The RNA-binding protein HuR is essential for the B cell antibody response

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Post-transcriptional regulation of mRNA by the RNA-binding protein HuR (encoded by Elavl1) is required in B cells for the germinal center reaction and for the production of class-switched antibodies in response to thymus-independent antigens. Transcriptome-wide examination of RNA isoforms and their abundance and translation in HuR-deficient B cells, together with direct measurements of HuR-RNA interactions, revealed that HuR-dependent splicing of mRNA affected hundreds of transcripts, including that encoding dihydrolipoamide S-succinyltransferase (Dlst), a subunit of the 2-oxoglutarate dehydrogenase (α-KGDH) complex. In the absence of HuR, defective mitochondrial metabolism resulted in large amounts of reactive oxygen species and B cell death. Our study shows how post-transcriptional processes control the balance of energy metabolism required for the proliferation and differentiation of B cells.

The generation of antibodies is a hallmark of the fight against infection and is a principal goal in the design of vaccines. B lymphocytes produce distinct antibody isotypes by the process of class-switch recombination (CSR). Antibodies arising from the extrafollicular differentiation of B cells into antibody-secreting cells (ASCs) can prevent pathogen spread at early stages of infection, while switched antibodies quickly recruit effector cells during memory responses. Somatic hypermutation of the immunoglobulin-encoding genes increases antibody affinity. This requires the positive selection of B cells, bearing high-affinity membrane immunoglobulins, by T cells within germinal centers (GCs), the specific anatomical structures formed in peripheral lymphoid organs during many immune responses.

Resting lymphocytes require glucose and glycolysis to survive but undergo metabolic reprogramming following antigen encounter to meet the energetic and biosynthetic demands of proliferation. Unlike the activation of T cells, which results in a disproportionate increase in glycolysis, the activation of B cells via membrane immunoglobulins is accompanied by a balanced increase in both glycolysis and oxidative phosphorylation. The molecular mechanisms that control this metabolic reprogramming in lymphocytes have come under intense scrutiny and have highlighted a link between metabolic flux and cell fate decisions.

The network of transcription factors required for the activation and subsequent differentiation of B cells has been elucidated in some detail. In contrast, despite the established role of splicing and polyadenylation in control of the expression of immunoglobulin-encoding genes, relatively little is known about how post-transcriptional regulation of gene expression influences the outcome of antigen encounter in B cells. The post-transcriptional fate of mRNA is mediated by its associated RNA-binding proteins (RBPs) and noncoding RNAs, which determine the timing and magnitude of protein expression. The B cell–specific deletion of the RNA-processing enzyme Dicer inhibits the germinal center reaction and for the production of class-switched antibodies in response to thymus-independent antigens. B cell death. Our study shows how post-transcriptional processes control the balance of energy metabolism required for the proliferation and differentiation of B cells.

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proinflammatory cytokine production during chronic inflammation and cancer\textsuperscript{17,18}. HuR is required for the maintenance of hematopoietic stem cells\textsuperscript{19} and the selection and chemotaxis of T cells\textsuperscript{20}.

Here we found that regulation of the B cell transcriptome by HuR was essential for normal antibody responses. B cell–specific deletion of HuR revealed an obligatory role for HuR in the GC reaction. Moreover, the production of class-switched antibodies induced by thymus-independent antigens was dependent on HuR. To identify the direct targets of HuR action, we mapped HuR-RNA interactions at the single-nucleotide level of resolution in primary B cells\textsuperscript{21,22}. The integration of gene-expression profiling by high-throughput mRNA sequencing (mRNA-seq) and the identification of translationally regulated mRNA by ribosomal profiling (or ‘footprinting’) (ribo-seq) established a direct correlation between changes in gene expression and in the translation of specific mRNAs bound by HuR. Our analysis indicated that HuR regulated the expression of genes encoding molecules related to energy metabolism in B cells and prevented the accumulation of fatal levels of reactive oxygen species (ROS) upon the activation of B cells.

**RESULTS**

**Increased HuR expression upon B cell activation**

We found that HuR was the only member of the Elavl family of RBPs expressed in splenic B cells (Fig. 1a). To understand the role of HuR during B cell development and activation, we generated mice with conditional, B cell–specific deletion of loxP-flanked Elavl1 alleles (Elavl1\textsuperscript{fl/fl}) by Cre recombinase expressed from the B cell–specific gene Mb1 (Mb1-Cre) (called ‘HuR-cKO’ mice here). Elavl1\textsuperscript{fl/fl}-Mb1-Cre mice served as a control (except where noted otherwise). Analysis of HuR expression by flow cytometry showed that in HuR-cKO mice, it was absent from the pro-B cell stage in the bone marrow (BM) and was undetectable in transitional and mature B lymphocytes (Fig. 1b–d). HuR was expressed similarly in all B cell populations present in the spleen (Fig. 1c,d). Quantitative RT-PCR analysis of GC B cells formed in the spleen of wild-type (C57BL/6) mice after immunization with the hapten NP (4-hydroxy-3-nitrophenylacetyl) coupled to keyhole limpet hemocyanin (KLH) showed a sixfold greater abundance of Elavl1 mRNA in GC B cells than in non-GC B cells (Fig. 1e). Intracellular flow cytometry indicated that HuR protein was twofold more abundant in GC B cells than in non-GC B cells (Fig. 1f). In vitro activation of B cells with lipopolysaccharide (LPS) or with antibody to the costimulatory receptor CD40 (anti-CD40) plus interleukin 4 (IL-4) and IL-5 increased the expression of HuR protein by up to threefold relative to its expression in unstimulated B cells (Fig. 1g). Together these data suggested that HuR expression increased during B cell activation in vitro and in vivo.

