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CCL28 Is Involved in Mucosal IgA Responses, Olfaction, and Resistance to Enteric Infections

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CCL28 is a mucosal chemokine that has been involved in various responses, including IgA production. We have analyzed its production in human tissues using a comprehensive microarray database. Its highest expression is in the salivary gland, indicating that it is an important component of saliva. It is also expressed in the trachea, bronchus, and in the mammary gland upon onset of lactation. We have also characterized a *Ccl28*$^{-/-}$ mouse that exhibits very low IgA levels in milk, and the IgA levels in feces are also reduced. These observations confirm a role for the CCL28/CCR10 chemokine axis in the recruitment of IgA plasmablasts to the lactating mammary gland. CCL28 is also expressed in the vomeronasal organ. We also detected olfactory defects (anosmia) in a *Ccl28*$^{-/-}$ mouse suggesting that CCL28 is involved in the function/development of olfaction. Importantly, *Ccl28*$^{-/-}$ mice are highly susceptible to *Salmonella enterica* serovar Typhimurium in an acute model of infection, indicating that CCL28 plays a major role in innate immunity against *Salmonella* in the gut. Finally, microbiome studies revealed modest differences in the gut microbiota between *Ccl28*$^{-/-}$ mice and their co-housed wild-type littermates. The latter observation suggests that under homeostatic conditions, CCL28 plays a limited role in shaping the gut microbiome.

Keywords: chemokines, vomeronasal organ, *Salmonella*, innate immunity, gut

Introduction

Chemokines are small secreted cytokines that represent an important family of chemotactic mediators. They arose through evolution from an ancestral chemokine gene that gave rise to a superfamily that has been divided into inflammatory and homeostatic chemokines depending on their expression patterns (Zlotnik and Yoshie 2012). While chemokines are best known for their chemotactic activities, they are now known to be involved in many other important processes, including development (Raz 2003), cancer and metastasis (Zlotnik and others 2011), and mobilization of hematopoietic stem cells (Stover and others 2017), among others. There are 48 human chemokines known, of which 4 represent chemokines with predominant expression in mucosal tissues (Hernandez-Ruiz and Zlotnik 2017).

These include CCL25, CCL28, CXCL14, and CXCL17. Probably the best characterized of these is CCL25, which is expressed in the small intestine and recruits CCR9^{+} T cells (Svensson and others 2002). The function of the rest has only recently started to be characterized. Based on the phenotype of a *Cxcl14*$^{-/-}$ mouse, Hara and Tanegashima (2012) concluded that CXCL14 is involved in metabolic responses and feeding behavior, whereas CXCL17 has emerged as an important macrophage chemotactic factor that recruits macrophages to mucosal tissues (Burkhardt and others 2014).

CCL28 is a mucosal chemokine produced by epithelial cells that binds a chemokine receptor called CCR10 (Wang and others 2000). It is expressed by epithelial cells in many mucosal tissues, including the respiratory tract, the digestive system, and the female reproductive system. One of its

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properties is wide-spectrum antimicrobial activity, including candidacidal activity mediated by a Histidine motif found at the carboxy terminus of its sequence (Hieshima and others 2003). CCL28 has been identified as responsible for the recruitment of IgA producing plasmablasts (Wilson and Butcher 2004) to the mammary gland upon the onset of lactation, allowing them to produce the IgA that will be transferred to the newborn in the milk. Supporting this conclusion is the fact that the *Ccl28* gene only exists in mammalian genomes (unpublished observation), suggesting mammalian-specific functions.

There have been 2 studies describing the phenotype of $Ccr10^{-/-}$ mice, and both reported that $Ccr10^{-/-}$ mice exhibit defects in IgA production (Morteau and others 2008; Hu and others 2011). Matsuo and others (2017) have recently reported the production and phenotyping of a $Ccl28^{-/-}$ mouse, which exhibits reduced IgA and an altered microbiota in the colon.

In the present study, we have also characterized a $Ccl28^{-/-}$ mouse. These mice develop normally but exhibit abnormalities in their reproductive behavior, which we have mapped to defects in olfaction. We also show that Ccl28 is expressed in the vomeronasal organ (VNO), suggesting that $Ccl28^{-/-}$ mice have abnormal development or function of this organ. This is consistent with reports that have documented that CCL28 is expressed in the nasal mucosa (Bourges and others 2007). In addition, $Ccl28^{-/-}$ mice also exhibit defects in IgA production in the milk, a finding that, as we mentioned above, mirrors the phenotype of $Ccr10^{-/-}$ mice. Finally, we observed that $Ccl28^{-/-}$ mice are highly susceptible to *Salmonella enterica* serovar Typhimurium in an acute infection model, indicating that CCL28 plays a pivotal protective role in enteric infections.

Taken together, these observations highlight the important roles that CCL28 plays in both mucosal homeostasis and in innate/adaptive immune responses.

Materials and Methods

Mice

Mice were obtained at the heterozygote stage ($Ccl28^{+/-}$) from Deltagen (www.deltagen.com/target/deltabase.html). Briefly, the *Ccl28* gene was replaced with a Neo cassette by homologous recombination resulting in a whole-body knockout mouse in the C57Bl/6 background. Unless otherwise specified, controls for most experiments were wild-type (WT) littermates obtained in the process of maintaining the $Ccl28^{-/-}$ mouse colony.

