GENOME WIDE MAPPING OF MURINE GENES CONTROLLING DIFFERENTIAL EXPRESSION OF CD5L AND CCR2

A Major Qualifying Project Report

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ABSTRACT

Listeria monocytogenes is gram positive bacterium which can cause overt infection in humans through consumption of contaminated food and beverages. Two host genes CD5l and CCR2 play a major role in the innate immune response to such infection. CD5l is a negative regulator of apoptosis in macrophages preceding infection to *L. monocytogenes*. CCR2 is a chemokine receptor which when triggered during infection plays a role in the initiation of monocyte recruitment from bone marrow in mammals. Both CCR2 and CD5l are differentially expressed in mouse strains that differ in their susceptibility to *L. monocytogenes* infection. Therefore differential expression of these genes could be contributing to susceptibility to infection. The purpose of this project is to determine what regulates these genes at a molecular level through mouse models. Possible transcription factors, regulatory elements, or mutations could all be the cause of differential expression of these genes. A genome wide mapping was performed using 42 N2 backcross mice. Linkage maps were created for all 19 chromosomes consisting of 4-6 markers for each spaced 20-30 Mb. A linkage between CD5l and CCR2 expression levels to markers on each chromosome was tested using genetic mapping software (Map Manager). High linkage scores for either of these traits CD5l or CCR2 to any of the 19 chromosomes could point to an area of the genome that contains mutations that control their expression.

TABLE OF CONTENTS

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BACKGROUND

Listeria Monocytogenes

      Listeria monocytogenes is a gram-positive bacterium **(Figure-1)** that can cause serious infection in humans through ingestion of contaminated food. The scientific name for such an infection is *Listeriosis*. This bacterium is rod shaped and mainly affects women that are pregnant, newborn children, and adults that suffer from weakened immune systems. In the United States alone *Listeriosis*, in its overt (serious) form, has approximately a 25% death rate. *Listeriosis* can also manifest in its host as either meningitis which can include encephalitis, or it can produce sepsis (Todar, 2008).

Figure 1: *Listeria Monocytogenes* Under Transmission Electron Microscopy. *L. monocytogenes* is a gram-positive rod shaped bacterium that can cause infection in humans through ingestion of contaminated food. It can also manifest into other clinical infections which can be life threatening (Todar, 2008).

      Humans are infected with *Listeria monocytogenes* mainly by ingesting raw, contaminated food, even if it was kept at low temperatures. Unlike most bacteria, *Listeria* has a strong affinity to multiply at temperatures near 4° C (**Figure 2**), and

therefore even properly stored food products can accumulate significant numbers of
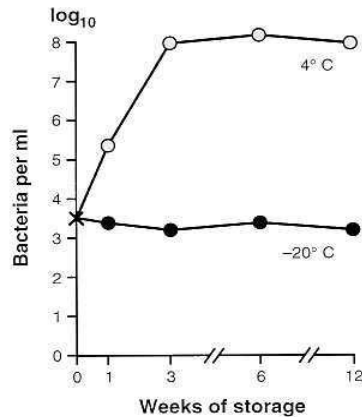
bacteria and cause infection (Todar, 2008).

Figure 2: The Reproduction Rate of *L. monocytogenes* at 4°C and -20°C after 12 Weeks of Storage in Broth. *L. monocytogenes* reproduces at a steadily fast pace for the first 3 weeks at 4°C (open circles) and continues to thrive for the next 9 weeks of testing, but does not replicate at -20°C (closed circles)   (Hof, Miscellaneous Pathogenic Bacteria).

The host immune system usually can attack and rid the body of this infection,

mainly because adults that have previously been infected by *Listeria* have T lymphocytes

specifically primed by *L. monocytogene* antigens. In the intestinal tract, *Listeria* will

hold on to D-galactose receptors found on the host cell through its own D-galactose

residues. This bacterium is also known to infect macrophages during infection. *Listeria*

will initially infect macrophages and then will move into the liver and infect the

hepatocytes (Todar, 2008).

The total number of listeriosis occurrences in the human population is unknown

due to the fact that most of these infections are asymptomatic. If symptoms are seen in

the infected patient, they usually can range from influenza-like indications, to meningitis

or even meningoencephalitis. Listeriosis in pregnant women can spread to and infect the

fetus which it does by entering the endothelial layer of the placenta.   This situation is known to cause women to abort the child, or it leads to the possibility of giving birth to an extremely ill newborn (Rebagliati, 2009).

Outbreaks of overt listeriosis from infection of *L. monoyctogenes* rarely occur, yet can be deadly. Such an outbreak occurred in Canada in 1981 which was eventually linked to the consumption of coleslaw. This outbreak took a toll on 34 pregnant women and their newborns as well as 7 adults. Of these seven adults, 6 displayed symptoms of meningitis and one showed symptoms of pneumonia. In the population of pregnant women, 5 had miscarriages while four fetuses died and 23 of the women showed premature birth of their child. The mortality rate seen in these children was approximately 27% (Rebagliati, 2009).

An outbreak also occurred in 1985 in California, where a total of 142 people developed overt (serious) cases of listeriosis from consuming pasteurized soft cheese that had been contaminated by *L. monocytogenes* during the manufacturing process by milk that had not yet been pasteurized (raw). 93 of the 142 infected were pregnant women in the perinatal stages of pregnancy, causing 30 of these fetuses to die, and 18 adults to also pass away. The other infected men and women suffered from flu like symptoms, and had no further complications after the infection had been treated (Todar, 2008).

It was not until the early 1960's that *Listeria monocytogenes* was found not only to infect animals but it was also abundant in humans. This infectious bacterium was also found in numerous other sources including 42 species of mammals, and approximately 17 bird species, both wild and domestic species. *Listeria monocytogenes* is also known to harbor itself within the intestinal tract of about 5-10% of humans and can also be found in

fish, ticks, flies, and crustaceans. Most of the humans that are host to this bacterium in the intestinal tract show no tell tale signs of infection (Todar, 2008).

L. monocytogenes has the ability to reproduce outside the cell as well as inside. Macrophages have mannose binding receptors which are sugar monomers of the aldohexose carbohydrates. This receptor's primary function is to bind bacteria by binding polysaccharides found on the surface of the bacterium and causing the polysaccharide wall to diminish. The bacterium (*L. monocytogenes*) is then taken in by the cell through induced phagocytosis and has the ability to survive in the phagolysosome through the capability of the bacteria to produce catalase and dismutase. Phagocytic oxidative burst will then be essentially subdued by this production of catalase and dismutase. *L. monocytogenes* can also destroy the phagolysosome and escape in the cell's cytoplasm. This escape is mediated by a toxin known as listeriolysin O or LLO. This toxin mimics the function of a hemolysin. The same common promoter regulates not only the hemolysin gene but other virulent genes which all can be found on the same chromosome. This promoter and the genetic material it regulates play a role in continuing the process which allows *L. monocytogenes* to survive within another cell (Todar, 2008).

CCR2

One of the traits used for the genome wide mapping of this project was expression of the chemokine receptor CCR2. This gene initiates monocyte recruitment through interaction with the Monocyte Chemoattractant Protein-1 (MCP-1) (which is the main ligand for CCR2). This initiation occurs during inflammatory diseases, and in response

to tumors and other infections such as *L. monocytogenes*. A chemokine receptor is a specific type of receptor located on the surface of specific cells. Each of these receptors performs signal transduction using a structure known as a G-protein. These receptors signal the cell to cause an influx of calcium ions, and this in turn causes the cell to perform chemotaxis where the cell will be attracted to an area that it has been triggered to proceed to. This can be seen when infection or inflammation occurs within the body of mammals, and cells containing CCR2 will be drawn from bone marrow where monocytes are produced. These monocytes will then adhere to inflamed or infected areas of the body (an innate immune response to the body's infection) (Gene Cards, 2009).

Monocytes are essentially complex leukocytes created in bone marrow and play a role in the expression of a number of chemokine receptors. These are then released into the circulation of mammals in response to infection and inflammatory diseases in the body. CCR2 can be found on a subset of these monocytes which are involved in immune response. It was found that mice lacking the gene CCR2 will have an increased susceptibility to certain pathogens, including *Listeria monocytogenes*. These mice lacking CCR2 are also protected from atherosclerosis, an inflammatory disease in the walls of the arteries, and encephalitis an acute inflammation of the brain tissue.

In mice containing CCR2 infected with *L. monocytogenes*, CCR2 mediates the production of dendritic cells (also known as TipDCs) through the recruitment of tumor necrosis factor (TNFα) and an inducible nitric oxide synthase (iNOS). These TipDCs infiltrate the spleen and help fight infection. The mice that lacked the CCR2 gene showed no recruitment of TipDCs which lead to bacterial growth that was uninhibited within the mouse. This result shows that signals transmitted by CCR2 were needed to

recruit TipDC precursors which originally came from the bone marrow and entered the circulation after infection (Pamer and Serbina, 2006).

CCR2 also contributes to monocyte homeostasis, meaning the stability of the internal environment. The specific monocytes that are recruited during infection to *L. monocytogenes* are known as Ly6C^{hi} monocytes. These monocytes eventually differentiate into the TipDCs that were talked about briefly in the previous paragraph. It was found that CCR2 mediated signals are necessary in defining the total amount of Ly6C^{hi} monocytes that circulate in the blood stream. It was found that CCR2 deficient mice had a diminished amount of Ly6C^{hi} monocytes in the bloodstream and an increased amount of CD11b^{+}LY6C^{hi} monocytes in the bone marrow. This result shows that Ly6C is a marker for circulating monocyte subsets in the bloodstream. This marker is specifically regulated by CCR2. It was also discovered that the CD11b^{+}LY6C^{hi} monocytes that were accumulating in the bone marrow of these CCR2 deficient mice that were not infected with *L. monocytogenes*, were able to differentiate themselves into TipDCs (Pamer and Serbina, 2006).

Monocyte recruitment during infection was studied by using CCR2 deficient and wild type mice infected with *L. monocytogenes*. The spleen, bone marrow, and blood were then tested for TNF producing cells and CD11b^{+}LY6C^{hi}. The results showed that in wild type mice, LY6C^{hi} monocytes were recruited out of the bone marrow by a principle ligand, which is not exclusive to CCR2, known as MCP-1. It was found that even in MCP-1 deficient mice, other related MCP chemokines will eventually pick up the slack and partially compensate for the loss of MCP-1. In the MCP-1 knockout mice, a large accumulation of TNF within the bone marrow was observed, similar to the bone marrow

tested for CCR2 deficient mice, demonstrating that these other MCP chemokines were playing a role in regulation. The amount of TNF-producing cells in the bone marrow of infected wild type mice exhibited only a slight increase (Pamer and Serbina, 2006).

