B-1a cells acquire their unique characteristics by bypassing the pre-BCR selection stage

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B-1a cells are long-lived, self-renewing innate-like B cells that predominantly inhabit the peritoneal and pleural cavities. In contrast to conventional B-2 cells, B-1a cells have a receptor repertoire that is biased towards bacterial and self-antigens, promoting a rapid response to infection and clearing of apoptotic cells. Although B-1a cells are known to primarily originate from fetal tissues, the mechanisms by which they arise has been a topic of debate for many years. Here we show that in the fetal liver versus bone marrow environment, reduced IL-7R/STAT5 levels promote immunoglobulin kappa gene recombination at the early pro-B cell stage. As a result, differentiating B cells can directly generate a mature B cell receptor (BCR) and bypass the requirement for a pre-BCR and pairing with surrogate light chain. This ‘alternate pathway’ of development enables the production of B cells with self-reactive, skewed specificity receptors that are peculiar to the B-1a compartment. Together our findings connect seemingly opposing lineage and selection models of B-1a cell development and explain how these cells acquire their unique properties.

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B-CLL is the most common form of adult leukemia in the western world. In the early 1980s, the T-cell antigen CD5 (Ly-1) was identified on the surface of cancerous B cells in patients with B-CLL. This observation led to the search for normal CD5+ B-cell counterparts to potentially determine the cancer cell of origin. As a result of these efforts, CD5+ B cells, otherwise known as B-1a cells, were discovered in mice. Further characterization of CD5+ B-1a B cells revealed that they are long-lived, self-renewing cells that predominantly reside in the pleural and peritoneal cavities where they produce natural polyreactive IgM antibodies with a biased, autoreactive repertoire. In contrast to conventional B-2 cells, B-1a cells produce antibodies with reduced junctional diversity and less somatic hypermutation. Furthermore, Igh VH gene rearrangements favor VH12 segment usage, generating antibodies that interact with phosphatidylcholine (PC), a major lipid in the protective mucus layer of the gastrointestinal tract that is also present in the membranes of diverse bacteria. Thus, the B-1a receptor repertoire is biased toward bacterial and self-antigens, which is important for mounting a rapid immune response to infection and in the clearing of apoptotic cells. Because B-1a cells are found in pre-immune mice, they function as an important first line of defense against bacterial pathogens. These characteristics distinguish B-1a cells from conventional B-2 cells, which have a highly diverse receptor repertoire that is important for mediating adaptive immunity.

Although B-1a cells were discovered in the early 1990s, their origin has been hotly debated since, and despite the efforts of numerous labs this remains an unresolved issue. The controversy has mainly been centered on two opposing models, the lineage model and the selection model. The lineage model proposes that a distinct B-1 progenitor cell gives rise to B-1a cells, while the selection model favors the idea that a common B-cell progenitor can acquire a B-1a or a B-2 fate depending on the type of antigen it recognizes. Support for the lineage model comes from early reconstitution experiments, which reveal that fetal tissues are much more efficient at generating B-1a cells in irradiated recipient mice than adult bone marrow counterparts. Furthermore, the first wave of B-1a cells was shown to originate in early embryos in an HSC-independent manner. However, cellular barcoding experiments demonstrate that a single progenitor cell can give rise to both B-1a and B-2 cells challenging the notion that B-1a cells arise from a distinct lineage. Moreover, the finding that B-1a cells have a restricted and biased receptor repertoire provides support for a selection model. Further support for the selection model comes from a study by Graf et al. that made use of a transgenic system to show that swapping B-2 and B-1a-specific B-cell receptors (BCRs) is sufficient to efficiently change a B-2 cell into a B-1a cell in the absence of any lineage constraints. The lineage switch is rapid, induces a proliferative burst, and cells migrate to their normal environments within the pleural and peritoneal cavities.

Investigations have also focused on expression of specific genes that influence development. For example, the Lin28b/let7 molecular switch influences CD5+ B-1a cell fate. The Lin28b and let7 miRNAs, respectively, promote and inhibit the expression of the transcription factor, Arid3a, which in turn can drive the development of CD5+ B-1a cells. Nonetheless, the BCR repertoire of the resulting cells do not include Ptc-specific antibodies that are characteristic of a typical B-1a cell compartment. Thus, enforced expression of Arid3a fails to fully explain how B-1a cells develop. Another transcription factor, BHLHE41, has also been shown to be important in B-1a cell biology. Specifically, cells deficient in this transcription factor lose B-1a cells expressing Vg12/Vg4 Ptc-specific receptors, have impaired BCR signaling, increased proliferation, and apoptosis. BHLHE41 therefore plays an important role in B-1a maintenance by regulating self-renewal and BCR repertoire; however, it is not known whether its forced expression can drive development of these cells.

