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IL-21 and tissue-specific signals instruct Tbet^{+}CD11c^{+} B cell development following viral infection

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Abstract

Tbet^{+}CD11c^{+} B cells, also known as age-associated (ABCs), are pivotal contributors to humoral immunity following infection and in autoimmunity, yet their *in vivo* generation is incompletely understood. We utilized a mouse model of systemic acute LCMV infection to examine the developmental requirements of ABCs that emerged in the spleen and liver. IL-21 signaling through STAT3 was indispensable for ABC development. In contrast, IFN-γ signaling through STAT1 was required for B cell activation and proliferation. Mice that underwent splenectomy or were deficient in lymphotoxin α generated hepatic ABCs despite the lack of secondary lymphoid organ contributions, suggesting that the liver supported *de novo* generation of these cells separately from their development in lymphoid organs. Thus, IFN-γ and IL-21 signaling have distinct, stage-specific roles in ABC differentiation, while tissue microenvironment provides additional cues necessary for their development.

Introduction

Co-expression of Tbet and CD11c marks the overlapping B cell subsets described in aging, infection, and autoimmunity, termed age-associated (ABCs), atypical memory, and double-negative B cells, respectively (1-4). Tbet^{+}CD11c^{+} B cells (hereafter, ABCs) contribute humoral protection against infections (5, 6), while also associated with autoimmune disease progression (7-12). Prior *in vitro* studies revealed that the ABC phenotype emerges following TLR7 and BCR stimulation combined with exposure to IFN-γ and IL-21 (12-14). IFN-γ primarily signals through STAT1, upregulating Tbet in cultured B cells, while IL-21 activates STAT1, STAT3, and STAT5, and induces CD11c expression (10, 14, 15). However, it is unclear how these signals are coordinated *in vivo* to drive the integrated ABC developmental program. Compared to other B cells, ABC exhibit unusual tissue localization, the basis of which is poorly understood. They persist as memory cells in the spleen and lung following infection (6, 16-18), and infiltrate disease-affected nonlymphoid tissues in

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autoimmunity (17, 19, 20). Understanding development and localization of ABCs will help reveal how they mediate tissue protection and damage.

We interrogated signals driving ABC generation using acute LCMV infection (6, 14). We found that STAT1 signaling ensured activation and proliferation of B cells, while the IL-21-STAT3 axis drove ABC differentiation. These cells emerged in a tissue-specific pattern that did not seem to result from B cell intrinsic differences across organs. Moreover, development of splenic and liver ABCs could be uncoupled, suggesting *de novo* generation in nonlymphoid tissues.

Materials and methods

Mice

Mice were maintained and used following guidelines by the Institutional Animal Care and Use Committee of Yale School of Medicine and Rutgers New Jersey Medical School. C57BL/6N animals were from Charles River. *Mb1*^{Cre} (020505), *Ifngr1*$^{fl/fl}$ (025394), *Ifnar* $^{fl/fl}$ (028256), *Stat3*$^{fl/fl}$ (016923), CD19^{Cre} (006785), *Tbet*$^{-/-}$(004648) and CD45.1 (002014) mice were from the Jackson Laboratory. *Stat1*$^{fl/fl}$ mice were from Lothar Hennighausen (NIH). *Il21r*$^{fl/fl}$ mice (13001) were from Taconic. Tbet-AmCyan mice were obtained from Jinfang Zhu (NIH). *LTα*$^{-/-}$ (Jax 002258) were from Andrew Wang (Yale University). CD19^{Cre} *Tbet* $^{fl/fl}$ mice were from Philippa Marrack (National Jewish Health). Mice were used at 6–8 weeks with sex- and age-matched controls.

Primary B cell culture

Splenic B cells were purified using a magnetic negative selection kit (EasyStep, StemCell Technologies) and cultured for 48 hours. When indicated, 10μg/ml of Fab fragment anti-IgM (Jackson Immuno), 100ng/ml of R848 (Invivogen), 50ng/ml of IL-21, 2 ng/ml of IL-12, 10ng/ml of IFNγ (all from Peprotech), and 3000 U/ml of IFNα (Pbl assay science) were used.

