Most B cells in non-lymphoid tissues are naïve.

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Summary

The current view of lymphocyte migration states that naïve lymphocytes re-circulate between the blood and the lymph via the lymph nodes but are not able to access non-lymphoid tissues. We examined B lymphocytes in peripheral tissues and found that the majority were phenotypically similar to naïve B cells in lymphoid tissue and were located within the parenchyma, not associated with blood vessels. The mutation rate within the Vh region of these cells was substantially less than the rate attributed to somatic hypermutation and was identical to that observed in naïve B cells isolated from the lymph nodes, demonstrating the presence of naïve B cells in the non-lymphoid organs. Further, using FTY720 treated mice, we showed that naïve B cells migrate through the peripheral tissues and, using pertussis toxin, that the entry of B cells was not controlled by chemokine-mediated signalling events. Overall, these results demonstrate that naïve B lymphocytes constitute the majority of the total B cell population in non-lymphoid tissues and suggest that these cells may re-circulate through the periphery as part of their normal migration pathway. This has implications for the current view of the role of naïve B cells in priming and tolerance.

Introduction

In order to function effectively, the immune system must integrate complex signals from specialised immune microenvironments (such as the lymph nodes) and immune effector sites throughout the body. This allows the maintenance of a balance between priming and tolerance and ensures that disease states, such as autoimmunity, do not develop. It is now well established that the initial induction of priming or tolerance in naïve lymphocytes occurs in the organised lymphoid tissues and requires rapid migration of cells to these sites after antigen challenge. Therefore, an understanding of the migration patterns of naïve T and B lymphocytes is central in dissecting the complex mechanisms by which different types of immune response occur.

Early studies by Gowans and colleagues [1-2] provided the foundations for our current understanding of naïve lymphocyte migration, indicating that these cells undergo continuous recirculation between the blood and the lymph via the lymph nodes. Further studies have reinforced these findings, demonstrating that naïve lymphocytes are able to enter the organised lymphoid tissue by binding to high endothelial venules (HEVs) [3-6]. Until recently, the discovery of these mechanisms and the absence of HEVs in peripheral tissues have resulted in the commonly held belief that naïve lymphocytes are not able to access non-lymphoid tissues easily or in any substantial numbers [7-8]. Studies demonstrating heterogeneity in the expression of tissue-specific homing molecules on memory and effector (but not naïve) lymphocytes have also added weight to this argument.

In contrast to this view, a recent study in normal adult mice has suggested that a significant number of CD4+ and CD8+ T lymphocytes in non-lymphoid tissues have a naïve phenotype [9]. In addition, earlier studies have suggested that naïve T lymphocytes may be found in the

peripheral tissues in the neonate [10] and under inflammatory conditions [8, 11]. However, these and other studies demonstrating entry of phenotypically naïve T cells to non-lymphoid tissues after adoptive transfer [12-14] have been hampered by either the absence of data indicating functional naivety or the difficulties associated with presenting conclusive phenotypic data in T lymphocytes.

The literature examining naïve B cell recirculation is less comprehensive, with the more recent migration studies focusing on T lymphocytes. However, given the importance of the role that B cells play in the generation of tolerance and effector responses, patterns of naïve B cell migration clearly warrant further investigation. In addition, the phenomenon of somatic hypermutation allows the definitive separation of B cells that have (memory or effector cells) or have not (naïve cells) been exposed to antigen. The mutation rate attributable to somatic hypermutation has been estimated at approximately $1\text{-}4x10^{-3}$ per base pair (bp) per cell generation [15-16], significantly higher than the spontaneous mutation rate [17]. Therefore, sequencing experiments allow the identification of naïve and effector/memory B cells.