In the fetus, B-cell development takes place in the liver and moves to the bone marrow after birth. Each stage of development is marked by a particular rearrangement event that drives differentiation forward. These recombination events occur in a stage-specific manner. The first step involves the joining of the immunoglobulin heavy-chain (Igh) Dh1 and Ih4 gene segments within pre-pro-B cells. Rearrangement continues at the pro-B cell stage, where V12-to-Dh1 joining is both initiated and completed. Rearrangement of the immunoglobulin light-chain (Igl) gene loci, lgl or ilg, occurs at the subsequent pre-B cell stage of development. Igh and Igl gene rearrangement is separated by a proliferative burst of large pre-B cells that allows individual cells that have successfully rearranged their heavy chain to clonally expand. At the following small pre-B cell stage, each B-cell undergoes a distinct Igh gene recombination event. Ultimately, this results in unique heavy- and light-chain pairs that expand the antigen receptor repertoire. The successful pairing of immunoglobulin heavy chain with surrogate light chain (SLC) forms the pre-B cell receptor (pre-BCR), which is required for expansion of large pre-B cells and subsequent differentiation to the small pre-B cell stage, where Iglk recombination occurs. Since SLC pairs poorly with autoreactive heavy chains, the pre-BCR provides a mechanism for negative selection of self-reactive B cells.

As noted early on, B-1 cell development is quantitatively unaffected in SLC-deficient mice, and in a small fraction of B-cell progenitors in the bone marrow Igh rearrangements occur prior to rearrangements at Iglk and independent of SLCs. In addition, in the absence of SLC and thus pre-BCR expression, an autoreactive BCR can drive B-cell development efficiently to the stage of the immature B cell, where BCR diversification and counterselection of autoactivity is achieved through the process of receptor editing. This has led to a model in which early B-cell development is driven by a positive signal from the pre-BCR (in the majority of progenitors) or an autoreactive BCR (in a minority of cells), with the pre-BCR having evolved as a surrogate autoreactive BCR.

Downstream of pre-BCR signaling, IL-7 receptor (IL-7R) signaling is extinguished at the small pre-B cell stage. This is noteworthy because the IL-7R signaling pathway is responsible for directing the sequential ordering of recombination events, i.e., heavy-chain gene followed by light-chain gene rearrangement in classical B-cell development. IL-7 is a cytokine secreted by stromal cells within the bone marrow where development takes place throughout the postnatal and adult life of the animal. In murine bone marrow, development past the pre-pro-B cell stage stringently requires IL-7R signaling. In contrast, B-cell development within the fetal liver can occur independent of IL-7R signaling. In terms of directing recombination, IL-7 and its downstream signaling component STAT5 have been shown to promote Ighk accessibility and recombination while actively inhibiting recombination of the Iglk locus. Activated STAT5 enters the nucleus and forms a complex with PRC2/EOZH2, which binds to the intronic enhancer of Iglk (iEiks) and induces H3K27me3-mediated repression to inhibit recombination of this locus. Strikingly, conditional deletion of STAT5 in pre-B cells results in a robust increase of Iglk recombination at the pre-B cell stage.

It is known that B-1a cells originate primarily from fetal tissues, however, it remains unclear what pathways drive B-1a cell development and lead to the acquisition of their unique characteristics. In addition, it has been shown that B-1a cells are efficiently generated in Il7−/− and Il7r−/− mice albeit at reduced numbers compared with wild-type mice. Given our finding that fetal liver (FL) pro-B cells have less active cytoplasmic
pSTAT5 than bone marrow (BM) pro-B cells\textsuperscript{48}, we asked whether this could influence V(D)J recombination and B-cell development.

Here, we show that low levels of IL-7R/pSTAT5 signaling in the fetal liver environment promote an "alternate pathway" of B-cell development, in which increased Igk rearrangement occurs at the pro-B cell stage of development. Productive rearrangement of Igk and Igk at this stage leads directly to cell surface expression of a mature BCR, bypassing the requirement for SLC and the selection of autoreactive receptors that are characteristic of B-1a cells. Extending earlier work, we demonstrate that while SLC-independent development leads to a significant reduction in B-2 cell numbers, B-1a cells with their characteristic Vj\textsubscript{12} anti-πC bias are still efficiently generated. Thus, reduced IL-7R/STAT5 signaling promotes an alternate pathway of development which favors the production of B-1a cells with self-reactive receptors characteristic of this B-cell subset. Together these data connect opposing lineage and selection models of B-1a cell development and explain how these cells acquire their unique properties.

