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B-1a Cell Diversity: Nontemplated Addition in B-1a Cell Ig Is Determined by Progenitor Population and Developmental Location

Nicol E. Holodick,* Teresa Vizconde,* and Thomas L. Rothstein*,†,‡

Natural Abs produced by B-1a cells are required for immediate protection against infection. The protective capacity of natural Abs is attributed to germline-like structure, which includes the relative absence of N-region addition. Previous studies have shown B-1a cell Ig from aged mice contains abundant nontemplated (N)-additions. B-1a cells have been shown to derive from a specific lineage-negative (Lin<sup>−</sup>)CD45R<sup>b&</sup>low/CD19<sup>+</sup> progenitor found both in fetal liver and adult bone marrow. In this study, we report identification of a fetal liver population characterized phenotypically as Lin<sup>−</sup>CD45R<sup>b&</sup>low/CD19<sup>+</sup>Mac-1<sup>+</sup>CD19<sup>high</sup>CD43<sup>low</sup> B-1a cells upon adoptive transfer to SCID recipients. These B-1a cells derived from the Lin<sup>−</sup>CD45R<sup>b&</sup>low/CD19<sup>+</sup> fetal liver population produce natural Ab that binds pneumococcal Ags, but this Ig contains substantial N-addition despite initial absence of TdT. Furthermore, we show extensive N-addition is also present in B-1a cells derived from the Lin<sup>−</sup>CD45R<sup>b&</sup>low/CD19<sup>+</sup> B-1 progenitor found in the bone marrow. Together these results demonstrate B-1a cell N-addition depends on the type of progenitor and the location of the progenitor during its development. These findings have implications for how regulation of different progenitors from fetal liver and bone marrow may play a role in the age-related increase in N-region addition by B-1a cells in normal animals. The Journal of Immunology, 2014, 192: 000–000.

M urine B-1a cells are defined by unique surface marker expression (IgM<sup>hi</sup>IgD<sup>lo</sup>CD45R<sup>lo</sup>CD5<sup>−</sup>Mac-1<sup>+</sup>CD19<sup>hi</sup>CD43<sup>lo</sup>) as well as distinct functional characteristics as compared with conventional splenic B-2 cells (1, 2). B-1a cells are found in the peritoneal cavity, spleen, and bone marrow. Functionally, B-1a cells exhibit unique signaling characteristics (2–4), are potent APCs (5), and constitutively secrete IgM, which is referred to as natural IgM (6–8). B-1a cells are essential for immediate protection against infection, and therefore survival from, infection by both bacterial and viral pathogens (9–11). The unique ability of B-1a cells to provide immediate protection against infection is attributed to natural IgM, which is germline-like due to minimal N-region addition with little somatic hypermutation, and includes biased V<sub>H</sub> gene usage in favor of V<sub>H11</sub> and V<sub>H12</sub> (1, 12–15). This unique germline structure of natural Ab is established during the early development of B-1a cells.

In general, B cell development begins with hematopoietic stem cells (HSC), which are self-renewing pluripotent cells found in fetal liver and adult bone marrow (16). B cell development continues through a series of differentiation steps dictated by expression of transcription factors, cytokines, and cell surface receptors. Proper Ig rearrangement allows for the B cell to progress through each stage of differentiation, culminating in a naïve B cell expressing a BCR, which is necessary for B cell survival and response to Ag (17). During Ig gene rearrangement, nontemplated (N) nucleotides may be added to joining sites, which increases diversity of the B cell Ag receptor. The process of N-nucleotide addition is mediated by the enzyme TdT (16–18), which is not expressed in the liver, spleen, or bone marrow during fetal life (19). The limitation of TdT expression until after birth correlates with little to no N-addition observed in fetal derived B cells (12).

Specifically, the B-1 cell population in mice originates mainly from fetal liver precursors and was thought to persist throughout adult life by self-renewal (20–22). Recently, Dorshkind and colleagues (23) identified a B-1 cell–specific progenitor with the phenotype, lineage-negative (Lin<sup>−</sup>)CD45R<sup>low</sup>/CD19<sup>+</sup>, found in low numbers in adult bone marrow and abundantly in fetal liver. Total Lin<sup>−</sup> bone marrow as well as fetal liver precursors can give rise to B-1a cells upon adoptive transfer (24–26). We and others have shown B-1a cell Ig from older mice contains more N-addition than B-1a cell Ig from younger mice (24, 27). Interestingly, an increase in N-addition in TdT transgenic mice produces Abs less protective against Streptococcus pneumoniae (28). This study suggests the increased diversity generated by N-addition can be detrimental for microbial protection. In the course of elucidating the relationship between Lin<sup>−</sup>CD45R<sup>low</sup>/CD19<sup>+</sup> progenitor cells and Ig N-addition diversity, we discovered a population of fetal liver cells, characterized as Lin<sup>−</sup>AA4.1<sup>−</sup>CD45R<sup>low</sup>/CD19<sup>+</sup>, that gives rise to B-1a cells containing abundant N-additions. Furthermore, we discovered the Lin<sup>−</sup>AA4.1<sup>−</sup>CD45R<sup>low</sup>/CD19<sup>+</sup> B-1 cell progenitor found in the adult bone marrow generates B-1a cells containing abundant N-additions, in keeping with our previous finding that Ig produced by bone marrow–derived (BMD) B-1a cells differs from that of native B-1a cells by expressing much

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Abbreviations used in this article: HSC, hematopoietic stem cell; Lin<sup>−</sup>, lineage negative; N, nontemplated; PC, phosphorylcholine; PPS3, pneumococcal capsular polysaccharide 3; PCC, phosphatidylcholine; SCF, stem cell factor; TPO, thrombopoietin; TSLP, thymic stromal lymphopoietin.

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more N-region addition (24, 25). These results identify a novel B-1a cell progenitor population, and indicate both the progenitor type and progenitor location determine N-region-mediated diversity.

