BMC Immunology

RESEARCH

Open Access

In vitro differentiation of common lymphoid progenitor cells into B cell using stromal cell free culture system

Pan Yang[1†], Xiaoling Chen[2†], Hao Wen[2], Meiling Yu[2], Haili Yu[3], Li Wang[2*], Liang Gong[1*] and Lintao Zhao[1*]

Abstract

The OP9 culture system is an important in vitro model for B cell development. However, the complex nature of operations and the intrinsic variability of stromal cell functionality, which can be influenced by factors such as radiation exposure or contamination, pose considerable challenges to their wider application. Currently, there exists a paucity of studies documenting in vitro B cell differentiation culture systems that exclude stromal cells, and the experimental methodologies available for reference remain limited. This report elucidates a robust stromal cell-free culture system. Specifically, bovine serum albumin (BSA) or fetal bovine serum (FBS), in conjunction with interleukin-7 (IL-7), Flt3 ligand (Flt3L), and stem cell factor (SCF), were incorporated into X-VIVO15 medium. This system proficiently facilitates the directed differentiation of common lymphoid progenitor cells (CLP), defined as lineage-CD127 + CD117^{low}sca-1^{low}CD135+, into B lymphocytes in vitro, achieving an amplification factor of up to one hundredfold.

We examined the roles of IL-7, Flt3L and SCF in differentiation of B cells from CLP in this culture system. Our findings indicate that IL-7 is a pivotal cytokine essential for B cell differentiation in vitro, demonstrating a notable synergistic impact when combined with SCF and FLT3L. Moreover, this system is capable of supporting the differentiation of hematopoietic stem cells (HSCs) and lymphoid-primed multipotent progenitor cells (LMPPs) into B cells in vitro. The findings substantiated the efficacy of the culture system in investigating the in vitro differentiation of bone marrow-derived progenitor cells into B cells and elucidated the specific roles of BSA, FBS and three cytokines (IL-7, FLT3L and SCF) in promoting efficient B lineage differentiation.

Keywords Vitro differentiation, Common lymphoid progenitor cells, B cell, stromal cell free culture system

[†]Pan Yang and Xiaoling Chen contributed equally to this work.

*Correspondence:
Li Wang
liwang118@tmmu.edu.cn
Liang Gong
gongliang@tmmu.edu.cn
Lintao Zhao
zhao72@tmmu.edu.cn

[1]Department of Respiratory And Critical Care Medicine, First Affiliated Hospital of Army Medical University, No. 30 Gaotan Yanzheng Street, Chongqing 400038, China
[2]Institute of Immunology PLA, Army Medical University, No. 30 Gaotan Yanzheng Street, Chongqing 400038, China
[3]Biomedical Analysis Center PLA, Army Medical University, Chongqing 400038, China

Introduction

B lymphocytes are integral to the immune system, engaging in the immune response through the synthesis of immunoglobulins and modulating this response by releasing cytokines. B cells are derived from pluripotent HSCs in fetal liver and bone marrow (BM) after birth. The BM contains all stages of B cells from early stage CLP cells to mature B cells. CLP cells are precursors with the capacity for lymphoid lineage specification, capable of differentiating into T lymphocytes, B lymphocytes, natural killer (NK) cells, dendritic cells (DC), and innate lymphoid cells (ILC) [1, 2]. B cell development is a highly regulated process that has been thoroughly investigated and characterized over the past decades. The generation of B-cells is contingent upon the intricate interactions between cellular and molecular networks involving hematopoietic stem cells and their specialized microenvironment. Within this microenvironment, hematopoietic stem cells undergo differentiation into common lymphoid progenitor cells, which then further mature into B-cells. During their developmental trajectory, B-cells can be classified into four distinct subsets, determined by the unique expression profiles of specific cell surface markers. These subsets are identified as components A (pre-pro B cells), B/C (pro B cells), and D (pre B cells). Immature B cells originate from a specialized subset of D cells that migrate from the bone marrow to the spleen, where they undergo peripheral maturation and subsequently differentiate into mature B cells and immunoglobulin-secreting plasma cells [3, 4, 5, 6].Elucidating the mechanisms that govern the ontogeny of B cells within the bone marrow is crucial for comprehensively understanding the immune system's functionality and its perturbations in various pathological conditions.

The development of an in vitro culture system that promotes the differentiation of CLP into B lymphocytes enhances the investigation of B cell ontogeny. OP9 stromal cells, sourced from murine bone marrow, are integral to this system. The co-culture of OP9 stromal cells with B cell progenitors effectively replicates the in vivo microenvironment essential for B cell maturation. This arrangement provides crucial cell-cell interactions and signaling molecules that are indispensable for the differentiation and maturation of B cells in vitro, thereby serving as a significant experimental platform for exploring the mechanisms governing B cell differentiation, development, and their functional attributes [7, 8, 9, 10]. Research has demonstrated that SCF, stromal cell-derived factor 1 (SDF-1), Flt3L, and IL-7 are essential mediators that promote the differentiation of HSCs into B lymphocytes within the bone marrow and support their longevity. These cytokines are produced by various cell types in the hematopoietic microenvironment, including osteoblasts [11, 12], CXC-chemokine ligand 12 high (CXCL12hi) reticular cells [13], and endothelial cells [14]. Moreover, OP9 stromal cells have been shown to facilitate the in vitro differentiation of B lymphocytes from embryonic stem cells without requiring external cytokine stimulation [15, 16]. Additionally, these stromal cells can also promote B lymphocyte differentiation from pluripotent stem cells in vitro; however, the efficiency of generating IgM + B cells remains relatively suboptimal [17, 18].

Nonetheless, the OP9-derived in vitro system for B cell differentiation and maturation exhibits multiple constraints: (1) Limitations of cellular origin and species: The OP9 cell line, which is derived from murine bone marrow, may not be ideal for examining the differentiation and development of human B cells. (2) Stromal cells demonstrate rapid proliferation, requiring irradiation and frequent replenishment, which complicates operational procedures. The process of irradiation can modify the supportive characteristics of lymphocyte development within stromal cell lines. Additionally, the optimal irradiation dosage necessary to achieve growth suppression without inducing cell mortality in stromal cells may differ from the conditions that result in alterations of their functional state [19]. (3) A multitude of investigations has established that the incidence of mycoplasma contamination in cell lines generally falls between 15% and 35%, with certain reports indicating rates soaring to 65–80% [20, 21, 22]. Such contamination can induce modifications in chromosomal abnormalities, metabolic processes, and cellular growth behavior, thereby jeopardizing the integrity and precision of experimental findings [23]. (4) Fetal bovine serum (FBS) is a commonly utilized element in cellular culture protocols; however, it incurs significant costs. Furthermore, discrepancies exist among various batches and brands of FBS, potentially leading to experimental inconsistencies. The possibility of contamination with toxins or viruses also poses a risk that could adversely affect the reproducibility and uniformity of research outcomes [24, 25]. These constraints may, to a certain degree, compromise the validity of conclusions derived from investigations into B-cell differentiation and development [26].

X-VIVO15 is a chemically defined, serum-free culture medium that contains pharmaceutical-grade human albumin, recombinant human insulin, and pasteurized human transferrin, thereby establishing a comprehensive and nutritionally balanced environment for cell culture applications. It has been employed to facilitate the proliferation of T cells [27], DC [28], and NK [29]. Nonetheless, research on its efficacy in promoting the differentiation of bone marrow HSC and CLP into B cells is scarce [30, 31], with existing publications lacking thorough experimental protocols.

