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## ISCHEMIC STROKE DAMAGE IS REDUCED BY INHIBITION OF IL-6 SIGNALING WITH TOCILIZUMAB

Jacob Hudobenko

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ISCHEMIC STROKE DAMAGE IS REDUCED BY INHIBITION OF IL-6  
SIGNALING WITH TOCILIZUMAB

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A

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Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

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of the Requirements

for the Degree of

MASTER OF SCIENCE

by

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Houston, Texas

August 2018

## ISCHEMIC STROKE DAMAGE IS REDUCED BY INHIBITION OF IL-6 SIGNALING WITH TOCILIZUMAB

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Abstract

### **Introduction:**

Stroke is the leading cause of serious long-term disability and is the 5<sup>th</sup> leading cause of death causing approximately 130,000 deaths in the United States every year {1}. Stroke is also the 2<sup>nd</sup> leading cause of death in American women {2}. Women are 33% more likely to require nursing home care than men, have a higher lifetime risk of stroke and are 13% less likely to receive thrombolytic (clot busting) treatment than men {2}. Interleukin 6 (IL-6) is a pro-inflammatory cytokine involved in the regulation of the immune system in multiple disease states such as rheumatoid arthritis and contributes to increased inflammation. In stroke patients, higher plasma IL-6 levels correlate with increased stroke severity and poor long-term prognosis {3}. Preliminary work in our lab has shown that tocilizumab, an FDA approved immunosuppressive drug that blocks IL-6 receptors to inhibit the pro-inflammatory effects of IL-6, reduced infarct size in young male mice following ischemic stroke. Based on this promising preliminary data, we now hypothesize that tocilizumab will reduce injury in aged male and female mice.

### **Methods:**

Experiments utilized aged (18-20 month) C57BL/6J male and female mice and examined neuroprotection and behavioral outcome after tocilizumab treatment. Mice were randomly assigned to the stroke or sham surgery group and then further subdivided into tocilizumab or IgG control treatment groups. Stroke was induced by 1 hour of transient right middle cerebral artery occlusion (MCAO) {6} under isoflurane anesthesia followed by reperfusion and then studied for 35 days. Four hours after the onset of ischemia, mice were given an intraperitoneal injection of

either tocilizumab (20mg/1kg) or an IgG control antibody. Behavior tests evaluated sensorimotor deficits, locomotor activity, working memory, gait and spatial learning {6, 9}. Mice were sacrificed and brain atrophy assessed using cresyl violet staining.

### **Results:**

Tocilizumab treatment reduced stroke damage by decreasing brain atrophy. In addition mortality and behavioral deficits were lessened in aged males on multiple behavior tests ( $P < 0.05$ ). Females did not show similar benefits with tocilizumab at a dose of 20mg/kg. However, tocilizumab did show acute neuroprotection in aged females at a dose of 100mg/kg three-days post-stroke, which is likely the result of higher levels of soluble IL-6 receptor in females post-stroke. The results support our hypothesis that inhibition of the IL-6 receptor is a potential therapeutic approach for stroke treatment.

### **Summary and Conclusions:**

Together, these results support our hypothesis that inhibition of the IL-6 receptor can be a novel therapeutic approach for stroke treatment. Interestingly, the neuroprotective dose of tocilizumab was different in males and females. This effect appears to be due to significantly higher soluble IL-6 receptor levels in females in the post-stroke phase and emphasizes the importance of determining efficacy in a sex-specific manner.

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## **Introduction**

Stroke is a devastating disease contributing to approximately 130,000 deaths annually in the United States {1}. While there has been a reduction in stroke mortality over the past decade, stroke remains the leading cause of serious long-term disability {1}. The destructive effect of stroke is more pronounced in women because they live longer and stroke is more prevalent with age {2}. Women have a higher lifetime risk of stroke, are more likely to be discharged to a nursing home and are less likely to receive thrombolytic (clot busting) treatment than men {2}. Currently there are approximately 7 million stroke survivors who live with severe disabilities because of stroke {1}. Stroke also contributes to a lower standard of living for those afflicted and the effects of stroke cost the United States approximately \$34 billion annually in related medical costs {1}.

The two main types of stroke are hemorrhagic and ischemic. Ischemic stroke, which accounts for 90% of strokes, is caused by obstructed blood flow in the vessels of the brain, usually because of a blood clot {1}. In a hemorrhagic stroke, ischemia is induced by a hemorrhage, or bleeding into the brain, leading to increased intracranial pressure. Increasing pressure reduces blood flow to affected parts of the brain and can lead to areas of ischemia.

There are currently very few treatment options for ischemic stroke. Tissue Plasminogen Activator (tPA) is one acute treatment option for ischemic stroke and works by dissolving the clot allowing the ischemic brain to be reperfused. This treatment however, is time sensitive and needs to be given within four and a half hours of stroke symptom onset or there is a high risk of hemorrhagic transformation, which can lead to worse outcomes and even death {6}. More recently, thrombectomy has been shown to be effective up to 24 hours following stroke onset {20}. Thrombectomy is most effective in a subset of patients with large vessel occlusion and core infarct below a specific threshold. This treatment option requires a specialized interventional team. Ultimately, these two treatment options still account for a minority of stroke patients, with the majority of patients limited to therapies aimed at reducing the risk of subsequent strokes. Due

to the limited current treatment options for ischemic stroke and morbidity to patients, there is an immediate need for new treatment options.

The time window for treatment also needs to be expanded beyond that of tPA treatment because many stroke patients, especially women, do not reach the hospital in time to receive tPA {2}. Although inflammation is a needed defense mechanism to protect from infection and clear debris after injury, the inflammatory response in stroke leads to worse outcomes for patients {3, 6, 7, 8, 9}. Since the inflammatory cascade post-stroke lasts longer than the effective therapeutic time window of tPA, targeting inflammation may be an effective new stroke therapy.

Levels of interleukin-6, a pro-inflammatory cytokine, are increased in the serum of stroke patients compared to controls. Higher levels of interleukin-6 in stroke patients correlate with larger infarct volume and worse long-term outcomes {3}. Interleukin-6 dysregulation contributes to autoimmune diseases, such as rheumatoid arthritis, and in fact a drug that targeted and diminished interleukin-6 signaling is already FDA approved for treating rheumatoid arthritis.

This drug, tocilizumab, is a humanized monoclonal antibody against the interleukin-6 receptor. The interleukin-6 receptor can be either membrane bound in a complex with the gp130 subunit or soluble. While the membrane bound form of the interleukin-6 receptor is found on limited cell types, such as neutrophils, monocytes, T-cells, hepatocytes and neurons, the gp130 subunit is found on most cell types. This means that soluble interleukin-6 receptor can allow more cells, that have a gp130 subunit but no membrane bound interleukin-6 receptor, to undergo interleukin-6 signaling. Interleukin-6 signals through the Janus activated kinase/Signal transducer activator of transcription pathway also known as the JAK/STAT pathway. Activation of the JAK/STAT pathway leads to the phosphorylation and activation of STAT3 which then translocates to the nucleus and upregulates genes responsible for inflammation, proliferation and apoptosis. Interleukin-6 signaling also leads to increased acute phase protein synthesis as well as stimulating production of neutrophils and supporting the growth of other immune cells like B and T-cells {13}. Increased interleukin-6 signaling leads to worse outcomes in stroke by promoting a

more pro-inflammatory environment through the activation and recruitment of peripheral immune cells to the site of injury. Tocilizumab binds to the interleukin-6 receptor, thereby blocking interleukin-6 so the associated inflammatory signaling is reduced, ameliorating rheumatoid arthritis symptoms and pathology {5}. Tocilizumab is therefore a possible promising new therapeutic candidate for ischemic stroke.

We hypothesized that tocilizumab treatment would reduce interleukin-6 signaling post-stroke reducing post-stroke peripheral inflammation leading to better stroke outcomes. Using this hypothesis, we designed a preliminary study to test whether tocilizumab treatment affected stroke outcomes acutely in young male mice after stroke. The results from this preliminary study in young male mice were promising, with reduced infarct volume with tocilizumab treatment, and were the basis for the project described in this thesis below. We examined long-term outcomes post-stroke in aged male and female mice treated with tocilizumab. Aged male and female mice were studied, as stroke is a sexually dimorphic disease being more prevalent and damaging in women most frequently in the elderly population {2}.

## **Methods/ Materials**

### **Animals**

This study utilized mainly aged (18-20 months old) male and female C57Bl/6J mice from Jackson laboratories along with a small preliminary cohort of young (3-4 months old) male C57Bl/6J mice, also from Jackson laboratories. Mice were housed at the McGovern Medical School in pathogen free housing and had access to water and food ad libitum. The young males were ordered from Jackson at approximately 2 months of age and were given a month to acclimate before use. The aged male and female mice were ordered at approximately 6-9 months of age and were kept in the animal facility until they were 18-20 months old. All procedures were performed in accordance with NIH guidelines for the care and use of laboratory animals and were

approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center. All mice utilized in this study were randomly assigned to both the surgical group and treatment group.

### **Drug dosing**

For the 35-day study aged male and female study along with the three-day young male study, mice were injected with either tocilizumab or IgG control intraperitoneally four hours after stroke onset at a dose of 20mg/kg. A cohort of aged females also received a dose of 100mg/kg of either tocilizumab or IgG control four hours post-stroke to analyze infarct volume three-days post-stroke.

### **Euthanasia**

Mice were euthanized at the 3 and 35-day post-surgery endpoints of the study, or earlier if instructed by the veterinarians in the animal facility, using an overdose of avertin (2,2,2-Tribromoethanol) injected intraperitoneally. The mice were confirmed to be under anesthesia by pinching the tail and paws with forceps before subsequent tissue harvesting. During subsequent tissue harvesting the mice had their brain and blood removed for analysis.