Materials and Methods

Mice
Male BALB/c ByJ and C57BL/6 mice of 6–8 wk age were obtained from The Jackson Laboratory. CB17-SCID mice of 6–8 wk of age were obtained from Taconic. Timed pregnant female mice were obtained from either The Jackson Laboratory (BALB/c-ByJ) or Taconic (Swiss Webster). TdT knockout mice on the C57BL/6 background were provided by A. Feeney (Scripps Research Institute). Mice were cared for and handled in accordance with National Institutes of Health and institutional guidelines.

Adoptive transfer
Fetal liver was obtained from either BALB/c-ByJ or Swiss Webster timed pregnant females at day 14, 15, or 18, as indicated. Fetal liver cell populations were sort purified using the Influx cell sorter (BD Biosciences), washed twice in 1× PBS, resuspended in 1× PBS, and then injected (i.v.) into recipient CB17-SCID mice at 0.2–1.0×10^6 cells per mouse in 0.2 ml. Four to 5 wk postinjection, the CB17-SCID recipients were euthanized and examined for B-1a cell development.

Cell purification and flow cytometry
Peritoneal washouts from wild-type and fetal liver chimaera mice were stained with immunofluorescent Abs and then analyzed on a LSRII flow cytometer or Influx cell sorter (BD Biosciences) with gating on live cells by forward side scatter. Images were constructed with FlowJo 6.0 software (Tree Star). The following Abs were obtained from BD Pharmingen: biotin-conjugated rat anti-mouse IgM (clone AF6-78); FITC-conjugated rat anti-mouse IgM (clone DS-1); PE-conjugated rat anti-mouse CD19 (clone ID3), CD43 (clone S7), Mac-1 (clone MI70), CD45R/B220 (clone RA3-6B2), CD23 (clone B3B4), PDL2 (clone TY25), and CD86 (clone GL1); PE-Cy5-conjugated rat anti-mouse CD5 (clone 53-7.3); and Alexa647-conjugated rat anti-mouse CD5 (clone 53-7.3); and PerCP-Cy5.5-conjugated rat anti-mouse CD45R/B220 (clone RA3-6B2). The following Abs were obtained from BioLegend: PE-Cy7-conjugated rat anti-mouse CD23 (clone B3B4) and Pacific Blue-conjugated rat anti-mouse IgD (clone 11-26c.2a). The following biotin-conjugated rat anti-mouse Abs were used to discriminate lineage-positive cells: Mac-1 (clone MI70); TER-119; CD8a (clone 53-6.7); Ly-6C/Ly-6G (clone RB6-8C5); CD5 (clone 53-7.3); and IgM (Southern Biotechnology Associates). PC liposomes were kindly provided by S. Clarke (University of North Carolina, Chapel Hill, NC).

Gene expression
Gene expression was assayed by TaqMan and standard PCR. Briefly, RNA was prepared from B cells using Ultraspec (BiotecX) and chloroform extraction. Following isolation, RNA was treated with DNase I (Ambion) to remove contaminating DNA. cDNA was prepared using avian myeloblastosis virus reverse transcriptase (Bio-Rad). For TaqMan expression analysis following culture, RNA was prepared from 10,000–100,000 cells (Cells-to-Ct; Ambion). Gene expression was measured by real-time PCR using TaqMan chemistry. Primer and probe sets were obtained from Applied Biosystems for TdT (Mm00493500_m1) and β-actin, which was used for normalization. Gene expression was also measured by standard PCR. The following primer sets were adapted from Ref. 29 and used for standard PCR: actin (5′-CCTAAGGCGAACCCTGAAG-3′; 5′-TCTCATGGTGCTATTAGGCA-3′) and TdT (5′-GAAGATGGGAACAACTCGAA-3′; 5′-CAGGTTGCTGGAACATTCTGGGAG-3′). Products of amplification were run on a 2% agarose gel.

Single-cell sequencing and analysis
Peritoneal washout cells and splenocytes were obtained from 8- to 14-wk old wild-type BALB/c-ByJ mice, wild-type C57BL/6 mice, TdT−/−/C57BL/6 mice, or chimeras 4–5 wk posttransplantation and were stained with fluorescence-labeled Abs to IgM, IgG, CD45R, CD5, and CD23. B cell populations (native peritoneal CD5+ B1 cells, IgM+CD45R+/CD5−; adoptive transferred peritoneal CD5+ B1 cells, IgM+CD45R+/CD5−; native peritoneal CD5− B2 cells, IgM+CD45R+/CD5−) were then purified using an Influx cell sorter (BD Biosciences). Postsort analysis of B cell populations showed populations to be ≥98% pure. Native peritoneal CD5+ B-1, adoptive-transfer-derived peritoneal CD5+ B-1, and native splenic B2 cells were sorted onto 48-well AmpliGrid slides (Avadix). Reverse transcription and PCR (Qagen OneStep RT-PCR) were carried out as described previously (24). The products were purified and then sequenced (Biotic Solutions) using the MsVHE primer. Sequences were then analyzed using an online sequence analysis tool for VDJ sequences (IMGT, the international ImMunoGeneTics information system). Each of the sequences analyzed and reported in this manuscript, from all populations, has a unique V, D, and J segment along with a unique CDR3. Sequences with identical V, D, and J segments as well as identical CDR3 regions were eliminated from consideration according to the criteria of Kantor et al. (30). As stated in previously published work, because it cannot be determined whether these sequences containing identical V, D, J, and CDR3 regions result from a single clonal expansion, or from analysis of multiple cells with identical rearrangements, we removed these sequences from the analysis presented in this work (30). Nevertheless, upon analysis, including these sequences containing identical V, D, J, and CDR3 regions, the results (significant differences) and conclusions of N-region addition remain the same.

