Increases in IgA\(^+\) B cells in Peyer’s patches during milk-borne mouse mammary tumor virus infection are influenced by Toll-like receptor 4 and are completely dependent on the superantigen response

Gabriel Cabrera, Claudia Vercelli, Dalia Burzyn, Noel Badano, Andrea Maglioco, Héctor Costa, Juliana Mundiñano, Gabriela Camicia, Irene Nepomnaschy\(\dagger\) and Isabel Piazzon\(\dagger\)

ILEX-CONICET, División Medicina Experimental, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Buenos Aires, Argentina

Mouse mammary tumor virus (MMTV) is a milk-borne betaretrovirus that has developed strategies to exploit and subvert the host immune system. Although mammary glands are the final target of infection, Peyer’s patches (PP) are the entry site of the virus. Herein, we show that the infection induces increases in the number of PP IgA\(^+\) B cells and higher expression of the \(\alpha\) circular transcript, which is a specific marker of the switch to IgA. In addition, IgA\(^+\) B-cell increases correlated with higher levels of cytokines related to IgA class switching, such as interleukin (IL)-5 and IL-6. Of interest, the increases in IgA\(^+\) B cells were lower in Toll-like receptor 4-deficient mice and were completely dependent on the presence of superantigen-reactive T cells. Our results point to a novel mechanism involved in MMTV infection and suggest that IgA\(^+\) B cells may play an important role in carrying the virus to the mammary glands.

INTRODUCTION

Mouse mammary tumor virus (MMTV) is a milk-borne betaretrovirus that causes mammary tumours in mice. MMTV is a pathogen that subverts the immune system, and several strategies of viral host exploitation have been described (Acha-Orbea et al., 2007; Czarneski et al., 2003). The virus enters through Peyer’s patches (PP), where it infects B cells and dendritic cells (DCs). These infected cells present a viral superantigen (SAg) to T cells expressing SAg-specific T-cell receptor V\(\beta\) chains. The resulting interaction causes a strong immune response that is critical for virus amplification (Cabrera et al., 2008; Czarneski et al., 2003; Held et al., 1994). Many experiments have shown that MMTV infection is not efficient in mice that do not generate an immune response to the SAg due to a deficiency in certain genes or cells (Beutner et al., 1996; Golovkina et al., 1992, 1993; Held et al., 1993, 1994).

Even when the PP are the entry site of the virus, the mammary glands are the final target of the infection. It is believed that infected lymphocytes transport the virus to the mammary glands, but it is still not clear which cells are responsible for the transfer (Acha-Orbea et al., 2007; Dzuris et al., 1997).

It has previously been shown that, in a model of MMTV infection in adult mice by subcutaneous injection of the virus in the footpad, there is an increase in the number of infected B lymphocytes in the popliteal lymph node, and this is critical for amplification of the virus reservoir (Held et al., 1993). It has also been reported that, in the absence of SAg-reactive T cells, there are no increases in the number of infected B cells (Held et al., 1993).

The model of adult infection has been useful to study many aspects of the interaction between MMTV and the immune system. However, there are some important differences between this model and natural infection with the milk-borne virus, including the age of the host and the characteristics of the immune tissue that interacts with the virus at the beginning of the infection.

Because the PP are part of the gut-associated lymphoid tissue (GALT), one of the differences from the immune responses in other sites is the production of IgA. This immunoglobulin is considered the main antibody isotype involved in the defence against pathogens in the mucosal immune system. IgA is the isotype produced in highest amounts in the body, and it is considered that about 80% of IgA-producing cells reside in the gastrointestinal mucosa (Mora & von Andrian, 2008). The PP are the main secondary lymphoid organ where B cells differentiate to IgA-producer cells (Butcher et al., 2008).
The PP have high levels of IgA class-switch-specific markers, such as germininal transcripts and circular transcripts (CTa) (Glick et al., 1991).

Some cytokines and other molecules have been associated with IgA production. In particular, interleukin (IL)-4, IL-5, IL-6, transforming growth factor β, retinoic acid and others have been highlighted as important factors for the generation of IgA antibodies (Fagarasan, 2008; McGhee et al., 2007; Mora & von Andrian, 2008).

