HHS Public Access

Author manuscript

Nat Immunol. Author manuscript; available in PMC 2011 November 01.

Published in final edited form as:

Nat Immunol. 2011 May ; 12(5): 434–440. doi:10.1038/ni.2012.

The P4-type ATPase ATP11C is essential for B lymphopoiesis in adult bone marrow

Owen M. Siggs[1], **Carrie N. Arnold**[1], **Christoph Huber**[2], **Elaine Pirie**[1], **Yu Xia**[1], **Pei Lin**[1], **David Nemazee**[2], and **Bruce Beutler**[1]

[1] Department of Genetics, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037

[2] Department of Immunology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037

Abstract

B lymphopoiesis begins in fetal liver, switching to bone marrow after birth where it persists for life. The unique developmental outcomes of each phase are well documented, yet their molecular requirements are not. Here we describe two allelic X-linked mutations in mice that caused a cell-intrinsic arrest of adult B lymphopoiesis. Mutant fetal liver progenitors generated B cells *in situ*, but not in irradiated adult bone marrow, highlighting a necessity for the affected pathway only in the context of adult bone marrow. The causative mutation was ascribed to *Atp11c*, which encodes a P4-type ATPase with no previously described function. Our data establish an essential, cell-autonomous and context-sensitive function for ATP11C, a putative aminophospholipid flippase, in B cell development.

Keywords

B cell development; fetal liver; IL-7; flippase

Introduction

B cell progenitors first arise in fetal liver, then in bone marrow shortly after birth, and give rise to three major mature populations[1]. Marginal zone (MZ) B cells localize to the splenic marginal zone and respond to blood-borne antigens independently of T cell help[2]. Follicular B cells, by contrast, respond to protein antigens in a T cell-dependent manner, and progressively undergo immunoglobulin (Ig) isotype switching and affinity maturation. B-1 B cells comprise a much smaller population, which predominates in the pleural and

Correspondence should be addressed to: Bruce Beutler, M.D., Professor and Chairman, Department of Genetics, The Scripps Research Institute, 10550 N. Torrey Pines Road, SP293, La Jolla, CA 92037, telephone: 858-784-8610, fax: 858-784-8444, bruce@scripps.edu.

Author Contributions

O.M.S. designed and performed experiments, analyzed data and wrote the paper under the guidance of B.B.; C.N.A. and E.P. identified the *spelling* phenotype, and assisted with immunization experiments; Y.X. and P.L. assisted with positional cloning and mutation identification, and C.H. measured immunoglobulin isotypes with reagents contributed by D.N..

peritoneal cavities and contributes most of the serum IgM during the early phases of infection[3].

Whereas MZ and B-1 B cells are predominantly self-renewing, follicular B cells require constant replenishment from bone marrow, as demonstrated in mice with a conditional deletion of *Rag2* (ref. [4]). Briefly, deletion of *Rag2* in newborn mice prevents almost all follicular B cell development, but leaves MZ and B-1 populations largely intact. Similarly in adult mice, *Rag2* deletion causes a progressive decline in follicular B cell numbers, yet MZ and B-1 populations persist. A similar phenomenon has been reported for mice with a mutation of *Il7* (ref. [5]). While redundant with FLT3 in the fetal liver[6–8], interleukin 7 (IL-7) becomes essential for B cell development in the bone marrow shortly after birth[7]. As a result, MZ and B-1 B cells predominate in the periphery of *Il7* mutant mice, while follicular B cell numbers are severely reduced.

In the absence of IL-7 or its common gamma chain (γ_C)-associated receptor component IL-7R α, B cell development is arrested in the adult bone marrow shortly after cells acquiresurface B220, yet before they express CD19 or CD24 (refs. [8,9]). Expression of the transcription factor early B cell factor (EBF) via STAT5 signaling appears to be important at this stage, since overexpression of EBF or constitutively active STAT5 can overcome the developmental arrest in *Il7r* mutant progenitors[9,10]. Other data suggests that rather than inducing transcription of *Ebf1*, STAT5 signaling instead promotes B cell progenitor survival through induction of *Mcl1* (ref. [11]). Whichever the mechanism, IL-7 and EBF are both key determinants of B cell differentiation[12,13], and little else is known of the molecules and microenvironments that induce and maintain their expression in the adult.

We (and our colleagues[14]) describe here an essential role for the previously uncharacterized P4-type ATPase ATP11C in early B cell differentiation. ATP11C was redundant during B cell development in the fetal liver, yet essential in the context of adult bone marrow, where it was required for optimal responses to IL-7 and sustained expression of *Ebf1*.

Results

A heritable B cell deficiency

During a forward genetic screen for non-redundant regulators of lymphopoiesis (adapted from [15]), we identified several mice from common *N*-ethyl-*N*-nitrosourea- (ENU) treated founders with low percentages of $CD19^+$ cells in blood (Fig. 1a). The phenotype, named *emptyhive*, was transmitted as an X-linked recessive trait. Affected male mice were outwardly normal in behavior, fertility, lifespan and appearance, but were notably hyperbilirubinemic. This aspect of the phenotype, which stems from effects of the mutation in the liver, has been considered separately (O.M.S., B. Schnabl, B. Webb & B.B., submitted).

Among B cell progenitors in the bone marrow, *emptyhive* mutants had reduced numbers of cells beginning at the pre-pro-B to pro-B transition (Hardy fraction A [$B220^+CD43^+BP\text{-}1^-CD24^-$] to B [$B220^+CD43^+BP\text{-}1^-CD24^+$][16]) (Fig. 1b), with a severe deficiency of immature B cells (Hardy fraction E [$B220^+CD43^-IgM^-IgD^+$]). In the spleen,

emptyhive mice had one-tenth the normal number of $CD19^+$ cells, largely due to a lack of follicular and transitional subsets (Fig. 1c,d), although numbers of MZ B cells and $Thy1.2^+$ cells and were normal. Numbers of B-1 cells, the predominant population of B cells in the peritoneal cavity, were reduced by a factor of three in mutant mice (Fig. 1d,e), while numbers of peritoneal B-2 cells were reduced by a factor of ten. B cells in the blood of mutant mice had undergone normal allelic exclusion at the *Igh* locus (Fig. 1f).

Despite a reduction in follicular B cell numbers, the B cells that remained appeared largely functional, and retained the capacity to produce all major immunoglobulin isotypes (Fig. 2a), as well as the ability to generate specific antibodies to T-independent and T-dependent immunogens (here, 4-hydroxy-3-nitrophenylacetyl (NP)-conjugated Ficoll and NP-chicken gamma globulin, NP-CGG, Fig. 2b,c, respectively). However, 50% less NP-specific IgM and high-affinity IgG1 was produced in response to NP-Ficoll and alum-precipitated NP-CGG immunization, respectively.

Cell-intrinsic failure of adult B lymphopoiesis

To determine the cellular origin of the *emptyhive* phenotype, irradiated *Rag1* mutant mice were reconstituted with an equal mix of mutant and wild-type bone marrow. Although the $Thy1.2^+$ compartment was reconstituted equally, less than 1% of $CD19^+$ cells were of mutant origin (Fig. 3a), indicating that the defect was intrinsic to *emptyhive* B cell progenitors but did not appreciably affect T cell development. Even in the absence of competition, bone marrow cells from *emptyhive* donors were unable to repopulate any peripheral B cell compartment in irradiated recipients (Fig. 3b,c), and unlike their non-irradiated counterparts (Fig. 2b), recipients of mutant bone marrow also failed to produce specific antibody after immunization with NP-Ficoll (Fig. 3d). These data suggested a failure of adult B cell development in *emptyhive* mice, consistent with the severe reduction of immature B cells in mutant bone marrow (Fig. 1b) and the age-dependent decline of follicular, but not MZ, B cells in mutant spleens (Fig. 3e). This effect was not due to an intrinsic difference between adult and fetal progenitors, since neither bone marrow nor fetal liver cells could reconstitute the B cell compartment in irradiated recipients (Fig. 3c). Rather, the results suggest that *emptyhive* B cell progenitors, irrespective of their source, cannot develop in the microenvironment of adult bone marrow.