Here in we report a more efficient and stable stromal-free cell culture methodology that successfully facilitates

the differentiation of bone marrow CLP into B lymphocytes. We offer a detailed experimental protocol and conduct a thorough analysis of the impacts of various cytokines, as well as different concentrations of serum or BSA, on B cell differentiation within this culture system. This research aims to provide a comprehensive reference for experimental approaches pertaining to B cell differentiation and maturation.

Results

X-VIVO15 is superior to other culture medium in supporting B cell development in stromal cell-free and serum-free culture system

Initially, drawing upon pertinent research regarding in vitro B cell culture [7, 9, 30, 32, 33], we performed a comparative evaluation of the differentiation potential of CLP into B lymphocytes across several culture media, namely X-VIVO15, RPMI-1640, IMDM, DMEM, and α-MEM. This assessment was conducted in the absence of OP9 stromal cells and serum supplementation. We purified bone marrow CLP, identified as lineage-CD127 + CD117^{low}sca-1^{low}CD135+, from male C57BL/6J mice aged 6 to 8 weeks through flow cytometric sorting (Fig. 1A). A total of 1000 CLP were dispensed into each well of a 96-well plate. Following this, various media [30, 31] supplemented with 1% BSA and a range of cytokines, including IL-7, FLT3L, and SCF, were introduced. On the seventh day, we noted a distinct aggregation of cells within the X-VIVO15 culture wells, while only a sparse population of cells was observed in the RPMI-1640, IMDM, DMEM, and α-MEM culture wells (Fig. 1B a-e). The X-VIVO15 medium facilitated an approximate 100-fold increase in total cell count over the week, culminating in the differentiation of roughly 4 × 10^4 CD19 + B220 + cells. Conversely, the alternative culture systems demonstrated negligible B cell production (Fig. 1C). These findings suggest that B cell generation is markedly diminished under culture conditions lacking OP9 stromal cells and serum. Thus, X-VIVO15 medium emerges as a potent system for promoting B cell differentiation in vitro.

BSA can act as an alternative to FBS in supporting B cell development in a stromal cell-free medium in vitro

Subsequently, we assessed the ability of CLP to undergo differentiation into B lymphocytes within an X-VIVO15 culture system, supplemented with 1% BSA or varying concentrations of FBS (1%, 5%, 10%, and 20%), while excluding OP9 stromal cells. Microscopic analysis demonstrated the formation of substantial cellular aggregates across all experimental groups, revealing only minor variations among them (Fig. 2Aa-e). Flow cytometric analysis revealed that the total cell yield from the 1% BSA X-VIVO15 culture system, along with the enumeration of CD19 + B220 + cells, was marginally elevated compared to that of the 1% FBS X-VIVO15 culture system. Nevertheless, no statistically significant differences were identified between the two culture conditions. Furthermore, an increase in FBS concentration correlated with a marked enhancement in both the overall cell yield and the population of CD19 + B220 + cells. Nevertheless, upon reaching a FBS concentration of 20%, there was a notable reduction in both the total cell number and the CD19 + B220 + cell count (Fig. 2B). These findings suggest that within the X-VIVO15 culture system, which lacks OP9 stromal cells, 1% BSA can serve as an effective alternative to an equivalent concentration of FBS for promoting the in vitro differentiation of CLP into B lymphocytes. Furthermore, an increase in FBS concentration correlated with a marked enhancement in both the overall cell yield and the population of CD19 + B220 + cells. Nevertheless, upon reaching a FBS concentration of 20%, there was a notable reduction in both the total cell number and the CD19 + B220 + cell count (Fig. 2B). These findings suggest that within the X-VIVO15 culture system, which lacks OP9 stromal cells, 1% BSA can serve as an effective alternative to an equivalent concentration of FBS for promoting the in vitro differentiation of CLP into B lymphocytes.

IL-7 is indispensable to the differentiation of CLP into B cells in stromal cell-free medium in vitro

Building upon the stromal cell-free X-VIVO15 culture system, we conducted an in-depth investigation into the impact of administering Flt 3 L in isolation, along with IL-7 or SCF, as well as various cytokine combinations, on the maturation of B cells (Fig. 3A-C). On day 7, the cell clusters in presence of IL-7 alone (20 ng/mL) is detectable via microscopy, indicating that the B cells derived from CLP differentiation are present in quantities five times greater than those of the initial progenitor cells. Conversely, the administration of SCF alone (40 ng/mL), FLT3L (100 ng/mL), or a combination of SCF and FLT3L results in the formation of only minimal cell aggregates when examined microscopically, akin to the findings observed in the PBS control group. The co-administration of Flt3L or SCF with IL-7 elicited a significant synergistic enhancement in cellular proliferation, with Flt3L exhibiting a more pronounced synergistic effect. The triadic combination of these factors led to the emergence of the most substantial cell aggregates (Fig. 3A). Consistently, absolute cell counts demonstrated that CLP cultured with either Flt3L or SCF in conjunction with IL-7 generated 15 to 20 times the quantity of B cells compared to the initial progenitor population. Moreover, the synergistic application of all three cytokines led to an extraordinary enhancement, resulting in a production of B cells that was 100-fold greater than that of the original

