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UNIVERSITY OF CALIFORNIA SAN DIEGO

CCL28 increases the production of reactive oxygen species during *Salmonella enterica* serovar Typhimurium infection

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

In

Biology

by

Steven Silva

Committee in charge:

Professor Manuela Raffatellu, Co-Chair
Professor Elina Zuniga, Co-Chair
Professor Matt Daugherty

2021

The thesis of Steven Silva is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

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LIST OF ABBREVIATIONS

aCCR3	rIgG2a anti-mouse C-C Motif Chemokine Receptor 3
aCCR10	rIgG2a anti-mouse C-C Motif Chemokine Receptor 10
CFU	Colony Forming Unit
CCL28	C-C Motif Chemokine Ligand 28
$Ccl28^{-/-}$	C-C Motif Chemokine Ligand 28 knock out
CCR3	C-C Motif Chemokine Receptor 3
CCR10	C-C Motif Chemokine Receptor 10
DCFDA	2'-7'-dichlorodihydrofluorescein diacetate
DMSO	Dimethyl Sulfoxide
FBS	Fetal Bovine Serum
FMO	Fluorescence Minus One
FVD	Fixable Viability Dye
LB	Luria-Bertani
MOI	Multiplicity of Infection
NAL	Nalidixic Acid
OD	Optical Density
NTS	Non-Typhoidal Salmonella
ns	Not Significant
PBS	Phosphate Buffered Saline
rIgG2A	rat Immunoglobulin G subclass 2A
ROS	Reactive Oxygen Species
STm	*Salmonella enterica* serovar Typhimurium
UI	Un-infected
WT	Wild Type

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ABSTRACT OF THE THESIS

CCL28 increases the production of reactive oxygen species during *Salmonella enterica* serovar Typhimurium infection

by

Steven Silva

Master of Science in Biology

University of California San Diego, 2021

Professor Manuela Raffatellu, Co-chair

Professor Elina Zuniga, Co-chair

The mucosal chemokine CCL28 plays a protective role during *Salmonella* infection by mechanisms that are not well understood. Using $Ccl28^{-/-}$ mice, our previous studies determined that CCL28 promotes the recruitment of neutrophils during *Salmonella enterica* serovar Typhimurium (STm) infection and enhances the survival of wild-type (WT) mice. Here we sought to investigate whether the CCL28 receptors CCR3 and CCR10 are expressed in neutrophils and whether their activation by CCL28 promotes neutrophil function. First, neutrophils were infected with STm in order to determine changes in surface and intracellular expression of CCR3 and

CCR10. Using flow cytometry, we observed that there is higher expression of intracellular and surface CCR3 when compared to CCR10 during STm infection. Furthermore, surface CCR3 expression increased after infection. The next step was to determine the mechanism by which CCL28 promotes survival of WT mice during STm infection. As neutrophils produce reactive oxygen species (ROS) as a form of defense during infection, we tested whether neutrophil ROS production would be increased in the presence of CCL28 during STm infection. Using flow cytometry, we discovered that in the presence of CCL28, neutrophil ROS production is increased during STm infection. However, when blocking CCR3 with a neutralizing antibody, neutrophil ROS production decreases. Thus, we conclude that ROS production in neutrophils stimulated with CCL28 during STm infection is primarily facilitated via CCR3.

Introduction

The immune system can be described as a network of highly specialized immune cells, broadly known as white blood cells or leukocytes, which assist in protecting the host from pathogens (Marshall et al., 2018). Usually, some immune cells circulate throughout the body as a form of homeostatic immune surveillance (Zhang and An, 2007). When invaded by an infectious agent, the immune system responds through a series of mechanisms to induce inflammation to combat the infection. Commonly, this inflammation manifests as redness, heat, and pain. Inflammation can be described as a byproduct of cellular injury that is marked by capillary dilatation which facilitates leukocyte infiltration, which serves as a mechanism initiating the elimination of a pathogen or damaged tissue (Freire and Van Dyke, 2013). This specific mechanism occurs via the production of chemical factors, including specialized mediators called cytokines (Ramesh et al., 2013).

Cytokines can be described as a diverse group of chemical factors that help promote or reduce inflammation through the binding of their receptors, which in turn influences the interaction and communication between cells (Mohan et al., 2017). Cytokines are grouped into families based upon their structural makeup or that of their receptors (Ramesh et al., 2013). Chemokines are a type of cytokine that are best known for their ability to influence the migration of cells, specifically leukocytes. They function by activating specific G protein-coupled receptors, which results in different activities ranging from the development and homeostasis of the immune system, to immune and inflammatory responses (Bennett et al., 2011). Depending on the protein sequence, different chemokines fall into different categories based on the cysteine sequences. C-C is a common motif that refers to chemokines whose cysteine sequences are juxtaposed (Hughes and

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Nibbs, 2018). The C-C motif chemokine ligand 28 (CCL28), also referred to as Mucosae-associated Epithelial Chemokine is considered a dual function chemokine as it has homeostatic functions and can be induced under inflammation (Mohan et al., 2017).

