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Specific Compartmentalization of IgA ASCs in Mouse Salivary Glands via Differential Expression of Chemokines and Chemokine Receptors

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SPECIFIC COMPARTMENTALIZATION OF IMMUNOGLOBULIN A ANTIBODY SECRETING CELLS IN MOUSE SALIVARY GLANDS VIA THE DIFFERENTIAL EXPRESSION OF CHEMOKINES AND CHEMOKINE RECEPTORS

by

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ABSTRACT

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The mucosal system, which forms a barrier between internal organ systems and the external environment, is frequently exposed to pathogenic microorganisms. Immunoglobulin A (IgA) antibody secreting cells (ASCs) localize in the lamina propria, and produce IgA antibodies which help protect mucosal tissues. The concept of a common mucosal immune system in which IgA ASCs have the ability to populate any mucosal site has been proposed (1, 2). However, recent research has suggested that IgA ASCs primed in different mucosal sites might possess different sets of chemokine receptors, and therefore migrate specifically to particular mucosal locations (3). In this study, the specific compartmentalization of IgA ASCs in two mouse salivary glands:

sublingual gland (SLG), and submandibular gland (SMG) was studied. It was observed that SLG had 12 times more IgA ASCs per gram of gland than that of SMG ($p<0.01$). This suggested that IgA ASCs migrated to the two salivary glands with different efficiencies. Since the migration of lymphocytes is mediated by interactions between tissue specific chemokines and chemokine receptors, I hypothesized that the specific compartmentalization of IgA ASCs in the SLG and SMG was mediated by the differential expression of IgA ASC attracting chemokines. Quantitative PCR was used and showed that SLG expressed high levels of CCL28 and its receptor CCR10, which correlated to the distribution of IgA ASCs in the two salivary glands. In agreement with QPCR data, reduced levels of IgA ASCs were found in the SLG of CCR10 deficient mice when compared to wild type (WT) mice. Adoptive transfer of CCR10 deficient mice with WT spleen cells reconstituted the WT phenotype. It was therefore concluded that the interaction between CCL28 and CCR10 play an important role in mediating the migration of IgA ASCs into SLG. These results suggested that the accumulation of IgA ASC to distinct salivary glands is a highly selective process. These data also suggested that homing within mucosal sites is not common but rather a highly regulated process with specific subsets of cells homing to different tissues within the mucosal immune system.

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INTRODUCTION

Immune System

The immune system is a complex network of cells and organs in the body that provides protection against invading pathogens. The immune system protects the body through both innate and adaptive mechanisms. Adaptive immunity generates the "memory" of the immune system in which a more rapid and vigorous immune response results in a subsequent exposure to an antigen. Self and non-self recognition is another characteristic of the immune system in which, ideally, only foreign object are attacked. The cells of the immune system originate in the bone marrow. Lymphocytes then migrate to the primary lymphoid organs where they develop before migrating to the secondary lymphoid tissues (SLT). In the SLT, lymphocytes are activated by contact with antigens and undergo further differentiation. B cells originate and mature in bone marrow, while immature T cells migrate to the thymus for maturation. After maturation, the naïve lymphocytes recirculate through the lymph nodes, spleen, and mucosal associated lymphoid tissues (MALT) continually to encounter foreign antigens. Lymphocytes activated in the MALT become part of the mucosal immune system, which provide protection to the mucosal surfaces (4).

Mucosal Immune System

The mucosal system consists of the nasal, bronchial, gastrointestinal and urogenitinal tracts, as well as the lachrymal, salivary, mammary glands, and the synovium of joints (5). The large surface area of the mucosal system is the entry site of many pathogens. It is, therefore, important that the mucosal tissues are protected by the immune system. The MALT comprises the mucosal immune system and protects the

mucosal tissues with both humoral and cell mediated immunity. The MALT is usually divided into several components according to the anatomical locations. They are the gut associated lymphoid tissue (GALT), the bronchus associated lymphoid tissue (BALT), the nasopharynx associated lymphoid tissues (NALT), the mammary glands and the genitourinary lymphoid tissues (6). Among all the components of MALT, GALT is the largest and most extensively studied. GALT consists of the Peyer's patches (PP), appendix and mesenteric lymph nodes (MLN), and submucosal lymphoid follicles. It is estimated that GALT contains up to 80% of the body's immunoglobulin producing cells (7).

On the surface of the gastrointestinal (GI) tract resides a specialized epithelial cell type called M cells. These cells bind to and transcytosis foreign antigens to the conjugated MALT. The antigens transported to the subepithelial dome region in the GALT interact with immune cells including dendritic cells, macrophages, T and B cells. The B cells activated in this germinal follicle generally become IgA producing cells, which are responsible for secreting immunoglobulin in all the mucosal sites. These IgA committed B cells, however, do not developed into plasma cells within the germinal center (GC) in the PP. But rather leave the GC, migrate to draining lymph nodes and home to the lamina propria at mucosal sites, where they are further activated by cytokines, mainly TGF-β, secreted by Th2 cells. Here the activated B cells will fully differentiate into plasma cells and produce dimeric IgA for secretion (8).

Secretory IgA (SIgA) is the predominant immunoglobulin that protects mucosal tissues. Mucosal sites are frequently exposed to pathogens, so interactions between antibody and antigen are constantly occurring and optimal immune protection is vital.

Secretory IgA, which lacks the ability to activate complement, protects these tissues by binding to pathogens, blocking the adhesion of pathogens to the mucosal surface, and preventing the penetration of antigens into the internal environment. This binding also prevents the over stimulation of the immune system, which may result in extensive inflammation in the mucosal sites (9). In addition, SIgA also protects the mucosae by neutralizing toxins and inactivating harmful enzymes (10).

In order to protect mucosal tissues, IgA is secreted through the epithelial layer of the mucosal surfaces. IgA antibodies are synthesized in dimeric form with a J chain connecting the two monomers. The J chain allows the transcytosis of IgA across the mucosal epithelium by binding to the poly-Ig receptor (pIgR) on the basolateral surfaces of the epithelial cells. After transcytosis, the ligand-binding region of pIgR is enzymatically cleaved from the cell and becomes the secretory component (SC) of the secreted IgA. SC has anti-microbial properties and is also important in protecting the IgA dimer from proteolytic cleavage (11).

Human IgA is grouped into two subclasses (IgA1 and IgA2), while the mouse only has a single isoform of IgA. The proportions of the two human IgA subclasses vary among different mucosal sites. The distal gastrointestinal tract is predominantly protected by IgA2; whereas IgA1 is the major IgA subclass at salivary glands and nasal associated lymphoid tissue (5). The distribution of the two IgA subclasses generally reflects the local antigen types (proteins or polysaccharides) IgA ASCs encountered. IgA1 is the predominant subclass in humans and is often generated in response to protein antigens, while IgA2 antibodies often bind polysaccharides (12).

To study the ontogeny of the mucosal immune system in human, many researchers have used the level of salivary immunoglobulins. It was believed that salivary glands are part of the common mucosal immune system and the antibodies levels in the saliva are representative of mucosal immune competence (13). Collection of saliva is simple and non-invasive, and the secretion can be easily analyzed by routine laboratory procedures. Therefore saliva has long been used to monitor the mucosal immune system (5, 13-17).

Based on the analysis of saliva, detection of SIgA has been reported to be as early as at birth, although most researchers agree that secretion of SIgA begins between 1 week and 2 months of age (18). During the first few weeks of life, salivary IgA levels increase rapidly and peak at 4 to 6 weeks of age. The initial peaks of salivary SIgA slowly decline to lower levels between 3 and 6 months of age and are believed to reach adult salivary SIgA levels at around 7 years of age. Interestingly, at birth, the proportion of the two IgA subclasses is different from adulthood, even though IgA1 remains the predominant subclass. At birth, almost 90% of the salivary SIgAs are of IgA1, which decreases to around 60% in first 3 months of life to approach adult proportions (19). The decline of the IgA1 proportion may be the result of changes in antigen type and load after birth, when more polysaccharide based antigens are introduced.

Common Mucosal Immune System

The concept of a common mucosal immune system involves an interconnective network within the mucosal immune system to provide effective protection to all mucosal tissues. In theory this would provide a mechanism by which immunization to a single mucosal site would provide protection to all mucosal tissues. This hypothesis is based on data showing that immunization in one mucosal site would not only induce a response at

the site of antigen encounter, but also in remote mucosal surfaces and exocrine glands. It was observed that after oral immunization, IgA antibodies specific for the antigen were detected not only in the gut, but also in the bronchial tract, the salivary, lachrymal and mammary glands (20). It has also been demonstrated that cells from the BALT can repopulate the spleen, bowel and lung of lethally irradiated rabbits with IgA ASCs (2). Vice versa, IgA ASCs from GALT had similar ability to repopulate the bronchial mucosa (21). Adoptive transfer of the precursors of the IgA plasmacytes from PP reconstituted the gut and bronchus of allogenic or autologous irradiated hosts. Other adoptive transfer experiments by McDermott *et al*. (20) also showed that thymidine-labeled donor MLN cells migrated to recipient gut, cervix and vagina, uterus, mammary glands, and MLN.

Goldblum *et al*. (22) hypothesized that IgA ASCs originating in the intestinal tract have the capacity to migrate to other mucosal site, which may explain the phenomenon of the common mucosal immune system. They showed that antigen specific IgAs for non-pathogenic *E. coli* appeared in mammary gland secretion of lactating females after oral feeding of the bacteria. The migration of IgA ASCs from the intestinal mucosa into the mammary gland results in the secretion of SIgA specific for antigens found the mother's intestine, which the infant will most likely encounter. In the newborn, which has an immature immune system and a not fully developed intestine, the passive immunity received from breastmilk provides important protection. The importance of breastmilk antibody is also supported by the studies of Weisz-Carrington *et al*. (23), in which they showed that oral immunization of ferritin resulted in IgA ASCs localized in lactating mammary gland, salivary glands, and respiratory tract. They further confirmed this theory by showing that adoptively transferred ferritin-specific IgA ASCs from mesenteric

lymph node homed to exocrine glands of nonimmunized recipients (23). All these evidence suggested that there is a common mucosal immune system.

Homing of Lymphocytes and Chemokines

Lymphocyte trafficking into specific tissues is critical in maintaining normal homeostasis of the immune system. Many aspects of the immune system depend on the proper homing of leukocytes. These range from naïve T and B cells homing to lymph nodes for activation to the homing and accumulation of leukocytes to sites of infection and inflammation. Autoimmune diseases might result when activated lymphocytes home to inappropriate locations and exert immune responses. For example, when lymphocytic infiltration develops in the salivary glands due to the expression of homing signals, Sjögren's syndrome occurs. Inflammatory bowel disease is the result of aberrant activation of both innate and adaptive immune responses in the gastrointestinal tract (24), while rheumatoid arthritis is the condition when the immune system is activated and attacks the joints. Therefore the homing and migration of lymphocytes must be strictly regulated. It is known that the homing of lymphocytes is mediated by a cascade of interactions, including the interactions between chemokines and adhesion molecule ligands secreted or expressed by tissues, and chemokine receptors and the adhesion molecules expressed by the lymphocytes. Chemokines, which are small protein chemoattractant cytokines (~12kDa), can be called the organizers of the immune system as they promote the specific localization of lymphocytes into distinct parts in the body. Members of this family have conserved amino acid sequences and their receptors are G protein coupled seven trans-membrane helices (25).

