Chapter 2

MATERIAL AND METHODS

2.1. Mouse techniques

2.1.1. Animal husbandry

All animal experiments were performed in accordance with the UK's 1986 Animals Scientific Procedure Act and local institute ethics committee regulations.

Bcl11b conditional knockout mice were generated by Dr. Pentao Liu. Briefly, Bcl11b conditional knockout targeting vector was constructed using recombineering, and the mouse strain *Bcl11b*$^{flox/flox}$ was made according to a standard gene targeting approach in ES cells. Then Cre-ERT2;*Bcl11b*$^{flox/flox}$ mice were derived from the cross between *Bcl11b*$^{flox/flox}$ mice and Cre-ERT2 transgenic mice. Cre-ERT2;*Bcl11b*$^{flox/flox}$ mice were in the mixed C57BL/6J and 129S5 genetic background. Most mice were NK1.1+ by flow cytometry, suggesting that they had inherited the C57BL/6 haplotype at the NK gene complex. The *Bcl11b-tdTomato* knock-in mouse strain, in which the *tdTomato* cassette was inserted into the 3' UTR of *Bcl11b*, was generated by Juexuan Wang in Dr. Pentao Liu's lab. *Bcl11b-tdTomato* knock-in mice are in the C57BL/6 genetic background. Both strains in the C57BL/6 and 129S5 genetic background have the H-2b haplotype at the MHC. *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ mice that are in the C57BL/6 background were obtained from Francesco Colucci at Babraham Institute.

2.1.2. Tamoxifen administration

1 g of tamoxifen (Sigma) was suspended in 5 ml of ethanol and then dissolved in 50 ml of sunflower oil to obtain a final tamoxifen stock at the concentration of 20 mg/ml. To dissolve the tamoxifen completely, the stock was sonicated on ice for 2 min (at 15 sec interval). 1 mg of tamoxifen in sunflower oil stock (50 μl) was administrated to each mouse by oral route each day for three consecutive days. After administration, mice were provided with food mash and their weights were closely monitored.

2.1.3. Intravenous trail vein injection

Donor cells were counted and re-suspended in PBS solution at a certain concentration for injection. Host mice were initially put under a heat lamp for 5 min to increase blood flow to the tail vein. Then they were transferred to a holding device to allow access to the tail vein. 100 μl of cell solution was loaded into a small syringe with a 27G needle. The needle was inserted into vein and the 100 μl cell solution was injected.

2.2. DNA methods

2.2.1. Extraction of DNA from primary cells

Primary cells were initially spun at 300xG for 5 min. Next cell pellets were incubated in 200 μl of lysis buffer [50 mM Tris (pH 7.5), 25 mM EDTA (pH 8), 100 mM NaCl, 0.5% SDS, and 1mg/mL Proteinase K (added fresh)] at 65^0C for 2 hours. After adding 200 μl of isopropanol, the suspension was mixed gently and incubated at -20^0C for 30 min to precipitate the DNA. The suspension was then spun at 16,000xG

for 10 min and washed twice with 70% ethanol. Finally, the DNA pellet was air dried before re-suspended in an appropriate volume of ddH_2O.

2.2.2. Extraction of DNA from tissues

Tissues (ear or tail biopsies) were incubated in 400 μl of lysis buffer as described above at 65^0C for 4 hours or overnight. Next, the buffer with tissue was vortexed vigorously and spun at 16,000xG for 10 min. Genomic DNA was precipitated by adding 500 μl of isopropanol into cell lysis buffer. After centrifugation, DNA was washed once with 500 μl of 70% ethanol and air dried before being re-suspended in an appropriate volume of TE buffer.

2.2.3. Genotyping PCR

Extensor Hi-Fidelity PCR Master Mix 2 (Thermo) was used for PCR amplification. 1 ng of DNA template, 1 μl of each primer (10 μM), and 2 μl of PCR-grade water were mixed with 5 μl of the PCR master mix and incubated in PTC-225 PCR machine (Peltier Thermal Cycler) with the following setting: 94^0C for 2 min, 30 cycles of 94^0C for 30 sec, 60^0C for 30 sec and 68^0C for 30 sec, 68^0 for 10 min. Primers used here are shown in Table 1.

