Immunological Characterization of the Peritoneal Cavity of the TLR5 Knockout Mouse

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Abstract:

Toll-like receptor 5 (TLR5) is a cell surface molecule associated with cells of the innate immune system, particularly those in proximity to the gut mucosa. TLR5 detects flagellin, the major protein comprising the flagella ("tails") of bacteria. Most current TLR5 research focuses on organized lymphoid tissue; its function in the peritoneal cavity (PerC), a location enriched with innate immune cells, has not been investigated. We have been comparing the immunobiology of normal ("wild type") mice with those lacking, or "knocked out" for, TLR5 (TLR5KO). Flow cytometry analyses revealed an increase in B1 B cells and a decrease in B2 B cells in the spleen of TLR5KO mice. Surprisingly, no differences were seen between PerC B cells of TLR5KO and wildtype mice. T cell analyses revealed a decrease in CD4+ helper T cells of the TLR5KO spleen; in the PerC, a decrease in CD8+ killer T cells was observed coinciding with a decrease in activated killer T cells. All spleen cell analyses revealed decreased amounts of macrophages relative to wildtype mice. These differences in the immune cells of TLR5KO mice invite further research of their immunobiology to advance understanding of the role of TLR5.

Introduction:

The immune system can be subdivided into innate versus adaptive responses. The innate immune response is the same for everyone, evidenced by cells like macrophages that are constantly roaming around in the body. This is the first, non-specific, line of defense. The innate immune response is triggered by pattern-recognition receptors (PRRs) which are not highly specific, unlike the B and T cell receptors of the cells of the adaptive immune response which are very specific (1). The adaptive immune response occurs after the innate immune response and involves receptors very specific to antigens expressed by the foreign invader (1). Essentially the innate immune response "sets the table" for the later, adaptive immune response. The innate immune response is essential for the survival of the host.

In the innate immune response, there are cell surface molecules that recognize patterns (PRRs), a subset of which are the toll-like receptors (TLRs). TLRs are expressed by macrophages that patrol various mucosal linings inside the body (1). These TLRs are mainly responsible for detection of invaders but also play key roles in other various bodily mechanisms. It is not fully understood how they impact these other mechanisms. TLR5 is found mainly in the gut mucosa and appears to play a role in the development of metabolic syndrome, wherein a cluster of conditions, including high blood pressure, high blood sugar, abnormal cholesterol levels, and increased fatty tissue deposits occur, increasing one's risk of developing type 2 diabetes and cardiovascular disease (2). When first exploring TLR5, it was found that this TLR was mainly responsible for detecting flagellin, the key protein found in the flagella ("tails") of bacteria (3). TLR5 ligation signals pathways that produce the cytokines IL-17 and IL-22 which promote mucosal defenses and tissue repair (4). Much of current TLR5 research focuses on its

involvement in metabolic syndrome and controlling pro-inflammatory responses (2). Mice lacking the gene encoding TLR5 (TLR5 "knockout" or TLR5KO) develop pro-inflammatory diseases like colitis (5, 6).

The goal of this research was to investigate and characterize the cellular composition of the peritoneal cavity (PerC) of TLR5KO mice. Prior research of the role of TLR5 in immunity has focused on the role of this molecule in organized lymphoid tissues, such as the spleen or lymph nodes (4**)**. Study of the immunobiology of the PerC is relevant as this body cavity is enriched for innate cells, notably macrophages as well as distinctive sets of B and T cells that are vital to innate and adaptive immunity. There are few prior PerC TLR5 studies, and none have characterized the fundamental immunobiology of this anatomical location (7). This is intriguing considering the association between the absence of TLR5, visceral adipose tissue (VAT) accumulation and increased basal inflammation (2). This work may provide insight into the regional immunological changes manifested by the lack of TLR5 expression.

To get the immunological characterization desired, flow cytometry (FACS) experiments were run to compare the immune cellular composition of TLR5KO mice with those of wildtype, C57BL/6J mice. In flow cytometry, the spleen/ PerC cells are stained with monoclonal antibody (mAbs) cocktails specific to cell surface markers that are characteristic to certain cells. Two mAb cocktails were used to differentiate between B and T cells. The B or T cell mAb cocktails detect cell surface markers that can be used to differentiate between the different types/stages of B/T cells. The mAbs are chemically linked to different fluorophores so when hit by the laser they become excited and emit light. The flow cytometer detects these signals and records their intensity and cellular distribution. The cell type is determined by knowing which fluorophore is chemically linked with each mAb. After all the cells are hit with the lasers in a sample, results are given showing how much each fluorescently labeled mAb was excited and percentages of the subpopulations of B and T cells are seen.

Materials and Methods:

Mice

C57BL/6J wildtype (strain #664) and TLR5KO mice were purchased from the Jackson Laboratory and housed in Rider University's mice colony.

