An ontogenetic switch drives the positive and negative selection of B cells

Xijin Xu[a], Mukta Deobagkar-Lele[a], Katherine R. Bull[a], Tanya L. Crockford[a], Adam J. Mead[b], Adam P. Cribbs[c], David Sims[c], Consuelo Anzilotti[a], and Richard J. Cornall[a,1]

[a]Medical Research Council Human Immunology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, OX3 9DS Oxford, United Kingdom; [b]Medical Research Council Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, OX3 9DS Oxford, United Kingdom; and [c]Medical Research Council, Weatherall Institute of Molecular Medicine, Centre for Computational Biology, Weatherall Institute of Molecular Medicine, University of Oxford, OX3 9DS Oxford, United Kingdom

Edited by Michael Reth, University of Freiburg, Freiburg, Germany, and approved January 6, 2020 (received for review September 3, 2019)

Developing B cells can be positively or negatively selected by self-antigens, but the mechanisms that determine these outcomes are incompletely understood. Here, we show that a B cell intrinsic switch between positive and negative selection during ontogeny is determined by a change from Lin28b to let-7 gene expression. Ectopic expression of a Lin28b transgene in murine B cells restored the positive selection of autoreactive B-1 B cells by self-antigen in adult bone marrow. Analysis of antigen-specific immature B cells in early and late ontogeny identified Lin28b-dependent genes associated with B-1 B cell development, including *Arid3a* and *Bhleh41*, and Lin28b-independent effects are associated with the presence or absence of self-antigen. These findings identify cell intrinsic and extrinsic determinants of B cell fate during ontogeny and reconcile lineage and selection theories of B cell development. They explain how changes in the balance of positive and negative selection may be able to adapt to meet the immunological needs of an individual during its lifetime.

B cell | selection | ontogeny

The development of different lymphocyte subsets changes during the life of an individual, but the mechanisms that underlie these changes are poorly understood. In the B cell compartment, early ontogeny is dominated by formation of B-1 B cells, an innate-like subset that produces the majority of secreted IgM and provides a first line of defense against pathogens (1, 2). B-1 B cells are readily detected in the peritoneal and pleural cavities, but they are able to recirculate throughout the secondary lymphoid organs (3). They develop predominantly from the fetal liver (FL) and neonatal liver (NL) but then self-renew throughout adult life (4–7). In this respect, B-1 B cells differ from conventional follicular (FO) and marginal zone (MZ) B-2 B cell subsets that are constantly replaced from precursors in the adult bone marrow (BM). B-1 B cells are often divided on the basis of the expression of the inhibitory receptor CD5 (8), into B-1a ($CD5^+$) and B-1b ($CD5^-$) subsets, although the significance of this difference is unknown (4). The developmental origin of B-1 B cells has been a matter of controversial debate, centered around independent theories relating to their lineage and their positive selection.

In terms of the B-1 B cell lineage hypothesis, it has been suggested that precursors with B-1 B cell potential may exist before the appearance of definitive hematopoietic stem cells (HSCs) (9), although the contribution of these precursors to the B-1 B cell pool is uncertain. More robust evidence tracing the progeny of HSCs with cellular barcoding has shown that while FL HSCs can efficiently give rise to B-1 B cells, each FL HSC has, over time, the potential to generate both B-1 and B-2 B cells (10). Studies of BM chimeras have also shown that adult BM HSCs can generate B-1 B cells (11, 12), although they are not as efficient as FL HSCs. Elsewhere, the Lin28b/let-7 signaling axis has been identified as a key pathway controlling the transition from fetal to adult hematopoiesis (13–15) and B-1 B cell development (6, 15, 16). Ectopic expression of a retrovirally encoded Lin28b transgene (tg) in adult BM HSCs increased $CD5^+$ B-1a B cell development (15), while expression of let-7b in FL pro-B cells blocked the development of B-1 B cells (17). These findings support the notion of hard-wired differences during ontogeny, but possibly downstream of the HSC commitment stage.

Several lines of evidence also suggest that B-1 B cells can undergo positive selection, which is linked to their B cell receptor (BCR) specificity. B-1 B cells are frequently autoreactive (2), have a restricted BCR repertoire (18), and often seem to depend upon a strong BCR signal for their differentiation (19). Mutations that decrease BCR signaling or its costimulation tend to reduce B-1 B cell differentiation (4), whereas mutations leading to the loss of the negative regulators of BCR signaling have the opposite effect (20, 21). Several BCRs exclusive to B-1 cells have been found to recognize self-carbohydrates and lipids (22), and it is proposed that these may give rise to antibodies that contribute to tissue homeostasis by clearing cellular debris. The transgenic expression of VH12/Vk4 and VH11/Vk14 (Vk9) BCRs specific for host phosphatidylcholine (PtC) will also result in the development of B-1 B cells rather than B-2 B cells (23). Recent data suggest B-1–like B cells can differentiate from splenic B cells that have converted their BCR specificity to PtC and are free to expand in *rag*-deficient hosts (24); however, it is unclear if this can occur in a normal physiological setting. Collectively, however, these results suggest

Significance

The early life of an individual mouse is characterized by the generation of a population of long-lived and frequently autoreactive B cells, called B1 cells, which are largely developed in the fetal liver, whilst adult life is characterized by continuous B cell production in the bone marrow, the development of normally short-lived B cells and the stringent elimination of any immature B cells that are dangerously autoreactive. In this study, we show that the change from positive to negative selection in response to self-proteins requires a Lin28b to Let-7 microRNA-dependent switch, which coincides with the transition from fetal liver to bone marrow B cell development.

Author contributions: X.X., M.D.-L., K.R.B., T.L.C., A.J.M., A.P.C., D.S., C.A., and R.J.C. designed research; X.X., M.D.-L., K.R.B., T.L.C., A.J.M., A.P.C., D.S., C.A., and R.J.C. performed research; X.X., M.D.-L., K.R.B., T.L.C., A.J.M., A.P.C., D.S., C.A., and R.J.C. analyzed data; and X.X., M.D.-L., C.A., and R.J.C. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, http://www.ncbi.nlm.nih.gov/geo (accession no. GSE135650).

[1]To whom correspondence may be addressed. Email: richard.cornall@ndm.ox.ac.uk.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1915247117/-/DCSupplemental.

First published February 4, 2020.

that BCR specificity and signaling have an important role in the differentiation of the B-1 B cell lineage.

Direct evidence for the positive selection of B-1 B cells by self-antigens during normal development came initially from a study showing that mice expressing an anti-Thy-1 BCR tg develop Thy-1–specific B-1 B cells in the presence of the self-antigen, but not in its absence (25). Later, we showed that positive selection can also occur in MD4 tg mice that express an anti-hen egg lysozyme (HEL) BCR tg (C57BL/6-Tg(MD4)4Ccg/J) (26). In the anti-HEL model, the anti-HEL–specific MD4 B-1 B cells are positively selected by coexpression of a membrane-bound intracellular HEL neo self-antigen ($mHEL^{KK}$), which is sequestered within cells by a C-terminal cytosolic dilysine ER retention motif (27). This contrasts with the situation in the absence of self-antigen, where developing MD4 B cells differentiate predominantly into follicular (FO) and marginal zone B-2 B cells. Importantly, exposure to the same antigen in later life causes negative selection, indicating that the capacity of B cells to undergo positive selection in response to $mHEL^{KK}$ changes during ontogeny (27, 28). Hence, this model allows us to investigate the basis of the ontogenetic switch associated with positive and negative selection in the context of a specific BCR.

Results

The Ontogenetic Lin28b/Let7 Switch Is Associated with Positive vs. Negative Selection Potential. We used the MD4/$mHEL^{KK}$ double tg mouse model to study the mechanism of B-1 B cell positive selection. Because B-1 B cell populations can arise from both FL and NL, we reconstituted lethally irradiated $CD45.1^+$ $mHEL^{KK}$ mice with either MD4 $CD45.2^+$ NL from 1-d-old donors or MD4 $CD45.2^+$ BM from mice aged 10 wk. In agreement with our previous findings, $B220^{lo}IgM^+IgD^-$ MD4 B-1 B cells were positively selected by antigen in mice reconstituted with NL, whereas all B cell subsets were negatively selected by the same antigen in mice reconstituted with adult BM (Fig. 1 *A* and *B*). As before (26), the positively selected B-1 B cells did not express the coreceptor CD5, although the significance of this is unclear. We can conclude from this study that B-1 B cell positive selection is intrinsic to the hematopoietic compartment in early ontogeny.

Since developing B cells first express a fully formed BCR at the immature B cell stage, we reasoned that the earliest intrinsic differences in the potential for positive vs. negative selection during ontogeny might manifest at this developmental stage. To explore this hypothesis, we flow-sorted and then compared $B220^+IgM^{a+}IgD^{a-}$ immature B cells from NL and adult MD4 single tg BM, in the absence of self-antigen (Fig. 1 *C*, *Upper*). Total RNA sequencing revealed a pattern of differential gene expression characterized by higher transcript levels of the RNA binding protein, Lin28b, and its downstream targets such as *Igf2bp3*, *H19*, and *Hmga2* in MD4 immature B cells from NL compared to MD4 immature B cells from adult BM (Fig. 1 *C*, *Left*). In agreement with the counterregulation of let-7 microRNAs by Lin28b and H19, microRNA sequencing showed that let-7 family members were >twofold enriched in MD4 immature B cells from adult BM compared to NL (Fig. 1 *C*, *Right*).

