A STUDY OF GENES EXPRESSED IN IMMUNOGLOBULIN M B CELLS FROM THE ZEBRAFISH KIDNEY.

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Abstract

Bony fish are the most primitive vertebrates to possess an adaptive immune system. The zebrafish (*Danio rerio*) is one example of these bony fish, which means they have an immune system similar to that of a human (composed of both an innate and an adaptive immune system). As such, zebrafish are an excellent model for studying the evolution of the adaptive immune system. The immune system is composed of many different types of cells; one of these populations is known as B cells. B cells can further be classified into developing, mature, plasma, or memory B cells. Each subset of B cells has unique characteristics and functions within the immune system. One of the major gaps in the current knowledge of the zebrafish adaptive immune system is a lack of information about what classes of B cells are present in zebrafish kidneys (the location where B cell development occurs). This knowledge could further increase the use of zebrafish as an animal model by allowing researchers to know what types of B cells were being used in an immune response, and how this could affect the observed results. This study used double transgenic fluorescent zebrafish, where the two fluorophores labeled and differentiated various cell populations in order to further characterize the types of B cells present in developing zebrafish immunity. Cell samples from the kidneys of zebrafish were analyzed to determine if cells that expressed both fluorophores were a pure population of plasma B cells. It was found that populations of double-fluorescent cells, which consisted of either IgM^{hi} cells or IgM+BLIMP+ cells, express both BLIMP and Pax5. This potentially indicates that Pax5 takes longer to stop being expressed than it does for BLIMP to start being expressed, allowing for the presence of a population of B cells which transiently express both BLIMP and Pax5.

Introduction

The zebrafish (*Danio rerio*) is one of the most primitive vertebrates to possess both an innate and adaptive immune system. Thus, it is one of the most primitive vertebrates to possess an immune system similar to that of humans, making the zebrafish an excellent model organism.[1] In particular, zebrafish are known to be an excellent animal model for certain human conditions, including the early stages of tuberculosis. Although they do not have lungs, the innate immune response of zebrafish to the Mycobacterium analog of the tuberculosis bacterium that infects humans is very similar to the initial stages of the human innate immune response.[2] By furthering the knowledge of the immune system of zebrafish, their efficacy as an animal model can be increased.

The adaptive immune system is composed of many different cell types. Two major cell types involved in this system are known as B cells and T cells. B cells are the cells that produce antibodies against pathogen. They are activated by interacting with T cells. B cells interact with pathogen, whereas T cells only interact with antigen-presenting cells (such as B cells). B cells can further be classified into subcategories of cells. These categories are developing B cells, mature B cells, plasma B cells, and memory B cells. Developing B cells do not yet have all the necessary genes expressed to effectively interact with pathogen. Mature B cells have finished their development, and have not yet interacted with their specific pathogen. Alternatively, mature B cells have just interacted with pathogen, but have not yet undergone differentiation. Once they interact with their pathogen by binding it with their B cell receptor, phagocytosing it,

digesting it, and presenting fragments of the pathogen on MHC II molecules, they can interact with an activated T cell. This interaction with the activated T cell activates the mature B cell. These activated mature B cells can further differentiate into plasma B cells or memory B cells. Memory B cells are long-lasting; they do not actively participate in the initial immune response to the pathogen. Instead, they play a critical role in secondary immune responses (when the individual is exposed to the same pathogen at a later point in time), when they can differentiate into plasma B cells. This is shown in Figure 1. Plasma B cells are the class of B cells that produce antibodies. Antibodies are molecules that bind to the pathogen of interest; they can neutralize a pathogen and mark it for destruction by phagocytic cells. Plasma B cells play a key role in the development of an individual's adaptive immune response against a pathogen.[3]

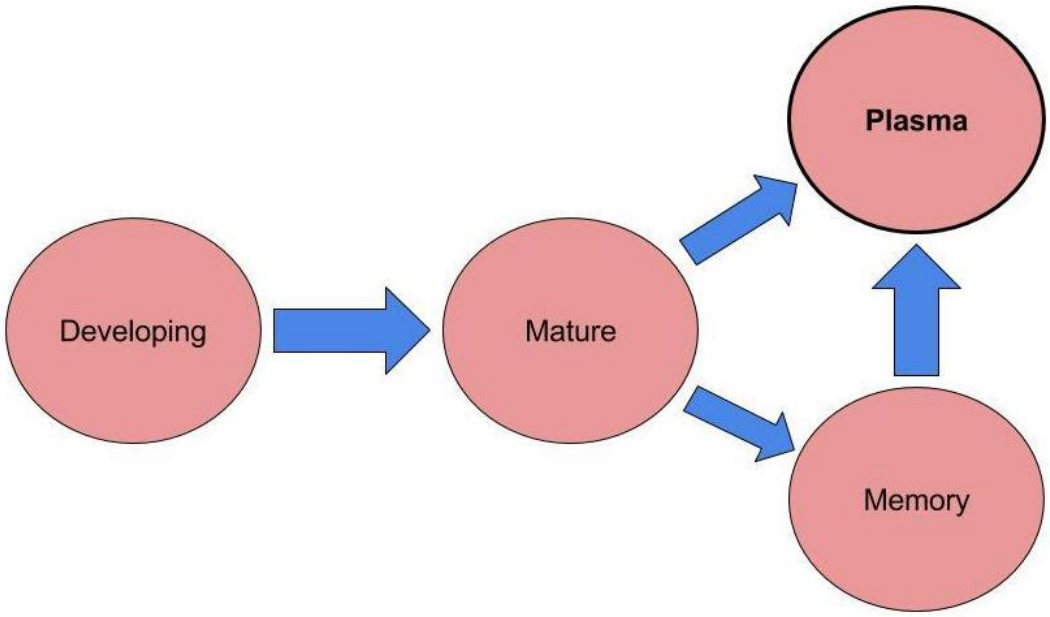


Figure 1. Summary of B cell development. This figure shows the various stages of B cell development and maturation. B cells start as developing B cells, then progress into mature B cells. Mature B cells then differentiate into either plasma B cells or memory B cells. Memory B cells can differentiate into plasma B cells.

