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New insights into the dual recruitment of IgA$^+$ B cells in the developing mammary gland

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ABSTRACT

In monogastric mammals, transfer of passive immunity *via* milk and colostrum plays an important role in protecting the neonate against mucosal infections. Here we analyzed the hypothesis that during gestation/lactation IgA$^+$ plasmablasts leave the intestinal and respiratory surfaces towards the mammary gland (MG). We compared the recruitment of lymphocytes expressing homing receptors α4β1 and α4β7 to expression of their vascular counter-receptors, VCAM-1 and MAdCAM-1. Furthermore, the expression of the chemokines responsible for the recruitment of IgA$^+$ plasmablasts was analyzed. Data confirmed that expressions of CCL28 and MAdCAM-1 in the MG increased during pregnancy and α4β1$^+$ and α4β7$^+$/IgA$^+$ cell recruitment in lactation correlated with increase of CCL28 expression. Interestingly, VCAM-1 expression was found in small blood vessels of the lactating porcine MG, while in mice VCAM-1 was expressed in large blood vessels within the MG. Thus, our results indicate that the recruitment of IgA$^+$ plasmablasts to MG is mediated by VCAM-1/α4β1 and MAdCAM-1/α4β7 in conjunction with CCL28/CCR10. They support the existence of a functional link between entero- and upper respiratory surfaces and MG, thereby, conferring protection against aero-digestive pathogens in the newborn.

1. Introduction

The mammary gland (MG) is an exocrine gland producing the lactating secretions which contain a variety of molecules including glycopeptides, lipoproteins, cytokines and antimicrobial peptides, as well as large amounts of immune cells and immunoglobulins. Transfer of passive immunity, mediated *via* colostrums and milk, is needed for protection of the newborn against infectious agents. In monogastric species such as human, mouse and swine, secretory IgA (sIgA) is the predominant isotype (Kraehenbuhl et al., 1979). The specificity of milk sIgA for intestinal and respiratory pathogens and/or dietary antigens (Macpherson et al., 2008) and the role of breast feeding for the prevention of neonatal infections is thought to be the result of antigenic stimulation at the digestive and upper respiratory tracts (entero-mammary and broncho-mammary immunological links) (Fishaut et al., 1981; Kleinman and Walker, 1979). The presence of sIgA in the milk is due to either transport from the blood of serum IgA into the milk or by sIgA secretion from IgA$^+$ B cells immigrated from the inductive site into the MG.

The hypothesis that IgA$^+$ plasmablasts selectively migrate into the MG was supported by observations showing that murine IgA$^+$ plasmablasts isolated from mesenteric lymph node (MLN) populated the small intestine and the MG (Roux et al., 1977), when adoptively transferred. In contrast, in rats a distinct population of IgA$^+$ plasmablasts other than those in the small intestine was found to populate the MG (Parmely, 1985). In pigs, labelling studies confirmed that Ig$^+$ B lymphocytes in MG originated from both MLN and inguinal lymph node (Salmon et al., 1984), whereas T cells originated predominantly in the MLN. However, no attention was given to the isotype and the lymphoblastic stage of these B cells. Furthermore, these results have to be carefully interpreted as the mammary location was assessed after adoptive transfer of IgA$^+$ B cells which had originated from both mucosal and systemic lymph nodes and thus potentially expressing a wide range of homing receptors. For example, in rat the cervical lymph node was thought to represent a systemic organ, while in mice cells in the same lymph node express a mucosal-addressing phenotype (Csencsits et al., 2002).

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Table 1
Primers designed in this study, localization in MAdCAM-1, sequence and annealing temperature (°C)

Primer sets	Localization	Primer sequence	Annealing temperature (°C)
FP1/RP1	Domains 1 and 2	(S) ACA CCA GCC TGG GCT CCG TAA AGT (AS) AGG AGC AGG GAC ATG GAT AAG ACA	62
FP2/FP2	Domain 2 and cytoplasmic domain	(S) CTG TTC CAA GTG ACA GAA CGC TGG (AS) AGA TGA GGA ACA CCA GGA GAA GCA	62
FP3/RP3	Both extremities	(S) ATA ACA CAT ATG GAG CAG GGC CTC GCC CTC CTG C (AS) ATA GGA TCC AGC CCA GAG GAG CGT AGG CCT GGT C	62
qPCRs/qPCRas	Domains 1 and 2	(S) AGC CTG GGC TCC GTA AAG TC (AS) TGG TCA GGG AAG GCG AAC AC	67