**B cell development following deletion of HuR in pro-B cells**

Normal proportions of B cell subpopulations were present in the BM, spleen and lymph nodes of HuR-cKO mice (Fig. 2a–c). We found

![Figure 1](image-url)
Figure 2 HuR-cKO mice have no major defects in B cell development. (a) Flow cytometry of B cell populations in the BM of control (Ctrl) and HuR-cKO mice (left) and quantification of pre-B cells (Pre B), immature B cells (Imm) and mature B cells (Mat) based on the expression of cell surface markers (far right). Numbers adjacent to outlined areas (left) indicate percent B220+IgM+ cells (top left) or B220+IgM+ cells (bottom right) (far left); IgD+IgM+ mature B cells (top left) or IgD−IgM+ immature B cells (bottom right) (middle); or CD25+CD19lo pre-B cells (right). (b) Flow cytometry of B cell subsets in the spleen of control and HuR-cKO mice (left) and quantification of those subsets based on the expression of cell surface markers (far right). Numbers adjacent to outlined areas (left) indicate percent IgD+IgM− cells (top left) or IgD−IgM+ cells (bottom right) (far left); IgMhiCD23− T1 B cells (top left), IgMhiCD23+ T2 B cells (top right) or IgM+CD23+ cells (bottom right) (middle); or CD21+CD23− marginal zone B cells (top left) or CD21−CD23− follicular B cells (bottom right) (right). (c) Quantification of CD19+ cells in the peripheral lymph nodes of control and HuR-cKO mice. (d) Flow cytometry of B cell subsets from the peritoneal cavity of control and HuR-cKO mice (left) and quantification of B-1 cells among those (far right). Numbers adjacent to outlined areas (left) indicate percent CD19+ B220lo B-1 cells (left) or CD19+ B220hi B-1 cells (right) (left column); or IgM+CD5− B-1a cells (left) or IgM+CD5− B-1a cells (right) (right column). (e) Flow cytometry assessing BrdU incorporation (right) by pre-B cells obtained from control and HuR-cKO mice 2.5 h after intraperitoneal injection of BrdU, followed tissue isolation and cell staining (gating at left). Numbers adjacent to outlined areas indicate percent CD25+CD19lo (pre-B) cells (left), or BrdU+ pre-B cells (right; mean ± s.d. of \( n = 8 \) mice per group). Flow cytometry assessing BrdU incorporation by mature follicular B cells (middle) and marginal zone B cells (right) in the spleen of control and HuR-cKO mice provided BrdU in the drinking water for 7 d (gating at left). Numbers adjacent to outlined areas indicate percent CD21+CD23− marginal zone B cells (top left) or CD21−CD23− follicular B cells (bottom right) (far left); or BrdU+ cells (right; mean ± s.d. of \( n = 5 \) mice per group). Each symbol (a, b, d, far right and c) represents an individual mouse (\( n = 8 \) per group per genotype); small horizontal lines indicate the mean. * \( P < 0.05 \), ** \( P < 0.005 \) and *** \( P < 0.0005 \) (Mann-Whitney test). Data are representative of more than three independent experiments (a−d) or two experiments (e, f).

B cells require HuR for normal antibody responses

HuR-cKO mice had significantly lower titers of immunoglobulins of all isotypes in the serum, apart from IgA, than did control mice (Fig. 3a). The titers of immunoglobulin G1 (IgG1) and IgG2b were 2.5-fold lower in HuR-cKO mice than in control mice, whereas the titers of IgG3 and IgM were 6- and 12-fold lower, respectively. The lower IgM titer could have resulted from the lower abundance of B-1 cells, as these contribute substantially to serum IgM. The biggest difference was in IgG2c titer, which were 200-fold lower in HuR-cKO mice than in control mice. The decrease in almost all antibody isotypes was probably reflective of a requirement for HuR in B cells for a functional response following an encounter with antigen.

To investigate the requirement for HuR during the response of B cells to antigen, we immunized control and HuR-cKO mice with the thymus-independent antigens Ficoll or LPS coupled to the hapten NP. NP-reactive IgM titers in serum were fivefold lower in HuR-cKO mice than in control mice in response to either antigen (Fig. 3b,c). We were unable to detect NP-reactive IgG3 antibodies in serum from HuR-cKO mice, in contrast to their detection in control mice (Fig. 3b,c). Next
Figure 3  B cells require HuR for responses to various classes of antigens. (a) Serum immunoglobulin (Ig) titers in immunized control and HuR-cKO mice (n = 10 per genotype). (b,c) Quantification of NP23-reactive IgM and IgG3 in serum collected from control and HuR-cKO mice (n = 6 (b) or 6–7 (c) per group) 7 d after immunization with NP-LPS (b) or NP-Ficoll (c); dashed lines indicate limit of detection. (d) Time-course analysis of NP23- or NP2-reactive IgM and IgG1 in serum from control and HuR-cKO mice immunized with NP-KLH at day 0 and day 42 (downward arrows). (e) Ratio of NP2-bound (high-affinity) IgG1 to NP23-bound (low- and high-affinity) IgG1 for the titers in d. (f) Quantification of GC B cells in the spleen of control and HuR-cKO mice (n = 7–8 per genotype) 7 d after immunization with NP-KLH (right); left, gating strategy: numbers adjacent to outlined areas indicate percent B220+CD95+PNA+ (GC) B cells. (g) Enzyme-linked immunospot assay of IgM+ and IgG1+ ASCs in the spleen of the mice in f (n = 7–8 per genotype). (h,i) Quantitation of IgM+ and IgG1+ASC in the spleen (h) and BM (i) of control and HuR-cKO mice (n=7–8 per genotype) 7 d after secondary immunization with NP-KLH at day 42 after primary immunization. Each symbol (a–c,g–i and f, right) represents an individual mouse; small horizontal lines indicate the mean. *P < 0.05, **P < 0.005, and ***P < 0.0005 (Mann-Whitney test). Data are from two independent experiments (a), are representative of three (b,c) or two (d,e) experiments (mean ± s.e.m. of n = 6 mice per group in d,e) or are from one experiment representative of two independent experiments (f).

we evaluated the ability of HuR-cKO B cells to participate in a thymus-dependent antibody response by assessing serum antibody responses following primary and secondary immunization with NP-KLH. Serum titers of NP-reactive IgM were on average 8.5-fold lower in B cells from HuR-cKO mice than in those from control mice (Fig. 3d). Titers of NP23-reactive IgG1 (where ‘NP23’ indicates 23 moieties of NP conjugated to LPS or Ficoll), which reflect the combination of both low-affinity antibodies and high-affinity antibodies, were 15-fold lower in HuR-cKO mice than in control mice (Fig. 3d). Memory responses, evaluated 7 d after secondary immunization, were clearly detectable in HuR-cKO mice in the form of IgM (Fig. 3d). In contrast, NP2-reactive IgG1 titers did not increase in the HuR-cKO mice (Fig. 3d), which suggested that memory cells expressing high-affinity IgG1 did not form or, if they did, their reactivation failed to generate ASCs. As a measure of affinity maturation, we determined the ratio of NP2-reactive IgG1 to NP23-reactive IgG1; these results indicated that NP-reactive antibodies in HuR-cKO mice failed to undergo affinity maturation (Fig. 3e).

