Peripheral Organs Key Role in Carrying Retroviral Infection to Extracellular Plasmablast B Cells Play a

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Extrafollicular Plasmablast B Cells Play a Key Role in Carrying Retroviral Infection to Peripheral Organs

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B cells can either differentiate in germinal centers or in extrafollicular compartments of secondary lymphoid organs. Here we show the migration properties of B cells after differentiation in murine peripheral lymph node infected with mouse mammary tumor virus. Naïve B cells become activated, infected, and carry integrated retroviral DNA sequences. After production of a retroviral superantigen, the infected B cells receive cognate T cell help and differentiate along the two main differentiation pathways analogous to classical Ag responses. The extrafollicular differentiation peaks on day 6 of mouse mammary tumor virus infection, and the follicular one becomes detectable after day 10. B cells participating in this immune response carry a retroviral DNA marker that can be detected by using semiquantitative PCR. We determined the migration patterns of B cells having taken part in the T cell-B cell interaction from the draining lymph node to different tissues. Waves of immigration and retention of infected cells in secondary lymphoid organs, mammary gland, salivary gland, skin, lung, and liver were observed correlating with the two peaks of B cell differentiation in the draining lymph node. Other organs revealed immigration of infected cells at later time points. The migration properties were correlated with a strong up-regulation of αβ integrin expression. These results show the migration properties of B cells during an immune response and demonstrate that a large proportion of extrafolliculary differentiating plasmablasts can escape local cell death and carry the retroviral infection to peripheral organs. The Journal of Immunology, 2001, 166: 6266–6275.

Following encounter with foreign Ag in the presence of T cell help, B cells start proliferating and differentiating into Ab-secreting cells (1). After cognate interaction with T lymphocytes, B cells either migrate to adjacent follicles or to extrafollicular sites of proliferation. In the spleen, extrafollicular B cell proliferation and plasma cell differentiation occur in the periarteriolar lymphocytic sheaths (2–4). In lymph nodes (LNs), extrafollicular plasmablast growth is localized in the medullary cords (5). Ig switch is observed in both germinal center and extrafollicular B cells (6). Although follicular B cell differentiation and germinal center formation have been studied in great detail, the fate of extrafollicularly differentiated B lymphocytes is less well defined. Most of the B cells proliferating in extrafollicular sites have been considered to secrete low affinity Abs and undergo rapid elimination by cell death soon after proliferation (7, 8). Apoptosis has been ascribed to a mechanism to exclude self-reactive B cells from affinity maturation in germinal centers (9, 10). However, rescue of extrafollicular plasmablasts from apoptosis by interaction with dendritic cells has been reported (11).

In this study, we have undertaken a systematic analysis of the fate of extrafollicularly differentiated plasmablasts in vivo using the Swiss strain (SW) of mouse mammary tumor virus (MMTV) as an antigenic model. After s.c. injection of MMTV into the hind footpad of BALB/c mice, the virus is transported to the draining popliteal (PO)-LN, where it productively infects naive B lymphocytes, which are the main target cells for MMTV infection (12, 13). MMTV(SW)-infected B cells present a viral superantigen (SAg) to Vβ6+ T cells, which leads to a strong T cell-B cell collaboration. The MMTV SAg-specific B cell response has the characteristics of a classical Ag response in terms of localization, phenotype, Ab secretion, and longevity. Having received cognate SAg-mediated T cell help, infected B cells strongly proliferate and differentiate into extrafollicular plasmablast B cells by day 5, followed by formation of germinal centers by day 10 (5). Extrafollicularly differentiating B cells are large plasmablasts that are localized in the medullary cords of the draining PO-LN and secrete polyclonal, as well as Ag-specific, Abs (5, 14). As previously shown, these cells have down-modulated B220 (CD45R) and are MHC class IIlow, IgD+, and syndecan-1 (CD138)high (15). The near-exclusive infection of all plasmablasts 6 days after MMTV infection, together with their phenotypic profile, allowed us to determine whether these cells had entered the recirculating pool of lymphocytes and migrated to peripheral tissues. Because infectious viral particles can be shed by both B and T cells (16), we treated mice after infection with the reverse transcriptase inhibitor AZT to block residual reinfection of peripheral organs (17). Therefore, detection of reverse-transcribed viral DNA in lymphoid and nonlymphoid organs reflected migration of infected cells exported from the draining PO-LN. The essential findings of the present study are: First, B cell plasmablasts leave the medullary cord of the draining PO-LN and carry the virus among other organs to the mammary gland. Second, recruitment of infected B lymphocytes to the periphery is synchronized with extrafollicular and follicular lymphocyte differentiation. Third, MMTV-infected plasmablast B cells express elevated levels of αβ integrin, which can mediate adhesion and entry into peripheral tissues.