FACS

Spleen and PerC cells were obtained from wildtype (C57BL/6J) and TLR5KO mice. The cells were stained at $1.0x10^6$ cells/mL per monoclonal antibody cocktail for 1 hour on ice, washed with cold PBS, centrifuged, the supernatant decanted, and pellets resuspended in 0.4 ml of FACS buffer for analysis. Samples were analyzed by a dual-laser, 7-color ATTUNENxT flow cytometer (ThermoFisher). Two mAb cocktails were used. The B cell specific stain one was made up of αIgM, αIgD, CD23, CD19, CD11b, CD1D (spleen cell-specific), and CD5 (PerC cell-specific). The T cell specific stain two was made up of CD62-L, CD25, CD8, PD-1, CD44, and CD4. The mAbs, purchased from ThermoFisher, were fluorescence-labeled with FITC, PE, PerCP, PeCy7, APC, Alexa 700, Cy7, and APC Cy7.

Results:

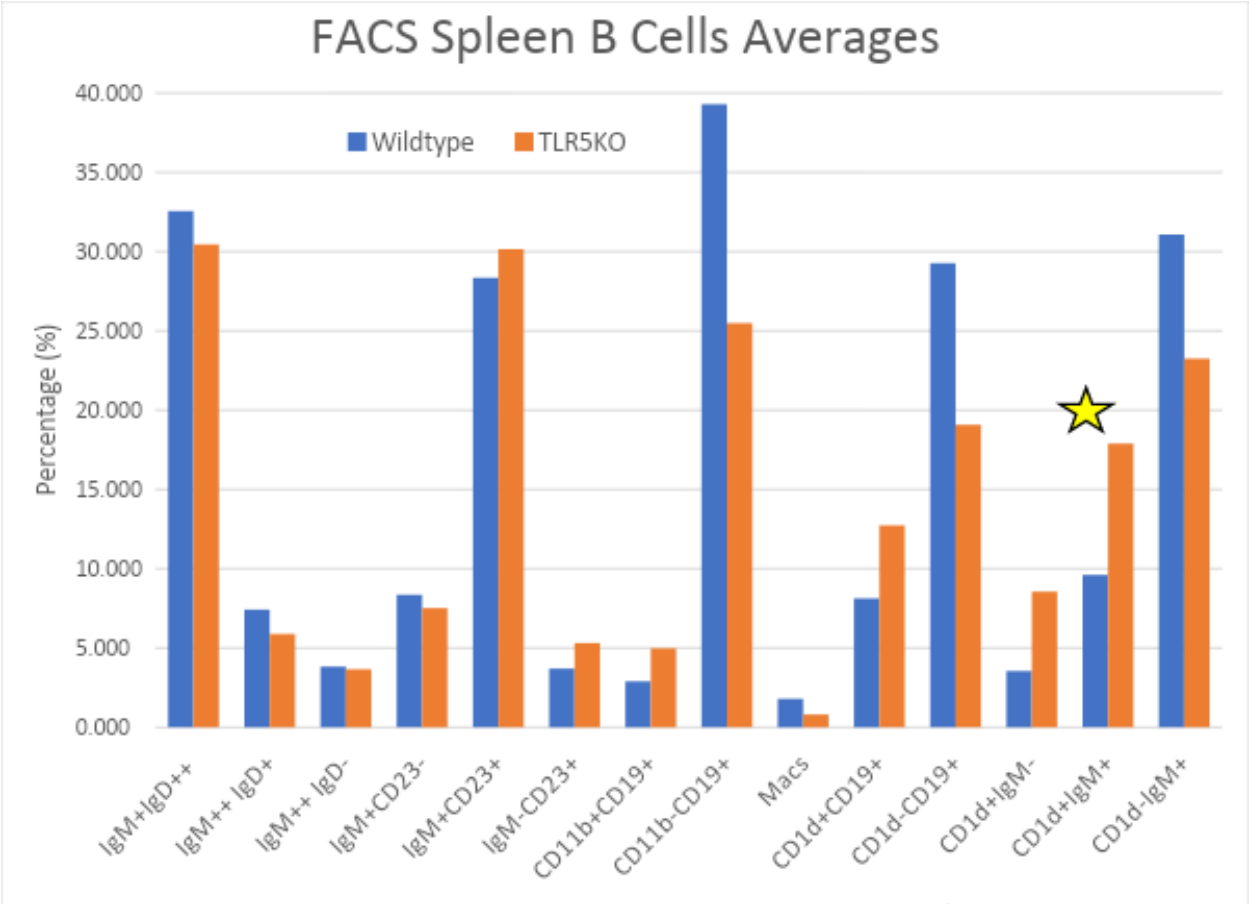
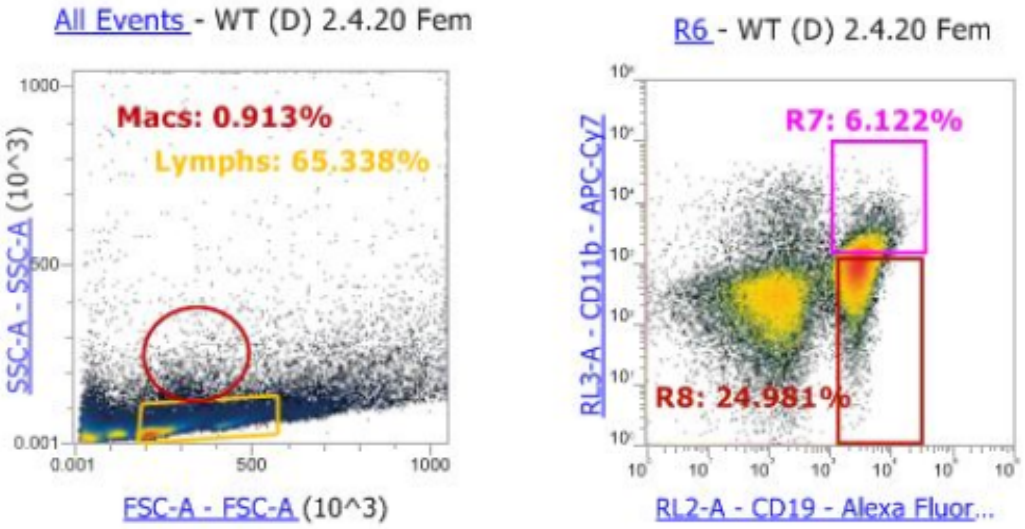
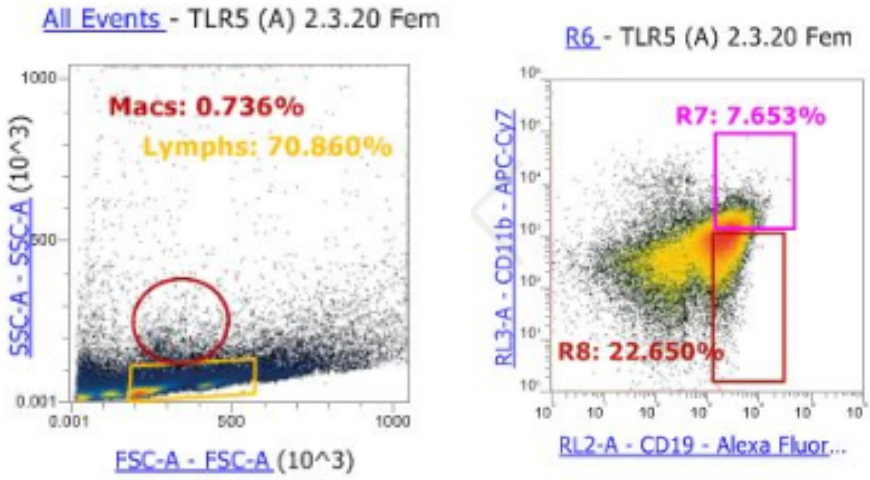