Affinity maturation takes place in GCs, where helper T cells select B cells with the highest affinity for antigen. GC formation at 7 d after immunization with NP-KLH was diminished in HuR-cKO mice relative to that in control mice (Fig. 3f). The number of GC B cells was more than sixfold lower in HuR-cKO mice than in control mice (Fig. 3f). Similarly, following administration of highly immunogenic sheep red blood cells, the GC response was defective over time in HuR-cKO mice (Supplementary Fig. 1c,d). Consistent with the diminished titers of serum antibodies, the number of NP-reactive IgM+ ASCs was twofold lower in the spleen of HuR-cKO mice immunized with NP-KLH than in that of control mice immunized in the same way, whereas the number of NP-reactive IgG1+ ASCs was 50-fold lower in HuR-cKO mice than in control mice (Fig. 3g). We found a similar lower number of NP-reactive IgM+ and IgG1+ ASCs in the spleen of HuR-cKO mice than in that of control mice at 7 d after secondary immunization with NP-KLH (Fig. 3h). Further analysis of ASCs in the BM showed that HuR-cKO mice and control mice contained a similar number of NP-reactive IgM+ ASCs, but NP-reactive IgG1+ ASCs were barely detectable in HuR-cKO mice (>100-fold fewer than in control mice) (Fig. 3i). These data showed that HuR was required for proper activation and/or differentiation of B cells following antigen encounter.

Regulation of the B cell transcriptome by HuR
To determine whether an early stage of B cell activation was affected by the absence of HuR, we measured changes in intracellular Ca2+ following crosslinking of cell surface IgM. The Ca2+ flux elicited after activation of B cells with either a highly crosslinking antibody to IgM F(ab’2) or a monoclonal antibody (B7.6) to IgM was similar for B cells from the lymph nodes of HuR-cKO mice and those of control mice (Supplementary Fig. 2a). Additionally, surface expression of CD40, the cytokine receptor CD25 (IL-2Rα), the activation marker CD69 and the costimulatory molecule CD86 (B7-2) was increased to a similar extent on cells from control and HuR-cKO mice in response to a variety of stimuli (Supplementary Fig. 2b,c), which indicated that at least by these criteria, B cell activation was normal in the absence of HuR.

To identify the molecular mechanisms regulated by HuR in freshly isolated splenic B cells (called ‘ex vivo’ B cells here) and in splenic B cells activated in vitro with LPS, we performed an integrated analysis of three high-throughput measurements of the B cell transcriptome. First, we searched for differences in mRNA expression in B cells from HuR-cKO mice relative to mRNA expression in B cells from control
Table 1 Pathway-enrichment analysis

| Pathway                                      | Total genes | DE genes | P             |
|----------------------------------------------|-------------|----------|---------------|
| mRNA processing                             | 456         | 58       | 5.98 x 10^-14 |
| microRNA regulation of DNA-damage response  | 69          | 14       | 6.54 x 10^-06 |
| G1-to-S cell-cycle control                   | 65          | 12       | 8.63 x 10^-05 |
| Senescence and autophagy                     | 109         | 15       | 2.00 x 10^-04 |
| Apoptosis                                    | 93          | 13       | 5.00 x 10^-04 |
| Androgen receptor signaling pathway          | 123         | 15       | 6.00 x 10^-04 |
| Cytoplasmic ribosomal proteins               | 78          | 11       | 1.20 x 10^-03 |
| BCR signaling pathway                        | 200         | 19       | 1.60 x 10^-03 |
| Electron transport chain                     | 101         | 12       | 2.40 x 10^-03 |
| Glycolysis and gluconeogenesis               | 50          | 8        | 2.60 x 10^-03 |
| DNA replication                              | 41          | 7        | 3.20 x 10^-03 |
| PlurNetWork                                  | 289         | 23       | 3.20 x 10^-03 |
| IL-5 signaling pathway                       | 80          | 10       | 3.30 x 10^-03 |
| TCA cycle                                    | 32          | 6        | 3.70 x 10^-03 |
| TCR signaling pathway                        | 143         | 14       | 3.70 x 10^-03 |
| Adipogenesis                                 | 133         | 13       | 5.10 x 10^-03 |
| Alzheimer’s disease                          | 77          | 6        | 6.50 x 10^-03 |
| Toll-like receptor signaling                 | 37          | 6        | 6.50 x 10^-03 |
| Cell cycle                                   | 95          | 10       | 8.10 x 10^-03 |
| Amino acid metabolism                        | 112         | 11       | 8.40 x 10^-03 |
| MAPK signaling pathway                       | 165         | 14       | 9.60 x 10^-03 |

Gene ontology analysis of ribo-seq data from LPS-activated B cells, by WebGestalt pathway-enrichment analysis, showing total genes in each pathway (for gene sets with a minimum of six genes) and genes expressed differently in HuR-cKO B cells versus control B cells (DE genes), as well as the adjusted P value for each (hypergeometric test and Benjamini-Hochberg multiple-test correction). PlurNetWork, mechanisms associated with pluripotency; TCR, T cell antigen receptor; MAPK, mitogen-activated protein kinase.

were due to compensatory regulatory mechanisms that affected the abundance or ribosome loading of mRNAs encoding other members of the Elavl family or of other AU-rich element–binding proteins (Supplementary Fig. 3).

