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Differential Molecular and Anatomical Basis for B Cell Migration into the Peritoneal Cavity and Omental Milky Spots

Simon Berberich,* Sabrina Dähne,* Angela Schippers,† Thorsten Peters,‡ Werner Müller,† Elisabeth Kremmer,§ Reinhold Förster,* and Oliver Pabst2*

The constitutive migration of B cells from the circulation into the peritoneal cavity and back is essential for peritoneal B cell homeostasis and function. However, the molecular machinery and the anatomical basis for these migratory processes have hardly been investigated. In this study, we analyze the role of integrins as well as the role of the omentum for B2 cell migration into and out of the peritoneal cavity of mice. We demonstrate that α4β1 integrin-mucosal addressin cell adhesion molecule 1 interaction enables B2 cell migration from the circulation into omental milky spots but not into the peritoneum. In contrast, α4β1 integrin mediates direct entry of B2 cells into the peritoneal cavity as well as their retention at that site, limiting B2 cell egress via the draining parathymic lymph nodes. Surgical removal of the omentum results in a 40% reduced immigration of B2 cells from the circulation into the peritoneum but does not impair B cell exit from this compartment. In conclusion, these data reveal the existence of alternative routes for B2 cell entry into the peritoneal cavity and identify integrins as key factors for peritoneal B2 cell homeostasis, mediating B2 cell migration into and out of the peritoneal cavity as well as their retention at this site. The Journal of Immunology, 2008, 180: 2196–2203.

Antibody production by adaptive immune responses requires several days for the activation, clonal expansion, and differentiation of conventional B2 cells and T cells. This vulnerable gap in the humoral response is bridged by the rapid production of natural Abs by specialized populations of B cells, including B1 cells. B1 cells are distinguished from conventional B2 cells by distinct functional properties, differentiation, phenotypes, and tissue distribution (1–4). In particular, the body cavities, which are the peritoneal and pleural cavity, contain a prominent population of B1 cells.

Besides B1 cells the peritoneal cavity (PerC) harbors a prominent population of B2 cells. Indeed, B2 cells constitute the major B cell population in the PerC of most mouse strains and represent the sole B cell subset in the body cavities of adult humans. However, the functional properties of these peritoneal B2 cells have hardly been investigated so far. Peritoneal B2 cells share some features of B1 cells in that they acquire B1 cell characteristics in response to the local environment such as expression of CD11b and the ability to produce natural IgM (5). Moreover, we have recently shown that the PerC affects the migratory properties of B2 cells: Splenic B2 cells that have been exposed to the peritoneal environment re-entered this compartment more efficiently after adoptive i.v. transfer compared with unmanipulated splenic B cells (6). Such enhanced propensity to re-enter the PerC after exposure to stimuli present in that compartment is accompanied by modulation of the chemokine receptors CXCR4 and CXCR5 and integrins. CXCR5 is instrumental for B1 as well as B2 cell entry into the PerC (6–8), whereas the importance of other molecules involved in this process has not been addressed so far.

As a port of entry for B cells into the PerC the omentum has been proposed (7). The omentum is a bilayered sheet of mesothelial cells connecting various organs including spleen (SPL) and stomach. Inside the omentum, multiple B cell follicles are present that, based on their white color, have been termed milky spots. Consistent with a function of the omentum in B cell homing into the PerC, CXCL13 is highly expressed in the milky spots (9) and CXCL13-CXCR5 signaling is essentially required for the migration of adoptively i.v. transferred B cells into the PerC (6, 7, 10). Moreover, in short-term i.v. homing experiments, B cell accumulation in the omentum precedes appearance of the cells in the PerC (7). Similarly, accumulation of B cells in the omentum accompanies exit of B cells from the PerC after LPS stimulation (9). However, no direct experimental evidence supporting a role for the omentum in either B cell entry or B cell egress has been reported so far. With regard to B cell egress, lymphatic drainage into the parathymic lymph nodes (PTLN) has been suggested as an alternative pathway for B cell exit from the PerC.