### **Transient stroke model**

All mice were randomly assigned to either stroke or sham surgical groups, which were then subdivided into either drug or vehicle treated groups. Thus, there were four groups for each cohort. Transient cerebral ischemia was induced by 60 minutes of reversible middle cerebral artery occlusion (MCAO) surgery performed in this study by Dr. Anjali Chauhan, a post-doctoral researcher in the McCullough lab. The MCAO is a sterile surgery where a midline incision is made in the ventral portion of the neck and then fat and muscle tissue is carefully separated to

reveal the right internal carotid artery. A monofilament (Doccol Corporation, Sharon, MA) is then inserted into the right internal carotid artery and advanced up to occlude blood flow to the right middle cerebral artery. After surgery, the mouse was placed into a recovery cage (with heating pad) for one hour until the mouse underwent a separate reperfusion surgery in which the monofilament was removed and blood flow was reestablished to the middle cerebral artery. While under anesthesia during both surgeries rectal temperatures are monitored and maintained at 37 degrees Celsius. Following the reperfusion surgery, the mice recovered with a heating pad placed under one half of the cage to aid the mouse in thermoregulation. Sham surgery control mice underwent the same surgical procedure with the exception that the monofilament was not used, so no occlusion took place. Four hours after surgery, the mice were given an intraperitoneal injection of either tocilizumab at a dose of 20mg/kg, 100mg/kg (high dose aged female cohort) or a vehicle injection of a normal human IgG control (R&D systems, Minneapolis, MN). The mice were then given daily subcutaneous injections of sterile saline and wet mash food for seven days after reperfusion. This post-surgical care regimen helps the mice recover and reduces mortality from dehydration, especially in aged mice post-stroke surgery. The mice were sacrificed at either three or thirty-five days post-surgery.

### **Behavioral testing**

We performed a battery of behavioral tests on the mice including, neurological deficit scoring, the Corner test, the Digigait, the Y-maze, the Barnes maze, and the Open-field test. Before the study began all mice were tested on the Y-maze, Corner and Open-field test to ensure no baseline biases existed. If the mice exhibited any bias they were not used in the study. All behavior tests were conducted and analyzed by investigator blinded to the treatment group.

### ***Neurological deficit scoring:***

Neurological deficit score was observed and recorded immediately after surgery and was also observed and recorded post-surgery every day for the first week post-surgery (day 1-7), day 10, day 14, day 20, day 28, and day 35 post-surgery before sacrificing the mice in the 35-day survival cohort. The score was taken every day in the 3-day survival cohort.

The neurological deficit score is a five-point stroke severity scale based on the researcher's observations of the mouse. Neurological deficit is scored from 0 to four with four being the highest level of deficit and 0 being no deficit detected. The scoring was recorded as follows: 0, no deficit detected; 1, torso turning to ipsilateral side when the mouse is held by the tail; 2, when mouse is held by the tail so that front legs are comfortably on the ground but the back legs are in the air the mouse circles only towards its affected side; 3, mouse spontaneously circles to affected side (cannot walk in straight line); 4, no spontaneous locomotor activity or if the mouse is barrel rolling (most severe deficit).

***Corner test:***

The corner test is a test for sensorimotor function. It examines both the stimulation of the vibrissae (whiskers), which is the sensory component and also the rearing, and turning away from the corner, which is the mouse's motor response. In the corner test, we used two boards (30x20x1 cm) that were placed in front of the mouse at a 30-degree angle with a tiny opening between them so that the mouse walks into the corner to escape. Once the mouse enters the corner, this hole is closed but the boards are still kept at a 30-degree angle. The small angle means that both sides of the mouse and therefore all the vibrissae (whiskers) should be stimulated. This causes the mouse to rear upward and turn away from the corner so that it is facing the open end of the two boards "escaping" the corner. The direction of the turn, either left or right, was recorded after each trial for a total of twenty trials per mouse. The percentage of right turns was calculated for each mouse. A mouse with no sensorimotor deficit should have close to a 1:1 ratio of left and right turns but a mouse with a deficit caused by the right middle cerebral artery occlusion surgery

would have a greater proportion of right turns than would be expected by chance alone. The corner test, like the Y-maze, was initially performed 7 days prior to surgery to ensure no initial biases in the mice. Two aged male mice had initial biases on the Corner test so were not used in this study. The corner test was performed on day 7, 14 and 20 post-surgery.

***Digigait:***

Digigait is a behavioral test used to examine differences in gait between animals at different speeds and inclines. We utilized digigait on day 21 post-surgery to examine long-term gait differences between experimental conditions. For this study, an incline of 0-degrees and a final belt speed of 10 cm/second was used. The mice were placed on the transparent treadmill, with a camera recording from below as seen in the picture in Figure 1. The belt speed began at 2 cm/second, and as the mouse became more comfortable on the behavioral apparatus, the speed was slowly increased to the testing speed of 10 cm/second. Once the speed reached 10cm/second video was recorded from below until the mouse walked continuously for 10 steps. Mice that were not able to complete the task because they could not continuously walk at 10 cm/second had their failure recorded and they were removed from the equipment to limit unnecessary stress. In our study two IgG control treated stroke male mice were not able to complete this behavior test. The recorded videos were then manually edited to shorter time segments and analyzed by the Mouse Specifics behavioral software to examine any long-term gait deficits post-surgery.

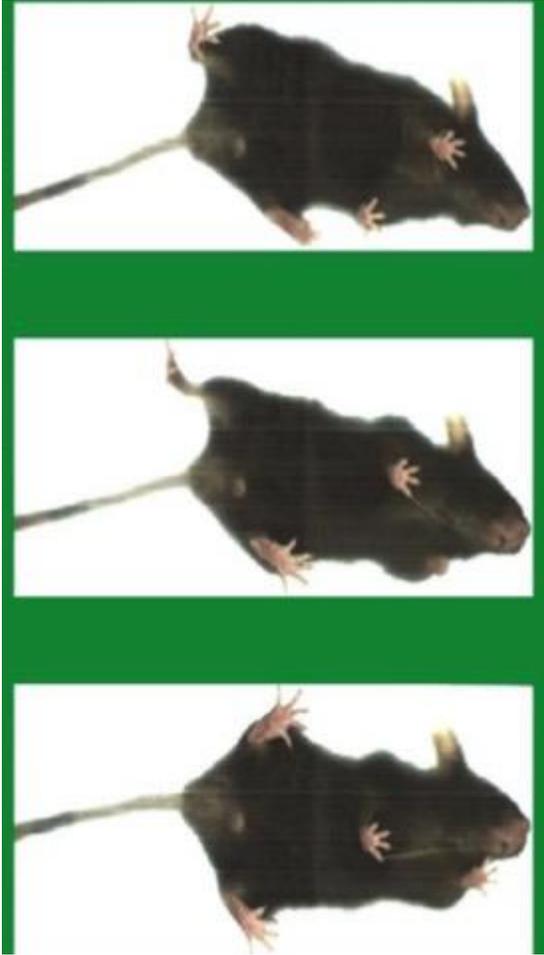


Figure 1: Picture of mouse on Digigait testing apparatus

***Y-maze:***

The Y-maze is a non-invasive behavioral test that examines short-term working memory. A picture of the Y-maze can be seen in Figure 2 below. During the behavioral test the mouse is first placed in the center of the Y-maze, where the three arms meet, with removable doors initially blocking off each arm. The test begins when the doors are removed and the mouse is allowed to move about and explore the Y-maze for five minutes. These five minutes are recorded from a camera mounted above the maze. The video is then analyzed by Noldus behavioral software (Leesburg, VA) to examine the amount of correct alterations the mouse completes and the errors that it makes. A correct alteration is based off of the idea that mice preferentially explore novel

areas. A correct alteration therefore is when the mouse travels from arm A to arm B to arm C or arm B, to C to A etc. An error is recorded as a direct revisit error if, for example, a mouse that enters arm A returns to the middle of the maze and then re-enters arm A without going into any other arms first. The Y-maze was initially performed 7 days prior to surgery to ensure no initial biases in the mice. No preliminary biases were seen in the mice used for this study. The Y-maze was performed on day 7 and 20 post-surgery.

