Sustained Treg Expansion by Repeated *in vivo* Injection of anti-TNFRSF25 mAb

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04-0031-00

Abstract

FOXP3⁺ regulatory T cells (Tregs) play key roles in maintenance of immune tolerance and autoimmune reactions. TNFRSF25 (also known as DR3) is one of the more recently discovered TNFRSF members, expressed by mouse Tregs, naïve CD4, CD8, NKT cells and TLR4-induced DC and macrophages. A significant Treg *in vivo* expansion by TNFRSF25 stimulation has been reported. Here, we show that by single dose i.p. injection of an agonistic mAb, 4C12, Treg expansion starts to be observed on day 3, reaches a peak (up to 30-40% of total CD4⁺ T-cells) on day 5-6, and then returns to baseline (5-7%) by day 10. Upon repeated 3-day interval injections, a high level (about 20%) of expanded Tregs can be sustained more than 10 days. As reported by other authors, followed by one dose 4C12 injection, the absolute number of CD8⁺T cells, B cells, dendritic cells and macrophages was not affected. In the *in vitro* study, 4C12 antibody did not significantly induce Treg expansion in a CD3/CD28/IL-2 culture system. Moreover, we characterized the TNFRSF25 expanded Tregs *in vivo*, and found that the expression levels of CD304, CD152, Helios, GARP, LAP (TGF-β1), FR4, GITR, and CD39 were significantly increased in comparison to the control. These data suggest that multiple injections of 4C12 can long term sustain expanded Tregs at a certain level, and the biological significance of Treg needs to be further studied.

Introduction

TNFRSF25, also known as DR3, lymphocyte-associated receptor of death (LARD), WSL-1, TRAMP, TR3 and APO3, is a 55 kD glycoprotein belonging to the TNF receptor superfamily (TNFRSF). TNFSF25 is expressed on CD4⁺, CD8⁺ T cells, NKT cells, and Tregs. The ligand of TNFRSF25, TL1A (also known as TNFSF15), is expressed by some endothelial cells and is rapidly induced on dendritic cells and macrophages/monocytes following TLR4 or FcgR signaling. Following binding to TL1A, TNFRSF25 signaling increases the sensitivity of T cells to endogenous IL-2 via the IL-2 receptor and enhances T cell proliferation. Interestingly, single injection of anti-TNFRSF25 agonist antibody is highly effective at reducing lung pathology when used prophylactically in an allergic disease model, and it promotes organ allograft survival. These data demonstrate that TNFRSF25 may function as a regulator of Tregs, which protect from disease pathological autoimmune responses. This knowledge may facilitate the clinical use of Treg therapy in human.

Material and Methods

4C12 induced Treg expansion *in vivo*

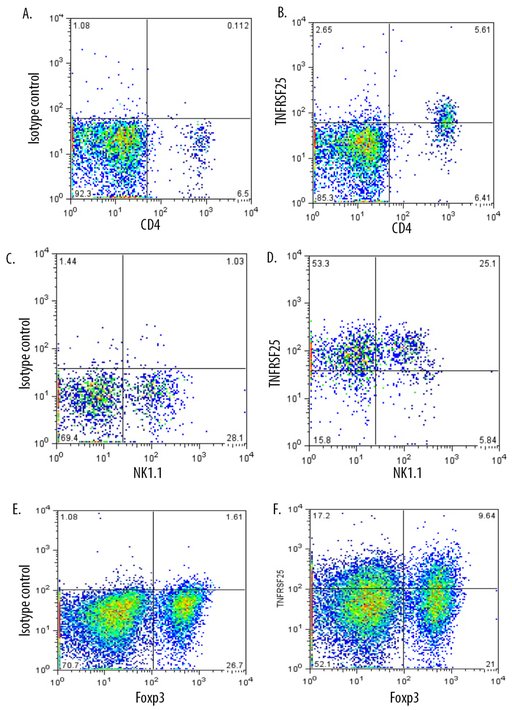
C57BL/6 mice were single or multiple dose i.p injected with 100 µg LEAF™ purified anti-mouse TNFRSF25 (clone 4C12) (3 mice) or LEAF™ purified Armenian hamster IgG (3 mice). On the indicated days, the blood was collected for immunofluorescence staining.

Immunofluorescence staining:

C57BL/6 mouse peripheral blood cells were surface stained with CD4 or indicated markers. Then the cells were fixed and permeabilized with BioLegend's FOXP3 Fix/Perm Buffer Set followed by intracellular staining with FOXP3. All the antibodies are from BioLegend.