Figure 1. Flow cytometric analyses shown for spleen B cells in TLR5KO and wildtype mice. The average percentages of each B cell subset seen is on the y-axis versus the mAbs used to gate and represent the different B cell subsets on the x-axis. Wildtype (n=15) and TLR5KO (n=14) averages calculated and shown in comparison to each other. T-tests were run to determine statistical significance in the differences seen. Statistically significant differences are marked with stars.

In the spleen, flow cytometric analyses showed t-test statistical significance in the increase of CD11b+ CD19+ cells in the TLR5KO. This gate is representative of B1 B cells. It would be expected that the "opposite" subset of B cells, CD11b- CD19+ cells, or B2 B cells, would show a decrease in the TLR5KO and this was the case. Intriguingly, the percentage of macrophages present in the TLR5KO spleen also decreased. Below is representative data of the macrophage and CD11b and CD19 gates of one TLR5KO and one wildtype mouse to be able to see these experimental differences directly. This greater percentage of CD11b+ CD19+ B1 B cells appeared to increase with aging (data not shown). Further research could be geared towards validating the increased spleen B1 B cell expansion of older aged TLR5KO mice.



Wildtype Spleen B Cells



TLR5KO Spleen B Cells

Figure 2. Flow cytometry results for the spleen B cells of one wildtype and one TLR5KO mice are shown above. Macrophage gates are represented by the red circles and the experimental decrease from 0.913% macrophages in the wildtype down to 0.736% macrophages in the TLR5KO can be seen. The CD11b+ CD19+ gate is represented by the pink R7 gates in the top right corner and the increase going from 6.122% to 7.653% in wildtype to TLR5KO is representative of the increase in B1 cells noted above. The CD11b- CD19+ gate is represented by the red R8 gates in the bottom right corner and the decrease going from 24.981% to 22.650% in wildtype to TLR5KO mice, validating the decrease in B2 cells mentioned above.

