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The malignant transformation potential of the oncogene STYK1/NOK at early lymphocyte development in transgenic mice

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ABSTRACT

B-cell Chronic Lymphocytic Leukemia (B-CLL) is a malignancy caused by the clonal expansion of mature B lymphocytes bearing a $CD5^{+}CD19^{+}$ (B1) phenotype. However, the origin of B-CLL remains controversial. We showed previously that STYK1/NOK transgenic mice develop a CLL-like disease. Using this model system in this study, we attempt to define the stage of CLL initiation. Here, we show that the phenotype of STYK1/NOK-induced B-CLL is heterogeneous. The expanded B1 lymphocyte pool was detected within peripheral lymphoid organs and was frequently associated with the expansions of memory B cells. Despite this immunophenotypic heterogeneity, suppression of B cell development at an early stage consistently occurred within the bone marrow (BM) of STYK1/NOK-tg mice. Overall, we suggest that enforced expression of STYK1/NOK in transgenic mice might significantly predispose BM hematopoietic stem cells (HSCs) towards the development of B-CLL.

1. Introduction

B-CLL is a heterogeneous disease that differs dramatically not only in its clinical severity but also in its mutational status and in its signaling events associated with the B cell receptor (BCR) [1,2]. B-CLL can be classified as mutated B-CLL (M-CLL) or unmutated B-CLL (U-CLL) according to the mutation status of immunoglobin heavy chain variable regions (IGHV) [3–5]. An M-CLL phenotype indicates that some clonal founder cells of CLL may, as with normal B lymphocytes, undergo somatic hypermutation of IGHV segments upon antigen encounter, either within or outside of the germinal center [3]. U-CLL can be regarded as expansions of leukemic clones that were repetitively stimulated by microbes or autoantigens. In the case of U-CLL, B cells typically are signaling-competent upon antigen ligation via their membrane (m) IgM [6] and express high levels of molecules typically associated with fatal outcome, including CD38 and/or ZAP-70) [7–11]. In contrast, IGHV germline mutations may eventually block BCR-mediated signaling cascades upon antigen engagement in M-CLL cells [6,12]. This signaling blockade might result either from the inability or high avidity of antigen recognition by their mutated BCRs. M-CLL cells that express low levels of CD38 and ZAP-70 are usually indolent [13].

STYK1/NOK (Serine/Threonine/Tyrosine Kinase 1/Novel Oncogene with Kinase-domain) was identified as an oncogene with high transformation potential in both cell culture systems and in transplantation experiments of immuno-deficient mice [14,15]. We recently showed that STYK1/NOK transgenic mice develop a leukemia resembling human B-CLL [16]. Using this model system, here we systemically examined the impacted stage of STYK1/NOK overexpression in STYK1/NOK transgenic mice with respective to B-CLL. We found that STYK1/NOK induced B-CLL is heterogeneous when detected with terminally differentiated B cell markers. STYK1/NOK-tg B lymphocytes show restricted and mutationally distinct V gene usage and had flawed CSR. Significant up-regulation of cell populations can be consistently found with early differentiation cell markers in BM. This study might provide an important clue for the further elucidation of B-CLL initiation in STYK1/NOK transgenic mice.

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Abbreviations:

B-CLL	B-cell Chronic Lymphocytic Leukemia
STYK1	Serine/Threonine/Tyrosine Kinase 1
NOK	Novel Oncogene with Kinase-domain
HSCs	Hematopoietic Stem Cells
CSR	Class Switch Recombination
BM	Bone Marrow
PMBCs	Peripheral Blood Mononuclear Cells
IGHV	Immunoglobin Heavy Chain Variable Region
LN	Lymph Node
ELISA	Enzyme Linked Immunosorbent Assay

2. Materials and methods

2.1. Generation and identification of STYK1/NOK transgenic mice

STYK1/NOK transgenic (tg) mice were generated with standard microinjection techniques as described previously [16]. Briefly, STYK1/NOK expression cassette was released from pcDNA3.0 -STYK1/NOK-HA [15] by *Pvu*I and *Sma*I double digestions, and gel-purified with a DNA gel extract kit (Vigorous, Beijing, China). About 25 microinjected oocytes were implanted into the oviduct of each pseudopregnant Kunming mouse. Kunming moouse is an outbred Swiss mouse line originally from India. Genomic PCR was conducted to identify the founder mice with two pairs of primers: 5′-tggcccgcctggcattatg cccagtacatg-3′(P1) and 5′-agccacaggatgaccccaagaaggatgagg-3′(P2) as well as 5′-tcctgaagtccctcctaccagcatcctaga-3’ (P3) and 5′-tcttcccaatcctccc ccttgctgtcctgc-3′(P4).

The animal experiments were conducted by strictly following the guidelines of the National Institute of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Both male and female transgenic mice were chosen for each analysis and we observed no differences that were sex related.

2.2. Antibodies

The following antibodies, each purchased from eBioscience, were used for flow cytometric analysis: Phycoerythrin (PE) anti-mouse c-Kit (CD117), PE anti-mouse CD5, Biotin anti-mouse Sca-1 (Ly-6A/E), Fluorescein isothiocyanate (FITC) anti-mouse CD19, FITC anti-mouse Sca-1 (Ly6A/E), Phycoerythrin-Cy7 (PE-Cy7) anti-mouse IgM, FITC anti-mouse CD23, PE anti-mouse CD27, Biotin anti-mouse IgM, Biotin anti-mouse IgD, Allophycocyanin-conjugated streptavidin, FITC streptavidin, PE streptavidin, PE anti-mouse CD43, PE-Cy7 anti-mouse CD45R (B220) and mouse hematopoietic lineage cocktails (anti-mouse CD3, anti-mouse B220, anti-mouse CD11b, anti-mouse TER-119 and anti-mouse Gr-1).

2.3. Hematoxylin-eosin staining and immunohistochemical analysis

After sacrifice, the major organs such as liver, spleen, lung, kidney and lymph nodes were isolated from WT hosts and STYK1/NOK-tg mice and analyzed for tumor cell infiltrations. The tissues were first fixed in 10% formalin, then dehydrated gradually in ethanol, followed by embedding in paraffin, and finally sectioned into 4 μm thickness. Glass slides containing the paraffin sections were deparaffinized in Xylene and rehydrated in diluted ethanol. After overstained with hematoxylin and destained in acidic alcohol, the sections were blued in bicarbonate and finally stained with eosin. Also, the paraffin-embedded specimens were sectioned and fixed on a glass slide for immunohistochemical analysis. The slides were first incubated with rabbit anti-STYK1/NOK antibody at a dilution of 1:200 at room temperature for 1 h. Subsequently, STYK1/NOK gene expression was detected with an immunohistochemistry (IHC) polymer detection kit (Zymed).