In this study, we report that the entry of B2 cells into omental milky spots and the PerC requires different sets of integrins, indicating that entry into both compartments is independent of each other. Exit of peritoneal B2 cells requires the down-modulation of α4 integrins that initiates increased appearance of B2 cells in the PTLN. Surgical removal of the omentum does not result in a detectable failure of B2 cells to exit from the PerC, indicating that the
omentum cannot be attributed a bottleneck function for B2 cells passing the PerC. Omental milky spots rather possess features of a secondary lymphoid structure, representing one of the available gateways for B2 cell circulation into and out of the PerC.

Materials and Methods

Mice

C57BL/6 and C57BL/6 expressing the EGFP³ protein under control of the β-actin promoter (11) were bred in the Central Animal Facility of Hanover Medical School under specified pathogen-free conditions or were purchased from Charles River Laboratories. β₂ integrin-deficient mice (C57BL/6 background) were a gift from K. Scharfetter-Kochanek (University of Ulm, Ulm, Germany) (12). β₇ integrin-deficient (C57BL/6 background) (13), ICAM-1-deficient (C57BL/6 background) (14), mucosal addressin cell adhesion molecule 1 (MAdCAM-1)-deficient (C57BL/6 background) and MAdCAM-1/V-CAM-1 double-deficient mice were bred at the Helmholtz Centre for Infection Research (Braunschweig, Germany). MAdCAM-1-deficient mice were generated in the laboratory of Dr. W. Müller and will be reported elsewhere. VCAM-1-deficient mice were generated by crossing mice homozygous for a loxp-flanked (floxed) Vcam1 gene (15, 16) and Mx-Cre-transgenic mice (17), resulting in a mouse in which Cre-loxP-mediated Vcam1 deletion can be induced by IFN injection. MAdCAM-1/V-CAM-1 double-deficient mice on a mixed C57BL/6 × 129 SvEv background were generated by crossing the MAdCAM-1-deficient mouse strain and the floxed VCAM-1 mouse. In the animals used for this study, deletion of the Vcam1 gene was induced by i.p. injection of 10⁶ U of IFN-α 1 day after birth. Cre-negative littermates served as a control group. All mice were used at the age of 8–12 wk. All animal experiments have been performed in accordance with institutional guidelines and have been approved by the local government.

Bone marrow chimeras

Bone marrow cells were isolated from C57BL/6 mice expressing the EGFP protein under control of the chicken β-actin promoter and purified by discontinuous Lympholyte M gradient centrifugation. C57BL/6 wild-type recipients were lethally irradiated with a single dose of 10 Gy and reconstituted by i.v. injection of 10⁷ syngenic bone marrow cells. Animals were analyzed 7–9 wk after reconstitution using a fluorescence stereomicroscope (Leica MZ 16 FA; Leica) and Leica Application Suite software.

Antibodies

The following Abs were used: anti-β₂ integrin-FITC, anti-CD23-PE (Caltag Laboratories), anti-α₇ integrin, anti-β₇ integrin, anti-L-selectin (clone PS/2), anti-CD19-allophycocyanin, anti-CD21/CD35-FITC, anti-CD31-FITC, anti-CD43-FITC, anti-CD62L-allophycocyanin, anti-MAdCAM-1, and anti-MECA-79 (BD Biosciences). Anti-β₂ integrin (clone PS/2) and anti-L-selectin (clone MEL-14) Ab was raised under standard conditions and purified by affinity chromatography. Unconjugated primary Abs were detected using Cy5- or Cy3-conjugated mouse anti-rat IgG (Jackson ImmunoResearch Laboratories). Biotinylated Abs were recognized by streptavidin coupled to PerCP (BD Biosciences).