Cell transfers and viral infections

B cells purified by magnetic negative selection from spleens and LNs of CD45.1 and C57BL/6N mice were mixed in equal numbers prior to i.v. injection of 1x10^7 total cells into each MD4 mouse. Mice were infected 24 hours after transfer. Mice were injected i.p. with 2 × 10^5 PFU LCMV or i.v. with 10^4 PFU LCMV-Armstrong.

Sham and splenectomy surgery

Buprenorphine (Ethiqa)- and bupivacaine-treated mice were anesthetized with isoflurane. The spleen (with blood vessels and pancreatic tissue) was exteriorized from a left-sided skin incision. Blood vessels were ligated and splenectomy performed. The incision was closed with nonabsorbable sutures and VetBond skin glue.

Flow cytometry and analysis

Spleens and surface lymph nodes were mechanically dissociated. Livers were digested with 2 mg/ml Collagenase IV (Worthington) and centrifuged in 33%/66% Percoll (Sigma).

Surface staining was performed as previously described (antibodies, Supplemental Table 1) (6). Foxp3/Transcription Factor Staining kit (eBioscience) was used for intracellular staining.

FTY720 administration

FTY720 (Millipore) in saline was administered via i.p. injections every 2 days at 1mg/kg.

Statistics

Data were analyzed using one-way and two-way multiple-comparison ANOVA in Prism 8 (GraphPad Software). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Results and Discussion

IL-21 signaling drives ABC differentiation

To dissect signals driving ABC differentiation, we cultured purified splenic B cells from C57BL/6N mice for 48 hours in the presence of various stimuli, including BCR and TLR7 agonists, and cytokines. Similar to previous findings, IFN-γ and IL-21 respectively elevated Tbet and CD11c expression, which was further amplified by BCR and TLR activation (Supplemental Fig 1A-B) (10, 13-15). CD11c was upregulated only with IL-21, whereas Tbet was induced by multiple type I cytokines including IFN-γ, IL-21, IFN-α, and IL-12 (Supplemental Fig 1A-C), suggesting that IL-21 drives emergence of the ABC phenotype.

To test the *in vivo* contribution of these cytokines, mice lacking B cell expression of IFNAR, IFNγR, or IL-21R (*Mb1*^{Cre} *Ifnar1*$^{fl/fl}$, *Mb1*^{Cre} *Ifngr*$^{fl/fl}$ and *Mb1*^{Cre} *Il21r*$^{fl/fl}$, respectively) along with *Mb1*^{Cre} controls were infected with LCMV. At day 10 p.i., the peak of ABC response (6), all groups of conditional knockout mice exhibited reduced frequencies of activated and proliferating splenic B cells marked by CD44 and Ki67 (gating strategies in Supplemental Fig 1D-E, Fig 1A-B, Supplemental Fig 2A), suggesting that type I IFNs, IFN-γ and IL-21 were required for optimal B cell activation. Among activated B cells, Tbet was decreased in all conditional knockouts, whereas CD11c induction was only impaired in the absence of IL-21R (Fig 1C). Mice lacking B cell expression of IL-21R but not IFNAR nor IFNγR had decreased ABC frequency among activated B cells compared to controls, indicating IL-21 is the principal cytokine driving their differentiation *in vivo* (Fig 1B, Supplemental Fig 2A). Although B cell-specific deficiency in either IFNγR or IL-21R impaired ABC development (Fig 1D), the former resulted mainly in defects in activation and proliferation, whereas the latter had an additional bottleneck in differentiation (Fig 1E). These data indicate distinct, stage-specific function of cytokines in ABC development, in contrast with previous models that postulating simultaneous roles for IFN-γ and IL-21 in upregulating Tbet and CD11c.