2.4. Immunoglobin heavy chain variable region (IgHV) analysis of B-CLL-like cells from STYK1/NOK-tg mice

Total RNA was extracted from aged STYK1/NOK-tg mice with enlarged lymph nodes in which B-CLL-like cells should be greatly enriched. One step RT-PCR was conducted using 1 μg total RNA as templates in combination with the following primers: (5′-MH1, 5′-SARGTNMAGCTGSAGSAGTC-3’; 5′-MH2, 5′-SARGTNMAGCTGSAGSAGTCWGG-3’; 5′-MH3,5′-CAGGTTACTCTGAAAGWGTSTG-3’; 5′-MH4, 5′-GAGGTCCARCTGCAA CARTC-3’; 5′-MH5, 5′-CAGGTC-CAACTVCAGCARCC-3’; 5′-MH6,5′-GAGGTGAASSTGGTGGAATC-3’; 5′-MH7,5′-ATGTGAACTTGGAAGTGTC-3′) and 3′ constant primers (3′-IgA, 5′-GATGGTGGGATTTCTCGCAGACTC-3’; 3′-IgE, 5′-TAAGGGGTA-GAGCTGAGGGTTCCT-3’; 3′-5′ACATTTGGGAAGGACTGACTCTC-3’; 3′-IgG1,5′-TAGACAGATGGGGGTGTCGTTTGGC-3’; 3′-IgG2A,5′-CTTGAC-CAGG CATCCTAGAGTCA-3’; 3′-IgG2B,5′-AGGGGCCAGTGGATA-GACTGATGG-3’; 3′-IgG3, 5′-AGGG ACCAAGGGATAGACAGATGG-3′). The reaction products were subcloned into pUC19 and then transformed into DH5α competent bacterial cells. After overnight incubation at 37 °C, 18 colonies were picked from ampicillin resistant LB agar plates and propagated. DNA sequencing results of the heavy chain regions were analyzed by pasting them into IMGT/V-Quest (www.imgt.org).

2.5. Enzyme linked immunosorbent assay (ELISA)

To evaluate antibody production, sheep red blood cells (SRBC) [17] were used as an antigen to immunize ten 8–12 week old wild type and ten age-matched STYK1/NOK-tg mice. Six animals from each group were selected for ELISA. For primary immunization, ~1 × 10^8 SRBC were injected intraperitoneally (i.p.) into each WT host and STYK1/NOK-tg mouse. Sera were taken from each immunized mouse at 1, 2, 3 and 4 weeks post-immunization. At the fourth week after primary immunization, mice were reboosted i.p. with a second dose of SRBC (~1 × 10^8). Sera were taken at one week after the second SRBC challenge.

The titers of immunoglobins of immunized STYK1/NOK-tg mice were determined by an alkaline phosphatase (AP)-based enzyme linked immunosorbent assay (ELISA) according to the manual instructions. To compare the primary humoral immune response in WT hosts and STYK1/NOK-tg mice, serum samples before and after one week SRBC immunization were evaluated for the productions of SRBC specific antibodies. The collected sera after primary and secondary immunizations were also quantified for the presence of SRBC-specific Ig isotypes.

2.6. Flow cytometric analysis

Isolated lymph nodes or spleens were minced in ice-cold 1xPBS. BM cells were harvested by flushing dissected femurs of STYK1/NOK-tg and control mice. Single cell suspensions were prepared by passing through a cell strainer. Peripheral blood mononuclear cells (PMBCs) were purified from the tail blood of WT hosts and STYK1/NOK-tg mice by using Lympholyte®-Mammal (Cedarlane). Fluorochrome-labeled antibodies were added to an 80 μl-staining buffer containing ~5 × 10^6 cells. The reaction mixture was then incubated at 4 °C for 30 min, washed three times with staining buffer, and re-suspended into 150 μL of OptiLyse®C (Beckman Coulter). For Biotin-conjugated antibody labeling, Allophycocyanin-conjugated streptavidin was added to the reaction mixture. All reaction products were subsequently detected by flow analysis on a FACSAria™ flow cytometer (BD Biosciences). The cells were first gated by FSC-H/SSC-H, the gated cell populations were subjected to the analysis by respective channels according to the fluorescence being labeled. The enrichment of lineage negative cells from mouse bone marrow was conducted using a mouse hematopoietic cell lineage depletion kit (R&D Systems) according to the manual

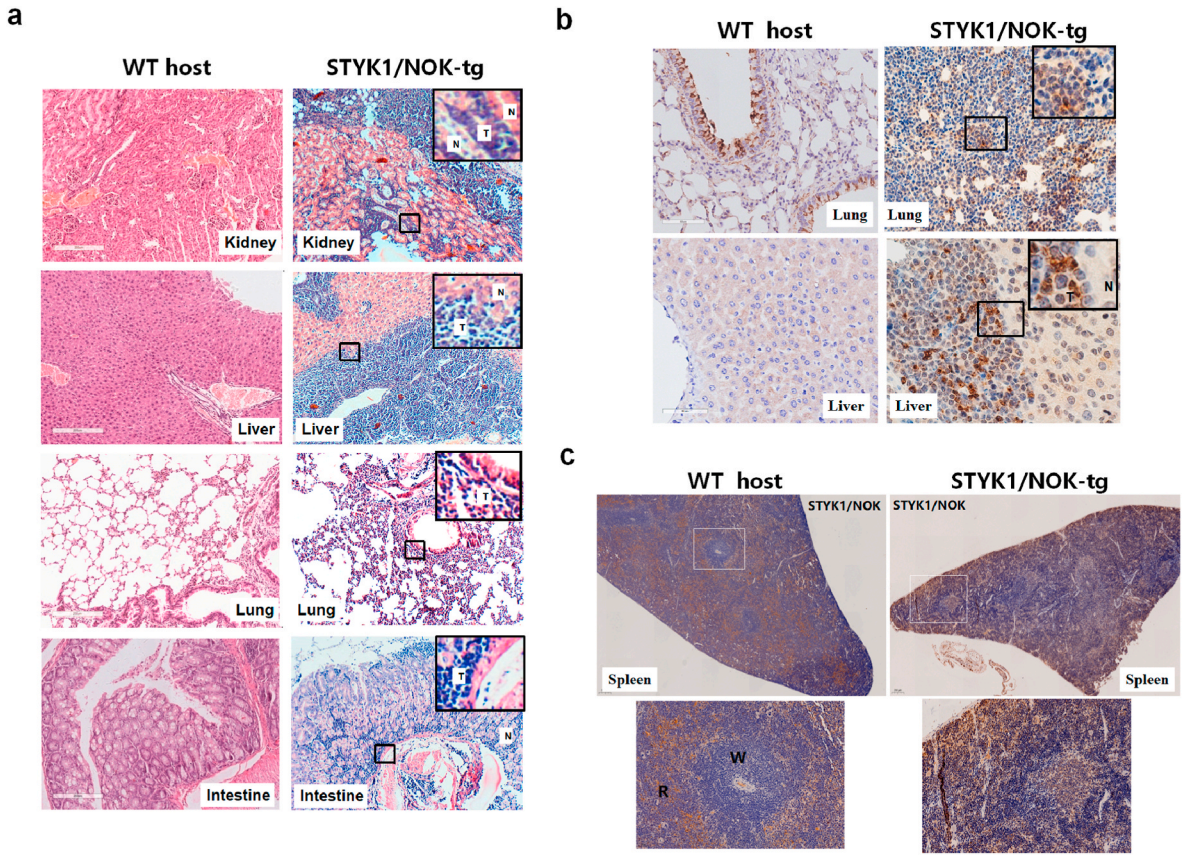

Fig. 1. Pathological analysis of major tissues of STYK1/NOK transgenic mice. (a) Representative Hematoxylin & Eosin (HE) staining. H&E staining was conducted on sections of kidneys, lungs, livers, and small intestines prepared from both WT controls and STYK1/NOK transgenic mice. (b) Representative immunohistochemical (IHC) staining of tissue sections prepared from lungs and livers of both WT hosts and STYK1/NOK-tg mice (n = 3). The enlarged window on the selected field was inserted into the right corner of each photo for the STYK1/NOK transgenic mouse. T, tumor and N, normal. (c) IHC of spleen sections from WT (n = 3) and STYK1/NOK-tg (n = 4) mice (magnification × 14). White squares indicate the enlarged regions. W, white pulp; R, red pulp. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

instructions.