From this information, MCP-1 was then tested at different times during infection of *L. monocytogenes*. This experiment showed that after 12-24 hours of infection, MCP-1 showed a significant increase in the serum and spleen. It was still questionable whether LY6C^{hi} monocytes were recruited from the bone marrow by MCP-1 ligand in serum and spleen. It is probable that local production of MCP-1 could be the result of bone marrow infection. It was also determined that CCR2 deficient mice infected with *L. monocytogenes*, showed large accumulations of monocytes in the bone marrow yet this was not caused by an increased intensity of infection. It was observed that CCR2 increases the release of LY6C^{hi} monocytes from the bone marrow of mice that were not CCR2 deficient but CCR2 does not need to be present to recruit monocytes to travel to the infected tissues. Thus the circulation of LY6C^{hi} monocytes to tissue was found to be independent of CCR2 (Pamer and Serbina, 2006).

"The results from this study indicate that the interaction between CCR2 and its principle ligand MCP-1 are necessary for the emigration of LY6C^{hi} monocytes from the bone marrow of mice infected with *L. monoctogenes* and or other inflammatory infections into circulation" (Pamer and Serbina, 2006).

Our laboratory previously assayed the expression of CCR2 in bone marrow macrophage cells in two strains of mice using quantitative RT-PCR (**Figure-3**). It is interesting to note that there is a large difference between the CCR2 expression levels in BALBc/ByJ mice (light blue in the figure) which is higher at each time interval up to 8

hours compared to C57BL/6J mice (dark blue in the figure) which show expression levels of CCR2 at a steady low rate from time 0 hours to 8 hours. Since CCR2 plays a role in triggering monocyte recruitment from bone marrow during infection, it is important for mice to have highly expressed amounts of this gene to fight against infection. Due to their high expression of macrophage CCR2, BALBc/ByJ mice would show a higher ability to fight infection than C57BL/6J mice. The amount of expression drops quickly after infection, which is also interesting.
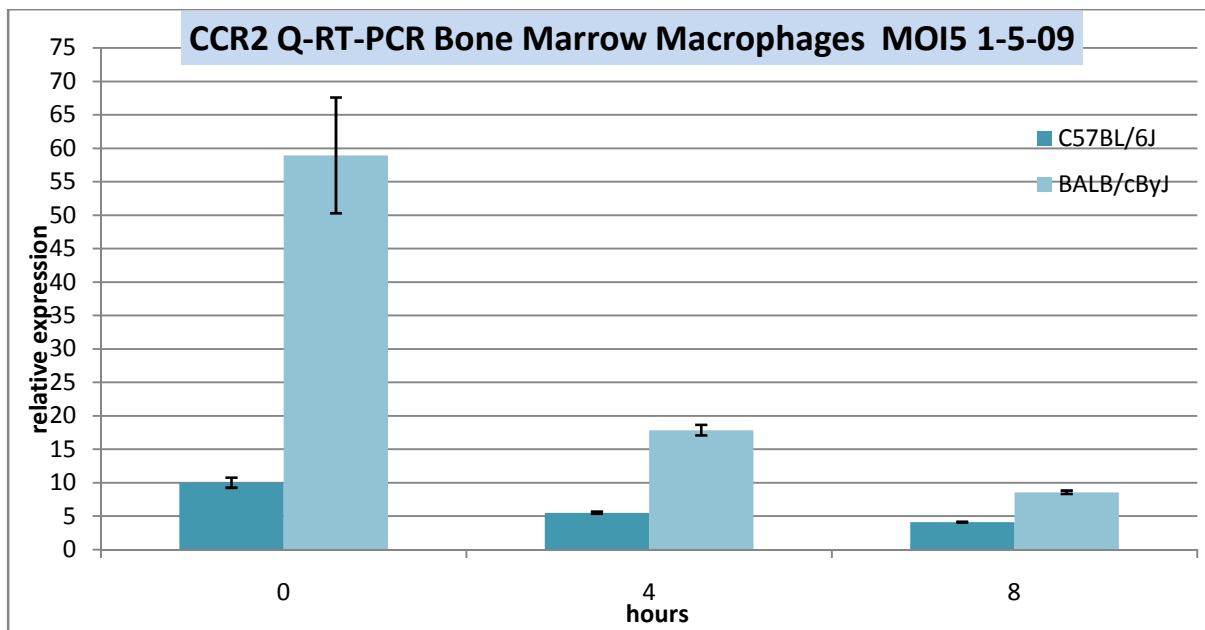

Figure 3: Assay of CCR2 Expression in Macrophages Isolated From Bone Marrow Using RT-PCR of BALB/cByJ (C) and C57BL/6J (B) Mice. (Figure provided by Boyartchuk lab)

CD5l

Differential expression of the gene that controls CD5l or also known as (SPα, AIM, API6) was also researched in this project. CD5l has been proven to promote cell survival after infection to *L. monocytogenes*. Liver X receptors or LXRs are transcription factors that help in the regulation of antimicrobial response, and mice that lack these receptors will be much more susceptible to infection. In Sean et al (2004) the major

determinant was found to be disturbed macrophage function within these mice. In this particular study, it was observed that LXR deficient mice showed little to no macrophages survival, they were more likely to become apoptotic at a more accelerated rate after encountering *L. monocytogenes*. CD51-deficient mice also experienced trouble getting rid of bacteria when they were observed *in vivo*. This is a result of the loss of function (regulation) of CD5l which when expressed in macrophages, inhibits apoptosis (Sean et al, 2004).

As discussed in the previous section, macrophages function as microbial assassins and play a role in inflammatory response. This response is regulated through the release of immune modulators, otherwise known as chemokines as well as through the release of cytokines (like the chemokine receptor CCR2). When defects occur in macrophage receptor functioning, susceptibility to microbial infection may occur. In Sean et al (2004), real time RT-PCR showed high expression of CD5l in the livers of wild type mice during *L. monocytogenes* infection, and a greater than 50 fold reduction of expression of CD5l during infection was observed in mice that were deficient in LXRs (LXR-null mice). "CD5l was found to be induced by *L.monocytogenes*, as well as by a synthetic LXR agonist, and is differentially expressed between LXRα deficient and LXRβ deficient mice" (Sean et al, 2004).

When transplantation of wild type bone marrow was introduced into LXRαβ deficient mice, a complete restoration of the expression of CD5l was witnessed during infection. It was found that the expression of CD5l during *L. monocytogene* infection, was dependent on LXRα. LXRs can act as a positive regulator of genes that play a role in an antimicrobial response as well as a negative role in this situation. "The linkage

between CD5l and LXRα is seen through differential interaction within the CD5l promoter, meaning CD5l is a direct target for regulation by LXRα heterodimers" (Sean et al, 2004).

Signaling of LXRs will initiate bacterial clearance and cause macrophage survival when in response to *L. monocytogene* infection. "When wild type and LXR-null mice were tested against infection, wild type mice macrophages demonstrated small amounts of viable intracellular bacteria, whereas LXR-null mice showed large quantities of intracellular bacteria within macrophages" (Sean et al., 2004). There was also an increased rate of apoptosis in LXR-null macrophages during infection. Wild type bone marrow was then transplanted and this was utilized to demonstrate the regaining of expression of CD5l in macrophages. It was shown that increased cell death (apoptosis) was directly due to infection of *L. monocytogenes* and CD5l played an important role in inhibiting this function in macrophages (Sean et al., 2004).

"Thus, CD5l is a negative regulator (inhibitor) of apoptosis in macrophages during infection, and is directly linked to innate immune response. CD5l is differentially regulated by LXRα and is induced during *L.monocytogenes* infection. This gene is a promoter of macrophage survival as well as antimicrobial activity during infection" (Sean et al., 2004).

Our laboratory previously investigated CD5l expression levels by RT-PCR in two murine strains. Differences in expression levels were noted for both BALB/c and C57BL/6ByJ (**Figure-4 and Figure-5**). Expression levels for CD5l found in both the spleen and liver tissues of these mouse strains were measured over a 24 hour period using RT-PCR in Figure 4. Expression in the bone marrow of male mice was measured at 0, 2,

and 4 hours using RT-PCR is shown in Figure 5. Expression levels of CD5l in the liver showed the greatest difference between BALB/c and C57BL/6ByJ(Y) mice. Expression in the spleen was close to identical. In the bone marrow of the male mice, C57BL/6ByJ mice again showed higher expression of CD5l.

