Genetic manipulation and immortalised culture of ex vivo primary human germinal centre B cells

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Abstract

Next generation sequencing has transformed our knowledge of the genetics of lymphoid malignancies. However, limited experimental systems are available to model the functional effects of these genetic changes and their implications for therapy. The majority of mature B cell malignancies arise from the germinal centre (GC) stage of B cell differentiation. Here we describe a detailed protocol for the purification and ex vivo expansion of primary, non-malignant, human GC B cells. We present methodology for the high efficiency transduction of these cells to enable combinatorial expression of putative oncogenes. We also describe alternative approaches for CRISPR/Cas9-mediated deletion of putative tumour suppressors. Mimicking genetic changes commonly found in lymphoid malignancies leads to immortalised growth in vitro, whilst engraftment into immunodeficient mice generates genetically customised, synthetic models of human lymphoma. The protocol is simple and inexpensive and can be implemented in any laboratory with access to standard cell culture and animal facilities. It can be easily scaled up to enable high-throughput screening and thus provides a versatile platform for the functional interrogation of lymphoma genomic data.

Introduction

Germinal centres (GC) are transient microanatomical structures that form within secondary lymphoid tissue following the encounter of a naïve B cell with cognate antigen in the context of T cell help[1]. Within the GC, B cells undergo intense proliferation and expansion associated with a transcriptional programme enforced in part by the transcription factor BCL6[2]. GC B cells undergo somatic hypermutation of their immunoglobulin genes followed by selection for variants that produce the highest affinity antibody. The GC reaction is essential for proper humoral immunity; however, the GC is also the origin of a majority of B cell lymphomas, most notably Diffuse Large B cell Lymphoma (DLBCL), Burkitt Lymphoma and Follicular Lymphoma[1,3]. Recent sequencing studies, which have collectively examined thousands of cases, reveal an extensive and heterogeneous catalogue of recurrent genetic alterations associated with these lymphomas[4-20]. A major challenge now exists to determine the functional contribution of each genetic alteration to lymphomagenesis and establish the potential implications for therapy.

To date, most functional studies of putative lymphoid cancer genes have relied on lymphoma cell lines or transgenic mice. However, lymphoma cell lines have an extensive mutational burden that can make it hard to interpret the effects of experimentally induced genetic changes. Transgenic mice are costly to create and poorly suited to high-throughput experiments. Moreover, the genetic requirements to transform mouse cells may differ from those of human cells[21]. Alternative experimental models are therefore required to study the genetic pathogenesis of lymphoma. We reasoned that, as the cell of origin of most B cell lymphomas, the human GC B cell would be a good model. However, technical difficulties in genetic manipulation and culture of these cells in vitro have limited the use of primary human GC B cells in functional and genetic experiments. We therefore developed and optimised a strategy for the ex vivo expansion, viral transduction and immortalisation of human GC B cells. We recently reported the first use of this experimental strategy as a tool to study the genetics of lymphoid malignancy, including high-throughput functional genetic screening and the creation of genetically customised lymphoma models[22] (**Figure 1**).

Development of the protocol.

To develop a protocol for ex vivo culture of GC B cells we built upon previously reported strategies to mimic the GC microenvironment, such as providing the cells with critical survival signals supplied in vivo by follicular dendritic cells (FDCs) and T follicular helper cells (TFHs). Previous reports had established a requirement for CD40 ligand (CD40Lg), in either cross-linked or membrane-anchored form, combined with soluble cytokine[23-25]. Subsequent studies utilised FDC-like cells (HK cells) either transfected with CD40Lg or in the presence of anti-CD40 antibody, plus cytokine[26,27]. Mouse knockout studies showed that the cytokine IL21 regulates B cell proliferation and differentiation; revealing its importance for the GC reaction in vivo[28,29]. We therefore transduced an immortalised FDC-like line YK6 with membrane bound CD40Lg and IL21[22]. When these cells were co-cultured with human GC B cells, we observed vigorous expansion of the GC B cells in ex vivo coculture for 7-10 days[22]. We had thus removed the requirement for the GC B cells to be grown in exogenous recombinant cytokine.

Human GC B cells are resistant to transduction with classical amphotrophic or VSVG pseudotyped virus, however we found that they express high levels of *SLC20A1*, the receptor for the Gibbon Ape Leukaemia Virus (GaLV) envelope[22]. Based on previous reports[30,31], we constructed a series of GaLV-MuLV fusion envelopes and identified GaLV-MTR as an envelope that allowed high efficiency transduction of human GC B cells using either lentivirus or retrovirus[22]. We used this system to screen common lymphoma driver genes to identify combinations that led to immortalised GC B cell growth in vitro or tumour formation in vivo[22]. Combined BCL2 and BCL6 expression emerged as one combination able to immortalise GC B cells in vitro. In contrast, a minimum of four oncogenic hits was required to generate models of high-grade B cell lymphoma grown in mice in vivo[22].

Applications of the protocol.

Our experimental system represents a simple and inexpensive approach for the ex vivo expansion and genetic modification of primary human GC B cells. We anticipate the primary users of this protocol will be researchers working to understand the functional genetics of B cell malignancies. The ability to manipulate the genetics of human GC B cells and to generate bespoke models of human lymphoma provides a new experimental approach for the interrogation of lymphoma genetics. For instance, we used GC B cells immortalised with

BCL6 and BCL2 as a genetically-defined cellular platform for a gain-of-fitness CRISPR screening[22]. This identified *GNA13* (a gene recurrently mutated in GC-derived lymphomas) as a potent suppressor of growth and survival in human GC B cells, a finding that was not evident when the same screen was performed in established lymphoma cell lines[22]. In a separate initiative, we used GC B cells transduced with MYC and DDX3X to study the role of *DDX3X* mutations in the development of Burkitt lymphoma[32].

The potential to expand human GC B cells ex vivo and to immortalise them will also present new experimental opportunities to immunology researchers. To date almost all mechanistic GC research has been conducted in murine cells using similar coculture systems developed for the creation and transduction of induced GC B cells from stimulated murine naïve splenic B cells[33]. Our culture system allows for similar experiments to be conducted in human GC B cells. Finally, we anticipate this strategy could also be used to investigate the interaction of B cells with viruses such as EBV. We have demonstrated that our system can be used to stably express individual viral genes in human GC B cells[34].

Comparison with other methods.

This protocol complements existing tools commonly used for the interrogation of lymphoma genetics but has some important differences. Firstly, in contrast to cell lines, putative oncogenic modifications are introduced into an otherwise genetically normal background. This allows for the creation of genetically-customised experimental models not currently represented in our existing cell line armamentarium. Secondly, cell lines have evolved over years in culture for optimal in vitro growth. Therefore, whilst they are well-suited to loss-of-fitness screening, it is rare that introduction of an oncogene or disruption of a tumour suppressor will lead to a gain-of-fitness phenotype. The converse is true when using primary cells; non-transformed, genetically normal GC B cells do not survive long-term in culture without the introduction of oncogenic genetic alterations. Therefore, they are ideally suited for screening of genetic and other factors that promote growth, survival and cellular transformation. Finally, a practical advantage over murine cells is the scalability of the system. Tens of millions of GC B cells can be purified from a single human tonsil. These are grown on feeder cells engineered to express components required to stimulate B cell growth, thereby avoiding the need to purchase the large quantities of costly recombinant CD40 ligand and cytokines that would otherwise be required for large-scale experiments.

Limitations.

Not every genetic alteration predicted to be oncogenic leads to a competitive fitness advantage in our system. Examples include the histone acetyltransferase CREBBP. Whilst well established as a tumour suppressor in GC lymphomas, the depletion of this gene was not associated with a competitive advantage in our CRISPR screens[22]. We postulate a number of explanations for this and similar findings. Firstly, there is increasing realisation that genetic corruption during lymphomagenesis may begin at an early stage of B cell development; thus, the impact of these early alterations may be much less evident when introduced into mature GC B cells. Secondly, our use of BCL2 and BCL6 to immortalise GC B cells, combined with strong CD40Lg and IL21 stimulation, may limit the "oncogenic space" available for further co-operating events. We envisage that changes to the oncogenic platform used to immortalise B cells or to the nature of the cytokine stimulation could address this limitation. Thirdly, viral overexpression or CRISPR knockout provide little control over the degree of target overexpression or knockdown and there may be genes where the optimal oncogenic window of expression is not achieved. Finally, our system has no ability to model the interaction between tumour cells and the host immune system. All these problems are of course shared with standard cell line models of lymphoma and represent some of the biggest challenges for the development of further improved preclinical models in the future.