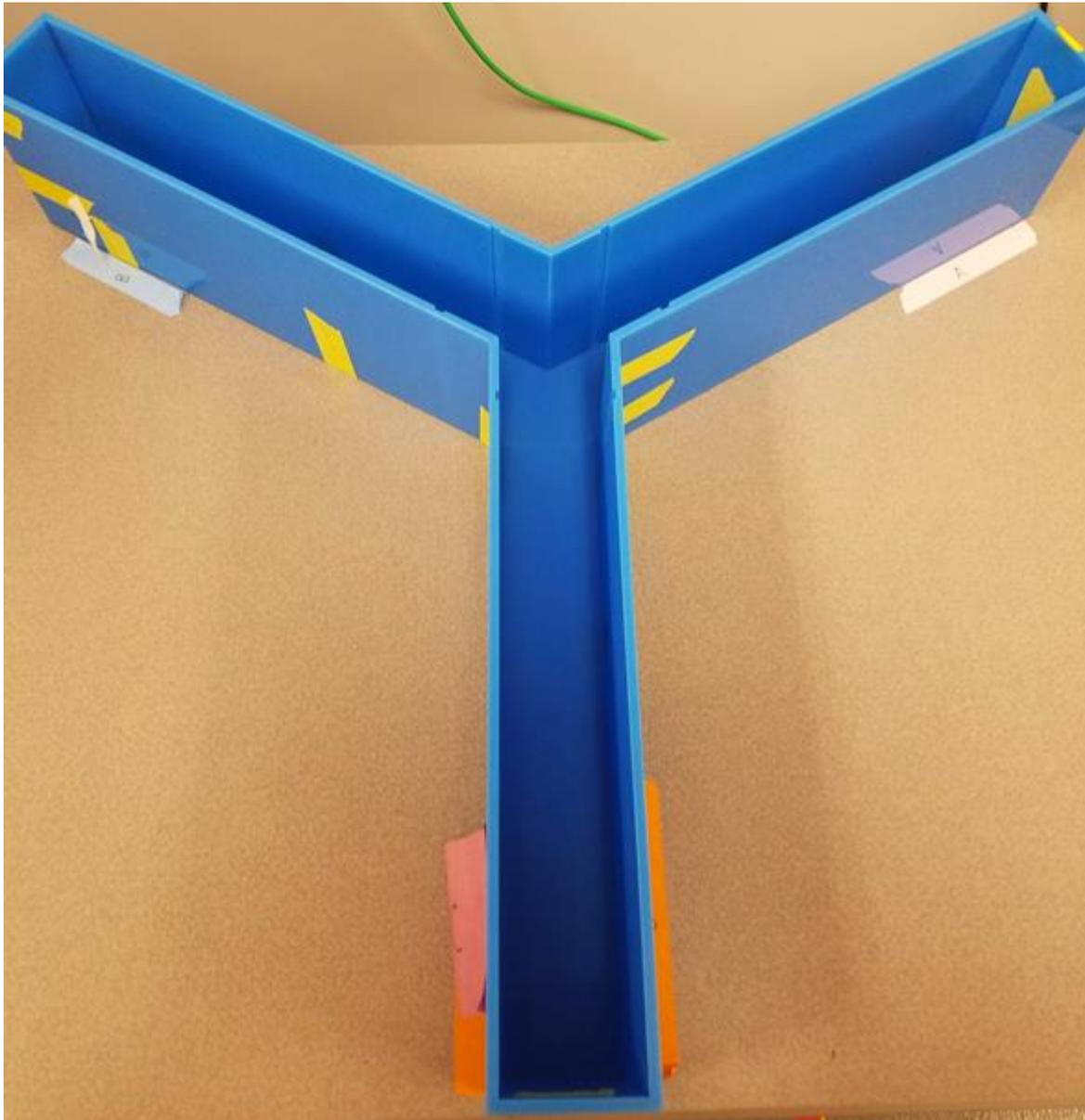


Figure 2: Picture of the Y-maze

***Barnes Maze:***

The Barnes maze is a non-invasive spatial learning and memory test based on the intrinsic tendency of rodents to escape an aversive stimulus as fast as possible. The Barnes maze (as can be seen in Figure 3 below) is an elevated circular platform with 20 equally spaced holes around its perimeter. One of the holes labeled below as the “goal box” has a platform underneath so that the mouse can climb down and hide in the darkness. Surrounding the Barnes maze on the walls (not shown in the picture below) are visual cues for orientation to allow the mouse to find the escape hole once it has learned where it is. During the training period, the lights were turned off in the room except for overhead lights directed at the maze to serve as an aversive stimulus to encourage the mouse to enter the escape hole. During the second and third training days, along with the testing phase, a buzzer was added as a negative stimulus to encourage the mice that remembered where the escape hole was to go to the hole quickly and enter it.

To examine long-term cognitive outcomes, training and testing took place over five days starting on day 27 through day 31 post-surgery. For the first three days (days 27-29) the mice were trained on the Barnes maze. During the first day of training the mice were put in a clear container in the center of the Barnes maze for 30 seconds so that they could visualize their surroundings. Once the 30 seconds had elapsed, the container with the mouse inside was slowly moved from the center of the maze to the escape hole with extra care taken not to stress the mouse. The mouse was left in the container until it entered the escape hole itself or 3 minutes had elapsed at which point it was nudged into the hole and left there for 1 minute. The second day of training the mouse was put in an opaque container in the center of the maze for 10 seconds so that they could not visualize their surroundings. The container was then lifted and a buzzer was turned on and the mouse was allowed to explore the maze on its own for three minutes or until it found and entered the escape hole itself. If the mouse did not enter the hole by the end of the three

minutes the mouse was covered with a clear container and slowly moved to the escape hole and left there until it entered the hole or three additional minutes expired after which the mouse was gently nudged into the hole and left there for one minute. Once the mouse entered the hole the buzzer was shut off for the minute the mouse was in the hole. This process was repeated three times for each mouse. The third day of training was the same as the second day but training was repeated twice rather than three times for each mouse. At the end of the third day of training if the mouse could not find the escape hole it was excluded from the study. One IgG control treated stroke male mouse was excluded as it could not find the escape hole at the end of the training. The fourth day the mice were allowed to rest.

The fifth day was the testing day and the testing trial was recorded from a camera mounted above the Barnes maze and analyzed using Noldus behavioral software. The test was similar to the second and third days of training where the mice were placed into an opaque container for 10 seconds in the middle of the maze so that they could not see anything around them. The container was then lifted, the buzzer turned on and the mouse was allowed to explore the maze freely for three minutes. There was only one testing trial per mouse. This trial was recorded and analyzed to determine if the mouse found the escape hole within the three minutes, how long it took them to find the escape hole, and how long they spent moving or not moving.

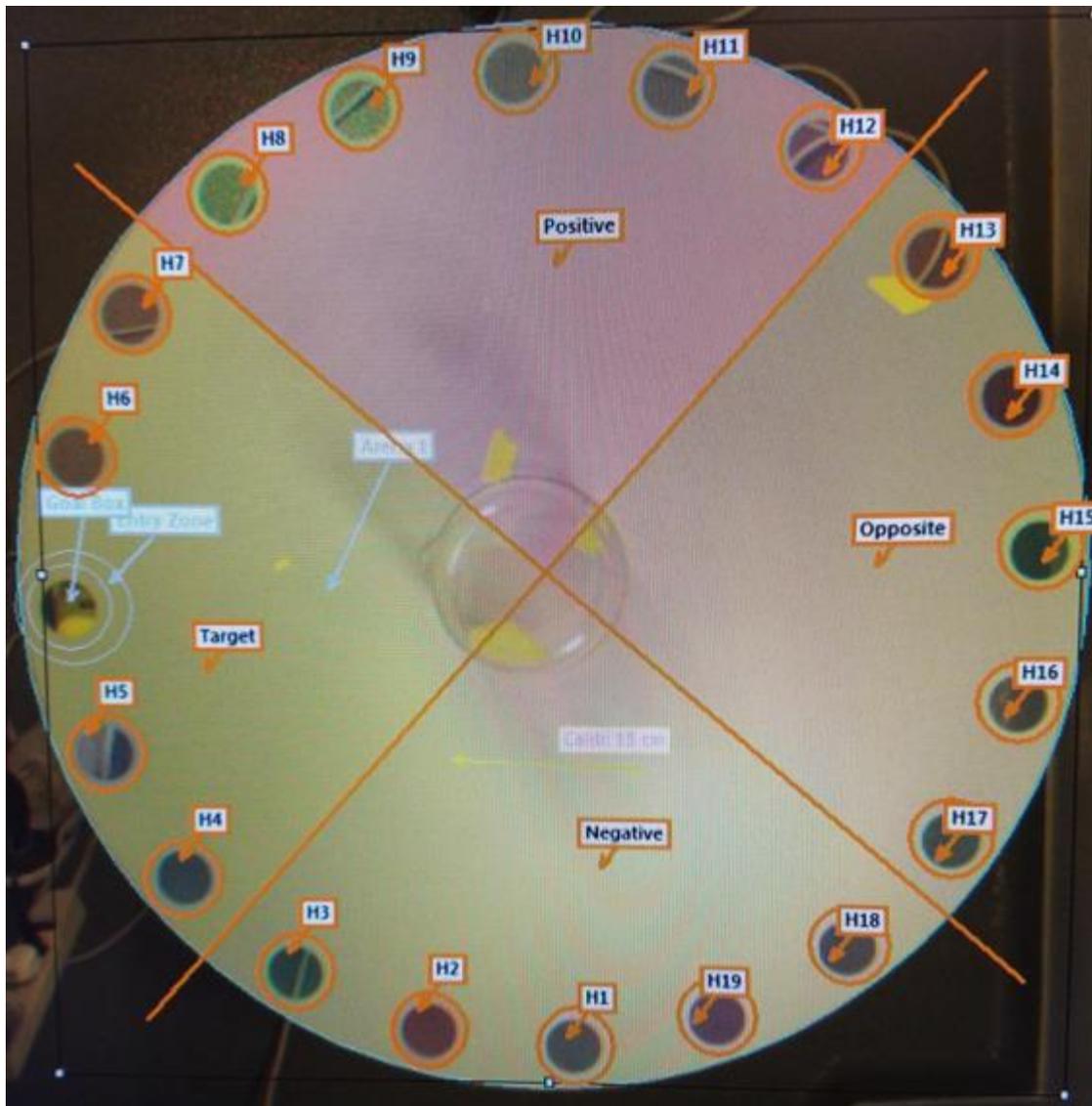


Figure 3: Picture of the Barnes maze

***Open-field test:***

The open-field test is a test of both general locomotor activity and anxiety like behaviors. The open-field, shown below in Figure 4, is a well-lit box where the mouse is free to explore. Mice with more anxiety will move less and spend more time in the periphery of the maze. Mice that are not anxious will move around more and spend more time exploring the entire open-field and therefore spend more time in the center of the open-field compared to anxious mice. During

testing mice are first placed in the upper left corner of the open-field box and allowed to freely explore for 20 minutes. The entire period is recorded by a video camera mounted above the open-field. The analysis of this study, using Noldus behavioral analysis software of the open-field data, focused on both the first five minutes of the test and the entire 20 minutes of the test to see if there were any differences between groups. The open-field test, like the Y-maze and corner test, was initially performed 7 days prior to surgery to ensure no initial biases in the mice. No preliminary biases were seen in the mice used for this study. The open-field test was performed on day 7 and 20 post-surgery.

