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Lambda Light Chain Revision in the Human Intestinal IgA Response[1]

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Abstract

Revision of antibody light chains by secondary rearrangement in mature B cells has the potential to change the specific target of the immune response. Here we show for the first time that light chain revision is normal and widespread in the largest antibody producing population in man; intestinal IgA plasma cells. Biases in the productive and non-productive repertoire of lambda light chains, identification of the circular products of rearrangement that have the characteristic biases of revision and identification of RAG genes and protein all reflect revision during normal intestinal IgA plasma cell development. We saw no evidence of immunoglobulin heavy chain revision probably due to inappropriately orientated recombination signal sequences, and little evidence of kappa chain revision, probably due to locus inactivation by the kappa deleting element. We propose that the lambda light chain locus is available and a principal modifier and diversifier of antibody specificity in intestinal IgA plasma cells.

Keywords

Human; Antibodies; Gene Rearrangement; Repertoire Development; Mucosa

Introduction

Precursors of IgA plasma cells are chronically challenged on the immunological front line, defending the body against potentially deadly micro organisms and toxins in the gut lumen. One of the strategies that IgA plasma cells use to meet this daily challenge is quantity; they generate between 3 and 5g of IgA that is transported into the gut lumen every day (1,2,3). Clonal expansion of plasma cell precursors is involved in generating sheer numbers of IgA plasma cells (4,5), but extensive clonal expansion would intrinsically compromise one of the defining characteristics of the adaptive immune system; diversity of receptors.

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Like antibody diversity generally, generation of IgA diversity begins in the bone marrow with RAG dependent, imprecise joining of single *V*, *D* [*IgH* only] and *J* segments of multiple alternatives at the *Ig* loci. *Ig* genes rearrange strictly hierarchically, starting at the *IgH* locus. If *IgH* rearrangement is successful, rearrangement at the *Ig* kappa light chain (κ)[3] locus is initiated and if rearrangement of *Igκ* is productive, the B cell matures expressing Igk. If rearrangement fails at both *Igκ* alleles, despite potential editing at the *Igκ* locus, rearrangement at the *Ig* lambda (*λ*) locus proceeds, and if this is successful an Igλ expressing B cell is generated (6,7). Most non-productive rearrangements at the κ loci are inactivated by recombination of the k-deleting element (KDE) that excises the constant region and the intronic enhancer from non-functional rearrangements, thus disabling somatic hypermutation which is dependent on the intronic enhancer (7,8).

Precursors of human intestinal IgA plasma cells that have been activated in GALT are expanded in germinal centers where somatic hypermutation changes approximately 5% of the V region sequence on average (9, 10). Analysis of the acquisition of and types of mutations with time in murine Peyer's patches and analysis of lineage trees from the germinal centers of human GALT does not suggest that the mechanism of hypermutation is different or more rapid in mucosal compared to peripheral sites (11, 12). The high frequency of mutation in GALT is thought to be due to a greater number of mutational cycles coupled with chronic challenge (12). There is evidence that adaptive and innate mechanisms are involved in generating the intestinal plasma cell response (13, 14). Therefore it is also possible that the high frequency of mutation reflects a higher tolerance threshold for mutations due to differences in selection pressure when B cells are driven by innate mechanisms. Selection for specificity can result in the elimination of sequences carrying mutations that reduce binding affinity. In the absence of antigenic selection, mutations that would affect affinity could theoretically accumulate, so long as they did not affect the structural integrity of the Ig molecule.

Receptor revision is a third potential mechanism of diversifying the antibody repertoire, though the nature and extent of revision is contentious. Receptor revision involves replacement of rearranged *IgH* or *IgL V* regions through additional DNA recombination events in mature peripheral B cells. Evidence of revision in man has been observed in autoimmunity and malignant human tissues (15, 16), though it has not been universally accepted to be part of normal B cell physiology. Expression of recombination activating genes (RAGs), which would be essential for revision, has been observed in germinal centers by some groups but not others (15, 17, 18). Data from a transgenic mouse model suggested that RAGs are not re-expressed in the periphery, but that expression may be retained by recent emigration from the bone marrow (19).

Our evidence that *IgL* revision modifies the normal intestinal IgA repertoire is based on comparison of the rearranged gene repertoire, the frequency of recombination excision circles (RECs) at the *Igλ* locus (λRECs), biases in J segment usage in λRECs and local expression of RAG-2 in GALT. All gene rearrangements whether primary or secondary, generate RECs. These do not divide but are passed on to single daughter cells through progressive cycles of division. Their frequency in intestinal IgA plasma cell precursors, together with estimates of proliferative cycles gained from the load of somatic mutations

(20, 21, 22, 23), indicates substantial revision. The gut is a site of generation of antibody diversity in many species and by different mechanisms (24, 25). We suggest that *IgL* revision is another mechanism that diversifies human intestinal IgA, thus enhancing the capacity to bind luminal antigens. Although autoreactivity would be a possible consequence of revision in other circumstances, this may not be relevant to IgA, first because it is relatively non-inflammatory compared to other isotypes and secondly because IgA secreted by intestinal plasma cells is largely destined for the gut lumen.

Materials and Methods

Human tissues and cells

Human ileum from right hemicolectomy specimens and isolated human lamina propria cells from macroscopically normal colon of 2 patients following therapeutic surgery for colon cancer, were used with the approval of the Local Research Ethics Committee, as described previously (5). Isolated cells were immunostained with RPE-conjugated CD38 (Dako UK) (1μg/ 100μl) then fixed and permeabilized with an IntraStain kit (DAKO,UK) and immunostained with anti-IgA-FITC (4μg/100μl) for intracytoplasmic Ig and sorted by FACS as single cells into 96-well plates. IgA immunoblasts, identified as triple positive cells immunostained by IgA-FITC (4μg/100μl) (Dako,UK), CD79b-RPE (BD,UK) (0.5μg/100μl) and α4bβ7-Cy5(BD,UK) (0.6μg/100μl), were isolated by FACS from buffy coats of 2 healthy donors. Small IgD+ B cells from the same buffy coats were sorted by FACS with mouse anti-human IgD (0.8μg/100μl) and goat anti-mouse - FITC (Dako,UK) for analysis of λRECs. Equivalently processed isotype and concentration matched controls were used throughout.

B cell clones

The FACS-isolated IgA^+, $CD79b^+$, $\alpha4\beta7^+$ cells (immumnostained as described above) at 20 or 50 cells per well in 96-well plates were cultured with EBV, irradiated allogenic feeder cells and 2.5μg/ml CpG (26). After 3 weeks, the culture supernatants were screened for Igλ and IgA secretion by ELISA and IgAλ positive cell lines cloned out on irradiated feeder cells with CpG.

