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Pro-B cells propagated in stromal cell-free cultures reconstitute functional B-cell compartments in immunodeficient mice

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Abbreviations:

BM – bone marrow

FL – fetal liver

NIP - 4-hydroxy-5-iodo-3-nitrophenyl

Abstract

Fetal liver (FL) and bone marrow (BM) derived pro-B cells were propagated long-term in stromal cell-free cultures supplemented with interleukin-7 (IL-7), stem cell factor and FLT3 ligand. Within a week, most cells expressed surface CD19, CD79A,  $\lambda 5$  and VpreB antigens and had rearranged immunoglobulin D-J heavy chain genes. Both FL and BM pro-B cells reconstituted the B-cell compartments of immunoincompetent Rag2-deficient mice, with FL pro-B cells generating follicular, marginal zone (MZB) and B1a B cells, and BM pro-B cells giving rise mainly to MZB cells. Reconstituted Rag2-deficient mice generated significant levels of IgM and IgG antibodies to a type II T-independent antigen; mice reconstituted with FL pro-B cells generated surprisingly high IgG<sub>1</sub> titers. Finally, we show for the first time that mice reconstituted with mixtures cell-free pro-B and pro-T cells propagated in stromal cell-free in vitro cultures mounted a T-cell-dependent antibody response. This novel stromal cell-free culture system facilitates our understanding of B-cell development and might be applied clinically.

## Introduction

In mammals, B cells develop from hematopoietic stem cells (HSCs), antenatally from the fetal liver (FL) and postnatally from the bone marrow (BM). Various stages of B-cell development are distinguishable by combinations of cell surface and intracellular markers, cell cycle profile and rearrangement status of IgH and IgL genes [1-4]. B-cell commitment is determined by the transcription factor Pax5 [5-7] one of whose target genes is CD19 [8]. The earliest B-cell committed precursor, or pro-B cell, proliferates rapidly, is CD19<sup>+</sup> CD117<sup>+</sup> [1, 9] and has its Ig<sub>H</sub> D–J genes rearranged [4] and are absent in Pax5-deficient [10], *Il7* or *Il7R* gene deleted mice [11, 12]. The receptor tyrosine kinases CD117 and CD135 and their corresponding ligands, SCF and FLT3L, are important for early B-cell development [13-17]. Blocking SCF binding with an anti-CD117 antibody inhibits pro-B cell proliferation in IL-7-containing stromal cell cultures [18] and CD135 or FLT3L-deficient mice have a dramatically reduced BM pro-B-cell compartment [19]. Thus Pax5 and cytokines are important for early B-cell development.

We previously showed that FL-derived pro-B cells could be grown long-term on stromal cells and IL-7 [20]. Here we present for the first time that this is possible for BM-derived pro-B cells. We have described an early (E) progenitor (P) with lymphoid (L) and myeloid (M) developmental potential, called EPLM, in the BM [21]. EPLM are B220<sup>+</sup>, CD117<sup>+</sup> but CD19<sup>-</sup> NK1.1<sup>-</sup>. When cultured either on OP9 stromal cells plus IL-7 or without stromal cells but with IL-7, SCF and FLT3L, EPLM from the FL or BM can be propagated long-term and differentiate into CD19<sup>+</sup> pro-B cells. Moreover, upon *in vivo* transplantation into immunodeficient *Rag2* gene

deficient hosts, these pro-B cells reconstitute a functional B-cell compartment.

Finally, when these mice are reconstituted with a mixture of *in vitro* stromal cell-free propagated pro-B and pro-T cells [22], a small, but functional adaptive immune system is generated.

## Results

### *Longterm growth of BM pro-B cells can be achieved by XXX*

It was previously shown that, using stromal cell-based cultures, only FL-derived pro-B cells could be grown long-term [20]. We recently identified a B220<sup>+</sup>CD117<sup>+</sup>CD19<sup>-</sup>NK1.1<sup>-</sup> BM progenitor having lymphoid and myeloid developmental potential, called EPLM [21]; now we tested their capacity to generate long-term growing pro-B cells. Thus, B220<sup>+</sup>CD117<sup>+</sup>CD19<sup>-</sup>NK1.1<sup>-</sup> (CD19<sup>-</sup>) and B220<sup>+</sup>CD117<sup>+</sup>CD19<sup>+</sup>NK1.1<sup>-</sup> (CD19<sup>+</sup>) cells from FL or BM were sorted (Fig.1A) and plated on OP9 stromal cells plus IL-7. After 6 days, cells were harvested every 3 - 4 days and re-plated on fresh stromal cells and IL-7. FACS staining 6-7 days after initiation of cultures showed in all cases that the cultured cells were >90% positive for CD19 expression and this expression was further increased to almost 100% and retained throughout the culture period (data not shown and Figure 1D). CD19<sup>-</sup> (EPLM) cells grew continuously with doubling times of about 30 hours, whereas CD19<sup>+</sup> cells proliferated for the first 7 days and then died (Fig 1B). RNAseq analysis on CD19<sup>+</sup> cells derived from CD19<sup>-</sup> EPLM grown for 12 days on OP9 plus IL-7 and freshly-isolated CD19<sup>+</sup>CD117<sup>+</sup> cells similarly grown for 5 days revealed that only 83 genes, none of which were B-cell related, were  $\geq 2$  fold differentially expressed (Supporting Information Table I). All 44 B-cell-related genes were similarly expressed (Table SII). Gene ontology analysis of the 83 genes did not identify genes that explained the growth difference observed. An additional RNAseq analysis of *ex vivo* isolated CD19<sup>+</sup>CD117<sup>+</sup> cells and EPLM derived CD19<sup>+</sup> cells maintained on OP9 stromal cells in the presence of IL-7 for two weeks revealed 3449

