

**AN EVALUATION OF REGULATORY T CELL POPULATIONS IN ORALLY  
TOLERIZED AND ANTIBIOTIC-TREATED**

A RESEARCH PAPER BY

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## **Materials and Methods**

### Dissection

All dissecting utensils were kept in 70% ethanol. Mice were sacrificed in a CO<sub>2</sub> tank. The spleen was removed and lymph nodes (LN) were extracted from Peyer's patches and around the small intestine. Spleen and LNs were placed in separate plates with 5 mL RPMI/FBS solution (5% FBS in incomplete RPMI).

### Release of cells

LNs and spleen were isolated by grating with the back side of a 5 mL syringe. The cell suspension was removed and placed in 15 mL conical tube. After 1-2 minute of incubation on ice, the cell suspension was transferred to a fresh tube to remove cell debris. The cell suspension was centrifuged for 5 min at 1500 rpm, 4° C. The supernatant was removed and pellet resuspended. 5 mL of RBC lysis buffer was added to each tube and then inverted to mix. The tubes were incubated for 5 minutes on ice. 5 mL of FBS/RPMI media was added to the suspension. This was centrifuged for 5 minutes at 1500 rpm, 4° C. The supernatant was removed and the pellet was resuspended in 5 mL FACS buffer (LN only) or 10 mL FACS (spleen only). The cells were then counted with a

hemacytometer. The cells were diluted to  $1 \times 10^6$  cells/mL with 10 mL FACS buffer.

### Staining for Tregs

The diluted cells were centrifuged for 5 min at 1500 rpm, 4° C. The supernatant was discarded. The pellet was flicked to resuspend it in the residual buffer.

Surface molecules were stained with anti-CD4 FITC and anti-CD25 PE and intracellular anti-FoxP3 Cy5 to identify Tregs.

*Stain of surface molecules:* Control tubes were prepared for surface molecules. Anti-CD4 FITC and anti-CD4 PE were added to appropriate control tubes. The tubes were incubated on ice for 10-15 minutes. 1 mL of cold FACS buffer was added to each to wash cells. They were then centrifuged at 5 min at 1500 rpm, 4° C. The supernatant was discarded and the cell pellet was resuspended by flicking. 600 uL of FACS n FIX buffer were added to each tube. The tubes were incubated on ice until analysis. A master mix of Fc block was prepared by adding 180 uL of staining buffer to 6 uL of Fc block antibody. 5 uL of this buffer/block mix was added to each sample. A surface antibody stain master mix was prepared by adding either 12 uL FITC-CD4 or 6 uL PE-CD25 to 360 uL of staining buffer. After vortexing, 10 uL of antibody/buffer mix was added to each sample. These were incubated for 30 minutes at 4° C. 1 mL/sample of cold FACS buffer was used to wash the cells. The tubes were centrifuged 5 min at 1500 rpm, 4° C. The supernatant was discarded. The pellet was resuspended before adding 1 mL of fixation/permeabilization working solution to each sample. The samples were