When a B cell differentiates from a mature/memory B cell to a plasma B cell, the genes actively expressed by the cell change. One gene expressed by mature/memory B cells is called Pax5, which is a transcription factor expressed during development of B cells.[4] Once a mature/memory B cell undergoes differentiation into a plasma B cell, Pax5 is no longer expressed; however, BLIMP (B lymphocyte-induced maturation protein)[5] and Xbp1 (X-box binding protein 1),[4] both of which are transcription factors, are expressed by the B cell.[4,6] Expression of BLIMP inhibits expression of Pax5, and Pax5 is known to inhibit Xbp1 expression.[6] This provides a clear way to distinguish between populations of mature/memory B cells and plasma B cells, since plasma B cells cannot express Pax5, and mature/memory B cells cannot express either BLIMP or Xbp1. This is shown in Figure 2.

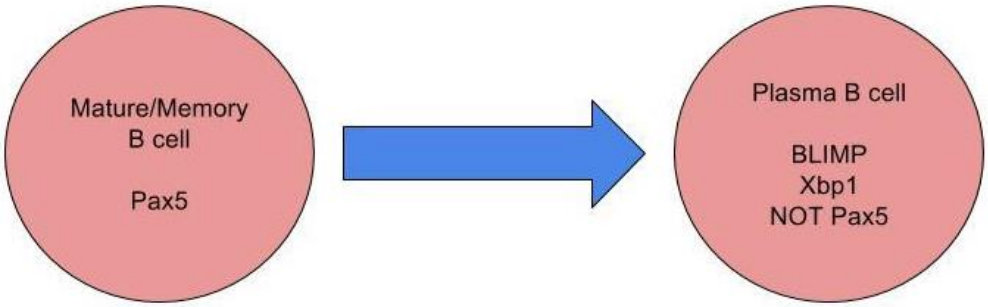


Figure 2. Summary of relevant transcription factors expressed by mature/memory B cells versus plasma B cells. This diagram indicates that mature/memory B cells express the transcription factor Pax5. This expression is stopped when a mature/memory B cell differentiates into a plasma B cell. Plasma B cells express the transcription factors BLIMP and Xbp1, but do not express Pax5.

The zebrafish used in this experiment were double transgenic.[4] This means that two extra genes are present in specific locations of the genome of the fish. Two different lines of double transgenic fish were used. One line of fish was IgM:mCherry; BLIMP:GFP; cells expressing both fluorophores (mCherry and GFP) in this line should be IgM+BLIMP+. The second line of fish was IgM2:mCherry; IgM1:GFP; the populations of cells expressing both fluorophores (mCherry and GFP) in this line should be composed of mature B cells, plasma B cells, and memory B cells. Since the two fluorophores of each line (mCherry and GFP) are linked to expression of the gene specified, when the gene is expressed in a cell, the gene for fluorescence would also be expressed, and the cell would express this fluorescence. Thus, when each fluorophore is observed in a cell, that is a visual indication that the linked gene is being expressed. This provided a clear way to identify the cells of interest in the zebrafish cell populations that were studied, since only the fluorescent populations would contain cells that were known to express certain genes of interest. In the case of these transgenic lines, only B cells and T cells would be fluorescent; IgM is only expressed in B cells, and BLIMP is expressed only in plasma B cells and some T cells.[5]

To isolate these fluorescent cell populations, flow cytometry was used. This allowed four different populations of cells to be isolated: cells that expressed neither fluorophore, cells that expressed GFP only, cells that expressed mCherry only, and cells that expressed both GFP and mCherry. Cells expressing neither fluorophore or GFP only are not necessarily B cells, since these populations are not linked to IgM expression (a gene only expressed in B cells). This does mean, however, that all B cells that are isolated are IgM+ B cells only, since mCherry expression is linked to expression of IgM in these transgenic zebrafish. The populations of cells expressing both fluorophores should express both IgM and BLIMP (in the IgM:mCherry; BLIMP:GFP line) or IgM (in the IgM2:mCherry; IgM1:GFP line). However, this does not mean that the genetic profile of this population can be deduced from this information. It is not known if the populations of cells expressing both fluorophores in either transgenic line are pure populations of plasma B cells.

This experiment looked at the genetic profile of cells expressing both fluorophores to determine if these double-fluorescent populations were a pure population of plasma B cells or a mixed population of plasma B cells and mature/memory B cells. By identifying a population of pure plasma B cells it would be possible to understand the expansion and retraction of the plasma B cell population during an immune response. This experiment also analyzed the genetic profile of other cell populations isolated according to their fluorescent expression (GFP+ only, mCherry+

only, and cells expressing neither fluorophore) to ensure that their genetic profile matched what was expected. Thus, this experiment had two questions:

1) Do the cell populations of double transgenic zebrafish isolated by flow cytometry express the genes they are expected to?
2) Are cell populations that express both fluorophores composed of a pure population of plasma B cells, or are they composed of a mixed population of both plasma B cells and mature/memory B cells?

To answer these questions, quantitative polymerase chain reaction (qPCR) was used to determine if specific genes of interest were expressed in these various populations, and if so, what their relative level of expression was (when compared to the expression of a baseline gene of interest).

If cells expressing both fluorophores are only plasma B cells, they should express BLIMP and Xbp1. They should not express Pax5. A population composed of only mature/memory B cells should express Pax5, but should not express BLIMP and/or Xbp1. Cell populations in which both Pax5 and Blimp/Xbp1 are expressed should therefore be composed of both plasma B cells and mature/memory B cells. We did not distinguish between mature B cells and memory B cells in these experiments.