2.2.4. TCR rearrangement PCR

Extensor Hi-Fidelity PCR Master Mix 2 (Thermo) was used for TCR rearrangement PCR amplification. 10 ng of genomic DNA template, 1 μl of each primer (10 μM) and 2 μl of PCR-grade water were mixed with 5 μl of the PCR master mix and incubated in PTC-225 PCR machine (Peltier Thermal Cycler) with the following setting: 94^0C for 2 min, 30 cycles of 94^0C for 30 sec, 60^0C for 30 sec and 68^0C for 1 min, 68^0 for 10 min. 5 μl of PCR product was loaded on 1% agarose gel

TCR rearrangement bands visualization. When further amplification of TCR rearrangement PCR products was necessary, 1-2 μl of PCR products were purified on the QIAprep spin column (QIAGEN), according to manufacturer's protocols. Finally, 1 μl of eluted PCR products was used as DNA template to repeat the PCR in the same condition. Primers used here are shown in Table 1.

2.3. RNA methods

2.3.1. Extraction of total RNA from cells

RNA from primary cells was extracted using the RNAqueous-Micro RNA isolation kit according to the manufacturer's protocol (Ambion). Briefly, cells were counted and pelleted by centrifuge at 400xG for 5 min. The supernatant was removed thoroughly by aspiration, and the cell pellet was resuspended in at least 100 μl of Lysis Solution by vortexing vigorously. Then, 50 μl of 100% ethanol was added and mixed thoroughly with the lysate. Next, the lysate/ethanol mixture was loaded onto a Micro Filter Cartridge Assembly and centrifuge for 10 sec at 16,000xG. Then the filter was washed with 180 μl of Wash Solution 1 once and 180 μl of Wash Solution 2/3 twice. The flow-through was discarded and the filter was centrifuged for 1 min at 16,000 G. The RNA eluted in 10 μl of preheated Elution Solution. 1/10th volume of 10X DNase I Buffer and 1 μl of DNase I were added into the RNA sample and mixed gently but thoroughly for DNA elimination. After incubation at 20 min at 37^0C, the DNase reaction was mixed with 2 μl of DNase Inactivation Reagent and left at room temperature for 2 min. Finally, the DNase Inactivation Reagent was pelleted and the RNA was transferred to a fresh tube. For RT-PCR or qRT-PCR experiments, RNA quality and quantity was verified using a Nanodrop ND-100 Spectrophotometer

(Thermo Scientific). For gene expression array experiments, RNA quality and quantity was determined using 2100 Bioanalyzer platform (Aglient Technologies).

2.3.2. First strand cDNA synthesis

SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) was used for first strand cDNA synthesis from RNA, according to the manufacturer's protocol. In brief, 1-2 μg of RNA was mixed with 1μl of oligo$(dT)_{20}$ (50 μM), 1 μl of dNTP mix (10 mM) and DEPC-treated water up to 10 μl. The reaction was incubated at 65^0C for 5 min and then place on ice for 1 min. Then the following cDNA Synthesis Mix [2 ul of 10X RT buffer, 4ul of $MgCl_2$ (25 mM), 2 μl of DDT (0.1 M), 1μl of RNaseOUT and 1 μl of SuperScript III RT] was prepared and mixed with the reaction gently and incubated for 50 min at 50^0C. The reactions were terminated at 85^0C for 5 min and chill on ice. Finally, 1 μl of RNase H was added to the reaction and incubated for 20 min at 37^0C for RNA elimination.

2.3.3. RT-PCR

RT-PCR was performed on cDNA using primers listed in Table 1. PCR amplification was performed using Extensor Hi-Fidelity PCR Master Mix 2 (Thermo). Similar to genotyping PCR, 1 μl of cDNA (about 10 ng) was used as template. It was mixed with 1 μl of each primer (10 μM) and 2 μl of PCR-grade water were mixed with 5 μl of the PCR master mix and incubated in PTC-225 PCR machine (Peltier Thermal Cycler) with the following setting: 94^0C for 2 min, 25-30 cycles of 94^0C for 30 sec, 60^0C for 30 sec and 68^0C for 30 sec, 68^0 for 10 min.