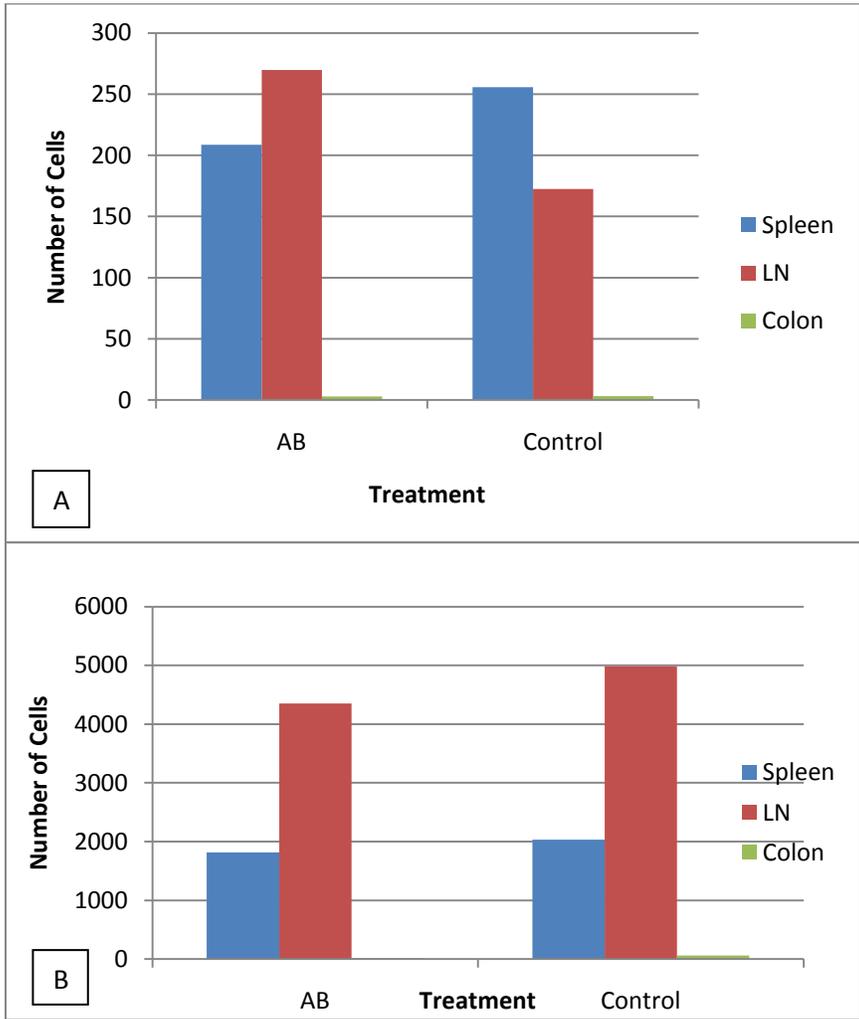
flicked to agitate. The tubes were incubated for 30 minutes at 4° C. The cells were resuspended with 400 mL FACS buffer and immediately analyzed by flow cytometry. Cells that stain for anti-CD4 Fitc/anti-CD25 PE must also stain for intracellular FoxP3 to be confirmed as a Treg.

*Intracellular staining for FoxP3:* The cells were washed with 2 mL of 1X permeabilization buffer. The cells were centrifuged 5 min at 1500 rpm, 4° C and supernatant discarded. 50 uL of antibody/buffer solution (1 uL of Fc block antibody + 200 uL of permeabilization buffer), except to controls. The tubes were incubated at 4° C for 15 minutes. 10 uL of anti-mouse FoxP3 antibody was added to each sample (3 uL FoxP3 per 3 samples + 30 uL of 1x permeabilization buffer). For FoxP3 negative control tubes, the same was performed as above except FoxP3 isotype antibody was used. The tubes were incubated at 4° C for 30 minutes in the dark. 2 mL of 1X permeabilization buffer was used to wash cells. The cells were centrifuged 5 min at 1500 rpm, 4° C and the supernatant discarded. The cells were resuspended in 400 uL of FACS Buffer. All cells were then analyzed by flow cytometry immediately.

## **Results**

The antibiotic studies were performed to demonstrate that treatment with Erythromycin does not affect the T cell population (either CD4+ T cells or regulatory T cells). CD4+ T cells were defined as cells that bound anti-CD4 (Fitc), anti-25(PE) antibodies. Tregs were defined as cells that bound anti-CD4, anti-CD25, and FoxP3, an intracellular transcription factor. CD4+ T cell and Treg cell population numbers remain consistent between organs despite antibiotic treatment (figure 1). The greatest number of CD4+ T cells was seen in the mesenteric lymph nodes and Peyer's patches. A minimal number of CD4+ T cells could be isolated from colon tissue.