Cell isolation and flow cytometry

Mice were sacrificed by CO₂ inhalation, and lymphoid organs were dissected and used for preparing single-cell suspensions by mincing through a 45-μm nylon mesh. Erythrocytes were lysed when necessary using a buffer containing ammonium chloride (1.7 M), potassium hydrogen carbonate (100 mM), and EDTA (1 mM). For isolation of peritoneal cells, the PerC was flushed with 10 ml of ice-cold PBS/3% FCS. Omental cells were isolated after incubation for 45 min with 1.5 mg/ml collagenase and 0.5 U/ml dispase (Roche) and mincing through nylon mesh. Cells were washed twice with PBS/3% FCS and stained with the Abs described above. Dead cells were excluded from further analysis by gating on DAPI- cells. Analysis was performed using a LSRII (BD Biosciences). Data were analyzed using WinList5.0 (Verity Software House) or FlowJo (Tree Star) software.

Whole mount microscopy

Omenta were dissected and fixed in 1% paraformaldehyde for 2 h, blocked with 5% of mouse or rat serum for 30 min, and incubated with anti-CD3-FITC, anti-B220-Cy5, and unconjugated anti-MAdCAM-1 or anti-MECA-79 Abs for at least 6 h. Specimens were washed twice for 30 min and, when appropriate, unconjugated Abs detected by Cy3-conjugated mouse anti-rat Ig. Omenta were washed twice for 30 min, refixed with 2% paraformaldehyde for 30 min, and mounted with Mowiol (Sigma-Aldrich). The whole staining protocol was performed at 4°C under continuous shaking. Slides were analyzed with a confocal laser scanning microscope (LSM 510 META; Zeiss). Images were created using Zeiss LSM5 and Imaris software (Bitplane).

Adoptive transfers

Splenocytes were labeled with CFSE or TAMRA (Molecular Probes). In brief, 2 × 10⁶ cells/ml were preincubated for 30 min in RPMI 1640 medium containing 25 nM HEPEs at 37°C. CFSE or TAMRA was added to a final concentration of 0.1 μM (CFSE) or 15 μM (TAMRA) for 10 min, followed by washing the cells in ice-cold PBS containing 3% FCS. For competitive adoptive transfer experiments, cell suspensions were adjusted to equal numbers of B cells labeled with each fluorochrome. Cell suspensions (10⁴ cells/recipient) were injected i.p. and/or i.v. into the lateral tail vein of 8- to 12-wk-old syngenic recipients. Selective effects of the fluorescent staining procedure on the migration of cells were excluded by using both combinations of CFSE- and TAMRA-labeled cells throughout.

For the in vivo neutralization of integrins and L-selecin, TAMRA-labeled splenocytes were incubated with purified anti-α₇ integrin (isotype: IgG2b, clone PS/2), anti-α₂ integrin (isotype: IgG2a, clone D4T32), or anti-L-selectin Ab (isotype: IgG2a, clone MEL-14) for 15 min at room temperature. In brief, 10⁵ cells were injected along with the Abs (anti-α₂ integrin: 325 μg/mouse; anti-α₇ integrin: 100 μg/mouse; anti-L-selectin: 100 μg/mouse) into wild-type recipients. As a control, cells were incubated with non-neutralizing isotype control Abs (IgG2a: clone 5D11D; IgG2b: clone 297-1).

In some experiments, 10³ TAMRA-labeled splenocytes were injected along with 10 μg of LPS (Sigma-Aldrich) i.p. into wild-type recipients and omenta were analyzed 2 h after transfer using confocal microscopy (see above).

Explanation of omentum

For the surgical removal of the omentum, mice were anesthetized using a mixture of ketamine and rompun. A 1-cm longitudinal incision was made in the skin and a small opening was made in the upper left quadrant of the abdomen (laparotomy). The omentum was dissected where it attaches to the most distal part of the stomach (curvature major). Sham operation consisted of abdominal incision and gentle manipulation of the omenta. The abdomen was closed in a single layer using discontinuous suture. Animals were used as adoptive transfer recipients 2 wk after surgery.

Statistics

Statistical analysis was performed on the original data with GraphPad Prism 4.0 software using the unpaired Student t test. Statistical differences for the mean values are indicated as follows: *p < 0.05; **p < 0.01; ***p < 0.001.