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3 Abbreviations used in this paper: LN, lymph node; SW, Swiss strain; MMTV, mouse mammary tumor virus; SAg, superantigen; PO, popliteal; IEL, intraepithelial lymphocyte.
Materials and Methods

BALB/c mice, 6- to 7-wk-old, were obtained from Harlan Olac (Bicester, UK.). Mtv-7 congenic BALB/c mice (BALB.D2) (18) were bred at the Swiss Institute for Cancer Research (Lausanne, Switzerland). Mice were housed under standard conditions and provided with food and water ad libitum. MMTV(SW) encoding a Vβ-specific SAg was recovered from the milk of lactating female mice as described previously (12). It was diluted 1:3 in PBS, centrifuged at 600 g for 10 min to skin and remove cells, and stored in aliquots at -70°C. Mice were injected s.c. into one hind footpad with MMTV(SW) (5 μl) and remove cells, and stored in aliquots at 2°C and 70% ethanol. Mice were injected i.p. into one hind footpad with MMTV(SW) encoding a Vβ-specific SAg. Where indicated, mice were i.p. injected with 2 mg AZT (Sigma, St. Louis, MO) 2 days after viral infection or before infection. AZT treatment was continued by dissolving AZT in the drinking water at a concentration of 1 mg AZT/ml water (19).

Flow cytometry and cell separation

The following mAbs were used (When no provider is indicated the antibody was produced in our laboratory): FITC-conjugated rat anti-CD3 (17A2; BD PharMingen, San Diego, CA), FITC-conjugated anti-B220 (6A3-6B2; Caltag, San Francisco, CA), FITC-conjugated goat anti-rat IgG (polyclonal serum; Caltag), PE-conjugated mouse anti-MHC class II E-OMA-1 (14-4-4S; BD PharMingen), biotin-conjugated rat anti-mouse syngeneic-1 (CD138) (281.2; BD PharMingen), biotin-conjugated hamster anti-mouse CD11c (N418), biotin-conjugated rat anti-mouse Mac 1 (M1/70; BD PharMingen), biotin-conjugated anti-β2m (M293) (BD PharMingen), biotin-conjugated anti-β1 (CD29) (HA2/5; BD PharMingen), biotin-conjugated and unconjugated anti-α2 (clone PS22, (23); clone R1-2, (24), biotin-conjugated hamster anti-α2 intrathelial lymphocyte (IEL) (clone 2E7; BD PharMingen), biotin-conjugated hamster anti-β1 (CD61) (clone 29C.2; BD PharMingen), Cy-Chrome-conjugated anti-B220 (CD45R) (clone RA3-6B2; BD PharMingen), APC-conjugated anti-B220 (RA3-6B2; BD PharMingen), streptavidin-Cy-Chrome (BD PharMingen), and streptavidin-APC (Molecular Probes, Eugene, OR). Lymphocytes were preincubated with anti-FcγRII mAb (2.4G2; BD PharMingen) and stained in one step in a mixture of FITC-, PE-, APC- and biotinylated Abs diluted in PBS/3% FCS. Alternatively, a combination of FITC- and PE-biotinylated and Tricolor-labeled Abs was used. If indicated, staining with anti-integrin mAbs was performed in PBS/3% FCS containing 0.25 mM Mn2+ . After incubation, cells were washed and stained with streptavidin-Cy-Chrome or streptavidin-APC. Acquisition was performed by a FACS Calibur (BD Biosciences, Mountain View, CA) cell analyzer. Dead cells were excluded from acquisition. Data were analyzed using BD CellQuest software (BD Biosciences).

For separation of B and T cells from secondary lymphoid organs after MMTV infection, tissues were homogenized, and cells were stained with PE-conjugated anti-MHC class II E-OMA-1 (14-4-4S), biotin-conjugated rat anti-mouse CD11c (N418), and biotin-conjugated rat anti-mouse Mac 1 (M1/70). B cells (FITC-conjugated anti-B220 (6A3-6B2) and T cells (FITC-conjugated anti-B220 (6A3-6B2) and CD3-PE) were stained with the same fluorochrome and were identified by the presence (B cells) or absence (T cells) of MHC class II expression. After incubation, cells were washed and stained with streptavidin-Cy-Chrome. B220hi/MHC class II+ B cells were collected and CD3- MHC class II- cells were sorted with MACS beads (BD Biosciences). Dendritic cells and macrophages were excluded by gating out Cy-Chrome-positive cells.