The following genes were used to analyze the genetic profile of these cell populations. IgM expression was checked to ensure that these B cells were in fact expressing IgM, as their fluorescence indicated. BLIMP was analyzed to see if plasma B cells were present. Likewise, Xbp1 was used to confirm the presence of plasma B cells and to confirm that it was coexpressed with BLIMP, as expected. Pax5 was studied to see where developing, mature, and memory B cells were located. TCRα (T cell receptor alpha) was used as a negative control; since it is expressed by T cells and not by B cells, it should not be expressed in populations expected to be composed only of B cells. EF1α (Elongation Factor 1 alpha) was the baseline gene to which gene expression was compared; this was critical in calculating the relative expression of each gene. EF1α is constitutively expressed in healthy cells; this made it a good baseline gene to use, since all living cells should express it.

Materials and Methods

Cells and Zebrafish

Cell samples were obtained from double transgenic zebrafish, which were created by the Page Lab at Point Loma Nazarene University.[4] Fish were raised at the University of California San Diego zebrafish facility, and were temporarily kept at Point Loma Nazarene University. Zebrafish were euthanized by being placed on ice, and their organs were then harvested. Kidney samples were placed in FACS (Fluorescence Activated Cell Sorting) buffer (PBS, 1% Fetal Calf Serum). Single-celled suspensions were then generated as previously described[4] and run through an Aria II flow cytometer (Becton Dickinson) to obtain populations of the specific cell types. RNA was isolated from the samples using a Qiagen RNeasy Mini Kit, and cDNA was synthesized from these RNA extractions using a SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) with oligo-dT primers. The RNA and cDNA samples were stored at -80°C to prevent sample degradation.

qPCR

Quantitative polymerase chain reaction (qPCR) was performed on the cDNA samples. qPCR is a technique that amplifies a specific gene of interest by making many copies of it. This determines whether or not a gene of interest is being expressed in a sample, and if so, how much of that gene's transcript is present; in other words, it can be used to determine how much

that gene is being used in that cell. To analyze the level of expression of a gene of interest, relative expression was used. This gives the level of expression of a gene of interest relative to the expression of another gene. The level of expression of this second gene is used as a baseline. The gene that was used for this purpose was Elongation Factor 1 alpha (EF1α). This gene is known as a "housekeeping gene" since it is required for cell growth and maintenance. EF1α needs to be present in living cells. Its expression levels also are known to remain fairly stable in varying conditions, so this made it a good gene to which expression of the selected genes of interest could be compared against.

The equation used to calculate relative expression is as follows:

$$\Delta C(t) = 2^{-(C(t) \text{ value of gene of interest} - C(t) \text{ value of baseline gene})}$$

The following primer sequences were used when performing qPCR. All primers are written from the 5' end to the 3' end.

EF1α-For	GAG AAG TTC GAG AAG GAA GC
EF1α-Rev	CGT AGT ATT TGC TGG TCT CG
IgM-For	AGC TTC TCT AGC TCC ACC AG
IgM-Rev	ATT TTG GTG AAA TGG AAT TG
BLIMP-For	GGC GTC TTT ACC TAG AAA CC
BLIMP-Rev	ATC TTT GGG GAC ATT TTC TG
Pax5-For	CTG ATT ACA AAC GCC AAA AC
Pax5-Rev	CTA AAT TAT GCG CAG AAA CG
Xbp1-For	TCA GAT TCA GAC TCC ACC AC
Xbp1-Rev	TGT CTC TTG CTG TCT GTG C
TCRα-For	TCG TTT TCA ATG TGC TGG TG
TCRα-Rev	GAT GAT CTG GAA TGG GAT GC

All qPCRs were performed on a Chromo4 Real-Time PCR Detector (Bio-Rad). Each qPCR was prepared using 2X Brilliant II SYBR® Green QPCR Master Mix (Agilent). The program used to perform all qPCRs is as follows:

1. Incubate at 95.0°C for 00:10:00
2. Incubate at 95.0°C for 00:00:10
3. Incubate at 55.0°C for 00:00:45
4. Incubate at 72.0°C for 00:00:10
5. Plate read
6. Goto line 2 for 44 more times
7. Incubate at 94.0°C for 00:01:00
8. Melting Curve from 55.0°C to 95.0°C, read every 1.0°C, hold 00:00:30
9. Incubate at 20.0°C forever
   END

Opticon Monitor 3 software (Bio-Rad) was used to analyze the melting curves and quantitation graphs obtained from each qPCR.

Results

Cells from zebrafish kidneys of two lines of transgenic fish were studied at various time points from three separate years (see Table 1). These time points were chosen because it was thought there would be a good population of plasma B cells from fish at these points in the immune response.

Transgenic Line	Year	Sort Date	Immune response studied	
IgM:mCherry; BLIMP:GFP	2013	July 12	day 28 post-boost	secondary
		July 19	day 35 post-boost	secondary
	2014	July 15	day 21 post-boost	secondary
		July 22	day 28 post-boost	secondary
		July 24	day 30 post-boost	secondary
IgM2:mCherry; IgM1:GFP	2016	June 10	day 35 post-vaccination	primary
		July 15	day 28 post-boost	secondary

Table 1. Vaccination and sort information for zebrafish kidney cells collected in 2013, 2014, and 2016. This table states the date on which each sample was collected, and which immune response each sample studied (primary response or secondary response).