Experimental design.

The protocol has four main stages. We also highlight alternative strategies that are available at each stage and could facilitate adaptation to individual experimental requirements.

Purification of human GC B cells from discarded tonsil tissue (Steps 1-17). Existing strategies to purify human GC B cells have predominantly employed flow cytometric cell sorting[35,36]. Flow cytometric approaches enable highly purified subsets of GC B cells, such as light zone and dark zone GC B cells, to be collected. This can be beneficial to address specific experimental questions however, only a relatively small number of cells can be sorted in a single day. Furthermore, the logistical requirement to book access to specialist sorting machinery is often incompatible with the unpredictable schedule associated with use of tissue derived from human patients. We therefore customised a strategy to purify cells based on magnetic beads. It does not require access to specialist equipment and was designed to be flexible, scalable and fast (**Figure 2**). We typically purify up to one hundred million GC B cells (purity >80-90%, **Figure 3**) from a single tonsil and cells are ready for culture in less than three

hours. By minimising the time spent manipulating cells we aim to minimise the stress imposed upon these comparatively fragile primary cells. We also provide details of how to purify either total B cells or memory B cells from tonsil tissue. We have successfully used commercially available magnetic bead-based kits (Miltenyi Biotec) to purify naïve or memory B cells from peripheral blood. These peripheral blood B cells can also be expanded in our coculture system; however, the comparatively small cell yield from peripheral blood (typically around $0.5x10^6$/10ml blood) has made tonsil tissue our preferred source of human B cells and the most practical source of GC B cells, a B cell subset that is not found in the peripheral blood. Some tonsils will contain B cells that are latently infected with EBV which may complicate the interpretation of long-term cultures. Thus, it is important to screen isolated GC B cells for the presence of EBV DNA, which we describe in detail in Box 1. We have used both male and female donors and have not observed sex differences in our experiments. However, donor sex should be considered an important biological variable and care taken to minimise the impact that sex differences may have when designing specific experiments.

Ex vivo expansion of human GC B cells (Steps 18-21). Human GC B cells are expanded by coculture with a human FDC-derived feeder line engineered to express CD40Lg and IL21. This combination of stimuli was selected to maximise proliferation and transduction efficiency. Feeder cells express all required stimulation, allowing large-scale and long-term experiments to be conducted without a need to culture in the presence of recombinant cytokines. In the main procedure we describe how to use YK6-CD40Lg-IL21 as the feeder system (**Figure 4A**). Whilst we have found CD40Lg to be indispensable, the cytokine provided can be adapted to suit individual experimental requirements. We found IL21 provided the most potent expansion ex vivo. However soluble IL4 is also able to support growth of human B cells in the presence of CD40Lg-expressing feeder cells (**Figure 4B**) and there may be experimental circumstances in which this cytokine might be preferred. Varying the cytokine stimulus provided allows adaptation of the procedure to suit the needs of specific experiments. In contrast to previous feeder systems developed to support mouse B cells[33], we did not detect an additive benefit if BAFF was also expressed by our feeder cells (**Figure 4C, Supplementary Figure 1A**).

We anticipate that users may wish to further adapt the feeder system for their own experimental needs. Therefore, we also provide details of how to generate customised feeder lines, including

the generation and use of an alternative, murine fibroblast feeder line (L-cells) transduced with CD40Lg (**Supplementary Figure 1B&C) (See Box 2)**. This feeder line, with the addition of soluble IL21, also supports the growth of B cells, although cell proliferation is not as vigorous as with YK6-CD40Lg-IL21 (**Figure 4D**). However, a murine feeder line may be advantageous for experiments involving next generation sequencing, since it allows reads arising from contaminating feeder cells to be filtered in silico. Others have used stably transfected murine L cells to support B cell growth[24,37], however we found that combinations of ligand and cytokine are more easily engineered in a stable fashion into both murine fibroblasts and YK6 cells using retroviral transduction (**Supplementary Figure 1D and Box2).**

Viral transduction and CRISPR knockout in human GC B cells. (Steps 22-27). Primary human GC B cells have previously been considered refractory to standard transduction or transfection techniques. Our optimised protocol uses modified viral envelopes to facilitate high efficiency transduction by either retrovirus or lentivirus[22]. This allows combinatorial expression of oncogenes or the incorporation of CRISPR/Cas9 technology. The main procedure describes the viral transduction of Cas9 and gRNA into primary GC B cells. This leads to efficient target deletion over a period of 5-9 days (**Figure 5**). Using this approach, the CRISPR machinery and any associated markers (eg GFP) become permanently integrated into the target cell genome, a feature that allows experimental tracking of deleted cells in screening experiments. However, in other situations, this residual genetic material and the ongoing expression of Cas9 protein may not be desirable. We therefore also present an alternative protocol based upon the transient nucleofection of ribonucleoprotein (RNP) complexes (**Box 3**, **Figure 6**). Both approaches lead to efficient depletion of the target protein (**Figure 5&6**) and either strategy may be selected depending upon the experimental aims. CRISPR knockout experiments should also include non-targeting gRNA as the appropriate control. Where experiments aim to overexpress mutant oncogenes, the wild type construct and the empty vector would be appropriate controls.

Strategies for B cell immortalisation and in vivo tumour formation (Steps 28-33). Genetic manipulation of human GC B cells can lead to immortalised growth in vitro. It also allows the establishment of genetically customised models of human lymphoma in immunodeficient mice (**Figure 7**). Therefore, genetic screening can be performed both in vitro and in vivo. In vitro experiments assess the ability of genetic alterations to drive proliferation, survival and immortalisation of B cells. In contrast, in vivo experiments assess the ability to drive

transformation to a malignant B cell, that grows in vivo in the absence of the feeder stimulation. We have shown how the combination of forced BCL2 and BCL6 expression is sufficient to drive B cell immortalisation in vitro (**Supplementary Figure 2**). These immortalised cells remain dependent on the YK6-CD40Lg-IL21 feeders and do not form tumours when injected into mice[22]. In contrast, further genetic hits were required to drive full transformation in vivo. The combination of MYC, BCL2, CCND3 and a dominant negative P53 emerged as the most powerful combination of genetic hits tested and led to tumour formation in all mice within 30 days of injection[22]. We anticipate that other combinations of genetic alterations able to drive B cell immortalisation and transformation will be tested and identified in future studies.

Immunodeficient mice such as NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice are excellent tools for cancer xenograft experiments as they permit the engraftment of human cells. They have been widely used for other research areas such as stem cell biology, humanised mice, and infectious disease research. Whilst we have only used the NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) strain for our experiments, alternatives might include other immunodeficient mice such as NOD.Cg-Rag1tm1MomIl2rgtm1Wjl/SzJ or NOD.Cg-PrkdcscidIl2rgtm1WjlTg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ. Future research may attempt to recapitulate the immune microenvironment by utilising humanised immune system mice or by the simultaneous engraftment of autologous T cells harvested from the same tonsil. Our experiments have predominantly used male mice. We have not compared the engraftment or transformation efficiencies between male and female mice; however, the sex of recipient mice should be considered an important biological variable when researchers are designing these xenograft experiments. We present a protocol here for subcutaneous injections, but researchers may also explore alternative routes of injections such as intravenous or intraperitoneal injection.