Results

Integrin expression on peritoneal B cells and their function in peritoneal B cell homeostasis

Integrins are a group of heterodimeric adhesion molecules that are centrally involved in the homing of lymphocytes into lymphoid tissues. Integrin function on hemopoietic cells is mediated by β₁, β₂, and β₇ integrins and the differential usage of these integrins critically contributes to the allocation of different immune cell subtypes to distinct immune compartments (18, 19). To investigate the function of integrins in the homeostasis of peritoneal B cells, we determined the expression level of integrins on peritoneal B1 and B2 cells and splenic B2 cells. β₁, β₂, β₇, and α₂ integrin were readily detected on peritoneal B1 cells as well as peritoneal and splenic B2 cells, although the different B cell subsets displayed divergent expression levels (Fig. 1A). Most evidently, peritoneal B2 cells expressed higher levels of β₂ and α₂ integrin and reduced expression of β₇ integrin compared with splenic B2 cells (Fig. 1A).

³ Abbreviations used in this paper: EGFP, enhanced GFP; MAdCAM-1, mucosal addressin cell adhesion molecule 1; MLN, mesenteric lymph node; PerC, peritoneal cavity; SPL, spleen.
Integrin expression by peritoneal B1 cells is dominated by expression of $\beta_1$ and $\alpha_4$ integrins that generally exceeds the expression levels observed on B2 cells (Fig. 1A). These results are consistent with a general role for integrins in regulating peritoneal B cell homeostasis. We next quantified B1 and B2 cells in the PerC of $\beta_2$ and $\beta_7$ integrin-deficient mice and in mice lacking the respective integrin ligands ICAM-1 and MadCAM-1. Numbers of B1 and B2 cells were generally only mildly affected in these mutants and only the number of B2 cells in the PerC of ICAM-1-deficient mice was evidently increased compared with that of wild-type controls (Fig. 1B). Because MadCAM-1 deficiency did not have any impact on the number of peritoneal B cells, we investigated the function of the $\beta_1$ integrin ligand VCAM-1 for peritoneal B cell homeostasis in MadCAM-1/Vcam-1 double-deficient mice. Because constitutive lack of VCAM-1 function is lethal, VCAM-1 was deleted postnatally by conditional gene deletion by Cre-recombinase induction. Interestingly, MadCAM-1/Vcam-1 double-deficient mice but not MadCAM-1 single-deficient controls displayed severely reduced numbers of peritoneal B1 and B2 cells (Fig. 1C), indicating that VCAM-1 might serve an essential function for homeostasis of peritoneal B cells.

**B CELL MIGRATION INTO THE PERITONEUM**

To directly explore the function of integrins for B2 cell entry into the PerC, we performed extensive adoptive transfer experiments. Splenocytes were isolated from wild-type and integrin-deficient mice and differentially labeled with fluorochromes. Mixtures of wild-type and integrin-deficient cells were adjusted to contain equal numbers of B cells and injected i.v. Sixteen to 17 h later, the homing efficiency of integrin-deficient cells into the splenic, mesenteric lymph nodes (MLN), peripheral lymph nodes, Peyer’s patches, blood, and the PerC was evaluated in comparison to wild-type cells. $\beta_1$ integrin deficiency selectively impaired B cell homing into peripheral lymph nodes and Peyer’s patches, whereas $\beta_2$ integrin deficiency reduced homing into Peyer’s patches and MLN (Fig. 2A, upper two panels). In contrast, none of both integrins affected the homing of B2 cells into the PerC. The redundancy inherent to the integrin/integrin ligand system may mask substantial contributions of distinct integrins for B cell homing. To rule out such compensatory effects, we next evaluated the homing of $\beta_2$ integrin-deficient B2 cells in recipients lacking the archetypical $\alpha_4\beta_2$ integrin ligand MadCAM-1. In these experiments, we observed that homing of $\beta_2$ integrin-deficient B2 cells into peripheral lymph nodes was further diminished in MadCAM-1 recipients (Fig. 2B, lower panel). Similarly, homing of B cells into MLN was almost completely abolished when $\beta_2$ integrin-deficient B cells were transferred into MadCAM-1-deficient recipients. These results indicate that $\beta_2$ integrin/MadCAM-1 interaction might compensate for residual homing of $\beta_2$ integrin-deficient B2 cells in wild-type recipients and vice versa. Homing of $\beta_2$ integrin-deficient B2 cells into the PerC was unaffected even in MadCAM-1-deficient recipients, suggesting that $\beta_2$ and $\beta_7$ integrins might be dispensable for homing of B2 cells into that compartment.

