Development of a New Mouse/Human FoxP3 Antibody using Rabbit Antibody Technology

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Abstract

T regulatory cells (Tregs) play a key role in immune system suppression during autoimmunity and tumor development. Tregs are classified as $CD4^+CD25^+FoxP3^+$, and exist as either natural Tregs (nTregs) derived from the thymus, or induced Tregs (iTregs) that develop from $CD4^+$ T effector cells in the periphery. Reduction in Treg numbers leads to autoimmunity; this is clearly shown by the *scurfy* mouse model in which FoxP3 has been deleted, Treg numbers are severely reduced, and mice succumb to autoimmunity early in life. In contrast, Tregs also play a debilitating role in cancer biology when Treg numbers are elevated, and the immune response is dampened allowing cancer cells to evade the immune system. Thus, the correct balance of Tregs is essential for maintaining immune homeostasis. FoxP3 has been widely used as a marker of Tregs, and many antibodies are available on the market that distinguish the Treg population in human or mouse cells.

We developed a novel FoxP3 antibody and accompanying Fixation and Permeabilization buffer system that is comparable to current clones, but recognizes both mouse and human FoxP3 equally well, eliminating the need for separate antibodies.

The mouse/human FoxP3 antibody was generated using our rabbit antibody technology, which allows for selecting high specificity, high affinity antibodies, lot to lot consistency and detection of both mouse and human nTregs and iTregs. This allows researchers to extend their mouse models into clinical studies without a change in the antibody clone, eliminating a variable and streamlining the process.

Methods

Cell culture of mouse and human Tregs

PBMCs were isolated using a standard Ficoll gradient separation protocol. To generate PBMC iTregs, PBMCs were placed into culture for 2 days in media (RPMI, 10% FBS, penicillin/streptomycin, Glutamax, sodium pyruvate, nonessential amino acids, and β-mercaptoethanol) containing Recombinant Human IL-2 (20 ng/mL; Catalog # 202-IL) and Human TGF-beta 1 (10 ng/mL; Catalog # 100-B). C57Bl/6 or Balb/c splenocytes were isolated, lysed, and used fresh.

To induce IL-2 and IFN-γ production in mouse splenocytes, PMA (50 ng/mL) and Ionomycin (200 ng/mL) were added for 4 hours with Monensin (3 μM). Intracellular staining was then carried out using the FlowX FoxP3 buffer set.

Staining Protocol for FoxP3 and intracellular cytokines

Wash human PBMCs or mouse splenocytes (about 1 x 10^6 cells per sample) with 2 mL of Flow Cytometry Staining Buffer (Catalog # FC001) or other BSA-containing buffer, by spinning at 300 x g for 5 minutes, using 5 mL flow cytometry tubes. Fc-block cells with blocking IgG (1 μg IgG/10^6 cells) for 10 minutes at 4 °C. Surface stain with CD4 and/or CD25 antibodies for 30 minutes at 4 °C. Wash cells 2x with cold 1x PBS. Resuspend cells in freshly made 1x Fixation Buffer using 0.5 mL/tube. Incubate at 4 °C for 30 minutes. Wash 2x with freshly made, cold 1x Permeabilization/Wash Buffer. Add FoxP3 antibody (or intracellular cytokines) to cells and incubate for 30 minutes at 4 °C. Wash cells 1x with cold 1x Permeabilization/Wash Buffer. Resuspend cells in Flow Cytometry Staining Buffer and analyze on a flow cytometer.

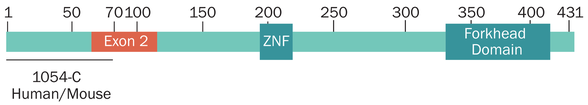

Figure 1. Schematic of the FoxP3 immunogen. The rabbit monoclonal antibody for FoxP3 was generated using amino acids 1-70 at the N-terminus of the FoxP3 protein. This monoclonal antibody cross-reacts in both mouse and human cells.

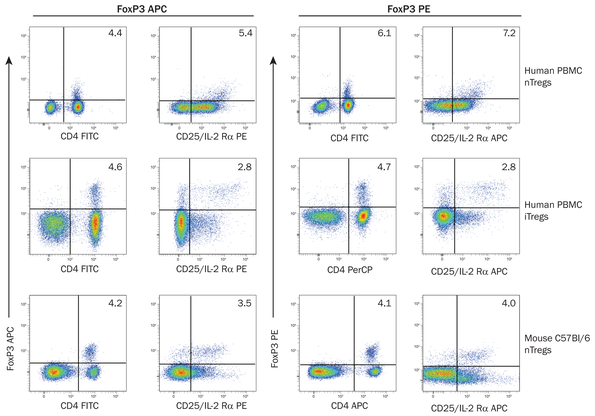

Figure 2. APC- and PE-conjugated FoxP3 Staining in Mouse and Human Tregs. To identify Tregs in human and mice, cells were surface stained with the indicated fluorochrome-conjugated CD25/IL-2 Rα (Catalog # FAB1020, FAB2438) and CD4 (Catalog # FAB3791, FAB554) antibodies as described in the Methods. The cells were then fixed and permeabilized using the FlowX™ FoxP3 Fixation & Permeabilization Buffer Kit (Catalog # FC012) and stained with R&D Systems APC- or PE-conjugated Anti-Mouse/Human FoxP3 Monoclonal Antibody (Catalog # IC8214).