In this study, by phenotypic and sequence analysis of immunoglobulin hypermutation, we demonstrate that the majority of the total B lymphocytes in non-lymphoid tissues are indistinguishable from their naïve lymph node counterparts. We go on to show, with the use of FTY720, that these cells actively migrate into peripheral tissues and that chemokine-mediated signalling is not necessary for their entry. These findings challenge the established belief that naïve B lymphocytes are unable to enter non-lymphoid tissue and indicate that a review of the involvement of naïve B cells in priming and tolerance induction would be timely.

Results

B cells in peripheral tissues have a naïve phenotype

Initial studies focused on examining the phenotype of B cells in lymphoid (peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN) and spleen (SPL)) and non-lymphoid (liver (LIV), lung and intestinal lamina propria (LP)) tissues. Lymphocytes were isolated from these tissues and B cells were identified by the expression of CD19 by flow cytometry. Naïve B lymphocytes were then identified by co-expression of IgM and IgD. As expected, a high proportion (74-91%) of B lymphocytes in the lymphoid tissues had a naïve phenotype (Fig. 1A). Surprisingly, when the analysis was repeated in non-lymphoid tissues, over 50% of the total B cell population in the LP and over 60% in the liver and lung co-expressed IgM and IgD (Fig. 1A and B), suggesting that the majority of B cells in peripheral tissues are, in fact, naïve. In addition, similar results were obtained using B220 rather than CD19 to identify B cells (Fig. S1). In order to further characterise the phenotype of these cells, they were isolated from the same tissues, stained with antibodies against B220 or CD19 and IgD and then with antibodies against molecules shown to differentiate naive and memory B cells [18] or molecules associated with activation (Table S1 and Fig. S2; Table S2, Fig. S3 and Fig. S4). With the exception of CD73 in the LP, the mean fluorescent intensity (MFI) for each marker was comparable or significantly less on IgD^+ B cells from the non-lymphoid tissues when compared to IgD^+ B cells from PLN (Table S1), indicating that they were phenotypically similar to the naïve B cells present in lymphoid tissue. In order to further characterise this unique B cell population in the peripheral tissues, we stained with antibodies against other molecules commonly associated with activation (Table S2). In the liver and lung, the expression of these molecules on IgD^+ B cells was not significantly different to that

observed in the organised lymphoid tissue, again indicating that this B cell population in the peripheral tissues was phenotypically similar to the naïve B cell population in the lymph nodes and spleen. A similar pattern could be observed in the LP, although the MFI for CD11a and CD86 was significantly less and significantly greater, respectively, when compared to the organised lymphoid tissues although neither have been shown to be significantly different between naïve and memory B cell populations [18]. Overall these results indicate that a large proportion of the B cells present in non-lymphoid tissue are phenotypically identical to the naïve B cell population in organised lymphoid tissue.

Naïve B cells in non-lymphoid tissues are not associated with blood vessels

Despite perfusing the mice before harvesting the tissues, there was still a possibility that the IgD^+ B cell population isolated from the peripheral tissues was present due to blood contamination: it was important to quantify the numbers of these cells in the parenchyma of the non-lymphoid tissues. Sections of lung, intestine, liver and spleen were stained to identify $B220^+/IgD^+$ B cells and $CD31^+$ blood vessels. $B220^+/IgD^+$ B cells that were not associated with blood vessels (Fig. 2A, Fig. S5A, Fig. S6) could be identified in all tissues. An analysis of the total area of $B220^+/IgD^+$ B cell staining showed that, with the exception of the lung, in all tissues the area of $B220^+/IgD^+/CD31^-$ staining was significantly greater than the area of $B220^+/IgD^+/CD31^+$ staining (Fig. 2B), indicating that there were more $B220^+/IgD^+$ B cells present in the parenchyma of the tissues than in or associated with the blood vessels. Manual cell counting using the same images gave similar results (Fig. S7). In addition, the observed proportion of area which was positive both for B220 and IgD but not associated with CD31 was significantly greater than the area expected if the three target molecules were independently distributed in all tissues (Fig. S5B),

indicating that co-localization of $B220^+$ and IgD^+ area in the tissue parenchyma was due to genuine co-expression of these molecules by individual B cells and not due to overlap of these stains by random chance.