Materials

Biological materials

- Fresh human tonsil tissue. !CAUTION Usually tonsil tissue derives from donors that have not been screened for blood-borne viral infection and so it should be handled at containment level 2 with appropriate precautions. The proper use and disposal of sharps is particularly important. Appropriate risk assessments must be in place. !CAUTION Appropriate ethical approval should be obtained prior to working with human tissue. For the experiments shown here, fresh, tonsil tissue was sourced from the Addenbrooke's ENT Department, Cambridge with written informed consent of the patient/parent/guardian and processed directly to preserve viability. Ethical approval for the use of human tissue was granted by the Health Research Authority Cambridgeshire Research Ethics Committee (REC no. 07/MRE05/44). !CRITICAL We routinely store surgically-excised tonsil tissue for up to 4 hours at 4°C without noticeable loss of cell viability. !CRITICAL We prefer paediatric tonsils because they contain a high proportion of lymphoid tissue, are less likely to contain latent EBV infected cells and are typically discarded at source in the operating theatre without requiring histological examination.
- Feeder cell line YK6-CD40Lg-IL21 (RRID CVCL_A2XK https://scicrunch.org/resolver/RRID:CVCL_A2XK) (Available on request from Hodson Laboratory). The generation of this feeder line was previously reported[22]. See **Box 2** for generation of alternative feeder systems. !CAUTION The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

- Lenti-X 293T Cell Line (Clontech Laboratories, cat. no. 632180)
  !CAUTION The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma
    - Mice: sex and age-matched immunodeficient mice such as NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice (Jackson laboratories, JAX ID 005557). !CAUTION All experimental procedures involving mice must conform to institutional and national regulations and be performed by appropriately trained researchers in dedicated experimental animal facilities. The procedure we describe here follows the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 and the results obtained here were undertaken following ethical

review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB-PPL number P846C00DB).

Reagents

GC B cell purification reagents

- Surgical Scalpels (FisherScientific; 11728353)

  !CAUTION Appropriate risks assessment should be made and followed when using sharps and unscreened human tissue.
- Syringes 5ml (Appleton Woods Ltd; GS575)
- Falcon® 70 µm Cell Strainer (Corning; 352350)
- Stainless steel fine tweezers (Onecall, cat. no. 1779183)
- UltraPure™ 0.5M EDTA, pH 8.0 (Thermo Fisher Scientific, cat. 15575020)
- human B cell negative selection isolation Kit II (MACS, Miltenyi Biotec, cat.no. 130-091-151)
- MACS LS columns (Miltenyi; 130-041-306)
- Gibco PBS (calcium- and magnesium-free; Thermo Fisher Scientific, cat. no. 10010-015)
- Anti-Human IgD-Biotin, Clone: IADB6 (SouthernBiotech, cat.no. 9030-08, RRID AB_2796594)
- Anti-Human CD44- Biotin, Clone: F10-44-2 (SouthernBiotech, cat.no. 9400-08, RRID AB_2796778)
- Lymphocyte Separation Medium (Lonza; 17-829E)

Flow cytometry and sorting reagents

- CD38 Mouse anti-Human, PE, Clone: HB7 (ebioscience, cat.no. 12-0388-42, RRID AB_1518748; https://scicrunch.org/resolver/RRID:AB_1518748)
- CD38-APC (17-0389-42 ThermoFisher Scientific, RRID AB_1834353; https://scicrunch.org/resolver/RRID:AB_1834353)
- CD19-PE (302254 Biolegend, RRID AB_2564142; https://scicrunch.org/resolver/RRID:AB_2564142)
- CD19-APC (302212, Biolegend. RRID AB_314242; https://scicrunch.org/resolver/RRID:AB_314242)
- CD20 Mouse anti-Human, APC, Clone: 2H7 (ebioscience, cat.no. 17-0209-41, RRID AB_10670628; https://scicrunch.org/resolver/RRID:AB_10670628)
- Brilliant Violet 421 anti-human CD19 Antibody [Clone: HIB19] (BioLegend, cat.no. 302234, RRID AB_11142678; https://scicrunch.org/resolver/RRID:AB_11142678)
- CD10 Mouse anti-Human, APC, Clone: 97C5 (MiltenyiBiotec, cat.no. 130-119-675, RRID AB_2733329; https://scicrunch.org/resolver/RRID:AB_2733329)
- DAPI (422801, Biolegend)

- 7-AAD (420403, Biolegend)
- CountBright™ Absolute Counting Beads (ThermoFisher, C36950)
- Software: FlowJo v.10.4.2

Transfection and Transduction reagents

- Opti-MEM media (Invitrogen, cat.no. 31985062)
- TransIT-293 (Mirus, cat.no. MIR 2700)
- Millex 33mm Durapore PVDF 0.45 µm syringe filter (Millipore, SLHV033RB)
- Polybrene infection reagent (10 mg/mL stock = 1,000×; INSIGHT biotechnology, cat.no. sc134220)
- HEPES (1M stock=40x; ThermoFisher, cat.no. 15630-056)

Mouse Injection reagents

- Corning Matrigel Matrix (Corning, product number 356234)
- BD SafetyGlide Needle, 25 Gauge (Product ID: A10012582)
- 10% Formalin (Any supplier)

Media reagents

- Advanced Roswell Park Memorial Institute medium (Advanced RPMI-1640, Invitrogen, cat.no.12633020)
- Roswell Park Memorial Institute medium (RPMI-1640, Invitrogen, cat.no.12633020)
- Gibco Penicillin-Streptomycin-Glutamine (100X) (Thermo Fisher Scientific, cat. no. 10378016)
- Gibco FBS (Thermo Fisher Scientific, cat. no. 10270-106)

Cytokines

- IL4 (0200-04-05, PeproTech)
- IL21(130-095-768, Miltenyi Biotec)

Consumables

- 15ml Falcon (Starlab; E1415-0200)

- Graduated filter tips (1,000, 200, 20 and 10µl; Starlab, cat. nos. S1122-1830, S1120-8810, S1120-1810 and S1120-3810)
- 10ml Serological pipette (VWR, order number 612-3700)
- 1.5-mL Protein LoBind microcentrifuge tubes (Fisher Scientific, cat. no. 13-698-794)

Plasmids

- phCMV-GaLV MTR (Addgene ID 163612)
- A gag-pol expressing lentiviral packaging vector such as pCMVDeltaR8.91 or pCMVDeltaR8.2 (Addgene ID 12263)
- A gag-pol expressing retroviral packaging vector such as pHIT60 or other CMV-driven gag-pol packaging plasmid eg pBS-CMV-gagpol (Addgene ID 35614)
- MSCV-BCL6-2A-BCL2-IRES-hCD2 (Addgene ID135305)
- MSCV-MYC-2A-BCL2-IRES-hCD2 (Addgene ID135306)
- MSCV-Cas9-2A-BFP (Addgene ID164662)
- gRNAs of interest expressed from lentiviral vector such as pKLV2-U6gRNA5(BbsI)-PGKpuro2AmCherry-W (Addgene ID 67977)
- Overexpression construct of interest expressed from retroviral backbone such as MSCV-IRES-GFP (Addgene ID 52107)

Equipment

- QuadroMACS Separator Magnet and stand (Miltenyi; 130-090-976)
- Class II microbiological safety cabinet (MSC; Envair, model no. Bio 2+)
- CO_2 incubator (Binder)
- Tissue culture plate (12-well plates, flat bottom; Corning; BC011)
- Tissue culture plate (24-well plates, flat bottom; VWR; 734-2325)
- Tissue culture plate (6-well plates, flat bottom; Corning; CC010)
- Tissue culture flask (T75, filter cap, 75 cm^2; Greiner Bio-One; 658175)
- Tissue culture treated culture dishes (100x20, Corning; BC153)
- Benchtop mini-centrifuge (Fisher Scientific, cat. no. 13-100-676)
- CoolCell® LX (biocision, order no. BCS-405)
- 1-mL Capacity cryovials (Thermo Fisher Scientific; 10577391)
- Polystyrene disposable standard serological pipettes (10 mL; Sigma; CLS4488)

- Polystyrene disposable standard serological pipettes (5 mL; Sigma; CLS4487)
- Irradiator (Caesium source)
- BD LSR II Flow Cytometer. We used BD FACS Diva 6.1.3 software to collect flow cytometry data and FlowJo 10.0.8 software for the analysis of flow cytometry data.

Reagent Setup

MACS Buffer

Add 10ml (2% (vol/vol)) of FBS, 1ml (1mM) EDTA to 500ml PBS and store at 4ºC. This solution can be stored at 4 °C for up to 2 months.

