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Abstract

The objective of this study was to determine what blood cell types express the B lymphocyte-induced maturation protein-1 (BLIMP-1) gene. Such findings would be instrumental in research regarding the aryl hydrocarbon receptor (AhR) which is important in immune responses. The experimental model was a transgenic mouse that expresses the enhanced yellow fluorescent protein (eYFP) under the control of the BLIMP-1 promoter. Testing was carried out in two parts; (1) testing mice for eYFP presence and (2) testing mice for phenotypic cell markers. Cell samples were tested via flow cytometry. During phase one of testing it was found that positive mice showed an average of 0.972% eYFP positive cells, and negative mice showed an average of 0.138% eYFP positive cells. In the second phase it was found that the eYFP+ cells express several different markers, including CD3, CD4, CD8, and, CD11b. Additional testing needs to be completed to further understand what these cells express, but this preliminary data is promising for the future of the AhR and cell trafficking.

Introduction

The aryl hydrocarbon receptor (AhR) is a promising component of the human body that may improve treatments for many autoimmune diseases, including graft vs. host and Crohn's. The receptor has had countless hours of research poured into it, and there is still more being conducted in an attempt to understand the receptor's workings and impacts on the body, particularly within the immune system and inflammatory response. It is known that the AhR is highly receptive to 2, 3, 7, 8-tetrachlorodibenzo p dioxin (TCDD), but the effects TCDD has on the AhR, and the implications this activation has on the human body are still not completely understood.

The aryl hydrocarbon receptor (AhR) was discovered 40 years ago and is promising in a multitude of medical arenas (Esser et al., 2018). After its original discovery, there was much research conducted to determine the workings of the AhR, but despite this, it is still not entirely understood what role this receptor plays within the body, especially regarding immune response (Sulentic & Kaminski, 2010).

The AhR is a ligand-activated protein receptor that is part of the Per-Arnt-Sim (PAS) protein family which regulates metabolism, circadian rhythms, hormone production, development, and immune function (McIntosh, Hogenesch, Bradfield, 2010). A unique and medically relevant component to the AhR is that there are many different ligands that will activate it. These ligands can come from plants, bacteria, or chemicals within the cell (Esser et al., 2018). One of the ligands the AhR has the highest affinity for is 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD has a longer half-life with the AhR, and as a result is the most widely used in research as a model (Ramos, 2019).

Enhanced yellow fluorescent protein (eYFP) is a commonly used transgenic marker in research. It is a valuable tool for tracking cells throughout the body and in research and has been used in many research laboratories, such as those tracking immune pathways and the trafficking of immune cells (Xiaoxi, et al., 2011; Bullock, et al., 2008). eYFP is expressed on the Blimp-1 (B lymphocyte induced maturation protein) promoter, which is used to induce the maturation and differentiation of B lymphocytes (Turner, et al., 1994). Beyond B cells, Blimp-1 expression is also seen in T cells, especially in CD4+ and CD8+ ones; however, this expression has been found to be restricted predominantly to activated T cells (Fu, et al., 2017).

Significance

In order to maintain optimal health, the body must properly defend against pathogens. One of the greatest exposure to these occurs within the GI tract. When pathogens enter the gut, antibodies bind to them to neutralize their attack and prevent advancement into other portions of the body (Macpherson, Geurking, McCoy, 2012). B cells and other immune cells gather in the lamina propria (LP) in preparation to intercept pathogens (Brandtzaeg et al., 1999). Many of these B cells migrate from other parts of the body, and some migrate selectively to the LP (Habetzion, et al., 2016). During the immune response, B cells secrete antibodies to defend against pathogens. Most of this antibody creation occurs within the LP (Ramos, 2019). Migration into the LP is crucial for proper immune defense so these antibody-secreting B cells are in the correct place to intercept pathogens.

The AhR plays a critical role in the migration of some immune cells. Previous research by others has demonstrated that the AhR regulates the migration of T cells into

the gut as well as T regulatory cell (Treg) differentiation (Qunintana et al., 2010). It is possible to activate the AhR with a wide array of foods and drugs, and some entire experiments have been conducted using food exclusively (Ramos, 2019).

Activation of the AhR in one study showed an increased average β7-integrin expression on CD3+ cells (Ramos). Previous research has also shown that when T-cells are primed and activated by the gut-associated lymphoid tissue, integrin α4β7 and CCR9 are upregulated, which directs them to the lamina propria (Habetzion, et al., 2016). In mice without β7, an immune igA response to cholera toxin could not be properly mounted (Schippers, et al., 2012). These models suggest that the AhR can change the expression of β7-integrin from T cells, which in turn impacts the migration of T cells to the LP.

If the expression of eYFP on specific T cells can be fully understood, then specific cells and coreceptors can be manipulated to better understand the trafficking of immune cells. This would be especially important with regards to tracking cells as they respond to the AhR, as well as understanding what types of T and B cells are being signaled by the AhR. If immune responses such as these can be better understood then those methods could be utilized therapeutically in autoimmune diseases such as Crohn's and graft vs. host. Although mice models aren't completely representative of effects in humans, these findings are promising for developments in autoimmune therapies.

Methods

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (National Research Council, 1996). The animal protocol was approved by the

Institutional Animal Care and Use Committee at UNC. All efforts were made to minimize suffering.

eYFP Transgenic Presence

Testing for eYFP was completed by cheek bleeding mice via lancelet, lysing blood in AKC buffer, then washing cells in medium before resuspending in cell staining buffer. Flow cytometry via the Attune NxT V6 flow cytometer was utilized to assess eYFP presence. Mice expressing >0.52% eYFP+ were deemed to be positive for the gene, as per previous work in the laboratory (Hufford, et al.).