differentially expressed genes (Table SIII). However, amongst them we could not identify particular candidates that might be responsible for the dissimilar growth capacity observed.

FL-derived EPLM grew better than BM-derived EPLM on OP9 with IL-7 (Fig.1C) with >95% of both becoming CD19<sup>+</sup> (Fig.1D) with ~25% BM and >60% FL-derived cells expressing CD117 (Fig.1D). RT-PCR analysis revealed that both expressed CD79a, CD79b, Igl11 ( $\lambda$ 5) and Vpreb1 (Fig.1E) and both had undergone D<sub>H</sub>-J<sub>H</sub> rearrangements (Fig.1F). Thus, BM and FL EPLM cultured on OP9 plus IL-7 give rise to pro-B cells proliferating for more than three months.

#### *Long-term propagation of BM and FL pro-B cells in stromal cell-free cultures*

FL pro-B cells propagated on stromal cells plus IL-7 have previously been shown to reconstitute the B-cell compartment of immuno-deficient mice [20, 29, 30]. However, it is unlikely that pro-B cells derived from co-culture settings will ever be approved for therapeutic purposes. Therefore, we developed a stromal cell-free culture system consisting of soluble IL-7, SCF and FLT3L. As shown in figure 2A and B, EPLM from FL and BM showed very robust growth under these stromal cell-free conditions. Indeed, the growth rate of BM-derived EPLM was identical under stromal cell and stromal cell-free conditions (Fig.2A). However, growth of FL EPLM was slightly slower without stromal-cell support (Fig.2B). Stromal cell-free cultured EPLM became CD19<sup>+</sup> with ~35% BM and >75% FL-derived cells expressing CD117 (Fig.2C and D). Moreover, cells cultured without stroma expressed CD79a and b, Igl11 ( $\lambda$ 5) and Vpreb1 (Fig.2E) and had their I<sub>gH</sub> chains D<sub>H</sub>-J<sub>H</sub> rearranged (Fig.2F). Thus, both BM and FL EPLM cultured with IL-7, SCF and FLT3L alone also give

rise to long-term proliferating pro-B cells. Moreover, as shown for FL-derived pro-B cells [20], BM-derived pro-B cells expressing the anti-apoptotic Bcl2 transgene cultured with or without stromal-cell support efficiently differentiated into IgM<sup>+</sup> B cells upon IL-7 removal (Figure S1).

*In vivo B-cell reconstitution by BM or FL EPLM-derived pro-B cells.*

To test whether in vitro-generated FL or BM-derived pro-B cells could reconstitute mice, 10<sup>7</sup> EPLM-derived pro-B from FL or BM of B6 CD45.1 mice cultured with or without stroma were transferred into sub-lethally irradiated CD45.2 B6 Rag2-deficient mice. After 5-10 weeks, FACS analysis of spleen or peritoneal cavity (PerC) cells of FL (Fig 3A) or BM (Fig 4A) reconstituted mice showed that expression of donor CD45.1<sup>+</sup> was restricted to CD19<sup>+</sup> B cells (first column) and in both cases, all CD19<sup>+</sup> cells were IgM<sup>+</sup> (second column). In figure 3, WT cytograms are shown in the first row. Based on CD5 expression, FL-derived pro-B cells propagated with or without stroma gave rise to a large fraction of B1a B cells especially in the PerC (Fig.3A third column cytograms). As expected, BM-derived pro-B cells generated few CD5<sup>+</sup> B cells (Fig.4A third column cytograms). Using combined expression of CD21 and CD23 to define CD21<sup>+</sup>/CD23<sup>-</sup> marginal zone B (MZB) and CD21<sup>+</sup>/CD23<sup>+</sup> follicular B cells (FB) spleen CD19<sup>+</sup> B cells derived from FL pro-B cells propagated with or without stroma were similar (Fig3A fourth column cytograms). Thus 40 – 60% were FB and 30 – 40% were MZB. The CD19<sup>+</sup>/CD21<sup>-</sup>/CD23<sup>-</sup> cells (lower left quadrant) most likely represent B1 B cells. Immunohistochemical analysis (Fig 3B) of reconstituted mice showed the typical B cell follicular structure seen in WT mice comprising an outer ring of IgM<sup>high</sup> (green) IgD<sup>low</sup> (blue) MZB cells surrounding