Analysis of differences in expression by mRNA-seq and ribo-seq followed by pathway-enrichment analysis showed enrichment for gene signatures related to various aspects of the cell cycle and energy metabolism in HuR-cKO B cells, relative to their abundance in control B cells (Table 1). HuR deficiency influenced the expression of genes encoding molecules related to glycolysis, the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, three pathways in which the expression of these genes was higher both in GC B cells and after activation of B cells with LPS than in naive B cells (Supplementary Fig. 4a–c). Blockade of these energy pathways through the use of 2-deoxyglucose, the inhibitor CPI-613 (which inhibits pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase (α-KGDH)) or oligomycin A decreased the survival, growth and proliferation of B cells after stimulation with mitogen (Supplementary Fig. 4d,e). Analysis of the global mRNA expression and translation of genes related to glycolysis, the TCA cycle and oxidative phosphorylation showed no difference in these parameters in ex vivo control B cells versus ex vivo HuR-cKO B cells (Supplementary Fig. 4f). In contrast, global mRNA expression and translation of these genes were higher in LPS-activated HuR-cKO B cells than in LPS-activated control B cells (Supplementary Fig. 4f), which suggested that HuR was important for regulating the reprogramming of energy metabolism only after activation of the cells. Correlation of data from mRNA-seq and ribo-seq for only those metabolism-related genes translated differently in LPS-activated HuR-cKO B cells than in LPS-activated control cells showed that all of these, with the exception of Dlst, which encodes dihydrodipamoine S-succinyltransferase (DLSL), had modestly increased expression and/or translation in control B cells relative to their expression and/or translation in HuR-cKO B cells (Fig. 4a and Supplementary Fig. 4g,h). The abundance of Dlst mRNA was greater in GC B cells than in naive B cells (Supplementary Fig. 4i), but the expression and translation of Dlst mRNA was significantly lower in LPS-activated B cells from HuR-cKO mice than in LPS-activated B cells from Elavl1fl/flMb1+/+ mice (Fig. 4b).
DLST is one of the three subunits of the α-KGDH enzymatic complex, which is essential for maintaining TCA-cycle flux and cell-energy supply. To understand the role of HuR in regulating Dlst mRNA, we examined mRNA-seq data and plotted the reads mapped across the Dlst locus. These mRNA-splicing profiles showed that a single mRNA transcript was generated after RNA splicing in ex vivo and LPS-activated Elavl1<sup>fl/fl</sup> B cells (Fig. 4c). In the absence of HuR, Dlst mRNA showed two alternative splicing events: retention of intron 10, and alternative inclusion of a cryptic exon between exons 10 and 11 (Fig. 4c). The iCLIP data showed that HuR bound to several locations along Dlst RNA (Fig. 4c and Supplementary Fig. 5a–c). Peak-calling analysis showed that HuR ‘preferentially’ bound to introns, including the poly-pyrimidine tract downstream of the 3′ splice site of the cryptic exon present within intron 10 (Supplementary Fig. 5d). Together these data demonstrated that the binding of HuR to Dlst pre-mRNA might promote the expression and translation of Dlst mRNA in HuR-cKO B cells. The modest change in translation of other components of cell energy pathways might have reflected a compensatory mechanism.

**Binding of HuR to introns modulates alternative intron use**

To gain mechanistic insight into the role of HuR in mRNA splicing in B cells, we further examined the HuR iCLIP data obtained with LPS-activated B cells. Analysis of unique read counts in all three iCLIP experiments showed that 75% of HuR-RNA crosslink sites mapped to introns (Fig. 5a and Supplementary Fig. 5e,f). Visualization of HuR crosslink sites near the exon-intron boundaries indicated that HuR ‘preferentially’ bound to introns and mostly bound between the branch point and the 3′ splice site (Fig. 5b). These data suggested that HuR might be a splicing regulator in B cells; thus, we investigated whether HuR modulates the splicing of pre-mRNA by further analysis of mRNA-seq data obtained with LPS-activated B cells. Analysis of differences in exon use did not reveal substantial differences in exon levels for protein-coding transcripts in the absence of HuR and failed to identify the alternative splicing events associated with Dlst mRNA (Supplementary Tables 1–5). Thus, we performed an ‘intronic’ analysis of the mRNA-seq data (Supplementary Fig. 6a), which showed that 530 introns belonging to 375 genes were used differently in LPS-activated HuR-cKO B cells than in control B cells.
Figure 6 Alternative splicing of Dlst in HuR-cKO B cells diminished the enzymatic activity of α-KGDH. (a) Design of PCR primers (top) spanning the exon 10–intron 10 junction and intron 10–exon 11 junction of Dlst, used to detect intron inclusion in \textit{Elavl1\textsuperscript{fl/fl}}Mb1\textsuperscript{+/+} and HuR-cKO B cells (middle). Bottom, quantification of intron inclusion in \textit{ex vivo} and LPS-activated HuR-cKO B cells, presented in arbitrary units (AU) relative to that in \textit{Elavl1\textsuperscript{fl/fl}}Mb1\textsuperscript{+/+} (HuR +) B cells. *P < 0.05 and **P < 0.0005 (unpaired t-test). (b) PCR across the exon 10–exon 11 junction of Dlst (top), and frequency of alternative exon inclusion (below), calculated after gel-density quantification of long amplics (alternative exon included) and short amplics (alternative exon skipped). (c) mRNA-seq analysis of Dlst reads in \textit{ex vivo} and LPS-activated \textit{Elavl1\textsuperscript{fl/fl}}Mb1\textsuperscript{+/+} and HuR-cKO B cells; results were normalized by library size factor. Adjusted P values: *P < 0.05 and **P < 0.0005 (Benjamini-Hochberg multiple test correction). (d) Quantification of ribosome ‘footprints’ mapped to \textit{Dlst} mRNA in \textit{ex vivo} and LPS-activated B cells (results normalized as in c). Adjusted P values: *P < 0.0005 (Benjamini-Hochberg multiple test correction). (e) Immunoblot analysis of DLST and β-actin (loading control) in lysates of \textit{ex vivo} and LPS-activated splenic B cells from control and HuR-cKO mice (numbers above lanes indicate experiment number). (f) Gel-density quantification of DLST abundance in \textit{ex vivo} and LPS-activated control and HuR-cKO B cells, presented relative to β-actin abundance. Each symbol represents a biological replicate (n = 3 per group); small horizontal lines indicate the mean. *P < 0.05 (unpaired t-test). (g) Enzymatic activity of α-KGDH in total extracts of \textit{ex vivo} \textit{Elavl1\textsuperscript{fl/fl}}Mb1\textsuperscript{+/+} and HuR-cKO B cells. *P < 0.05 (unpaired t-test). Data are from two independent experiments (a,b,g; mean and s.d. of n = 6 (\textit{ex vivo}) or 4 (LPS-treated) biological replicates (a,b) or n = 4 samples per group (g)), two experiments (c,d; mean and s.d. of n = 3–4 (c) or 4–5 (d) samples per group) or three independent experiments (e,f).