An analogous experimental setup was used to investigate the function of $\beta_2$ and $\beta_7$ integrins for B2 cell exit from the PerC following i.p. transfer of differentially labeled splenocytes. These experiments showed that $\beta_7$ integrin is not involved in B2 cell exit from the PerC, whereas $\beta_2$ integrin deficiency reduced the ability to leave the PerC 1.5-fold in wild-type as well as MadCAM-1-deficient recipients (Fig. 2B).

Since neither $\beta_2$ nor $\beta_7$ integrin appeared to play a major role in the migration of B cells into the PerC, we extended our studies to $\alpha_4$ integrin and L-selectin. $\alpha_4$ integrin can associate with the $\beta_1$ integrin chain to form $\alpha_4\beta_2$ heterodimers (VLA-4) and the $\beta_7$ integrin chain resulting in $\alpha_4\beta_7$ dimers (LPAM-1). $\alpha_4$ integrin and L-selectin function was impaired in vivo by neutralizing Abs (see Materials and Methods). Wild-type splenocytes were fluorescently labeled, preincubated with the neutralizing Abs or the same amount of isotype control Abs, and constant cell numbers were transferred along with neutralizing amounts of Ab into wild-type...
function for grin in retaining cells in the PerC. Because we did not observe any
2 integrin might mediate immigration of B2 cells into the PerC. Likewise, α4 integrin neutralization increased the number of i.p. transferred cells recovered into wild-type (A) or i.p. (B) wild-type mice and differentially labeled with CFSE and TAMRA. Mix
tures containing equal numbers of B cells were injected i.v. (A) or i.p. (B) into wild-type (A and B, upper two panels) or MadCAM-1-deficient mice (A and B, lower panel). Sixteen to 17 h after adoptive transfer, the ratio of integrin-deficient to wild-type B cells was determined in different compartments by flow cytometry. Filled circles represent individual recipients and horizontal bars show mean values. PLN, Peripheral lymph node; PP, Peyer’s patches; n.d., not determined.

β1 integrin and β2 integrin are dispensable for B cell homing into the peritoneal cavity. The migration efficiency of β3 integrin-deficient or β3 integrin-deficient B cells in comparison to wild-type B cells was determined by competitive adoptive transfer experiments. Splenocytes were isolated from β3 integrin-deficient or β3 integrin-deficient as well as wild-type mice and differentially labeled with CFSE and TAMRA. Mix
tures containing equal numbers of B cells were injected i.v. (A) or i.p. (B) into wild-type (A and B, upper two panels) or MadCAM-1-deficient mice (A and B, lower panel). Sixteen to 17 h after adoptive transfer, the ratio of integrin-deficient to wild-type B cells was determined in different compart
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α4β7 integrin mediates homing of B2 cells into omental milky spots
Migration of B cells into the PerC has been proposed to route through the omentum. The murine omentum is rich in B cell follicles, i.e., milky spots, and might contribute to homing of B cells into the PerC. However, experimental evidence for such function of the omentum is mostly indirect. We thus investigated the distribution of milky spots in vivo. To this end, wild-type C57BL/6 mice were lethally irradiated and reconstituted with syngeneic bone marrow constitutively expressing the GFP. Eight weeks after reconsti
tution, bone marrow chimeras were sacrificed and the distribution of fluorescent cell aggregates in the omentum and other tissues was inspected using a fluorescent stereomicroscope. Fluorescent aggregates corresponding to milky spots were strictly confined to the omentum as morphologically separate tissues, whereas no such structures were present at other sites throughout the peri
toneum (Fig. 4A).

