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Expression of duck *CCL19* and *CCL21* and *CCR7* receptor in lymphoid and influenza-infected tissues

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Abstract

Ducks are the natural host and reservoir of influenza viruses. We are interested in their immune responses to these viruses, to understand host-pathogen interactions and to develop effective agricultural vaccines. We identified duck homologues of the chemokines *CCL19* and *CCL21* and cloned their cognate receptor, *CCR7*. Conservation of key features, and expression in lymphoid tissues suggests that these chemokines are the direct orthologues of their mammalian counterparts. Mammalian *CCL19* and *CCL21* are responsible for the homing of dendritic cells and naïve lymphocytes to secondary lymphoid tissues. The contribution of local tertiary lymphoid tissues may be important during influenza infection in ducks. Consistent with leukocyte recruitment, *CCL19* and *CCL21* transcripts are abundant in lung tissues at 1 day post-infection with highly pathogenic avian influenza A/Vietnam/1203/04 (H5N1) (VN1203). In contrast, expression in lung or intestine tissues infected with low pathogenic A/mallard/BC/500/05 (H5N2) (BC500) is not significant. Recruitment and aggregation of leukocytes is visible in the vicinity of major airways 3 days after infection with VN1203. Chemokine gene expression may serve as a useful marker to evaluate duck immune responses to natural infections and vaccine strains.

Keywords

Adaptive immunity; ducks; avian influenza; chemokines; dendritic cells

1. Introduction

Ducks are the natural host and animal reservoir of influenza viruses (Webster et al., 1992). Typically they are unharmed by influenza, and show no disease signs (Kida et al., 1980). Immune responses to influenza strains that replicate in the lung, are robust and protective (Kida et al., 1980). In contrast, immune responses to low pathogenic avian influenza that replicates in the intestine, are relatively weak (Kida et al., 1980), and ducks can chronically

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shed virus or become infected with other strains in the environment (Latorre-Margalef et al., 2009; Sharp et al., 1997). The factors that initiate a successful adaptive immune response in ducks are largely unknown. A better understanding of duck immune responses, in particular, activation of dendritic cells and naïve T cells is critical for the development of effective adjuvants and vaccines.

Because of their essential role in the trafficking of leukocytes to lymphoid tissue for the priming of T cells and initiation of the adaptive immune response, we sought to characterize two duck chemokines with homology to *CCL19* and *CCL21* that were identified in a sequencing project (Xia et al., 2007). Mammalian *CCL19* and *CCL21*, acting through a common receptor, *CCR7*, orchestrate trafficking of naïve lymphocytes and dendritic cells to secondary lymphoid tissues (Forster et al., 2008). Through sequence analyses, we demonstrate that the duck *CCL19* and *CCL21* are orthologues of the mammalian chemokines. We identify the duck *CCR7* receptor gene and show it is highly conserved. Both chemokines and receptor are expressed in lymphoid and mucosal tissues of the duck, consistent with their involvement in leukocyte recruitment. We also detect increased transcript abundance for *CCL19* and *CCL21* in tissues infected with highly pathogenic avian influenza, but not a low pathogenic strain. We demonstrate recruitment of leukocytes by histology to VN1203 infected lung tissue, consistent with the initiation of an effective immune response.