2.7. Statistical analysis

Data were presented as mean ± standard error mean (SEM) from at least 3 samples and subjected to two-tailed, unpaired student's *t*-test. A p value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Histological analysis of selected tissues/compartments of STYK1/NOK-tg mice

Our previous report has shown that STYK1/NOK can induce B-CLL like disease in transgenic mice. However, how organs and tissues affected by this genetic alteration are currently unknown. Here, we employed a variety of techniques, including anatomical analysis, hematoxylin and eosin (H&E) staining and immunohistochemistry analysis to approach this issue. First, anatomical analysis showed frequent enlargements of major organs such as liver, spleen, kidney and lung (Suppl Table 1). H&E staining of tissue sections revealed amassed infiltrations of tumor cells with the appearance of mature B lymphocytes in several major organs, including kidney, lung, liver and intestine in STYK1/NOK-tg mice (Fig. 1a). Moreover, immunohistochemical analysis further demonstrated the enhanced expressions of STYK1/NOK proteins in the leukemic cell-infiltrated tissues, such as lung and liver (Fig. 1b). Immunohistochemical analysis of the enlarged spleen sections revealed the diffuse expansion of the STYK1/NOK^{+} neoplastic lymphocytes obliterated the normal framework of white pulp and red pulp (Fig. 1c).

Some of the aged STYK1/NOK-tg mice (typically over 6 month old) spontaneously developed lymphadenectasis, as evidenced by the visual enlargements of lymph nodes of the neck, axillary, or inguinal regions. These animals often had symptoms that resembled human B-CLL such as weight loss, fatigue, sweating, pruritus and skin nodules.

Whole blood tests revealed that the majority of STYK1/NOK-tg mice present disease phenotypes at or above 6 months of age with total numbers of lymphocytes reaching greater than $\sim 9 \times 10^9$ cells/L. This increase almost doubled levels observed in WT host control ($\sim 5 \times 10^9$ cells/L) (Suppl Fig. 1). However, on occasion, blood tests revealed that CLL-like disease features could onset as early as 3.5 months post birth in STYK1/NOK-tg mice (Suppl Fig. 1).

3.2. Evaluation of antibody production in STYK1/NOK-tg mice

Next, we evaluated the effect of STYK1/NOK on production of B lymphocytes by measuring immunoglobin (Ig) productions by standard ELISA. With the exception of T-independent isotypes (IgM and IgG3), the overall levels of serum IgG1, IgG2a, IgG2b, IgA, κ and λ were markedly elevated in STYK1/NOK-tg mice relative to WT host controls (Fig. 2a). These results indicated that STYK1/NOK-induced B leukemic cells overproduced not only the Ig light chain isotypes but also H chain isotypes. This suggested that flawed Ig class switch recombination (CSR) had occurred.

To assess antigen-specific antibody production, sera were withdrawn from STYK1/NOK-tg and WT host mice immediately prior to or after one-week challenge with sheep red blood cells (SRBC)—a typical T-