Sequence analysis reveals no difference between tissue resident IgM^+/IgD^+ B cells and their naïve lymph node counterparts

Despite the extensive flow-cytometric analysis indicating the presence of B cells with a naive phenotype in non-lymphoid tissues, there was still the possibility that the cells we had identified represented a memory/effector B lymphocyte population with a novel phenotype. It is well established that maturation of affinity of B cell responses is achieved by somatic hypermutation of immunoglobulin heavy and light chain genes and the selection of high affinity cells. This phenomenon allows the differentiation, by sequence analysis and enumeration of point mutations, of B cells that have (effector/memory) or have not (naïve) met antigen. If the B cells that we had so far identified in our studies were truly naïve, we would expect them to have a mutation rate similar to naïve B cells in secondary lymphoid organs and in any case far less than that expected from hypermutated immunoglobulin genes. Therefore, in order to ensure that these cells had not undergone somatic hypermutation, we extracted genomic DNA from flow-sorted $B220^+/IgM^+/IgD^+$ cells and sequenced a 1.2Kb fragment spanning the 3'-flank of rearranged V_HJ558 genes to the 3'-end of the IgH intronic enhancer [19]. We found that the mutation frequency in non-lymphoid tissues was not different from the mutation frequency of cells with the same surface phenotype from PLN (Table 1). In addition, in all non-lymphoid tissues the number of mutations per bp was $0.02\text{-}0.04 \times 10^{-3}$ (Table 1). In previous studies, the mutation rate attributed to somatic hypermutation during antigen-specific responses has been calculated at1-

$4x10^{-3}$ per cell division [15-16]. Our results show that the $B220^+/IgM^+/IgD^+$ B cells we have identified in non-lymphoid tissues had undergone substantially less mutations than B cells undergoing somatic hypermutation after a single division. This indicated that the $B220^+/IgM^+/IgD^+$ B cells present in the non-lymphoid tissues were genuinely naive. Taken together, the results from the experiments described here suggest that naïve B cells are not only present in the non-lymphoid tissues, but that they also constitute the majority of B cells in the periphery.

Naïve B cells migrate through peripheral tissues

Having established that the majority of the total B cells in non-lymphoid tissues were naïve, we went on to examine whether they represented a resident population or whether passage through the non-lymphoid tissues was part of the naïve B cell migration pathway. The primary chemokine receptor involved in the entry and retention of lymphocytes in the lymph nodes is CCR7 with its ligands, CCL21 and CCL19 present on HEVs (CCL21) and stromal cells (CCL21 and CCL19) within the T cell zones [20]. Exit from the lymph nodes is facilitated by the expression of sphingosine-1-phosphate receptor 1 ($S1P_1$) which appears to mediate its effects by overriding CCR7 retention signals [21]. Since blocking of $S1P_1$ with the immunosuppressant FTY720 prevents egress of lymphocytes from the lymph nodes, use of this molecule would allow us to establish whether peripheral tissue naïve B cells moved through the lymph nodes as part of their normal migration pathway. Therefore, mice were treated with FTY720 in their drinking water for three days before lymphocyte isolation from perfused organs and staining for CD19 or B220, IgM and IgD. As expected, total B cell numbers significantly increased in the MLN and PLN and significantly decreased in the peripheral tissues (Fig. 3A) after FTY720

treatment. When the naïve B cell population was examined alone, again as expected, the numbers significantly increased in the MLN and PLN (Fig. 3B). In all the peripheral tissues examined naïve B cell numbers significantly decreased, indicating that these cells were no longer able to follow their normal migration pathway from (presumably) the blood to the non-lymphoid tissues (Fig. 3B).