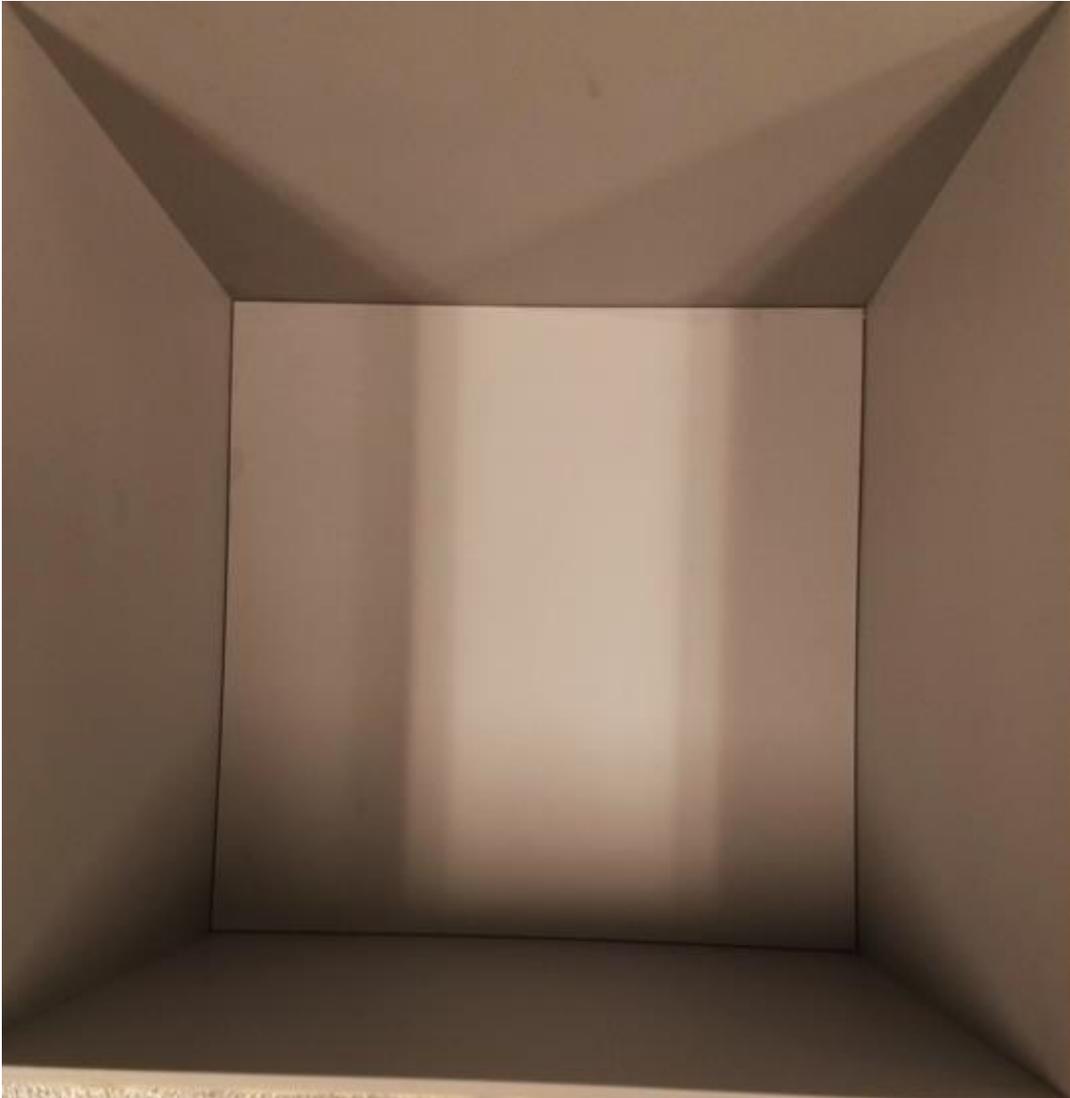


Figure 4: Picture of the Open-field testing arena

### **Tissue harvesting**

At the conclusion of the study at either 3 or 35-days post-surgery the mice were euthanized as previously described above and their tissues were harvested. After being put under anesthesia using an intraperitoneal injection of an overdose of avertin, the mice had their blood drawn using an external heart stick with a heparinized 25-gauge needle to remove between 500 and 1,000 microliters of blood. This blood was transferred to a 1.5 mL Eppendorf tube and put on ice until all of the mice in that group had their blood drawn (no longer than 1-2 hours). At which

point the blood was then transferred to a centrifuge maintained at four degrees Celsius and spun for 14 minutes at 14,000 rotations per minute. The supernatant plasma was then transferred to a fresh Eppendorf tube and kept at -80 degrees Celsius until it was used for subsequent ELISA experiments.

The mouse then underwent transcardial perfusion with at least 60mL of cold sterile 1X heparinized (0.2%) phosphate buffered saline (PBS). Care was taken to use the same hole in the heart as was initially used to remove the blood to reduce the possibility of leakage, to avoid incomplete perfusion. The liver was visualized while transcardial perfusion was taking place to approximate when the mouse was well perfused. Brains used for TTC (2,3,5-triphenyl-2H-tetrazolium chloride) were then removed.

Mice used for cresyl violet infarct and atrophy analysis were also transcardially perfused with 60 mL of 4% paraformaldehyde (PFA) in 1X PBS to fix the brain. After the PBS and PFA perfusion was complete, the brain was removed and kept in a 30% sucrose solution at 4 degrees Celsius for two days to dehydrate the tissue. The brains were then kept at 4 degrees Celsius for two+ days in a 4% PFA solution. The brains were sectioned on a microtome into 30-micrometer thick coronal sections for long-term storage in ethylene glycol at -20 degrees Celsius until being used.

### **2,3,5-triphenyl-2H-tetrazolium chloride (TTC) staining**

TTC staining measured the infarcted or damaged area of the brain in the 3-day post-surgery endpoint cohort. After the mice were euthanized and perfused with 1X-heparinized PBS the brains were removed and put in the freezer at -20 degrees Celsius for approximately 30 minutes. This procedure hardens the brain for easier sectioning. Once the brain was firm, it was sectioned into five 1.5-2.0 millimeter sections and placed into fresh TTC solution, covered with aluminum foil to protect it from light and kept at 37 degrees Celsius for 10 minutes. After 10 minutes the sections were visually examined to make sure red color had developed. Once color

was confirmed the sections were transferred to a 4% PFA solution overnight at 4 degrees Celsius, after which images were taken of each section for infarct measurement.

The 1.5% TTC solution was prepared fresh in sterile 1X PBS. For example, 0.225 grams of TTC powder would be dissolved in 1X PBS to make 15 mL of TTC solution.

The infarct volume on each image was then measured using Sigma Scan Pro software. As can be seen in Figure 5 below, the red portion of the brain slice is live tissue and the white area is the infarcted tissue. Measurements between each hemisphere of the same brain on multiple sections were compared to help determine the percentage of infarcted tissue for the ipsilateral side (stroke side) based on structure size on the contralateral side for each mouse. These percentages were then compared between tocilizumab and IgG control treated male and female mice subjected to stroke.

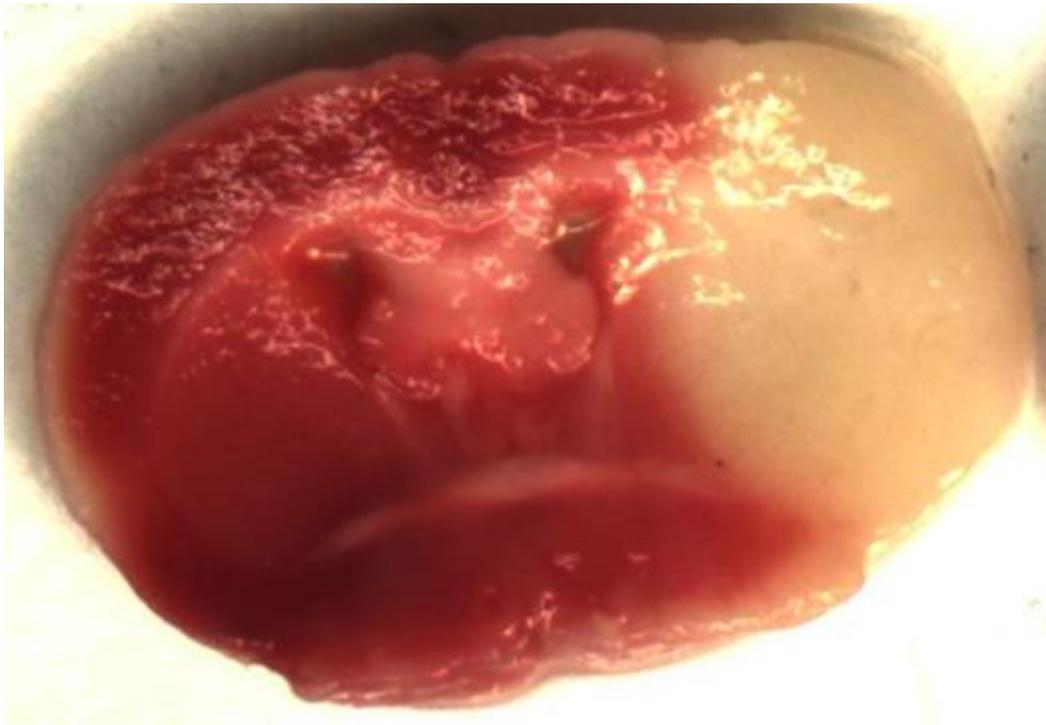


Figure 5: TTC image, red is live tissue and white is infarct volume

### **Cresyl Violet staining**

Cresyl violet staining measured 35-day post-surgery brain atrophy; a picture of this stain can be seen in Figure 6. After brains were perfused, dehydrated and preserved in PFA the brains were sectioned on a microtome into 30 um sections. Eight equally spaced sequential sections (360 um apart) were then mounted onto a microscope slide and stained with cresyl violet solution. Images were taken of the cresyl violet stained tissues and these images were analyzed using Sigma Scan Pro software (San Jose, CA) to calculate the percentage of atrophy in the ipsilateral hemisphere compared to the contralateral hemisphere. These percentages were then compared between tocilizumab and IgG control treated male and female mice subjected to stroke.

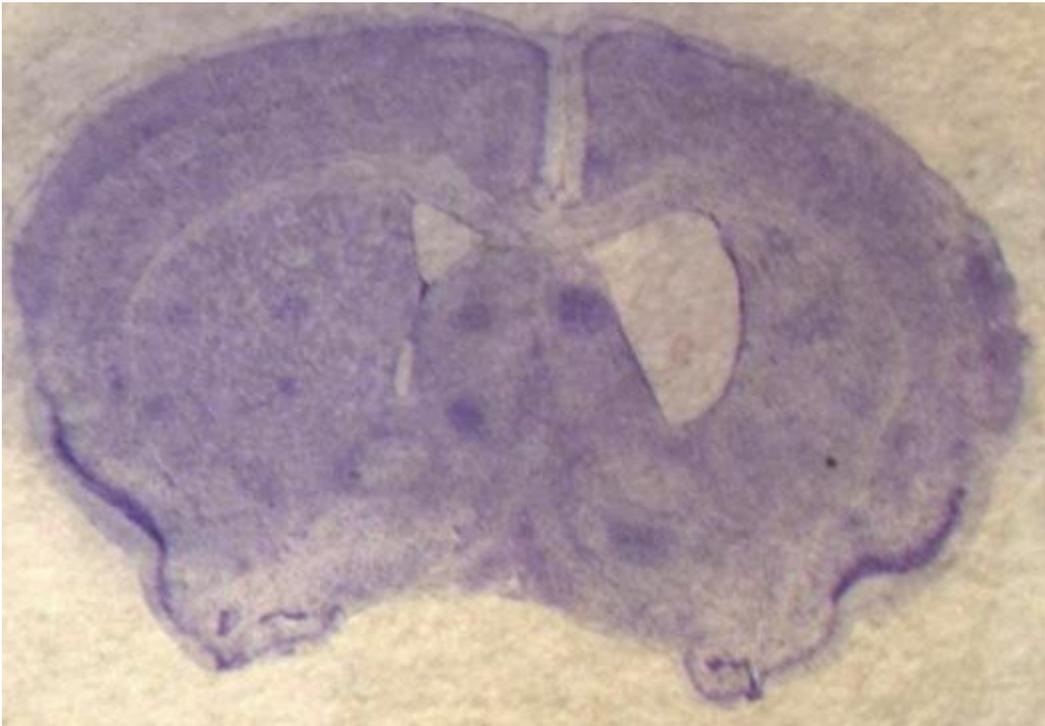


Figure 6: Cresyl violet staining image showing brain atrophy 35-days post-stroke

### **Enzyme linked immunosorbent assays (ELISA)**

This study used both human and mouse ELISA kits to measure plasma levels of lipopolysaccharide binding protein (LBP) and also plasma levels of soluble interleukin-6 receptor levels. The ELISA was run based on the instructions of the manufacturer when first establishing the correct plasma dilution for each ELISA and then again when completing the assays.

