Memory T Cells Are Significantly Increased in Rejected Liver Allografts of Rhesus Monkeys

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The rhesus monkey (RM) is an excellent preclinical model in kidney, heart, and islet transplantation that has provided the basis for new immunosuppressive protocols for clinical studies. However, there remain relatively few liver transplantation (LT) models in nonhuman primates. In this study, we analyzed the immune cell populations of peripheral blood mononuclear cells (PBMCs) and secondary lymphoid organs along with livers of normal RMs and compared them with those of rejected LT recipients following withdrawal of immunosuppression. We undertook 5 allogeneic ABO compatible orthotopic LTs in monkeys using 5 normal donor monkey livers. We collected tissues including lymph nodes, spleens, blood, and recipient livers, and we performed flow cytometric analysis using isolated immune cells. We found that CD4 or CD8 naïve T cells were normally seen at low levels, and memory T cells were seen at high levels in the liver rather than lymphoid organs or PBMC. However, regulatory cells such as CD4+ forkhead box P3+ T cells and CD8+ CD28– cells remained in high numbers in the liver, but not in the lymph nodes or PBMC. The comparison of CD4/8 T subpopulations in normal and rejected livers and the various tissues showed that naïve cells were dramatically decreased in the spleen, lymph node, and PBMCs of rejected LT monkeys, but rather, the memory CD4/8 T cells were increased in all tissues and PBMC. The normal liver has large numbers of CD4 regulatory T cells, CD8+ CD28–, and myeloid-derived suppressor cells, which are known immunosuppressive cells occurring at much higher levels than those seen in lymph node or peripheral blood. Memory T cells are dramatically increased in rejected liver allografts of RMs compared with those seen in normal RM tissues.

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Liver transplantation (LT) remains the gold standard treatment for end-stage liver disease along with acute fulminant hepatic liver failure and hepatocellular carcinoma.[1] The liver is a unique anatomical and immunological organ with the liver's lymphocyte population selectively enriched in natural killer (NK) cells and natural killer T cells (NKTs), which play critical roles in the first lines of immune defense against invading pathogens as well as modulation of liver injury and recruitment of circulating lymphocytes.[2] These unique features have underpinned early graft acceptance rates following LT, which have seen a significant increase not only because of the unique nature of the liver but also due to the development and use of novel targeted immunosuppressive drug regimens. However, disappointingly the rates of late graft failure still remain high and largely unchanged over the last decade.[1] Clearly then, new therapeutic strategies should be developed and used to improve the outcome of LT focusing on the use of the very unique nonhuman primate (NHP) model.

Abbreviations: ACR, acute cellular rejection; APC, allophycocyanin; Cy7, cyanine 7; DC, dendritic cell; ELISPOT, enzyme-linked immunosorbent spot; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; FOXP3, forkhead box P3; H & E, hematoxylin-eosin; HLA, human leukocyte antigen; IFN, interferon; IHC, immunohistochemistry; Lin, lineage; LT, liver transplantation; mAb, mitochondrial antibody; mDC, myeloid or conventional dendritic cell; MDSC, myeloid-derived suppressor cell; MHC, major histocompatibility complex; NHP, nonhuman primate; NK, natural killer; NKT, natural killer T cell; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; pDC, plasmacytoid dendritic cell; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; POD, postoperative day; RM, rhesus monkey; SSC, standard sodium citrate; Treg, regulatory T cell.

Despite rodents offering some advantages for experimental research, including ease of genetic manipulation and a vast array of biological tools and resources, they still do not provide a comprehensive model for all transplantation research. The inbred nature of laboratory rodents such as their short life span and the scarcity of murine homologues to human pathogens restricts the successful transfer of immunological discoveries made in murine models to the clinical setting[3,4] which makes them less ideal for this purpose than large animal models. However, NHPs share significant genetic homology as well as anatomical, physiological, hematological, and immunological characteristics with humans, therefore offering a unique opportunity to carry out mechanistic studies in a species that more closely mimics human biology.[3] Rhesus monkeys (RM; *Macaca mulatta*) and baboons have been shown to be excellent organ transplantation models. Specifically in regards to kidney, heart, and islet allotransplantation and xenotransplantation, preclinical RM models have provided the basis for new immunosuppressive protocols for application to clinical studies.[5-9] Many preclinical studies of kidney and islet allotransplantation have provided effective guidelines or developed broadly accepted therapeutic strategies, which have then been moved into the clinic.[7,10] There are also many reports of biomarkers and new drug applications especially in kidney transplantation that quite readily could be tested in such preclinical models.[11-13]

However, there are relatively few LT models of NHPs[14-17] that have been undertaken for such. This is especially true when it comes to investigating immune cell populations, which are important in monitoring the immune status of the recipient following LT. In this regard, it is therefore necessary to understand the background status of the immune cell populations in lymphoid organs, liver, and peripheral blood of normal RMs to ensure that we have adequate knowledge to compare them with our transplanted animals. Fortuitously, Messaoudi et al. reviewed recent advances in NHP innate and adaptive immune systems, specifically in the RM,[3] and they suggested valuable diverse markers for the immune cell population of RMs which we are able to use.

In order to establish the normal ranges of immune cell populations of the liver, secondary lymphoid organs, and peripheral blood, we analyzed the immune cell populations of peripheral blood mononuclear cells (PBMCs) and secondary lymphoid organs and the livers of normal RMs and then compared them with those of rejected LT recipients following withdrawal of immunosuppression. From this, we demonstrate that the liver has rather unique immunological properties compared with other organs and establish the basis for undertaking further trials to establish novel immunosuppressive strategies targeted for LT.

Materials and Methods

EXPERIMENTAL ANIMALS

RMs (*Macaca mulattas*) were used for this study, and 1 donor was used for each recipient with all of them being male (Table 1). Recipient and donor pairs were selected

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Hwajung Kim participated in research design, performance of experiments (immune cells analysis), data analysis, and the writing of the article. Hyeyoung Kim participated in research design, performance of experiments (liver transplantation), and data analysis. Sun-Kyung Lee participated in the performance of experiments (immune cells analysis). Xue-Li Jin participated in the performance of experiments (immune cells analysis). Tae Jin Kim participated in research design and data analysis. Chanho Park participated in the performance of experiments (immune cells analysis and enzyme-linked immunosorbent spot). Jae-Il Lee participated in research design and data analysis. Hyo-Sin Kim participated in performance of experiments (liver transplantation) and data analysis. Suk Kyun Hong participated in the performance of experiments (liver transplantation) and data analysis. Kyung Chul Yoon participated in the performance of experiments (liver transplantation) and data analysis. Sung Woo Ahn participated in the performance of experiments (liver transplantation) and data analysis. Kyoung-Bun Lee participated in data analysis for immunohistochemistry. Nam-Joon Yi participated in the performance of experiments (liver transplantation) and data analysis. Jaeseok Yang participated in research design and data analysis. Kwang-Woong Lee participated in the performance of experiments (liver transplantation) and data analysis. Wayne J. Hawthorne participated in data analysis and the writing of the article. Kyung-Suk Suh participated in research design, performance of experiments (liver transplantation), data analysis, and the writing of the article.