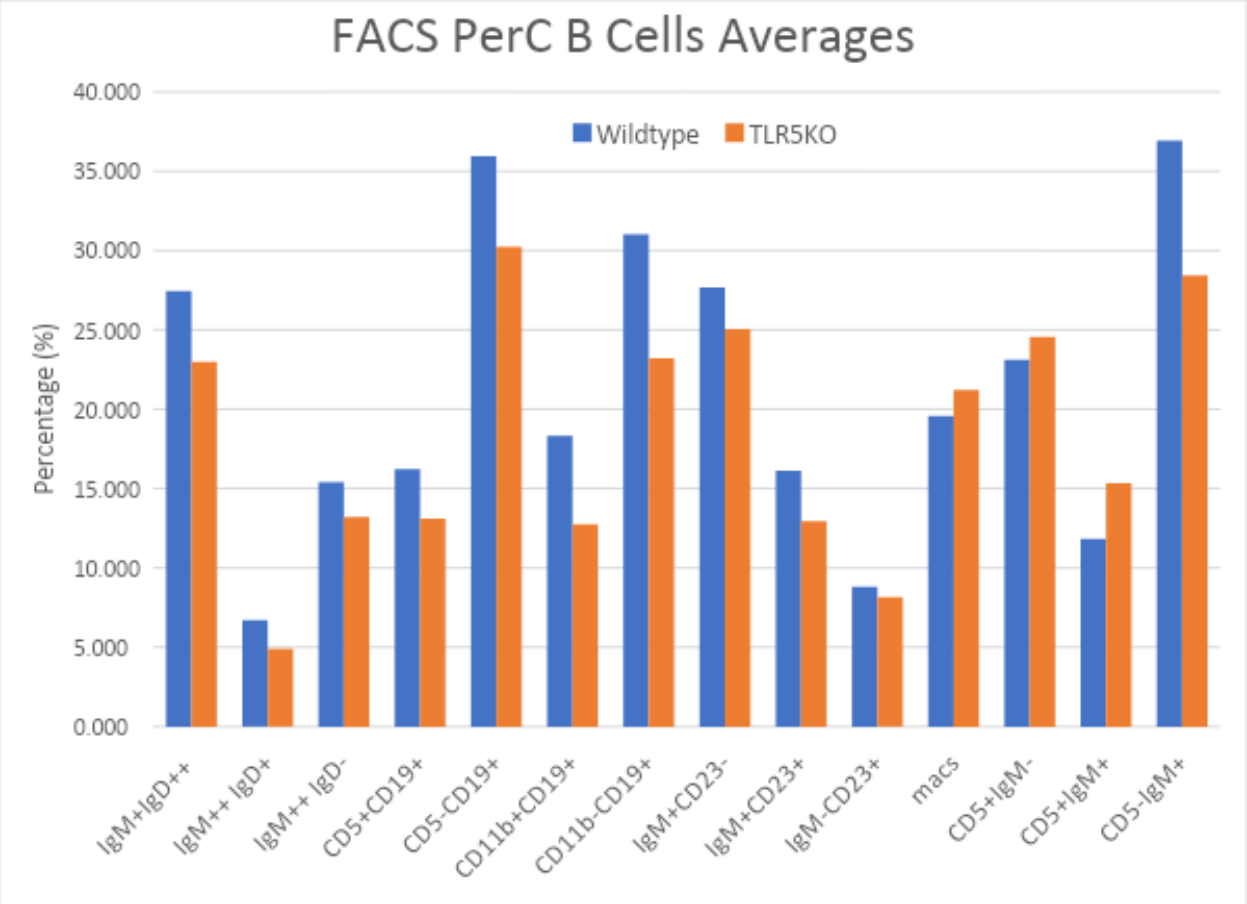


Figure 3. Flow cytometric analyses shown for PerC B cells in TLR5KO and wildtype mice. The average percentages of each B cell subset seen is on the y-axis versus the mAbs used to gate and represent the different B cell subsets on the x-axis. Wildtype (n=15) and TLR5 KO (n=14) averages calculated and shown in comparison to each other. T-tests were run to determine statistical significance in differences seen. Statistically significant differences are marked with stars.

Considering the differences in B1/B2 cell composition found in the spleens of TLR5KO mice, we expected similar results in the PerC. Our hypothesis was based on the established fact that the PerC is enriched for B1 B cells in wildtype mice (8). With the PerC being more enriched for macrophages and innate immune cells, these results were surprising. The decrease in CD5- IgM+ B cells (B2 cells) at the far right of figure 3 above was the only B cell subset that approached statistical significance. Thus, the PerC B cell immunobiology looks to be similar for both TLR5KO and wildtype mice. The only place to attempt to flesh out some differences would be possibly looking into further experimentation examining that CD5- IgM+ B2 B cell subset that was approaching statistical significance.

