Video Article

Isolation of Double Negative αβ T Cells from the Kidney

Maria N. Martina[1], Samatha Bandapalle[2], Hamid Rabb[2], Abdel R. Hamad[1]

[1]Department of Pathology, Johns Hopkins University School of Medicine

[2]Department of Medicine, Johns Hopkins University School of Medicine

Correspondence to: Abdel R. Hamad at ahamad1@jhmi.edu

URL: https://www.jove.com/video/51192
DOI: doi:10.3791/51192

Keywords: Immunology, Issue 87, Double Negative (DN) αβ, T cells, CD45+ T cell isolation, renal lymphocytes, non-lymphoid-tissues, T cells purification, Ischemia Reperfusion Injury, Acute Kidney Injury, Tissue Resident Lymphocytes, Lymphoproliferative Disorders, Erythematosus Lupus

Date Published: 5/16/2014

Citation: Martina, M.N., Bandapalle, S., Rabb, H., Hamad, A.R. Isolation of Double Negative αβ T Cells from the Kidney. *J. Vis. Exp.* (87), e51192, doi:10.3791/51192 (2014).

Abstract

There is currently no standard protocol for the isolation of DN T cells from the non-lymphoid tissues despite their increasingly reported involvement in various immune responses. DN T cells are a unique immune cell type that has been implicated in regulating immune and autoimmune responses and tolerance to allotransplants[1-6]. DN T cells are, however, rare in peripheral blood and secondary lymphoid organs (spleen and lymph nodes), but are major residents of the normal kidney. Very little is known about their pathophysiologic function[7] due to their paucity in the periphery. We recently described a comprehensive phenotypic and functional analysis of this population in the kidney[8] in steady state and during ischemia reperfusion injury. Analysis of DN T cell function will be greatly enhanced by developing a protocol for their isolation from the kidney.

Here, we describe a novel protocol that allows isolation of highly pure ab CD4+ CD8+ T cells and DN T cells from the murine kidney. Briefly, we digest kidney tissue using collagenase and isolate kidney mononuclear cells (KMNC) by density gradient. This is followed by two steps to enrich hematopoietic T cells from 3% to 70% from KMNC. The first step consists of a positive selection of hematopoietic cells using a CD45+ isolation kit. In the second step, DN T cells are negatively isolated by removal of non-desired cells using CD4, CD8, and MHC class II monoclonal antibodies and CD1d α-galcer tetramer. This strategy leads to a population of more than 90% pure DN T cells. Surface staining with the above mentioned antibodies followed by FACs analysis is used to confirm purity.

Video Link

The video component of this article can be found at https://www.jove.com/video/51192/

Introduction

Peripheral αβTCR$^+$CD3$^+$CD4$^-$CD8$^-$double-negative (DN) T cells are divided into various subsets that possess distinct phenotypes and functions[1-4]. DN T cells are poorly understood but increasingly being implicated in pathophysiological immune responses in different disease models[4-6].

DN T cells are a unique immune cell type that is increasingly implicated in the regulation of various immune and autoimmune responses and the modulation of allotransplant tolerance. [4-6,9] They are rare in the peripheral blood and secondary lymphoid organs (spleen and lymph nodes). However, they are major residents of the normal kidney and gut epithelium[10-12]. Very little is known about their function[7] in the kidney in the steady state and under pathological conditions such as acute kidney injury (AKI) associated with kidney transplant.

Because of the intricate roles of different immune cells in regulating immune responses including alloresponses, defining the role of each player is critical in understanding alloresponses and designing new therapeutics. Given the significant numbers of DN T cells present in the kidney under physiologic and different disease conditions, DN T cells are likely to play a critical role in regulating immune and autoimmune responses in mice and humans, and alloresponses in transplant recipients. Accumulating though still scattered evidence implicates DN T cells in both pathogenic and immunosuppressive functions but it is poorly understood why and how they exhibit a specific harmful or suppressive function and how the environment influences them.