### **Gut permeability test**

Gut permeability of mice was tested 3-days post-surgery using a 4,000 Dalton Fluorescein isothiocyanate (FITC) dextran, made by dissolving 50 mg FITC dextran in 1 mL of sterile water. On the morning of the third day post-surgery the mice were fasted for 5-hours. Mice were then orally gavaged 6 mg FITC dextran/ 10 grams of body weight. One hour after oral gavage the mice were euthanized and their blood was drawn using an external heart stick with a heparinized needle. This blood was transferred to a 1.5 mL Eppendorf tube and put on ice until all mice in that group had their blood drawn (approximately 1 hour). This blood was then transferred to a centrifuge maintained at four degrees Celsius and spun for 14 minutes at 14,000 rotations per minute. The supernatant plasma was then transferred to a fresh Eppendorf tube and protected from the light with aluminum foil.

Fifty microliters of plasma from each sample was then added to a 96-well plate in duplicate, along with a blank water control, a normal mouse plasma sample control (no FITC gavage), and seven FITC standards ranging from 16 mg/mL to 0.001024 mg/mL of FITC dextran; all in duplicates. The level of FITC dextran in each plasma sample was then measured at an excitation wavelength of 470 nm and emission wavelength of 520 nm, using an EnSpire plate reader. The seven standards, along with the two blank controls, were used to create a standard curve in Microsoft Excel to find the amount of FITC dextran. Higher levels of plasma FITC dextran correlates with a higher level of gut permeability.

### **Lung bacterial colony-forming units (CFUs) measurement**

The level of bacterial infection in mice post-surgery was assessed at 35-days post-surgery using both plasma LBP levels and the amount of bacterial colony forming units found in lung homogenates. The mice were transcardially perfused with sterile 1X PBS and one lung lobe was removed (same lobe from each mouse). The lungs were weighed and suspended in a sterile 1% saponin 1X PBS solution at 50 mg of lung tissue per 1 mL saponin solution. This tissue was then gently homogenized in the saponin solution. Fifty microliters, or the equivalent of 2.5 mg of lung tissue, of this homogenate was then plated on a 5% sheep blood agar plate (Carolina Biological Supply, Burlington, NC) and incubated at 37 degrees Celsius for 16 hours after which the number of colony-forming units (CFUs) was counted. The number of CFUs counted on the blood agar plates after the 16-hour incubation was then multiplied to get the number of CFUs per mL of saponin solution, in order to express the lung bacterial burden.

### **Temperature Probe Insertion**

Three days prior to stroke or sham surgery all mice underwent a temperature probe (IPTT300 BMDS, Seaford, DE) implantation surgery. Prior to implantation all temperature probes were tested three times at three different temperatures (23, 37 and 45 degrees Celsius) to ensure accuracy. Temperature probes were implanted subcutaneously in the back of the mouse between the anterior shoulder blades. After the mouse was anesthetized and a proper sterile field constructed, a small incision was made between the anterior shoulder blades. A sterile blunt pair of scissors was then inserted into the hole and advanced towards the posterior of the mouse. The scissors were then opened slightly to provide a space under the skin for the temperature probe to rest. The scissors were then closed and retracted out of the incision. The temperature probe was then implanted by placing the large gauge syringe that held the probe into the incision and then pushing the probe into place. Physical manipulation was then applied if the probe did not sit perfectly between the shoulder blades. The incision was then sutured shut and the mouse was taken off of isoflurane and put in a recovery chamber for observation. Before mice underwent the

sham or stroke surgery three days later they were checked for weight loss or temperature variation, either hypothermic or hyperthermic. Exclusion criteria were established to omit any mouse that deviated from normal weight or body temperature; no mice needed to be excluded based on these criteria.

### **Statistical data analysis**

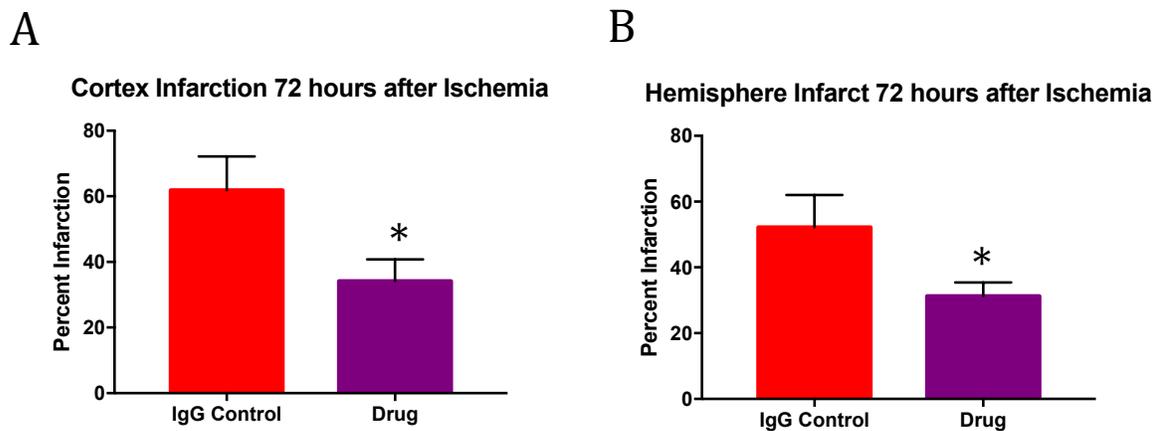
A two-tailed unpaired T-test was used for young male infarct volume and for high dose aged female infarct volume. A one-way ordinary ANOVA with Sidak's multiple comparison test was used for the Y-maze, Digiait, 35-day atrophy, interleukin-6 receptor ELISAs (human and mouse), LBP ELISA and lung Colony-forming unit counts. A log-rank Mantel Cox test was used for both mortality and the Barnes maze. Lastly, a 2-way ANOVA with Tukey's multiple comparisons test was used for the Corner test. Results were considered statistically significant if  $P < 0.05$ .

## **Results**

### **Administration of tocilizumab reduced brain infarct volume acutely in young male mice**

To test tocilizumab's effectiveness as a treatment for ischemic stroke we first subjected young male mice (2-3-month-old C57BL/6J from Jackson labs) to 60 minutes of cerebral ischemia followed four hours later by an intraperitoneal injection of either tocilizumab (20mg/kg) or a human IgG control. After three days, neurological deficit scoring, open-field testing and the corner test was performed before sacrifice and infarct analysis. Treatment with tocilizumab led to a 21% reduction in brain infarct volume three days post-stroke compared to IgG control treated young males in both the cortex and total hemisphere (\* P = 0.0279 and 0.0443) as can be seen in **Figure 7**.

### **Administration of tocilizumab reduced brain infarct volume acutely in young male mice**



**Figure 7:** Young male infarct volume three-days post-stroke. Both cortex and total hemisphere infarct volume were significantly reduced with tocilizumab treatment. A) Cortex infarct. B) Total hemisphere infarct. N= 9 and 11, IgG Control and Drug, respectively. \* P= 0.0279 for cortex and 0.0443 for hemisphere, using a two-tailed unpaired T-test.

## **Administration of tocilizumab improved motor and sensory behavioral outcomes in aged male mice post-stroke**

Because stroke is more prevalent with aging {1} and is sexually dimorphic {2} we tested whether tocilizumab would reduce behavioral deficits after stroke in aged male and female mice (18 to 20 month C57BL/6J, Jackson labs). Mice were subjected to either 60 minutes of cerebral ischemia via the MCAO surgery or a sham surgery control. Four-hours after initiation of reperfusion, mice received an intraperitoneal injection of either tocilizumab (20mg/kg) or a human IgG control. Mice were then studied over the next thirty-five days, during which a battery of behavior tests were performed.

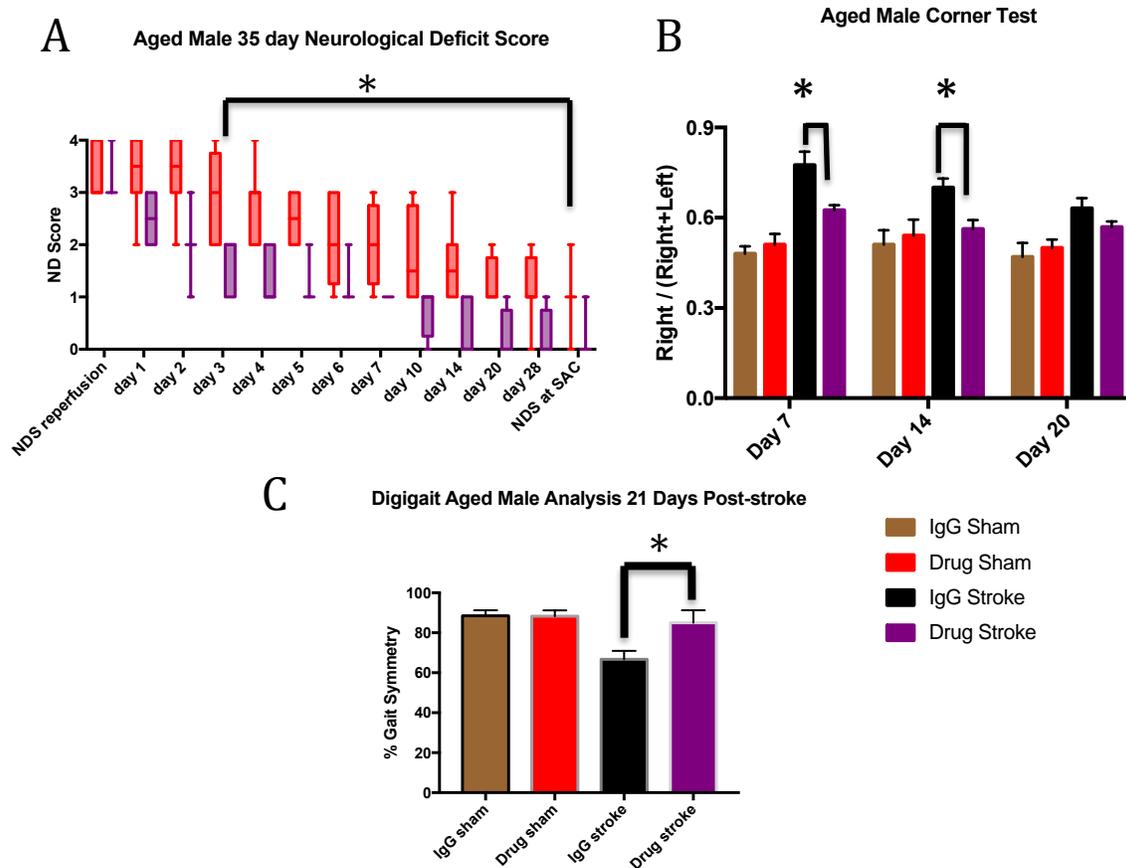
Neurological deficit scores were tested frequently throughout the study. A significant difference ( $P < 0.05$ ) was seen between the tocilizumab and IgG control treated aged males post-stroke, where tocilizumab treatment led to faster improvements in recovery, starting on day three post-stroke and continuing until the end of the study (**Figure 8A**).

