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Comment on "Gut-associated lymphoid tissue contains the molecular machinery to support T-cell-dependent and T-cell-independent class switch recombination"

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To the editor: Although recently confirmed in an activation-induced cytidine deaminase (AID) reporter mouse model,[1] AID expression and immunoglobulin A (IgA) class switch recombination (CSR) in the intestinal lamina propria (LP) remain a matter of debate.[2] In an article published in the last issue of *Mucosal Immunology*, Barone *et al.*[3] revisited AID expression in the human intestine. Barone *et al.* observed AID expression in intestinal follicles but not LP.[3] In addition, Barone *et al.*[3] detected a proliferation-inducing ligand (APRIL) and its receptors TACI and BCMA in both intestinal follicles and LP.[3] As previously published data show that human APRIL elicits AID expression and IgA CSR in the absence of T-cell help to B cells by CD40 ligand,[4,5] Barone *et al.* conclude that APRIL and its receptors are part of a molecular mechanism that promotes IgA CSR in intestinal follicles by both T-cell-dependent (TD) and T-cell-independent (TI) pathways.[3] Given the additional involvement of APRIL in plasma cell survival,[6] Barone *et al.* further suggest that APRIL promotes plasma cell survival in the LP.[3]

Although plausible these conclusions are not demonstrated by the data. Indeed, Barone *et al.*[3] solely document intestinal expression of APRIL, but provide neither functional nor molecular evidence of APRIL involvement in follicular IgA CSR or LP plasma cell survival. Furthermore, Barone *et al.*[3] provide neither functional nor molecular evidence to demonstrate the involvement of APRIL, TACI, and BCMA in TD or TI routes of B-cell activation within intestinal follicles. We also question the approach used by Barone *et al.*[3] to study the expression of AID, a hallmark of ongoing CSR,[5] in the human LP.

Barone *et al.*[3] stained intestinal tissues for AID and APRIL through immunohistochemistry (IHC) and compared the results of these stainings with those obtained by our group through immunofluorescence analysis (IFA).[5] This comparison is inaccurate, because IHC and IFA are fundamentally different methodologies that deal with paraffin-embedded and frozen tissues, respectively. Different tissue processing modalities can affect sensitivity. Indeed, the sensitivity of IHC can be attenuated by antigen retrieval procedures, which modify the antigenic structure of the protein under study due to its exposure to high temperatures. Limited sensitivity explains the detection by Barone *et al.*[3] of AID in follicular B cells, but not in LP B cells or tonsillar subepithelial B cells. A sensitivity issue may also explain the finding of APRIL in crypt but not lumen-facing epithelial cells.[3] Although we agree with the former observation,[5] the lack of APRIL in lumen-facing epithelial cells differs not only from our results,[5] but also from the results of Shang *et al.*[7] and data available in the Swedish Human Proteome resource program (http://www.proteinatlas.org/show_image.php?image_id=1635790). Because of these reasons and the glaring lack of supporting functional data, the lack of APRIL expression by lumen-facing epithelial cells should not be used as an argument to question the role of bacterial Toll-like receptor ligands in APRIL release by intestinal epithelial cells.[5] In this regard, Barone *et al.*[5] fail to recognize that the flagellin receptor Toll-like receptor-5 promotes APRIL secretion rather than APRIL expression by epithelial cells.[3]

Antigen retrieval procedures can affect not only the sensitivity, but also the specificity of IHC. Indeed, Barone *et al.*[3] detected AID in $CD68^+$ LP macrophages, and attribute this finding to the poor specificity of an EK2-5G9 antibody to AID (anti-AID) used in some of our studies.[5] We reject this claim, because we used IFA instead of IHC to visualize AID. IFA specifically detected AID in LP-activated B cells, plasmablasts, and plasma cells co-expressing IgA, BSAP (Pax5), IRF4, Blimp-1 and/or CD138,[5] but not CD68 (B. He and A. Cerutti, unpublished data). Importantly, we validated and further extended our IFA-based results by means of fluorescence *in situ* hybridization (FISH) in both CD40-sufficient and CD40-deficient individuals,[5] whereas Barone *et al.*[3] did not. Moreover, the specificity of EK2-5G9 for both follicular and extrafollicular B cells, including tonsillar subepithelial B cells, is consistent not only with results by Cattoretti *et al.*,[8] but also with additional data from our group generated with anti-AID antibodies different from EK2-5G9 (B. He and A. Cerutti, unpublished data).