2.3.4. qRT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was used to measure the relative amounts of mRNA expression of gene of interest again βActin as an internal control. At first, primers were tested for their amplification efficiency. Amount of cDNA samples was determined with a Nanodrop ND-100 Spectrophotometer (Thermo Scientific). The PCR reactions were carried out with either the Taqman or SYBR Green system in an ABI PRISM 7900HT (Applied Biosystems), according to the manufacturer's manuals.

2.3.5. Taqman

50 μl of cDNA (10 ng/μl) was mixed with 12.5 μl of Absolute QPCR mix (Thermo) with 1 μl of each primer (10 μM), 0.5 μl of probes (0.25 μM; MWG, Ebersberg, Germany). PCR reactions were conducted in triplicate. Probes were labeled with reported dye (FAM) at the 5' end and the quencher dye TAMRA at the 3' end. Primers and probes used are listed in Table 1.

2.3.6. SYBR Green

50 μl of cDNA (10 ng/μl) was mixed with 12.5 μl of SYBR GreenER qPCR SuperMix (Invitrogen) with 1 μl of each primer (10 μM). PCR reactions were conducted in triplicate. SYBR Green I DNA fluorescent dye was used with high affinity to dsDNA and an excitation wavelength of 488 nm. Primers used are listed in Table 1.

2.4. Protein methods

2.4.1. Protein extraction

Cell pellets were re-suspended in 100-300 μl of RIPA buffer [50mM Tris (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, NaCl (150 mM), EDTA (1mM), 1X Cocktail protease inhibitors (Roche), Na_3VO_4 (1mM), and 1 mM NaF] and transferred to a 1.5 ml tube. The lysate was pipetted up and down several times to be dissolved. After boiled at 99^0C for 5 min, the lysate was chilled on ice for 10 min and spun for 15 min at 16,000xG. Then the supernatant was collected and frozen. Also, 10 μl of the supernantant was collected in a separate tube for protein concentration assay.

2.4.2. BioCinchominic Acid (BCA) assay

Bovine Serum Albumin (BSA) (Sigma) was made at required amounts of 0.1 mg, 0.2 mg, 0.5 mg, 1.0 mg and 2.0 mg and used as standards for the assay. The BCA reagents were mixed in a ratio of 50:1 (A:B) respectively, according to manufacturer's protocol. 200 μl of the reagent mix was added to 5 μl of standard or sample in a 96-well plate. Samples were diluted in water (from 1:4 to 1:16) before measurement. Each standard or sample was measured for three times. After incubation at room temperature for 20 min, the light absorbance of reactions was measured at a wavelength of 570 nm using a DYNATECH-MR5000 plate reader. The readout of standards was used to plot a graph. Finally, the slope of the graph was used to calculate the concentration of the samples.

2.4.3. SDS-PAGE

The protein samples were diluted to a final concentration of 30 μg/ml using RIPA buffer and Loading Buffer [125 mM Tris-HCl (pH 6.8), 2.5% SDS, 20%

glycerol, 0.002% Bromophenol blue and 5% b-mercaptoethanol]. After boiling at 95^0C for 5 min, samples were chilled on ice for 5 min and then loaded to SDS-PAGE Criterion precast Tris-HCl resolving gels (Biorad). The gels were run at 100 V for 90-120 min at room temperature using Biorad powepac 300 in Running Buffer (3.03 g Tris, 14.4 g Glycine, 1 g SDS and ddH_2O to 1 L).

2.4.4. Immunoblotting

Protein samples were immunoblotted to PVDF membranes (Millipore-immobilon FL) with wet transfer tanks (Biorad), according to the manufacturer's protocols. Briefly, the gels were placed in a cassette facing the PVDF membranes. The concentration of methanol in Transfer Buffer [192 mM Glycine, 25 mM Tris, 0.1% SDS, 5-20 v/v 100% Methanol, pH 8.3) varied depending on the size of proteins of interest. For high molecular weight proteins, transfer buffer with low percentage of methanol was used while for low molecular weight protein that with high percentage of methanol was used. Transfer was performed with electric current applied at 400 mA for 1 hr.