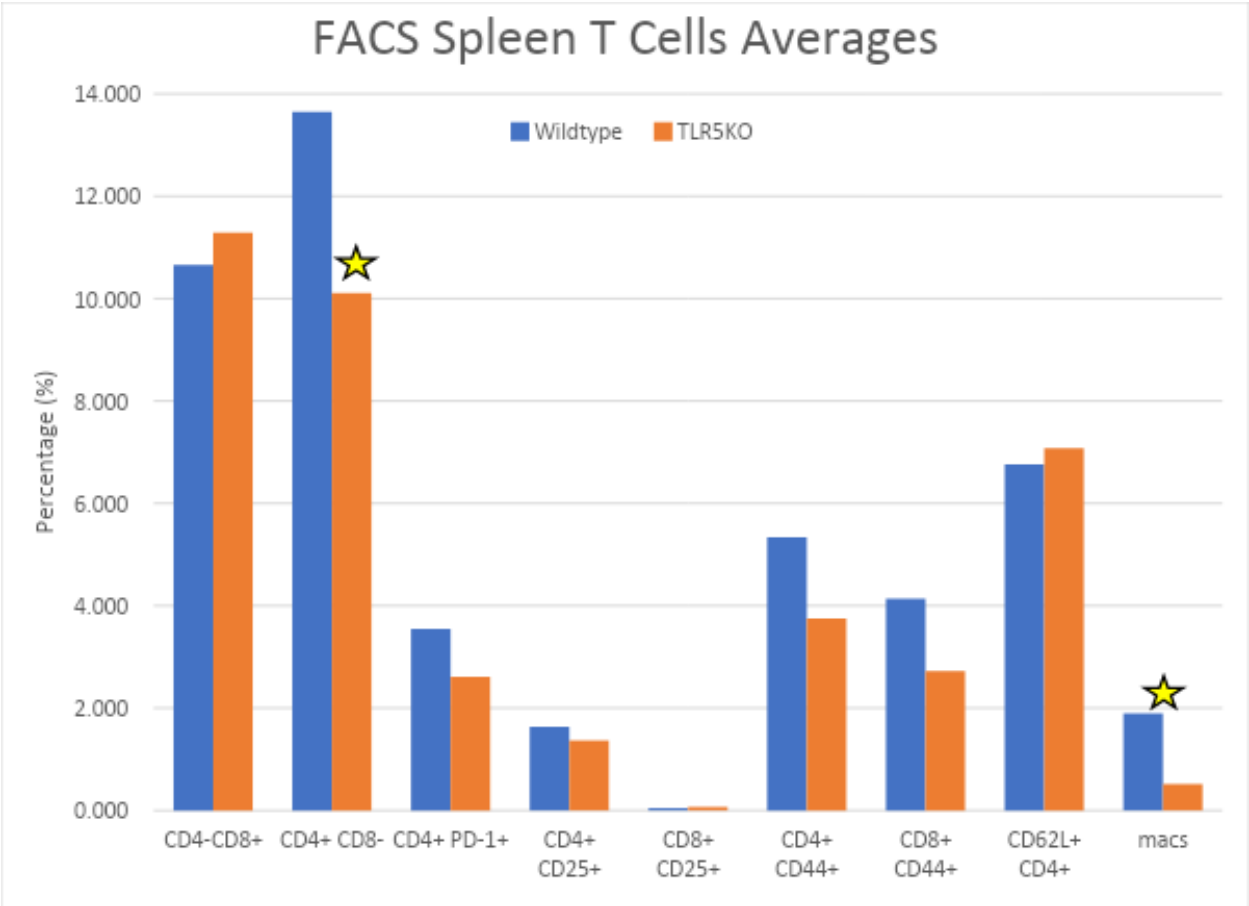
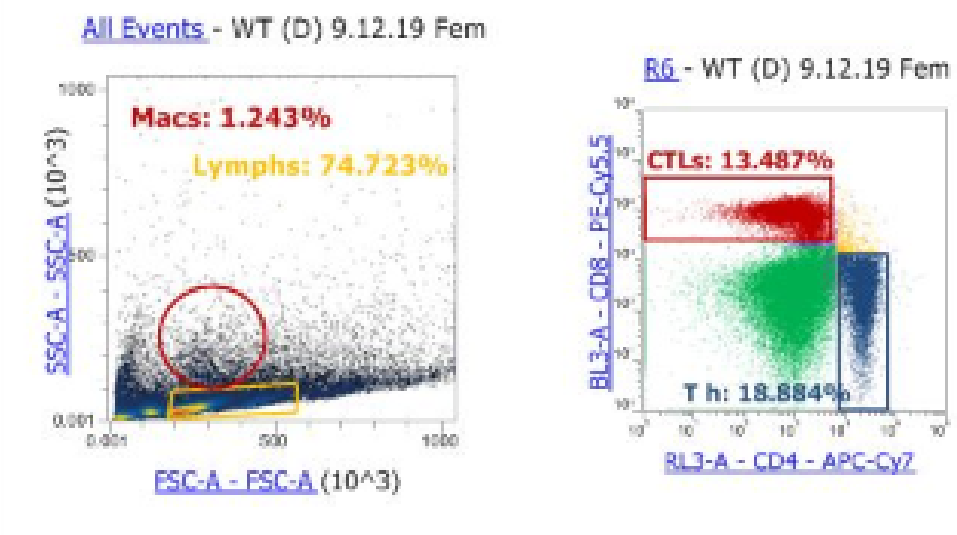
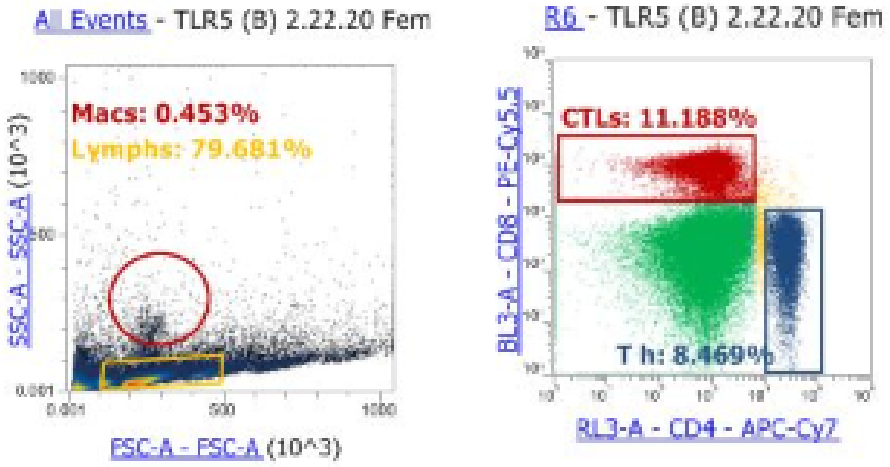


