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B-1-cell subpopulations contribute differently to gut immunity

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B-1 cell subpopulations contribute differently to gut immunity

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Abbreviations: BM, bone marrow; GC, germinal center; LP, lamina propria; NT, non transgenic; PC, plasma cell; PEC, peritoneal cavity; PP, Peyer's patches; SC, single cell; SHM, somatic hypermutation

Summary

In mice, B-1 (B1a/B1b) cells are mainly located in the peritoneal cavity. B-1 cells are well known for their role in the early stages of antibody-mediated immune responses against pathogenic invasion as well as for the production of natural IgM antibodies. Although such B cells have been claimed to give rise to intestinal plasma cells producing IgA, a clear role of B-1 cells in IgA production in the gut-associated tissues is still not defined. Here, we employed the transgenic L2 mouse model characterized by the lack of B-2 cells and presence of B-1 cells as major B-cell subpopulation. The oligoclonality of the antibody repertoire in this mouse allowed us to take typical B1a cell VH sequences as indicators of the presence of IgM-producing B-1a cells in Peyer's patches as well as in lamina propria (LP). However, amongst the IgAVH sequences recovered from the same tissues, none of the sequences showed B1a-cell specificity. Interestingly, all IgAVH sequences derived from the LP of L2 mice displayed extensive numbers of nucleotide exchanges, indicating somatic hyper mutation and affinity maturation. This suggests that the contribution of natural unmutated IgA by B-1a cells to intestinal immunity is negligible.

Introduction

Immunoglobulin A (IgA) is the major class of antibody present in mucosal tissues of mammals. IgA constitutes a key defense mechanism against invasion by inhaled or ingested pathogens. IgA is also found at significant concentrations in the serum of many species, where it mediates the elimination of pathogens that have breached the mucosa [1].

Among the various mucosa-associated lymphoid tissues (MALTs), IgA-producing cells are present in highest numbers in gut-associated lymphoid tissues (GALTs) constituted by Peyer's patches (PPs), isolated lymphoid follicles (ILFs) and solitary intestinal lymphoid tissue (SILT) [2, 3]. B cells, present in the B-cell follicles of such organized structures, form germinal centers (GCs) upon antigen encounter. Under the influence of various cellular and molecular factors - T cells and cytokines for instance - these B cells are thought to switch from IgM to IgA [4].

Multiple pathways of IgA induction have been reported. The T-cell dependent (TD) pathway of IgA induction is well known and, it is clear that IgA can also be induced in T-cell independent (TI) manner (e.g. in T-cell deficient mice, $CD40^{-/-}$ mice, $CD28^{-/-}$ mice etc.) [5-7]. However, the precise contribution of TD and TI pathways to IgA production is not known.

An important contribution of B-1 cells to TI responses has been suggested [6, 8, 9]. Peritoneal cavity (PEC) B-1 cells have also been claimed to contribute significantly to IgA-producing plasma cell (PC) pool in the lamina propria (LP) of the gut [6, 10, 11]. Importantly, according to phenotype, origin and function, PEC B-1 cells can be divided into B-1a and B-1b subpopulations [12, 13]. Phenotypically, B-1a cells are characterized as $B220^{lo}CD19^{hi}IgM^{hi}IgD^{lo}CD43^{+}Mac\text{-}1^{+}CD5^{int}$. B-1b cells share all the aforementioned markers with B-1a cells except CD5. With respect to differential contribution of these two B-1-cell

subtypes to IgA production, recently, we could show that most of the IgA-secreting cells in the PEC of unmanipulated mice belonged to B-1b-cell subpopulation [14]. Additionally, IgA derived VH regions from B-1b cells also contained frequent single nucleotide exchanges indicative of somatic hyper mutation (SHM) [14]. Thus, a contribution of PEC B-1b cells to IgA production and hence to gut-associated immunity is quite likely. However, in spite of evidential suggestions for the contribution of B-1 cells to the intestinal PC pool, their contribution under non-manipulated conditions remains uncertain. This is partly due to the lack of appropriate markers to distinguish PCs according to their origin. In this context, a sequence based approach to address this question should become very useful.

In the present work, to investigate the participation of PEC B-1 cells in the gut-associated IgA production we have made use of the transgenic mouse model known as the L2 mouse line [15]. Mice of this line are characterized by the expression of a transgenic λ light chain obtained from the plasmacytoma MOPC315 ($\lambda 2^{315}$). PEC of these mice contains almost exclusively $CD5^+$ B-1a cells, while the development of conventional B-2 cells in bone marrow (BM) is inhibited [15]. MZ B cells are also present in the spleen of L2 mice in addition to B-1a cells [16].