Immature and mature B cells were also entirely absent from the bone marrow of *emptyhive*-reconstituted recipients (Fig. 3b), with very few progressing beyond the $B220^+CD19^-$ pre-pro-B stage. This developmental arrest was even more severe than that observed in non-irradiated *emptyhive* or *Rag1* mutant mice, implying that a failure of IgM rearrangement was not the cause of developmental arrest in *emptyhive* mutants (Fig. 3b). Few mutant progenitors were capable of inducing expression of cytoplasmic IgM (cμ, Fig. 3f), consistent with the interpretation that the *emptyhive* phenotype originated from a defect upstream of *Rag*-mediated recombination.

Compromised responsiveness to IL-7

Because IL-7 is known to be essential for adult, but not fetal B cell development[5], we hypothesized that a specific failure of IL-7R signaling in B cell progenitors could explain

the *emptyhive* phenotype. To examine the competence of IL-7R signaling in mutant progenitors, surface IgM^- precursors were purified by flow cytometry and cultured *ex vivo* in the presence of IL-7. *emptyhive* progenitors formed lymphoblasts with similar kinetics to wild-type in response to IL-7, indicating that IL-7R-induced proliferation was largely intact (Fig. 4a). *In vitro* IL-7 treatment also leads to the differentiation of low frequencies of surface IgM^+ cells, which unexpectedly also emerged in *emptyhive* mutant cultures (Fig. 4b). Absolute numbers of IL-7-induced IgM^+ cells were nonetheless lower than wild-type: a deficiency exaggerated by as much as two-fold at high IL-7 concentrations (Fig. 4b).

Since these data suggested a qualitative or quantitative difference in the IL-7 response of *emptyhive* progenitors, we examined expression of EBF: an early transcriptional regulator of B cell differentiation[12] whose expression is lost in the absence of IL-7R signaling[9]. We measured *Ebf1* transcription in sorted pre-pro-B cells by RT-PCR, and found it to be severely reduced in *emptyhive* mutant progenitors (Fig. 4c). We also measured transcription of *Igll1*: a target of EBF[12] encoding the λ5 component of the surrogate light chain (SLC) portion of the pre-BCR[17]. Neither transcription of *Igll1* (Fig. 4c), nor surface expression of λ5 (Fig. 4d) was compromised in *emptyhive* mutant progenitors, suggesting that the lack of *Ebf1* transcript was either transient (and isolated to adult pre-pro-B cells or a subset thereof), or insufficient to affect *Igll1* expression.

Consistent with their intact IL-7 responsiveness *in vitro*, IL-7Rα was expressed in equivalent amounts on the surface of *emptyhive* mutant progenitors (Fig. 4e). Furthermore, in the absence of IL-7, FLT3 signaling is thought to account for residual B lymphopoiesis in fetal liver[18]. While *Flt3*$^{-/-}$*Il7*$^{-/-}$and *Flt3*$^{-/-}$*Il7r*$^{-/-}$double mutants lack all peripheral B cells[6,18], *emptyhive Flt3*$^{-/-}$double mutant mice had similar numbers of $CD19^+$ splenocytes as *emptyhive* single mutants (Fig. 4e). This result implied that IL-7R signaling remained intact in *emptyhive* fetal liver progenitors, at least to a degree sufficient for fetal B lymphopoiesis in the absence of FLT3.

We also found no major role for programmed cell death as the cause of the *emptyhive* phenotype. Homozygosity for the *Fas*^{lpr} mutation[19] or a null allele of *Bcl2l11* (ref. [20]) could not correct the B cell deficiency in blood or bone marrow (Supplementary Fig. 1a-c), although partial rescue was observed in the presence of the B lineage-restricted *EμBCL2-22* transgene[21] (Supplementary Fig. 1a-c). Despite a relative increase of early B cell progenitors ($B220^+IgM^-IgD^-$, Supplementary Fig. 1b,c), numbers of mature B cells ($B220^+IgM^+IgD^+$) in the bone marrow of *EμBCL2* transgenic mutants were nevertheless reduced as severely as they were in the absence of the transgene (Supplementary Fig. 1c). In the spleen, numbers of follicular and marginal zone B cells were increased (Supplementary Fig. 1d), likely due to an increased half-life[4,21], which was consistent with the increased frequency of recirculating B cells observed in blood (Supplementary Fig. 1a).

Partial correction of B cell deficiency by a BCR transgene

pre-BCR signaling is an essential step in the progression of B cell development, signaling productive rearrangement at the *Igh* locus, triggering proliferation and differentiation of preB cells, and initiating recombination of the kappa light chain allele[22]. The presence of recombined heavy and light chain transgenes renders pre-BCR signaling redundant, and

effectively bypasses the developmental stages that surround it. The hen egg lysozyme (HEL)-specific MM4 transgene[23], for example, is induced at the pre-pro-B stage, bypassing *Rag*-mediated recombination and expression of endogenous heavy and light chain genes and diverting progenitors directly to the surface IgM^+ stage (Fig. 5a). Combining the *emptyhive* mutation with the MM4 transgene partially increased $CD19^+$ cell frequencies in the blood (Fig. 5a) as well as numbers of IgM^+ cells in the bone marrow (Fig. 5b). Since the MM4 transgene is a co-integration of heavy and light chain elements, this correction could reflect the effect of either recombined chain, or both in combination. We directly tested whether recombined heavy or light chains could rescue the *emptyhive* B cell defect in isolation using the 'switch- HEL' (SW_{HEL}) system[24], which consists of a HEL-specific V_H10 heavy chain knockin and the LC2 light chain transgene. Significant correction of the *emptyhive* B cell deficiency was achieved only in the presence of both a heavy chain knockin allele and light chain transgene (Fig. 5c). This result is in contrast with SLC or *Rag1*-deficient mutants, whose respective B cell deficiencies can be fully or partially corrected by light or heavy chain transgenes alone[25],[26], and reveals that bypassing intermediate stages of B cell development (namely those within the $B220^+CD19^+IgM^-IgD^-$ subset) could partially, but not completely, overcome the B cell deficiency in *emptyhive* mutant mice. IL-7R and pre-BCR signaling are both important during these stages[22], and this may therefore reflect a failure of one or both pathways during the development of *emptyhive* mutant precursors.

Mutation of an uncharacterized P4-type ATPase

To identify the mutation responsible for the *emptyhive* phenotype, mutant males were outcrossed to C3H/HeN females and backcrossed to their F1 daughters. A total of 25 F2 males (11 mutant and 14 wild-type phenotype) were typed at 128 polymorphic microsatellite markers. Phenotypic linkage to the X chromosome was detected (maximum LOD score of 8.37 at DXMit68) (Fig. 6a), and fine mapping with a further 105 meioses confined the mutation to a 13.51Mb interval between DXMit68 and DXMit74 (Fig. 6b). Coding exons and flanking splice junctions of the 81 annotated protein-encoding genes in the mutant interval were sequenced by capillary sequencing, with 85.6% high-quality coverage (Phred score>30) of all nucleotides (129589/151336). A single hemizygous transition mutation was identified in *Atp11c* (C2113T, CAG→TAG, Q655*) (Fig. 6c). Since this mutation (in exon 19 of 30) occurs before the penultimate exon, the mutant transcript is most likely targeted by the nonsense-mediated decay pathway shortly after transcription[27], although this has yet to be confirmed. In addition, we subsequently identified a mutant pedigree which phenocopied *emptyhive* (named *spelling*), and after sequencing *Atp11c* in these mice identified a second mutant allele (T1214A, ATA→AAA, I355K), which introduced a charged residue into the fourth of ten membrane-spanning domains (Fig. 6c, Supplementary Fig. 2). Compound heterozygosity for both alleles caused a B cell deficiency as severe as either parental strain (Fig. 6d,e), indicating that both *emptyhive* and *spelling* phenotypes were caused by X-linked recessive loss of function alleles of *Atp11c* (see Fig. 6f for protein schematic).