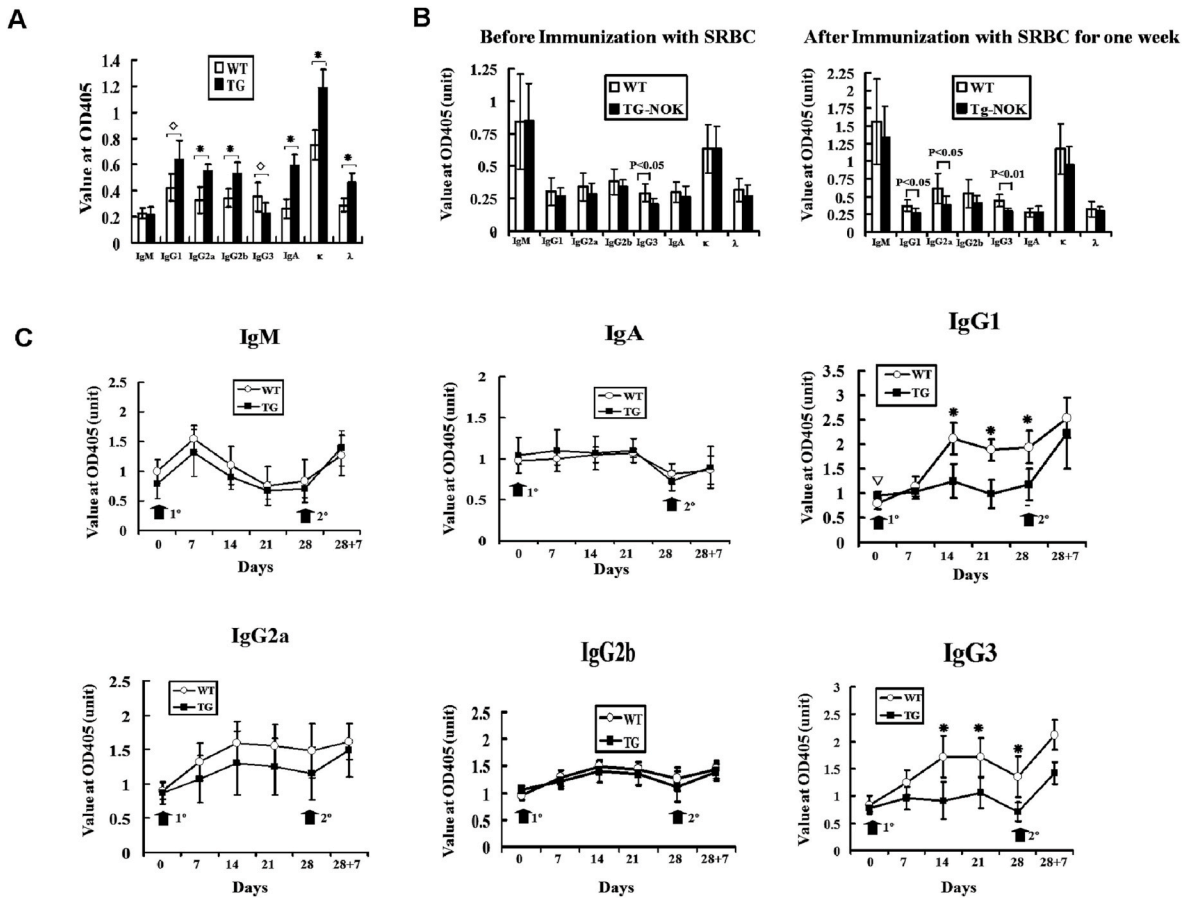

Fig. 2. Analysis of immunoglobin production and antigen specific humoral responses. (a) The relative serum levels of immunoglobins and their switch variants were analyzed with ELISA tests. Sera were collected from STYK1/NOK-tg (n = 6) and age-matched control mice (n = 6) and diluted 1:1000. A 96-well ELISA plate was first coated with the fixed amounts of goat anti-mouse Ig capture antibodies. The diluted sera were then added into each well. The reaction mixtures were assayed with a standard ELISA kit. The results were recorded with a spectrometer at OD405. Open diamonds, $p < 0.05$; black stars, $p < 0.01$. (b) ELISA analysis to determine the relative levels of SRBC-specific immunoglobins and their switched variants before and after one-week of SRBC immunization. ELISA details and numbers of mice were as described in Panel A. (c) Evaluation of the production of antigen-specific Ig isotypes and isotype switch variants following primary (1°) and secondary (2°) immunization with SRBCs. Sera were collected from STYK1/NOK-tg (n = 6) and age-matched WT control mice (n = 6) at each time point and diluted 1:1000 with PBS. The 96-well plate was coated with SRBCs before the addition of the diluted serum. The reaction mixtures were assayed with a standard ELISA kit. Solid stars indicates $p < 0.01$.

dependent response. SRBC-specific IgG1, IgG2a and IgG3 responses were significantly reduced as compared with those of WT mice (Fig. 2b). The production of SRBC-specific IgG1 and IgG3 were also markedly decreased after primary immunization (Fig. 2c). There were no statistically significant differences observed for the primary anti-SRBC production of IgM, IgA, IgG2a or IgG2b (Fig. 2c). However, after a second boost, memory B cell activities in STYK1/NOK-tg mice were markedly elevated to levels comparable to that of the WT littermates (Fig. 2c).

3.3. STYK1/NOK-tg mice display a strong immunologic memory response

The above results indicated that humoral immune responses were suppressed in STYK1/NOK-tg mice. They also indicated that STYK1/NOK-induced B leukemic cells might retain strong memory B cell characteristics.

With respect to this latter phenotype and in addition to the clonal expansion of B1 lymphocytes, human B-CLL patients usually express low levels of surface IgM and IgD as well as antigen-experienced memory B lymphocytes [18,19]. CD27 is a defined cell surface marker for memory B lymphocyte [20,21]. Based on the differential expression of IgM, IgD and CD27, memory B cells can be divided into four groups: $IgM^+IgD^+CD27^+$, $IgM^-IgD^-CD27^+$, $IgM^-IgD^+CD27^+$ and $IgM^+IgD^-CD27^+$ [21]. CD23, an activation B cell marker, has been shown to be up-regulated in human B-CLL [9,22].

To examine the level of memory B cells in STYK1/NOK-tg mice, four-color flow cytometric analysis of peripheral blood and lymphoid tissues was conducted by employing PE-Cy7 anti-IgM, Biotin anti-IgD, PE anti-CD27 and FITC anti-CD23. Representative results (Suppl Fig. 2) revealed significant differences of memory B cell accumulation within diverse cellular compartments of STYK1/NOK-tg mouse. For example, in blood and spleen, the relative proportions of IgM^+IgD^+ B lymphocytes were markedly decreased (Suppl Figs. 2a and 2b), whereas no differences within memory B cells were observed in other compartments. In contrast, an increase in IgM^+IgD^+ B lymphocytes was detected in the lymph nodes of the same STYK1/NOK-tg mice. This corresponded with an enhancement of $IgM^+IgD^+CD27^+CD23^+$ memory B cells in that compartment (Suppl Fig. 2c).

The data indicate that over-expression of STYK1/NOK is able to activate both CD27 and CD23 memory B cell markers in transgenic mice. Collectively, the above results further indicate that the memory B cell immunophenotypes of STYK1/NOK-tg mice resemble those of human B-CLL.

Table 1
The oligoclones of IgH chains from the potential B-CLL cells of STYK1/NOK-tg mouse.

Sample No.	IMGT VH sequence	VH family	% homology to GL	N1	DH name	N2	JH name	DH RF	CDR3	CDR3 length	PI
4250–3	VH1-64	VHJ558	96.88	gaggggga	D1-1	ttc	J2	2	SREGDYYYGSSSYFDC	16	4.15
4250–4	VH5-12	VH5	98.98	–	D2-4	cccc	J4	3	ARHDSPYAMDY	11	5.61
4250–7	VH1-64	VHJ558	99.65	gaggccgt	D1-1	c	J3	3	AREAVYYGSPWFAY	14	6.14
4250–17	VH5-12	VH5	98.26	ggag	D1-1	cc	J1	1	ARRSSTTVVAPYWYFDV	17	8.22
4250–18	VH1-82	VHJ558	96.88	ggcc	D1-1	aact	J2	3	AAYYYGRRTYYFDY	14	8.17

IMGT stands for international ImMunoGeneTics information system with web address www.imgt.org. VH, IgH chain variable region; GL, Germline; N, nucleotides; —, not applicable; DH, Ig H chain D region; RF, reading frame; CDR3, third complementarity determining region; pI, isoelectric point.

3.4. STYK1/NOK-tg B lymphocytes show restricted and mutationally distinct V gene usage

To determine the mutated and/or unmutated germline IGHV genotypes, we analyzed the status of IGHV usage in STYK1/NOK-tg mice. Total RNAs were extracted from the enlarged lymph nodes of elder STYK1/NOK-tg mice suffering severe lymphadenectasis of the neck. The majority of cells present in the enlarged lymph nodes were considered to be clonally expanded B-CLL cells.

Using appropriate primers (Methods), we amplified IGHV regions with RT-PCR and subsequently sequenced the RT-PCR products. By comparing the derived sequences with those in the IMGT database, we identified five IGHV clones that likely derived from clonal B-CLL lymphocytes. As shown in Table 1, the CDR3 lengths, that are normally 11.5 amino acids on average [23], were abnormally long in STYK1/NOK-tg lymphocytes; ie, lengths of 11, 16, and 17 amino acids in one representative clone and 14 amino acids in two other clones (Table 1).

VH sequence analyses (Table 1) further revealed that three clones (#3, #7 and #18) belonged to the VHJ588 family, while the other two (#4 and #17) were members of the VH5 family. Two (#4 and #17) VH5