2. Materials and methods

2.1. Animals and tissues for RNA extraction and histology

White Pekin ducks were euthanized by lethal injection of Euthanol and tissue samples removed and frozen in liquid nitrogen. For isolation of putative lymph nodes, we anesthetized ducks with a 2:1 ratio of ketamine and Rompun (active ingredient Xylazine) and injected India ink to track lymphatic tissue in the sublingual area. Sublingual, clavicular and mesenteric lymphatic tissues were obtained from 3 mature ducks. Brain, heart, lung, kidney, duodenum, spleen tissues were obtained from 2 mature ducks, and a bursa was obtained from a 26 day-old duck. RNA was extracted using TRIzol® Reagent (Invitrogen). RNA samples from influenza-infected ducks were obtained from a previous study (Barber et al., 2010) in which we infected ducks with either A/Vietnam/1203/04 (H5N1) or A/mallard/British Columbia/500/05 (H5N2). A/Vietnam/1203/04 (H5N1) was generated by reverse genetics (Salomon et al., 2006) and A/mallard/British Columbia/500/05 (H5N2) was an environmental isolate. All animal experiments were approved by the Animal Care and Use Committee of St. Jude Children's Research Hospital and performed in compliance with relevant institutional policies. VN1203 was handled in biosafety level 3+ and BC500 in biosafety level 2 facilities approved by the United States Department of Agriculture and Centers for Disease Control and Prevention. Duck lung samples infected with VN1203 were collected at time of necropsy, fixed in 10% neutral-buffered formalin. Paraffin-embedded tissues were sectioned 5 micrometers thin. Sectioning and hematoxylin-eosin staining was performed by the Veterinary Core Pathology Laboratory at St. Jude Children's Research Hospital.

2.2. Sequencing cDNA clones encoding *CCL19* and *CCL21* and *CCR7*

In an expressed sequence tag (EST) project to identify immune relevant genes in duck spleen (Xia et al., 2007), two clones (4B6 and 17C4) bearing significant homology to mammalian CC chemokines were isolated. The sequences were determined and deposited in GenBank, for *CCL19* (GenBank ID: AY682098) and *CCL21* (GenBank ID: HQ174214). A 716 bp fragment of *CCR7* was amplified from duck spleen mRNA (Mesa et al., 2004) using degenerate primers (Table 1). The full-length sequence of *CCR7* was obtained by 5'

and 3' rapid amplification of cDNA ends (RACE) using the BD SMART™ RACE cDNA Amplification Kit. The assembled sequence was used to predict the mRNA sequence for the *CCR7* gene (**GenBank ID: ACB46528**). CLUSTALW at DDBJ was used for sequence alignments (<http://www.ddbj.nig.ac.jp/search/clustalw-e.html>). An arrayed cDNA library (Xia et al., 2007) was screened with overlapping primers creating 40-mer probes for *CCR7* and *CCL19*. Probes were ³²P-labelled using the Random PrimeIt kit (Stratagene). One cDNA clone, 140I24, hybridized to the *CCR7* probe, and partial sequencing confirmed that it carries the full-length cDNA sequence already determined by RACE. Two clones, 109O21 and 17F21, hybridized to *CCL19*, and their sequences were identical to the 4B6 *CCL19* sequence.

2.3. Isolation and sequencing of a genomic clone for duck *CCR7*

We screened a genomic library (Moon and Magor, 2004) using the overgo probes for *CCR7* and *CCL19*. We obtained clone 249G7 that hybridized with *CCR7* and restriction mapped it, and subcloned and sequenced a 10 kb *NheI* fragment using the EZ: TN< KAN> transposon insertion kit (Epicentre, Madison, WI). We determined the remaining coding region sequences by primer walking within clone 249G7, and the entire *CCR7* contig was deposited under **GenBank ID: EU418503**. No clones hybridized with the *CCL19* probe.

2.4. Semi-quantitative amplification using reverse-transcription PCR

RNA was quantified using a Nanodrop ND-1000 UV-vis spectrophotometer. DNAase digestion with DNase I AmpGrade was performed and first-strand cDNA was synthesized using 1 µg of RNA and Superscript III™ reverse transcriptase (Invitrogen). Reverse-transcription PCR was performed for *CCL19*, *CCL21* and *CCR7* using 50 ng of cDNA template from organ tissues or 100 ng from lymphatic network tissues. PCR amplifications were performed for 35 cycles for *CCL19*, *CCL21* and *CCR7*, 30 cycles for *GAPDH* and 25 cycles for immunoglobulin light chain (*IgL*), at an annealing temperature of 65°C for *CCL19* and 60°C for others.

