# 3. MATERIALS

## 3.1. Chemicals and reagents

| | |
|---|---|
| Acrylamide (30%) Bisacrylamide (0.8%) | Roth, Karlsruhe, Germany |
| Agar Noble | Difco Laboratories, USA |
| Ammonium persulfate (APS) | Sigma, Steinheim, Germany |
| Bromphenol Blue | Sigma, Steinheim, Germany |
| Bovine serum albumine (BSA) | Fluka, Steinheim, Germany |
| Chlorophorm | Merck, Darmstadt, Germany |
| Chloroquine | Sigma, Steinheim, Germany |
| Coomassie-Brilliant Blue G-250 | Merck, Darmstadt, Germany |
| Complete protease inhibitor cocktail (50x) | Roche, Mannheim, Germany |
| Diethylpirocarbonat (DEPC) | Sigma, Steinheim, Germany |
| Dithiotreitol (DTT) | Sigma, Steinheim, Germany |
| DNA from salmon sperm | Sigma, Steinheim, Germany |
| dNTPs (0.1M) | MBI Fermentas, St.Leon Rot, Ger. |
| [α-$^{32}$P] dCTP (3000 Ci/mmol) | Amersham Biosciences, Munich, Ger. |
| Ethanol, 99% | Merck, Darmstadt, Germany |
| Ethidium bromide | Sigma, Steinheim, Germany |
| ExpressHyb™Hybridization Solution | BD Biosciences Clontech |
| 5-Fluorouracil | GRY-Pharma, Kirchzarten, Germany |
| Giemsa stain | Sigma, Steinheim, Germany |
| 4-hydroxy tamoxifen | Sigma, Steinheim, Germany |
| Lennox L Broth Base (LB Broth Base) | Gibco, Invitrogen,Karlsruhe,Germany |
| L-glutamine (200mM, sterile) | Gibco, Invitrogen,Karlsruhe,Germany |
| Lipopolysacharide (LPS) | Sigma, Steinheim, Germany |
| Isopropanol | Roth, Karlsruhe, Germany |
| 2-mercaptoethanol (50mM, sterile) | Gibco, Invitrogen,Karlsruhe,Germany |
| May-Grünwald stain | Sigma, Steinheim, Germany |
| Methanol, 99% | Roth, Karlsruhe, Germany |
| peqGOLD TriFast™ | Peqlab Biotechnology, Erlangen, Ger |
| Polybrene | Sigma, Steinheim, Germany |
| Poly (I) poly (C) | Sigma, Steinheim, Germany |
| Polyoxyethylensorbitanmonolaurat (Tween 20) | Sigma, Steinheim, Germany |
| Propidium iodide (PI) | Sigma, Steinheim, Germany |
| Sodium azide | Sigma, Steinheim, Germany |
| Sodium chloride | Roth, Karlsruhe, Germany |
| Sodium hydroxide | Roth, Karlsruhe, Germany |
| Sodium lauryl sulphate (SDS) | Sigma, Steinheim, Germany |
| Sodium hydrogenphosphate | Roth, Karlsruhe, Germany |
| Tetra-methyl-ethylenediamine (TEMED) | Gibco, Invitrogen,Karlsruhe,Germany |
| Tris (hidroxymethyl) aminomethan (Tris) | Roth, Karlsruhe, Germany |

## 3.2. Buffers

When not otherwise stated, deionised (Millipore) water was used as solvent.

| | |
|---|---|
| Annexin V staining buffer (10x) | 0.1M Hepes (pH 7.4), 1.4M NaCl, 25mM $CaCl_2$ |
| APS solution | 10% APS in water, prepared fresh |
| BD PharMLyse™ erythrocyte lysis buffer | BD Biosciences, Pharmingen, Karlsruhe, Germany |
| Biocoll separating solution, density 1.090 | Biochrom AG, Berlin, Germany |
| Blocking solution (Western blotting) | 5% low-fat milk in TBS, 0.1% Tween 20 |
| Bradford solution | 100mg Coomassie G250, 50ml (v/v) 95% ethanol, 100ml (w/v) 85% phosphate acid in 1L $H_2O$; filtered before use |