Chemokines are grouped and named by the number and location of the conserved N terminal cysteines. There are four groups of chemokines: first, the CC chemokines, which have two adjacent cysteines at the amino end; second, the CXC chemokines with the two cysteines separated by a single amino acid; third, the C chemokines, which has only one conserved cysteine at the amino terminus; and the last group CX3C chemokines, which the two N terminus cysteines are separated by three amino acids. These chemokines act on different sets of receptors to orchestrate lymphocyte migration. There are ten known CC receptors (CCR), designated CCR1-10, and they bind to CC chemokines CCL1-28. CXC chemokines 1-16, on the other hand, bind to CXCR1-6 (25).

After binding to their cognate receptors, chemokines, exert their functions in two steps: first, they increase the binding avidity of the adhesion molecule integrin receptors expressed on the lymphocyte membrane allowing firm adhesion of the lymphocyte. Second, chemokines are also thought to be involved in lymphocyte extravasation and migration via concentration gradients of the chemokine molecules (4). Extravasation of lymphocytes out of blood vessels occurs in four steps. The first step is the rolling step and selectins are involved. Selectins are glycoproteins that are expressed on both endothelium and the lymphocyte membrane. Selectins bind to heavily glycosylated molecules such as mucins to promote the adhesion and rolling of lymphocytes along the epithelium. This interaction is not strong enough to stop the lymphocyte, but allows the lymphocyte to slow down for the activation in the second step. In the normal state, lymphocytes express integrins with low binding avidity to endothelium expressed cell adhesion molecules (CAMs). In the presence of appropriate chemokines, integrins on the lymphocyte that express the cognate chemokine receptors exert conformational changes,

resulting in firm attachment to the endothelial wall and rolling is arrested. In the third step, lymphocytes diapedese across the endothelial wall, which is, again, mediated by the interactions between the integrins and adhesion molecules expressed on the lymphocyte and the endothelial cells. In the forth step the lymphocyte migrates toward the source of the chemokine (4).

As understanding of the lymphocyte migration process increases, the molecular nature of homing to mucosa tissue is being better understood. It has been shown that gut associated mucosal venules express the mucosal addressin MAdCAM-1, which is an immunoglobulin superfamily adhesion molecule. MAdCAM-1 has been shown to bind $\alpha 4\beta 7$ integrin expressing lymphocytes and mediate homing to mucosal surfaces (26). Two main sets of mucosal attracting chemokines and their receptors have also been identified, these are CCL28 and its receptor CCR10; and CCL25 and its receptor CCR9 (3). Some IgA ASCs have also been shown to respond to CXCL12, which binds to receptor CXCR4.

CCL28 and CCR10

CCL28, also known as mucosae-associated epithelial chemokine (MEC), is a potent chemoattractant for CCR10 expressing IgA ASCs (3, 27). CCL28 is also called "a common mucosal chemokine" by Lazarus *et al*. (27) because it selectively attracts IgA ASCs, but not IgG or IgM ASCs, into almost all mucosal surfaces. CCL28 exerts its function by enhancing the $\alpha 4$ integrin dependent adhesion of IgA ASCs to MAdCAM-1 and vascular CAM-1 (VCAM-1), which are expressed in various mucosal sites (3). CCL28 is secreted at high levels by epithelial cells in various mucosal tissues including the salivary gland, colon, lactating mammary glands, and bronchial tissues (28-30).

Expression of CCL28 in small intestine has also been reported and anti-CCL28 blocking antibody can prevent the homing of IgA ASCs into both small and large intestine (3).

Among all the mouse mucosal tissues, salivary glands have the highest CCL28 mRNA level, as determined through dot blot and Northern blot analysis methods (30). Immunohistochemistry also shows that salivary glands express high level of CCL28, which correlates with the findings that high concentrations of CCL28 are found in human saliva (28). The high expression of CCL28 in salivary glands may mediate the migration of IgA ASCs into this mucosal site. It has been shown that IgA ASCs from mouse parotid glands, which is one of the major salivary glands, express CCR10 at high levels and respond to CCL28 in chemotaxis assays (28). These findings suggest that CCL28 may be important in attracting IgA ASCs to the two mouse salivary glands *in vivo*.

CCL25 and CCR9

CCL25, a CCR9 chemokine ligand, is selectively secreted by the thymus and small intestinal epithelial cells (31). Another name for CCL25 is thymus-expressed chemokine (TECK) as it is a chemoattractant for circulating and resident gut-homing T cells (32). It has been demonstrated that IgA ASCs from the spleen, intestinal lymphoid tissues, and the small intestine respond and migrate to CCL25 in chemotaxis assays (33). This suggests that CCR9 is probably induced during the development of IgA ASCs in the GALT. Similar to CCL28, CCL25 can also promote the adhesion of IgA ASCs to MAdCAM-1 and VCAM-1, which is important in the extravasation of IgA ASCs into the intestinal lamina propria (3). Although CCL25 is not highly expressed in mucosal tissues other than the small intestine (31), its ability in attracting IgA ASCs should not be

ignored, as it may also contribute in the specific homing of IgA ASCs into other mucosal tissues.

CXCL12 and CXCR4

CXCR4 and its ligand CXCL12, also known as stromal cell-derived factor 1 (SDF-1), is important in the localization of plasma cells in the splenic red pulp and in the bone marrow (34). It has been reported that IgA ASCs in both small and large intestine express CXCR4, and some IgA ASCs migrate to CXCL12 (3). CXCL12 can activate VCAM-1 binding to the integrin $\alpha4\beta1$. Glodek *et al*. (35) showed that CXCL12 promotes the sustained activation of $\alpha4\beta1$ in immature B cells in bone marrow, but not in mature, peripheral circulating B cells, and prevents the premature release of B cell precursors from bone marrow.

Unique IgA ASCs Migration Patterns

The differential secretion of CCL25 and CCL28 by mucosal tissues may promote the migration of antigen-specific IgA ASCs after oral or nasal immunization. For example, after oral immunization, antigen-specific IgA ASCs are detected in both intestinal and non-intestinal mucosal tissues, such as salivary glands, respiratory tract, and the mammary glands (36). However, after nasal immunization, distribution of antigen-specific IgA ASCs is only restricted the upper aerodigestive tract and the urogenital tract (37, 38). These results suggest that the differential expression of chemokines may contribute to the specific compartmentalization of IgA ASCs in the salivary glands.

Salivary Glands

In mammals, salivary glands are exocrine glands that produce saliva. There are three major pairs of salivary glands; they are the parotid glands, the submandibular glands (SMG), and the sublingual glands (SLG). In humans, they are located at different places in the mouth and the secretions from the three salivary glands are chemically different. Saliva consists of serous and mucous fluids. The serous fluid contains amylase to digest carbohydrates, and the mucous fluid serves as lubricant. In humans the parotid glands are the largest and they are located in the subcutaneous tissues of the face overlying the mandibular ramus and anterior and inferior to the external ear. Parotid glands produce almost purely serous saliva. SMG are located beneath the floor of the mouth and secrete a mixture of serous and mucous saliva. SLG are located anterior to the SMG, and the secretion is mainly mucous in nature (39).

IgA ASCs in Pig Tonsils and Mouse Salivary Glands

In the pig, the pharyngeal (Ph) and palatine (Pa) tonsils are located in different regions of the upper aerodigestive tract and form part of the Waldeyer's ring. It was previously believed that they played redundant roles in protecting the respiratory tract. However it has recently been observed that one of the tonsils has more abundant IgA ASCs (40). After intranasal immunization, antibody secreting cell response was induced in the Ph tonsil but not in the Pa tonsil, suggesting that these two tonsils may represent different compartments in the mucosal immune system (41). Analysis of the chemokines and adhesion molecules in these tissues revealed the differential expression of CCL28 and VCAM-1 in the two tonsils, where Ph expressed higher concentrations of both of the

adhesion molecules. The results correlated with differential compartmentalization of IgA ASCs in the tissues (40).

Being part of the mucosal system, the salivary glands produce large amount of IgA antibodies. However, not much is known about the specific localization of IgA ASCs in the salivary glands and the migration events of IgA ASCs into the glands have not been described. Similar to the pig tonsils, I found that the concentration of IgA ASCs in one of the mouse salivary glands, the SLG, was surprisingly higher than that of in the SMG. Since the localization pattern of IgA ASCs in the two mouse salivary glands were very similar to the pig tonsils, I hypothesized that the differential expression of epithelial chemokines (CCL28 and CCL25) and addressins (MAdCAM-1 and VCAM-1) controls the specific localization of the IgA ASCs into distinct mucosal sites. In this thesis, the distribution of IgA ASCs in the two mouse salivary glands was shown and its correlation with the mucosal attracting chemokines, chemokine receptors, and adhesion molecule levels was analyzed. My results demonstrated that CCR10 and its ligand CCL28 were expressed at high levels in SLG, which correlated with high numbers of IgA ASCs in the salivary gland. CCR10 knock out (KO) mice, which are deficient in the CCL28 receptor CCR10, had less IgA ASCs localized in SLG when compared with WT mice. CCR10KO mice adoptive transferred with WT cells reconstituted the SLG with high levels of IgA ASCs but not the SMG. Together these results suggest that differential expression of chemokines and chemokine receptors contributed to the distinct migration patterns of IgA ASCs into salivary tissues.

It has previously been suggested that the salivary glands are part of the common mucosal immune system and that the IgA ASCs within this system should migrate

equally well to all mucosal tissues. Here, I demonstrated the differential expression of CCL28 mediated the specific localization of IgA ASCs in the mouse SLG but not the SMG. These results suggest that migration within the mucosal immune system is not common but in some cases is highly specific to individual glands. Understanding the mechanisms of IgA ASC localization into specific tissues has important implication in the design and use of mucosal vaccines, as well as increasing our understanding of the basic science of lymphocyte homing and migration.

MATERIALS AND METHODS

Immunohistology

Salivary gland samples were collected from animals upon necropsy and frozen in OCT freezing medium (Tissue-Tek, Elkhart, IN). For immunohistology, 8μm frozen sections were air dried, fixed in cold acetone for 10 minutes and stained with FITC labeled anti-IgA (Clone C10-3), APC labeled anti-CD4 (clone GK1.5) and PE labeled anti-CD45R (clone RA3-6B2). Staining was visualized using confocal microscopy.

