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FULL LENGTH ARTICLE

Ubiquitin-specific peptidase 18 regulates the differentiation and function of Treg cells

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Abstract Ubiquitin-specific peptidase 18 (USP18) plays an important role in the development of CD11b^{+} dendritic cells (DCs) and Th17 cells, however, its role in the differentiation of other T cell subsets, especially in regulatory T (Treg) cells, is unknown. In our study, we used *Usp18* KO mice to study the loss of USP18 on the impact of Treg cell differentiation and function. We found that USP18 deficiency upregulates the differentiation of Treg cells, which may lead to disrupted homeostasis of peripheral T cells, and downregulates INF-γ, IL-2, IL-17A producing CD4^{+} T cells and INF-γ producing CD8^{+} T cells. Mechanistically, we also found that the upregulation of Tregs is due to elevated expression of CD25 in *Usp18* KO mice. Finally, we found that the suppressive function of *Usp18* KO Tregs is downregulated. Altogether, our study was the first to identify the role of USP18 in Tregs differentiation and its suppressive function, which may provide a new reference for the treatment of Treg function in many autoimmune diseases, and USP18 can be used as a new therapeutic target for precise medical treatment.

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Introduction

Ubiquitin-specific peptidase 18 (USP18) removes ubiquitin from its substrates. The Usp18 is a 43-kDa protein that is homologous to ubiquitin-specific proteases (UBPs) and is therefore also known as Ubp43. It was first cloned from mice expressing the acute myelogenous Leukemia fusion protein AML1-ETO by Liu et al[1,2] USP18 is expressed in several tissues with different levels. For instance, USP18 is highly expressed in liver, spleen and thymus, but clearly detectable in lung tissue, adipose tissue and bone marrow.[1,3] USP18 is also expressed in lymphocytes and hematopoietic cells, including splenic B and T cells. In T cells, USP18 is highly expressed in naïve or memory, as well as highly maintained in Th0, Th1, Th17 cells and natural regulatory T cells but decreased in Th2 cells and inducible regulatory T cells.[4] USP18 is a negative regulator of type I and type III interferon signaling,[5,6] which plays an essential role in the innate antiviral response. What's more, USP18 was found involved in the development of DCs[7] and Th17 cells.[4] However, its role in the differentiation of other T cell subsets remains to be explored.

As a critical subset of T cells, regulatory T cells (Tregs) mediate peripheral tolerance and maintain the immune homeostasis. In the early stage, the concept of 'suppressor' T cells has been arised.[8,9] Next, Sakaguchi identified a unique $CD4^+CD25^+$ T population and named it regulatory T cells [Tregs].[10] Tregs are classified into natural Tregs and induced Tregs. The former develops in the thymus and the latter is derived from naïve $CD4^+$ T cells in the periphery. However, the effect of USP18 on Treg development and its suppressive function is still unknown.

Inducible co-stimulator (ICOS, CD278) is a member of the CD28 family of costimulatory molecules,[11] and is lowly expressed in resting naïve T cells but is rapidly upregulated after activation by TCR ligation and CD28.[12] ICOS is expressed in unpolarized $CD4^+$ T cells, activated Th1, Th2, Th17, Tfh and Treg lineages.[12–16] As one of the signature molecules in Treg cells, ICOS plays an important role in the survival and maintenance of $CD4^+Foxp3^+$ regulatory T cells.[16] Besides, $ICOS^{hi}$ Tregs suppress proliferation of T cell through secreting IL-10 and transforming growth factor-β (TGF-β) whereas $ICOS^{low}$ Tregs through TGF-β secretion.[17] However, whether USP18 affects the function of Tregs via ICOS remains elusive.

As a T cell growth factor, IL-2 has the ability to grow and expand T cells in culture,[18] and maintains the homeostasis of T regulatory (Treg) cells in the periphery.[19–21] As the IL-2 receptor α-chain, CD25 plays a critical role of maintaining the number of regulatory T cells and the function of $CD4^+$ regulatory T cell. Mice deficient for the IL-2 receptor have decreased numbers of thymus and periphery regulatory T cells,[22] and develop a lethal phenotype characterized by excessive T cell proliferation.[23]

In our study, *Usp18* knockout mice were used to investigate the role of USP18 in Treg differentiation and its suppressive function. Interestingly, we found that loss of USP18 results in the upregulation of Tregs which inhibits the differentiation of naïve $CD4^+$ and $CD8^+$ T cells into activated effector T cells and leads to the reduced production of cytokines. Mechanistically, we found that the upregulation of Tregs was due to the upregulation of CD25, a crucial marker of Tregs. Finally, although the expression of ICOS in Tregs of *Usp18* KO mice is upregulated, the suppressive function *in vitro* is decreased. Altogether, our study is the first to illustrate the role of USP18 on the differentiation and suppressive function of Tregs in mouse model as well as the underlying mechanism.

