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Control of B-1a cell development by instructive BCR signaling

Taras Kreslavsky[1,#], **Jason B. Wong**[2], **Maria Fischer**[1], **Jane A. Skok**[2], and **Meinrad Busslinger**[1,#]

[1]Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Campus-Vienna-Biocenter 1, A-1030 Vienna, Austria

[2]Department of Pathology, New York Medical Center, New York University, New York, USA

Summary

B-1a cells remain one of the most enigmatic lymphocyte subsets. In this review, we discuss recent advances in our understanding of the development of these cells and their regulation by the transcription factors Bhlhe41 and Arid3a as well as by the RNA-binding protein Lin28b. A large body of literature supports an instructive role of BCR signaling in B-1a cell development and linage commitment, which is initiated only after signaling from an autoreactive BCR. While both fetal and adult hematopoiesis can generate B-1a cells, the contribution of adult hematopoiesis to the B-1a cell compartment is low under physiological conditions. We discuss several models that can reconcile the instructive role of BCR signaling with this fetal bias in B-1a cell development.

Introduction

B-1 cells are an innate-like B lymphocyte subset that was discovered over 30 years ago [1]. These cells, which populate the peritoneal and pleural cavities, omentum and spleen, are believed to provide the first line of defense against pathogens and participate in the maintenance of tissue homeostasis [2]. While both fetal and adult precursors possess B-1 cell potential, B-1 cells are predominantly generated during fetal and neonatal life and can self-renew throughout the lifetime of the organism [3]. This is in stark contrast to conventional follicular (FO) and marginal zone (MZ) B cells (collectively called B-2 cells) that are constantly replenished from the adult bone marrow (BM). Unlike B-2 cells, B-1 cells can spontaneously differentiate into plasma cells that are believed to be the major source of secreted IgM ('natural antibodies') in unchallenged mice [2].

Most B-1 cells express the surface marker CD5 and are termed B-1a cells, while the minor subset of $CD5^-$ B-1 lymphocytes is referred to as B-1b cells. The developmental relationship and exact division of labor between these two cell types is not fully understood, but it was suggested that B-1a cells are the source of natural IgM in the steady state, while B-1b cells

[#]To whom correspondence should be addressed: busslinger@imp.ac.at (phone: [+43/1] 797 30 – 3150; fax: [+43/1] 797 30 – 223150), taras.kreslavsky@imp.ac.at.

undergo T cell-independent responses to bacterial pathogens [4]. B-1a and B-1b cells share a common $CD23^{-}$ $CD43^{+}$ $B220^{lo}$ cell surface phenotype [2], and CD5 remains the only marker that allows the distinction of the two cell types. Of note, CD5 expression follows a continuum from high to low rather than a discrete bimodal distribution. Comparison of gene expression profiles indicate that B-1a and B-1b cells are more similar to each other than to MZ or FO B cells (unpublished observations), suggesting a close relationship between the two subsets. The expression of CD5 is induced proportionally to the strength of T cell receptor (TCR) signaling during positive selection of T cells [5], where CD5 functions as a negative regulator of antigen receptor signaling [6]. Importantly, CD5 also negatively regulates B cell receptor (BCR) signaling in B-1a cells [7], suggesting that the gradient of CD5 expression reflects the different strengths of BCR signaling received by the cell. However, the CD5 expression level seems to be also developmentally regulated, as discussed below.