| | |
|---|---|
| CasyTon® isotonic buffer | Scharfe System, Reutlingen, Germany |
| Cell Dissociation Buffer (enzyme free) | PAA Laboratories, Pasching, Germany |
| CIAP buffer | Invitrogen, Karlsruhe, Germany |
| DEPC-$H_2O$ | 1ml DEPC desolved in 1L $H_2O$, incubated over night and autoclaved |
| DNase-buffer | Invitrogen, Karlsruhe, Germany |
| Electrophoresis buffer | 25mM Tris; 250mM glycine, pH 8.3; 0.1% SDS |
| FACS buffer | 2 % FCS, 2mM EDTA; 0.1% $NaN_3$ in PBS |
| Formalin (neutral) | 4% formaldehyde in PBS |
| Formalin (acidic) | 4% formaldehyde in 1% acetic acid |
| Hepes | Cambrex, Verviers, Belgium |
| Hebs buffer (Hepes buffered saline) (2x) | 50mM Hepes, 280mM NaCl, 1.5mM$Na_2HPO_4$ , pH 7.05 |
| Loading buffer (DNA) 10x | 0.4% Bromphenol Blue; 0.4% xylencyanol; 50% glycerol; 0.1M EDTA |
| MOPS buffer (10x) | 0.4M MOPS, 50mM sodium acetate; 10mM EDTA in DEPC-$H_2O$; pH 7.0 (set with NaOH) |
| PBS | PAA Laboratories, Pasching, Germany |
| PCR buffer | Bioline, Luckenwalde, Germany |
| Ponceau stain | 0.1% Ponceau in 5% acetic acid |
| RIPA buffer | 50mM Tris/HCl pH 7.4; 50mM NaCl; 10mM EDTA; 1% IGEPAL; 0.1% SDS; 0.25% sodium deoxycholate |
| RNA-Premix | 1.3x MOPS; 3M formaldehyde (pH 4-7); 64% deionised formamide |
| Restriction enzyme buffers (10x) | MBI Fermentas, St. Leon-Rot, Germany |
| RNA loading buffer | 1mM EDTA pH 8.0; 0.25% Bromphenol Blue; 0.25% xylene cyanol; 50% glycerol |
| RNA-Premix | 1.3x MOPS ; 3M formaldehyde (8,39%) ; 64% formamide |
| SDS denaturing and loading buffer (4x) 0.2M | Tris/HCl, pH 6.8; 8% SDS; 40% (w/v) glycerol; 0.004% Bromphenol Blue; 0.1M DTT |
| SSC (20x) | 3M NaCl; 0.3M sodium citrate, pH 7.0 |
| TBS (Tris buffered saline) (10x) | 0.5M Tris base, 9% NaCl, pH 7.6 |
| TBS/T buffer | 1x TBS buffer, 0.1% Tween |
| $T_4$–DNA-Ligase buffer (10x) | MBI-Fermentas, St. Leon-Rot, Germany |
| $T_4$-DNA-Polymerase buffer (10x) | MBI-Fermentas, St. Leon-Rot, Germany |
| TAE-buffer (50x) | 2M Tris; 57% acetic acid; 100mM EDTA; pH 8.0 |
| Taq-Polymerase Puffer (10x) | Bioline, Luckenwalde, Germany |
| TE-buffer | 10mM Tris/HCl, pH 7.5; 1mM EDTA |
| Transfer buffer 5x (Western) | 200mM glycine; 250mM Tris; 0.185% SDS; pH 8.3 |
| Transfer buffer 1x (Western) | 1:1:3 (v/v) 5x transfer buffer:methanol:water |
| Separating buffer (Western) (4x) | 1.5M Tris/HCl pH 8.8; 0.4% SDS |
| Stacking buffer (Western) (4x) | 0.5M Tris/HCl pH 6.8; 0.4% SDS |
| Stripping solution (Northern) | 0.5% SDS |
| Stripping solution (Western) | 50mM Tris, 2% SDS, 100mM 2-mercaptoethanol, pH 6.8 |
| Washing buffer I (Northern) | 2x SSC; 0.05% SDS |
| Washing buffer II (Northern) | 0.1x SSC; 0.1% SDS |