To investigate the homing of adoptively transferred cells into the omentum, we established a method that allows analyzing the number and distribution of cells in the omentum. To this end, cells in the omentum were stained in whole mounts, i.e., in the intact tissue. Subsequently, confocal microscopy was used to reconstruct the three-dimensional architecture of the tissue and to quantify the number of transferred TAMRA+ cells within individual omental milky spots (Fig. 4B, left panel). Using this technique, we deter
ined the homing efficiency of β2 integrin-deficient B cells as well as wild-type B cells in the presence of neutralizing anti-α4 integrin, anti-α4β7 integrin, and anti-L-selectin Abs in vivo following i.v. transfer. Interestingly, genetic as well as Ab-mediated impairment of β2 integrin function inhibited the homing of B cells into omental milky spots (Fig. 4B, right panel). In contrast, inhibition of L-selectin did not decrease homing of B cells into the omentum but leads to an accumulation within that tissue. This effect might be attributed to the impaired B cell entry into peripheral lymph nodes following L-selectin blockade, resulting in overall increased numbers of B cells within the circulation.
We noticed binding of MECA-79 Ab recognizing sialy-LewisX and this process was virtually completed after 16 h. At this time point, most cells will have made stable contacts with local niches. In contrast, at later time points after transfer, most cells will have made stable contacts with local matrix, resulting in fewer cells entering the omentum despite overwhelming numbers of B cells in the PerC.

To test this hypothesis, we transferred differentially labeled splenocytes in two waves: TAMRA-labeled cells were i.p. transferred and allowed to settle in the PerC and omentum of wild-type recipients. Twenty-four hours later, CFSE-labeled cells were injected into the same recipients and the mice were sacrificed 1 day after the second transfer. Confocal microscopy of these recipients revealed that cells injected at both time points populated the omentum with the same kinetics and pattern as described for first-wave immigrants (Fig. 5A). This indicates that there is no space limitation to B cell immigration into the omentum and egress from the PerC. We therefore propose that the rapid entry of i.p. transferred cells into the omentum reflects a wave of freely “disposable” cells that did not yet adhere to local niches. In contrast, at later time points after transfer, most cells will have made stable contacts with local matrix, resulting in fewer cells entering the omentum despite overall high numbers of B cells in the PerC.

To further characterize the interrelation of B cells in the PerC and the omentum, we tracked the positioning of i.p. transferred cells in the omentum by three-dimensional reconstruction of confocal images. Fluorescently labeled, adoptively transferred splenocytes were abundantly scattered throughout the omentum 1 h after i.p. transfer without any obvious enrichment in either milky spots or along CD31-expressing vessels (Fig. 5A). Two hours after transfer, the cells started to aggregate in the region of omental milky spots and this process was virtually completed after 16 h. At this time point, the majority of all omentum-resident cells was confined to milky spots, with only a few cells scattered in the surrounding regions. Notably, at all times, virtually all transferred cells detected in the omentum expressed B220 (data not shown), suggesting that almost exclusively B cells rapidly enter milky spots from the PerC in this experimental setup. At all time points analyzed, adoptively transferred cells, by far exceeding in number the cells in the omentum, could be isolated by peritoneal lavage (data not shown). This indicates that only a minority of cells settled in the omentum, whereas the majority associated with other niches within the PerC. We therefore propose that the rapid entry of i.p. transferred cells into the omentum reflects a wave of freely “disposable” cells that did not yet adhere to local niches. In contrast, at later time points after transfer, most cells will have made stable contacts with local matrix, resulting in fewer cells entering the omentum despite overall high numbers of B cells in the PerC.

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Since B2 cell homing into the omentum after i.v. transfer depends on β7 integrin, we investigated whether this integrin would also mediate homing of B2 cells into the omentum after i.p. transfer. Quantifying the number of adoptively transferred cells in the recipients’ omentum 2 h after i.p. injection, we did not notice any difference in the frequency of wild-type and β7 integrin-deficient cells in the recipients’ omentum (data not shown). This shows that in contrast to the i.v. route the entry of cells into the omentum from the PerC but does not depend on β7 integrin.