2.5. Quantification of gene expression by qRT-PCR amplification

To measure *CCL19* and *CCL21* gene expression in lymphatic network tissues and influenza-infected tissues we used qRT-PCR using the 7500 Fast Real Time PCR instrument (Applied Biosystems) and the FastStart TaqMan® Probe Master (Roche). Primers and probe sets (Integrated DNA Technologies (IDT)) were designed using Primer Express version 3.012.5. qRT-PCR amplifications were done using 12.5 ng of cDNA template for lymphatic tissues and spleen, and 6.25 ng of cDNA for mock-infected or influenza-infected spleen and lung tissues. We used 62.5 ng of cDNA template to detect influenza M gene in intestine samples. PCR conditions were 95°C for 10 min, and 40 cycles of denaturation at 95°C for 15 s followed by annealing-extension at 60°C for 1 min. All samples were run in triplicate, each assay repeated at least once. Expression levels of the target genes were normalized to the housekeeping gene *GAPDH* using the relative quantitation method ($\Delta\Delta CT$), and the analysis done using the 7500 Fast system software version 1.4 (Applied Biosystems).

3. Results

3.1 Avian *CCL19* and *CCL21* chemokines and *CCR7* receptor resemble their mammalian homologues

To look for conserved features, we aligned the deduced amino acid sequences for duck *CCL19* and *CCL21* with those of chicken, human, mouse and rat (Fig. 1A and B). The first two cysteines in the mature chemokine sequences are adjacent to each other, thus identifying these as CC chemokines. The DCCL motif, present in *CCL19* family members, is

conserved. The D7 of this motif is involved in ligand binding (Ott et al., 2004a). C8 and C9 form disulphide bonds with cysteines C34 and C55, which are also conserved. The *N*-glycosylation site, seen in the murine ortholog (Yoshida et al., 1997) is absent in duck CCL19. Duck and chicken CCL19 have a putative cleavage site between H21 and G22, removing a 21 amino acid signal peptide that has two additional unique cysteines. The predicted signal peptide cleavage site for duck CCL21 is between G23 and V24 and the mature protein is 88 amino acids long. The amino acid identity of duck and chicken CCL19 is 76.3% whereas its identity with the human protein is only 41.7%. Duck CCL21 shows 75.9% amino acid identity with a putative chicken CCL21 and only 35.1% identity to human CCL21.

Avian CCL21 is lacking most of the carboxyl-terminal extension seen in the mammalian CCL21 proteins (Hromas et al., 1997; Nagira et al., 1997). This region of CCL21 binds heparin sulphate residues to immobilize the chemokine to the lumen of the high endothelial venules (de Paz et al., 2007; Hirose et al., 2002; Patel et al., 2001) and is cleaved to release a gradient of soluble chemokine (Schumann et al., 2010). However, we predict that the avian CCL21 possesses the ability to bind heparin sulphate because the shorter region is overall very rich in basic amino acids and carries the motif BXBBXBBB, where B represents a basic amino acid and X any amino acid. The BXBB motif of RANTES is sufficient for heparin-binding (Proudfoot et al., 2001). Although the avian chemokines share low sequence identity with the mammalian chemokines, they have many of the conserved features and similar structure.

To confirm the identity of the duck chemokines, we generated a phylogenetic tree using related CC chemokines. Our phylogenetic analysis shows that although avian *CCL19* and *CCL21* are distant from their mammalian homologues they do group with their respective orthologues, and group separately from the *CCL20* chemokines (Fig. 1C). While clear relationships of most chicken CC chemokines to those in human and mouse cannot be discerned, Kaiser and colleagues previously noted that chicken *CCL19* and *CCL21* had direct orthologous relationships with mammalian CC chemokine sequences (Kaiser et al., 2005b). The sequence and phylogenetic analyses suggest the duck chemokines are *bona fide* *CCL19* and *CCL21* orthologs.