IgM:mCherry; BLIMP:GFP fish

2013 fish

The first transgenic line of fish that was studied was IgM:mCherry; BLIMP:GFP. The earliest cells of this line that were studied were from 2013. There were two sorts from zebrafish kidneys collected on July 12 and July 19 (see Table 1). Each date refers to a different group of fish that were euthanized and dissected. The four cell populations that were analyzed were: mCherry-GFP- (double negative, abbreviated as DN), mCherry-GFP+ (abbreviated as GFP+), mCherry+GFP- (abbreviated as mCherry+), and mCherry+GFP+ (double positive, abbreviated as DP). The cells that were expected to be present in each population were as follows: 1) any cells that were not B cells (potentially T cells) in the DN population, 2) T cells in the GFP+ population, 3) developing, 4) mature, and 5) memory B cells in the mCherry+ population, and 6) plasma B cells in the DP population. Previous data has indicated that cells which express mCherry do express IgM. Therefore the B cells present in the mCherry+ and DP populations should only be IgM+ B cells.[4] Cells from the DP population of this transgenic line of fish were expected to be a pure population of plasma B cells because these cells express BLIMP (see Figure 2).

A control was run using primers for the T cell receptor alpha chain (TCRα) to ensure that T cells were not present in the mCherry+ and DP populations. Figure 3a shows that TCRα was only expressed in the GFP+ and DN populations, which are expected populations for T cells to occur. TCRα is not expressed in the DP or the mCherry+ populations, further establishing the validity of the experimental setup and results.

All figures contain a kidney control. This control was kidney cells from unimmunized wild-type zebrafish in order to obtain a baseline value of expression in the kidney to which the expression of the genes of interest in these various cell populations could be compared against. This would

allow us to determine the level of expression change before and after an immune response. Samples for which “n.d.” is written indicate that the gene of interest was not tested on that cell population, since samples were limited and it was already known that the gene of interest would not normally be expressed in that cell population.[4]

After it was verified that T cells were not present where they were not supposed to be, qPCR was performed to see in which populations Blimp and Pax5 were expressed. As shown in Figure 3b, BLIMP was expressed in the DP and GFP+ populations, which is to be expected of this cell line (BLIMP expression is linked to GFP fluorescence). This means that plasma B cells are present in the DP populations. BLIMP expression for the GFP+ cells from July 12 was not high enough to be significant; it is assumed that this result is due to the small number of cells that were in the population. Since the cells from these transgenic zebrafish link BLIMP expression with GFP fluorescence, it is highly likely that BLIMP is expressed in this population as expected.

Pax5 expression was also examined. Figure 3c shows that Pax5 was expressed in low levels in the DN population, and that it was also expressed in the mCherry+ and DP populations. It was expected that Pax5 would be expressed in low levels in the DN population because B cells start to express Pax5 before they start to express IgM. A t-test was run to determine if the difference in level of expression of Pax5 between the mCherry+ samples and the DP samples was significant, and a p-value of 0.035 was obtained. This means that the level of Pax5 expressed in the mCherry+ samples was significantly higher than the level of Pax5 expressed in the DP samples. A t-test was also performed to determine if the level of Pax5 expression in the DP samples was significantly different from the level of Pax5 expression in the DN cells. A p-value of 0.00182 was obtained; indicating that Pax5 expression in the DP cells was significantly higher than the level of Pax5 expression in the DN cells.

These results indicate that all of the DP populations contain B cells that express BLIMP, and also contain B cells that express Pax5. Since BLIMP and Pax5 cannot be coexpressed in the same B cell, this is evidence that the DP population is a mixed population containing both mature/memory and plasma B cells.

