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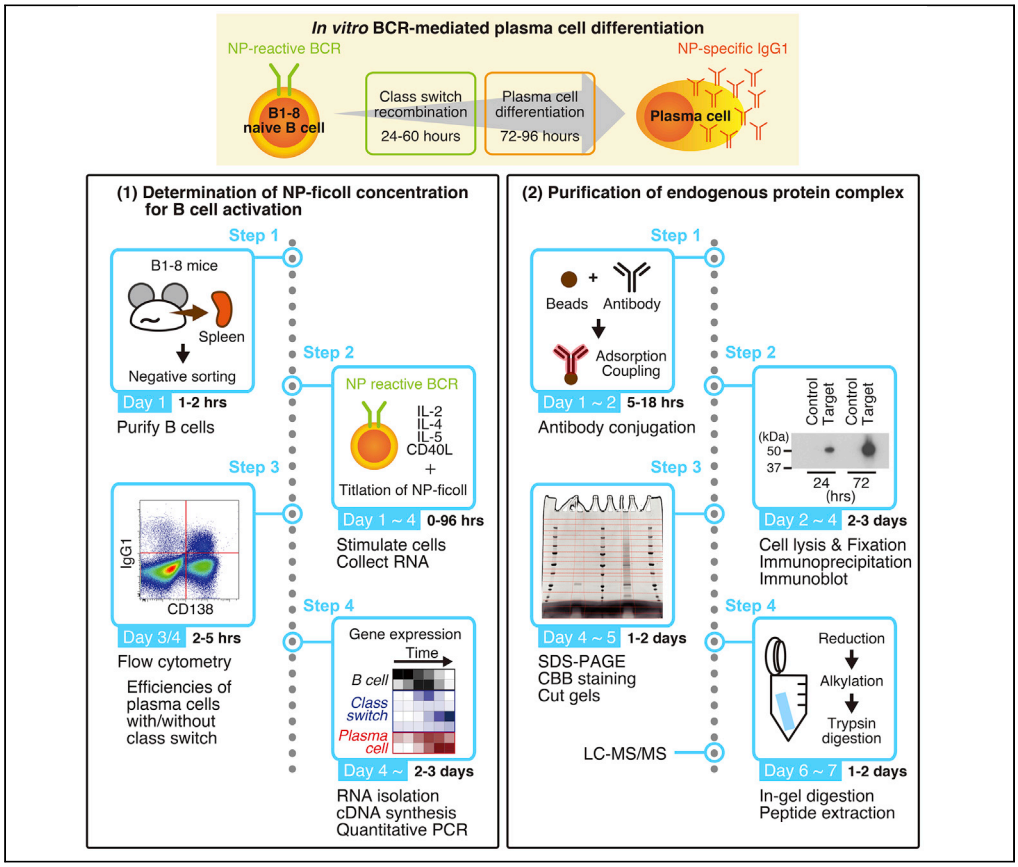
Protocol for *in vitro* BCR-mediated plasma cell differentiation and purification of chromatin-associated proteins



Molecular-level understanding of plasma cell (PC) differentiation has been modeled using LPS stimulation *in vitro*. However, this system does not involve the B-cell receptor (BCR)—a critical component of B cell biology. Here, we present a protocol for *in vitro* PC differentiation system dependent on BCR signaling that easily scales up for cell number-demanding applications, including protein complex purification. We describe how to set up this system and detail applications for endogenous complex purification of chromatin-associated proteins.

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Highlights

In vitro plasma cell differentiation system via B-cell receptor signaling

Antibody conjugation of protein A or G beads for immunoprecipitation

Purification of endogenous protein complexes and chromatin-associated proteins

Gel extraction and digestion of proteins in preparation for LC-MS/MS analysis

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SUMMARY

Molecular-level understanding of plasma cell (PC) differentiation has been modeled using lipopolysaccharide (LPS) stimulation *in vitro*. However, this system does not involve the B-cell receptor (BCR)—a critical component of B cell biology. Here, we present a protocol for *in vitro* PC differentiation system dependent on BCR signaling that easily scales up for cell number-demanding applications, including protein complex purification. We describe how to set up this system and detail applications for endogenous complex purification of chromatin-associated proteins.

For further details on the use and execution of this protocol, please refer to Sciammas et al. (2011) and Ochiai et al. (2018, 2020).

BEFORE YOU BEGIN

The antibody response, a component of adaptive immunity, is critical for pathogen elimination. As such, this response has evolved numerous mechanisms to control the quantity and quality of antibody that is specific to a pathogen. Quantity is controlled by the regulated allocation of short-lived and long-lived antigen-specific plasma cells (PC) that are endowed with a gene program to enable high-level secretion of antibodies. In contrast, quality of antibody is controlled by changing the constant regions (Class Switch Recombination; CSR) of antigen specific receptors, thereby allowing diverse effector functions, e.g., complement lysis vs opsonization. A second modification of antibody quality, known as affinity maturation, is mediated by Germinal Center B cells (GCBs) that are specialized in targeting somatic hypermutation to the VDJ or VJ segments of heavy and light chains, respectively, to enable high affinity variants to be selected. The B cell-intrinsic Gene Regulatory Networks (GRNs) that orchestrate the cell fate decisions that enable these processes to be executed are of intense interest and clear progress has been made. It is expected that a mechanistic understanding of the complete network and its dynamics will improve our overall understanding of how the antibody response develops as well as how it may be manipulated in different vaccine platforms.

The antibody response is initiated in naïve resting B cells upon B Cell Receptor (BCR) binding to pathogen components (antigen) which, with critical cues from several cell types, leads to clonal expansion and differentiation into GCBs and PCs to produce the aggregate antibody response. While *in vivo* analyses have quantitated the number and rate of these cell transitions, very few cells are available for mechanistic

studies of signaling and GRNs. Scaling up the number of mice is a costly option and still highly limiting for some experimental systems involving activity and composition of protein complexes. In contrast, *in vitro* systems offer an inexpensive option to model critical B cell state transitions that are easily scalable for even the most demanding experimental platforms. The obvious benefits of these systems also include defined conditions and analysis of multiple time points. However, all *in vitro* systems to date do not involve BCR signaling and instead rely on Toll-like receptor (TLR) signaling. Populations such as GCBs, Memory B cells (MBs), and long-lived PCs do not form under these conditions. One notable *in vitro* system exception relies on CD40 and Baff receptor stimulation from engineered support cells to generate short-lived PCs, GCBs, and MBs (Nojima et al., 2011). On one hand, because the precursor frequency of TLR- or CD40-responsive cells vastly outnumbers those responsive to antigen, these systems enable polyclonal stimulation of the majority of cells *in vitro*, which makes them amenable to cell number-demanding experimental interrogation. An important note, however, is that the relationships between TLR and BCR signaling are complex and in some contexts display antagonistic effects (Akkaya et al., 2018; Chen-Bettecken et al., 1985; Richard et al., 2008). One important example is that the generation of short-lived PCs by LPS is strongly inhibited if F(Ab')2 anti-IgM is supplemented in the LPS cultures (Chen-Bettecken et al., 1985). Thus, although important cell types are generated in these *in vitro* systems, navigation of the GRNs that coordinate these cell fate decisions may not fully recapitulate those occurring *in vivo*. Furthermore, although many *in vitro* studies have circumvented the precursor frequency problem of antigen responsive cells by stimulating with polyclonal BCR stimulation, e.g., F(Ab')2 anti-IgM or IgD-dextran, these conditions do not promote PC differentiation (Brunswick et al., 1988).

With these issues in mind, we sought to generate a BCR responsive *in vitro* system that would enable mechanistic evaluation of B cell fate decisions e.g., that of undergoing CSR vs. PC differentiation with downstream cell number-demanding experimental interrogation – including ChIP-seq and protein complex identification. Our system combines B cells from B1-8 VDJ heavy-chain knockin mice and stimulation with cognate antigen (see below). Under these conditions, short-lived PCs and class switched cells were efficiently generated, which allowed us to gain important mechanistic insight regarding GRNs involving the essential transcription factor (TF) IRF4 and the protein complexes in which it is assembled. Interestingly, while these conditions worked with polymerized antigen (bioconjugated to Ficoll), soluble antigen resulted in abortive activation. We propose that the combination of any BCR VDJ knockin and its polymerized cognate antigen is amenable to study B cell activation and PC differentiation.