In vitro cell culture
Fetal liver was obtained from Swiss Webster timed pregnant females at day 18. Fetal liver cell populations were sort purified using the Influx cell sorter (BD Biosciences), washed once in complete medium (RPMI 1640 containing 10% FBS, penicillin/streptomycin, 10 μg/ml, and 2-ME), resuspended in complete medium, and then plated for 1 d with 100-ng/ml stem cell factor (SCF), 100-ng/ml Flt3-L, and 50-ng/ml thrombopoietin (TPO). After the 24-h culture, the cells were collected for analysis by flow cytometry. Alternatively, sorted cells were washed once in complete medium, resuspended in complete medium, and then plated in the bottom of a 6-well 0.45-μm transwell plate at 10,000 cells/well with one of the two cytokine mixtures, as indicated: 1) 20 ng/ml SCF, 10 ng/ml Flt3-L, 10 ng/ml rhimyoclonal lymphopoietin (TSLP), 20 ng/ml IL-3, and 20 ng/ml IL-6 (IL-7); or 2) 20 ng/ml SCF, 10 ng/ml Flt3-L, 10 ng/ml TSLP, and 10 ng/ml IL-7 (IL-7). The upper chambers of the transwell plates were seeded with OP9 cells (American Type Culture Collection) the day before culture in α-MEM without ribonucleosides and deoxyribonucleosides containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 20% FBS. The cells were cultured for 3 or 9 d and then collected for analysis by cell counting and flow cytometry.

ELISA
Serum was collected from individual naive chimera mice 4–5 wk posttransplantation or from 3- to 5-mo-old BALB/c-ByJ mice at the time of euthanasia. The serum was analyzed for total IgM by ELISA, according to the manufacturer’s instructions (Bethyl Laboratories). IgM-specific phosphorylcholine (PC) and pneumococcal capsular polysaccharide 3 (PPS3) secreted Ab levels were assayed by conjugate coating 96-well plates with PC-ByJ (Biosearch Technologies) or PPS3 (American Type Culture Collection) at 5 μg/ml in 1× PBS. All plates were washed with PBS Tween (0.05%) and blocked with 1% BSA for 1 h at room temperature. Bound IgM was measured using HRP-conjugated goat anti-mouse IgM (Bethyl Laboratories). IgM standards were included on each plate, and PC/PPS3-specific Ab levels were interpreted as volume equivalent of IgM, as described in (31).

Results
Peritoneal IgM+CD45R+/CD5+ B-1 cells develop from Lin+CD45R+CD19− fetal liver cells
Fetal liver cells were collected at day 14, 15, or 18 of embryonic life from either BALB/c-ByJ or Swiss Webster mice. The B-1 cell–specific progenitor described by Dorshkind and colleagues (23), Lin−CD45Rhigh/CD19+, was sorted purified and adoptively transferred by i.v. inoculation into adult CB17-SCID mice. At the same time, we identified among Lin− fetal liver cells a CD45R+CD19− population that was transferred to separate CB17-SCID recipients. Four to 5 wk posttransfer, recipient mice were euthanized, at which time peritoneal washouts and spleens were collected for analysis. To identify donor-derived cells in the CB17-SCID recipients, allotypic differences at the Igh locus between the donor and recipient (IgM) and recipient (IgMb) strains were used. We found the Lin−CD45Rhhigh/CD19− fetal liver cell population reconstituted IgM+CD45R+/CD5+ and IgM+CD45R−/CD5− B-1 cell populations in the peritoneal cavity (Fig. 1), with resultant B-1 cells amounting to ∼40% of peritoneal cells, which is compared with
native B-1a cells from unmanipulated 3-mo-old mice and B-1a cells derived from Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>−</sup> fetal liver cells wherein B-1a cells constituted 41 and 53% of peritoneal cells, respectively (Fig. 1). In contrast, neither fetal liver cell population reconstituted peritoneal B-2 cells, nor splenic B or T cells, to any significant extent. Total fetal liver cells (nonlineage depleted) reconstituted a significant proportion of splenic and peritoneal T cells, as previously reported (data not shown) (32). The total number of each population in fetal liver was assessed at 15, 16, and 18 d of gestation. We found the number of Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>−</sup> cells exceeded the number of Lin<sup>−</sup>CD45R<sub>low</sub>/CD19<sup>+</sup> cells at each time point (Supplemental Fig. 1). These results demonstrate there is a population of fetal liver cells defined as Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>−</sup>, which can preferentially repopulate the peritoneal cavity with IgMa<sup>+</sup>CD45R<sub>low</sub>CD5<sup>+</sup> lymphocytes.

Peritoneal B lymphocytes derived from Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>−</sup> fetal liver cell phenotype as B-1a cells

Beyond IgM, CD45R, and CD5, peritoneal B-1a cells express additional cell surface markers in a distinctive pattern consisting of IgD<sub>low</sub>/CD19<sup>high</sup>Mac-1<sup>−</sup>CD43<sup>−</sup>CD23<sup>low</sup>, in contrast to B-2 cells that are IgD<sub>high</sub>/CD19<sup>low</sup>Mac-1<sup>−</sup>CD43<sup>−</sup>CD23<sup>high</sup> (1, 2, 33). To verify the identity of fetal liver Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>−</sup> progeny, peritoneal IgM<sup>+</sup>CD45R<sub>low</sub>CD5<sup>+</sup> cells were immunofluorescently stained and analyzed. We found IgM<sup>+</sup>CD45R<sub>low</sub>CD5<sup>+</sup> cells derived from both Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>−</sup> and Lin<sup>−</sup>CD45R<sub>low</sub>/CD19<sup>+</sup> adoptively transferred populations differed from native splenic B-2 cells by expressing less IgD and more CD19 (Fig. 2A), which is comparable to native B-1a cells. Similarly, we demonstrated, like native B-1a cells, a much higher proportion of Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>−</sup> and Lin<sup>−</sup>CD45R<sub>low</sub>/CD19<sup>+</sup> derived IgM<sup>+</sup>CD45R<sub>low</sub>CD5<sup>+</sup> cells expressed Mac-1 and CD43, and a much lower proportion expressed CD23, as compared with native splenic B-2 cells (Fig. 2B). In sum, IgM<sup>+</sup>CD45R<sub>low</sub>CD5<sup>+</sup> B cells derived from Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>−</sup> fetal liver cells express cell surface markers in a pattern that is characteristic of native peritoneal B-1a cells and is also observed in IgM<sup>+</sup>CD45R<sub>low</sub>CD5<sup>+</sup> B cells derived from fetal Lin<sup>−</sup>CD45R<sub>low</sub>/CD19<sup>+</sup> B-1 cell progenitors. These results indicate peritoneal IgM<sup>+</sup>CD45R<sub>low</sub>CD5<sup>+</sup> cells derived from Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>−</sup> fetal liver cell phenotype as authentic B-1a cells.