PCR Amplification of *Igκ, Igλ* and *IgH* gene rearrangements

Single gut plasma cells were analysed by amplification of rearrangements involving 7 *Vκ* (27) and 10 *Vλ* families (12, 28). Similarly, 6 *IgH* (29) and 10 *Vλ* family gene rearrangements were retrieved from B cell clones and IgA^+, $CD79b^+$, $\alpha4\beta7^+$ B cells. PCR products were purified and cloned into the pGEM-T Vector (Promega) and sequenced by LARK Technology,UK. Sequences analyzed are accessible from EMBL/ Genbank under accession numbers (AJ972151-AJ972375, AM941072-1110, AM943489-3516).

Amplification of λRECs from IgD^+ and IgA^+, $CD79b^+$, $\alpha4\beta7^+$ B cells

The primers to amplify λRECs were designed according to IMGT database (http://imgt.cines.fr) to recover the signal joints from the circular DNA (Table I) with a multiplex nested PCR program as described (10). PCR products were purified and cloned into the pGEM-T Vector (Promega) and then sequenced by LARK Technology,UK. The frequency

of λRECs in IgD^+ B cells and IgA immunoblasts was performed by doubling dilutions starting from 400 cells to less than one cell per reaction. Each dilution was tested with 4 replicates in each of 3 separate experiments. Limiting dilution was further carried out with 2 cells per reaction for IgD^+ and 20 cells per reaction for IgA immunoblasts with 40 reactions for each analysis.

Isolation of germinal center cells from human gut tissue by laser capture microdisection

Frozen sections of human gut tissue (8μm) on PALM membrane slides (P.A.L.M. Microlaser Technologies, GmbH, Germany), were fixed with 70% pre-cooled (-20°C) ethanol for 2 or 3 minutes, and then stained with 1% cresyl violet acetate for 20 seconds. The stained slides from specimens from two individuals were stored at -80°C until use. The replicate germinal centers from entire well orientated follicles where zonation was clearly apparent, were dissected by laser capture microdissection and put into PALM adhesive Caps tubes containing 100μl of mRNA lysis/ Binding Buffer RLT (QIAGEN:RNeasy Mini Kit, Germany) with 10% carrier-N (ExpressArt Pico RNA Care reagents, Germany) to protect mRNA from degradation. The mRNA were isolated and reverse transcribed using a QIAGEN:RNeasy Mini Kit (Germany).

Detection gene expression in microdissected fragments

A semi-nested PCR was carried out in 50 μl volumes containing 5μl cDNA from germinal center cells, 1μmol 5’ primer, 1μmol of each 3’ primer, 200 μm of each dNTP and 2.5 mM $MgCl_2$ in *Taq* DNA polymerase 1x reaction buffer (Promega, Southampton, UK) and 1 unit of Taq DNA polymerase (Promega). 2μl of first round PCR product was used for the second round. Both rounds of PCR were performed as 30 cycles of 94°C for 30 minutes, 60°C for 1 and 72°C for 2 minutes, followed by the extension at 72°C for 5 minutes. PCR sense primer for RAG-2 is 5'GCAGCCCCTCTGGCCTTC 3'. External and internal anti-sense primers were 5'TTTCAGACTCCAAGCTGCCT 3' and 5'AGCGAAGAGGAGGGAGGTAG 3' respectively. Namalwa B cell line (European collection of cell cultures, Wilshire, UK) was used a positive control for RAG-2. The expression GAPDH gene was detected in both Namalwa and microdissected gut germinal centers. Activation induced deaminase (AID) expression was detected in the germinal center cells microdissected by LCM using a nested PCR (30) as an additional positive control.

Immunohistochemistry

RAG-2 was visualized in 3μm paraffin sections of 4 different specimens of human terminal ileum using rabbit anti human RAG-2 raised to amino acids 1-300 of RAG-2 (4μg/ml) and goat anti human RAG-2 raised against the C-terminus of RAG-2 (4μg/ml) (both from Santa Cruz Biotechnology) after antigen retrieval using Dako antigen retrieval solution. Staining controls were species and concentration matched antibodies with other specificities. DakoAntibody reactivity was visualized with the EnVision kit (Dako) or anti goat biotinylated antibody followed by ExtrAvidinPeroxidase (Sigma) and developed with DAB plus (Dako, Denmark). Slides immunostained using goat anti-RAG-2 were double stained with CD20 and an EnVision kit (Dako, UK).

Statistical methods—Comparisons of observed versus expected numbers were carried using c^2 tests in excel. Observed differences were considered to be statistically significant at $p \leq 0.05$.

Results

IgL rearrangements at the κ locus in intestinal IgA plasma cells

Single IgA plasma cells were sorted by FACS from cell suspensions of lamina propria cells, and *IgL* gene rearrangements were amplified by PCR. Sequence characteristics of 164 different rearrangements at the *Igκ* and *Igλ* loci of 125 single IgA plasma cells were analysed. There were no biases in the repertoire of *IgVκ* gene segments used, either by comparison with existing studies of blood B cells, or by comparison of productive versus non-productive rearrangements (Fig. 1A). A bias against *Jk1* segment rearrangements was observed in non-productive rearrangements (Fig. 1B). This has been observed before in a study of blood B cells (6,8) and is considered to be a consequence of editing during B cell development rather than revision in mature B cells.

The majority of productive rearrangements at the *Igκ* locus had undergone somatic hypermutation, whereas the majority of non-productive rearrangements had not (Fig 1C). Failed rearrangements at the *Igκ* locus are most often inactivated by the KDE which stops hypermutation of any remaining rearrangement by excision of the intronic enhancer (Fig 1D). Therefore, simply the presence of a mutated *Igκ* allele implies that it probably is or was functional (31). The presence of 2 mutated, in-frame rearrangements in a cell could reflect revision if an allele that has become non-productive as a consequence of somatic hypermutation is replaced functionally by secondary rearrangement on the other allele. Alternatively, this could reflect allelic inclusion where both in-frame alleles are used (31). Only 4 cells out of the 125 single cells studied showed evidence of either revision of *Igκ* rearrangements or allelic inclusion. Three cells contained 2 mutated in-frame *Igκ* rearrangements each. In one of these cells, one allele had acquired stops as a consequence of somatic hypermutation, rendering it non-productive (Fig. 1E). This suggests that previously functional *Igκ* rearrangement rendered non-productive by somatic hypermutation, could have been replaced by *Igκ* rearrangement on the second allele. One cell had a productive *Igλ* rearrangement and a mutated productive *Igκ* rearrangement, implying either replacement of previously used (mutated) *Igκ* rearrangement with *Igλ*, or allelic inclusion. Therefore revisions from *Igκ*, and allelic inclusion probably occur in intestinal IgA plasma cells, but are relatively rare.

Evidence for extensive *Igλ* revision

The repertoire of *IgVλ* segments in productive rearrangements from single IgA plasma cells was not significantly different from other studies of naïve B cells (either peripheral or mucosal) or from previously analysed populations of plasma cells (12, 28) (data not shown). However, the ratio of productive to non-productive rearrangements was dramatically skewed towards productive rearrangements in the most commonly used Vλ familes Vλ1 and Vλ2 and towards non-productive rearrangements in the Vλ5 family and related Vλ9 segment (Fig. 2). No biases in the other, less commonly used of the 10 Vλ families were apparent.