Mice were bred to obtain homozygote *Ccl28*-deficient mice ($Ccl28^{-/-}$) or WT mice with 2 functional copies of *Ccl28*. All mice used were housed in the same specific pathogen-free facility with a 12-h dark/12-h light cycle with autoclaved bedding and irradiated food. All breeding and handling of the mice were performed under an approved IACUC protocol.

Genotyping polymerase chain reaction

Ear punches were collected from 3-week-old mice upon weaning. DNA was extracted from the ear punches using a kit (Bioland Scientific, Paramount, CA). Primers specific to the Ccl28-targeting construct were used in a 35-cycle conventional polymerase chain reaction (PCR) to determine the genotype of each mouse. The amplified DNA products were analyzed on a 1% agarose gel. Primers: *Ccl28* forward 5′-AAGATGAGGACAGGCTGGTACTCTG-3′, *Ccl28* reverse 5′-CTACAGTTGCAGACATGTTGG-3′, Neo forward 5′-GGGTGGGATTAGATAAATGCCTGCTCT-3′, Common reverse 5′-ATTTCGCATGTCCTTGCTGAGGGAC-3′.

Collection of mouse samples for studies

Several biological samples were collected from WT, $Ccl28^{+/-}$, or $Ccl28^{-/-}$ mice for secreted molecule studies by enzyme-linked immunosorbent assay (ELISA). Serum was collected following centrifugation of blood collected through cardiac punch. Fecal samples were collected from the colons of mice during postmortem dissection. Milk was collected from the stomachs of 9-day-old mice during postmortem dissection. All samples were stored at −20°C until their analysis by ELISA.

Isolation of the mouse VNO

VNO isolation was performed according to instructions from the JOVE video ''An Effective Manual Deboning Method To Prepare Intact Mouse Nasal Tissue With Preserved Anatomical Organization'' (DOI 10.3791/50538). Briefly, mice were euthanized with lethal doses of CO_2 followed by cervical dislocation. To gain access to the soft palate on the roof of the mouth, scissors were used to separate the upper and lower jaws from each other. The upper portion of the skull was removed from the rest of the animal and placed, soft palate side down, in a Petri dish of cold 1 × PBS until ready to perform complete dissection. Using a dissection microscope, the soft palate was cut open and pulled back to reveal the underlying bony structure containing the VNO. Using scissors, the vomer bone was then cut at the caudal and rostral side and gently removed using fine forceps; the VNO was attached. Gentle dissection separated the VNO from the vomer bone. Any remaining cartilage or bone fragments still attached were carefully removed. The VNO was then placed in RNALater for RNA extraction.

IgA ELISAs

IgA ELISAs were performed using the Ready-SET-Go! Mouse IgA ELISA Kit (eBioscience, San Diego, CA). Assays were performed according to the manufacturer's instructions and the absorbance at 450 nm was read using a BioTek ELx800 machine (BioTek, Winooski, VT). Data were processed in Excel and analyzed using GraphPad Prism (www.graphpad.com).

CCL28 ELISA

CCL28 ELISA was performed using the Mouse CCL28 ELISA MAX Deluxe Kit from BioLegend (BioLegend, San Diego, CA). Assays were performed according to the manufacturer's instructions and the absorbance at 450 nm was read using a BioTek ELx800 machine (BioTek). Data were processed in Excel and analyzed using GraphPad Prism (www.graphpad.com).

Quantitative real-time PCR analysis

Total RNA was extracted from mouse tissues using TRIzol (Invitrogen, Carlsbad, CA) and subsequently purified using Qiagen's RNEasy columns and DNase digest (Qiagen, Valencia, CA). Equal concentrations of RNA were used for each

tissue sample in a reverse transcription reaction to synthesize cDNA (Qiagen). Each reaction used cDNA synthesized from 1 μg of total RNA, gene-specific primers, and gene-specific UPL probes to quantitatively detect *Ccl28* and control gene transcripts in each tissue sample. The results were processed in Excel and analyzed using GraphPad Prism (www.graphpad.com).

Olfaction test

These assays were performed as described (Del Punta and others 2002; Kurtenbach and others 2013). Briefly, an Oreo cookie was hidden under the corncob bedding in the back left corner of a standard mouse cage. The mice to be tested were placed in the cage at the opposite corner. Time was measured, in seconds, starting from the time the mice were placed in the cage until they took a bite of the cookie. If after 400 s a mouse had not found the hidden cookie, time was stopped, the mouse was removed from the cage and the animal was assumed to have defects in olfaction compared with WT controls. Fresh cages and fresh cookies were used for each experiment to prevent crosscontamination. Control mice were either WT or heterozygous $Ccl28^{+/-}$ littermates of $Ccl28^{-/-}$ mice.