CCL28 is predominantly found and produced constitutively in mucosal tissues, and is known as a chemoattractant as it influences the migration of immune cells expressing C-C motif chemokine receptor 3 (CCR3) and/or C-C motif chemokine receptor 10 (CCR10) (Lee et al., 2021). Furthermore, one of the functions of CCL28 is to recruit B and T lymphocytes to sites of infection, as these cells express CCR3 and CCR10 on the surface (Mohan et al., 2017). This is significant because B and T lymphocytes are key components of the immune response, and as such CCL28 helps mediate a strong reaction against pathogens (Merad and Martin, 2020; Mohan et al., 2017). Under homeostatic conditions, B and T lymphocytes expressing CCR10 migrate towards CCL28. CCL28 also influences the migration of CCR10$^+$ IgA plasmablasts to the mammary gland and other tissues (Burkhardt et al., 2019). The result supports that the chemokine CCL28 promotes the secretion of the IgA antibodies into milk (Burkhardt et al., 2019; Matsuo et al., 2018; Mohan et al., 2017). Thus, CCL28 can either function as a homeostatic chemokine or can also lead to the migration of immune cells to the site of inflammation/infection.

Meanwhile, previous research has demonstrated that CCL28 is highly expressed in various disease conditions (Lee et al., 2017, Hansson et al., 2008). For instance, previous research has identified that patients suffering from ulcerative colitis, an inflammatory bowel disease resulting in inflammation in the digestive tract, have a higher expression of CCL28 (Lee et al., 2021). The study included 25 patients diagnosed with ulcerative colitis and 25 healthy controls. When the levels of serum CCL28, and colon biopsies were compared between both groups it was noted that those diagnosed with ulcerative colitis had a significantly higher level of CCL28 (Lee et al., 2021).

Furthermore, in a different study, individuals suffering from *Helicobacter pylori*- induced gastritis had significantly higher levels of CCL28 when compared to healthy controls. Using an enzyme-linked immunosorbent assay on gastric tissue it was determined that *Helicobacter pylori* infected individuals had 2.8 times more CCL28, when compared to uninfected individuals (Hansson et al., 2008). Collectively, these studies demonstrate that CCL28 plays a role during inflammation of the gut.

Salmonella enterica is a Gram-negative bacterium that causes a wide range of gastrointestinal and systemic diseases (Andino and Hanning, 2015). Human disease occurs via direct or indirect contact with various contaminated food products derived from an infected animal such as meat, poultry, eggs, or water. In the United States alone, Non-typhoidal *Salmonella* (NTS) infections are reported to be around 1.2 million per year with 23,000 hospitalizations, and 450 deaths (Mukherjee and Hooper, 2015). Hence, NTS infections remain common and a significant public health issue (Smith et al., 2016). *Salmonella enterica* serovar Typhi can spread to systemic sites and cause typhoid fever (Johnson et al., 2018). In contrast, *Salmonella enterica* serovar Typhimurium (STm) leads to acute intestinal inflammation in both healthy human and animal hosts, as a response to the bacterium invading the mucosa. It is important to note that STm infection in mice resembles that of typhoid fever in humans (Broz et al., 2012). When using mice as a model for NTS infection, the antibiotic streptomycin is administered prior to infection to reduce the microbiota and have STm better colonize the mice. Thus, leading to an inflammatory diarrheal response upon oral infection with *Salmonella* resembling that of NTS rather than typhoid fever (Tsolis et al., 2011).

Our lab has been studying the relationship between CCL28 and STm. Being that CCL28 is predominantly found and produced in mucosal tissue, our goal is to establish and understand the

role and mechanism of CCL28 during mucosal infection. Although various studies have established that CCL28 is involved in inflammatory diseases such as ulcerative colitis (Lee et al., 2021, Hansson et al., 2008) its mechanistic funcon in the context of health and disease remains largely unknown. This in large part is because *Ccl28* knockout mice (*Ccl28*^{-/-}) have only recently been generated (Burkhardt et al., 2019; Matsuo et al., 2018). To establish and understand the role of CCL28 during mucosal infection, wild-type (WT) and *Ccl28*^{-/-} mice were infected with STm using the NTS model. We noticed that *Ccl28*^{-/-} mice succumbed to STm from 24 to 72 hours while WT mice did not die (Burkhardt et al., 2019). Therefore, STm colony forming units (CFU) were enumerated from several tissues 72 hours post-infection. Colony forming units are a unit used in microbiology to estimate the number of viable bacteria within a sample. It was observed that the STm CFU recovered from the gastrointestinal tract and systemic sites were significantly higher in the *Ccl28*^{-/-} mice vs. WT littermates. Hence, we were able to determine that CCL28 plays a protective role during STm infection (Perez-Lopez et al., 2021).

Once this protective role was established, our next goal was to determine the mechanism by which this protection occurs. It was hypothesized that protection against STm is due to the recruitment of immune cells to the site of infection. Thus, intestinal tissue was analyzed for different leukocyte populations. Knowing that CCL28 recruits B and T cells as they express CCR3 and CCR10 (Mohan et al., 2017, Lee et al., 2021), these cell populations became our first point of interest. However, there was no difference in B and T cell numbers in the gut of *Ccl28*^{-/-} mice after STm infection, when compared to their WT littermates (Perez-Lopez et al., 2021). Surprisingly, we noted that ~50% fewer neutrophils were recruited to the gut of *Ccl28*^{-/-} mice after STm infection, in comparison to their WT littermates (Perez-Lopez et al., 2021). Neutrophils can be described as the most abundant leukocyte of the innate immune system and are produced in the

bone marrow (Rosales, 2018). They constantly monitor the organism for signs of microbial infection and, if detected, respond through the trapping and killing of the pathogens (Rosales, 2018). This is typically done through a series of anti-microbial activities consisting of reactive oxygen species (ROS) production, neutrophil extracellular trap formation, pro-inflammatory cytokine production, degranulation, and phagocytosis (Kobayashi et al., 2018).