metallophilic macrophages (red) with IgM<sup>positive</sup>IgD<sup>high</sup> FB inside. Mice reconstituted with BM pro-B cells cultured by the two methods also showed no obvious differences in CD21 and CD23 expression (Fig4A fourth column cytograms). However, unlike FL-derived pro-B cells, >70% of BM pro-B cell-derived B cells were MZB and only 15 – 20% FB (Fig3B fourth column cytograms).

Spleens of FL-derived pro-B cell reconstituted mice contained around  $5 \times 10^6$  B cells with no difference between cells cultured with or without stroma (Fig.4B). In contrast, spleens of mice reconstituted with BM-derived pro-B cells contained only  $0.5 \times 10^6$  B cells irrespective of whether they had been propagated with or without stroma. Thus FL-derived pro-B cells seem to be much more efficient at reconstitution than BM-derived cells.

To test this more stringently, competitive reconstitution experiments were performed. Sub-lethally irradiated CD45.2 B6 Rag2-deficient mice were reconstituted with a 1:1 mixture of  $5 \times 10^6$  CD45.2 FL-derived and CD45.1 BM-derived pro-B cells. After 8 weeks, FACS analysis of one representative mouse (Fig 4C) showed that 44% of splenocytes were CD19<sup>+</sup> of which 97.5% (42.9/44) were FL and 2.5% (1.1/4.4) BM-derived. Similar results were obtained in more than three independent experiments. Thus FL-derived pro-B cells are superior to BM-derived ones also in competitive transplantation settings.

In order to identify genes that might be responsible for these observed differences between FL- and BM-derived pro-B cells we performed RNA-sequencing analysis of the two populations. This analysis identified 218 genes differentially expressed more than 2-fold and with high significance (Table SIV). We identified the gene *Lin28B*, as the one most highly expressed in FL-derived pro-B cells compared to BM-derived pro-B (34-fold). It was recently shown that enforced *Lin28B* gene expression in adult

HSC converted them functionally into FL HSC [31] and that BM-derived pro-B cells gained FL-derived pro-B cell properties [32, 33]. To test whether *Lin28B* expression also influenced reconstitution efficiency, we introduced a MigR1 retrovirus encoding *Lin28B* into BM-derived pro-B cells propagated on OP9 stromal cells for two weeks. Then  $5 \times 10^6$  *Lin28B* transduced and  $5 \times 10^6$  non-transduced BM-derived pro-B cells were co-transferred into sub-lethally irradiated Rag2-deficient recipients. Only 2% CD19<sup>+</sup> cells were found in the spleens of these mice of which half expressed *Lin28B* (GFP) (Figure S2). Thus *Lin28B* expression did not improve the in vivo B-cell generating capacity of BM-derived pro-B cells. However, practically all CD19<sup>+</sup> cells were IgM<sup>+</sup> and > 80% *Lin28B*<sup>+</sup> cells were CD5<sup>+</sup>, thereby resembling B1 B cells. Concerning CD5 expression, *Lin28B* over-expression also converted BM pro-B cells to ones phenotypically resembling FL cells.

#### *Pro-B cell derived B cells mount a T-cell independent immune response*

To test whether the B-cell compartments of Rag2-deficient reconstituted mice were functional, 8 weeks after cell transfer they were immunized with the T-cell independent antigen NIP-Ficoll. Serum anti-NIP titers were determined one week before and two weeks after immunization. All reconstituted mice mounted a good IgM anti-NIP response with IgM titers comparable to those in immunized wild type B6 mice (Fig.5A). Mice reconstituted with BM-derived pro-B cells showed a rather low, but significant, IgG anti-NIP response (Fig.5B). However, the IgG anti-NIP response of mice reconstituted with FL pro-B cells was as high or even higher than wild type B6 mice (Fig.5B). Thus the pro-B cell reconstituted B-cell compartments were functional.