Analysis of the microbiota

Fecal samples were collected from $Ccl28^{-/-}$ mice and wild-type, cohoused littermate mice. The samples were snap frozen in liquid nitrogen, and then DNA was later extracted using the QIAamp DNA Stool Kit (Qiagen) according to the manufacturer's instructions with modifications as previously described (Behnsen and others 2014). DNA extracted from fecal samples was amplified by PCR of 16S rDNA (V4 region) with primers 515F and 806R modified by the addition of barcodes for multiplexing, then sequenced on an Illumina MiSeq system (UC Davis HMSB Facility). Sequences were processed and analyzed by employing the QIIME pipeline v1.9.1 with default settings, except as noted. In brief, paired-end sequences were joined, quality filtered, and chimera filtered (usearch61 option; RDP gold database); operational taxonomic units were picked *de novo* (''pick_otus'' options: enable_rev_strand_match True, otu_picking_method usearch61) at 97% similarity, employing the SILVA rRNA gene database v123 (align_seqs:template_fp core_alignment_SILVA123.fasta; ''filter_alignment'' options: allowed_gap_frac 0.80, entropy_threshold 0.10, suppress_ lane_mask_filter True); taxonomy was assigned with the RDP classifier (''assign_taxonomy'' options: assignment_method rdp, confidence 0.8, rdp_max_memory 24000, reference_ seqs_fp 97_otus_16S.fasta, id_to_taxonomy_fp consensus_ taxonomy_7_levels.txt). Samples were rarefied to 10,000 reads and then alpha (Shannon index) and beta (unweighted and weighted UniFrac) diversity were assessed through QIIME. Prism 7 software (GraphPad) was used for statistical analyses (Mann–Whitney *U* test).

Salmonella *infection*

Mice were infected as previously described using an acute infection model (Barthel and others 2003; Raffatellu and others 2008, 2009). Briefly, mice were pretreated with streptomycin (0.1 mL of a 200 mg/mL solution in sterile water) intragastrically before oral inoculation with fully virulent *Salmonella enterica* serovar Typhimurium strain IR715 (1×10^9 colony-forming units (CFUs)/mouse) and mouse survival was monitored at 24, 48, and 72 h postinfection.

In vitro *growth curves*

Salmonella enterica serovar Typhimurium strain IR715 cultures were grown overnight at 37°C in LB supplemented with 50 μg/mL of nalidixic acid (Nal). The following day, cultures were diluted 1:100 in LB and grown at 37°C for 3 h, subsequently diluted to $\sim 0.5 \times 10^6$ CFU/mL in 1 mM potassium phosphate buffer (pH 7.2) as previously described (Hieshima and others 2003), and incubated at 37°C in the presence or absence of recombinant human or murine CCL28 (BioLegend). After 2 h, samples were plated onto LB Nal agar to enumerate viable bacteria.

Results

CCL28 is a mucosal chemokine highly expressed in salivary glands

CCL28 has been recognized to be a mucosal chemokine. However, a comprehensive study of its expression in the human body has not been reported. We analyzed the expression of *CCL28* in the BIGE (Body Index of Gene Expression) database that represents 130 tissues from 4 human males and 4 females (Lee and others 2005; Roth and others 2006). As shown in Fig. 1, the highest expression of *CCL28* in the human body is in the salivary glands. In support of this conclusion, CCL28 is readily detectable in saliva from normal humans (Hieshima and others 2003; Hernandez-Molina and others 2015). The second site of strong expression is the mammary gland, a finding that supports a role for CCL28 in lactation [it has been reported to mediate the recruitment of $CCR10^+$ IgA-producing plasmablasts to the maturing mammary gland (Wilson and Butcher 2004)].

We have confirmed that *Ccl28* is induced in the mouse mammary gland upon the onset of lactation (not shown). Rounding up the top expression sites are trachea and bronchus, and the thyroid gland. Hybridization values for the top 14 human tissues that exhibit significant expression of *CCL28* are shown in Table 1. Other noteworthy sites of expression include tongue superior [*CCL28* is expressed by the lingual epithelium (Hevezi and others 2009)], colon, urethra, small intestine, and skin. CCL28 has been recognized to be a mucosal chemokine that plays a role in both lung and digestive tract physiology (Hernandez-Ruiz and Zlotnik 2017). In the digestive tract, Matsuo and others (2017) have shown that it plays a role in IgA production and likely shaping of the microbiome through its antimicrobial activity and in the lung it may play a role in eosinophil recruitment during allergic inflammation (John and others 2005).

However, this expression pattern suggests that its main role in the human digestive tract may be as an important component of saliva, of which an estimated 1 L is produced every day, and it continuously covers the oral cavity and esophagus. It is also expressed in the small intestine and colon; however, its expression in these tissues likely involves only specific subsets of epithelial cells.

CCL28 is required for the recruitment of IgA plasmablasts to the mammary gland

We obtained a $Ccl28^{-/-}$ mouse, which was produced by replacing the *Ccl28* mouse gene with a *Neo* cassette by homologous recombination, resulting in a whole-body *Ccl28* knockout mouse. We confirmed that *Ccl28* expression was not

FIG. 1. Expression of CCL28 in the human body (BIGE database). Hybridization Units in the *Y* axis to tissue-specific cDNAs (*X* axis) correspond to probeset 224027_at from the U133 2.0 Affymetrix human genearray. Significant expression was observed in (highest to lowest) salivary gland; mammary gland; trachea; bronchus; and thyroid.

Table 1. Significant Expression of *CCL28* in Human Tissues (from the BIGE Database)

Human tissue	*CCL28 expression*[a]
Salivary gland	823.06 ± 158.35
Mammary gland	251.50 ± 177.23
Trachea	122.46 ± 32.63
Bronchus	79.02 ± 24.89
Thyroid	92.85 ± 14.29
Tongue superior	47.58 ± 43.64
Nipple	43.653 ± 29.18
Colon	42.64 ± 7.36
Tongue	35.56 ± 10.41
Skin	32.64 ± 16.39
Stomach pyloric	32.35 ± 8.85
Urethra	29.06 ± 6.46
Small intestine	27.92 ± 2.48
Kidney medulla	27.17 ± 6.00

[a]Hybridization Units of probeset 224027_at (Affymetrix U133 2.0 genearray) corresponding to human *CCL28* to cDNAs from each tissue in the BIGE database (20); mean ± standard deviation. Background hybridization units averaged a mean of 20.