Atp11c is a previously uncharacterized gene encoding a P4-type ATPase, expressed most prominently in the liver, but also by many other cell types[28] (Supplementary Fig. 3). Little is known about mammalian P4-type ATPases in general, although mutation of one (ATP8B1) can cause progressive intrahepatic cholestasis in man[29]. While P-type ATPases are typically

involved in cation transport[30], the P4 subtype includes inwardly-translocating lipid flippases thought to enrich aminophospholipids at the cytoplasmic leaflet of lipid bilayers[31],[32]. Because P4-type ATPases have previously been implicated in phosphatidylserine (PS) asymmetry in yeast[31], we hypothesized that increased surface PS exposure and consequent PS-mediated phagocytosis could account for diminished numbers of B cells in *Atp11c* mutant mice[33]. PS expression on λ5-expressing cells was examined using Annexin V, yet no increase in surface expression was observed (Supplementary Fig. 4). Another requirement for phospholipid asymmetry could conceivably occur at the level of lipid rafts: membrane domains specialized in signal transduction[34] and potentially important for IL-7R or pre-BCR[35] signaling. Total surface expression of the raft-associated ganglioside G_{M1} (a target of the B subunit of cholera toxin) did not differ between wild-type and mutant cells (Supplementary Fig. 4), although closer examination of membrane microdomains around the IL-7R or pre-BCR will be needed to assess assembly of the rafts themselves.

Although not an aminophospholipid, phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3) is a phospholipid vital for signaling initiated at the IL-7R and pre-BCR[36]. Mutation of *Cd19* or *Pik3cd* cripples the function of phosphatidylinositol-3-kinase (PI3K) and generation of PtdIns(3,4,5)P_3 at the cytoplasmic leaflet of the pre-BCR signalosome, resulting in an impairment of MZ and B-1 B cell development[37],[38]. Conversely, in the absence of the PtdIns(3,4,5)P_3 phosphatase PTEN, an excess of PtdIns(3,4,5)P_3 leads to an expansion of MZ and B-1 subsets[37]. Since mutations of both positive (*Pik3cd*, *Pik3r1*, *Btk*, *Pik3ap1*, *Plcg2* and *Blnk*) and negative (*Pten* and *Ptpn6*) regulators of PtdIns(3,4,5)P_3 availability are known to affect B cell development[39], and since phosphatidylinositol asymmetry may be disrupted in *emptyhive* progenitors, we combined the *emptyhive* mutation with mutations of *Cd19*, *Cd45*, *Inpp5d* and *Ptpn6*. Neither genetic enhancement (*Inpp5d*, *Ptpn6* mutation) nor diminishment (*Cd19*, *Cd45* mutation) of PtdIns(3,4,5)P_3 availability altered the *emptyhive* phenotype (Supplementary Fig. 5), implying that a disruption of phosphatidylinositol asymmetry at the cytoplasmic leaflet of the pre-BCR was an unlikely cause of B cell deficiency in *Atp11c* mutant mice.

A context-sensitive requirement for ATP11C

Having identified the causative mutation of the *emptyhive* phenotype, we were able to directly compare fetal B lymphopoiesis among wild-type and *Atp11c* mutant siblings *in utero*. Since IgM^+ cells only appear after ~E16 (ref. [40]), we examined E18.5 fetal liver cells, and found no difference in the number or frequency of $B220^+IgM^-$ or $B220^+IgM^+$ cells in *emptyhive* embryos (Fig. 7a,b). This finding provided further evidence that fetal B cell development was intact in *emptyhive* mice, and that residual B cells in mutant mice were of pre-adult origin. Despite this result, mutant fetal liver cells were unable to reconstitute any B cell lineage in irradiated adult recipients (Fig. 3c), indicating that the B cell deficiency was cell-autonomous, yet microenvironment-dependent.

Discussion

Our results define a key role for *Atp11c* in adult, but not fetal B cell development. MZ and B-1 B cell compartments were both relatively intact in mutant mice, whereas follicular B

cell numbers declined with age, closely resembling that seen after postnatal deletion of *Rag2* (ref. [4]). Most strikingly, fetal liver B cell progenitors developed normally in *Atp11c* mutant fetal liver, but not in the adult bone marrow, revealing a cell-intrinsic, but context-dependent requirement for ATP11C.

Few genes have been reported to act in such a way[1], with *Il7* and *Il7r* being the prototypical examples[7]. *Il7*, *Il7r* and *Atp11c* are all essential for adult, but not fetal B cell development, with a cell-intrinsic requirement for both *Il7r* and *Atp11c* to sustain *Ebf1* transcription in pre-pro-B ($B220^+CD43^+BP\text{-}1^-CD24^-$) cells, and promote their differentiation to the pro-B ($B220^+CD43^+BP\text{-}1^-CD24^+$) stage[9]. But there are many key distinctions, the most notable being that T cell development proceeds in *Atp11c* mutants, but not in the absence of IL-7 or IL-7Rα[8,41,42]. Even among B cell progenitors, *Igll1* transcription and λ5 expression are intact in *Atp11c*, but not *Il7r* mutants[9]. *Ebf1*$^{-/-}$ mice show a similar arrest in B cell development, yet they too fail to transcribe *Igll1*, and since these mice lack all peripheral IgM^+ cells it does not appear to be an adult-restricted defect[12]. *Atp11c* mutant progenitors can clearly proliferate and differentiate *ex vivo* in the presence of IL-7, as well as during fetal development in the absence of FLT3 signaling[6,43], and unlike *Il7r* mutant fetal liver cells (which can give rise to mature B cells in the bone marrow of irradiated adults[7]), have their developmental potential determined by their environment, rather than their source.

While ATP11C is clearly not essential for all IL-7-dependent phenotypes, it may still be quantitatively or qualitatively important, affecting only a given threshold or branch of IL-7R signaling, or migration of B cell progenitors to a stromal depot of IL-7. Another interpretation is that ATP11C has only a transiently important role in early B cell development. Temporal restriction of IL-7R expression appears to be critical, since retroviral overexpression arrests B cell development at the pre-pro-B stage and inhibits *Ebf1* expression[44], implying that transient downregulation of IL-7R is important for the progression of B cell development.

IL-7 is also necessary for sustaining expression of EBF and the progression of B cell development beyond the pre-pro-B stage, but not because of IL-7R signaling in pre-pro-B cells themselves. Instead, signaling during the common lymphoid precursor to pre-pro-B transition induces high and persistent expression of *Ebf1*, presumably via a series of feedback loops at the *Ebf1* promoter[45], and allows maturation of pre-pro-B cells even in the absence of IL-7 (refs. [9,10,46]). Even though *Ebf1* appears not to be expressed in *Atp11c* mutant precursors, an EBF target gene is (*Igll1*), suggesting that ATP11C may be important not for the initial induction of *Ebf1*, but instead to sustain *Ebf1* expression through a feedback loop[45]. Whatever the cause, a failure to sustain *Ebf1* expression has broad consequences for the regulation of early B cell development[13].