Instruction of B-1a cell lineage choice by BCR signaling

Many innate-like lymphoid lineages, such as NKT cells, some γδ T cell subsets and nonconventional αβ intraepithelial lymphocytes (IELs), are believed to be self-reactive [8]. Likewise, several lines of evidence suggest that B-1a cells are autoreactive [2], have a restricted BCR repertoire [9] and require a strong BCR signal for their differentiation [10]. Consequently, gene mutations that attenuate BCR signaling or co-stimulation pathways interfere with B-1a cell differentiation [11], whereas the loss of negative regulators of BCR signaling results in an expanded B-1a cell compartment [12–14]. A number of B-1a-specific BCRs, which recognize self carbohydrates and lipids either in their native or oxidized forms, have been identified to date [15]. It is believed that natural antibodies with these specificities can play a role in the maintenance of tissue homeostasis by clearing apoptotic cells and cellular debris, while simultaneously providing a first line of defense against pathogens that have similar epitopes in their membranes or cell walls. For example, a substantial fraction of the murine B•1a cells express the $V_H12/V\kappa4$ and $V_H11/V\kappa14(V\kappa9)$ BCRs [16, 17] that recognize phosphatidylcholine (PtC), which is present in the plasma membrane of host cells as well as in some pathogenic bacteria [18]. Transgenic expression of either of these BCR receptors promotes the development of B-1a cells at the expense of B-2 cells, revealing an important role of the BCR specificity in B-1a cell lineage choice [16, 17]. Direct evidence for the selection of B-1a cells on self-antigens is provided by a transgenic mouse model that expresses the heavy chain of a BCR recognizing the cell-surface protein Thy1 [19]. While this transgene efficiently induces the development of B-1a cells in Thy1-sufficient mice, B-1a cells are not generated on a Thy1-deficient background, clearly indicating that the positive selection of B-1a cells can be driven by self-antigen [19]. Importantly, a recently published ‘preview’ of data from Klaus Rajewsky’s lab indicates that mature FO B cells are still developmentally plastic and can be converted to the B-1 cell lineage by simply replacing a B-2 cell-specific BCR with a B-1a-typical BCR recognizing PtC [20]. Taken together, these results strongly suggest an instructive role of BCR signaling in B-1a cell lineage determination.

B-1a cell development

While the role of BCR signaling in B-1a cell development is clearly documented, the exact developmental path of these cells is still hotly debated. It has been suggested that precursors prior to the appearance of definitive hematopoietic stem cells (HSCs) may have B-1a cell potential [21, 22], but the contribution of these progenitors to the B-1a cell pool still remains to be shown. It has also been proposed that HSCs, including those in fetal liver (FL), are unable to generate B-1a cells [23]. However, an elegant study using cellular barcoding to trace the progeny of HSCs clearly demonstrated that FL HSCs can efficiently give rise to B-1a cells and that a single FL HSC has both B-1a and B-2 cell potential [24]. Several studies of BM chimeras also demonstrate that B-1a cells can be generated from adult BM hematopoiesis [25, 26], although this process may be less efficient than for FL HSCs. While BM-derived B-1a cells seem to express lower levels of CD5 than their FL-derived counterparts [25, 27], they are otherwise phenotypically normal [25, 26] and frequently express the B-1a-specific V_H12 BCR [27]. Of note, the lower CD5 expression likely explains why some studies conclude that B-1a cells cannot be generated from adult BM HSCs [24, 28]. Intriguingly, the low CD5 expression does not merely reflect attenuated BCR signaling due to a possible BCR repertoire change, as $V_H12/V\kappa4$ transgenic B-1a cells are normally $CD5^{hi}$, but express lower CD5 levels in BM chimeras (unpublished observations).

While B-1a cell potential is clearly present in adult BM, several lines of evidence indicate that this potential is quite limited under physiological conditions. Early experiments demonstrated that in neonatal mice, which have been transiently depleted of B cells prior to reconstitution with adult peritoneal cells, the B-1a cell compartment remains largely of donor origin for several months after transplantation [3], demonstrating that endogenous BM hematopoiesis minimally contributes to the B-1a cell pool under these conditions. Moreover, recent fate mapping experiments with the HSC-restricted *Pdzk1ip1*-CreER line in tamoxifen-treated adult mice indicated that the contribution of adult hematopoiesis to the B-1a cell compartment is below 10% during a 46-week observation period [29].

While FL HSCs efficiently generate B-1a cells, cells with a B-1a cell surface phenotype cannot be detected in FL [27] and first emerge in the postnatal spleen only several days after birth [30]. These transitional B-1a cells (TrB-1a) express the early B cell marker CD93 and have an otherwise mature IgM^+ $CD5^+$ $B220^{lo}$ B-1a cell phenotype [30]. In addition, B-1-specified progenitors (Lin^- IgM^- $CD93^+$ $CD19^+$ $B220^{neg\text{-}lo}$) have been described [31, 32] and, although they seem to be committed to the B cell lineage as indicated by the expression of Pax5 and its target gene *Cd19* [33], they are thought to be functionally equivalent to the uncommitted pre-pro-B cells (Lin^- IgM^- $CD93^+$ $CD19^-$ $B220^+$ $CD43^+$; referred to as B-2 progenitors) that give rise to B-2 cell development [31, 32]. The B-1-specified progenitors are more frequent in fetal and neonatal mice and, when sorted from fetal and adult BM, predominantly generate B-1 cells in injected mice [31]. These results are difficult to reconcile with the instructive role of BCR signaling discussed above. Our bioinformatic analysis of recently published RNA-seq data obtained from these B-1 progenitor populations [33] suggests a possible explanation for the apparent conundrum. All RNA-seq samples from the adult B-1 cell progenitors contained reads corresponding to V gene segments of immunoglobulin heavy- and light-chain genes, and two of the four samples additionally