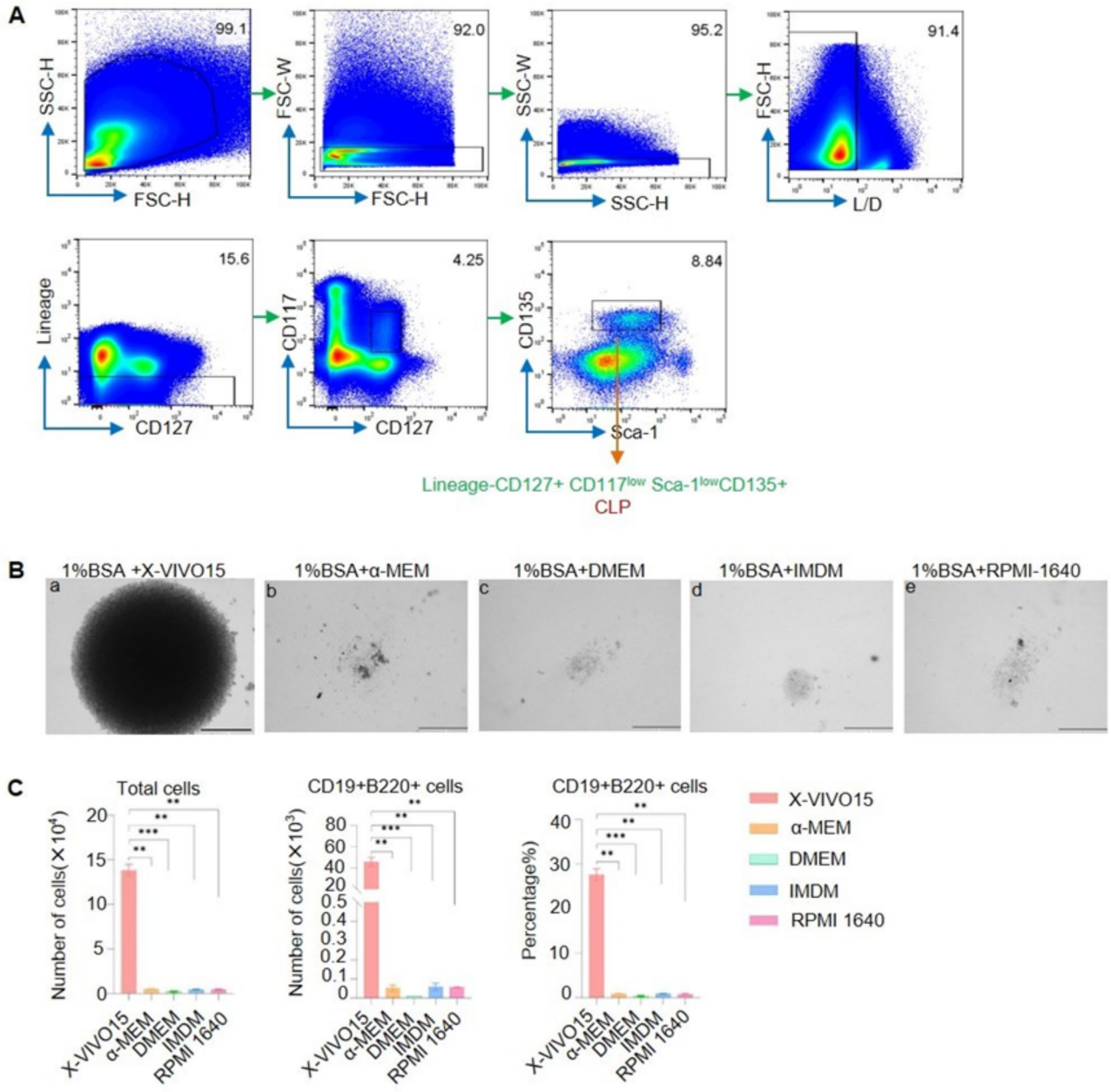

Fig. 1 Expansion of in vitro CLP cells in X-VIVO15, RPMI-1640, IMDM, α-MEM and DMEM on day 7. (**A**) Gating strategy for the isolation of CLP (Lineage-CD127 + CD117^{low} Sca-1^{low} CD135+) from bone marrow on the MoFlo Astri os EQ. (**B**) Morphology of in vitro mouse CLP in 5 medium systems on day 7. B scale bars indicate 750 μm. (**C**) The total numbers of cells and CD19 + B220 + cells obtained in the 5 medium systems on day 7. The percentage means the cell frequency of CD19 + B220 + cells among total cells. ***P*<0.01. ****P*<0.001

progenitor (Fig. 3C). Flow cytometric analysis revealed that IL-7 was sufficient to enhance the viability and differentiation of CLP cells, with CD19 + B220 + cells arising from this differentiation constituting roughly 50% of the total viable cell population. Moreover, the absolute count and proportion of CD19 + B220 + cells produced by the synergistic combination of SCF, Flt-3 L, and IL-7 were markedly elevated compared to those yielded by IL-7 alone or when combined with either SCF or Flt-3 L (Fig. 3B-C). In summary, the findings indicate that IL-7 plays a critical role in the in vitro maturation of CLP into fully developed B cells within the X-VIVO15 culture environment, independent of OP9 stromal cell support. Additionally, both SCF and Flt-3 L exhibit a synergistic effect that significantly promotes the differentiation and proliferation of CLP into B cells. Importantly, the optimal outcomes are achieved through the combined application of these three factors, corroborating previous studies.

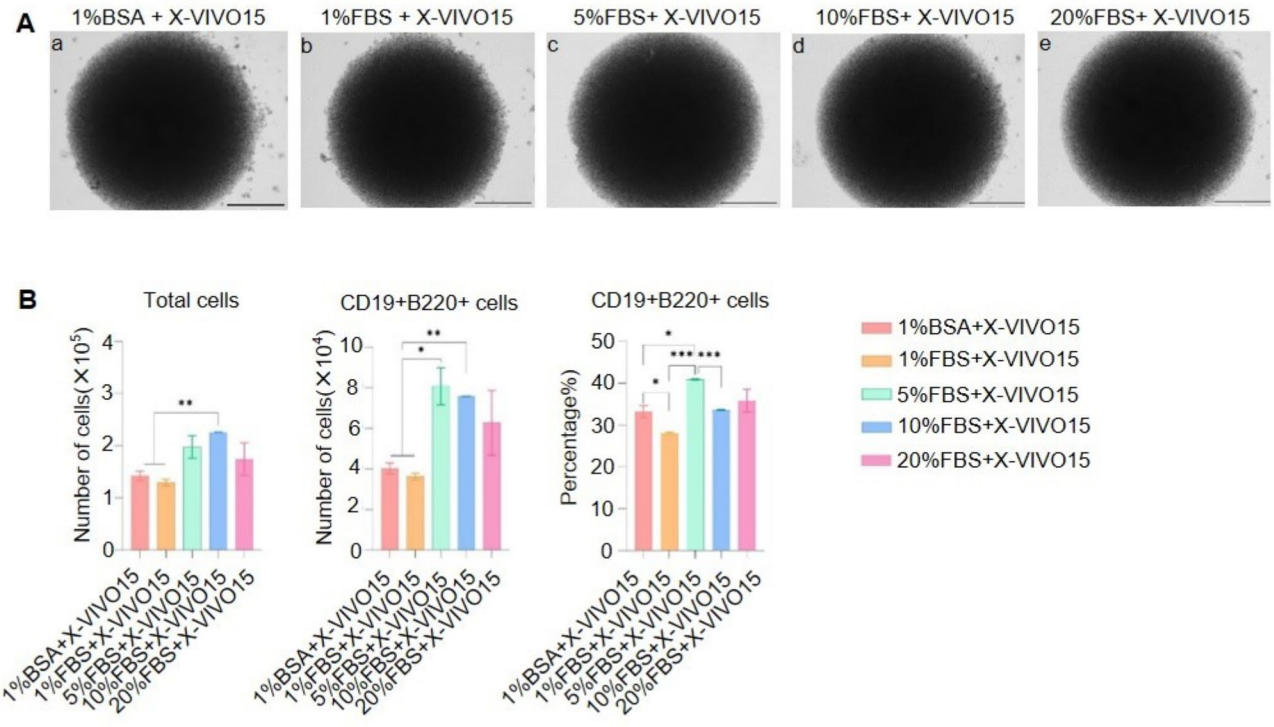

Fig. 2 Expansion of in vitro CLP cells in X-VIVO15 system containing different concentrations of FBS and BSA on day 7. (**A**) Morphology of in vitro mouse CLP in 1%BSA X-VIVO15, 1%FBS X-VIVO15, 5%FBS X-VIVO15, 10%FBS X-VIVO15 and 20%FBS X-VIVO15 on day 7. B scale bars indicate 750 μm. (**B**) The total numbers of cells and CD19 + B220 + cells obtained in the 5 medium systems on day 7. The percentage means the cell frequency of CD19 + B220 + cells among total cells. **P*<0.05. ***P*<0.01. ****P*<0.001

Dynamically analyzed B cell differentiation in vitro stromal cell-free culture system

We performed a comprehensive analysis of B cell in vitro differentiation utilizing the X-VIVO15 culture system, which is supplemented with 1% BSA, IL-7, SCF, and FLT3L. We assessed the cellular growth within each pore at days 0, 3, 5, and 7 utilizing a light microscope (Fig. 4A). In parallel, we performed flow cytometric analysis of the cultured cells on these corresponding days, employing antibodies that target B220, CD19, CD43, and IgM. The cell populations were classified as follows: Pre-B cells (B220 + CD19 + CD43- IgM-), Pro-B cells (B220 + CD19 + CD43 + IgM-), Immature B cells (B220^{low} CD19 + IgM + CD43-), and Mature B cells (B220 ^{high} CD19 + IgM + CD43-) (Fig. 4C- D). Additionally, Fig. 4B provides flow gating diagrams that delineate the various stages of B cell differentiation. The findings revealed that on day 0, the generation of stage B cells was minimal. By day 3, around 50% of the viable cell population had matured into CD19 + B220 + cells, primarily consisting of Pro-B cells. By day 5, more than 90% of the viable cells had progressed to CD19 + B220 + cells, with a significant increase noted in the CD19 + B220 + IgM + cell subset. By day 7, the cumulative cell count in the culture exceeded 100,000. The proportion of CD19 + B220 + cells within the viable population remained relatively consistent when compared to day 5; however, there was a pronounced increase in the absolute cell numbers. Importantly, the quantity of IgM + CD19 + B220 + cells saw a significant rise, representing 13% of the overall cell population and 18% of the viable cells. Additionally, both the absolute values and frequencies of these four cell subsets demonstrated substantial increases (Fig. 4D). The results suggest that this culture system proficiently replicates the differentiation pathway of bone marrow-derived B cells. Therefore, it presents a robust alternative for examining the influence of various parameters on the differentiation and maturation of B cells.