HuR modulates mRNA expression and translation via splicing
Analysis of all 375 genes identified above with different intron use in HuR-cKO B cells versus control B cells (group 1) showed no global change in the expression and translation of these genes in the absence of HuR (Supplementary Fig. 6c). Individually, 64 of those 375 genes were expressed differently in LPS-activated HuR-cKO B cells than in LPS-activated \textit{Elavl1\textsuperscript{fl/fl}}Mb1\textsuperscript{+/+} B cells and their mRNAs were bound to HuR (group 2) (Fig. 5d). A similar data correlation showed that 71 of the 375 genes were both translated differently in LPS-activated HuR-cKO B cells than in LPS-activated \textit{Elavl1\textsuperscript{fl/fl}}Mb1\textsuperscript{+/+} B cells and the mRNA bound to HuR (group 3) (Fig. 5e). Only 25 of those 71 genes were both expressed differently and translated differently in HuR-cKO B cells than in \textit{Elavl1\textsuperscript{fl/fl}}Mb1\textsuperscript{+/+} B cells (group 4) (Fig. 5f). When we analyzed the expression of genes in groups 1, 2 and 3 globally, we observed no differences in mRNA abundance in HuR-cKO B cells versus \textit{Elavl1\textsuperscript{fl/fl}}Mb1\textsuperscript{+/+} B cells (Fig. 5g). In contrast, global translation of these mRNAs was significantly lower in HuR-cKO B cells than in \textit{Elavl1\textsuperscript{fl/fl}}Mb1\textsuperscript{+/+} B cells (Fig. 5h), which suggested that even though HuR-dependent regulation of alternative splicing might not necessarily affect the overall level of mRNA, HuR was required for mRNA translation. Global expression and global translation of the genes in group 4 were both up to 50% lower in HuR-cKO B cells than in \textit{Elavl1\textsuperscript{fl/fl}}Mb1\textsuperscript{+/+} B cells (Fig. 5h). Closer examination of individual genes indicated that both mRNA expression and mRNA translation of 76% of the genes in group 4 (19 of 25), including \textit{Dlst}, were lower in the absence of HuR (Fig. 5i,j). In summary, analysis of differences in intron use and its correlation with differences in expression as analyzed by mRNA-seq and ribo-seq allowed us to discover the alternative intron events associated with the binding of HuR. Visualization of read coverage revealed high complexity in mRNA splicing associated with the 25 genes in group 4 and detected up to eight different alternative splicing events associated with \textit{Slc25a19} (which encodes the mitochondrial thiamine pyrophosphate carrier TPC) in the absence of HuR (Supplementary Fig. 6d,e). Genetic deletion of either \textit{Dlst} or \textit{Slc25a19} has been associated with diminished α-KGDH enzymatic activity and alterations in the TCA cycle\textsuperscript{23,24}.

HuR regulates splicing of Dlst mRNA
To understand the role of HuR in mRNA splicing, we selected \textit{Dlst} mRNA for detailed analysis and evaluated the use of intron 10 by two RT-PCR assays. HuR-cKO B cells showed more intron retention than did \textit{Elavl1\textsuperscript{fl/fl}}Mb1\textsuperscript{+/+} B cells (Fig. 6a). Read-coverage analysis of the \textit{Dlst} locus with mRNA-seq data from HuR-cKO B cells identified a previously unknown alternative exon–splicing site located after exon 10 (Fig. 6c). This alternative exon 10b was previously annotated in Ensembl as the start exon of predicted transcript ENSMUST00000165575; however, evidence of the existence of this transcript is lacking. The detection of reads mapped across the exon 10–alternative exon 10b junction suggested that this was an alternative exon inclusion rather than an alternative first exon. RT-PCR confirmed that conclusion and indicated that over 50% of \textit{Dlst} transcripts in HuR-cKO B cells included the alternatively spliced...
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Figure 7 The enzymatic activity of α-KGDH is required for the survival and proliferation of B cells. (a) Viability of splenic B cells obtained from C57BL/6 mice and activated for 96 h with LPS and IL-4 in the presence of various doses (horizontal axes) of PESP (top) or SP (bottom). *P < 0.05, **P < 0.01 and ***P < 0.001, control (0) versus each dose (unpaired t-test). (b) Flow cytometry analyzing dilution of the dye CellTrace Violet (CTV) in the cultures in a (left), and quantification of cells in each generation, calculated on the basis of dye dilution (right). (c) In vitro proliferation of Dlst+/+ and Dlst−/− splenic B cells after activation for 96 h with LPS and IL-4 (analyzed by dye dilution as in b). *P = 0.0162 (two-way analysis of variance). (d) Quantification of IgG1+ cells in the cultures in c (right), assessed as CellTrace Violet dilution versus IgG1 surface expression (left). Each symbol (right) represents an individual mouse (n = 6 per genotype); small horizontal lines indicate the mean. Numbers adjacent to outlined areas (left) indicate percent IgG1+ cells. *P < 0.05 (unpaired Student’s t-test). (e) Analysis of CSR in the cultures in a and cells cultured in medium alone (Med). Numbers in outlined areas (left) indicate percent IgG1+ cells. *P < 0.05, **P < 0.01 and ***P < 0.001, untreated cells versus each other condition (unpaired Student’s t-test). Each symbol (a,e) represents an individual mouse. Data from one experiment representative of two independent experiments (a,b,e; mean and s.d. of n = 4 mice per group) or from two independent experiments (c,d; mean ± s.d. of n = 6 mice per genotype).