To validate these findings beyond the tg model, we next compared differentially expressed genes from three distinct datasets, namely, (*i*) our dataset of MD4 immature B cells comparing NL vs. BM and datasets derived (or curated) from the Immgen database (www.immgen.org) that compared FL vs. BM gene expression in (*ii*) pro-B cells (Hardy Fractions B-C) and (*iii*) immature B cells (Fraction E) from nontg mice. *Lin28b*, *H19*, *Igf2bp3*, and *Arid3a* were among 13 genes up-regulated in FL or NL in common to all three groups (*SI Appendix*, Fig. S1). Of these, Arid3a has been described as a transcriptional regulator required for B-1 B cell selection (17). We also compared the subset of mouse genes expressed at a higher level in MD4 immature NL vs. adult BM B cells with a published dataset of 1,527 predicted Lin28b target genes based on Lin28b binding with PAR CLIP in a human cell line (29). We found enrichment for these genes in the NL ($P = 0.00130$ and enrichment score 0.328) (*SI Appendix*, Fig. S2*A*). Gene ontogeny showed that the NL differentially expressed genes were associated with biochemical processes including translation repressor mRNA binding, cell division, DNA repair, and ribosomal RNA maturation, consistent with the prosurvival and oncogenic effects associated with Lin28b (*SI Appendix*, Fig. S2*B*).

In conclusion, the MD4 NL and BM expression profiles are consistent with the formation of a bimodal switch involving Lin28b and let-7. The consistency of the transcriptomic signal between WT, nontg animals with a varied BCR repertoire and MD4 tg mice expressing a single specific BCR shows that the Lin28b/let-7 bimodal switch is independent of BCR specificity (Fig. 1*D*).

Switching Is Associated with the Relocation of B Cell Development from Liver to BM. To explore the timing of the Lin28b/let-7 switch, we measured the expression of Lin28b protein in immature MD4 B cells from the liver and BM at different times after birth by flow cytometry. Lin28b expression was consistently high in immature B cells from the liver for up to 3 wk postnatally while, from 2 wk onward, it was present at lower and diminishing levels in immature B cells from the BM (Fig. 2 *A* and *B*). The findings suggest that the switch from Lin28b to Let7-dependent profiles is linked to the site of B cell development.

To investigate the synchronicity of the switch from Lin28b to let-7, we performed qPCR on RNA from single and pooled immature MD4 NL and adult BM B cells (20 individual cells and 100 cell pools) and T and myeloid cells from adult BM (100 cell pools) (Fig. 2*C*). The Lin28b target Igf2bp3 was highly expressed in the pooled MD4 immature B cell NL sample and was detectable in 19/20 individual immature MD4 B cells from NL but only 4/20 immature MD4 B cells from adult BM (χ^2 23.01, $P < 0.0001$) (Fig. 2*C*). A similar pattern existed for Arid3a (χ^2 7.6, $P = 0.015$) (Fig. 2*C*). In the pooled samples, Lin28b and H19 transcripts were expressed only in the MD4 immature B cell NL, but at lower levels and, probably as a consequence, reached the threshold for detection in only some single immature B cells from NL (Fig. 2*C*). Overall, these data support the idea that, while the Lin28b/let-7 switch is developmentally unidirectional, it is also binary at a single-cell level and has a strong association with location.

To further define the timing of the switch, we flow-sorted immature MD4 B cells and sequenced total RNA from 1-d NL, 3-wk BM, and 10-wk BM. We identified 264 transcripts that were significantly higher and 240 transcripts that were lower in immature MD4 B cells from NL compared to 10 wk adult BM (fold change >2 and $P < 0.05$) (Fig. 2*D*). In contrast, by similar criteria only 39 transcripts were higher and 28 transcripts lower in immature MD4 B cells from 3-wk BM compared to adult BM. Of these transcripts, just 27/39 and 19/28 maintain the pattern of differential expression from the NL stage (Fig. 2*D*). Therefore, using this model, we could show that the transition from early (FL dependent) to late (BM dependent) ontogeny is largely complete by 3 wk after birth.

Expression of Lin28b Restores Positive Selection of B Cells from Adult BM. To investigate whether ectopic expression of Lin28b could reverse the let-7 driven pattern of adult lineage in B cells, we obtained LSL-*Lin28b* tg mice, which had been backcrossed onto a C57BL/6 background (a gift from Johannes Schulte, Charité Hospital, Berlin). LSL-*Lin28b* mice contain a conditional *Lin28b* tg in the *Rosa26* locus, downstream of CAG promoter and LoxP-flanked transcription termination sites (30). Crossing these mice with B6.C(Cg) $Cd79a^{tm1(cre)Reth}$/EhobJ (*Mb1-cre*) mice results in deletion of the transcription termination site and persistent expression of Lin28b in B cells due to CD79-dependent expression of Cre from the prepro-B cell (Hardy Fraction A) stage onwards (31). In agreement with other studies, where transplanted

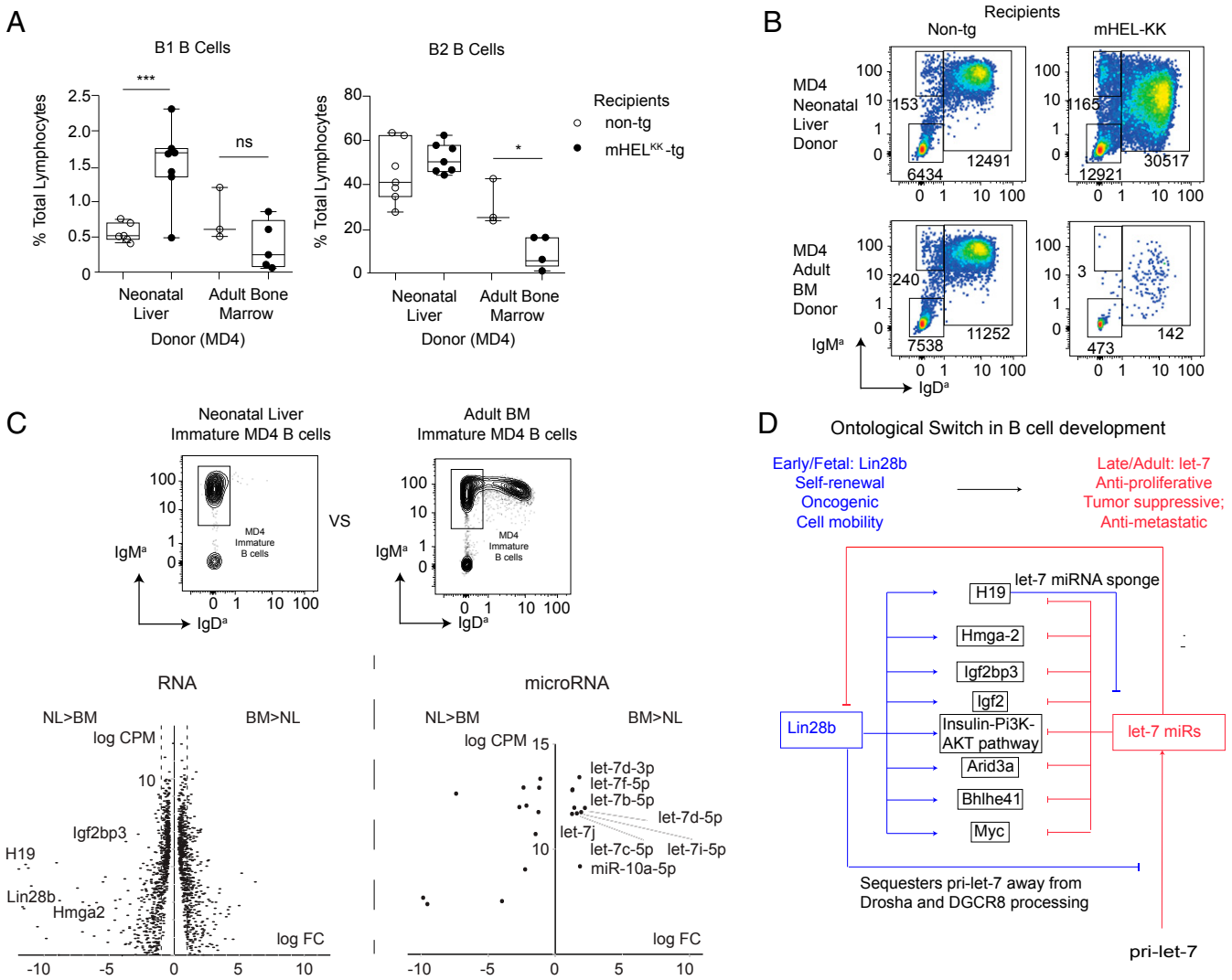

Fig. 1. Lin28b/let-7 switch in B cell ontogeny is linked to selection and independent of BCR specificity. (*A*) Peritoneal B220^{lo}CD19^{+}IgM^{++}IgD^{-} B-1 (*Left*) and B220^{+}CD19^{+} B-2 (*Right*) B cells shown as percent of total CD45.2^{+} lymphocytes in irradiated CD45.1 allotype nontg (open circles) or mHEL^{KK} tg (filled circles) mice reconstituted for 8 wk with CD45.1 allotype MD4 NL or MD4 adult BM. Representative of three experiments. Circles are individual mice, bars show mean and range, and boxes 95% confidence limits. Comparison by unpaired *t* test, where ns, not significant, $*P < 0.05$ and $***P < 0.001$. (*B*) Representative flow cytometry of the donor-derived peritoneal CD45.2^{+} lymphocytes, from chimeras established as in *A*, highlighting MD4 B220^{lo}CD19^{+}IgM^{a++} B-1 and B220^{+}CD19^{+}IgM^{a+} follicular B cells with absolute cell counts. (*C*) Gates used to sort B220^{+}IgM^{a+}IgD^{a-} immature MD4 B cells from NL and adult BM (*Upper*), and volcano plots showing differences (>twofold) in the two populations by RNA-seq (*Lower Left*) and microRNA (*Lower Right*) in the two populations. (*D*) Lin28b vs. let-7 counter regulation in early and late ontogeny affects multiple downstream pathways.

adult BM had been transduced with a Lin28b tg (15), irradiated chimeras reconstituted with adult BM from LSL-*Lin28b*/*Mb1-cre* mice had a higher frequency of CD19^{+}B220^{lo} B-1 B cells in the peritoneal cavity than mice reconstituted with control *Mb1-cre* adult BM, and as high as in mice reconstituted with NL (*SI Appendix*, Fig. S3).