Stromal cell-free culture system also supported the development of HSC and LMPP into B cells

To evaluate the effectiveness of the stromal cell-free X-VIVO15 system in facilitating the in vitro differentiation of early hematopoietic precursor cells into B lymphocytes, we isolated HSCs (Lineage-CD117 + Sca-1 + CD150 + CD135-) using flow cytometric techniques. LMPPs (Lineage-CD117 + scal-1 + CD150-CD135+) and CLP were each seeded with 1000 stem progenitor cells in individual wells of a 96-well plate containing a 1% BSA X-VIVO15 culture medium. The subsequent differentiation capacity of these cell populations into B cells was then evaluated. Figure 5A and B

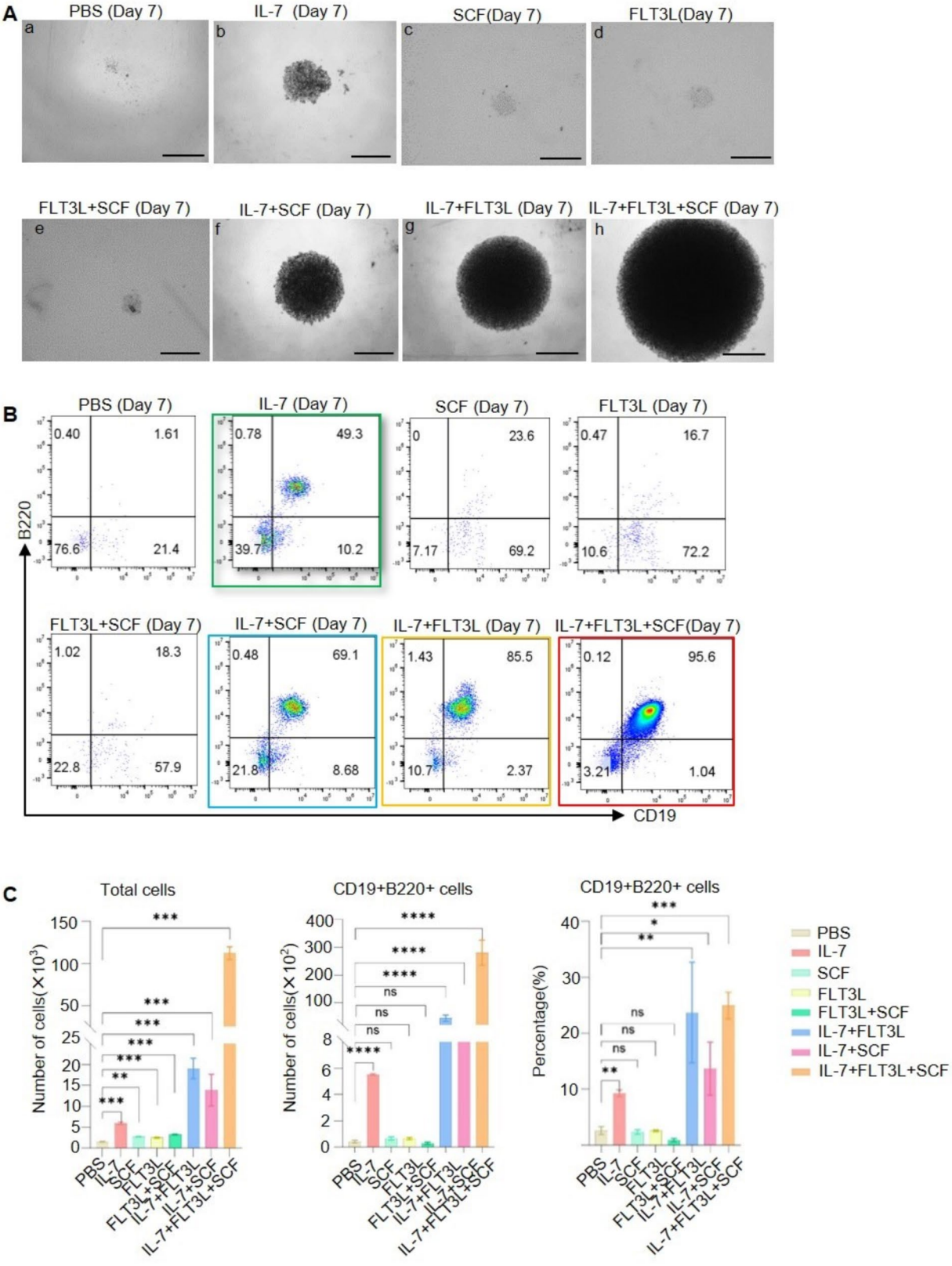

Fig. 3 (See legend on next page.)

(See figure on previous page.)

Fig. 3 Comparison of B-cell production from CLP in the presence or absence of the indicated cytokines(s) supplementation. (**A**) Microscopic observation of B lymphopoiesis of CLP in vitro. (**B**) Flow cytometry analysis of in vitro B lymphopoiesis in culture (Gated on live cells). The total cells derived from culture in the absence of cytokine supplementation (PBS), IL-7 and FLT3L or SCF, FLT3L and SCF, IL-7, FLT3L and SCF for 7 d were analyzed via flow cytometry with antibodies against B220, CD19. (**C**) The total cells were analyzed by flow cytometry in the media. The absolute number and percentage of B220 + CD19 + cells under the different culture media. NS, not significant. The percentage means the cell frequency of CD19 + B220 + cells among total cells. **P*<0.05. ***P*<0.01. ****P*<0.001. *****P*<0.0001

illustrate that differentiation from CLP leads to the generation of approximately 60% CD19 + cells relative to the total cell population, with an almost negligible fraction of CD11b + cells. In contrast, differentiation from LMPP yields a lower proportion of CD19 + cells, approximately 40%, while producing about 8% CD11b + cells. The differentiation from HSC results in an even smaller percentage of CD19 + cells, around 20%, with the CD11b + cell proportion being approximately 22%. Importantly, the absolute count of CD19 + cells derived from CLP is significantly higher than that obtained from both HSC and LMPP. Conversely, the absolute quantity of CD11b + cells produced by LMPP exceeds that generated by both HSC and CLP. The findings suggest that the culture system employed for these acellular cells exhibits extensive utility in the in vitro differentiation of diverse hematopoietic progenitor cell lineages into lymphoid and myeloid immune cell populations.

Discussion

The maturation of B lymphocytes within the bone marrow has been a central topic of investigation for an extended period. The in vitro differentiation of CLP into B cells is of paramount significance, as it not only deepens our comprehension of the intrinsic mechanisms governing the immune system but also presents novel therapeutic avenues and targets for addressing immune-mediated disorders.