2.4.5. Primary antibody incubation

The PVDA membranes were incubated in blocking solution (5% milk) for 1 hr at room temperature to reduce background signal in the consequent steps. Then primary antibodies were added into blocking solution. All primary antibodies were incubated overnight at 4^0C whilst shaking.

2.4.6. Secondary antibody incubation and detection

After the primary antibodies were removed, the membranes were rinsed once with 1X PBS-0.1% Tween (Sigma) (PBST) and incubated in the blocking buffer with the appropriate secondary antibody (1:2000 dilution) for 45 min. The secondary

antibodies used were all conjugated to horseradish peroxidase (HRP) (DAKO). The membrane was then washed twice with 1X PBST for 5 min each to prevent on specific binding. ECL (Amersham) was used as a substrate for the HRP enzyme. The semi-luminescence was detected with Hyper-film (Amersham).

2.5. Flow cytometry and cell sorting

2.5.1. Single cell suspension

Bone marrow cells were flushed out from the femurs using a 26G syringe with fluorescent-activated cell sorting (FACS) Stain Buffer [DPBS (Invitrogen) with 2% FCS (GIBCO)]. Spleen or thymus were place in FACS Stain Buffer and gently homogenized with a syringe. The red blood cells were removed with ACK lysis buffer (Lonza). Blood was collected into EDTA tubes (Sarstedt). In vitro cultured cells were collected and washed with FACS Stain Buffer. Cells were filtered through a 30 μm mesh to obtain single-cell suspension.

2.5.2. DN thymocytes enrichment

CD4 (L3T4) and CD8 (Ly-2) MicroBeads (Miltenyi Biotec) were used in accordance to the manufacturer's protocols for the enrichment of $CD4^-CD8^-$ population from thymocytes. Briefly, cells were counted and a certain number of cells were pelleted by centrifuge at 300xG for 10 min. The cell pellet was re-suspended in 90 μl of MACS Buffer [PBS pH 7.2, 0.5% BSA and 2 mM EDTA] per 10^7 total cells, after supernatant was completely removed. 5 μl of CD4 and CD8 MicroBeads were mixed with the cells and incubate for 15 min at 4-8^{0}C. Cells were washed twice by adding 1-2 mL of MACS Buffer per 10^7 cells and centrifuged at 300xG for 10 min. Cell pellets were resuspended up to 10^8 in 500 ul of MACS Buffer. Then cell

suspension was then applied onto the MACS Separator Column (Miltenyi Biotec). Unlabeled cells that pass through the column were collected and the columns were washed for 3 times. Cells were pelleted by centrifuging the total effluent, re-suspended in FACS Stain Buffer, and stained for cell surface antigens.

2.5.3. Staining of cell-surface antigens

For all cells, Fc receptors were blocked with anti-CD16 (2.4G2) prior to antibody labelling. Antibodies to the following antigens were used: CD3ε (145-2C11), CD4 (L3T4), CD8α (53-6.7), CD25 (PC61), CD44 (IM7), CD122 (TM-β1), CD27 (LG.3A10), CD11b (M1/70), CD45.2 (104), TCRβ (H57-597), CD117 (2B8), NK1.1 (PK136), CD49b (DX5), NKp46 (29A1.4), Ly49C/I (5E6), Ly49G2 (4D11), Ly49D (4E5). All antibodies were from BD Biosciences or eBioscience. Cells were incubated with antibody for 30 minutes at 4° C before being washed. In some cases, biotinylated antibodies were revealed by incubation with fluorochrome-conjugated streptavidin for a further 20 minutes at 4° C. Cells were washed and resuspended prior to data acquisition using a FACSCalibur (BD Biosciences), LSR II (BD Biosciences) or a FC 500 (Beckman Coulter) with dead cells excluded based on scatter profile or DAPI inclusion. Analysis was performed using FlowJo (Tree Star) software. Sorting was performed using a MoFlo (DAKO) or FACS Aria (BD Biosciences).