To discover whether B cell-specific ectopic Lin28b expression throughout an animal's lifetime could reestablish the conditions necessary for the positive selection of B-1 B cells by self-antigens, we generated MD4 LSL-*Lin28b*/*Mb1-cre* mice by breeding. We transplanted irradiated nontg or mHEL^{KK} tg recipients with MD4 adult BM or MD4 LSL-*Lin28b*/*Mb1-cre* adult BM or 3-d-old MD4 NL. After 8 wk of reconstitution, HEL-binding self-reactive MD4 B-1 B cells were positively selected from MD4 NL and LSL-*Lin28b*/*Mb1-cre* adult BM in recipient mice expressing mHEL^{KK} (Fig. 3*A* and *SI Appendix*, Fig. S4*A*). In contrast, and as seen before, self-reactive MD4 B cells derived from adult BM were not increased in the B-1 compartment by self-antigen (Fig. 3 *A* and *B*). The ectopic expression of Lin28b in adult BM B cells also reestablished the positive selection of high anti-HEL IgM^{a} titers and HEL-specific IgM^{a} secreting plasma cells by mHEL^{KK}, features normally seen in mice reconstituted with MD4 NL but not adult BM (Fig. 3 *C* and *D*). It has been proposed but not proven that these plasma cells derive from the B1 B cells, and, as in previous studies (27) anti-HEL B-1 B cells were not detectable in splenic B cell populations (*SI Appendix*, Fig. S4*B*). Collectively, these results show that the persistent expression of Lin28b is sufficient to reestablish positive selection by self-antigens even in B cell precursors originating from adult BM, thus recapitulating NL B cell precursor phenotypes.

Lin28b Is Permissive but Not Limiting in Positive Selection by Antigen. Within the time frame of the reconstitution of mixed chimeras, the ectopic expression of Lin28b restored positive selection of B-1

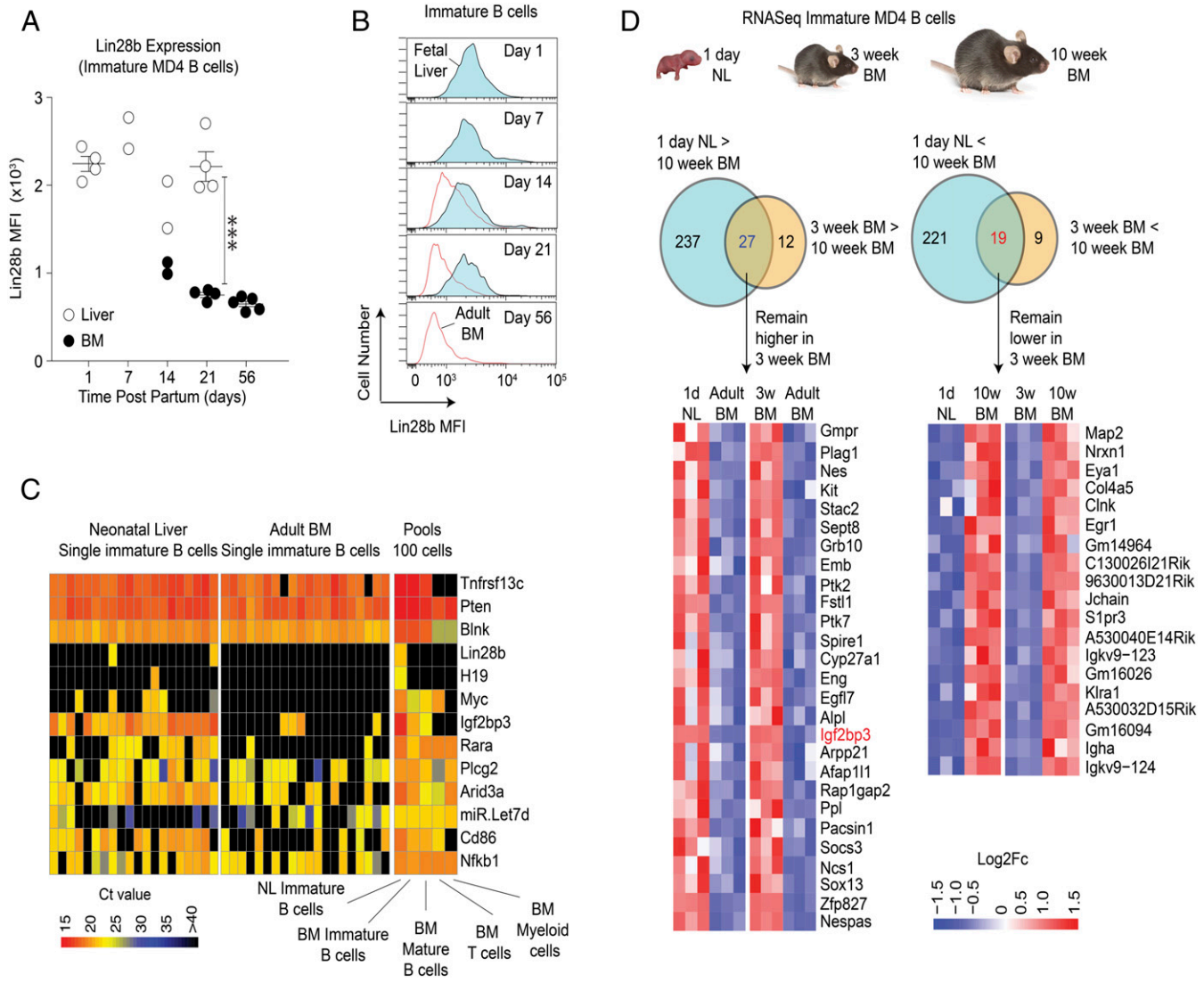

Fig. 2. The Lin28b/let-7 bimodal switch is associated with the transition from liver to BM. (*A*) Mean fluorescence intensity (MFI) of Lin28b in B220^{+}IgM^{a+}IgD^{a-} immature MD4 B cells in the NL and BM measured by intracellular staining and flow cytometry. Circles are individual mice. Representative of three experiments. Bars shows means and 95% confidence limits, with comparison by unpaired *t* tests, ***$P < 0.0001$. (*B*) Representative Lin28b fluorescence profiles in immature B cells stained as in *A*. (*C*) Heatmap showing relative abundance of selected differentially expressed transcripts in single-cell samples from B220^{+}IgM^{a+}IgD^{a-} immature MD4 B cells from NL ($n = 20$) and adult BM ($n = 20$) and 100 cell pools. Gene expression analysis uses the Biomark platform. Cells were sorted and assessed for the presence of the indicated transcripts. Each column represents a single cell or 100 cells. Expression data for each gene is displayed as relative Ct values across all cells assessed. (*D*) RNA was generated and sequenced from 100 cell B220^{+}IgM^{a+}IgD^{a-} immature MD4 B cell samples from 1-d-old NL, 3-wk BM, and 10-wk BM missense (*Upper*). Venn diagrams of number of transcripts elevated (*Left*) and suppressed (*Right*) in 1-d NL (blue) and 3-wk BM (orange) immature B cell relative to 10-wk BM (*Middle*). Heatmaps of transcripts that are elevated (*Left*) and suppressed (*Right*) in both 1-d liver and 3-wk BM relative to 10-wk BM. Columns represent samples from individual mice.