Beyond the transcriptional consequences, what is the substrate of ATP11C, and how might it affect IL-7 responsiveness and the sustained expression of *Ebf1*? The aminophospholipids (phosphatidylserine and phosphatidylethanolamine) are primary candidate substrates based upon the study of ATP11C orthologs in yeast[31,32], and lipid reconstitution experiments will be important to determine which specific variants are trafficked by ATP11C, and in which membrane compartments and/or domains. Further work will follow to define how this

translates into an aberrant sensitivity to IL-7, and particularly why it does so only in the context of B cell development in the adult bone marrow. Presumably this outcome is related to phospholipid asymmetry during IL-7R signal transduction, or perhaps because of impaired migration to an IL-7-rich stromal niche. Identification of the substrate and affected pathway should expose a vital target for the specific regulation of adult B cell development, while *Atp11c* mutant strains will provide key experimental models to study membrane asymmetry and the origins of B cell immunity and malignancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank M. Gutierrez for animal care; T. Robinson, S. Kalina & C. Ross for genotyping; C. Goodnow (John Curtin School of Medical Research) & R. Brink (Garvan Institute) for mice; A. Feeney for discussions; D. La Vine for panel 5E; and reviewers for their valuable critiques. Supported by the Bill & Melinda Gates Foundation and National Institute of Allergy and Infectious Diseases Broad Agency Announcement Contract HHSN272200700038C (to B.B.), a General Sir John Monash Award (to O.M.S.), and an Irvington Institute Fellowship of the Cancer Research Institute (to C.N.A.).

References

1. Hardy RR, Hayakawa K. B cell development pathways. Annu Rev Immunol. 2001; 19:595–621. [PubMed: 11244048]
2. Lopes-Carvalho T, Foote J, Kearney JF. Marginal zone B cells in lymphocyte activation and regulation. Curr Opin Immunol. 2005; 17:244–250. [PubMed: 15886113]
3. Tung JW, Herzenberg LA. Unraveling B-1 progenitors. Curr Opin Immunol. 2007; 19:150–155. [PubMed: 17303402]
4. Hao Z, Rajewsky K. Homeostasis of peripheral B cells in the absence of B cell influx from the bone marrow. J Exp Med. 2001; 194:1151–1164. [PubMed: 11602643]
5. Carvalho TL, Mota-Santos T, Cumano A, Demengeot J, Vieira P. Arrested B lymphopoiesis and persistence of activated B cells in adult interleukin 7(-/)- mice. J Exp Med. 2001; 194:1141–1150. [PubMed: 11602642]
6. Sitnicka E, et al. Complementary signaling through flt3 and interleukin-7 receptor alpha is indispensable for fetal and adult B cell genesis. J Exp Med. 2003; 198:1495–1506. [PubMed: 14610045]
7. Kikuchi K, Kondo M. Developmental switch of mouse hematopoietic stem cells from fetal to adult type occurs in bone marrow after birth. Proc Natl Acad Sci USA. 2006; 103:17852–17857. [PubMed: 17090683]
8. von Freeden-Jeffry U, et al. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J Exp Med. 1995; 181:1519–1526. [PubMed: 7699333]
9. Kikuchi K, Lai AY, Hsu CL, Kondo M. IL-7 receptor signaling is necessary for stage transition in adult B cell development through up-regulation of EBF. J Exp Med. 2005; 201:1197–1203. [PubMed: 15837809]
10. Dias S, Silva H, Cumano A, Vieira P. Interleukin-7 is necessary to maintain the B cell potential in common lymphoid progenitors. J Exp Med. 2005; 201:971–979. [PubMed: 15767371]
11. Malin S, et al. Role of STAT5 in controlling cell survival and immunoglobulin gene recombination during pro-B cell development. Nat Immunol. 2010; 11:171–179. [PubMed: 19946273]
12. Lin H, Grosschedl R. Failure of B-cell differentiation in mice lacking the transcription factor EBF. Nature. 1995; 376:263–267. [PubMed: 7542362]
13. Busslinger M. Transcriptional control of early B cell development. Annu Rev Immunol. 2004; 22:55–79. [PubMed: 15032574]

14. Yabas M, et al. ATP11c is critical for phosphatidylserine internalization and B lymphocyte differentiation. Nat Immunol. 2011 this issue.
15. Nelms KA, Goodnow CC. Genome-wide ENU mutagenesis to reveal immune regulators. Immunity. 2001; 15 :409–418. [PubMed: 11567631]
16. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J Exp Med. 1991; 173:1213–1225. [PubMed: 1827140]
17. Karasuyama H, et al. The expression of Vpre-B/lambda 5 surrogate light chain in early bone marrow precursor B cells of normal and B cell-deficient mutant mice. Cell. 1994; 77:133–143. [PubMed: 8156589]
18. Jensen CT, et al. FLT3 ligand and not TSLP is the key regulator of IL-7-independent B-1 and B-2 B lymphopoiesis. Blood. 2008; 112:2297–2304. [PubMed: 18566323]
19. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature. 1992; 356:314–317. [PubMed: 1372394]
20. Bouillet P, et al. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science. 1999; 286:1735–1738. [PubMed: 10576740]
21. Strasser A, et al. Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. Proc Natl Acad Sci USA. 1991; 88:8661–8665. [PubMed: 1924327]
22. Herzog S, Reth M, Jumaa H. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. Nat Rev Immunol. 2009; 9:195–205. [PubMed: 19240758]
23. Brink RA, et al. Immunoglobulin M and D antigen receptors are both capable of mediating B lymphocyte activation, deletion, or anergy after interaction with specific antigen. J Exp Med. 1992; 176:991–1005. [PubMed: 1402669]
24. Phan TG, et al. B cell receptor-independent stimuli trigger immunoglobulin (Ig) class switch recombination and production of IgG autoantibodies by anergic self-reactive B cells. J Exp Med. 2003; 197:845–860. [PubMed: 12668643]
25. Pelanda R, Schaal S, Torres RM, Rajewsky K. A prematurely expressed Ig(kappa) transgene, but not V(kappa)J(kappa) gene segment targeted into the Ig(kappa) locus, can rescue B cell development in lambda5-deficient mice. Immunity. 1996; 5:229–239. [PubMed: 8808678]
26. Spanopoulou E, et al. Functional immunoglobulin transgenes guide ordered B-cell differentiation in Rag-1- deficient mice. Genes Dev. 1994; 8:1030–1042. [PubMed: 7926785]
27. Chang YF, Imam JS, Wilkinson MF. The nonsense-mediated decay RNA surveillance pathway. Annu Rev Biochem. 2007; 76:51–74. [PubMed: 17352659]
28. Su AI, et al. A gene atlas of the mouse and human protein-encoding transcriptomes. Proc Natl Acad Sci USA. 2004; 101:6062–6067. [PubMed: 15075390]
29. Bull LN, et al. A gene encoding a P-type ATPase mutated in two forms of hereditary cholestasis. Nat Genet. 1998; 18:219–224. [PubMed: 9500542]
30. Kühlbrandt W. Biology, structure and mechanism of P-type ATPases. Nat Rev Mol Cell Biol. 2004; 5:282–295. [PubMed: 15071553]
31. Tang X, Halleck MS, Schlegel RA, Williamson P. A subfamily of P-type ATPases with aminophospholipid transporting activity. Science. 1996; 272:1495–1497. [PubMed: 8633245]
32. Holthuis JC, Levine TP. Lipid traffic: floppy drives and a superhighway. Nat Rev Mol Cell Biol. 2005; 6:209–220. [PubMed: 15738987]
33. Leventis PA, Grinstein S. The distribution and function of phosphatidylserine in cellular membranes. Annu Rev Biophys. 2010; 39:407–427. [PubMed: 20192774]
34. Dykstra M, Cherukuri A, Sohn HW, Tzeng SJ, Pierce SK. Location is everything: lipid rafts and immune cell signaling. Annu Rev Immunol. 2003; 21:457–481. [PubMed: 12615889]
35. Guo B, Kato RM, Garcia-Lloret M, Wahl MI, Rawlings DJ. Engagement of the human pre-B cell receptor generates a lipid raft-dependent calcium signaling complex. Immunity. 2000; 13:243–253. [PubMed: 10981967]

