ transplants [26–28], and can result in acute cellular rejection with T_{TotMem} predominating in peripheral blood and in graft histology [27]. Memory cells can be cross-reactive with graft antigens and cause rejection with reduced need for antigen presentation and co-stimulation, so called 'heterologous immunity' [23,29]. This represents a clinically-relevant problem, e.g., when weaning of

immunosuppression is attempted. Moreover, memory cells have been found to represent a barrier to Tx tolerance [23,30]. Successful suppression of memory T cells can promote transplant tolerance [31].

The ideal panel of conjugated mAbs used to identify lymphocyte subsets by their cell surface antigens remains a topic of debate. In our studies, we opted for differentiation of T_N and T_{TotMem} by mAbs against CD45RA and CD62L, a strategy that is well-established in humans [29,32] and NHP [33–36], and that we previously successfully applied in cynomolgus monkeys [37]. However, Pitcher *et al.* postulated that memory T cells in rhesus monkeys can best be determined by anti-CD95 and CD28 staining [16]. The different strategies, however, stained very significantly overlapping cell populations [16,38], leading us to conclude that CD45RA and CD62L can be reliably used in baboon studies.

In conclusion, after birth the immune system of infant baboons is relatively lymphopenic and naïve, and the numbers of T-lymphocytes significantly increase with a transient appearance of memory T cells. We hereby confirmed that neonatal NHP undergo LIP to establish T-lymphocyte homeostasis. After artery patch Tx in baboons of 3 months of age, an age at which natural Abs against ABO-blood group and pig antigens are still practically undetectable [22], the T cell immune system was able to readily and actively mount an immune response against donor-type antigens. On the basis of the assays performed, we therefore did not identify a T cell 'window of opportunity' for tolerance induction. However, the PBMC MLR is a crude functional assay, and more subtle signs of immune tolerance, or opportunities to achieve so, may have been missed. More detailed investigations in this regard are warranted. IS including lymphocyte depletion was associated with the expansion of memory T cells that may actually form an obstacle if immunologic tolerance is the goal.

Authorship

DJvdW and EMD: participated in research design, in the performance of the research, and in the writing of the paper. CLM, ME, and CL: participated in the performance of the research, and in the review of the paper. RFW: provided blood samples for the study and reviewed the paper. JNMIJ: participated in research design, and in the review of the paper. FGL and DKCC: participated in research design, and in the writing of the paper.

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