B cells from adult BM precursors but did not enhance it beyond that observed with NL precursors. As reported previously (27), the positive selection of B-1 B cells by mHEL^{KK} occurs ∼2–5 times more efficiently in unmanipulated mice compared to those reconstituted with FL or NL (Figs. 3*A* and 4*A*). To explore the role of Lin28b in unmanipulated mice, we bred MD4mHEL^{KK}LSL-*Lin28b*/*Mb1-cre* mice, in which the persistent ectopic expression of Lin28b in the B cell lineage would prevent the switch to Let7 throughout the life of the animals. We then compared MD4/mHEL^{KK} and MD4/mHEL^{KK}LSL-*Lin28b*/*Mb1-cre* mice and MD4 and I MD4/LSL-*Lin28b*/*Mb1-cre* controls at 8 wk of age. Consistent with the data from the chimeras, the lifelong expression of Lin28b did not increase the number of MD4 HEL-specific B-1 B cells selected by the self-antigen beyond that seen in MD4/mHEL^{KK} controls (Fig. 4 *A* and *C*). However, it did increase the anti-HEL IgM^{a} titers in the presence of self-antigen (Fig. 4*B*) and the number of non-HEL–binding B-1 B cells in the absence of the self-antigen (Fig. 4*C*). It is not possible at this stage to determine whether these effects are due to the expression of Lin28b in early development or its enforced abnormal expression in the later stages of B cell development, where it might support the survival of B cell subsets. Collectively, this and the previous experiment suggest that Lin28b has a necessary and permissive effect during positive selection, in as much as it allows adult BM precursors to exhibit NL/FL-like selection behavior. However, additional factors must be limiting for positive selection, since persistent expression in adulthood does not augment the inherent selection potential beyond that seen in NL/FL precursors. The extent of positive selection may be affected not just by the precursor selection potential but also by the age of the mice, the

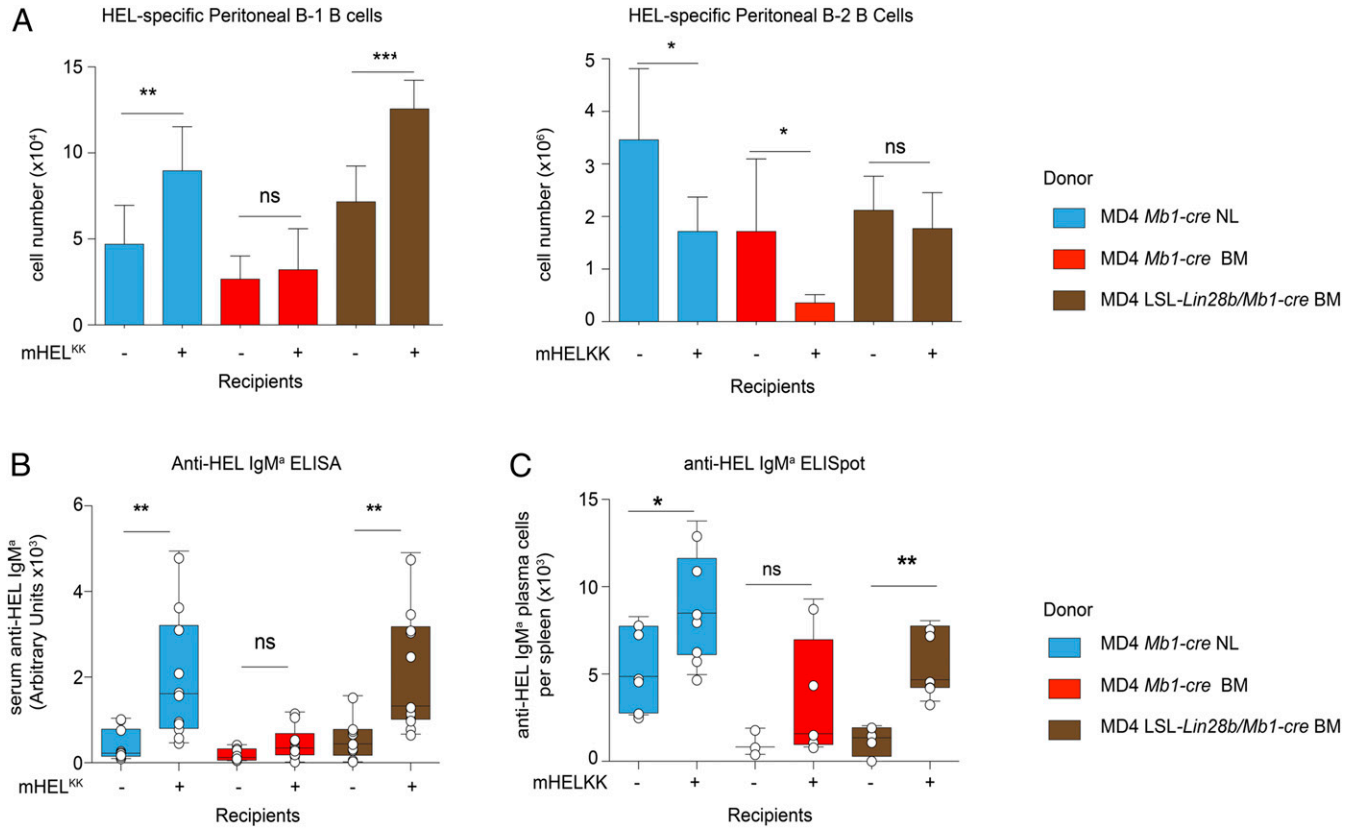

Fig. 3. Lin28b restores positive selection in adult BM. (*A*) Numbers of HEL^{+}IgM^{a+}IgD^{a-}B220^{lo}CD19^{+} peritoneal B-1 (*Left*) and HEL^{+}IgM^{a+}IgD^{a+}B220^{+}CD19^{+} B-2 (*Right*) B cells in irradiated nontg and mHEL^{KK} tg mice reconstituted for 8 wk with MD4 *Mb1-cre* NL (blue, $n = 11$ and $n = 11$), MD4 *Mb1-cre* BM (red, $n = 8$ and $n = 10$), or MD4 LSL-*Lin28b*/*Mb1-cre* BM (brown, $n = 9$ and $n = 12$). Data pooled from three independent experiments. Columns represent means, bars 95% confidence limits. (*B* and *C*) Serum anti-HEL IgM^{a} titres (*B*) and splenic anti-HEL IgM^{a} secreting plasma cell numbers (*C*) in mice described in *A*. Circles are individual mice, bars show mean and range, and boxes 95% confidence limits. Comparisons by unpaired *t* test, where ns, not significant; $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$.

tissue-specific selection site and other cell intrinsic and extrinsic factors.

Lin28b-Dependent Pathways during Ontogeny. To identify the downstream Lin28b-dependent targets, we compared gene expression by RNA-sequencing (seq) in MD4 immature B cells from LSL-*Lin28b*/*Mb1-cre* and *Mb1-cre* adult BM (Fig. 5*A*). This analysis highlighted the Lin28b-dependent transcripts previously associated with the early ontogeny signature in our RNA-seq data from MD4 immature B cells from NL vs. BM (Figs. 1*D* and 5*A*).

We then went on to use the same approach to identify those additional elements that might play a specific role in early and late ontogeny and the development of B-1 B cells, including the role of Lin28b. To focus on Lin28b-dependent and Lin28b-independent pathways, we performed two further comparisons by RNA-seq: (*i*) immature MD4 B cells from single tg MD4 NL vs. single tg MD4 adult BM to identify all ontogenetic factors; and (*ii*) B220^{lo}CD19^{+}IgM^{a+}IgD^{a-} peritoneal MD4 B-1 B cells from MD4/mHEL^{KK} double tg mice vs. naïve B220^{+}CD19^{+}IgM^{a+}IgD^{a+}CD21^{mid}CD23^{+} MD4 splenic FO B cells from MD4 single tg mice to identify factors associated with subset selection. We then made pairwise comparisons between these two subsets and the subset of Lin28b-dependent targets identified from the MD4 immature B cells from LSL-*Lin28b*/*Mb1-cre* vs. *Mb1-cre* adult BM in Fig. 5*A*. In validation of our model, 592/1,000 transcripts expressed >twofold in B-1b B cells compared to FO B cells from Immgen were also >twofold higher in MD4 B-1 B cells compared to MD4 FO B cells in our model, with a high correlation in expression levels ($t = 55.512$, df = 590, $P < 2e\text{-}16$, and $r = 0.9161379$).

In the first analysis, we looked at transcripts that were up-regulated, by Lin28b (LSL-*Lin28b*/*Mb1-cre* vs. *Mb1-cre*) in immature B cells in BM (Fig. 5*B*, green circle), or immature B cells in NL relative to BM (Fig. 5*B*, red circle), or in B-1 B cells relative to FO cells (Fig. 5*B*, blue circle), hypothesizing that this would enrich for factors common to early ontogeny, positive selection, and B-1 B cell differentiation. We identified 10 genes with increased expression in NL compared to adult BM that were also Lin28b dependent, including *Lin28b* itself, *Igf2bp3, Arid3a, Stac2, Plag1, Myo15b, Plaur*, and *Lst1* (Fig. 5*B*). These genes may work together with Lin28b, functioning as "the permissive gate" for B-1 B cell positive selection, directly enhancing positive selection or indirectly reducing negative selection. Two of these transcripts, *Bhlhe41* and *Sspn,* were also up-regulated in B-1 B cells. Bhlhe41 has recently been recognized as a transcription factor required for B-1 B cell development (12). *Sspn* encodes Sarcospan, a 25-kDa transmembrane component of the dystrophin–glycoprotein complex, with roles in maintaining muscle function and Akt-dependent signaling (32). Although the role of Sarcospan in B cell function is unknown, like *Bhlhe41*, expression of *Sspn* is elevated in B-1a and B-1b B cells and plasma cells in the Immgen database.