RNA collection and analysis

Sublingual and submandibular salivary glands were collected from mice. RNA was extracted from the samples using TRIZOL reagent according to manufactures instructions (Invitrogen Carlsbad, California). Quantitative PCR (QPCR) was performed in duplicate on each sample using a Verso 1-step QRT-PCR ROX kit (Thermo Fisher Scientific, Waltham, MA), with 1 cycle of cDNA synthesis at 50^{o}C for 15 min, 1 cycle of Thermo-Start activation at 95 ^{o}C for 15 min, then 40 cycle of denaturation at 95 ^{o}C for 15 sec and annealing/extension at 60 ^{o}C for 60 sec. GAPDH specific primers with VIC-MGB probe were used as an endogenous control. Primer and probe for the IgA constant region, chemokines (CCL28, CCL25, CXCL12, and VCAM), and the chemokine receptors (CCR10 and CCR9) were designed and manufactured by Applied Biosystems, Foster City, CA. All the primers were used in conjunction with FAM labeled probe. QPCR assays were performed on an Applied Biosystems 7300 Real-Time PCR System and the results were analyzed using Relative Quantification software from Applied Biosystems. This software generated amplification curves of the endogenous control

(GAPDH) and these values are then used to standardize any differences in RNA concentrations between samples.

IgA mRNA levels from all the salivary gland samples were assessed by assigning the IgA mRNA level in the submandibular gland of each mouse to an arbitrary value of 1 and then comparing the IgA mRNA level from the sublingual gland of the same mouse. Differences are determined as fold increase or decrease to this calibrator sample. The resultant values from WT, CCR10KO and adoptive transferred CCR10KO mice were averaged separately.

Chemokines and the chemokine receptor mRNA levels were measured and compared from 8 WT mice using the same calibration method as with IgA mRNA levels.

Isolation of protein from salivary glands

Sublingual and submandibular salivary glands from 3 to 6 month old female mice were collected and immediately placed in round bottom tubes in ice. Samples not used immediately were stored in -80^{o}C for future protein extraction. Brij 150 lysis buffer (1ml 1M Tris, 0.4ml 0.5M EDTA, 3ml 5M NaCl, 8.75ml Brij 96 10%, 1.25ml NP40 10%, 85.6ml H2O) with protease inhibitors was added into the tubes with approximately 1ml Brij buffer per each 100μl tissue volume. The samples were then homogenized into the buffer and transferred into 1.5ml Eppendorf tubes, followed by centrifugation in 4^{o}C at full speed. Supernatants containing the protein were transferred into new Eppendorf tubes and stored at -20^{o}C in a non-frost-free freezer. The protein samples were used in both ELISA and Bradford assays.

ELISA

ELISA plates (Nun International, Rochester, NY) were incubated overnight at 4^{o}C with 50 μl of 2μg/ml anti-mouse IgA antibody (clone C10-3, BD Pharmingen) for IgA ELISA or cholera toxin for antigen specific ELISA. Wells were washed 3 times with PBS and incubated for 1/2 hr at RT with 200 μl blocking buffer (0.17M boric acid, 0.12M sodium chloride, 0.05% Tween 20, 1mM EDTA, 0.25% BSA, 0.05% sodium azide). Wells were again washed 3 times and incubated for 1 hr at RT with serial dilutions of 100 μl 1:2 diluted protein samples in blocking buffer or 1:1000 diluted reference mouse IgA antibody (M18-254, BD Pharmingen). After 3 washes, wells were incubated for 1 hr at RT with 100 μl 1:1000 diluted AP-goat anti-mouse IgA (Zymed). Wells were washed 6 times and incubated with 50 μl AP substrate solution (SouthernBiotech). The plates were read at OD 405 nm. Total IgA concentrations in each protein sample were determined by constructing a standard curve of the known values. Concentrations of antigen specific IgA were represented as absorbance at OD 405 nm.

Bradford protein assay

Total protein levels from the extracted samples were determined by Bradford assay. 4μl extracted protein samples and known concentrations of BSA samples were mixed with 200μl Bradford reagent in 750μl Eppendorf tubes at RT. 2 μl of the mixtures were then loaded onto NanoDrop ND-1000 (NanoDrop Technologies) and read at 280nm. Total protein contents in the samples were determined by comparing with the standard curve of the BSA samples.

IgA content

To normalize for the effect of unequal amounts of protein extracted in each sample, IgA content in each sample was represented as pg of IgA (calculated from ELISA assay) per μg of total protein. Resultant values from sublingual and submandibular glands of WT, CCR10KO, and CCR10KO mice received adoptive transferred were averaged and standard error of the mean were determined.

CCR10KO adoptive transfer

CCR10KO mice were developed by and obtained from Olivier Morteau (Perlmutter Laboratory and Department of Pediatrics, Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA). Spleen cells from 5 female WT mice and 5 female CCR10KO mice were collected separately by mashing spleens between two microscope slides. The slides were washed with RPMI-1640 medium (HyClone) and the cells-RPMI mixtures were filtered through nylon into 15ml centrifuge tubes. The tubes were then centrifuged at 1700 rpm for 5 minutes. Supernatants were discarded and the cell pellets were diluted with RPMI to $2.0x10^8$ cells/ml. 5 CCR10KO mice were injected intraperitoneally with 200 μl WT spleen cells and another 5 CCR10KO mice were injected with 200ul CCR10KO spleen cells as controls. The 10 adoptively transferred mice were sacrificed 2 weeks following adoptive transfer and their sublingual and submandibular glands were collected. RNA and protein were extracted from the salivary glands for analysis of IgA levels by QPCR and IgA protein levels.

Oral and nasal cholera toxin immunization

For oral cholera toxin (CT) immunization, 7 mice were deprived from food and water 2 hours prior to the immunization. Then 250μl of sodium bicarbonate solution (8 parts HBSS to 2 parts 7.5% sodium bicarbonate) was administered orally to the mice. Half an hour later, the mice were orally immunized with 250μl CT solution (10μg CT in 250μl PBS). For nasal immunization, 10μl CT solution (10μg CT in 10μl PBS) was pipetted into nasal cavities of 6 WT mice. The two groups of mice were boosted with CT 2 weeks after the first immunization and they were sacrificed 2 weeks following the second immunization. Total protein was extracted from the salivary glands of the two immunized group for ELISA and Bradford assays.

RESULTS

IgA ASCs selectively accumulated in the SLG when compared to the SMG

Immunohistological analysis of sections of the salivary glands from WT mice revealed that there were higher concentrations of IgA ASCs localized in sublingual glands when compared with the submandibular gland (Figure 1). To confirm this finding and to measure the amount of IgA in the two salivary glands quantitatively, QPCR was used. Primers for IgA ASCs were designed from the constant region of IgA, which should only be expressed by IgA ASCs. IgA mRNA levels as determined by QPCR provided measurement of the amount of IgA in the two salivary glands. The IgA QPCR results from 8 female mice showed that sublingual gland contained 12 times more IgA mRNAs than submandibular gland ($p=0.00314$) (Figure 2). All the samples were run in duplicate.

Levels of mRNA expressions do not always represent the amount of protein synthesized. For example, IgA ASCs in the two salivary glands might process the IgA mRNAs differently. To quantify the amount of IgA proteins produced by the two salivary glands, IgA ELISA assays were used. Total protein was extracted from salivary gland samples, and the protein extracts were used in both ELISA and Bradford assays. The resultant values from the IgA ELISA assays were normalized to the total amount of protein in the individual samples. The amounts of IgA per microgram of protein from the two salivary glands were averaged separately and the SEM was determined. IgA ELISA assays of the salivary glands of three WT female mice, after normalizing with total protein content, showed that sublingual gland had an average of 738pg IgA per μg of protein, while the submandibular gland had only 55 pg IgA per μg protein (Figure 3).

The result was statistically significant (P= 0.018). The ratio of IgA per μg of protein in the sublingual gland and the submandibular gland was about 12:1, which was the same as IgA QPCR results. The results from the QPCR and ELISA assays were consistent. Immunohistology, QPCR, and ELISA assays all suggested that IgA ASCs accumulated preferentially in the SLG when compared to the SMG.

Differential expression of chemokines and adhesion molecules in the two salivary glands

Chemokines can be considered the organizers of the immune system and they promote the specific localization of lymphocytes into distinct parts in the body. The mucosal chemokines CCL28 and CCL25 have been shown to mediate the homing of IgA ASCs into mucosal sites. In an effort to understand the role of these chemokines in the accumulation of IgA ASCs into the salivary glands, I next examined expression levels of these chemokines and other mucosal adhesion molecules in the two salivary glands. QPCR results showed that CCL28 mRNA levels were 2.5 times higher in SLG when compared with SMG ($p<0.01$), and the mRNA for the receptor for CCL28, CCR10, was 19 times higher in SLG ($p=0.029$). On the other hand, SMG expressed 5.5 times more mRNA for CCL25 ($p<0.01$) than was present in SLG. Expression levels of the mRNA for the receptor for CCL25, CCR9, were consistent with the result of CCL25. The SMG was shown to have 2 times more CCR9 mRNA ($p=0.022$) than in SLG. The expression level of CXCL12 mRNA was also higher in the SMG, being expressed almost 3 times higher than in the SLG ($p<0.01$). The chemokine CXCL12 is an activator of VCAM-1 binding molecule $\alpha4\beta1$(35) and our results showed that SMG had 3.5 times more

VCAM-1 mRNA than SLG ($p<0.01$). However, the expression level of β1 mRNA contradicted the result from CXCL12 and VCAM-1. Higher level of β1 mRNA was observed in SLG, which expressed 9 times more than SMG ($p=0.016$). Interestingly, MAdCAM-1 mRNA was expressed in very low levels in both salivary glands. Expression of the MAdCAM-1 binding molecule β7 showed no statistical difference between the two salivary glands, suggesting that MAdCAM-1 is probably not important in mediating lymphocyte migration into the glands (Figure 4A and 4B).

CCR10KO mice had similar IgA levels in the two salivary glands

The chemokine and chemokine receptor expression level results showed that in WT mice the SLG contained high levels of CCL28 and CCR10 mRNA, which correlated with the higher number of IgA ASCs in that salivary gland. I therefore hypothesized that the interactions between CCL28 and CCR10 were important in mediating IgA ASCs migration into the SLG. To test this hypothesis, the levels of IgA ASCs in the two salivary glands of CCR10KO mice were measured. Based on immunohistology observations, the numbers of IgA ASCs in the two salivary glands were visually identical. They both had low numbers of IgA ASCs (Figure 5). IgA QPCR results in CCR10KO mice showed that IgA mRNA levels were not statistical different between the two salivary glands (Figure 6). Protein levels, as determined by IgA ELISA assays further showed that SLG and SMG in CCR10KO mice contained similar amounts of IgA antibody. In CCR10KO mice, SLG contained only 50 pg IgA per μg protein and SMG contained 16 pg IgA per μg protein (Figure 7). These results highly suggested that CCL28/CCR10 interactions play important roles in mediating IgA ASCs homing to SLG.

Adoptive transfer reconstituted SLG of CCR10KO mice with WT IgA mRNA

IgA QPCR and ELISA results from CCR10KO mice were highly suggestive that CCL28 mediated the migration of IgA ASCs to the SLG via CCR10. To confirm this, I performed adoptive transfer experiments, in which splenocytes from WT donor mice were adoptively transferred into CCR10 deficient recipients. Since CCR10KO salivary glands should express normal levels of CCL28, introducing WT IgA ASCs that expressed CCR10 into CCR10KO mice would theoretically reconstitute SLG with higher levels of IgA ASCs.