exhibited a strong enrichment of *Ighv11*-2 and *Igkv14*-126 sequences (Supplemental Figure 1A,B) that constitute the PtC-specific $V_H11/V\kappa14(V\kappa9)$ BCR, which is found in a large fraction of mature B-1a cells [9, 27]. Analysis of the sequence reads spanning the V(D)J junctions revealed the presence of productive *Igh* and *Igk* rearrangements and identified in-frame V_H11 and $V\kappa14$ CDR3 sequences (Supplemental Figure 1C,D), which have previously been described in the case of V_H11 [9]. These analyses suggest that the sorted B-1 cell progenitor populations [33] contained at least some cells that expressed a fully rearranged B-1a-specific BCR and could thus expand upon cell transfer in recipient mice. Hence, we conclude that most experimental evidence is consistent with a scenario, in which commitment to the B-1a cell lineage takes place upon BCR expression and is first manifested by the appearance of TrB-1a cells in the postnatal spleen.

The results described above indicate that any model of B-1a cell development should take into account the following facts. First, the BCR plays an instructive role in the choice of the B-1a cell lineage. Second, both fetal and adult hematopoiesis have the potential to generate B-1a cells. Third, the actual contribution of adult hematopoiesis to the B-1a cell compartment is low in wild-type mice. Below we discuss three non-mutually exclusive hypotheses, which can explain these facts, together with recent advances in our understanding of the transcriptional regulation of B-1a cell development.

Distinct effects of autoreactive antigen receptors in fetal and adult lymphopoiesis

B-1a cells are not unique in their preferential fetal and neonatal origin as a number of innate-like T cell subsets are also generated early in ontogeny [34]. Such preferential production of innate-like lymphocytes early in life may be explained by a higher necessity for a first line of defense in an immunologically ‘naïve’ organism and may rely on a common mechanism. One shared feature of innate-like T cells and B-1a cells is their known or suspected self-reactivity. While autoreactive antigen receptors are thought to be largely eliminated from the repertoire, it is conceivable that T and B cells of fetal and neonatal origin may better tolerate strong antigen receptor signaling than their adult counterparts and may therefore be more efficiently diverted to innate-like lineages instead of being eliminated by tolerance mechanisms, such as negative selection (Figure 1; see also accompanying review by Joan Yuan). Indeed, this was directly demonstrated in an antigen-driven model of B-1 cell differentiation [35], where self-antigen diverts developing BCR-transgenic B cells to the B-1 lineage in chimeric mice generated by transplantation of FL cells, whereas the transgenic B cells are deleted in mice transplanted with BM progenitors [36].

Role of premature *Igk* gene rearrangements in B-1a cell development

Another common theme in the differentiation of innate-like T and B-1a cells may be the timing of expression of a ‘complete’ antigen receptor. The majority of αβ T cells first rearrange the *Tcrb* locus in DN thymocytes followed by expression of the pre-TCR and subsequent differentiation to the DP thymocyte stage, where the *Tcra* locus is then recombined [37]. In contrast, γδ T lymphocytes, which are highly enriched for cells with innate-like properties, rearrange both the *Trcg* and *Trcd* loci in DN thymocytes, do not