We identified 254 genes that are up-regulated in NL relative to adult BM but unaffected by expression of the Lin28b tg, including 228 genes that are only expressed at a higher level in NL relative to adult BM and 26 that are also expressed at a higher level in B-1 B cells relative to FO B cells (Fig. 5*B*). In theory, the

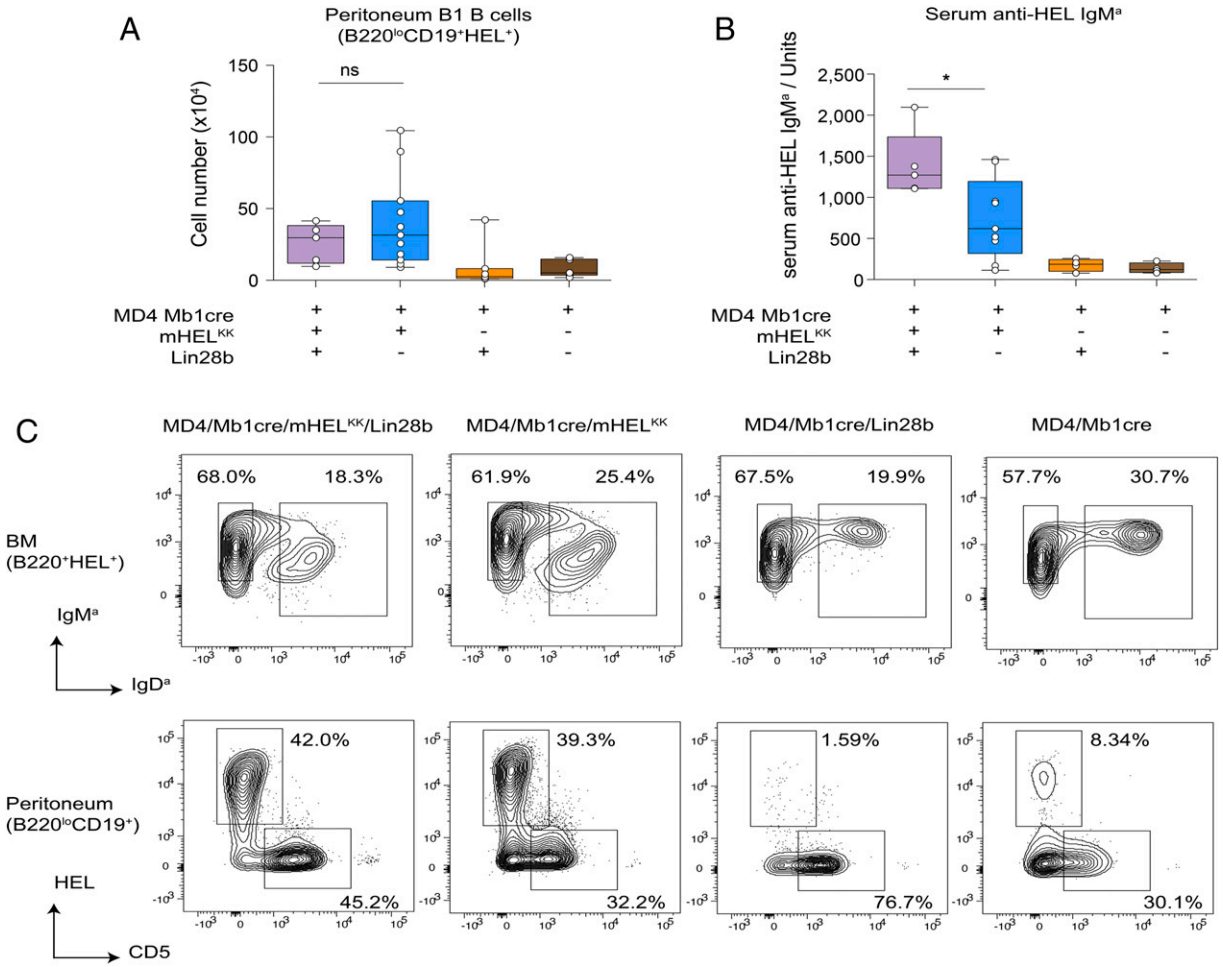

Fig. 4. Increased and lifelong Lin28b does not enhance the endogenous positive selection. (*A* and *B*) Numbers of HEL-specific peritoneal B-1 B cells (*A*) and serum IgM^a anti-HEL titers (*B*) in 8- to 12-wk adult MD4/mHEL^{KK}/LSL-*Lin28b*/*Mb1-cre* (violet, $n = 5$), MD4/mHEL^{KK}/*Mb1-cre* (blue, $n = 9$), MD4/LSL-*Lin28b*/*Mb1-cre* (orange, $n = 9$), and MD4/*Mb1-cre* (brown, $n = 4$) mice. Circles are individual mice, bars show mean and range, and boxes 95% confidence limits. Comparisons by unpaired *t* tests, where ns, not significant; $*P < 0.05$. (*C*) Representative flow cytometry of B cell development in BM (*Upper*) and peritoneum (*Lower*) of the indicated mice, gating on B220$^+$HEL$^+$ and B220$^+$IgM$^+$CD19$^+$ lymphocytes respectively, and highlighting MD4 IgM^{a+}IgD^{a-} immature and IgM^{a+}IgD^{a+} recirculating FO B cells (*Upper*) and HEL-binding IgM^{a+}CD5$^-$ anti-HEL B-1b and non-HEL binding IgM^{a-}CD5$^+$ nontg B-1a B cells (*Lower*).

subset of 26 genes may include candidates that are involved in both B-1 B cell differentiation and their maintenance but are Lin28b independent. This list includes genes that may be involved in modulating the threshold for B cell survival and negative or positive selection, including genes involved in BCR signaling or costimulation (Fig. 5*B*); for example, *Sh2d1b*, which encodes the SLAM adaptor Eat-2a, is involved in BCR signaling and costimulation (33). Some, such as *Nid1*, which encodes the basement membrane protein nidogen-1, are highly enriched in B-1 B cells compared to other B cell subsets (ImmGen database and Fig. 5*B*). Whether these genes play a role individually or collectively in B-1 B cell selection and function remains to be further studied.

We then examined transcripts that were higher in the absence of Lin28b (LSL-*Lin28b*/*Mb1-cre* vs. *Mb1-cre*) in immature B cells in BM (Fig. 5*C*, green circle), or in immature B cells in BM relative to NL (Fig. 5*C*, red circle), or in FO B cells relative to B-1 B cells (Fig. 5*C*, blue circle). In this case, there were no differentially expressed genes common to all groups. Fifteen genes were suppressed in the presence of Lin28b and in NL including *Slamf9*, *S1pr3*, and *Il2ra*. *S1pr3* encodes the sphingosine phosphate receptor 3, which regulates the retention of MZ B cells and is required for the egress of GC B cells from lymph nodes (34, 35). The 16 transcripts enriched in adult BM immature B cells and FO B cells encode proteins associated with negative regulation of the BCR and downstream signaling, including Dusp6, which negatively regulates ERK signaling, and CD72, which inhibits BCR signaling and promotes B-1 B cell development in a SHP-1 phosphatase-dependent manner (21, 36). Other transcripts include *Icoslg*, which is linked to antigen presentation and T cell help, important for FO B cell function (37).

Discussion

Our findings show that a Lin28b/let-7 dependent switch occurs during B cell ontogeny, and that this is independent of BCR specificity, but dictates the susceptibility of developing B cells to positive and negative selection by self-antigen. The switch from Lin28b to Let7 regulation occurs mainly, but not exclusively, at the same time as the transition of the site of hematopoiesis from the NL to BM. In this way, B cell selection may be tuned to generate autoreactive B-1 B cells with regulatory or innate functions during early fetal or neonatal life, but once the adult repertoire is populated, stringent and rigorous elimination of autoreactive B cells could be enforced so as to minimize the risk of collaboration with autoreactive T cells.

The Lin28b/let-7 signaling axis is now well established as a key pathway controlling the transition from fetal to adult hematopoiesis