Nevertheless, the in vitro differentiation of B lymphocytes utilizing common lymphoid progenitor (CLP) methodologies continues to encounter several methodological hurdles. Firstly, the prevalence of CLP is exceedingly low, estimated at approximately 0.02% of the total bone marrow population [34]. Furthermore, a significant proportion of existing studies has employed OP9 stromal cells in conjunction with serum-supplemented media, yielding CD19 + or B220 + B cells with frequencies ranging from 6–60% [8, 35]. However, the expansion efficiency of these B cell populations remains inconsistent, potentially due to the inherent instability of the stromal cells and serum or the specific formulation of the culture medium. The existing literature on B cell differentiation in vitro in the absence of stromal cells is notably limited. This includes culture media such as opti-MEM, RPMI-1640 [32], and serum-free, stromal cell-free culture systems utilizing immobilized recombinant kit-L [26]. However, the descriptions of these experiments lack sufficient detail, which undermines experimental reproducibility and scientific rigor, escalates research costs, diminishes experimental efficiency, and has consequently led to the underutilization of these methods. We introduce a comprehensive, reproducible, and economically viable stromal cell-free 1% BSA X-VIVO15 in vitro culture system that facilitates the differentiation of CLP into B cells solely with the addition of IL-7. Moreover, the application of a triad of cytokines enhances the system's capacity to efficiently generate CD19 + B220 + and IgM + CD19 + B220 + cells, which can be utilized in a broad spectrum of investigations pertaining to B cell differentiation.

FBS is widely utilized as a supplement in cell culture due to its rich nutrient profile. Nevertheless, its application presents several drawbacks, such as ethical dilemmas surrounding animal experimentation, potential contamination risks, and substantial costs. Therefore, it is crucial to explore viable alternatives to FBS. Research has compared the impacts of FBS with those of human serum albumin and BSA on cell cultures. Findings from these investigations reveal that cell proliferation and growth in various cell lines, including C2C12, are notably enhanced, and the multipotency of mesenchymal stromal cells is preserved when BSA is employed in the culture medium. These observations indicate that BSA could serve as a promising substitute for FBS in cell culture applications [36, 37, 38]. This can be ascribed to the role of BSA as both a stabilizer and a carrier that enhances cell proliferation and viability, in addition to its capacity as an antioxidant, which mitigates cellular stress and injury. The X-VIVO15 medium is a serum-free formulation meticulously crafted for the cultivation of lymphocytes and primary cells. It facilitates the in vitro differentiation of hematopoietic stem cells into B cells [30, 39] and promotes the proliferation of T cells and dendritic cells (DCs) [40, 41]. Our research demonstrates that the X-VIVO15 culture medium can effectively replace stromal cells, allowing for the in vitro differentiation of hematopoietic progenitor cells into B cells. X-VIVO15 contains numerous additional nutrients compared to other types of media, including: (1) L-glutamine, a key amino acid in energy metabolism that supports lymphocyte proliferation; (2) Recombinant insulin, which regulates cell metabolism and promotes cell growth and survival; (3) Recombinant transferrin, which binds and transports iron ions to meet the iron metabolism requirements of

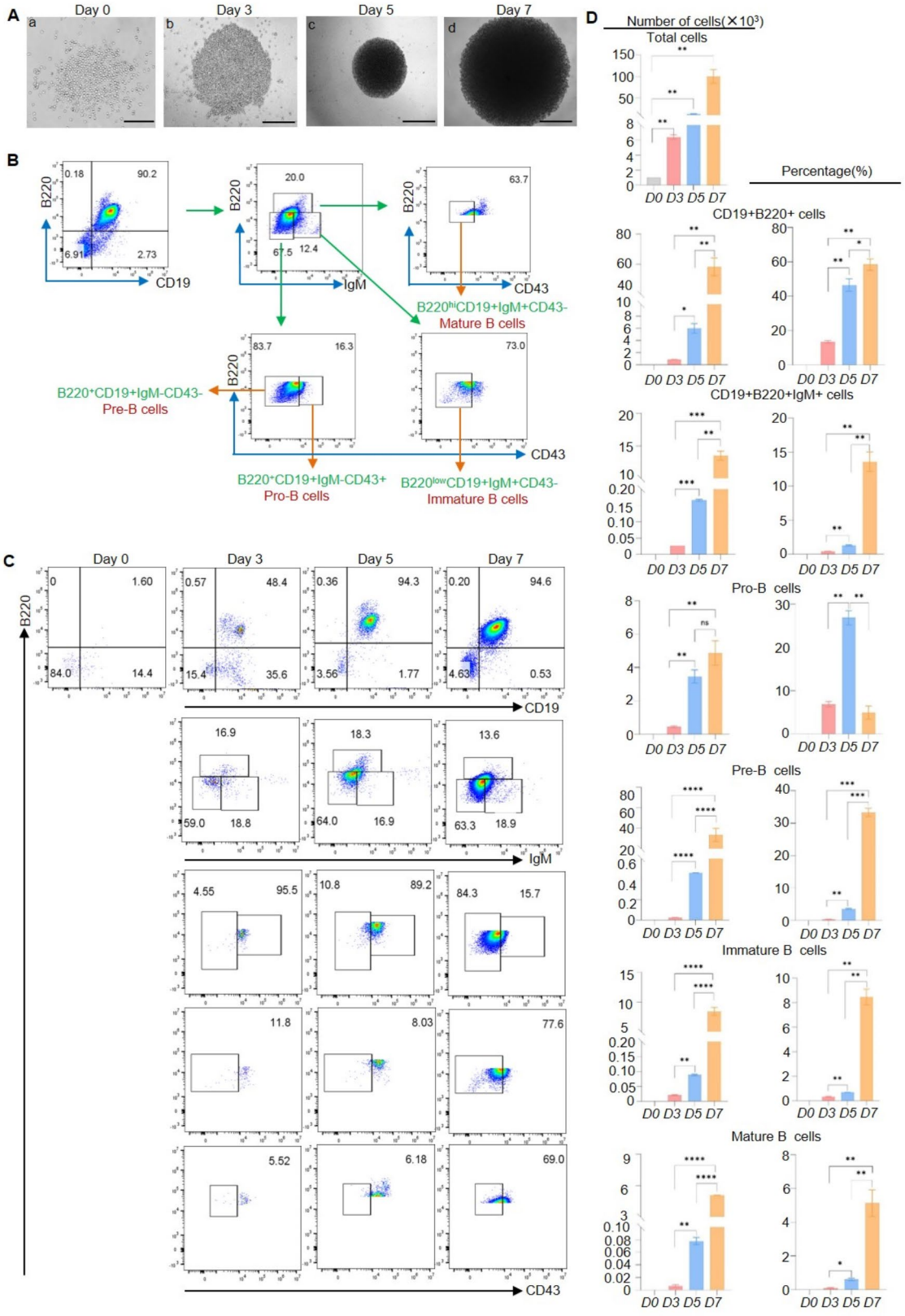

Fig. 4 (See legend on next page.)

(See figure on previous page.)