Neutrophil production of ROS is induced as a result of acute infection or inflammation, which is typically brought on by pathogens (Paiva and Bozza, 2014; Ray et al., 2012). ROS are highly unstable reactive molecules containing oxygen, which mediate pathogen killing through oxidative damage to biocompounds directly. ROS leads to DNA, RNA, and/or protein damage, ultimately leading to cell death if oxygen radical formation levels exceed those of antioxidants (Paiva and Bozza, 2014). Neutrophil ROS production can also cause cell damage via non-oxidative means, by inducing autophagy, and neutrophil and T-lymphocyte responses (Ray et al., 2012).

Furthermore, it has been established that neutrophils express several chemokine receptors, including CXCR1, CXCR2, CCR1, and CCR2 (Bonecchi, 2020). Previous literature has also demonstrated that under inflammatory conditions neutrophils undergo phenotypic changes. These phenotypic changes include the up-regulation of CCR1 and CCR3 (Hartl et al., 2008). As previously mentioned, CCL28 influences the migration of immune cells expressing CCR3 and CCR10 (Mohan et al., 2017), thus establishing a connection between CCL28, CCR3, and neutrophils. However, the expression of these chemokine receptors and the function neutrophils play at the site of inflammation is poorly understood. Our objective was to determine whether CCR3 and CCR10 are expressed in uninfected neutrophils and in STm infected neutrophils *in vitro*. Then, we wanted to determine whether CCL28 enhances neutrophils anti-microbial activity during STm infection, specifically ROS production.

Using flow cytometry, we found that expression of CCR3 is increased on the surface during STm infection when compared to uninfected (UI) neutrophils, while intracellular expression remained at similar levels. Moreover, we noticed higher expression of surface and intracellular CCR3 when compared to CCR10 during STm infection. We also discovered that in the presence of CCL28, neutrophil ROS production is increased during STm infection. However, when blocking CCR3 with a neutralizing antibody, ROS production decreases. Hence, we conclude that ROS production in neutrophils stimulated with CCL28 during STm infection is primarily facilitated via CCR3.

MATERIALS AND METHODS

Bacterial Preparation

Salmonella enterica serovar Typhimurium (STm) wild-type strain IR715 was grown overnight (i.e. 14 hours prior to infection) in 5mL of Luria-Bertani (LB) broth supplemented with Nalidixic Acid (Nal) (50ug/ml) at 37° C on a roller (~50 cycles per minute). The next day, the Optical Density 600 (OD) was measured using BioSpectrometer (Eppendorf). When measuring the OD, our control consisted of 1,000 μl of LB broth supplemented with Nal and the bacteria were diluted 1:10 in LB broth supplemented with Nal at a final volume of 1,000 μl. Once the OD was measured, the OD was multiplied by the dilution factor (10) and 5×10^8 CFU/ml (a lab established conversion factor of OD to CFU/mL) to determine the concentration of bacteria. 1×10^9 CFU were collected into a 1.5 ml eppendorf tube, washed with phosphate buffered saline (PBS; Invitrogen) and centrifuged at 4ºC at 300 x g for 5 minutes, and resuspended in 1 ml of PBS to have a final concentration of 1×10^9 CFU/ml. 100 μl of this stock was placed into another eppendorf to have 1 $\times 10^8$ bacteria. This aliquot was centrifuged at 4ºC at 300 x g for 5 minutes, washed with PBS, and resuspended in 80 μl of phenol red-free RPMI media and placed in ice. The bacteria were then opsonized by adding 20 μl of normal mouse serum (isolated from the blood of uninfected mice using Z-gel tubes, Sarstedt) to the 80 μl of STm for 30 min at 37 ºC and 5% of CO_2. STm was added to neutrophils at a multiplicity of infection (MOI) = 10 as further described below.

In vitro neutrophil enrichment

Neutrophils were obtained from the bone marrow (2 femurs and tibias) of ~8-10-week-old female C57BL/6J Jackson wild-type mice. Bone marrow cells were flushed out with sterile PBS using a 10 ml syringe with a 0.45 mm x 16mm needle into a 50 ml conical tube. Once the femurs and tibia were completely washed out, the bone marrow suspension was passed through a 100 μM strainer and lysed with 1x red blood cell lysis buffer. The red blood cell lysis buffer was made at 10x concentration using 8.04 g NH_4Cl, 0.84 g $NaHCO_3$, 0.37 g EDTA in 100 ml Millipore water. The cells were centrifuged at 4ºC at 300 x g for 8 minutes, resuspended in 500 ul of PBS, and then transferred to a 5 ml polystyrene round-bottom tube. Approximately 5 x 10^7 bone marrow cells are collected after red blood cell lysis. Using the EasySep[TM] Mouse Enrichment Kit (Stemcell) the neutrophil enrichment process began. Instructions for the kit were followed with some modifications as follows. 25 μl of Rat Serum were then added to the bone marrow cells followed by 25 μl of Neutrophil Enrichment Cocktail. The sample was mixed and incubated on ice for 15 minutes. The bone marrow cells were then washed by being topped off with 2 ml of PBS, and then centrifuged at 300 x g for 10 minutes. The supernatant was then discarded, and the bone marrow cells were then re-suspended in 500 ul of PBS. 25 μl of Biotin Selection Cocktail was then added to the sample and incubated in ice for 15 minutes. Once the incubation was completed, 75 μl of Magnetic Particles, that were vortexed for 30 seconds, were added to the sample. The mixture was then incubated on ice for 10 minutes. At the end of the incubation period, the sample was then topped off with PBS to have a final volume of 2.5 ml and placed in an EasySep[TM] magnet for 3 minutes. At the end of the incubation period, the neutrophil enriched cell suspension was poured into a new 5 ml polystyrene round-bottom tube. 10 μl the enriched neutrophil cell suspension was

mixed at a concentration 1:1 with Trypan Blue (Invitrogen) and counted using Countess II (Lifesciences).