**Results**

Igk rearrangement is increased in fetal liver pro-B cells. Fetal liver (FL) and bone marrow (BM)-derived progenitor cells are differentially dependent on IL-7 for development, and we have found that FL-derived pro-B cells have significantly lower levels of the downstream signaling component, phospho-STAT5 compared with their bone marrow-derived pro-B cell counterparts\textsuperscript{48}. Thus, we asked whether alterations in phospho-STAT5 levels correlate with altered regulation of Igk recombination in these two anatomic locations. To explore this, we analyzed recombination from ex vivo-derived bone marrow (5–6 wk adults) and FL (E17–18) pro-B cells using semiquantitative PCR analysis (Fig. 1a). This assay uses a degenerate Vx primer with a common reverse primer downstream of Jk gene segments to measure recombination of a Vx with each of the four Jk segments\textsuperscript{49}. As shown in Fig. 1a, FL pro-B cells have increased Igk recombination relative to BM pro-B cells.

To address the question of altered Igk recombination between these two cell types in a more quantitative manner, we made use of a real-time PCR assay that quantifies Jk1 rearrangement by assessing the retention of germline Igk\textsuperscript{50}. In this assay, a product cannot be generated after recombination, because the sequence to which the upstream primer binds is lost or inverted during the recombination process. Quantitation of the amplified product is determined as a ratio between the single copy gene β-actin and the germline Igk band. Control tail DNA, in which no Igk rearrangement occurs, is set at 100%. Consistent with the semiquantitative experiments described above, FL pro-B cells have lower levels of Igk germline retention (61%) relative to bone marrow pro-B cells (87%) (Fig. 1b).

To determine the proportion of cells that are actively undergoing recombination at a single-cell level, we used three-dimensional (3-D) immunofluorescent in situ hybridization (FISH) assay that visualizes recombination by the association of γ-H2AX DNA repair foci with the individual alleles of antigen receptor loci\textsuperscript{51,52}. In these experiments, we used two Igk BAC DNA probes that hybridize to the distal Vx24 (RP23–101G13) gene region and the Cx region (RP24–387E13) in combination with an antibody against the phosphorylated form of γ-H2AX (Fig. 1c). We found significantly increased association of γ-H2AX foci with the Igk locus in FL versus bone marrow-derived pro-B cells (16.7% versus 3.0%). Consistent with our previous experiments, the frequency of DNA breaks in FL pro-B cells (16.7%) is again at an intermediate level compared with BM pre-B cells (30%) where Igk recombination typically occurs (Fig. 1d). These numbers reflect the combined data from two independent experiments (Supplementary Table 1).

It is known that activated STAT5 forms a complex with PRC2 and binds to the intronic enhancer of Igk, iEx to inhibit Igk recombination\textsuperscript{49,53,54}. To determine if the increase in early Igk rearrangement could be caused by reduced STAT5-mediated repression, we analyzed enrichment of STAT5 binding on iEx via STAT5-ChIP-qPCR. As shown in Fig. 1e, we found that FL pro-B cells have significantly less STAT5 bound to iEx compared with BM pro-B cells, and binding levels more closely resemble those in BM pre-B cells where Igk recombination normally occurs. Taken together, these findings indicate that in the FL versus BM environment, Igk rearrangement occurs at higher levels in pro-B cells due to a decrease in STAT5-mediated repression of the Igk locus.

Antigen receptor loci undergo large-scale contraction through chromatin looping, which enables rearrangement between widely dispersed gene segments. Locus contraction is tightly linked both to recombination status and usage of distal gene segments\textsuperscript{49,53}. To address the contraction status of the Igk locus, we measured the distance separating the distal Vx24 and Cx probes in interphase cells. Our analyses indicate that FL pro-B cells are significantly more contracted than wild-type (WT) double-positive (DP) T cells, a cell type in which Igk rearrangement does not occur (Fig. 2). Indeed, the level of contraction is comparable with that seen in wild-type bone marrow-derived pre-B cells, where Igk gene rearrangement is known to occur.