DMEM/10% FBS/ 1%GPS

Add 50ml (10% (vol/vol)) of FBS and 5.5ml (1% (vol/vol)) of GPS to 500ml DMEM. Can be stored at 4 °C for up to 4 weeks.

Advanced RPMI/20% FBS/1% GPS

Add 100ml (20% (vol/vol)) of FBS and 6ml (1% (vol/vol)) of GPS to 500ml Advanced-RPMI. Can be stored at 4 °C for up to 4 weeks.

RPMI/ 10% RPMI/ 1% GPS

Add 50ml (10% (vol/vol)) of FBS and 5.5ml (1% (vol/vol)) of GPS to 500ml RPMI. Can be stored at 4 °C for up to 4 weeks.

Freezing Medium (FBS/10%DMSO)

Add 5ml (10% (vol/vol)) of DMSO to 45ml of FBS. This solution can be stored at 4 °C for up to 4 weeks.

Flow cytometry Buffer (PBS/2%FBS)

Add 1ml (10% (vol/vol)) of FBS to 50ml of PBS. This solution can be stored at 4 °C for up to 2 months.

Maintenance and irradiation of YK6-CD40Lg-IL21 feeder cells

1 Culture YK6-CD40Lg-IL21 in RPMI 10%FBS. !CRITICAL It is important to switch to Advanced RPMI 20% FBS when coculturing with GC B cells (as described in the procedure).

2 Split cells 1-2 times a week when confluent. We split cells by first washing cells with PBS and then detaching with trypsin (we usually use 2ml trypsin per T75cm flask and find cells take approximately 5 minutes to detach). We neutralize trypsin with 8 ml media (total volume 10 ml) and spin down cells by centrifugation (400g, 4 min, RT). We usually split cells 1:5.

3 To irradiate cells, resuspend cells from step 2 in RPMI 10% FBS in a falcon and irradiate with 30Gy. Density of resuspended cells is not critical at this step.

!CRITICAL Irradiation is essential for longer term co-cultures or if cultures are going to be used in animal experiments. Short term B cell co-cultures (up to 10 days) can be performed on feeder cells that have not been irradiated.

4 To freeze irradiated cells, spin down cells by centrifugation (400g, 4 min, RT) and resuspend pelleted cells in freezing medium (see reagent setup). We freeze approximately 10^7 cells per vial, which usually corresponds to 4 vials per confluent T75cm flask (which corresponds to the number of cells required to plate one 12-well plate). Place vials into a CoolCell® Cell Freezing Container and move to -80 overnight, then transfer to liquid nitrogen.

PAUSEPOINT Cells can be frozen indefinitely in liquid nitrogen.

5 To thaw feeders, place vial into a 37ºC water bath until almost all thawed, then transfer to a 15ml falcon containing 5ml media. Top up to 13ml and centrifuge down cells to wash out DMSO (400g, 4min, RT). Gently resuspend pellet in RPMI 10%FBS and plate out 1 vial per 12-well plate. CRITICAL STEP An example of the appearance of a feeder layer is shown in **Figure 4A** and shows both the appropriate confluency and an example of overconfluent feeder cells.

Generation of GaLV-pseudotyped Virus Timings 4-5 days

!CAUTION Wear appropriate PPE (CL2) and dispose appropriately of any material that has been in contact with virus. All work should be conducted at Biocontainment level 2 in accordance with appropriate local safety rules.

1. Plate Lenti-X HEK293T packaging cells (approximately 5 x 10^6 cells) 16-24 hours before transfection at a confluency of around 20-30% in a 10cm dish (use a total volume of 10ml of DMEM, 10% FBS). !CRITICAL Viral titre is dependent on the health of the packaging cells. We discard packaging cells that have been in culture for more than 4 weeks and any culture that has been allowed to overgrow.

2. On the day of transfection, mix Opti-MEM and TransIT-293, vortex and leave at RT for 5-10min. For each 10cm dish, mix 1ml Opti-MEM with 18 or 33 µl TransIT-293 for retrovirus or lentiviral transduction, respectively. !CRITICAL Cells should be 50-70% confluent, evenly spread and fully adherent at the time of transfection.

3. Add packaging plasmids and retro/lentiviral constructs to the combined Opti-MEM and TransIT-293 mixture, as indicated in the tables below, vortex and leave for a further 15-30min. The following reagent quantities are for transfection of a single 10cm dish. Quantities should be scaled up or down proportionally by surface area for transfection of other plate sizes.

Retrovirus	
Opti-MEM	1ml
Transit293	18µl
pHIT60	1µg
GaLV MTR	1µg
Retroviral construct	4µg

Lentivirus	
Opti-MEM	1ml
Transit293	33µl
pCMVDeltaR8.91	8.3µg
GaLV MTR	2.8µg
Lentiviral construct	11µg

4. Gently add mixture dropwise to the 10cm dish, gently rock the plate to distribute the transfection mix, and place in incubator. !CAUTION When adding the virus mixture dropwise to the 10cm dish, be careful not to use too much force as otherwise packaging cells will be dislodged. !CAUTION There is no need to change the media after transfection.

5. Harvest Virus 36-48h after transfection by removing medium and filtering through a low-binding 0.45μM syringe filter to remove any residual packaging cells. If needed, replenish media on cells (10ml) and perform a second harvest 16hrs later. !CAUTION Virus does not usually need to be concentrated to achieve a high transduction efficiency. If concentration is required, we routinely use Lenti-X Concentrator for both lentivirus and retrovirus.

6. Freeze viral supernatant by placing direct into -80°C freezer. PAUSEPOINT Viral titre is optimal when used fresh but can also be stored at -80 °C (for at least up to 1 year). When needed, thaw viral supernatant at 37°C in a water bath until completely thawed immediately prior to transduction.

Procedure

Purification of GC B cells from discarded human tonsil

Timing Steps 1 – 17, 2-3 hours

!CAUTION Tonsil tissue derives from human patients who have not usually been screened for blood-borne viral infection and should be handled at containment level 2 with appropriate precautions. Particular care should be paid to the proper use and disposal of sharps. !CAUTION Appropriate ethical approval should be obtained prior to working with human tissue.

!CAUTION Tonsils may be stored at 4ºC for several hours without any loss of viability. However, viability starts to decrease rapidly once the tissue has been disaggregated, so steps 1-16, required prior to culture or freezing, should be completed quickly.

1 Start with approximately 1cm^3 of lymphoid tissue dissected from freshly collected human tonsil. Dice the tissue into small 3-5mm pieces in a sterile petri dish using a sterile scalpel and discard any attached mucosal tissue. (**Figure 2**).

2 Place the tissue pieces in a 70micron cell strainer in a 10cm petri dish and add 10ml media or PBS. Gently disaggregate the small tonsil pieces through the cell strainer using the rough end of a 5ml syringe plunger. (**Figure 2**).

3 Transfer cell suspension to a 15ml falcon and rinse the 10cm dish and strainer with 5ml of media to collect remaining cells and add to falcon. Centrifuge at 400g, 4 min, RT. The total pellet should be between 0.5 and 1ml in size and contain visible red cells. (**Figure 2**).

4 Remove and discard supernatant and gently resuspend pelleted cells in 10ml of MACS Buffer. Spin at 400g, 4min, RT !CAUTION Tonsils are not sterile when they are collected from surgery. This and subsequent washing steps reduce the risk of bacterial contamination of culture.

5 Remove and discard supernatant and gently resuspend pelleted cells in 20ml MACS Buffer.

6 Prepare two 15ml falcon tubes each with 5ml of lymphocyte separation medium (warmed up to RT). Carefully apply 10ml of cell suspension into each tube to form an upper layer on top of separation media. When layering, tilt the falcon tube, put the stripette tip at the top of the falcon and slowly release the cells without disturbing the layers. Spin at 800g, 20min with brakes switched off at RT (20ºC). A thick white interface of lymphoid cells should be visible. CRITICAL STEP We find that 15ml falcons provide a more easily harvestable interface layer than 50ml falcons. (**Figure 2**).

7 Aspirate and discard most of the media without disturbing the lymphocyte layer. Take a p1000 and transfer the lymphocyte layer to a new 15ml falcon. Top up to 13ml with MACS buffer, resuspend and count cells.