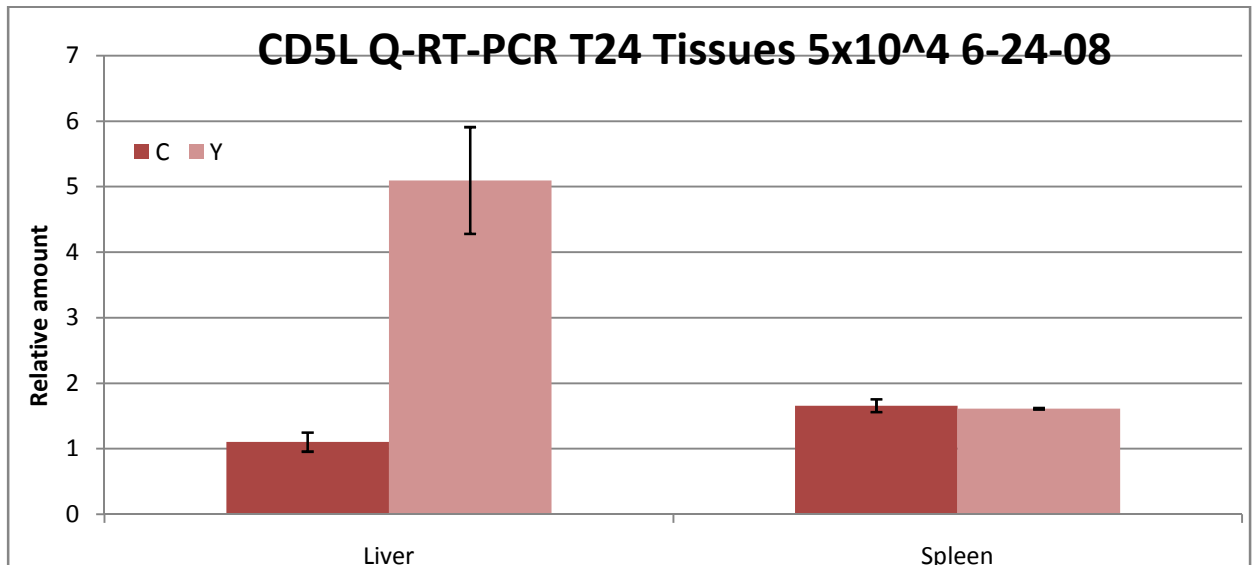**Figure 4:** Assay of CD5l expression levels in liver and spleen tissue of BALB/c (dark brown) and C57BL/6ByJ (light brown) mice after 24 hours using RT-PCR. (Figure provided by Boyartchuk lab)
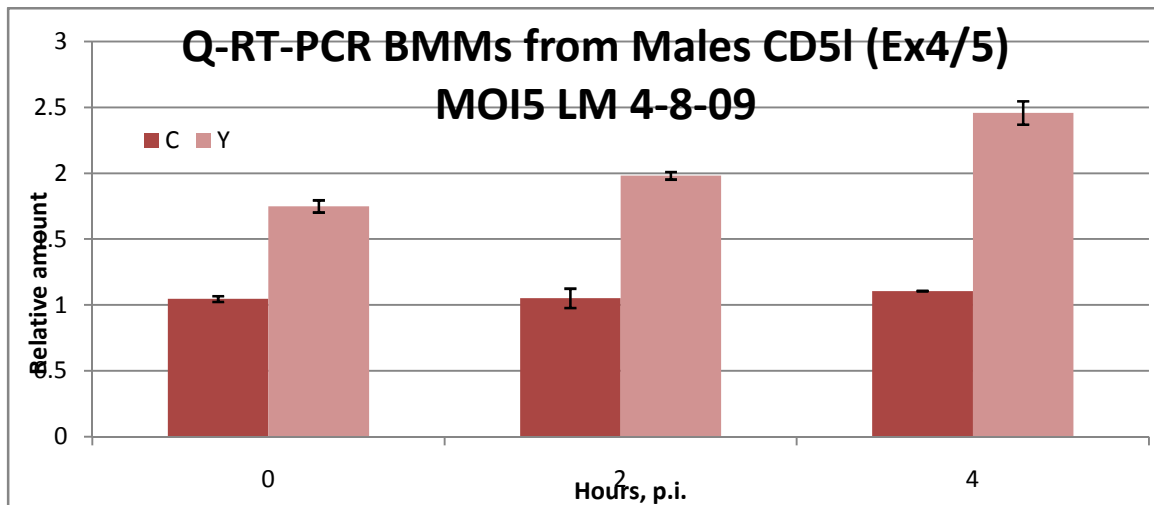

Figure 5: Assay of CD5l expression levels in bone marrow macrophages of BALB/c (dark brown) and C57BL/6ByJ (light brown) male mice measured at 0, 2, and 4 hours using RT-PCR. (Figure provided by Boyartchuk lab)

PROJECT PURPOSE

The purpose of this genetic mapping project is to determine what, at a molecular level, controls expression levels of CCR2 and CD5l in mice. It is evident that certain tissues express this genetic trait more than others in different murine strains, and this is an important factor in different strains having greater susceptibility to infection than others. C57BL/6ByJ mice are less susceptible to infection from Listeria since expression levels of CCR2 and CD5l in the livers of these mice are much higher than levels seen in BALB/c mice.

Choice of Backcross Mice for Analysis

The 42 mice chosen for this genome wide mapping assay were N2 backcross generation. Heterozygous F1 male hybrids formed by mating of C57BL/6ByJ and BALB/cByJ strains were backcrossed to BALB/cByJ females to create these N2 mice. When one performs a linkage study, that person must choose a breeding scheme after an outcross has been performed between two parental strains creating an F1 hybrid strain. The next step is to choose whether to utilize a backcross which would cross F1 generation back to one of the parental strains or to intercross the F1 generation with each other. When performing a large scale mapping experiment (meaning multiple loci spread across one or more chromosomes) such as the one done in this project, a backcross is usually the breeding scheme chosen.

The benefit of this type cross is that it creates a much easier means of handling the large amounts of data that would be obtained from genotyping, and the deficiency of a backcross involves the lower mapping resolution created from such a large sample size.

An intercross is usually utilized when one focuses on a smaller region, possibly a section of one chromosome where a higher resolution of data is necessary. In an intercross, the three moving parts (genotypes) that are possible are C, B, or H (heterozygous), whereas in a backcross there is only two moving parts and these involve either C or H, making it much easier to navigate through a large amount of data and be able to make assumptions form recombinant genotypes.

In this genome wide mapping experiment, a backcross was utilized to cross males from the F1 hybrid generation to BALB/cByJ females (homozygous). This cross creates the N2 generation liters of mice that were then utilized for experimentation in genotyping through PCR (polymerase chain reaction) to pinpoint the chromosome that each of the two traits (CD5L and CCR2) has the highest occurrence on (Silver, 1995).

The two key traits explored during this experiment, described above, were regulation of the gene that controls CD5L expression which plays a role in cell survival of macrophages during infection to *L.monocytogenes*, as well as CCR2 which is a chemokine receptor that encodes two isoforms of a specific receptor for monocyte chemoattractant protein which is known to mediate monocyte chemotaxis. The purpose of this experiment was to perform a genome wide mapping of the 42 mice from the N2 generation that would allow us to possibly pinpoint chromosomal locations of factors that could control of the differential expression of CD5l and CCR2. Finding the elements, genes, or mutations that regulates differential expression of the genes CD5l and CCR2 would allow us to understand how these mechanisms are activated when infection to *L. monocytogenes* occurs.

Materials and Methods

Tail Section DNA Extraction

HotSHOT genomic DNA preparation was utilized to extract DNA from mouse tail snips for each of the 42 mice utilized. Hot SHOT stands for hot sodium hydroxide and tris. When preparing to extract DNA from tissue, the correct amount of tail must be utilized. Too much tissue will lead to unsatisfactory PCR (polymerase chain reaction) results. 0.5 to 5 µl of product (DNA) should be utilized for PCR reaction, so for this project 3 µl of DNA was utilized for each reaction. Any floating tissue or debris found in the solution after heating does not affect the quality of the DNA extracted, the tail will look very similar after the heating procedure yet the DNA is in the solution. After the HotSHOT process, the DNA for each mouse should be stored at 4° C. The amount of tissue that should be utilized during this process was about the size of the letter (**v**) on this page. The tail snip was then placed in a 1.7 ml eppendorf tube, and 80 µl Alkaline Lysis Reagant was added. The tube was then placed in a centrifuge, and spun down at 10 kg for 30 seconds. The heating block was preheated to 95° C, and the tube containing the lysis reagent and tail were placed in the block and heated at 95° C for 1 hour. The sample was then placed in the centrifuge and spun down at 10Kg for 30 seconds after heating. 80 µl of Neutralization Buffer was then added to the sample, and it was then vortexed and spun down in the centrifuge again at 10Kg for 30 seconds. The DNA was now ready for use and was placed at 4° C for storage. The following table details the concentrations of each ingredient utilized to create each buffer. ddH$_2$O was added to create a final volume

of 200 ml for each buffer. The pH of the Alkaline Lysis Reagant is to be 12 while the pH

for the Neutralization Buffer will be 5 (Biotechniques, 2000).

Table 1: Constituents of the Alkaline Lysis Reagent.
This is the first buffer utilized in the HotSHOT DNA extraction method.

Alkaline Lysis Reagant		
Reagent	Final Conc.	Amount for 200 ml
NaOH	25 mM	200 mg
EDTA	0.2 mM	14.88 mg

Table 2: Second Buffer.
This buffer is added after the tail had been heated for 1 hour
in a heating block at about 95° C.

Neutralization Buffer		
Reagent	Final Conc.	Amount for 200 ml
Tris-HCl	40 mM	1.3 g

Polymerase Chain Reaction (PCR)

The DNA polymerase utilized in the PCRs performed during this genome wide

mapping experiment was Taq DNA Polymerase, and the thermal cycling steps are shown

in **Table 3**. These steps were used for all PCR samples throughout the entire genome

wide mapping experiment. The primers that were utilized have complementary regions

of nucleotide arrangements that were complementary to the DNA region that had been

selected for amplification (Gene Almanac, 2009).

Table 3: PCR Thermocycling Program.
This table shows the set up utilized for each temperature cycle during the PCR process.

Step 1	Incubate at 95 Degrees C	3:30
Step 2	Incubate at 95 Degrees C	:20
Step 3	Incubate at 60 Degrees C	:30
Step 4	Incubate at 72 Degrees C	:40
Cycle to step 2 for 39 more times		
Step 5	Incubate at 72 Degrees C	10:00
Step 6	Incubate at 4 Degrees C	Forever

Primers were manufactured by Invitrogen to specific sequences unique to specific

genetic regions. These primers were originally created to differentiate between various

mouse strains in 1992. Preparation of each primer is detailed in the Materials and Methods section.   Each marker utilized was spaced 20-30 Mb down the chromosome to allow the genetic software to create a linkage between each. Anywhere from 3-8 primers were used for each of the 19 chromosomes. The largest chromosome, number 1, is approximately 197 Mb in length, and the shortest chromosome (19) is 61 Mb in length. The longer the chromosome, the more primers were needed to create linkages and map the entire length. A Genetic Map of all primers used and their locations on each chromosome can be seen in Figure 14 of the results section.

PCR was performed utilizing 3 µl of template DNA from each mouse sample (tail snip) for each reaction. Reaction components that included Taq DNA Polymerase Buffer, dNTPs, ddH$_2$O, and Taq DNA Polymerase were all prepared in a master mix which involved using excel to determine the proper amount of each agent. This was decided by the number of samples being utilized for each primer and the total number of primers which were used to genotype.   An example PCR template for 24 samples can be seen under **Table 4**. Two extra samples were added to each sample total to create extra volume to make up for lost volume during pipetting. Extra volume was then added to the total number of samples, which depended on the number of primers being used. This technique created sufficient master mix volumes for each primer, allowing the author to run a PCR with the exact volumes. This ensured the best product after the process had been finalized in the thermal cycling machine.