STAT1 and STAT3 interactions orchestrate ABC development

IL-21 and IFNs activate STAT signaling in B cells, but how this promotes ABC development is unclear. *Mb1*^{Cre} *Stat1*$^{fl/fl}$ mice lacking STAT1 expression in B cells had decreased activated (CD44^{+}Ki67^{+}) B cells compared to *Mb1*^{Cre} controls and *Mb1*^{Cre} *Stat3*$^{fl/fl}$ mice upon infection with LCMV (Fig 2A-B), despite comparable overall and activated B cells

prior to infection (Supplemental Fig 2B). STAT1-deficient activated B cells further had decreased Tbet expression and unexpected upregulation of CD11c, in contrast to their STAT3-decifient counterparts with decreased expression of both Tbet and CD11c (Fig 2C). An activation defect in total B cells was mainly responsible for reduced ABCs upon STAT1 deletion (Fig 2D), as it did not affect Tbet and CD11c expression by activated B cells (Fig 2A-B). While the downstream mechanism underlying STAT1-mediated B cell proliferation remains to be elucidated, this observation can be recapitulated *in vitro* (data not shown) and is in line with observations in other B cell subsets (21) and in patients with loss-of-function STAT1 mutations having fewer ABCs in circulation (22). In contrast, *Mb1*^{Cre} *Stat3*$^{fl/fl}$ mice had defective ABC differentiation (Fig 2A-B) , suggesting STAT3 drives their formation without significant effects on activation and proliferation. Likewise, patients with STAT3 loss-of-function mutations have decreased circulating ABCs (22).

To parse STAT1 and STAT3 orchestration of ABC development in the cytokine milieu, we cultured *Mb1*^{Cre} *Stat1*$^{fl/fl}$ STAT1- and *Mb1*^{Cre} *Stat3*$^{fl/fl}$ STAT3-deficient B cells, with both demonstrating reduction in Tbet expression upon IL-21 stimulation versus *Mb1*^{Cre} controls. By contrast, IFN-γ and IFN-α induction of Tbet was only reduced in *STAT1*-deficient B cells (Fig 2E and Supplemental Fig 2C). These data suggest that STAT1 and STAT3 mediate Tbet expression in B cells, with IL-21 signaling requiring both, whereas IFNs signaled via STAT1. CD11c expression was abolished in the absence of STAT3 but unaffected in cells lacking STAT1 (Fig 2F-G), suggesting that STAT3 drives CD11c expression in B cells, consistent with findings in IL-21R-deficient ABCs (Fig 1C). Furthermore, in *Mb1*^{Cre} control cells, IFN-γ or IFN-α was inhibitory to IL-21-mediated induction of CD11c in the absence of TLR stimulation (Fig 2G and Supplemental Fig 2C). However, IFN-γ- and IFN-α-stimulated STAT1-deficient B cells were insensitive to suppression of CD11c.These results suggest that co-activation of STAT1 and STAT3 by IL-21 induced the ABC phenotype, while additional IFN signaling through STAT1 upregulated Tbet at the cost of CD11c (Fig 2H).

The environment of the spleen, not that of lymph nodes, is conducive to ABC development

ABCs are more abundant in the spleen than in lymph nodes (LNs) (18), suggesting the latter lacks tissue-specific developmental signals. To address this, we performed splenectomy or sham surgery on *Tbet*-AmCyan reporter mice (23), and four weeks later, i.v. infected them with LCMV (24). The spleens of mice receiving sham surgery showed robust ABC response at day 10 p.i., approximately 4-fold higher than LNs (Fig 3A). LNs of splenectomized mice were deficient in ABCs, suggesting they rarely develop in LNs regardless of splenic presence. Splenic tissue restriction of ABCs was unlike other B cell subsets such as germinal center or plasma cells, which showed no tissue developmental preference (Supplemental Fig 3A).