36. Fruman DA, Cantley LC. Phosphoinositide 3-kinase in immunological systems. Semin Immunol. 2002; 14:7–18. [PubMed: 11884226]
37. Anzelon AN, Wu H, Rickert RC. Pten inactivation alters peripheral B lymphocyte fate and reconstitutes CD19 function. Nat Immunol. 2003; 4:287–294. [PubMed: 12563260]
38. Janas ML, et al. The effect of deleting p110delta on the phenotype and function of PTEN-deficient B cells. J Immunol. 2008; 180:739–746. [PubMed: 18178811]
39. Miosge LA, Goodnow CC. Genes, pathways and checkpoints in lymphocyte development and homeostasis. Immunol Cell Biol. 2005; 83:318–335. [PubMed: 16033527]
40. Raff MC, Megson M, Owen JJ, Cooper MD. Early production of intracellular IgM by B-lymphocyte precursors in mouse. Nature. 1976; 259:224–226. [PubMed: 814466]
41. Peschon JJ, et al. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. J Exp Med. 1994; 180:1955–1960. [PubMed: 7964471]
42. Grabstein KH, et al. Inhibition of murine B and T lymphopoiesis in vivo by an anti-interleukin 7 monoclonal antibody. J Exp Med. 1993; 178:257–264. [PubMed: 8315381]
43. Vosshenrich CA, Cumano A, Müller W, Di Santo JP, Vieira P. Thymic stromal-derived lymphopoietin distinguishes fetal from adult B cell development. Nat Immunol. 2003; 4:773–779. [PubMed: 12872121]
44. Purohit SJ, et al. Determination of lymphoid cell fate is dependent on the expression status of the IL-7 receptor. EMBO J. 2003; 22:5511–5521. [PubMed: 14532123]
45. Roessler S, et al. Distinct promoters mediate the regulation of Ebf1 gene expression by interleukin-7 and Pax5. Mol Cell Biol. 2007; 27:579–594. [PubMed: 17101802]
46. Kikuchi K, Kasai H, Watanabe A, Lai AY, Kondo M. IL-7 specifies B cell fate at the common lymphoid progenitor to pre-proB transition stage by maintaining early B cell factor expression. J Immunol. 2008; 181:383–392. [PubMed: 18566404]

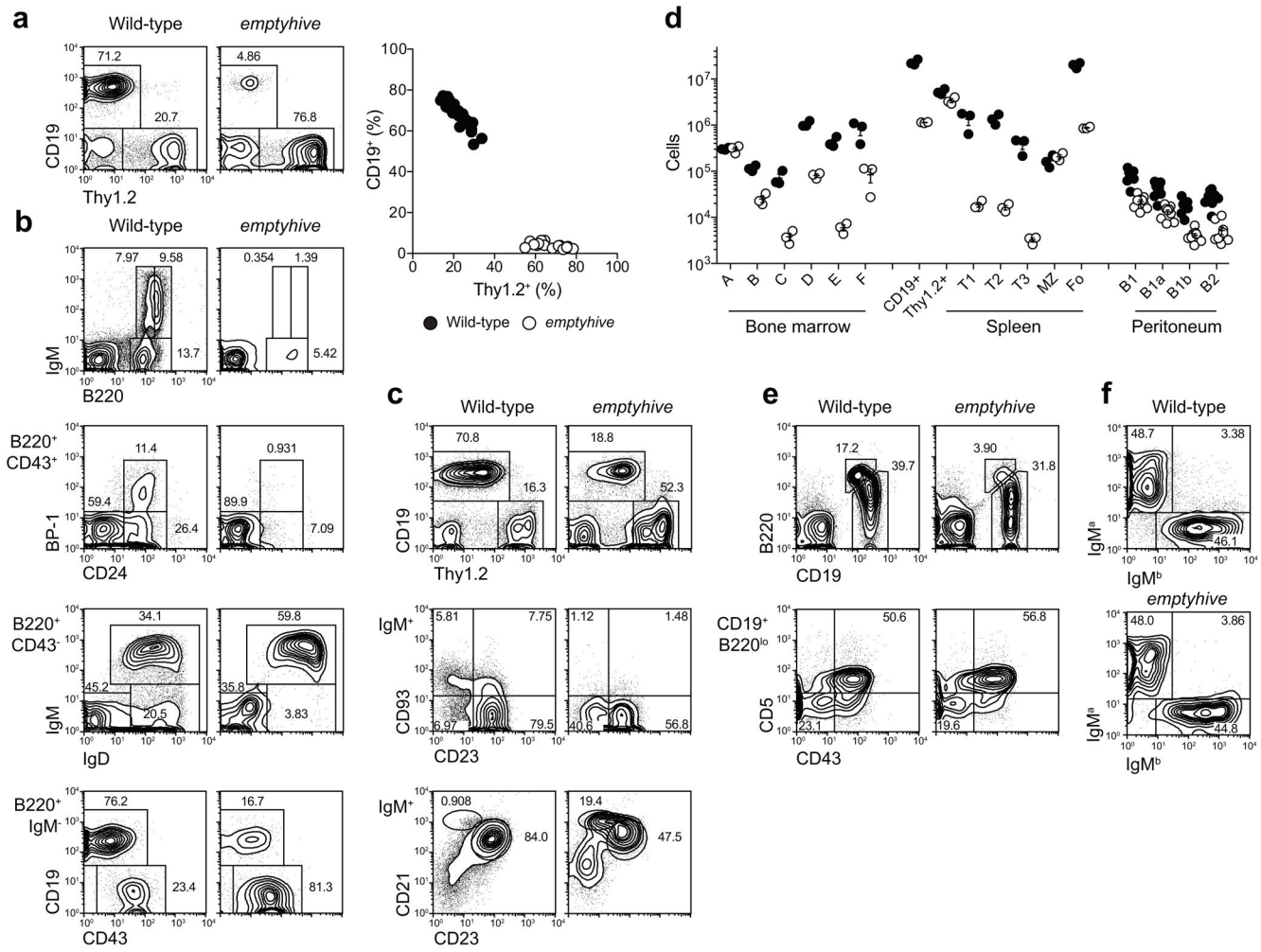

Figure 1.

A heritable B cell deficiency. (**a**) Frequencies of $CD19^+$ and $Thy1.2^+$ blood lymphocytes in the *emptyhive* pedigree. Percentages (**b**, **c, e**) and numbers (**d**) of B cell subsets in bone marrow (**b**), spleen (**c**) and peritoneal cavity (**e**). Hardy fractions in bone marrow (A-F) were gated as follows: A ($B220^+CD43^+BP\text{-}1^-CD24^-$); B ($B220^+CD43^+BP\text{-}1^-CD24^+$); C ($B220^+CD43^+BP\text{-}1^+CD24^+$); D ($B220^+CD43^-IgM^-IgD^-$); E ($B220^+CD43^-IgM^-IgD^+$); F ($B220^+CD43^-IgM^+IgD^+$). The C' fraction ($B220^+CD43^+BP^-1^+CD24^{hi}$) was not resolved. Fractions A and B-D may also be gated as $CD19^-$ and $CD19^+$ populations among $B220^+IgM^-$cells, respectively (**b**). IgM^+ splenocytes were divided into the following subsets: T1 ($CD93^+CD23^-$); T2 ($CD93^+CD23^+IgM^{int}$); T3 ($CD93^+CD23^+IgM^{hi}$); MZ ($CD93^-CD23^-IgM^{hi}CD21^{hi}$); Fo ($CD93^-CD23^+$). Peritoneal lymphocytes were divided into B-2 ($CD19^+B220^{hi}$), B-1 ($CD19^+B220^{lo-int}$), B-1a ($CD5^+CD43^+$) and B-1b ($CD5^-CD43^-$) subsets. (**f**) IgM allotype expression on $CD19^+$ blood lymphocytes from wild-type and *emptyhive* mice on a (C57BL/6 × BALB/c)F2 ($IgM^{a/b}$) background. Data are representative of three (**a** to **e**), or one (**f**) independent experiments using three mice per genotype. Each symbol represents an individual mouse.