Somatic hypermutation, which particularly affects *IgV* in gut plasma cells could generate biases in PCR by mutating the primer binding sites (32). Productive and non-productive rearrangements may differ in their susceptibility to mutation in certain sequences, which might generate the observed biases. Therefore, to eliminate the possibility that the dramatic biases in *IgL* rearrangements observed by PCR were an artefact of PCR, B cell clones were prepared. B cells from blood that expressed integrin α4β7, CD79b and IgA, were considered to be precursors of mucosal IgA plasma cells in transit through the blood, on their way back to the mucosal lamina propria (IgA immunoblasts). They were sorted and transformed with EBV, then cloned at limiting dilution with feeder cells and CpG (26). Emerging clones were screened for Igλ and IgA production and 19 different IgAλ clones were generated. Heavy and light chain gene rearrangements from these clones were analysed and detailed in Table II. These clones showed the same bias toward productive rearrangements in Vλ1 and Vλ2 and towards non-productive rearrangements in the Vλ5 family as observed previously in individual and populations of gut plasma cells (10) (Fig. 2). This demonstrates beyond doubt that the skewed ratio originally observed by PCR is real and that the mechanism generating it is common, so that its effects are clearly apparent in a sample of 19 cell lines.

Frequency and composition of λRECs in IgA immunoblasts and plasma cells

Since the skewed ratios of productive and non-productive rearrangements in IgA immunoblasts and intestinal plasma cells had not been seen in mature naïve B cells, we hypothesised that the skewing may be a consequence of widespread receptor revision. If this hypothesis is correct, IgA immunoblasts and plasma cells should firstly contain more λRECs than expected if the only λRECs present were those generated during B cell development in the bone marrow. Secondly, λRECs from IgA plasma cells/ precursors should be biased towards the most distal J segment usage compared to naïve B cells, but might not show biases in V segment rearrangement since the profile of used segments appears unaffected by the revision process.

λRECs amplified by PCR (Fig. 3) were observed in 28.6% of IgD+ cells, determined by limiting dilution analysis. We plotted the number of cells expected to contain a single λRECs within an exponentially expanding population starting from the observed number of cells containing λRECs in the naïve IgD+ B cell population (Fig. 4A). To determine the number of IgA immunoblasts expected to contain λRECs, we first deduced how many cycles of replication they are likely to have been through using the observed frequency of hypermutation in these cells. The average mutation frequency in *IgH* and *IgL* chain, in intestinal plasma cells or IgA immunoblasts studied was 4.9% over 280bp of V region sequence (Fig. 4B). There are several estimates of the rate of hypermutation and therefore we calculated the number of cell divisions required to generate the observed hypermutation frequency of 4.9% using a recent study that defined a range of from 0.07 to 0.11mutations/ 100bp/ cycle (23), and also a conservative rate of hypermutation widely used in models of the germinal center reaction, and supported by earlier studies, of 0.5 mutations /V segment / cycle (20, 21, 22). The number of cycles of replication derived from these figures is from 44.5 to 70 cycles and 27.4 respectively. Therefore the number of cells expected to contain a single λREC ranges from to 9.4×10^8 to 4.1×10^{21} cells (Fig. 4A). Although this is a wide window, the observed frequency of λRECs in IgA immunoblasts of 1 per 89 cells

determined by limiting dilution analysis is approximately 7 orders of magnitude higher than the most conservative estimate within the range of expected λREC frequency.

If no revision had occurred, the observed proportion of cells containing λRECs in IgA immunoblasts (1/89) would be reached after approximately 4.7 cell divisions (Fig. 4A). This figure can also be visualised in the doubling dilution analysis of λREC frequency in IgD+ cells and IgA immunoblasts in Fig. 4C. However, if this were correct and no revision had occurred, the hypermutation mechanism would have to introduce mutations at a rate of approximately 1 mutation per 100 nucleotides per division into this population, which is approximately 6 times faster than the most rapid rates estimated, and is not substantiated by lineage tree analysis of germinal center responses in human GALT (11 and DD-W, unpublished data) or studies of the hypermutation mechanism in murine Peyer's patch cells (12).

The frequency of J segment recombination signal sequences (RSS), that mediate the recombination event, in the λREC sequence was strongly biased towards replacement with J2/ 3 in the IgA immunoblast ($P<0.001$) and intestinal plasma cell populations ($P<0.02$), compared to approximately equal proportions of J1 and J2/3 in the λRECs from IgD+ cells from blood, confirming that they are consequences of revision (Fig. 5A). The V segment, and V family replacements observed in the λRECs showed approximately the same repertoire as all other populations analysed (Fig 5 B and C).

Identification of RAG

RAG protein expression in Peyer's patches of the terminal ileum was assessed by immunohistochemistry. Expression characteristics observed using two different antibodies generated in different species and to different epitopes of RAG-2 are illustrated. RAG-2 was detected on subsets of germinal center B cells, including germinal center centroblasts (Fig. 6A and C). In addition, RAG-2 expression by a subset of intraepithelial reddish/ brown cells was observed (Fig 6B).

The expression of RAG-2 in germinal centers was confirmed by RT-PCR of cDNA derived from germinal centers isolated from Peyer's patches by laser capture microdissection. PCR for AID served as a positive control for germinal center origin of the microdissected fragments (Fig. 6 D).

Discussion

The data presented here highlight a role of secondary *Igλ* rearrangements as modifiers of the antigen binding repertoire of IgA. Potential revision of *IgH* is thwarted by the removal of the D segments with appropriately orientated flanking RSS, that mediate recombination from D to J and from V to D, by the initial rearrangement process. It is theoretically possible to use embedded RSS in *IgVH* for receptor revision (33), but there is no evidence from examination of junctional regions of *IgH* rearrangements from IgA plasma cells that this occurs (JS, unpublished observations). Editing of *Igκ* has been described (34), but failed *Igκ* rearrangements in man are generally inactivated by the KDE which removes the potential for future rearrangement events in mature B cells (6,7,8). Examples of revision of *Igκ* have been

described here, but they are relatively rare. In contrast, the *Igλ* locus has 'nested' V and J segments that can recombine to generate new rearrangements on the same allele and there is no known mechanism for inactivation of the locus. The intact *Igλ* locus is also available for secondary rearrangements following Igk expression.

Revision of *Igλ* does not substantially alter the profile of Vλ usage. Vλ 1 and 2 families are dominant in the used repertoire in naïve B cells, in intestinal IgA plasma cells, IgA immunoblasts and the λRECs. In contrast, the non-productive repertoire of rearrangements involves predominantly Vλ5 and Vλ9 families in the IgA+ populations studied only. We have previously shown that the non-productive rearrangements involving Vλ5 and 9 are biased towards rearrangement with Jl2 and 3, implying that the non-productive rearrangements involving Vλ5 and 9 are the products of secondary rearrangements (10). The data on the whole are consistent with the hypothesis that rearrangements from Vλ 1 and 2 families can be replaced by revision, but those from Vλ5 and 9 can not, so that they accumulate in the non-productive repertoire.