8 Divide cell suspension into separate 15ml falcon tubes so each tube contains 2 x 10^8 cells. Pellet cells by centrifuging at 400g, 4min, 4°C. The pellet should now be white in colour but can have some red blood cells remaining which should be eliminated during the next purification step. !CRITICAL Keep cells and MACS Buffer cold from now on !CAUTION Set aside a small aliquot for use as pre-purification flow cytometry control.

9 Remove supernatant and gently resuspend cells in 800µl MACS Buffer. Add 100µl of B cell depletion cocktail, 1µl of biotinylated anti-IgD and 1µl of biotinylated anti-CD44. For purification of alternative subsets of B cells other than GC B cells, perform as follows: 1) Total B cells: omit both anti-IgD and anti-CD44 antibodies. 2) Non-naïve B cells (including GC and memory B cells): omit anti-CD44 antibody. This will substantially increase cell yield.

10 Incubate on ice for 25min with intermittent gentle mixing.

11 Add 10ml MACS Buffer and pellet cells by centrifugation (400g, 4min, 4°C).

12 Remove and discard supernatant. Flick to resuspend cells. Add 800µl MACS Buffer and flick. Add 200µl anti-biotin beads and mix gently.

13 Incubate at 4°C (in fridge) for 15min.

14 Add 10ml MACS Buffer and pellet cells by centrifugation (400g, 4min, 4°C). During the centrifugation, assemble the MACS magnetic stand with the LS columns. Prime each column by adding 3ml of MACS buffer. Discard flow-through.

15 After spinning, remove supernatant, gently resuspend cells in 1ml MACS Buffer and add to LS column. Collect flow-through into a 15ml falcon on ice. Add 3 x 3ml MACS Buffer. Wait for the MACS Buffer to pass through the columns before adding more buffer. !CAUTION Remove any visible clumps before adding to column. !CAUTION We have found that up to 200 x10^6 cells can be applied to a MACS LS column, however greater numbers can lead to blocking of the column and loss of sample. **?Troubleshooting**

16 Pellet cells from the collected flow-through by centrifugation (400g, 4min, 4ºC). Discard the supernatant, the pellet comprises the purified GC B cells that are the starting point for GC B cell culture in step 20. Set aside 10^6 cells for flow cytometry analysis (step 17). (**Figure 2**).

PAUSEPOINT Purified GC B cells can be frozen for storage at this stage. To freeze, pellet purified GC B cells by centrifuging (400g, 4min, 4ºC), resuspend in freezing media and add to cryovials. Place vials into a CoolCell® Cell Freezing Container and move to -80 overnight, then transfer to liquid nitrogen tanks. We freeze approximately $12x10^6$ cells per vial; this represents the number of GC B cells required to set up a single 12-well plate of culture. To thaw, see Step 19.

17 Assess purity of GC B cells by flow cytometry as follows:

- Spin down (400g, 4min, 4ºC) 1 x 10^6 cells previously set aside in Steps 8 and 16 (representing before and after purification)
- Resuspend the pellet in 100 µl FACS Buffer with added 0.1-10 µg/ml of the primary labelled antibody (CD20, CD19, CD38, CD10) and incubate for 30 min at 4ºC in the dark.
- Wash cells once in FACS Buffer and resuspend in 200-500 µl FACS Buffer
- Analyse by flow cytometry, collecting more than 10,000 events, to assess the purity of GC B cells (Example results are shown in **Figure 3**).

!Caution A cell viability stain such as DAPI should be included to exclude dead cells (**Supplementary Figure 3A**).

?Troubleshooting.

PAUSE POINT

Ex vivo expansion of GC B cells Timing Steps 18-21, 2 hours hands-on, 2 days total

!CRITICAL Maintenance of YK6-CD40Lg-IL21 feeder cells is described in the Reagent setup section. Alternative coculture systems can be used – see **Box2**

18 Plate freshly irradiated or thawed irradiated YK6-CD40Lg-IL21 feeder cells into desired cell culture vessel in RPMI-Advanced with 20% FBS (if using a 12-well plate, plate 1-5 x 10^5 cells in 1ml medium per well). Incubate cells overnight to allow feeder cells 16-24 hours to adhere to the bottom of the wells.

!CRITICAL See **Figure 4A** for examples of correct and over-confluency of the feeder cells.

!CAUTION If using fresh GC B cells this step should be performed the day before cell isolation (Step 1-17).

19 If using frozen GC B cells, place vial in a 37 °C water bath until almost thawed. Then transfer to a 15ml falcon tube containing 5ml media. Top up to 13ml and wash out DMSO by spinning at 400g, 4min, RT. Then gently resuspend pellet in media (RPMI-Advance 20% FBS) and proceed to step 20. If using cells straight after isolation, proceed directly to step 20. !CAUTION Freshly purified GC B cells used direct from step 16 expand quicker than those that have been frozen. However, use of frozen cells may be preferred for logistical reasons. !CAUTION For examples of the appearance of healthy B cells grown on YK6-CD40Lg-IL21, in comparison with unhealthy cells grown on YK6 alone, please refer to **Figure 4A**.

20 Add GC B cells (10^6 cells in 1ml per well of a 12 well plate), on to the top of the feeder cells plated in step 18. Do not remove the existing media. Leave undisturbed for 48-72h before proceeding to passage (step 21), transduction (step 22) or RNP nucleofection (Box 3).

Passage of GC B cells Step 21, TIMING 15 min hands-on, cells can be cultured for 7-10 days without immortalisation

21 Passage GC B cells every 3-4 days as follows:

- Gently resuspend cells (feeders and GC B cells) with a p1000
- Transfer a split (one quarter to one eight of the original cell volume) into a fresh TC culture plate or flask that has been preplated with irradiated feeder cells as described in step 18
- Top up to original culture volume with fresh media (RPMI Advance 20%FBS).

!CAUTION When splitting cells, irradiated feeders can be added in fresh media (RPMI-Advance 20% FBS) at the same time as GC B cells ($10x10^6$ cells per 12-well plate) to avoid the need for preplating. ! CAUTION GC B cells can be expanded short-term (7-10days) in culture after isolation before proliferation slows, differentiation occurs and cells die. !CAUTION Transduction with viral constructs that permit GC immortalisation such as BCL2-2A-BCL6 is required for long-term growth. !CAUTION If antibiotics are required, we have found MycoZap PR (Lonza) to be effective and well-tolerated by GC B cell cultures. **?Troubleshooting**.

!CRITICAL We recommend screening of cultures for latent EBV infection. This is best done on GC B cells that have been in co-culture for 7-10 days. Refer to **Box1** for details of how to screen for latent EBV infection.

Transduction of GC B cells with GaLV-pseudotyped Virus

Timings Steps 22-27, 4 hours hands-on, 3days total

CRITICAL When using fresh GC B cells, we find the optimal transduction time to be after 48 hours of culture. However, an extra 24 hours is beneficial when using GC B cell that have been stored frozen in DMSO since these initially proliferate more slowly. At the time of transduction, GC B cells should be visibly larger in comparison to day 1 and should be firmly stuck to the feeder cells (See Figure 4A for examples of successful and unsuccessful cultures).
!CAUTION When transducing with more than one construct the viral supernatants from each can be pooled for simultaneous transduction.

!CRITICAL CRISPR knockout using transient nucleofection of RNP can be used in place of this section (see Box 3 for details). Viral overexpression of putative oncogenes can be combined sequentially with CRISPR deletion using nucleofection of RNP by undertaking steps 1-21 followed by Box 3.

22 Collect fresh viral supernatant or thaw frozen viral supernatant (see Reagent Setup).
23 Add 25µM HEPES (1:40) and 10µg/ml Polybrene (1:1000) to the fresh or thawed viral supernatant.

24 Spin the undisturbed plate containing cultured GC B cells from step 20 at 400g, 4min, RT and gently remove and discard most of the supernatant with a p1000, leaving B cells, feeder cells and about 100µl of media in the well if using a 12-well plate.