Due to their low abundance in the kidney, improved methods of isolation are necessary.

There is currently no standard protocol for isolation of DN T cells from the non-lymphoid tissues. Our protocol describes a novel method for isolation of DN T cells from the kidney; however, the method can also be used for various non-lymphoid tissues.

Protocol

1. Preparation of Instruments, Culture Media and Reagents

1. Instruments: Prepare the reagents under sterile conditions and use under a laminar flow hood.
2. Prepare 1 L of RPMI tissue culture medium, add 5% of fetal bovine serum, 2.1 g of bicarbonate, 10 ml glutamate 100x, 10 mg of HEPES, 1 mg sodium pyruvate and 10 ml of 100x penicillin/streptomycin.
   Note: the recommended tissue culture medium, RPMI, is interchangeable with DMEM.
3. Prepare 5% collagenase “D” solution (5 ml of collagenase “D” to 9 ml of tissue culture medium).
4. Make Percoll solution at three different concentrations: 100%, 80% and 40%. The following concentrations are calculated for 1 sample. Keep solutions at room temperature.
   1. Percoll solution: add 9 ml of undiluted Percoll (from stock solution); add 1 ml of PBS 10x.
   2. Percoll solution: add 8 ml of Percoll 100%; add 2 ml of PBS 1x.
   3. Percoll solution: add 4 ml of Percoll 80%; add 4 ml of PBS 1x.
5. Make 1 L of fluorescence-activated cell sorting (FACS) buffer; add 5 ml 10% BSA (0.1% BSA), 1 ml 10% sodium azide (0.1%), 2 ml EDTA agent, make up to 1,000 ml with 1x PBS.

2. Preparation of Kidney Digestion

1. Having acquired any necessary institutional approval, sacrifice and exsanguinate the mice with standard methods and following the current regulations and animal care guidelines.
   1. Anesthetize the mouse with pentobarbital (0.07 mg/kg).
   2. Proceed to exsanguination.
   3. Place the mouse in a supine position on a surgical table.
   4. Spray the abdominal area with 70% ethanol.
2. Add 10 ml of collagenase D solution to the Petri dish.
3. Remove and decapsulate both kidneys from each mouse.
   1. Open the abdomimal cavity by making a midline incision through the abdominal skin and peritoneum from the sternum to the pubis.
   2. Expose the left kidney by moving the intestine laterally to the side.
   3. Carefully separate the left capsule with the forceps.
      Note: The capsule appears as a transparent layer surrounding the kidney and partially covered by perinephric adipose tissue.
   4. Remove the left kidney by cutting the pedicle vessels using a surgical scissor.
   5. Repeat same procedure for the right kidney.
      Note: The right kidney is more cranial and closer to the midline (pedicels are shorter).
4. Place the kidney into the Petri dish.
5. Cut the kidney tissue into small pieces (1-2 mm in dimension) using a regular metal shaping blade and place the pieces into the collagenase solution for 30-45 min in the incubator at 37 °C (**Figure 1**).

3. Preparation of Kidney Mononuclear Cells (KMNC) from the Kidney Digestion

1. Obtain a cell suspension of the kidney digestion by mechanically disrupting the tissue using a strainer (70 µm) and resuspend in 25 ml of tissue culture medium and mix.
2. Centrifuge at 400 x g for 10 min at 4 °C to pellet the cell suspension.
3. Re-suspend the pellet in 4 ml of 40% Percoll solution and gently overlay onto 4 ml of 80% Percoll solution with a transfer plastic pipette, very slowly and carefully. This will result in two phases clearly separated by a translucent layer. On the top will be a yellow layer corresponding to the lipids.
4. Centrifuge at 1,500 x g for 30 min at room temperature with the centrifuge in brake off mode.
5. Remove by aspiration the yellow and thick top layer (about 1-2 ml).
6. Collect only the slight whitish translucent layer from the interface of the two phases with a transfer pipette. Very carefully transfer this suspension into a 15 ml conical tube containing 2 ml of tissue culture medium and then add 13 ml of medium to make a total volume of 15 ml.
7. Centrifuge at 400 x g for 10 min at 4 °C.
8. Resuspend the samples in 1 ml of the medium.
9. Count the number of KMNC using trypan blue exclusion on a hemocytometer and express the results as numbers of KMNC per ml per Kidney.
10. Wash once as above.