Materials and methods

Mice

Usp18 KO mice on the C57 BL/6 background were donated by Prof. Bo Zhong's lab in Wuhan University of China as described before.[24] 8-Week-old *Usp18* KO mice and sex-matched littermates were used in all experiments. All mice were fed and housed in a specific pathogen-free condition and all experiments were performed according to the protocols from the Chinese Council on Animal Care and approved by the Ethics Committee of Animal Experimentation of Tongji Medical College (Wuhan, China).

Cells preparation

Spleens, thymuses and LNs from *Usp18* KO and WT mice were harvested and mashed into cell suspensions in DMEM containing 2% FBS, and RBCs in splenocytes were lysed with ACK (TIANGEN, RT122-01).

Flow cytometry analysis of cell surface molecule and intracellular molecule

Cell suspensions were incubated with anti-mouse CD16/CD32 (Biolegend, 101,319). For cell surface flow cytometry, cells from spleens, thymuses and LNs were stained with specific antibodies for surface antigens as follows: PE-anti-CD4 (Biolegend, 100,408), Pacific Blue-anti-CD4 (Biolegend, 100,531), Brilliant Violet 510-anti-CD8a (Biolegend, 100,751), APC/Cy7-anti-TCRβ (Biolegend, 109,220), 7-AAD (BD Pharmingen™, 559,925), APC-anti-CD25 (Biolegend, 102,012), PerCP/Cy5.5-anti-CD44 (Biolegend, 103,032), PE/Cy7-anti-CD62L (Biolegend, 104,418), PE-anti-CD278 (ICOS) (Biolegend, 107,705), APC-anti-CD304 (Neuropilin-1, Nrp1) (Biolegend, 145,206), PE/Cy7-anti-CD279 (PD-1) (Biolegend, 109,110). For intracellular staining, cells were fixed and permeabilized with Fixation/Permeabilization Kit (eBioscience, 00—5123, 00—5223), washed with Permeabilization Buffer (eBioscience, 00—8333) and stained with PE-Cy7-anti-ki67 (eBioscience, 25-5698-82), PE-anti-CTLA4 (Biolegend, 106,306), AF488-anti-Foxp3 (Thermo Scientific™, 53-5773-82), PE-anti-Foxp3 (Biolegend, 126,404). For apoptosis analysis, cells were stained with FITC-AnnexinV (Biolegend, 640,906) in AnnexinV Binding Buffer (Biolegend, 422,201). Samples were analyzed by LSRII multicolor flow cytometer (BD Biosciences CA, USA)

and data analysis was performed using the FlowJo software (Tree Star, USA).

T cell stimulation and intracellular cytokine staining

2×10^6 lymphocytes from spleens, thymuses and LNs were plated in round-bottomed 96-well plates in 1 ml RPMI complete media containing PMA (50 ng/ml, Sigma, P1585-1 MG), GolgiStop (1:1000, BD Biosciences, 554,724) and ionomycin (1 μM, CST, 9995S). After culturing for 5 h in 37 °C, 5% CO_2, cells were collected and stained with PE-anti-CD4 (Biolegend, 100,408), Brilliant Violet 510-anti-CD8a (Biolegend, 100,751), PerCP/Cy5.5-anti-CD44 (Biolegend, 103,032), APC/Cy7-anti-TCRβ (Biolegend, 109,220). Then cells were fixed, permeabilized and stained with APC-anti-IL-4 (Biolegend, 504,106), PE/CY7-anti—IFN—γ (Biolegend, 505,826) and Brilliant Violet 421-anti-IL-17A (Biolegend, 506,925).

Cell sorting and Treg suppression assay

Splenic $CD4^+$ naïve T cells and Treg cells were sorted by Naïve $CD4^+$ T Cell Isolation Kit (Miltenyi, 130-104-453) and $CD4^+$ $CD25^+$ Reg T Cell Isolation Kit (Miltenyi, 130-091-041). $CD4^+$ naïve T cells were labled with CellTrace™ Violet (Thermo Scientific™, C34557, C34571) at a concentration of 5 μM for 10 min at 37 °C, followed by incubation with Treg cells at a different ratio in a U-bottom 96-well plate that pretreated with anti-CD3/CD28 antibodies (Biox Cell, BE-0002, BE0015). After 72 h, cells were collected to stain with 7-AAD and APC-anti-CD4 antibody (Biolegend, 100,412). Samples were then analyzed by flow cytometer (BD Biosciences CA, USA) and data analysis was performed with FlowJo software (Tree Star, USA).

Bone marrow chimera generation

To generate mixed bone marrow (BM) chimeras, CD45.1 recipient mice were pre-treated drinking water with gentamycin (Biofroxx, 1453GR005, 480,000 U/L) and erythromycin (Biosharp, 375,000 U/L) for one week, and then were irradiated (7 Gy) 4 h prior to BM cell transfer. BM cells (2.5×10^6) were obtained from WT or *Usp18* KO mice and mixed with CD45.1 congenic BM cells (1:1) and were injected i.v. into irradiated recipient mice. After transfer, recipient mice continued to receive antibiotic-containing drinking water for four weeks and were analyzed eight weeks later.