The surprisingly high IgG anti-NIP titers in FL pro-B reconstituted mice prompted us to determine their isotypes. Thus, the IgG<sub>2A</sub> and IgG<sub>2B</sub> anti-NIP titers were low (Fig 5C) and comparable to those in immunized wild type B6 mice whereas IgG<sub>1</sub> and IgG<sub>3</sub> titers were considerably higher. Although high IgG<sub>3</sub> titers are observed in other T-cell independent responses, switching to IgG<sub>1</sub> was thought to be a highly T-cell dependent phenomenon requiring IL-4 [34-36]. In addition to T cells, mast cells [37, 38], basophils [39], eosinophils [40] and ILC2s [41] may produce IL-4. ILC generation is largely dependent on IL-7 and Rag2/common gamma chain (Rag2 $\gamma$ ) double-deficient mice are practically devoid of ILCs [42, 43]. Therefore, Rag2 and Rag2 $\gamma$  double-deficient mice were reconstituted with 10<sup>7</sup> FL-derived pro-B cells and immune responses analyzed as above. Both Rag2 and Rag2 $\gamma$  double-deficient mice showed a very significant IgM and IgG anti-NIP response (Fig.6A and B), similar and/or even higher than wild type B6 mice. Moreover, both types of reconstituted mice showed a very strong IgG<sub>1</sub> and IgG<sub>3</sub> anti-NIP response (Fig.6 C and D). Thus the IgG class switching observed in immunized reconstituted mice does not seem to be regulated by ILCs.

*Reconstitution of the adaptive immune system by in vitro-propagated pro-B cells and pro-T cells*

Recently, we described a stromal cell-free culture system for the long-term propagation of pro-T cells that could be used to reconstitute the T-cell compartment of T-cell deficient mice [22]. However, reconstituted mice contained few regulatory T cells and developed a wasting disease preventable by the co-transfer of mature Treg cells or co-transfer of pro-T cells transduced with a retrovirus encoding *Foxp3-IRES-*

*GFP*. Simultaneous reconstitution with a mixture of *in vitro*-propagated pro-B cells and a non-transduced and *Foxp3* transduced pro-T cells in a 4:1 ratio resulted in T but no B-cell reconstitution (not shown). Therefore, sub-lethally irradiated B6 Rag2-deficient mice were first reconstituted with  $10^7$  FL-derived pro-B cells and 4 weeks later with  $10^7$  pro-T cells of which  $2.5 \times 10^6$  were *Foxp3*<sup>+</sup>. Six weeks after pro-T cell-transfer, a significant B and T-cell reconstitution was seen in peripheral blood cells (data not shown). Because FL pro-B cells partially reconstituted the B-cell compartment of B-cell deficient, T-cell proficient,  $\mu$ Mt mice and mounted a T-cell dependent immune response (Figure S3) we tested whether the established B and T cells were functional and could cooperate. Reconstituted mice were therefore immunized with the T-cell dependent antigen NIP-OVA. Rag2-deficient mice reconstituted with pro-B cells alone were used as controls and serum anti-NIP titers determined one week before and two weeks after immunization. No IgM or IgG anti-NIP response was observed in control pro-B cell-reconstituted Rag2-deficient mice (Fig.7A). However, mice reconstituted with both pro-B and pro-T cells mounted a strong IgM and relatively weak, but significant, IgG anti-NIP response (Fig.7A). Thus the reconstituted adaptive immune system was functional.

At 10 – 14 weeks after pro-T cell-transfer, the extent of reconstitution was assessed by FACS analysis. In one such mouse, spleen lymphocytes comprised 10% B cells, 10% CD8 T cells and 20% CD4 T cells (Fig.7B) with ~10% CD4 T cells expressing GFP, indicating they were Tregs. Spleen cell numbers of 7 individual mice 14 weeks after pro-T cell-transfer (Fig.7C) show they contained around  $3.5 \times 10^6$  CD19<sup>+</sup> IgM<sup>+</sup> B cells,  $1 \times 10^6$  CD8 T cells and  $3 \times 10^6$  CD4 T cells of which  $0.5 \times 10^6$  were GFP positive; derived from *Foxp3* transduced pro-T cells. Thus transfer of *in vitro*-

propagated pro-B and pro-T cells into Rag2-deficient mice resulted into reconstitution of a small, but functionally active, adaptive immune compartment.

## Discussion

To our knowledge, this is the first report describing the long-term propagation of BM-derived pro-B cells and their use in reconstituting a functional immune system in immune-deficient recipients. Pro-B cells from Pax5<sup>-/-</sup> mice were previously shown to have multi-lineage developmental potential [44] and we identified an equivalent B220<sup>+</sup>CD117<sup>+</sup>CD19<sup>-</sup>NK1.1<sup>-</sup> EPLM cell with lymphoid and myeloid developmental potential (called EPLM) in the BM of WT mice [21]. Herein we show that BM-derived EPLM cultured on OP9 stromal cells plus IL-7 differentiated into CD19<sup>+</sup>, CD79a<sup>+</sup>, CD79b<sup>+</sup>, Igl11<sup>+</sup> and Vpreb1<sup>+</sup> pro-B cells with rearranged D<sub>H</sub>-J<sub>H</sub> genes. Unlike freshly isolated CD19<sup>+</sup> CD117<sup>+</sup> pro-B cells, those derived from EPLM could be cultured long-term *in vitro*. Gene expression profiling did not reveal an obvious explanation for this difference in growth capacity but could possibly be regulated via RNA modification or at the translational level [45-47].