Other phenotypic characteristics have been described for peritoneal B-1a cells, which include surface expression of CD86 (5) and PD-L2 (34), and binding of phosphatidylycholine (PtC) (35).
As with native B-1a cells, we found many more B-1a cells derived from both Lin-CD45R-CD19- and Lin-CD45R<sup>low</sup>-CD19- fetal liver cells compared with splenic B-2 cells and Lin-CD45R<sup>low</sup>-CD19- fetal liver cells derived from SCID mice transplanted with either Lin-CD45R-CD19- fetal liver cells were analyzed for surface markers typically found on peritoneal B-1a cells. These cells were compared with peritoneal B-1a and splenic B-2 cells from 3 mo-old BALB/c-ByJ mice in addition to peritoneal IgM<sup>+</sup>CD45R<sup>low</sup>-CD19<sup>+</sup> cells derived from SCID mice transplanted with bone marrow progenitors. Lin<sup>+</sup>CD45R<sup>low</sup>-CD19<sup>+</sup> fetal liver progenitors gave rise to cells producing lower levels of PC-specific and PPS3-specific IgM as compared with mice transplanted with bone marrow progenitors. Despite small disparities, no statistically significant differences were observed in the amount of total PC-specific, or PPS3-specific IgM between mice transplanted with either fetal liver population or Lin<sup>+</sup> bone marrow. Regardless, these results demonstrate both fetal liver B-1a progenitors are capable of producing total IgM, PC-specific, and PPS3-specific serum IgM, which are hallmarks of B-1a cell natural Ab.

**B-1aFN and B-1aBP cells produce Ig with substantial N-region addition unlike B-1aFP cells**

Native peritoneal B-1a cells contain few N-region additions at both the V-D and D-J junctions (55% of sequences with no N-additions), as well as differential V<sub>H</sub>, D<sub>4</sub>, and J<sub>5</sub> gene usage, in comparison with splenic B-2 cells (24, 30). To examine the IgH structure of fetal liver Lin-CD45R-CD19- B-1a progeny, fetal liver Lin-CD45R<sup>low</sup>-CD19- B-1a progeny, and bone marrow Lin-CD45R<sup>low</sup>-CD19- B-1a progeny, B-1a cells from SCID recipients were single cell sorted 4 wk following adoptive transfer. Ig H chains were then amplified with promiscuous primers, sequenced, and analyzed (30). All sequences are provided as Supplemental Fig. 4. As expected, B-1aFP cells produced Ig containing very few N-region additions, with 57% of 159 analyzed H chains characterized by zero nontemplated sequences at both the V-D and D-J junctions (Fig. 4A). Surprisingly, however, we found B-1aFN and B-1aBP cells expressed Ig containing abundant N-region additions. Only 12% of the 278 H chains analyzed from B-1aFN and 6% of the 33 H chains analyzed from B-1aBP cells contained zero N-additions at both junctions, which are both significantly different from B-1aFP sequences by χ<sup>2</sup> analysis (p < 0.0001). This level of N-addition is similar to that which characterizes B-2 cells, and B-1 cells produced by adoptive transfer of Lin<sup>+</sup> bone marrow (24, 25). Furthermore, we found N-addition lengths were significantly longer in B-1aFN and B-1aBP cells as compared with B-1aFP cells at the V-D junction (p < 0.0001), the D-J junction (p < 0.0001), and for the sum of...
Lincreased N-addition present in B-1aFN and B-1aBP cells. In sum, cells used JH4 less frequently (21%) than B-1aFN cells (31%) usage was similar between B-1aFN and B-1aFP cells, B-1aFN cells in N-addition, which is reflected in average CDR3 length. B-1aBP cells, both of which differ dramatically from B-1aFP individual mice, which demonstrated reconstitution by flow cytometry time. The results represent an average of serum samples taken from in-serum from 3-mo-old BALB/c-ByJ mice was also evaluated at the same specific IgM, and (4–5 wk posttransfer. The serum was evaluated for (Fig. 4B) with the exception of some differences Lincreased in the absence of TdT, albeit significantly fewer N-additions than found in comparable B-1a cells from wild-type. Results shown in Fig. 5D demonstrate B-1a cells express Ig with sub-
stantial, B-2-like levels of N-addition (Fig. 5A, 5B). The absence of TdT gene expression in fetal liver LinCD45RCD19 progenitors was further confirmed by real-time PCR. As a control for positive and negative TdT expression, Hardy fractions A–D were sort purified from bone marrow cells. As previously reported, we found TdT is expressed in fractions B (Pro-B) and C (late Pre-B) from the bone marrow, but there is little to no expression in fractions A (Pre-Pro-B) and D (small Pre-B) (29). As shown in Fig. 5C, LinCD45RCD19 and LinCD45RCD19 progenitors from bone marrow express TdT, but LinCD45Rlow/CD19+ and LinCD45RCD19 cells from fetal liver do not express TdT. Serial dilution studies indicate that real-time PCR detects TdT transcripts from as few as 250 LinCD45Rlow/CD19 bone marrow progenitors, a population that is TdT positive as a whole. This number represents 0.1% the number of adoptively transferred cells, but could be less, because not all LinCD45Rlow/CD19 bone marrow progenitors may express TdT, so the detection limit may be fewer than 250 TdT cells. Together these results demon-
strate both LinCD45Rlow/CD19 and LinCD45RCD19 progenitors found in the bone marrow express TdT, whereas all progenitors found in the fetal liver do not express TdT, which might be expected from the generally acknowledged absence of TdT during fetal development.

Despite the lack of TdT expression found in the LinCD45RCD19 fetal liver progenitors, extensive N-addition was observed in the B-1aFN cells with 88% of sequences contain-
ing N-additions at one or both junctions (Fig. 4A). These results call into question whether a TdT-independent mecha-

nism of N-addition exists. Therefore, we evaluated B-1a cell Ig obtained from TdT knockout (TdT−/−) mice for N-insertions. Results shown in Fig. 5D demonstrate B-1a cells express Ig with N-additions in the absence of TdT, albeit significantly fewer N-additions than found in comparable B-1a cells from wild-type mice. These results suggest a TdT-independent mechanism could be partially at work after transfer of LinCD45RCD19 fetal liver cells into adult SCID mice to produce B-1aFN cells with extensive N-additions (Fig. 4A).