Through analysis of somatic hypermutations in *IgV* segments from IgA plasma cells and the rates at which they are introduced, we know that at least $9x10^8$ cells are generated from a single precursor (Fig. 4), though not all will survive the germinal center response since some mutations are likely to be functionally deleterious and the cells carrying them will die during the selection process (35). Since replacement of light chain can result in changes in antigen binding (36), light chain revision would add to the spectrum of specificites in the chronically dividing B cells that sustain the extensive lamina propria plasma cell population.

The requirement for cognate B cell -T cell interaction during germinal center formation guards against the generation and propagation of autoantibody responses (14). Receptor revision generates new specificities within germinal centers once this initial regulatory barrier is crossed, which is potentially dangerous in the context of autoimmunity. In the periphery high affinity BCR enhances B cell survival through selection, which would form a second level of regulation to prevent autoreactivity (14), though this process may not regulate B cell survival in GALT germinal centers (14). However, it is possible that the consequences of generating a new specificity in an IgA response in a mucosal microenvironment are not as hazardous as an equivalent event in the periphery, partly because of the relatively passive functional properties of IgA. Most autoantibodies associated with disease processes are IgM or IgG isotypes which are complement fixing, and are more 'proinflammatory' than IgA. In addition, most IgA is produced beneath the intestinal epithelium and is transported into the gut lumen (2), where diversity at the expense of potential autoimmunity may not be a problem. One example of secondary light chain rearrangement has been identified in somatically mutated IgG involving kappa and lambda light chains. The authors propose that light chain revision and somatic hypermutation may both be involved in affinity maturation (37).

It is possible that *IgL* revision could occur in germinal centers of GALT because the high load of mutations in *IgV* generates non-functional or autoreactive variants. *Igλ* and receptor revision have been associated with autoimmunity, though this was not necessarily associated

with mucosal surfaces, and may be a consequence of aberrant activity of a normal phenomenon described here.

The expression of RAG in secondary and tertiary lymphoid organs has been debated partly due to divergent findings (15–19, 38). Even amongst studies that have identified expression of RAGs in secondary lymphoid tissues, expression has been reported in Ig negative cells, consistent with centroblasts as observed here (17) and also in centrocytes (38). Whether these contrasting data reflect differential expression in diverse sites or technical factors is unclear. We observed expression of RAGs in the cytoplasm and not in the nucleus, and our observations may not directly reflect rearrangement activity in centroblasts or in the epithelium, if the protein is partitioned. However, the the presence of RAGs indicates that local activity is certainly possible. The expression of RAGs in cytoplasmic foci in centroblasts of the germinal center is consistent with re-expression of RAGs and potential for revision during a T cell -dependent B cell response. Interestingly, a recent study identified induction of RAG gene expression by IL-6; a factor associated with plasma cell development (39).

In conclusion, we propose that revision of *Igλ* occurs during the development of intestinal IgA plasma cells and is an intrinsic component of the human IgA response that is likely to occur in germinal centers of GALT. We consider this to be an additional level of diversification of mucosal Ig in B cells that have proliferated extensively. Revision events may occur purely to diversify the repertoire, or to remove non-functional or autoreactive variants generated by a high load of somatic mutations. Whatever the initiating event, an increase in diversity is the likely outcome.

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Abbreviations used

κ	kappa light chain
λ	lambda light chain
KDE	kappa deleting element
REC	recombination excision circle
λREC	recombination excision circle resulting from rearrangement at the Igλ locus
RSS	recombination signal sequence
PC	plasma cell

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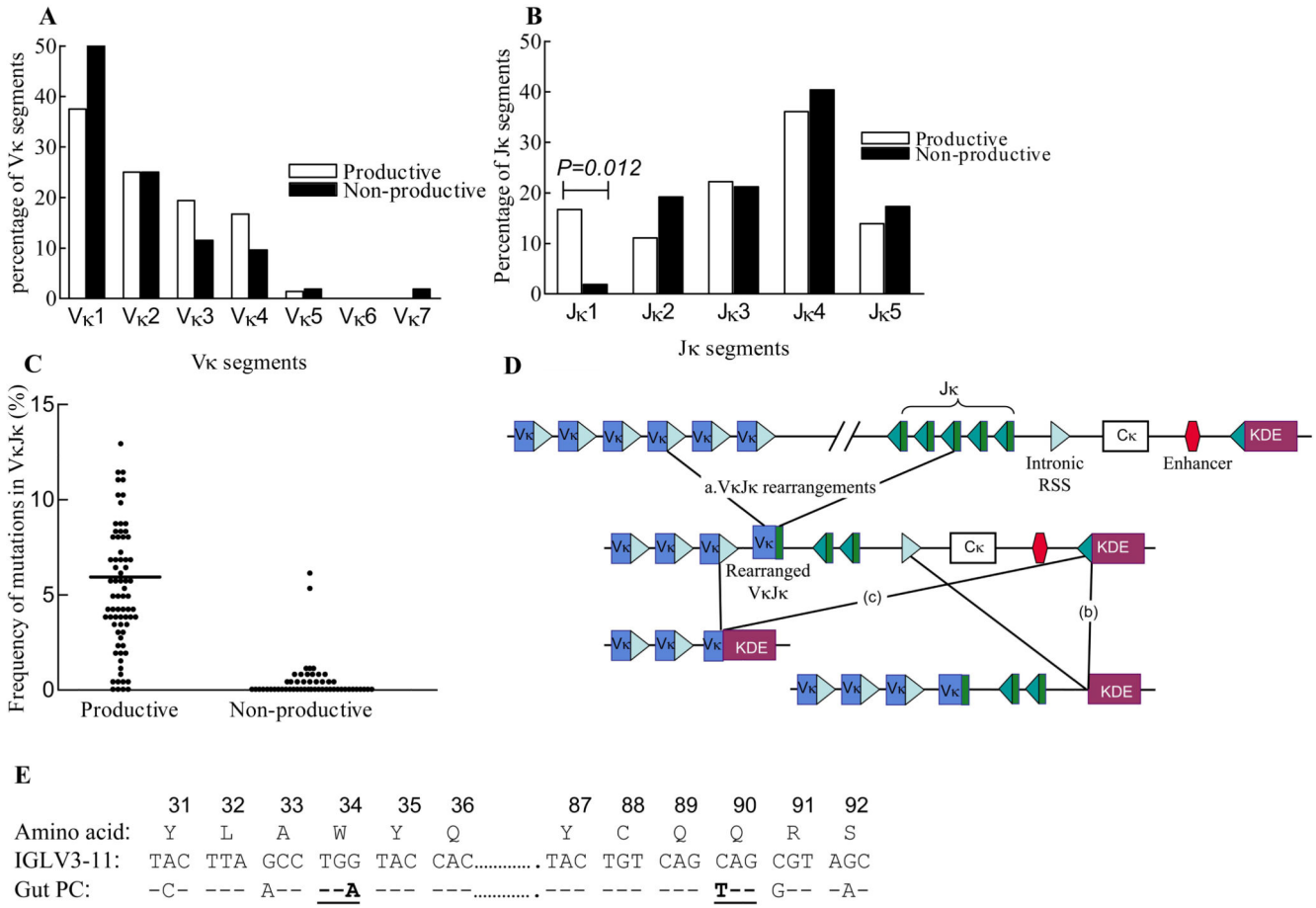

Figure 1. Characteristics of Igk rearrangements from single IgA plasma cells.