Figure 4. Flow cytometric analyses shown for spleen T cells in TLR5KO and wildtype mice. The average percentages of each T cell subset seen is on the y-axis versus the mAbs used to gate and represent the different T cell subsets on the x-axis. Wildtype (n=15) and TLR5KO (n=14) averages calculated and shown in comparison to each other. T-tests were run to determine statistical significance in differences seen. Statistically significant differences are marked with stars.

In the spleen, flow cytometric analyses revealed a decrease of CD4+ CD8- cells in the TLR5KO. This gate is representative of helper T cells. Interestingly, a flip in the CD4+/CD8+ ratio of cells was seen for the TLR5KO mice. In the wildtype mice there was an average CD4+/CD8+ ratio of 1.28 indicating more helper T cells present than killer T cells but in the TLR5KO mice the average ratio flips to 0.90 which indicates more killer T cells present than helper T cells. This coincides with the statistically significant decrease in helper T cells in the TLR5KO but it stands out that the amount of helper T cells dropped below the amount of killer T cells. Internal validation of the experiments performed can be seen here as the macrophages for the spleen were again decreased with both the B and T cell staining cocktails. Below is representative data of the macrophage and CD4 CD8 gates of a TLR5KO versus a wildtype mouse. TLR5KO mice had a trend of increasing helper T cell activation with increasing age. Further research should be done regarding the helper/killer T cell ratio inversion and helper T cell number to assess what this might imply for the B cell subset differences reported above.



Wildtype Spleen T Cells



TLR5 KO Spleen T Cells

Figure 5. Flow cytometry results for the spleen T cells of one wildtype and one TLR5KO mice are shown above. Macrophage gates are represented by the red circles and the experimental decrease from 1.243% macrophages in the wildtype down to 0.453% macrophages in the TLR5KO can be seen. The CD4+ CD8- gate is represented by the blue helper T cell gates in the bottom right corner and the decrease going from 18.884% to 8.469% in wildtype to TLR5KO is representative of the decrease in helper T cells noted above. The T helper/ T killer ratio inversion is seen here as well where the wildtype T cells show populations of 18.884% helper T cells versus the lower 13.487% killer T cells whereas the TLR5KO T cells show populations of 8.469% helper T cells versus the now higher populated 11.188% killer T cells.