To date, long-term growth of pro-B cells required co-culture on stromal cells, yet the specific role of stromal cells in this culture system was unknown. Clearly IL-7, IL-7R (CD127), SCF and its receptor (CD117) as well as FLT3 and its receptor (CD135) all play a role in B-cell development and/or pro-B cell growth [11, 12, 15, 16, 18, 19]. Indeed, we now show that EPLMs efficiently grow and differentiate into pro-B cells when cultured in the combined presence of IL-7, SCF and FLT3L without contact with stromal cells. This indirectly suggests that SCF and FLT3 can substitute for stromal cells.

Pro-B cells maintained on stromal cells plus IL-7 could reconstitute the B-cell compartment of immunodeficient mice [20] and herein we extend these findings. Both

FL and BM EPLM-derived pro-B cells propagated either on stromal cells or stromal cell-free generated a significant B-cell compartment upon transfer into Rag2-deficient mice. However, FL-derived pro-B cells were about 40 fold more efficient at B-cell reconstitution than their BM-derived partners.

Although pro-B cells expressing the *Lin28B* can acquire characteristics of FL-derived pro-B cells [32] and *in vitro*-propagated *Lin28B*-expressing BM-derived pro-B cells could generate CD5 positive B cells *in vivo*, there was no improvement in the *in vivo*-reconstitution capacity of BM pro-B cells.

An enlarged MZB compartment is frequently observed in B lymphopenic mice [48-52] suggesting that newly-formed B cells first fill the MZB compartment. This phenomenon could be due either to a specialized environment within the splenic marginal zone or that only B cells expressing certain BCR home to and expand in this anatomical location [53].

When immunized with the T-independent antigen NIP-Ficoll, all reconstituted mice mounted an anti-NIP response indicating their B cell compartments were functional.. Mice reconstituted with BM-derived pro-B cells had a slightly lower IgM anti-NIP titer than WT mice possibly due to poor B cell reconstitution and showed a significant IgG anti-NIP response although still at least 10 fold lower than WT mice. In marked contrast, Rag2-deficient mice reconstituted with FL pro-B cells mounted an IgM and IgG anti-NIP response that was identical, or even higher than, WT mice suggesting that fetal progenitor-derived B cells are the main anti-NIP responders in a primary immunization. In B6 mice, the primary anti-NIP antibodies use predominantly  $\lambda 1$  light chains [54-56]. The anti-NIP response of pro-B cell-reconstituted mice was likewise dominated by  $\lambda 1$ -containing antibodies (data not shown) indicating that the

anti-NIP B-cell repertoires in pro-B cell reconstituted mice are identical to those of WT mice.

Surprisingly, FL pro-B cell-reconstituted mice had a high IgG<sub>1</sub> anti-NIP response an antibody class normally associated with IL-4. Since reconstituted mice were devoid of T cells, the question arose which cell type was responsible for IL-4 production [34-36]. Pro-B cell reconstituted Rag2 $\gamma$  double-deficient mice, deficient in ILCs, also mounted high IgG<sub>1</sub> response suggesting that ILCs were not the source of IL-4 [42, 43]. Mast cells, basophils and/or eosinophils, could be the source of IL-4 [37-40]. FL pro-B cells were also able to reconstitute a functional B-cell compartment in T-cell containing, B-cell deficient  $\mu$ Mt mice (Figure S3). This result might seem surprising given that the T-cell compartments in these mice may not be tolerant to mature B cells.

Pro-T cells propagated *in vitro* under stromal free conditions can reconstitute the T-cell compartments of T-cell deficient mice [22]. Here we show that the combined transfer of stromal cell-free-propagated pro-B and pro-T cells into Rag2-deficient mice results in the generation of a functional adaptive immune repertoire capable of mounting a T-dependent antibody response. For efficient reconstitution, pro-T cell transfer had to be performed 2-3 weeks after the pro-B cell reconstitution suggesting that when transferred together, T cells develop that are not tolerant to B cells and therefore eliminate them.

Overall the findings described herein show that progenitor lymphocytes can be readily propagated under stromal free conditions *in vitro* and that these cells can be used to reconstitute mice with mature functional lymphocytes. Based on these results the establishment of stromal cell-free culture systems for human lymphocyte progenitors might be of great interest since unlike stromal cell-propagated lymphocyte



progenitors, stromal cell-free propagated cells could be potentially used for therapeutic purposes in patients with B and/or T-cell deficiencies.