The tissue-specific migration of lymphocytes is governed by a combination of adhesion molecules as well as cytokines, chemokines and hormones. Indeed, the migration of T and IgA^+ B cells in the intestinal tract is mediated firstly by the interaction of the mucosal addressin cellular adhesion molecule 1 (MAdCAM-1) and its ligand α4β7 (Berlin et al., 1993) followed by a chemokine/receptor interaction. For example, the interaction between CCL25 and CCR9 mediated the migration of plasmacytoid dendritic cells (Wendland et al., 2007) and T cells and a large proportion of IgA^+ plasmablasts to the small intestine (Bowman et al., 2002; Zabel et al., 1999), while the interaction of CCR10 and CCL28 allows the remaining IgA^+ B cells to migrate to other mucosal tissues expressing CCL28 (Kunkel et al., 2003). However, less is known about the migration of IgA^+ B cells in extra-intestinal epithelial tissues although it is thought that VCAM-1/α4β1 interacts with the help of CCL28 expressed in epithelial tissues including the MG (Berri et al., 2008; Meurens et al., 2006, 2007b; Pan et al., 2000; Wilson and Butcher, 2004). In addition, CCL28 is secreted in swine (Berri et al., 2008) and human milk (Hieshima et al., 2003) and in human saliva (Hieshima et al., 2003). This chemokine is specifically chemoattractive for IgA plasmablasts *via* CCR10 expressed by IgA^+ but not IgG^+ plasmablasts (Kunkel et al., 2003) and anti-CCL28 antibodies partially block the migration of IgA^+ plasmablasts to murine MG and gut (Feng et al., 2006; Wilson and Butcher, 2004). We previously showed that MAdCAM-1 is expressed by endothelial cells in the murine MG and was correlated with $\beta7^{high}$ T cell homing (Tanneau et al., 1999). Interestingly, after a maximum expression at the end of gestation, the expression of MAdCAM-1 decreased during lactation whereas the number of IgA^+ B cells increased (Tanneau et al., 1999), suggesting that additional factors, such as chemokines might be implicated in the homing of IgA^+ B cells to the MG. Moreover, in β7 knockout mice, the recruitment of $CD3^+$ T cells towards the MG was dramatically decreased, while no changes in the number of IgA^+ B cells between wild-type and β7 knockout mice (Tanneau et al., 1999) were detectable. This supports our hypothesis that chemokines are involved in the recruitment of IgA^+ B cells in the MG.

In order to investigate the specific mechanisms responsible for the homing of IgA^+ B cells from the aero-digestive tract to the MG during lactation, we looked at the expression of vascular addressins in the MG in relation to homing receptors expressed by IgA^+ B cells, as well as to the mRNA expression of epithelial chemokines in the porcine and murine developing MG. Our findings indicate that IgA^+ B cells are recruited from the intestine towards the end of gestation by the expression of both mucosal addressins and chemokines in MG tissue. They confirm the previously made observations that the intestine could be linked to the MG and provide insight into the molecular mechanisms potentially responsible for the phenomena. Our results also highlight the importance of developing novel vaccination strategies that could induce strong mucosal immune responses in the intestine before or during pregnancy to ensure optimal recruitment of IgA^+ plasmablasts to the MG.

2. Materials and methods

2.1. Animals and tissue samples

Multiparous miniature histocompatible $SLA^{d/d}$ sows were used for biopsies of MG in virgin sows (day 0) and at different stages of gestation (50, 80, 90 and 114 days), lactation (2, 8, 15 and 21 days) and involution (24 and 30 days after farrowing). Sows were anesthetized with fluothane–O_2 inhalation and biopsies were taken after skin incision from the subcutaneous tissue of MG pairs using 8 mm skin biopsy punches (V Kruuse Company, Marslev, Denmark). Then, several biopsies, representative of posterior MG in individual animals, were either snap-frozen immediately in liquid nitrogen for tissue sections or placed in a tube containing 1 ml of Trizol reagent (Invitrogen, Cergy Pontoise, France) for RNA extraction and stored at −80 °C. Mice MG biopsies were collected and sampled as previously described (Tanneau et al., 1999). Animals were cared for in accordance with the guidelines of the Institutional Animal Care and Use committee at INRA (France).

2.2. Messenger RNA expression analysis using PCR

Total RNA, isolated from homogenized tissues using Trizol reagent (Invitrogen, Cergy Pontoise, France) and RNeasy Mini Kit (Qiagen, Courtaboeuf, France) was prepared as previously described (Meurens et al., 2007a). Primers used for murine (Hromas et al., 1999; Tashiro et al., 1993; Vicari et al., 1997; Wang et al., 2000) and swine (Tanneau et al., 1999) chemokines as well as reference genes have been previously described.

Three sets of porcine MAdCAM-1primers, based on the published sequence (GenBank accession no. NM001037998), were designed using Clone Manager 8 (Scientific & Educational Software, Cary, NC, USA) for conventional PCR and real-time PCR (qPCR) (Table 1). The pig MAdCAM-1 protein had two N-terminal Ig-like domains (D1 and D2), a mucin-like domain and a third Ig-like domain (D3) found in rodent but not in human MAdCAM-1 (Tachedjian et al., 2006). In the protein, the AA sequence LDT, located in D1, is known to participate in α4β7 lymphocytes binding (Tachedjian et al., 2006) (Fig. 5B). Two sets of housekeeping gene primers, cyclophilin (Oswald et al., 2001) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Meurens et al., 2006) were used to assess the uniformity of the RT reactions. Their suitability as reference genes was confirmed by the lack of variation observed between animals from a same group and physiological stages in the MG.

Conventional PCR reactions were performed in an automated DNA thermal cycler (Mastercycler, Eppendorf, Hamburg, Germany). Samples were subjected to 35 cycles of amplification consisting of denaturation during 30 s at 94 °C, primer annealing to the template at optimal temperature for 1 min and primer extension at 72 °C for 2 min. Finally, 10 min final extension was performed at 72 °C. PCR products (10 μl) were examined by electrophoresis in 1.5% agarose gels, stained with ethidium bromide, visualised under a transil-