express the pre-TCR and are directly selected by signaling from the γδ TCR. Moreover, 'premature' expression of a pre-rearranged αβ TCR in transgenic mice leads to the development of cells with innate-like properties [38]. A small number of wild-type thymocytes is also thought to take this developmental route, and more cells do so in pre-TCR-deficient mice [39]. Akin to conventional αβ T cells, the majority of developing B cells first rearrange the immunoglobulin heavy-chain (*Igh*) locus in pro-B cells leading to expression of the pre-BCR and subsequent differentiation to small pre-B cells that rearrange the immunoglobulin light-chain (*Igk* or *Igl*) loci [37] (Figure 2). The sequential ordering of *Igh* and *Igk* recombination is enforced by IL-7 receptor (IL-7R) signaling through activation of the transcription factor STAT5. In pro-B cells, IL-7R signaling actively suppresses rearrangements at the *Igk* locus, as evidenced by a strong increase of *Igk* recombination in pro-B cells lacking IL-7Rα or STAT5 [40]. IL-7R signaling and its STAT5-mediated repression of the iEκ enhancer are lost during the subsequent pre-BCR transition, which activates *Igk* recombination in pre-B cells [41]. While B lymphopoiesis in the adult BM strictly depends on IL-7, it can take place in the FL in the absence of IL-7, consistent with the observation that B-1a cell differentiation is less dependent on this cytokine [42, 43]. Interestingly, a recent report revealed a strongly increased frequency of 'premature' *Igk* rearrangements in wild-type FL pro- B cells as a result of their reduced IL-7R/STAT5 activity [44] (Figure 2). Strikingly, the number of B-1a cells and their BCR repertoire are largely normal in pre-BCR-deficient mice, which are, however, severely deficient in B-1b and B-2 cells [44]. Hence, the simultaneous recombination of *Igh* and *Igk* loci in FL pro-B cells can directly generate a mature B cell receptor, thus bypassing the pre-BCR checkpoint [44] (Figure 2), which is consistent with a previous report demonstrating that a transgenic autoreactive BCR can efficiently replace the pre-BCR in early B lymphopoiesis [45]. In this context, it is important to note that pre-BCR signaling is known to counterselect against the generation of autoreactive BCRs [46], as potentially autoreactive heavy chains, such as V_H11, may pair poorly with the surrogate light chains, thus preventing pre-BCR assembly [47]. Instead, the alternative pre-BCR-independent pathway of FL pro-B cells seems to promote the efficient generation of B-1a-specific autoreactive BCRs, which may explain the fetal bias of B-1a cell development in the context of instructive BCR signaling.

Age-dependent BCR repertoire selection by clonal competition of B-1a cells

The clonal evolution of the B-1a BCR repertoire was recently analyzed by high-throughput sequencing of the complementarity-determining region 3 (CDR3) encoded by the V(D)J-rearranged *Igh* transcripts of splenic and peritoneal B-1a cells [9]. This elegant study revealed that the BCR repertoire of B-1a cells is continuously shaped by selection throughout the lifetime of a mouse. In neonatal life, the BCR repertoire is very diverse with even contribution of individual small B-1a cell clones to the overall diversity [9] (Figure 3). In contrast, the BCR repertoire of adult mice is dominated by a few large B-1a cell clones expressing certain BCRs, including those with PtC-binding specificity, which restricts the BCR repertoire of adult B-1a cells (Figure 3) [9]. Importantly, this oligoclonality is still observed under germ-free conditions and is thus not driven by microbiota-derived antigens, but likely results from competition of B-1a cell clones that have been selected on self-antigens [9].

These observations suggest a further explanation for the fetal bias of B-1a cell development in addition to the cell-intrinsic mechanisms described above. As the B-1a cell compartment of adult mice largely consists of cells of fetal and neonatal origin, which have undergone competition-based selection, it is conceivable that the low contribution of adult hematopoiesis to the B-1a cell pool can be explained in part by the failure of newly generated B-1a cells to efficiently compete with the already established B-1a cell clones (Figure 3). While this hypothesis requires experimental testing, it is consistent with published data. Neonatal mice, which are transiently depleted of their B cells, are known to readily restore their B-1a cell compartment, possibly by differentiation from endogenous HSCs [3]. However, endogenous hematopoiesis fails to contribute to the B-1a cell pool when the B cell depletion is accompanied by transfer of peritoneal B-1a cells from adult mice [3]. Hence, the presence of mature B-1a cells can interfere with the contribution of newly developing cells to the B-1a cell compartment, possibly through a competition-based mechanism. Interestingly, ~80% of the B-1a cell clones in the spleen of adult mice appear to be of postnatal origin, as they have N-nucleotide insertions in their CDR3 sequences [9] due to the postnatal expression of terminal deoxynucleotidyl transferase [48]. It is thus conceivable that the contribution of adult hematopoiesis to the B-1a cell pool is relatively high at the clonal level. However, the clone sizes of these B-1a cells may remain small due to inefficient competition with the established B-1a cell clones of fetal and neonatal origin, which is consistent with the observed low (< 10%) contribution of adult HSCs to the entire B-1a cell compartment [29].