Since both spleen and LNs are rich in IL-21 and Tfh cells promoting ABC development (6), the lack of their robust LN differentiation could be due to tissue-specific differences or a unique splenic precursor population, since the latter is home to diverse B cell subsets such as immature/transitional and marginal zone B cells. To test if ABCs preferentially arise from unique splenic progenitors, we transferred equal numbers of B cells purified from the spleens or LNs of congenically marked mice into MD4 recipients expressing an irrelevant

BCR transgene (25). At day 10 p.i. with LCMV, splenic ABCs were composed of roughly 50% of B cells of spleen- and of LN-origin (Fig 3B), suggesting that the splenic as opposed to the LN environment was conducive to development of ABCs.

ABCs differentiate *de novo* in the liver

ABCs emerge in the liver following *Ehrlichia* and hepatitis virus infections (26, 27); however, the origin of the hepatic population is unclear. Hepatic ABCs accumulate at day 10 p.i. with LCMV, with absence of ABCs in circulation confirming liver infiltration (Supplemental Fig 3B). To determine when ABCs seeded the liver during infection, we infected Tbet-reporter mice and treated them every other day with FTY720, a functional antagonist of S1PR1, to block lymphocyte egress into circulation (Fig 4A). While FTY720 did not significantly affect generation of splenic ABCs, consistent with development of this resident population (6, 18), ABCs were largely absent from the liver of treated mice regardless of the timing of injection, starting from day 2 or 6 p.i. (Fig 4A). Thus, the liver population was likely seeded later in infection by circulating precursor B cells.

To determine whether the liver population resulted from differentiated ABCs leaving splenic residence or from *de novo* hepatic differentiation, we analyzed splenectomized Tbet-reporter mice at day 10 p.i. with LCMV. We found robust hepatic ABC response despite lack of splenic or LN ABC generation (Fig 4B), suggesting that hepatic subset did not result from splenic ABC translocation. To further assess this possibility, we infected lymphotoxin α-deficient ($LT\alpha^{-/-}$) mice lacking LNs and Peyer's patches. Splenic generation of ABCs was impaired in $LT\alpha^{-/-}$ mice (Fig 4C), likely due to the reduction of Tfh cells necessary for development of the splenic subset (Supplemental Fig 3C) (6, 28). Yet, development of the liver subset was unaffected in $LT\alpha^{-/-}$ mice. These data, along with the experiments using FTY720, indicated that ABCs differentiated *de novo* in the liver from precursor circulating B cells. While consistent with clonal overlap between blasting B cells in the spleen and liver (26), our work further interrogated the inter-dependency of splenic and hepatic ABCs and elucidated their developmental point of divergence.

We next infected $Mb1^{Cre}$ $Stat3^{fl/fl}$ and $Mb1^{Cre}$ control mice and examined hepatic development of ABCs. At day 10 p.i., STAT3-deficient animals had reduced ABCs in the liver (Fig 4D), confirming STAT3-signaling governs their differentiation across tissues. This contrasts with LNs, which generate few ABCs despite abundant STAT3-activating cytokines produced by Tfh cells (6).

Therefore, we propose that unique environmental signals underlie the tissue-specific development of ABCs. Further clarifications of these site-specific requirements are necessary for therapeutic targeting of ABCs in vaccination and autoimmunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ABCs	age-associated B cells
LCMV	lymphocytic choriomeningitis virus
LNs	lymph nodes
p.i.	post infection
i.p.	intraperitoneal
i.v.	intravenous

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Key points

Cytokines driving ABC emergence have stage-specific functions.

ABCs differentiate *de novo* in the liver.

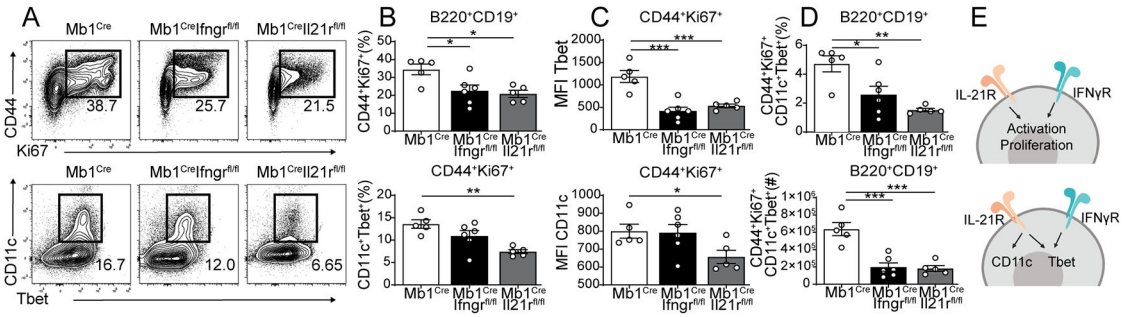

Figure 1. IFN-γ and IL-21 play distinct roles in driving genesis of ABCs.