The sequencing of transcribed heavy chains from PEC B-1a cell pool of L2 mice showed pronounced oligoclonality. In addition, some repertoire dominating sequences could be found repeatedly in different mice [17, 18]. Thus, a substantial fraction of B-1a cells expresses identical BCRs on their surface providing an excellent molecular marker for such cells. This particular trait of L2 mice was used here to establish the presence of B-1 cells in gut-associated tissues of L2 mice.

Results

The number of B cells in the PPs of L2 mice is reduced

After their development in BM, conventional B cells migrate to secondary lymphoid organs for further maturation in B-cell follicles. However, due to blocked development of B cells in the BM, L2 mice show considerable reduction in the number and percentage of B cells in spleen and lymph nodes [17]. Consistently, in comparison to non-transgenic (NT) littermates, the percentage of total B cells ($CD19^+$) in the PPs of L2 mice was reduced by four- to five-fold (Fig. 1A). In terms of absolute numbers of B cells in the PPs of these mice, a 30-40 fold reduction was observed while total cellularity as well as the overall size of the PPs was also reduced in comparison to NT controls (Fig. 1B). Additionally, the percentage of IgM expressing B cells was approximately six-fold lower (Fig.1A and 1B). Notably, approximately 70% of total B cells in the PPs of L2 mice were observed to be positive for the transgenic λ light chain (data not shown).

Normally, B-1 cells constitute a very small percentage of B cells in the PPs of mice. In contrast, B-1 cells ($CD19^+CD43^+$) constituted 40% of total B cells in PPs of L2 mice (Fig. 1A). However, in comparison with NT mice, the absolute number of B-1 cells was significantly reduced (Fig. 1B). B-1a cells ($CD5^+CD19^+$) accounted for 28% of total B cells in PP of L2 mice (Fig. 1A) indicating that the remaining $CD19^+CD43^+$ B cells might be B-1b cells.

In contrast to the perturbed B-cell compartment in the PP of L2 mice, the percentage of T cells ($CD19^-CD5^+$) showed a relative increase by approximately three-fold (Fig. 1A) although the absolute number of T cells was decreased significantly (Fig. 1B). BM chimera study done after adoptive transfer of a mixture of bone marrow from L2 mice and normal congenic Thy1.1 mice showed a normal development of T cells of L2 origin (Supporting Information Fig. 1A-D).

Germinal centers are formed in the PPs of L2 mice

B-2 cells represent the major subset of B cells recruited into the GCs of GALT in normal mice and humans [19]. On the other hand, B-1 cells are present at very low percentages in the PPs of mice [20]. Hence, to investigate the effect of B-2 cell deficiency on the GC formation, cell preparations from PPs of L2 mice were stained with peanut agglutinin (PNA), which binds to germinal center B cells [21]. A significant percentage of $CD19^{+}PNA^{+}$ B cells were observed in the PPs of such mice (Fig. 1A). IgA-positive B cells constituted a major proportion of such GC B cells (Fig. 1A). Comparatively, a very small fraction of IgM^{+} B cells was found to be positive for PNA (Fig. 1A). This is consistent with the finding by Casola and Rajewsky [19], who reported the occasional presence of GCs in the PPs of LMP2A knock-in mice with B-1 cells as the predominant B-cell population.

Histological sections of PPs of L2 mice showed an anatomy that was comparable with that of NT mice (Fig. 1B). However, consistent with flow cytometric analysis, the number of IgM^{+} cells appeared to be reduced in the follicular region (Fig. 1A and 1B).

Peritoneal B1a cell-associated specificities are found in the PP of L2 mice

PEC B-1 cells have been claimed to migrate to LP where they take part in IgA production [6, 22, 23]. Do they also migrate to PPs? If yes, then there should be some overlap between the repertoires of B-1 cells from these two compartments. To investigate the possible commonality, previously established PEC B-1a-cell ($IgM^{+}CD5^{+}$)-associated IgVH sequences of L2 mice [17, 18] were used as molecular markers to track the presence of such B cells in the PP of L2 mice.

Using this concept, IgMVH sequences derived from bulk sorted GC B cells ($CD19^{+}PNA^{Hi/Int}$) from PPs of L2 mice were analyzed. Out of 43 such IgMVH sequences, four were found to be identical (100% CDR3 region nucleotide sequence match) to already known (a dataset of around 800 sequences) PEC B-1a cell derived IgMVH sequences of L2 mice (Table 1, Sort I). One of

these sequences (PP1) was among the dominantly found PEC B-1a-cell-derived IgMVH sequences [17].