4. Isolation of Hematopoietic (CD45+) Cells from KMNC (Step I)

1. Resuspend the cells up to 10^7 into 90 µl of running buffer.
2. Add 10 µl of CD45 microbeads and incubate for 15 min at 4 °C.
3. Add 1 ml of the running buffer to stop the reaction. Centrifuge at 400 x g for 10 min at 4 °C and resuspend in 500 µl of running buffer.
4. Prepare the magnetic column by placing at the magnet and rinse with 3 ml of running buffer.
5. Pass the cell suspension through the magnetic column and collect the unlabeled cells that pass through the column.

6. Wash the cells three times with 3 ml of running buffer. Then collect the effluent.
7. Remove the column from the separator and pipette 5 ml of running buffer onto the columns and flush out the labeled fraction immediately. This fraction contains the CD45+ cells. Count the cells.
8. To calculate the absolute number of different T cells subsets into kidneys multiply the total number of KMNC by the percentage of positive cells determined by flow cytometry (**Figure 2**).

5. Isolation of DN T Cell from CD45+ Preparation by Negative Selection (Step II)

1. Add 2.5 µl (0.5 mg/ml, 1.25 µg) of each of the following biotinylated antibodies: anti-CD4, anti-CD8, anti-Fc receptor (CD16/32), anti-MHC class II (I-A b), anti-CD1d PBS-57 tetramer for every 10^7 hematopoietic cells.
2. Incubate cells for 30 min in cold room.
3. Add 1 ml anti-Biotin microbeads.
4. Incubate for 30 min in a cold room.
5. Place the tube in the magnet and proceed to depletion.
6. Count the viable cells to determine the total number of DN T cells in the negative fraction by using a hemocytometer.
7. Check purity of DN T cells with flow cytometry by following this gating strategy: first gate CD45+, second gate TCRαβ+. Exclude CD1d+ cells. Plot CD4+ vs. CD8+ to identify DN T cells (**Figure 3**).
8. Multiply the total number by the percentage determined in purity.
   Note: Using this protocol results in a population of more than 90% pure DN T cells.

Representative Results

Wild type C57BL/6 (B6) kidney contains approximately 1.5-2.1 x 10^6 mononuclear cells per kidney. Approximately less than 10% are hematopoietic $CD45^+$ cells. For the preparation of kidney mononuclear cells (KMNC), the kidneys are cut into small pieces as shown in **Figure 1** followed by digestion using 5% collagenase.

This is followed by performing a density gradient centrifugation to collect KMNC layer **(Figure 2, left panel)**. KMNC were then subjected to positive selection using CD45+ magnetic microbeads as described in section 4 (STEP I). The column bound cells were then eluted and analyzed for $CD45^+$ by FACS. This results in enrichment of $CD45^+$ cells to about 80 to 95% as analyzed by flow cytometry **(Figure 2, middle panel)**. Among enriched hematopoietic $CD45^+$ cells, about 40-60% was αβ TCR+ (data not shown), about 30-60% were DN T cells **(Figure 2, right panel)**.

The final step involved subjecting enriched $CD45^+$ cells to negative selection to label and remove CD4+, CD8+, MHC class II+ and CD16/32+ cells using biotin specific mAbs and anti-biotin microbeads and magnetic columns. This result in highly purified (90-95%) DN T cells (**Figure 3**). Following this protocol it was possible to obtain up to 0.5 x 10^6 hematopoietic $CD45^+$ magnetically labeled cells per mouse (two kidneys).