Fig. 4 Serum-free and stromal cell-free culture of CLP cells ex vivo. (**A**) Microscopic observation of B lymphopoiesis of CLP in vitro. 1,000 sorted CLP were cultured in stromal free and serum free conditions with 20 ng/ml IL-7、100ng/mL FLT3L and 40ng/mL SCF for the indicated times; a, b, c, d demonstrated the microscopic manifestations of CLP differentiation into B cells on day 0, day 3, day 5 and day 7, respectively; a, b scale bars indicate 150 μm; c, d scale bars indicate 750 μm. (**B**) Diagram of B cell flow gating strategy. (**C**) In vitro B lymphopoiesis of CLP in the presence of IL-7, FLT3L and SCF. The development of B-lineage cells was analyzed via flow cytometry with antibodies against B220, CD19, CD43 and IgM during cultured for 7 d. (**D**) The increase in the number of total cell, CD19 + B220+, CD19 + B220 + IgM + cells and each cell population under culture. The percentage mean each cell accounted for the total cell frequency. **P*<0.05. ***P*<0.01. ****P*<0.001. *****P*<0.0001

cells; and (4) Pharmaceutical-grade albumin, which provides nutritional support while minimizing batch variation. We hypothesize that these components are crucial for the development of CLP. Although these elements are absent in other media, we suggest that OP9 stromal cells may partially compensate for this deficiency. However, factors such as batch variation and the condition of OP9 cells—affected by irradiation, digestion, and passage—may impact their secretion ability. Furthermore, the secretion levels of these factors may not fully satisfy the requirements for CLP differentiation and proliferation, resulting in significantly inferior outcomes compared to the use of X-VIVO15 in this culture system.

In this cultural environment, we observed a differentiated B cell population that was 100 times greater than that of the initial progenitor cell cohort. The efficiency of inducing CD19 + cells in this study was nearly 40 times higher than that of previous induction using the OP9 free system [7, 32], significantly surpassing earlier findings. To date, no studies have systematically evaluated the comparative efficacy of X-VIVO15 relative to other standard culture media in facilitating B cell differentiation and maturation in vitro in the absence of OP9 stromal cells. We postulate that B cells exhibit rapid proliferation under in vitro conditions, and traditional media may be insufficient for supporting extensive cellular expansion. Consequently, the secreted factors from stromal cells are critical for enhancing the synergistic proliferation of B cells. We propose that X-VIVO15 can independently substitute for stromal cells, thereby providing this necessary synergistic effect. This methodology streamlines experimental workflows, minimizes contamination risks, and reduces variability linked to different preparations of irradiated stromal cells. Considering the accessibility, cost-efficiency, and safety profile of BSA, we have opted for a culture system that integrates BSA with X-VIVO15. Researchers have the option to select an appropriate culture system utilizing BSA or to employ varying concentrations of FBS alongside X-VIVO15, enabling experiments designed for specific parameters. For example, in scenarios where an increased cell yield is necessary for sequencing purposes or when examining variations in signaling molecules related to B-cell differentiation pathways, a culture system consisting of 10% FBS in combination with X-VIVO15 can be utilized.

Cytokines are integral to the differentiation of hematopoietic progenitor cells into mature immune cells. Our research has shown that that the absence of IL-7 leads to the inability of CLP to successfully differentiate into B cells. This finding underscores the central role of IL-7 in CLP differentiation. Furthermore, in the presence of IL-7, both Flt3L and SCF significantly enhance CLP differentiation. Therefore, we propose that these two factors act synergistically. The CD19 + B220 + cells identified in the 1% BSA X-VIVO15 culture system on day 3 were predominantly Pro-B and Pre-B cells, which persisted through day 5. Notably, there was a substantial proliferation of IgM + CD19 + B220 + cells during this interval. By day 7, the proportion of IgM + CD19 + B220 + cells reached approximately 18% of the total cell population, representing a significant enhancement compared to the sub-5% frequency documented in prior studies concerning stromal cell-supported hematopoietic stem cell generation of IgM + B cells [17, 18]. Moreover, although SCF and FLT3L exhibit a synergistic effect, their ability to facilitate B cell progenitor development is markedly restricted when administered alone. Conversely, the combination of these factors with IL-7 considerably augments the differentiation potential of CLP into mature B cells. This investigation systematically evaluates the impact of three different cytokines—both singularly and in combination—on the differentiation of CLP into B cells within the 1% BSA X-VIVO15 culture system in vitro, thereby offering critical insights for future scholarly research.

Moreover, we conduct a comprehensive analysis of the potential of the 1% BSA X-VIVO15 culture system for the exploration of differentiation pathways in various bone marrow hematopoietic precursor cells, including HSC and LMPP. Our findings reveal that both HSC and LMPP are capable of differentiating into CD19 + B cells and CD11b + myeloid cells within this culture environment. This observation indicates that these progenitor populations exhibit multidirectional differentiation potential, enabling concurrent maturation into lymphoid and myeloid lineages, consistent with prior research. In contrast, CLP demonstrate a limited differentiation capacity, restricted solely to lymphoid lineage development. This culture system effectively promotes the maturation of diverse bone marrow hematopoietic precursors into functional B cells, thereby serving as an exceptional platform for elucidating the differentiation mechanisms of hematopoietic precursors into B cells across various developmental stages.