PCR and virus-specific hybridization

DNA was amplified with the oligonucleotide MS10 (AGGTGGGCTCA CAATCAAGCGC), which reacts with all the open reading frame molecules and with the MMTV/SW-7-specific oligonucleotide Vβ83 (GC GACCACTCGTATTCTTC) complementary to the long terminal repeat positions 705–725. A total of 500 ng of DNA from cells extracted from the draining PO-LN was analyzed by PCR. The number of cycles used for amplification of viral DNA from non-draining tissue was 26 cycles (1 min annealing step at 94°C, 1 min extension step at 72°C, and 1 min at 94°C). For each cycle, 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C; and finally an extension step for 10 min at 72°C) in 1× PCR buffer containing 20 mM Tris-HCl (pH 8.55), 16 mM (NH4)2SO4, 2.5 mM MgCl2, 150 μg/ml BSA, 0.2 mM of each of the four dNTPs, and 2.5 U of Taq polymerase (Biotaq; Bioprobe International, Richmond, CA); a total of 25 μl of each oligonucleotide was added per PCR. For each experiment, the optimal number of cycles to stay in the linear range was determined. PCR on DNA from draining PO-LN was performed with 22 cycles because the number of infected cells was much higher. A total of 30 cycles of PCR with specific primers for Mtv-6, -8, and -9 were used to quantify DNA isolated from LN cells as described before (15). For FACS-sorted cells, 25 ng (30 cycles) were used. To determine the numbers of viral copies per nanogram of DNA, DNA was extracted from lymphocytes derived from BALB.D2 mice containing two copies of the endogenous provirus Mtv-7 per cell. The Sag sequence of Mtv-7 was amplified from 10-fold dilutions of DNA starting with 50 ng DNA mixed with 450 ng DNA of BALB/c mice, keeping the total DNA concentration constant. Results in Fig. 4 are represented as the amount of Mtv-7 DNA that gives an identical PCR product with two copies of MMTV per infected lymphocyte, we estimated the number of MMTV(SW)-infected cells in 103 total cells. The PCR product was detected by liquid hybridization using a radioactive oligo-probe as follows: 1 μl of specific primer (5’-CAAGGGAGCTCACTCTGGCG-3’), 11 μl H2O, 1 μl phosphotyrosine kinase (10 U/μl), 5 μl of [γ-32P]ATP (2 pmol/μl), and 2 μl of hybridization buffer (0.5 mM Tris (pH 7.6), 0.1 M MgCl2, 50 mM DTT, 1 mM EDTA, and 1 mM spermidine) were incubated for 45 min at 37°C. The reaction was stopped by adding 180 μl of 10 mM Tris/0.5 mM EDTA + 1 μl EDTA (0.5 M). The oligo-probe was washed three times by using a Sephadex G50 column (Pharmacia, Uppsala, Sweden), and 1 μl was added to 10 μl PCR product together with 7 μl of H2O and 2 μl of a solution containing 1.5 M NaCl and 25 mM EDTA. The annealing reaction was performed with 1 cycle consisting of 5 min at 98°C and 1 cycle consisting of 15 min at 55°C. PCR products were separated on a 6% denaturing polyacrylamide gel, which was dried and then exposed to Kodak X-OMAT film (Eastman Kodak, Rochester, NY).

Immunoprecipitation

Extracellular plasmablast B cells were isolated from draining PO-LN 6 days after infection by incubating LN cells with biotinylated anti-syngeneic-1 Ab. After washing and incubating with streptavidin-conjugated MACs beads for 15 min at 4°C, lymphocytes were resuspended in PBS/5% FCS. After loading on to a MACS separation column (Miltenyi Biotech, Bergisch Gladbach, Germany) and extensive washing, bound cells were removed from the column in 1 ml PBS and analyzed by flow cytometry. The purity of each cell population collected from columns ranged from 90% to 95%. A total of 106 purified plasmablast B cells were surface labeled by incubating with 5 μl [32P]ATP (0.5 mCi) for 20 min in a glass vial coated with 200 μl of iodogen solution (0.5 mg/ml chloroform; Pierce, Rockford, IL) on ice. A total of 500 μl of 1-Tyrosoin solution (0.3 mg/ml PBS) was added and incubated for several minutes on ice. Cells were washed in 1 ml PBS and lysed in 600 μl lysis buffer (150 mM NaCl, 50 mM Tris (pH 7.5), 1 mM CaCl2, 1 mM MgCl2, 1% Nonidet P-40, and 0.02% NaN3) and EDTA-free protease inhibitors (Boehringer Mannheim, Mannheim, Germany; 1 tablet/10 ml lysis buffer). Insoluble material was removed by centrifugation at 12,000 × g for 15 min. Supernatant was preclarified by three sequential 2-h incubations with 60 μl packed protein A-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) conjugated to anti-β1 (M293; BD PharMingen; 1 μg/20 μl) and anti-β2m, mAb (CD29; BD PharMingen; 1 μg/20 μl) and a 2-h incubation with unconjugated protein A-Sepharose. The preclarred extract was immunoprecipitated with 80 μl protein A-Sepharose coated with anti-α2 mAb (PS22; 1 μg/20 μl). Samples were fractionated by 6% SDS-PAGE and visualized by exposure to Kodak X-OMAT film.