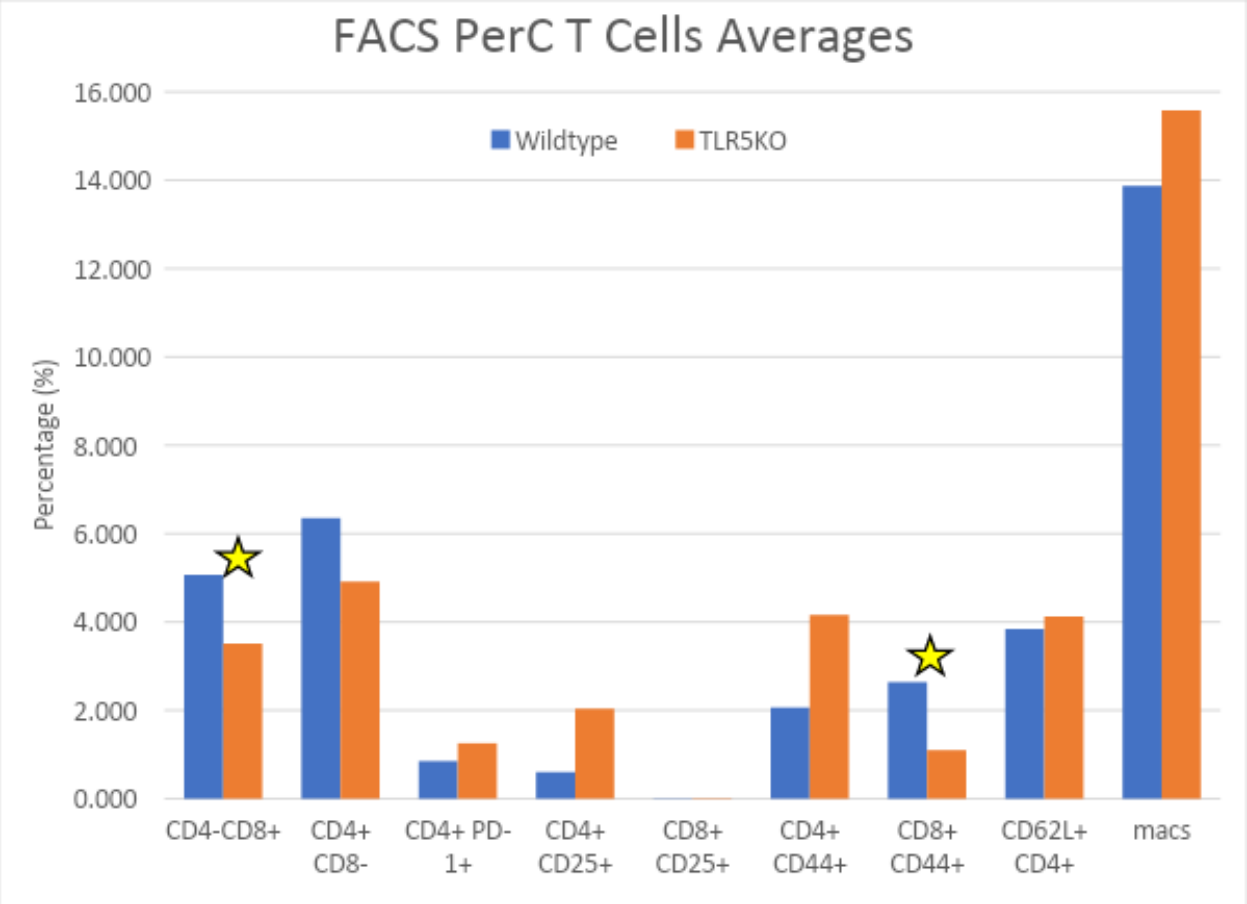
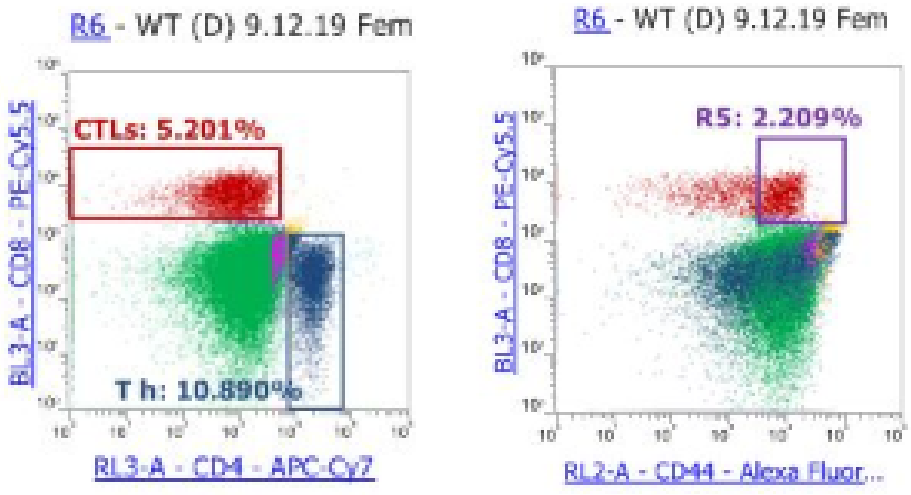
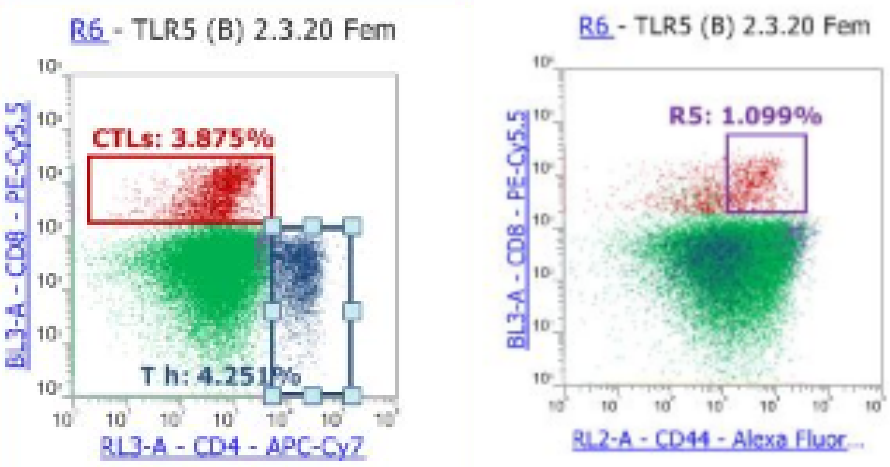