ROS scavengers ‘rescue’ B cell proliferation and CSR

Analysis of B cell cultures showed that cell viability was reduced in the absence of HuR (Fig. 8a,b). The viability of HuR-cKO B cells was not restored by blockade of caspase-dependent apoptosis or programmed necrosis through the use of the caspase inhibitor Q-VD-Oph or necrostatin-1, respectively, but was restored by the H2O2 scavenger catalase (Fig. 8b,c). Consistent with those results, fluorescence of the ROS reporter dye H2DCFDA showed that viable HuR-cKO B cells produced more ROS than did control B cells (Fig. 8b). The addition of catalase diminished H2DCFDA fluorescence (Fig. 8b), indicative of lower ROS concentration.

HuR-cKO B cells failed to proliferate when cultured with mitogens in vitro (Fig. 8d and Supplementary Fig. 8). Supplementation of the
medium with catalase as well as ROS scavengers such as sodium pyruvate, N-acetyl cysteine or EUK134 enhanced the proliferation of HuR-cKO B cells in response to a variety of stimuli (Fig. 8d and Supplementary Fig. 8). Quantification of the B cells recovered showed that HuR-cKO B cells and control B cells underwent a similar number of cell divisions; however, there were fewer HuR-cKO cells in each division (Fig. 8e), which indicated that the ROS scavengers did not completely restore proliferation. The addition of ROS scavengers led to a recovery in the frequency and number of IgG1+ and CD138+ (ASC) HuR-cKO B cells (Fig. 8f,g). However, NAC and EUK134 marginally inhibited the yield of IgG1+ and CD138+ cells in cultures of control B cells (Fig. 8f,g). Together our data indicated that HuR-cKO B cells contained elevated amounts of ROS and these compromised B cell survival. Mitigating ROS allowed the HuR-cKO B cells to proliferate, undergo CSR and form ASCs in vitro.

**DISCUSSION**

Here we found that HuR was dispensable for B cell development but that the GC response and the production of class-switched antibodies following immunization with thymus-independent antigens was highly dependent upon HuR. Our *in vitro* experiments indicated that HuR might control cell metabolism upon B cell activation to limit ROS production, but further confirmation in the context of the GC response is required.

The regulation of intracellular ROS is important during the activation of lymphocytes. A greater abundance of ROS is found in activated B cells from virus-infected mice. B cells deficient in the voltage-gated proton channel HVCN1 produce a lower abundance of ROS and have attenuated antibody responses, and the administration of antioxidants to mice increases B cell apoptosis and decreases B cell proliferation. However, lack of ROS production in mice deficient in the gp91phox subunit of the NADPH oxidase complex has been linked to modestly increased responses to T cell–independent type 2 antigens. Thus, a general picture is emerging for B cells in which ROS production can be separated in space and time. Low levels of ROS have mitogenic properties, whereas high levels will induce cell death. administration of antioxidants to mice increases B cell apoptosis and decreases B cell proliferation. However, lack of ROS production in mice deficient in the gp91phox subunit of the NADPH oxidase complex has been linked to modestly increased responses to T cell–independent type 2 antigens. Thus, a general picture is emerging for B cells in which ROS production can be separated in space and time. Low levels of ROS have mitogenic properties, whereas high levels will induce cell death.

The presence of hydroxyl radicals also enhances the deamination of cysteine to uracil, a reaction that is necessary for CSR. CSR requires control of ROS levels by DNA damage–sensing proteins such as p53.
and Atm. p53 limits ROS production in B cells and promotes switching to IgG2a. Atm expression during the repair of double-stranded DNA breaks decreases ROS levels via the regulation of cell energy pathways. Atm−/− mice have an impaired antibody response and elevated ROS that is restored by treatment with the antioxidant N-acetyl-L-cysteine. Furthermore, the Atm-specific inhibitor KU-55933 decreases cellular ATP levels by diminishing TCA cycle intermediates and oxidative respiration. Thus, we suggest that diminished energy metabolism during CSR might limit progression through the cell cycle and might prevent the accumulation of ROS until double-stranded DNA breaks are repaired. By sustaining the TCA cycle, HuR ensures provision of the reducing equivalents required for ATP synthesis and/or ROS scavenging.

Evidence has accumulated demonstrating that increased glycolysis is part of a metabolic switch during activation of cells of the immune system. Although the TCA cycle is the main provider of reducing equivalents for oxidative phosphorylation, its regulation during lymphocyte activation remains largely unexplored. We used CPI-613 to inhibit PDH and α-KGDH, two key enzymes of the TCA cycle; this resulted in a block in the growth and proliferation of B cells. It has been reported that CPI-613 induces an oxidative burst responsible for inhibition of the enzymatic activity of α-KGDH. This observation underscores the importance of regulating ROS production during B cell activation and is strengthened by our finding that phosphate analogs of α-KGDH diminished the viability and proliferation of B cells. The number of IgG1+ cells recovered after activation of B cells in vitro in the presence of 10 mM PESF was fivefold lower than the number recovered in the absence of PESF, even though cell proliferation was less affected by the presence of PESF, findings consistent with our results obtained with Dlst−/− B cells, which would suggest that B cells undergoing CSR are more susceptible to ROS than are cells not undergoing CSR.

Notably, Dlst−/− B cells did not recapitulate the in vitro phenotype of HuR-cKO B cells. This probably reflects as-yet-unidentified roles for other targets of the HuR splicing program that are important during B cell activation. Among the 375 genes identified as HuR dependent was Slc25a19; deletion of this gene also diminishes the enzymatic activity of α-KGDH.

The α-KGDH complex has three subunits: α-oxoglutarate dehydrogenase (encoded by Ogdh), DLST (encoded by Dlst), and dihydrolipoamide dehydrogenase (encoded by Dld). Dlst is a haploinsufficient gene, and Dlst−/− mice show increased ROS production and cell death in the brain. The APP670/671 mutation of DLST in humans diminishes enzyme activity and causes Alzheimer’s disease associated with mitochondrial dysfunction and cell death. Decreased DLST diminishes the provision of reducing equivalents and succinyl-CoA required for GTP synthesis by matrix-substrate-level phosphorylation. Increased ROS levels in the absence of HuR may be due to increased translation of mRNA from genes encoding components of the oxidative respiratory chain, but diminished matrix-substrate-level phosphorylation may additionally contribute to ROS production by increasing ADP levels. Furthermore, α-KGDH activity generates O2−, which acts as a negative regulator of α-KGDH enzymatic activity. Thus, oxidative stress may promote cell death due to inhibition of flux through the TCA cycle.