25 Add viral supernatant to the wells containing cultured GC B cells and gently resuspend cells and virus with a p1000. !CAUTION For retroviral constructs such as MSCV-BCL2-2A-BCL6 or MSCV-BCL2-2A-MYC, 500µl of raw supernatant per well of a 12-well plate is sufficient to achieve >50% transduction efficiency. !CAUTION Larger inserts, eg Cas9 may be more difficult to transduce. Transduction on subsequent days might be necessary or alternatively concentration with Lenti-X concentrator. We find that lentiviral transduction is less efficient than retrovirus and therefore we use 1-2ml supernatant per well of a 12-well plate. !CAUTION Appropriate controls such as MSCV-Empty vector or WT vector may be included at this step.

26 Spin at 1500g for 90min at 32°C. For retroviral transduction, remove most of the supernatant immediately after centrifugation and replace with fresh media. For lentivirus, leave for 4-12 hours, then briefly spin at 4min, 400g, RT and replace with fresh media (Advanced-RPMI, 20% FBS).

27 Confirm transduction efficiency 48-72 hours post transduction by flow cytometry for fluorescent protein or cell surface marker. For example, to confirm expression of our BCL2-2A-BCL6 or BCL2-2A-MYC constructs, we stain cells for the surface protein CD2 which is expressed as a transduction marker from these plasmids. The procedure for flow cytometry staining is described in **step 17.**
!CAUTION Expression of overexpressed fluorescent protein or cell surface marker will be detectable from 24-48 hours but may continue to increase up to day four. **?Troubleshooting**.

Culture of immortalised B cells Timings: Passage takes 15 min, cells can be cultured long term if immortalised

CRITICAL Examples of oncogene combinations that lead to immortalised growth in vitro include either MSCV-BCL6-2A-BCL2 or MSCV-MYC-2A-BCL2. **Supplementary Figure 2** shows an example of the phenotype of GC B cells on day of isolation and post-transduction with these oncogenes (Day 5 and long-term, Day 73). We have cultured GC B cells that have been transduced with these combinations for over 6 months. Other genetic combinations may also provide a similar outcome.

28 If B cells have been immortalised, they will proliferate vigorously and require passage every 3-4 days. Passage as described in Step 21. Typically, a 4 to 8-fold split is required. Cells transduced with the combination of BCL2 and BCL6 remain dependent on the presence of YK6-CD40Lg-IL21 but other oncogenic combinations may render cells feeder-independent. Culture cells long-term in vitro or proceed to the next step to undertake xenograft experiments 3-7 days following transduction.

Generation of synthetic lymphoma in mice Timings Steps 29-33, 3 hours hands on, >30 days total

CRITICAL An example oncogene combination that robustly forms synthetic tumours is the combined transduction of BCL2, P53dd, MYC & CCND3. If co-transduced with this combination of oncogenes, CD20-positive, polyclonal tumours usually develop in around 30 days. Other combinations may also provide a similar outcome. Combinations of three oncogenes may result in tumour growth over a longer interval – eg 90 days and this may be associated with the selection of spontaneous mutations and development of clonal or oligoclonal tumours. !CAUTION When working with multiple constructs, viral supernatant can be pooled for simultaneous transduction or performed on consecutive days.

29 At approximately 3-7 days after transduction, harvest transduced cells (10^7 cells/mouse) by centrifugation (400g, 4min, 4°C) and wash in cold PBS, 400g, 4min, 4°C.

30 For each mouse, mix 100ul cell suspension with 100ul cold Matrigel and keep the tube on ice. !CRITICAL Aliquot Matrigel in small volumes and thaw on ice in the fridge. When preparing cell mixture, keep pipettes and Eppendorf tubes on ice. Matrigel will solidify if at RT. Keep syringes and needles for injections on ice until immediately before use. Make up cell mixture for an additional 1-2 mice to account for pipetting loss.

31 Inject 200ul of the cell and Matrigel mixture subcutaneously (10^7 cells/mouse) into left or right flank of 8-12 week old NSG mice with a 25-27G needle. IP or IV injections without Matrigel are also possible but we use subcutaneous injection in Matrigel to allow easy visualisation of tumour growth.

32 Monitor tumour size daily. Once tumours have reached 12mm in size, mice should be humanely killed by an approved method that follows the Animals (Scientific Procedures) Act 1986 (UK). We cull mice by exposure to gradually rising CO2 followed by cervical dislocation.
!CAUTION All experimental procedures involving mice must conform to institutional and national regulations and be performed by appropriately trained researchers in dedicated experimental animal facilities. The procedure we describe here follows the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 and the results obtained here were undertaken following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB-PPL number P846C00DB).

33 Process tumours immediately after killing mice. We cut tumour tissue into small 5mm cubed pieces. We snap freeze fragments at -80ºC for subsequent use in RNA and protein assays and fix fragments in 10% formalin buffered saline for histology. We also produce a single cell suspension (by undertaking steps 1- 3) and then freeze 10^7 viable cells/vial in freezing media (as described in step 4 in Maintenance and irradiation of YK6-CD40Lg-IL21 feeder cells) or culture in vitro (as described in steps 18-21) or retransplant into further NSG mice (as described in steps 29-33), either immediately or after DMSO storage. **Figure 7** **?Troubleshooting**.

Box 1 Exclusion of latent EBV infection

Some tonsils will contain B cells that are latently infected with EBV. The outgrowth of these cells may complicate the interpretation of long-term cultures. Thus for long-term experiments we use cells from batches that we have previously shown to be negative for EBV.

We screen our GC B cells for the presence of EBV DNA using the following qPCR assay. The assay is more sensitive after 7-10 days of culture, so we tend to screen cells after passage as described in steps 18-21.

We preferentially utilise tonsil material from younger donors (<3 years old) as we have found it uncommon to detect evidence of latent EBV in tonsil tissue from this age group.

Additional materials required

EBV qPCR reagents

- EBV-negative control genomic DNA (such as from Lenti-X 293T)
  - EBV-positive control genomic DNA (such as from Namalwa, ATCC® CRL-1432™RRID:CVCL_0067)
- PCR Plate, 384-well (Thermo Fisher Scientific, cat. no. AB1384W)
- Fast SYBR Green Master Mix (Thermo Fisher Scientific, cat. no. 4385612)
- qPCR hood and machine

Sequencing primers for EBV screening

- qRT-PCR Primers – (originally described in[38])

  EBV F QP1L 5' – GCCGGTGTGTTCGTATATGG – 3'

  EBV R QP2L 5' – CAAAACCTCAGCAAATATATGAG – 3'

Procedure

1. Serially dilute genomic DNA from Namalwa cells (positive control) and Lenti-X 293T cells (negative control) in water.
2. Working in a qPCR hood, mix 5ul Fast SYBR Green Master Mix, 0.4ul of Forward and Reverse EBV PCR primers (10uM), 100ng of genomic DNA from GC B cells or controls from step 1 and top up to 10ul with water.!CAUTION We typically set up four replicates per reaction in a 384 well qPCR plate.
3. Seal plate using MicroAMp clear adhesive film and spin down at 100g, 1min, RT.
4. Use the following qPCR conditions per manufacturer's instructions.

Cycle no.	Denature	Anneal/Extend
1	95°C, 20sec	
2-40	95°C, 1sec	60°C, 20 sec

END BOX

Box 2 Alternative Feeder Systems

The main protocol utilises the YK6-CD40Lg-IL21 feeder system, however the stimulation provided may be tailored to individual experimental needs. The creation of customised feeder lines is possible using the following procedure.

Additional materials required

- Viral supernatant: CD40Lg-Puro virus can be prepared as described in Reagent Setup, but a packaging construct encoding ecotrophic envelope or amphotrophic should be substituted for the GaLV envelope if transducing murine L-cells or YK6 cells respectively (**Supplementary Figure 1D**).

Suitable cell line – eg YK6 (RRID CVCL_A2XJ) or L cells (ATCC CRL-2648, RRID:CVCL_4536)

- Antibiotic selectable viral vector expressing ligand or cytokine – eg MSCV-CD40Lg-IRES-Puromycin or MSCV-IL21-IRES-Blasticidin (plasmids available from the Hodson lab)
- Ecotrophic retroviral envelope to transduce L cells eg pHCMV-EcoEnv (Addgene #15802).