**Bypass of the pre-BCR checkpoint.** Classical bone marrow B-cell development starts with Igh recombination at the pre-pro to pro-B cell stages followed by Igk/Igl4 rearrangement at the small pre-B cell stage. However, JHT mice that lack Jh segments as well as the Igk intrinsic enhancer, and are therefore unable to undergo Igk rearrangement, but can rearrange Igk/Igl4 independent of Igh rearrangement (Fig. 3a)\textsuperscript{29,54,55}. This is also true for SLC-deficient mice, and a fraction of B cells undergoing development in the bone marrow of wild-type mice\textsuperscript{56}. Given our finding that a significant number of pro-B cells rearrange Igk in the FL environment, we reasoned that productive heavy and light-chain gene rearrangement in pro-B cells could lead to expression of a mature BCR, bypassing the requirement for a pre-BCR. To address this question as a proof of principle, we examined FL B cells from mice that are deficient for a component of SLC, lambda5 (Igl4\textsuperscript{−/−}), in conjunction with a transgene that has a prearranged functional heavy chain (B1.8). Igl4\textsuperscript{−/−} mice display a strong developmental block at the pro-B cell stage and made very few IgM+ cells (0.59% of B cells). In contrast Igl4\textsuperscript{−/−} B1.8 and B1.8 mice produce similarly high percentages of IgM + B cells (12 and 13.5%, respectively). Importantly, B1.8 mice have a substantial pre-B cell compartment which is not observed in Igl4\textsuperscript{−/−}. B1.8 mice despite a similar output of IgM+ B cells which express IgK protein (Fig. 3b). This shows that having a productive heavy- and light chain can bypass the requirement for pairing with SLC to form immature B cells that express the mature BCR. Together these data demonstrate that in the FL expression of Igk and Igk at the pro-B cell stage can bypass the developmental block induced by surrogate light-chain deficiency by forming B cells with a mature BCR.

**B-1a cells are efficiently generated in Igl4\textsuperscript{−/−} mice.** B-1a cells are primarily generated from fetal tissues, and we have shown that FL-derived B cells frequently undergo early Igk recombination allowing cells to bypass the pre-BCR selection stage. Thus, we next asked whether an absence of SLC could impact the generation of B-1a cells\textsuperscript{12,23}. Here, we analyzed the B-2 as well as
the B-1 compartment, which consists of both B-1a and B-1b cells. B-1b cells are distinct from B-1a cells in that they lack CD5 expression and have a memory function in protecting against bacterial infections. As expected, peritoneal B-2 cells were significantly decreased compared with controls. Furthermore, although B-1b cells were also significantly decreased, the B-1a cell compartment remained intact. This was reflected in cell percentage, as well as total cell numbers (Fig. 4a, b). These data indicate that while B2 and B1b cell development is strongly promoted by SLC, B-1a cells do not depend on a pre-BCR checkpoint. It should be noted that B1.8 transgenic mice could not be used to analyze the B-1a cell compartment in SLC-deficient mice, because the B1.8 pre-rearranged heavy chain does not support B-1a cell development.
Two different subsets of B-1a cells that segregate different functions of the B-1a compartment have been identified by the plasma cell alloantigen 1 (PC1). B-1a cells produce higher amounts of natural IgM, and can undergo plasma cell differentiation, while PC1hi B-1a cells produce less natural IgM, have Ptc-reactive BCRs, and can produce IL-10. In order to determine whether or not both of these subsets of B-1a cells were represented in B-1a cells generated in SLC-deficient mice, we analyzed PC1 expression. B-1a cells from Igll1−/− mice were found to have similar proportions of PC1lo and PC1hi B-1a cells as in wild-type mice (Fig. 4c). This suggests that not only is the B-1a compartment size normal in SLC knockout mice but also the PC1lo and PC1hi subsets are maintained.

B-1a cells cannot be detected in FL, and first appear in the transitional B-cell population 1 week after birth. To further analyze the impact of surrogate light-chain deficiency on the generation of B1a cells, we examined transitional B-1a cells in spleens from 10–11 days old neonatal mice. As expected, the proportion of B-2 in cells in this population was severely reduced, while the proportion of B-1a cells was found to increase. This is reflected by the finding that total numbers of transitional B-2 cells are severely depleted compared with B1a cells in Igll1−/− versus wild-type mice (Fig. 5a–c). Thus, even at this early stage of life, transitional B-1a cells from SLC-deficient mice are found at only moderately reduced levels compared with their wild-type counterparts, indicating that the presence of adult Igll1−/− B-1a cells is not a result of clonal expansion by self-renewal over time.

B-1a cell development is inhibited in Stat5b-CA mice. Our data suggest that low phospho-STAT5 signaling induces early Igk recombination in FL pro-B cells, which promotes the efficient generation of B-1a cells in SLC-deficient mice. To further validate the role of IL-7R/STAT5 signaling in B-1a cell development, we next asked whether increased STAT5 signaling could have the opposite effect on B-1a cell development and impair the generation of this compartment. To address this question, we used mice expressing a constitutive active form of STAT5 (Stat5b-CA).