Table 4: Example PCR Master Mix Template for 24 Samples.

Two extra "samples worth" were added for extra volume, and 4 Primers
(26x4=104, 104 + 8=112 extra samples for extra volume).

Genotyping: 15ul Total Volume										
400nm primers	**Taq DNA Polymerase**									
			ul	**Final**						
Buffer	10	x	1.5	1	x			**Buffer**	**168**	
primersF+R	5	uM	1.2	0.4	uM			*primer*	*134*	
dNTPs	25	mM	0.12	0.2mM				**dNTPs**	**13.4**	
BlueTaqDenville 1u/ul			0.3	0.03u/ul				**Taq**	**33.6**	
Water			8.88					**Water**	**995**	
Template			3	3	ul	For		*Template*	*336*	
Total			**15**			**112**	**Samples**	**Total**	**1680**	
				Final				**Total Volume per Primer:**		**280.8**
PCR cycles				1	x			**Buffer**	**39**	
	95C	3\`30\`\`		0.4	uM			**primer**	**31.2**	
40cycles	95C	20\`\`		0.2mM				*dNTPs*	*3.12*	
40cycles	60C	30\`\`		0.03u/ul				**Taq**	**7.8**	
40cycles	72C	40\`\`						**Water**	**231**	
	72C	10\`		3	ul	**For**		**Template**	**78**	
	4C	forever				**26**	**Samples**	*Total*	*390*	

DNA template wasthawed out at room temperature before use and stored at 4°C overnight. After thawing was completed, the DNA was placed in an ice bath and vortexed vigorously before dispersing 3µl from each sample into sections of a 96 well PCR plate that had previously been marked off for where the location each sample was going to be placed. The plate was then covered and kept in an ice bath.

The master mix was made using ddH_2O which was added to a 1.7ml eppendorf tube. Thermo Polymerase Buffer was thawed at room temperature, vortexed, and then the proper amount was added according to the excel sheet. The eppendorf tube was then kept in an ice bath. dNTPs were thawed and quickly placed back at 4°C because of its poor stability at room temperature. These were vortexed and then added to the master mix at correct volumes according to the excel spreadsheet. Taq DNA Polymerase was not taken out of the -20°C freezer until use. Once this component was added at correct volume to the master mix, it was quickly placed back into the freezer to again ensure stability. During the preparation as well as after completion, the 1.7 ml eppendorf tube containing the master mix was kept on ice at all times in a pre-made ice bath.

Once the master mix was completed, specific amounts of each primer being utilized were added to separate 1.7 ml eppendorf tubes and kept on ice. This was also determined using the spreadsheet. The amount of primer was determined by the number of mouse DNA samples being genotyped per primer. Next the master mix was vortexed vigorously and evenly dispersed into one or each of the primer tubes according to the spreadsheet. With the primer and master mix now dispersed in the same eppendorf tube, the tube was vortexed vigorously and 12µl is added to each well in the PCR plate that contains DNA according to organizational placement of the samples for each primer.

The plate was then capped using a rubber heat stable top and vortexed to mix the master mix/primer with the DNA template in each well. The plate was then spun down at 10Kg for 10 seconds in a large centrifuge and placed in the PCR thermo heat cycler according to the PCR set up seen in **Table 4** of the results section.

Once the cycling procedure was complete, the plate was taken out of the thermo cycler and spun down again at 10Kg for 10 seconds in a large centrifuge. The PCR products were then placed in a 4^oC freezer and kept there until the 4% agarose gel was prepared and ready for use.

PCR Primer Design

Primers were manufactured by Invitrogen to specific sequences unique to specific genetic regions. These primers were originally created in 1992 to differentiate between various mouse strains. The vials were spun down using a centrifuge at 10Kg for 5 minutes to make sure the pellet was brought back down to the bottom of the vial. To create stock primers, 100 µM (micromolar) concentrations must be reached. The nM (nanomolar) concentration on the vial of each FWD and REV marker is multiplied by a value of 10 and this many µl of ddH_2O is added to each FWD and REV vial. The vials were then vortexed and placed in 4° C environment until further use. To create primer for utilization in PCR, 10 µl from the 5' vial and 10 µl from the 3' vial were mixed together along with 180 µl of ddH_2O. This creates a 5µM (micromolar) dilution mixture of the marker now ready for use in PCR. This marker mixture was vortexed before distribution for use, and was also kept at 4° C when stored.
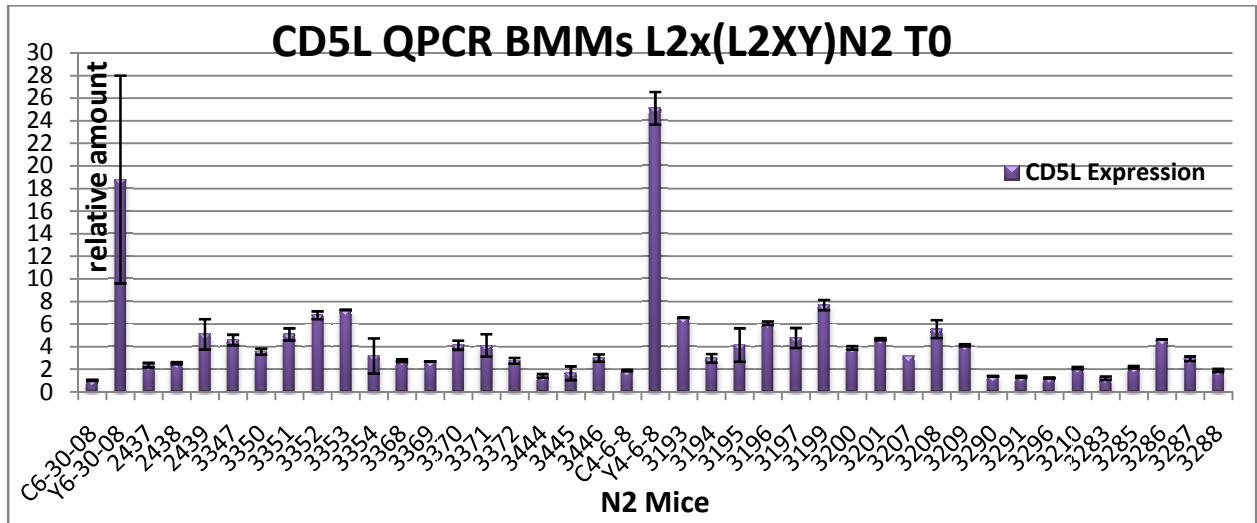
Linkage Analysis

       Linkage analysis software Map Manager was utilized to establish linkage between gene expression phenotypes and the genotypes that were determined through PCR methods and gel electrophoresis. We analyzed two traits: expression levels of CD5l and CCR2. The values for CD5l were determined by measuring levels of expression in macrophages of all 42 N2 mice compared to the baseline value found in C mice (BALB/c). The same measurement was performed for CCR2. Macrophage populations were obtained from the bone marrow of these mice. RT-PCR (Real Time Polymerase Chain Reaction) was performed to determine levels of expression in each of the mice, which was then compared to the baseline seen in BALB/C mice. Expression levels of CCR2 and CD5l in macrophages of N2 backcrossed mice (performed previously in our laboratory) can be seen in the results section in Figures 3 and 4. Marker regressions were run utilizing this data to determine significance levels of each marker used and linkage analysis maps were constructed by Map Manager which allowed the author to determine areas along the chromosome that may not have been mapped but may display high significance meaning an area that might contain a mutation that controls differential expression of CD5l or CCR2.

RESULTS

CCR2 and CD51 Expression Levels

  Relative amounts of expression levels of CD5l in bone marrow macrophages of all 42 N2 backcross mice using RT-PCR and the values were graphed in **Figure 6**. These expression levels were measured before genotyping to be sure that backcross progeny maintained differences in expression of genes of interest. These numerical values for levels of gene expression were entered for each mouse as a specific trait in the genetic software Map Manager and were labeled CD5l. It can be seen in (Figure 6) that C57BL/6ByJ (Y) mice or also referred to as B mice, show the greatest amount of CD5l expression in macrophages which can be seen under the label Y6-30-08 and Y4-6-8. These values were compared to the baseline amounts seen in BALB/cByJ (C) mice. Expression levels are spread out at different amounts across all N2 backcross mice.

  Relative amounts of expression of CCR2 in macrophages (monocytes are precursors to macrophages) were taken from the bone marrow of the N2 backcross mice using RT-PCR and can be seen in **Figure 7**. These expression levels were determined to create a linkage analysis correlating these values with the genotypes that were later determined for specific markers on each chromosome. This analysis was done by the genetic software Map Manager that generates LRS (likelihood ratio statistic) values for each marker in the map.

Figure 6: Relative amount of CD5l expression measured in bone marrow macrophages from each of the N2 mice compared to the baseline positive control BALB/C mice.

Figure 7: Relative amount of CCR2 expression in bone marrow macrophages of each of the N2 mice compared to the baseline positive control B6/J mice.

Genotyping by PCR

Genotyping of these 42 N2 backcross mice was performed to generate a genetic map for each of the 19 autosomes. Data was entered into Map Manager to create a linkage map that establishes the relationship between markers used for each chromosome. Genotyping was performed using PCR and gel electrophoresis to visualize the PCR product under UV light. Mouse tail snips were utilized to extract DNA which was later used as the template in the PCR reactions. A Hot SHOT (Hot Sodium Hydroxide and Tris) method was utilized to extract DNA from the tissue. This method is detailed in the Materials and Methods. 4-5 tail snips were taken from each mouse to ensure there was enough DNA to supply numerous reactions. The DNA that was created from this process became the template for amplification, and numerous copies of the DNA region were processed.

Genotyping was performed on all 42 backcross mice for multiple markers utilized at each chromosome. **Figures 8 through 13** show the PCR gels under UV illumination. The first two bands (seen in the first two lanes of each example gel photo) are the positive controls B6/J (B Mice) and then BALB/C (C Mice). Each successive band is part of the N2 backcross population detailed above each photo. When 2 bands are visualized, a heterozygous genotype is determined, and when a single band is seen, a homozygous or in the case for this N2 generation, any mouse that is not heterozygous is a C genotype. This is due to the way the mice were crossed. A C female was crossed to a heterozygous male. Because the mother does not contain the B genotype, all of these mice will be C or heterozygous. These UV illuminated photos were utilized to genotype

all 42 mice using 3-8 markers per chromosome, depending on length of the chromosome and on the genetic software and if it was able to create a linkage between primers.