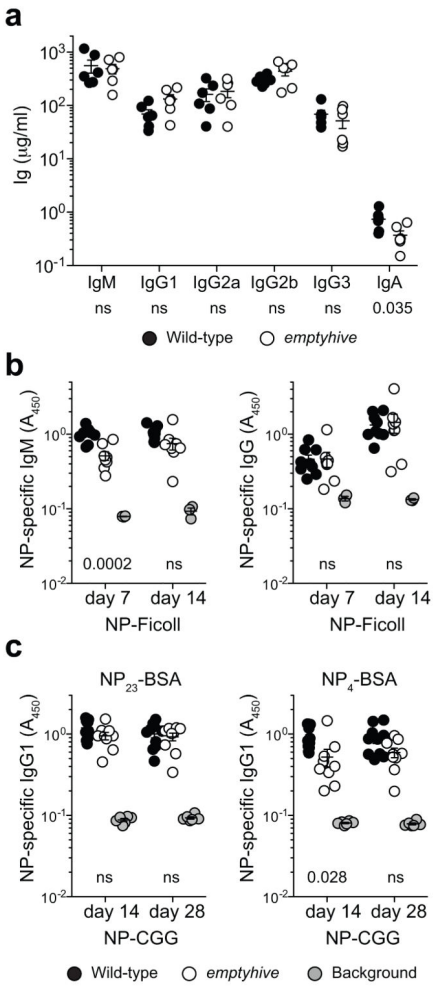

Figure 2.

Immunoglobulin secretion in *emptyhive* mice. (**a**) Total immunoglobulins as measured in the serum of 12–24 week-old naive mice. NP-specific antibodies were measured 7, 14 or 28 days after immunization of 12 week-old mice with NP-Ficoll (**b**) or alum-precipitated NP-CGG (**c**). Different capture antigens were used to discriminate between total (NP_{23}-BSA) and high-affinity (NP_4-BSA) IgG1 in response to NP-CGG. Each symbol represents an individual mouse from one experiment. *P* values indicated under plots were calculated by unpaired *t*-tests comparing wild-type and *emptyhive* groups. ns, not significant ($P > 0.05$).

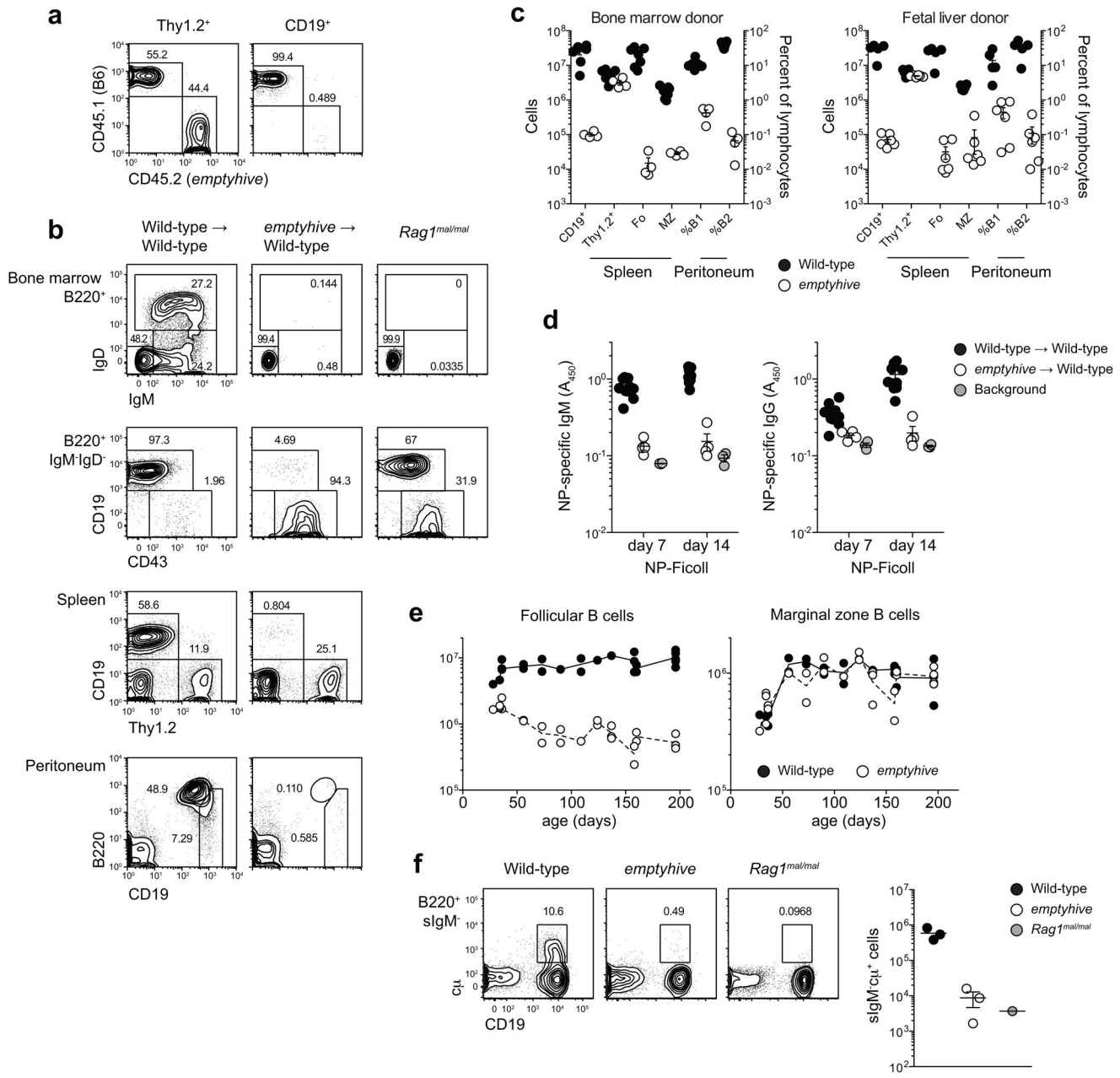

Figure 3.

A cell-intrinsic failure of adult B cell development. (**a**) A 1:1 mix of *emptyhive* (CD45.2) and wild-type (CD45.1) bone marrow was used to reconstitute irradiated *Rag1* mutant recipients, and lymphocyte repopulation was measured eight weeks later in blood. Unmixed bone marrow (**b**, **c**) or E16.5 fetal liver cells (**c**) from either mutant or wild-type donors (CD45.1) was used to reconstitute irradiated wild-type recipients (CD45.2), and donor-derived reconstitution of bone marrow, spleen and peritoneal B cell subsets was analyzed eight weeks later. (**d**) Bone marrow chimeras were immunized with NP-Ficoll 8 weeks after reconstitution, and NP-specific immunoglobulin titers measured 7 and 14 days later. (**e**) Absolute numbers of follicular and marginal zone B cells as a function of age. (**f**)

Intracellular IgM (cμ) expression in $B220^+$ bone marrow cells negative for surface IgM. Plots are representative of 6 (**a**), 4–7 (**b**), or 3 (**f**) mice per genotype, from one (**a**-**e**) or two (**f**) independent experiments. Each symbol represents an individual mouse.