In these mice, we found that although B-2 and B-1b cell development were not significantly affected, all three independent littermate controlled Stat5b-CA mice exhibited a decrease in the B-1a compartment. This is reflected in cell proportions as well as the total cell numbers (Fig. 6a, b). Thus, STAT5 signaling inhibits B-1a cell development underscoring the importance of
B-1a cells express PtC-specific receptors. B-1a cells are known to have a strong bias for immunoglobulin rearrangements that confer specificity to PtC (VH12/Vk4). In addition, the pre-BCR is known to select against B-1a-specific autoreactive heavy chains as a result of a defect in pairing of the latter with SLC26,27. Given these facts, we next asked whether B-1a cells that develop independent of SLC would promote the generation of B-1a-specific rearrangements. To address this question, we analyzed peritoneal cavity B cells for VH12 usage and specificity against PtC. As shown in Fig. 6c, B-1a cells from Igll1−/− mice express VH12 rearranged receptors. Importantly, these VH12+B-1a cells bind PtC containing liposomes, inferring...
that they have the canonical V_{H}12/V_{K}4 gene segment bias (Fig. 6c). In addition, there is not a significant difference in the proportion of B-1a cells that have specificity against PtC (Fig. 6d). Thus, an absence of SLC supports the generation of these self-reactive B-1a cells.

**Igll1^−/−** and wild-type B-1a cells have similar repertoires. To determine how SLC deficiency affects gene segment usage, we sequenced the BCR repertoires of B-1a cells from WT and SLC-deficient mice. Briefly, peritoneal cavity B-1a cells were sorted, lysed, and the rearranged Igh products amplified by PCR. Illumina adapters and barcodes were added, and the rearrangements sequenced by next-generation sequencing. BCR repertoires were analyzed by IMGT/High-V-QUEST, and visualized using IMGT/StatClonotype64–67. These analyses revealed that B-1a cells from Igll1^−/− mice have a similar overall V_{H} usage compared with those of wild-type mice (Fig. 7). This shows that B-1a cells that develop in the absence of SLC have a wide distribution of gene segment usage, and thus do not represent a clonal expansion of a few rare progenitors. A diverse repertoire was also observed for light-chain gene segment usage (Fig. 8). As expected, V_{H}12 (V_{H}12-3) usage was found to be high in wild-type B-1a cells, and calculated proportions were comparable with our flow-cytometry experiments in the cells from Igll1^−/− mice (Fig. 6c). V_{K}4, which pairs with V_{H}12 to confer PtC specificity, has a reduced representation in SLC-deficient B-1a cells, but it is still found at significantly higher frequency than in wild-type B-2 cells (Fig. 8).

These rearrangement studies support the data from our flow-cytometry analysis and underscore the finding that B-1a cells generated in the absence of SLC maintain a V_{H}12/V_{K}4 gene segment bias.

To confirm that B-1a cells generated in SLC-deficient mice were from fetal origin, we analyzed Igh sequence for N-additions. B-1a cells are known to predominantly come from fetal origins and as a consequence of low Tdt activity at this stage, they are known to have reduced N-additions compared with adult bone marrow-derived B cells. Our analysis revealed that Igll1^−/−
peritoneal cavity B-1a cells had similarly reduced levels of N-additions as wild-type controls (Fig. 9). The paucity of N-additions supports the idea that B-1a cells are efficiently generated in SLC-deficient mice, maintaining key B-1a cell traits like VH12 and VK4 gene segment bias and reduced levels of junctional diversity.

Discussion

Although it was known that B-1a cells predominantly develop in the FL, the pathways driving development and the mechanisms underlying the generation of B cells with a repertoire skewed toward autoreactivity were not previously known. Indeed, this has been a subject of debate for many years. Here, we now reveal that a reduction in IL-7 signaling in the FL environment alleviates Igk repression and promotes early rearrangement of this locus in pro-B cells. Productive heavy and light-chain gene rearrangement at the pro-B cell stage can lead directly to the expression of a mature BCR, thereby bypassing the requirement for a pre-BCR check-point. Since PtC-specific V_H gene rearrangements pair poorly with SLC26 and the pre-BCR selects against autoreactive receptors27, SLC-independent development provides an explanation for how B cells with autoreactive, PtC-specific receptors are generated. In more general terms and along Jerne’s idea that the immune system selects antibody mutants from an initial self-reactive repertoire68, B-cell progenitors can be initially positively selected by the expression of autoreactive BCRs or their evolutionary surrogate32,33. This is followed by negative selection of autoreactivity through receptor editing. The skewing of the B-1a receptor repertoire toward autoreactivity likely reflects the imprint of the initial positive selection of B-1a cells through autoreactive BCRs rather than SLC (Fig. 10).