Our approach of integrating global analysis of the expression and translation of mRNA comparing control and HuR−/− cKO primary B cells has revealed a role for HuR in the regulation of metabolism after B cell activation. We applied a bioinformatics approach to identify differences in intron use based on read counting within intron ‘bins’ defined as windows between annotated exons. We identified 530 introns from 375 genes that were expressed differently in the absence of HuR. HuR loss was associated with aberrant intron inclusion that compromised, for Dlst, the abundance and translation of mRNA but might also affect the isoform of translated proteins. Quantitative analysis of full-length transcripts and alternative protein isoforms in the presence and absence of HuR should address this. We suggest that HuR, like other splicing regulators, promotes the correct splicing of pre-mRNA and provides a quality-control mechanism for the transcriptome. In the context of B cell activation, depletion of HuR promotes an imbalance in energy metabolism that led to the fatal accumulation of ROS and impaired B cell proliferation and CSR.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Access code** GEO: mRNA-seq, GSE62129; ribo-seq, GSE62134; iCLIP, GSE62148.

**Note:** Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

A.F.C. provided advice on experimental design; M.D.D.-M. and M.T. designed and performed experiments; M.D.D.-M., S.E.B., K.F. and M.T. designed and performed all high-throughput sequencing experiments; M.D.D.-M., E.M.-C., M.G.-P., S.R.A. and K.Z. participated in bioinformatics analysis; T.C. and J.U. designed the iCount pipeline for iCLIP analysis; V.I.B. provided the inhibitors SP and PESF and advice on their cellular application and action; W.A.H. helped with procedures involving Dlst−/− mice; S.H. and G.E.G. provided Dlst−/− mice generated by Lexicon Pharmaceuticals; D.L.K. provided Elav1tm1Dkon mice; and M.D.D.-M. and M.T. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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Mouse strains and animal procedures. Mice on the C57BL/6 background used in this study were CD79a<sup>tm1cre</sup>Reth (ref. 51) and Elavl1<sup>tm1Dxon</sup> PSl<sup>+</sup>- mice (generated by Lexicon Pharmaceuticals)<sup>34</sup>. Mice were immunized by intraperitoneal administration of sheep red blood cells (2 × 10<sup>6</sup> per mouse), NP-Ficoll (25 μg), NP-LPS (25 μg) or alum-precipitated NP-KLH (100 μg). For<sup>in vivo</sup> experiments, the number of animals was decided on the basis of preliminary studies with small cohorts to assess the magnitude of the changes (two to three mice per genotype). Six to ten mice (12–16 weeks of age) were considered a sufficient number of mice in final studies to reach sufficient statistical power analysis. No animals were excluded due to a lack of responsiveness to immunization. Randomization, but not experimental 'blinding' to sample identity, was used for these studies. All animal procedures at the Babraham Institute were approved by the Animal Welfare and Experimentation Committee of Babraham Institute and the UK Home Office. The Local Ethical Committee for Animal Experiments of KU Leuven, Belgium approved experiment with Dlts<sup>+</sup>- mice (license number 000/2014).

**Online Methods**

Reagents, antibodies and oligonucleotides. This information is provided in Supplementary Tables 6 (reagents), 7 (antibodies) and 8 (oligonucleotides).

Enzyme-linked immunosorbent and enzyme-linked immunospot assay. Serum immunoglobulins and NP-specific antibodies were detected by enzyme-linked immunosorbent assay<sup>9</sup>. NP-specific antibody endpoint titers were used as a measure of relative concentration. NP-specific ASCs were detected by enzyme-linked immunospot assay as described<sup>9</sup>.

Flow cytometry. B cell populations were analyzed with anti-B220, anti-CD19, anti-CD21, anti-CD23, anti-c-kit, anti-CD138, anti-CD93, anti-IgG1, anti-IgD, anti-IgM, anti-CD95, anti-CD40, anti-CD69, anti-GL7 and anti-CD86 (Supplementary Table 7).

Protein separation and immunoblot analysis. Total cell extracts were prepared by incubation of cells in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate) supplemented with protease inhibitors. 10% polyacrylamide-SDS gels were loaded with 20 μg of total protein extract per lane. Gel electrophoresis and protein transfer to nitrocellulose membranes was performed before detection of HuR, DLST and β-actin by immunoblot analysis with mouse monoclonal anti-HuR, rabbit monoclonal anti-DLST or rabbit polyclonal anti-β-actin (Supplementary Table 7) and visualization by enhanced chemiluminescence or IRDye detection at 694 or 794 nm.

RNA extraction and RT-PCR assays. Total RNA was extracted from purified B cells with TRIZol (LifeTech). 1 μg of RNA was treated with DNase I before reverse transcription into cDNA. Dlts alternative splicing events were analyzed by RT-PCR with specific primers (Supplementary Table 3). Intron retention was assessed by quantitative PCR, whereas alternative exon inclusion was quantified after gel densitometry of the PCR products. Quantitative PCR assays were performed with Platinum SYBR Green qPCR SuperMix (Life Technologies). Expression of mRNA from Dlts and Elavl1 was quantified by the comparative threshold cycle (ΔΔCT) method and results were normalized to the expression of 18S RNA.