B1-8 B-cell receptor (BCR)-knockin mouse

B1-8-type VDJ knockin mice were generated separately in two labs, B1-8i from the Rajewsky group (Sonoda et al., 1997) and B1-8^{hi} from the Nussenzweig group (Shih et al., 2002). These mice were created by knocking in a rearranged VDJ derived from the NP (4-hydroxy-3-nitrophenylacetic)-reactive B1-8 hybridoma, composed of V_H186.2, DFL16.1 and J_H2 (Bothwell et al., 1981; Reth et al., 1978) into the JH4 region of the Ig heavy chain locus. Use of these mice in their *in vivo* studies provided important observations of B cell dynamics during the antibody response including germinal centers and memory (Ersching et al., 2017; Ise et al., 2018; Kometani et al., 2013; Roco et al., 2019; Victora et al., 2010). Furthermore, the Nussenzweig lab provided evidence that B cell fate could be influenced by BCR signal strength (Shih et al., 2002). Sciammas et al. reasoned that use of these mice would enable a BCR-dependent *in vitro* model of B cell differentiation (Sciammas et al., 2011). In this system, B cells from B1-8 mice underwent both PC differentiation and CSR upon stimulation with cognate antigen, NP (Ochiai et al., 2013; Sciammas et al., 2011). Furthermore, this strategy has also proven useful for cell number-demanding applications such as protein complex purification and ChIP-seq (Ochiai et al., 2018; Ochiai et al., 2020).

Protein complex purification in B cells

Most proteins do not function in isolation and instead are assembled into complexes containing multiple subunits. Affinity-based protein complex purification has emerged as a powerful tool to identify interacting members and reveal novel biological function. Two strategies of purification are mainly used, immunoprecipitation (IP) of epitope-tagged fusion proteins or endogenous proteins.

Identities of proteins in resulting samples are analyzed by mass spectrometry. Each strategy has advantages, and it is recommended to tailor those advantages with the scientific goal. Tagged fusion proteins are produced by recombinant DNA technology in expression vectors that are then stably expressed in cells or mice; in this scenario background is expected to be low because of the highly specific tag immunoaffinity reagents. This approach is also amenable to structure / function analyses by introducing mutations. For example, we have previously identified complexes of TF BACH2 in BAL17 cells, an immortalized murine mature B cell line, by tag purification. FLAG-HA-tagged BACH2 was stably expressed in BAL17 using virus transduction, and was analyzed by sequential tag-specific IP using anti-FLAG and anti-HA antibodies. The purified BACH2 complex was analyzed by mass spectrometry to identify interacting factors as well as post-translational modifications of BACH2 (Ando et al., 2016; Tanaka et al., 2016). BACH2 represses plasma cell differentiation in mature B cells (Muto et al., 2004; Ochiai et al., 2006). This approach revealed molecular mechanism of BACH2-mediated gene repression (Tanaka et al., 2016) as well as the important finding that BACH2 protein which is inducibly degraded upon BCR signaling (Tamahara et al., 2017). In contrast, purification of endogenous complexes using antibodies directed to the protein of interest has the advantage of understanding the basis of the complex in its native state, and avoids complications sometimes associated with recombinant DNA-based cell engineering.

Cell differentiation, including PC differentiation, is often accompanied by a dynamic transition of protein function. To analyze such dynamics during PC differentiation, it is preferable to utilize primary cells rather than immortalized cell lines which are in a fixed cell state. For example, the protein levels of IRF4 are dynamically increased during the course of PC differentiation (Sciammas et al., 2006). Specifically, using the BCR-induced cell system described above, we found that IRF4 protein levels dramatically increase as cells navigate the GRNs specifying the CSR gene program to the PC program (Sciammas et al., 2011). Importantly, to understand the consequences of this change of IRF4 abundance, we used ChIP-seq to identify that IRF4 preferentially recognizes low affinity DNA binding sites when expressed at high levels. Furthermore, purification of endogenous IRF4 during PC differentiation revealed that it exists in two distinct complexes comprised of the chromatin regulators PC4 or IKAROS (Ochiai et al., 2018; Ochiai et al., 2013; Ochiai et al., 2020). Thus, the *in vitro* PC differentiation system we developed enabled us to reveal the dynamics of IRF4-mediated PC differentiation at the level of both DNA binding protein interactions which has led to a more detailed understanding of PC differentiation. In this protocol, we describe how to set up *in vitro* plasma cell differentiation using B1-8 mice, and the purification of an endogenous protein complex.

Buffer preparation

Timing: 1–2 days

1. Cell culture medium should be freshly made and warmed in the absence of stimulators.
2. Prepare concentrated stock of stimulators, such as NP-ficoll and cytokines. These should be added to warmed medium prior to resuspension of purified B cells for culture.
3. Prepare reagents used for protein complex purification without inhibitors or crosslinkers, which should be added immediately before usage.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
B220-APC (RA3-682)	eBioscience	Cat#103212
IgG1-biotin (A85-1)	BD Biosciences	Cat#553441
CD138-PE (281-2)	BD Biosciences	Cat#553714

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Goat TrueBlot; Anti-Goat IgG HRP	Rockland	Cat#18-8814-33
IRF4 (M-17)	Santa Cruz	Cat#sc-6069
Normal goat IgG	Santa Cruz	Cat#sc-2028
Normal rabbit IgG	Santa Cruz	Cat#sc-2027
PC4	Abcam	Cat#ab84459
αTubulin	Santa Cruz	Cat#sc-5286
Chemicals, peptides, and recombinant proteins		
RPMI-1640	Sigma-Aldrich	Cat#R8758
FBS	Sigma-Aldrich	Cat#172012
HEPES (1 M)	Thermo Fisher/Gibco	Cat#15630-080
Sodium Pyruvate (100 mM)	Thermo Fisher/Gibco	Cat#11360-070
Non-Essential Amino Acids (100 X)	Thermo Fisher/Gibco	Cat#11140-050
L-Glutamine (200 mM)	Thermo Fisher/Gibco	Cat#25030081
Penicillin/Streptomycin (10,000 U/mL)	Thermo Fisher/Gibco	Cat#15140-122
β-Mercaptoethanol	Thermo Fisher/Gibco	Cat#21985-023
NP-Ficoll	Biosearch Technologies	Cat#F1420
Recombinant mouse IL-2	R&D Systems	Cat#402-ML-020
Recombinant mouse IL-4	BD Biosciences	Cat#550067
Recombinant mouse IL-5	R&D Systems	Cat#405-ML-025
Recombinant mouse CD40L	R&D Systems	Cat#1163-CL
BSA (bovine serum albumin)	Sigma	Cat#A8022
Sodium azide	Wako	Cat#195-11092
cOmplete ULTRA Tablets, Mini, EDTA-free (Protease Inhibitor Cocktail)	Roche	Cat#5892791001
L-Cysteine	Wako	Cat#073-00737
Dimethyl pimelimidate dihydrochloride powder	Sigma	Cat#8388
DMSO (dimethyl sulfoxide)	Sigma-Aldrich	Cat#D2650
DSP (dithiobis(succinimidyl propionate))	Thermo Fisher	Cat#22585
DTME (dithiobismaleimidoethane)	Thermo Fisher	Cat#22335
DL-DTT	Sigma-Aldrich	Cat#9779
Dynabeads Protein A	Thermo Fisher/Invitrogen	Cat#10001D
Dynabeads Protein G	Thermo Fisher/Invitrogen	Cat#10003D
2NA (EDTA· 2NA)	Dojindo	Cat#345-01865
EGTA (ethylene glycol bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid)	Nacalai Tesque	Cat#15214-92
16% Formaldehyde (w/v), Methanol-free	Pierce	Cat#28908
Glycine	Wako	Cat#073-00737
HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid)	Dojindo	Cat#342-01375
KCl (potassium chloride)	Wako	Cat#163-03545
LiCl (lithium chloride)	Wako	Cat#125-01161
NaCl (sodium chloride)	Wako	Cat#197-01667
NP-40 (Nonidet(R) P40 substitute)	Nacalai Tesque	Cat#23640-94
PhosSTOP (phosphatase inhibitor cocktail)	Roche	Cat#4906837001
Sodium borate	Wako	Cat#194-01415
Sodium deoxycholate	Sigma-Aldrich	Cat#D6750
SDS (sodium dodecyl sulfate)	Wako	Cat#191-07145
Tris (hydroxylmethyl) aminomethane	Wako	Cat#011-16381
Acetonitrile (for LC/MS)	FUJIFILM Wako Pure Chemical Corporation	Cat#012-19851
Acrylamide (for proteomics)	FUJIFILM Wako Pure Chemical Corporation	Cat#019-08011
Ammonium bicarbonate (for proteomics)	FUJIFILM Wako Pure Chemical Corporation	Cat#012-21745
Dithiothreitol	Thermo Fisher Scientific	Cat#20291