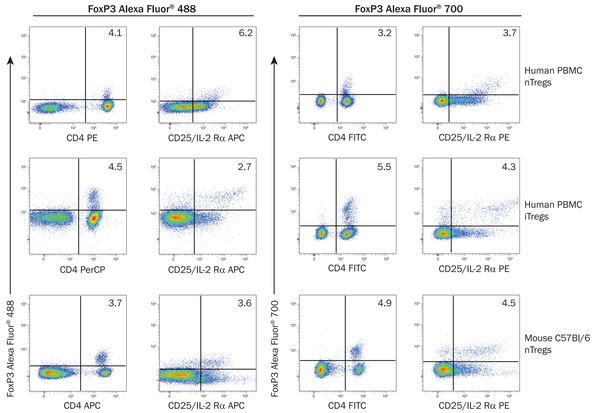

Figure 3. Alexa Fluor®488- and Alexa Fluor®700-conjugated FoxP3 Staining in Mouse and Human Tregs. To identify Tregs in human and mice, cells were surface stained with the indicated fluorochrome-conjugated CD25/IL-2 Rα (Catalog # FAB1020, FAB2438) and CD4 (Catalog # FAB3791, FAB554) antibodies as described in the Methods. The cells were then fixed and permeabilized using the FlowX™ FoxP3 Fixation & Permeabilization Buffer Kit (Catalog # FC012) and stained with R&D Systems Alexa Fluor 488- or Alexa Fluor 700-conjugated Anti-Mouse/Human FoxP3 Monoclonal Antibody (Catalog # IC8214).

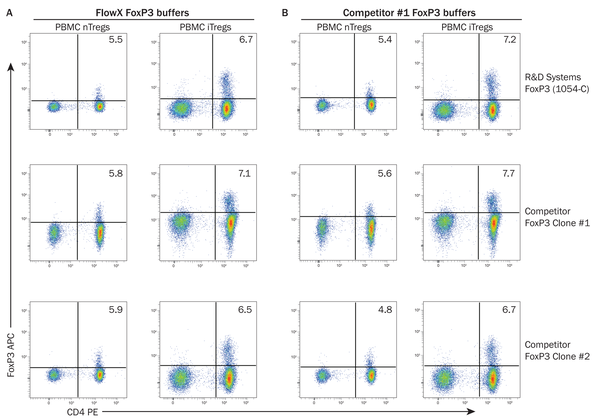

Figure 4. Comparison of R&D Systems APC-conjugated FoxP3 Antibody to Other FoxP3 Antibodies on the Market using Human nTregs and iTregs. PBMCs were stained as described in the Methods using **A.**) R&D Systems FlowX FoxP3 Fixation & Permeabilization Buffer Kit (Catalog # FC012) or **B.**) another vendor's buffer set. PE-conjugated Anti-Human CD4 (Catalog # FAB3791P) was included as a costain with R&D Systems APC-conjugated Anti-Mouse/Human FoxP3 Monoclonal Antibody (Catalog # IC8214A) or with two other APC-conjugated FoxP3 antibodies obtained from a different vendor.

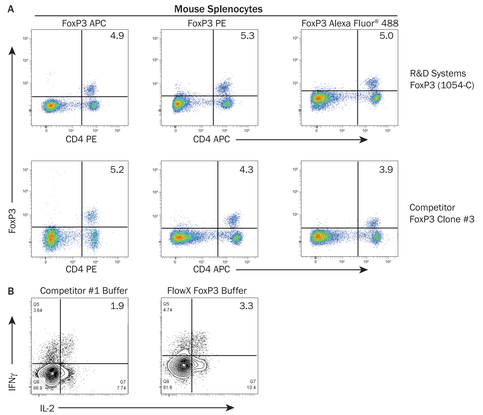

Figure 5. R&D Systems FoxP3 Antibody/Buffer Performance and Compatibility in Mouse Splenocytes. A.) C57Bl/6 mouse splenocytes were stained as described in the Methods with R&D Systems APC-, PE-, or Alexa Fluor 488-conjugated Anti-Mouse/Human FoxP3 Monoclonal Antibody (Catalog # IC8214) or with another vendor's fluorochrome-conjugated FoxP3 antibodies using R&D Systems FlowX FoxP3 Fixation & Permeabilization Buffer Kit (Catalog # FC012). Fluorochrome-conjugated CD4 (Catalog # FAB554) was used as a costain with FoxP3 to identify nTregs. **B.**) Balb/c splenocytes were stimulated with PMA and Ionomycin for 4 hours with Monensin to induce IL-2 and IFN-γ production. The stimulated cells were fixed and permeabilized using the FlowX FoxP3 Fixation & Permeabilization Buffer Kit (Catalog # FC012) or another vendors buffer set. The cells were then stained with a fluorochrome-conjugated Anti-Mouse IL-2 Monoclonal Antibody (Catalog # IC402) and an Anti-Mouse IFN-γ Monoclonal Antibody (Catalog # IC485) to detect IL-2 and IFN-γ production by flow cytometry.

Summary

- R&D Systems FoxP3 antibody is comparable and/or better than other FoxP3 antibodies on the market.
- One antibody can be used to detect FoxP3 in two different target species
- A variety of antibodies work with the R&D Systems FlowX FoxP3 Fixation & Permeabilization Buffer Kit. Specifically, staining of intracellular cytokines using this kit looks virtually identical to the staining obtained with another vendor's buffer set.
- Characteristics of R&D Systems Rabbit Anti-Mouse/Human FoxP3 Monoclonal Antibody:
  - High specificity
  - High affinity
  - Shows lot to lot consistency
  - Allows detection of both mouse and human nTregs and iTregs