2.5.4. CD1d stain

Prior to cell-surface antigen staining, CD1d:Ig proteins (BD Bioscience) were loaded with α-GalCer (Kyowa Hakko Kirin Company) by mixing antigen and CD1d:Ig together in PBS, pH7.2 and incubating at 37^0 C overnight. 1 μg of peptide-loaded CD1d:Ig protein was subsequently mixed with 1μg of PE-conjugated A85-1

mAb (BD Bioscience) at a ration of 1:1 of dimmer: A85-1 mAb were incubated for 60 minutes at room temperature (RT) in the dark. Next, 1 μg of purified mouse IgG1 isotype control mAb A111-3 (BD Bioscience) was added to the staining cocktail and incubated for 30 minutes at room temperature RT in the dark. Finally cells were stained with the prepared stain cocktail, plus anyother cell-surface marker-specific antibodies to be used, as described above.

2.5.5. Staining for intracellular antigens

After cell-surface antigen staining, cells were fixed by adding 100 μl of Fixation Solution (eBioscience), while vortexing the tube. The tube was incubated in the dark at room temperature for 20 min and cells were washed twice with 1 ml of Permeabilization Buffer (eBioscience). Then, cells were resuspended in 100 μl of Permeabilization Buffer. Fluorochrome-labeled anti-cytokine mouse antibodies were added into tubes and mixed well. After incubation in the dark at room temperature for 20 min, the cell suspension was washed once with 1 ml of Permeabilization Buffer. Cells were resuspended in FACS Stain Bufferand ready for analysis on a flow cytometer.

2.6. Cell culturing

2.6.1. Culture of OP9 and OP9-DL1 stromal cells

OP9 stromal cells were cultured in α-MEM (Sigma) with 10% FCS (heat inactivated at 56^0C for 30min), 1% penicillin/streptomycin, and 2 mmol/L L-glutamine (Life Technologies). OP9-DL1 stromal cells were cultured in α-MEM (Sigma) with 20% FCS, 1% penicillin/streptomycin, and 2mmol/L L-glutamine (Life Technologies). Cells were passaged every 2 to 3 days by trypsinization (0.25%

trypsin) (Invitrogen). A confluent monolayer (70%-80%) of OP9-DL1 cells was prepared 24 hours prior to co-culture.

2.6.2. Culture of T cells

T cells were co-cultured with OP9-DL1 in T Cell Culture Media [RPMI-1640, 10% FCS, 1% penicillin/streptomycin, and 2 mmol/L L-glutamine, 5 ng/ml Flt-3 lignad, 5 ng/ml IL-7]. Half volume of culture media was replaced with fresh T cell Culture Media every three days. Every week, T cells were disassociated from OP9-DL1 stromal cells by being pipetted gently and passing through 30 μn mesh, then were transferred to fresh OP9-DL1 culture. All cytokines used in this study were purchased from PeproTech, if not specified otherwise.

2.6.3. Culture of myeloid cells

Myeloid cells were co-cultured with OP9 in Myeloid Cell Culture Media [IMDM, 10% FCS, 1% penicillin/streptomycin, and 2 mmol/L L-glutamine, 5 ng/ml Flt-3 ligand, 1 ng/ml IL-7 and 10 ng/ml IL-3, IL-6, stem cell factor (SCF), and macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), and granulocyte/macrophage colony-stimulating factor (GM-CSF)]. Half volume of culture media was replaced with fresh Myeloid Cell Culture Media every three days. Every week, myeloid cells were disassociated from OP9 stromal cells by being pipetted gently and passing through 30 μn mesh, and were transferred to fresh OP9 culture.

2.6.4. Culture of B cells

B cells were co-cultured with OP9 in B Cell Culture Media [IMDM, 10% FCS, 1% penicillin/streptomycin, and 2 mmol/L L-glutamine, 5 ng/ml Flt-3 lignad, 5 ng/ml IL-7]. Half volume of culture media was replaced with fresh B Cell Culture Media

every three days. Every week, B cells were disassociated from OP9 stromal cells by being pipetted gently and passing through 30 μm mesh, and were transferred to fresh OP9 cultures.

2.6.5. Culture of NK cells

NK cells were co-cultured with OP9 in NK Cell Culture Media [RPMI-1640, 10% FCS, 1% penicillin/streptomycin, and 2 mmol/L L-glutamine, 30 ng/ml IL-15]. Half volume of culture media was replaced with fresh NK Cell Culture Media every three days. Every week, NK cells were disassociated from OP9 stromal cells by being pipetted forcefully and passing through 30 μn mesh, and were transferred to fresh OP9 cultures.