Figure's 8-10 show the marker D10 MIT 230 which was utilized to genotype chromosome 10 approximately 89 Mb down the chromosome. This marker showed the highest significance value for CCR2 once marker regressions were performed and linkage analysis was observed. Figures 11-13 show the marker D6 MIT 361, located 76 Mb down chromosome 6, which showed the highest level of significance for the trait CD5l. All gels were run as such and look similar to each example photo shown below. Some bands were not as visible and some were not visible at all when the agarose gel was exposed to UV light. This could be due to a number of situations. Master mixes may not have been prepared correctly, in some cases where bands were visible yet dim, product may have seeped out as precipitate during the PCR process and some sample may have been lost. On occasion, a sample was lost when loading it into the wells of the agarose gel before electrophoresis was performed (pipetting error). Another situation could be that since 4% agarose gels could be reused and stored at 20°C for up to about a month, some gels may have been overused or possibly contaminated from bacteria causing impaired visualization of bands in the gel under UV.
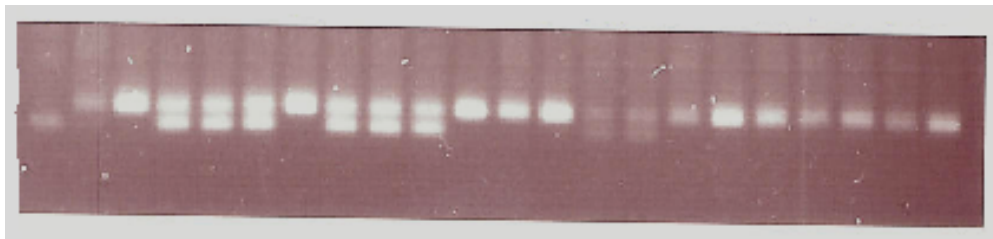

Figure 8: D10 MIT 230 (3193-3288); Photo taken after the gel containing samples 3193-3288 were observed under UV.
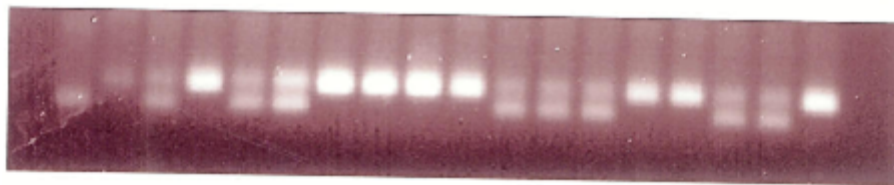
Figure 9: D10 MIT 230 (2437-3446); Photo taken after mice 3437-3446 were observed in 4% agarose gel under UV.
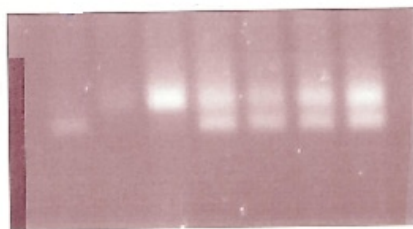

Figure 10: D10 MIT 230 (3534-3656); Photo shows last 5 samples that were observed under UV for D10 MIT 230 being 3534-3656.
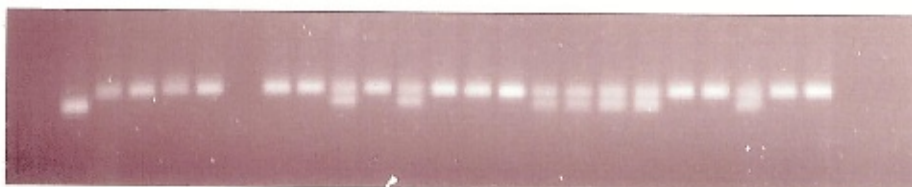

Figure 11: D6 MIT 361 (3193-3288); Photo shows gel exposed to UV light for mice 3193-3288.
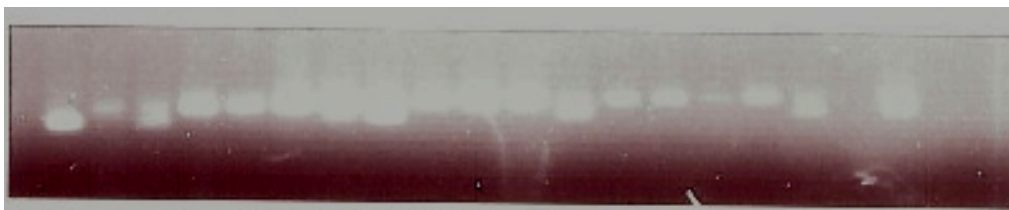

Figure 12: D6 MIT 361 (3437-3446); Photo was taken under UV light for samples 3437-3446. Contrast for this gel was not as proficient.
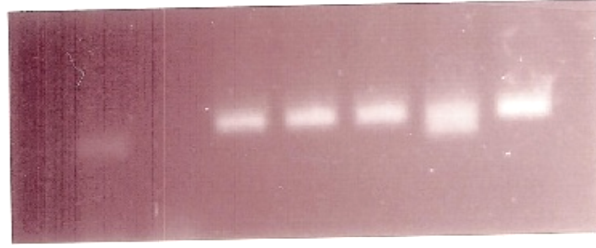
Figure 13: D6 MIT 361 (3534-3656); Samples 3534-3656 are observed under UV light in this photo.

Genetic Map

A Genetic Map was then constructed for all 19 chromosomes to be able to observe the markers utilized and to create linkages between all markers by looking at spacing parameters. These maps were produced through Map Manager Software by selecting the drop down map key and selecting each specific chromosome that had been genotyped. This Genetic Map is shown in **Figure 14**. All markers used to genotype each chromosome through PCR and gel electrophoresis is detailed in this diagram. Some chromosomes needed more than 3-4 markers to create linkages which could possibly be a result of some chromosomes having more length than others. Chromosome 1 is the largest and the length of each gradually diminishes until the shortest chromosome which is 19 is reached.

```
29.1      ┬ D1 MIT 411
2.4       ┼ D1 MIT 132        D1 MIT 494
33.5
          ┼ D1 MIT 451                              ┬ D2 MIT 37
32.5                                      28.3      ┼ D2 MIT 45
          ┼ D1 MIT 356                    31.8
29.1                                               ┴ D2 MIT 48
          ┴ D1 MIT 293

          ┬ D3 MIT 62
33.5
          ┼ D3 MIT 227
41.7      ┼
          ┼ D3 MIT 29
36.2
          ┴ D3 MIT 350
```

Chromsome 1   **Chromosome 2**

Chromosome 3

```
15.1     ┬ D4 MIT 214                              ┬ D5 MIT 386
         ┼ D4 MIT 178            32.5
22.9     ┼ D4 MIT 181                              ┼ D5 MIT 233
4.8                             29.3
                                                   ┼ D5 MIT 83
64.1     ┼                      16.3               ┴ D5 MIT 24

         ┴ D4 MIT 12
```

```
                                          ┬ D6 MIT 159
                            30.0
                                          ┼ D6 MIT 354
                            21.2
                            9.9           ┼ D6 MIT 361
                            17.3
                                          ┼ D6 MIT 149
                            24.2
                                          ┴ D6 MIT 25
```

Chromosome 4   **Chromosome 5**

Chromosome 6

Chromosome 7:
- 9.6 — D7 MIT 246
- 17.3 — D7 MIT 90
- 20.1 — D7 MIT 238
- 30.0 — D7 MIT 259

Chromosome 8:
- 15.1 — D8 MIT 4
- 12.4 — D8 MIT 231
- 17.3 — D8 MIT 242
- 17.8 — D8 MIT 14

Chromosome 9:
- 5.9 / 2.5 / 15.1 — D9 MIT 247
- 10.1 — D9 MIT 11
- D9 MIT 106
- D9 MIT 243

Chromosome 7   **Chromosome 8**
Chromosome 9

Chromosome 10:
- 9.9 — D10 MIT 167
- 12.4
- 23.6 — D10 MIT 180
- D10 MIT 194

Chromosome 11:
- 27.5 — D11 MIT 226
- — D11 MIT 185
- 35.9
- — D11 MIT 339
- 26.7 — D11 MIT 358
- 20.6 — D11 MIT 258

Chromosome 12:
- 20.6 — D12 MIT 147
- — D12 MIT 114
- 15.5 — D12 MIT 101
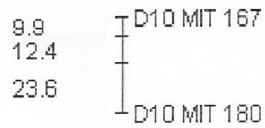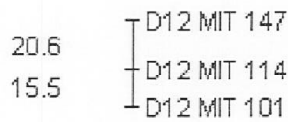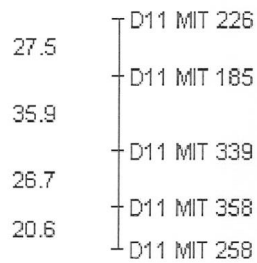
Chromosome 10   **Chromosome 11**
Chromosome 12

Chromosome 13:
- — D13 MIT 218
- 37.4
- — D13 MIT 88
- 50.0
- — D13 MIT 254
- 38.6
- — D13 MIT 226

Chromosome 14:
- 21.8 — D14 MIT 109
- — D14 MIT 50
- 26.7
- — D14 MIT 14
- 41.7
- 9.6 / 8.4 — D14 MIT 101
- 26.7
- — D14 MIT 266
- D14 MIT 113

Chromosome 15:
- 24.2 — D15 MIT 226
- — D15 MIT 100
- 15.5 — D15 MIT 159

Chromosome 13   **Chromosome 14**
Chromosome 15

21.2 ⌐D16 MIT 34
7.6   ⌐D16 MIT 84   D16 MIT 76

15.1 ⌐D17 MIT 22
4.9   ±D17 MIT 152   D17 MIT 38

Chromosome 16   **Chromosome 17**

                                               ⌐D19 MIT 59
                                   36.2
                                               ⌐D19 MIT 42
9.9   ⌐D18 MIT 110   D18 MIT 149   50.0
15.5 ⌐D18 MIT 51   D18 MIT 4
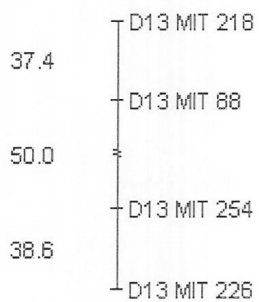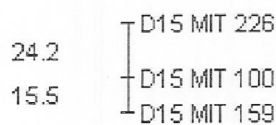   ⌐D19 MIT 10