**Figure 1**

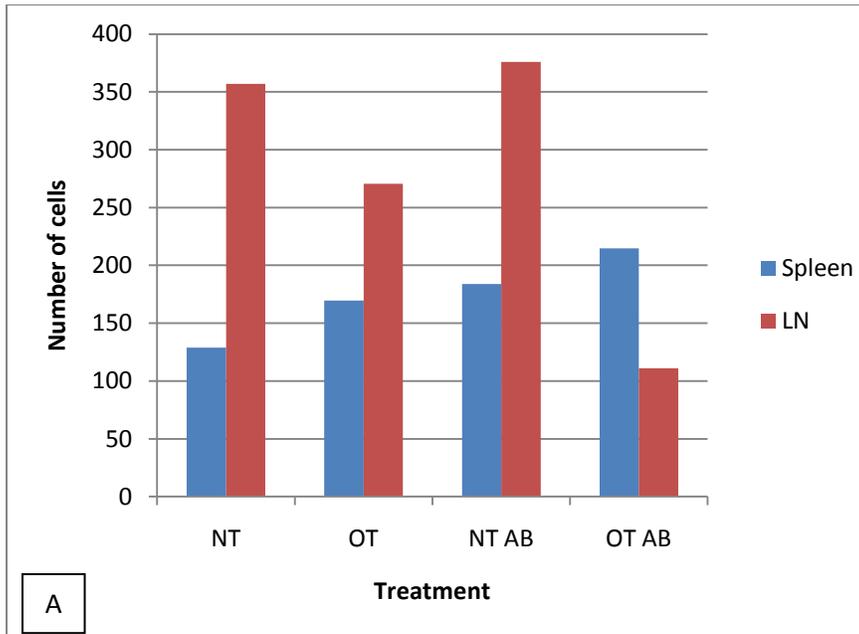


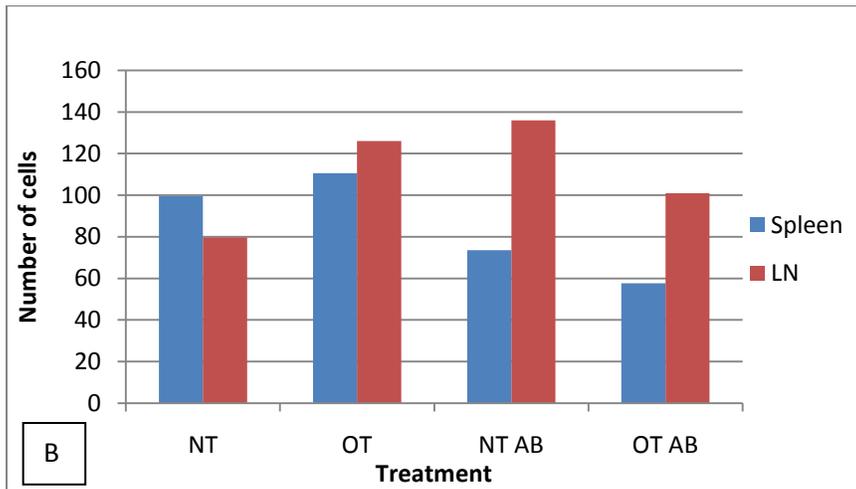
**Figure 1:** Erythromycin treatment does not influence total numbers of CD4+ T cells or Tregs in Balb/C mice. Two groups of mice (n=3) were treated with (Antibiotic) or without (No antibiotic) Erythromycin (300 ug/day) for ten days. A) Numbers of LAB were determined pre- and post treatment to confirm antibiotic effectiveness. Cells were harvested from the spleen, mesenteric lymph nodes (LN) and colon and stained for expression of CD4 and CD25. Numbers of CD4+ T cells were enumerated from flow cytometric analysis of 10,000 total cells (B). Tregs were identified by staining with antibodies to CD4, CD25, and FoxP3. Statistical analysis was performed using a student's t-test, but no statistical difference was determined between groups.

A student's t-test was performed and confirmed that there was no significant difference between antibiotic-treated and control groups. The antibiotic studies served as a control experiment that showed Erythromycin does not disturb T cell populations. The numbers of CD4+ T cells and Treg cells were not affected by antibiotic treatment.