Another modulator of IgA production is Toll-like receptor (TLR) 4. TLRs are pattern-recognition receptors that interact with conserved microbial products. TLRs function as sensors of infection and are critical for the initiation of innate and adaptive immune responses. It has been reported that TLR4-deficient C3H/HeJ mice have more potent IgA responses than TLR4-sufficient C3H/HeN mice (Kaneko et al., 2005; Kiyono et al., 1980).

Interestingly, it has been shown that MMTV is able to interact with B cells and DCs through TLR4. This interaction is important for the generation of the immune response induced by the virus and it favours the infection of DCs. In particular, we have shown that the interaction of MMTV with TLR4 expressed on DCs induces an increase in the expression of costimulatory molecules on these cells, promotes the recruitment of DCs to the PP and augments the expression of the MMTV cell-entry receptor, the transferrin receptor (CD71) (Burzyn et al., 2004).

IgA+ cells migrate to other compartments of the mucosal system, such as the mammary glands, which are the final target of MMTV infection (Acha-Orbea et al., 2007; Dzuris et al., 1997; McDermott & Bienenstock, 1979; Weisz-Carrington et al., 1977).

Because PP B cells are prone to IgA class switching and these cells could be involved in carrying the virus to the mammary gland, the aim of this study was to investigate whether milk-borne MMTV infection causes alterations in PP IgA+ B cells and to determine the influence of TLR4 and the SAg response.

RESULTS

IgA+ B cells in the PP after MMTV infection

Because class switching to IgA typically occurs in the PP, we analysed by FACS whether milk-borne natural infection with MMTV induces changes in the IgA+ B−-cell subset in the PP. Fig. 1(a) depicts a representative dot-plot of IgA expression in B220+ cells 10 days after infection. We found that the absolute number of IgA+ B220hi cells increased progressively in the PP of MMTV-infected mice during at least the first 10 days of infection (Fig. 1b).

B220lo IgD− cells have been described as plasmatic cells or plasmablasts (Youngman et al., 2002). IgD− indicates that B cells have switched to downstream Ig classes. Thus, IgA+ B220lo B cells will hereafter be referred to as plasmablasts or plasmatic cells. Our experiments showed that the absolute number of PP IgA+ B220lo cells also increased progressively in MMTV-infected mice (Fig. 1c).

These results indicated that MMTV infection caused progressive increases in both IgA+ B220hi and IgA+ B220lo plasmablasts or plasmatic cells in the PP during at least the first days of infection.

Class switching to IgA in the PP

Class-switch recombination (CS) is a biological mechanism that occurs in activated B cells. During this process, the
μ constant region of the heavy chain is replaced with a downstream isotype, determining that B cells change the production of antibody from one class to another.

CS occurs in activated B cells by deletion of a loop-shaped fragment of DNA, followed by ligation of the broken DNA ends. The excised circular fragment contains a promoter that is still responsive to specific cytokines regulating the transcription of CTα, which can be used as molecular markers of CS, as they are detectable when CS is occurring and they quickly disappear after CS is finished (Kinoshita et al., 2001). High levels of CTα can be found in the PP (Glick et al., 1991).

To determine whether the increases in IgA+ B cells were due to CS in situ in the PP, we analysed by RT-PCR the presence of CTα transcripts in the PP at different days after MMTV infection (Fig. 2a). The different sizes of the CTα transcripts correspond to different splice variants of the transcript (Bergqvist et al., 2006; Kinoshita et al., 2001). Product lengths are shown in Fig. 2(c). MMTV infection statistically significantly increased the expression of CTα normalized to β-actin in PP from day 2 to day 9 (P<0.05) (data not shown). Fold increase of CTα expression in PP of MMTV-infected mice ranged from 1.7 to 2.3 (Fig. 2d).

The increase of CTα from day 2 of infection correlated with the subsequent increase in IgA+ B cells in the PP, suggesting that MMTV infection induces CS to IgA at the site of virus entry.