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Potential conflict of interest: Nothing to report.

TABLE 1. Information of Recipient, Graft Survival, and Complications After Transplantation in RMs

Recipient				Donor				Group	Outcome	
Identification Number	Weight, kg	Blood Group	Age, Months	Identification Number	Weight, kg	Blood Group	Age, Months	Treatment	Survival	Diagnosis
21-14	3.6	B	47	21-13	3.4	B	45	No IS	POD 5	ACR
203	5.5	AB	67	205	4.58	B	62	No IS	POD 6	ACR
1105	5.5	AB	36	1171	4.68	B	35	Conventional IS	POD 52	Rejection
17RM29	6.2	AB	63	17RM28	5.8	B	63	Conventional IS	POD 57	Ongoing
17RM27	5.4	B	56	17RM25	5	B	56	Conventional IS	POD 22	Ongoing

by blood typing and cross-match testing according to our previous study.[18] We designed the allogeneic ABO-compatible orthotopic LT model in the RM to mimic that used in human LTs. The surgical procedures were the same as those used for human deceased donor LT but without venovenous bypass using standard cavaocaval anastamosis. Immunosuppression was based on our clinical LT triple-immunosuppressive regimen with a calcineurin inhibitor (tacrolimus, for 50 days with 1 mg/bid; trough level, 5-10 ng/mL), rapamycin (for 38 days with 0.5 mg/kg; trough level, 3-8 ng/mL), and steroids (for 28 days with 1 mg/kg), but we did not use basiliximab induction therapy.

All animals received the utmost humane standards of care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital.

COLLECTION OF IMMUNE CELLS FROM THE LIVER, SPLEEN, AND LYMPH NODES

For the normal animals, baseline samples collected were spleen, lymph nodes, and blood samples at the time of their liver donation. The native liver from the LT recipient was also taken as a normal control. Briefly, all tissues (lymph nodes, spleen, liver) were dissected and placed in Roswell Park Memorial Institute 1640 medium and kept on ice until processed. For spleen and lymph nodes lymphocyte purification, the tissues were gently squashed through a 100-μm cell strainer (Thomas Scientific, Swedesboro, NJ) and washed in phosphate-buffered saline (PBS) supplemented with 0.2% heat-inactivated bovine serum. To isolate lymphocytes from the liver, it was dissected and incubated in Roswell Park Memorial Institute 1640 medium with 200 U/mL collagenase (Sigma-Aldrich, St. Louis, MO) and 30 U/mL DNase (Roche, Basel, Switzerland) for 1.5 hours at 37 °C under continuous shaking. Undigested tissue was removed by centrifugation at 800 rpm for 1 minute, and the fluid containing single cells was collected, transferred into a new tube, and washed with PBS supplemented with 0.2% human serum.[19]

PBMC ISOLATION AND FLUORESCENCE-ACTIVATED CELL SORTING

Freshly drawn ethylene diamine tetraacetic acid anticoagulated blood samples were collected from healthy RMs, and their PBMCs were isolated using Ficoll separation (Ficoll-PaqueTM PLUS, GE Health Sciences, Uppsala, Sweden). To monitor immune cell populations, washed PBMCs were surface-stained with the following antibodies:

1. Anti-CD45-BV510 was used for lymphocyte gating, and anti-CD3-phycoerythrin (PE)–cyanine 7 (Cy7), CD4–peridinin chlorophyll protein (PerCP), CD8–allophycocyanin (APC)–H7, CD28-PE, CD95-APC, CD20–fluorescein isothiocyanate (FITC), and CD27-V450 (BD Bioscience, San Diego, CA) were used to monitor T cells, memory T cells, and B cells.
2. Anti–lineage (Lin) 3-FITC and CD123-PE, human leukocyte antigen (HLA)–DR-PE-Cy7 (BD Bioscience), and CD11c-APC (e-bioscience, San Diego, CA) were used for dendritic cell (DC) analysis.
3. Anti-CD56-FITC, CD3-V450, HLA-DR-PE-Cy7, CD20-APC-Cy7, CD11b-PE, CD14-PerCP, and CD33-APC (Milteny Biotech, Auburn, CA) were used to monitor NK or NKT or monocyte.

For analysis of forkhead box P3 (FOXP3) expression (eBioscience, San Diego, CA), PBMCs were used, and the staining protocol was essentially the same as surface-staining with anti-CD4 and anti-CD25, except for permeabilization, which was performed using the kit provided with the antibody according to the manufacturer's instructions. Staining patterns were visualized by flow cytometry