The clonal outgrowth of B-1a cells is reminiscent of the behavior of transformed cells, which suggests that B-1a cells may be prone to malignant transformation. Indeed, while the origin of B cell chronic lymphocytic leukemia (CLL) in humans remains unclear [49, 50], mouse models that recapitulate genetic aberrations or dysregulated gene expression characteristic of human CLL have shown that leukemia can originate from B-1a cells as evidenced by the expression of B-1a cell-specific BCRs by leukemia cells and by cell transfer experiments [51–55]. Strikingly, experimental overexpression of oncogenes or deletion of tumor suppressor genes is not strictly required to induce CLL development, as two transgenic mouse lines, each expressing a distinct B-1a-specific BCR, spontaneously develop CLL [55, 56]. Hence, the transgenic expression of specific autoreactive BCRs, which leads to the excessive generation of B-1a cells, can predispose to CLL in aged mice. As CLL develops in these mouse models at a late age (> 15 months), which is close to the normal lifespan of *M. musculus*, it appears that the benefit of B-1a cells to provide a first-line defense against pathogens [2] may outweigh the small risk of CLL development. This may, however, be a more serious problem for mammals with a longer lifespan, such as humans.

Transcriptional control of B-1a cell development

Our understanding of the transcriptional network that controls the identity and function of B-1a cells is still rudimentary. While some transcription factors, such as Ebf1 [57], Oct2 [58] and IκBNS [30], are important for both B-1a and B-2 cells, their ectopic expression (Ebf1) or loss (Oct2, IκBNS) more severely affect the development of B-1a cells. Here we

discuss two transcriptional regulators, Arid3a and Bhlhe41, whose function is more restricted to B-1a cell development.

The Lin28b – Let-7 – Arid3a signaling axis has recently emerged as a key pathway controlling B-1a cell development, as discussed in detail in an accompanying review of this issue (review by Joan Yuan). The RNA-binding protein Lin28b is expressed in fetal but not adult hematopoiesis, and its ectopic expression in adult BM HSCs strongly enhances B-1a cell development, which indicates an important role for Lin28b in the developmental switch from fetal to adult hematopoiesis [24, 59, 60]. While Lin28b can bind to thousands of mRNAs [61], it functions as a negative regulator of the Let-7 microRNA family by binding to their precursor RNAs, which prevents microRNA processing [62]. Ectopic expression of Let-7b in FL pro-B cells interferes with the development of B-1a cells, indicating that the inhibition of Let-7 expression may be required for efficient B-1a cell generation [59]. A predicted target of negative regulation by the Let-7 microRNA is the mRNA encoding the transcription factor Arid3a [59]. Indeed, ectopic expression of Arid3a in adult pro-B cells enhances B-1a cell generation, while RNAi-mediated knockdown of its expression in FL pro-B cells interferes with B-1a cell differentiation [59]. Consistent with these findings, conditional inactivation of *Arid3a* results in a severe decrease of peritoneal B-1a cells [63]. Functional characterization of Arid3a by identifying critical downstream target genes will ultimately be required to fully understand the function of this pathway in controlling B-1a cell development.

In a search for regulators that are specifically expressed in mature B-1a cells [57], we recently identified the transcription factor Bhlhe41 (also known as Dec2 or Sharp1), which is highly and specifically expressed in postnatal and adult B-1 cells [27]. Consistent with this selective expression pattern, we identified an essential role for Bhlhe41, with a lesser contribution of its homolog Bhlhe40, in the differentiation and homeostasis of B-1a cells by analyzing *Bhlhe41*$^{-/-}$ and *Bhlhe40*$^{-/-}$ *Bhlhe41*$^{-/-}$ mice [27]. B-1a cells in these mutant mice are strongly reduced, which is already evident when the first IgM-expressing TrB-1a cells emerge in the postnatal spleen [27]. Mutant B-1a cells exhibit an abnormal cell surface phenotype and a drastically altered BCR repertoire, as exemplified by loss of the PtC-specific $V_H12/V\kappa4$ BCR [27]. Transgenic expression of a pre-rearranged $V_H12/V\kappa4$ BCR failed to rescue the mutant phenotype and revealed defective BCR signaling, enhanced proliferation and increased cell death of the mutant B-1a cells [27]. At the molecular level, Bhlhe41 directly represses the expression of cell cycle regulators and inhibitors of BCR signaling while promoting pro-survival signaling by the IL-5 receptor [27]. Hence, Bhlhe41 controls multiple aspects of B-1a cell biology by regulating the development, BCR repertoire and self-renewal of B-1a cells.