Figure 6. Flow cytometric analyses shown for PerC T cells in TLR5KO and wildtype mice. The average percentages of each T cell subset seen is on the y-axis versus the mAbs used to gate and represent the different T cell subsets on the x-axis. Wildtype (n=15) and TLR5KO (n=14) averages calculated and shown in comparison to each other. T-tests were run to determine statistical significance in differences seen. Statistically significant differences are marked with stars.

In the PerC, there was a decrease of CD4- CD8+ cells in the TLR5KO. This gate is representative of killer T cells. There also was a decrease in the percentage of activated killer T cells defined as a decrease in the CD8+ CD44+ gate for the TLR5KO mice. There also appeared to be more activated T helpers, specifically the CD4+ CD25+ population in the TLR5KOs but this data did not reach statistical significance. Representative data of the CD4 CD8 and the CD8 CD44 gates of one TLR5KO and one wildtype mouse are provided below. Interestingly, PerC T cell data contrast that of spleen T cell. Further research, particularly in aging animals, will be necessary to see if our initial observations hold up.



Wildtype PerC T Cells



TLR5KO PerC T Cells

Figure 7. Flow cytometry results for the PerC T cells of one wildtype and one TLR5KO mice are shown above. The CD4- CD8+ gate is represented by the red cytotoxic T lymphocytes (CTLs) gates in the top left corner and the decrease going from 5.201% to 3.875% in wildtype to TLR5KO is representative of the decrease in killer T cells mentioned prior. The CD8+ CD44+ gate is represented by the purple R5 gates in the top right corner and the decrease going from 2.209% to 1.099% in wildtype to TLR5KO is representative of the decrease in activated killer T cells noted earlier.

Discussion:

Flow cytometric analyses of TLR5KO mice revealed statistically significant differences in the B and T cell profiles of the spleen and PerC when compared to wildtype mice. In the spleen there was an increase in B1 B cells, a decrease in B2 B cells, and a decrease in

macrophages present in TLR5KO mice. The increase in B1 B cells was more common and showed an increasing trend in the older aged TLR5KO mice. Surprisingly, contrary to our hypothesis, there was no differences seen between the B cell subsets of the normally macrophage and B1 B cell enriched PerC of the TLR5KO mice and wildtype mice (8). In the spleen T cell profiles, a decrease in helper T cells and a decrease in macrophages was seen. The decrease in macrophages was consistent in both B and T cell profiles of the spleen. The decrease in helper T cells was marked in that it flipped the helper T cell/ killer T cell ratio relative to wildtype mice. There was increased helper T cell activation seen with increasing age, indicating the need for future research focused on older aged TLR5KO mice which seem to have greater differences with their normal counterparts. In the PerC T cell profiles, there was a decrease in both killer T cells present and activated killer T cells in the TLR5KO mice. The PerC T cell data looks to contrast that of the spleen T cell data in terms of decreased killer T cells in the PerC versus decreased helper T cells in the spleen.

An increase in the weight of TLR5KO mice was both visibly observed and experimentally determined by recording animal weights (data not shown). The TLR5KO mice were expected to have increased weights than the wildtype due to links between the absence of TLR5KO and increased visceral adipose tissue (VAT), characteristics found to correlate with increased basal inflammation (2). The weight increase became more evident with the increasing age of the mice implying that the increasing VAT accumulation and basal inflammation may not ever be controlled in TLR5KO mice. Male mice were heavier than age-matched female mice indicating that gender may play a role in the VAT accumulation and increased basal inflammation.

Overall, TLR5KO mice do present different immunological B and T cell profiles in the spleen and PerC when compared to wildtype mice. Future research could be performed that looks for direct impacts on immune responses by performing enzyme-linked immunosorbent assays (ELISAs) to measure humoral (antibody) responses and CFSE proliferation assays to measure cellular immunity in TLR5KO mice. ELISAs can be used to assess B and T cell immune responses *in-vivo* after exposure to various antigens that stimulate different arms of the immune response. CFSE proliferation assays could be used to assess *in-vitro* lymphocyte responses and determine if the observed differences in subset composition correlate with the differences in functional biology. Such research would allow for future connections to be made between the immunological profiles of the TLR5KO mice described herein and their capacity for immune responses. The increasing B and T cells differences in the spleen/PerC and the weight effects seen as TLR5KO mice age, suggest that the TLR5 response may serve in maintaining a healthy, low inflammatory, low-fat immunobiology. This has implications for cancer, a disease associated with increasing age and VAT accumulation and the associated increase in basal inflammation. The TLR5 flagellin response likely has a role in shaping the host microbiome, an increasingly important component of human health.

References:

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