## 3.3. Antibodies

### 3.3.1. Antibodies used for Fluorescence Activated Cell Sorting (FACS) and Magnetic Cell Sorting (MACS)

| *Antigen* | *Fluorochrome* | *Producer* |
|---|---|---|
| Annexin V | APC, FITC | BD Biosciences PharMingen, San Diego, USA |
| Cd3e (Cd3 ε-chain) | biotin | BD Biosciences PharMingen, San Diego, USA |
| Cd4 (L3T4) | FITC | BD Biosciences PharMingen, San Diego, USA |
| Cd8a (Ly-2) | PE | BD Biosciences PharMingen, San Diego, USA |
| Cd11b (Mac-1 α-chain) | APC, biotin | BD Biosciences PharMingen, San Diego, USA |
| Cd11c (HL3) | biotin | BD Biosciences PharMingen, San Diego, USA |
| Cd45R (B220) | APC, biotin | BD Biosciences PharMingen, San Diego, USA |
| Ter-119 | biotin | BD Biosciences PharMingen, San Diego, USA |
| Gr1 (Ly-6G) | PE, biotin | BD Biosciences PharMingen, San Diego, USA |
| Cd117 (c-kit) | PE | BD Biosciences PharMingen, San Diego, USA |
| Sca1 (Ly-6/E) | FITC | BD Biosciences PharMingen, San Diego, USA |
| Ccr3 | PE | R&D Systems, Wiesbaden, Germany |
| Pan-NK (DX5) | PE | BD Biosciences PharMingen, San Diego, USA |
| F4/80 | FITC, PE | Serotec, Düsseldorf, Germany |

For detection of biotinilated antibodies by FACS, streptavidin coupled with FITC, PE or APC was used (PharMingen, San Diego, USA).

For magnetic labeling of cells for MACS, antigen-specific biotinilated antibodies were used in the first step and streptavidin-coupled magnetic beads in the second step (Miltenyi Biotech, Bergisch Gladbach, Germany).

### 3.3.2. Antibodies used for Western blotting

| *Antigen* | *Origin* | *Producer* |
|---|---|---|
| Gklf (H-180) | rabbit | Santa Cruz Biotechnology, CA, USA |
| Icsbp (C-19) | goat | Santa Cruz Biotechnology, CA, USA |
| $p21^{Waf1}$ (M-19) | rabbit | Santa Cruz Biotechnology, CA, USA |
| Actin (I-19) | goat | Santa Cruz Biotechnology, CA, USA |
| Estrogen receptor (F-region) (F3-A6) | mouse | Euromedex, Souffelweyersheim, France |

For detection of primary antibodies, secondary anti-rabbit, anti-mouse and anti-goat antibodies coupled with horseradish peroxidase were used (Santa Cruz, Biotech., USA).

## 3.4. Recombinant cytokines and proteins

| *Recombinant protein:* | *Origin:* | *Concentration:* | *Producer* |
|---|---|---|---|
| SCF | rat | 50ng/ml | PeproTech, NY, USA |
| IL-3 | murine | 5ng/ml | PeproTech, NY, USA |
| Il-5 | murine | 50ng/ml | R&D Systems, Wiesbaden, Germany |
| GM-CSF | murine | 5ng/ml | PeproTech, NY, USA |
| M-CSF | human | 100ng/ml | PeproTech, NY, USA |
| G-CSF | human | 100ng/ml | PeproTech, NY, USA |
| Flt-3 ligand | murine | 100ng/ml | PeproTech, NY, USA |
| TPO | human | 100ng/ml | PeproTech, NY, USA |
| Interferon γ | murine | 100U/ml | PeproTech, NY, USA |
| Retronectin™ | recombinant human fibronectin fragments | $4mg/cm^2$ | Takara Bio, Otsu, Shiga, Japan |

## 3.5. Enzymes

| | |
|---|---|
| Alkaline Phosphatase (CIAP) | MBI-Fermentas, St. Leon-Rot, Germany |
| Restriction enzymes | MBI-Fermentas, St. Leon-Rot, Germany |
| RNase A (10mg/ml) | Roche, Mannheim, Germany |
| PowerScript™ Reverse Transkriptase | BD Biosciences Clontech, Mountain View, CA, USA |
| Taq Polymerase | Bioline, Luckenwalde, Germany |
| $T_4$-DNA-Ligase | MBI-Fermentas, St. Leon-Rot, Germany |

## 3.6. Kits

| | |
|---|---|
| ECL™Western blotting detection reagent | Santa Cruz Biotechnologies, CA, USA |
| NucleoSpin™ ExtractII kit | Macherey-Nagel, Düren, Germany |
| QIAGEN™Plasmid Maxi kit | Qiagen, Hilden, Germany |
| QIAexII™Gel extraction kit | Qiagen, Hilden, Germany |
| Rediprime™II | Amersham Biosciences, Munich, Germany |
| PowerScript™ reverse transcriptase | BD Biosciences Clontech, Mountain View, CA, USA |
| Dneasy™ Tissue Kit | Qiagen, Hilden, Germany |
| E.Z.N.A™ Tissue DNA Mini Kit | Peqlab, Erlangen, Germany |
| Mouse lineage panel® | BD Biosciences,Pharmingen, San Diego, USA |
| FastStart DNA Master$^{PLUS}$ SYBR Green1 kit | Roche Diagnostics, Mannheim, Germany |