BIGE, Body Index of Gene Expression (Database of human microarray data).

detected in some organs where *Ccl28* is normally expressed (salivary gland, small intestine) (using quantitative PCR, data not shown). Initial phenotyping of this mouse indicated that it develops normally, and is fertile. Given the reported role of CCL28 in the recruitment of IgA^+ B cell plasmablasts, we measured the levels of immunoglobulins in serum, milk, and feces of $Ccl28^{-/-}$ mice or WT littermates. As shown in Fig. 2, there was no significant difference in IgA levels in the serum of $Ccl28^{-/-}$ mice, an ~50% drop in IgA levels in the feces of $Ccl28^{-/-}$ mice, but the biggest decrease in IgA levels was observed in milk from $Ccl28^{-/-}$ mice. All other immunoglobulin isotypes were normal (data not shown). These observations indicate that $Ccl28^{-/-}$ mice has a similar phenotype to $Ccr10^{-/-}$ mice, which also exhibit a paucity of IgA in the milk (Morteau and others 2008; Hu and others 2011) and confirm that the CCL28/CCR10 axis is responsible for the recruitment of IgA plasmablasts to the mammary gland upon the onset of lactation.

We have also studied peripheral B cell subsets in the $Ccl28^{-/-}$ mouse and have observed no abnormalities in splenic or peritoneal B cell subsets (data not shown).

CCL28 expression is required for efficient olfaction

Interestingly, however, $Ccl28^{-/-}$ mice displayed abnormal reproductive behavior. Both males and females were observed mounting their same-sex cagemates during the daytime. A review of the literature indicated that similar behavior has been observed in mice where 16 V1R genes were deleted (Del Punta and others 2002). V1r and V2r genes encode 7 transmembrane receptors that represent a superfamily of genes involved in the detection of pheromones that are part of the olfactory system in mice and are expressed in the VNO (Del Punta and others 2002). These observations suggested that $Ccl28^{-/-}$ mice may exhibit abnormal olfaction. To test this hypothesis, we used a behavioral test that depends on olfaction (cookie test) (Dawson and others 2005; Kurtenbach and others 2013). In this test, an Oreo cookie is hidden under the bedding of a cage and a mouse is placed in the cage. Time measurements are taken from the time the mouse is placed in the cage until it finds the cookie. As shown in Fig. 3, $Ccl28^{-/-}$ mice show a marked deficiency in this test, indicating that they have deficient olfaction. We then hypothesized that CCL28 may be necessary for efficient development of the mouse olfactory system (VNO). As shown in Fig. 4, CCL28 is expressed in the VNO. Taken together, these observations suggest that CCL28 may play a role in its function/development.

CCL28 is involved in the host response against enteric infections

CCL28 is expressed in the normal small intestine and colon (Table 1) and an increase in CCL28 expression has been associated with epithelial inflammation (Wang and others 2000; Ogawa and others 2004); in line with these findings, CCL28 expression is increased in the colon of patients with inflammatory bowel disease and returns to normal levels when patients are treated with infliximab (anti-TNFα) (Arijs and others 2011). We, therefore, hypothesized that CCL28 may play a role in protection against the enteric pathogen *Salmonella enterica* serovar Typhimurium. Human infections with nontyphoidal *Salmonella* serovars are characterized by rapidly induced acute inflammation in the terminal ileum and colon (Zhang and others 2003). This acute infection can be modeled in mice by pretreatment with streptomycin 24 h before infection

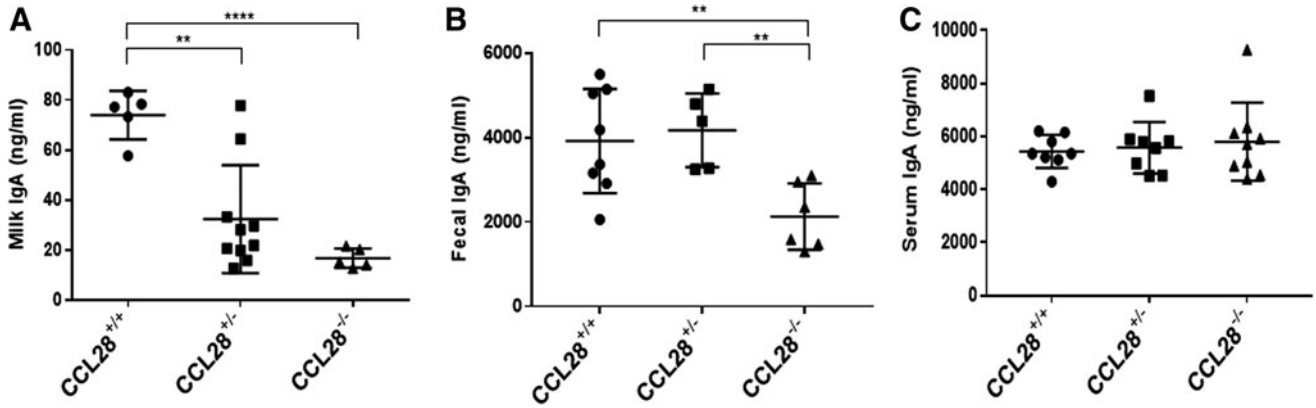

FIG. 2. **(A)** IgA levels in milk from $Ccl28^{+/+}$ WT, $Ccl28^{+/-}$ heterozygote, or $Ccl28^{-/-}$ mice. **(B)** IgA levels in feces from $Ccl28^{+/+}$ WT, $Ccl28^{+/-}$ heterozygote, or $Ccl28^{-/-}$ mice. **(C)** IgA levels in serum from $Ccl28^{+/+}$ WT, $Ccl28^{+/-}$ heterozygote, or $Ccl28^{-/-}$ mice. Shown is a representative experiment (out of 3). $^{**}P < 0.01$; $^{****}P < 0.001$.