## Materials and methods

### *Mice*

Female C57BL/6 CD45.1 and CD45.2, C57BL/6 Rag2-deficient [23], and C57BL/6 Bcl-2 transgenic mice [24] with 5–8 weeks of age were used. The appearance of vaginal plugs was counted as day 0 of gestation and embryos were taken at day E17.5. All mice were bred and maintained in our animal facility under specific pathogen-free conditions. Animal experiments were carried out within institutional guidelines (authorization numbers 1886 and 1888 from Kantonales Veterinäramt, Basel).

### *Cell lines, cell culture, and supplements*

The OP9 stromal cell line [25] was cultured as a monolayer in IMDM supplemented with 2% FBS,  $5 \times 10^{-5}$  M  $\beta$ -mercaptoethanol, 1 mM glutamine, 0.03% w/v Primatone (Quest, Naarden, The Netherlands), and 100 U/mL penicillin. For pro-B cell culture, CD117<sup>+</sup>B220<sup>+</sup>CD19<sup>-</sup>NK1.1<sup>-</sup> cells were sorted from the FL or the femoral BM of adult mice and cultured at  $10^4$ /ml in supplemented IMDM either on a semi-confluent layer of 30 Gy  $\gamma$ -irradiated OP9 stromal cells in the presence of 100U/ml IL-7, or without stromal cell support but in the presence of 100U/ml IL-7, 50ng/ml FLT3L, and 100ng/ml SCF. IL-7 was derived from culture supernatant of J558L cells transfected with murine IL-7 cDNA. Polyhistidin-tagged SCF was purified from transfected Rosetta pLacI bacteria using Ni-NTA-agarose beads (Qiagen, Venlo, NL). A vector expressing a human FLT3L-Fc fusion protein was expressed in Chinese hamster ovary cells. The supernatant was passed over a protein A-Sepharose (GE Healthcare,

Chalfont St. Giles, GB) column in order to purify the protein. Pro T cells were cultured as previously described [22].

#### *Antibodies, flow cytometry, and sorting*

FITC-, PE-, allophycocyanin-, or biotin-labeled mAbs specific for CD117, B220, CD19, NK1.1, IgM, Ig $\kappa$ , CD5, CD45.1, CD21, CD23, CD4, CD8 $\alpha$ , and TCR $\beta$  were either purchased from BD Biosciences (Franklin Lakes, NJ, USA) or eBiosciences (San Diego, CA, USA), or purified from hybridoma culture supernatants according to standard procedures. Staining of the cells was performed as described before [26]. Flow cytometry was done using a FACS Calibur (BD Biosciences) and data were analyzed using the CellQuest Pro (BD Biosciences) or FlowJo Software (Treestar). For cell sorting, a FACS Aria IIu (BD Biosciences) was used (>98% purity).

#### *Transfer of cultured progenitor cells*

Recipient mice were  $\gamma$ -irradiated using a Cobalt source (Gammacell 40, Atomic Energy of Canada, Ltd) 4h prior to reconstitution. The indicated number of pro-B or pro T cells was then injected into the tail vein.

#### *Immunohistochemical analysis*

To analyze pro-B-cell derived B-cell localization in the spleen, the 5 $\mu$ m snap frozen and acetone-fixed sections were incubated with FITC-labelled anti-IgM (clone M41, self-made), APC-labelled anti-IgD (clone 1.19 self-made), and biotinylated anti-MOMA-1 (Vector, Burlingame, CA), which was revealed with PE-conjugated streptavidin (SouthernBiotech, Birmingham, AL). Confocal microscopy images were

taken with a LSM 510 Meta (Zeiss, Oberkochen, D) and analyzed using the ImageJ software and the Fiji image processing package.

#### *NIP-specific antibody responses*

Reconstituted mice were immunized subcutaneously with 100 $\mu$ g NIP-Ficoll or NIP-OVA in a 1:1 CFA emulsion. Serum IgM and IgG against NIP was analyzed at day 14 using ELISA as described in [27].

#### *PCR analyses*

The PCR conditions for amplifying  $D_HJ_H$  rearrangements were described elsewhere [4, 6, 28]. The primers used were  $D_H$  5'-TTCAAAGCACAATGCCTGGCT-3' and  $J_H3$  5'-GTCTAGATTCTCACAAGAGTCCGATAGACCCTGG-3'. Oligonucleotide primers used for CD79A verification from pro-B cDNA were 5'-TGTTTGGGTCCCGGATGCCA-3' and 5'-CACGCGGAGGTAAGTACCACA-3', for CD79B 5'-TCTTCTCAGGTGAGCCGGTA-3' and 5'-TATGGTTGGCGCTGTCACAT-3', for IGLL1 5'-AGTAGGACAGACTCTGGGCA-3' and 5'-GGCTGACCTAGGATTGTGAGC-3', for VPREB1 5'-CTCCGGGTCCAAAGATACGAC-3' and 5'-GCTCATAGCAACACCGCAGAA-3', and for beta-actin 5'-GAAGTCTAGAGCAACATAGCACAGCTTCTC-3' and 5'-GTGGGAATTCGTCAGAAGGACTCCTATGTG-3'.