Gating for eYFP presence was done in three stages. The first was to run all cells and isolate the lymphocyte population, eliminating erythrocytes and any other cells. The second gate was created using FSC-A vs. FSC-H and eliminated all doublets and remaining cell fragments, so only single lymphocytes were read. The final gate took this single-cell population and used eYFP-H vs. SSC-A to measure the eYFP presence in the cells.

eYFP Phenotyping

T cell markers were assessed by euthanizing the mice via carbon dioxide, then extracting blood from the heart. This blood was then lysed via AKC lysis buffer, washed in cell medium, and then cell counts were performed using a hemocytometer. Once the cell counts were completed, 100,000 cells were transferred into tubes before antibodies were added. Following antibody addition and incubation, flow cytometry was run to assess for T cells markers and confirm eYFP presence.

Gating for cell phenotyping was conducted in five stages, with the first three being similar to that for eYFP transgenic presence testing. Following eYFP assessment,

eYFP positive cells were gated for in an SSC-A vs. CD3 plot. CD3 positive cells were gated and then placed in a CD4 vs. CD8 quadrant.

Results

eYFP Transgenic Presence

A total of 23 mice were assessed for eYFP transgenic presence via live blood collection as detailed above. Samples were then analyzed via flow cytometry (Table 1). Of the 23 total mice, there were 9 confirmed positive, 13 confirmed negative, and one inconclusive. Negative mice were either euthanized or used as negative controls in other experiments. Positive male mice were either used as stud mice for breeder cages if needed or as positive controls. Positive female mice were used as positive controls in later experiments. Positive mice showed an average of 0.972% eYFP positive cells, and negative mice showed an average of 0.138% eYFP positive cells.

Mouse	*eYFP %*	*eYFP Status*	*Mouse*	*eYFP %*	*eYFP Status*
19.8.2 LR	0.551	Positive	24.5.2L	0.389	Negative
19.8.2L	0.546	Positive	24.5.2R	0.187	Negative
19.8.2R	0.537	Positive	25.1.1-	1.103	Positive
19.8.2-	0.019	Negative	25.1.1L	0.077	Negative
24.3.2R	0.178	Negative	26.2.1-	0.346	Negative
24.3.2-	0.673	Positive	26.1.2-	0.042	Negative
24.3.2L	0	Negative	26.1.2L	0.068	Negative
24.3.1R	0.371	Negative	26.4.2L	1.302	Positive
24.3.1L	0	Negative	27.1.1-	1.160	Positive

24.3.1-	N/A	Inconclusive	27.1.1L	1.322	Positive
24.5.1-	1.556	Positive	27.1.1R	0.048	Negative
24.5.1L	0.076	Negative			

Table 1. Mouse identifier, eYFP reading from flow cytometry, and conclusion

eYFP Phenotyping

Mice with confirmed eYFP positive or negative were screened for phenotypes. These included CD3, CD4, CD8, CD11b, and GR1. Additionally, flow cytometry gating was used to assess for mice that were double or triple positive. Isotype controls and negative stains were run for all mice and antibodies. Of the four cell samples stained for CD3, three were confirmed eYFP positive, and one was a confirmed negative. The samples showed eYFP positive and negative as previously determined when assessed following necropsy. All four samples were positive for CD3 and eYFP but not for CD3, CD8, and eYFP.

Three mice were stained for CD3, CD4, and CD8. Two of these were positive for eYFP. One sample showed 5.106% of cells as CD3+CD8+eYFP positive. This occurred in the mouse that was 10 months old. Of the other two samples, one was 0.697% positive for the CD3+CD8+eYFP staining, and the other was inconclusive.

Mice stained for CD4, CD8, and a CD11b/GR1 combo were found to be positive for the antibodies they were stained for. The cells stained for CD4 were found to be positive for CD4 and CD11b. Similarly, cells stained for CD11b/GR1 were also found to be positive for CD4.

Mouse	*Antibody*	*eYFP %*	*eYFP+CD3 %*	*CD3, CD8, eYFP*	*Conclusion*
27.1.1L	CD3	3.385	7.842	0	T-cell w/ eYFP
27.1.1R	CD3	0.035	5.023	0	T-cell
27.1.1-	CD3	2.069	8.721	0	T-cell w/ eYFP
24	CD3	2.868	3.621	0	T-cell w/ eYFP
27.1.1-	CD3/CD4/CD8	1.803	12.064	0.697	Cytotoxic T-cell w/ eYFP
24	CD3/CD4/CD8	3.796	3.982	5.106	Cytotoxic T-cell w/ eYFP
27.1.1R	CD3/CD4/CD8	0.133	3.545	N/A	T-cell

Table 2. Mouse identifier, antibody tested for, percent eYFP positive cells from flow cytometry, percent eYFP and CD3 positive cells, percent CD3+CD8+eYFP cells, and conclusion

Discussion

Although the sample size is small, this study also suggests that eYFP positive cells do not generally express double or triple positive for CD3/CD4/CD8. As expected, this does suggest that eYFP cells are positive for the CD3 marker, which is the general marker for T-cells. The mouse that was CD3+CD8+eYFP suggests that eYFP cells are cytotoxic T-cells. The CD4 expression suggests that those cells are T-helpers, and those that express CD4 and CD11b are T-helpers and monocytes/macrophages.

Conclusion

This study continues to support that eYFP transgenic testing via cheek bleeding is a viable and accurate way to test for eYFP presence in mice. Preliminarily, this data suggests that eYFP positive cells in transgenic mice are T-cells, T-helper cells,

monocytes, and sometimes are cytotoxic T-cells. More testing needs to be run to confirm these findings.

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