Statistical analysis

Two-paired Student's t tests were carried out with Prism GraphPad Prism 6 Software (San Diego, CA) to assess the statistical significance. The difference was considered significant when $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ $^{****}P < 0.0001$, ns, no significance.

Results

USP18 deficiency alters the homeostasis of peripheral T cells, but not the development of T cells in thymus

To analyze the physiological role of USP18 in T cells, we first analyzed thymic development of $CD4^+$ and $CD8^+$ T cells, and no significant change of the frequency was observed between *Usp18* KO and WT mice (Fig. 1A—C). Meanwhile, we did not find significant difference in the absolute numbers of $CD4^+CD8^-$ (SP4) and $CD4^-CD8^+$ (SP8) thymocytes in *Usp*18 KO and WT mice (Fig. 1B and C). Next, we analyzed the T cell homeostasis in the periphery, and the frequencies as well as the total numbers of $CD4^+$ and $CD8^+$ T cells in spleen and lymph nodes (LNs) were unaltered between *Usp18* KO and WT mice (Fig. 1A—C). However, we observed a higher frequency of naïve $CD4^+$ T cells in spleen and naïve $CD8^+$ ($CD44^{lo}CD62^{hi}$) T cells in spleen and LNs in *Usp18* KO mice than that in WT mice (Fig. 1D—G). On the contrary, the frequencies of activated effector $CD4^+$ in spleen and activated effector $CD8^+$ ($CD44^{hi}CD62^{lo}$) T cells in spleen and LNs were reduced in *Usp18* KO mice (Fig. 1D—G). Accordingly, these results indicated that USP18 is critical for the homeostasis of peripheral T cells but not for the thymocyte development.

USP18 deficiency upregulates the differentiation of Treg cells

Because regulatory T (Treg) cells play an essential role in maintaining the naïve T cell pool, we further investigated the effect of USP18 deficiency on Treg cells. We found that the percentage of $CD4^+CD25^+$ Treg cells was significantly increased in spleen, LN and thymus of *Usp18* KO mice compared with that of WT mice (Fig 2A and C). Similarly, the frequency of $CD4^+Foxp3^+$ Treg was also increased in spleen and LN except for the thymus of *Usp18 KO* mice (Fig. 2B and D). What's more, the change of cell number of $CD4^+CD25^+$ or $CD4^+Foxp3^+$ was in keeping with that of percentage (Fig. 2C and D). Furthermore, we tested the expression of CD25 and Foxp3 by flow cytometry. Higher level of CD25 was found in spleen, LN and thymus of *Usp18* KO mice compared with that of WT mice (Fig. 2E and G). However, no significant difference in the Foxp3 expression was observed between *Usp18* KO and WT mice (Fig. 2F and H). To further dissect the mechanism of the increase of Tregs, the apoptosis and proliferation of Treg were measured by Annexin V and Ki67 staining respectively. No obvious difference was found in the percentage of Annexin V^+ and Ki-67^+ of Treg as well as the expression of Annexin V and Ki-67 (Fig. 2I—L). Together, these findings identified USP18 deficiency up-regulates the generation of Tregs both in central and peripheral tissues.

To further determine whether USP18 affects the development of Treg is intrinsic, we generated mixed bone marrow chimera mice with WT and *Usp18* KO mice to remove the secondary impact on KO Treg cells from environment. Interestingly, analyses of $CD45.2^+$ T cells showed that the percentage of $CD4^+CD25^+$ Treg cells was increased in spleen and LNs of *Usp18* KO mice than that of WT mice,