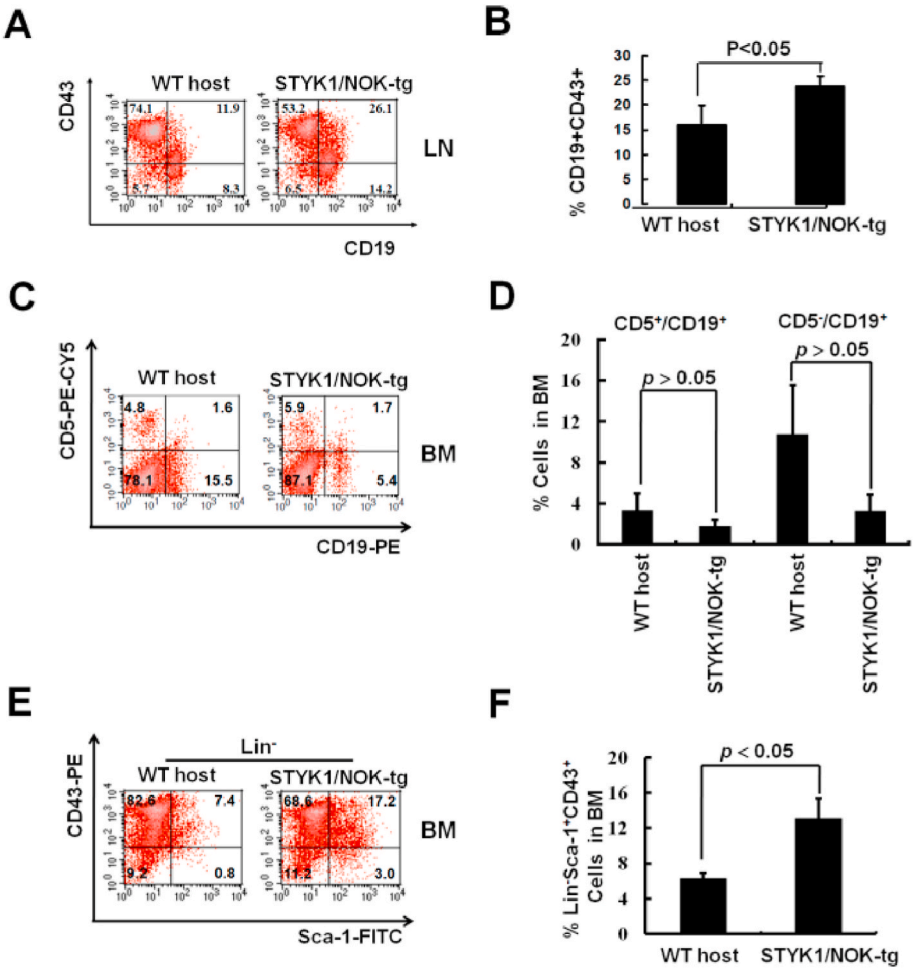

Fig. 3. Evaluation of the phenotypic differences of lymph nodes and BMs of WT hosts versus STYK1/NOK-tg mice. (a) Two color flow cytometric analysis of lymph nodes of WT hosts and STYK1/NOK-tg mice labeled with PE anti-CD43 and FITC anti-CD19. (b) Quantitation of the $CD43^+/CD19^+$ lymph node cells from WT host and STYK1/NOK-tg mice. Result are shown as means plus standard error of mean (SEM) from three independent tests. (c) Two color analysis of BM from WT hosts and STYK1/NOK-tg mice as subjected to PE anti-CD19 and PE-Cy7 anti CD5 double staining. (d) Quantitation of $CD19^+$ ($CD5^-CD19^+$ and $CD5^+CD19^+$) cell populations. Statistical analysis was performed using student *t*-test. (e) Two color analysis of lineage-depleted BM cells of WT and STYK1/NOK-tg mice stained with PE anti-CD43 and FITC anti-Sca-1. Each plot is representative of at least 3 independent assays that produced similar results. (f) Quantitation of the $Lin^-Sca\text{-}1^+CD43^+$ population in WT hosts versus STYK1/NOK-tg mice. Statistical analysis was performed with student *t*-test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

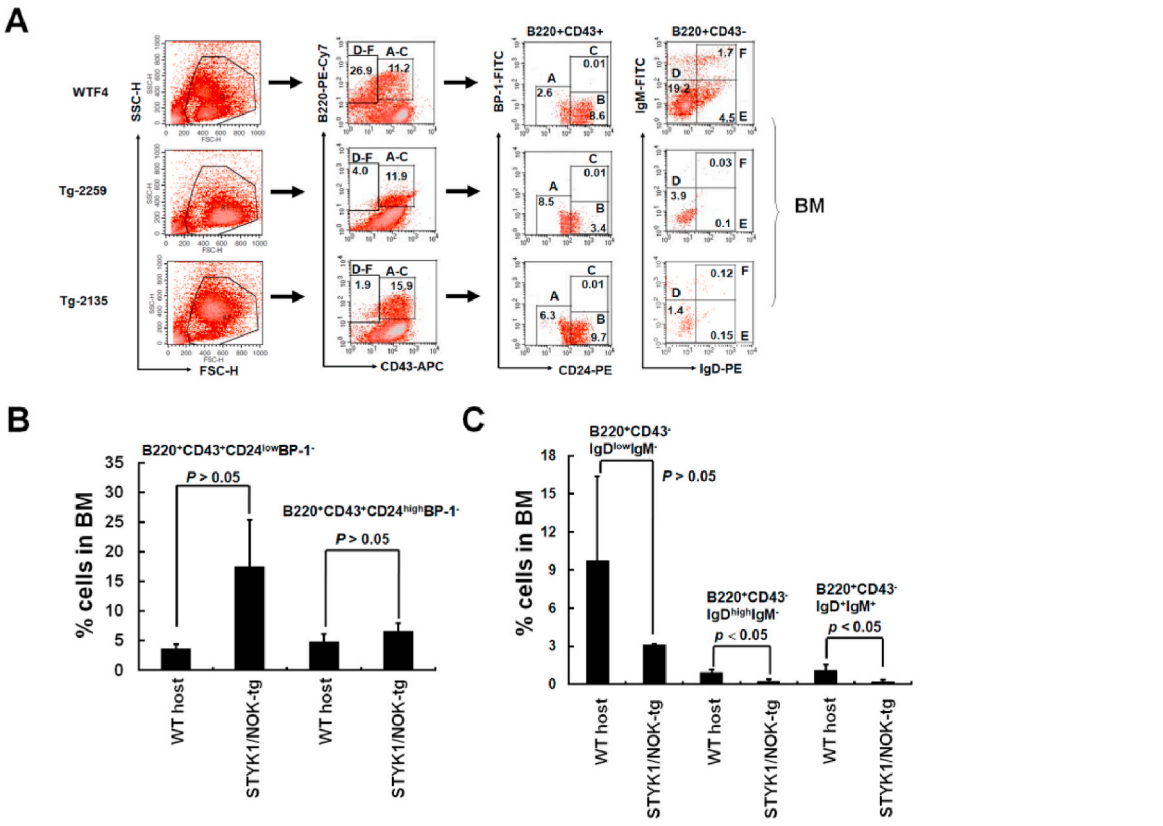

Fig. 4. Four color cytometric analysis shows divergence within early B lineage BM of WT host and STYK1/NOK-tg mice. (a) BM from WT hosts and STYK1/NOK-tg mice were stained with PE-Cy7 anti-B220 and biotin anti-CD43 in combination with either FITC anti-BP-1 and PE anti-CD24 or FITC anti-IgM and PE anti-IgD. Results from each of the 6 "Hardy" fractions (Fr. A to F) are indicated. For biotin-conjugated antibody labeling, APC-conjugated streptavidin was added to the final reaction mixture. (b) Quantitative analysis of $CD24^{+}$/BP-1^{-}populations (Hardy Fr. A and B). Statistical analysis was performed using student *t*-test. (C) Quantitative analysis of cell populations doubly stained with PE anti IgD and FITC anti IgM in Hardy Fr. D, E and F, respectively. Statistical analysis was performed using student t tests. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

sequences and one (#7) VHJ588 sequence showed less than 2 % differences from their germline genes deposited in the IMGT/V-Quest database—a result previously observed in human CLL ([24]). This indicated that these three clones can be considered unmutated (U-CLL) B-CLL. However, with the characteristic of M-CLL, two J558-expressing clones (#3 and #18) manifested extensive somatic hypermutation, with a 3.12 % difference from WT Vh-J558. This also was observed in B-CLL, which were reported as the first examples of M-CLL ([25]).