Chemokine-mediated signalling is not required to enter the liver or lung parenchyma

Chemokine signalling is critical for lymphocyte trafficking through tissues under both normal and inflamed conditions [22-23]. For example, studies have shown that the expression of the chemokine receptors CCR9 and CCR10 is necessary for lymphocyte homing to the intestine and skin, respectively [24] and, as discussed, signalling through CCR7 is necessary for lymphocyte entry into the lymph nodes. Given the findings of these studies, we wondered whether chemokine-mediated signalling might be required for lymphocyte entry into other peripheral sites such as the liver or lungs. To this end pertussis toxin (PTX) treated, CFSE-labelled B cells were transferred into recipient mice and after 2 days the mice were sacrificed and lymphocytes isolated from both lymphoid and non-lymphoid tissues. Numbers of CFSE-labeled cells were determined as a percentage of the total B cell population within each organ. As expected, in the lymph nodes, the percentage of CFSE-labeled B cells was significantly decreased in the PTX-treated mice compared to the controls (Fig. 4A and B) indicating that chemokine-mediated signaling was required for B cells to enter these tissues. However, in the liver and lung, B cell numbers were significantly increased in the PTX-treated mice relative to the controls (Fig. 4B) suggesting that, in the absence of directed lymph node migration, there is no absolute requirement for chemokine mediated signaling for B cells to access the liver and lung. A similar

pattern could be seen when absolute numbers of $CFSE^+$ cells were plotted (Fig. S8). No data was obtained for B cell migration to the LP since <5% of $B220^+$ cells expressed CCR9. We would, therefore, expect few cells to migrate efficiently to the intestine, whether PTX treated or otherwise.

Discussion

The data presented here suggests that, when examined by phenotypic and sequence analysis, the majority of the B cells found in non-lymphoid tissues are similar to the naïve B cells in organised lymphoid tissue. There are two possible explanations for this: either the naïve B cell population in lymphoid tissue is not truly naive or naïve B cells constitute a large proportion of the B lymphocytes in peripheral tissue. Given the numerous studies performed using naive B cells from lymphoid tissue, the former explanation seems unlikely. The other possibility, that the majority of B cells in the non-lymphoid tissues are in fact naïve, is in conflict with the established models of lymphocyte recirculation.

The use of techniques for analysis of surface phenotype to identify populations of memory and naïve B cells is well established [18, 25] and we were unable to differentiate between naïve B cells from non-lymphoid and organised lymphoid tissue using this method. The exception to this was CD62L expression which decreased on naïve B cells in the SPL, LIV and LP. This is in contrast to what has been shown to occur on memory B cells, in that CD62L is present at higher levels. Why naïve B cells express lower levels of CD62L in these tissues is unclear; CD62L is known to be shed upon engagement [26] and this molecule may be playing a role in either extravasation into the underlying tissue, or in the migration of B cells within the tissue. In the intestinal LP, the picture was a little less clear than in the lung and liver, since levels of

expression of both CD73 and CD86 were higher when compared to cells from lymphoid tissue. Other leukocytes from the intestine have an unusual phenotype and function [27-28], so it is possible that intestinal B cells may also be phenotypically different to naïve B cells from the systemic immune system. For example, the expression of the ectoenzyme CD73 [29] by these cells may contribute to the regulated immune environment present in normal intestine.

Although phenotypic analysis is a useful first step to screen for the presence of naïve lymphocytes in non-lymphoid tissue, the results obtained using this method are not conclusive. Therefore, we used somatic hypermutation as a more robust method of distinguishing between naïve and memory/effector B cells [19]. Our results show that the mutation frequency in the B cells from the non-lymphoid tissues is no different from that in the PLN. It is possible that the cells we have identified represent a subset of B cells that have encountered antigen in a T-cell independent manner without undergoing somatic hypermutation. For example, B1b cells have been shown to expand in a T-cell independent manner in response to microbial stimulation [30]. However, somatic hypermutation has been shown to occur in the absence of T cell help [31] and, specifically, somatic hypermutation has been demonstrated in B1b cells [32]. In addition, both B1a and B1b cells can be characterised by their low levels of expression of B220 and IgD [32]. The B cells identified in our work have comparable levels of expression of these molecules with the naïve population present in organised lymphoid tissue. In addition, B220 and IgD are both progressively down regulated during plasma cell development so that mature plasma cells are negative for the surface expression of both molecules [33], although the possibility remains that some of the cells we observed were very immature plasma cells.