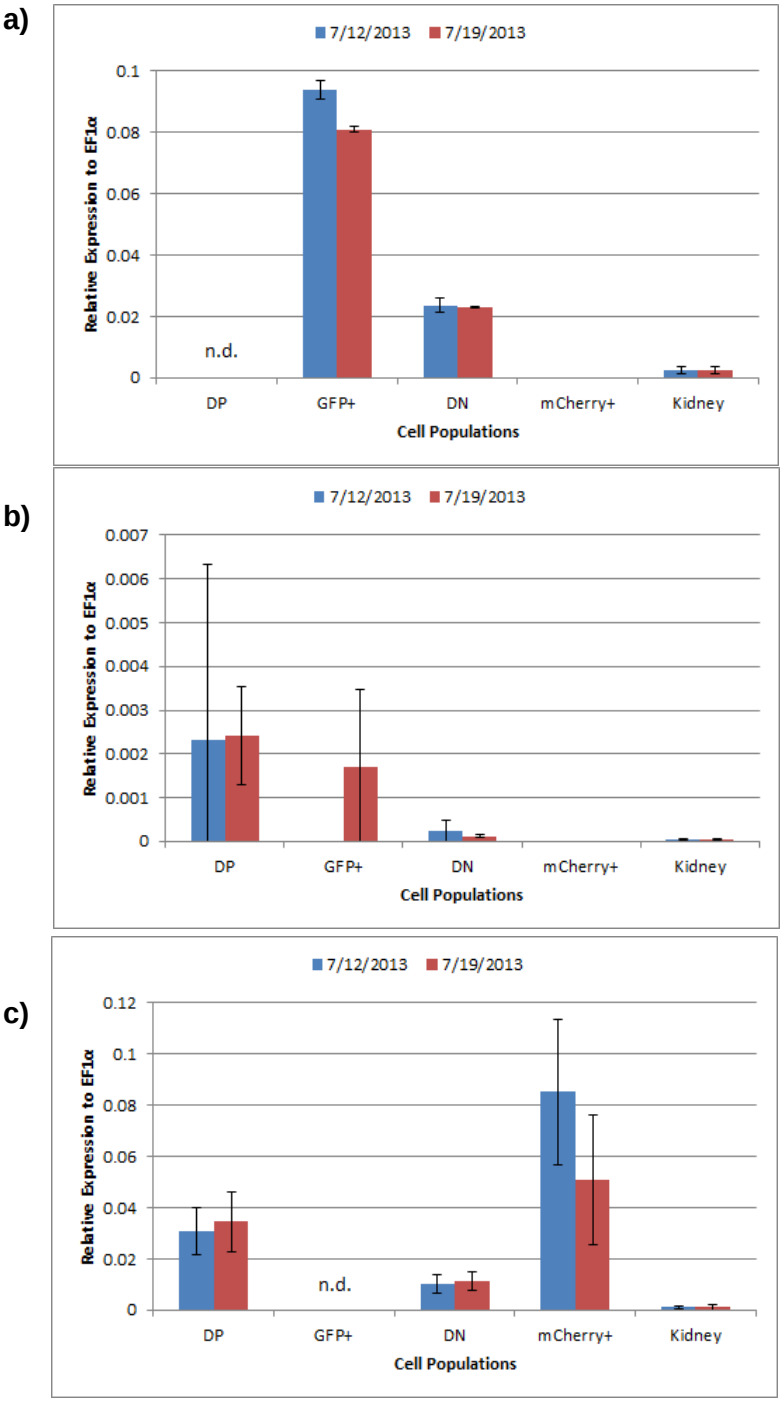


Figure 3. Expression of TCRα, BLIMP, and Pax5 relative to EF1α in zebrafish kidney cells from 2013. qPCR was performed on zebrafish kidney cells from 2013 to analyze the relative expression of various genes of interest. The baseline gene against which relative expression was calculated was EF1α.The abbreviation "n.d." refers to no data (no data was collected for this sample). **a)** Expression of TCRα was present in the DN and GFP+ populations. **b)** BLIMP was found to be expressed in both the GFP+ and DP populations. **c)** Pax5 was expressed in the DN, mCherry+, and DP populations.

2014 fish

The next-oldest sort that was studied was from 2014. These fish were also from the IgM:mCherry; BLIMP:GFP transgenic line. Cells were collected on July 15, July 22, and July 24 (see Table 1). A control was again run using the gene TCRα to ensure that T cells were not present in the populations that were assumed to contain only B cells. The cell populations obtained from July 22, 2014 did not sort for the DN or GFP+ cells. When qPCR was performed on this set of cells, DN cells from July 15, 2014 were used as a negative control. The "missing" populations of the DN and GFP+ cells from July 22 are indicated as "n.d." in the following figures to show that these data points are not zeros, but rather data that was never collected.

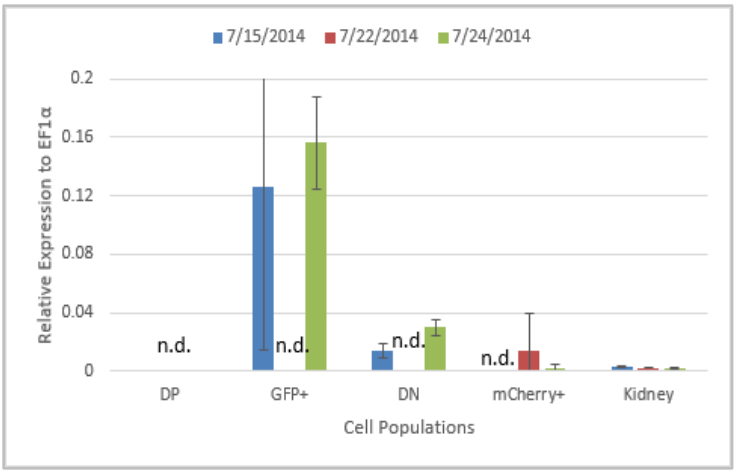
Figure 4a shows that TCRα was mainly expressed in the DN and GFP+ populations, as expected. The expression of TCRα in the mCherry+ population from July 22 has a very large error bar. Error bars are presented as the standard deviation of the triplicate of the sample, and the standard deviation for TCRα expression in the mCherry+ population was very large. This is because only one out of the three wells tested produced any product, resulting in a high standard deviation. This perceived expression of TCRα is likely due to the presence of less than 5% DN cells in the mCherry+ population, which occurred during cell sorting on the flow cytometer. This data likely is not valid due to the lack of consistency in expression.

After it was verified that the cell populations were mostly pure, the presence of BLIMP in these populations was examined. Figure 4b shows that BLIMP was expressed in the GFP+ populations from July 15 and July 24, and was also expressed in the DP populations from July 22 and July 24. The expression of BLIMP in the DP populations indicates there are plasma B cells present in these populations of DP cells. The presence of BLIMP in the mCherry+ cells was not tested, since previous testing verified that mCherry+ only cells did not express BLIMP (unpublished data of Page lab). This is shown by the abbreviation "n.d." on the graph, indicating that the cell population was not tested for expression of the gene of interest.

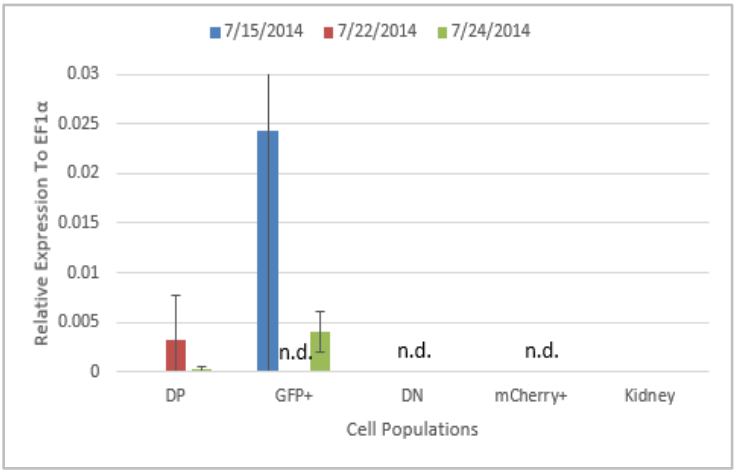
Pax5 expression was also examined in this sort. Figure 4c shows that Pax5 was expressed in both the mCherry+ and DP populations. The DP cells from July 15 did not express BLIMP or Pax5; however, these cell populations were so small that results are inconclusive. The presence of BLIMP and Pax5 in the cell populations from July 22 and July 24 strongly suggests that both BLIMP and Pax5 are expressed by cells in the DP population. Pax5 expression in the mCherry+ population is expected, but was not expected to be present in the DP population if the DP population is composed of only plasma B cells.