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Formic acid (for LC/MS)	FUJIFILM Wako Pure Chemical Corporation	Cat# 067-04531
Precast Gel (Long Life Gel) for Electrophoresis; 5%–20%	Oriental Instruments	Cat# HOG-0520-13
Precision Plus Protein™ Dual Color Standards	Bio-Rad	Cat#1610374
Trypsin	Promega	Cat#V5280
Ultrapure water (for LC/MS)	FUJIFILM Wako Pure Chemical Corporation	Cat# 214-01301
Critical commercial assays		
B Cell Isolation Kit, mouse	Miltenyi Biotec	Cat#130-090-862
LS Columns	Miltenyi Biotec	Cat#130-042-401
Experimental models: organisms/strains		
B1-8^{hi} (B57/BL6 background)	Shih et al., 2002	N/A

MATERIALS AND EQUIPMENT

Cytokine stock solutions	
100 μg/mL IL-2	20 μg in 200 μL of filtrated 0.1% BSA/PBS solution, store at −80°C. (Timing: 15 min)
50 μg/mL IL-4	50 μL of 200 μg/mL IL-4, add 150 μL of filtrated 0.1% BSA/PBS solution, store at −80°C. (Timing: 15 min)
10 μg/mL IL-5	10 μL of 100 μg/mL IL-5, add 90 μL of filtrated 0.1% BSA/PBS solution, store at −80°C. (Timing: 15 min)
100 μg/mL CD40 ligand	25 μg in 250 μL of filtrated 0.1% BSA/PBS solution, store at −80°C. (Timing: 15 min)
10 μg/mL NP-Ficoll	5 μL of 1 mg/mL in 495 μL of PBS, store at 4°C. (Timing: 5 min)

Note: For storage of IL-2, IL-4, IL-5 and CD40 ligand, make 10–20 μL aliquots in microfuge tubes.

Cell culture medium

Timing: 10 min

Reagent	Final concentration	Amount
RPMI1640	N/A	Up to 100 mL
FBS	10%	10 mL
1 M HEPES	10 mM	1 mL
100 mM Sodium Pyruvate	1 mM	1 mL
100 X Non-Essential Amino Acids	1 ×	1 mL
200 mM L-Glutamine	2 mM	1 mL
100 X Penicillin/Streptomycin	100 units/mL Penicillin 100 μg/mL Streptomycin	1 mL
55 mM β-mercaptoethanol	50 μM	100 μL
100 μg/mL IL-2	10 ng/mL	10 μL
50 μg/mL IL-4	5 ng/mL	10 μL
10 μg/mL IL-5	1.5 ng/mL	15 μL
100 μg/mL CD40 ligand	0.2 ng/mL	2 μL
NP-Ficoll	-	-

Note: Each cytokine should be supplemented prior to culture. If IL-2 is prepared using cell expression system, such as the baculovirus expression system, the final concentration of IL-

2 could be 100 U/mL (Sciammas et al., 2011). The final concentration of NP-Ficoll will differ depending on the valency of NP to Ficoll and it is recommended to test and identify the optimal concentration ahead of time, as peak plasma cell responses are inhibited beyond a certain concentration (Ochiai et al., 2018; Sciammas et al., 2011).

FACS wash and staining buffer

Timing: 5 min

FACS buffer	2.5 g BSA, 0.25 g sodium azide, fill up to 500 mL with PBS, store at 4°C.

Buffer stocks used in this protocol

1 M Tris	12.11 g Tris, adjust to each pH with HCl, fill up to 100 mL with ddH_2O. Autoclave sterilization, store at 20°C–25°C. (Timing: 1 h)
5 M NaCl	29.22 g NaCl in 100 mL ddH_2O. Autoclave sterilization, store at 20°C–25°C. (Timing: 1 h)
0.5 M HEPES (pH 8.0)	23.83 g HEPES, adjust to pH 8.0 with 10N NaOH, fill up to 100 mL with ddH_2O. Autoclave sterilization, store at 20°C–25°C. (Timing: 1 h)
0.5 M EDTA (pH 8.0)	18.61 g $Na_2EDTA\cdot 2H_2O$, adjust to pH 8.0 with NaOH, fill up to 100 mL ddH_2O. Autoclave sterilization, store at 20°C–25°C. (Timing: 1 h)
0.1 M EGTA	9.4 g EGTA, fill up to 200 mL ddH_2O. Autoclave sterilization, store at 20°C–25°C. (Timing: 1 h)
1 M LiCl	4.24 g LiCl, fill up to 100 mL ddH_2O. Autoclave sterilization, store at 20°C–25°C. (Timing: 1 h)
1 M MgCl2	20.33 g $MgCl_2\cdot 6H_2O$, fill up to 100 mL ddH_2O. Autoclave sterilization, store at 20°C–25°C. (Timing: 1 h)
10% NP-40	5 mL, fill up to 50 mL ddH_2O, store at 20°C–25°C. (Timing: 5 min)
10% (w/v) SDS	5 g, fill up to 50 mL ddH_2O, store at 20°C–25°C. (Timing: 30 min)
60% (w/v) Sucrose	60 g, fill up to 100 mL ddH_2O, store at 20°C–25°C. (Timing: 5 min)
1 M DTT	3.09 g DTT in 20 mL ddH_2O. Store 20–100 μL aliquots in microfuge tubes at −20°C. (Timing: 15 min)

Buffers used for antibody conjugation

0.2 M sodium borate (pH 9.0)	7.6 g $Na_2B_4O_7\cdot 10H_2O$, adjust to pH 9.0 with HCl, fill up to 100 mL with ddH_2O. Store at 20°C–25°C. (Timing: 1 h)
0.1 M Tris (pH 8.0)	A ten-fold dilution of 1 M Tris-HCl (pH 8.0) with ddH_2O. Store at 20°C–25°C. (Timing: 5 min)

Buffers used for ReCLIP

Timing: 10 min for 2 × cross link buffer

Timing: 5 min for quench buffer

Timing: 15 min for 5 × RIPA buffer

Timing: 5 min for elution buffer

2 × Cross link buffer

Reagent	Final concentration	Amount
DTME	0.5 mM	1.6 mg
DSP	0.5 mM	2.0 mg
PBS	N/A	Up to 5 mL

Note: First dissolve DSP in 100–150 μL DMSO and then combine with DTME prior to diluting in PBS. Prepare fresh prior to use.

Note: DTME and DSP should be kept at 4°C in the presence of $CaSO_4$, also known as Drierite, to avoid humidity. Prior to use, recover these reagents to 20°C–25°C.

Quench buffer		
Reagent	Final concentration	Amount
Cystein	5 mM	6 mg
1 M Tris (pH 7.5)	20 mM	200 μL
ddH_2O	N/A	Up to 10 mL

Note: Prepare fresh prior to use.

5 × RIPA buffer		
Reagent	Final concentration	Amount
1 M Tris (pH 7.4)	250 mM	25 mL
5 M NaCl	750 mM	15 mL
10% NP-40	5%	50 mL
Sodium deoxycholate	2.5%	2.5 g
10% SDS	0.5%	5 mL
0.5 M EDTA	5 mM	1 mL
ddH_2O	N/A	Up to 100 mL

Note: Store at 20°C–25°C. Prior to use, dilute with ddH_2O supplemented with 1 × proteinase inhibitor cocktail and 1 × PhosSTOP.

Elution buffer		
Reagent	Final concentration	Amount
1 M Tris (pH 8.0)	250 mM	20 μL
5 M NaCl	0.2 M	40 μL
10% SDS	2%	200 μL
1 M DTT	50 mM	50 μL
ddH_2O	N/A	Up to 1 mL

Note: Prepare fresh prior to use.

Buffers used for ChIP

Timing: 5 min for 10 × crosslink buffer

Timing: 10 min for quench buffer

Timing: 10 min for lysis buffer

Timing: 5 min for nuclei lysis buffer

Timing: 5 min for dilution buffer

Timing: 5 min for wash buffer 1

Timing: 5 min for wash buffer 2

Timing: 5 min for wash buffer 3

Timing: 5 min for TE

10 × Crosslink buffer		
Reagent	Final concentration	Amount
16% Formaldehyde	11%	687.5 µL
5 M NaCl	100 mM	20 µL
0.5 M EDTA	1 mM	2 µL
0.1 M EGTA	0.5 mM	5 µL
0.5 M HEPES (pH 8.0)	50 mM	100 µL
ddH_2O	N/A	Up to 1 mL

Note: Prepare prior to use with fresh 16% Formaldehyde.

Quench buffer	
2.5 M Glycine	18.77 g Glycine, fill up to 100 mL with ddH_2O. Autoclave sterilization, store at 20°C–25°C.

Note: Use with 2.5 mM final concentration.