(A,B) CD44 and Ki67 expression in $B220^+$ $CD19^+$ and CD11c and Tbet expression in $B220^+$ $CD19^+$ $CD44^{hi}$ $Ki67^+$ splenocytes in mice at day 10 p.i. with i.p. LCMV (A) with quantification (B). (C) Expression of Tbet and CD11c in $B220^+$ $CD19^+$ $CD44^{hi}$ $Ki67^+$ splenocytes. (D) Frequencies and numbers of splenic ABCs. (E) Model. Data representative of two independent experiments with 5 or 6 mice per group. Means ± SEM, one-way ANOVA.

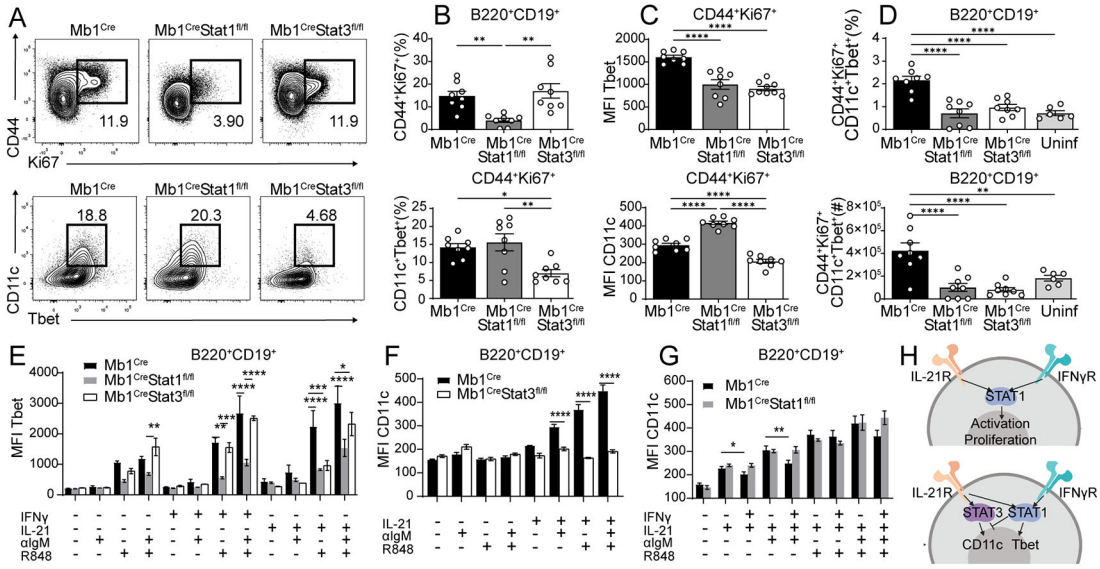

Figure 2. STAT1-STAT3 interactions in ABCs following viral infection.

(A,B) CD44 and Ki67 expression in B220^{+} CD19^{+} and CD11c and Tbet expression in B220^{+} CD19^{+} CD44^{hi} Ki67^{+} splenocytes in mice at day 10 p.i. with i.p. LCMV or uninfected *Mb1*^{Cre} mice (A) with quantification (B). (C) Expression of Tbet and CD11c in B220^{+} CD19^{+} CD44^{hi} Ki67^{+} splenocytes. (D) Frequencies and numbers of splenic ABCs. (E-G) Tbet and CD11c expression of purified cultured B cells. (H) Model. Data pooling two experiments representative of four independent experiments each with 2 to 6 mice per group(A-D) or representative of three experiments with 2 to 3 biological replicates (E-G). Means ± SEM, B-D: one-way ANOVA, E-G: two-way ANOVA.