Having established that the IgM^+/IgD^+ B cells in the non-lymphoid tissues were most likely to constitute a population of naïve cells, we went on to examine how these lymphocytes might be getting in to the tissues. The observation that FTY720 treatment depleted these cells from the non-lymphoid organs indicates that they are also functionally naïve; it is well established that this treatment prevents the movement of naïve lymphocytes out of the lymph nodes [21]. We propose, therefore, that naive B cells re-circulate through the peripheral tissues as part of their normal migration pathway. Whilst it is possible that these cells undergo apoptotic events within peripheral tissues instead, this seems unlikely given that the PTX experiments indicate that under normal circumstances they would be trafficking through to the lymph nodes. However, we accept that the studies presented here do not definitively demonstrate re-circulation of these cells through the tissues.

If, as we propose, naïve B cells do in fact re-circulate through the non-lymphoid tissues, it is important to consider how they gain access to these sites. Our preliminary experiments using PTX indicate that B cells do not use chemokine receptor-ligand interactions to achieve this. This is in concordance with previous studies suggesting that naïve T cells do not utilise these pathways [9]. It seems likely, therefore, that other, G-αi-independent, signalling allows access into the peripheral tissues. For example, it is possible that expression of the lower affinity conformation of LFA-1 by naïve B cells would allow extravasation from the vasculature by a G-αi-independent, chemokine-independent mechanism.

Having established that a large proportion of the total B cells in the peripheral tissues are naïve, a question not addressed in this study is why these cells would migrate through non-lymphoid tissues. One possibility is the regulation of autoreactive T cells directly in the local tissues [34].

In addition, naïve B cells produce IL-10 upon TLR [35], but not BCR, ligation [36]. Thus, there may be a role for naïve B cells in direct regulation of immune responses in the intestine. Other studies have shown resting splenic B cells to be capable of expanding the regulatory T cell population through production of TGF-β3. This ability is reduced upon activation [37]. Another possible role for naïve B cells in peripheral tissues, therefore, may be the maintenance of local tolerance by supporting the regulatory T cell population. In either case, the migration of naïve B cells through the peripheral tissues will ensure that they come into contact with foreign and tissue-specific autoantigens outside of the lymph nodes. Given the specialised microenvironments in organised lymphoid tissues, the outcome of antigen binding in the peripheral tissues via the BCR may be somewhat different to the outcome of activation in the lymph nodes.

The evidence presented in this study suggests that naïve B cells migrate through non-lymphoid tissues and, in fact, make up the majority of the B cell population in these organs. These findings, combined with the results from previous studies that have indicated that naïve T lymphocytes may also access the peripheral tissues [8-11] suggest that the established paradigm of naïve and memory lymphocyte recirculation may need to be redefined. Clearly, further studies are required. Certainly, the finding that the majority of B lymphocytes in non-lymphoid tissues are naïve seems likely to be functionally significant and future studies based around this hypothesis may shed light on the role that the non-lymphoid tissues may play in modulation of immune responses.

Methods

Mice

Female C57BL/6 mice, aged 6-10 weeks were purchased from Harlan UK and kept on site under specific pathogen-free conditions.

FTY720

FTY720 (Cayman Europe) was dissolved in PBS and administered to mice in their drinking water daily at 2 μg/ml for three days prior to sacrifice. Mice were also given an initial dose of 6 μg FTY720 by gavage prior to placing them on the treated water.