Lysis buffer		
Reagent	Final concentration	Amount
1 M Tris (pH 7.5)	25 mM	125 µL
1 M KCl	0.25 mM	1.25 µL
1 M $MgCl_2$	7.5 mM	37.5 µL
10% NP-40	0.5%	250 µL
60% Sucrose	30%	5 mL
ddH_2O	N/A	Up to 10 mL

Note: Prior to use, supplement with 1 × Proteinase inhibitor cocktail.

Nuclei Lysis buffer		
Reagent	Final concentration	Amount
1 M Tris (pH 8.1)	25 mM	50 µL
0.5 M EDTA	10 mM	20 µL
10% SDS	1%	100 µL
ddH_2O	N/A	Up to 1 mL

Note: Prior to use, dilute with ddH_2O supplemented with 1 × proteinase inhibitor cocktail and 1 × PhosSTOP.

Dilution buffer		
Reagent	Final concentration	Amount
1 M Tris (pH 8.1)	16.7 mM	167 µL
0.5 M EDTA	1.2 mM	24 µL
5 M NaCl	167 mM	334 µL
10% SDS	0.01%	10 µL
10% Triton-X	1.1%	1.1 mL
ddH_2O	N/A	Up to 10 mL

Note: Prior to use, dilute with ddH_2O supplemented with 1 × Proteinase inhibitor cocktail and 1 × PhosSTOP.

Wash buffer 1		
Reagent	Final concentration	Amount
1 M Tris (pH 8.1)	20 mM	167 μL
0.5 M EDTA	2 mM	40 μL
5 M NaCl	150 mM	300 μL
10% SDS	0.1%	100 μL
10% Triton-X	1%	1 mL
ddH_2O	N/A	Up to 10 mL

Wash buffer 2		
Reagent	Final concentration	Amount
1 M Tris (pH 8.1)	20 mM	167 μL
0.5 M EDTA	2 mM	40 μL
5 M NaCl	500 mM	1 mL
10% SDS	0.1%	100 μL
10% Triton-X	1%	1 mL
ddH_2O	N/A	Up to 10 mL

Wash buffer 3		
Reagent	Final concentration	Amount
1 M Tris (pH 8.1)	10 mM	100 μL
0.5 M EDTA	1 mM	20 μL
1 M LiCl	250 mM	2.5 mL
10% NP-40	1%	1 mL
ddH_2O	N/A	Up to 10 mL

TE		
Reagent	Final concentration	Amount
1 M Tris (pH 8.0)	10 mM	100 μL
0.5 M EDTA	1 mM	20 μL
ddH_2O	N/A	Up to 10 mL

Note: Autoclave sterilization, store at 20°C–25°C.

Reagents used for preparing mass spectrometry samples

Timing: 5 min for fixation solution

Timing: 10 min for CBB staining solution

Timing: 5 min for ammonium bicarbonate

Timing: 5 min for acrylamide

Timing: 5 min for reduction solution

Timing: 5 min for alkylation solution

Timing: 5 min for digestion solution

Timing: 5 min for trypsin stock solution

Fixation solution		
Reagent	Final concentration	Amount
MeOH	40%	40 mL
Acetic acid	10%	10 mL
ddH_2O	N/A	Up to 100 mL

CBB staining solution		
Reagent	Final concentration	Amount
MeOH	20%	20 mL
Acetic acid	5%	5 mL
CBB (either G-250 or R-250)	0.1%	100 mg
ddH_2O	N/A	Up to 100 mL

Note: Store at 20°C–25°C.

Ammonium bicarbonate	
1 M ammonium bicarbonate	79.06 g Ammonium bicarbonate, dissolved in 1 L Ultrapure water

Note: Used to prepare Reduction and Alkylation solutions and Digestion buffer.

Acrylamide	
1.1 M acrylamide	78.19 g Acrylamide, dissolved in 1 L Ultrapure water

Note: Used to prepare Alkylation solution.

Reduction solution		
Reagent	Final concentration	Amount
1 M DTT	10 mM	10 µL
1 M ammonium bicarbonate	25 mM	25 µL
Ultrapure water	N/A	Up to 1 mL

Note: Prepare just prior to use.

Alkylation solution		
Reagent	Final concentration	Amount
1.1 M acrylamide	55 mM	50 µL
1 M ammonium bicarbonate	25 mM	25 µL
Ultrapure water	N/A	Up to 1 mL

Note: Prepare just prior to use.

Digestion solution		
Reagent	Final concentration	Amount
1 M ammonium bicarbonate	50 mM	50 µL
Acetonitrile	10%	100 µL
Ultrapure water	N/A	Up to 1 mL

Note: Prepare just prior to use.

Trypsin stock solution	
100 μg/mL trypsin	100 μg trypsin, in 1 mL ice-chilled 5% acetic acid, prepared in Ultrapure water.

Note: Store 5–10 μL aliquots in microfuge tubes at −80°C. Dilution in Digestion buffer at 1/100 just prior to use.

STEP-BY-STEP METHOD DETAILS

Determination of NP-Ficoll concentration for B-cell activation

Timing: 3–4 days

Purified B cells from B1-8 mice are stimulated with the T-cell dependent cytokines IL-2, IL-4, IL-5, sub-optimal CD40 ligand and cognate antigen NP-Ficoll. We note that in the absence of NP-Ficoll, minimal plasma cell and CSR responses are observed. The amount of NP-Ficoll is absolutely critical as it displays a biphasic response, where plasma cell differentiation is inhibited at higher concentrations despite not inhibiting proliferation. Within a critical window, increasing the amount of NP-Ficoll alters the strength of BCR signaling and influences the efficiency of plasma cell differentiation as well as CSR (Ochiai et al., 2018; Sciammas et al., 2011). Any valency of NP-Ficoll can be used; however, due to their effects on BCR signal strength, each exhibit different dose response relationships to B cell differentiation and thus require titration (Figure 1A) (Ochiai et al., 2018; Sciammas et al., 2011). The concentration of NP-Ficoll can be determined using RT-PCR and flow cytometry; the genetic event related to CSR is observed at around 24–60 h using RT-PCR. $CD138^+$ plasma cells with/without class switched $IgG1^+$ can be detected from 72 h using flow cytometry. It is recommended to examine the frequency of $CD138^+$ cells with/without $IgG1^+$ by titrating NP-Ficoll in a first experiment. Then, using the selected concentration of NP-Ficoll, the genetic event will be confirmed.

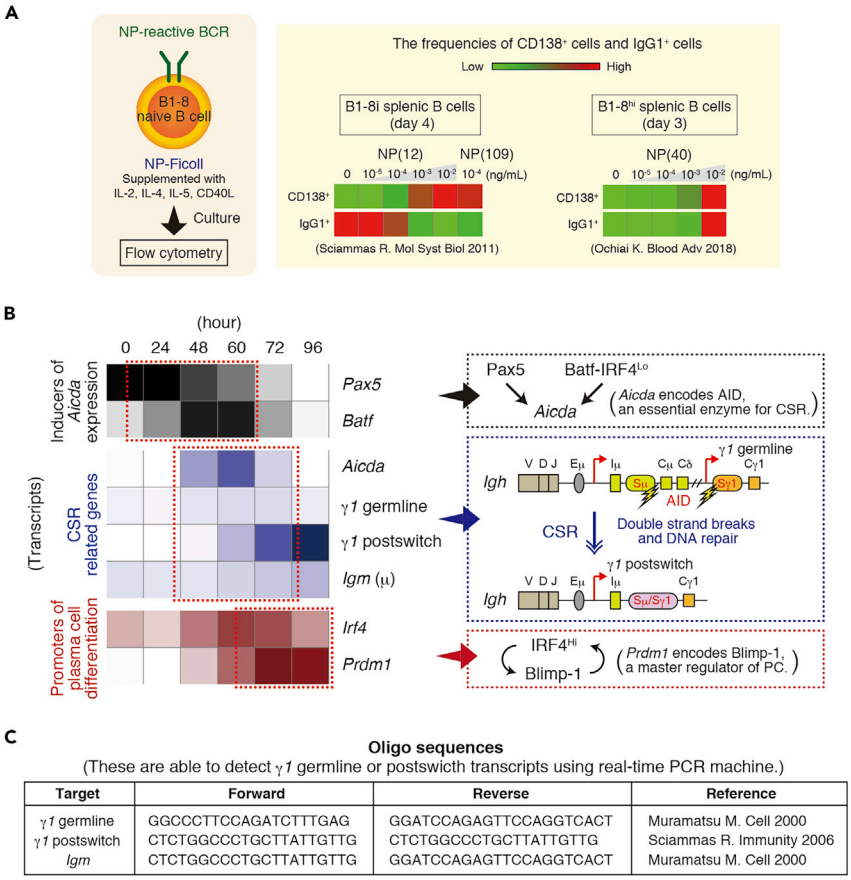
Note: We have found that the amount of NP-Ficoll used differs when analyzing B1-8i versus B1-8hi *in vitro* responses (Figure 1A).