#### *Statistical analysis*

Statistical analysis was performed with Prism 6.0g software (GraphPad Software, Inc.). Two-tailed unpaired Student *t* tests were used for statistical comparisons. If not

differently indicated, data are presented as mean values  $\pm$  SEM from three independent experiments. ns not significant or  $P > 0.05$ , \*<sup>or +</sup>  $P \leq 0.05$ , \*\*<sup>or ++</sup>  $P \leq 0.01$ , \*\*\*<sup>or +++</sup>  $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ .

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#### Disclosure of Conflicts of Interest

The authors declare no commercial or financial conflict of interest.

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## Figure Legends

### Figure 1

Establishment of EPLM-derived long-term proliferating pro-B cell lines from BM and FL. FL and BM-derived CD19<sup>-</sup>B220<sup>+</sup>CD117<sup>+</sup>NK1.1<sup>-</sup> cells were sorted and maintained on OP9 stromal cells plus IL-7 (A) Gating strategy for sorting of CD19<sup>+</sup> and CD19<sup>-</sup>B220<sup>+</sup>CD117<sup>+</sup> BM cells. (B) In vitro growth capacity of sorted CD19<sup>+</sup> and CD19<sup>-</sup> BM cells on OP9 stromal cells plus IL-7, measured by XXX. (C) Comparison of the in vitro growth of FL and BM-derived pro-B cells for about 40 days. (D) Representative FACS plots showing B220, CD19 and CD117 expression in FL and BM-derived pro-B cells cultured for 12 days. (E) RT-PCR analysis of *Cd79a*, *Cd79b*, *Igll1*, *Vpreb1*, and *Actb* expression in FL and BM-derived pro-B cells cultured for 12 days. (F) Genomic D<sub>H</sub>-J<sub>H</sub> rearrangement analysis of FL and BM-derived pro-B cells cultures for 12 days. Data shown are from single experiments/analyses which are representative of XXX experiments/analyses performed.

### Figure 2

Establishment of EPLM-derived long-term proliferating pro-B cell lines from BM and FL under stromal cell-free conditions. BM or FL-derived CD19<sup>-</sup>B220<sup>+</sup>CD117<sup>+</sup>NK1.1<sup>-</sup> cells were sorted and maintained either on OP9 stromal cells plus IL-7 or in the presence of IL-7, SCF and FLT3L without stromal cells. (A) Comparison of the growth capacity of BM-derived pro-B cells cultured in the absence (black squares) or presence (white circles) of stromal cells for about 40 days, assessed by XXXX. (B) Comparison of the growth capacity of FL-derived pro-B cells cultured in the absence

(black squares) or presence (white circles) of stromal cells for about 40 days. (C) Representative FACS plots showing B220, CD19 and CD117 expression on BM-derived pro-B cells cultured for 14 days with or without stromal cells, as indicated. (D) Representative FACS plots showing B220, CD19 and CD117 expression on FL-derived pro-B cells cultured for 14 days with or without stromal cells, as indicated. (E) RT-PCR analysis of *Cd79a*, *Cd78b*, *Igll1*, *Vpreb1* and *Actb* expression in FL and BM-derived pro-B cells cultured for 14 days in the presence of IL-7, SCF and FLT3L without stromal cells. (F) Genomic D<sub>H</sub>-J<sub>H</sub> rearrangement analysis of FL and BM-derived pro-B cells cultured for 14 days in the presence of IL-7, SCF and FLT3L without stromal cells. Data shown are from single experiments/analyses which are representative of XXX experiments/analyses performed.

### Figure 3

In vivo reconstitution potential of FL-derived pro-B cells propagated in vitro. Sub-lethally irradiated CD45.2 B6 Rag2-deficient mice were injected intravenously with 10<sup>7</sup> CD45.1 FL-derived pro-B cells propagated in the presence or absence of stromal cells. (A) Representative FACS analysis of B-cell populations in the spleen and peritoneal cavity (PerC) of WT controls and recipient mice 8 weeks after cell transfer of the indicated pro-B cells. (B) Representative picture of a staining for IgM (green), IgD (blue), and metallophilic macrophages (MOMA-1 in red) on a spleen section of mice reconstituted with FL-derived pro-B cells propagated on OP9 stromal cells (20× magnification). In total, 28 individual mice were injected: 14 mice with FL-derived pro-B cells propagated with OP9 stromal cells and 14 mice with FL-derived pro-B cells propagated without stromal cells.