The idea that B-1 cells can develop in a SLC-independent manner is not novel. In fact, Kitamura et al. observed that in contrast to B-2 cells, B-1 cells were essentially unaffected by SLC deficiency, however, the distinct effect on the B-1a versus B-1b
cell compartment was not investigated\textsuperscript{28}. Our analyses demonstrate that while B-2 and B-1b cell development is severely impaired in Igll\textsuperscript{−/−} mice, B-1a cells can be generated at wild-type levels. Furthermore, SLC-independent B-1a development can occur through a mechanism that involves reduced IL-7R/STAT5 signaling and early Igk rearrangement. We found that B-1a cell development is selectively impaired in Stat5b-CA mice, providing additional support for the idea that SLC-independent

**Fig. 6** Constitutive phospho-STAT5 signaling selectively inhibits B-1a cell development. **a** Representative flow-cytometry plots of peritoneal cavity cells from wild-type and Stat5b-CA mice. B-2 (CD19\textsuperscript{+}B220\textsuperscript{hi}) and B-1 (CD19\textsuperscript{+}B220\textsuperscript{lo}) cell gates are displayed as a percentage of CD19\textsuperscript{+} cells (left). B-1a (CD19\textsuperscript{+}B220\textsuperscript{lo}CD5\textsuperscript{+}) and B-1b (CD19\textsuperscript{+}B220\textsuperscript{lo}CD5\textsuperscript{−}) cell gates are displayed as a percentage of the B-1 cells (right). **b** Graphs display the total cell numbers of B-2, B-1b, and B-1a cells from the peritoneal cavity of wild-type and Stat5b-CA mice. Each dot represents an individual mouse, and lines connect pairs of littermates from three different litters of mice. **c, d** SLC-independent B-1a cells express receptors with phosphatidylcholine (PtC)-specific V\textsubscript{H}12 gene rearrangements. Peritoneal cavity B-1a cells from wild-type and Igll\textsuperscript{−/−} mice highlight the proportion of B-1a cells that are either **c** V\textsubscript{H}12\textsuperscript{+}, PtC\textsuperscript{+} or **d** PtC\textsuperscript{+}. The left side shows representative flow-cytometry plots. The right side is a graphical summary of the mice from all the experiments. Each dot represents an individual mouse and lines connect pairs of littermates. P-values were calculated using a two-tailed T-test. Source data are provided as Source Data File.
**Fig. 7** *Igll1*−/− B-1a cells have a similar V<sub>H</sub> gene segment V<sub>H</sub> usage to wild-type controls. Representative synthesis graphs generated from IMGT/StatClonotype that compare differences in V gene segment usage between wild-type (blue) and *Igll1*−/− (red)-derived B-1a cells (CD19<sup>+</sup>B220<sup>lo</sup>CD5<sup>+</sup>). The red box highlights V<sub>H</sub>12 (V<sub>H</sub>12-3) gene segment (n = 2 independent experiments). The graph combines a bar graph for the normalized proportions of each gene segment and the differences in proportions with significance and confidence intervals (CI).
**Fig. 8** Wild-type and Igll1−/− B-1a cells have a similar V<sub>k</sub> gene segment usage to wild-type controls and maintain V<sub>k</sub>4-91 enrichment. Representative synthesis graphs generated from IMGT/StatClonotype that compare differences in Igk V gene segment usage between Igll1−/− (red) versus wild-type (blue) peritoneal cavity B-1a cells CD19<sup>+</sup>B220<sup>lo</sup>CD5<sup>+</sup> (left side) or Igll1−/− (red) peritoneal cavity B-1a cells vs wild-type (blue) peritoneal cavity B-2 cells CD19<sup>+</sup>B220<sup>+</sup> (right side). The red box highlights V<sub>k</sub>4 (V<sub>k</sub>4-91) gene segment \( (n = 2 \text{ independent experiments}) \). This graph combines a bar graph for the normalized proportions of each gene segment and the differences in proportions with significance and confidence intervals (CI).
B-1a cell development is favored by a decrease in IL-7 signaling. It should be noted that although Igk rearrangement can occur prior to Igh rearrangement, it is not a prerequisite for SLC-independent B-1a cell development. Indeed, a rearranged Igh that cannot associate with SLC could persist at the pro-B cell stage until subsequent productive Igk rearrangement generates a BCR independent of IGH-SLC pairing. Taken together, our findings point to a model in which B-1a cells typically develop in a SLC-independent manner. Interestingly, earlier work analyzing expression patterns of genes relevant to rearrangement in the yolk sac, para-aortic splanchnopleura, and spleen demonstrates that at E9–11, early lymphoid progenitor cells express Rag2 and VpreB, but lack expression of Igfl169. The delayed expression of the SLC component, lambda5 (Igfl1), points to the possibility that there is an early window in which B-cell development can occur in the absence of SLC. This stage coincides with the stage at which the earliest B-1 progenitor cells are detected (E9 in the yolk sac and para-aortic splanchnopleura)70,71. Thus, we speculate that the reason why early progenitors become B-1 cells is because they develop in a SLC-independent manner. The observation that SLC-deficient mice generate B-1a cells with autoreactive receptors supports this model72. The generation of SLC-independent autoreactive receptors does not need to occur at high frequency, because cells that acquire a B1a cell receptor undergo a rapid proliferative burst and thus even a few cells have the ability to give rise to a robust B1a cell population70. In sum, assignment of a SLC-independent pathway of B-cell development to B-1a cells provides a new perspective on B-1a development, reconciling old lineage and selection models (Fig. 10).