Note: To perform RT-PCR, you require additional days for RNA purification, cDNA synthesis, and PCR analysis.

1. Perform negative enrichment of splenic B cells via magnetic separation according to manufacturer's protocol.
(https://www.miltenyibiotec.com/US-en/products/b-cell-isolation-kit-mouse.html#130-090-862)
   a. Homogenize mouse spleen in 2–3 mL of Red Blood Cell Lysis Buffer, wash with 2–3 mL of MACS buffer, and transfer to 15 mL centrifugation tube.
   b. Centrifuge (300 xg, 4°C, 5 min) and resuspend in 5–10 mL of MACS buffer.
   c. After magnetic separation, collect unbound cells according to the manufacturer's protocol. (https://www.miltenyibiotec.com/JP-en/products/ls-columns.html#130-042-401)
   d. Count cells.

Note: Keep about 1×10^6 before adding antibody cocktail of the B cell isolation kit, and compare with sorted cells for the enrichment of B220 positivity. It is expected to achieve ~95% enrichment purity.

   e. Incubate with 10 μL of diluted antibody in FACS buffer. Anti-B220 is diluted 1 in 100, and blocking Fc receptors (anti-CD16/CD32) is not necessary.



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Oligo sequences

(These are able to detect γ1 germline or postswicth transcripts using real-time PCR machine.)

Target	Forward	Reverse	Reference
γ1 germline	GGCCCTTCCAGATCTTTGAG	GGATCCAGAGTTCCAGGTCACT	Muramatsu M. Cell 2000
γ1 postswitch	CTCTGGCCCTGCTTATTGTTG	CTCTGGCCCTGCTTATTGTTG	Sciammas R. Immunity 2006
Igm	CTCTGGCCCTGCTTATTGTTG	GGATCCAGAGTTCCAGGTCACT	Muramatsu M. Cell 2000

Figure 1. Evaluation of gene expression during the course of PC differentiation

(A) (Left) Schematic of cell stimulation. (Right) The frequencies of $CD138^+$ plasma cells and $IgG1^+$ cells in B1-8i or B1-8^{hi} mice splenic B cells, stimulated with different valency of NP-Ficoll. Data are from Sciammas R. 2011 or Ochiai K. 2018.

(B) Visualization of qRT-PCR for indicated transcripts (Left), and schematic of these factors and co-relation with CSR and PC differentiation (Right). Each transcript was normalized by β-2 microglobulin.

(C) Oligo sequences which can detect γ1 germline or postswitch transcripts using real-time PCR.

f. Wash unbound antibody with 1 mL FACS buffer: Centrifuge (300 xg, 5 min) and remove supernatant.

2. Prepare culture medium supplemented with cytokines/stimulators (excluding NP-Ficoll).
3. Plate cells in culture dish at 0.5 × 10^6 cells/cm^2 in a volume of 0.5–1.0 × 10^6 cell/mL).

Note: When we examine the condition of cell differentiation efficiency, we normally use a 24 well plate with round bottom, 1 mL/well culture.

4. Add NP-Ficoll at empirically derived amount.

Important: In our hands, a final concentration of 0.01 ng/mL is effective for maximal PC differentiation whereas a 100 fold less for maximal CSR (Sciammas et al., 2011). For PC, a useful range is 0.001–0.1 ng/mL with a valency of ~10 moles of NP to 1 mole of Ficoll or about 10–50 fold less when using a valency of ~100 moles of NP to mole of Ficoll. In contrast, for maximal CSR, use 100–1,000 fold less NP-Ficoll.

5. Culture for up to 96 h (4 days) before analysis.

Important: The onset of CSR and PC differentiation is around 48 h (day 2) and peak generation is 96 h (day 4). Prominent cell death is observed after day 4.

6. Examine the frequency of IgG1$^+$ and/or CD138$^+$ cells by flow cytometry (preferred).
   a. Wash 1–2 × 10^6 cells with 1 mL FACS buffer: Centrifuge (300 xg, 5 min) and remove supernatant.

   Note: Blocking Fc receptors (anti-CD16/CD32) is not necessary.

   b. Incubated with 10 μL of diluted antibody in FACS buffer.

   Note: Anti-CD138 is diluted 1 in 30 and anti-IgG1 is diluted 1 in 100.

   c. Incubate (at least 30 min and up to 2 h).
   d. Wash unbound antibody with 1 mL FACS buffer: Centrifuge (300 xg, 5 min) and remove supernatant.

   Optional: Dead cells are excluded using a viability dye such as DAPI, PI, or 7-AAD.

   Note: In our hands, with the higher dose of NP-Ficoll, PC frequencies, as determined by anti-CD138 staining, were in the range of 50% of live cells. Similarly, at the lower dose of NP-Ficoll, frequencies of cells expressing surface IgG1 were 20%.

   Note: Alternatively, analyzing the expression of key genes including those for Blimp-1 and Irf4 (PC) or Aicda and germline IgG1 transcripts (CSR) over the 4 day time course can help determine the suitable NP-Ficoll concentration (Figures 1B and 1C). Genes examined for their expression in Figure 1B are described in below.

   i. Inducers of *Aicda* expression

Pax5; transcription factor which maintains B cell identify, and activates *Aicda* expression (Gonda et al., 2003; Nera et al., 2006).
Batf; transcription factor which activates *Aicda* expression collaborating with IRF4 (Ise et al., 2011; Ochiai et al., 2018).

   ii. CSR related genes

Aicda; encodes AID, an essential enzyme for CSR (Chaudhuri et al., 2003; Muramatsu et al., 2000).
γ1 germline; *γ1* transcripts prior to CSR, detected using γ1-forward and reverse primers (Muramatsu et al., 2000).
γ1 postswitch; *γ1* transcripts after CSR, detected using Igm-forward and γ1-reverse primers (Sciammas et al., 2006).
Igm; transcripts of μ, detected using Igm-forward and reverse primers (Muramatsu et al., 2000).

   iii. Promoters of plasma cell differentiation

IRF4*;* transcription factor required for both CSR and PC. Activates *Prdm1* expression (Gonda et al., 2003; Sciammas et al., 2006).
Blimp-1*;* a master regulator of PC, encoded by *Prdm1*. Activates *Irf4* expression (Minnich et al., 2016; Shapiro-Shelef et al., 2003).

Purification of endogenous protein complex from activated B cells

Timing: 3–4 days

Prior to designing the complex purification, it is necessary to prepare the specific antibodies against a target protein. The efficiency of immunoprecipitation (IP) protein purification is directly influenced by the quality of antibody and a key factor in the success of endogenous protein purification is the antibody conjugation. The contamination of immunoglobulin (Ig) in mass spectrometry samples

strongly interferes with the detection of peptides, which can be minimized in the Ab conjugation step. In addition, it is important to prepare enough cells for IP. Mouse splenic B cells are much smaller than cells from other tissues and therefore result in low protein yields. To obtain detectable signals for mass spectrometry, we normally start with 1×10^8 cells per IP reaction; this requires purification of B cells pooled from multiple B1-8 mice as the typical yield from one mouse is $2–4 \times 10^7$. In our experience, it is expected to obtain 1×10^8 splenic B cells from about 4 mice. Upon stimulation, cell number does not alter at 24 h, but it becomes about double at 72 h. Although we normally do not sort cells after stimulation further, you may sort with specific surface markers. In that case, you will need increased number of cells for complex purification.

Finally, the specificity of IP samples must be evaluated in LC-MS/MS, ideally with target-deficient B cells as a control. However, in cases where the target proteins are critical for cell survival, it is difficult to do so. In our previous analyses, we purified the IRF4 complex (Ochiai et al., 2018) and the PC4 complex (Ochiai et al., 2020) from B1-8^{hi} B cells stimulated for 72 h. Both IRF4 and PC4 are necessary for cell survival and/or plasma cell differentiation upon B cell activation. Therefore, we utilized an IgG IP as the control for αIRF4 and αPC4 IP.

7. Antibody (Ab) adsorption and conjugation to protein A/G dynabeads
   It is recommended to prepare the antibody-conjugated beads the day before.