Results

MMTV infection is synchronized with extracellular and follicular B cell differentiation in the draining PO-LN

To study the amplification of MMTV in the draining PO-LN during extracellular and follicular B cell differentiation, we determined the reverse-transcribed retroviral DNA copies 3–23 days after virus injection. PCR with primers amplifying exogenous MMTV as well as endogenous Mtv-7 sequences were performed from 500 ng DNA extracted from 103 lymphocytes of the PO-LN. The number of copies of DNA from endogenous Mtv-7 is two, and the number from infectious exogenous MTV sequences is two to three (25). Therefore, serial dilutions of Mtv-7 congenic BALB/c (BALB.D2) DNA mixed with a constant amount of BALB/c DNA were used to allow the estimation of exogenous MMTV DNA in draining PO-LNs (Fig. 1B). In parallel, PCR analysis with primers specific for endogenous Mtv-6, -8, and -9 sequences served as internal controls to confirm equal amounts of DNA in each sample.
After MMTV injection into the hind footpad, we observed two peaks of viral amplification in the draining PO-LN. The first one was maximal at day 6 after MMTV infection (Fig. 1A), when extrafollicular B cell differentiation in the medullary cords is at a maximum (5, 15). The second peak of viral DNA amplification was at day 11, when follicular differentiation and germinal center reaction of B cells takes place in the draining PO-LN (5).

Because peak infection in the draining lymph node occurs around day 5–6 and 11, we attempted to identify the lymphocyte subset being infected 6 and 14 days after MMTV injection in the draining PO-LN. As shown previously and in Fig. 1, germinal center reaction peaks around days 10–12 (5). We chose day 14 for PCR analysis because, at this time point, practically no extrafollicular plasmablasts are found in the LN (15). Staining of draining PO-LN cells with Abs to B220 and MHC class II revealed four subsets of lymphocytes: B220low class IIlow, CD3+, CD11c−, Mac1− B cells, which appeared as large blast cells in forward-side scatter profiles (15); B cells expressing B220high, class IIhigh, CD3−, CD11c+, Mac1+, which were either naive or activated follicular B cells; CD3+, B220+, class II+, CD11c+, Mac1− T cells; and B220high, class IIlow, CD3−, CD11c−, Mac1− B cells. DNA from equal numbers of sorted cells of these four subsets were analyzed by PCR with primers specific for reverse-transcribed exogenous MMTV. B220low plasmablast B cells were highly infected with MMTV at day 6, whereas only a few small B220high B cells, class IIlow B cells, or T cells contained viral DNA (Fig. 2A). The PCR signal was ~1000 times weaker for these cells, indicating that only about one cell in a thousand was infected compared with complete infection of the plasmablasts. However, at day 14 after infection, viral DNA was mainly detectable in DNA extracted from B220high B cells and, to a minor extent, from class IIlow B cells (Fig. 2B). These observations supplement our earlier studies, indicating that infected B cells isolated from draining PO-LN 2 wk after MMTV infection are mainly germinal center-derived B cells (26). Therefore, 14 days after infection, follicularly differentiated B cells represent the major reservoir of MMTV in the draining LN, whereas infected plasmablasts are no longer detectable in the draining LN.

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show that a significant proportion of extrafollicularly differentiated plasmablasts are able to reach the recirculating lymphocyte pool and transport MMTV to peripheral lymphoid organs.

**MMTV-infected cells selectively migrate to lymphoid and nonlymphoid organs**

Based on these observations, we determined whether recirculating MMTV-infected plasmablasts also entered nonlymphoid organs. Therefore, lymphoid and nonlymphoid organs of BALB/c mice were removed 3–24 days after MMTV infection, and extracted DNA was analyzed by PCR using primers specific for reverse-transcribed exogenous MMTV. Results in the presence or absence of AZT were identical (data not shown). As demonstrated in Fig. 4, we observed two major migration patterns. Either infection became detectable just after the emigration of extrafollicular plasmablasts on days 5–6 (Fig. 4, A, D, and F) or between days 14 and 20 (Fig. 4, H and K). These two major patterns consistently allowed subdivision. In mammary gland and skin, three peaks of infection were found. The first peak appeared 5–6 days after s.c. injection of MMTV, whereas the second was found by day 14 (Fig. 4, A and B). At day 24, not only mammary gland and skin, but also all other organs that we had tested, were strongly infected (Fig. 4, A–K). In addition, viral DNA containing cells appeared in lung, bone marrow, spleen, axillary, and contralateral PO-LN by day 6 (Fig. 4, C and D). Although viral DNA was isolated from draining PO-LN and analyzed by PCR (25 ng) with 30 cycles (D). As controls, 25 ng (1), 2.5 ng (2), 0.25 ng (3), and 0.025 ng (4) BALB. D2 DNA were diluted with BALB/c DNA to a total amount of 25 ng DNA (E). The experiment was performed twice with similar results.