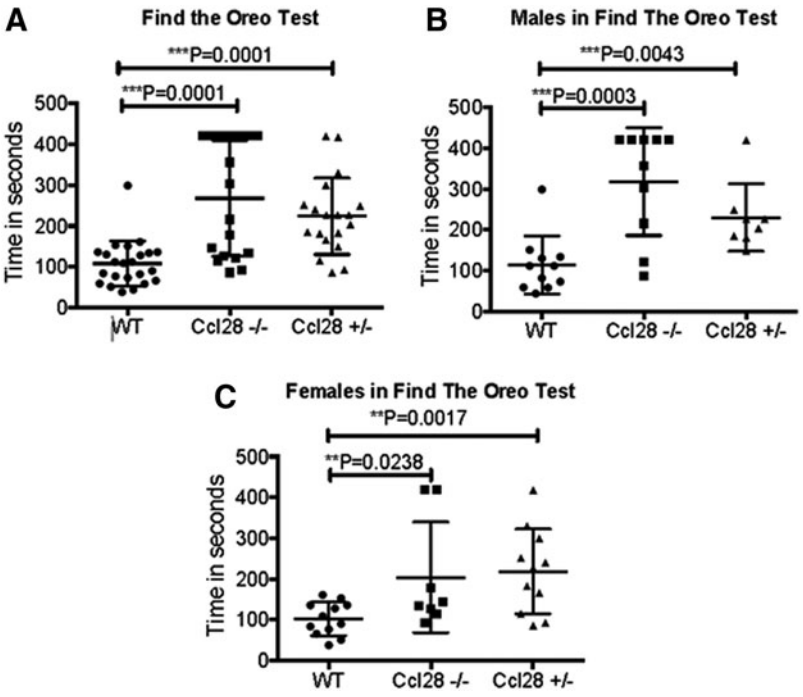

FIG. 3. Find the Oreo Test Results for WT, *Ccl28*$^{+/-}$, and *Ccl28*$^{-/-}$ Mice. **(A)** Combined data from male and female mice that were tested for the time (in seconds) needed to find the Oreo cookie buried in the cage bedding. **(B)** Time (in seconds) it took WT, *Ccl28*$^{+/-}$, and *Ccl28*$^{-/-}$ male mice to find the Oreo. **(C)** Time (in seconds) it took *Ccl28*$^{+/-}$ and *Ccl28*$^{-/-}$ female mice to find the Oreo. For both genders, *Ccl28*$^{+/-}$ and *Ccl28*$^{-/-}$ mice took significantly longer to locate the Oreo compared with WT littermates, indicating anosmia. These experiments were repeated twice, with at least 6 mice per group each experiment, using fresh mice for each experiment to control for learned behavior. WT, wild type.

(colitis model) (Raffatellu and others 2008, 2009). We sought to investigate a possible role for CCL28 in resistance against *Salmonella* using this model. To this end, we infected WT and *Ccl28*$^{-/-}$ littermate mice with *Salmonella enterica* serovar Typhimurium orally and followed the course of the infection to evaluate the survival of the mice. As shown in Fig. 5, *Ccl28*$^{-/-}$ mice are extremely susceptible to *Salmonella* in this acute infection model. *Ccl28*$^{-/-}$ mice started to succumb to the infection within 24 h following the bacterial inoculation, and only 40% of the *Ccl28*$^{-/-}$ mice survived by 72 h postinfection, whereas 100% of the WT mice were alive at this time point postinfection (Fig. 5). There was also a trend toward higher CFU in the spleen of *Ccl28*$^{-/-}$ mice (Table 2 shows CFUs in the spleen from a representative experiment). These data indicate that CCL28 is a pivotal chemokine mediating innate immunity against *Salmonella* infection in the gut.

Microbiome changes in Ccl28$^{-/-}$ *mice*

CCL28 has been reported to have antimicrobial activity (Hieshima and others 2003). We, therefore, sought to analyze the gut microbiome of *Ccl28*$^{-/-}$ mice and compare them to their cohoused wild-type littermates. Although the intrasample Eubacterial diversity was increased in *Ccl28*$^{-/-}$ mice, the difference was not significant ($P < 0.095$; Fig. 6A). Similarly, although the intersample community composition is trending toward being different between the 2 groups of mice (Fig. 6B), they are not distinct groupings, and the overall community structures fully overlap (Fig. 6C). When analyzed at the phylum level, the gut microbiota reflects what is usually observed under homeostatic conditions: a predominance of the phyla Bacteroidetes and Firmicutes (Fig. 6D). Although there was a slight average increase in the abundance of Firmicutes with a concomitant decrease of