   Note: Take care to determine whether the antibody species and/or isotype is compatible with protein A/G dynabeads. Use magnetic field to separate supernatant and beads.
   Protocol using 5 μg antibodies; sufficient for 1×10^8 cells/IP.
   a. Transfer 50 μL dynabeads into 1.5 mL tube.
   b. Wash dynabeads twice with cold PBS.
   c. Resuspend the beads to the manufacturer-prepared original 1:1 slurry concentration in PBS, typically, 50 μL.
      (https://www.thermofisher.com/order/catalog/product/10001D)
      (https://www.thermofisher.com/order/catalog/product/10003D#/10003D)
   d. Combine antibodies with protein A or G dynabeads supplemented with NaCl.

Reagent	Amount
Antibodies	5 μg
A/G Dynabeads	50 μL
5 M NaCl	1.5 μL

   e. Rotate (20°C–25°C, 1 h).
   f. Remove supernatant.
   g. Wash beads in 10 bead volumes of 0.2 M Sodium borate (pH 9.0), typically 500 μL.
   h. Remove supernatant.
   i. Resuspend in 10 bead volumes of 0.2 M Sodium borate, typically 500 μL.
   j. Crosslink with Dimethyl pimelimidate dihydrochloride at a final concentration of 20 mM from a stock concentration of 0.2 M dimethyl pimelimidate dihydrochloride in 0.2 M sodium borate.
   k. Rotate (20°C–25°C, 30 min).
   l. Remove supernatant.
   m. Stop reaction by diluting with 10 volumes of 0.1 M Tris (pH 8.0), typically 500 μL.
   n. Remove supernatant.
   o. Resuspend in 10 volumes of 0.1 M Tris (pH 8.0), typically 500 μL.
   p. Incubate (20°C–25°C, more than two hours)

   Note: It is recommended to incubate for 12–15 h.

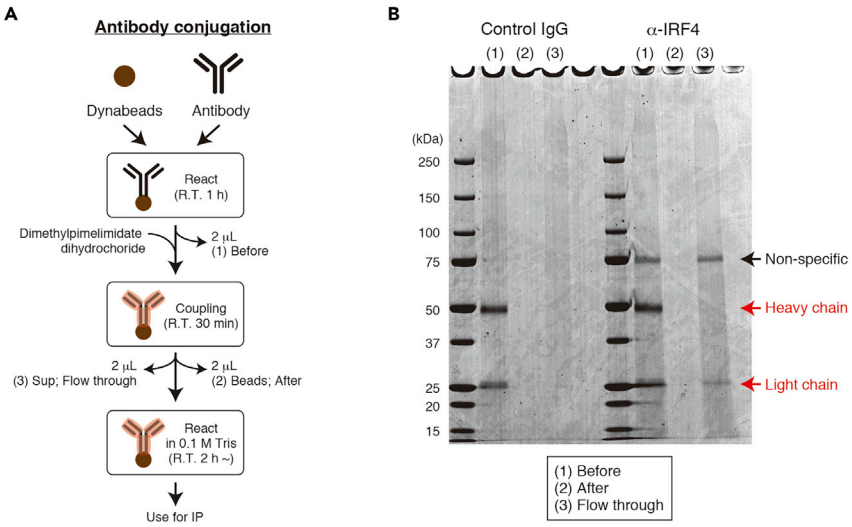


Figure 2. Antibody coupling used for endogenous IP

(A) The workflow of antibody conjugation. Collected samples, (1) to (3), are examined in B.

(B) Evaluation of antibody coupling. Load collected samples (1) to (3) on a SDS-PAGE gel, followed by coomassie staining. Bands observed around 27 kDa and 50 kDa are immunoglobulin (Ig) light and heavy chains, respectively. (1) Before conjugation: Ig bands were detected. (2) After conjugation: Ig bands have disappeared. (3) Flow through: non-specific bands were observed around 25 kDa and 75 kDa with flow through of αIRF4 antibodies conjugation.

q. Wash with 10 volumes of PBS, and remove supernatant.
r. Resuspend Ab-conjugated beads in PBS as a 50:50 slurry; typically 50 μL.
s. Store at 4°C (use within a few days).

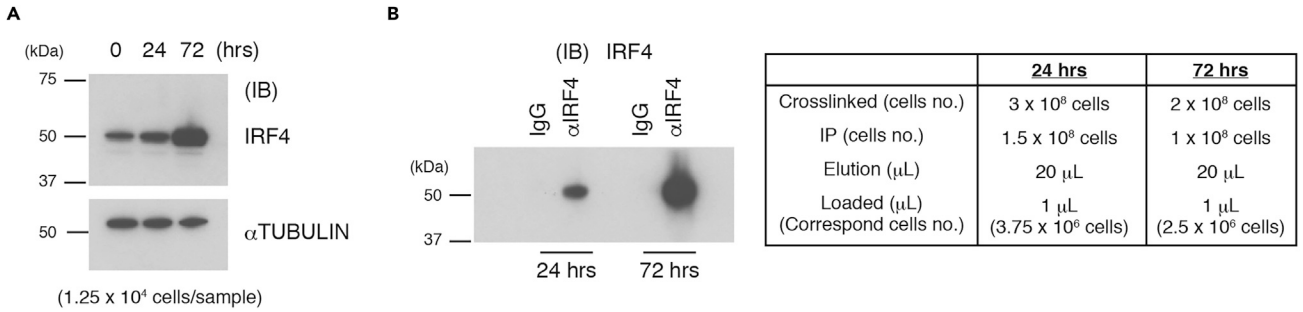
Note: Retain 2 μL at each step to confirm conjugation by SDS-PAGE and Coomassie blue staining (Figures 2A and 2B).

8. ReCLIP (Reverse Cross-Link Immuno-Precipitation) using whole cell extracts

This method is modified from (Smith et al., 2011). Cells are treated with reversible crosslinkers, DTME and DSP, which stabilize normally labile or dynamic molecular interactions. In the original protocol, target protein and interacting proteins were separated by reverse cross-linking using 1 × RIPA supplemented with 50 mM DTT. Our protocol modified this separation step, and eluted both target protein and interacting proteins (see step t. Elution). Therefore, the IP efficiency can be determined using immunoblot prior to LC-MS/MS (Figures 3A and 3B).

Protocol using 2×10^8 cells, which are divided into two samples (IgG and target protein) at step p.

a. Wash cells three times: suspend in 40 mL PBS and centrifuge (300 xg, 20°C–25°C, 5 min).
b. Resuspend pellet in 5 mL PBS.
c. Add 5 mL of 2 × Cross link buffer and mix gently.
d. Incubate in water bath with mild shaking (20°C–25°C, 30 min).
e. Centrifuge (1,710 xg, 20°C–25°C, 5 min) and remove supernatant.
f. Resuspend in 5 mL of ReCLIP quench buffer.
g. Incubate in water bath with mild shaking (20°C–25°C, 5 min).
h. Centrifuge (1,710 xg, 20°C–25°C, 5 min) and remove supernatant.
i. Resuspend with ice-cold PBS and centrifuge (1,710 xg, 4°C, 5 min).
j. Remove supernatant and add 1 mL of RIPA buffer, and transfer to 1.5 mL tube.
k. Incubate on ice for 10 min.
l. Sonicate using a Bioruptor: 10 cycles of 30 s ON and 30 s OFF.
m. Centrifuge (17,800 xg, 4°C, 10 min) and transfer supernatant into new 1.5 mL tube.
n. Incubate lysate with 100 μL unconjugated protein A/G dynabeads (4°C, 1 h).



	24 hrs	72 hrs
Crosslinked (cells no.)	3 x 10^8 cells	2 x 10^8 cells
IP (cells no.)	1.5 x 10^8 cells	1 x 10^8 cells
Elution (μL)	20 μL	20 μL
Loaded (μL) (Correspond cells no.)	1 μL (3.75 x 10^6 cells)	1 μL (2.5 x 10^6 cells)

Figure 3. An example of IP evaluation

(A) Immunoblot analysis of IRF4 protein expression in BCR activated B1-8^{hi} splenic B cells at indicated time. αTUBULIN; internal control. 1.25 × 10^4 cells were loaded per sample.

(B) Evaluation of IRF4 IP 24 and 72 h post BCR activation. Left: Immunoblot analysis using αIRF4 antibodies for IP. IgG; control IgG. Goat TrueBlot was used for secondary antibody staining. To detect IP samples in IB, the TrueBlot series (Rockland) reduced detection of immunoglobulin heavy and light chains. (Right) Table indicates the details of IP procedure and sample amounts loaded for immunoblot analysis.