**MMTV-infected plasmablast B cells express high levels of α4 and β7**

The striking organ-specificity of MMTV-infected plasmablasts prompted us to investigate whether the expression profile of adhesion molecules on B cells was modulated upon viral infection and SAg-mediated immune response. Using four-color flow cytometry, we analyzed four different populations derived from draining PO-LN 6 days after MMTV-infection (Fig. 5): extrafollicular B220highMHC class II high plasmablasts (1), follicular B220highMHC class IIlow B cells (2), B220 MHC class II– non-B cells (3), and B220high/MHC class IIlow B cells (4). When testing anti-α4 mAb PS/2 and anti-β7 mAb HA2/5, a small number of B220highMHC class II high B cells was detectable in naive animals, and most of them consisted of either α4β1 or α4β1high cells (Fig. 5A). All other lymphocyte populations derived from naive LN were mainly α4β1high cells. We then examined the expression levels of α4 and β7 on lymphocytes isolated from the draining PO-LN of 6-day MMTV-infected BALB/c mice (Fig. 5B). Interestingly, almost all B220low plasmablast B cells coexpressed α4 and β7 chains, indicating a noticeable up-regulation of α4 integrin expression upon MMTV infection. A total of 18.6% of B220highMHC class IIlow cells (4) from MMTV-infected LN coexpressed α4 and β7, whereas only 4.8% of follicular B cells (2) displayed α4β7 expression. Similar results were obtained when we performed flow cytometry with anti-β7 mAb 9E6G7, which recognizes an epitope of the activated β7– molecule (data not shown). This indicates that β7 was expressed in an activated conformation on plasmablasts. Because extrafollicular plasmablasts have lost migration toward chemokine gradients (27), this observation might also explain tissue migration in the absence of chemokine responsiveness.

We found considerable differences in expression levels of adhesion molecules not only for α4 and β7 integrin but also for α IELs, β7–, and β7– chains when plasmablast B cells and B220high B cells from MMTV-infected LNs were compared with naive LN cells (Table I). Upon MMTV infection, both B220low plasmablast B cells and B220high B cells had very low levels of α IELs and β7 and had up-regulated β7 (Table I). However, only B220high B cells from MMTV-infected LNs were found to reveal elevated expression levels of syndecan-1. They had also strongly down-modulated L-selectin that corresponds to the activation state of plasmablast B...
cells. Taken together, our data demonstrate that the MMTV-induced immune response can modulate the pattern of adhesion molecules on activated B cells, explaining the newly acquired migration patterns. Because β1 and β7 integrin chains, the known heterodimer partners of α4 integrin, did not increase to the same magnitude as α4 integrin on plasmablast B cells, we investigated whether α4 was coexpressed with another β-chain or was present alone on MMTV-activated plasmablast B cells. Day 6 plasmablast B cells from MMTV-infected draining PO-LN were purified by magnet separation, surface labeled by iodination, and solubilized, and preclearing was performed with protein A-Sepharose conjugated to anti-β1 and anti-β7 mAbs (Fig. 6, lane 1) followed by preclearing with unconjugated protein A-Sepharose (Fig. 6, lane 2). The subsequent anti-α4 immunoprecipitate revealed three prominent species of 152 kDa, 81 kDa, and 61 kDa and a faint band of 130 kDa under nonreducing conditions (Fig. 6, lane 3). These correspond to murine α4 integrin (155 kDa), the two breakdown products of disulfide-linked α4 integrin (81 kDa and 66 kDa), and β1 integrin (130 kDa). The quantitative differences of α4 and β1 bands could be presumably explained by different efficiency in iodination of both molecules or, alternatively, in the presence of α4 homodimers or monomers. Taken together, we were...
unable to detect a new β-chain associated with α4 integrin but clearly demonstrate coexpression of α4β1 integrin heterodimers on almost all plasmablast B cells of MMTV-infected LN.

Discussion

To exploit the fate of extrafollicularly differentiated plasmablast B cells, we studied the differentiation and the migration route of retrovirally infected plasmablasts in mice. Our results demonstrate that, at the peak of differentiation in the medullary cords, MMTV-infected plasmablasts escape from the draining LN and migrate to peripheral sites of the body, but mainly to the mammary gland, skin, lung, liver, and spleen. They have modulated their expression pattern of adhesion molecules with high expression levels of α4β1 and β2 integrin, indicating a role in entry into target organs.

In vitro and in vivo studies have demonstrated that Ag-stimulated B cells differentiate into enlarged plasmablasts, express syndecan-1, and down-modulate B220 and IgD (28, 29). Although follicular germinal center B cells express normal levels of B220, after secondary stimulation the appearance of B220high B cells has been reported, which most likely belong to germinal center-derived plasma cells (30). In MMTV infection, extrafollicular B cell differentiation takes place before germinal centers develop. This is distinct from conventional T-dependent Ag responses in the draining LN (5) and provided the tool to discriminate extrafollicular from follicular B220low plasmablast B cells. All other parameters of the B cell response after MMTV infection are analogous to classical Ag responses as described before (5).