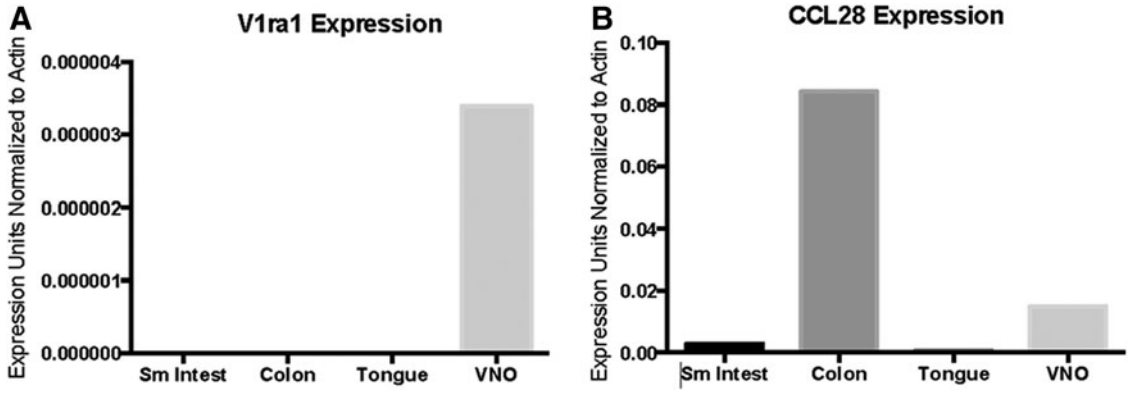

FIG. 4. Expression of the VNO-specific gene *V1ra1* and *Ccl28* in WT mouse mucosal tissues by qPCR. **(A)** Expression of the VNO-specific gene Vomeronasal Receptor A1 (*V1ra1*) was used as positive control to confirm the successful isolation of mouse VNO tissue. **(B)** Expression of *Ccl28* in the VNO in addition to confirming its previously reported expression in mucosal tissues, including small intestine (Sm Intest) and colon. Representative experiment (out of 2) is shown. qPCR, quantitative polymerase chain reaction; VNO, vomeronasal organ; WT, wild type.

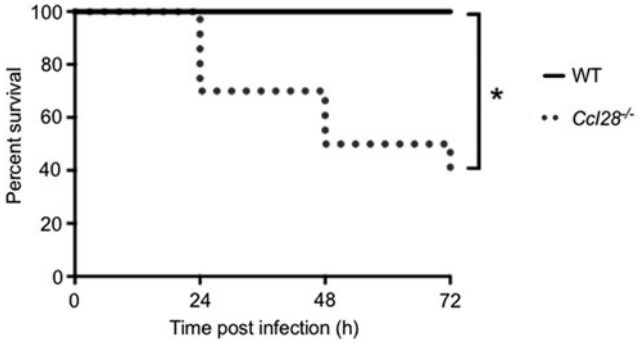

FIG. 5. Survival curve of *Ccl28*$^{-/-}$ ($n=10$) and WT ($n=7$) littermate control mice infected orogastrically with 1×10^9 fully virulent *Salmonella* (IR715). Data shown are combined results from 2 independent experiments. *$P<0.05$. WT, wild type.

Bacteroidetes in the absence of Ccl28, the differences were not significant (Fig. 6D). Elsewhere, we did see a few significant differences among taxa that each represented on average ≤1% of the microbiota. In *Ccl28*$^{-/-}$ mice, there were decreases of ~2-fold in the phylum Proteobacteria and ~10-fold in the family Peptostreptococcaceae; conversely, we saw an increase of ~6-fold in the Lachnospiraceae genus UCG-006. Taken together, these data suggest that although there are small changes in the microbial composition in the gut of *Ccl28*$^{-/-}$ mice, the absence of Ccl28 did not have a big impact on the gut microbiota of cohoused littermates.

Discussion

Chemokines represent a superfamily of chemotactic cytokines that have been studied for the last 30 years. While initially their chemotactic activities were the main focus of studies on chemokines, they are now known to be involved in multiple biological processes. Of the 48 human chemokines (Zlotnik and Yoshie 2012), 4 exhibit a predominantly mucosal expression pattern (Hernandez-Ruiz and Zlotnik 2017). These include CXCL14, CCL25, CCL28, and CXCL17. The best characterized of these are CCL25 and CCL28. However, even for these better-characterized mucosal chemokines, many questions remain unanswered. In this study, we have focused on the function of CCL28 through the characterization of its expression pattern in humans as well as the functional characterization of a *Ccl28*$^{-/-}$ mouse.

Table 2. Colony-Forming Units at 72 H Postinfection

	CFUs per mg of spleen	
Mouse No.	*WT*	*Ccl28*$^{-/-}$
1	1481	
2	2735	
3	1804	
4		272487
5		6835
6		[a]
7		21546390
8		[a]
9	392857	
10	492958	
11	8019	
12	1222	
13		[a]
14		[a]
15		56250000
16		[a]
17		[a]
Mean	10478	1225710

[a]Mouse was moribund and had to be sacrificed before 72 h postinfection (pi)

$P=0.1091$ (Mann–Whitney test).

CFU, colony-forming units; WT, wild type.

The first important observation we report in this study is that the main expression site of CCL28 in humans is in the salivary glands (Fig. 1). This important observation is confirmed by the fact that it is normally abundant in human saliva (Hernandez-Molina and others 2015). Matsuo et al. first reported the production of CCL28 by human salivary glands and further mapped it to epithelial cells of the parotid glands (Hieshima and others 2003). Our survey of the BIGE database confirms these data and further indicate that the salivary glands are the main site of expression of CCL28 in the human body. Other studies have documented that it is expressed in the small intestine (Bourges and others 2007), and female reproductive tissues (Cha and others 2011). However, data from the BIGE database indicate that the expression of CCL28 in the salivary glands is much higher than in other mucosal tissues (Fig. 1).