Comparison of productive and non-productive rearrangements involving the Vκ families (A) and individual Jk segments (B). The only bias observed was a tendency not to observe Jk1 in the non-productive rearrangements, consistent with editing of these rearrangements as seen in another study (7,8). In most cells, the somatic hypermutation mechanism was only active on productive rearrangements of VκJκ (C), presumably due to the activity of the KDE in non-productive rearrangements. The activity of the KDE is illustrated in D, where a VκJκ rearrangement is identified by 'a'. The KDE can inactivate this rearrangement if it is non-productive. The KDE can either recombine with an RSS in the intronic sequence between Jκ and Cκ identified by 'b', or with an upstream V segment identified by 'c'. Evidence for revision or allelic inclusion was apparent in 3 different plasma cells where each had two mutated in-frame alleles. In one in-frame rearrangement involving the germline V segment V3-11, had acquired stops by somatic hypermutation, which may have been replaced by rearrangement at the second allele (E).

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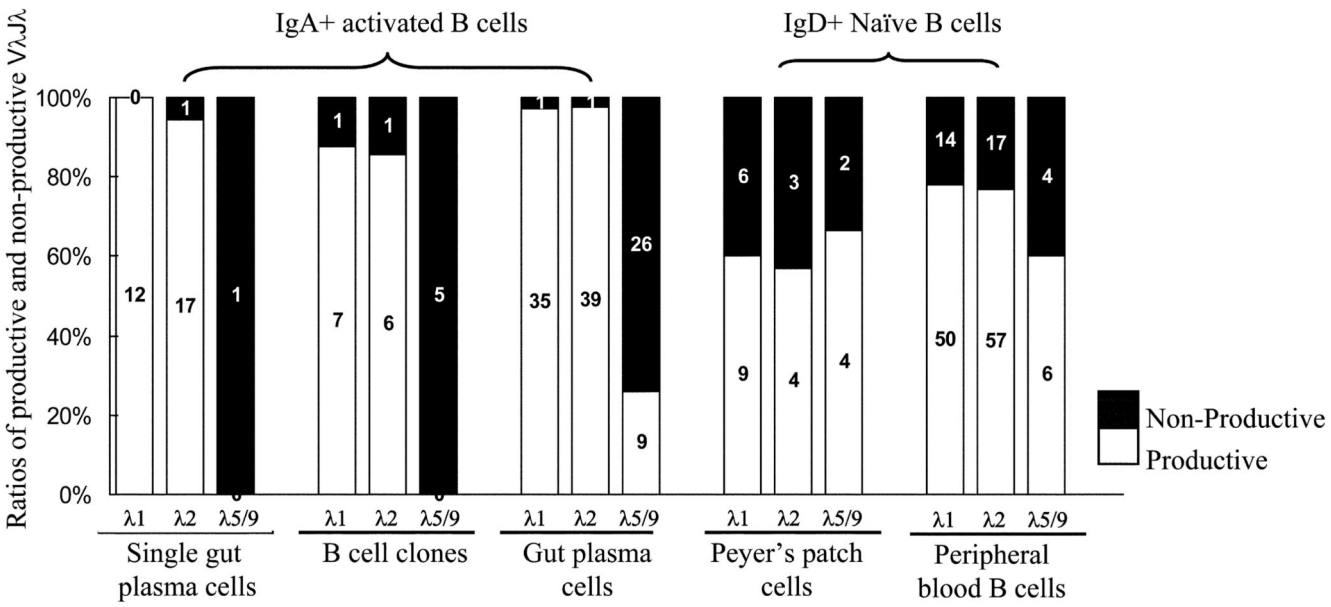

Figure 2. Biased ratio of productive: non-productive Igλ rearrangements in IgA plasma cells compared to mature naïve B cells.

Ratio of productive (open bars) to non-productive (black bars) rearrangements in the Vλ1 and 2 families and the Vλ5 family and related Vλ9 segment. Data from single B cells and from B cell lines in this study are shown alongside data from populations of IgA plasma cells and naïve B cells, including Peyer's patch cells from other studies (10, 28). The actual number of sequences contributing is written inside the bars. In IgA plasma cells and immunoblasts, there is a bias towards productive rearrangements in the Vλ 1 and 2 families and towards non-productive rearrangements in the Vλ5 and 9 families. This skewing is not apparent in naïve B cells and is therefore a consequence of revision and not editing.