In vitro neutrophil chemokine receptor quantification

   After neutrophil enrichment, 5 x 10^5 neutrophils were seeded in a round bottom 96-well plate in 100 ul of RPMI media supplemented with 10% FBS (Gibco) and 1 mM HEPES (Invitrogen). Each biological replicate was split into 4 conditions: surface stain, intracellular stain, FMO-CCR3, and FMO-CCR10. STm was prepared and added as described above. When indicated, the neutrophils were infected for 1 hour and incubated at 37ºC and 5% of CO_2. 30 minutes into the 1 hour infection, gentamycin (100 μg/ml; Gibco) was added to the neutrophils. The neutrophils were spun down (300 x g for 5 minutes at 4 ºC) and washed twice with PBS. A Live-Dead stain was prepared in the dark using fixable viability dye (FVD-APC-Cy7) at a concentration of 1:1000 in PBS. Each condition including the FVD single-color control was re-suspended in 100 ul of the FVD stain while the other single-color controls were re-suspended in PBS and incubated for 15 minutes in the dark on ice (all staining was done under these conditions). The neutrophils were spun down (300 x g for 5 minutes at 4ºC), supernatant was discarded, and then re-suspended in 200 μl of FACS buffer (PBS in 0.5% bovine serum albumin) twice. The neutrophils were spun down once more and resuspended in Fc block prepared at a concentration of 1:100 in FACS buffer. All conditions including single-color controls were re-suspended in 50 μl of the Fc block and incubated for 15 minutes. Meanwhile, surface stain antibody mix was prepared and protected from the light. The surface stain consisted of CD45-PacificBlue (clone: M1/70, 1:400, Biolegend), CD11b-BV510 (clone: M1/70, 1:200, Biolegend), Ly6G-FITC (clone 1A8, 1:400, Biolegend), CCR3-PE (polyclonal, 1:200, R&D Systems), and CCR10-APC

(polyclonal, 1:200, R&D Systems). At the end of the incubation period, 50 µl of the appropriate antibody mix were added to each well (into the Fc blocked samples) and mixed by pipetting. The surface stain was then incubated for 20 minutes. The neutrophils were then spun down (300 x g for 5 minutes) and re-suspended in 200 µl of FACS buffer twice, and spun down once more. The cells were then re-suspended in 200 µl Fixation Buffer (Biolegend) and incubated for 15 minutes. After the incubation, the cells were spun down (300 x g for 5 minutes) twice, and spun down once more with FACS buffer. The cells were then re-suspended in 200 µl of permeabilization buffer (Biolegend) and immediately spun down. The CCR3 and CCR10 intracellular stain antibody mixes were prepared in permeabilization buffer at 2x the final concentration. 50 µl of the CCR3 (1:200, R&D Systems) mix were then added to the appropriate wells (FMO-CCR10 and CCR3 single-color control). 50 µl of the CCR10 (1:200, R&D Systems) mix were then added to the appropriate wells (FMO-CCR3 and CCR10 single-color control). Meanwhile, cells that were not being stained intracellularly received an additional 50 µl of permeabilization buffer. The cells were then incubated for 15 minutes. Once the incubation was complete, the cells were spun down (300 x g for 5 minutes), washed once with permeabilization buffer, and twice with FACS buffer. The 200 µl samples were transferred to flow tubes and topped with another 100 µl of FACS buffer to have a final volume of 300 µl. The single-color controls were topped with 300 µl of FACS buffer to have a final volume of 500 µl. Expression of surface and intracellular CCR3 and CCR10 were quantified using BD Facs Canto II with Diva software and analyzed with FlowJo v10.8.

In vitro neutrophil ROS staining, infection, and stimulation

After neutrophil enrichment, 5 x 10^5 neutrophils were seeded in a round bottom 96-well plate with 100 μL of culture media (phenol red-free RPMI media supplemented with 10% fetal bovine serum (FBS; Gibco), and 1mM HEPES (Invitrogen)). As 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) is light sensitive the following was performed in the dark. DCFDA was resuspended in Dimethyl Sulfoxide (DMSO) to have a stock solution of 1 mM DCFDA. DCFDA was then diluted in culture media for a working stock at 100 μM DCFDA. Meanwhile, the DMSO vehicle control was made by diluting DMSO in culture media. Neutrophils were then incubated with 25 μM concentration of DCFDA in a final volume of 200 μl, at 37ºC and 5% of CO2 for 30 minutes, while the vehicle control was incubated with 2.5% DMSO in a final volume of 200 μl, at 37ºC and 5% of CO2 for 30 minutes. The neutrophils were centrifuged at 300g for 5 minutes and washed with PBS to remove excess DCFDA and resuspended in 100 μl of culture media. Opsonized STm was then prepared as described above and added at an MOI of 10. For stimulation, neutrophils were incubated with the following concentrations of chemokine and antibody: CCL28 (50 nM), aCCR3 (5 μg/10^6 cells), aCCR10 (5 μg/10^6 cells), rIgG2A (5 μg/10^6 cells) (all stock solutions were resuspended in PBS then diluted in culture media for working solution; R&D Systems). The chemokine, neutralizing antibodies, and STm were added alone or in combination in a final volume of 200 μl for 4 hours 37ºC and 5% of CO_2. ROS production was then immediately quantified using the BD Facs Canto II with Diva software and analyzed with FlowJo v10.8.