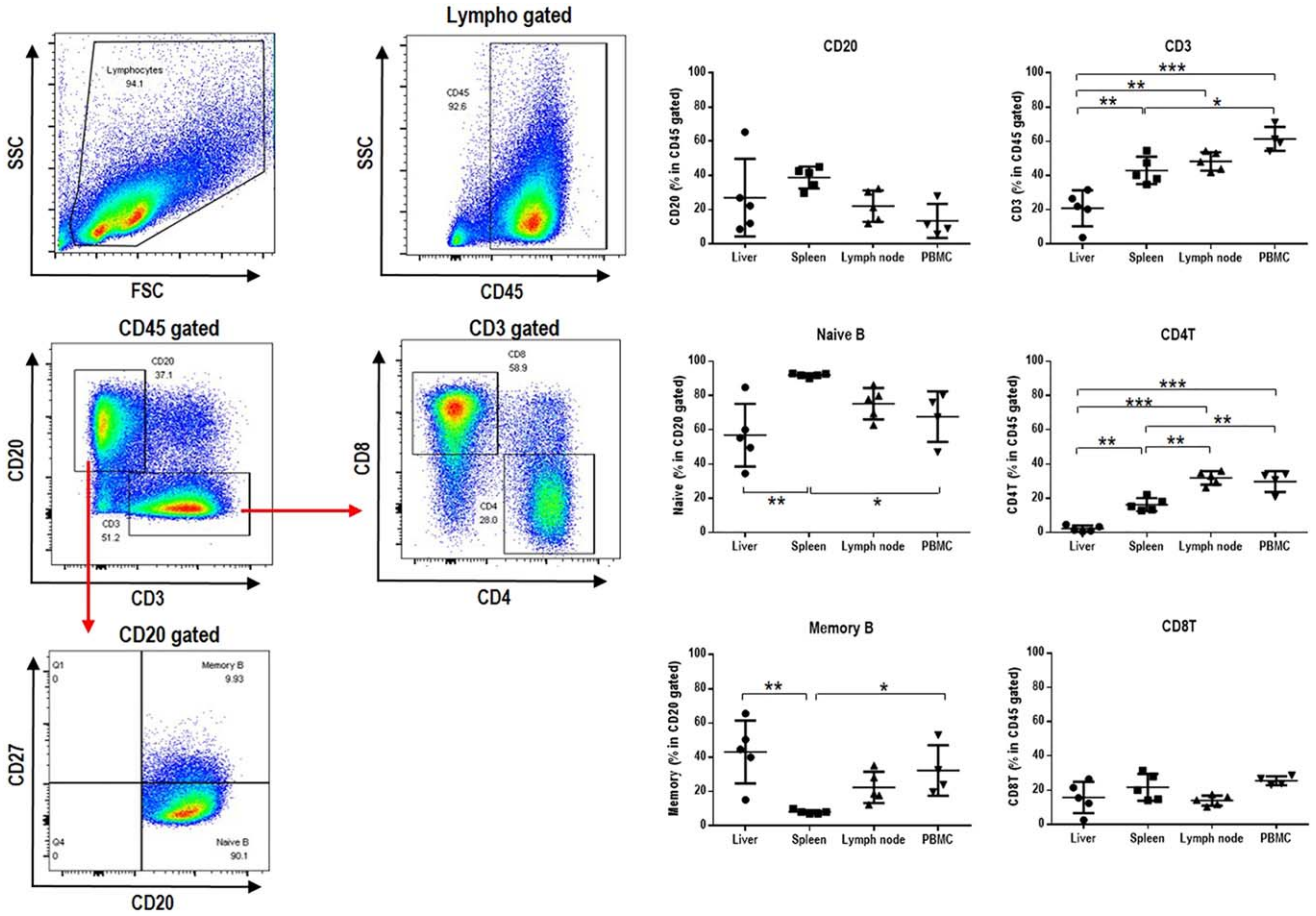

FIG. 1. Normal rhesus macaques have low levels of T and B cell populations in their livers compared with the spleen, lymph node, or PBMC. To analyze T and B cell populations, we gated with the CD45+ population as an immune cell subset and then divided it into CD3 and CD20, respectively. Naïve or memory B cells were measured by CD27– or CD27+ among CD20+ populations. (A) CD4T or CD8T cells were divided from the CD3 gated population. The CD3+ T cell population seen in rhesus macaque livers was significantly lower when compared with their spleen, lymph node, or PBMC (43.0% ± 8.0%, $P < 0.01$; 48.2% ± 5.5%, $P < 0.01$; and 61.4% ± 7.0%, $P < 0.001$). Most of the CD3+ T cells in the liver were CD8+ T cells (73.9% ± 9.2%), and CD4+ T cells were at low levels of 12.6 ± 8.2. CD4+ T cells in the liver was lower than the spleen, lymph node, and PBMC (each $P < 0.01$, 0.001, 0.001). Most B cells in their spleens were of the naïve phenotypes (91.9% ± 1.1%) rather than memory phenotypes (8.1% ± 1.1%). (B) There were no significant differences of B cell subtypes seen between the liver, lymph node, or PBMC (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

(fluorescence-activated cell sorting [FACS] Canto II [HTS], BD Bioscience). Data analysis was performed using FACSDiva software (BD Bioscience).

T AND B CELL SUBSET ANALYSES

To analyze T and B cell populations, we gated with the CD45+ population as an immune cell subset and then divided it into CD3 and CD20, respectively. Naïve and memory B cells were measured as CD27– or CD27+ among CD20+ populations. CD4T and CD8T cells were divided from the CD3 gated population (Fig. 1A). The CD4+ and CD8+ T cells from among the CD3+ gated cells were divided into CD28 and CD95; naïve or memory phenotypes of CD4T or CD8T subpopulations were measured by their expression of CD28 and CD95. Naïve cells were defined as a CD28+ CD95– expressing subset, the effector memory cells were defined as CD28–CD95+, and central memory cells were defined as CD28+ CD95+ (Fig. 2A).

INNATE CELL ANALYSIS

Innate cells such as NK, NKT, monocyte, myeloid-derived suppressor cells (MDSCs), and DCs were