In addition, a biased usage of DH, but not JH, segments were observed (Table 1). All five CDR3 have high contents of tyrosine and serine, ranging from 3 to 8 residues. The isoelectric points (pIs) for the five proteins ranged from acidic (#3, #4 and #7) to basic (#17 and #18).

Overall, the results showed that B-CLL cells from STYK1/NOK-tg mice had oligoclonal potential, indicating that the origin of B-CLL might come from a transformation event initiated at a primitive stage before lymphocyte commitment in STYK1/NOK-tg mice.

3.5. *Immunophenotypic heterogeneity of STYK1/NOK-induced B-CLL in peripheral lymphoid organs*

As observed in human B-CLL [26–28], the symptoms and the immunophenotypes of STYK1/NOK-induced B-CLL varied markedly, indicating that the disease progression in STYK1/NOK-tg mice might be a dynamic event. Consistent with previous observations [29,30], increased populations of $IgD^{+}IgM^{+}$ B lymphocytes were often detected within enlarged lymph nodes of STYK1/NOK-tg (Suppl Fig. 3). In contrast, no enhancement of $IgD^{+}IgM^{+}$ naive B lymphocytes were observed in peripheral blood or in BM compartments (Suppl Fig. 3). However, the peripheral blood population of $IgD^{-}IgM^{+}$ mature B lymphocytes were markedly expanded (Suppl Fig. 3). This elevated $IgD^{-}IgM^{+}$ population was also found in STYK1/NOK-tg spleens, but $IgD^{+}IgM^{+}$ naive B lymphocytes were not always increased in this compartment (Suppl Fig. 3).

To further analyze the immunophenotypic diversities of STYK1/NOK-tg mice, additional cell surface markers were chosen. For example, analysis of IgM and CD5 co-expression indicated that the $IgM^{+}CD5^{+}$ population was most often expanded in the lymph nodes, yet only slightly increased within BM, but not always in spleen (Suppl Fig. 4a).

In agreement with earlier reports [31,32], expanded B1 and reduced $IgD^{+}IgM^{+}$ populations also were detected within lymph nodes and BM, respectively (Suppl Figs. 4b and 4c). We then employed CD24 and BP-1 surface markers to estimate the proportion of immature B lymphocytes in BM as previously described [33,34]. Direct correlation was observed neither in immature (BP-$1^{-}CD24^{+}$) B cells in the BM, nor in $IgD^{+}IgM^{+}$ (follicular) or B-1 cells in peripheral lymphoid organs (Suppl fig. 4a, 4b and 4d).

These results indicated that alterations in the immunophenotypes of B lymphocytes might occur dynamically during disease progressions in STYK1/NOK-tg mice.

3.6. *Suppression of normal B lymphocyte lymphopoesis in the BM of STYK1/NOK-tg mice*

Analysis of CD43 and CD19 expression in lymph nodes revealed that immature $CD43^{+}CD19^{+}$ B lymphocytes were markedly expanded within STYK1/NOK-tg lymph nodes (Fig. 3a & b). This indicated that lineage