Humans produce around 1 L of saliva a day, and it ''bathes'' the digestive tract. This observation suggests that it may have important effects in the oropharyngeal cavity, for example, where its antimicrobial activity may be important against pathogens such as *Candida albicans* (Hieshima and others 2003). In support of this, we have reported lower levels of CCL28 in saliva from patients with Sjögren's syndrome (Hernandez-Molina and others 2015), suggesting that its absence could account for the greater incidence of Candidiasis in patients with this disease (Yan and others 2011). If this is correct, then supplementing artificial saliva with CCL28 may bring therapeutic benefit to these patients. Surprisingly, the BIGE database also indicates that CCL28 is expressed in the thyroid gland (Fig. 1 and Table 1). In support of this, CCL28 has been detected in a study of genes associated with thyroid carcinoma (Huang and others 2017). However, there is currently no information on a possible function of CCL28 in the thyroid.

Ccl28 starts to be expressed in the mammary gland upon the onset of lactation where it chemoattracts CCR10$^+$ IgA plasmablasts, which will produce the IgA that will be present in the milk that will be transferred to infants (Lazarus and others 2003; Wilson and Butcher 2004). Two previous studies have documented that *Ccr10*$^{-/-}$ mice have very low or no IgA in the milk (Morteau and others 2008; Hu and others 2011). This observation led to the conclusion that Ccl28 normally recruits IgA plasmablasts to the mammary gland upon the onset of lactation (Lazarus and others 2003; Wilson and Butcher 2004). In support of this conclusion, we observed that the *Ccl28*$^{-/-}$ mouse also exhibits minimal IgA levels in the milk and lower levels in the gut (Fig. 2).

One of these previous studies (Hu and others 2011) reported similar results for gut IgA in a *Ccr10*$^{-/-}$ mouse, but found that the repertoire of the fecal IgA antibodies in the

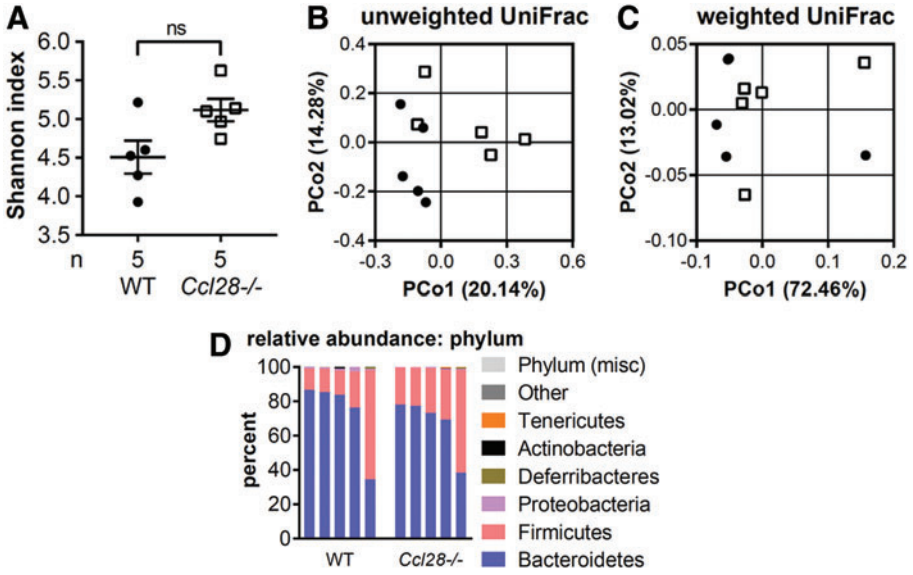

FIG. 6. 16S sequence analysis of fecal DNA from WT and *Ccl28*$^{-/-}$ mice. **(A)** Alpha diversity (Shannon index) in the gut microbiota of WT (*black circles*) or *Ccl28*$^{-/-}$ (*white squares*) mice. **(B, C)** Beta diversity represented by a PCoA (principal coordinate analysis) plot based on the **(B)** unweighted or **(C)** weighted UniFrac metric. **(D)** Bar chart of phylum relative abundance for each sample. ns = no significant change (Mann–Whitney-*U*) relative to WT control. WT, wild type. Color images are available online.

CCR10$^{-/-}$ mice was limited compared with WT mice. The similar IgA phenotype (to *CCR10*$^{-/-}$ mice) exhibited by *Ccl28*$^{-/-}$ mice confirms that the CCL28/CCR10 axis mediates the recruitment of IgA plasmablasts to the lactating mammary gland. The latter point is important because another chemokine (CCL27), is also able to bind CCR10 (Homey and others 2000). Similarly, CCL28 has also been reported to be able to bind CCR3 (Pan and others 2000). Taken together, our data indicate that there is no other CCR10 ligand that can substitute for CCL28 in this function, and that the CCL28/CCR10 chemokine axis recruits IgA plasmablasts to the lactating mammary gland.