## 3.7. Cell culture media

### 3.7.1. Basic components and supplements

| | |
|---|---|
| DMEM high glucose | PAA Laboratories, Pasching, Germany |
| IMDM (with L-glutamine) | PAA Laboratories, Pasching, Germany |
| Fetal calf serum (inactivated 30 min on 56°C) | Biochrom, Berlin, Germany |
| Horse serum (inactivated 30 min on 56°C) | Biochrom, Berlin, Germany |
| L-Glutamine | Cambrex, Verviers, Belgium |
| MethoCult M3134 | Stem cell technologies, USA |
| Penicillin/Streptamycin | Cambrex, Verviers, Belgium |
| Trypsin/EDTA (1x) | PAA Laboratories, Pasching, Germany |

### 3.7.2. Media

| | |
|---|---|
| Macrophage medium | 500ml DMEM;<br>10% FCS; 5% HS; 0.2mM glutamine;<br>0.1mM sodium pyruvate<br>0.05mM β-mercaptoethanol;<br>100U/ml penicillin<br>100µg/ml streptomycin<br>20% conditioned medium from L929-cells |
| Phoenix medium | 500ml DMEM<br>10% FCS; 0.2mM glutamine;<br>100U/ml penicillin<br>100µg/ml streptomycin<br>pH set to 7.9 with 1M NaOH, and then 10ml 1M HEPES added (final conc. 20mM); sterile filtered |
| NIH 3T3 medium | 500ml DMEM<br>10% FCS; 0.2mM glutamine;<br>100U/ml penicillin<br>100µg/ml streptomycin |

| | |
|---|---|
| Eosinophil medium | 500ml IMDM<br>10% FCS; 0.2mM glutamine;<br>100U/ml penicillin<br>100µg/ml streptomycin |

## 3.8. Bacterial media and supplements

| | |
|---|---|
| Ampicillin (100µg/ml) | Sigma-Aldrich, Steinheim, Germany |
| Kanamycin (50µg/ml) | Invitrogen |
| LB-Medium | 2% (w/v) LB Broth Base; 0.5% (w/v) NaCl; |
| LB-Agar | 2% (w/v) LB Broth Base;<br>0.5% (w/v) NaCl;<br>1.5% (w/v) Agar |

For all experiments *Escherichia coli* strain DH5α (Stratagene) was used.

## 3.9. Oligonucleotides

### 3.9.1. Primers for RT-PCR and Northern blotting probes

| | | |
|---|---|---|
| *Klf4:* | sense: | 5' GCCGCGCATGTGCCCCAAGATT 3' |
| | antisense: | 5' CACCCACAGCCGTCCCAGTCACAG 3' |
| *Icsbp:* | sense: | 5' GCGGGCAGCGTGGGAACC 3' |
| | antisense: | 5' CCACCAGCTTGCCCCCGTAGTAGA 3' |
| *$p21^{Waf1}$:* | sense: | 5' ATGCGAATTCATGTCCAATCCTGGTGATGTCCGACCTG 3' |
| | antisense: | 5' CTAATGCGGCCGCTCAGGGTTTTCTCTTGCAGAAGACC 3' |
| *p53:* | sense: | 5' CATCACCTCACTGCATGGAC 3' |
| | antisense: | 5' CCGTCATGTGCTGTGACTTC 3' |
| *Gapdh:* | sense: | 5' GGGGTGAGGCCGGTGCTGAGTAT 3' |
| | antisense: | 5' CATTGGGGGTAGGAACACGGAAGG 3' |
| *Il-5Rα*: | sense: | 5' GAAGGAAAAACTGATCGCCAATAA 3' |
| | antisense: | 5' TTCCCATGACTTCAAATCCAACC 3' |
| *Gata1:* | sense: | 5' ATCCGCCCCAAGAAGCGAATGA 3' |
| | antisense: | 5' TTGTAGGCGATCCCAGCAGAGGTC 3' |

### 3.9.2. Primers used for real-time PCR

| | | |
|---|---|---|
| *Mxd1:* | sense: | 5' ACGGAGGAACAAGCCCAAGAAGAA 3' |
| | antisense: | 5' AGAGCGCTCCGAAGACACCACAGA 3' |
| *Marco:* | sense: | 5' AAAAGGGGGCTGCAGGTCGTGAT 3' |
| | antisense: | 5' GGTCCCCTTTGTCTCCCTTGGTG 3' |
| *Ceruloplasmin:* | sense: | 5' TGTCATTTGGGCAGAAGTAGG 3' |
| | antisense: | 5' GCCCAATAAGCCCAGTAAATA 3' |
| *Cd177*: | sense: | 5' CGCTGCCCACTCTGCCTTTCTAAT 3' |
| | antisense: | 5' ATAACACCCCTCGCCAGCCTCAC 3' |