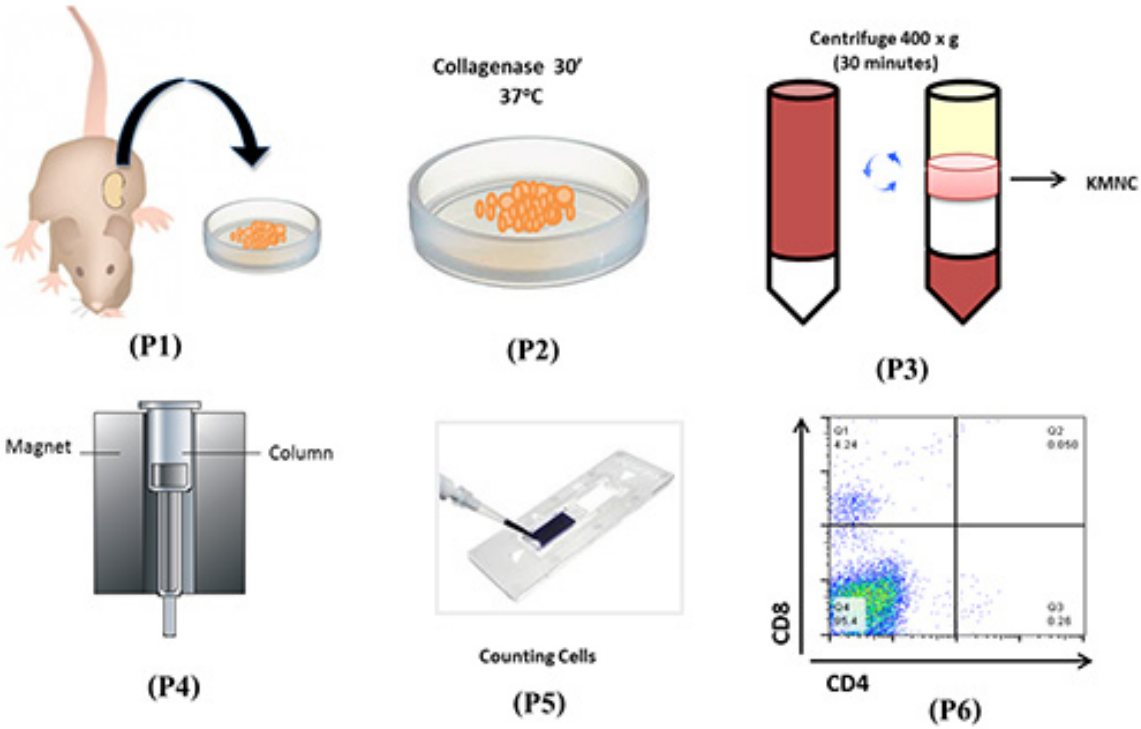

Figure 1. Preparation of the kidney for digestion with 5% collagenase solution. The mouse kidney is cut into small pieces of 1-2 mm and placed in collagenase D 5% solution for 30 min for digestion. Please click here to view a larger version of this figure.