Spleen cells, which are rich in lymphocytes, were collected from WT mice and injected intraperitoneally into CCR10KO mice. Two weeks following adoptive transfer salivary glands were collected and analyzed for IgA protein and mRNA levels. Control adoptively transferred CCR10KO mice received the same treatments but with injection of CCR10KO spleen cells rather than WT cells. The number of IgA ASCs and amount of IgA in the SLG were compared between the WT and control adoptive transferred groups. The same comparison was also used to compare SMG in both groups of mice. IgA QPCR results showed that in SLG, WT adoptively transferred CCR10KO mice had 2.4 times more IgA mRNA than control CCR10KO transferred mice ($p=0.01$). However, there was no statistical difference in amount of IgA mRNA in the SMG of the two adoptively transferred groups. ELISA results confirmed these findings. CCR10KO mice receiving WT spleen cells produced increased amount of IgA in the SLG, with an average 80 pg IgA per μg of protein versus 50 pg IgA per μg protein in control KO transferred mice ($p=0.02$). IgA protein levels in the SMGs of adoptively transferred mice showed no statistical differences, as seen in mRNA level (Figure 8). The adoptive

transfer experiments clearly suggested that CCL28 and CCR10 interaction play a major role in mediating the migration of CCR10-expressed IgA ASCs into the salivary gland.

Oral CT immunization resulted in increased antigen specific IgA accumulation in SMG while nasal immunization increased antigen specific IgA in SLG

Since SLG expressed high levels of the "common mucosal chemokine" (27) CCL28, and SMG expressed high level of intestinal expressing chemokine CCL25, it is possible that the two salivary glands might attract different subsets of IgA ASCs that were primed in different mucosal sites. To study the influence of the site of antigen activation to the migration of IgA ASCs, preliminary experiments were done using mice which were immunized either orally or nasally. Following immunization the amount of total IgA and antigen specific IgA were measured in the two salivary glands by ELISA assays. In the orally immunized group, SMG contained 1.2 ng IgA per μg protein, while SLG produced an average of 8.8 ng IgA per μg protein ($p<0.01$). The ratio of total IgA between SMG and SLG was 1:7.5, which was smaller than the non-immunized WT (1:12), suggesting SMG had an increased amount of IgA production when compared to the non-immunized group. On the other hand, in the nasal immunized group, SLG contained increased amounts of IgA. The SLG of the nasally immunized group contained 11.8 ng IgA per μg protein, while SMG contained 0.2 ng IgA per μg protein ($p<0.01$). The ratio of total IgA between the two salivary glands was 1:52.4, which was much larger than the WT (Figure 9A). These experiments showed that oral immunization increased total IgA accumulation in SLG.

In preliminary experiments, aimed at determining antigen specific IgA levels in the tissues of immunized animals, CT specific ELISA results were calculated as absorbance at OD 405nm. In the orally immunized group, the absorbance of antigen specific ELISA assays was 0.017 in the SLG and 0.045 in the SMG. These results showed no statistical difference between the two salivary glands. In nasally immunized mice the SLG had a much higher absorbance than SMG. SLG had an average absorbance 0.589, while SMG was 0.133 ($p<0.01$) (Figure 9B). The results of this CT specific ELISA suggested that nasal immunization resulted in a preferential accumulation of antigen specific IgA ASC in the SLG. Oral immunization resulted in a non-statistical trend toward increased antigen specific IgA in the SMG. These results were highly suggestive that IgA ASCs migrating to the two salivary glands may be activated in different mucosal tissues, resulting in different expression patterns of chemokine receptors and adhesion molecules.

DISCUSSION

The concept of a common mucosal immune system suggests that IgA ASCs activated in any one mucosal site have the ability to home to other mucosal surfaces. Based on this concept, the mucosal sites should have similar homing mechanisms for IgA ASCs. In this study I showed that within the mouse salivary glands, the number of IgA ASCs was much higher in SLG when compared to SMG. Three different analysis methods, immunohistology, QPCR, and ELISA, were used and they all confirmed that SLG had more IgA than SMG. This observation raised the question: how did one of the salivary glands come to possess more IgA ASCs than the other? Since chemokines are the mediators for lymphocyte homing, I hypothesized that the differential expression of chemokines by the salivary glands contributed to the specific compartmentalization of IgA ASCs in mouse salivary glands.

CCL28 and CCL25 are two major chemokines that mediate the homing of IgA ASCs into different mucosal tissues (30) therefore the expression levels of these two chemokines in the two salivary glands were studied. QPCR results showed differential expression of the mRNAs for these chemokines and cognate receptors, as well as the adhesion molecule VCAM-1 in the two salivary glands. Since salivary glands have been previously shown to express high levels of CCL28 (30), a role for CCL28 in the two salivary glands was predicted. QPCR results showed that SLG expressed 2.5 times more CCL28 mRNA than SMG, and CCR10 was 19 times higher in SLG than SMG. The higher level of CCL28 and CCR10 correlated with the number of IgA ASCs in SLG, and adoptive transfer experiments were performed which confirmed that CCR10 was indeed important in attracting IgA ASCs to SLG.

Although more CCL28 was expressed in SLG, the ratio of the CCL28 mRNA expressed between the two salivary glands (1:2.5) was much smaller than the mRNA ratio of CCR10 (1:19) and the level of IgA protein (1:12) in the two glands. The proportion of CCL28 mRNA represented the amount of chemokine transcription in the two glands, while the ratio of CCR10 represented the numbers of lymphocytes that expressed this chemokine receptor, including CCR10 expressing IgA ASCs in the two salivary glands. These results suggested that the accumulation of CCR10 expressing cells was not proportional to the level of CCL28 chemokine expression. A possible explanation could be that there was a threshold of chemokine needed for lymphocytes to efficiently migrate to, or be retained in a tissue. It could be that when the chemokine level exceeds that critical mass, lymphocytes would migrate to, and stay in, a tissue. To test this hypothesis, CCL28 chemotaxis assays could be used, in which numbers of IgA ASCs migrating to different concentrations of CCL28 are measured and compared. This experiment may explain why chemokine receptor expression is not proportional to chemokine levels.

The ratio of CCR10 in the two salivary glands (1:19) was higher than the ratio of IgA protein (1:12). This discrepancy could be due to lymphocytes other than IgA ASCs that express CCR10, being recruited into the tissue by the higher concentration of CCL28. In this study, I only focused on IgA ASC. But to gain a better understanding of the mucosal immune system in the salivary glands, other cell types such as T cells should also be studied.

Since the SMG in CCR10KO mice had similar numbers of IgA ASCs as WT mice, CCR10 interactions with CCL28 seemed to have no effect on IgA migration into SMG.

Surprisingly, high expression levels of CCL25 were detected in the SMG. This chemokine has previously been shown to be selectively expressed by thymus and small intestinal epithelial cells (31). IgA ASCs localizing in the SMG may be attracted by CCL25. To test this hypothesis CCR9 KO mice, which have been previously described could be used. To determine if CCL25 plays a role in IgA ASCs migration to the SMG, the number of salivary gland IgA ASCs could be measured from the CCR9 KO mouse and compared to the numbers of IgA ASC found in the SLG of WT mice. If the number of IgA ASCs is reduced in the SMG of CCR9KO mice, adoptive transfer experiments could then be performed to confirm that CCR9 interaction with CCL25 are important in mediating IgA ASCs migration into mouse SMG. Results from such experiments would not only confirm that the homing of IgA ASCs is highly regulated by differential expression of tissue specific chemokines, but also demonstrate that the IgA ASCs migrating to the two salivary glands express different sets of chemokine receptors and potentially represent different populations of IgA ASCs.

In that CCL28 expression appears to be differentially expressed in the two salivary glands it brings up the question of what regulates the expression of this chemokine. It has been reported that enteral stimulation is important in inducing the expression of CCL28 in small intestine and lung (42). For example, in intravenously fed animals, CCL28 expression decreased significantly in small intestine and lung when compared with control mice, suggesting that the expression of this chemokine requires enteral stimulation (42). Studies by Ogawa *et al*.(43) demonstrated that the expression of CCL28 in human colon epithelium can be upregulated by the proinflammatory cytokines IL-1α and TNF-α, as well as bacterial flagellin, bacterial infection, and *n*-

butyrate via a NF-κB activation pathway. Similar CCL28 upregulation patterns are also observed in human airway epithelial-like cells A549. O'Gorman *et al*. (44) showed that IL-1β and TNF-α induced the phosphorylation of NF-kB in the A549 cells, which resulted in the upregulation of CCL28. These results suggested that CCL28 may function as an inflammatory chemokine that is regulated by proinflammatory cytokines via NF-kB pathway. To study the expression mechanism of CCL28 in SLG and SMG, several experiments could be done. First, the proinflammatory cytokines, such as IL-1, could be measured and compared between the two salivary glands. Higher levels of these cytokines may be measured from SLG, which expressed higher levels of CCL28. Conversely, inhibition of these cytokines in the mouse may alter the expression level of CCL28. Since the expression of CCL28 is believed to be regulated by the NF-kB pathway, the second experiment would require the inhibition of NF-kB. If reduced levels of CCL28 are observed in SLG, the involvement of NF-kB in regulating the expression of CCL28 in the salivary could be confirmed. Third, since decreased enternal stimulation reduces CCL28 in small intestine and lung (42), reduction of the CCL28 levels in the two salivary glands may also observed in the salivary glands. If reduction of CCL28 production appears different in the two salivary glands in parenteral fed animals, it would further suggest that the two salivary glands serve different functions in the common mucosal immunological system.

Although CCL25 was discovered before CCL28, not much is known about the regulation of CCL25 expression. Research by Hermsen *et al*. (42) has shown that enteral stimulation is also important for normal CCL25 expression. CCL25 protein level is significantly reduced when animals were fed intravenously (42). Adding to this

knowledge, recent research has shown that bile may contribute to the high CCL25 levels in small intestine (45). CCL25 mRNA is also significantly decreased in animal models of experimental obstructive jaundice. Ericsson *et al*. (46) reported that in murine small intestinal epithelial cells, CCL25 expression is independent of lymphotoxin β receptor signaling pathway and is not upregulated by inflammatory stimuli. Genetic analysis further revealed that the CCL25 promoter contains several putative binding sites for the intestinal epithelial-associated Caudal-related homeobox (Cdx) transcription factors, suggesting Cdx transcription factors may play regulatory roles for CCL25 expression (46). Since the SMG expressed similar chemokine profile as in the small intestine, the regulation mechanism of CCL25 in SMG may also be similar. Reduced CCL25 levels may be observed in the SMG of parenteral feeding animals.

In this study, QPCR was used to quantify the amount of IgA mRNA and the expression levels of chemokines, chemokine receptors, and adhesion molecules. Although QPCR is a highly sensitive method and provides accurate quantitative results for comparison between the two salivary glands, the properties of each cell type were not shown. QPCR did not demonstrate the percentage of IgA ASCs that expressed CCR10, CCR9, or different integrins in each salivary gland. To assess this flow cytometry could be used. If it shows that one salivary gland has a higher concentration of CCR10 bearing IgA ASCs, while the other gland has more CCR9 IgA ASCs, it will confirm that the IgA ASCs migrate to the two salivary glands are indeed different, and that these IgA ASCs may be primed at different locations and therefore process different sets of chemokine receptors.