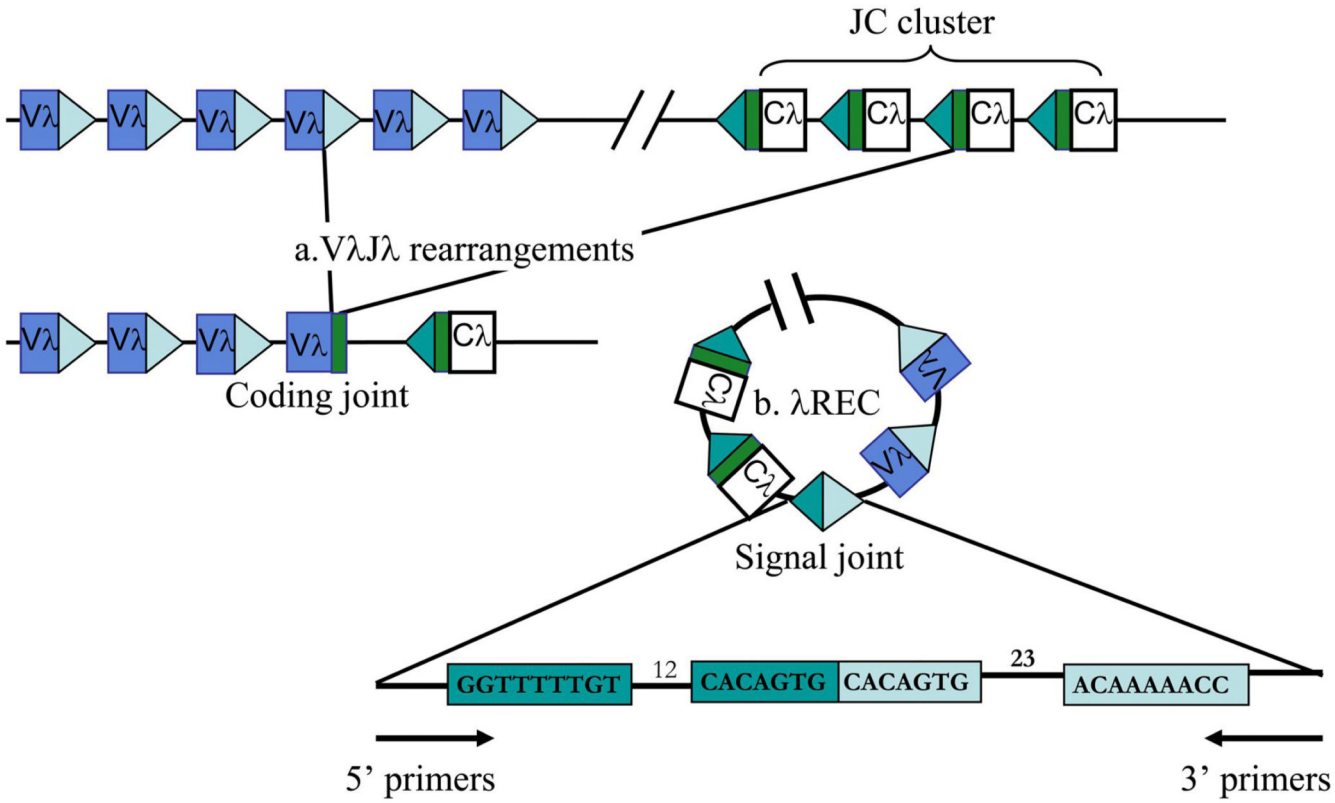

Figure 3. Illustration of generation and detection of λRECs.

When Vλ and Jλ segments rearrange, illustrated by 'a', a coding joint is formed between Vλ and Jλ. The intervening sequence forms a circular DNA, which we term the Igλ recombination excision circle (lREC), that includes a signal joint characterized by a pair of RSS in opposite orientations (illustrated by triangles). The binding sites of 5'- and 3'-primers used to amplify λRECs are shown as arrows.

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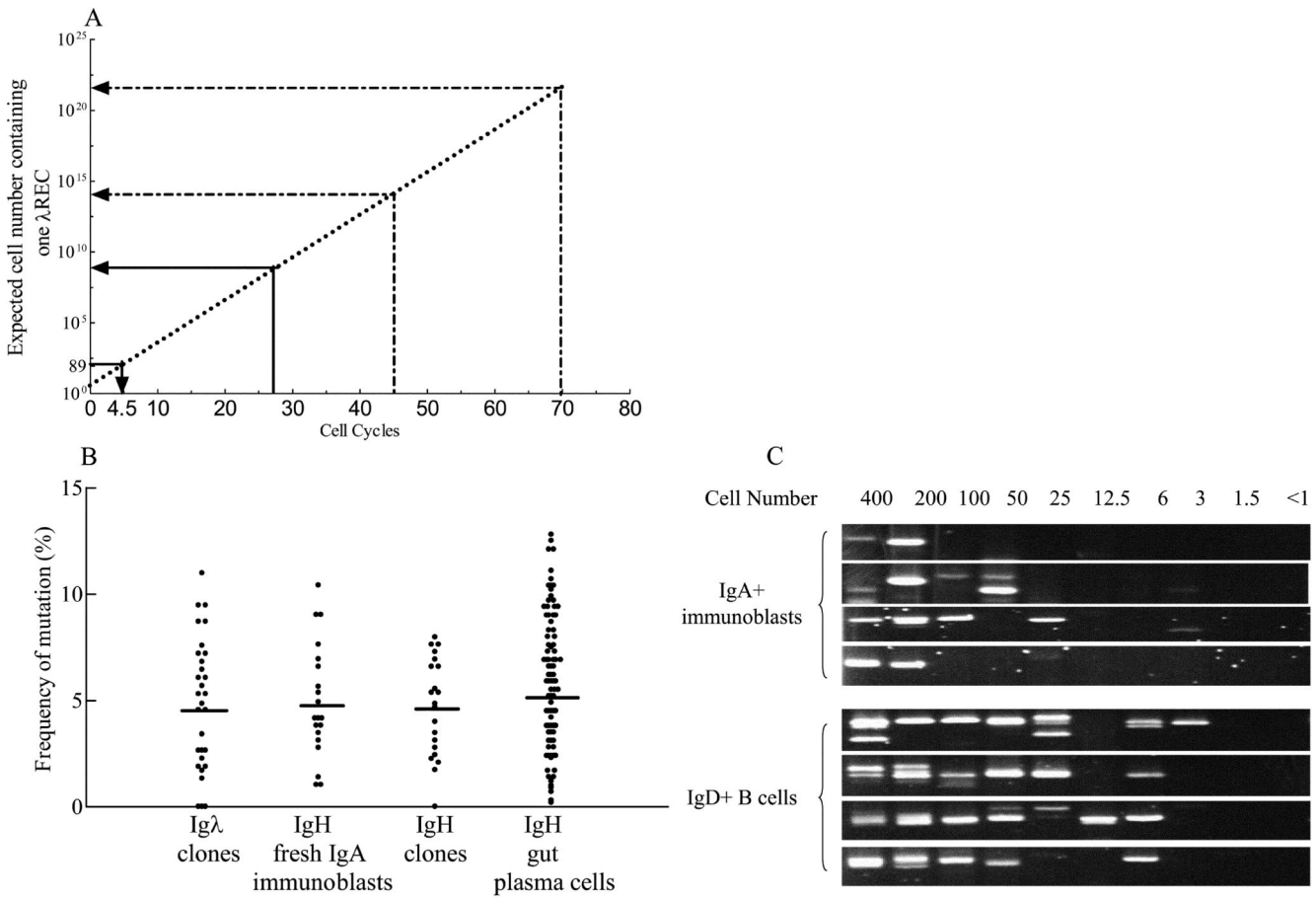

Figure 4. Calculation of expected and observed frequency of λRECs.

The graph in (A) illustrates the decreasing frequency of λRECs in an exponentially expanding population (y axis) which starts with a frequency of λRECs of 1 in 3.5 cells, as observed in mature naïve IgD+ B cells, as cell cycles progress (x axis). The observed frequency of λRECs is the experimental observation of 1 λREC per 89 IgA immunoblasts, which would be derived from 4.7 cycles of proliferation if revision did not occur. Three expected frequencies of λRECs are illustrated. Two use the range of hypermutation frequencies of 0.07 to 0.11 mutations/ 100 bp/ cycle to generate the 4.9% observed mutations over 44.5 to 70 cycles of replication respectively (23). This would result in 1 λREC in between 1.2 x 10^{14} and 4.1 x 10^{21} cells. The third expected frequency of λRECs is derived from an estimate of 0.5 mutations/ V segment/ cycle, which would generate 4.9% mutation frequency over 27.5 cycles resulting in 1 λREC per 9.4 x 10^{8} cells (22). (B) The frequencies of hypermutation in the IgH and IgL from the IgA immunoblast clones, IgH from the untransformed IgA immunoblasts from which clones were made, and IgH from gut plasma cells. The frequencies of hypermutation were on average 4.9%. The gels in (C) illustrate the frequency of λRECs when analysed by PCR. Cells for PCR were diluted by doubling dilutions from a starting cell number of 400 IgD+ lymphocytes or IgA immunoblasts per well. The top 4 rows are IgA immunoblasts, the bottom 4 rows are IgD+ lymphocytes from the same individual. The IgA immunoblasts are between 4 and 5 doubling

dilutions (analogous to doubling during cell cycles) behind the IgD+ cells if it is assumed that revision does not occur, consistent with figures calculated from limiting dilution studies.