Expression of IL-5 and IL-6 mRNA in the PP during MMTV infection

It is known that production of IL-5 and IL-6 in PP is related to the IgA class switch (Mora & von Andrian, 2008). In order to evaluate whether the increases in IgA+ B cells correlated with increases in IL-5 and IL-6, we studied by RT-PCR the
expression of these cytokines in the PP after MMTV infection (Fig. 2b). Product lengths are shown in Fig. 2(c). MMTV infection statistically significantly increased the expression of IL-5 normalized to β-actin from day 5 to day 9 (P<0.05) (data not shown). Fold increase of IL-5 expression in PP of MMTV-infected mice ranged from 2.0 to 2.4 from day 5 to day 9 (Fig. 2e). In addition, MMTV infection statistically significantly increased the expression of IL-6 normalized to β-actin from day 2 to day 9 (P<0.05) (data not shown). In this case, fold increase of IL-6 expression in PP of MMTV-infected mice ranged from 1.7 to 2.7 (Fig. 2f).

Thus, the results showed that MMTV infection induced increases in the expression of cytokines related to CS to IgA in PP of infected pups.

**Influence of TLR4 on the increases in IgA+ B cells in the PP**

It has been demonstrated that MMTV is able to interact with TLR4 expressed on B cells (Rassa et al., 2002) and DCs (Burzyn et al., 2004). This interaction affects important events during MMTV infection.

We studied whether the absence of a functional TLR4 affects the MMTV-induced increase in IgA+ cells in the PP. TLR4-deficient C3H/HeJ mice and the respective controls, C3H/HeN mice, were foster-nursed for 6 days on BALB/cJ females infected with MMTV(BALB6), and the PP IgA+ B-cell populations were studied by FACS (Fig. 3).

As shown in Fig. 3, MMTV infection induced increases in the absolute number of both IgA+ B220high and IgA+ B220low cells in TLR4-deficient mice. However, these increases were significantly lower than in the C3H/HeN control mice (P<0.05).

**Influence of the SAg response on PP IgA+ B-cell increases**

Considering that it is well-known that the SAg response is critical for an efficient infection and bearing in mind that IgA+ B cells may be relevant in the outcome of the infection, we wondered whether the SAg response is important for determining the increases in IgA+ B cells in the PP.

In order to address this question, we infected littermates that either possessed or did not possess SAg-reactive T cells. To perform this, we took advantage of the fact that there are different endogenous MMTVs (Mtvs) in the germline of mouse strains. Most Mtvs do not produce virus particles but they still express the SAg, causing the deletion of SAg-cognate T cells during thymic negative selection. The SAg of the endogenous Mtv-7 virus has the same specificity as the exogenous MMTV(BALB6) virus used in our experiments and, therefore, Mtv-7+ mice do not possess T cells reactive to the MMTV(BALB6) SAg (Cabrera et al., 2008; Golovkina et al., 1997).

![Fig. 3. Increases in PP IgA+ B cells are influenced by the presence of a functional TLR4. C3H/HeN and C3H/HeJ TLR4-deficient mice were nursed on MMTV(BALB6)-infected (MMTV+) or uninfected (MMTV−) mothers for 6 days. Expression of IgA and B220 in PP cells was analysed by FACS. PP cells were gated for live cells in the forward–sideward scatter. Absolute number of IgA+ B220high cells (a) and IgA+ B220low cells (b) in PP is shown. Data are presented as means±SD (n=4). *P<0.05. The experiment was performed three times with similar results.](http://vir.sgmjournals.org)

![Mtv-7+ and Mtv-7− littermates were obtained by back-crossing BALB/cJ (Mtv-7−/−) × AKR/J (Mtv-7+/+) F1 × BALB/cJ mice. Mice that possessed SAg-reactive T cells (Mtv-7+/−) or littermates that did not possess SAg-reactive T cells (Mtv-7−/−) were foster-nursed on MMTV(BALB6)-infected mothers, and the PP IgA+ B-cell subsets were analysed by FACS (Fig. 4). Our results indicated that the increases in both IgA+ B220high and IgA+ B220low cells were only produced in mice that bore SAg-reactive T cells and therefore were dependent on the immune response caused by the SAg.](http://vir.sgmjournals.org)