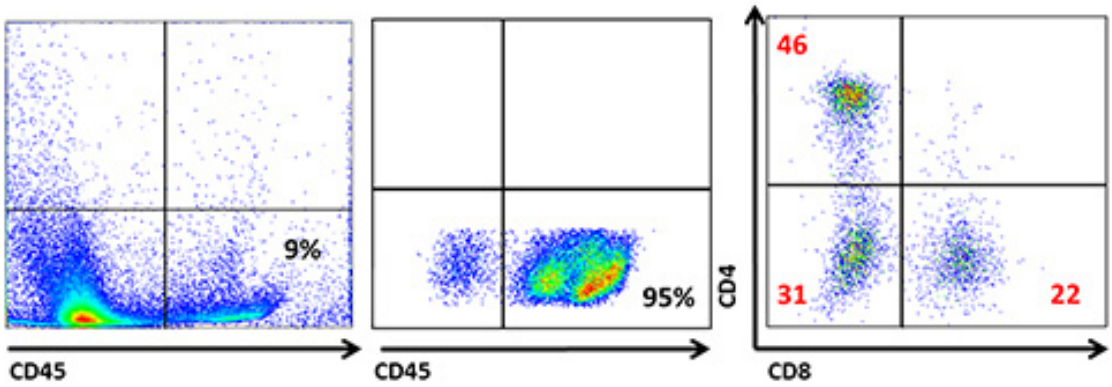

Figure 2. Strategy for enrichment of CD45+ hematopoietic cells from the Kidney. Left: CD45 staining of kidney MNC before enrichment using CD45$^+$ kit by positive selection. **Center**: CD45 staining of kidney MNC after enrichment using CD45$^+$ kit by positive selection. **Right**: CD4 & CD8 profile of αβTCR+ cells among gated CD45$^+$ cells different subsets of T cells. Please click here to view a larger version of this figure.

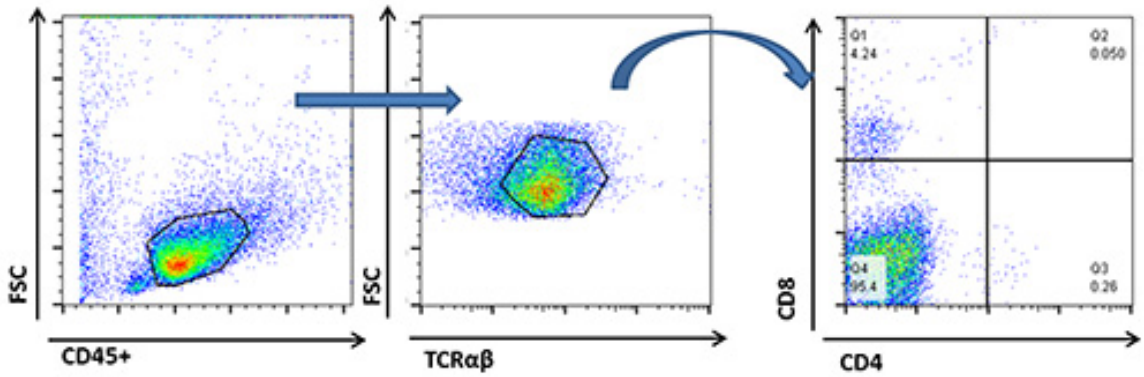

Figure 3. Purity of isolated DN T cells. Left: CD45 staining of isolated kidney DN T cells by negative selection. **Center**: TCRαβ staining of isolated kidney DN T cells by negative selection. **Right**: CD4 & CD8 staining among isolated DN T cells by negative selection. Please click here to view a larger version of this figure.