Since Pax5 and BLIMP are both expressed in the DP population, this is further evidence that the DP population of IgM+BLIMP+ cells are a mixed population. The results obtained from the 2013 sort are no longer an isolated set of data; since the expression of both BLIMP and Pax5 in the DP populations has been quantified in sorts from 2013 and 2014, the evidence supporting the hypothesis that the DP population of B cells in this transgenic line of zebrafish is a mixed population of mature and/or memory B cells and plasma B cells is stronger.

a)



b)



c)

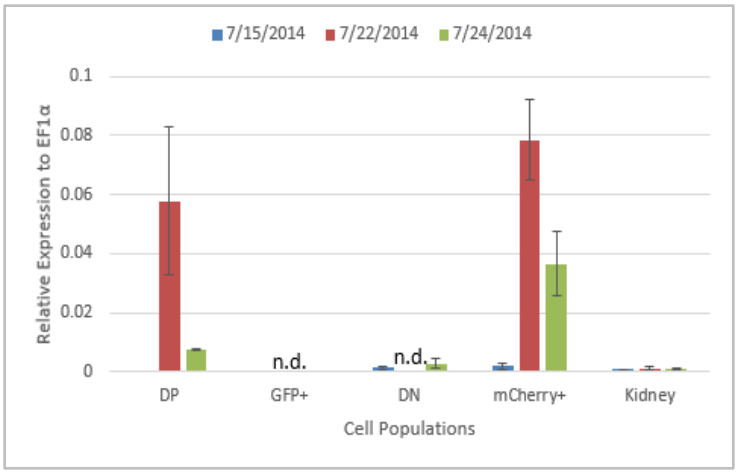


Figure 4. Expression of TCRα, BLIMP, and Pax5 relative to EF1α in zebrafish kidney cells from 2014. qPCR was used to analyze expression of specific genes relative to EF1α in zebrafish kidney cells. **a)**TCRα expression was present in the DN and GFP+ populations. **b)** BLIMP was expressed in the GFP+ and DP populations. **c)** Pax5 expression was observed in the mCherry+ and DP populations.

IgM2:mCherry; IgM1:GFP fish
2016 fish

The most recent sort of cells that were studied was from 2016. Rather than being IgM:mCherry; BLIMP:GFP, these fish were from the IgM2:mCherry; IgM1:GFP transgenic line. Samples were collected on June 10 and July 15 (see Table 1). Their DP population of cells was known to express IgM (since they express mCherry and GFP). However, expression of IgM alone does not determine what types of B cells are present in this DP population, since BLIMP is not one of the markers that is used to indicate fluorescence (unlike the IgM:mCherry; BLIMP:GFP line of fish, where DP cells must express BLIMP). B cells in this DP population could be mature B cells, memory B cells, or plasma B cells.

The 2016 sorts contained a unique population of cells. This subset of cells expressed both mCherry and GFP; however, they expressed both of these fluorophores at such high levels so as to be considered their own population, distinct from other cells expressing both fluorophores. This is shown in Figure 5.

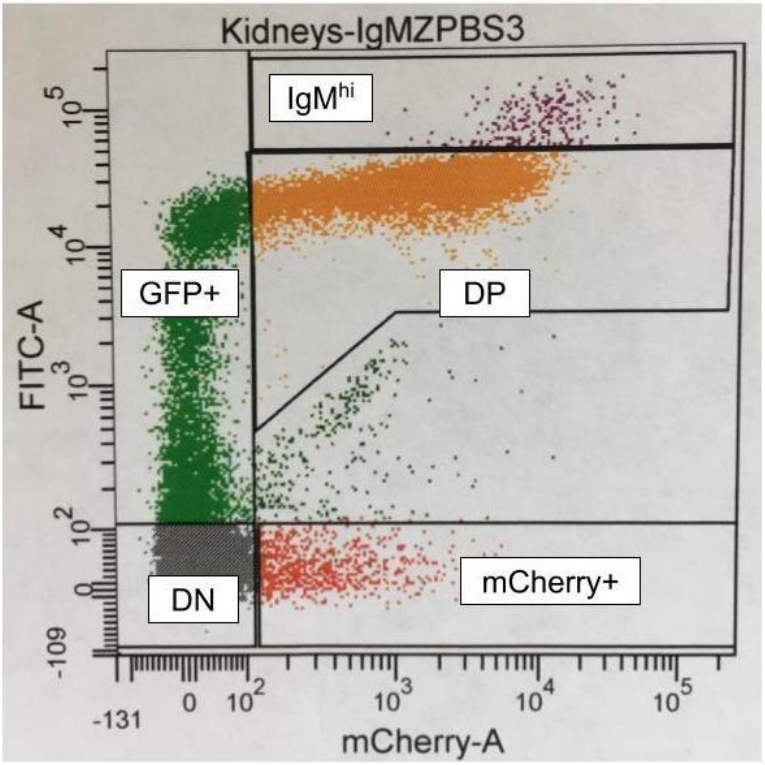


Figure 5. FACS plot depicting the location of IgM^{hi} cells in IgM2:mCherry; IgM1:GFP zebrafish. IgM^{hi} cells expressed high levels of both GFP and mCherry fluorescence.

To ensure that these cells were actually IgM+ B cells, IgM expression was studied. As seen in Figure 6a, this population of cells expresses IgM at extremely high levels. IgM expression in this population of cells, dubbed IgM^{hi} cells, dwarfs the IgM expression of the “regular” double-fluorescent cells. Unfortunately, it was found that the IgM^{hi} cells from July 15, 2016 did not provide valid data for any of the genes of interest (indicated by “n.d.”). These samples had C(t) values that were too high to give conclusive data; however, data from the DP populations of July 15 had C(t) values that were within a range that could be analyzed, and thus could be considered significant.