**DISCUSSION**

Immune responses generated in PP and other mucosal lymphoid tissues have unique features that distinguish them from responses in other sites. It is well-known that, in the microenvironment of the PP, B cells are prone to IgA class switching. Because IgA+ B cells are able to migrate to different mucosal tissues, including the mammary gland, which is the final target of MMTV infection, in this paper we addressed the question of whether natural milk-borne infection induces the generation of IgA+ B cells in PP.
Using the model of MMTV infection in adult mice, it has been reported that B220\textsuperscript{low} cells are the main population infected in the draining popliteal lymph node after MMTV infection (Ar\d{a}vin et al., 1999). In addition, by performing mammary-gland injections of the virus, it has been shown that MMTV spread within the mammary gland requires B cells (Golovkina et al., 1998). The results presented herein show that MMTV induces a significant increase in the absolute number of both IgA\textsuperscript{+} B220\textsuperscript{high} cells and IgA\textsuperscript{+} B220\textsuperscript{low} plasmablasts or plasmatic cells in PP during the first days of milk-borne infection.

It has been reported that high levels of molecular markers of IgA CS, such as CT\textsubscript{X}, can be detected in PP (Butcher et al., 1982; Glick et al., 1991). Our results indicate that the level of CT\textsubscript{X} expression is increased in the PP of MMTV-infected mice during the first 9 days, showing that virus infection induces IgA CS in situ in the PP.

Several reports have demonstrated that IL-5 and IL-6 are involved in the generation of IgA\textsuperscript{+} B cells in the PP (Fagarasan, 2008; McGhee et al., 2007; Mora & von Andrian, 2008). Accordingly, our data show that the expression of IL-5 mRNA is augmented in PP of infected mice from day 5 to at least day 9 of MMTV infection, and that the levels of IL-6 mRNA are also increased at all infection time points analysed.

It has been reported that TLR4-deficient C3H/HeJ mice have elevated IgA responses (Kaneko et al., 2005; Kiyono et al., 1980). Interestingly, in this paper we show that the increase in IgA\textsuperscript{+} B cells is significantly lower in TLR4-deficient mice than in the wild-type controls (\(P<0.05\)).

Whether the interaction of MMTV with TLR4 is beneficial for the virus or for the host has been discussed in the literature (Jude et al., 2003; Otten et al., 2002; Ross & Rassa, 2002). It has been suggested that the presence of a functional TLR4 prevents the generation of a CD8 antiviral response (Jude et al., 2003). Importantly, it has been reported that infection efficiency is reduced in TLR4-deficient mice, suggesting strongly that TLR4 interaction is beneficial for the virus (Jude et al., 2003; Otten et al., 2002; Ross & Rassa, 2002).

Because in the absence of a functional TLR4 there are lower increases in IgA\textsuperscript{+} B cells and also because MMTV infection is less efficient in this context, the data presented herein suggest that IgA\textsuperscript{+} B cells could be important for the success of the infection.

It has been reported that PP B cells are infected during MMTV infection (Acha-Orbea et al., 2007). Considering that MMTV causes B-cell activation (Rassa et al., 2002) and that it has been documented extensively that IgA class switching occurs in activated B cells in PP (Craig & Cebra, 1971; Fagarasan, 2008; Mora & von Andrian, 2008), it is most likely that PP IgA\textsuperscript{+} B cells are infected during milk-borne infection.

It is well-known that the response to the viral SAg is necessary for virus amplification and an efficient infection. It has been shown that the interaction of T cells with SAg-expressing B cells is a critical event that increases the number of infected B cells (Held et al., 1993). Importantly, our experiments performed with littermates with or without SAg-reactive T cells showed that the SAg response is necessary for the increases in IgA\textsuperscript{+} B cells in PP.

Although the importance of the SAg response during MMTV infection has been highlighted by several reports, it has not been described that this response induces IgA class switching in PP B cells. This observation, together with the fact that in the absence of a SAg response the MMTV infection is inefficient, supports the hypothesis that IgA\textsuperscript{+} B cells play an important role in the life cycle of the virus during MMTV natural infection.