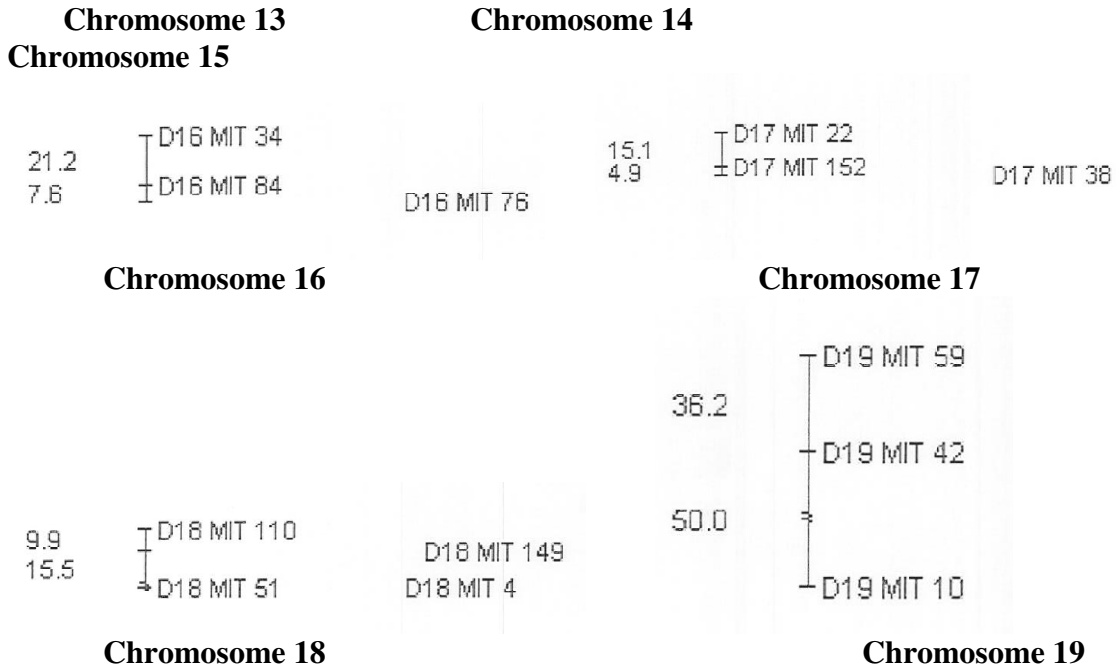**Chromosome 18**   **Chromosome 19**

Figure 14: Genetic Map of all Markers (primers) Utilized on all 19 Chromosomes to Genotype and Create Linkages.

Linkages

Genetic software Map Manager was next used to correlate all genotypes that were observed at each marker with expression levels of CD5l and CCR2 in the macrophages of these mice that were entered in as two separate traits. The expression levels were shown previously in Figures 6 and 7. Linkages were created through correlating this data to determine significance levels of markers utilized in genotyping for each trait. Significance levels were found through performing separate marker regressions on each trait in Map Manager. The software identified numerous markers for each trait that showed the highest amount of significance. These markers can be seen for CCR2 in Table 6 as well as graphically in Figure 15 and Figure 19. The markers for CD5l can be

seen in Table 7 and graphically in Figures 16 and 18. The greatest level of significance

observed for CCR2 was a stat level of 7.3 on marker D10 MIT 230 located on

chromosome 10. For trait CD5l, the highest stat level (significance) was 8.0 seen on

marker D6 MIT 361 located on chromosome 6.

Table 6: CCR2 Linkage.
Results of the marker regression performed on each

Chromosome	Locus	Stat	%	P	Cl	Add
1	D1 MIT 494	6.4	16	0.01124	90	-2.13
6	D6 MIT 25	4.4	12	0.03566	128	-1.93
10	D10 MIT 230	7.3	19	0.00679	80	2.25

chromosome for each marker utilized.
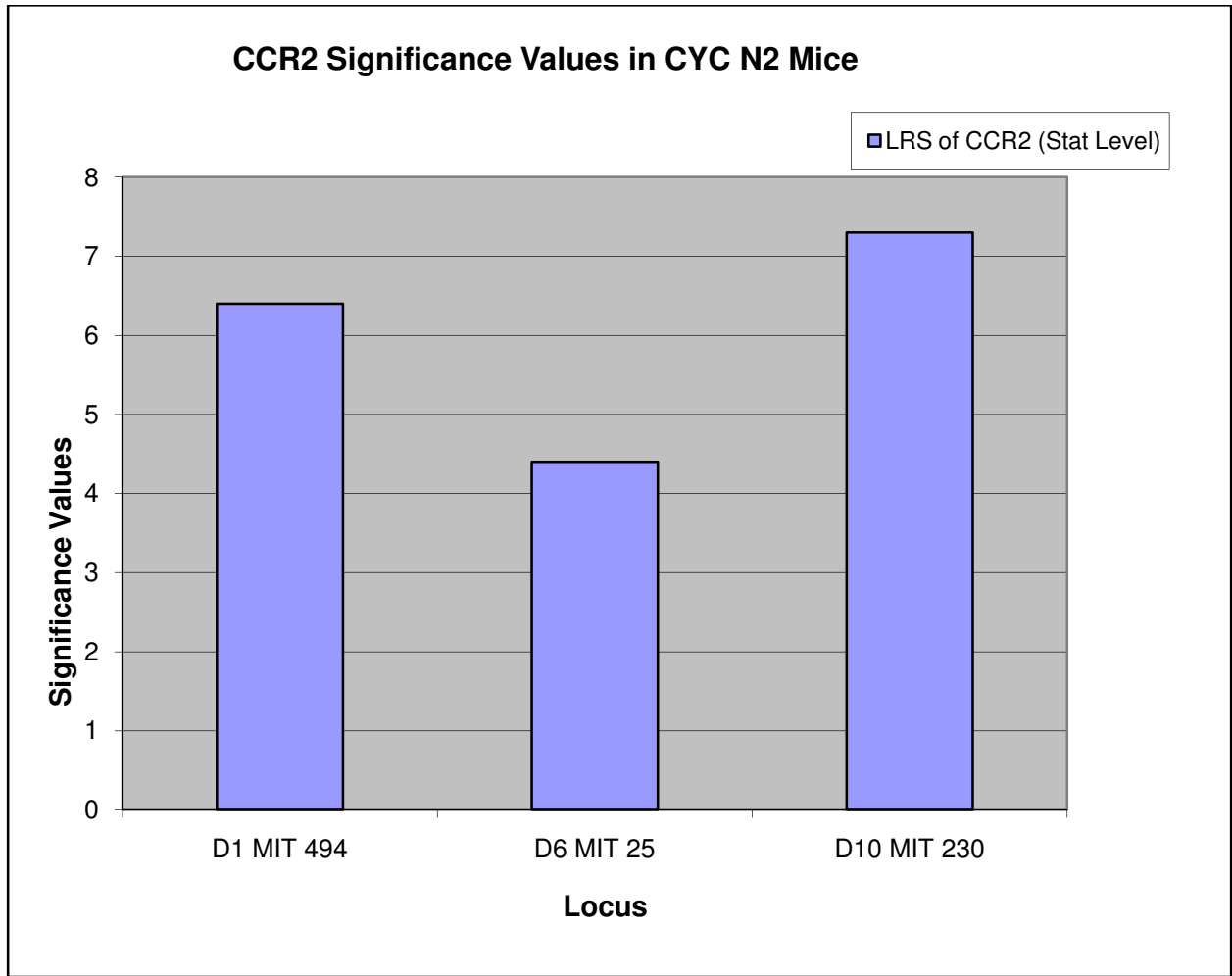
Figure 15: CCR2 Significance Levels. Determined through Map Manager for CCR2.

Chromosome	Locus	Stat	%	P	Cl	Add
1	D1 MIT 411	5.8	15	0.01563	98	-1.41
1	D1 MIT 132	6.6	16	0.01047	88	-1.5
1	D1 MIT 494	7	17	0.00806	83	-1.53
5	D5 MIT 83	7	17	0.00801	83	-1.53
6	D6 MIT 354	4.7	12	0.03034	120	-1.27
6	D6 MIT 361	8	20	0.0046	73	-1.66
6	D6 MIT 284	7.7	19	0.00567	77	-1.63
7	D7 MIT 246	4.1	10	0.04351	137	1.18
7	D7 MIT 27	7.1	17	0.00776	82	1.54
11	D11 MIT 339	5.1	13	0.02344	111	-1.31
14	D14 MIT 266	7	18	0.00817	83	1.49
15	D15 MIT 226	6.6	16	0.01043	88	-1.48