**Surgical removal of the omentum reduces homing of i.v. transferred B cells into the PerC but does not impact accumulation of i.p. transferred cells in lymphoid organs**

The divergent role of β7 integrin for B2 cell entry into the omentum and the PerC (cf Figs. 2A and 4B) demonstrates that B2 cell entry into the PerC does not necessarily route through the omentum. We next directly addressed the function of the omentum in B cell homing into the PerC through surgical removal of that tissue.
Discussion

B2 cells constitutively egress from the PerC entering the circulation and subsequently other lymphoid compartments such as the SPL. Similarly, B cells are able to enter the peritoneum from the circulation. In this study, we analyzed the molecular and anatomical basis for these migration processes. As summarized in Fig. 7, a divergent set of integrins mediates the migration of B2 cells from the circulation into omental milky spots and into the PerC. Migration of B2 cells from the circulation into omental milky spots requires $\alpha_4\beta_1$ integrin, whereas $\alpha_4\beta_2$ integrin mediates the direct entry of B2 cells into the PerC and their retention in the peritoneum (Fig. 7).

Immigration of B cells into the PerC as well as egress from this site have been suggested to route through the omentum (7, 9). However, here we show that surgical removal of the omentum does not abolish B cell homing into the PerC nor exit from this site. Thus, the omentum cannot be attributed a bottleneck function for B2 cells entering or exiting the PerC and alternative pathways leading B cells into and out of the PerC must exist.

Accumulation of B2 cells in milky spots can be observed already 1 h after adoptive i.v. transfer (this study and Ref. 7). Thus, B cells can be observed in milky spots before detectable numbers of B cells in the PerC. This indicates that B cell entry into milky spots is a more rapid process in comparison to B cell entry into the PerC. B2 cell entry into milky spots from the circulation does not depend on L-selectin (Fig. 4B), but is strictly dependent on CXCL13 (7) and, as shown in this study, on $\beta_2$ integrin-MAdCAM-1 interaction. Consistently, CXCL13 is detectable in milky spots (9). Moreover, we observed that CXCL13 could be detected on the luminal side of MECA-79-binding venules in omental milky spots (data not shown). In addition, CD31-expressing vessels vascularizing the milky spots express MAdCAM-1 and resemble classical high endothelial venules by virtue of their MECA-79 stain (Fig. 4C). Thus, the molecular constituents enabling B cell entry into milky spots are similar to those of B cell transmigration into Peyer’s patches (20, 21).

Apart from entering milky spots from the circulation, B2 cells can also colonize this tissue from the peritoneal side. However, this process is rather scarce under constitutive conditions as most of the peritoneal B2 cells make stable contacts to the local matrix via the interaction of adhesion molecules with their ligands. Recently, mobilization of peritoneal B1 cells in response to i.p. injection of LPS has been shown. LPS triggered the down-modulation of $\alpha_4$ integrin and CD9 on peritoneal B1 cells, initiating massive egress from the PerC and accumulation of B1 cells in the omentum (9). In the current study, similar effects could be observed for B2 cells following i.p. injection of LPS or after i.p. transfer of B2 cells (Fig. 5). In both cases, numerous B cells accumulate within omental milky spots, indicating that B2 cells that do not make stable contacts to the local matrix are present in the PerC. Thus, migration of both, peritoneal B1 and B2 cells, into the omentum might be a result of freely available nonadherent B cells in the PerC. The molecular mechanisms and the rate of peritoneal B2 cell migration into milky spots are unknown. In contrast to B2 cell entry into milky spots from the circulation, $\beta_2$ integrin is dispensable for B2 cell entry from the peritoneal side and one can only speculate on a putative role for CXCL13-CXCR5 interaction in leading B2 cells into milky spots.