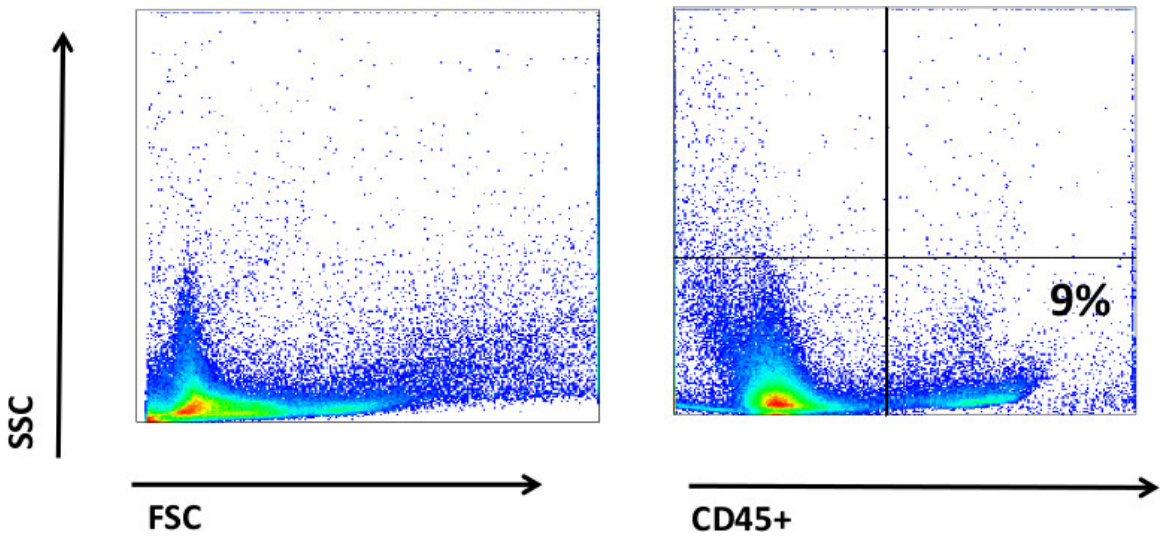

Figure 4. Lymphocytes in the kidney before purification. Left: total cells in kidney tissue before CD45 cells purification. **Right**: CD45 staining in kidney tissue before purification. Please click here to view a larger version of this figure.

Discussion

There is increasing interest in DN T cells since they are being implicated in different pathologic conditions such as autoimmune disorders, cancer, graft tolerance, and primary diseases of the kidney including acute kidney injury (AKI), glomerulonephritis[8,13]. Therefore, there is need to better understand and characterize the pathophysiologic functions of DN T cells. However, currently there is a lack in understanding of these cells' function as compared to CD4 and CD8 T cells. A major reason is paucity of DN T cells in secondary lymphoid organs and hence difficulty

in obtaining sufficient cells for *in vitro* analysis and adoptive transfer experiments. DN T cells are primarily located in non-lymphoid tissues such as the kidney[8] but the lack of reliable methods for their isolation from solid organ is impeding the effort to investigate their function. Therefore, our novel protocol for isolation of DN T cells from the kidney is expected to expedite research into the function of DN T cells. DN T cells accumulate in lymph nodes in large numbers in Fas deficient (lpr) or FasL deficient (gld) mice and they can be easily isolated from lymphoid organs [9,14,15], but it still remains a challenge to isolate these cells from other tissues.

According to this protocol, kidneys from two mice yield approximately 0.5 x 10^6 hematopoietic cells ($CD45^+$) and around 0.2 x 10^6 DN T cells. This number is sufficient to perform *in vitro* culture, functional assays and/or FACs analysis. The purity of the DN T cell population must be confirmed by flow cytometry. This protocol provides a population of DN T cells that can be as pure as 98%. While this protocol is designed for isolation of DN T cells from the kidney, it can be modified for isolation of T cells from other non-lymphoid organs such as heart, thyroid etc.

Since DN T cells represent a small population, it is sometimes necessary to increase yield by performing additional rounds of purification. However if a higher number of DN T cells is required (higher than 0.5 x 10^6 cells) we recommend performing sorting separation in order to reduce time and reagent cost.

In summary, this protocol describes a novel method for isolation of DN T cells from the kidney. It is composed of a preparation (via digestion and enrichment of KMNC) step followed by positive selection and then negative selection for depletion of non-desired cell populations. Therefore, there is minimal direct manipulation of DN T cells during the isolation process. It is noteworthy that there are existing protocols for isolation of DN T cells from peripheral blood in mice and humans and from secondary lymphoid organs, similar to those used for isolation of conventional T cells.

Because it has been shown that some DN T cells express FcR-γ, an alternative option is not to include CD16/32+ in the cocktail [16,17]. Successful isolation of DN T cells requires great attention to detail. Particularly crucial steps include: tissue digestion, cutting of the renal tissue into small pieces, and correctly identifying and collecting the light layer in the density gradient. Overall, several rounds of practice and some manual dexterity are required to achieve the desired result.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work is supported by NIH PBS (R21 AI095484). We thank NHI tetramer core facility for CD1d tetramer.