Because extrafollicular plasmablast B cells have differentiated from naïve B cells, which are the major target cells of MMTV infection (5, 13, 15), reverse-transcribed retroviral DNA in B cells is a highly sensitive genetic marker that can be traced by specific PCR. In a PO-LN draining the site of MMTV injection at day 6, ~2 × 10^6 cells of 2 × 10^7 total cells were infected (Fig. 1). As previously described, B cells but not T cells are the target cells for early MMTV infection in mice (14). Our results demonstrate that, at day 6, >99% of infected cells are plasmablasts, and only 0.1% B220high B cells, CD3+ T cells, or class IIlow cells contain provirus DNA (Fig. 2A). This low level of infection in the sorted T cells might represent contaminating B cells. To detect infected B cells distributed all over the body, DNA samples were analyzed by specific hybridization of PCR amplification products. This allowed us to detect five infected plasmablast B cells among 10^5 total cells of lymphoid or nonlymphoid tissues. Our results confirm that the number of virus-infected plasmablasts in the draining PO-LN dramatically increased by day 6 and were strongly decreased by day 9 (15). This was most likely not a result of further differentiation because the total number of B cells in the draining LN decreased at the same time. In addition, MMTV had no lytic effect on target cells, and, therefore, disappearance of plasmablast B cells could either reflect elimination by cell death of proliferating B cells that had not been positively selected in germinal centers or export of cells out of the draining LN. From earlier studies, it is known that B cells participating in primary immune responses are rapidly eliminated by cell death soon after proliferation (7, 31). Similarly,
in vitro-stimulated B cells have been shown to undergo apoptosis after a burst of proliferation (29). However, our results clearly demonstrate that 5–10 × 10^6 MMTV-infected plasmablaster B cells are detectable outside the draining PO-LN (Fig. 3), indicating that these cells had escaped from local cell death and emigrated out of the draining LN. By blocking reverse transcription 2 days after infection with AZT, we were able to block reinfection without reducing the SAg (19). Using this tool, we ruled out that the presence of infected plasmablaster B cells outside the primary responding LN was a result of peripheral infection rather than export of infected cells from the draining LN. Although we cannot exclude that most extrafollicular plasmablaster B cells underwent apoptosis soon after proliferation, the number of plasmablaster B cells that survived was sufficient to transport the virus to peripheral lymphoid and nonlymphoid organs, such as the mammary gland, consisting of glandular and adipose tissue. Germinal center-derived Ab-secreting plasma cells are known to migrate out of the draining LN to the bone marrow (32, 33). However, recirculating Ab-producing B cells are not found in nondraining LN and bone marrow before day 22 after viral infection (34). In contrast to this study, MMTV-primed plasmablaster B cells secreting virus-specific Abs (5) were found among total cells of axillary LN, contralateral PO-LN, bone marrow, and spleen (Figs. 3 and 4) and, after enrichment by FACS, were also found in mesenteric LN (Fig. 3) by day 6. The discrepancy between the two studies can be explained by the higher frequency of MMTV-infected plasmablaster due to their strong expansion by viral SAg-mediated T cell-B cell collaboration and the highly sensitive method required to trace plasmablaster B cell by PCR and virus-specific hybridization. Infected plasmablaster B cells appear in the periphery at the same time as extrafollicular B cell proliferation peaks in the draining LN. We postulate that extrafollicularly differentiated plasmablaster B cells are no longer retained in the medullary cords of the LN and leave the draining LN via the efferent lymphatics. This hypothesis is supported by the finding that extrafollicular plasmablaster B cells have lost responsiveness to homeostatic chemokines and have down-modulated most CC and CXC receptors specific for chemokines secreted in the draining LN (27). Loss of chemokine receptor function has also been observed in developing B cells as well as during differentiation into germinal center B cells (35). Therefore, chemokines that have been shown to arrest naïve B cells (36) or attract Ag-primed B cells to the follicle (37, 38) might fail to induce retention of extrafollicular plasmablaster B cells.

We have previously demonstrated that the germinal center reaction in the follicles of MMTV-primed LNs started by days 10–12 (5). In our present study, we observed a second peak of viral infection in the draining LN 11 days after MMTV infection (Fig. 1). Viral DNA was predominantly found in follicularly differentiating B cells (B220high/MHC class IIhigh) and plasma cells (B220high/int/MHC class Iint) (Fig. 2). Interestingly, follicular differentiation of B cells was accompanied by export of MMTV infection to lymphoid organs and mucosal tissue (Fig. 4, G and H). This was even more striking in the mammary gland, in which we observed a second peak of infection by days 12–14 (Fig. 4, A and B). This strongly indicates that two independent waves of emigration of B cells appear during the primary immune response to MMTV. The first is dependent on extrafollicular B cell differentiation in the medullary cords, and the second is at the time when the germinal center reaction occurs. At later time points, all tissue of infected mice contain retroviral proviruses, and infection is maintained for life.