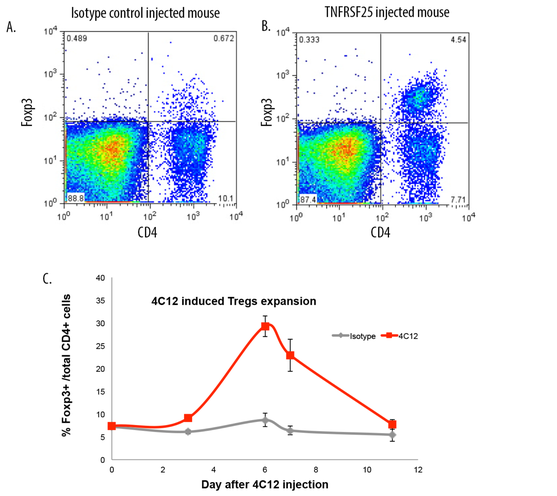
Results

Figure 1. TNFRSF25 is mainly expressed on CD4, NKT, and Treg cells



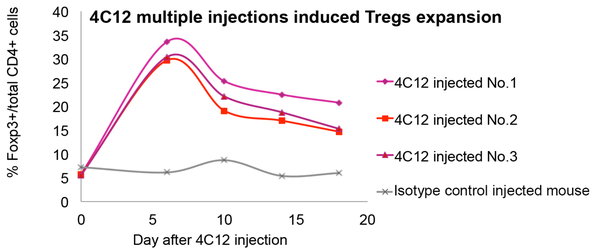
(A and B). C57BL/6 mouse splenocytes were stained with CD4 and TNFRSF25 or isotype control. (C and D). C57BL/6 mouse liver cells were stained with CD45, CD3, NK1.1 and TNFRSF25 or isotype control. (E and F). C57BL/6 mouse peripheral blood were stained with CD4 and TNFRSF25 or isotype control. Then the cells were fixed and permeabilized followed intracellular staining with Foxp3.

Figure 2. Single administration of anti-mouse TNFRSF25, clone 4C12 induces Treg expansion *in vivo*



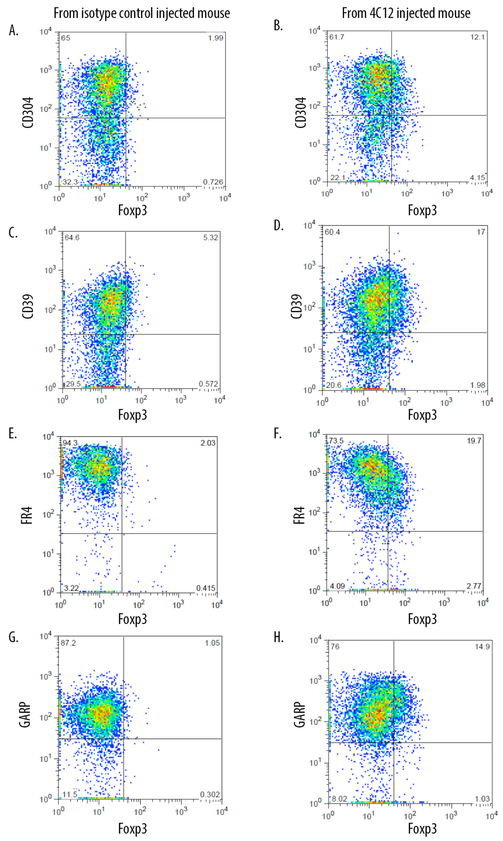
C57BL/6 mice were administered a single dose (100 µg) of LEAF™ purified HTK888 (left) or LEAF™ purified anti-mouse TNFRSF25 (clone 4C12) (n=3). On day 0, 3, 6, 7, 11, the blood was collected and stained with CD4, followed by intracellular staining with Foxp3-PE. Data was analyzed as a percentage of Foxp3-positive cells in total CD4-positive cells. Error bars are +/- SD. ***p < 0.001 between 4C12 and isotype injection. (A). HTK888 injected mouse. (B). Anti-mouse TNFRSF25 injected mouse. (C). Percentage of Foxp3-positive cells in total CD4-positive cell population.

Figure 3. Multiple doses of anti-mouse TNFRSF25 injection sustains Treg expansion *in vivo*



Every 3 days C57BL/6 mice were injected (i.p.) with 100 µg LEAF™ purified anti-mouse TNFRSF25 (clone 4C12) (n=3) or LEAF™ purified armenian hamster IgG (n=1). On day 0, 6, 10, 14, and 18, the blood was collected and stained with CD4, followed by intracellular staining with Foxp3. Data was analyzed as a percentage of Foxp3-positive cells in total CD4-positive cell population.

Figure 4. Characterization of anti-TNFRSF25 expanded Tregs



Peripheral blood cells from single dose of isotype control (A, C, E, G) or anti-TNFRSF25 injected (B, D, F, H) mice were surface stained with CD4 and CD304, CD39, FR4, or GARP followed by intracelluar staining with FOXP3. Data were analyzed by gated on CD4-positive cells.
CD152, Helios, LAP (TGF-β1), and GITR also showed positive on anti-TNFRSF25 expanded CD4⁺/FOXP3⁺ cells.

Conclusion

1. Single or multiple injections of anti-mouse TNFRSF25 (clone 4C12) agonistic antibody can expand and sustain Tregs *in vivo*.

2. Treg cells developed by anti-TNFRSF25 agonistic antibody injections express higher level of CD304, CD39, FR4 and GARP, suggesting that the expanded cells may be induced Tregs.