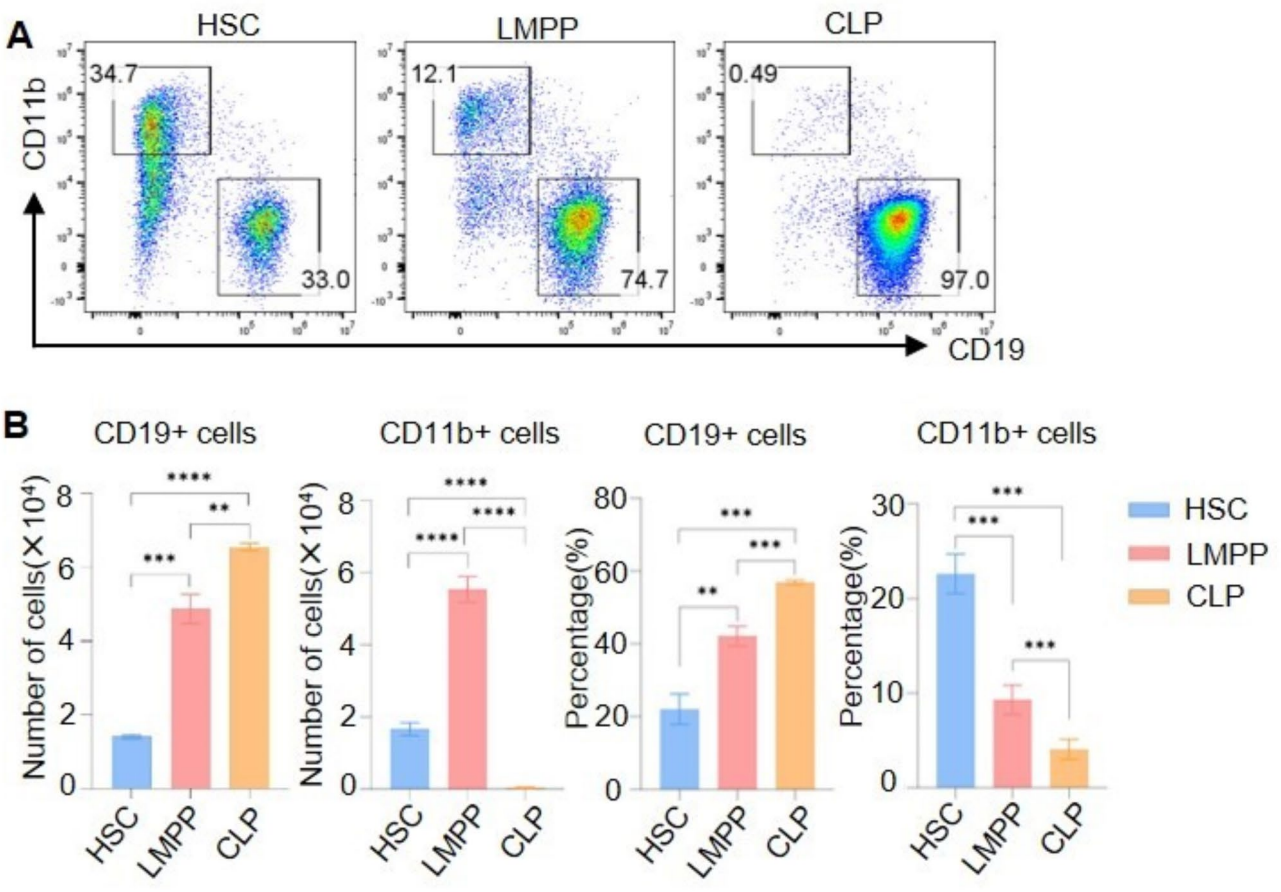

Fig. 5 In vitro B lymphopoiesis of HSCs, LMPPs, CLP in the 1%BSA X-VIVO15 complete medium. (**A**)The development of B-lineage cells was analyzed via flow cytometry with antibodies against CD11b and CD19 during cultured for 7 d. (**B**) The increase in the number of CD19 + and CD11b + cells under culture. The percentage mean each cell accounted for the total cell frequency. ***P*<0.01. ****P*<0.001. *****P*<0.0001

In summary, the culture system that employs BSA or FBS in conjunction with X-VIVO15 operates independently of stromal cells, providing a simplified and user-centric methodology. This system proficiently promotes the differentiation of HSCs, LMPPs, and CLP into B cells across diverse developmental stages. These results suggest that this culture system presents substantial promise for investigating the differentiation of precursor cells into B cells at various stages within the bone marrow microenvironment.

Materials and methods

Animals

Six-week-old C57BL/6J male mice (SPF (Beijing) Biotechnology, China) were kept in specific pathogen-free conditions with light/dark cycles of 12 h, 60% humidity, 23 ± 3 ℃, and free access to water. The mice were euthanized by cervical dislocation.

Chemicals, antibodies and analytical flow cytometry

Recombinant mouse stem cell factor (SCF), mouse Flt3 ligand (Flt3L) and mouse IL-7 were purchased from Cell Signaling Technology (Danvers, MA, USA). BSA were purchased from Sigma-Aldrich (St Louis, MO, USA). Penicillin/Streptomycin, FBS, L-glutamine, IMDM, a-MEM, RPMI 1640, DMEM was purchased from GIBCO (Grand Island, NY, USA). X-VIVO15 was purchased from Lonza Group Ltd.(Basel, Switzerland). BSA and β-mercaptoethanol was purchased from Sigma-Aldrich(St. Louis, MO, USA). Erythrocyte Lysate purchased from BioSharp(Hefei, China).

Specific antibodies were purchased from the following commercial sources: anti-mouse lineage markers(145-2C11; RB6-8C5; RA3-6B2; Ter-119; M1/70;), anti-B220(RA3-6B2), anti- CD19(6D5), anti- CD43(S11), anti- IgM(RMM-1), anti-Sca-1(D7), anti- CD117/c-kit (2B8), anti-CD127/IL-7R(S18006K), anti- CD135(A2F10), anti- CD4 (GK1.5), anti- CD8 (53 – 6.7), anti- CD44 (IM7), anti- CD25 (PC61), anti- CD45 (30-F11), anti- CD150 (TC15-12F12.2), anti-CD48(HM48-1)

and Purified anti-mouse CD16/32(93) from BioLegend (San Diego, CA, USA); anti-CD34(581) from BD Pharmingen(San Diego, CA, USA); and FIXABLE VIABILITY DYE EF780 (L/D) from Invitrogen(Carlsbad, CA, USA). OP9DL1 was purchased from ATCC(Manassas, VA, USA).

Analysis and cell sorting was done on an MoFlo Astrios EQ (BD Biosciences) and CytoFLEX LX (BECKMAN COULTER).

FACS staining and purification of bone marrow cells

Mice were euthanized through cervical dislocation and subsequently immersed in 75% ethanol for a duration of 5 min. The femur and tibia from the hind limbs were then procured and stored in frozen phosphate-buffered saline (PBS). Bone marrow cells were harvested using a 1 mL syringe, with washing performed until the femur exhibited a white appearance. The freshly isolated sterile bone marrow cells were treated with erythrocyte lysis buffer, followed by the application of purified anti-mouse CD16/32 antibodies to block non-specific binding. Subsequently, the cells were stained with anti-CD117, anti-CD127, anti-Lineage, anti-Sca-1, and anti-CD135 antibodies prior to analysis and sorting for CLP using a MoFlo Astrios EQ flow cytometer.

In vitro B lymphopoiesis in stroma free culture system

For evaluation of B cell potential, 1,000 CLP were cultured in round-bottom 96-well plates in 200μL 1% BSA X-VIVO15 complete medium (containing 55 mM β-mercaptoethanol, 0.5% Penicillin/Streptomycin, 1% BSA buffer, 2mM L-glutamine). This medium was supplemented with 20 ng/ml IL-7, 40 ng/ml SCF, and100 ng/ml Flt-3 L.

Additionally, we performed a comparative analysis of 1% BSA X-VIVO15 complete medium against various culture media, including DMEM, IMEM, RPMI-1640, and α-MEM, to assess their effectiveness in supporting in vitro B cell culture. We also evaluated the performance of 1% BSA X-VIVO15 complete medium against X-VIVO15 medium supplemented with different concentrations of FBS (1%, 5%, 10%, and 20%) to compare their abilities to culture B cells in vitro. CLP were cultured in 8 culture media as follows: DMEM plus 1% BSA, RPMI-1640 plus 1% BSA, IMDM plus 1% BSA, α-MEM plus 1% BSA, X-VIVO15 plus 1% FBS, X-VIVO15 plus 5% FBS, X-VIVO15 plus 10% FBS, X-VIVO15 plus 20% FBS. These media contain the same variety of cytokines and other additional ingredients as 1% BSA X-VIVO15 complete medium. Cells were then maintained at 37 °C and 5% CO2 for over 7d.

The culture medium should be replaced with half of its volume every 2 to 3 days. Cocultures were evaluated by FACS staining with anti-CD19, anti-B220, anti-CD43, anti-IgM and L/D for B cell cultures at day 7. Samples were analyzed on an CytoFLEX LX (BECKMAN COULTER).

Statistical analysis

All experiments were performed at least three times. GraphPad Prism software version 10 (La Jolla, CA, USA) was utilized for One-way ANOVA with subsequent Dunnett's multiple comparison test to compare multiple groups while two-tailed unpaired t-test was utilized for two groups. The presented data represent the means ± s.d.

Acknowledgements

Not applicable.

Author contributions

Contributions： LTZ, LG and LW contributed to the conception and design of the study. LTZ, PY, XLC, and HW contributed to data acquisition. LTZ, LG, PY, XLC, MLY, HLY and LW analyzed and interpreted the data. LTZ, LW, and PY drafted the manuscript. LTZ, PY, and LG performed statistical analyses. All authors read and approved the final manuscript.

Funding

This study was funded by National Nature Science Foundation of China (No. 82073169).