After IgM expression was confirmed, BLIMP expression was studied in this population. Figure 6b shows that BLIMP is expressed in both the DP cells as well as the IgM^{hi} cells. There are large error bars for these expression levels, so definite conclusions about the populations of B cells present cannot be drawn based solely on levels of BLIMP expression.

Xbp1 expression was also examined. This transcription factor is expressed with BLIMP, and is another indication of whether plasma B cells are present (Figure 2). Figure 6c shows that Xbp1 is expressed in the DP and IgM^{hi} populations. This is a clear indicator that plasma B cells are present in these two populations; this also supports the presence of BLIMP expression (since BLIMP and Xbp1 are coexpressed in individual cells).

Pax5 expression was also examined in these cell populations. Pax5 is not expressed in cells expressing BLIMP and Xbp1, so presence of Pax5 would indicate that plasma B cells are not the only B cells in these populations. Figure 6d shows that Pax5 is present in the double-fluorescent populations of cells and in the IgM^{hi} population from June 10. Pax5 expression in the IgM^{hi} cells is presented with a large error bar; this is because only two out of the three wells tested produced product, and one of the wells had such a small amount of product that its expression was inconclusive. More experiments would need to be done to confirm Pax5 expression in the IgM^{hi} cells. However, Pax5 is clearly expressed in the DP cell populations.

The definitive expression of both Xbp1 and Pax5 indicate that both plasma B cells and mature/memory B cells are present in the DP cell populations. It is likely that both plasma B cells and mature/memory B cells are present in the IgM^{hi} cells as well, but further experiments will need to be done to confirm these results. This is similar to the conclusion drawn from the data collected from the 2013 sorts, which is corroborated by the data presented from the 2014 sorts. Cell populations expressing both GFP and mCherry in either the IgM:mCherry; BLIMP:GFP or the IgM2:mCherry; IgM1:GFP double transgenic lines of zebrafish appear to be composed of both mature/memory B cells and plasma B cells, as evidenced by the expression of both BLIMP and Pax5 in these populations.

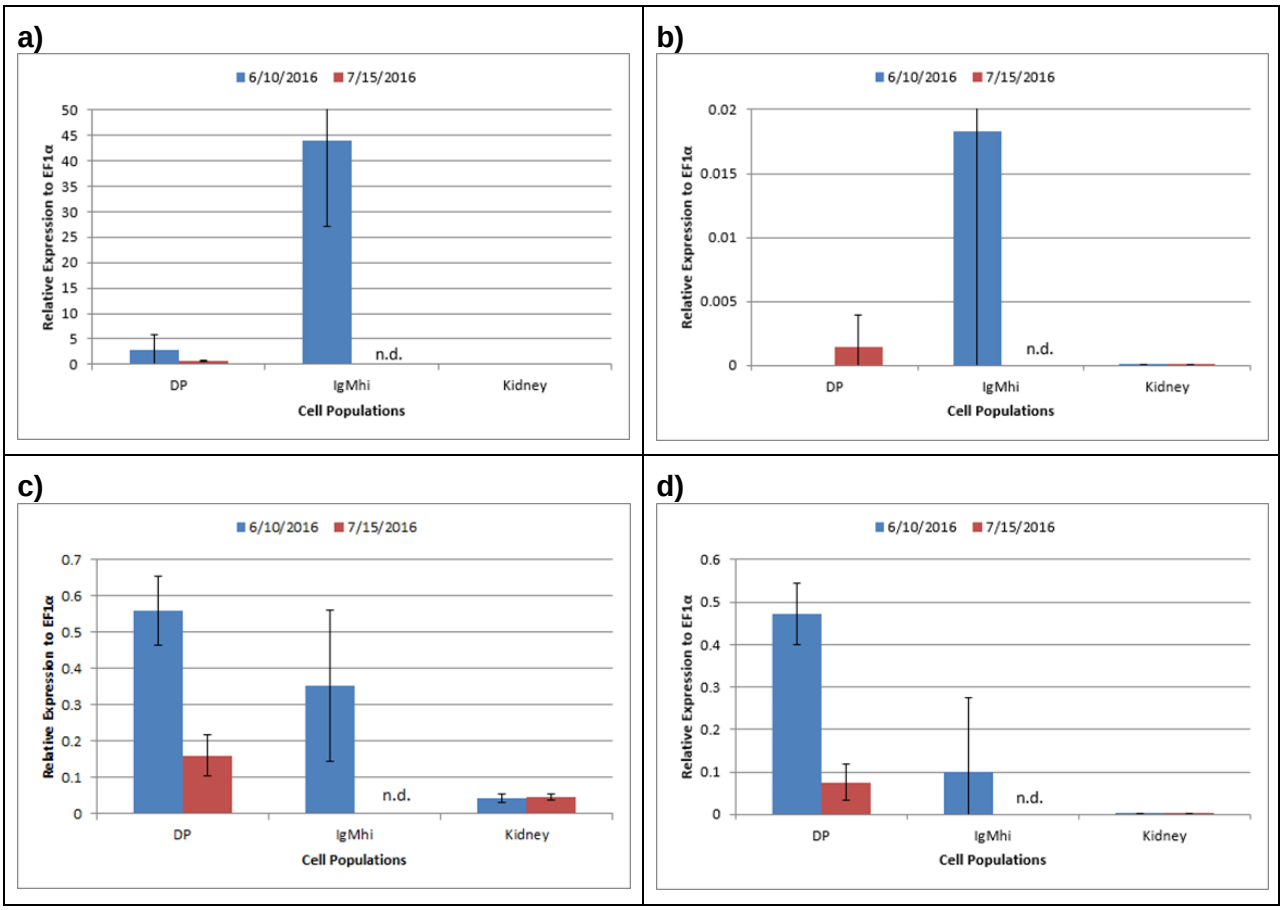


Figure 6. Expression of IgM, BLIMP, Xbp1, and Pax5 relative to EF1α in zebrafish kidney cells from 2016. qPCR was used to analyze expression of specific genes of interest relative to the expression of EF1α in zebrafish kidney cells. **a)** IgM expression was greatly elevated in the IgM^{hi} cell population when compared to the DP population. **b)** The expression of BLIMP was observed in the DP population from July 15 and in the IgM^{hi} population from June 10. **c)** Xbp1 expression was present in both DP populations and the IgM^{hi} population from June 10. **d)** Pax5 was expressed in the DP populations and in the IgM^{hi} population from June 10.