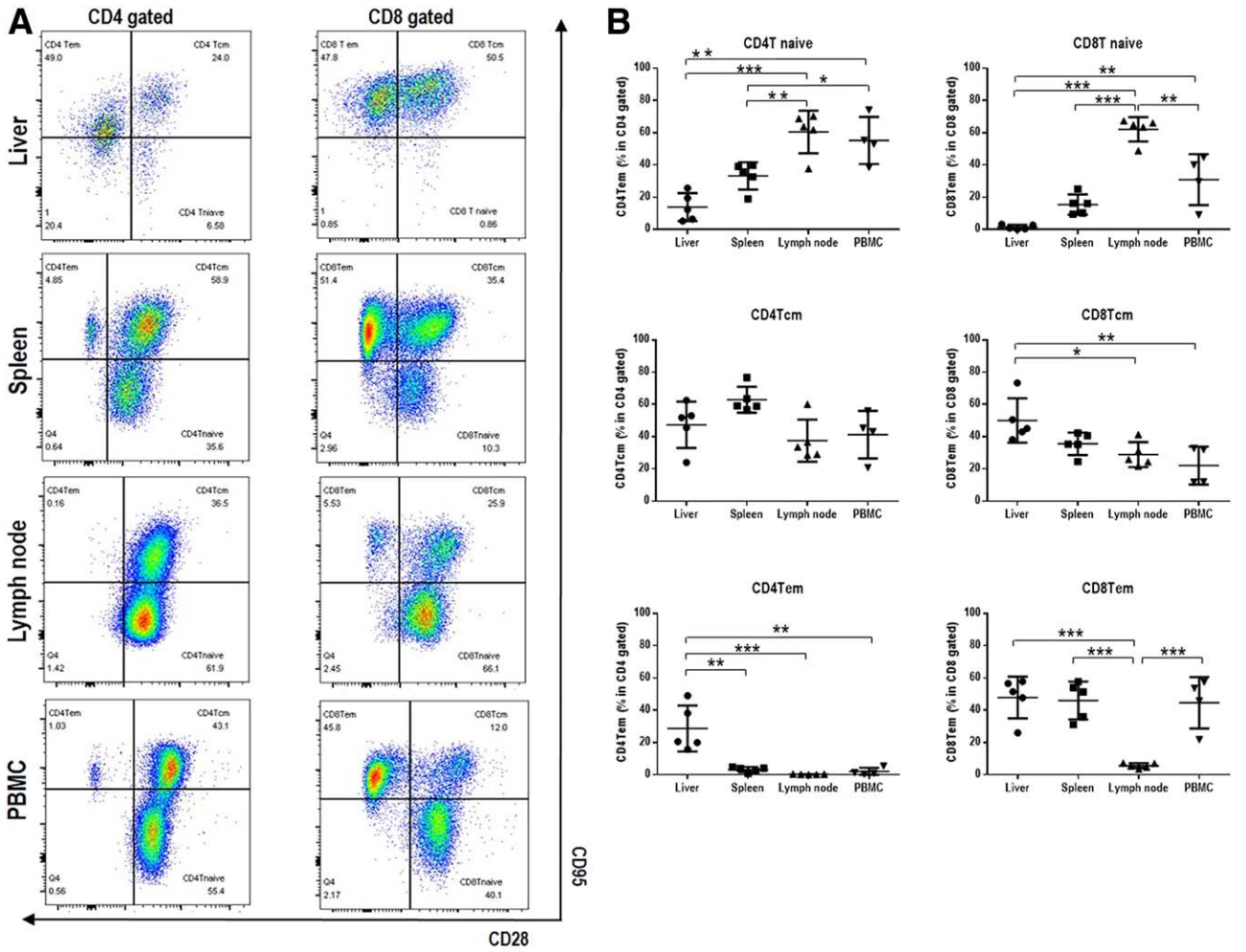

FIG. 2. Normal rhesus macaque CD4 or CD8T subpopulations in the liver, spleen, lymph node, and PBMCs. Naïve and memory phenotypes of CD4T or CD8T subpopulations were measured by CD28 and CD95, and the CD4+ and CD8+ cells were divided into CD28 and CD95. (A) Naïve cells were defined as CD28+ CD95–, effector memory cells were defined as CD28– CD95+, and central memory cells were defined as CD28+ CD95+. Most CD4T or CD8T cells in the liver existed as memory phenotypes such as effector memory T cells (28.7% ± 14.2% or 48.0% ± 12.9%) or central memory T cells (47.4% ± 14.4% or 50.0% ± 13.8%), and naïve CD4T or CD8T cells were significantly minor populations (13.9% ± 8.7% and 1.5% ± 1.4%, respectively) compared with other secondary lymphoid organs and PBMC. (B) However, lymph nodes have abundant naïve CD4T and CD8T cells (60.5% ± 13.2% or 62.2% ± 7.6%) with small numbers of CD4T or CD8T effector memory cells (2.1% ± 2.3% or 5.7% ± 1.6%) compared with the liver and others tissues (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

analyzed in accordance with previous studies.[3,20] NK and NKT cells were identified as CD3– CD56+ or CD3+ CD56+ cells from the CD45 gated cells (Fig. 3A). MDSCs were identified as CD11b+ CD33+ cells from among the CD3– CD56– CD20– HLA-DR– population. Monocytes were identified as CD14+ cells from among the CD20– HLA-DR+ populations. DCs were identified as Lin mitochondrial antibody (mAb) cocktail (anti-CD3, −14, −19, −20) negative, and HLA-DR+ populations and were divided into 2 distinct populations:

1. Myeloid or conventional dendritic cells (mDCs), which were identified as $CD11c^{pos}$ $CD123^{dim}$.[3,14]
2. Plasmacytoid dendritic cells (pDCs), which were identified as $CD11c^{neg}$ $CD123^{brigt}$ like human DCs subsets.[3,14]

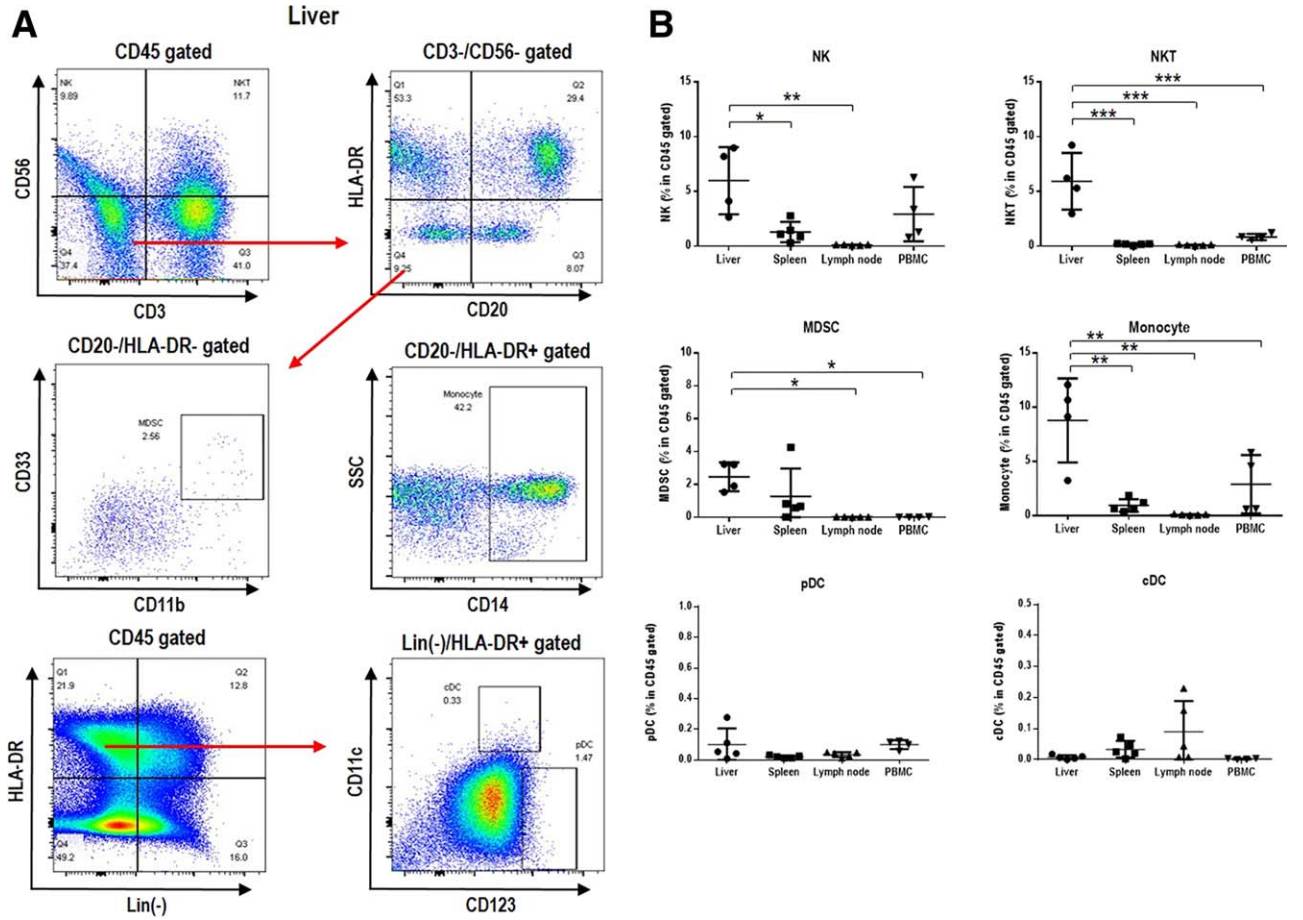

FIG. 3. Normal rhesus macaque innate cell populations in the liver, spleen, lymph node, and PBMC. NK or NKT cells were identified as CD3– CD56+ or CD3+ CD56+ cells from CD45 gated cells. MDSC were identified as CD11b+ CD33+ cells from the CD3– CD56– CD20– HLA-DR– cells. Monocytes were identified as CD14+ cells from CD20+ HLA–DR+ populations. (A) DCs were identified as Lin mAb cocktail (anti-CD3, −14, −19, −20) negative and HLA-DR positive populations and were divided into 2 distinct populations:

1. mDC, which were identified as $CD11c^{pos}CD123^{dim}$.
2. pDCs, which were identified as CD11c(−) CD123 high the same way as human DCs subset.