In addition to IgMVH sequences derived from germinal centre B cells, the IgMVH sequences derived from non germinal centre transgenic B cells ($\kappa^-IgM^+PNA^-$) from the PP of L2 mice were also examined independently. To determine the correct frequency of each B-cell-derived sequence, the IgMVH sequences from single cells (SCs) were analyzed by applying SC RT-PCR. Among 29 of such sequences, 2 were found to be identical to already known B-1a-cell sequences from PEC of L2 mice (Table 1, Sort II). Again, one of these sequences (PP1.2) could be attributed to the sequence found most frequently amongst PEC B-1a-cell-derived IgMVH sequences of L2 mice [17]. All these sequences, which were found to be identical to PEC B-1a-derived sequences, lacked the presence of N/P nucleotides at the V-D/D-J junctions, (Table 1) representing another characteristic of many B-1-cell-derived VH sequences.

On the other hand, analysis of 17, 9 and 17 IgAVH sequences derived respectively from bulk sorted GC ($CD19^+$ PNA^+)/non GC ($CD19^+$ PNA^-) or SC sorted GC ($\kappa^-IgA^+PNA^+$) PP B cells of L2 mice showed a complete absence of any overlap (100% CDR3 region nucleotide sequence match) with PEC B-1a cell associated sequences (data not shown). All these IgAVH sequences contained N/P nucleotides at V-D/D-J junctions and were found to be very heterogeneous (data not shown) suggesting a high variability among these specificities indicative for a strong selection.

IgA-expressing B cells are reduced in the LP of L2 mice

LP is considered the main effecter site where the final differentiation of IgA^+ B cells to IgA-secreting PCs takes place [24]. BM-derived B-2 as well as PEC-derived B-1 cells have been suggested to be the origin of these IgA-producing plasma B cells [6]. To study the status of the B-

cell compartment in LP under conditions favorable to B1 cells, as in the case of L2 mice, the B-cell population of this compartment was analyzed by flow cytometry. In comparison with NT littermates, the percentage of total B cells ($B220^+$), showed a five- to six-fold decrease in the LP of L2 mice (Fig. 2A). The B-1a type of B cells ($CD5^+B220^+$) constituted nearly 50% of all such B cells (Fig. 2A). On the other hand, the relative percentage of LP T cells had increased by four-fold.

Intracellular staining with anti-IgA showed the presence of a significant percentage of IgA-expressing cells in the LP of L2 mice (Fig. 2A). Most of such LP IgA^+ cells in the LP had a PC phenotype ($CD19^-IgA^+$).

A direct effect of the reduction in numbers of B lineage cells was observed. Reduced levels of serum IgM and IgG were detected in L2 mice [15]. Quite consistent with that, in comparison with NT controls, intestinal wash-outs of L2 mice showed approximately three- to four-fold reduction in the amount of secretory IgA (Fig. 2B). Most of this intestinal IgA was associated with λ light chain indicating the presence of transgenic IgA-producing PCs in the LP of L2 mice (data not shown). In addition to intestinal IgA, serum IgA level was also reduced significantly in L2 mice in comparison with that of NT controls (Fig. 2B).

Reduction in the levels of intestinal IgA could be well correlated with the reduced numbers of IgA-secreting PCs in the LP of L2 mice. As determined by ELISPOT assay, the number of IgA-secreting PCs was reduced by approximately three-fold in comparison with that of NT controls (Fig. 2B). Furthermore, immunohistological staining of intestinal sections of L2 as well as NT littermates with anti-mouse IgA showed the presence of a reduced number of IgA-positive PCs in the LP of L2 mice in comparison with NT controls (Fig. 2C).

Life span of IgA^+ cells in the LP of L2 mice

Reduced number of IgA-producing B cells in the LP led to the speculation that B-1-cell-derived PCs might be short lived. To test this hypothesis, mice were fed with BrdU via drinking water. Intestinal sections of the BrdU fed mice stained with anti- BrdU and anti-mouse IgA showed comparable levels of $BrdU^{+}IgA^{+}$ cells in the LP of L2 and NT mice after one and six weeks of BrdU feeding (Fig. 3). This result excludes the possibility of a short life span being the reason for the reduced number of IgA-producing PCs in the LP of L2 mice.

Presence of B-1a-cell-specific sequences in the LP of L2 mice

The presence of B-1a-cell-associated specificities in the PP of L2 mice was suggestive of these B cells being present in LP as well and serving as precursors for IgA-producing B cells. To investigate that possibility, IgMVH sequences derived from bulk sorted transgenic B cells ($CD19^{+}IgM^{+}\kappa^{-}$) from the LP of L2 mice were analyzed. Out of these 35 analyzed sequences, two were identical to already known PEC B1a-cell-derived IgMVH sequences from the PEC of L2 mice (Table 1, Sort III). One (LP1) of these two sequences had been observed among IgMVH sequences derived from PP B cells of L 2 mice as well (Table 1, Sort III).