Statistical analysis

All statistical analysis was conducted in Prism v8 (GraphPad). Statistical significance was determined by using one-way ANOVA (multiple comparisons) or *t* test (single comparisons) for the *in vitro* experiments. Differences were considered statistically significant if the *P* value was ≤ 0.05.
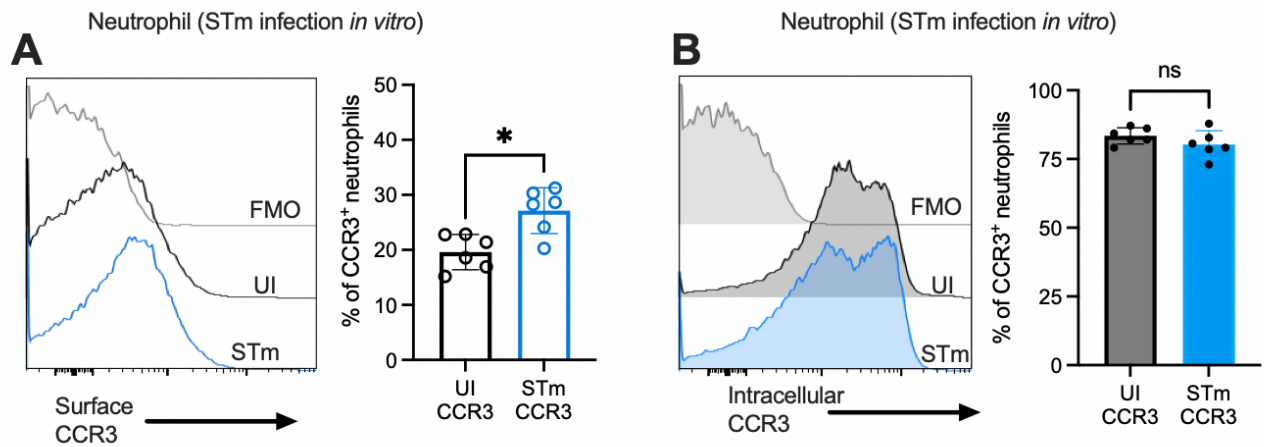
RESULTS

Figure 1. Surface and intracellular expression of the CCL28 receptor CCR3 on neutrophils during *Salmonella enterica* serovar Typhimurium (STm) infection.
Bone marrow neutrophils were uninfected (UI) or infected with opsonized STm at MOI=10 for 1h. (A) Surface CCR3 (open) or (B) intracellular CCR3 (closed) were detected using flow cytometry. Left panels show representative histograms of (A) surface CCR3 and (B) intracellular CCR3 (gated on singlets/ scatter/ live/ CD45$^+$/ CD11b$^+$/ Ly6G$^+$ cells) of FMO controls (gray), uninfected (black) and STm infected (blue). Right panels demonstrate the percent of CCR3$^+$ neutrophils from the bone marrow. Data shown consists of 3 independent experiments (n=6). Error bars represent mean $\pm$ standard deviation. ns, not significant; * ($P \leq 0.05$)

It has been established that CCL28 influences the migration of leukocytes expressing at least one of its two receptors, CCR3 and CCR10. Previous literature has also stated that inflammatory conditions tend to cause phenotypic changes in neutrophils, which result in an increased expression of CCR3 (Hartl et al., 2008). Therefore, I was interested in determining whether infection with STm led to an increased expression of surface and intracellular CCR3 in neutrophils. Hence, neutrophils were obtained from the bone marrow of C57BL/6J wild-type mice, and uninfected (UI) or infected with opsonized STm for an hour at an MOI = 10. The cell suspensions were then stained and analyzed using flow cytometry in order to see the levels of surface and intracellular expression of CCR3. Neutrophil surface expression of CCR3 significantly

increased during STm infection when compared to uninfected (Fig. 1A). The expression of CCR3 was ~20% in uninfected neutrophils, and went up to ~35% in STm-infected neutrophils. In contrast, when looking at the expression of intracellular CCR3, there was no significant difference between uninfected neutrophils and those infected with STm (Fig. 1B).
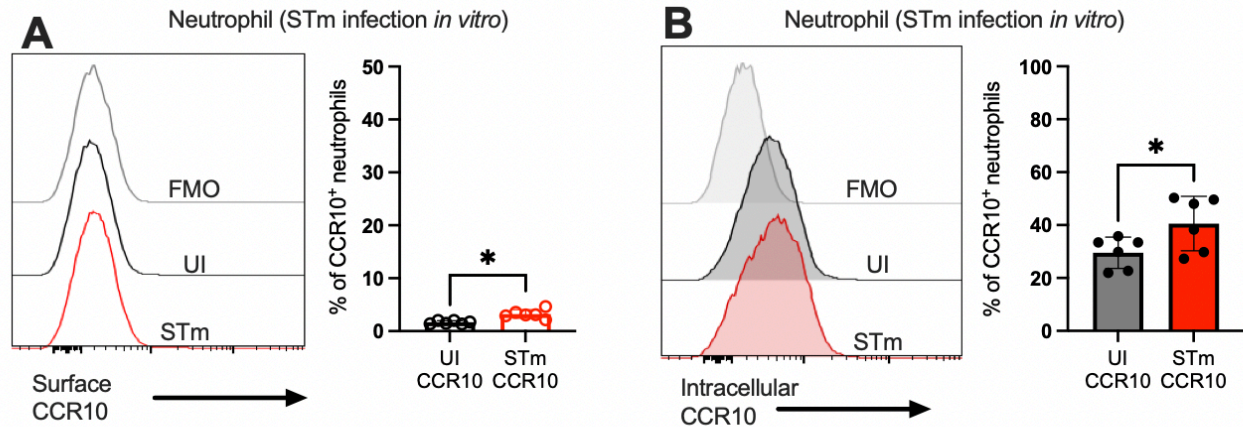