Discussion

In this study, the genetic profile of B cells of double-transgenic fluorescent zebrafish was analyzed in order to determine if cells expressed the genes that were expected according to their fluorescence, and whether populations of cells expressing both fluorophores were a pure population of plasma B cells.

Analysis of all fluorescence combinations (DN, GFP+, mCherry+, and DP) of cells of fish from the IgM:mCherry; BLIMP:GFP line indicated that these populations do express the expected genes. mCherry+ cells express Pax5, indicating that they are B cells, and GFP+ cells express BLIMP. mCherry+GFP+ cells express BLIMP (IgM expression was confirmed by previous studies).[4] Samples from IgM2:mCherry; IgM1:GFP fish also indicated that the DP populations do express the expected genes, namely IgM. Samples of other populations from this cell line were not available for analysis at this time, but previous experiments already determined that the DN, GFP+, and mCherry+ populations from IgM2:mCherry; IgM1:GFP fish expressed the genes that are predicted according to their fluorescence (unpublished data of Page lab).

In both IgM:mCherry; BLIMP:GFP and IgM2:mCherry; IgM1:GFP double transgenic fish, populations expressing both fluorophores expressed the transcription factors BLIMP and Xbp1 (expressed only in plasma B cells) as well as the transcription factor Pax5 (expressed in mature and memory B cells and not in plasma B cells). These results were unexpected; it was expected that only plasma B cells would be present in the DP populations of zebrafish kidneys since the DP cells express BLIMP. It is known that BLIMP and Pax5 cannot be actively coexpressed in the same B cell.[4] However, differences in the time required to turn expression of a gene on or off could allow for this perceived transient expression of both transcription factors in the same population. Mature B cells that are in the process of differentiating into plasma B cells may begin to express BLIMP before expression of Pax5 is completely turned off. This extended time required for Pax5 expression to stop might be the mechanism by which a B cell can appear to express both BLIMP and Pax5 at the same time. To confirm that the DP cells are actually co-expressing BLIMP/Xbp1 and Pax5, in situ hybridization could be used to determine if both BLIMP/Xbp1 and Pax5 are actively expressed. An alternative explanation for this phenomenon would be that the DP cells were contaminated with mCherry+GFP- cells during cell sorting. Since the isolated DP cell populations were so small (between 500 and 1000 cells on average), performing purity checks was not feasible. Purity checks were performed on all other cell populations and those cell populations were found to be more than 98% pure. It is highly likely that the DP cell populations that were analyzed are just as pure, although it is not possible to confirm this. A third potential explanation is that zebrafish are able to coexpress BLIMP/Xbp1 and Pax5 in the same cell, unlike mammals. There is currently no way to test this explanation.

Zebrafish have been highly utilized as an animal model for immunology research. However, knowledge of the development and maturation of B cells in zebrafish has been lacking. By demonstrating that both mature/memory and plasma B cells may be present in the kidney in these double-fluorescent transgenic lines, this knowledge can be used to improve analysis of flow cytometry data (providing a clearer understanding of which cells are present in the double-positive populations, and how that could affect the strength of the perceived immune response). Increasing this range of knowledge of the B cell populations in zebrafish increases their viability as an animal model, and can allow for them to be used in more situations in which changes in the B cell response are important; by furthering baseline knowledge, this makes it easier to notice and track changes in the B cell populations of zebrafish when they are subjected to a stimulus that causes an immune response.

By demonstrating that both BLIMP and Pax5 (and Xbp1 in IgM2:mCherry; IgM1:GFP fish) are present in the double-fluorescent populations of each of these transgenic lines, these results indicate that the types of B cells present in the kidney are potentially independent of the transgenic line of fish (i.e. this may be an innate characteristic of the zebrafish immune system).

BLIMP, Xbp1, and Pax5 are expressed in both the primary and secondary immune response of IgM2:mCherry; IgM1:GFP fish (Figure 6). BLIMP and Pax5 are expressed in the secondary immune response of IgM:mCherry; BLIMP:GFP fish (Figure 3, Figure 4). Samples from the primary immune response of IgM:mCherry; BLIMP:GFP fish were not available for analysis; future experiments will analyze samples from the primary immune response of this transgenic line to determine if both BLIMP and Pax5 are expressed in the kidney during the primary immune response. Future experiments will also analyze samples from IgM:mCherry; BLIMP:GFP fish from 2016 in order to further confirm the results presented here. Further work will also look deeper at samples from the primary immune response in zebrafish to determine if there is any significant temporal difference in BLIMP and Pax5 expression when compared to the secondary immune response. Plasma B cells are created by differentiation of mature or

memory B cells after their activation by a T cell. The zebrafish kidney contains B cells that are ready to interact with pathogen (mature and memory B cells) as well as B cells that have been activated, undergone differentiation, and are producing antibodies against their specific pathogen (plasma B cells). The presence of cells which express transcription factors associated with each of these categories of B cells in both the primary and secondary immune response suggests that the immune system of the zebrafish is continually producing new B cells in order to create as many B cells that could recognize the pathogen as possible.

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