In conclusion the omentum receives B2 cells from two different sources: 1) Blood-borne B2 cells enter milky spots via vessels resembling high endothelial venules and 2) peritoneal B2 cells apparently crawl throughout the tissue toward milky spots. The subset composition of omental B cells reflects these different influxes: B1 cells constitute 35.0 ± 7.7% of all peritoneal B cells but only 19.6 ± 8.8% (n = 11 mice analyzed) of all omentum-resident B cells and are hardly detectable in blood. Constitutive entry of B cells into milky spots needs to be balanced by corresponding numbers of B2 cells leaving the milky spots. Although we did not investigate this process, one might speculate that B2 cells exiting from milky spots might enter the circulation and/or the PerC. Therefore, the omentum might serve as a relay station for B cells present in the circulation and the PerC (Fig. 7), allowing transit of B cells from the circulation into the PerC. However, accumulation of i.v. transferred B2 cells in the PerC is drastically delayed compared with accumulation in milky spots, indicating that such a process appears to be counteracted by the exit of omental B2 cells back into the circulation. Thus, despite the fact that the omentum may enable entry of B2 cells from the circulation into the omentum, it certainly does not represent a highway for B2 cell migration and should rather be regarded as a separate lymphoid compartment.
Our results obtained by surgical removal of the omentum as well as the divergent role of β2 integrin in mediating entry of i.v. transferred B2 cells into the omentum but not into the PerC indicate the existence of alternative routes for B2 cell entry. Neutralizing anti-α4 integrin Ab reduces the immigration of splenocytes into the PerC. α5 integrin forms heterodimers with both the β1 and the β2 integrins. Because genetic ablation of β2 integrin does not impair homing of i.v. transferred B2 cells into the PerC, this observation argues for an essential role of α4β1 integrin in B cell migration into the PerC. α4β1 integrin interacts with VCAM-1 and indeed we observed reduced numbers of peritoneal B cells in MadCAM-1/- VCAM-1 double-deficient mice but not MadCAM-1/- single-deficient mice. The anatomical site for α4β1 integrin/VCAM-1-mediated B2 cell entry is unknown and one can only speculate that postcapillary venules expressing VCAM-1 might mediate this process. Notably, VCAM-1 is not expressed constitutively in the omentum (22), further supporting the idea that α4β1 integrin-mediated B2 cell entry from the circulation into the PerC does not route through this structure. α4β1 integrin also retains B2 cells in the PerC as revealed by reduced numbers of i.p. transferred B2 cells in the PerC in the presence of neutralizing anti-α4 integrin Ab. This effect might add to the reduced appearance of B cells in the PerC after i.v. transfer. However, it is unlikely to fully account for the observed effect, because both i.p. and i.v. transfers revealed a similar degree in reduction after anti-α4 integrin Ab treatment. We thus suggest that α4 integrin-mediated adhesion of peritoneal B2 cells to local niches might limit the availability of peritoneal B2 cells for exit as well as migration into the omentum. Therefore, modulation of α4 integrin function might constitute a critical regulatory element in peritoneal B2 cell homeostasis.

Egress of B2 cells from the PerC appears to route through the draining PTLN. Indeed, filtration of i.p. injected particulate material by PTLN is known for more than one century (23) and also accumulation of i.p. injected macrophages in these lymph nodes has been described already in 1970 (24, 25). Recently, accumulation of peritoneal B cells within the PTLN could be demonstrated in FTY720-treated mice. Because FTY720 is known to impair the egress of lymphocytes from lymph nodes, this finding indicates that B cell existing from the PerC pass the PTLN (26). Likewise, i.p. transferred B cells are more frequent in the PTLN compared with other nondraining lymph nodes (Fig. 6B). However, at present, we cannot rule out alternative pathways bypassing the PTLN.

In conclusion, our data reveal that divergent molecular mechanisms mediate entry of B2 cells into the PerC and into omental milky spots. Peritoneal and ometal B2 cells exchange between both compartments but there is no linear relationship that would qualify the omentum as a major port of entry for B2 cells into the PerC. Instead, α4β1 integrin-mediated homing into the PerC and retention of B2 cells inside this compartment might represent a major mechanism controlling peritoneal B2 cell homeostasis.

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Disclosures

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