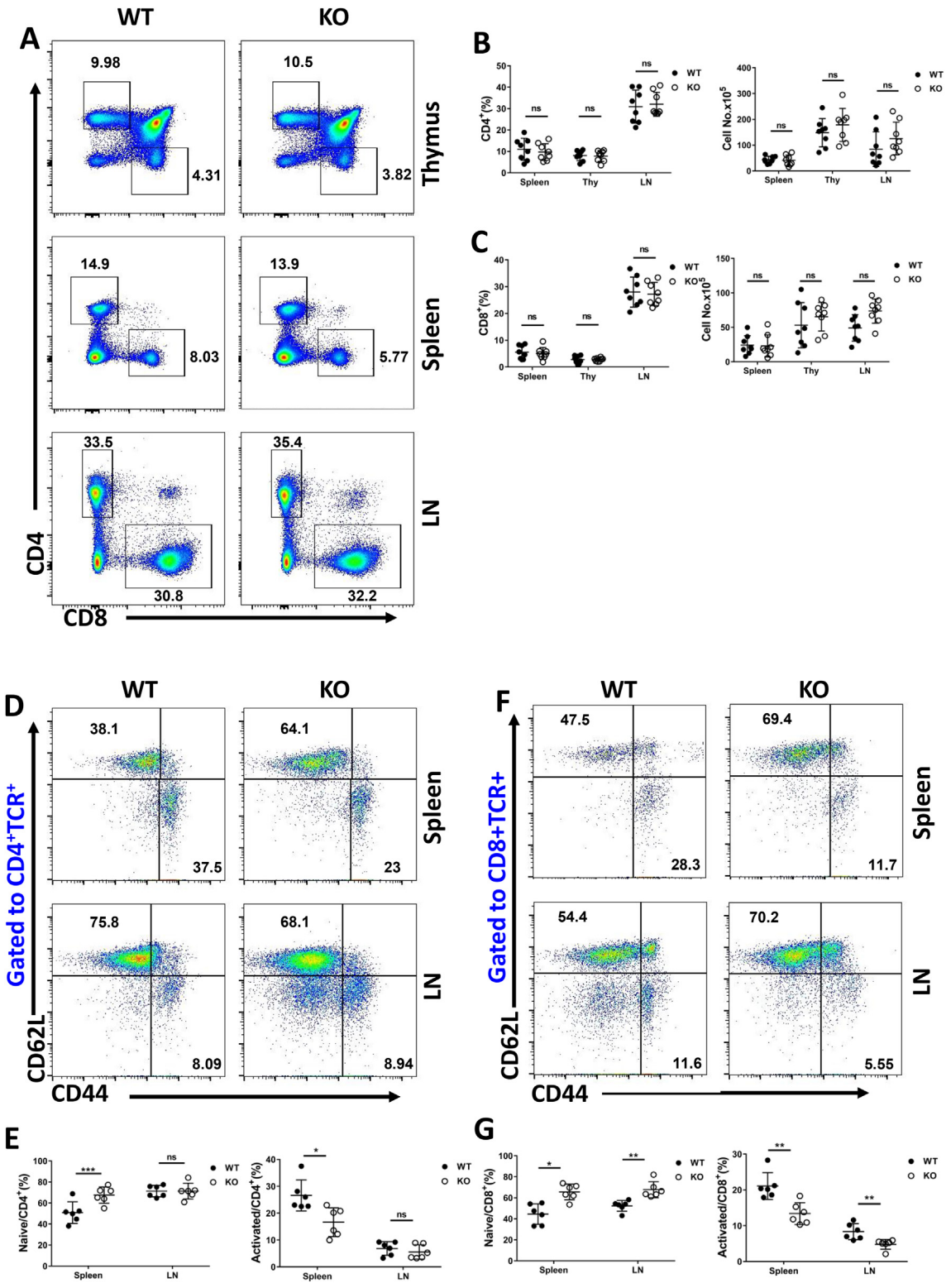

Figure 1 USP18 deficiency alters the homeostasis of peripheral T cells, but not the development of T cells in thymus. Flow cytometry analyzing the expression of CD4 and CD8 in lymphocytes of thymus, spleen and LN derived from *Usp18* KO ($n = 8$) and WT ($n = 8$) mice. Shown are representative dot plots **(A)** as well as percentages and absolute numbers of $CD4^+$ **(B)** and $CD8^+$ T cells **(C)**. Expression of CD44 and CD62L on $CD4^+TCR^+$ or $CD8^+TCR^+$ T cells of spleen and LN derived from *Usp18* KO ($n = 6$) and WT ($n = 6$) mice was analyzed by flow cytometry. Shown are representative dot plots and percentages of $CD62L^{hi}CD44^{lo}$ naïve $CD4^+$ T cells and $CD62L^{lo}CD44^{hi}$ activated $CD4^+$ T cells **(D–E)**. Dot plots and percentages of naïve and activated $CD8^+$ T cells are shown in **(F–G)**. Data are representative of three independent experiments and values are expressed as mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$, ns, not significant.