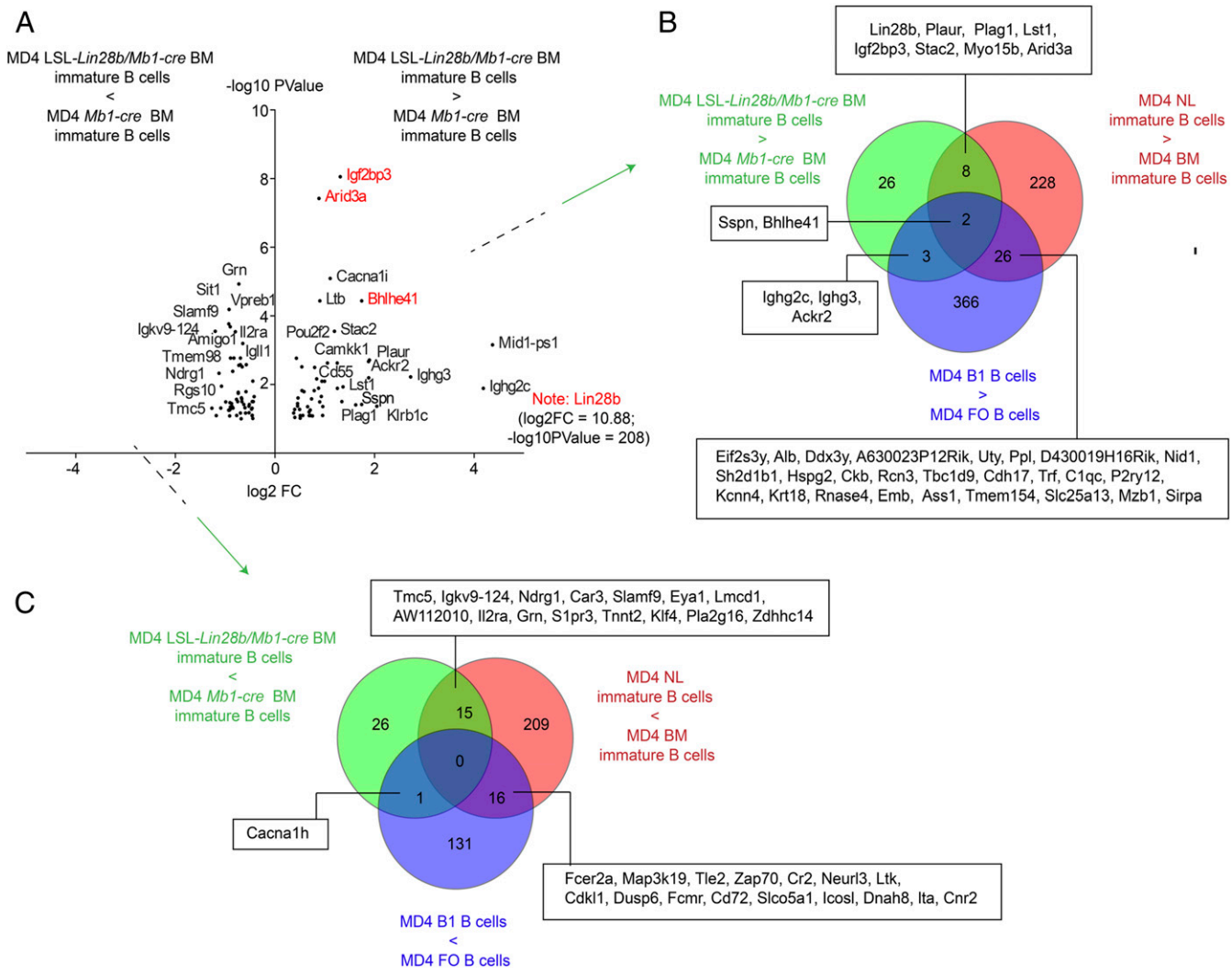

Fig. 5. Lin28b-dependent and Lin28b-independent mRNA during ontogeny and in autoreactive B-1 B cells. (*A*) Volcano plots comparing mRNA expression in immature MD4 B cells form MD4/LSL-*Lin28b/Mb1-cre* adult BM and MD4/*Mb1-cre* adult BM. Representative of three independent experiments (adjusted $P < 0.05$). (*B* and *C*) Venn diagrams comparing the pattern of mRNA transcripts up-regulated (*B*) or down-regulated (*C*) in 100 sorted cells from: immature MD4 B cells from MD4/LSL-*Lin28b/Mb1-cre* adult BM relative to MD4/*Mb1-cre* adult BM (green circles and text, adjusted $P < 0.05$); immature MD4 B from NL relative to adult BM (red circles and text, adjusted $P < 0.05$ and fold change >2); and peritoneal MD4 B-1 B cells vs. splenic MD4 FO B cells from adult mice (blue circles and text, adjusted $P < 0.05$ and fold change >4).

and B-1 B cell development (13–15). Although the exact mechanisms are unclear, Lin28b can potentially interact with thousands of mRNAs (29) and, importantly, suppresses the Let-7 microRNA family by preventing the processing of its RNA precursors (29). In this way, the counterregulation of Lin28b and let-7 forms a binary switch affecting broad classes of genes; our single cell analysis, although somewhat limited by sensitivity, supports this idea. Inactivation of the polycomb group gene and tumor suppressor Ezh2 in adult mouse HSCs has been linked to reactivation of Lin28b expression and fetal-like lymphopoiesis (38). This suggests that the fetal hematopoietic molecular program is kept in check at the chromatin level during adulthood, probably restricting oncogenic transformation.

Our data using LSL-*Lin28b/Mb1-cre* BM without MD4 or HEL transgenes confirms, in a model of lifelong expression, previous findings showing that ectopic expression of a retrovirally encoded Lin28b tg in adult BM HSCs enhanced $CD5^+$ B-1a B cell development (15). Our experiments with the MD4 tg mice suggest that the switch from Lin28b to let-7 dependence occurs concurrently with the transfer of hematopoiesis from the NL to BM. The reduced B-1 B cell output at 2–3 wk after birth has previously been shown to coincide with an increase in the reliance on IL-7 receptor (IL-7R) signaling during B cell development (39). Consistent with this, B-1 B cell development is less dependent on IL-7 (40, 41). Since our data also show that IL-7R mRNA transcripts are 12-fold lower in FL immature B cells compared to adult BM (http://www.ncbi.nlm.nih.gov/geo, accession no. GSE135650), we speculate that IL-7 dependence may be linked to the site of hematopoiesis. How differences in B cell development or ancillary signals during B-1 B cell development in early ontogeny in the FL might compensate for reduced IL-7–dependent signaling needs to be explored in the future.

Two transcription factor targets stabilized by Lin28b and highlighted by our analysis, Arid3a and Bhlhe41, have previously been shown to be critical for B-1 B cell development. Expression of Arid3a tg in adult pro-B cells increases B-1 B cell generation while RNAi-mediated knockdown or conditional inactivation of Arid3a in FL pro-B cells reduces $CD5^+$ B-1a B cell numbers in the peritoneum (17, 42). The transcription factor Bhlhe41, previously identified as highly expressed in mature B-1a B cells

(17), is also required for B-1 B cell generation and, possibly, survival, since B-1a cells are reduced in *Bhlhe41*- and *Bhlhe40*/*Bhlhe41*-deficient mice (12). Bhlhe41 represses the expression of several cell cycle regulators and inhibitors of BCR signaling, while increasing the survival of B-1a cells by enhancing expression of the IL-5 receptor (12). In this way, Bhlhe41 appears to control several aspects of B-1 B cell biology, including development, BCR repertoire, and self-renewal of these cells. Our findings suggest that Bhlhe41 may also be important in linking positive selection to the downstream programs of differentiation and maintenance of the autoreactive cells, since it is almost uniquely enriched downstream of Lin28b, in the fetal/neonatal stage of B cell ontogeny and in B-1 B cells that have undergone positive selection.

Expression of the Lin28b tg restores positive selection in adult BM chimeras in our experiments, implying that it is necessary or permissive, but the extent of positive selection in this case, or with transplanted NL, remains ~5-fold lower than that seen in nonchimeric MD4/mHEL^{KK} double tg mice. This may be due to the intrinsic weakness of BM transplantation as a means to study B cell development. Alternatively, it is possible that, as already discussed, B cells developing in the normal physiological environment of the FL/NL gain access to additional or intrinsic factors that support positive selection in the environment, which may be Lin28b independent. The fact that there is no additional positive selection derived from the expression of Lin28b tg in this situation implies that the expression of Lin28b is necessary, but other factors limit the size of the response. The expression of a Lin28b tg beyond the immature B cell stage might have an additional effect of supporting the expansion of positively selected MD4 B-1 B cell clones in the periphery but, by the same argument, this does not seem to be limiting.

Recent data shows that when splenic B cells from adult mice manipulated to express PtC-specific VH12/Vk4 de novo are transferred into rag-deficient mice they proliferate, probably after encountering the PtC self-antigen, differentiate into cells akin to B-1 B cells, and are characterized by expression of *Bhlhe41* (12). This form of positive selection would not normally be expected to occur in adult mice because of two tolerance checkpoints: negative selection of B-2 B cells at the immature B cell stage (43), which is evident in the spleens of unmanipulated VH12/Vk4 tgs (24), and the negative selection of naive B-2 cells, which encounter T-dependent antigen for the first time in the absence of cognate PtC-specific T cell help (43). The second of these tolerance mechanisms is driven by the BCR-dependent upregulation of Bim and competition for a limited amount of BAFF within the normal B cell repertoire (44). It is interesting to speculate that the positive selection of B-1 B cells by self-antigen might depend in normal physiological settings on relative B cell deficiency, where survival signals from BAFF can compensate for BCR activation and are not limiting. This is precisely what might happen in early ontogeny, as the repertoire develops and fills its niche, and in radiation chimeras as they reconstitute. In this situation, Lin28b-dependent pathways may operate within the B cell repertoire during early ontogeny to make negative selection less likely by enhancing the expression of a raft of factors associated with proliferation and differentiation that are also prooncogenic and normally suppressed in adults.

Questions remain about the extent to which B-1 B cells might be susceptible to inherent selection by virtue of a unique BCR in early ontogeny without the need for positive selection by antigen at the immature cell stage. The majority of developing B cells first rearrange the Ig heavy-chain (HC) locus in pro-B cells leading to expression of the pre-BCR including the surrogate light chain (SLC); subsequently, they proliferate and differentiate into small pre-B cells that rearrange the light-chain (LC, Igκ, and Igλ) loci and form the definitive BCR, which is first expressed in immature B cells. This sequence of recombination is supported by IL-7R signaling through activation of the transcription factor STAT5. In pro-B cells, IL-7R signaling suppresses rearrangements at the Igκ locus, as shown by an increase of Igκ recombination in pro-B cells lacking IL-7Rα or STAT5 (45). The reduced B-1 B cell output at 2–3 wk after birth coincides with an increased reliance on this IL-7R signaling pathway (39), and our data confirm that the IL-7R mRNA transcripts are 12-fold higher in immature adult BM B cells compared to fetal B cells.