Table 7: CD5l Linkage.
Results of linkage analysis of CD5l expression to
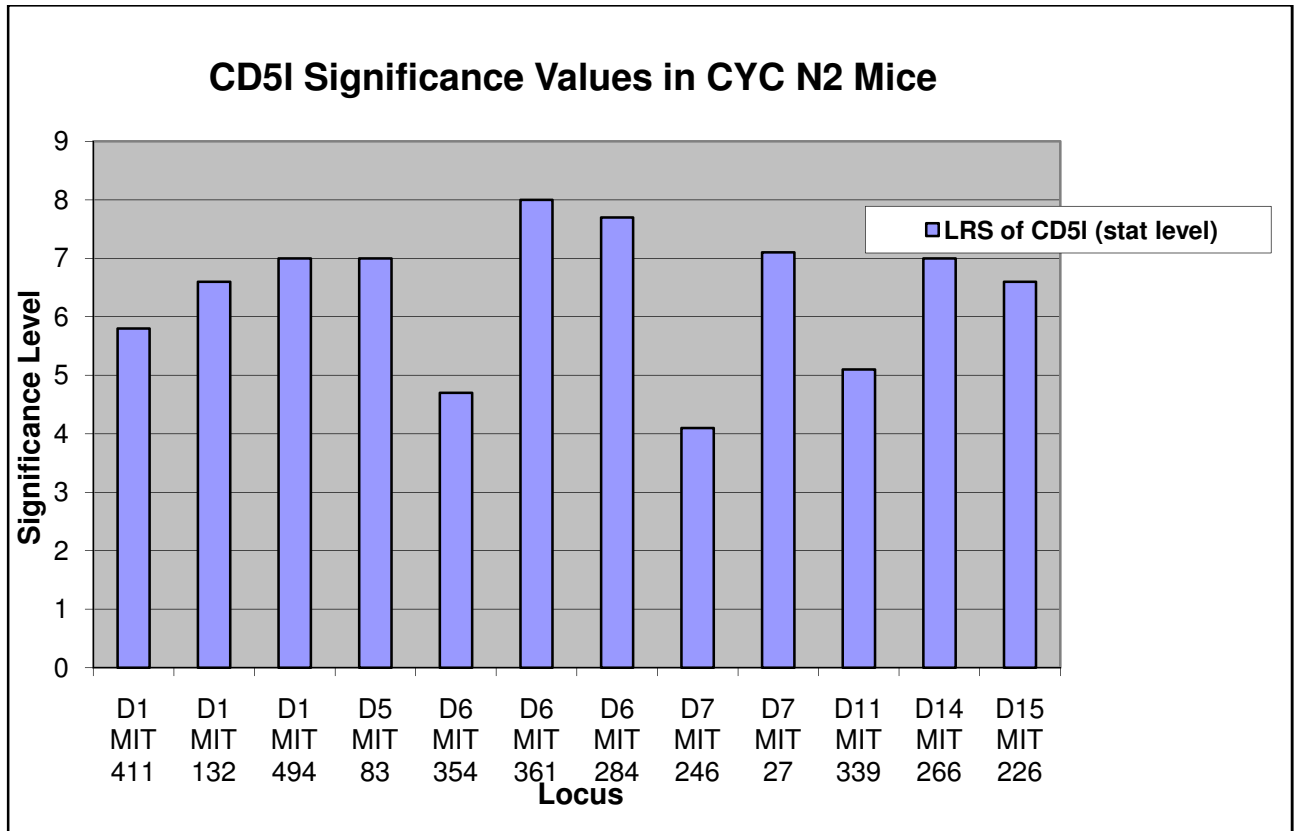each informative marker using marker regression.

Figure 16: LRS linkage significant scores obtained by marker regression analysis for CD5l expression trait.

As a positive control, a previous genome mapping project was used to display highly significant linkage values that allow one to determine and further explore the exact area of the genome which controls differential expression of IFNb. The region controlling IFNb expression was pinpointed to be located in chromosome 7. Numerous markers were then utilized to fine map the chromosome 7 region in these particular mice. Differential expression of IFNb was determined to be controlled by a locus at marker D7 MIT 229 which possessed a stat level of 35.9 which means there is highly significant linkage. D7 MIT 229 is located on chromosome 7 at approximately 52 Mbp down the chromosome. This data is displayed in **Table 8** and in **Figure 17**. This is seen as an

example of a definitive linkage in contrast to the linkages and significance values that

were seen for both CD5l and CCR2.

Chromosome	Locus	Stat (level)	%	P	Cl	Add
7a	D7MIT27	13.7	50	0.00022	54	3.08
7a	D7MIT229	9.3	49	0.00023	78	3.23
7	D7MIT340	7.1	15	0.00775	81	1.67
7	D7MIT270	18	34	0.00002	36	2.44
7	D7MIT27	28.4	48	0	26	2.9
7	D7MIT81	26.4	46	0	27	2.82
7	D7MIT229	35.9	57	0	22	3.12
7	D7MIT158	24.8	44	0	28	2.74
7	D7MIT145	14.4	28	0.00015	43	2.25

Table 8: (**Positive Control) IFNT4 Trait from BCN2 54(BCN2 54).**
Significance levels of Interferon Beta was analyzed
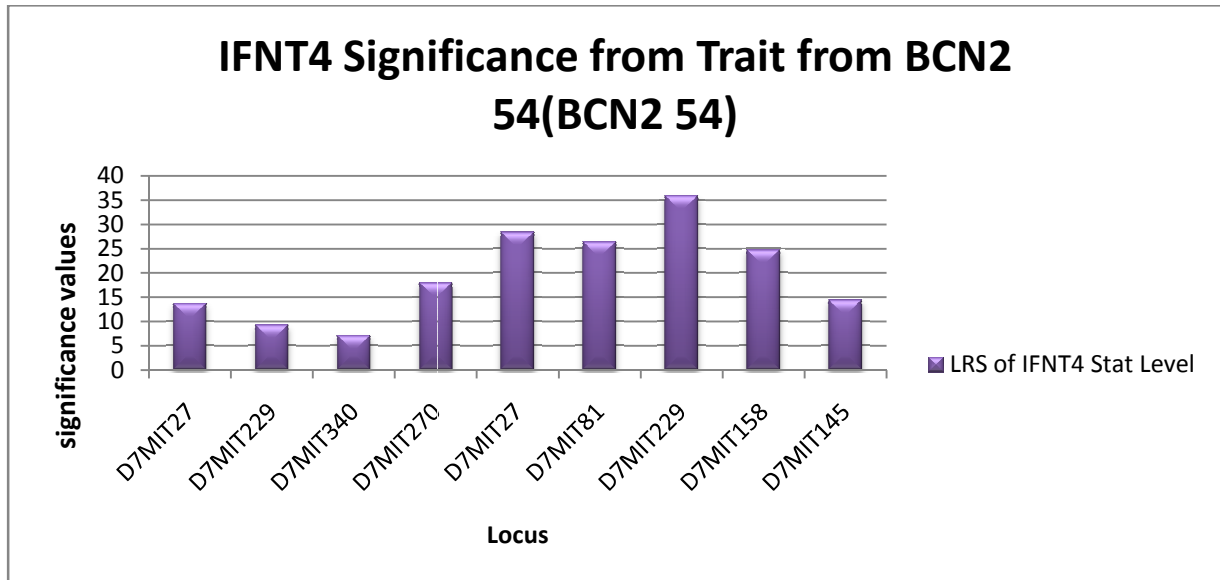using a genome wide mapping approach.

IFNT4 Significance from Trait from BCN2 54(BCN2 54)

Figure 17: Positive Control Data. LRS Scores for Loci on Mouse Chromosome 7 in search of the area that controls IFNb expression.

Figure 18 shows a linkage analysis map created by Map Manager on the

chromosome that contained the marker D10 MIT 230 which displayed the highest

significance value for CCR2. Figure 18 also shows a linkage analysis done on

chromosome 6 which contained the marker (D6 MIT 361) that showed the highest

significance value for the trait CD5l. These maps are essentially the same data that can

be found after a marker regression is run for both traits, meaning they were developed by

the software Map Manager by correlating the genotypes entered for each marker with the

expression levels seen in macrophages of CD5l and CCR2. These graphs also serve

another key purpose. Linkage analysis not only shows the significance values of the

markers that were used to genotype each chromosome but it also develops an estimated

guess of the areas that were not genotyped by a marker which may show higher values.

This is seen on the bold line in each interval graph which is otherwise known as the LRS

(likelihood ratio statistic). If higher values are predicted in a certain area of the

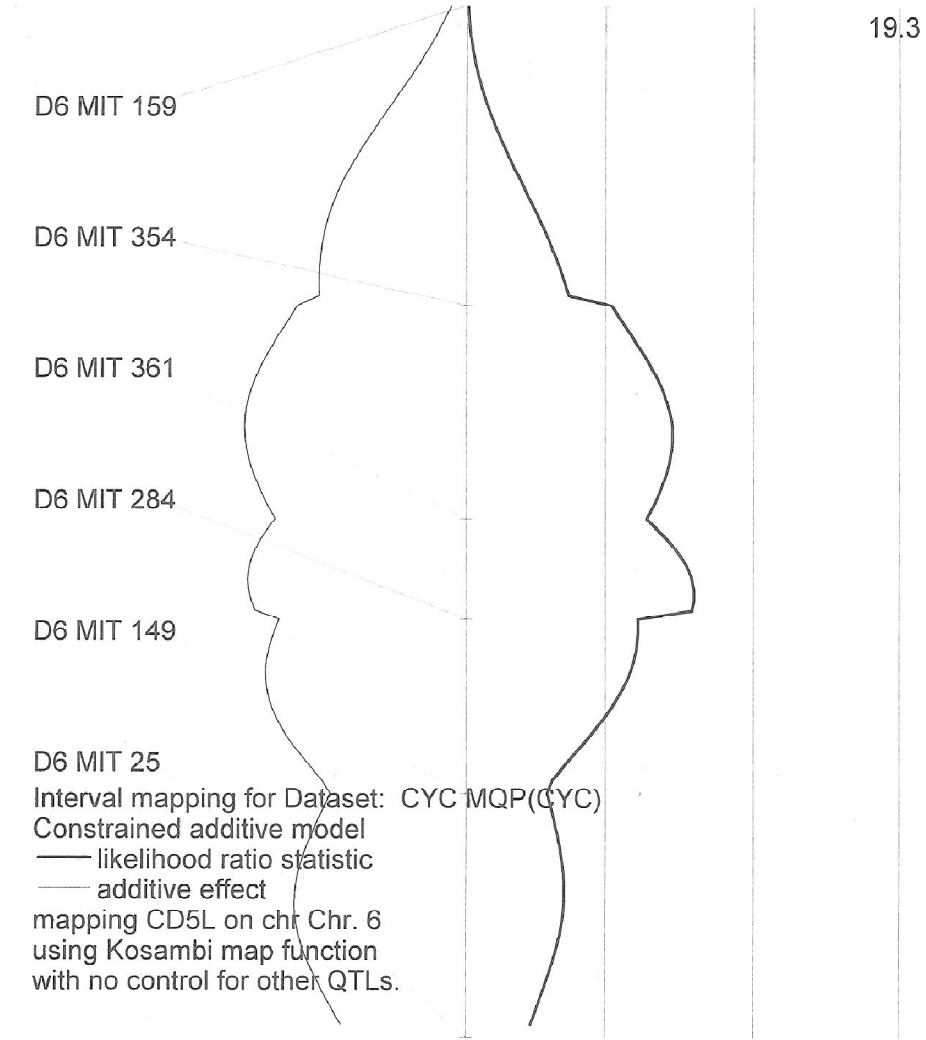chromosome, other markers may be used to map this region.