Barone *et al.*[3] back up their claim that there is no AID in the LP by comparing the presence of AID transcripts in microdissected tonsillar germinal centers and LP tissue through quantitative PCR. The sequence of the primers used to amplify AID mRNA is not disclosed. This issue aside, we contend that in this specific setting quantitative PCR is misleading, because based on the false assumption that germinal centers and LP have comparable B-cell densities and AID expression levels. Instead, germinal centers have a much higher

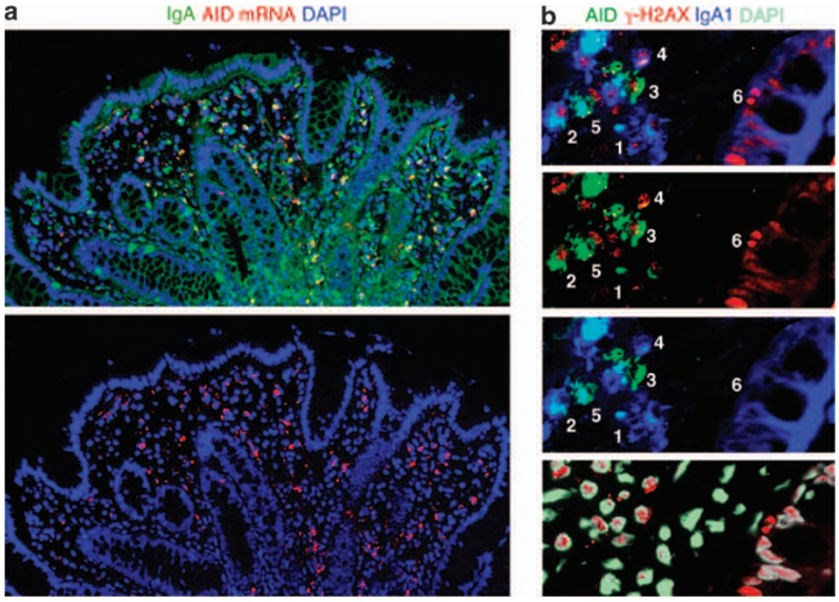

Figure 1 Some LP B cells express AID and the switch DNA region-targeting nuclear protein γ-H2AX. (**a**) Human LP from the small intestine of a patient with CD40 deficiency stained for IgA (green), AID mRNA (red) by IFA. DAPI (blue) counterstains nuclei. Original magnification, ×10. Orange- or yellow-appearing LP cells co-express IgA and AID mRNA. (**b**) Human colon LP from a healthy subject stained for AID (green), nuclear γ-H2AX (red), and IgA (blue). Cyan-appearing LP cells co-express IgA and AID protein. Bottom panel shows colocalization of nuclear γ-H2AX with DAPI-stained nuclear DNA (cyan). 1, AID^- γ-$H2AX^+IgA1^+$ LP B cell; 2, AID^+ γ-$H2AX^+IgA1^+$ LP B cell; 3, AID^+ γ-$H2AX^+IgA1^-$ LP B cell; 4, AID^+ γ-$H2AX^+IgA1^+$ LP B cell showing AID and γ-H2AX colocalization in the nucleus; 5, AID^+ γ-$H2AX^-IgA1^+$ LP B cell; 6, AID^- γ-$H2AX^+IgA1^+$ crypt epithelial cells. Cycling crypt epithelial cells contain γ-H2AX because γ-H2AX is generally involved in DNA repair during cell proliferation. Original magnification, ×40. One of several experiments yielding similar results.

B-cell density than the LP, which consists of a mixed population of B cells and non-B cells. Furthermore, the vast majority of germinal center B cells express AID, whereas only a fraction of LP B cells does.[5,9] Moreover, individual germinal center B cells are likely to express more AID than individual LP B cells. Thus, the observation that germinal centers contain more AID RNA than the LP is completely expected and should not allow one to conclude that the LP lacks AID. Similarly, naive B cells exposed to CD40 ligand and interleukin-4 contain negligible AID RNA compared with germinal center B cells, because only a fraction of naive B cells induce AID and undergo CSR upon *in vitro* stimulation (B. He and A. Cerutti, unpublished data). A more appropriate method to detect AID in scattered LP B cells is FISH (**Figure 1a**). Alternatively, one can PCR amplify AID mRNA from purified LP B cells.[6] At any rate, even the data provided by Barone *et al.*[3] seem to be consistent with the presence of AID in the LP, because $CD20^+$ B-cell-enriched contain more AID mRNA than $CD20^+$ B-cell-poor LP samples.[3] This difference would be visually more evident in a graphic lacking germinal center data.