To ensure that the cognate receptor for CCL19 and CCL21 is also present in ducks, we used degenerate primers to amplify the chemokine receptor *CCR7*, and RACE to obtain the full sequence from cDNA. The predicted amino-acid sequence is 90.1% identical to chicken *CCR7* and 67.6% identical to human *CCR7* (Fig. 2A). As a G-protein coupled receptor it has seven transmembrane domains and a highly conserved region between amino acids 150 and 159, including the DRY motif required for protein activation. K128, K135 and Q225 (equivalent to mammalian K130, K137 and Q227) involved in ligand binding and receptor activation (Ott et al., 2004b) are conserved, although residues in the vicinity differ greatly between avian and mammalian sequences. Mutational analyses of human residues indicated that K130 was important for receptor activation by CCL19 and CCL21, while K137 was necessary for binding of CCL21, as well as chemotaxis in response to this chemokine. Q227 was important in receptor activation via CCL19 and CCL21 binding, though mutation did not affect chemokine binding. The duck residue D301 replaces human N305, which is required for receptor activation and chemotaxis (Ott et al., 2004b). Thus the residues required for interaction with both respective ligands are present in the duck *CCR7* homologue.

To determine the genomic organization for *CCR7*, we sequenced across the coding region on fosmid clone 249G7. A comparison of the gene organization of duck *CCR7* to those of chicken, mouse and human shows this gene is highly conserved between avian and

mammalian species (Fig. 2B). Using our cDNA sequence for comparison, we assigned the sequence to three exons, with exon 1 encoding only 3 amino acids, exon 2 encoding 16 amino acids, while the remaining 355 residues are in exon 3. The intron-exon splice sites for exons 1, 2 and 3 create junctions with +1, +1 and 0 nucleotide overhangs, respectively. Similar splice junctions are also seen for chicken *CCR7*, although intron 1 is much shorter than in duck or the mammalian receptors. The exon organization and splicing junctions of avian *CCR7* resemble its mammalian homologues.

3.2. *CCL19*, *CCL21* and *CCR7* transcripts are abundant in duck lymphoid tissues

To assess the distribution of the chemokines and their receptor in duck tissues, we determined their gene expression by RT-PCR (Fig. 3). We saw highest expression of *CCL19* in spleen and lung, and lower expression in kidney, duodenum and bursa. *CCL21* expression is highest in spleen, but it is also expressed in heart, lung, kidney, duodenum and bursa. Duck *CCR7* mirrored the expression pattern for *CCL19*, with highest expression in spleen and lung. Duck secondary lymphoid organs, spleen and bursa, showed high expression of these chemokines, in keeping with their predicted role in leukocyte recruitment. The high expression of *CCL19* and *CCR7* in lung, suggests that it also contains significant lymphoid tissue.

The expression of *CCL19*, *21* and *CCR7* in kidney was unexpected, so we also examined the expression of *IgL* as a marker of the presence of lymphocytes. All tissues except heart and brain were positive for *IgL* expression. *IgL* was abundantly expressed in kidney, as previously seen for liver (Magor et al., 1994), which has been attributed to numerous foci of lymphoid tissue present in many duck organs. We believe that leukocytes in blood in these tissues probably contributed little to the gene expression because heart (with the most blood) showed no *CCL19* or *CCR7* expression. Also, isolated peripheral blood leukocytes showed a different profile of chemokine gene expression than seen in these tissues, with expression of *CCR7* and *CCL19* and no expression of *CCL21* (data not shown). These data suggest that kidney contains significant lymphoid tissue.