Note: This step removes bead-only reactive material from the lysate. Sample is divided at the next step, and use equal volume as total Ab conjugated beads. For example, if you divide into two tubes to perform IP using 50 μL of conjugated control IgG or 50 μL of target protein in each tube, use 100 μL of uncojugated beads for this step.

o. Apply magnetic field to the tubes in order to magnetically trap the beads; transfer supernatant to a new 1.5 mL tube.
   Important: At this step, retain 1/10 volume to use as an input control, typically 50 μL.
p. Divide sample equally into two 1.5 mL tubes, typically less than 500 μL/tube.
q. Add 50 μL of conjugated beads into each 1.5 mL tube.
   Tube 1; Control IgG-conjugated beads
   Tube 2; Target protein-specific Ab-conjugated beads
r. Rotate (4°C, at least 2 h).

Note: If necessary, incubation can be extended to 12–15 hours.

s. Wash with 500 μL of 1× RIPA buffer at least twice.
t. Elution
   i. Add 10 μL of elution buffer.
   ii. Incubate at 37°C for 20 min, then 70°C for 10 min.
   iii. Insert into the magnetic field to trap beads and retain the supernatant.
   iv. Transfer the supernatant into a new 1.5 mL tube.
   v. Add 10 μL of 2 × SDS sample buffer (Final volume; 20 μL).

Note: If you prefer to prepare highly-dense immunopreciptated samples, you may use a higher concentration of SDS sample buffer, such as 5 × SDS sample buffer, to reduce the final volume.

9. ChIP (Chromatin-immunoprecipitation) of nuclear extracts
   This method is modified from standard ChIP assays, using formaldehyde as a crosslinker. It could be useful if ReCLIP is not suitable to analyze chromatin-related proteins. In our case, we purified the PC4 complex using this method. PC4 regulates chromatin compaction by interacting with linker histones and heterochromatin proteins (Das et al., 2010; Das et al., 2006).

   Note: Compared with ReCLIP, the higher number of cells might be required to obtain the protein complex using sample amount of conjugated beads. For example, we carried out PC4 IP with around 6–10 × 10^8 cells using this method, whereas we carried out IRF4 IP with around 1 × 10^8 cells using ReCLIP. It should be noted that cell number required for IP is also affected by other factors, such protein expression level and antibody efficiency for IP.

a. Suspend cells in culture medium at 4 × 10^6 cells/mL.
b. Add 1/10 volume of 10 × crosslink buffer (final concentration of 1×) and mix gently.
c. Incubate (20°C–25°C, 10 min).
d. Quench with 1/20 volume of 2.5 M Glycine.
e. Incubate (20°C–25°C, 5 min).
f. Add >2 volumes of ice-cold PBS.
g. Centrifuge (1,710 xg, 4°C, 5 min) and remove supernatant.
h. Add ice-cold PBS as in step f.
i. Centrifuge (1,710 xg, 4°C, 5 min) and remove supernatant.
j. Resuspend in Lysis buffer at 20 × 10^6 cells/mL.
k. Incubate on ice for 10 min.
l. Centrifuge (1,710 xg, 4°C, 5 min) and remove supernatant.
m. Resuspend in Nuclear Lysis buffer at 20 × 10^6 cells/mL, and divide lysate into 300 μL volumes in 1.5 mL t tubes.
n. Incubate (20°C–25°C, 10 min).

Note: This step dissolves nuclear membrane and increases the sonication efficiency. After incubation, keep sample tubes at 4°C. To avoid SDS crystal precipitation, put sample tubes on a plastic eppen stand which is kept on ice.

o. Sonicate using a Bioruptor: 10 cycles of 30 s ON and 30 s OFF.

Note: Sonication conditions may vary depending on cell type; these have been optimized for *in vitro* activated B cells.

p. Centrifuge (17,800 xg, 4°C, 15 min) and transfer supernatant into a new 1.5 mL tube.
Important: At this step, retain 1/10 volume to use as an input control.
q. Dilute with 4 volumes of ChIP dilution buffer.
r. Incubate lysate with 100 μL of unconjugated protein A/G dynabeads (4°C, 1 h).

Note: Same as step 8.n.

s. Apply magnetic field to the tubes to magnetically trap the beads; transfer supernatant to a new tube and equally split into two 1.5 mL tubes.
t. Add 50 μL of conjugated beads into each 1.5 mL tube.
Tube 1; Control IgG-conjugated beads
Tube 2; Target protein-specific Ab-conjugated beads
u. Rotate (4°C, 2 h).
v. Wash with PBS or ChIP wash buffer three times, followed by two washes with TE, and elute as described in ReCLIP section (see step 8s).

Optional: Elution using ChIP elution buffer (final 1% SDS, 100 mM $NaHCO_3$) allows proceeding to ChIP qPCR or ChIP-seq, after reverse crosslinking and DNA purification.

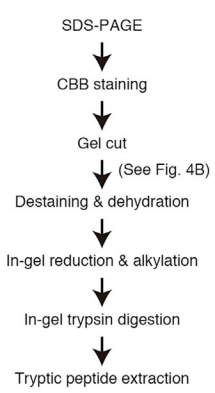
10. Check the IP efficiency by immunoblot (Figures 3A and 3B).

Sample preparation for LC-MS/MS

Timing: 2–3 days

Protein identification by LC-MS/MS requires efficient digestion of proteins into peptides and removal of molecules such as detergents or salts which can damage LC columns or interfere with ionization in MS. Many methods to these ends have been published, each of which has pros and cons. We usually subject samples to SDS-PAGE followed by in-gel digestion. Although this method

A



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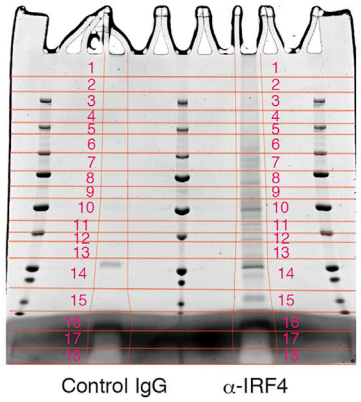

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Complex	Method	Cell no. /IP (Ab volume)	LC-MS/MS Score	LC-MS/MS Peptide (IRF4 or PC4)
IRF4 (Lot 1)	ReCLIP	1 x 10^8 (20 μg)	IgG; 31 αIRF4; 7,351	IgG; 1 αIRF4; 313
IRF4 (Lot 2)	ReCLIP	0.9 x 10^8 (20 μg)	IgG; N.D. αIRF4; 3,906	IgG; N.D. αIRF4; 208
PC4 (Lot 1)	ChIP	6 x 10^8 (20 μg)	IgG; 22 αPC4; 2,527	IgG; 1 αPC4; 140
PC4 (Lot 2)	ChIP	10 x 10^8 (20 μg)	IgG; N.D αPC4; 1,084	IgG; N.D αPC4; 70

Figure 4. Sample preparation for mass spectrometry

(A) The workflow for preparing mass spectrometry samples.

(B) Actual marks for gel extraction. Cells: B1-8^{hi} splenic B cells stimulated for 72 h. IP samples: control IgG, αIRF4.

(C) Outcomes of protein complex purification by mass spectrometry. These data have been reported in our previous reports; IRF4 complexes (Ochiai et al., 2018), PC4 complexes (Ochiai et al., 2020). Used antibodies: αIRF4 (Santa Cruz #sc-6067; no longer available), αPC4 (Abcam #84459). N.D.: not detected.

is time-consuming, it is easier and more consistent compared to other methods. In addition, SDS-PAGE serves for protein fractionation which can reduce protein complexity in an MS/MS run. See the workflow in Figure 4A.

11. Protein separation by SDS-PAGE

    The procedure is not particularly different from ordinary SDS-PAGE except that we must beware of contamination of human keratin. Electrophoresis apparatuses and running buffer should be clean.

    a. Prepare SDS-PAGE using a 5%–20% gradient polyacrylamide gel.
    b. Apply the entire volume of samples and 1.5–2 μL of the protein marker in every other lane.
    c. Cover the top of electrophoresis apparatus to avoid contamination of dust. Start electrophoresis at a constant voltage of 70–80 V.
    d. Stop run when the electrophoresis front is 2–3 cm above the gel bottom.

12. CBB (Coomassie Brilliant Blue) staining of the gel

    Other protocols will work, but heating or microwaving should be avoided.

    a. Disassemble the apparatus and remove the region of the gel below the electrophoresis front.
    b. Put the gel in a clean plastic container (with a cover), and rinse the gel briefly with Milli-Q water.
    c. Fix the gel in the fixation solution with gentle shaking (30 min or longer).
    d. Replace the solution with Milli-Q water, and add 2–3 mL of CBB staining solution.
    e. Shake the container gently until protein bands in the sample lanes become visible (20°C–25°C, 1–2 h or longer).