Figure 2. Surface and intracellular expression of the CCL28 receptor CCR10 on neutrophils during *Salmonella enterica* serovar Typhimurium (STm) infection.
Bone marrow neutrophils were uninfected (UI) or infected with opsonized STm at MOI=10 for 1h. (A) Surface CCR10 (open) or (B) intracellular CCR10 (solid were detected using flow cytometry. Left panels show representative histograms of (A) surface CCR10 and (B) intracellular CCR10 (gated on singlets/ scatter/ live/ CD45$^+$/ CD11b$^+$/ Ly6G$^+$ cells) of FMO controls (gray), uninfected (black) and STm infected (red). Right panels demonstrate the percent of CCR10$^+$ neutrophils from the bone marrow. Data shown consists of 3 independent experiments (n=6). Error bars represent mean $\pm$ standard deviation. * ($P \leq 0.05$)

As leukocytes expressing CCR10 have also been shown to have chemotactic activity towards CCL28, I wanted to see if CCR10 showed a similar trend when compared to CCR3. Therefore, neutrophils were obtained from the bone marrow of C57BL/6J wild-type mice, and mock treated or infected with opsonized STm for an hour at an MOI = 10. The cell suspensions were then stained and analyzed using flow cytometry in order to see the levels of surface and intracellular expression of CCR10. When looking at the expression of CCR10 on the surface of

the neutrophil, there is a small but significant difference between the levels of expression of CCR10 in uninfected neutrophils and those infected with STm. The levels of expression went from ~1.5 % in uninfected neutrophils to ~3.5% in neutrophils infected with STm (Fig. 2A). Furthermore, expression of intracellular CCR10 significantly differed between uninfected neutrophils and those infected with STm (Fig. 2B). Uninfected neutrophils expressed ~30% intracellular CCR10. Meanwhile, those infected with STm expressed ~45% intracellular CCR10. Thus, we conclude that surface expression of CCR3 and CCR10 is increased during STm infection, however CCR3 expression is much higher. Furthermore, we noted that intracellular expression of both receptors during STm infection is higher than surface expression.
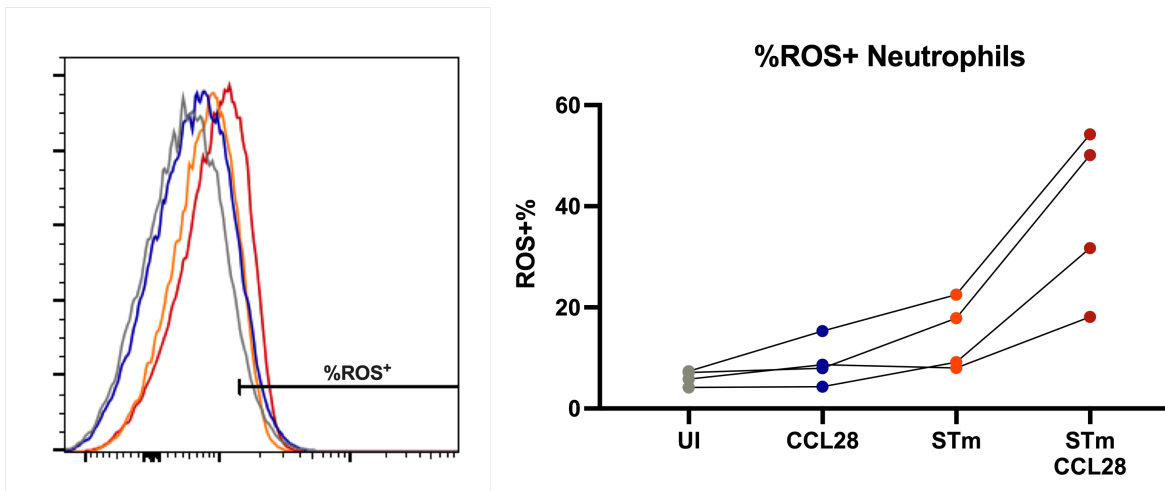

Figure 3. CCL28 increases Reactive Oxygen Species (ROS) production in neutrophils during *Salmonella enterica* serovar Typhimurium (STm) infection.
Purified neutrophils treated with ROS detection dye, 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) for 30 min, were uninfected (UI) or infected with *Salmonella enterica* serovar Typhimurium (STm) for 4 hours in the absence or presence of CCL28. Left panel shows representative histograms of ROS production in neutrophils under different conditions: uninfected (grey), CCL28 (blue), STm (orange), and STm with CCL28 (red). Data shown consists of 2 independent experiments (n = 4).