The way in which differences in B cell development or ancillary signals during B-1 B cell development in early ontogeny might compensate for reduced IL-7–dependent signaling is unknown, but positive selection at the pre-BCR stage might be an important factor. While adult B cell development requires pre-BCR signaling, some autoreactive HC associated with B-1a B cell development, including VH11 PtC-specific HC, do not need to associate with SLC to support B-1a cell development, provided they coexpress Lin28b or downstream Arid3a (42). In adult BM, an autoreactive anti-MHC class I 3–83 BCR HC and LC tg targeted to the endogenous loci can also support B cell development in the absence of SLC but in the presence of the cognate MHC self-antigen (46). Such autoreactive B cells would normally be subject to negative selection by receptor editing at the immature cells stage or competition with naïve cells for BAFF in the periphery. This is likely to be why the positively selected 3–83 BCR tg B cells from adult BM can only survive in immunodeficient mice and if editing of the LC gene removes the autoreactivity (46). It is interesting to speculate that a similar process of positive selection might be enhanced in early ontogeny if negative selection is reduced by B cell intrinsic factors driven by Lin28b or by incomplete recognition of antigen. Our findings provide a framework for exploring these underlying mechanisms and the role of antigen and ontogeny in both positive and negative selection.

Materials and Methods

Animal Experiments. All experiments were subject to the UK Animal (Scientific Procedures) Act 1986, with UK Home Office licensing housing and procedures as described previously (47).

Mice. All experiments were conducted in a C57BL/6 (B6) background. MD4 (C57BL/6-Tg(MD4)4Ccg/J), and mHEL^{KK} and OT-II (C57BL/6-Tg(TcraTcrb) 425Cbn/Crl) mice were as described previously (27) and maintained on a B6 background. B6.C(Cg)*Cd79a*$^{tm1(cre)Reth}$/EhobJ were obtained from Jackson Laboratories. LSL-*Lin28b* mice were a gift from Johannes H. Schulte and had been backcrossed >4 generations from 129 to C57BL/6; In chimera experiments, B6 mice were irradiated with two doses of 4.5 Gy spaced by 3 h and injected with at least 5×10^6 neonatal or BM cells of the indicated genotype. Mice were reconstituted for 8–10 wk before immunization or analysis. All experiments included age and sex-matched littermate control animals.

Ex Vivo Culture and Stimulation. Cell suspensions from mouse BM were washed and suspended in 2% Fetal Calf Serum (FCS) Roswell Park Memorial Institute (RPMI) 1640 Medium. Cells were warmed at 37 °C for 3 or 5 min and incubated at 37 °C (10^6 cells per well in 96w U bottom plates) with the indicated doses of medium alone, sHEL, or anti-IgM F(ab)$_2$ (Jackson Immunoresearch). Subsequent surface and intracellular staining were performed using the BD Cytofix/Cytoperm protocol.

Flow Cytometry. Cell suspensions from mouse liver, BM (one femur and tibia), spleen, thymus, mesenteric lymph nodes, and peritoneal cavity were processed as previously described (47), and data were acquired on a FACSCanto10c (BD) and analyzed with FlowJo Software (Tree Star).

The following anti-mouse antibodies were used: B220 (RA3-6B2, BioLegend), BAFFR (7H22-E16,BD), BP-1 (6C3, BioLegend), CD3e (145-2C11, eBioscience), CD4 (GK1.5, eBioscience), CD5 (53-7.3, BioLegend), CD8 (53-6.7, BioLegend), CD9 (MZ3, BioLegend), CD11b (M1/70, eBioscience), CD19 (6D5, 1D3, BioLegend), CD21/CD35 (7E9, BioLegend), CD23 (B3B4, BioLegend), CD24 (M1/69, BioLegend), CD25 (PC61, Biolgend), CD43 (S7, BioLegend), CD44 (IM7, BioLegend), CD45.1 (A20, BioLegend), CD45.2 (104, BioLegend), CD69 (H1.2F3, BioLegend), CD80 (16-10A1, BioLegend), CD86 (GL1, BioLegend), CD93 (AA4.1, BioLegend), DAPI (BIOTIUM), Gr-1 (RB6-8C5, eBioscience), IgDa (AMS-9.1, BioLegend), IgD (11-26c, BioLegend), IgMa (DS-1, MA-69,

BioLegend), IgM (Il/41, RMM-1, BioLegend), TCRγδ (GL3, BioLegend), Live dead dye (Zombie Aqua Dye, BioLegend), Live dead dye (Zombie NIR, BioLegend), pERK (4B11B69, BD), pPLCγ2 (K86-1161, BD), Lin28b (AP1485C, ABGENT), and anti-rabbit IgG (BioLegend).

Measurement of Serum Anti-HEL IgM^a and Splenic IgM^a-Secreting Plasma Cells. Anti-HEL IgM^a was measured from sera by ELISA in 96-well plates coated with 1 mg/mL HEL in carbonate buffer, pH 9.6. Serum IgM^a was detected by biotinylated anti-IgM^a (DS-1; BD Pharmingen), followed by avidin-alkaline phosphatase (Sigma-Aldrich). Anti-HEL IgM^a-secreting cells were measured in ELISpot in 96-well plates coated with 1 mg/mL HEL in carbonate buffer, pH 9.6 (43). Spots of bound antibody were revealed with biotinylated anti-IgM^a (DS-1; BD Biosciences) followed by avidin-alkaline phosphatase (Sigma-Aldrich).

RNA-seq and Transcriptomic Analysis. RNA from bulk RNA-seq assays was isolated using RNA-queous micro Kit (Ambion). The mRNA libraries were prepared with the Illumina TruSeq Stranded mRNA Sample Prep Kit (Illumina Inc.). For polyA selection assays, the mRNA fraction was selected from the total RNA provided before conversion to cDNA. For ribosome depletion assays, the ribo-depleted fraction was isolated from the total RNA provided before conversion to cDNA. After that, second-strand cDNA synthesis incorporated dUTP. The cDNA was end-repaired, A-tailed, and adapter-ligated. Prior to amplification, samples underwent uridine digestion. The prepared libraries were size selected, multiplexed, and quality controlled before paired-end sequencing over one lane of a flow cell on HiSeq2500 or HiSeq4000. Data were collected as fastq files.

One hundred cell RNA-seq samples were processed with the Smart-seq2 protocol and analyzed using CGAT Pipelines and tools (https://github.com/CGATOxford/CGATPipelines), as previously described (47). Gene ontogeny analysis was performed using the goseq package with the sampling method (48). Gene set enrichment analysis was performed with Fast Gene Set Enrichment Analysis (fgsea) (Sergushichev 2016 BioRxiv https://www.biorxiv.org/content/10.1101/060012v2) with gene rankings based on transformed log2 fold change and human genes from the target datasets lifted over to homologs in mouse using biomaRt.

Quantitative Real-Time RT-PCR. Total RNA was isolated from individual samples using RNA-queous micro Kit (Ambion). RNA (1–5 μg) was subjected to DNaseI treatment (Invitrogen) and reverse-transcribed using the SuperScriptIII First-strand Synthesis System (Thermo Fisher). Real-time quantitative RT-PCR was performed on 4× diluted cDNA using SYBR Green system. Analyses were performed on Applied Biosystems StepOnePlus Real-Time PCR Systems per manufacturer's instructions. CD19 levels were used to normalize the data. Results were analyzed using the ΔΔCt method. The following primers were used: CD19_mRNA_F – TCATCACCCCTTGCCTAAGA; CD19_mRNA_R – AGTAGGTTCACAGGTCCCAA; Lin28b_mRNA_F – GCACCAAGAGAAGTGGGAGG; Lin28b_mRNA_R – CTGCAAACGCTTTCGTGGAA.

Large-Scale Single-Cell qPCR. Single cells were sorted into 5 μL of RT-STA mix directly for preamplification. The RT-STA mix contained 2× cells direct reaction mix (Invitrogen), 0.2 U/μL SUPERase (Ambion), 0.2× assay mix, 0.12× SuperScript III RT/Platinum Taq Mix (with RNaseOUT Ribonuclease Inhibitor) and topped up with Tris-EDTA (TE) buffer (Invitrogen). Gene expression quantification used the Biomark HD platform (Fluidigm). Gene filter procedure was as follows. For each gene, data with CtCall = FAILED and Ct_Quality < Ct_threshold were removed.

Statistical Analysis. GraphPad Prism Software was used for statistical analyses, and unpaired, two-tailed Student's *t* tests were used for statistical comparison between groups, unless otherwise specifically mentioned.

Data Submission. RNA-seq data generated for this study have been deposited in the Gene Expression Omnibus under accession code GSE135650. Other supporting data are supplied in *SI Appendix*.

ACKNOWLEDGMENTS. We thank N. Ashley, P. Sopp, and C. Waugh for assistance with single-cell experiments and flow cytometry; staff at the Oxford Functional Genomics Facility for animal care; and Dr. Johannes H. Schulte (Charité Hospital) for mice. This work was supported by the Medical Research Council (MRC) and the China Scholarship Council. R.J.C. is a Principal Investigator of the MRC Human Immunology Unit.