SC RT-PCR was also employed. In addition to determining the frequency of IgMVH sequences, it was intended to investigate the possible overlap of these specificities among LP, PP and PEC from the same pool of L2 mice. Thus, the IgMVH sequences derived from sorted $\kappa^{-}IgM^{+}CD19^{+}$ SCs of LP origin, $\kappa^{-}IgM^{+}PNA^{-}$ SCs of PP origin and $\kappa^{-}IgM^{+}CD19^{+}$ SCs of PEC origin from the same pool of L2 mice were compared. Analysis of 20, 25, and 29 IgMVH sequences derived from LP, PP and PEC B cells respectively, showed the presence of one sequence which was common to all the three compartments (Fig. 4). Though some of the SC derived sequences from PEC and PP were found repeatedly, each of LP derived sequences was unique.

None of the IgAVH sequences derived from bulk sorted $\kappa^{-}CD19^{+}IgA^{+}$ LP B cells was found to be identical to B-1-cell-associated specificities from L2 mice though one out of a total of 32 LP IgAVH sequences matched with IgMVH sequences derived from PP (data not shown).

B-1b-cell-specific sequences in the LP of L2 mice

B-1a cells ($CD5^{+}CD19^{hi}$) constitute around 90% of total B cells in the PEC of L2 mice [25]. The remaining ones can be considered to be B1b cells as almost all of the B cells are $CD19^{hi}CD43^{+/-}$ [25]. Keeping this in mind, IgMVH sequences derived from bulk sorted $\kappa^{-}CD5^{-}IgM^{+}$ B-1b cells from the PEC of L2 mice were analyzed to generate a data set that can be used for comparison. Comparison of 63 of these sequences with 56 LP derived IgMVH sequences showed the presence of 3 sequences which were found to be present in both compartments (Table 1, Sort IV). Interestingly, a few among the PEC B-1b-cell-derived IgMVH sequences also showed single nucleotide exchanges characteristic for SHM and out of 63 IgMVH sequences only one lacked N/P nucleotides at V-D/D-J junction (data not shown).

When tested for the specificity towards gut bacteria, the Ig derived from sorted PEC B1a and B1b cells as well as secretory serum Ig from L2 mice showed a binding comparable with that of NT controls (Supporting Information Fig. 2A-C).

Analysis of LP PC-derived IgA and IgM VH sequences of L2 mice

Thus far, only surface IgM^{+} cells from LP had been analyzed. Such cells might not represent the terminally-differentiated pool of PCs. Rather they might be derived from B cells of ILFs [2] or SILTs [3]. To study the terminally-differentiated PC-associated IgVH repertoire from LP of L2 mice, sorting of intracellularly stained cells could not be considered since the staining procedure might interfere with downstream processes of amplifying the VH transcripts. Therefore, a

different strategy was developed. Lymphoid cells from LP were depleted of $B220^+$ B cells by FACS. Since B220 is down regulated in PCs, non-plasma B cells should be excluded. Additionally, LP lymphocytes were also depleted of $CD3^+$ cells by FACS to further enrich PCs. According to these criteria, the $CD3^-B220^-$ lymphoid population was bulk sorted from the LP of L2 and age-matched NT mice and used for the PCR amplification of IgA and IgMVH transcripts.

The analysis of such IgAVH sequences from L2 mice showed an extensive presence of N/P nucleotides at the V-D and D-J junctions in almost all of the analyzed sequences (Fig. 5 and Table 2). The frequencies of N/P nucleotide additions at the V-D and D-J junctions in these sequences were comparable to that of NT mice (Table 2). In addition, SHM was observed throughout the IgAVH regions (Fig. 5). All of the analyzed IgAVH sequences from LP PCs of L2 mice (33 out of 33) showed the presence of nucleotide exchanges. In comparison to NT mice, the sequences from L2 mice were less heterogeneous. In case of L2 mice 11 out of 33 sequences (33%) were found repeatedly whereas, in case of NT mice, only 6 out of 42 sequences (14%) were found repeatedly (data not shown).

The PC derived IgMVH repertoire of L2 mice appeared to be rather non heterogeneous. A high percentage of IgMVH sequences were found repeatedly (6 out of 14). Nucleotide exchanges were also found (data not shown). However, the frequency of mutation was lower among these sequences than for IgA. Only 6 out of the 14 different sequences showed the presence of nucleotide exchanges.

Out of 14 analyzed IgMVH sequences derived from LP PCs of L2 mice, two matched with IgMVH sequences (B1b1 and B1b2) derived from PEC B-1b cells of L2 mice (Table 1, Sort IV).