Our previous research findings have shown that CCL28 plays an important role in controlling STm infection. To determine whether CCL28 enhances neutrophil effector function, we infected bone marrow neutrophils with STm, in the presence or absence of CCL28 stimulation,

and quantified bacterial killing. We found that CCL28 stimulation strongly increased neutrophil bacterial killing (Perez-Lopez et al., 2021). However, the mechanism by which this is achieved is largely unknown. As neutrophils are well-known to exhibit antimicrobial activity against bacteria, including STm, through ROS production (Rosales, 2018), we sought to determine if neutrophils stimulation with CCL28 during STm infection would lead to an increase in ROS production. Therefore, neutrophils were obtained from the bone marrow of C57BL/6J wild-type mice and treated with 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). DCFDA is a dye that measures ROS production within a cell. DCFDA diffuses into the cell, and is then deacetylated by cellular esterases to a non-fluorescent compound. In the presence of ROS, this non-fluorescent compound is oxidized into 2',7'-dichlorodihydrofluorescein diacetate, a fluorescent molecule. Uninfected and STm-infected neutrophils were treated with CCL28 in order to determine if CCL28 stimulation during infection would increase ROS production in neutrophils. DMSO was used as a vehicle control for DCFDA. When comparing the uninfected neutrophils to those stimulated with CCL28, there was a slight increase in ROS production in neutrophils stimulated with CCL28. Neutrophils that were infected with STm had an even larger increase in ROS production when compared to the uninfected and CCL28 treated neutrophils. Interestingly, we found a trend in which neutrophils that were infected with STm and treated with CCL28 had a larger increase in ROS production when compared to the other three conditions (Fig. 3), suggesting that the enhanced killing of STm after CCL28 stimulation may be in part due to enhanced ROS production.
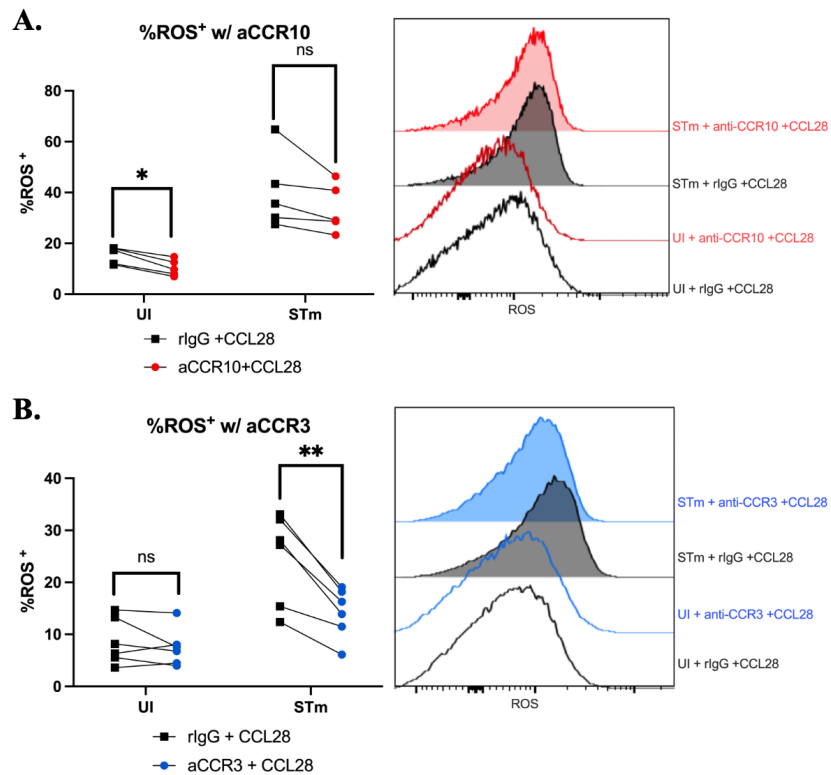
Figure 4. CCR3 receptor on neutrophils facilitates Reactive Oxygen Species (ROS) production in the presence of CCL28 during *Salmonella enterica* serovar Typhimurium (STm) infection.
Purified neutrophils treated with ROS detection dye, 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) for 30 minutes, were infected with STm for 4 hours in the absence and/or presence of CCL28. The neutralizing antibodies (A) anti-CCR10 or (B) anti-CCR3 were used to block their respective receptor. The isotype, rIgG2A was used as a control. Data shown consists of 4 independent experiments (n =5). ns, not significant; * ($P \leq 0.05$), **($P \leq 0.01$)

After noting that there is a trend in which ROS production increases in the presence of CCL28 during STm infection (Fig. 3), our next goal was to determine the mechanism by which this occurs. As previously mentioned, CCL28 influences the migration of immune cells expressing CCR3 and/or CCR10 (Lee et al., 2021). Moreover, we found that STm infection in neutrophils causes an increase in surface expression of both CCR3 and CCR10 (Fig. 1A, Fig. 2A). As such, we wanted to determine if either of these receptors facilitated the increase in ROS production in response to CCL28 (Fig. 3). Thus, neutralizing antibodies were used to block these receptors to

see whether they had any effect on the neutrophil ROS production. Uninfected and STm-infected neutrophils were treated with CCL28 and either neutralizing antibodies rIgG2a anti-mouse CCR3 (aCCR3), neutralizing antibody rIgG2a anti-mouse CCR10 (aCCR10) or isotype control rIgG2a.

Uninfected neutrophils that were treated with CCL28 and aCCR10 had significantly less ROS production than uninfected neutrophils treated with CCL28 and rIgG2A (Fig. 4A). However, STm infected neutrophils treated with CCL28 and aCCR10 showed no significant difference in ROS production, when compared to those treated with CCL28 and rIgG2A (Fig. 4A). These results indicate that enhanced neutrophil ROS production caused by CCL28 stimulation during STm infection was not mediated by CCR10. Being that there is low expression of surface CCR10 during infection, it makes sense that blocking this receptor had no major effects on ROS production.

When we investigated the effects of aCCR3 on neutrophil ROS production, there was no significant difference between uninfected neutrophils that were treated with CCL28 and aCCR3 compared to uninfected neutrophils treated with CCL28 and rIgG2A (Fig. 4B). Interestingly, among the STm infected neutrophils stimulated with CCL28, aCCR3 significantly decreased expression of ROS compared to rIgG2A isotype control ($p \leq 0.001$) (Fig 4B). These results suggest that CCR10 does not play a pivotal role in neutrophil ROS production during infection, as there was no significant decrease in ROS production when aCCR10 was added. However, CCR3 facilitates the increase in neutrophil ROS production mediated by CCL28 during STm infection. In conclusion, CCL28-induced ROS production during STm infection is predominantly facilitated through the CCR3 receptor.