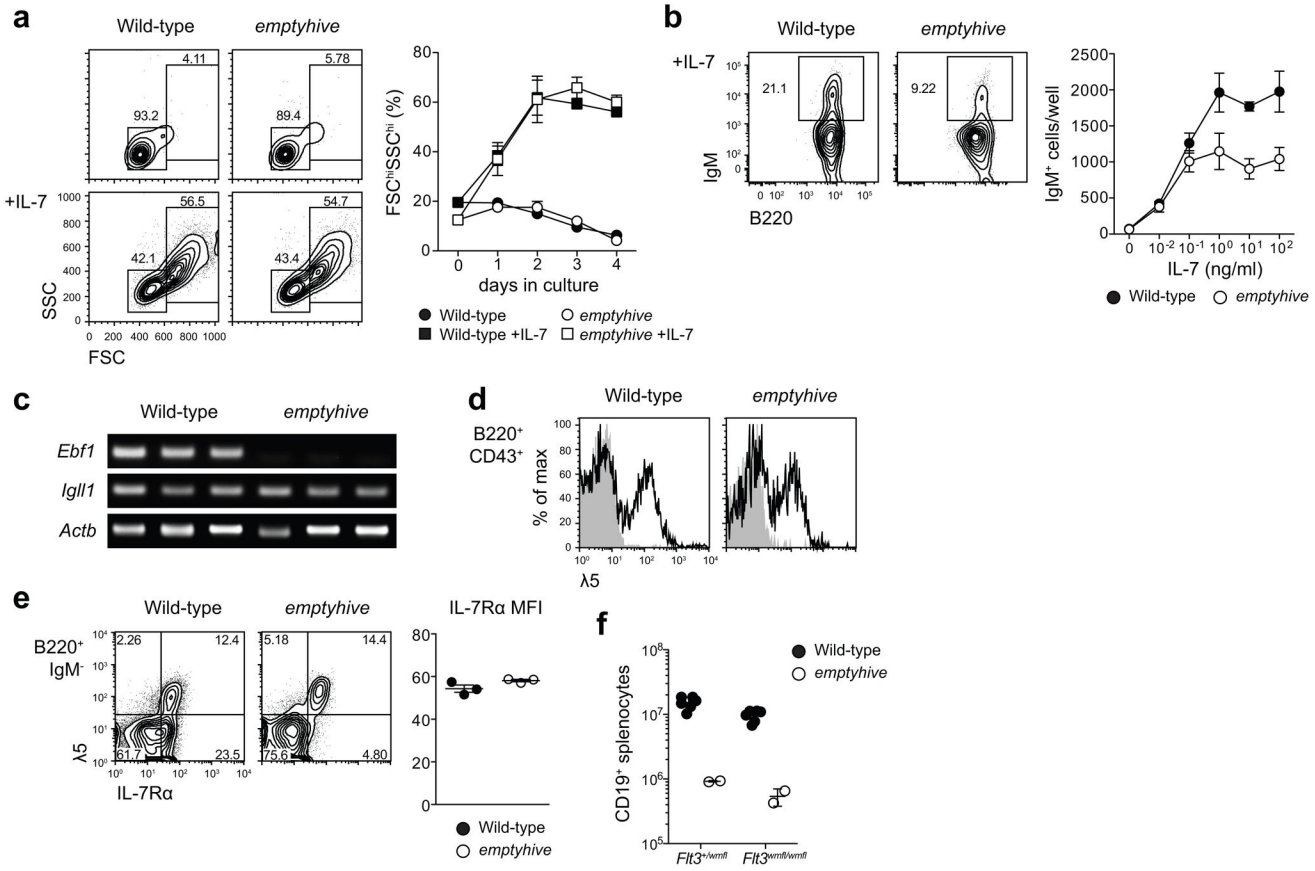

Figure 4.

Sensitivity to IL-7 and a failure to sustain expression of *Ebf1*. (**a**) B220$^+$ surface IgM$^-$bone marrow cells were sorted by flow cytometry and cultured *ex vivo* in the presence or absence of 100 ng/ml IL-7, and percentages of 7-AAD$^-$ lymphoblasts (FSC^{hi}SSC^{hi}) were measured at daily intervals. Symbols and error bars represent the mean and standard error of three mice per genotype. (**b**) B220$^+$ surface IgM$^-$ bone marrow cells sorted from wild-type (CD45.1) or *emptyhive* (CD45.2) mice were cocultured in the presence of various concentrations of IL-7. Following four days of culture, frequencies and numbers of 7-AAD$^-$surface IgM$^+$ cells were measured. Symbols and error bars represent the mean and standard error of three mice per genotype. (**c**) RT-PCR PCR of cDNA from pre-pro-B cells (7-AAD$^-$B220$^+$IgM/IgD$^-$CD19$^-$NK1.1$^-$Ly6C$^-$). Each lane represents an individual mouse. (**d**) λ5 (CD179b) expression on the surface of B220$^+$CD43$^+$ bone marrow lymphocytes. Shaded histograms represent rat IgG2a isotype control, while solid histograms show λ5 staining. (**e**) IL-7Rα (CD127) expression on the surface of B220$^+$IgM$^-$ bone marrow lymphocytes (dot plots, left), or B220$^+$IgM$^-$λ5$^+$ (mean fluorescence intensity, right). (**f**) Splenic B cell numbers in *emptyhive* mice with combined mutation of *Flt3*. All data are representative of two independent experiments, each with three mice per genotype.

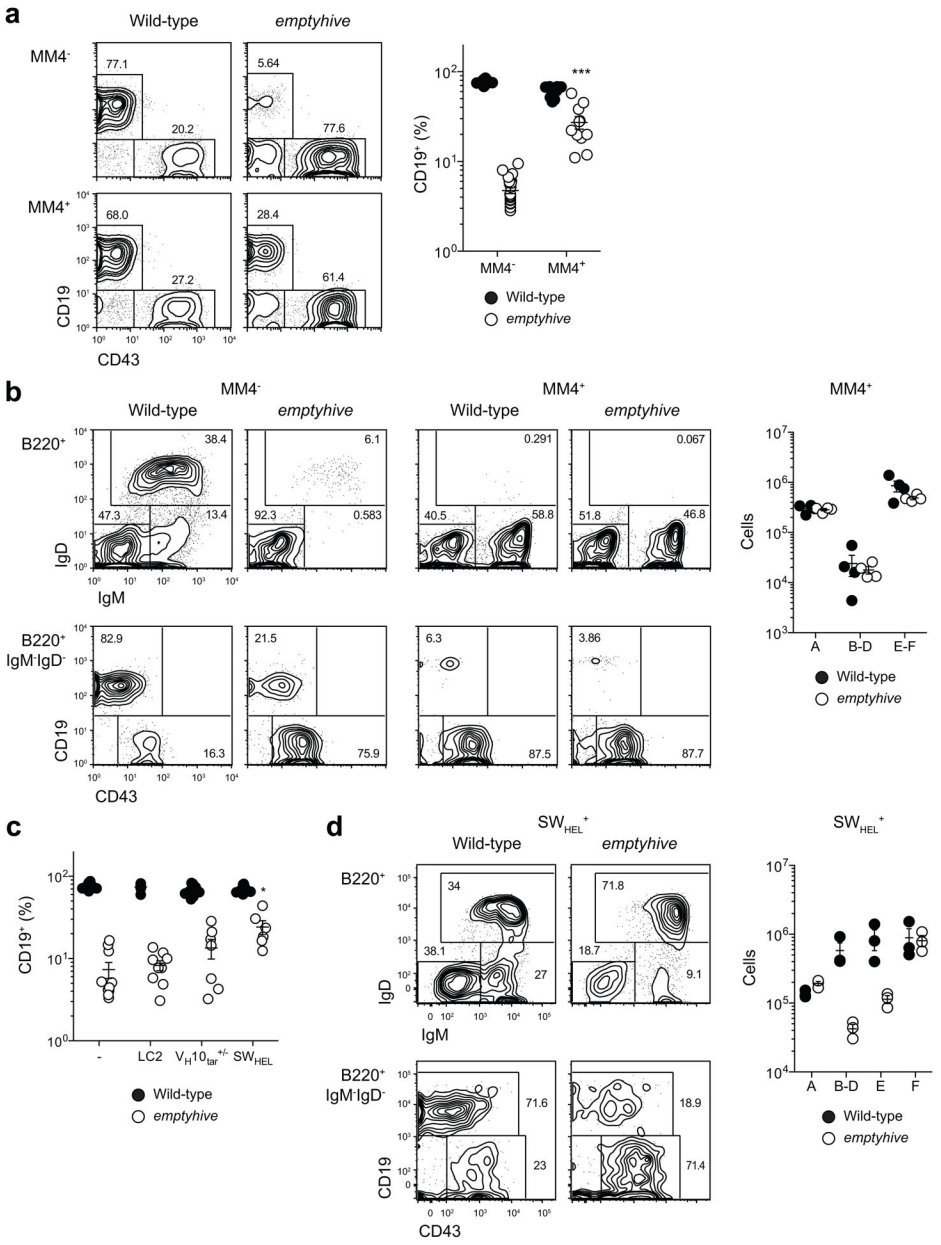

Figure 5.