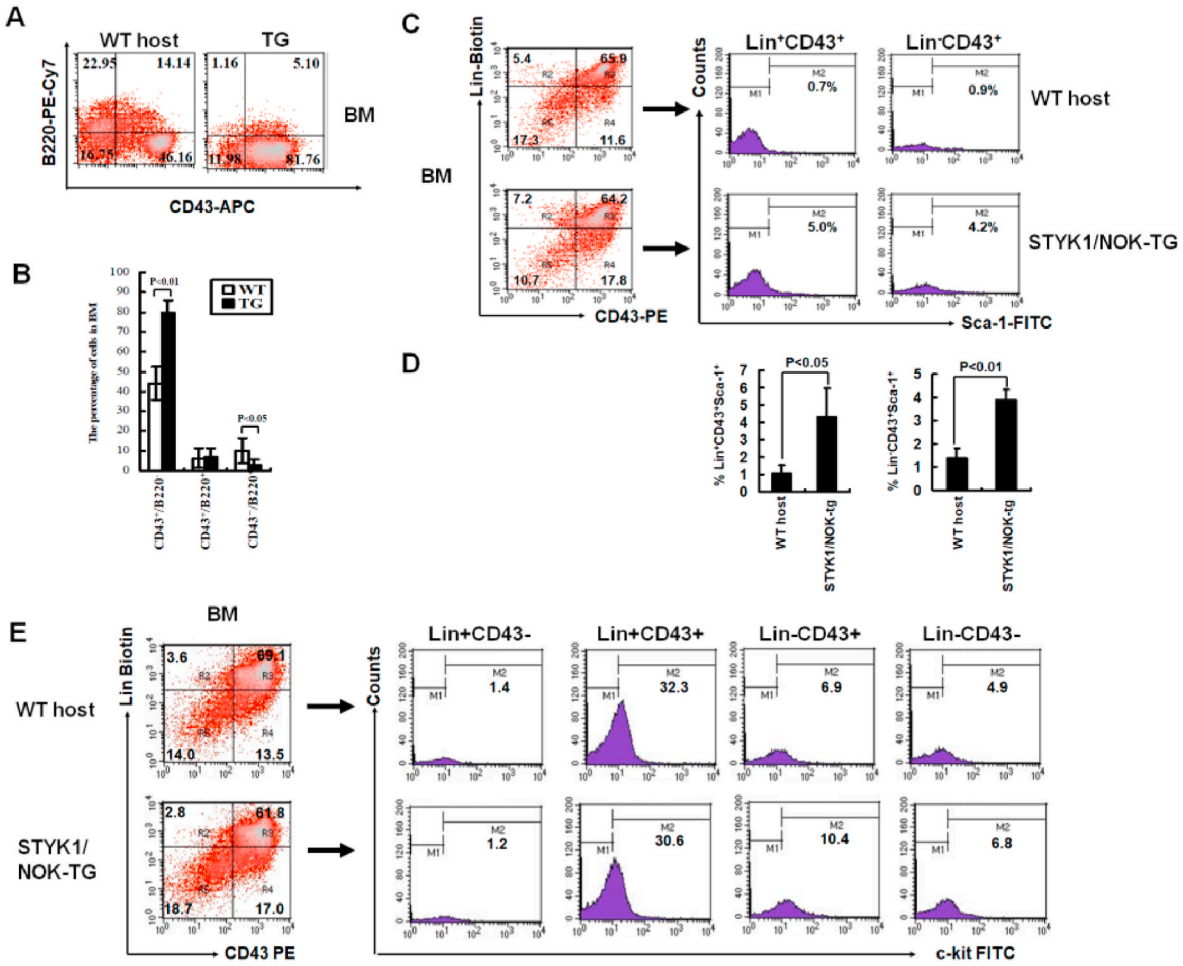

Fig. 5. Identification of the expanded population of Hematopoietic Stem Cells (HSC) in STYK1/NOK-tg mice. (a) Two color flow cytometric analysis of BM from WT hosts and STYK1/NOK-tg mice labeled with PE-Cy7 anti-B220 and Biotin anti-CD43 antibodies. For biotin-conjugated antibody labeling, Allophycocyanin-conjugated streptavidin was added to the reaction mixtures prior to their analysis by flow cytometry. (b) Quantitation of B220 and CD43 expression in BM cells from 9 STYK1/NOK-tg (solid bars) and 9 WT controls (opened bars) was performed. Statistical analyses were conducted using student's t tests. (c) Three color cytometric analysis of BM cells performed using Biotinylated anti-lineage (Lin), PE anti-CD43 and FITC anti-Sca-1 antibodies. The reaction mixtures were first assayed for the expression of lineage markers and CD43. Then the gated populations were selected for further analysis of Sca-1 expression. (d) Quantitation of the $Lin^{+}CD43^{+}Scal\text{-}1^{+}$ and $Lin^{-}CD43^{+}Scal\text{-}1^{+}$ subpopulations of BM as described in c. Results are provided as means plus standard error of means for 3 independent tests. The data were representative of at least 2 independent experiments with similar results. (e) Assessment of c-kit^{+} populations in BM of STYK1/NOK-tg and age-matched WT hosts. Single color cytometric analysis was conducted by labeling BM cells with FITC anti-*c*-kit antibodies. Then, the reaction mixtures were gated for the analysis of c-kit expression. Quantitation was performed using eight STYK1/NOK-tg and six WT host mice and subjected to statistical analyses with student's t tests. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

development of B lymphocytes might be affected in the BM of STYK1/NOK-tg.

To check B cell lymphopoesis directly, we performed CD5 and CD19 double staining in BM samples from both WT hosts and STYK1/NOK-tg. Although no statistical differences were observed in $CD5^{+}CD19^{+}$ subsets, a decrease in $CD5^{-}CD19^{+}$ cell populations was readily evident in STYK1/NOK-tg relative to WT hosts (Fig. 3c & d). However, after depletion of the lineage positive cells from the BM, the $Lin^{-}CD43^{+}Sca\text{-}1^{+}$ subpopulation was markedly expanded in STYK1/NOK-tg mice (Fig. 3e & f).

To further address whether there was a maturational defect during B lymphocyte development in STYK1/NOK-tg mice, the six fractions of B cell-committed stages within BM, as defined by Hardy et al. [35,36], were analyzed. We employed four color cytometric analyses including antibodies specific for B220, CD43, BP-1 and CD24 (or IgD and IgM) to measure potential variations within each of the six Hardy fractions.

As shown in Fig. 4a and b, increases within the most immature (pre-Pro-B) fraction A were often observed in STYK1/NOK-tg mice. Also, maturational defects were detected in downstream lineages (fractions D-F) (Fig. 4c). Regardless of lymphadenectasis, STYK1/NOK-tg mice consistently accumulated $B220^{-}CD43^{+}$ cells within their BMs (Fig. 5a and b).

These results strongly suggest that, in BM, STYK1/NOK overexpression might bias B cell differentiation at the pre-Pro stage. This would result in the accumulation of STYK1/NOK transformed $B220^{-}CD43^{+}$ progenitor cells which might be accountable for the phenotypes detected in peripheral lymphoid organs.

3.7. The impact of STYK1/NOK on hematopoietic stem cell (HSC) progenitors in STYK1/NOK transgenic mice

To determine whether STYK1/NOK directly impacts HSC development, BMs were analyzed for the expression of both B220 and CD43 as well as for the defining HSC markers, Sca-1 and c-kit. Three color reagents specific for lineage (Lin) markers, CD43 and Sca-1 were employed to examine the population levels of HSC in WT hosts versus STYK1/NOK-tg mice. As shown in Fig. 5c and d, significant increases in both $Lin^{+}CD43^{+}Sca\text{-}1^{+}$ and $Lin^{-}CD43^{+}Sca\text{-}1^{+}$ subpopulations were