It has been shown that the small intestine selectively expresses CCL25 and attracts IgA ASCs expressing CCR9 (33), whereas colon and bronchial tissues express high level of CCL28 and attract CCR10 expressing IgA ASCs (27). In this study, I showed that each of the two mouse salivary glands expressed high levels of one of the two IgA ASC attracting chemokines. Since the mouth is the most common entry site for intestinal pathogens, localization of IgA ASCs specific for those pathogens in salivary glands would provide the greatest protection to the body.

The differential accumulation of IgA ASC in the two salivary glands could also be useful in studying the influence of where a B cell is primed and where it ultimately localizes. Experiments were performed in which WT mice were immunized with a specific antigen either orally or nasally. Then the amount of antigen-specific IgA antibody was measured from the two salivary glands. In preliminary studies I demonstrated that oral immunization tended to produce more antigen specific IgA in SMG, while nasal immunization resulted in antigen specific IgA accumulation in SLG. These preliminary results suggested that the chemokine profiles of each salivary gland resulted in specific subsets of IgA ASC, defined by site of antigen exposure, accumulating in each salivary gland.

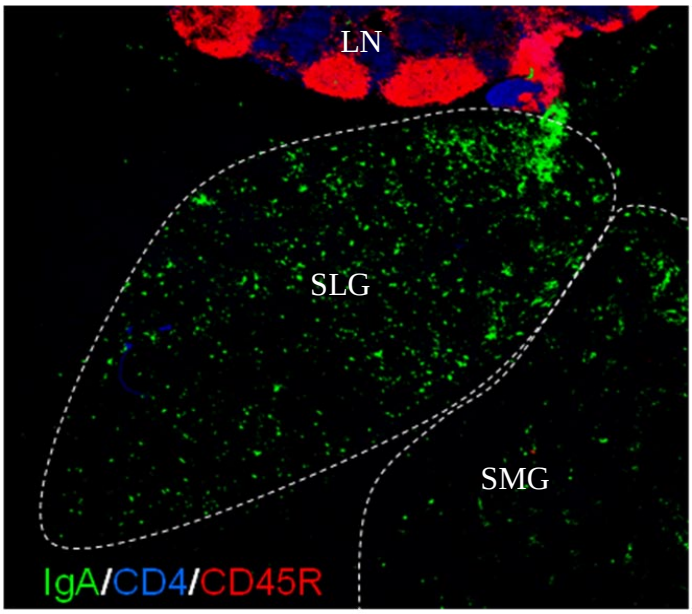
In this study I have shown that the chemokine expression profile of the SMG and SLG leads to dramatic differences in the efficiency in which IgA ASC accumulated in the tissues. Through the use of CCR10KO mice it may now be possible to determine the physiological importance of IgA ASC accumulation in the SLG. This could be tested by infecting CCR10KO mice and WT mice with oral pathogens and measuring the pathogenesis of disease in the two groups of mice. Since there are less IgA ASCs in the

salivary glands of CCR10KO mice to protect the oral cavity, these mice may have a more serious case of oral infection than the WT mice. These experiments would show the importance of localized IgA ASCs in providing tissue specific protection.

In conclusion, I showed that the accumulation of IgA ASC to distinct salivary glands is a highly selective process dependant on the differential expression of CCL28 and its interaction with the chemokine receptor CCR10. Furthermore the differential localization of IgA ASC into different tissues may reflect the site of antigen priming of B cells.

IgA ASC accumulate preferentially in the SLG compared to the SMG

(A)



(B)

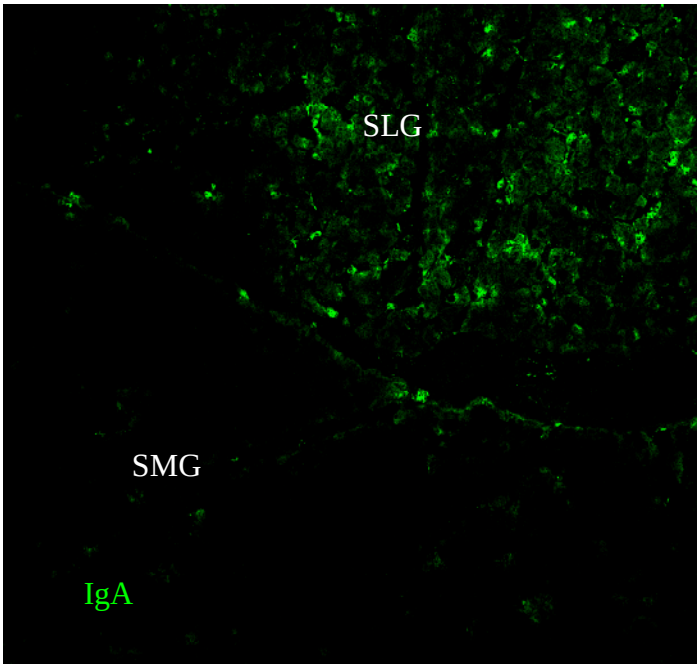


Figure 1. Immunohistology of mouse salivary glands cross sections showing that high concentration of IgA ASCs accumulate in mouse SLG. (A) Immunohistology showing SLG, SMG and cervical lymph node. High concentration of IgA ASCs (green) was found in SLG, but not in lymph node, where CD4+ cells (blue) and CD45R cells (red) were found. (B) Immunohistology with IgA ASCs stained with FITC showing both SLG and SMG. SLG had higher concentration of IgA ASCs when compared with SMG.

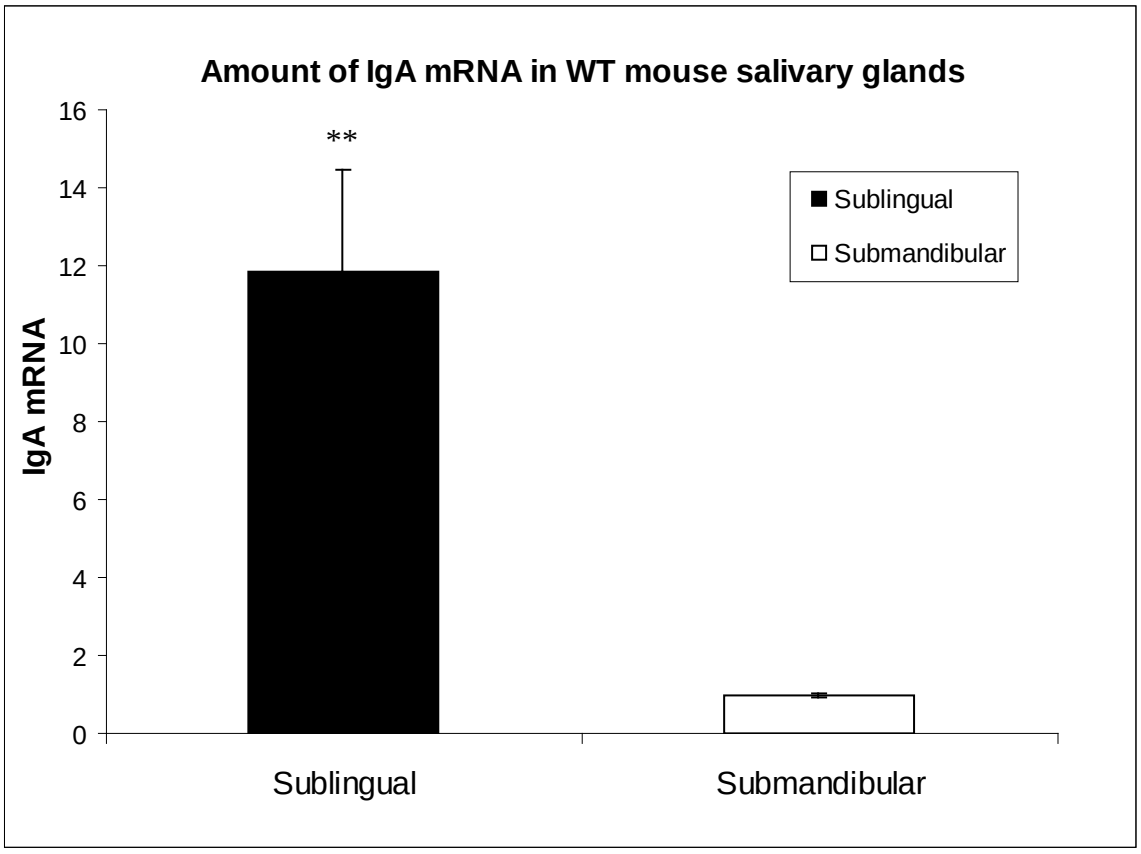


Figure 2. SLG contains 12 times more IgA mRNA than SMG. QPCR data represent the mean IgA mRNA levels in the SLG and SMG of 8 WT mice. The result was statistically significant (**$p<0.01$) and the bar represents the SEM.

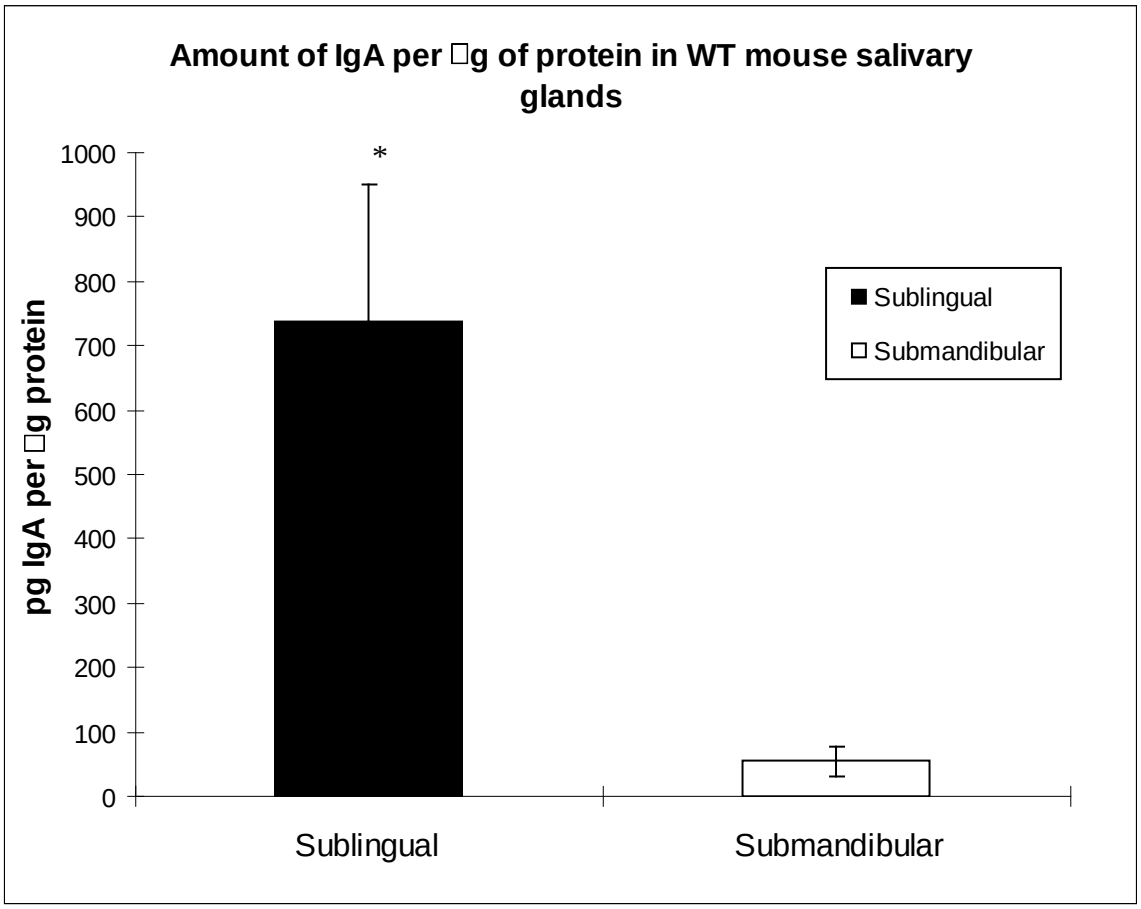


Figure 3. SLG of WT mice contain 12 fold more IgA protein than SMG. Data represent the mean protein levels from three WT female mice and the bar depicts SEM. The result was statistically significant with *p=0.018.