References

1. Zhang, Z. X., Yang, L., Young, K. J., DuTemple, B., & Zhang, L. Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. *Nat Med*. **6**, 782-789, doi:10.1038/77513 (2000).
2. Ford, M. S., Young, K. J., Zhang, Z., Ohashi, P. S., & Zhang, L. The immune regulatory function of lymphoproliferative double negative T cells *in vitro* and *in vivo*. *J Exp Med*. **196**, 261-267 (2002).
3. Ford McIntyre, M. S., Young, K. J., Gao, J., Joe, B., & Zhang, L. Cutting edge: *in vivo* trogocytosis as a mechanism of double negative regulatory T cell-mediated antigen-specific suppression. *J Immunol*. **181**, 2271-2275 (2008).
4. Young, K. J., Yang, L., Phillips, M. J., & Zhang, L. Donor-lymphocyte infusion induces transplantation tolerance by activating systemic and graft-infiltrating double-negative regulatory T cells. *Blood*. **100**, 3408-3414 (2002).
5. Chen, W., Ford, M. S., Young, K. J., & Zhang, L. Infusion of *in vitro*-generated DN T regulatory cells induces permanent cardiac allograft survival in mice. *Transplant Proc*. **35**, 2479-2480 (2003).
6. Young, K. J., DuTemple, B., Phillips, M. J., & Zhang, L. Inhibition of graft-versus-host disease by double-negative regulatory T cells. *J Immunol*. **171**, 134-141 (2003).
7. Mohamood, A. S. *et al.* Gld mutation of Fas ligand increases the frequency and up-regulates cell survival genes in CD25+CD4+ TR cells. *Int Immunol*. **18**, 1265:1277 (2006).
8. Ascon, D. B. *et al.* Normal mouse kidneys contain activated and CD3+CD4- CD8- double-negative T lymphocytes with a distinct TCR repertoire. *J Leukoc Biol*. **84**, 1400-1409, doi:10.1189/jlb.0907651 (2008).
9. Hamad, A. R. *et al.* B220+ double-negative T cells suppress polyclonal T cell activation by a Fas-independent mechanism that involves inhibition of IL-2 production. *J Immunol*. **171**, 2421-2426 (2003).
10. Rabb, H. *et al.* Pathophysiological role of T lymphocytes in renal ischemia-reperfusion injury in mice. *Am J Physiol-Renal*. **279**, F525-F531 (2000).
11. Burne, M. J. *et al.* Identification of the CD4(+) T cell as a major pathogenic factor in ischemic acute renal failure. *J Clin Invest*. **108**, 1283-1290, doi:10.1172/JCI12080 (2001).
12. Yokota, N., Daniels, F., Crosson, J., & Rabb, H. Protective effect of T cell depletion in murine renal ischemia-reperfusion injury. *Transplantation*. **74**, 759-763, doi:10.1097/01.TP.0000020413.44576.65 (2002).
13. Crispin, J. C. *et al.* Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. *J Immunol*. **181**, 8761-8766 (2008).
14. Igarashi, S., Takiguchi, M., Kariyone, A., & Kano, K. Phenotypic and functional analyses on T-cell subsets in lymph nodes of MRL/Mp-lpr/lpr mice. *Int Arch Allergy Appl Immunol*. **86**, 249-255 (1988).
15. Dowdell, K. C. *et al.* Somatic FAS mutations are common in patients with genetically undefined autoimmune lymphoproliferative syndrome. *Blood*. **115**, 5164-5169, doi:10.1182/blood-2010-01-263145 (2010).

16. Juvet, S. C. *et al.* FcRgamma promotes T cell apoptosis in Fas-deficient mice. *J Autoimmun.* **42**, 80-93, doi:10.1016/j.jaut.2012.12.002 (2013).
17. Thomson, C. W. *et al.* FcR gamma presence in TCR complex of double-negative T cells is critical for their regulatory function. *J Immunol.* **177**, 2250-2257 (2006).