Pertussis toxin (PTX), CFSE and adoptive transfer

For PTX treatment, splenocytes were resuspended in PBS at 10 x 10^6 cells/ml and incubated with (or without) 20 ng/ml PTX (Sigma) for 10 minutes at 37°C. At the same time, the cells were labelled with 5 mM CFSE (Molecular Probes), washed once in RPMI containing 10% FBS (Invitrogen), and a further two times in PBS prior to injecting 15 x 10^6 cells IV into recipient mice. Two days later mice were sacrificed and cells isolated from various organs. Transferred $CFSE^+$ B cells were identified using CD19 and CFSE. Total recovered $CFSE^+$ $CD19^+$ B cells were determined by multiplying the total cell count by the percentage of $CFSE^+$ $CD19^+$ cells obtained by flow cytometry.

Lymphocyte isolation and flow cytometry

Lymphocytes from the various tissues were isolated as previously described [9]. Briefly, single cell suspensions from PLN, MLN and Spleen were made by mashing the tissues between frosted glass slides. For liver and lung tissue, the organs were first cut into small pieces. These organs (PLN, MLN, spleen, liver and lungs) were then incubated in HBSS containing 1.3mM EDTA for

20 minutes. The tissues were then incubated in a collagenase solution (RPMI/5% FBS/1mM $MgCL_2$/1mM $CaCl_2$/150 U/ml collagenase) for 30 minutes. After incubation, the tissues were then forced through a 40mm mesh. PLN, MLN and spleen were then kept on ice until needed. Liver and lung suspensions were spun down, resuspended in 67% Percoll solution (23% RPMI and 67% Percol, v/v), underlayed beneath a 44% Percoll solution and spun at 2000 rpm for 20 minutes. Cells at the interface were taken, washed and kept on ice until needed. For isolation of lamina propria cells, the small intestines of mice were first flushed through with HBSS before cutting the intestine into small pieces. These pieces were then washed three times in CMF solution (1X HBSS/1X HEPES Bicarbonate/2% FBS) followed by incubation with DTE solution (CMF/10% FBS/154mg dithioerithritol (DTE) per ml) for 20 minutes. This was performed three times, discarding the supernatant after each incubation. Gut pieces were then treated as with the lung and liver tissues. Following isolation, lymphocytes were stained with a cocktail of antibodies containing unlabelled anti-CD16/32 (2.4G2), anti-IgD-FITC (11-26c.2a), anti-B220-PerCP/Cy5.5 (RA3-6B2) and one of the following: PE-labelled anti-CD11a (2D7), -CD40 (3/23), -CD44 (IM7), -CD69 (H1.2F3), -CD62L (MEL-14), -CD73 (TY/23), -CD80 (16-10A1), -CD86 (GL1), -CD95 (Jo2), -anti-MHC Class II (AF6-120.1), -anti-CCR9 (clone 242503, RnD Systems) or biotinylated anti-IgM (R6-60.2) followed by Streptavidin-PE (eBioscience). Unless otherwise stated, all antibodies were purchased from BD Pharmingen. For some experiments anti-CD19 (1D3) was used instead of anti-B220. Cells were collected on an EPICS-MCL (Beckman Coulter) and analysed using FlowJo software (Treestar). For cell sorting, lymphocytes were stained with unlabelled antiCD16/32, FITC-labelled anti-IgD, biotinylated

anti-IgM followed by PE-labelled Streptavidin and PerCP/Cy5.5-labelled anti-B220. Cells were sorted on a FACS Vantage (Becton Dickinson) and were routinely ≥ 98% pure.