iCLIP. iCLIP experiments were performed as described<sup>23</sup> with lyses of splenic B cells obtained from C57BL/6 mice and activated in vitro for 48 h with LPS. Splenic B cells were irradiated with ultraviolet light for crosslinkage of protein-RNA (150 mJ/cm<sup>2</sup>; Stratallinker 2400). After washing of cells with ice-cold PBS, total cell extracts were obtained with RIPI buffer and sonication (three times). Extract supernatants were treated for 3 min at 37 °C with RNase I (0.167 U/ml). Hu-RNA complexes were immunoprecipitated using 2 μg mouse monoclonal anti-HuR (Supplementary Table 8) coupled to protein G Dynabeads. Extracts of HuR-CKO B cells were used as a negative control. After washing of samples twice with high-salt buffer (50 mM Tris-HCL, pH 7.4, 1 M NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate) and once with PKN washing buffer (20 mM Tris-HCL, pH 7.4, 10 mM MgCl<sub>2</sub> and 0.2% Tween-20), one tenth of each sample was labeled with <sup>32</sup>P<sub>3</sub>IPATP with PKN, whereas an RNA linker was ligated to the rest of the sample after dephosphorylation of RNA. RNA-protein complexes were separated by SDS-PAGE and were transferred to nitrocellulose membranes. Hu-RNA complexes with a molecular size above 55 kilodaltons were isolated after visualization by autoradiography. RNA was extracted by incubation of the nitrocellulose fragment for 10 min at 37 °C with proteinase K in PK buffer (100 mM Tris-HCL, pH 7.5, 50 mM NaCl and 10 mM EDTA). 200 μl of PK buffer containing urea (7 M) was added to each sample, followed by further incubation for 20 min at 37 °C. RNA was isolated by phenol-chloroform extraction and ethanol precipitation. RNA was retro-transcribed into cDNA with RCLIP primers (Supplementary Table 9) and SuperScript III reverse transcriptase. After purification of cDNA with 6% TBE-urea gels, cDNA was circularized and amplified by PCR with Solesa PS/Ip7 primers. cDNA libraries were thus prepared and analyzed. RCLIP primers included a seven-base-long ‘barcode’ at the 5′ end (three known bases plus four unknown nucleotides). An additional two bases (AT) were added to the 3′ end. These ‘barcodes’ allowed us to ‘multiplex’ different cDNA libraries for Illumina sequencing and identify PCR duplicate reads.

High-throughput sequencing and library preparation. mRNA-seq libraries were obtained with a TruSeq Stranded mRNA Sample Prep Kit (Illumina). Splenic B cells from individual control or HuR-CKO mice were independently processed for RNA extraction (<i>ex vivo</i> samples) or were stimulated for 48 h with LPS in RPMI medium plus 1 μM sodium pyruvate.

Published mRNA-seq libraries were used for analysis of the expression of genes encoding molecules involved in glycolysis, the TCA cycle and electron transport in naive and GC B cells (GEO accession code GSE47705)<sup>53</sup>. Ribosomal profiling ('footprinting') (<i>ribo-seq</i>) assays were performed with an ARTSeq Ribosome Profiling Kit (Epimcrite, Illumina). <i>Ex vivo</i> or LPS-activated B cells were treated with cycloheximide (100 μg/ml) 3 min before the preparation of cell extracts.

cDNA libraries were sequenced with either GAIIx (iCLIP) or HTSSeq<sub>2000</sub> (mRNA-seq and ribo-seq) Illumina technology. The type of sequencing performed was as follows: iCLIP, 40 bp, single end; mRNA-seq, 100 bp, single end; and ribo-seq, 50 bp, single end.
Bioinformatics and statistical analysis. Mapping and peak call analysis of HuR iCLIP data was performed as described. RCLIP primers were used to generate the cDNA libraries from HuR iCLIP experiments. Data ‘demultiplexing’ involved the identification of the three known bases of the 5′ ‘barcode’. Quality analysis of sequencing data was done with the FastQC tool. Data analysis was done in the iCount pipeline. First, the four unknown bases of the 5′ ‘barcode’ were used for removal of PCR duplicate reads. The 3′ ‘barcode’ (AT) was used to define the length of the reads. Both parameters were important for the identification of unique reads. Then, reads were trimmed for removal of the 5′ and 3′ ‘barcodes’ along with any adaptor sequence before mapping to the genome with Bowtie software for the alignment of short DNA sequences. Data were originally annotated to the mm9 NCBI assembly of the mouse genome, but was ‘lifted’ over to mm10 NCBI assembly of the mouse genome with the Galaxy platform. Once genome annotation was completed, nucleotide position –1 (relative to the first base annotated, set as position 0) was located and was used for data visualization in the UCSC genome viewer. This base was the HuR-crosslink–binding site. The number of cDNA counts associated with a single HuR-crosslink site was quantified on the basis of read length and unique 5′ ‘barcode’ sequence. Peak call analysis was performed for the identification of unique HuR-crosslink–binding sites with a false-discovery rate of <0.05. Peak call analysis was performed in iCount by genome-wide analysis of 30 base windows defined after annotation at single-nucleotide resolution of HuR-RNA interactions (15 nucleotides flanking a crosslink site). Peak enrichment analysis and false-discovery rate calculation were performed with the allowance of 100 permutations and multiple-hypothesis testing to correct for multiple comparisons.

Raw sequencing data from mRNA-seq and ribo-seq was ‘demultiplexed’, analyzed with FastQ and mapped to the mm10 genome annotation with TopHat software (splice junction mapper for mRNA-seq reads). Analysis of differences in gene expression for mRNA-seq and ribo-seq data sets was performed with the DESeq and DESeq2 software packages of the R project for statistical computing. Data were adjusted to a negative binomial distribution after correction by library size factor. Analysis of differences in expression, calculation of $P$ values and Benjamini-Hochberg multiple test correction of the $P$ values were performed to obtain final adjusted $P$ values. The DEXSeq package was used for analysis of exon and intron use. First, we defined exons and introns through use of the Ensembl exon annotation for protein-coding transcripts (GRCm38.72 genome assembly of the Ensembl database), and we independently counted reads within the exon and intron bins through the use of HTSeq. Read counting along exon bins was required for estimation of the library size factors. We then used these size factors to normalize the intron bins and assessed differences in intron use with DEXSeq. A hypergeometric statistical test followed by Benjamini-Hochberg correction of the $P$ values was performed for pathway-enrichment analysis. iCLIP sequencing data were visualized with the UCSC genome browser. The Integrated Genome Browser (Integrative Genomics Viewer) was used for read coverage analysis and generation of ‘sashimi plots’ of mRNA-seq and ribo-seq data.

Unpaired $t$-tests or Mann-Whitney tests were performed for statistical analysis of non-sequencing data. Additional details about sample size and statistical tests are provided in the figure legends.

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