3.3. *CCL19* and *CCL21* transcripts are rare in tissues from duck lymphatic network

Ducks are reported to have lymph nodes (Berens von Rautenfeld and Burdras, 1983), which are absent in chickens and all other non-mammalian vertebrates (Hofmann et al., 2010). We were unable to identify anything morphologically resembling lymph nodes, so we tracked the sublingual lymphatic network using an injection of India ink in the duck palate. We isolated the sublingual lymphatic tissue, which should contain the thoracic lymph nodes, and homogenized it intact. We obtained other lymphatic network tissues from the clavicular and mesenteric regions. We amplified *IgL* as a positive control, confirming the presence of lymphocytes in the lymphatic network tissue. Using RT-PCR amplification, *CCL19* was barely detectable in sublingual lymphatic tissue, and *CCL21* and *CCR7* transcripts were weakly amplified in all lymphatic tissues, while all transcripts were robustly amplified in spleen (Fig. 4A). To quantify the relative expression of *CCL19* and *CCL21* in the lymphatic network tissues compared to spleen we used qRT-PCR (Fig. 4B). The amplification of duck *CCL19* was 100-fold higher in spleen than for sublingual lymphatic tissue, while *CCL21* was 400-fold higher in spleen than in sublingual lymphatic tissue. The low transcript abundance of chemokine genes in putative lymph nodes compared to spleen, suggests the lymph nodes of ducks, if they exist, do not contribute significantly to leukocyte recruitment.

3.4. *CCL19* and *CCL21* transcript abundance in influenza-infected tissues

Because of an apparent lack of lymph nodes, we reasoned that generation of tertiary lymphoid tissue in the infected tissues might contribute to local immunity, important in many viral infections (Carragher et al., 2008). Mice that lack conventional lymphoid organs

generate inducible BALT in their lungs following influenza infection (Moyron-Quiroz et al., 2006; Moyron-Quiroz et al., 2004), and *CCL19* and *CCL21* are essential for this leukocyte recruitment (Rangel-Moreno et al., 2007). To determine if *CCL19* and *CCL21* were expressed during an infection, we measured their gene expression following infection with a low pathogenic avian influenza environmental isolate (BC500) or a recombinant highly pathogenic avian influenza virus recovered from a fatal human infection (VN1203). We observe greater *CCL19* and *CCL21* transcript abundance in VN1203 infected duck lungs at 1 dpi compared to mock infected (Fig. 5A). However, we observe no significant change in *CCL19* or *CCL21* transcript abundance in response to BC500 in lung tissues. This correlates with viral load, since the relative abundance of the influenza M gene transcript by qRT-PCR is 10^4 times higher for VN1203 than BC500 infected lung (data not shown). We also examined expression of *CCL19* in spleen tissue from infected ducks. *CCL19* transcript is approximately 8-fold more abundant in spleen at 1dpi with VN1203 than in mock-infected tissue, therefore much less than the chemokine expression induced in lung (data not shown). *CCL19* expression in VN1203 infected lung and spleen is still increased at 3 dpi, however, it is lower than that seen at 1dpi (data not shown). *CCL19* and *CCL21* transcript abundance is increased in lung and spleen following infection with a highly pathogenic virus, but not a low pathogenic virus.

Because low pathogenic avian influenza replicates in the intestine, we also examined the expression of the chemokine transcripts in intestinal tissue. We observe no significant increase in *CCL19* and *CCL21* transcript abundance in BC500-infected intestine tissues compared to mock-infected intestine tissue at 1 or 3 dpi (Fig. 5B and C). We confirmed that these ducks were infected by recovering live virus from eggs inoculated with material from cloacal swabs on day 3. We also analyzed the tissues for presence of influenza using real-time PCR amplification of the M gene. The influenza M gene transcript in BC500-infected lung and intestine tissues was at the limit of detection using a very small amount of cDNA template (6.25 ng), but was readily amplified using more template (62.5 ng) (data not shown). Thus the VN1203 virus replicated to a much higher titre in lung tissue, than BC500 in either tissue. BC500 infection does not increase chemokine gene expression in infected tissues, which may be due to lower titre, or lower pathogenicity of the virus.

3.5. Leukocyte accumulation in VN1203 infected duck lung

To visualize leukocyte recruitment in response to influenza infection, we examined VN1203 infected duck lung tissues by histology (Fig. 6). We observe a visible thickening of the airway epithelium with evidence of leukocyte infiltration and migration into the lumen (Fig. 6A). Leukocytes are accumulating in the interstitial spaces concentrated near the airway epithelium, migrating out of the blood vessels and accumulating in the perivascular spaces. A leukocyte aggregate is forming near the major airway (Fig. 6B and 6C). While we have not analyzed the cellular composition of these aggregates, they appear to be iBALT nodules early in the initiation of lymphocyte follicles.