Immunohistology and image analysis

Whole organs from perfused mice were embedded in OCT (Tissue TEK, BDH) medium and snap frozen in isopentane pre-cooled to -70°C. To ensure architectural integrity, the lungs and small intestine were inflated with 1 ml 30% OCT medium in PBS following perfusion, before snap freezing and sectioning. 10% rat serum in PBS was applied to each section for 1 hour at room temperature before staining. Tissues were stained with pre-titrated FITC-labelled anti-IgD and PE-labelled anti-B220 and, for some experiments, biotinylated anti-CD31 (clone MEC13.3) (all from BD Pharmingen). Biotinylated anti-CD31 was detected with Streptavidin-AMCA (Vector Laboratories) or Streptavidin-AlexaFluor 633 (Invitrogen). The slides were mounted using Vectashield (Vector Laboratories) with or without DAPI. Negative control slides were prepared in conjunction with each positively stained slide. Between 6 and 10 grey scale images per tissue of each stain from each mouse were captured using a Leica DMR-A fluorescence microscope (Leica) fitted with appropriate single-color filters and a Hamamatsu Orca-ER camera (Hamamatsu) and *Q-fluoro* software (Leica). Images were analysed as described previously using *ImageJ* [38]. Briefly, a region of interest (ROI) was identified for each image (e.g. ROIs in the intestine included the mucosa and excluded the epithelium and unstained area outside the tissue) and levels of background staining (threshold levels) in all colour channels were obtained from negative control slides. These were then applied to the positively stained images so that all pixels with intensity above threshold were counted as positive and all those below were counted as negative. The proportion of area of each single colour or combination of colours within the

image was calculated as described [38-39]. Preferential co-localization of fluorochromes was determined by comparisons of the observed proportional area of each colour combination with those predicted from a null hypothesis that each element of positive staining (e.g. B220+ and CD31+) was randomly and independently distributed (the "expected" area of staining [38]). In addition, manual cell counts of $B220^{+}IgD^{+}$ cells were carried out for each image.

Sequencing

Genomic DNA was extracted from 10^5 sorted cells as per manufacturers guidelines (Machery Nagel). An initial PCR was performed to amplify an ~1.2 Kb fragment spanning the J-C intron of V_HJ558 family members, using primers specific for V_HJ558 (GGA ATT CGC CTG ACA TCT GAG GAC TCT GC) and a sequence located at the 3' end of the IgH intronic enhancer (GAC TAG TCC TCT CCA GTT TCG GCT GAA TCC) [19]. PCR conditions consisted of one cycle at 95°C, 35 cycles of 95°C for 15 seconds, 59°C for 30 seconds and 72°C for two minutes, followed by a single cycle of 72°C for 10 minutes. No product could be visualised after a first round cycle, so 1 μl of the first round PCR reaction was used as template in a second round PCR reaction, using an internal 5' primer (JH4 - TAT GCT ATG GAC TAC TGG) and the above primer located at the 3' end of the IgH intronic enhancer. PCR products were then cloned into the pGEM-T vector (Invitrogen) and competent E.coli DH5α (Invitrogen) transformed. Colonies containing the insert were grown overnight and plasmid mini-preps (Qiagen) obtained. Plasmids were either sequenced directly, or subjected to a further round of PCR (25 cycles). In this case, positive PCR products were cleaned up (Machery Nagel) and then sequenced. Sequencing using the JH4 primer was performed by The Sequencing Service (University of Dundee, UK) using HotStarTaq DNA Polymerase (Qiagen).

Statistics

All statistical tests were performed using SPSS. For the immunohistology results, the area of positive staining was calculated for each staining combination and the data were compared using a Wilcoxon signed ranks test. Observed areas of staining were compared to expected areas using a Wilcoxon signed ranks test. Manual cell counts of $B220^{+}IgD^{+}$ cells in the tissue parenchyma and associated with blood vessels were also compared using a Wilcoxon signed ranks test. For all other results, data was tested for normal distribution using an F-test and was analysed for statistical significance using ANOVA.

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Conflict of interest.

The authors have no conflict of interest to declare.

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Figure Legends

Figure 1. B cells in peripheral tissues have a naïve phenotype.