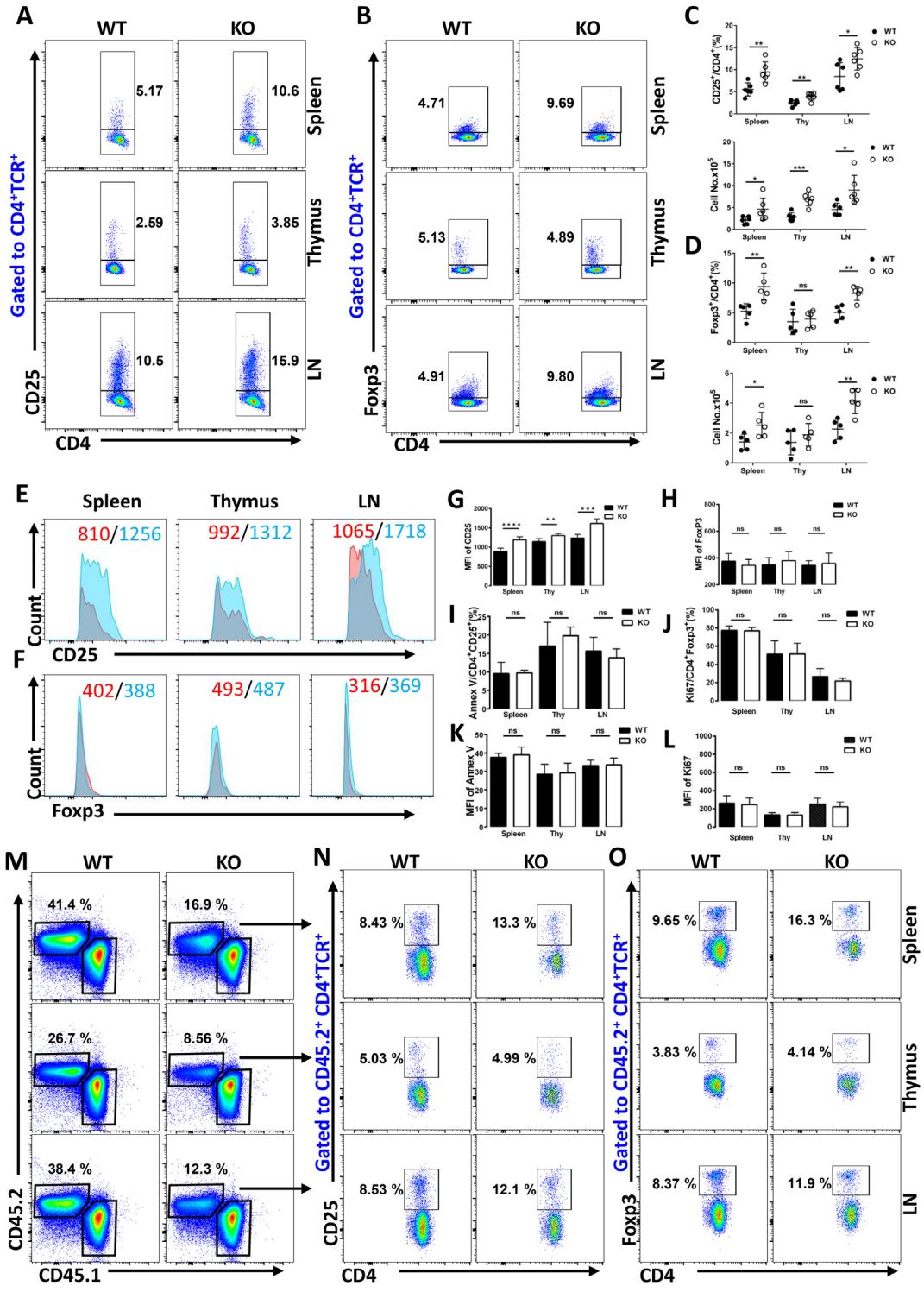

Figure 2 USP18 deficiency upregulates the differentiation of Treg cells. Representative figures shown the expression of CD25 in $CD4^+$ T cells from the spleen, thymus and LN of WT and *Usp18* KO mice **(A)** Percentages and absolute numbers of $CD4^+CD25^+$ T cells from the spleen, thymus and LN of WT ($n = 6$) and KO littermates ($n = 6$) **(C)** Representative figures, percentages and absolute numbers of $CD4^+Foxp3^+$ T cells from the spleen, thymus and LN of WT ($n = 6$) and KO littermates ($n = 6$) as indicated in **(B—D)** Flow cytometry analyzing expression of CD25 in $CD4^+CD25^+$ T cells **(E—G)** and Foxp3 in $CD4^+Foxp3^+$ T cells **(F—H)** from the spleen, thymus and LN of WT ($n = 6$) and *Usp18* KO littermates ($n = 6$). Flow cytometry analyzing the apoptosis and proliferation of $CD4^+CD25^+$ and $CD4^+Foxp3^+$ T cells from the spleen, thymus and LN of WT ($n = 4$) and KO littermates ($n = 4$) **(I—L)**. Shown are representative dot plots from one of three independent experiments. Flow cytometry analysis of CD25 and Foxp3 expression in $CD45.2^+CD4^+$T cells of spleen, thymus and LN in mixed bone marrow chimeras ($n = 5$) eight weeks after transfer **(M—O)** $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns, not significant.