NK and NKT cells were seen at significantly higher levels in the liver (4.9% ± 3.6% and 4.8% ± 3.4%) compared with other lymphoid organs and PBMC. MDSCs were also seen at high levels in the liver (2.7% ± 0.9%) when compared with lymph node or PBMC and they both had few MDSCs, which were seen at <0.02%. (B) Monocytes were abundant in the liver (8.2% ± 3.6%) compared with the spleen (0.9% ± 0.6%) or lymph node (0.1% ± 0.1%) or PBMC (2.9% ± 2.7%; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

REGULATORY T CELL ANALYSIS

Haanstra et al. showed that the CD4+ $CD25^{high}$ cells of RM have similar phenotypic and functional characteristics to those of the natural regulatory T cells (Tregs) in humans.[21,22] CD4 Tregs were identified as FOXP3+ populations from among the CD4+ $CD25^{high}$ population. CD8 Tregs were identified as CD8+ CD28– T cells from among the CD45+ gated cells.[23,24]

ENZYME-LINKED IMMUNOSORBENT SPOT ANALYSIS

The numbers of donor-reactive interferon (IFN) γ–secreting cells were measured by enzyme-linked immunosorbent spot (ELISPOT). The 96-well filtration plates (Merck Millipore, Darmstadt, Germany) were coated overnight with purified anti-rhesus IFNγ antibodies (MT126L, Mabtech, Nacka Strand, Sweden) at

4 °C. Blocking was performed with 1% bovine serum albumin in PBS for 1 hour at room temperature, after which plates were washed with washing buffer (0.05% Tween-20 in PBS). Splenocytes (1×10^6 cells/well) were cultured with 25Gy-irradiated donor blood mononuclear cells for 48 hours at 37 °C in a CO_2 incubator. Biotinylated anti-rhesus IFNγ antibody (mAB7-B6-1, Mabtech, Nacka Strand, Sweden) was then added, followed by incubation for 1 hour at room temperature. After washing, streptavidin-horseradish peroxide was added, followed by incubation for 1 hour at room temperature. After washing, 100 μL 3-amino-9-ethylcarbazole substrate (BD Bioscience) was added to each well, and the reactions were allowed to proceed for 5 minutes. The ELISPOT plates were analyzed using an ImmunoSpotTM 3B instrument (CellularTechnologies, Cleveland, OH).

IMMUNOHISTOCHEMISTRY ANALYSIS

The liver graft was collected at the study end point from the recipient at the time of graft rejection. Multiple consecutive cross-sectional pieces were obtained from the graft to evaluate morphological changes and immune cell infiltration. These multiple liver pieces were fixed in 10% buffered formalin, and 5-μm sections were cut from the paraffin-embedded tissue for hematoxylin-eosin (H & E) and IHC. For immunohistochemistry (IHC), antigen retrieval was performed using pressure cooking at 125°C for 60 seconds and then at 90°C for 10 seconds. The slides were incubated for 30 minutes with the primary antibody—polyclonal anti-human CD4, Santa Cruze or anti-human CD8, Abcam or anti-human neutrophil elastase (Abcam, Cambridge, UK)—which were diluted 1:50, 1:200, or 1:500, respectively. After washing with a wash buffer (×10) containing tris (hydroxymethyl) aminomethane (Tris)/hydrochloric acid and sodium chloride (catalog number S3006, Dako, Glostrup, Denmark) and peroxidase blocking, the tissue was incubated with an Envision+ System–labeled polymer/horseradish peroxidase anti-rabbit antibody (catalog number SH25-500D, Dako).[25]

STATISTICAL ANALYSIS

Statistical analysis was performed with Prism 6 for Windows (GraphPad, San Diego, CA). Data are shown as mean ± standard error of the mean and significance of differences was analyzed using a 1-way analysis of variance and Tukey's correction.

Results

Five RMs weighing a mean of 5.2 ± 1.0 kg received LTs from 5 donor animals weighing a mean of 4.7 ± 0.9 kg. All 5 transplants were successful with immediate graft function as seen by liver function tests. Of the 5 LTs, 2 were performed without the use of immunosuppression and 3 cases with conventional immunosuppression which included tacrolimus, rapamycin, mycophenolate mofetil, and steroids. The 2 control animals that had no immunosuppression lost their liver grafts from rejection on day 5 and 6 after transplantation. The 3 animals that received conventional immunosuppression showed no features of rejection and also had normal liver function tests. However, liver function test and C-reactive protein levels increased following withdrawal of immunosuppression on postoperative day (POD) 50 and immediately signs of rejection were seen from POD 51 onward until the graft was removed at completion of the experiment within several days, and the others still have normal liver functions with immunosuppression (data not shown).

NORMAL RHESUS MACAQUES HAVE LOW LEVELS OF T AND B CELL POPULATIONS IN THEIR LIVER COMPARED WITH THE SPLEEN, LYMPH NODE, OR PBMC

In the normal nontransplanted rhesus macaque livers, the CD3+ T cell population was significantly lower (20.81% ± 10.54%) than those in the spleen, lymph node, or PBMC (43.0% ± 8.0%, $P < 0.01$; 48.2% ± 5.5%, $P < 0.01$; and 61.4% ± 7.0%, $P < 0.001$). The majority of the liver CD3+ T cells were CD8+ T cells (73.9% ± 9.2%) with CD4+ T cells seen at the level of 12.6% ± 8.2%. Interestingly, the CD4+ T cell population of the liver was significantly lower than those seen in the spleen ($P < 0.01$), lymph node ($P < 0.001$), and PBMC ($P < 0.001$). On further analysis, we found that most B cells from their spleens were of naïve phenotypes (91.9% ± 1.1%) rather than memory phenotypes (8.1% ± 1.1%). However, there were no differences seen in the B cell subtypes of their livers, lymph nodes, and PBMCs (Fig. 1B).

NORMAL RHESUS MACAQUE CD4 OR CD8T SUBPOPULATIONS IN THE LIVER, SPLEEN, LYMPH NODE, AND PBMCS

Most CD4 or CD8 T cells present in the normal liver were effector memory T cells (28.7% ± 14.2% or

48.0% ± 12.9%) or central memory T cells (47.4% ± 14.4% or 50.0% ± 13.8%), and the percentages of naïve CD4T and CD8T cells were significantly lower (13.9% ± 8.7% and 1.5% ± 1.4%, respectively) than those in the secondary lymphoid organs or PBMCs. Compared with normal livers, the normal RM spleens had a significantly higher percentage of CD4T central memory cells (63.0% ± 8.0%), but the CD4T effector memory cells were seen at low levels (3.2% ± 1.7%), which were in similar percentages as seen in the lymph node and PBMCs. Their lymph nodes had abundant naïve CD4T or CD8T cells (60.5% ± 13.2% or 62.2% ± 7.6%) and small proportions of CD4T or CD8T effector memory cells (2.1% ± 2.3% or 5.7% ± 1.6%). Their PBMCs had a higher number of naïve CD4T cells (55.3% ± 14.6%), but their CD4T effector memory cells were present at significantly lower levels (2.1% ± 2.3%) than those in the liver ($P < 0.01$; Fig. 2B).