LinCD45RCD19 fetal liver cell characteristics AA4.1 is expressed early during B cell development (37). Furthermore, it has been shown that c-Kit along with AA4.1 marks early lym-
phoid progenitors in the mouse (38). The LinCD45Rlow/CD19 B-1 cell progenitor reported by Dorshkind and colleagues (23) expresses AA4.1 in both fetal liver and bone marrow. To ad-
dress similarities and differences between the CD19− and

the two junctions (p < 0.0001). These results are summarized in Fig. 4C. Thus, LinCD45RCD19 fetal liver cells and LinCD45Rlow/CD19 bone marrow cells function differently from LinCD45Rlow/CD19 fetal liver cells in giving rise to different kinds of B-1a cells; progeny of the former two add nontemplated nucleotides at H chain V-D and D-J junctions, whereas progeny of the latter largely do not.

CDR3 length was also evaluated in B-1aFN, B-1aFP, and B-1aBP cells (Fig. 4C). We found B-1aFN cells expressed Ig with significantly longer average CDR3 length (10.81 ± 2.49) as compared with B-1aFP cells (10.14 ± 2.44) (p = 0.0073). B-1aBP cells expressed Ig with significantly longer average CDR3 length (12.45 ± 2.57) as compared with B-1aFP cells (10.14 ± 2.44) (p < 0.0001) and as compared with B-1aFN cells (10.81 ± 2.49) (p = 0.0006). These results are consistent with the in-
excreased N-addition present in B-1aFN and B-1aBP cells. In sum, Lin−CD45RCD19 fetal liver cells give rise to B-1aFN cells and LinCD45Rlow/−CD19 bone marrow cells give rise to B-1aBP cells, both of which differ dramatically from B-1aFP cells in N-addition, which is reflected in average CDR3 length.

VH, D, and JH gene usage was also analyzed in B-1a cell sequences derived from the fetal liver progenitor populations, and, unlike N-addition, these aspects of Ig structure differed little. Overall, we found VH gene usage was similar between B-1aFN and B-1aFP cells (Fig. 4B) with the exception of some differences in V11 (J558 family) and VH5 (7183 family) gene usage. B-1aFN cells used VH1 more frequently (32%) than B-1aFP cells (17%) (p = 0.0008). Conversely, B-1aFP cells used VH5 more frequently (25%) than B-1aFN cells (16%) (p = 0.02). Although JH gene usage was similar between B-1aFN and B-1aFP cells, B-1aFN cells used JH4 less frequently (21%) than B-1aFN cells (31%) (p = 0.0136) (Fig. 4B). No significant differences in DJ usage were observed between B-1aFN and B-1aFP cells.

TdT expression is limited to progenitors found in the bone marrow

The process of N-nucleotide addition is mediated by the enzyme TdT (18). Because B-1a cells derived from Lin−CD45R−CD19− fetal liver cells produced Ig containing levels of N-addition indistinguishable from splenic B-2 cells, B-1a progenitors were examined for TdT. LinCD45R−CD19− and LinCD45Rlow/−CD19− cells from day 15 or day 18 fetal liver cells and LinCD45Rlow/−CD19− bone marrow cells were sorted purified, after which TdT tran-
scripts were evaluated by PCR. As expected, we found bone marrow LinCD45Rlow/−CD19− and bone marrow LinCD45Rlow/−CD19− progenitors expressed TdT transcripts, in keeping with the level of N-addition found in the Ig of their B-2 cell or B-1a cell progeny, respectively (Fig. 5B). In direct contrast, we found fetal liver LinCD45R−CD19− progenitors did not express TdT, despite the fact that their B-1aFN progeny produced Ig with sub-
stantial, B-2-like levels of N-addition (Fig. 5A, 5B). The absence of TdT gene expression in fetal liver LinCD45R−CD19− progenitors was further confirmed by real-time PCR. As a control for positive and negative TdT expression, Hardy fractions A–D were sort purified from bone marrow cells. As previously reported, we found TdT is expressed in fractions B (Pro-B) and C (late Pre-B) from the bone marrow, but there is little to no expression in fractions A (Pre-Pro-B) and D (small Pre-B) (29). As shown in Fig. 5C, LinCD45Rlow/−CD19− and LinCD45R−CD19− cells from fetal liver do not express TdT. Serial dilution studies indicate that real-time PCR detects TdT transcripts from as few as 250 LinCD45Rlow/−CD19− bone marrow progenitors, a population that is TdT positive as a whole. This number represents 0.1% the number of adoptively transferred cells, but could be less, because not all LinCD45Rlow/−CD19− bone marrow progenitors may express TdT, so the detection limit may be fewer than 250 TdT cells. Together these results demon-
strate both LinCD45Rlow/−CD19− and LinCD45R−CD19− progenitors found in the bone marrow express TdT, whereas all progenitors found in the fetal liver do not express TdT, which might be expected from the generally acknowledged absence of TdT during fetal development.

Despite the lack of TdT expression found in the Lin−CD45R−CD19− fetal liver progenitors, extensive N-addition was observed in the B-1aFN cells with 88% of sequences contain-
ing N-additions at one or both junctions (Fig. 4A). These results call into question whether a TdT-independent mecha-

nism of N-addition exists. Therefore, we evaluated B-1a cell Ig obtained from TdT knockout (TdT−/−) mice for N-insertions. Results shown in Fig. 5D demonstrate B-1a cells express Ig with N-additions in the absence of TdT, albeit significantly fewer N-additions than found in comparable B-1a cells from wild-type mice. These results suggest a TdT-independent mechanism could be partially at work after transfer of Lin−CD45R−CD19− fetal liver cells into adult SCID mice to produce B-1aFN cells with extensive N-additions (Fig. 4A).