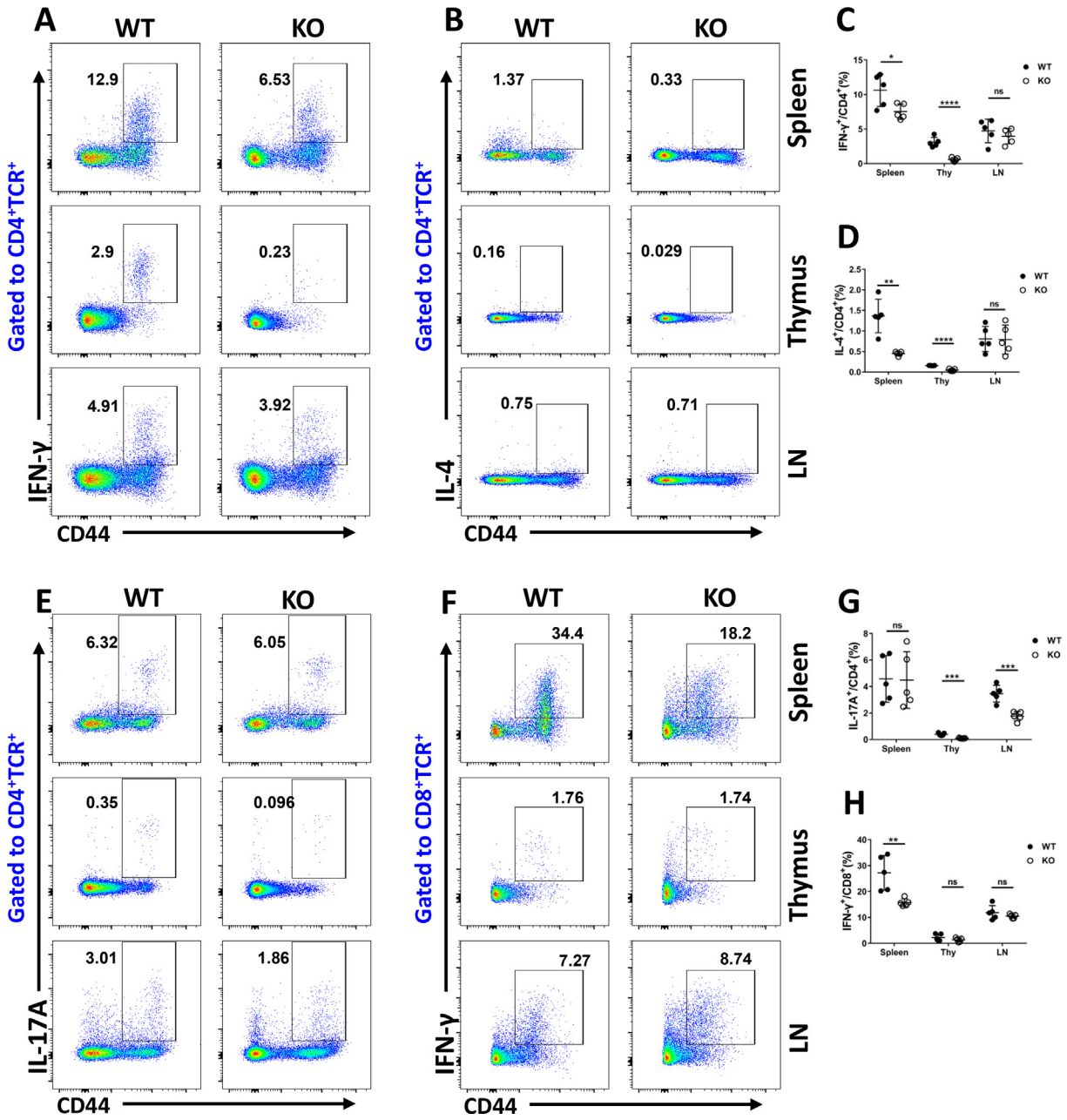

Figure 3 USP18 deficiency decreases the ability of cytokine production by effector T cells. Flow cytometry analyzing the levels of IFN-γ, IL-4 and IL-17A secreted by stimulated $CD4^+$ and $CD8^+$ T cells from the spleen, thymus and LN of WT ($n = 5$) and *Usp18* KO mice ($n = 5$) **(A–H)**. Shown are representative dot plots and mean values (±SD) of percentages from three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ns, not significant.

but no difference in the thymus (Fig. 2M and N). Similarly, the frequency of $CD4^+Foxp3^+$ Treg cells in *Usp18* KO mice were also higher in spleen and LNs, and equally in the thymus compared to WT mice (Fig. 2O). These observations collectively indicate an intrinsic role of USP18 in mediating the differentiation of Treg cells.

USP18 deficiency decreases the ability of cytokine production by effector T cells

Next, we detected the ability of effector T cells in *Usp18* KO mice to produce cytokines. After stimulated with PMA, GolgiStop and ionomycin for 5 h, lymphocytes from spleens, LNs and thymus of *Usp18* KO and WT mice were collected to detect intracellular cytokines with flow cytometry. We found that significantly decreased production of IFN-γ and IL-4 in $CD4^+$ T cells of spleen and thymus from *Usp18* KO mice compared with that of WT mice, but not for that of LN (Fig. 3A–D). The production of IL-17A in $CD4^+$ T cells of thymus and LN was also significantly reduced in *Usp18* KO mice, but no change in $CD4^+$ T cells of spleen (Fig. 3E and G). In $CD8^+$ T cells, reduced production of IFN-γ in spleen of *Usp18* KO mice was found, but we didn't found significant difference in thymus and LN (Fig. 3F and H). These results