An additional problem relates to the inaccurate discussion of the literature on the inductive function of the LP. For example, Barone *et al.*[3] do not discuss recent studies showing LP AID expression and both TD and T1 LP IgA CSR events in an AID-reporter mouse model.[1] In addition, Barone *et al.*[3] do not mention the fact that APRIL triggers IgA1 CSR in addition to IgA2 CSR,[5] which may explain the presence of APRIL not only in IgA2-rich areas such as the colon LP,[5] but also in IgA1-rich lymphoid areas such as mucosal follicles.[3,5,10] Finally, Barone *et al.*[3] do not discuss earlier evidence showing that APRIL cooperates with antigen, cytokines, and microbial molecular patterns to induce not only direct IgM-to-IgA1 CSR, but also sequential IgA1-to-IgA2 CSR and B-cell proliferation.[5,11–14] This evidence is consistent with the local presence of clonally expanded B cells in the LP[15] and with the expression of growth-associated nuclear proteins such as Ki-67 (http://www.proteinatlas.org/show_image.php?image_id=282924) and γ-H2AX (**Figure 1b**) in some LP B cells. Of note, the histone γ-H2AX targets AID-induced double-strand switch DNA breaks in B cells transiting through the G1 phase of the cell cycle.[16]

In summary, we contend that Barone *et al.*[3] provide neither functional nor molecular evidence to demonstrate that APRIL signals IgA CSR in B cells from intestinal follicles through TD and TD pathways involving TACI and BCMA. We also question the conclusion that the human intestinal LP lacks AID, because the experimental approaches used by Barone *et al.*[3] neither allow an accurate detection of LP AID nor can be compared with approaches successfully used in earlier works.[1,5,17,18]

DISCLOSURE

The authors declared no conflict of interest.

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Reply to "Gut-associated lymphoid tissue contains the molecular machinery to support T-cell-dependent and T-cell-independent class switch recombination"

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To the editor: Few would dispute that AID is expressed in gut-associated lymphoid tissue (GALT) of both humans and mice inside and outside germinal centers.[1,2]

Most GALT contains germinal centers that are acknowledged sites of T-cell-dependent class switch recombination (CSR). In addition, analysis of sites of immunoglobulin A (IgA) CSR in CD40 −/− mice identifies the GALT as the sole location of germinal center-independent IgA CSR.[2] APRIL (A PRoliferation Induced Ligand) is able to induce activation-induced cytidine deaminase (AID) expression and CSR to IgA independent of T-cell help.[3] In our recent study,[4] we show for the first time that human GALT contains cells unequivocally expressing APRIL and its receptors, in association with AID. These generally accepted statements justify the title of our publication in *Mucosal Immunology*.

In contrast, as highlighted by the letter from He *et al.*,[5] there is no agreement as to whether the lamina propria can act as a site of IgA CSR, partly due to the lack of agreement on whether AID is expressed in lamina propria. One consistent finding in an otherwise argumentative area is that PCR-based methods in four laboratories did not detect AID message in mucosal lymphoid tissue that was specifically checked to be free of GALT in mice or humans.[1,2,6,7] In our current paper, quantitative real-time-PCR for AID performed with three sets of commercially available primers (see Materials and Methods[4]) show negligible expression of AID in the lamina propria. Although studies using AID reporter mice and isolated cells from mucosa that was not checked to be free of lymphoid tissue claim AID expression in the lamina propria,[8–10] they cannot simply negate the consistently published results from several other groups.[1,2,4,6,7]

We have no reason to doubt the validity or sensitivity of our method for immunohistochemical analysis of AID expression. Unlike immunofluorescence, this method that is recommended by the supplier of the EK2 5G9 antibody (Cell Signaling) allows accurate assessment of the histological context and cell morphology. The characterization of the false-positive staining artefact in lamina propria was an important stage in our own analysis, because this artefact resembles the cells illustrated by He *et al.*[11] and again in their letter. If occasional cells in lamina propria expressed nuclear AID with the intensity observed in germinal centers, we concede that our method may have missed them. However, we did not see evidence of extensive and intense cytoplasmic expression of AID in large lamina propria B cells as illustrated by He *et al.* at either protein or RNA levels. He *et al.* argue that our immunohistochemistry and PCR are not sufficiently sensitive, but they illustrate cells that should be easily detected. The contention that our technique is not sensitive is not supported by any quantitative data.

Lamina propria expression of AID in B cells is a central component of the model proposed by He *et al.*[11] They have suggested that GALT supports CSR to IgA1 subclass only and that GALT-derived IgA1 class switched cells then home to the lamina propria where APRIL secreted by epithelium, in response to TLR5 ligation, would induce CSR to IgA2. This elegant hypothesis could explain the relatively higher expression of IgA2 in colon compared with small intestine. This hypothesis is, however,