After confirming that Erythromycin does not affect T cell populations, a series of experiments were performed to assess if orally-tolerized mice had altered Treg populations and how a disrupted probiotic flora caused Treg populations to change (figure 2). These studies would confirm the role of Tregs and probiotics as a mechanism in oral tolerance. These experiments were termed "oral tolerance studies."

**Figure 2**





**Figure 2:** *CD4+ and Treg cell populations cannot be determined in orally tolerized (OT) or antibiotic-treated (AB) Balb/C mice. Two groups of mice (n=3) were treated with (Antibiotic) or without (No antibiotic) Erythromycin (300 ug/day) for ten days. A) Numbers of LAB were determined pre- and post treatment to confirm antibiotic effectiveness. Cells were harvested from the spleen, mesenteric lymph nodes (LN) and colon and stained for expression of CD4 and CD25. Numbers of CD4+ T cells were enumerated from flow cytometric analysis of 10,000 total cells (B). Tregs were identified by staining with antibodies to CD4, CD25, and FoxP3 to calculate Tregs. Statistical analysis was performed using a student's t-test, but no statistical difference was determined between groups.*

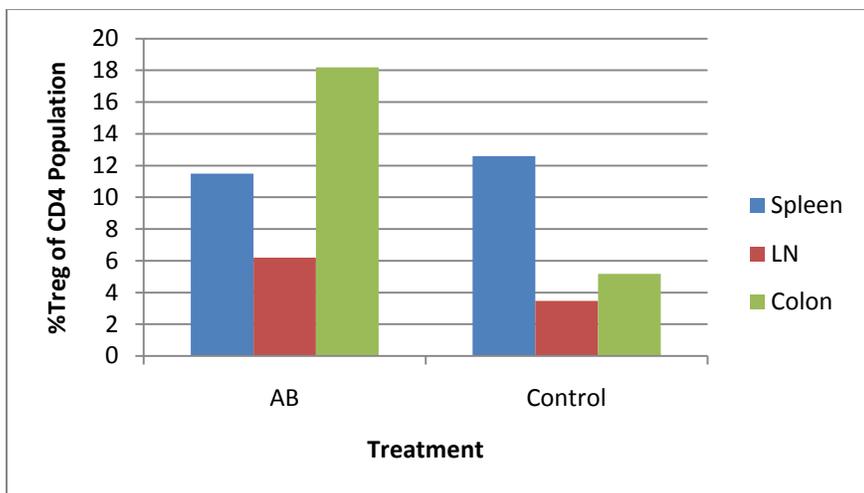
The results of these studies were not conclusive to any of the goals of the experiment because tolerance could not be induced in the mice. However, it would be expected that the orally-tolerized mice would have elevated Tregs (figure 2b). The role of Tregs has been examined in multiple laboratories by comparing IL-10 and TGF- $\beta$  expression—both indicators of Treg activity—between germ-free (GF) and specific pathogen-free (SPF; maintain probiotics) mice. The GF mice had significantly decreased levels of both indicators of Treg

function and were not able to be orally tolerized. This provides correlative evidence to the role of probiotics and Tregs in a tolerance mechanism. The goal of the present study was to extend this work by enumerating Treg cell numbers directly through flow cytometry. By orally treating antibiotic and control mice with ovalbumin (OVA) and demonstrating oral tolerance in control mice, one could analyze to what extent Treg cells play in oral tolerance. It has been demonstrated in multiple laboratories that germ-free mice are not able to undergo oral tolerance (Sudo, 1997; Ishiwaka, 2008). Therefore, it would be expected that antibiotic-treated mice would not be able to demonstrate oral tolerance and would not have altered Tregs. Alternatively, orally tolerized mice should have elevated Tregs. Non-tolerized, control mice should not prove tolerant and would not have elevated Tregs.