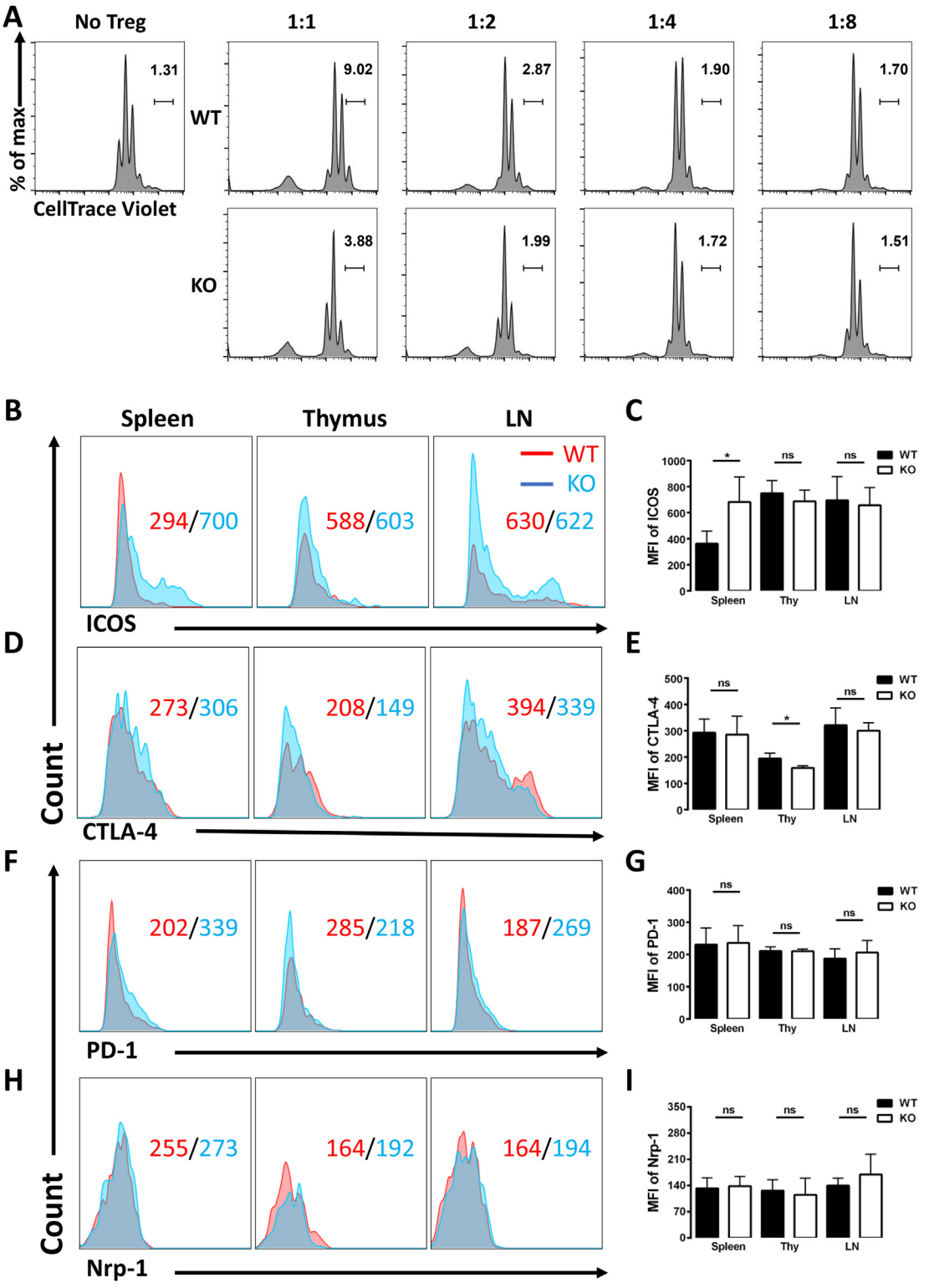

Figure 4 USP18 deficiency downregulates the suppression function of Treg cells *in vitro*. Purified Treg cells of spleens from WT and *Usp18* KO mice were cultured with CellTrace Violet-labeled naïve $CD4^+$ T cells from WT mice and anti-CD3/CD28 antibodies at the indicated ratio for 72 h *in vitro*. The proliferation of naïve $CD4^+$ T cells was measured by flow cytometry. Shown are representative images from at least two independent experiments **(A)**. Flow cytometry analyzing the expression of ICOS **(B—C)**, CTLA-4 **(D—E)**, PD-1 **(F—G)** and neuropilin-1(Nrp1) **(H—I)** in $CD4^+CD25^+$ and $CD4^+Foxp3^+$ Treg cells from the spleen, thymus and LN of WT ($n = 5$) and KO littermates ($n = 5$). Shown are representative images and mean values (±SD) from three independent experiments. *$P < 0.05$; ns, not significant.

indicated that USP18 plays an essential role of maintaining the ability of cytokine production by effector T cells.