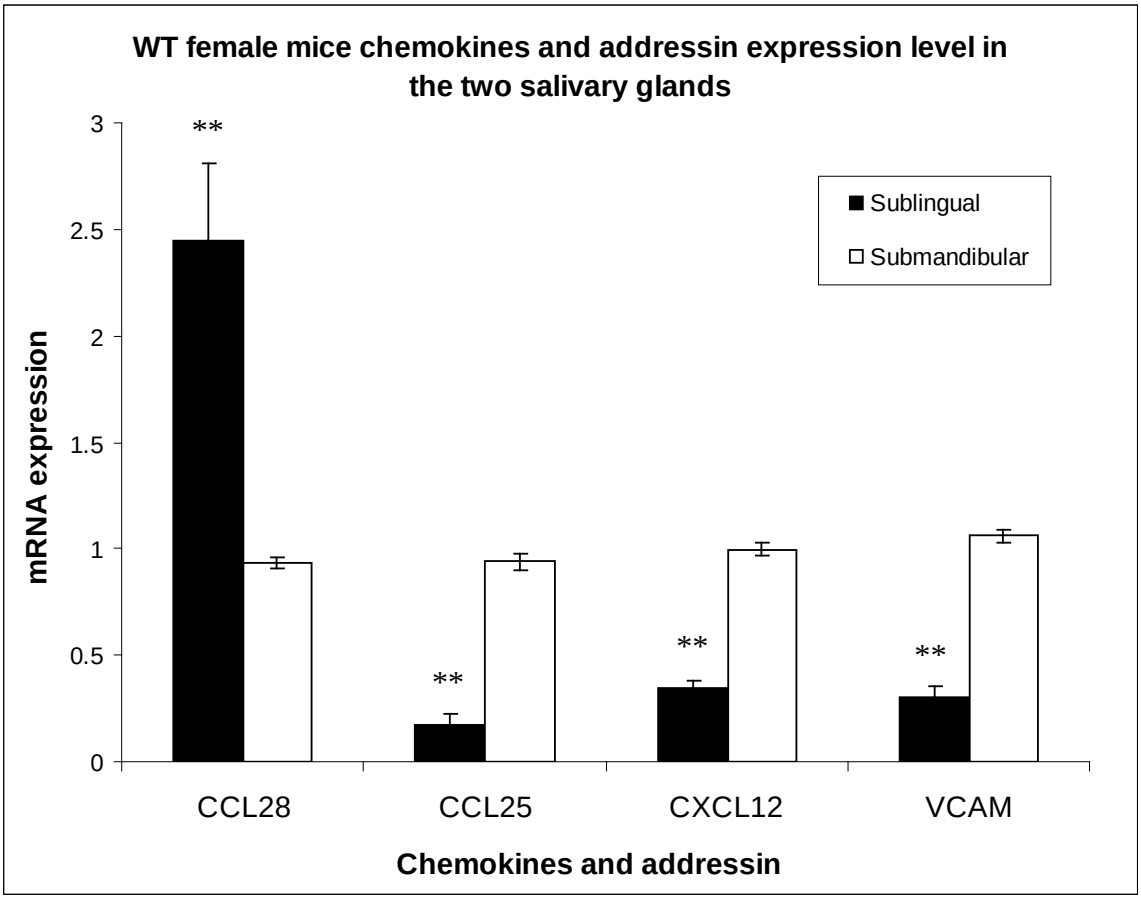


Figure 4A. Differential expressions of chemokines and vascular addressins in the salivary glands of wild type mice. SLG expresses 2.5 times more CCL28 than SMG, while CCL25, CXCL12, and VCAM-1 levels are all higher in SMG. Data represent the means and the bar depicts the SEM. All data were statistical significant with **$p<0.01$.

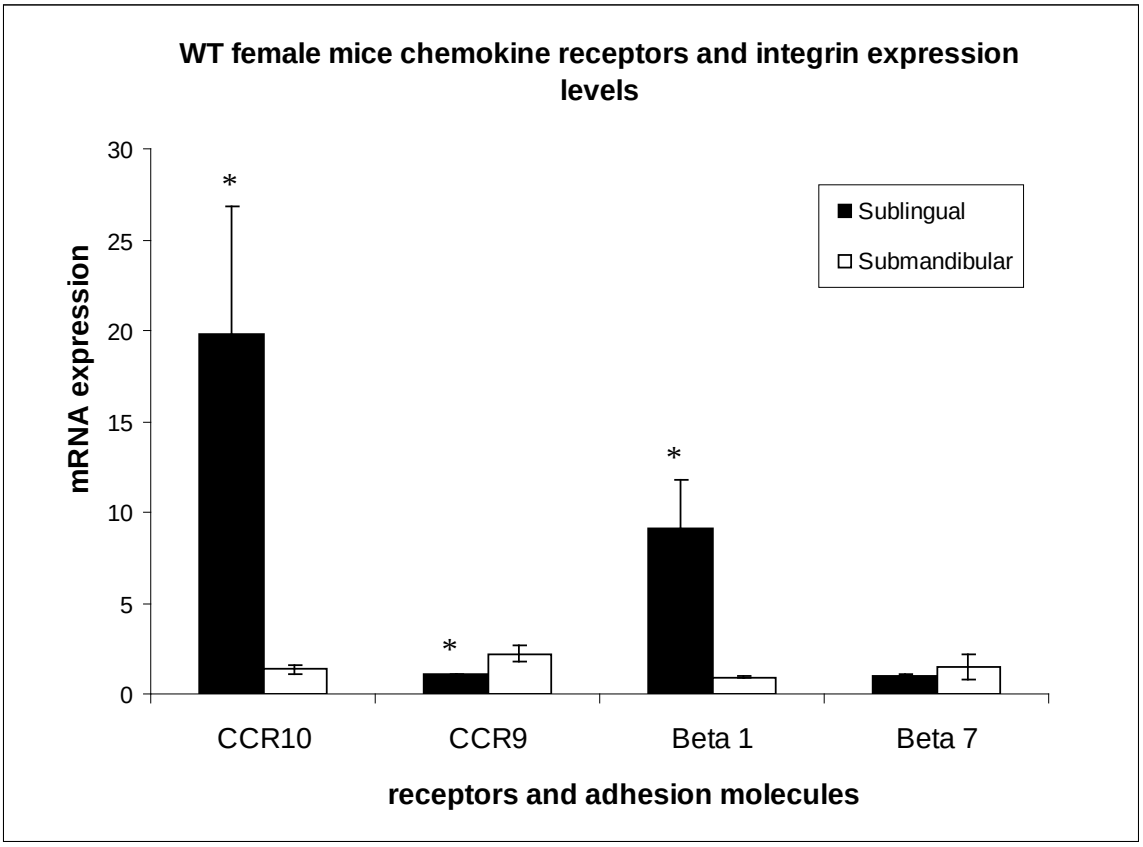


Figure 4B. Differential expression of chemokine receptors and integrin molecules in WT mouse salivary glands. CCR10 and β1 were expressed at high levels in SLG, while CCR9 was high in SMG. β7 showed no differences between the two salivary glands. Data represent the mean and bar depicts SEM. *$p<0.05$.

CCR10KO mice have similar numbers of IgA ASCs in SLG and SMG

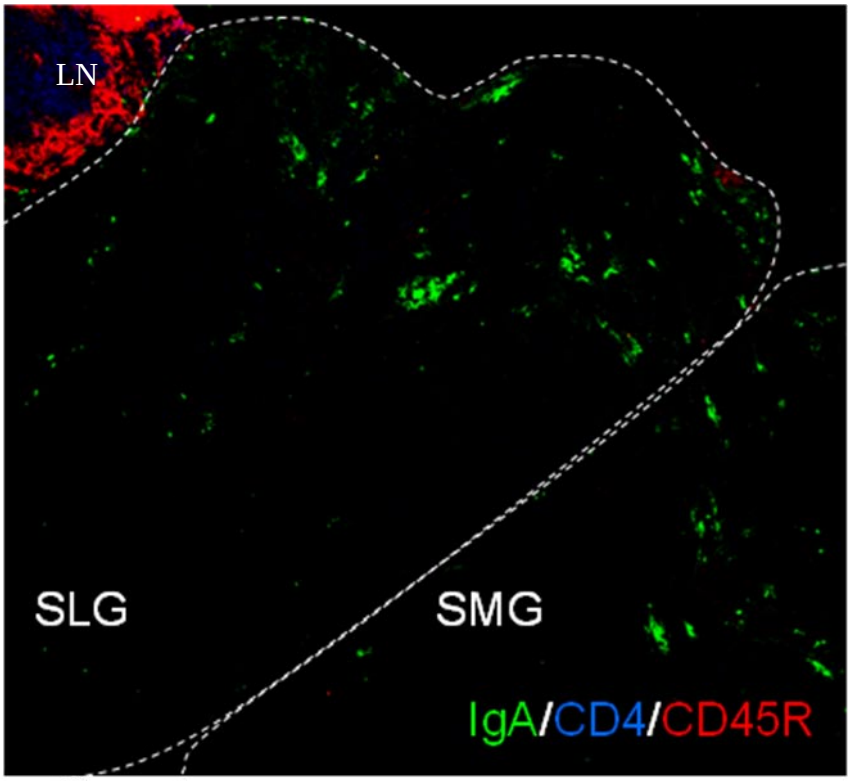


Figure 5. Immunohistology showing similar numbers of IgA ASCs in CCR10KO mouse salivary glands. Similar, low concentrations of IgA ASCs (green) were found in SLG and SMG. CD45R and CD4 T cell lymphocyte cell numbers and distribution in the lymph node (upper right corner) was unaffected.