NORMAL RHESUS MACAQUE INNATE CELL POPULATIONS IN THE LIVER, SPLEEN, LYMPH NODE, AND PBMC

The percentages of NK and NKT cells were significantly higher in the liver (4.9% ± 3.6% and 4.8% ± 3.4%, respectively) than those in other lymphoid organs or PBMC. MDSCs were seen at higher levels in the liver (2.7% ± 0.9%) than in the lymph node or PBMC, which both had extremely low levels of MDSCs < 0.02%. Monocytes were abundant in the liver (8.2% ± 3.6%) when compared with the spleen (0.9% ± 0.6%), lymph node (0.1% ± 0.1%), or PBMC (2.9% ± 2.7%). Interestingly, DCs were a very minor population with a proportion of <1% in the liver, spleen, lymph node, and PBMC, and there were no significant differences among these tissues (Fig. 3B).

NORMAL RHESUS MACAQUE REGULATORY CELL POPULATIONS OF THE LIVER, SPLEEN, LYMPH NODE, AND PBMC

FOXP3+ CD4 Tregs were seen at higher levels in the liver (0.4 ± 0.3) compared with the lymph node (0.1 ± 0.0; Fig. 4). CD8+ CD28– Tregs were also abundantly found in the liver (35.0 ± 6.4) compared with the spleen (20.0 ± 6.5), lymph node (4.2 ± 0.8), or PBMC (12.3 ± 6.0). CD8+ CD28+ T cells in the liver were seen at higher levels than those in the spleen

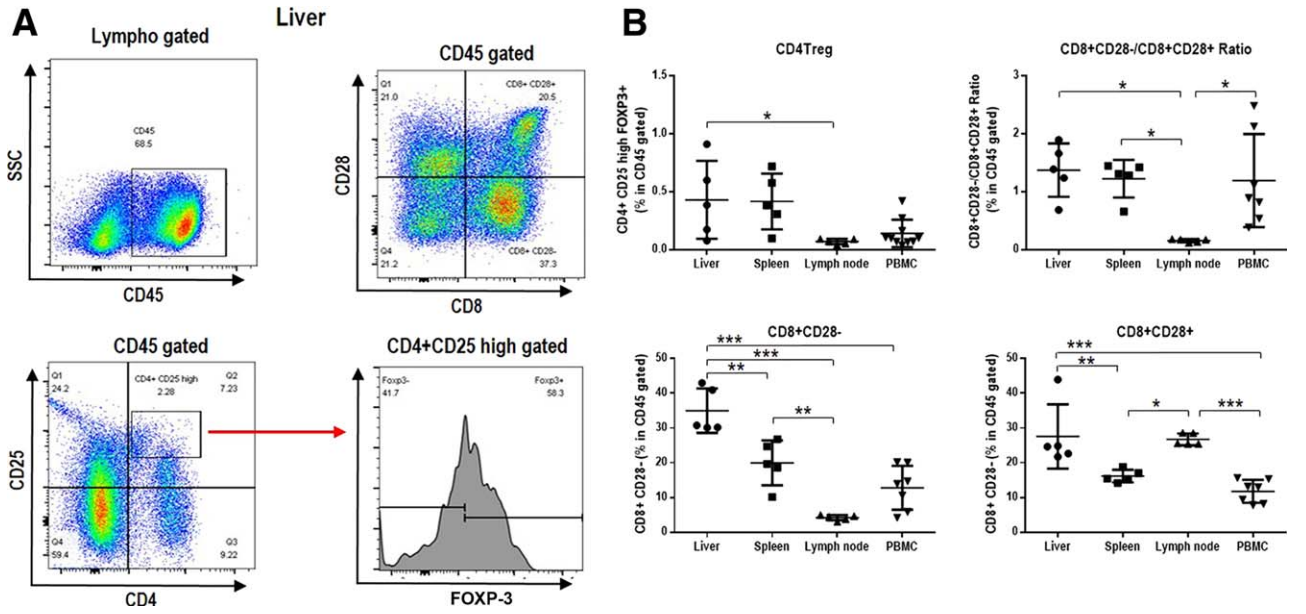

FIG. 4. Normal rhesus macaque regulatory cell populations of the liver, spleen, lymph node, and PBMC. CD4 Tregs were identified as FOXP3+ populations from among the CD4+ $CD25^{high}$ cell population. (A) The CD8 Tregs were identified as CD8+CD28– T cells from the CD45+ gated cells. FOXP3+ CD4 Tregs were seen at significant numbers in the liver (0.4% ± 0.3%) when compared with the lymph node (0.1% ± 0.0%). (B) CD8+ CD28– Tregs were also abundant in the liver (35.0% ± 6.4%) when compared with the spleen (20.0% ± 6.5%), lymph node (4.2% ± 0.8%), and PBMC (12.3% ± 6.0%; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