USP18 deficiency downregulates the suppression function of Treg cells *in vitro*

To identify whether USP18 is crucial for the function of Treg cells, we performed a suppression assay *in vitro*. Isolated and purified Treg cells of *Usp18* KO or WT mice were co-cultured with naïve $CD4^+$ T cells from WT mice in the present of anti-CD3/CD28 for 72h, and then the proliferation of T naïve cells was detected. We found that USP18-deficient Treg cells were less effective than WT Treg cells in inhibiting the proliferation of naïve T cells *in vitro* (Fig. 4A). To further investigate the mechanism of how USP18 control the suppressive function of Treg cells, we detected several signature molecules in Treg cells related to suppressive function such as ICOS, CTLA-4, PD-1 and neuropilin-1(Nrp1). We found that the expression of ICOS in *Usp18* KO Tregs in spleen was significantly increased compared with that of WT Tregs in spleen, but no obvious difference in thymus and LN (Fig. 4B and C). However, Tregs in *Usp18* KO mice exhibited slightly decreased expression of CTLA-4 in thymus compared with that of WT mice, but not for that in spleen and LN (Fig. 4D and E). What's more, we didn't found any significant difference of PD-1 and neuropilin-1(Nrp1) in spleen, thymus and LN between *Usp18* KO and WT mice (Fig. 4F—I). Accordingly, these findings suggested that USP18 is critical for the suppressive function of Tregs although the expression of ICOS is upregulated.

Discussion

So far, previous studies indicate that USP18 plays an essential role in the innate antiviral response and is involved in the development of DCs[7] and Th17 cells,[4] but there is no report to explore the role of USP18 in Tregs. In our study, we first use the *Usp18* knock out animal model to investigate the effect of USP18 deficiency on Treg differentiation and suppressive function. Overall, USP18 is critical for the homeostasis of peripheral T cells and negatively regulates the differentiation of Tregs by downregulating the expression of CD25. What's more, USP18 plays an essential positive role in maintaining the suppressive function of Tregs. However, using the germ-line deletion mice for *Usp18*, we cannot exclude the influence of other immune cells. In addition, it caused embryonic lethality that *Usp18* KO mice were backcrossed to C57BL/6 mice to over five generations.[25] So, our further study is aimed to explore the function of USP18 on Tregs by building the model of conditioned knockout mice through crossing $Usp18^{flox/flox}$ mice with $Foxp3^{YFP\text{-}Cre}$ mice and further validate our results in the bone marrow chimera mice model.

The transcription factor Foxp3, which cooperates with other transcription factors play an essential role in the development and function of $CD4^+CD25^+$ Treg cells, can induce upregulation of CD25, cytotoxic T lymphocyte—associated antigen 4 (CTLA-4) and the glucocorticoid-induced TNF receptor, and inhibite IL-4, IFN-ɣ, and IL-17 is produced by effector T cells.[26] Previous researches have shown that the IL-2 signaling transmitted by high-affinity IL-2R is essential for the homeostasis of Treg cells, promotion of survival and immunosuppressive by inducing Foxp3 and CD25 expression.[26,27] Our study illustrated the mechanism that USP18 deficiency upregulates the differentiation of Treg cells through upregulating the expression of CD25, and decreases the ability of cytokine production by effector T cells. Besides, the result that decreased percentage of producing-IL-17 cells in *Usp18* KO mice is also in accordance with the finding before.[4] We found that changes in CD25 expression were inconsistent with changes in Foxp3, which may be caused by the deubiquitination of Foxp3 by USP18, similarly to the effect of USP21 on Foxp3.[28] It is worthy of figuring out the defined mechanisms.

Strangely, but interestingly, we found that USP18 deficiency downregulates the suppressive function of Tregs, whereas the expression of ICOS is upregulated, which may be caused by the inflammatory stimulation in the microenvironment. In addition, the influence of the increased percentage of Tregs in *Usp18* KO mice maybe outweighs that of downregulated suppressive function. Further investigation is also needed.

In summary, we first report here an important role of USP18 in regulatory T cell differentiation and function. These results are helpful for understanding and treatment of Treg function associated diseases. Treg dysfunction exists in all kinds of autoimmune diseases, and USP18 could be used as a new therapeutic target for precise medical treatment.

Authors contribution

L. Yang drafted the initial manuscript. C. Liu designed the study, reviewed and revised the manuscript. L. Yang, D. Kang, P. Jiang and Na. Li performed the flow cytometry assay. L. Yang and Y. Jing carried out the cell sorting and Treg suppression assay. L. Yang analyzed the data and generated figures. X. Zhou, Y. Chen and L. Westerberg assisted with the manuscript. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

Conflict of Interests

The authors have no financial conflict of interest.

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Abbreviations

USP18	Ubiquitin-specific peptidase 18
DC	Dendritic cells
Treg	Regulatory T cell
KO	knockout
UBPs	ubiquitin-specific proteases
PMA	Phorbol 12-myristate 13-acetate
ICOS	inducible costimulatory
TGF-β	transforming growth factor-β

CTLA-4 cytotoxic T lymphocyte—associated antigen 4
Nrp1 neuropilin-1
PD-1 programmed cell death protein 1
LN lymph node

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