4. Discussion

Here we identify the genes encoding duck chemokines *CCL19* and *CCL21* and examine their tissue expression. Conservation of key features of the amino acid sequences and structure, albeit with low homology to mammalian chemokines, suggests that these are the duck homologues of the mammalian homeostatic chemokines, which control the trafficking of lymphocytes and dendritic cells. We have also identified the putative receptor for these chemokines, *CCR7*. The genomic organization, conserved amino acid sequence and features suggest that the duck *CCR7* is the direct orthologue of the mammalian *CCR7* receptor. *CCL19* and *CCR7* are most abundantly expressed in lymphoid and mucosal tissues, while *CCL21* is more widely expressed. We also saw increased transcript abundance of *CCL19*

and *CCL21* in lung tissue infected with highly pathogenic avian influenza, compared to mock infected tissues.

CCL19 and *CCR7* are expressed in lymphoid and mucosal tissues, while *CCL21* was expressed in all tissues except brain. The broader distribution of *CCL21* expression, is also seen in human tissues (Nagira et al., 1997). *CCL19* and *CCR7* show similar tissue distribution, with highest expression in lymphoid tissues. The expression pattern suggests that duck *CCL19* may play a more important role in lymphoid tissue than *CCL21*. In mouse, *CCL21* is necessary and sufficient for lymphocyte and dendritic cell trafficking (Britschgi et al., 2010). However, in gradients of directly opposing signals, mDCs migrate preferentially towards *CCL19*, and *CCL19* was shown to be 10–100-fold more potent than *CCL21* (Ricart et al., 2010).

In mammals, *CCL19* and *CCL21* transcript levels in lymph nodes greatly exceeds expression in spleen. In ducks, we observed 100-fold greater relative transcript abundance for *CCL19* in spleen, and 400-fold more expression of *CCL21* in spleen, than in tissues from the lymphatic network previously thought to contain lymph nodes. Given the low transcript abundance for the chemokines and their receptor, we infer that putative duck 'lymph nodes' do not contribute significantly to lymphocyte recruitment and maturation. The sublingual lymphatic network, which lies along the neck, should contain the thoracic lymph nodes described previously (Berens von Rautenfeld and Burdras, 1983), but the 'nodes' were not visible by eye. Perhaps this was because the animal was a naïve duck. Previously, prior to isolation of lymph nodes the duck had received repeated injections of BSA in IFA (Magor et al., 1994). However in a later study, cervical lymph nodes were isolated in naïve and even neonatal ducks, and *Ig* expression detected very early in ontogeny (Bando and Higgins, 1996). Both studies used the presence of *Ig* transcripts to identify the 'lymph node' as a lymphoid tissue. However, *Ig* transcripts could be present simply from B lymphocytes migrating through the lymphatic network in the absence of lymph nodes. Our data, showing insignificant expression of the chemokine genes in lymphatic network tissues, suggests that lymph nodes are absent in ducks.

We observed a significant increase in relative transcript abundance for *CCL19* and *CCL21* in VN1203 influenza-infected duck lung tissues at 1 dpi. One duck showed much greater chemokine expression than the other two, which may be due to the distribution of leukocyte aggregates in the tissue, or genetic differences between individual ducks. We examined two VN1203 infected ducks by histology, and while the leukocyte infiltration seen at day 3 was extensive in both, leukocyte aggregates were distributed randomly near major airways. Mammalian *CCL19* and *CCL21* are induced in influenza-infected lung and recruit lymphocytes and dendritic cells to establish tertiary lymphoid tissue (Rangel-Moreno et al., 2007). We speculate that tertiary lymphoid tissue in lungs of ducks may contribute to their survival against highly pathogenic avian influenza virus. Studies using splenectomized, lymphotoxin alpha knockout mice (which lack lymph nodes, Peyer's patches and spleen but have iBALT) showed these mice were resistant to higher titres of influenza virus than normal mice, suggesting they suffer less pathology than a systemic immune response would generate (Moyron-Quiroz et al., 2004).