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The animal experiments were approved by the Laboratory Animal Welfare and Ethics Committee of Army Medical University (AMUWEC2020760 、AMUWEC20242078). This research does not involve human subjects.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 9 January 2025 / Accepted: 25 February 2025
Published online: 04 March 2025

References

1. Karsunky H, Inlay MA, Serwold T, et al. Flk2 + common lymphoid progenitors possess equivalent differentiation potential for the B and T lineages. Blood[J]. 2008;111(12):5562–70.
2. Adolfsson J, Mansson R, Buza-Vidas N, et al. Identification of Flt3 + lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell[J]. 2005;121(2):295–306.
3. Morrison SJ, Spradling AC. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell[J]. 2008;132(4):598–611.
4. Ghosh D, Jiang W, Mukhopadhyay D et al. New insights into B cells as antigen presenting cells. Curr Opin Immunol[J]2021,70(129–37.
5. Singh H, Medina KL, Pongubala JM. Contingent gene regulatory networks and B cell fate specification. Proc Natl Acad Sci U S A[J]. 2005;102(14):4949–53.
6. Nutt SL, Kee BL. The transcriptional regulation of B cell lineage commitment. Immunity[J]. 2007;26(6):715–25.
7. Kawano Y, Petkau G, Stehle C et al. Stable lines and clones of long-term proliferating normal, genetically unmodified murine common lymphoid progenitors. Blood[J] 2018,131(18):2026–35.

8. Tsapogas P, Zandi S, Ahsberg J, et al. IL-7 mediates Ebf-1-dependent lineage restriction in early lymphoid progenitors. Blood[J]. 2011;118(5):1283–90.
9. Seehus CR, Aliahmad P, de la Torre B et al. The development of innate lymphoid cells requires TOX-dependent generation of a common innate lymphoid cell progenitor. Nat Immunol[J] 2015,16(6):599–608.
10. Yu J, Choi S, Kim H et al. Generation of an osteoblast-based artificial niche that supports in vitro B lymphopoiesis. Exp Mol Med[J] 2017,49(11):e400.
11. Calvi LM, Adams GB, Weibrecht KW, et al. Osteoblastic cells regulate the Haematopoietic stem cell niche. Nature[J]. 2003;425(6960):841–6.
12. Zhang J, Niu C, Ye L et al. Identification of the Haematopoietic stem cell niche and control of the niche size. Nature[J] 2003,425(6960):836–41.
13. Tokoyoda K, Egawa T, Sugiyama T et al. Cellular niches controlling B lymphocyte behavior within bone marrow during development. Immunity[J] 2004,20(6):707–18.
14. Kiel MJ, Yilmaz OH, Iwashita T et al. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell[J] 2005,121(7):1109–21.
15. Lagergren A, Mansson R, Zetterblad J et al. The Cxcl12, Periostin, and Ccl9 genes are direct targets for early B-cell factor in OP-9 stroma cells. J Biol Chem[J] 2007,282(19):14454–62.
16. Taguchi T, Takenouchi H, Shiozawa Y et al. Interleukin-7 contributes to human pro-B-cell development in a mouse stromal cell-dependent culture system. Exp Hematol[J] 2007,35(9):1398–407.
17. Carpenter L, Malladi R, Yang CT et al. Human induced pluripotent stem cells are capable of B-cell lymphopoiesis. Blood[J] 2011,117(15):4008–11.
18. Cho SK, Webber TD, Carlyle JR et al. Functional characterization of B lymphocytes generated in vitro from embryonic stem cells. Proc Natl Acad Sci U S A[J] 1999,96(17):9797–802.
19. Montecino-Rodriguez E, Dorshkind K. Stromal cell-dependent growth of B-1 B cell progenitors in the absence of direct contact. Nat Protoc[J]. 2006;1(3):1140–4.
20. Drexler HG, Uphoff CC. Mycoplasma contamination of cell cultures: incidence, sources, effects, detection, elimination, prevention. Cytotechnology[J]. 2002;39(2):75–90.
21. Roth JS, Lee TD, Cheff DM et al. Keeping it clean: the cell culture quality control experience at the National center for advancing translational sciences. SLAS Discov[J] 2020,25(5):491–7.
22. Drexler HG, Uphoff CC, Dirks WG et al. Mix-ups and Mycoplasma: the enemies within. Leuk Res[J] 2002,26(4):329–33.
23. Huff LM, Horibata S, Robey RW, et al. Mycoplasma infection mediates sensitivity of Multidrug-Resistant cell lines to Tiopronin: A cautionary Tale. J Med Chem[J]. 2020;63(3):1434–9.
24. Liu S, Yang W, Li Y, et al. Fetal bovine serum, an important factor affecting the reproducibility of cell experiments. Sci Rep[J]. 2023;13(1):1942.
25. Yang N, Sin DD, Dorscheid DR. Various factors affect lipopolysaccharide sensitization in cell cultures. Biotechniques[J]. 2020;69(2):126–32.
26. Kawano Y, Petkau G, Wolf I, et al. IL-7 and immobilized Kit-ligand stimulate serum- and stromal cell-free cultures of precursor B-cell lines and clones. Eur J Immunol[J]. 2017;47(1):206–12.
27. Chen B, Shi Y, Smith JD, et al. The role of tumor necrosis factor alpha in modulating the quantity of peripheral blood-derived, cytokine-driven human dendritic cells and its role in enhancing the quality of dendritic cell function in presenting soluble antigens to CD4 + T cells in vitro. Blood[J]. 1998;91(12):4652–61.
28. Kugler A, Stuhler G, Walden P, et al. Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. Nat Med[J]. 2000;6(3):332–6.
29. Calmeiro J, Mendes L, Duarte IF et al. In-Depth analysis of the impact of different Serum-Free media on the production of clinical grade dendritic cells for Cancer immunotherapy. Front Immunol[J].2020,11(593363.
30. Nagai Y, Garrett KP, Ohta S, et al. Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment. Immunity[J]. 2006;24(6):801–12.
31. Kouro T, Medina KL, Oritani K et al. Characteristics of early murine B-lymphocyte precursors and their direct sensitivity to negative regulators. Blood[J] 2001,97(9):2708–15.
32. Miller JP, Izon D, DeMuth W, et al. The earliest step in B lineage differentiation from common lymphoid progenitors is critically dependent upon Interleukin 7. J Exp Med[J]. 2002;196(5):705–11.
33. Wang H, Pierce LJ, Spangrude GJ. Distinct roles of IL-7 and stem cell factor in the OP9-DL1 T-cell differentiation culture system. Exp Hematol[J]. 2006;34(12):1730–40.
34. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell[J]. 1997;91(5):661–72.
35. Yang Y, Xu J, Chen H, et al. MiR-128-2 inhibits common lymphoid progenitors from developing into progenitor B cells. Oncotarget[J]. 2016;7(14):17520–31.
36. Escobedo-Lucea C, Bellver C, Gandia C et al. A xenogeneic-free protocol for isolation and expansion of human adipose stem cells for clinical uses. PLoS One[J] 2013,8(7):e67870.
37. Johal KS, Lees VC, Reid AJ. Adipose-derived stem cells: selecting for translational success. Regen Med[J]. 2015;10(1):79–96.
38. Rajala K, Lindroos B, Hussein SM et al. A defined and xeno-free culture method enabling the establishment of clinical-grade human embryonic, induced pluripotent and adipose stem cells. PLoS One[J] 2010,5(4):e10246.
39. Bruserud O, Glenjen N, Ryningen A et al. In vitro culture of human acute lymphoblastic leukemia (ALL) cells in serum-free media; a comparison of native ALL blasts, ALL cell lines and virus-transformed B cell lines. Leuk Res[J] 2003,27(5):455–64.
40. Xu H, Wang N, Cao W, et al. Influence of various medium environment to in vitro human T cell culture. Vitro Cell Dev Biol Anim[J]. 2018;54(8):559–66.
41. Lellahi SM, Azeem W, Hua Y et al. GM-CSF, Flt3-L and IL-4 affect viability and function of conventional dendritic cell types 1 and 2. Front Immunol[J].2022,13(1058963.

Publisher's note