Evolutionary conservation of the B-1a cell lineage

The existence of a human B cell population, that is homologous to the peritoneal B-1a cells of the mouse, remains an area of hot debate. To date, such a human B cell type has not been unequivocally identified [2, 64–68]. Although some candidate B cell populations have been suggested [2, 64], they have been identified on the basis of cell surface markers and functional properties (such as spontaneous antibody secretion) that are not only specific for

B-1a cells, but are also shared with activated B-2 cells [66, 68]. To our knowledge, the mouse is the only mammalian species, in which B-1a cells have unequivocally been identified. Even within the genus *Mus*, $CD5^+$ B cells in the peritoneum appear to be restricted to the subspecies *M. musculus domesticus*, as these cells could not be detected in many other *M. musculus* subspecies [69]. Future research on the evolutionary conservation of the B-1a cell type should ideally take advantage of the published gene expression signature of murine B-1a cells [57, 70] rather than solely relying on cell surface markers and few functional properties.

Conclusions

Since their discovery more than 30 years ago, the innate-like B-1a cells have remained an enigmatic lymphocyte subset. Recent publications have shed new light on the development of these cells and their transcriptional regulation. We believe that the bulk of the literature is consistent with an instructive role of BCR signaling in B-1a cell lineage commitment and development. This process is more efficient in fetal than in adult B lymphopoiesis, possibly because fetal B lymphocytes better tolerate strong autoreactive BCR signaling, which may divert them to the B-1a cell lineage. Premature *Igk* rearrangements in FL pro-B cells efficiently generate autoreactive BCRs by a pre-BCR independent pathway, which may further contribute to the fetal bias of B-1a cell development. At the molecular level, the RNA-binding protein Lin28b as well as the transcription factors Arid3a and Bhlhe41 have been identified as key regulators of B-1a cell development, although their downstream transcriptional programs remain to be fully explored.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Papers of particular interest, published within the period of review have been highlighted as:

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Highlights

- The BCR specificity plays an instructive role in the B-1a cell lineage choice.
- The clonal composition of B-1a cells is shaped throughout the lifetime of a mouse.
- Premature *Igk* rearrangements in fetal pro-B cells can promote B-1a cell development.
- Bhlhe41, Aridr3a and Lin28b emerged as key molecular regulators of B-1a cells.