Discussion

Evidence for the contribution of B-1 cells to the intestinal B-cell compartment is several-fold [6,10,26]. However, due to the complexity of the cell markers to distinguish particular B-cell subpopulations, the evidences provided thus far should not yet be considered conclusive. For instance, total PEC cells were taken [26] or IgM^{hi} cells were sorted and used for transfer [6]. In both cases, one could argue that B-2 cells were present in significant numbers in the donor cell population. Similarly, using B-1-cell-specific cellular markers also does not solve the problem as such markers might be down regulated under certain circumstances or up-regulated under others [27]. Hence, we argued that the only unchangeable marker for a B cell is its receptor. Since it is known that the IgH repertoire of B-1 B cells comprises specificities typical for this B-cell subpopulation, the database established by us for PEC and splenic B-1-cell-associated specificities from L2 mice [17, 18] could be used as marker for B-1 cells in the GALTs of such mice. This assumption turned out to be correct.

Several VH sequences could be recovered from intestinal tissue-associated B cells that had been observed before amongst PEC B-1 cells of L2 mice. In the PPs, B1a sequences were observed amongst IgM expressing germinal center B cells as well as non-germinal center B cells. In particular, two of the B1a-cell-associated VH sequences that usually dominate in the peritoneum of L2 mice could be recovered, indicating a strong overlap of the repertoires of the two compartments.

Similar to peritoneum, several sequences were recovered repeatedly from PP B cells of L2 mice. This suggests that under the constraints of the transgenic light chain the repertoire of the IgM-expressing cells is rather restricted. In contrast, sequences of IgA-bearing B cells apparently were

very heterogeneous. In addition, none of the previously described sequences could be recovered amongst IgAVH sequences. This suggests a strong selection pressure that drives the switch to IgA. Only B cells with a specific receptor for intestinal antigens should be expanded and switch. This might support minor populations that did not become apparent in the peritoneum where selection is believed to be strongly driven by self-antigens. On the other hand, IgA-producing B cells could be derived from B-1b cells or the few B-2 cells that are observed in the periphery of L2 mice. They might be expanded in the PP due to their non-self antibody repertoire. At the moment, these possibilities cannot be distinguished.

Expression of the transgene influences the numbers of B cells found in the PPs. Although the numbers of PPs present in L2 mice were normal, most of them were quite small in size. Thus, the lack of B-2 cells cannot be compensated completely by B-1 cells or the few B-2 cells present in L2 mice. On the other hand, the data clearly demonstrate the potential of B-1a cells to contribute to IgM^+ B-cell repertoire of germinal centers. No gross histological difference between PP from normal and L2 mice could be observed except for the decrease in IgM-bearing cells.

A similar situation is found in the LP of L2 mice. Although lower numbers of Ig-positive cells could be observed, the gross appearance was normal. IgA-expressing PCs could be observed at lower numbers but were clearly present. This was reflected also in a lower concentration of luminal IgA. It could be excluded that the lower number of such PCs was due to a shorter half-life indicating that most likely the lack of B-2 cells cannot be compensated by B-1 cells present in the periphery of L2 mice.

It is unclear why the B cells present in the L2 mice do not occupy the potential empty niches in such mice. Obviously, homeostasis is disturbed. Besides B cells, the number of T cells is also reduced in the PP of L2 mice even though the transgene should not influence the latter cellular compartment. On the other hand, recently, an influence of B cells on the differentiation of T cells

has been unraveled [28, 29]. Thus, originating from the block in the development of B cells in the BM of L2 mice the interrelationship of these two cell types might further explain the reduction found in both peripheral lymphocyte compartments.

When the VH repertoire of LP B cells of L2 mice was compared with the data base obtained for B-1a cells from the peritoneum and the PP of L2 mice, only sequences derived from the IgM^+ population were overlapping. In addition, IgMVH sequences established for PEC B-1b cells could also be recovered amongst the IgM repertoire in LP. This clearly shows that B-1 cells are able to contribute at least to the IgM^+ B-cell compartment in the LP. This might be representing B cells rather than PCs since they still express the surface markers B220 or CD19 and might be mainly restricted to ILFs or SILTs. However, an overlap between IgMVH sequences from PEC B-1b cells and from LP-derived PCs expressing IgM could also be detected. This demonstrates that in principle, B-1b cells could contribute to the LP PC pool and intestinal Ig repertoire.

Importantly, no B-1-cell-derived VH sequence could be detected amongst IgA-expressing cells from the LP of L2 mice. Thus, a contribution of B-1 cells to this compartment remains unresolved. Although overlaps between IgM and IgA associated VH sequences derived from $CD19^+IgM^+$ and $CD19^+IgA^+$ LP B cells respectively could be observed indicating a relationship between such cells, these VH sequences had not been found to be associated with B-1 cells in L2 mice so far.