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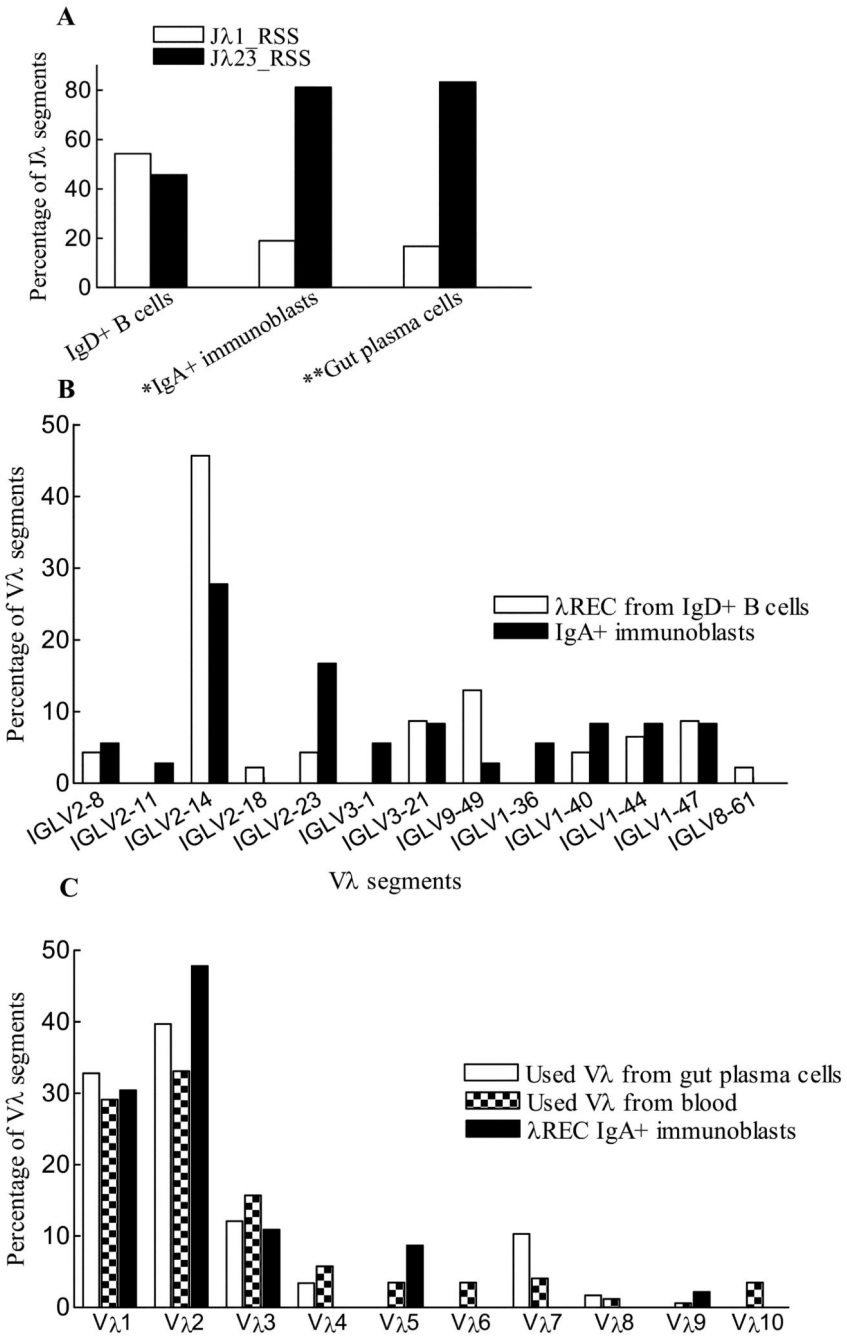

Figure 5. Analysis of J and V segment rearrangements in λRECs.

J segment involvement in λRECs from IgD+ B cells, IgA immunoblasts and IgA plasma cells from the gut (A). Jl1 is most common in the λREC from IgD+ cells, whereas there is a significant tendency to see Jl2/3 in the λRECs from IgA immunoblasts *(P<0.001) and plasma cells **(P<0.02). In contrast there is no strong bias in Vλ segments in λRECs, other than a tendency not to see the commonly rearranged Vλ2-14 segment in the λRECs from IgA immunoblasts (B). Similar profile of Vλ familes in productive rearrangements from naive B cells (28) and plasma cells (10) and in the λRECs from the IgA immunoblasts (C).