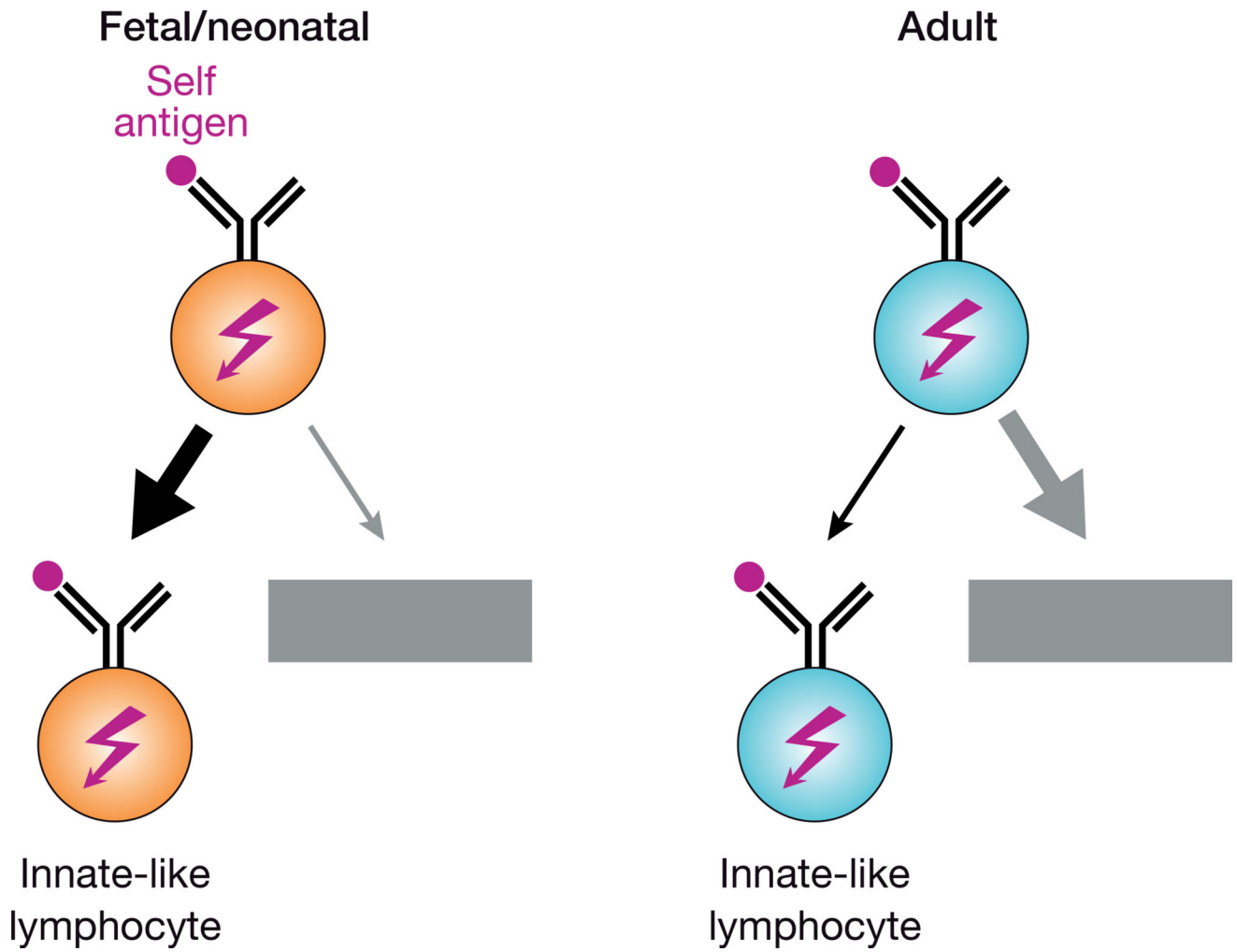

Figure 1. Distinct effects of autoreactive antigen receptors in fetal and adult lymphopoiesis

Fetal and neonatal lymphocytes expressing autoreactive antigen receptors seem to tolerate strong signaling, which may promote their efficient diversion to innate-like T or B cell lineages [36]. In contrast, adult autoreactive lymphocytes are efficiently eliminated by tolerance mechanisms such as negative selection, receptor editing or anergy induction.