Lin−CD45R−CD19− fetal liver cell characteristics AA4.1 is expressed early during B cell development (37). Furthermore, it has been shown that c-Kit along with AA4.1 marks early lym-
phoid progenitors in the mouse (38). The Lin−CD45Rlow/−CD19− B-1 cell progenitor reported by Dorshkind and colleagues (23) expresses AA4.1 in both fetal liver and bone marrow. To ad-
ress similarities and differences between the CD19− and

FIGURE 3. Lin−CD45R−CD19− fetal liver-derived B-1a cells produce natural IgM Ab that binds pneumococcal Ags. Serum was obtained from CB17 SCID mice transplanted with Lin−CD45R−CD19− fetal liver cells, Lin−CD45Rlow/−CD19− fetal liver cells, or total Lin− bone marrow cells 4–5 wk posttransfer. The serum was evaluated for (A) total IgM, (B) PC-specific IgM, and (C) PPS3-specific IgM levels by ELISA. As a reference, serum from 3-mo-old BALB/c-ByJ mice was also evaluated at the same time. The results represent an average of serum samples taken from inindividual mice, which demonstrated reconstitution by flow cytometry analysis. The number of individual serum samples analyzed per group is as follows: 19 Lin−CD45R−CD19− fetal liver cell chimera mice, 14 Lin−CD45Rlow/−CD19− fetal liver cell chimera mice, and 6 total Lin− bone marrow chimera mice.

A

PC

PPS3

BALB/c-ByJ

Lin-CD45R-CD19- FL

Lin-CD45R+B-CD19+ FL

Lin-BM

B

C

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CD19⁺ fetal liver progenitor cells, AA4.1 and c-Kit surface expression was examined on fetal liver Lin⁻CD45⁻CD19⁻ and Lin⁻CD45⁻CD19⁺ cells by immunofluorescent staining. In contrast to Lin⁻CD45⁻CD19⁻ bone marrow-derived B-1a cells that are AA4.1 positive (99%), we found Lin⁻CD45⁻CD19⁻ fetal liver cells are largely negative for AA4.1 expression, with only 4.4% positive (Fig. 6). However, c-Kit was partially and similarly expressed on Lin⁻CD45⁻CD19⁻ fetal liver cells that are AA4.1 positive (99%).

**FIGURE 4.** IgH sequence analysis of Lin⁻CD45⁻CD19⁻ fetal liver and Lin⁻CD45⁻CD19⁻ bone marrow–derived B-1a cells shows abundant N-addition. (A) N-region addition analysis at the D-J and V-D junctions in B-1a cells derived from Lin⁻CD45⁻CD19⁻ and Lin⁻CD45⁻CD19⁻ fetal liver cells, Lin⁻CD45⁻CD19⁻ bone marrow cells, and total Lin⁻ bone marrow is shown. (B) V, D, and J gene segment usage in B-1a cells derived from Lin⁻CD45⁻CD19⁻ or Lin⁻CD45⁻CD19⁻ fetal liver cells is displayed. (C) Average number of N-additions at the V-D, D-J, or sum of the two junctions is shown along with CDR3 length. Results are based on three to five independent experiments with sequences combined from each independent experiment.
To further respond to TSLP in an in vitro culture system (23) and are sen-
dependent experiments. (26) the cytokine mixture reported by Montecino-Rodriguez et al. (23), which included SCF, Flt-3L, TSLP, IL-3, and IL-6. Cultures were harvested at days 3 and 9. Results shown in Fig. 6C demonstrate Lin’ CD45R’ CD19’ fetal liver cells start to gain AA4.1 in the absence of IL-7 but gain more AA4.1 expression in the presence of IL-7 by day 9 of culture. However, Lin’ CD45R’ CD19’ fetal liver cells only acquire significant levels of CD54R and CD19 expression in the presence of IL-7 at day 9 (Fig. 6D). These results demonstrate Lin’ CD45R’ CD19’ fetal liver cells are able to progress forward in development by acquisition of AA4.1 in the presence of TSLP with or without IL-7, but only in the presence of TSLP and IL-7 do they acquire CD45R and CD19 expression.

We evaluated whether isolated Lin’ CD45R’ CD19’ fetal liver cells upregulate TdT in vitro in the presence or absence of IL-7, using the culture conditions described in Fig. 6C and 6D. The Lin’ CD45R’ CD19’ fetal liver cells did not express TdT in the absence or presence of IL-7 after 3 or 9 d of culture (no detectable TdT mRNA expression by real-time PCR analysis). Lin’ CD45Rlow’CD19’ bone marrow cells were used as a positive control for TdT expression (Supplemental Fig. 3).

We also assessed Lin’ CD45R’ CD19’ fetal liver cells for the presence of the hematopoietic stem cell–enriched population referred to as LSK (Lin’ Sca-1’ c-Kit’) (26). Results demonstrate an average of 1.8% (±0.57 SEM) of Lin’ CD45R’ CD19’ fetal liver cells express Sca-1 and c-Kit (Supplemental Fig. 2A, 2B). The results show a small number of HSC is present within the Lin’ CD45R’ CD19’ fetal liver population. Previously reported data suggest B-1a cell–specific progenitors present in the fetal environment arise independently of HSCs (39).

**Discussion**

N-addition plays a key role in Ag receptor diversity and Ab effector fitness. Anti-PC Abs derived from B-1a cells have been shown to be protective against *S. pneumoniae* infection (9, 36), and the prototypic B-1a anti-PC Ab, T15, has no N-addition (36, 40). Conversely, TdT transgenic mice vaccinated with heat-killed *S. pneumoniae* generated an anti-PC response, but these anti-PC Abs were not protective against *S. pneumoniae* infection (28). These findings highlight the importance of Ab structure in terms of N-addition in the protection afforded by natural Ab.

In this study, we identify two progenitor populations, distinguished by phenotype and location, that give rise to B-1a cells expressing Ig characterized by abundant N-region addition. This differs from the accepted paradigm that B-1a cells produce protective germine-like Ig with few N-additions (1, 41). The first progenitor population, described herein, is a population of fetal liver cells, defined as Lin’ CD45Rlow’CD19’ fetal liver progenitors.