On the corner test, which measures sensorimotor function, aged males treated with tocilizumab post-stroke had significantly reduced deficits on testing days 7 and 14 ( $P = 0.0059$  and  $0.0136$ ) compared to IgG control treated males (**Figure 8B**).

Gait symmetry, analyzed on the Digigait, was consistent between both male sham surgical groups and tocilizumab treated males post-stroke. Gait symmetry was significantly reduced in IgG control treated males post-stroke compared to tocilizumab treated males (\*  $P = 0.0260$ ), which helps to show the benefits of tocilizumab treatment after stroke (**Figure 8C**).

All three of these behavior tests were also performed post-stroke on aged female mice. In contrast to the males, the 20mg/kg tocilizumab dose showed no significant benefit in females.

## Administration of tocilizumab improved motor and sensory behavioral outcomes in aged male mice post-stroke



**Figure 8:** Motor, mobility and sensory deficits were improved in aged males post-stroke with tocilizumab treatment compared to IgG control treatment. N= 9, 8, 5, and 5 for drug treated stroke, IgG control treated stroke, drug treated sham and IgG control treated sham respectively. A) (Red: IgG control, purple: tocilizumab) Tocilizumab treatment led to significantly reduced neurological deficit scores starting on day three post-stroke and remained significant till the conclusion of the study on day 35. \* P<0.05, Two-way ANOVA Sidak's multiple comparison test. B) Sensorimotor deficits were significantly reduced with tocilizumab administration on testing days 7 and 14 post-stroke. \* P= 0.0059 and 0.0136 respectively, Two-way ANOVA Tukey's multiple comparison test. C) Gait symmetry was significantly worse in IgG treated stroke controls compared to both the tocilizumab treated stroke aged males and both sham surgery groups 21-days post-surgery. \* P= 0.0260, One-way ordinary ANOVA with Sidak's multiple comparison test.

## **Administration of tocilizumab improved cognitive behavioral outcomes in aged male mice post-stroke**

Cognitive impairment occurs in 20-80% of stroke patients {10}. While there is not a complete understanding of the mechanisms after stroke that contributes to cognitive impairment, damage to key areas of the brain, such as the hippocampus, contribute to cognitive decline {10}. We tested our experimental groups on the Y-maze to examine working memory and the Barnes maze to examine memory consolidation.

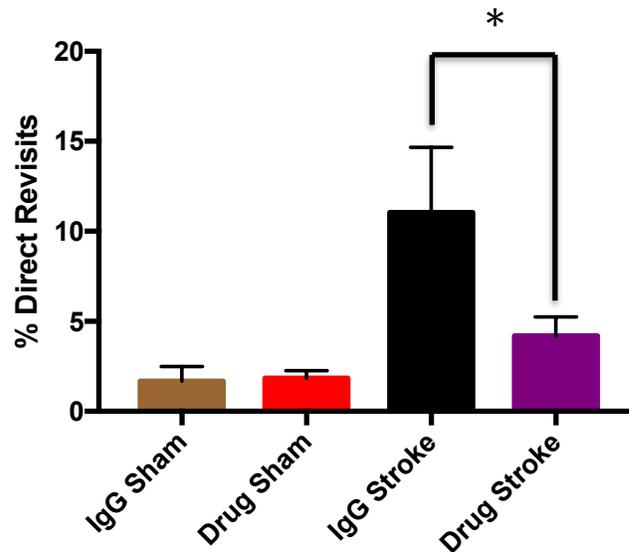
On the Y-maze 7-days after surgery the percent direct revisit error was examined as a measure of working memory. In this test, a high percent of direct revisit error correlates with worse working memory. We found that post-stroke, tocilizumab treated aged males had a percent direct revisit error consistent with both sham surgical groups. Post-stroke IgG control treated males had a significantly higher percent direct revisit error compared to both sham surgical groups and the tocilizumab treated stroke males ( $P= 0.0135$ ) (**Figure 9A**).

For the Barnes maze, mice were tested for memory consolidation two days after completing training. During testing the majority of males from both the drug and IgG control sham surgical groups along with the tocilizumab treated stroke group were able to find the escape hole within the allotted three minutes (80%, 100% and 75% respectively, within the first minute of the test). However, significantly fewer (12.5%) of the IgG control treated stroke males were able to find the escape hole within the three minutes allotted, and those that did took much longer (approximately 2 minutes) than the tocilizumab treated stroke and both sham groups. This led to a significant difference in escape from the Barnes maze between the tocilizumab treated and IgG control treated males post-stroke ( $P= 0.0081$ ), suggesting that tocilizumab treatment reduces post-stroke cognitive impairment (**Figure 9B**).

Administration of tocilizumab improved cognitive behavioral outcomes in aged male mice post-stroke

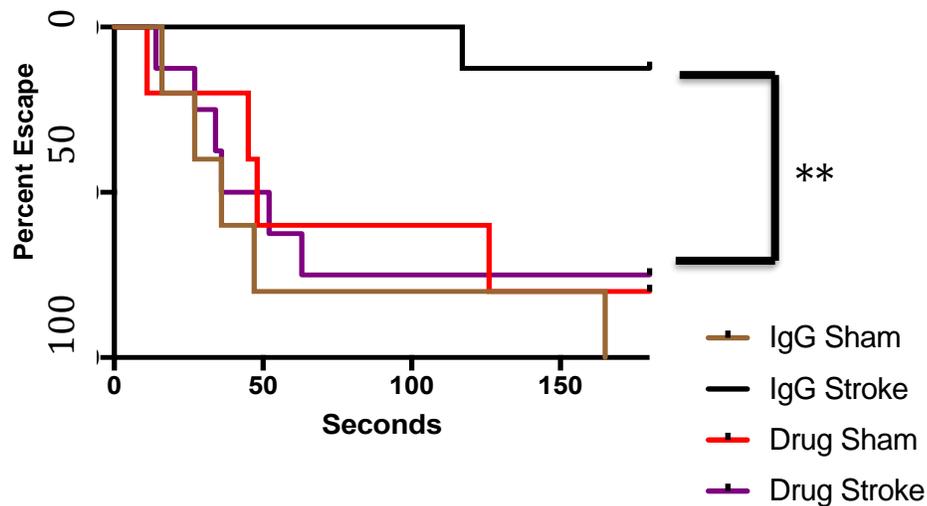
A

Aged Male Percent Direct Revisit Error



B

Aged Male Barnes Maze Escape



**Figure 9:** Working memory on the Y-maze 7-days post-stroke and memory consolidation on the Barnes maze 31-days post-stroke was significantly improved with tocilizumab treatment in aged males. A) Percent direct revisit error on the Y-maze 7-days after surgery was significantly higher in the IgG control treated stroke group compared to all of the other three experimental groups. \*P= 0.0135, One-way ordinary ANOVA with

Sidak's multiple comparison test. B) On the Barnes maze 31-days after surgery the percentage of IgG control treated stroke males who could locate the escape hole within the 3-minute testing trial was significantly lower than all of the other three experimental groups. **\*\*P= 0.0081**, long-rank Mantel Cox test. Tocilizumab treated stroke males were not statistically different from the two sham surgical groups.

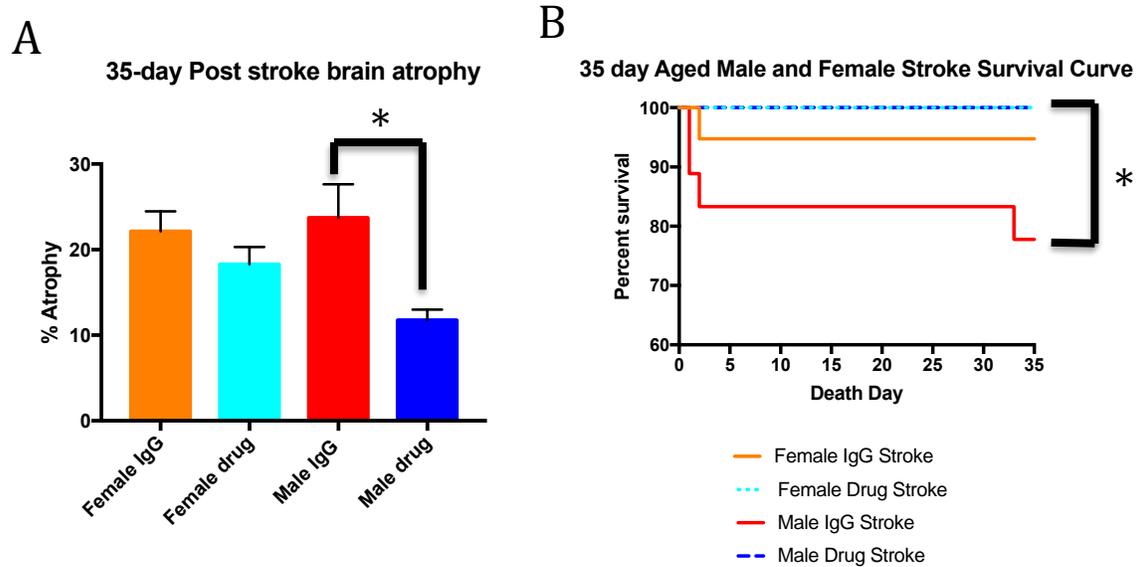
	<b>Males</b>	<b>Females</b>
<b>Open-field test</b>	<b>Not Significant</b>	<b>Not Significant</b>
<b>Neurological Deficit Score</b>	<b>*P&lt; 0.05</b>	<b>Not Significant</b>
<b>Tail-Suspension</b>	<b>Not Significant</b>	<b>Not Significant</b>
<b>Corner Test</b>	<b>**P= 0.0059 and *P= 0.0136</b>	<b>Not Significant</b>
<b>Digigait</b>	<b>*P= 0.0260</b>	<b>Not Significant</b>
<b>Y-maze</b>	<b>*P= 0.0135</b>	<b>Not Significant</b>
<b>Barnes maze</b>	<b>**P= 0.0081</b>	<b>Not Significant</b>

**Table 1:** Review of behavior tests in the 20mg/kg tocilizumab 35-day post-stroke study. There were no significant differences between the IgG control treated and tocilizumab treated aged stroke females. Multiple behavior tests had significant differences between the IgG control treated and tocilizumab treated aged stroke males, which are marked in red.