DISCUSSION

Initial studies suggested that neutrophils did not express CCR3 (Perez-Lopez et al., 2021). However, these studies did not look at CCR3 expression in neutrophils in the context of inflammatory disease. A other study demonstrated CCR3 expression on human neutrophils isolated from the lung of patients with chronic lung disease and on neutrophils from bronchoalveolar lavages of mice infected with influenza (Rudd et al., 2019). These seemingly contradictory publications can be resolved by our results. Our study demonstrated that neutrophils express both surface and intracellular CCR3, and expression is increased during STm infection (Fig. 1) Interestingly, intracellular CCR3 expression is significantly higher when compared to surface expression. Furthermore, the expression of CCR10 in neutrophils (Fig. 2) has not been previously reported. Our research showed surface expression of CCR10 in neutrophils followed a similar trend to CCR3, although at lower levels of expression. Collectively our data suggest that CCR3 and CCR10 are stored intracellularly and relocate to the surface during infection. Thus, our findings could explain why initial studies did not see surface expression of CCR3 and CCR10 on neutrophils.

Overall, our results indicate that CCL28, through CCR3, is protective during STm infection. Our lab has also focused on *Acinetobacter baumannii* in the context of a pneumonia model. Whereas CCL28 played a protective role during STm infection, CCL28 was not protective in lung infection with *Acinetobacter*. *Ccl28*$^{-/-}$ mice infected with *Acinetobacter* survived the entirety of the experiment (10 days), while WT littermates succumbed to *Acinetobacter* by 48 hours. These results demonstrated that CCL28 can be harmful during lung infection. Although previous scientific literature has shown that neutrophils are essential in controlling Acinetobacter

during infection (García-Patiño et al., 2017, Grguric-Smith et al., 2015, van Faassen et al., 2007) it appears that an excess recruitment of neutrophils to the *Acinetobacter*-infected lung is detrimental. This could possibly be a result of the antimicrobial properties neutrophils possess, such as ROS production, which also cause damage to the host.

Oxidative stress is the imbalance between the production and accumulation of ROS where the presence of ROS exceeds the ability of a biological systems to detoxify these reactive products. ROS production is a balancing act. During regulated levels of ROS production, ROS is beneficial as it helps combat pathogens such as bacteria, fungi, and viruses. This beneficial role can be observed within individuals suffering from chronic granulomatous disease. These individuals are unable to produce O_2^- due to a defect in the NADH oxidase system, which then leads to cases of persistent infection (Pizzino et al., 2017). Although beneficial in such context, too much ROS production can also have negative consequences. In excess, ROS can lead to the destruction of cellular structures, such as membranes, lipids, ultimately leading to cell death. If not controlled, excess ROS production has been attributed to several diseases (Pizzino et al., 2017).

Our data supports the hypothesis that CCL28 is beneficial during STm infection as it promotes ROS production of neutrophils. Others have found that CCL28 is increased during *Helicobacter pylori* infection (Hansson et al., 2008). Furthermore, human colon epithelial cells increase *Ccl28* mRNA in response to bacterial flagellin or infection (Ogawa et al., 2004). Broadly, these studies suggest that CCL28 is a conserved response to bacterial infection, and our data supports a novel mechanism by which CCL28 promotes clearance of infection. Understanding how to regulate neutrophil ROS production via CCL28 may have relevance to a multitude of gut

infections. Therefore, the regulation of CCL28 can be used in clinical settings as a novel form of therapy during gut infections.

Our findings could potentially have implications also for COVID-19, a disease caused by the novel coronavirus SARS-CoV-2. Through the course of the pandemic, it has been established that the inflammatory response induced by SARS-CoV-2 is a leading factor of disease severity and overall death in SARS-CoV-2-infected patients (Del Valle et al., 2020). Recent studies have found that CCL28 is highly induced in both adult patients with severe COVID-19 symptoms (i.e. organ damage) and children suffering from COVID-19-associated multisystem inflammatory syndrome (Gruber et al., 2020; Yan Yan et al., 2021), suggesting that the chemokine CCL28 is involved in the immune response elicited by SARS-CoV-2. Furthermore, it has been established that patients with severe COVID-19 symptoms have higher levels of ROS, through ROS production markers such as lipid peroxidation, and neutrophil to leukocyte ratio (Laforge et al., 2020). Following the observations made during *Acinetobacter* infection, one can predict that excess ROS production observed during COVID-19 infection can be enhanced by CCL28, which also facilitate the migration and/or retention of neutrophils to the lung, further increasing ROS production.

Overall, my thesis contributes to demonstrating that CCL28 plays an important role during the mucosal immune response to pathogens via the involvement of neutrophils through CCR3. Furthermore, it establishes that neutrophils ROS production can be reduced through the use of a CCR3 neutralizing antibody. Depending on the site of infection, whether the lung or the gut, the results yielded can be implemented in the context of diseases where neutrophils are involved, leading to advancements in therapies that regulate or exacerbate the neutrophil ROS response. For

instance, in the context of lung infection a CCR3 neutralizing antibody might be used to control ROS production to alleviate the severity of tissue damage that can occur. In the case of gut infection, CCL28 might be used as a means to increase the immune response to enhance pathogen clearance.

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