Once plasmablaster B cells enter the pool of recirculating lymphocytes around day 6, they migrate to a range of lymphoid and nonlymphoid organs such as the spleen, mammary gland, skin, salivary gland, and lung. The affinity of lymphoblasts to secretory tissue such as mammary and salivary glands has been reported in earlier studies (39, 40). The detection of radioactivity in lactating mammary glands after transfer of radiolabeled rat T cells into naive animals led to the conclusion that T cells play a role in maternal-to-neonatal transfer of immunity (41). However, the in vivo relevance of T cell vs B cell migration to the mammary gland has not been clarified, and studies of naive lymphocytes instead of immunoblasts, as well as cells isolated from lymph or blood instead of lymphoid organs, have to be interpreted with caution. Transfer of both T cells and B cells from MMTV-infected donors to naive recipients was found to induce infection of the mammary gland (42–44). Whether this was a result of virus shedding, a SAg response, or migration to the mammary gland has not been shown. Here we demonstrate that, in the first weeks after infection, the presence of MMTV DNA in the mammary gland is the result of cellular export of infected B cells from the draining LN and is neither from virus shedding nor recruitment of T cells to the mammary gland. Because only 0.1% of T cells in the draining PO-LN are infected 6 and 14 days after MMTV infection (Fig. 2) by virus-specific PCR, we cannot rule out migration of T cells to the mammary gland. However, we found that radiolabeled plasmablaster B cells, but not T cells, from draining PO-LN that were at day 6 adoptively transferred into naive recipient mice entered the mammary gland (42–44). Clearly, tissue-specific homing requires the interaction of adhesion receptors with the corresponding ligand. Therefore, we analyzed the expression profile of homing receptors on plasmablaster B cells from MMTV-infected LNs. In the present study, we show first that α4β7 integrin is expressed on almost all

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**Table I. Expression of adhesion molecules on naive and MMTV-activated B cells**

<table>
<thead>
<tr>
<th></th>
<th>αIEL</th>
<th>α4</th>
<th>β3</th>
<th>β1</th>
<th>β2</th>
<th>L-Selectin</th>
<th>Syndecan-1</th>
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<tbody>
<tr>
<td>Naive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmablaster</td>
<td>15.5</td>
<td>18.9</td>
<td>0</td>
<td>62.1</td>
<td>8.4</td>
<td>58.8</td>
<td>54.1</td>
</tr>
<tr>
<td>B220high</td>
<td>± 5.6</td>
<td>± 8.7</td>
<td>± 6.5</td>
<td>± 3.8</td>
<td>± 2.2</td>
<td>± 4.7</td>
<td>± 0.8</td>
</tr>
<tr>
<td>Small</td>
<td>2.6</td>
<td>10.3</td>
<td>1.6</td>
<td>19.2</td>
<td>1.1</td>
<td>59.5</td>
<td>86.1</td>
</tr>
<tr>
<td>B220high</td>
<td>± 0.9</td>
<td>± 5.9</td>
<td>± 10.1</td>
<td>± 0.3</td>
<td>± 15.5</td>
<td>± 6.1</td>
<td>± 0.2</td>
</tr>
<tr>
<td>Small</td>
<td>0.9</td>
<td>14.4</td>
<td>1.1</td>
<td>16.1</td>
<td>2.4</td>
<td>86.6</td>
<td>88.6</td>
</tr>
<tr>
<td>B220high</td>
<td>± 0.3</td>
<td>± 4.1</td>
<td>± 5.2</td>
<td>± 0.6</td>
<td>± 10</td>
<td>± 5.6</td>
<td>± 0.9</td>
</tr>
</tbody>
</table>

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Data show profiles gated on B220high/MHC class IIhigh B cells and B220low/int MHC class Iint B cells. Data are presented as the mean of three independent experiments with five animals ± SD.