The percentage of Treg cells was calculated from the total population of CD4<sup>+</sup> T cells in both the antibiotic (figure 3) and oral tolerance studies (figure 4). This calculation was performed by dividing the average number of Tregs for each treatment and source by the total number of CD4<sup>+</sup> T cells from the same source. The quotient was multiplied by 100 to express this value as a percent. There was very little change in the percentage of Tregs in the antibiotic study. This would be expected because antibiotic treatment without any attempt at tolerance should not induce a Treg response. Unfortunately, oral tolerance could not be induced in the oral tolerance study. This greatly limits the conclusions one could draw from the data. One would expect to see an increased percentage of Treg cells in orally

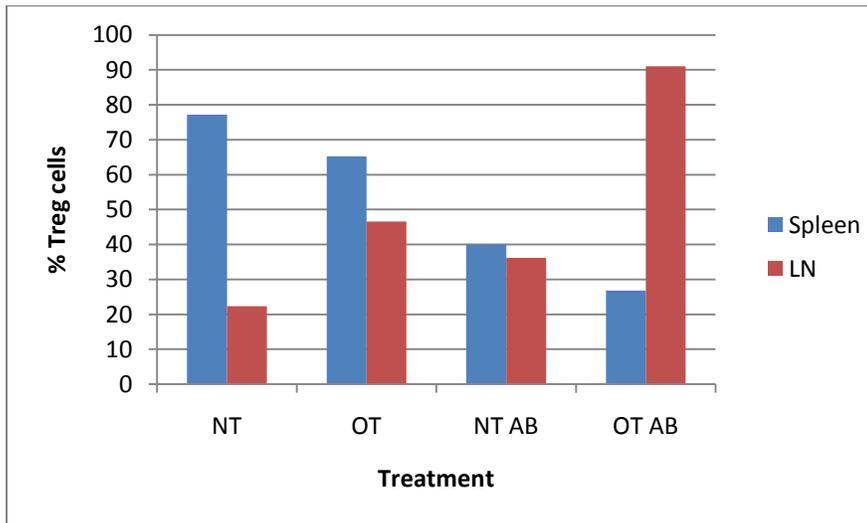
tolerized mice. Non-tolerized mice would demonstrate a baseline percentage of Tregs. Antibiotic-treated mice should not be able to demonstrate oral tolerance, as discussed above with germ-free mice, and so should not show higher percentages of Tregs. The data was very limiting because tolerance could not be induced.

**Figure 3**



**Figure 3:** *The percentage of Tregs in the CD4+ population did not change during treatment in the antibiotic study. Treg percentages were calculated as a percentage of CD4+ T cells. The ratio of Treg/CD4+T cells does not change which demonstrates the lack of effect of antibiotic treatment on Tregs.*

**Figure 4**



**Figure 4:** *The percentage of Tregs in the CD4+ population is significantly higher in OT AB mice . Treg percentages were calculated as a percentage of CD4+ T cells. Antibiotic-treated mice would not be expected to have elevated Treg cells, as discussed above, because germ free mice are unable to be orally tolerant. This is likely an artifact of a particularly low level of CD4+ T cells in these mice, not elevated Treg cells.*

## **Discussion**

Oral tolerance must first be induced and proven before the antibiotic studies can have any authority. These experiments must be able to replicate the results of other laboratories regarding Treg expression in mice with altered probiotic flora. However, achieving tolerance has proven difficult.

Elucidating a mechanism behind Treg activation is the next goal. One suspected way in which Tregs could be activated is through intestinal dendritic cells (DCs). Future extensions of the above work may include a possible for DCs in the gut as they interact with adaptive cells of the immune system.

## References

Ishikawa H, Tanaka K, Maeda Y, Aiba Y, Hata A, Tsuji NM, Koga Y, Matsumoto T. Effect of intestinal microbiota on the induction of regulatory CD25<sup>+</sup> CD4<sup>+</sup> T cells. *Clin & Experim Immunol* 2008; **153**: 127-35.

Sudo N, Sawamura S, Tanaka K, Aiba Y, Kubo C, Koga Y. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol* 1997; **159**:1739–45.