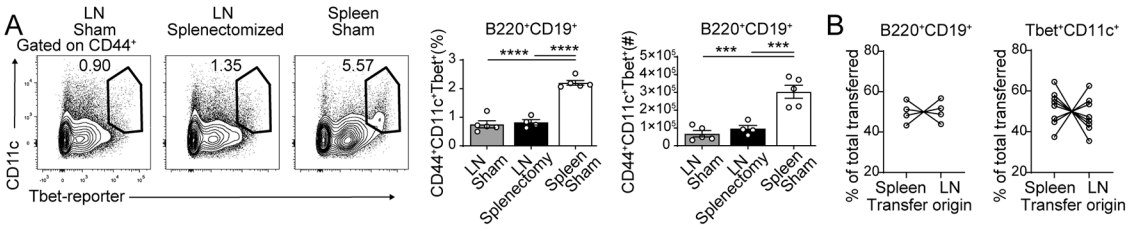

Figure 3. Tissue environments contribute to organ-specific generation of ABCs.

(A) CD11c and Tbet expression in B220^{+} CD19^{+} CD44^{hi} cells and quantifications of ABCs from LNs and spleens of *Tbet*-AmCyan reporter mice at day 10 p.i. with i.v. LCMV. (B) 5x10^{6} of CD45.1 purified splenic B cells and 5x10^{6} of CD45.2 purified LN B cells were transferred into CD45.1/CD45.2 MD4 recipient mice 24 hours prior to i.p. infection with LCMV. Percentage of each population among total transferred cells in total splenic B cells (left) and ABCs (right). Data representative of two independent experiments with 4 to 5 mice per group (A) or with 4 to 7 mice per group (B). Means ± SEM, one-way ANOVA.

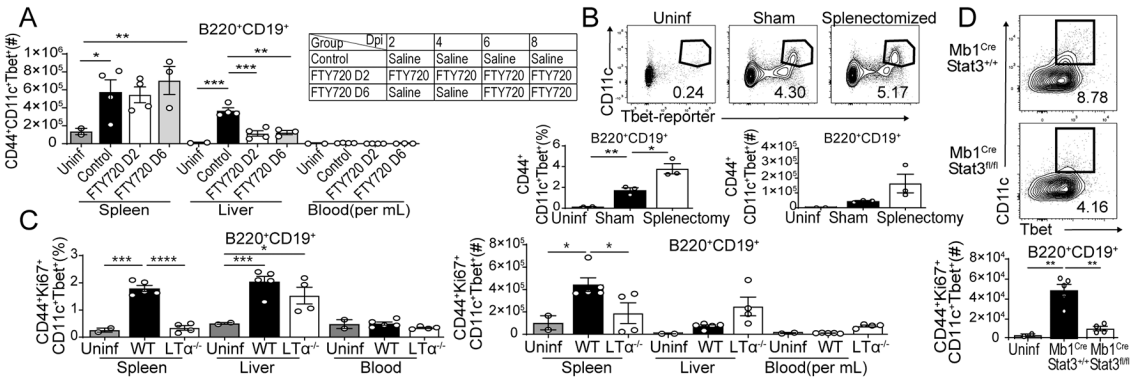

Figure 4. Hepatic ABCs arise from circulating B cells following viral infection.

(A) Saline and FTY720 administration to *Tbet*-AmCyan reporter mice following i.v. LCMV infection and Tbet^{+}CD11c^{+} B cell numbers at day 10 p.i. (B-D) CD11c and Tbet expression of liver B220^{+} CD19^{+} CD44^{hi} cells (top of B and D) and frequencies and numbers of hepatic ABCs (bottom of B and D). Data are representative of three independent experiments (A), or two independent experiments with 2 to 5 mice per group (B-D). Means ± SEM, one-way ANOVA.