A high heterogeneity was observed amongst IgA-producing PCs indicating a strong selection and extended diversity. This was obviously amplified by somatic hypermutation. All of the VH sequences derived from IgA-producing PCs displayed nucleotide exchanges. In addition, some of the VH sequences associated with IgM also displayed SHM. In agreement with the current view, fewer nucleotide exchanges were found for such VH regions since such clones are supposed to be less expanded and had less chance to mutate. Since B-1 cells were originally claimed not to be

able to undergo isotype switching and SHM, our finding was surprising. However, more recently, PEC B-1b cells were shown to differ severely from B1a cells. They could be shown to differentiate into memory B cells [8]. In addition, we could show that in PEC a subpopulation of B-1b cells exists that expressed AID which apparently resulted in class switch to IgA and SHM [14]. Therefore, it is clear that such cells in the appropriate microenvironment are able to differentiate in a fashion similar to B-2 cells and contribute to IgA-producing PCs. This demonstrates a clear difference between B-1a and B-1b cells. It appears that B-1b cells occupy a niche in between the extremes of B-1a on one side and B-2 cells on the other side.

Additional evidence for the strong selection pressure acting on the IgA positive cells in L2 mice can be deduced from the fact that endogenous κ is more often found to be associated with IgA than IgM-producing cells in PP (data not shown). Only in gut-associated tissues can a high number of such cells be detected and not in the other anatomical locations. It remains to be determined where these cells are derived from. The possibility of expansion of the few B-2 cells was already mentioned. Another possibility would be antigen-driven receptor editing. However, in preliminary experiments Rag-expression could not be detected in these organs.

Quite possibly, B-1b cells from PEC or the PP could be the source of the IgA-producing PCs found in the LP. However, no repertoire overlap between IgM or IgA from PEC B-1b cells and IgA-producing LP PCs of L2 mice could be observed. This is obviously owed to the extreme diversity amongst such VH specificities [30]. The repertoire restriction inflicted by the transgenic light chain observed for the B-1a-cells, does not hold for the B-1b-cells. Therefore, likelihood of a contribution by B-1b cells to the PC pool in the LP seems to be very high, despite the absence of a repertoire overlap.

In conclusion, in the present work, additional important knowledge on the physiology of B-1 cells could be revealed. The L2 mice remain a valuable tool that will further contribute to the understanding of this still enigmatic B-cell subpopulation.

Materials and methods

Mice: Heterozygous L2 [15] mice and NT littermates on BALB/c background were used at 8-14 weeks of age for all the experiments. Transgenic progenies were identified by PCR, using tail biopsies and transgene specific primers [25]. $Rag1^{-/-}$ mice on BALB/c background used for adoptive transfer were from our animal facility (Helmholtz Institute for Infection Research; Braunschweig, Germany) and Thy1.1 mice were kindly provided by Prof. Jochen Huehn (Department of experimental medicine, Helmholtz Institute for Infection Research; Braunschweig, Germany) All experiments were carried under the permission of the ethical committee of the local authorities of Lower Saxony LAVES.

Flow cytometry and cell sorting: Monoclonal antibodies against mouse B220 (RA3-6B2), CD19 (1D3), CD5 (53-7.3), CD43 (S7), IgM (DS-1), IgA (C10-3), were purchased from Pharmingen. Anti - mouse κ (187.1) and λ (9A8) antibodies were made in-house from the respective hybridoma supernatants. Above-mentioned antibodies were conjugated with allophycocyanin/FITC/PE/Biotin. To reveal biotinylated antibodies, Streptavidin-allophycocyanin (Pharmingen) or Streptavidin-PE (SouthernBiotech) was used. For the staining of germinal center (GC) cells, FITC coupled PNA (Sigma) was used. Flow cytometry was carried out using a FACSCalibur ® (Becton Dickinson) and data were analyzed using CellQuest software (Becton Dickinson). Using PI staining, dead cells were excluded from the analysis. Using MOFLO (Cytomation) or FACSAria® (Becton Dickinson) cell sorting was performed. Reanalysis showed that cells were >90% pure. For intracellular IgA staining of LP lymphocytes, cells were first stained for surface marker (CD19), then fixed with 2% paraformaldehyd (Sigma) at room temperature for 20 minutes, washed twice with PBS, stained intracellularly with FITC

coupled rat anti-mouse IgA (C10-3, Pharmingen) diluted in 0.5% saponin (Sigma) at 4°C for 45 minutes, washed twice with saponin/PBS and twice with FACS buffer before subjecting them to flow cytometry.