Methods
Mice. Igfl1−/− (Jackson Laboratory 002401) and Stat5b-Ca mice were maintained on a C57Bl/6 background, as previously described68,69,72. B1a mice21 were crossed onto a Igfl1−/− background. These mice were housed and cared for in accordance with IACUC guidelines and protocols approved by NYUMC (protocol #: IA15-01468) and U of Minnesota (protocol #: 1502–32347A).

Flow cytometry and antibodies. BM and FL cell populations were isolated from C57Bl/6 mice via cell sorting and analyzed by flow cytometry. B- and T-cell antibody include: anti-CD45R/B220 (clone: RA3–68B cat. no. 53092), anti-CD19 (clone ID3 cat. no. 557655), anti-IgM6 (clone AE6–76 cat. no. 533520), anti-IgM (clone II/41 cat. no. 533473, 743324), anti-IgK (clone 187.1 cat. no. 562476, 562888), anti-CD117/c-Kit (clone 288 cat. no. 553366), anti-CD2 (clone RM2–5 cat. no. 553111), anti-CD25/IL2Ra (clone PC61 cat. no. 553866), anti-CD93 (clone AA4.1 cat. no. 553110), anti-CD93 (clone AA4.1 cat. no. 561990) anti-CD90.2/Thy1.2 (clone 53–7.3 cat. no. 553022), anti-CD2 (clone RM2–5 cat. no. 553111), anti-CD25/IL2RA (clone PC61 cat. no. 553866), anti-CD93 (clone AA4.1 cat. no. 561990) anti-CD90.2/Thy1.2 (clone 53–2.1 cat. no. 25-0902-82), anti-CD5 (clone 53–7.3 cat. no. 553022), anti-Vj5.12 (clone 5C5)34 anti-TCRB (clone 11, early lymphoid progenitor cells express Igll1, points to the possibility that there is an early window in which B-cell development can occur in the absence of SLC. This stage coincides with the stage at which the earliest B-1 progenitor cells are detected (E9 in the yolk sac and para-aortic splanchnopleura)70,71. Thus, we speculate that the reason why early progenitors become B-1 cells is because they develop in a SLC-independent manner. The observation that SLC-deficient mice generate B-1a cells with autoreactive receptors supports this model72. The generation of SLC-independent autoreactive receptors does not need to occur at high frequency, because cells that acquire a B1a cell receptor undergo a rapid proliferative burst and thus even a few cells have the ability to give rise to a robust B1a cell population70. In sum, assignment of a SLC-independent pathway of B-cell development to B-1a cells provides a new perspective on B-1a development, reconciling old lineage and selection models (Fig. 10).

Fig. 9 Wild-type and Igfl1−/− peritoneal cavity B-1a cells have similar low levels of N-additions as compared with wild-type counterparts. Cumulative frequency graph demonstrates the proportion of cells that have a certain number of N-additions between VH and VD gene segments for wild-type peritoneal cavity B-1a cells CD19+B220loCD5+ (blue), Igfl1−/− peritoneal cavity B-1a CD19+B220loCD5+ cells (green), and wild-type bone marrow immature B cells CD19+B220lo+Mint (black) (n = 2 independent experiments).