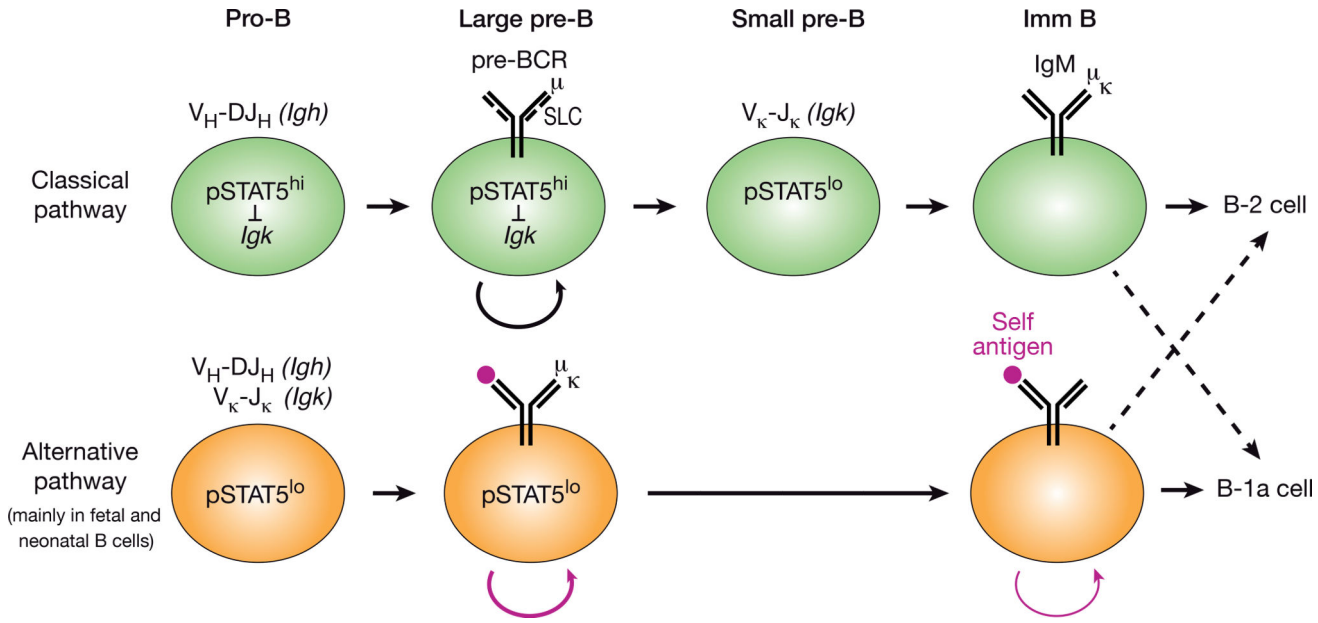

Figure 2. Generation of autoreactive BCRs by premature *Igk* rearrangements in fetal pro-B cells

In the classical pathway of B cell development, pro-B cells experience strong IL-7R signaling, which activates the JAK/STAT pathway leading to active, phosphorylated (p) STAT5. *Igk* recombination is suppressed by pSTAT5 in IL-7R^{+} pro-B cells, which undergo VH-DJH recombination at the *Igh* locus, and is induced only after the pre-BCR transition in small IL-7R^{-} pre-B cells containing low pSTAT5 levels. In contrast, IL-7R/pSTAT5 signaling is low already in fetal and neonatal pro-B cells, leading to efficient *Igk* rearrangements concomitant with *Igh* recombination, which directly generates mature BCRs. If an autoreactive BCR is generated, it is stimulated by self-antigen (red dots), which results in proliferative cell expansion and commitment to the B-1a cell lineage by bypassing the pre-BCR stage. The model of the alternative developmental pathway is based on recently published data [44].

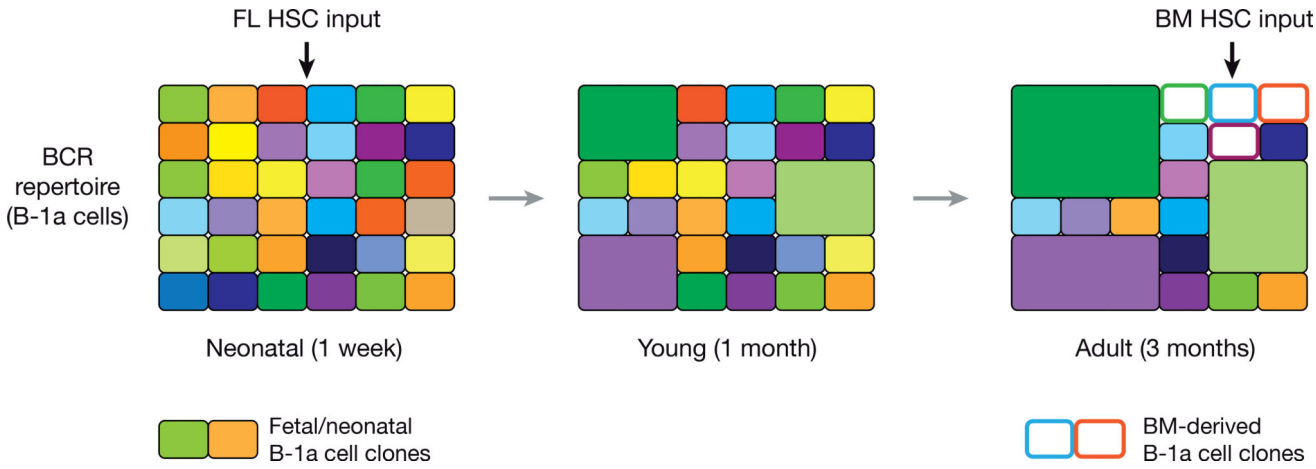

Figure 3. Clonal evolution of the BCR repertoire of B-1a cells throughout adult life

The schematic diagrams of the B-1a BCR repertoire in neonatal, young and adult mice are based on data obtained by high-throughput CDR3 sequencing of rearranged *Igh* transcripts [9]. Each rectangle represents a unique CDR3 sequence, and the size denotes the relative frequency (clone size) of an individual sequence. B-1a cell clones derived from FL HSCs are shown as filled colored rectangles, while B-1a cell clones originating from BM HSC are indicated by white rectangles.