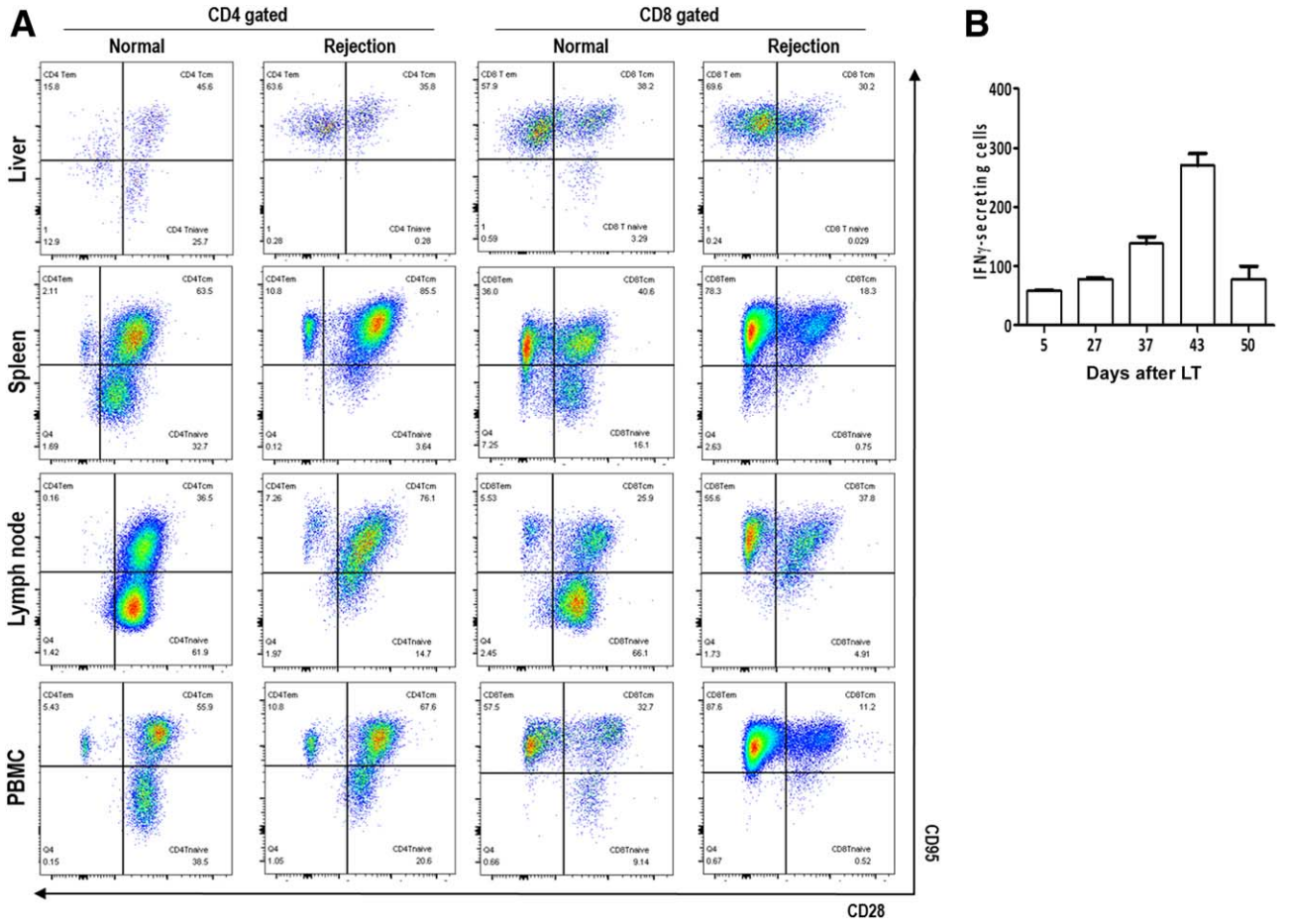

FIG. 5. The CD4/8 T subpopulation in recipients after liver TPL rejection. Naïve CD4T or CD8 T cells of the rejected recipient were seen at dramatically decreased levels in the liver, spleen, lymph node, and PBMC when compared with the controls where there was no rejection seen. (A) Also both effector and central memory CD4T or CD8T cells were increased in all tissues and also the PBMC. There were no other differences seen in other subpopulations from the rejected recipient. The frequencies of IFNγ-secreting cells in the spleens from LT recipients with immunosuppression were measured upon stimulation with irradiated donor mononuclear cells by ELISPOT analysis. (B) Even without any sign of rejection, the frequency of alloreactive IFNγ-secreting cells gradually increased with time after LT, suggesting the accumulation of donor-reactive T cells in the immune system.

or PBMC, but they were seen at the same level with no significant difference between the liver and lymph node. However, the ratio of CD8+ CD28– versus CD8+ CD28+ T cells was higher in the liver (1.4 ± 0.5) than in the lymph node (0.2 ± 0.0).

THE CD4/8 T SUBPOPULATION IN RECIPIENTS AFTER LIVER TPL REJECTION

Distinct features of rejection became apparent in the recipient as soon as immunosuppression was withdrawn on POD 50 (data not shown). The changes of the recipient immune cell subsets were estimated in comparison to those of the normal monkeys. The percentages of naïve CD4 T cells from the rejected LT recipients were dramatically decreased in the liver (0.3%), spleen (3.6%), lymph node (14.7%), and PBMC (20.6%) compared with those in the controls (25.7%, 32.7%, 61.9%, and 38.5% respectively). The percentages of naïve CD8 T cells from the rejected LT recipients were also dramatically decreased in the liver (0.0%), spleen (0.8%), lymph node (4.9%), and PBMC (0.5%) compared with those in the controls (3.3%, 16.1%, 66.1%, and 9.1% respectively). Of specific importance is the fact that the numbers of both effector and central memory