Fig. 10 The model of classical versus alternate B-cell development. The classical B-cell developmental pathway is favored in the bone marrow where high levels of STAT5 signaling inhibit Igk recombination at the pre-B cell stage. At the large pre-B cell stage, a productive heavy-chain pairs with SLC and pre-BCR signaling at this stage leads to proliferative expansion and positive selection. Autoreactive heavy-chains pair poorly with SLC, and will not experience positive selection in this manner. Following Igk recombination (blue) at the small pre-B cell stage, self-reactive B cells can undergo receptor editing to further select against self-reactive BCRs. The alternate B cell developmental pathway is favored in fetal liver where low levels of STAT5 signaling in pre-B cells promotes Igk recombination at the same stage as Igh recombination (blue). This enables the generation of B cells expressing a mature BCR instead of a pre-BCR. Here, self-reactive B cells are initially positively selected by self-antigens. Although these B cells can potentially undergo receptor editing to modify their BCRs, the initial positive selection of autoreactive B cells skews the B-1a BCR repertoire toward autoreactivity.
the antibodies were obtained from BD Pharmingen, except anti-CD90.2, anti-TCRB, and anti-ENPP1/PC1 (eBioscience). Fluorescent DPOC/CHOL liposomes were acquired from FormuMax. The gating strategy was as follows: bone marrow pro-B cells B220+CD19-μIgM- /-κi/CD25 /-γM- , BM pre-b cells B220+CD19+/IgM- /-κi/CD25 /-γM- , and thymic DP cells Thy1.2+/TCRB- /-CD4- /-CD8- . FL pro-b cells were sorted on CD2+ instead of CD35 , because CD25 is not expressed in fetal B cells. CD2 expression is correlated with cytoplasmic µ heavy chain, and can be used to identify pre-b cells and efficiently sort pro-b cells from the FL [72] . From the peri-tonic cavity, B-2, B-1a, and B-1b were identified as followed: B-2 cells B220+/CD19- , B-1a cells B220+/CD19+/CD5+ , and B-1b cells B220+/CD19+/CD5- . To obtain FL pre-b precursor cells, FLs from E15.5 EmGc mice were used to lineage depletion using the following biotin-labeled antibodies: anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-TCRβ5/6, anti-CD49b (DX5), anti-Ly6G/Ly6C (RB6-8C5), anti-ENPP1/PC1 (eBioscience). Fluorescent DPOC/CHOL liposomes were analyzed in triplicate and represent three biological replicates.

Intracellular staining for Igk. FL E17.5 B cells were stained for surface markers (CD19, B220, IgM, Igk, CD12, and CD127), followed by permeabilization/fixed and secondary staining for Igk and IgK in accordance with the manufacturer’s instructions (BD 557414). Antibodies with different conjugated fluorophores were used to distinguish between surface IgM/IgK (BD 554714 and 562476) and intracellular IgM/IgK by FlowJo software.

Semiquantitative Igk rearrangement. Prepared by proteinase K digestion at 55 °C for 4 h, 85 °C for 20 min. V κ jk joins were amplified using a degenerate V κ primer (5'-GGGTCGACGATCCCAGTGGCAGTGGCTGCGWG- GRAC-3'), and a Jκ primer (5'-ATGCGCCTGAACCTGAATGAAGGCCTC-3'). Acidic gene was amplified as a loading control (AID F: 5'-GCCACCTTGCGAACAGTGC-3', AID R: 5'-CGGCGGACGCAGTACAGC-3'). Bands were run through 1.5% agarose gel electrophores were treated by standard dA tailing followed by adapter ligation using Quick Ligase (NEB) mixed with pre-annelled NEXTflex DNA Barcodes (Bio Scientific). QC was carried out by tapestation, and quantified by qPCR (Kapa Biosystems). Samples were pooled and sequenced on an Illumina HiSeq 4000 (2 x 150 PE reads). Nucleotide sequences were compared with the reference sequences from IMGT, the international ImMunoGeneTics information system (http://www.imgt.org) and analyzed using IMGT/HighV-QUEST [27], a web portal allowing the analysis of thousands of sequences on IMGT/V-QUEST [28]. IMGT/StatClonotype was used to analyze statistically significant gene segment usage between samples [26]. Igk N-addition information from the region between V and D gene segments was extracted from IMGT/HighV-QUEST and graphed in R.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data that support findings of this study have been deposited in the Gene Expression Omnibus (GEO) database under the accession code GSE101411.

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Author contributions
J.B.W., S.L.H., K.J., and J.A.S. designed the experiments. J.B.W., S.L.H., L.M.H.-H., M.M., and K.J. performed the experiments and conducted data analysis. J.B.W., J.A.S., and K.R. wrote the paper, with input from M.M., S.B.K., M.R.C., and M.A.F.

Competing interests
The authors declare no competing interests.

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