2.6.6. Culture of Lymphokine-activated killer (LAK) cells

Splenic NK cells were enriched using the NK Isolation Kit (Miltenyi) and cultured for 6-9 days at 1 x 10^6 cells/ml in LAK Culture Media [RPMI 1640 medium containing 10% FCS/50 μM 2-mercaptoethanol/2.0 mM L-glutamine and 100 ng/ml IL-2]. The cells were split every 2 days and supplemented with fresh IL-2. Purity was always >90%. For culturing reprogrammed cells ex vivo, whole splenocytes were cultured without pre-enrichment.

2.6.7. Culture of tumour cell lines

RMA and RMA-s, two mouse lymphoma cell lines were cultured in RMA Culture Media [RPMI 1640 medium containing 5% FCS/50 μM 2-mercaptoethanol /2.0 mM L-glutamine]. B16, a mouse melanoma cell line, was cultured in B16 Culture Media [DMEM medium containing 5% FCS/50 μM 2-mercaptoethanol/2.0 mM L-glutamine]. Cells were passaged every 2 to 3 days by trypsinization (0.25% trypsin) (Invitrogen).

2.6.8. Activation of unprimed T cells

T cells were activated using Dynabeads Mouse T-Activator CD3/CD28 (Invitrogen), according to its protocol. Briefly, 1 X 10^5 purified T cells were mixed with washed 2µl Dynabeads Mouse T-Activator CD3/CD28 to obtain a bead-to-cell ratio of 1:1 in T cell media [RPMI 1640 medium containing 10% FCS/50 µM 2-mercaptoethanol/2.0 mM L-glutamine and 3 ng/ml hIL-2].

2.6.9. OHT treatment in vitro

Thymocytes or splenocytes were cultured in T Cell Media with 1 µM OHT (Sigma) at 37^0C for 48 hrs. After this time, cells were washed and resuspended with fresh media without OHT.

2.7. Gene expression analysis

RNA was extracted using the RNAqueous Micro Kit (Ambion) from FACS sorted cells. Quality and quantity of RNA samples was tested with the Bioanalyzer. Total RNA was amplified using the Illumina Total Prep RNA Amplification Kit (Ambion) according to the manufacturer's instructions. The biotinlated cRNA (1.5 µg per sample) was applied to Illumina Mouse-6 Expression BeadChips and hybridized overnight at 58^0C. Chips were washed, detected and scanned according to the manufacture's instruction and the scanner output imported into BeadStudio software (Illumina).

2.8. Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as previously described (Forsberg et al., 2000). Control IgG and the CSL antibody were purchased from Abcam. Genomic DNA was purified with Qiaquick PCR purification kit (QIAGEN)

and specific genomic DNA regions were quantified by real-time quantitative PCR with Taqman (ABI) or SYBR Green (Invitrogen). Input DNA was used as a standard curve to quantify concentration of DNA recovered after IP. The amount of DNA recovered from each ChIP sample was presented as a relative to the control.

2.9. Tumour killing assays

B16F10 melanoma (H-2b), RMA lymphoma and RMA-S lymphoma (H-2b TAP-1-deficient variant) cells were maintained as above. For killing assays, target cells were washed and incubated with 0.1 μCi $Na_{25}1CrO_4$ (Perkin Elmer) for 45 min at 37° C. The target (T) cells were then washed and added in triplicate to effector (E) cells at the indicated E:T ratio. Plates were incubated for 4 hr at 37° C before the supernatant was tested for chromium release in a scintillation counter. Percent specific lysis was calculated as (experimental release – spontaneous release)/(maximum release – spontaneous release) x 100.

2.10. Calcium flux

Briefly, 1 X 10^6 cells after cell surface antigen staining were re-suspended in 1 ml of Cell Loading Medium (CLM) [RPMI containing 2% FCS]. Next, cells were loaded with Indo-1 (Invitrogen) at a final concentration of 1.5 μM and incubated at 37^0C for 45 minutes in the dark. After wash with washing buffer [DMEM containing 2% FCS], cells were gently resuspended in CLM and stored for 1 hour at RT in the dark before flow cytometric analysis.