1. K. Hayakawa, R. R. Hardy, D. R. Parks, L. A. Herzenberg, The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. *J. Exp. Med.* **157**, 202–218 (1983).
2. N. Baumgarth, The double life of a B-1 cell: Self-reactivity selects for protective effector functions. *Nat. Rev. Immunol.* **11**, 34–46 (2011).
3. K. M. Ansel, R. B. Harris, J. G. Cyster, CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. *Immunity* **16**, 67–76 (2002).
4. R. Berland, H. H. Wortis, Origins and functions of B-1 cells with notes on the role of CD5. *Annu. Rev. Immunol.* **20**, 253–300 (2002).
5. T. Kreslavsky, J. B. Wong, M. Fischer, J. A. Skok, M. Busslinger, Control of B-1a cell development by instructive BCR signaling. *Curr. Opin. Immunol.* **51**, 24–31 (2018).
6. T. A. Kristiansen, S. Vanhee, J. Yuan, The influence of developmental timing on B cell diversity. *Curr. Opin. Immunol.* **51**, 7–13 (2018).
7. P. A. Lalor, L. A. Herzenberg, S. Adams, A. M. Stall, Feedback regulation of murine Ly-1 B cell development. *Eur. J. Immunol.* **19**, 507–513 (1989).
8. E. Burgueño-Bucio, C. A. Mier-Aguilar, G. Soldevila, The multiple faces of CD5. *J. Leukoc. Biol.* **105**, 891–904 (2019).
9. E. Montecino-Rodriguez, H. Leathers, K. Dorshkind, Identification of a B-1 B cell-specified progenitor. *Nat. Immunol.* **7**, 293–301 (2006).
10. T. A. Kristiansen *et al.*, Cellular barcoding links B-1a B cell potential to a fetal hematopoietic stem cell state at the single-cell level. *Immunity* **45**, 346–357 (2016).
11. N. E. Holodick, K. Repetny, X. Zhong, T. L. Rothstein, Adult BM generates CD5+ B1 cells containing abundant N-region additions. *Eur. J. Immunol.* **39**, 2383–2394 (2009).
12. T. Kreslavsky *et al.*, Essential role for the transcription factor Bhlhe41 in regulating the development, self-renewal and BCR repertoire of B-1a cells. *Nat. Immunol.* **18**, 442–455 (2017).
13. M. R. Copley *et al.*, The Lin28b-let-7-Hmga2 axis determines the higher self-renewal potential of fetal haematopoietic stem cells. *Nat. Cell Biol.* **15**, 916–925 (2013).
14. R. G. Rowe *et al.*, Developmental regulation of myeloerythroid progenitor function by the Lin28b-let-7-Hmga2 axis. *J. Exp. Med.* **213**, 1497–1512 (2016).
15. J. Yuan, C. K. Nguyen, X. Liu, C. Kanellopoulou, S. A. Muljo, Lin28b reprograms adult bone marrow hematopoietic progenitors to mediate fetal-like lymphopoiesis. *Science* **335**, 1195–1200 (2012).
16. Y. S. Li *et al.*, A developmental switch between fetal and adult B lymphopoiesis. *Ann. N. Y. Acad. Sci.* **1362**, 8–15 (2015).
17. Y. Zhou *et al.*, Lin28b promotes fetal B lymphopoiesis through the transcription factor Arid3a. *J. Exp. Med.* **212**, 569–580 (2015).
18. Y. Yang *et al.*, Distinct mechanisms define murine B cell lineage immunoglobulin heavy chain (IgH) repertoires. *eLife* **4**, e09083 (2015).
19. S. Casola *et al.*, B cell receptor signal strength determines B cell fate. *Nat. Immunol.* **5**, 317–327 (2004).
20. A. Hoffmann *et al.*, Siglec-G is a B1 cell-inhibitory receptor that controls expansion and calcium signaling of the B1 cell population. *Nat. Immunol.* **8**, 695–704 (2007).
21. C. Pan, N. Baumgarth, J. R. Parnes, CD72-deficient mice reveal nonredundant roles of CD72 in B cell development and activation. *Immunity* **11**, 495–506 (1999).
22. C. J. Binder, Natural IgM antibodies against oxidation-specific epitopes. *J. Clin. Immunol.* **30** (suppl. 1), S56–S60 (2010).
23. L. W. Arnold, C. A. Pennell, S. K. McCray, S. H. Clarke, Development of B-1 cells: Segregation of phosphatidyl choline-specific B cells to the B-1 population occurs after immunoglobulin gene expression. *J. Exp. Med.* **179**, 1585–1595 (1994).
24. R. Graf *et al.*, BCR-dependent lineage plasticity in mature B cells. *Science* **363**, 748–753 (2019).
25. K. Hayakawa *et al.*, Positive selection of natural autoreactive B cells. *Science* **285**, 113–116 (1999).
26. C. C. Goodnow *et al.*, Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* **334**, 676–682 (1988).
27. H. Ferry, M. Jones, D. J. Vaux, I. S. Roberts, R. J. Cornall, The cellular location of self-antigen determines the positive and negative selection of autoreactive B cells. *J. Exp. Med.* **198**, 1415–1425 (2003).
28. H. Ferry, T. L. Crockford, J. C. Leung, R. J. Cornall, Signals from a self-antigen induce positive selection in early B cell ontogeny but are tolerogenic in adults. *J. Immunol.* **176**, 7402–7411 (2006).
29. R. Graf *et al.*, Identification of LIN28B-bound mRNAs reveals features of target recognition and regulation. *RNA Biol.* **10**, 1146–1159 (2013).
30. J. J. Molenaar *et al.*, LIN28B induces neuroblastoma and enhances MYCN levels via let-7 suppression. *Nat. Genet.* **44**, 1199–1206 (2012).
31. E. Hobeika *et al.*, Testing gene function early in the B cell lineage in mb1-cre mice. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 13789–13794 (2006).
32. J. L. Marshall, R. H. Crosbie-Watson, Sarcospan: A small protein with large potential for Duchenne muscular dystrophy. *Skelet. Muscle* **3**, 1 (2013).
33. S. G. Tangye, B. C. van de Weerdt, D. T. Avery, P. D. Hodgkin, CD84 is up-regulated on a major population of human memory B cells and recruits the SH2 domain containing proteins SAP and EAT-2. *Eur. J. Immunol.* **32**, 1640–1649 (2002).
34. J. R. Muppidi, E. Lu, J. G. Cyster, The G protein-coupled receptor P2RY8 and follicular dendritic cells promote germinal center confinement of B cells, whereas S1PR3 can contribute to their dissemination. *J. Exp. Med.* **212**, 2213–2222 (2015).
35. K. Tedford *et al.*, The opposing forces of shear flow and sphingosine-1-phosphate control marginal zone B cell shuttling. *Nat. Commun.* **8**, 2261 (2017).
36. S. Shojaee *et al.*, Erk negative feedback control enables pre-B cell transformation and represents a therapeutic target in acute lymphoblastic leukemia. *Cancer Cell* **28**, 114–128 (2015).
37. D. Liu *et al.*, T-B-cell entanglement and ICOSL-driven feed-forward regulation of germinal centre reaction. *Nature* **517**, 214–218 (2015).

38. M. Oshima *et al*., Ezh2 regulates the Lin28/let-7 pathway to restrict activation of fetal gene signature in adult hematopoietic stem cells. *Exp. Hematol.* **44**, 282–296.e3 (2016).
39. K. Kikuchi, M. Kondo, Developmental switch of mouse hematopoietic stem cells from fetal to adult type occurs in bone marrow after birth. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 17852–17857 (2006).
40. L. Erlandsson *et al*., Impaired B-1 and B-2 B cell development and atypical splenic B cell structures in IL-7 receptor-deficient mice. *Eur. J. Immunol.* **34**, 3595–3603 (2004).
41. D. G. Hesslein, S. Y. Yang, D. G. Schatz, Origins of peripheral B cells in IL-7 receptor-deficient mice. *Mol. Immunol.* **43**, 326–334 (2006).
42. K. Hayakawa *et al*., Crucial role of increased Arid3a at the pre-B and immature B cell stages for B1a cell generation. *Front. Immunol.* **10**, 457 (2019).
43. C. C. Goodnow *et al*., Self-tolerance checkpoints in B lymphocyte development. *Adv. Immunol.* **59**, 279–368 (1995).
44. R. Lesley *et al*., Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF. *Immunity* **20**, 441–453 (2004).
45. S. Malin *et al*., Role of STAT5 in controlling cell survival and immunoglobulin gene recombination during pro-B cell development. *Nat. Immunol.* **11**, 171–179 (2010).
46. C. Eschbach *et al*., Efficient generation of B lymphocytes by recognition of self-antigens. *Eur. J. Immunol.* **41**, 2397–2403 (2011).
47. C. Anzilotti *et al*., An essential role for the Zn^{2+} transporter ZIP7 in B cell development. *Nat. Immunol.* **20**, 350–361 (2019).
48. M. D. Young, M. J. Wakefield, G. K. Smyth, A. Oshlack, Gene ontology analysis for RNA-seq: Accounting for selection bias. *Genome Biol.* **11**, R14 (2010).