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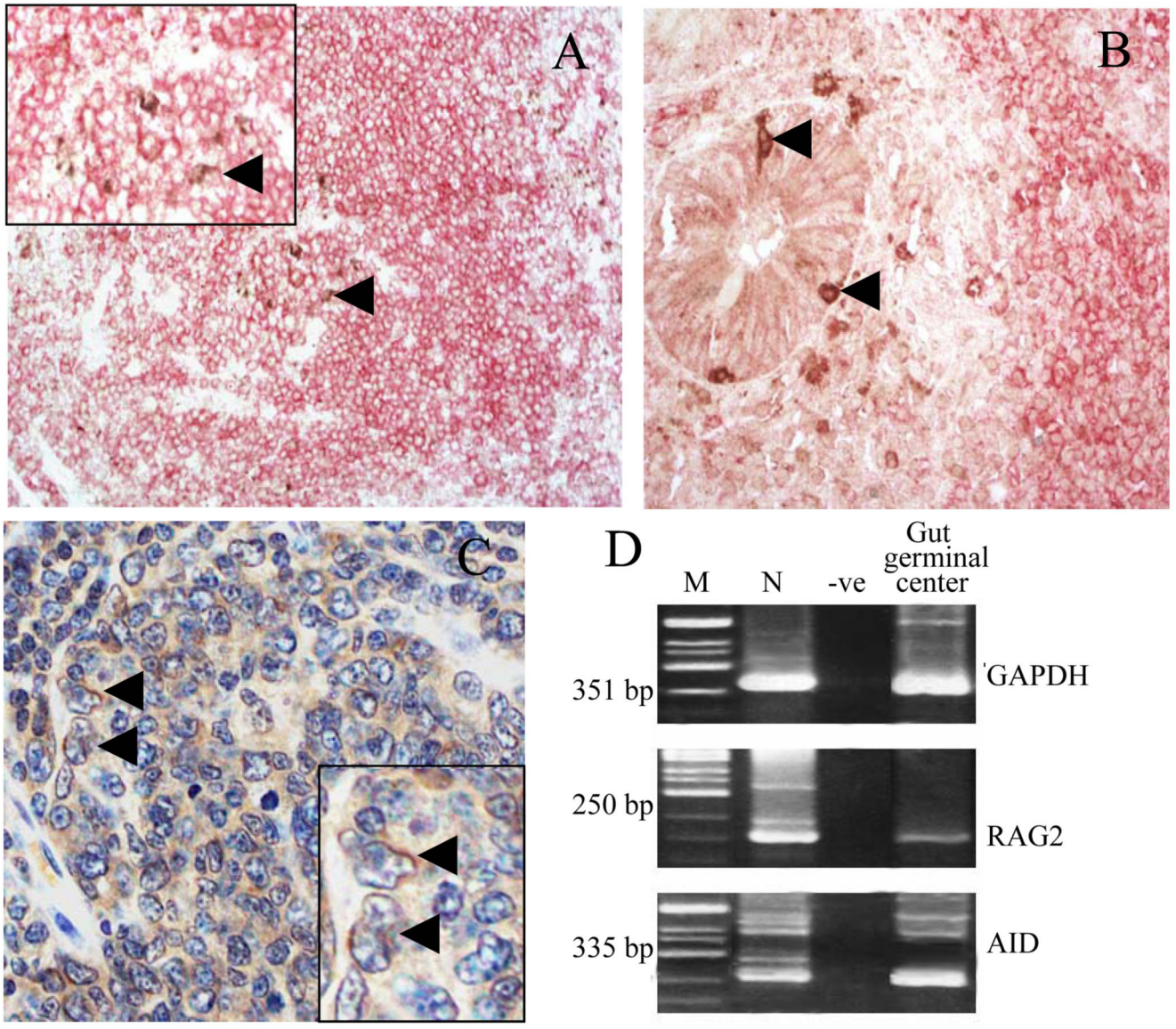

Figure 6. RAG-2 expression in Peyer's patches.

A and B, double immunostain with goat anti-RAG-2 (brown) and CD20 (red) on paraffin sections of normal human Peyer's patches. (A) Detection of RAG-2 positive B cells in the germinal center of a Peyer's patch (arrowed with higher power detail in insert). (B) RAG-2 expressing cells infiltrating the epithelium adjacent to the Peyer's patches (arrowheads). (C) Rabbit anti-RAG-2 also identified germinal center cells (arrows and in insert) and a subset of intraepithelial lymphocytes (not illustrated) consistent with the staining illustrated in B and C. D. Example of amplification of RAG-2 gene transcripts by RT-PCR from laser capture microdissected samples of germinal centers RNA. Molecular weight marker is identified by 'M', the cell line Namalwa (N) that rearranges light chain genes in vitro (unpublished) is a positive control. AID transcripts are the positive control for laser microcapture of germinal center cells.

Table I

Primers use for detection of λREC.

Name of primers	Primer sequences
5'Vλ External primers	
CirVL1E	gaggaggagcctccactctca
CirVL2E	catggtggaaagggaaggagc
CirVL3E1	ctggagggaagggcaacctga
CirVL3E2	ggaacataagggcagggatag
CirVL4E1	aggttagctccacaggcactga
CirVL4E2	agcagacatctcagtgtcatc
CirVL5E1	atgtctgcctgtgaaatgcgag
CirVL5E2	acaaccagccaacacttcagg
CirVL5E3	actgagacttcagacgaatgg
CirVL78E	agagactgggtgaggcacct
CirVL9E	cacacagggaacatatgctgtg
CirVL9E	cacacagggaacatatgctgtg
CirVLPE	ggcctgtggctgagtctcactt
5'Vλ Internal primers	
CirVL1N	agcctccactctcaggcagg
CirVL2N	aagggaaggagctgagagag
CirVL3N1	ggaagggcaacctgaaaggctc
CirVL3N2	agggcagggataggactggaa
CirVL4N2	tcagtgtcatcaggaggct
CirVL4N1	ccacaggcactgaattcagc
CirVL5N1	tgaaatgcgagcaaggtaggac
CirVL5N2	acacttcagggtcatcaggtg
CirVL5N3	acttcagacgaatggatggga
CirVL78N	tgaggcacctggcagtgaac
CirVL9N	tatgctgtgaattagaactcc
CirVLPN	gtggctgagtctcacttcctcc
3'Jλ External primers	
CirJL1E	cctgcctcatgtctaggtcc
CirJL23E	ggccatgtggactccctcatga
CirJL6E	aggaagtgatattcaggtcag
CirJL7E	tccagtaaagtgtgcatccatgc
3'Jλ Internal primers	
CirJL1N	ctcatgtctaggtcccagcc
CirJL23N	tggactccctcatgagcagatg
CirJL6N	ttcaggtcagtagaaggtgacc
CirJL7N	tgtgcatccatgcatggagg

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Table II

Details of heavy and light chain rearrangements from EBV clones derived from IgA+, CD79b+, α4β 7+ cells from blood.

Cell line	Rearranged IGLV Genes	No. Mutations in IGLV	Used IGHV Gene	No. Mutations in IGHV
1	IGLV2-14	18	IGHV3-23	26
2	IGLV1-47	14	IGHV3-7	12
3	IGLV2-14	12	IGHV3-74	12
	IGLV3-1(NP)[+]	16		
4	IGLV1-40	16	IGHV4-31	19
	IGLV3-16 (NP)	25		
5	IGLV4-69	0	IGHV3-33	3
	IGLV5-52 (NP)	0		
6	IGLV2-8	7	IGHV1-18	10
	IGLV5-37 (NP)	14		
7	IGLV2-14	7	IGHV3-30	12
	IGLV5-37 (NP)	12		
8	IGLV2-11	5	IGHV4-61	9
9	IGLV3-25	5	IGHV3-23	3
	IGLV5-52 (NP)	0		
10	IGLV1-44	15	IGHV1-18	22
	IGLV3-16 (NP)	20		
11	IGLV1-36	23	IGHV3-53	26
12	IGLV3-1	15	IGHV3-23	11
13	IGLV1-51	19	IGHV3-33	12
	IGLV7-46 (NP)	23		
14	IGLV3-16	29	IGHV4-39	30
15	IGLV1-51	7	IGHV3-48	8
	IGLV1-44 (NP)	3.5		
16	IGLV3-19	19	IGHV3-74	11
	IGLV5-45 (NP)	17		
17	IGLV2-11	9	IGHV3-33	30
18	IGLV2-8	4.5	IGHV3-13	4
19	IGLV1-40	6	IGHV4-41	3

[+]NP = non-productive allele.