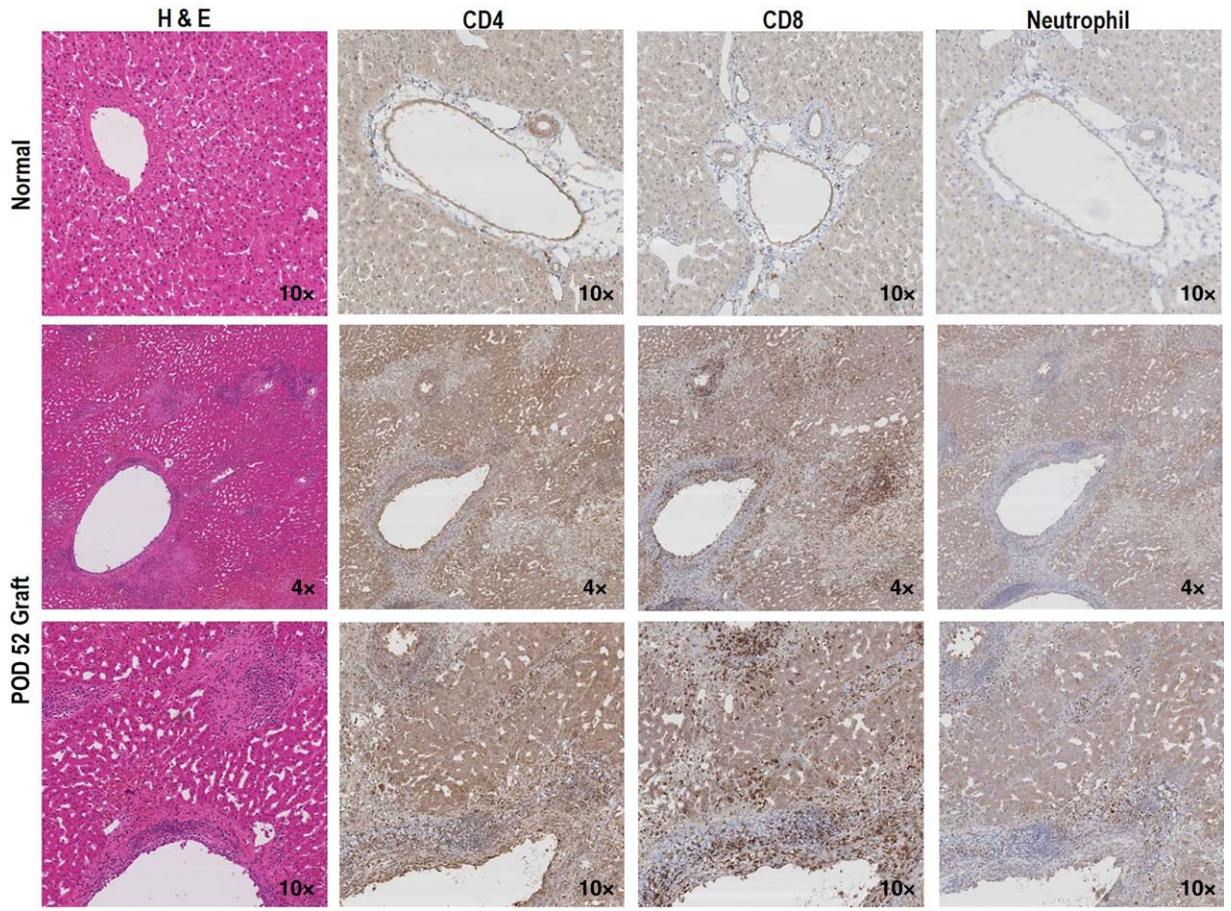

FIG. 6. CD8 T cells dominantly infiltrate into the rejected liver graft. This figure shows the histology from a rejected liver graft from POD 52. The graft shows marked lymphoplasma cell infiltration in the portoperiportal area, and some bile ducts revealed infiltration of lymphocytes. Most of the central vein and portal venules revealed severe endothelialitis with edematous change of vascular wall. Most of the infiltrative lymphocytes were CD8+ T cells. Histological findings suggest severe acute cellular rejection.

CD4T and CD8T cells were increased in all tissues and PBMC (Fig. 5A). There were no differences in other subpopulations in tissues and blood from rejected LT recipients.

The frequencies of IFNγ-secreting cells in the spleens from LT recipients with immunosuppression were measured upon stimulation with irradiated donor mononuclear cells by ELISPOT analysis. Even without any sign of rejection, the frequency of alloreactive IFNγ-secreting cells gradually increased with time following LT, suggesting the accumulation of donor-reactive T cells in the immune system (Fig. 5B). After withdrawal of immunosuppression, the frequency of splenic alloreactive IFNγ-secreting cells dramatically dropped presumably due to the recruitment of donor-reactive T cells into the liver.

CD8 T CELLS DOMINANTLY INFILTRATE INTO THE REJECTED LIVER GRAFT

Following withdrawal of all immunosuppressive treatment by POD 52, grafts demonstrated marked lymphoplasma cell infiltration in the portoperiportal area of the liver with some but not all bile ducts revealing infiltration by lymphocytes. However, at this time point, degenerative changes of cholangiocytes and loss of bile ducts were not present. Most of the central vein and portal venules revealed severe endothelialitis with edematous change to the vascular walls. Sinusoidal spaces were mildly dilatated with some neutrophilic aggregates. Most of the infiltrating lymphocytes were identified as CD8+ T cells (Fig. 6). The histological

findings were consistent with severe acute cellular rejection.

Discussion

LT has rather unique advantages over other solid organ transplants because it can be spontaneously accepted even indefinitely in some species such as murine models. They have even been shown to be able to be transplanted across major histocompatibility complex (MHC) incompatible barriers as seen in some porcine transplant studies.[26-29] In addition, LTs have also been shown to be able to induce donor-specific tolerance in otherwise immune-competent recipients.[28,29] Racanelli and Rehermann undertook a review on the liver and showed that it is truly a unique anatomical and immunological site,[2] and these unique features may provide LT with appropriate cell populations that can potentially provide tolerizing properties to other conjointly transplanted organs. Although these remarkable properties were described many years ago, the specific mechanisms responsible for the "liver tolerizing effect" remain obscure.[30] As such, to understand these mechanisms would provide a major breakthrough in transplantation immunology because it could be beneficially applied to prevent rejection of the liver and other solid organ transplants in humans. The NHP LT model is therefore a valuable tool to be able to provide such unknown information and to be able to provide a better understanding of the mechanisms of tolerance and therefore provide a means to use this to for a novel therapeutic approach. To gain a better understanding of the immune response in LT, we clearly need to understand and define the immune cell properties of normal monkeys. This study is a landmark paper that for the first time defines the diverse immune cell subsets that are seen in the normal tissues of RM. More importantly, it clarifies which cells form the basis of those cells involved in rejection and potentially for developing tolerance in LT.

The basic concepts of graft tolerization are thought to be based on induction of Tregs following transplantation with FOXP3+ Tregs found in operationally tolerant patients after LT.[20,31] Ciubotariu et al. suggested that human regulatory CD8+CD28– T-suppressor cells exhibit suppressive functions and inhibit T-helper cell activation and proliferation by allogeneic cells.[32,33] In our study, we found that regulatory CD4T cells or CD8T cells preferentially resided in the liver rather than in any other tissues including the spleen, lymph node, or PBMC. Interestingly, MDSCs which are known as suppressive regulators of the immune responses,[34] also resided in high numbers in the liver when compared with the spleen, lymph node, and PBMC.

Because of the multifactorial and rather complex processes involved, it is still unclear how naïve, effector, and memory T cells interact to induce allograft rejection. It has been suggested that the trafficking pathways of these T cell subsets are different and that this affects their respective behavior during transplantation. Naïve T cells, which lack adhesion molecules and chemokine receptors required to enter peripheral tissues,[26,35] are precluded from recognizing donor peptide/MHC complexes expressed by the allograft. Instead, naïve T cells recirculate via blood and lymph through the secondary lymphoid organs, and it is here where they encounter donor antigens presented by DCs.[36] In our results, we have shown that lymph nodes and peripheral blood have high levels of both CD4 or CD8T cells in the form of naïve cells but the liver has large numbers of memory cells with relatively few naïve cells. Clearly, this difference in cell populations and the process of naïve cell presentation are of great importance to the better understanding of how the liver can force tolerization processes to occur when transplanted.

Additionally, several studies have shown that the presence of alloreactive memory T cells are a major barrier to the induction of tolerance in kidney or islet allografts[37-39] and tried to suppress alloreactive memory T cells. Koyama et al. showed that they could increase central memory CD8T cells in recipients following appropriate conditioning. This conditioning consisted of depletion of the various T cell subsets by low-dose total body irradiation, thymic irradiation, antithymocyte globulin, and anti-CD154 antibody followed by a brief course of a calcineurin inhibitor. They more effectively achieved cellular depletion by the addition of treatment with humanized anti-CD8 monoclonal antibody (cMT807), and these recipients successfully achieved mixed chimerism and tolerance.[38] Oura et al. also showed that effector memory CD4T cells were increased in their induction treatment group, which was treated with anti-CD40 antibody for 2 weeks, but there was no increase of memory cells when they compared them with their longterm treatment group which was treated with anti-CD40 antibody for 6 months following LT on cynomolgus monkeys.[17]

Consistent with these findings, we also found that effector memory CD4T or CD8T cells were increased

in peripheral blood secondary lymphoid organs as well as the liver, but naïve cells were decreased in the LT recipients after rejection.

In summary, we have shown that the normal liver has large numbers of C4 Tregs or CD8+ CD28– or MDSC, which are known immune suppressive cells that favor the liver as an organ uniquely capable of providing an environment to encourage tolerance far more than secondary lymphoid organs or peripheral blood. As such, tolerance regimens can be developed in this rather unique RM LT model and then potentially directly applied to clinical therapy.

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