Accumulating evidence indicates that mucosal IgA\textsuperscript{+} B cells can be generated by T-dependent as well as T-independent mechanisms (Fagarasan, 2008; Mora & von Andrian, 2008). As in the absence of SAg-reactive T cells the
Mice were bred in the animal facility of the ILEX-CONICET, División Medicina Experimental, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Buenos Aires, Argentina. The mice were housed according to the policies of the Academia Nacional de Medicina (National Research Council, 1996). All animal experiments were approved by the ethical committee of the ILEX-CONICET.

Experimental model. Eight-day-old mice were foster-nursed on non-infected BALB/c females or MMTV(BALB6)-infected mothers for the number of days indicated in the text or figures. The mice were sacrificed, their PP were dissected and single-cell suspensions were prepared by homogenization through a stainless-steel mesh. Flow cytometry or RT-PCR was performed as described below.

Flow cytometry. Cells were incubated with Fc-γ-block antibody (anti-mouse CD16/32; Pharmingen) to prevent non-specific binding of antibodies to Fc-γ receptors. For staining, 1 x 10^6 cells were incubated with the following monoclonal antibodies (Pharmingen) and subjected to FACS analysis: FITC-conjugated anti-IgA (clone C10-3) and PE-conjugated B220 clone (RA3-6B2). Scans were acquired on a FACScan flow cytometer (Becton Dickinson). Data were analysed by using CELLQuest software (Becton Dickinson Immunocytometry Systems).

RT-PCR. RNA from PP-cell suspensions of pups was extracted by using a Qiagen RNeasy Mini kit according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA by using an Access RT-PCR System kit (Promega) according to the manufacturer's instructions. PCRs were performed by using specific primers as described elsewhere: for CTx, 5'-CCAGGCAATGGTTGGAATGAGAT-AAGATG-3' and 5'-AATGTGTGCTTGGCAGAAGT-3' (Bergqvist et al., 2006; Kinoshita et al., 2001); for IL-6, 5'-TTCCTCTGTGCA-AGAGACT-3' and 5'-TGTATCCTCTGGAAGAAGC-3' (Burzyn et al., 2004); for IL-5, 5'-AAGATGCTTCTGCAAGTCA-3' and 5'-ACACC-AAGAACCTCTTGGCA-3' (Goto et al., 1999; Van Wauwe et al., 2000); for β-actin, 5'-TCATGAGGTTGAGGTACATC-3' and 5'-CCT-AGAAGCATTTGCGGCAAGATG-3' (Burzyn et al., 2004).

PCR was carried out as follows. For CTx amplification, the PCR program consisted of 30 x 94 °C, 40 s at 60 °C and 40 s at 72 °C for 36 cycles in PCR buffer containing 100–500 ng DNA, 0.5 U Taq polymerase (Invitrogen), 10 mM Tris/HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.1 µM of each primer and 0.2 mM of each deoxynucleotide in a final volume of 50 µl. For IL-5 and IL-6 amplification, the PCR program consisted of 40 x 94 °C, 40 s at 60 °C and 40 s at 72 °C for 30 cycles in the same PCR buffer used for CTx amplification. For β-actin amplification, the PCR program consisted of 30 x 94 °C, 20 s at 60 °C and 30 s at 72 °C for 22 cycles and the same PCR buffer used for CTx amplification. These conditions gave linear DNA amplification. The PCR products were analysed by electrophoresis on 2% agarose gels with ethidium bromide. Bands corresponding to different PCR products were quantified by using Scion Image software.

Statistical analysis. Levels of significance were determined by using a two-tailed Student's t-test, and a confidence level of >95% (P<0.05) was used to establish significance.

ACKNOWLEDGEMENTS

This work was supported by ANPCyT PICT 506305, CONICET and FUNDALEU. We thank C. D. Pasqualini for helpful discussions.

REFERENCES


METHODS

Mice. The following strains of mice were used: BALB/c (H-2b, Mtv-7b) mice; MMTV(BALB6)-infected BALB/c mice carrying an exogenous virus that encodes a SAg with major specificity for Vj6b; AKR/J (H-2k Mtv-7k) mice, C3H/HeN and C3H/HeJ TLR4-deficient mice.

Mtv-7^+/+, and Mtv-7^-/- mice were generated by crossing (BALB/c x AKR/J)F1 x BALB/c mice, and their phenotype was determined by FACS analysis of the presence or absence of Vj6^- T cells.