To determine whether Lin’ CD45R’ CD19’ fetal liver cells are preprogrammed to differentiate into Lin’ CD45Rlow’ CD19’ progenitors, day 18 Lin’ CD45R’ CD19’ fetal liver cells were placed in culture with or without a cytokine mixture of SCF, TPO, and Flt-3L. After 24 h, the cells were assessed for expression of CD19, CD45R, AA4.1, and c-Kit expression by immunofluorescent staining. We found Lin’ CD45R’ CD19’ fetal liver cells did not acquire CD45R, CD19, or AA4.1 during culture in medium alone or with SCF, TPO, and Flt-3L (Fig. 6B). Furthermore, c-Kit expression on Lin’ CD45R’ CD19’ fetal liver cells did not change in the absence or presence of cytokine stimulation. These data demonstrate Lin’ CD45R’ CD19’ fetal liver cells are not preprogrammed to become AA4.1’Lin’ CD45Rlow’CD19’ cells within a 24-h period.

It has been shown bone marrow B-1 cell progenitors are responsive to TSLP in an in vitro culture system (23) and are sensitive to IL-7 signaling in the bone marrow (26). To further investigate whether the Lin’ CD45R’ CD19’ fetal liver cells can acquire AA4.1, CD45R, and/or CD19, we cultured these cells using two previously described in vitro culture systems either with or without IL-7. For cultures with IL-7, we used the cytokine mixture reported by Esplin et al. (26), which included SCF, Flt-3L, TSLP, and IL-7. For cultures without IL-7, we used the cytokine mixture reported by Montecino-Rodriguez et al. (23), which included SCF, Flt-3L, TSLP, IL-3, and IL-6. Cultures were harvested at days 3 and 9. Results shown in Fig. 6C demonstrate Lin’ CD45R’ CD19’ fetal liver cells start to gain AA4.1 in the absence of IL-7 but gain more AA4.1 expression in the presence of IL-7 by day 9 of culture. However, Lin’ CD45R’ CD19’ fetal liver cells only acquire significant levels of CD54R and CD19 expression in the presence of IL-7 at day 9 (Fig. 6D). These results demonstrate Lin’ CD45R’ CD19’ fetal liver cells are able to progress forward in development by acquisition of AA4.1 in the presence of TSLP with or without IL-7, but only in the presence of TSLP and IL-7 do they acquire CD45R and CD19 expression.

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FIGURE 6. Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>+</sup> fetal liver cells express little to no AA4.1 and are responsive to IL-7. (A) Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>+</sup> and Lin<sup>−</sup>CD45R<sub>low</sub>/CD19<sup>+</sup> fetal liver cells were assessed for AA4.1 and cKit expression at day 18 by immunofluorescent staining. (B) Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>+</sup> fetal liver cells were sorted from day 18 embryos. The cells were placed in culture overnight with SCF, TPO, and Flt3L. Expression of AA4.1 and cKit was determined following the 24-h culture. The results shown are representative of three independent experiments. (C, D) Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>−</sup> (black bars) and Lin<sup>−</sup>CD45R<sub>low</sub>/CD19<sup>+</sup> (gray bars) fetal liver cells were sorted from day 18 embryos. The cells were placed in culture with a cytokine mix containing IL-7 (SCF, Flt3-L, TSLP, IL-7) or lacking IL-7 (SCF, Flt3-L, TSLP, IL-6, IL-3). After 3- and 9-d expression of AA4.1 (C) or CD45R and CD19 (D), expression was determined. The results shown are an average of three independent experiments.

represent a fetal liver cell population shown to be capable of giving rise to B-1a cells with abundant N-addition and hence enhanced diversity.

The second progenitor population described in this work is the aforementioned Lin<sup>−</sup>CD45R<sub>low</sub>/CD19<sup>+</sup> progenitor as it develops in the adult bone marrow rather than the fetal liver. Bone marrow Lin<sup>−</sup>CD45R<sub>low</sub>/CD19<sup>+</sup> progenitor cells give rise to authentic B-1a cells, and these B-1aBP cells produce Ig with abundant N-addition. This is in direct contrast to the phenotypically identical Lin<sup>−</sup>CD45R<sub>low</sub>/CD19<sup>+</sup> progenitors obtained from fetal liver, which give rise to B-1a cells expressing Ig with minimal N-addition. Thus, Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>−</sup> fetal liver cells, and Lin<sup>−</sup>CD45R<sub>low</sub>/CD19<sup>+</sup> bone marrow cells, each give rise to N<sup>+</sup> B-1a cells, and either of these progenitor populations, or both, may contribute to the age-related increase in B-1a cell Ab N-addition documented elsewhere (22, 24). Moreover, our results highlight two variables that determine enhanced, N-addition-mediated B-1a diversity, namely the nature of the B-1a progenitor population (in particular, CD45R<sup>−</sup>CD19<sup>−</sup> versus CD45R<sub>low</sub>/CD19<sup>+</sup>) and the location in which the B-1a progenitor develops (in particular, fetal liver versus adult bone marrow).

Because B-1aFN and B-1aBP cells displayed Ig with high-level N-addition, we evaluated fetal liver and bone marrow progenitors for TdT expression. We found no expression of TdT in Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>−</sup> or Lin<sup>−</sup>CD45R<sub>low</sub>/CD19<sup>−</sup> fetal liver cells, whereas TdT was expressed in Lin<sup>−</sup>CD45R<sub>low</sub>/CD19<sup>+</sup> bone marrow progenitors. The importance of progenitor expression of TdT for B-1a N-addition is emphasized by our finding that, unlike TdT-negative Lin<sup>−</sup>CD45R<sub>low</sub>/CD19<sup>−</sup> fetal liver cells, TdT-positive Lin<sup>−</sup>CD45R<sub>low</sub>/CD19<sup>+</sup> bone marrow cells give rise to B-1aBP progeny expressing Ig with abundant, B-2 cell-like levels of N-addition (Fig. 4A). These results are consistent with the generally accepted notion that TdT is not expressed in hematopoietic tissue during fetal life but is abundantly expressed in adult animals (19, 29, 40). Nevertheless, these results raise the question of how Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>−</sup> cells function to generate B-1aFN progeny characterized by N-replete Ig in the absence of TdT. It may be that another route to N-addition beyond TdT exists, or that Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>−</sup> progenitors acquire TdT upon adoptive transfer to SCID recipients. It is unlikely to result from repertoire skewing produced by selective pressure because both Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>−</sup> and Lin<sup>−</sup>CD45R<sub>low</sub>/CD19<sup>+</sup> progenitors experienced the same SCID environment upon adoptive transfer.