Lymphocytes were isolated from the indicated organs of perfused, uninfected mice and stained with antibodies to CD19, IgM and IgD. (A) Representative plots are gated (CD19+) B cells. Numbers within each plot represent the percentage of gated B cells falling into each indicated quadrant. (B) Collated data show the percent of B cells within the whole lymphocyte population (open bars) and the proportion of those B cells co-expressing IgM and IgD (closed bars). n = 5-9 different experiments, with 2-4 organs combined per experiment for lung, liver and LP. Error bars represent the SD.

Figure 2. Naïve B cells present in non-lymphoid tissues are not associated with blood vessels.

(A) $B220^+$ IgD^+ cells (indicated by arrows) within the parenchymal tissues of the indicated organs. B220 (red), IgD (green) and CD31 (blue). Scale bar in the top left hand corner represents 10μm. (B) With the exception of the lung, there was significantly more $B220^+/IgD^+/CD31^-$ area than $B220^+/IgD^+/CD31^+$ area in all tissues ($B220^+/IgD^+/CD31^-$ area, black diamonds; $B220^+$/IgD+/$CD31^+$ area, open triangles). Data were compared using a Wilcoxon Signed Ranks test. SPL * $p=0.08 \times 10^{-6}$; LIV * $p=0.02 \times 10^{-5}$; LP * p=0.01; LUNG p=0.149. Each data point represents one image (40 images from LIV, 25 images from LUNG and 35 images from LP). LIV and LP taken from 4 mice, LUNG taken from 3 mice. Prior to thresholding, the analysis was carried out on unmanipulated images.

Figure 3. Naïve B cells migrate through peripheral tissues.

Mice were treated with FTY720 in their drinking water for three days after which lymphocytes were isolated from the perfused organs and stained for CD19 or B220, IgM and IgD. Data represents the absolute number of B cells (A) or the absolute number of IgM^+/IgD^+ cells (B) in treated (open bars) or untreated (closed bars) controls. n>8 mice for both treated and untreated groups. Error bars represent SD. Data were tested for normal distribution using an F-test and then compared using ANOVA. *, $p<0.05$; **, $p<0.005$.

Figure 4. Chemokine-mediated signalling by B cells is not required to enter the liver or lung parenchyma.

MACS-enriched B cells were CFSE-labeled and incubated without or with PTX prior to injecting 15×10^6 cells into recipient mice. Lymphocytes from perfused organs were isolated two days later. Very few transferred cells, treated or otherwise, could be isolated from LP. (A) Representative flow cytometry histograms showing CFSE labelled B cells in organised lymphoid tissues, liver and lung after adoptive cell transfer without (top row) and with (bottom row) PTX treatment. (B) Transferred CFSE-labeled cells incubated without (closed bars) or with (open bars) PTX represented as a percentage of the total B cell population in the given organ. Error bars represent the SD. Data were tested for normal distribution using an F-test and then compared using ANOVA. Asterisks represent significance compared to the untreated control (*, $p<0.05$; **, $p=0.05$); n=4 for treated and untreated groups.

Table legends.

Table 1. Sequence analysis reveals no difference between tissue resident IgM^+/IgD^+ B cells and their naïve lymph node counterparts.

Data show the number of sequences obtained for each tissue, the percent of sequences with less than three mutations across the 500 bp fragment, and the number of mutations per 100 bp +/- SD. Data were tested for normal distribution using an F-test and then compared using ANOVA. No significant difference was observed between sequences obtained from PLN versus any other tissue. 1-2 x 10^5 sorted cells were obtained from the combined tissues of two mice (for PLN, Liver and Lung) or eight mice (for LP).

Table 1.

	PLN	Lung	Liver	LP
Number sequences	18	17	11	20
% with < 3 mutations	89	94	82	90
Mutations/100 bp	0.2 +/- 0.3	0.2 +/- .03	0.4 +/- 0.3	0.3 +/-0.2