Lung tissues infected with highly pathogenic avian influenza VN1203 had much greater relative abundance of *CCL19* and *CCL21* transcripts than tissues infected with low pathogenic BC500. The human *CCL19* promoter has functional NF- κ B and ISRE sites and responds to components elicited downstream of the TLR7 and RIG-I pathways (Pietila et al., 2007). Duck *RIG-I* expression was 200-fold higher in tissues infected with VN1203, compared to BC500 (Barber et al., 2010). Because *CCR7* ligands recruit and prime the T cells towards a Th1 phenotype (Marsland et al., 2005), and induce scanning behavior to

enable T cells to find their cognate ligands (Kaiser et al., 2005a), we might predict that initiation of T cell responses to VN1203 would be superior to those generated by BC500. Should this prove to be the case, the expression of these chemokines can serve as one marker for evaluation of immune responses to vaccines and or natural infections.

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Highlights

- > We identify duck orthologues of lymphoid chemokines CCL19, CCL21 and CCR7 receptor.
- > CCL19/21 and CCR7 are expressed in duck lymphoid and mucosal tissues.
- > Putative duck lymph nodes had no chemokine expression.
- > CCL19/21 transcripts increased in influenza-infected lung.
- > Leukocytes are recruited to infected duck lung tissue.

Fig. 1.

Duck chemokines CCL19 and CCL21 resemble their mammalian orthologues. The amino acid sequences were aligned using CLUSTALW and edited using BOXSHADE. Black shading indicates amino acid identity, grey shading indicates similarity and gaps are represented with a dot. The DCCL motif is boxed, the cleavage site is indicated with an arrow, and the cysteines with asterisks. (A) Alignment of CCL19 amino acid sequences (GenBank ID: duck [AAU04400](#), chicken [XP424980.2](#), mouse [NP036018.1](#), rat [NP001102131.1](#), and human [NP006265.1](#)). (B) Alignment of CCL21 amino acid sequences (GenBank ID: duck [HQ174214](#), chicken [CR522995.1](#), mouse [NP035254](#), rat [NP001008513.1](#) and human [NP002980](#)). (C) Phylogenetic tree constructed with amino acid sequences of CCL19, CCL21 and CCL20 of human, mouse, rat, chicken and duck. The bootstrap values for 1,000 replicates are shown. The tree was generated using CLUSTALW. Sequences aligned include those above and CCL20 sequences (Genbank accession: chicken CCL20 [NP989769](#), human CCL20 isoform 2 [NP001123518](#) and mouse CCL20 isoform 2 [NP001153210](#)).

Fig. 2.

Duck *CCR7* chemokine receptor resembles the mammalian orthologs in sequence and gene organization. (A) Alignment of *CCR7* amino acid sequences (Genbank ID: for duck **ACB46528**, chicken **XP425875**, mouse **NP031745**, rat **NP955783**, and human **NP001829**). Sequences were aligned using CLUSTALW and edited using BOXSHADE, black shading indicates amino acid identity, grey shading indicates similarity and gaps are represented with a dot.

(B) Schematic of gene organization of duck, chicken, mouse and human *CCR7*.

Fig. 3. *CCL19*, *CCL21* and *CCR7* transcripts are expressed in lymphoid and mucosal tissues. Transcripts for *Ig*, *CCL19*, *CCL21*, *CCR7*, *GAPDH* were analyzed by reverse transcription PCR. cDNA quality and quantity was assessed using *GAPDH*. Immunoglobulin light chain (*IgL*) transcripts show the presence of lymphocytes in these tissues. Tissues were analyzed from two ducks. Br: brain, H: heart, L: lung, K: kidney, D: duodenum, Bu: bursa, S: spleen, N: negative control.