Preparation of LP lymphocytes (LPLs): To obtain LPLs, the whole small intestine was flushed with PBS to remove the luminal content. All the PPs were excised, gut was opened longitudinally and cut into 3 mm long pieces, then treated twice with PBS-3mM EDTA for 10 minutes each, twice with Ca^{2+} free RPMI 1640 (Gibco/BRL) for 15 minutes each and then was digested with RPMI containing 20% FCS and 50U/ml collagenase type VIII (Sigma). Each step was carried under 37°C, 5% CO_2 and 95% humidity with constant stirring using a magnetic stirrer. Cell suspension was filtered through 100µm nylon mesh, centrifuged, resuspended in 40% isotonic Percoll™/RediGrad™ (Amersham Biosciences), overlaid on 70% isotonic percol, and then centrifuged at 2000g. Interface was collected and washed with IMDM.

RT-PCR: Total RNA from bulk-sorted cells was prepared with TRIzol™ Reagent (GIBCO) following manufacturer's protocol. cDNA from DNase1 treated RNA (Amersham biosciences) was prepared using oligo-$d(T)_{12-18}$ (Amersham biosciences) and Superscript II $RNaseH^-$ reverse transcriptase (Invitrogen) and further PCR amplified using HotstarTaq™ DNA polymerase (Qiagen) and following primers: Igµ/α VH region, for VHcons 5'GAGGTGCAGCTGCAGGAGTCTGG3' rev Cµ1/Cµ2 5'ATGGCCACCAGATTCTTATCAGA3'/5'CATTTGGGAAGGACTGA3' or Cα1/Cα2 5'ATCAGGCAGCCGATTATCAC3'/5'GAGCTGGTGGGAGTGTCAGTG3'. PCR conditions were: 94°C for 20 s, annealing at 50°C for 40 s

SC RT-PCR: SC RT-PCR was done exactly as described previously [18].

Sequencing of IgVH chain and sequence analysis: Sequencing of IgVH chain and sequence analysis was done exactly as described previously [14].

Sequence data: IgA and IgM VH sequences derived from sorted, PP B cells , PEC B cells and LP B cells of L2 and NT mice were submitted to GenBank (accession number JX657351 – JX657674).

ELISA: Ig concentrations in the serum/intestinal lavage were measured using standard ELISA method. In brief, 96 well plates (MaxiSorb TM Immunoplates, Nunc) coated over night with 2 µg/ml (in coating buffer) goat anti-mouse IgA (α chain specific, Sigma) antibodies at 4^{o}C were blocked for 1 h with 3% BSA in 0.05% Tween 20 and then added with appropriately diluted sera/intestinal lavage from each mouse. After incubating for 2 h at RT, bound IgA was detected with 0.5 µg/ml of biotinylated goat anti-mouse IgA (Sigma) antibody which was then revealed by using horseradish peroxidase (HRP) conjugated streptavidin (BD). Using o-Phenylendiamin (OPD) as substrate, bound HRP activity was visualized and the results were read using an ELISA-reader (BioRad 3550- UV microplate reader) at a wavelength of 490 nm.

ELISPOT assay: Detection of antibody secreting cells (ASCs) amongst cells isolated from LP was done using ELISPOT assay. In brief, single-cell suspensions at serial dilutions of 1:5 were plated on goat-anti-mouse IgA (α chain specific, Sigma) antibody coated ELISPOT plates (Millipore) and incubated overnight at 37°C in 5% CO_2 and 95% humidity. Biotin conjugated goat-anti-mouse IgA (α chain specific, Sigma) was added after washing with PBS 0.01% Tween 20, and bound antibodies were developed with Streptavidin-horseradish peroxidase using AEC (3-amino-9-ethyl-carbazole; Sigma A-5754) in DMF (N,N-Dimethylformamide; Sigma D-4551) diluted in 0.1 M acetate solution and added with H_2O_2 as substrate. By washing plates with

double distilled water spot development was stopped. Spots marking ASCs were counted after air drying.

BrdU treatment and histology of paraffin embedded tissues: BrdU purchased from Sigma (B5002) was given in drinking water at a concentration of 0.6 mg/ml for a period of 6 weeks. Mice were analysed after one or 6 weeks. Intestine was taken out, flushed with PBS,and then cut into 1cm long pieces, fixed for two hours in Bouin´s solution (Sigma Aldrich). Afterwards, they were embedded in paraffin. Using BrdU labelling kit (Immunohistochemistry System, Calbiochem), 4µm thick intestinal sections were stained for BrdU. Biotin was revealed using Fluorophore provided with TSA^{TM} FLUORESCENCE SYSTEMS signal amplification kit (Perkin Elmer, cat. No. NEL704A). FITC conjugated goat anti-mouse IgA (Caltag Laboratories) was used in combination with anti-BrdU. Images were visualized using Zeiss LSM META 501 confocal microscope with Zeiss LSM browser software.