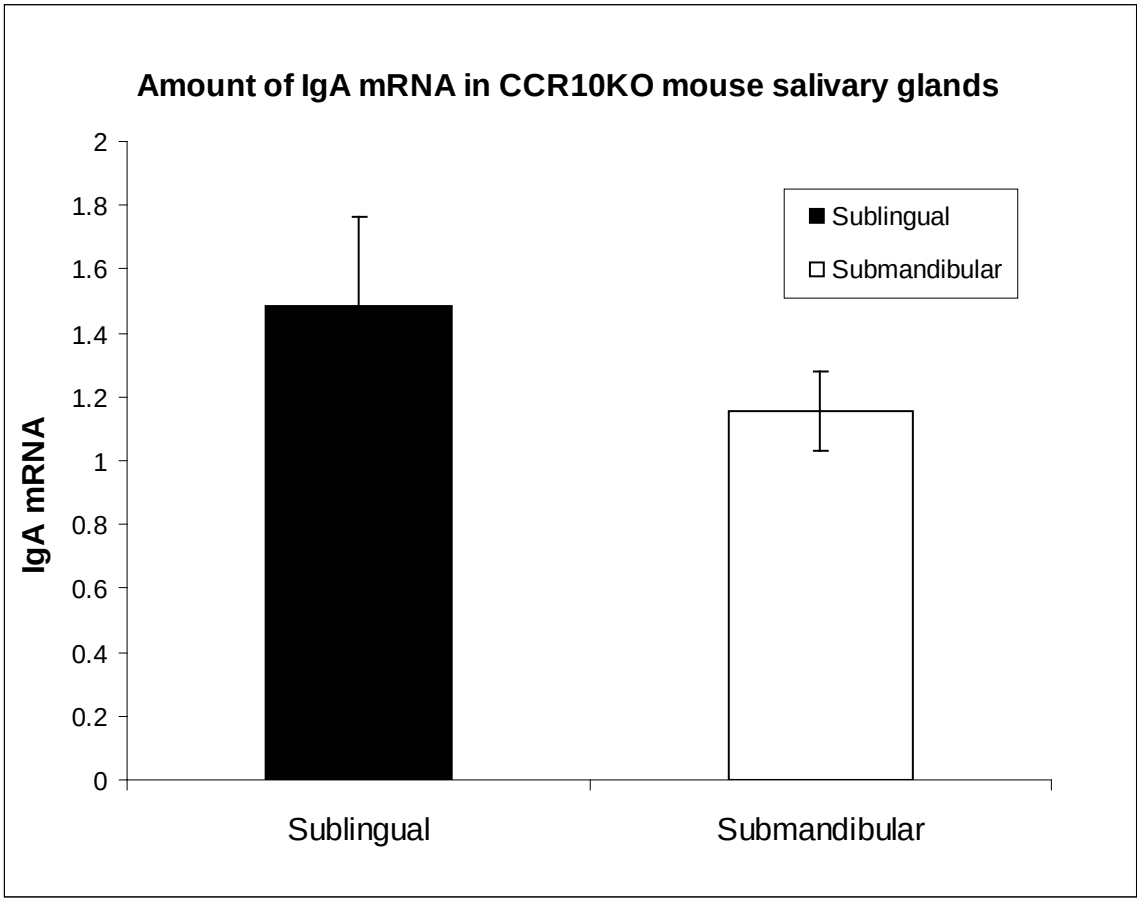
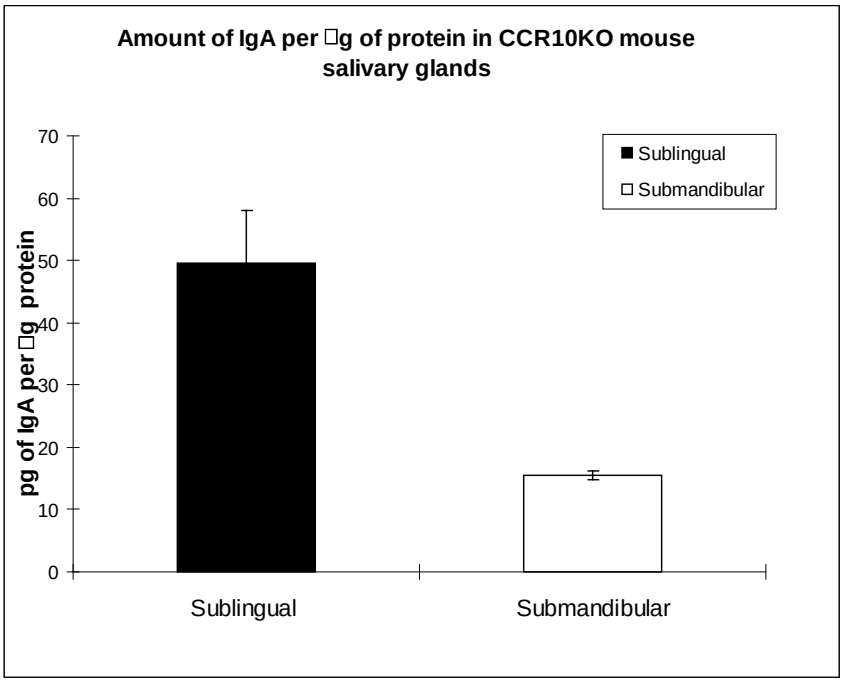


Figure 6. IgA mRNA levels are similar in the SLG and SMG in CCR10KO mice. Data represent the mean IgA mRNA levels from each tissue. Bars represent the SEM. No statistical differences in the levels of IgA mRNA in the two CCR10KO mouse salivary glands were observed.

(A)



(B)

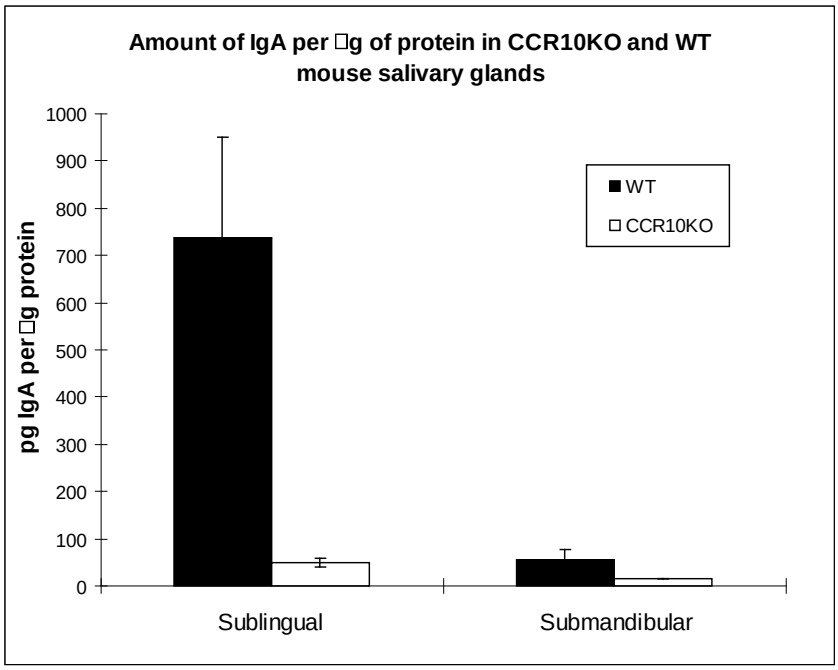


Figure 7. IgA protein levels are reduced in CCR10KO mice in SLG and SMG when compared to WT mouse. (A) In CCR10KO mice SLG IgA protein was found at higher concentrations than in SMG, but the ratio and the amount were greatly reduced when compared with WT salivary glands. (B) Comparison of the amount of IgA protein between WT and CCR10KO salivary glands showed that SLG of CCR10KO mouse produced similar amounts of IgA to SMG of WT mouse.

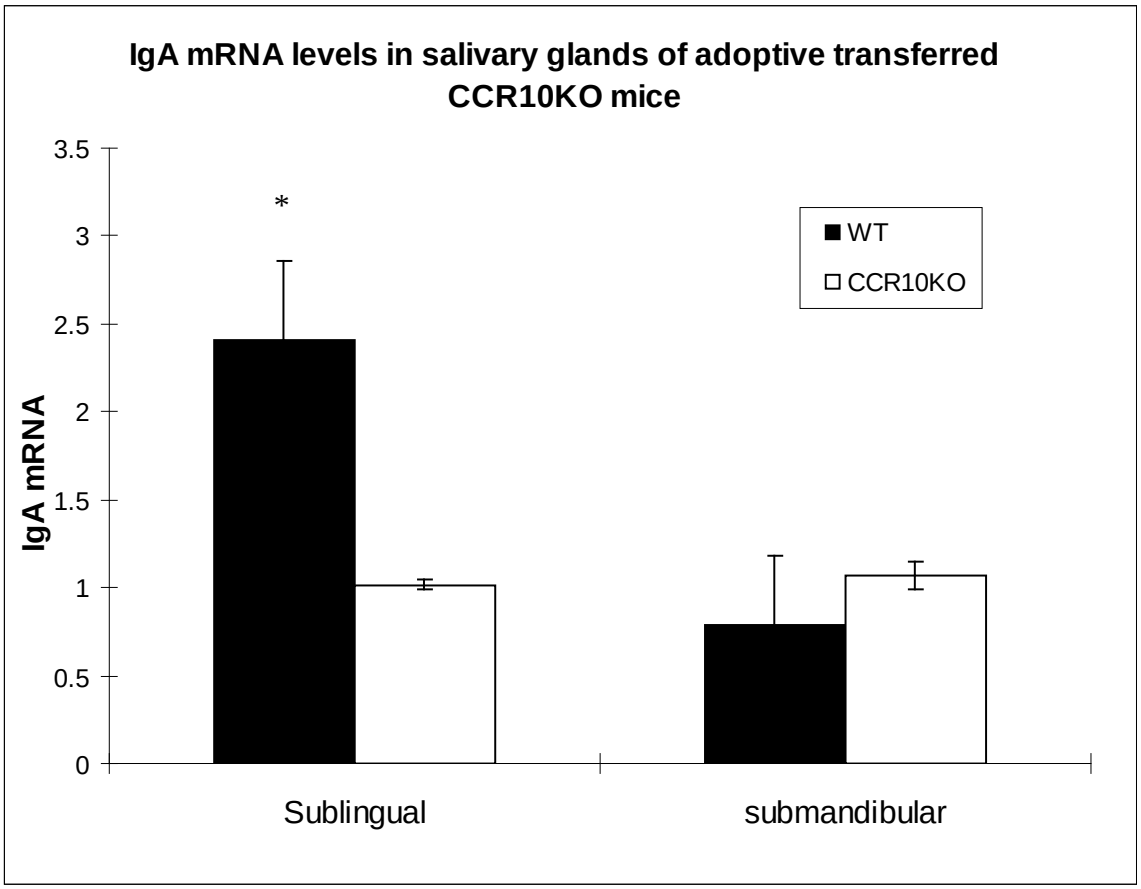


Figure 8A. Adoptive transfer of WT cells reconstituted the SLG of CCR10KO mice as measured by increased levels of IgA mRNA. The amount of IgA mRNA in the SLG was increased when WT lymphocytes were transferred into CCR10KO mice, when compared to the control group (CCR10KO lymphocytes transferred into CCR10KO recipients). This increased was not observed in SMG. There was no statistical difference in the amount of IgA mRNA between the two adoptive transferred groups in the SMG. *$p<0.05$