Fig. 4. *CCL19*, *CCL21* and *CCR7* transcripts are barely detectable in lymphatic tissues in comparison to spleen. (A) *CCL19*, *CCL21* and *CCR7* gene expression was analyzed by reverse transcription PCR. cDNA quality and quantity was assessed using *GAPDH*, while IgL transcripts showed the presence of lymphocytes. Lanes correspond to SL: sublingual, CL: clavicular, and ML: mesenteric lymphatic tissues, S: spleen, N: negative control. Tissues were analyzed from three ducks. (B) Relative expression of *CCL19* and *CCL21* transcripts in lymphatic tissues and spleen by qRT-PCR normalized to *GAPDH*. Error bars show RQmin/max at a 95% confidence level. Tissues from one duck were analyzed and one representative of two assays is shown.

Fig. 5.

CCL19 and *CCL21* are up-regulated in duck lung infected with VN1203, but not BC500. Fold expression of *CCL19* and *CCL21* mRNA in (A) duck lung at 1 dpi, duck intestine at (B) 1dpi or (C) 3 dpi, compared to a mock-infected animal. Duck lung or intestine cDNA was analyzed by qRT-PCR and normalized to *GAPDH*. Error bars show $RQ_{\min/\max}$ at a 95% confidence level. One representative of two assays is shown.

Fig. 6. Leucocyte infiltration and aggregation in duck lung following VN1203 infection. Light micrographs of hematoxylin/eosin stained lung sections at day 3 post infection showing leukocyte infiltration (A) and aggregate formation (B) and enlarged in (C). A: airway, B: inducible BAL, E: epithelium, F: follicle, SM: smooth muscle, V: vessel. Sections are from one of two VN1203 infected ducks examined by histology.

Table 1

PCR primers and probes

Gene	Primer sequence (5' to 3')
CCR7	TAY TTC AAG MGG CTS AAG ACC ATG
Fragment	ARG AAR GGR TTG ASG CAG CA
CCR7	GCC AGG GCC AGG TTC AGC AGG
RACE	CCA TCA GCA TCG ACA GGT AC
CCR7	TTG AAG GCT GAG ATG GTC TTG ACC
Overgo	ACA ACA GCG TCA TGC TGG TCA AGA
CCL19	CAG GAA GGT CCC AAA TAA AGG CAA
Overgo	CTT CTT CAG GGC CTA ATT GCC TTT
CCL19	CGG TGG CAA CAA CGT CCT GGA <u>CTC GAG</u> ATT GCC TTT ATT TGG GAC CTT C
CCL21	ATG GCT CTG CGC ATC CTT CTG TTA GGC CTG CTT CCG CTT CAG
CCR7	AAG CGG CTG AAG ACC ATG AC CTG GGG ACG GAA GCG GTG GG
GAPDH	CCG TGT GCC AAC CCC CAA TGT CT GCC CTA CAG CAG CAG CCT TCA TC
IgL	CTG GGC CAG CCC AAG GTG TCT CC GCG TCA GGT AGC TGC TGG CCA TGA AC
CCL19	TGC CAG GAA GGT CCC AAA TA GCT TCA TGA GCT TCA GAT TCA CA 56FAM-TGG CCC CAG CAC CCA TAT CGG-31ABkQ
CCL21	GAA ACA TCA GCT CCC TGT GCT T GAC CAA AGT CCG TGT CTG AGA TC 56-FAM-CAG ACC CCG CTG GTG TGC CAA-31ABkQ
GAPDH	AAA TTG TCA GCA ATG CCT CTT G TGG CAT GGA CAG TGG TCA TAA 56FAM-ACC ACC AAC TGC CTG GCG CC-31ABkQ
Inf A matrix	GAC CRA TCC TGT CAC CTC TGA C AGG GCA TTY TGG ACA AAK CGT CTA TGC AGT CCT CGC TCA CTG GGC ACG