    Note: Staining is complete when two pink bands in the protein marker lane turn purple (Protein marker used: Precision Plus Protein™ Dual Color Standards from Bio-Rad). If staining looks insufficient, refresh water with staining solution.

    f. Replace the solution with Milli-Q water to reduce background staining.

    Pause point: The gel can be stored at 4°C at any step after fixation.

13. Cutting the gel

a. Cut the entire lane of interest into gel pieces using a clean surgical knife.

Note: It may be difficult when protein bands are faint. We usually scan a gel with a flatbed image scanner, enhance contrast in sample lanes in the image using a photo retouch software, draw vertical and horizontal lines to assist cutting off gel pieces, and print it out on an overhead projector sheet (Figure 4B). The gel is superposed on the sheet laid on a tracing light box. Number of gel pieces from one lane depends on sample complexity, where four is probably the maximum to give a gel size that a microfuge tube can contain.

b. Make many small scores in the gel pieces by sticking them with the knife.
c. Transfer every gel piece into numbered 1.5- or 2-mL tubes.

Pause point: Gel pieces can be stored at 4°C for at least a few days. For a longer storage, add 30% acetonitrile/0.1M ammonium bicarbonate (ABC) to suppress mold growth.

14. Destaining and dehydration of the gel pieces
    a. Add an appropriate volume of 30% acetonitrile (ACN)/0.1 M ABC to every tube.

Note: The volume should be adjusted so that the gel is soaked in the solution. E.g., soak a gel completely with 300 μL 30% ACN, and add 30 μL 1M ABC.

Note: Dilute 100% ACN with Milli-Q water to prepare 30% ACN.

b. Vortex or rotate the tubes until the gels are thoroughly destained.

Note: For deeply stained gels, refresh the solution several times until the gel becomes colorless.

c. Replace the solution with 50% ACN.
d. Dehydrate the samples by vortex or rotation (20°C–25°C, 5–10 min)
e. Remove solution.
f. Repeat dehydration in 100% ACN (20°C–25°C, 5–10 min)
g. Remove solution.

Note: the gel pieces should appear white.

h. Dry the gel pieces in a centrifugal vacuum concentration.

Pause point: Dried gel pieces can be stored at 4°C for more than a week.

15. In-gel reduction and alkylation of protein
    a. Add an appropriate volume of Reduction solution to each tube; soak gels completely.
    b. Incubate the samples (56°C, 1 h).
    c. Remove Reduction solution.
    d. Add Alkylation solution; soak gels completely.
    e. Incubate the samples with vortex or rotation (20°C–25°C, 45 min).
    f. Remove Alkylation solution
    g. Add Milli-Q water; 0.5–1 mL.
    h. Rinse the samples by vortex or rotation (20°C–25°C, 5–10 min).
    i. Refresh water and wash the gel pieces once more.
    j. Dehydrate the samples in 0.5–1 mL 30% ACN (20°C–25°C, 5–10 min).
    k. Remove solution (Repeat h. once).

Note: Increase count or length of step e-h for complete washing if necessary.

l. Dehydrate in 0.5–1 mL 50% ACN (20°C–25°C, 5–10 min)

m. Remove solution.
n. Dehydrate in 0.5–1 mL 100% ACN (20°C–25°C, 5–10 min)
o. Remove solution thoroughly.
p. Dry the gel pieces in a centrifugal vacuum concentration.

Pause point: Dried gel pieces can be stored at 4°C for more than a week.

16. In-gel trypsin digestion
    a. Chill the samples on ice.
    b. Prepare 1 ng/μL trypsin in ice-chilled Digestion buffer.
    c. Add 10–30 μL of diluted trypsin to each sample.

Note: Chilling the samples and solution are important to suppress trypsin activity before the enzyme enters the gel.

    d. Keep the tubes on ice (10-15 min).

Optional: If necessary, repeat adding a small amount of Digestion buffer (without trypsin) until the gel pieces are fully reconstituted and their surfaces become wet.
    e. After the gel pieces are reconstituted, incubate the sample tubes on ice (more than an hour)
    f. Trypsin digestion: Place the sample tubes into an air incubator (37°C, 10–12 h).

17. Tryptic peptide extraction
    For each sample, peptides are recovered completely by extracting three times. Three extracts are combined, concentrated, and finally suspended in a small volume of 0.5% Formic acid solution.
    a. **First extraction**: Add 70% ACN to each tube, typically 100–300 μL.
    b. Vortex or sonicate for 15–20 min.
    c. Transfer the extracts to new tubes and reduce the volume to 10–20 μL in a centrifugal vacuum concentration.
    d. **Second extraction**: Add freshly prepared 75% ACN containing 1% Formic acid, typically 100–300 μL.
    e. Vortex or sonicate for 15–20 min.

Note: When the first extract is almost dried, transfer the second extract to the same tube and reduce the volume to 10–20 μL in a centrifugal vacuum concentration.

    f. **Third extraction**; Repeat above step d and e.
    g. Combine all three extracts, and reduce the volume to 10–20 μL in a centrifugal vacuum concentration.
    h. Add 6 μL of 2.5% Formic acid, and vortex for 1–2 min.
    i. Adjust the volume to 30 μL by adding Milli-Q water and transfer the samples to 0.3-mL vials.

Pause point: Samples can be stored at −30°C for at least a week.

Run each sample on mass spectrometry to identify peptides.

EXPECTED OUTCOMES

Figure 4C shows expected outcomes of protein complex purification. Compared with ReCLIP, ChIP-based protein purification may require a large number of cells to obtain detectable peptide amount.

LIMITATIONS

Using this *in vitro* BCR-mediated differentiation system, it is possible to examine cell dynamics of CSR and short-lived PCs, but not MBs and long-lived PCs. Also, it is difficult to separate each fraction of activated B cells, such as marginal zone or follicular B cells.

TROUBLESHOOTING

Problem 1

Cell enrichment is poor (step 1).

Potential solution

We typically achieve ~95% enrichment purity ; if you attain <90% purity this may be due to inaccurate cell count affecting quality of antibody labeling, inappropriate antibody dilutions, poor washing of unbound Ab, or use of too few magnetic beads. When cell enrichment is poor, titrate cell number, antibody concentration, and bead numbers used in enrichment. Also, wash cells twice after antibody labeling. If cell enrichment still does not increase, access Miltenyi kit protocol for further troubleshooting information.

Problem 2

The high frequency of cell death is observed (step 6).

Potential solution

Poor cell viability in culture may be due to inadequate stimulation conditions or inefficient reagents. Check FCS lots, since lot variability can affect cell viability and activation / differentiation potential. Check quality and concentration of costimulation reagents. Also, ensure that cells are cultured at an appropriate density for the chosen dish/flask; typically between 0.5–1 million cells/mL

Problem 3

The frequency of cell differentiation is poor (step 6).

Potential solution

If the costimulation reagents have been validated, poor cell differentiation is most likely due to improper concentration of NP-Ficoll. Titrate concentration of NP-Ficoll, and identify the ideal concentration by analyzing cell differentiation phenotype in each condition after 3–4 days in culture. Too little or too much NP-Ficoll will impede plasma cell formation. Note that cell proliferation alone is not a sufficient test as they are not linearly related.

Problem 4

The IP efficiency is low (step 10).

Potential solution

If target protein is properly detected in immunoblot using input samples, low IP efficiency is due to reagents or procedures. If you use the commercial antibodies, the efficiency may differ by each lot. 1 × RIPA buffer contains 0.1% SDS, and the usage of milder detergent, such as NP-40, may increase the efficiency. Longer reaction time for immunoprecipitation, such as 12–15 h (4°C), also increase the efficiency. Note that this may increase immunoprecipitation of non-specific protein as well.

Problem 5

Ig chains are highly detected in eluted samples (step 10 or 12).

Potential solution

This could be caused by unsuccessful Ab conjugation at Step 7. Prepare new buffers. Particularly, crystal precipitation often occurs in 0.2 M sodium borate solution, which is used for antibody conjugation. Use fresh crosslinker, dimethyl pimelimidate dihydrochloride. For the usage of Ab conjugation, it is recommended to store at −20°C after opening and use within six months.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kyoko Ochiai (kochiai@med.tohoku.ac.jp).

Materials availability

This study did not generate new unique technique.

Data and code availability

This study did not generate datasets or code.

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AUTHOR CONTRIBUTIONS

K.O. and R.S. have been working on the regulation of B cell-to-plasma cell differentiation using B1-8 *in vitro* plasma cell differentiation system. H.S. performed LC-MS/MS analysis. T.I. provided the original protocol for antibody conjugation. M.C.F. and E.P.S. provided experimental insight on B-cell purification and stimulation. K.O., H.S., T.I., and K.I. conceived the complex project. All authors were involved in writing the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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