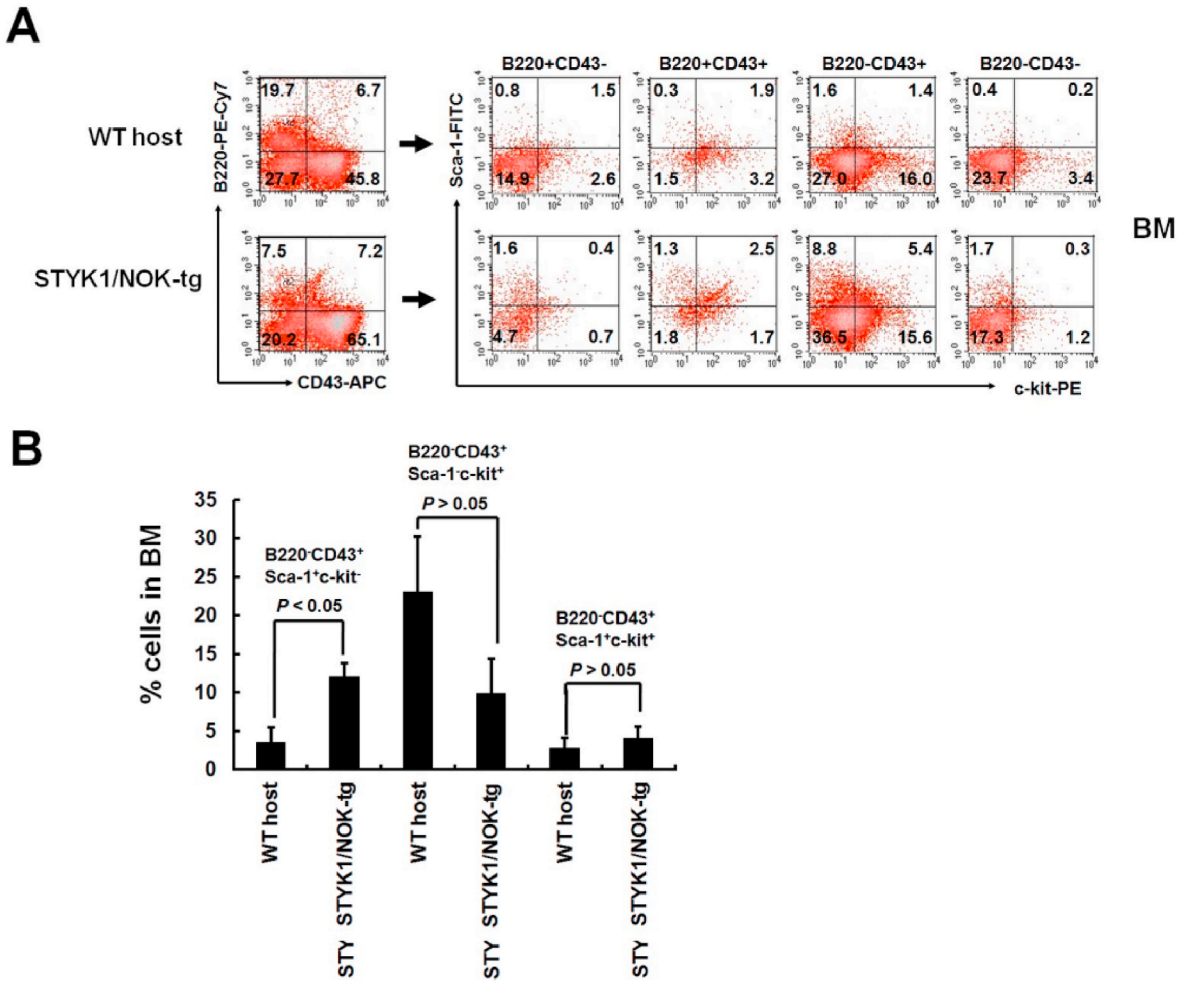

Fig. 6. Assessment of Sca-1 and c-kit cell populations within the BM of WT host and STYK1/NOK-g mice. (a) Four color cytometric analysis of BM cells from WT host and STYK1/NOK-tg mice was performed using PE-Cy7 anti-B220, biotin anti-CD43, PE anti-*c*-Kit and FITC anti-Sca-1. The BM cells were first analyzed with PE-Cy7 anti-B220 and biotinylated-CD43. Then, the gated cells were displayed as log plots of FITC anti-Sca-1 versus PE anti-*c*-Kit. For biotin-conjugated antibody labeling, APC-conjugated streptavidin was added to the reaction mixture last. (b) Quantitation of different subpopulations of $B220^{-}CD43^{+}$ cells in the BM of WT and STYK1/NOK-tg mice. Statistical analyses provided by student's t tests. Results are representation of at least three independent tests. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

observed in STYK1/NOK-tg mice. Unexpectedly, c-kit was significantly down-regulated in STYK1/NOK-tg mice (Fig. 5e). Consistently, four color staining revealed expansions of $B220^{-}CD43^{+}Sca\text{-}1^{+}c\text{-}kit^{-}$ subpopulations in BM of STYK1/NOK-tg mice (Fig. 6).

4. Discussion

A review by Garcia-Munoz et al. [37] highlighted ten important "clues" as to the origin of B-CLL. However, which of them is the most critical determinant is currently unknown. B-CLL was thought to be a type of "blood cancer" produced by the clonal expansion of mature B lymphocytes. This suggested that B-CLL could derive from the flawed development of follicular mantle B cells. However this hypothesis has been challenged by the observation that ~50 % of B-CLL patients carry IGHV mutations [38], whereas follicular mantle B cells have virtually no IGHV mutations [39]. Also it was proposed that B-CLL cells were derived from marginal zone (MZ) B cells, as evidenced by the surface IgM^{high}-IgD^{low} phenotype shared by both B-CLL and MZ B cells [40]. However, this assumption also was challenged by the fact that MZ B cells rarely express CD5 and/or CD23. In addition, B-CLL cells were proposed to be antigen-experienced, memory B cells due to their expression of memory B cell surface markers such as CD23 and CD27 [9]. This conclusion contrasts with recent evidence demonstrating that B-CLL can be driven by antigen-independent, autonomous signaling [41].

The studies mentioned above have not excluded the possibility that receptor restriction in B-CLL might represent a "marked" event occurring prior to the exit of the transformed progenitor cells from bone marrow under certain circumstances. By analyzing IGHV usage from the enlarged lymph nodes of STYK1/NOK-tg, we observed that STYK1/NOK might induce an oligoclonal rather than a monoclonal expansion of malignant B lymphocytes, and that leads to skewed VH1 and VH5 usage (Table 1). In agreement with several earlier studies, Crossen et al. [42] uncovered three distinct clones from a female B-CLL patient, indicating a potential oligoclonal character of B-CLL. A more detailed cohort study by Sanchez et al. [43] demonstrated that oligoclones could be identified in about 14 % of atypical B-CLL. Indeed, the presence of oligoclonal B lymphocytes was found even more frequently in monoclonal B lymphocytosis (MBL), a disease believed in most instance to be an early stage of B-CLL [44,45]. More recently it has been demonstrated that a patient's HSC could independently mature into either monoclonal or oligoclonal B-CLL [46].

According to the cancer stem cell hypothesis, only a minor portion of cancer cells with stem cell characteristics is capable of undergoing self-renewal [47]. The current study indicates that STYK1/NOK might promote the expansions of a small fraction of primitive stem cells within the BM. These primitive stem cells may endow them with self-renewal and

accordingly, skewed differentiation toward B-CLL. It was previously demonstrated that up-regulation of Sca-1 is associated with many types of malignancies and plays a positive role in the renewal of cancer stem cells [48–50]. Alternatively, Sca-1 can also function as a negative regulator by blocking T cell proliferation [51]. In contrast, deleting Sca-1 by gene targeting in transgenic mice produced a state of T cell "hyper-proliferation" [52]. Furthermore, mistimed expression of Sca-1 in immature T cells typically results in the impairment of T cell differentiation [53].

Thus, up-regulation of Sca-1 by STYK1/NOK in primitive stem cells might be a critical event for B-CLL development. C-Kit has been demonstrated to be a critical determinant for HSC development, for HSC homing within the bone marrow 'niche' and for normal lymphopoesis [54,55]. We suggest that absence of c-Kit in STYK1/NOK-induced cancer stem cells might be important for the creation of a suitable microenvironment for their abnormal self-renewal and differentiation. The expression of CD43 also has been showed to be a key factor associated with human B-CLL [56,57]. Our results indicated that up-regulation of CD43 could be the key feature of STYK1/NOK-induced B-CLL. Consistent with that hypothesis, we detected $CD43^{+}$cells in virtually all lymphoid tissues, from central lymphoid bone marrow to peripheral lymph nodes in STYK1/NOK-tg mice. CD43 expression on B cell surfaces can promote B cell proliferation and extend B cell survival [58]. Thus, the increased $CD43^{+}$ cell populations may propagate the severity of STYK1/NOK transgenic-induced malignancy.

Although our results suggest that the enforced expression of STYK1/NOK in STYK1/NOK-tg prompts the initiation of B-CLL during HSC developments, the limitations of the current study may also be apparent. The proliferation and differentiation of mouse HSC from STYK1/NOK-tg could further be characterized with multiple functional assays. These include transplantation assay, long-term culture initiation, and colony-forming unit assays. In addition, it would be informative to carry out rescue assays by knocking-down STYK1NOK expression in STYK1/NOK-tg and/or in leukemia cell in transplanted immunodeficient mice. Such future approaches will extend our understanding of the functions of the STYK1NOK oncogene *in vivo*. Moreover, a systematical study on the development of immune organs and status of various immune cell populations of STYK1/NOK-tg mice will provide great insights on our understanding about how genetic alternations by STYK1NOK can cause B-CLL.

Finally, our data suggest that STYK1/NOK induced B-CLL-like disease in transgenic mice might induce oligo-clonal expansion of B1 cells. They further suggest that STYK1/NOK most likely targets HSC within the bone marrow to initiate B-CLL.

Ethics approval

The ethics approval for the animal study was issued by the Institutional Review Board of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (the approval No. ACUC-A01-2017-011).

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Data availability

The data that has been used is confidential.

CRediT authorship contribution statement

Yin Yang: Resources, Methodology, Investigation, Funding acquisition, Data curation. **Li Liu:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Haley O. Tucker:** Writing – review & editing, Validation, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101709.

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