Immunohistochemistry: 4-5 cm long pieces of proximal jejunum portion were cut, flushed with PBS, opened longitudinally and rolled up in Tissue Tec O.C.T Compound (Sakura Finetek) medium, embedded in the same medium and frozen immediately on dry ice or at -80°C. 8µm thick cryostat sections were fixed with acetone, rehydrated with TBST (0.1M Tris, pH7.5, 0.15 M NaCl, 0.1% Tween 20), preincubated with TBST containing 5% rat serum, blocked with 0.01% avidin/PBS and 0.005% biotin/PBS, and stained with an appropriate mixture of fluorescent dye-coupled antibodies: anti-IgM-Cy3 (HB88), B220-Cy5 (TIB146), anti-IgA FITC (Caltag), and anti-λ (9A8) in 2.5% serum/TBST. Above antibodies except anti-λ were kindly provided by Dr. Oliver Pabst (Institute of Immunology, MHH, Hannover). Nuclei were stained with DAPI (1µg/ml DAPI/TBST) and sections were mounted with MOWIOL (PolySciences) Images were visualised using an Axiovert 200 M microscope with Axiovision software (Carl Zeiss

MicroImaging, Inc.) or Zeiss LSM META 501 confocal microscope with Zeiss LSM browser software.

Statistics: Paired two-tailed Student`s *t*-test was applied to determine the statistical significance (*p* value).

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Figure legends

Figure 1. PPs of L2 mice exhibit reduced number of B cells. (A) Flow cytometric analysis of PP cells from L2 and NT mice. Values in quadrants represent the percentage of live lymphocytes. Each dot plot represents the results of one of four independent experiments. (B) Absolute numbers of total cells, total B cells ($CD19^+$), IgM^+ B cells ($CD19^+IgM^+$), B1a cells ($CD19^+CD5^+$) and T cells ($CD19^-CD5^+$) in the PPs. Data are shown as mean ± SD of 3-4 mice per group and are representative of three independent experiments. Paired two-tailed Student`s *t*-test was applied to determine the statistical significance (*p* value). (C) Immunohistology (20× original magnification) of PP showing the presence of B-cell follicles ($B220^+IgM^+$). Images shown are representative of 3 stainings performed.

Figure 2. LP of L2 mice contains IgA^+ PCs. (A) Flow cytometric analysis of LP cells stained for intracellular IgA and surface CD19 (top). The staining for $CD5^+$ B1a cells is also shown (bottom). Values in quadrants represent the percentage of lymphocytes. Each dot plot represents the results of one of four independent experiments. (B) Amount of secretory IgA in the intestinal wash-out and serum of L2 and NT mice measured by ELISA. The number of IgA-secreting cells among the LP lymphocytes is shown (bottom). Data are shown as mean ± SD of 3-4 mice per group and are representative of three independent experiments. Paired two-tailed Student`s *t*-test was applied to determine the statistical significance (*p* value). (C) Immunhistology (10× original magnification) of LP showing the presence of IgA^+ PCs. Images shown are representative of 4 stainings performed.

Figure 3. Life span of LP IgA$^+$ PCs of L2 mice and NT mice are comparable. (A) Immunohistology (40× original magnification) of paraffin-embedded intestinal sections stained for IgA and BrdU. Images shown are representative of 4 stainings performed (B) Quantitative data showing the percentage of BrdU$^+$IgA$^+$ cells among total IgA$^+$ cells present in the LP. The percentage was calculated after counting the number of total IgA$^+$ cells and BrdU$^+$IgA$^+$ cells and expressed as the percentage of BrdU$^+$ IgA$^+$ cels among total IgA$^+$ cells. Data are shown as mean ± SD of 3 mice per group and are representative of two independent experiments.

Figure 4. PEC B-1a-cell-associated IgMVH specificity was found to be present among PP and LP B cells of L2 mice. CDR3 nucleotide sequence found commonly among SC-sorted B cells of PP, LP and PEC origin. The numbers under each organ in the table represent the number of times this sequence was found in that particular organ in one SC RT-PCR experiment. The number of sequences analyzed from each organ is denoted by ´´n`` in the parentheses between the representative dot plots and the table.

Figure 5. LP PC-derived IgAVH sequences of L2 mice display high frequencies of SHM. Part of the IgAVH region sequences containing the CDR3 and 36 upstream nucleotides. cDNA was derived from sorted B220$^-$CD3$^-$ LP lymphocytes. The arrow indicates the beginning of the CDR3 sequence at position Cys 92.