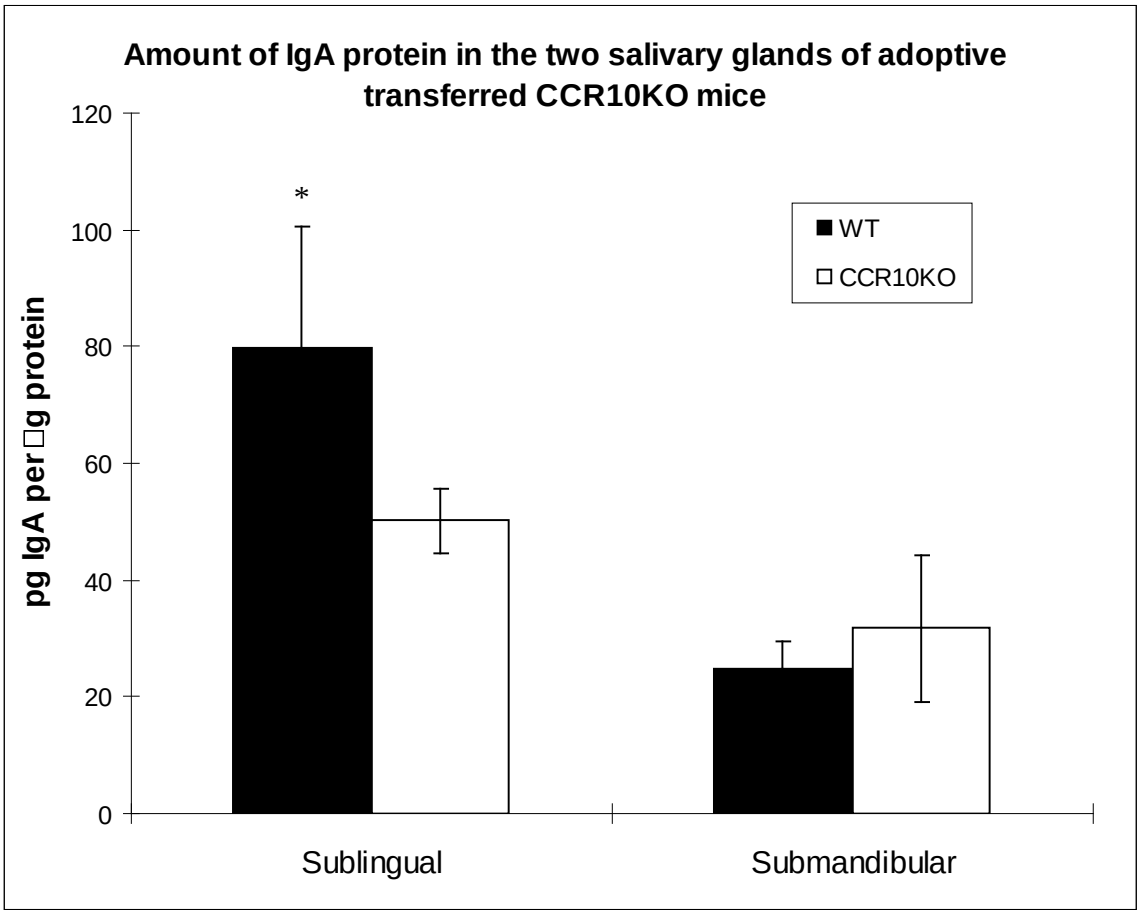


Figure 8B. SLG of WT transferred CCR10KO mice produced increased amounts of IgA protein. Adoptively transferred CCR10KO mice, receiving WT cells, produced higher amounts of IgA when compared to the control group. There was no statistical different of IgA production in SMG of the two transferred group. *$p<0.05$.

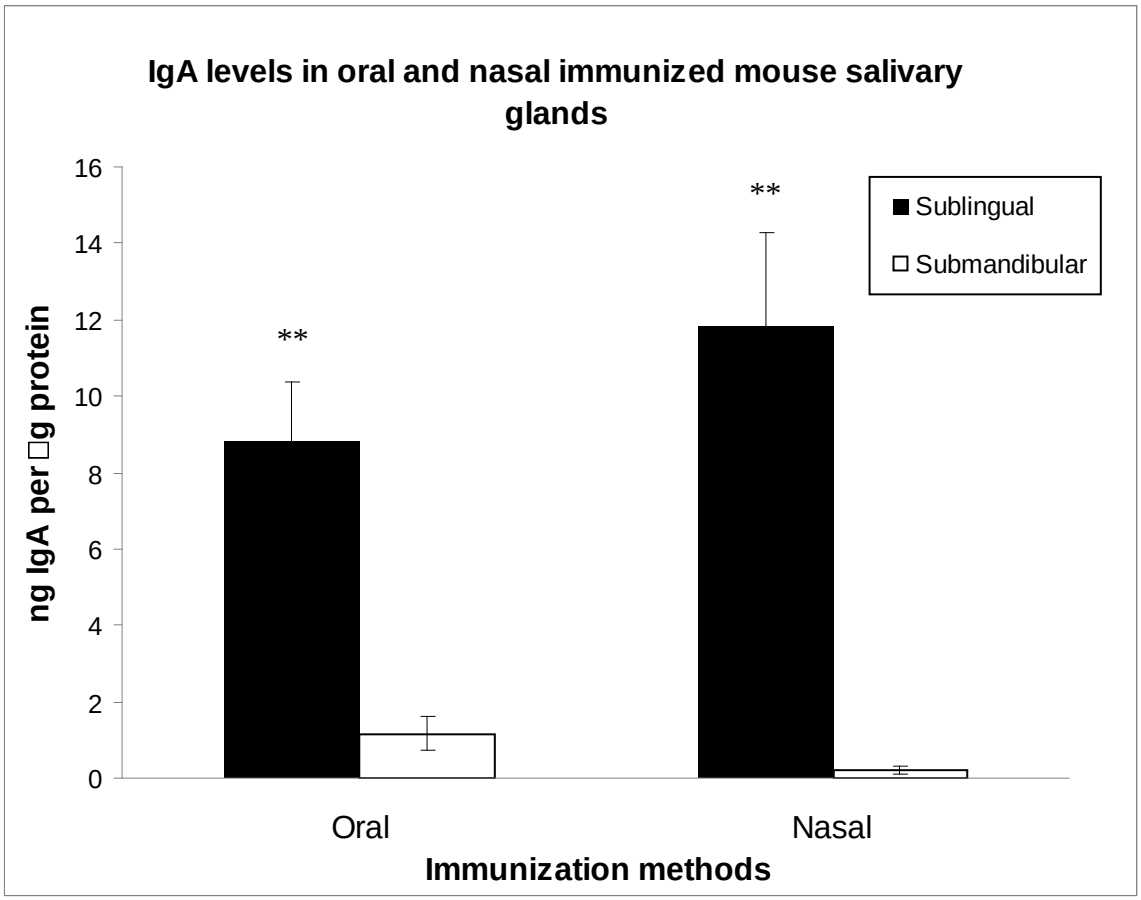


Figure 9A. Amount of IgA in the salivary glands of orally and nasally CT immunized mice. Orally immunization increased the amount of total IgA in SMG and reduced the ratio of IgA between the two salivary glands from 1:12 in non-immunized WT mice to 1:7.5. Nasal immunization raised the amount of total IgA in the SLG and enlarged the ratio of IgA between the two salivary glands to 1:52.4. **$p<0.01$.

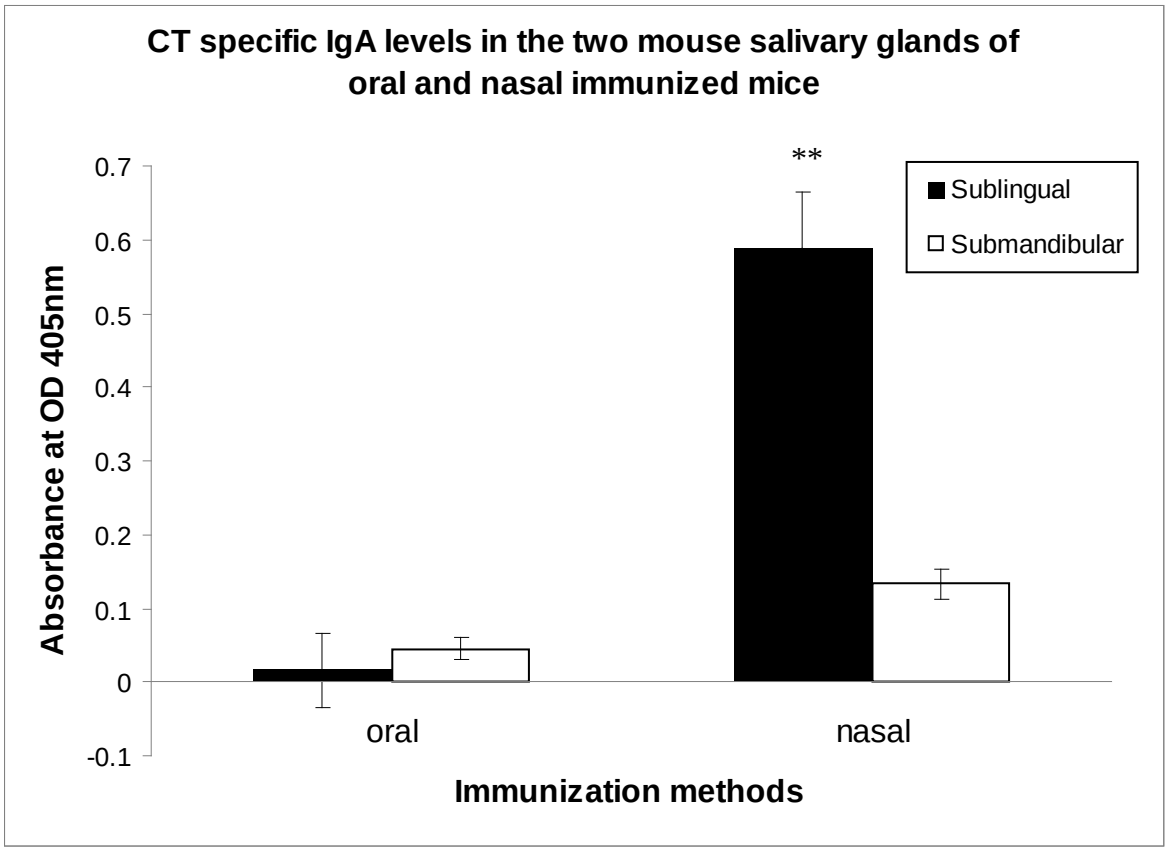


Figure 9B. CT specific IgA levels in mouse salivary glands of orally and nasally immunized mice. The SMG contained more antigen specific IgA than SLG after oral immunization while nasal immunization generated more antigen specific IgA in SLG than in SMG. **$p<0.01$.

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