Partial correction of the *emptyhive* phenotype by a BCR transgene. The *emptyhive* mutation was combined with the MM4 transgene (**a, b**) or the LC2 light chain transgene, V_H10_{tar} knockin allele, or both (SW_{HEL}) (**c, d**). Frequencies of $CD19^+$ cells in blood (**a**, **c**), or B cell subsets in bone marrow (**b**, **d**) as determined by flow cytometry. An unpaired *t*-test was performed in (**a**) with comparison to the *emptyhive* non-transgenic group. For (**c**), a one-way ANOVA followed by Bonferroni post test was used, with reference to the *emptyhive* non-transgenic group. $*P < 0.05$, $***P < 0.001$. Each symbol represents an individual mouse, and data are representative of two independent experiments.

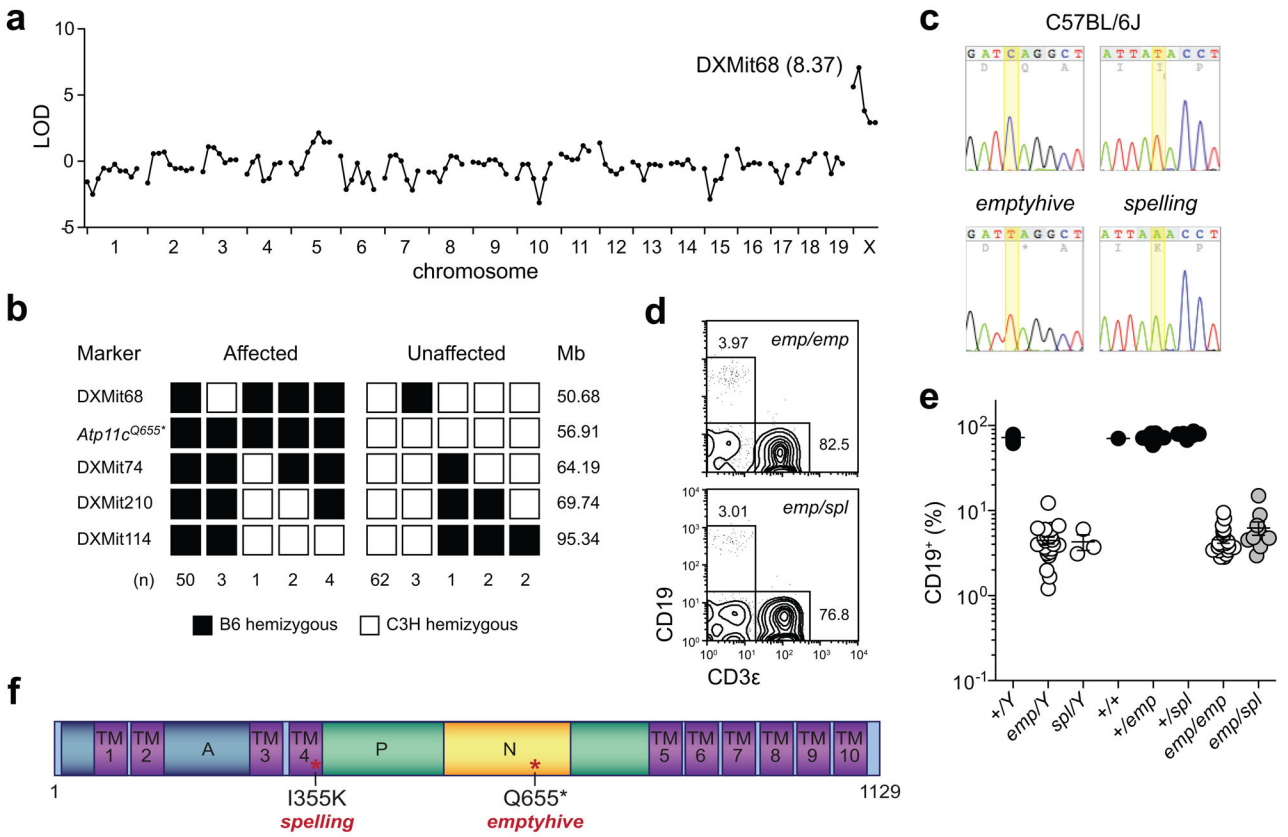

Figure 6.

The *emptyhive* phenotype is caused by a recessive mutation of *Atp11c*. Chromosomal (**a**) and fine (**b**) mapping of the *emptyhive* phenotype. (**c**) Sequence traces of the mutated nucleotides (C2113T, Q655*; T1214A, I355K) in *Atp11c* as detected in *emptyhive* and *spelling* hemizygous males. (**d**, **e**) Allelism test of *spelling* and *emptyhive* alleles. Progeny from an $Atp11c^{spl/Y} \times Atp11c^{emp/+}$ mating were tested for phenotypic complementation by measuring the frequency of B cells in blood. Each symbol represents an individual mouse. (**f**) Predicted ATP11C protein domain structure. TM, transmembrane domain; P, phosphorylation domain; A, actuator domain; N, nucleotide binding domain.

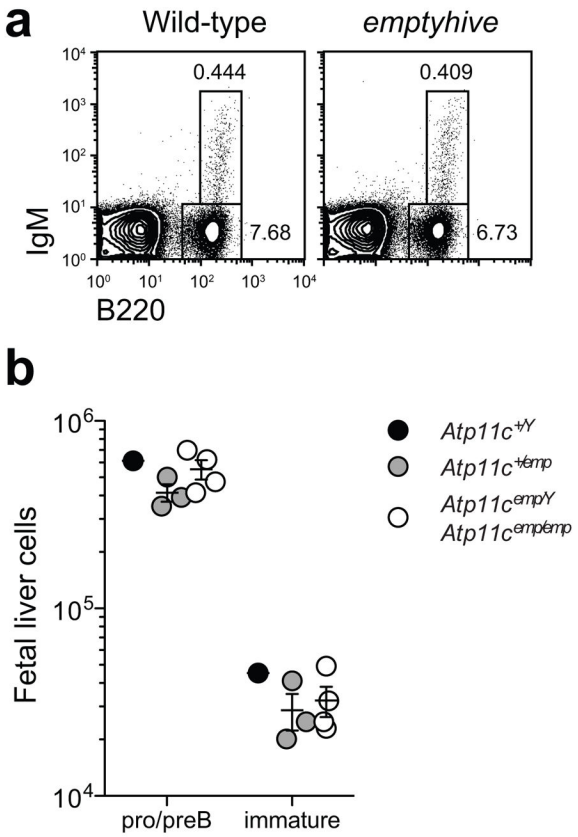

Figure 7.

Intact B lymphopoiesis in fetal liver. Percentages (**a**) and absolute numbers (**b**) of fraction A-D ($B220^+IgM^-$) and immature ($B220^+IgM^+$, fraction E) B cells in the fetal liver of E18.5 embryonic siblings from *Atp11c*$^{emp/Y}$ × *Atp11c*$^{+/emp}$ matings. Each symbol represents an individual embryo, and data are representative of two experiments with 8–14 embryos per experiment.