#### **Administration of tocilizumab reduced brain atrophy and mortality in aged male mice chronically**

Beyond behavioral improvements tocilizumab treatment led to significantly reduced brain atrophy 35-days post-stroke ( $P = 0.0120$ ) and mortality over the course experiment ( $P = 0.0239$ ) in aged males (**Figure 10A and 10B**). In aged females there was no significant difference seen between the two stroke treatment groups in either atrophy or mortality at the same dose of 20mg/kg.

## Administration of tocilizumab reduced brain atrophy and mortality in aged male mice chronically



**Figure 10:** At a dose of 20mg/kg tocilizumab treatment led to significantly reduced brain atrophy and mortality in aged males post-stroke. A) Tocilizumab treatment in aged males led to significantly reduced brain atrophy 35-days post-stroke compared to IgG treated controls. \*P= 0.0120, One-way ordinary ANOVA with Sidak's multiple comparison test. In aged females tocilizumab treatment (20mg/kg) did not lead to a significant difference post-stroke. B) Aged male mortality was significantly reduced with tocilizumab treatment post-stroke. \*P= 0.0239, log-rank Mantel Cox test.

## Administration of tocilizumab reduced bacterial burden in aged male mice post-stroke

After observing the reduction in mortality post-stroke in aged males with tocilizumab treatment we sought to determine the cause. From previous studies in our own lab and other published studies, post-stroke infection (especially pneumonia) is a major contributor to worse long-term prognosis and can contribute to higher risks of mortality. Pneumonia can arise from gut-derived bacteria that translocate to the lungs {11}. Interestingly, increased interleukin-6 signaling reduces intestinal tight junction integrity therefore increasing gut permeability {12}. We hypothesized that when tocilizumab blocks interleukin-6 signaling throughout the body it

should also block the interleukin-6 mediated reduction in intestinal tight junction integrity and lead to reduced gut permeability. We predict that preventing this increase in gut permeability would reduce post-stroke gut bacterial derived infection risk, thus potentially explaining one component by which tocilizumab reduced mortality following stroke in our studies.

We tested to see if there was a reduction in post-stroke infection by examining the level of lipopolysaccharide binding protein in the plasma of our experimental mice 35-days post-surgery. Lipopolysaccharide binding protein (LBP) binds to lipopolysaccharide from gram-negative bacteria and interacts with immune cells, such as macrophages, to initiate an inflammatory response to the infection. Thus, an elevated plasma level of LBP correlates with increased infection. In our aged males, post-stroke tocilizumab treatment (mean 65,001 $\pm$ 6,212 ng/mL) led to a significant reduction in plasma LBP levels compared to IgG control treated males (mean 86,703 $\pm$ 2,355 ng/mL) ( $P= 0.0297$ ) (**Figure 11A**).

To confirm the reduction in infection post-stroke with tocilizumab treatment, we examined the number of bacterial colony-forming units (CFU) in the lungs 35-days post-surgery. Tocilizumab treatment (mean 83.33 $\pm$ 44.17 CFU/mL) led to a significant reduction in the number of colony-forming units in the lungs 35-days post-stroke compared to IgG control (mean 624.3 $\pm$ 188.6 CFU/mL) treated males ( $P= 0.0389$ ) (**Figure 11B**).

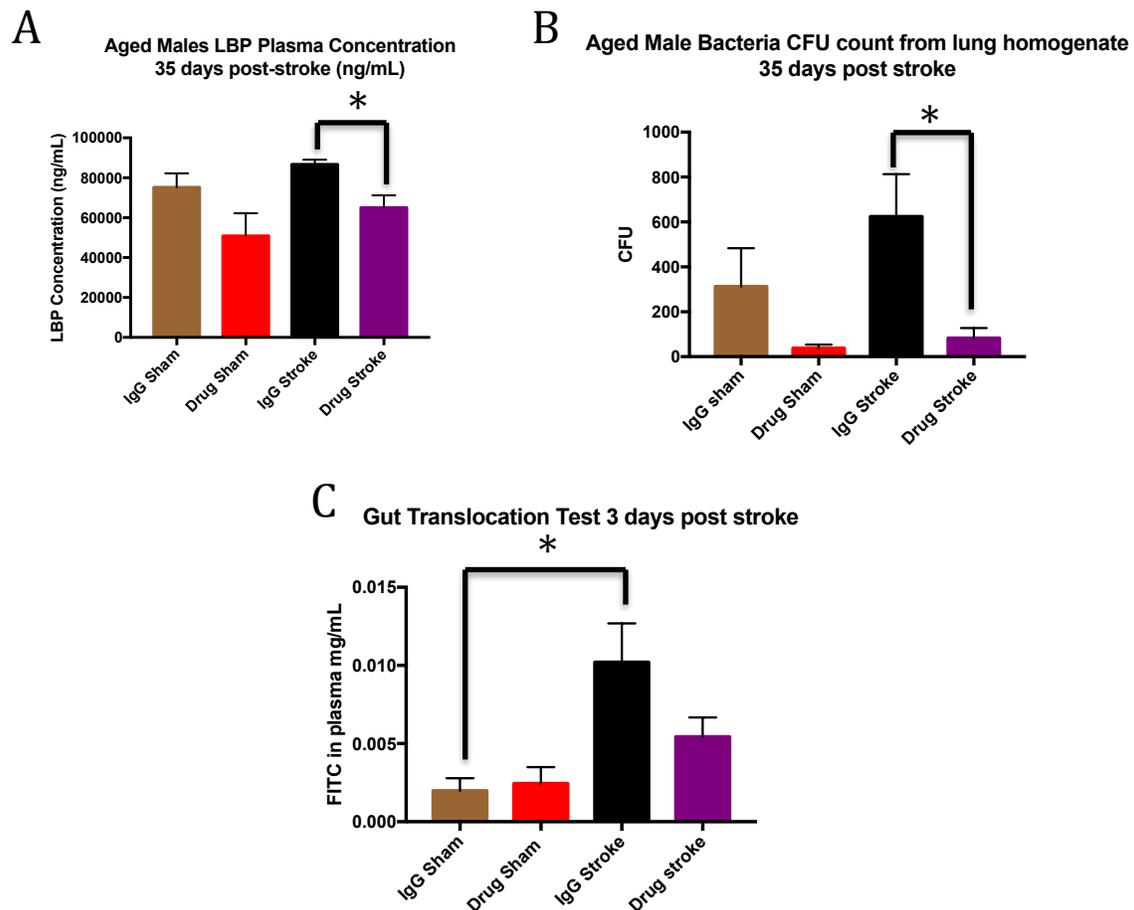
The 35-day post-surgery time point used for both the LBP and the lung bacterial CFU experiments is important due to the fact that a human stroke patient is at the highest risk of infection during the first 30-days after stroke. Our finding that tocilizumab is beneficial at reducing post-stroke infection 35-days after stroke strengthens its potential as new therapy in the clinic.

Since there were significant reductions in infection risk demonstrated by both plasma LBP levels and bacterial CFU in the lungs, we tested the hypothesis that post-stroke tocilizumab treatment leads to reduced gut permeability. To test this hypothesis, we orally gavaged FITC dextran (4,000 Da) on the third day after stroke/sham and then tested the plasma for FITC

fluorescence one-hour later. There was no significant difference in FITC fluorescence between tocilizumab treated and IgG treated aged males post-stroke ( $P= 0.2001$ ) (**Figure 11C**).

There was however, a significant increase in FITC fluorescence and therefore gut permeability in IgG control treated stroke compared to IgG control treated sham males ( $P= 0.0219$ ). Interestingly, there was no significant difference between the tocilizumab treated sham and tocilizumab treated stroke males ( $P= 0.7015$ ) or between the IgG control treated sham and tocilizumab treated stroke males ( $P= 0.5839$ ) (**Figure 11C**). The lack of significant difference between either sham group and the tocilizumab treated stroke males helps to show that while there was no significant difference between the two stroke treatment groups, the IgG control treated males post-stroke display a trending higher level of gut permeability than those treated with tocilizumab after stroke.