#### Figure 4

In vivo reconstitution potential of BM-derived pro-B cells propagated in vitro and comparison to the corresponding potential of FL-derived pro-B cells. Sub-lethally irradiated CD45.2 B6 Rag2-deficient mice (n=21) were injected intravenously with  $10^7$  CD45.1 BM-derived pro-B cells propagated in the presence (14 mice injected) or absence (7 mice injected) of stromal cells. (A) Representative FACS analysis of B-cell populations in the spleen and peritoneal cavity (PerC) of WT controls and recipient mice 8 weeks after cell transfer of the indicated pro-B cells. (B) Absolute numbers of CD19<sup>+</sup> IgM<sup>+</sup> cells in the spleens of mice injected with BM-derived pro-B cells and FL-derived pro-B cells, as determined by flow cytometry (Figure 3).

Unpaired student's t-test. Data shown above are mean  $\pm$  SEM and each circle or square is an individual mouse. ns: not significant or  $P > 0.05$ , \*\* $P \leq 0.01$  and \*\*\*\* $P \leq 0.0001$ . (C) CD19, IgM, and CD45.1 expression from a spleen of a CD45.2 B6 Rag2-deficient mouse 8 weeks after transfer of  $5 \times 10^6$  CD45.2 FL-derived pro-B cells and  $5 \times 10^6$  CD45.1 BM-derived pro-B cells. Both pro-B cells were propagated stromal cell-free. A representative FACS analysis out of 3 independently performed experiments is shown.

#### Figure 5

T-cell independent responses of mice reconstituted with pro-B cells propagated in the absence or presence of stromal cells. B6 Rag2-deficient mice were reconstituted with BM or FL derived pro-B cells propagated with or without stromal cells and immunized with NIP-Ficoll 8 weeks after cell transfer. Wild-type B6 (WT) mice were used as controls and statistical significance compared to WT is indicated below the after immunization data points (black squares). Titers were defined as the serum

dilutions that gave 2 times background OD values in the ELISA. Titers of the indicated antibodies were determined in sera taken (A, B) 1 week before immunization (open symbols) and 2 weeks after immunization closed symbols). (A) IgM anti-NIP titers. (B) IgG anti-NIP titers. (C) Anti-NIP IgG subclass titers in the serum of mice reconstituted with FL-derived pro-B cells and WT mice. ns: not significant or  $P > 0.05$ , \* or  $^+ P \leq 0.05$ , \*\* or  $^{++} P \leq 0.01$ , \*\*\* or  $^{+++} P \leq 0.001$ , \*\*\*\*  $P \leq 0.0001$ . \* represents significance compared with levels in pre-bleed.  $^+$  represents significance compared with titer of WT mice. Each symbol represents an individual mouse. Unpaired student's t test;  $n = 5-8$ . Data shown above are mean  $\pm$  SEM and collective data from two independent experiments are shown.

#### Figure 6

Anti-NIP responses of WT mice, Rag2-deficient and Rag2 $\gamma$  double-deficient mice reconstituted with FL pro-B cells propagated on stromal cells and immunized with NIP-Ficoll 8 weeks after cell transfer. Titers were defined as the serum dilutions that gave 2 times background OD values in the ELISA. (A, B) Titers were determined in sera taken 1 week before immunization (open symbols) and 2 weeks after immunization (closed symbols). (A) IgM anti-NIP titers. (B) IgG anti-NIP titers. (C) IgG1 anti-NIP titers. Sera from B6 mice immunized with NIP-Ficoll or NIP-OVA were used as positive controls. (D) IgG3 anti-NIP titers. Sera from B6 mice immunized with NIP-Ficoll or NIP-OVA were used as positive controls. Collective data from two independent experiments are shown and each symbol represents an individual mouse. ns: not significant, \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ , \*\*\*\*  $P \leq 0.0001$ . Unpaired student's t test;  $n = 5-8$ . Data shown above are mean  $\pm$  SEM.



## Figure 7

In vivo lymphoid reconstitution and immune responses in mice injected with in vitro propagated pro-B and pro-T cells. Sublethally irradiated Rag2-deficient mice were reconstituted with FL-derived pro-B cells and 4 weeks later with a 3:1 mixture of in vitro propagated pro-T cells [22] and Foxp3-transduced in vitro propagated pro-T cells. Mice were immunized with NIP-OVA 11 weeks after pro-B cell transfer and splenocytes analyzed by flow cytometry 3-7 weeks later. (A) IgM (left) and IgG (right) anti-NIP responses of reconstituted and immunized B6 Rag2-deficient mice. Sera were collected 1 week before and 2 weeks after immunization. (B) Representative FACS analysis of splenic B-cell and T-cell populations 10-14 weeks after pro-T cell transfer. (C) Absolute numbers of T cells and B cells found in the spleens of reconstituted mice. ns: not significant or  $P > 0.05$ , \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ , \*\*\*\*  $P \leq 0.0001$ . Unpaired student's t test; n = 7-9. Numbers above data points indicate mean  $\pm$  SEM. Each symbol represents an individual mouse and data pooled from two independent experiments are shown.