D6 MIT 159

D6 MIT 354

D6 MIT 361

D6 MIT 284

D6 MIT 149

D6 MIT 25
Interval mapping for Dataset: CYC MQP(CYC)
Constrained additive model
──── likelihood ratio statistic
──── additive effect
mapping CD5L on chr Chr. 6
using Kosambi map function
with no control for other QTLs.

19.3

Figure 18: CD5l Interval Mapping. Interval mapping of chr 6 for CD5l trait.

41

17.6

D10 MIT 167

D10 MIT 194

D10 MIT 230

D10 MIT 180
Interval mapping for Dataset: CYC MQP(CYC)
Constrained additive model
—— likelihood ratio statistic
—— additive effect
mapping ccr2 on chr Chr. 10
using Kosambi map function
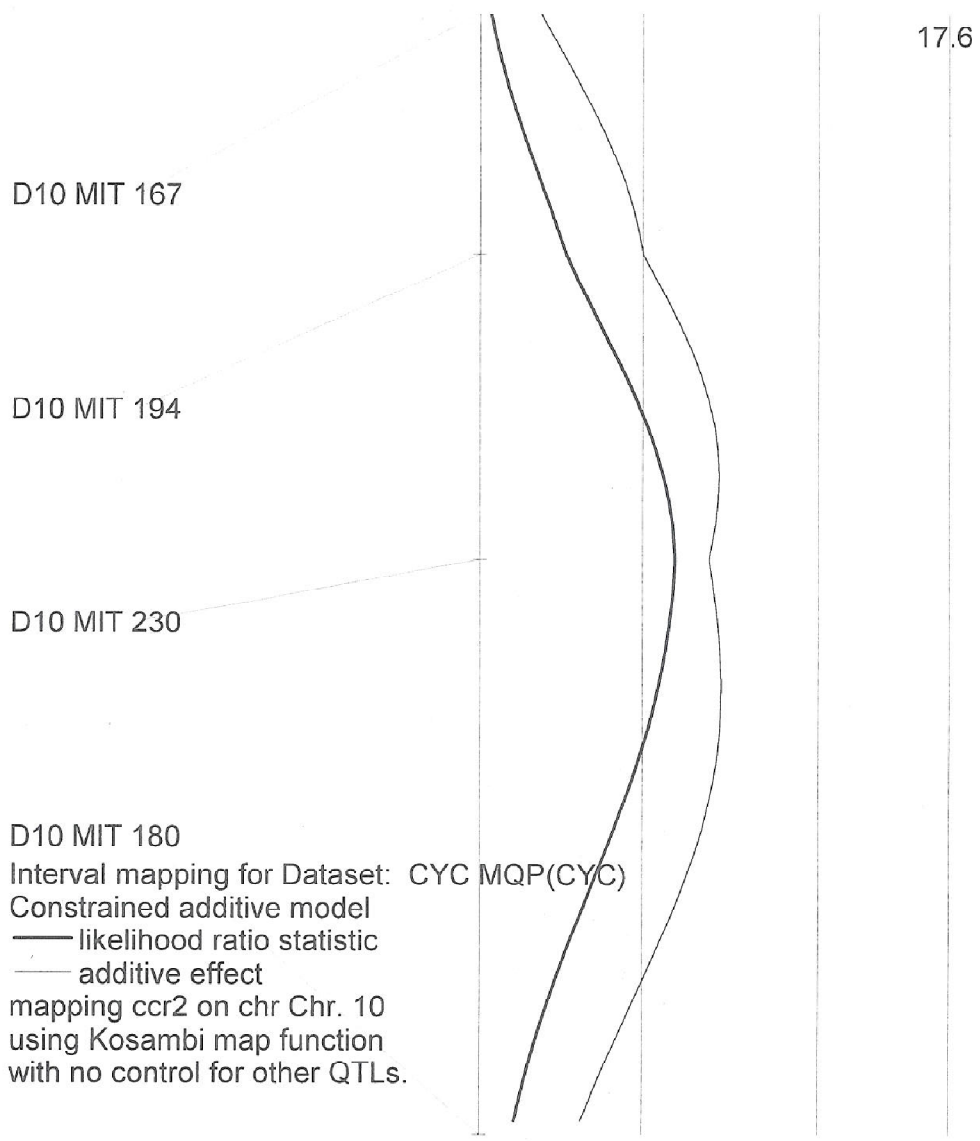with no control for other QTLs.

Figure 19: CCR2 Interval Mapping. Interval mapping of chromosome 10 for CCR2 trait.

DISCUSSION

Cis and trans acting elements are the two possibilities when determining what controls expression of a specific target gene. Trans acting factors are possibly proteins that will attach themselves to Cis acting elements to regulate the expression of a specific gene downstream. Some specific properties of trans acting factors are that they can attach themselves to RNA polymerase which in turn allows the initiation complex to become stable. Trans acting factors also bind themselves to specific target promoters which contain specific sequences that these factors recognize, as well as positive trans regulators which can bind to some of these promoters and commence transcription (McClean, 1998).

Numerous Trans acting factors that bind successively to different areas, each possessing a different property could possibly trigger initiation of transcription. Some enhancers have sites designed specifically for multiple Trans acting factors to bind to and influence transcription of the target gene. This means that it may be necessary for specific genes that contain these enhancers to have numerous complexes which allow for initiation of this genes expression (McClean, 1998).

Transcription factors are also Trans acting factors which can affect gene expression through binding of a ligand which activates the transcription factor to then control gene expression as well as through spatial regulation, temporal regulation, phosphorylation, or through an environmental signal which in turn triggers the transcriptional factor to come in contact and act together with nuclear DNA. Transcription factors also have defined, specific motifs which make up a small section of

the protein but which place each transcription factor in a different category of Trans acting factors. (McClean, 1998).

Cis acting elements are factors that affect gene expression and are found within the gene itself or a short distance from the target gene. For example a promoter is a cis acting element. It is usually located right in front of the gene. Mutations in the promoter can affect the strength with which transcription factors bind to it and therefore influence the level of transcription. A protein that is specific for a particular sequence in Cis will bind and regulate the gene through its binding activation. The presence of one of these elements is usually enough to regulate the gene once it has been activated (McClean, 1998).

When observing genes through use of a genome wide mapping to determine how it's expression is regulated, one needs to determine whether differences in expression of target genes in question are controlled through initiation of elements and factors in Cis or in Trans. This can be determined by looking at where linkage is highest and if this correlates with where the target gene is located. If linkage for a specific differential expression trait is highest on a different chromosome from that of the target gene, then one can deduce that regulation of this gene is in Trans. If expression is determined to be greatest on the same chromosome as the target gene, then it can be determined that the gene is probably regulated in Cis.

CD5l is located on chromosome 3 at approximately 87,161,897 bp, while CCR2 is found on chromosome 9 at about 124,016,922 bp. Once the genotyping was completed and the genotypes of the different markers at each of the 19 chromosomes were entered into the gene mapping software, which were spread out to map each chromosome with a

44

20-30 Mb distance between each primer. Expression levels of CCR2 and CD5L were measured in macrophage RNA samples from each of the 42 backcross animals. Numeric values representing the levels of CCR2 and CD5L in each of these samples were entered into Map Manager mapping program and correlated with experimentally determined genotypes using marker regression approach. The highest level of correlation between gene expression level and genotype was found on chromosome 10 at about 89,554,985 Mbp's for CCR2, and the on chromosome 6 at approximately 76,907,674 Mbp's for CD5l. Neither of these locations corresponds to the location of these genes (CD5l and CCR2). This suggests that genetic mutations that control differences in expression of these genes are not in the coding sequence of genes themselves (in Cis) but are in genes located in Trans (Genome Bioinformatics, 2009).

If our genetic mapping results are correct, the mutation that controls CCR2 expression is in a gene located around 89Mb on Chromosome 10, and mutation that controls CD5L expression is in around 80 Mb on Chromosome 6. Therefore the future experiments would involve identification of candidate genes at these locations that could control expression of CCR2 and CD5L. There are hundreds of genes at these locations. Therefore we have to use certain criteria to select those that could affect expression of these specific target traits.

1. Genes that affect expression levels of other genes are transcription factors, signaling molecules and their receptors and signal transduction molecules. Therefore for our analysis we will select those genes that have one of these functions.

2. In order to induce differences in CCR2 or CD5L expression, our candidate gene has to have different properties in mice that show differences in CCR2 or CD5L expression. Such differences could be mutations or differences in expression of regulatory genes themselves. It is difficult to find mutations without laborious sequencing of all candidate genes. However it is easy to identify differences in expression of a large number of genes by using Gene Chip technology. Even though by analyzing differences in expression we run a risk of missing the mutations that do not cause changes in gene expression, it is still a reasonable first pass approach.

A Gene Chip array could also be used in the future. This technique allows simultaneous analysis of expression levels of all known genes in the genome. It is created by building up short DNA sequences on the special glass through light directed chemical synthesis. This is done by building a foundation of substrate to nucleotides at specific sites on the glass. Polymerization is inhibited by a "X" group which is detached from the nucleotide once it is exposed to UV light. A filter is then added which only allows specific nucleotides to be exposed to the UV allowing them to add the next nucleotide to the chain. Through changing of the location of the filter, one can build up specific sequences of approximately 20 nucleotides long to be screened. cDNA probes generated from total pool of mRNA are hybridized to the Gene Chip allowing for screening many thousands of sequences at a time. By analyzing levels of gene expression in macrophages of C57BL/6ByJ and BALB/c mice we can determine if there are any candidate genes that are differentially expressed between the two strains and are located next to the markers that showed the highest linkage in our experiments. For

example, if there is a known transcription factor located around 80 Mb on chromosome 6, and it's expressed at a higher level in C57BL/6ByJ macrophages than in BALB/c macrophages, we can hypothesize that this factor controls expression of CD5l.

Once this gene or number of candidate genes have been identified, the next step could be to place them in a cell line and both inhibit as well as over express them. If inhibition of this gene affects expression of CD5l or CCR2 (target genes) in a negative way meaning lowering expression, this would allow one to determine that this is the gene that is differentially expressing that specific target gene which it was thought to be associated with. The same goes for over expression of the chosen candidate genes once placed in the cell line. If the target gene is overly expressed through over expression of the candidate gene, then this would indicate that this candidate gene is what is differentially expressing the target gene.

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