To address the possibility of a TdT-independent mechanism of N-addition, we evaluated B-1a cell Ig from TdT<sup>−/−</sup> mice. We found a limited number of N-additions in native B-1a cells, in the absence of TdT (Fig. 5C). These results are congruent with previously published data showing ~3% of the VH5 sequences analyzed from splenic B cells of TdT knockout mice display N-additions (42). As suggested by Gilfillan et al. (42), it is probable in the absence of TdT N-additions are inserted during nonhomologous recombination at the time of the joining process, or before the joining process, by DNA polymerase misincorporation of nucleotides. Therefore, it is plausible that a TdT-independent mechanism could be at work after transfer of Lin<sup>−</sup>CD45R<sup>−</sup>CD19<sup>−</sup> fetal liver cells into adult SCID mice to produce B-1a cells with N-additions.

However, the data presented in this work support at most only a minor role for a TdT-independent mechanism for N-addition in the Ig of B-1aFN cells. The N-additions seen in B-1aFN cells are much more extensive (average N-length at both junctions = 3.8) than those seen in B-1a cells from TdT<sup>−/−</sup> mice (average N-length at both junctions = 0.6). Thus, a TdT-independent mechanism of N-addition may contribute to N-addition in Abs produced by B-1a.
cells derived from TdT-negative progenitors, but is unlikely to be fully responsible.

Interestingly, induction of TdT expression by IL-7 has been demonstrated in bone marrow pro-B cells (43). IL-7 receptor knockout studies have shown mouse fetal B cell development is not dependent upon IL-7, whereas B cell development in the adult is IL-7 dependent (44). These previous reports suggest B cell development in the fetal liver environment takes place in the absence of IL-7. In context with the data presented in this study showing TdT-negative progenitors transferred into adult SCID mice produce B-1a cells expressing Ig with abundant N-additions, it is reasonable to hypothesize that, upon transfer into the adult environment, fetal progenitors are exposed to IL-7 (along with other factors), which upregulates TdT expression. The absence of TdT expression in Lin−CD45R−CD19− fetal liver cells cultured with IL-7 does not rule out induction of TdT in vivo after transfer to SCID mice because in vitro conditions cannot completely reproduce the natural bone marrow environment. Together our results along with previous findings suggest the numerous N-additions observed in B-1a cells derived from TdT-negative Lin−CD45R−CD19− fetal liver cells are most likely the result of TdT upregulation in the adult environment in combination with minimal contribution of TdT-independent N-addition. In this scheme, the key functional difference between Lin−CD45R−CD19− and Lin−CD45Rlow/−CD19+ progenitor populations lies in responsiveness to the adult bone marrow milieu in terms of TdT upregulation.

Peritoneal B-1a cells from young (3-mo-old) mice express Ig with little N-addition, similar to the level of N-addition found in B-1aFP progeny of Lin−CD45Rlow/−CD19+ fetal liver progenitors, whereas native B-1a cells do not begin to express Ig with substantial N-addition until well into adulthood. Several groups have shown total lineage-negative adult bone marrow can give rise to B-1a cells, inferring that B-1a cells are generated de novo throughout adulthood (24–26). Notably, hematopoietic stem cells, and common lymphoid progenitors, largely fail to give rise to B-1a cells beyond the second week of life (45–47). These studies imply mainly bone marrow cells other than HSC and common lymphoid progenitors produce N+ B-1a cells in the adult. This focuses attention on the behavior of the Lin−AA4.1+CD45R−CD19− and Lin−AA4.1+CD45Rlow/−CD19+ progenitor populations, which could provide a source of non-HSC B-1a progenitors in the adult.

Lin−CD45R−CD19− cells may correspond to an earlier stage of development than Lin−CD45Rlow/−CD19− cells because the former lack AA4.1 and acquire CD45R and CD19 during in vitro culture with IL-7. We have shown AA4.1+Lin−CD45R−CD19− fetal liver cells lack TdT but presumably acquire it in the adult bone marrow, where resident AA4.1+Lin−CD45Rlow/−CD19− cells express TdT constitutively (although the phenotypically identical AA4.1+Lin−CD45Rlow/−CD19− cells developing in the fetal liver appear refractory to TdT induction). Thus, there are at least two ways in which the AA4.1+Lin−CD45R−CD19− progenitor cells described in this work may give rise to N+ B-1a cells in vivo: (1) AA4.1+Lin−CD45R−CD19− progenitor cells may differentiate into AA4.1+Lin−CD45Rlow/−CD19+ progenitor cells that give rise to N− B-1a cells in life but migrate to the bone marrow in adulthood, where, having acquired TdT, they generate N+ B-1a cells; or (2) AA4.1+Lin−CD45R−CD19− progenitor cells are quiescent early in life but migrate to the bone marrow in adulthood, where they acquire TdT and generate N+ B-1a cells, or they give rise to TdT+ AA4.1+Lin−CD45Rlow/−CD19+ progenitors that generate N+ B-1a cells. At present it is not possible to differentiate between these mechanisms. We hypothesize option 2 is the more likely scenario because results presented in this work demonstrate the following: 1) AA4.1+Lin−CD45R−CD19− progenitor cells do not acquire CD19 or AA4.1 without IL-7 in vitro (Fig. 6C, 6D), and 2) AA4.1+Lin−CD45Rlow/−CD19+ fetal liver cells do not give rise to N+ B-1a cells upon transfer to the adult (Fig. 4). These results suggest bone marrow migration of fetal liver AA4.1+Lin−CD45Rlow/−CD19− cells cannot account for generation of N+ B-1a cells.

In sum, our data suggest Lin−CD45R−CD19− fetal liver cells, which grow out B-1aFP cells in SCID mice, add to the adult pool of B-1a cells present in older animals. Their relationship to Lin−CD45Rlow/−CD19+ progenitors in adult bone marrow, which also generate B-1a cells with abundant N-additions, remains uncertain.

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Disclosures

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References