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MMTV-infected plasmablast B cells and, second, that expression levels are higher on both plasmablast B cells of MMTV-infected mice compared with rare B220\(^{hi}\)MHC class II\(^{hi}\) B cells of naive control animals. Mn\(^{2+}\) treatment of cells did not enhance the efficiency of 9EG7 mAb staining, indicating that \(\alpha_4\beta_1\) heterodimer is present on MMTV-infected B cells in the activated conformation (data not shown). The loss of chemokine receptor function and the induction of activated \(\alpha_4\beta_1\) integrin molecules explain the observed migration properties. MMTV infection induced a dramatic increase in \(\alpha_4\beta_1\) coexpressed with \(\beta_1\) (Fig. 5B), whereas, in naive control mice, most plasmablast B cells were either \(\alpha_4^{-}\beta_1^{-}\) or expressed \(\beta_1\) integrin without \(\alpha_4\) (Fig. 5A). We confirmed by immunoprecipitation that both \(\alpha_4\) and \(\beta_1\) integrins were expressed on plasmablast B cells (Fig. 6). We found VCAM-1, a ligand for \(\alpha_4\beta_1\) integrin, to be expressed on mammary gland vascular endothelium, and homing of adoptively transferred plasmablast B cells to the mammary gland was dependent on \(\alpha_4\beta_1\). Our data clearly demonstrate that \(\alpha_4\beta_1\) integrin expression can be modulated by MMTV infection. Whether this is a result of viral infection or SAg-mediated T cell-B cell interaction is unclear. In different antigenic models of mice and humans, an altered expression of \(\alpha_4\beta_2\) or \(\alpha_4\beta_3\) on activated/memory B and T cells has been reported (45–48). *Mycobacterium tuberculosis* infection leads to expansion of \(\alpha_4\beta_1^{bighlep}\beta_2^{-}/\alpha_2^{-}\) cells (49), whereas staphylococcal enterotoxin B induces down-modulation of \(\alpha_4\beta_1\) on activated T cells (50), indicating an influence of the Ag stimulus on integrin expression. Elevated expression of \(\alpha_4\) integrin (CD49d) is not only correlated with a memory phenotype of T cells (51, 52) but is also dependent on the local tissue microenvironment (53) and the differentiation into either type 1 or type 2 lymphokine-secreting subsets (54). Taken together, many pathogens can induce \(\alpha_4\beta_1\) and \(\alpha_4\beta_2\) integrin expression on B and T lymphocytes, but expression levels can differ depending on cellular subsets, localizations, and Ags. Therefore, elevated levels of \(\alpha_4\beta_1\) integrin on plasmablast B cells are not a unique effect of MMTV and, as in other Ag models, can mediate entry of infected cells into tissues that express VCAM-1 on endothelial cells. \(\beta_1\) expression on B lymphocytes plays not only an essential role in homing to particular organs but also in increased cell proliferation and rescue from apoptosis, as demonstrated for T cells (55–57). Therefore, \(\alpha_4\beta_1\) expression on plasmablast B cells might play a key role in rescue from activation-induced cell death in the draining PO-LN. Additionally, these cells have elevated expression levels of \(\beta_2\) and syndecan-1. The interaction of \(\beta_2\) with VCAM-1 has been shown to increase the avidity of \(\beta_2\) (58), suggesting that migration of extrafollicular plasmablast B cells to peripheral organs is controlled by complex molecular interactions involving cross-talk between different integrins. Because plasmablast B cells have down-modulated L-selectin, the affinity for high endothelial venules in secondary lymphoid organs should be low. We were able to isolate MMTV-infected plasmablast B cells from axillary and mesenteric LNs (Fig. 3), indicating entry independent of L-selectin via afferent lymphatics. It must be emphasized that, by analyzing total LN cells 6 days after MMTV infection, viral DNA was detectable only in axillary and contralateral PO-LN but not in mesenteric LN. Therefore, trafficking of extrafollicular plasmablast B cells to secondary lymphoid organs differs in terms of quantity and may be influenced by additional parameters.

How long MMTV-infected plasmablast B cells survive in a particular environment outside the draining LN remains unclear. Studies examining the longevity of plasma cells indicate that a substantial fraction can survive for extended periods of time in bone marrow and spleen (59, 60). These cells have been considered to survive in the absence of Ag and to spontaneously secrete Abs ex vivo (61). Plasma cells that home to the bone marrow in response to the hapten (4-hydroxy-3-nitrophenyl)acetyl have been shown to be selected for high-affinity variants (6, 62). Therefore, the bone marrow seems to play a major role in affinity-driven clonal competition independent of germinal centers. This is supported by the fact that, in germinal center-deficient mice, affinity maturation can occur (63). In a recent manuscript, B220\(^{hi}\)CD138\(^{+}\) B cells have been described as one of three memory B cell subsets that can persist through at least 42 days of a secondary immune response in spleen and bone marrow and secrete specific switched isotype Ab (28). Similarly, B220\(^{-}\)CD19\(^{+}\) B cells containing hypermutated Ig genes have been isolated from peripheral blood of quasimonomoclonal mouse model (64, 65), indicating that this cell population is circulating.

What is the general consequence of survival of infected B cells for the virus? In many viral infections, B cell differentiation is critical for amplification and persistence. During EBV infection, activation to the blastoid stage is an essential step in the process of latency. Some activated B cell blasts can exit the cell cycle and reach a resting state, allowing persistence of EBV in these cells (66). We hypothesize that a similar mechanism acts in MMTV infection, allowing survival of some latently infected B cells. In this model, MMTV infection of naive B cells leads to differentiation into cycling extrafollicular B cell blasts. A proportion of these blasts exits the draining LN and enters peripheral tissue via \(\alpha_4\beta_1\), resulting in persistent infection of lymphoid and nonlymphoid tissue. It is possible that these cells exit the cell cycle and belong to a subtype of B220\(^{hi}\) memory B cells that have been described recently (28). This model is supported by the observation that adoptive transfer of MMTV-infected syngeneic plasmablast B cells in AIZT-treated recipient mice leads to a long-lasting deletion of viral SAg-reactive T cells, whereas transfer of allogeneic MMTV SAg-presenting B cells induces a reversible deletion due to rapid elimination of allogenic donor cells (our unpublished observations).

To complete the MMTV life cycle, B cell differentiation requires T cell-B cell collaboration (25, 67, 68). Both B and T cells have been demonstrated to be principally capable of shedding MMTV particles (16). However, in the first two weeks of MMTV infection, we never observed a dramatic increase of viral load in peripheral organs when mice were not treated with AIZT. This indicates that infection of peripheral organs reflects emigration of...
References


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B CELL MIGRATION AFTER RETROVIRAL INFECTION