Antibody to detect CD40Lg: CD154-APC (clone 24-31 Ebioscience, RRID AB_314832)

- Puromycin (Thermo cat # A1113803)
- Blasticidin (Thermo cat # A1113903)

Procedure

1. Transduce feeder cells such as L cells with retroviral CD40Lg-Puro.
2. 48 hours after transduction, select for transduced cells by adding puromycin 2-5µg/ml to the medium for 48-72 hours.
3. Perform limiting dilution of the transduced cells in 96-well plates to generate single cell clones.
4. Test 10-20 clones by flow cytometry for surface expression of CD40Lg (CD154) and for the ability to support B cell growth in the presence of exogenous IL21.
5. Repeat steps 1-4 on the optimal clone to transduce a blasticidin-selectable retroviral construct containing a desired cytokine such as IL21.

END BOX

Box 3 CRISPR knockout using RNP nucleofection

The main protocol describes the use of viral transduction to introduce Cas9 and gRNA into primary B cells. An alternative approach is the transient nucleofection of RNP complexes assembled in vitro (Lonza, V4XP-3032). We have applied this approach to B cells from step 20 that have been in coculture for 48 hours. The effect of different gRNA to Cas9 molar ratios and the beneficial effect of the electroporation enhancer is shown in **Figure 6**.

Additional materials required:

- sgRNAs. These can be designed using the IDT online design tool (https://eu.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM). However, for simple knockout of coding genes we typically use predesigned sgRNA sequences from two published genome-wide libraries[39,40].
- P3 Primary Cell 4D-NucleofectorTM X Kit S – (Lonza cat # V4XP-3032)
- Custom sgRNAs (IDT)
- Alt-R S. p. Cas9 Nuclease V3 (IDT cat # 1081058)
- Alt-R Cas9 Electroporation Enhancer (IDT cat # 1075915)
- Amaxa Nucleofector 4D (Lonza cat # AAF-1002B with AAF-1002X)

Procedure

1. Prepare 12-well cell culture plates with feeders and 1ml of B cell culture medium (10% FBS-Advanced-RPMI) per well and incubate at 37°C (will be required for step 14).
2. Warm B cell culture medium (80 µl/sample) to 37°C in a water bath or heat block (will be required for Step 14).
3. Allow Nucleofection Solution P3 (with Supplement provided in the Nucleofectior Kit S) to reach RT.
4. Dilute 1µl from stock solution of Alt-R Sp Cas9 Nuclease V3 in 4 µl PBS to a final concentration of 2µg/µl (Total volume 5µl).
5. Dilute 0.5µl of Alt-R CRISPR-Cas9 sgRNA in 1.5µl PBS (Total volume 2µl)

!CAUTION These dilutions produce a molar ratio of sgRNA to Cas9 protein of 2:1 which we have found to be optimal when combined with 2µg of Cas9 for primary B cells (Figure 6)

6. For each electroporation, mix 1µl of diluted Cas9 (Step 4) and 1µl diluted sgRNA (Step 5) in a 1.5 ml Eppendorf and incubate at RT for 20min. During the incubation, harvest and count B cells (10^6 cells are required per condition) and pellet by centrifugation (3min at 500g) in a Falcon tube.

!CRITICAL Increasing the amount of Cas9 used (up to 5µg) did not significantly improve knockout efficiency in primary B cells.

7. Carefully remove all supernatant from the cells.

8. Resuspend B cells in 20µl of Nucleofector Solution P3 buffer per 10^6 cells

!CAUTION Time in Nucleofection Solution should be minimised, however in our experience exposure times of 5-10min do not impair primary B cell viability.

9. Add 0.4µl Electroporation Enhancer to each tube prepared in Step 6 and mix by gentle pipetting. !CRITICAL We have found knockout efficiency to be greatly increased by the use of the Electroporation Enhancer (**Figure 6**).

10. Add 20µl of cells suspended in Nucleofector Solution P3 buffer into tubes from Step 8 and mix gently by pipetting.

11. Transfer 22µl of each reaction into each well of the Nucleocuvette strip taking care to avoid air bubbles.

12. Turn on the electroporator, select the wells to be electroporated, the buffer used (P3) and programme EO117.

13. Insert Nucleocuvette strip into 4D-nucleofector and press start.

14. Immediately after electroporation, remove Nucleocuvette strip from the instrument, add 80µl pre-warmed media (from Step 2) to each well and transfer cells back into co-culture with feeder cells (from Step 1).

15. Analyse cells by flow cytometer or other technique 3 days after electroporation (**Figure 6**).

END BOX

Timing

The below schedule can be used to generate synthetic lymphoma models in NSG mice

Day 0 Plate out feeders (See Reagent Setup)
Plate out HEK293T viral packaging cells (See Reagent Setup)

Day 1 GC B cell isolation from discarded tonsil tissue (Steps 1-21)
Transfect HEK293T cells with desired oncogenic constructs (See Reagent Setup)

Day 3 Transduction of GC B cells with oncogenic cocktail (Steps 22-28)

Day 7 Injection of GC B cells into NSG mice (Steps 29-32)

Day 30+ Harvest tumours (Steps 33)

The below schedule can be used for in vitro CRISPR experiments

Day 0 Plate out feeders (See Reagent Setup)
Plate out HEK293T viral packaging cells (See Reagent Setup)

Day 1 GC B cell isolation from discarded tonsil tissue (Steps 1-21)
Transfect HEK293T cells with viral constructs (See Reagent Setup)

Day 3 Transduction of GC B cells with oncogenic cocktail and Cas9-BFP (Steps 22-26)
Or consider alternative CRISPR knockout protocol using RNP nucleofection (**Box3**)

Day 5 Analyse cells on a Flow cytometer to verify sufficient (~50%) Cas9. (Step 27)
If BFP <50%, re-transduce with Cas9-BFP (Steps 22-26)

Day 6 Transduction of GC B cells with gRNA-GFP lentiviral construct. (Steps 22-26)

Day 8+ Flow cytometry analysis for gRNAs. Analyse cells by flow cytometry every week to determine dropout or enrichment of gRNAs and harvest a cell pellet at each timepoint. (Step 27)

Troubleshooting

For troubleshooting guidance see Table 1.

Table 1: Troubleshooting

Step	Problem	Possible Reason	Possible solution
15	Slow or no flow through the MACS column	Too many labelled cells applied to column	Check number of cells applied. Use fewer cells.
17	Poor purity for GC B cells	Low GC B cell frequency in the donor.	Check percentage of GC B cells in the pre-purified sample set aside in step 8.
		Too much custom antibody added relative to amount of beads added.	Check antibody concentrations. If needed titrate the antibody to bead ratio.
		Column insufficiently exposed to magnet	Make sure column is properly applied to the magnetic stand.
21	Slow growth of GC B cells	Problems with freeze/thawing GC B cells left for too long in suspension before adding into coculture.	Repeat using freshly purified GC B cells.
		Mycoplasma infection	Minimise time between disaggregating tonsil and adding into coculture.
			Screen feeders and B cells for mycoplasma, discard culture or treat for infection.
21	Contamination by bacterial or fungal microorganisms	Washing steps during purification omitted	Include all washing steps (1-17). Consider prophylactic antibiotic eg MycoZap-PR (Lonza)
27	Transduction efficiency is low	Health of packaging cells is suboptimal	Use freshly thawed packaging cells. At the point of transfection, they should be evenly spread across the plate with few floating cells.

		Packaging plasmid has recombined during bacterial transformation.	Perform restriction digest and electrophoresis. Use fresh plasmid preparation.
		Expression of the construct is toxic to GC B cells.	Consider using an inducible expression construct.
		The vector is too large for efficient packaging.	Consider concentrating viral supernatant or transducing on consecutive days
33	No tumour formation	Combination of oncogenes used is insufficient to transform GC B cells	Consider alternative genetic combinations.