### Administration of tocilizumab reduced bacterial burden in aged male mice post-stroke



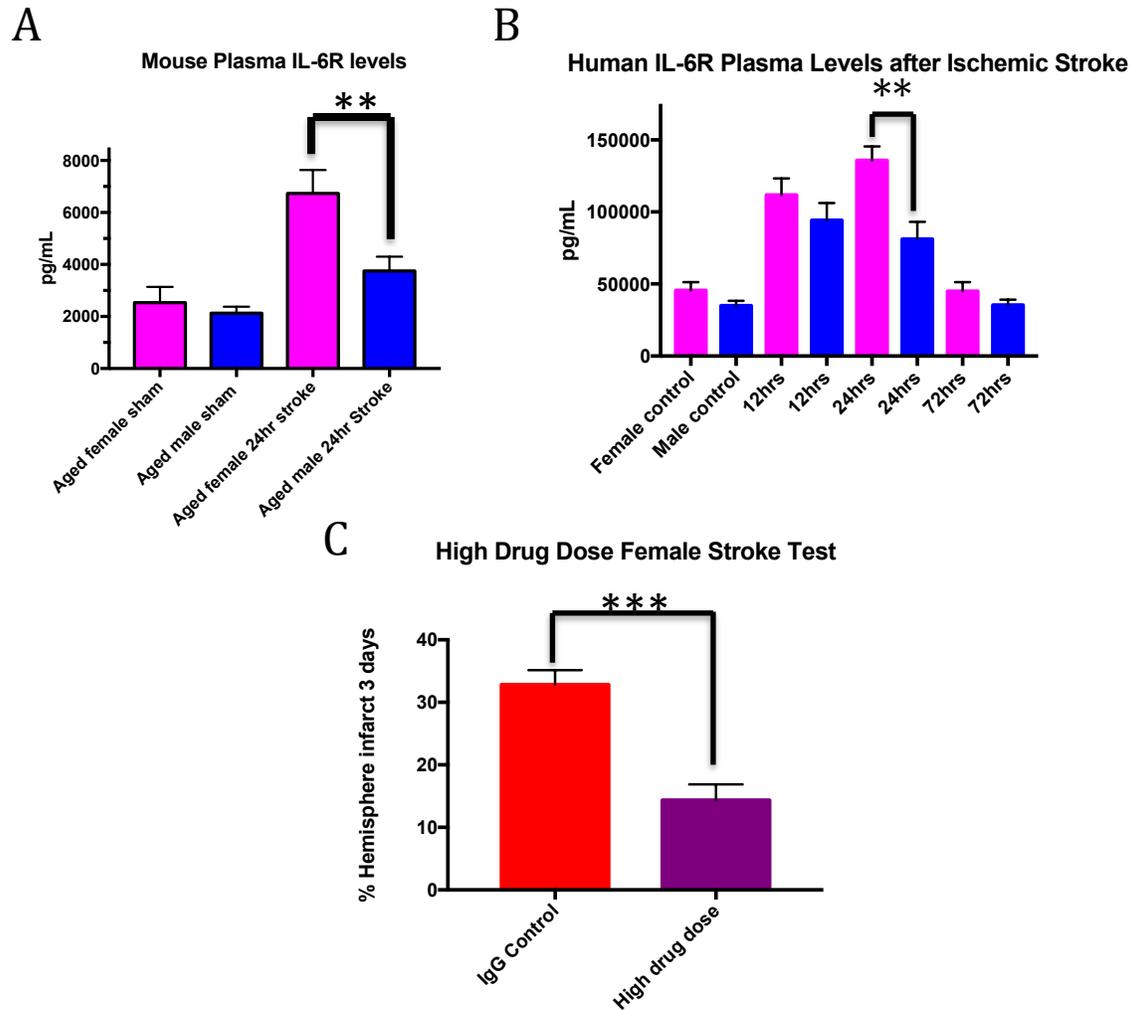
**Figure 11:** Post-stroke infection risk, measured by plasma LBP levels and by lung homogenate CFU counts, was significantly reduced post-stroke with tocilizumab treatment in aged males. A) Tocilizumab treatment reduced plasma lipopolysaccharide binding protein levels, a marker of bacterial translocation, significantly after stroke (\* $P=0.0297$ , One-way ordinary ANOVA with Sidak's multiple comparison test) and tocilizumab had a trend in reduction in sham surgical groups. B) Bacterial colony-forming unit counts were significantly reduced with tocilizumab treatment post-stroke. \* $P=0.0389$ , One-way ordinary ANOVA with Sidak's multiple comparison test. CFU counts also had a trend in reduction with tocilizumab treatment in sham surgical groups. C) There was no significant differences in the level of FITC dextran in the plasma after gavage between treatment groups after stroke. Sham surgery IgG treated aged males had a significantly lower level of plasma FITC compared to IgG treated aged males post-stroke ( $P=0.0219$ ).

**Administration of tocilizumab at a higher dose (100mg/kg) reduced brain infarct volume acutely in aged female mice**

While our findings up to this point support a beneficial effect of tocilizumab (20 mg/kg) treatment for ischemic stroke in aged males, our study showed no significant benefits from tocilizumab treatment in aged females. Although the level of soluble interleukin-6 receptor was similar at baseline between males and females, 24-hours post-stroke, females had a significantly higher level of plasma interleukin-6 receptor ( $P= 0.0070$ ) (**Figure 12A**). Utilizing human stroke patient plasma, we also found the same trend where baseline plasma interleukin-6 receptor levels were similar between sexes, whereas females had significantly higher levels than men at 24-hours post-stroke ( $P= 0.0047$ ) (**Figure 12B**).

Based on these findings, we proposed that the ineffectiveness of tocilizumab in our female mice might be due to the higher levels of plasma interleukin-6 receptor in females post-stroke. If so, we predicted that a higher dose of tocilizumab might be required to achieve the same therapeutic benefit as seen in males. We tested this prediction using a small aged female cohort in which a high dose of tocilizumab (100mg/kg) was administered intraperitoneally four-hours after stroke on brain infarct volume three days later. The high dose of tocilizumab led to a significant reduction in hemisphere infarct when compared to IgG control treated females ( $P= 0.0005$ ) (**Figure 12C**).

**Administration of tocilizumab at a higher dose (100mg/kg) reduced brain infarct volume acutely in aged female mice**



**Figure 12:** Females have higher soluble interleukin-6 receptor in their plasma after stroke than males. A) Aged female and male mice have no difference in plasma IL-6R levels pre-stroke. Post-stroke IL-6R increases for both aged males and females, but females have a significantly higher IL-6R level than males 24 hours post-stroke.  $**P=0.0070$ , One-way ordinary ANOVA with Sidak's multiple comparison test. B) Human samples show the same relationship with IL-6R, sex and stroke as seen in mouse stroke model samples. At baseline, no difference in plasma IL-6R level between sexes but after stroke women have a significantly higher level of soluble IL-6R than male patients.  $**P=0.0047$ , One-way ordinary ANOVA with Sidak's multiple comparison test. C) A 5X higher dose of tocilizumab (100mg/kg) than was used in the 35-day study given to aged females 4-hours after stroke onset led to a significant reduction in infarct volume compared to IgG control treated females three-days post-stroke.  $N=5$  and  $6$  for IgG control and drug treated females respectively.  $***P=0.0005$ , Two-tailed unpaired T-test.

## Discussion and Future Objectives

Ischemic stroke is a common and devastating disease with 130,000 deaths in the United States annually and is the leading cause of serious long-term disability {1}. In this study, we examined the effectiveness of tocilizumab, an FDA approved drug for rheumatoid arthritis treatment that blocks interleukin-6 signaling, as a potential new therapy for ischemic stroke. The data supports our hypothesis that tocilizumab is beneficial post-stroke in an aged male and female mouse model.

Tocilizumab at a dose of 20mg/kg led to neuroprotection in aged male, but not female mice. We speculated that this disparity might be due to the higher levels of soluble interleukin-6 receptor in aged females post-stroke compared to males. The results from a high dose (100 mg/kg) aged female cohort showed that females treated with the high dose of tocilizumab had significantly less brain infarct than the IgG control treated mice. This finding is similar to our finding of acute neuroprotection in males with lower dose of tocilizumab. Further experiments are needed to confirm the ‘permanence’ of the neuroprotection with high dose tocilizumab in aged females. We therefore plan to perform a 35-day survival study with the 100-mg/kg dose to see if this leads to reduced behavioral deficits in the aged female mice.

Ours and other studies indicate that high interleukin-6 levels in the periphery are detrimental for stroke outcomes. However, other studies have shown that blocking interleukin-6 signaling in the brain can actually increase injury by reducing post-stroke angiogenesis {19}. Thus there appears to be a critical difference between the effects peripheral and central interleukin-6 signaling on stroke outcome. Tocilizumab cannot pass the blood-brain barrier (BBB) under normal conditions {18}. However, the post-stroke barrier is characterized by phases of increased “leakiness”. If tocilizumab crosses the BBB during one of these periods, it could potentially lead to worse recovery by impairing beneficial interleukin-6 signaling. Therefore,

another future objective is to determine if tocilizumab enters the brain through the weakened blood-brain barrier following stroke.

Treatment with tocilizumab post-stroke leads to reduced peripheral infection long-term (35-days later). While the difference between gut permeability in our experiment was not significant between tocilizumab treated and IgG control treated male mice post-stroke, I speculate that reduced gut permeability is responsible for the reduction in LBP levels and lung bacterial CFU counts. We tested gut permeability at one time-point (3-days post-stroke) and with one size FITC dextran (4,000 Da), so it is possible we missed the critical time window or used the wrong size molecule to capture tocilizumab's effect. We plan to examine gut epithelial cell permeability differences in-vitro with tocilizumab and our IgG control to see if tocilizumab helps reduce gut permeability. We will additionally examine molecular markers of epithelial barrier weakness, such as Claudin-2, which is activated by interleukin-6 {12}.

The main future direction is to elucidate what cell types are responsible for the upregulation of soluble interleukin-6 receptor in females compared to males. To do this we will utilize flow cytometry focusing specifically at neutrophils, monocytes and T-cells in aged males and females post-stroke to determine which cell types are responsible. Once we know the cell type or types responsible, we will pursue the differences that exist in these cell populations between the sexes, which could be causing females to express more soluble interleukin-6 receptor. Unpublished research in our lab has previously demonstrated sex differences in post-stroke neutrophils. After stroke, a higher number of neutrophils infiltrate the brain in males than females. This might be an explanation as to why females have a higher level of soluble interleukin-6 receptor, since there will be a larger number of peripheral neutrophils shedding the receptor whereas in males those neutrophils trafficked into the brain and are no longer in the periphery to shed the receptor. To test this hypothesis, we will use flow cytometry to look at total

peripheral neutrophil counts (present in the blood) post-stroke in aged male and female mice to see if females have a higher number.

In summary, our data supported our initial hypothesis that blocking post-stroke interleukin-6 signaling with tocilizumab would lead to better stroke outcomes. Tocilizumab treatment led to early neuroprotection post-stroke in aged male and female mice as well as long-term neuroprotection in aged male mice. Long-term post-stroke behavior deficits and infection risk were also diminished in aged males treated with tocilizumab compared to IgG treated controls. Unexpectedly, we found that aged females do not benefit from tocilizumab treatment at the same dose as males but do benefit from a higher dose. This dose discrepancy appears to be due to the significantly higher levels of plasma interleukin-6 receptor compared with males post-stroke. Our promising new data, along with the fact that tocilizumab is already an FDA approved treatment for human use, strengthens the potential for tocilizumab as a novel therapy for ischemic stroke. My work also supports the assertion that proper drug dosing needs to be based off of more than just weight. Patient sex and other unknown variables need to also be considered for the best patient outcomes. Thus, the main conclusions from this study are that tocilizumab is a promising new experimental therapy for ischemic stroke. Additionally, basic and translational research needs to be conducted in both female and male cohorts.

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