A *Ccl28*$^{-/-}$ mouse is viable and develops normally. However, we noticed abnormal reproductive behavior. Both males and females were observed mounting their same-sex cagemates during the daytime. When we reviewed the literature, we noticed that similar behavior has been observed in mice engineered to have defects in the VNO, which mediates olfaction in mice (Del Punta and others 2002). We, therefore, tested the olfactory ability of *Ccl28*$^{-/-}$ mice and observed abnormal olfactory ability suggesting anosmia (Fig. 3). Since *Ccl28* is expressed in both olfactory epithelial mucosa (Bourges and others 2007; Nagakubo and others 2016) and in the VNO (Fig. 4), we hypothesize that it may be required for normal development and/or function of the olfactory system. Its absence during development in the *Ccl28*$^{-/-}$ mouse is likely responsible for this abnormal behavior and anosmic phenotype.

We also tested the ability of the *Ccl28*$^{-/-}$ mouse to handle infection with *Salmonella* in an acute infection model. In this model, infection develops in a few days and therefore resistance is dependent on innate immunity mechanisms operational in the gut and does not depend on the development of IgA responses (Behnsen and others 2014) because of its short duration (just a few days). As shown in Fig. 5, *Ccl28*$^{-/-}$ mice are very susceptible to *Salmonella* in this acute infection model and succumb to the infection rapidly (starting at 24 h). To explain this surprising phenotype, we hypothesized that CCL28 may be involved in an important mechanism of resistance to *Salmonella* in 2 possible ways: first, CCL28 may be active against *Salmonella* through its wide-spectrum antimicrobial activity (Hieshima and others 2003). Second, it may be necessary for the recruitment of certain cell(s) of the immune system to the infected intestine that mediate innate resistance to *Salmonella.* If the latter is the case, we predict that these cells should express either CCR10 or alternatively CCR3 [which has also been reported to bind CCL28 (Pan and others 2000)].

We favor the second hypothesis, because we have observed that recombinant CCL28 does not show significant antimicrobial activity against the *Salmonella* strain we used for this model (data not shown), and a knockout mouse of the also mucosal chemokine Cxcl17 does not exhibit enhanced susceptibility to *Salmonella* in the same acute infection model (data not shown) even though Cxcl17 (like Ccl28) also exhibits broad antimicrobial activity (Burkhardt and others 2012).

Furthermore, we have recently reported the identification of a novel B cell-associated cytokine called interleukin 40 (IL-40). An *Il-40*$^{-/-}$ mouse exhibits significant defects in gut IgA production, especially in the gut (Catalan-Dibene and others 2017). We have tested *Il-40*$^{-/-}$ mice in the same *Salmonella* infection model, and observed that *Il-40*$^{-/-}$ mice did not show increased susceptibility. By 72 h after infection, 100% of *Il-40*$^{-/-}$ mice were alive (data not shown), whereas, as shown in Fig. 5, only 40% of *Ccl28*$^{-/-}$ mice were alive at the same time point (72 h) postinfection. These observations confirm that IgA is not involved in the mechanism of susceptibility to *Salmonella* observed in the *Ccl28*$^{-/-}$ mouse. Taken together, these observations strongly suggest that CCL28 is part of an important innate immunity mechanism operating in the gut that renders mice resistant to *Salmonella* in this acute infection model.

Finally, we analyzed the fecal microbiome of *Ccl28*$^{-/-}$ mice. Although we did detect some differences in the microbiome of the *Ccl28*$^{-/-}$ mice relative to cohoused wild-type littermates (Fig. 6), they were relatively modest and the only significant changes were among a few taxa that individually represent ≤1% of the microbiota. A recent report also showed only minor changes in the microbiota of another *Ccl28*$^{-/-}$ mouse and cohoused wild-type mice (not littermates) (Matsuo and others 2018). This study identified

a modest but significant expansion of class Bacilli in *Ccl28*$^{-/-}$ mice (Matsuo and others 2018), which we did not observe in our colony. Importantly, microbiota analyses are known to be influenced by several factors, including housing and diet, even when mice have the same genetic background (Rausch and others 2016). While Matsuo and others (2018) compared *Ccl28*$^{-/-}$ mice with WT mice from a different colony that were cohoused at weaning, our control mice were WT littermates that were housed together for an extended period of time before obtaining the samples for analyses.

From another perspective, it is not uncommon for animals in different facilities to be colonized with different strains of the same microbial taxa, so the susceptibility profiles of the microbes could also be different. Another factor that could influence microbiota studies is the fact that in the gut, CCL28 acts like an inflammatory chemokine; that is, its expression in the normal gut is detectable but not high. Instead, it is strongly induced in gut epithelial cells during inflammation (Arijs and others 2011). Furthermore, as shown in Fig. 1 and Table 1, CCL28 is normally more abundant in the oral cavity than in the intestinal gut. Therefore, its antimicrobial role may be more important in shaping the microbiome of the oral cavity than in the intestinal mucosa. Its particular ability to kill *C. albicans* (Hieshima and others 2003) also lends support to this hypothesis.

We conclude that CCL28 is a very versatile multifunctional chemokine. Our data indicate that it is involved in several processes, including recruitment of IgA plasmablasts to the mammary gland, an unexpected function in the olfactory process, and finally, it represents a critical factor in gut innate immunity against *Salmonella*. Our data confirm that the recruitment of IgA plasmablasts to the mammary gland is mediated by the CCL28/CCR10 chemokine axis. Further studies are needed to fully understand the role of CCL28 in the olfactory process and its role in host defense against enteric infections.

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Author Disclosure Statement

No competing financial interests exist.

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