Anticipated results

When processing lymphoid tissue from a single paediatric tonsil (Steps 1-17) we expect to harvest around 50-100 x10^6 purified cells with a purity of around 80-95% (**Figure 3**). We have found there can be quite marked variability between different human donors. When placed into coculture we expect to see very vigorous proliferation beginning at around 48-72 hours, associated with an increase in cell size (**Figure 4A**). When cells are transduced with an oncogene combination that leads to immortalisation (eg BCL6 and BCL2) we expect to see rapid outgrowth of the doubly-transduced cells such that transduced cells will take over almost all the culture within 7-10 days. These cells continue to expand indefinitely if maintained on YK6-CD40Lg-IL21 feeder cells and can be used as a cellular platform for functional genetic experiments as we reported recently[22]. When transferred into NSG mice, we typically see tumour formation in around 30 days when four potent oncogenes are combined. Slower tumour formation (up to 100 days) is observed when three oncogenes are expressed. These tumours are generally clonal suggesting that further spontaneously-arising co-operating genetic events have occurred during the interim period[22]. We anticipate that this protocol can be used to identify other combinations of overexpressed or deleted genes that will lead to immortalised growth or tumour formation and consider that the range of combinatorial genetic experiments that could be performed using this culture system is essentially unlimited.

Data availability statement

All raw data underlying Figures are provided as Source Data Files. Other examples of data can be made available upon reasonable request.

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Author Contributions

RC, JG, MDR, CG and DH contributed to the development and optimization of the protocols described in this manuscript. RC and DH wrote the manuscript with input from all authors.

Conflicts of Interest

RC - Consultancy for Karus Therapeutics. DH - Research funding from Gilead Sciences.
The remaining authors declare no competing interests.

Figure legends

Figure 1 A schematic overview of the expansion, immortalisation and use of human GC B cells cultured on FDC-like feeder cells for the functional interrogation of lymphoma genetics (1) and creation of in vivo lymphoma models (2). Scale bar represents 100μm.

Figure 2 Summary of the GC B cell purification strategy. Panels show tonsil dissection in a 10cm diameter dish (step 1), disaggregation through a cell strainer (step 2), typical appearances of cell pellets before density gradient centrifugation (step 3), layering onto separation media (step 6), the post-centrifugation white lymphoid layer positioned between the 4 and 5ml markers (Step 6) and the post-purification lymphocyte pellet (Step 16).

Figure 3 Example of successful GC B cell enrichment. Typical Flow cytometry plots showing staining of cells pre- and post- purification using a magnetic bead-based strategy to enrich GC B cells. CD38, CD20, CD19 & CD10 markers were used to assess cell purity. Yield and cell purity may vary between different donors. Refer to Supplementary Figure 3A for Gating Strategy.

Figure 4 Examples of the use of YK6-CD40Lg-IL21 and modified feeder systems. (**A**) Images showing the appearances of YK6-CD40Lg-IL21 feeder cells (top panel) plated at the desired confluence (left) and over confluence (right). Bottom panel shows a successful culture where GC B cells that have increased in size and proliferated vigorously on YK6-CD40Lg-IL21 cells (left) compared to small round unhealthy B cells on a background of feeder cells expressing neither CD40Lg nor Il21 (right). Scale bar represents 100μm for all images apart from 50μm for YK6-CD40lg-IL21 + B cells. (B) Primary human B cells were cultured on YK6 control and YK6-CD40Lg using three different conditions throughout the time period indicated: no cytokines, in the presence of IL4 (10 ng/ml) or IL21 (50 ng/ml). Bar graph illustrates number of viable B cells (± s.e.m., n = 6) over three timepoints. (**C**) Primary human B cells were cultured on YK6-CD40Lg and YK6-CD40Lg-BAFF for 10 days using two different conditions: no cytokines, and in the presence of IL21. Bar graph illustrates number of viable B cells (± s.e.m., n = 6) at Day 10. (**D**) Generation of alternative feeder lines. Primary human B cells were cultured on L cells control and L-cells engineered to express CD40Lg using two different conditions: no cytokines and in the presence of IL21 (50 ng/ml). This was compared

to the same conditions using YK6 cells as seen in (B). Bar graph illustrates number of viable B cells (± s.e.m., $n = 6$) over three timepoints. Viable cells were determined by flow cytometry and cell counting beads. Refer to Supplementary Figure 3B for Gating Strategy. Source data are provided as a Source Data file.

Figure 5 Gene knockout using virally transduced Cas9 and gRNA. Primary B cells were transduced with BCL2-BCL6 and Cas9-BFP and subsequently with gRNAs targeting CD19 (2 gRNAs), CD38 (3 gRNAs) and non-targeting controls (3 gRNAs). Staining for CD19 and CD38 was performed on the indicated timepoints after gRNA transduction and gated on double positive Cas9-BFP and gRNA-GFP expressing cells. Blue and red histograms show CD38 and CD19 expression, respectively, in cells transduced with gRNAs and non-targeting control (one donor shown as representative). Bar graph illustrates percentage of CD38 (blue) and CD19 (red) positive cells over time. Error bar represent mean ± SEM of four independent biological experiments. Refer to Supplementary Figure 3C for Gating Strategy. Source data are provided as a Source Data file.

Figure 6 Gene knockout using transient nucleofection of RNP complexes. Primary B cells were thawed 2 days prior to electroporation with Cas9 and CD19 gRNAs at the molar ratio indicated with and without Electroporation Enhancer (IDT) (4µM). B cells were stained for CD19 three days after electroporation. Histogram shows a representative flow cytometry histogram of CD19 expression. Bargraph illustrates percentage of CD19 positive cells (mean ± SEM, n=4). Refer to Supplementary Figure 3D for Gating Strategy. Source data are provided as a Source Data file.

Figure 7 Typical histological and immunohistochemical appearances of an example "synthetic" human tumour. This tumour was generated by transduction of ex vivo GC B cells with MYC, BCL2 & BCL6 and injected subcutaneously into 9-week old male NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice in Matrigel. The tumour was harvested 108 days after injection. Scale bar represents 100µm. Different images of the same tumour have been shown previously[22]. Staining for the following antibodies was performed by the Human Research Tissue Bank, Pathology Lab, Cambridge University Hospitals NHS according to their standard protocols: H&E (CellPath, VFM Harris' Haematoxylin and Eosin Y Stain), CD79A (Leica, clone JCB117), CD20 (Leica, clone L26), PAX5 (Leica, clone 1EW), MYC (Abcam, clone

Y69), P53 (Leica, clone DO-7), Ki67 (Dako clone MIB-1), CD10 (Leica, clone 56C6), MUM1 (Leica, clone EAU32), BCL2 (Dako, clone 124), CD138 (Leica, clone MI15), CD3 (Leica, clone LN10), EBER (Leica, EBER probe).

Supplementary Figure legends

Supplementary Figure 1 Alternative feeder systems. (A) Flow cytometry analysis for the expression of CD40Lg (CD154) and BAFF (transduction marker CD8a) on immortalised FDC-like cells (YK6) engineered to express CD40Lg and BAFF. FSC, forward scatter. (B) Image showing the appearance of the murine feeder cell line (L-cells) at the desired confluence. Scale bar represents 400μm. (C) Flow cytometry analysis for the expression of CD40Lg (CD154) on L-cells engineered to express CD40Lg. FSC, forward scatter. (D) L-cells were transduced with a GFP construct using an amphotrophic, ecotropic or GaLV_MTR fusion construct. Transduction efficiency was determined by expression of GFP two days after transduction. FSC, forward scatter.

Supplementary Figure 2. Phenotype of immortalised B cells. Flow cytometry analysis for the expression of the GC B cell markers CD38, CD20, CD19, CD22 and CD95 at day of isolation, Day 5 after transduction, and long term culture with either *BCL2* and *BCL6* or *BCL2* and *MYC.* Red histograms show GC B cells compared to primary human naïve B cells (blue). Day of isolation and Day 5 after transduction represents a different donor to long-term culture.

Supplementary Figure 3 Flow cytometry gating strategy. Gating strategy for (A) live, CD38, CD20, CD10, CD19 positive cells as seen in Figure 3. (B) live, CD19 positive cells and the indicated Cell Counting Beads were used to calculate cell number as seen in Figure 4. (C) live, BFP+GFP+ (Cas9/gRNA) expressing cells with knockdown of CD19 or CD38 or control as seen in Figure 5. (D) live cells with knockdown of CD19 as seen in Figure 6.

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