*Cd36:* sense: 5’ GATCGGAACTGTGGGCTCATT 3’
antisense: 5’ GTGGTCCTCGGGGTCCTG 3’

*p21$^{Waf1}$:* sense: 5’ GCTCATGGCGGGCTGTC 3’
antisense: 5’ CTGCGCTTGGAGTGATAGAAAT 3’

*Gapdh:* sense: 5’ CTGACGTGCCGCCTGGAGAAAC 3’
antisense: 5’ CCCGGCATCGAAGGTGGAAGAG 3’

### 3.9.3. Primers for PCR fragments used for cloning

The primers for creating the constitutive expression constructs are designed with *NcoI* site added in the sense primer and *ClaI* site in the antisense primer (*Icsbp* and *Klf4*) or *EcoRI* site in the sense primer and *NotI* site in the antisense primer (*Klf4$^{\Delta Zn}$* and *p21$^{Waf1}$*):

*Icsbp:*
sense: 5' GGAA**CAAGCC**ATGGTCGCCACCCATGTGTGACCGGAACGG 3'
antisense: 5’ CCTTCC**ATCGAT**TTAGACGGTGATCTGTTGATTTTCTC 3’

*Klf4:*
sense: 5' GGAA**CAAGCC**ATGGTCGCCACCCATGAGGCAGCCACCTGGCGAGTCTG 3'
antisense: 5’ CCTTCC**ATCGAT**TTAAAAGTGCCTCTTCATGTGTAAGGC 3’

*Klf4$^{\Delta Zn}$:*
sense: 5' GGAACAAG**GAATTC**GCCGCCACCATGAGGCAGCCACCTGGCGAGTC 3'
antisense: 5’ CCTTCATG**GCGGCCGC**TCAAGTGTGGGTGGCTGTTCTTTTCCGGG 3’

*p21$^{Waf1}$:*
sense: 5' GGAACAAG**GAATTC**GCCGCCACCATGTCCAATCCTGGTGATGTCC 3'
antisense: 5’ CCTTCATG**GCGGCCGC**TCAGGGTTTTCTCTTGCAGAAGACC 3’

The PCR fragments for creating the inducible expression constructs (fused with the ER$^{T2}$ sequence on the C-terminus) are designed to have the stop codon removed, Kozak sequence (underlined) and *EcoRI* site added in sense primers and *NotI* site in the antisense primer (highlighted):

*Icsbp:*
sense: 5' GGAACAAG**GAATTC**GCCGCCACCATGTGTGACCGGAACGGCGGG 3'
antisense: 5’CCTTCATG**GCGGCCGC**CGACGGTGATCTGTTGATTTTCTCTAAAAAAG 3’

*Klf4:*
sense: 5' GGAACAAG**GAATTC**GCCGCCACCATGAGGCAGCCACCTGGCGAGTC 3'
antisense: 5’ CCTTCATG**GCGGCCGC**CAAAGTGCCTCTTCATGTGTAAGGCAAGGTG 3’

*Klf4$^{\Delta Zn}$:*
sense: 5' GGAACAAG**GAATTC**GCCGCCACCATGAGGCAGCCACCTGGCGAGTC 3'
antisense: 5’ CCTTCATG**GCGGCCGC**CAGTGTGGGTGGCTGTTCTTTTCCGGG 3’

*Klf4$^{\Delta Nterm}$:*
sense: 5' GGAACAAG**GAATTC**GCCGCCACCATGTGTGACTATGCAGGCTGTGGC 3'
antisense: 5’ CCTTCATG**GCGGCCGC**CAAAGTGCCTCTTCATGTGTAAGGCAAGGTG 3’

p21$^{Waf1}$:
sense: 5' GGAACAAG**GAATTC**GCCGCCACCATGTCCAATCCTGGTGATGTCC 3'
antisense: 5’ CCTTCATG**GCGGCCGC**CGGGTTTTCTCTTGCAGAAGACC 3’

*ER$^{T2}$*, *NotI* site added in sense primer and *XhoI* site in antisense primer (highlighted):

sense: 5' GGAACAAG**GCGGCCGC**TCTGCTGGAGACATGAGAGCTGC 3'
antisense: 5' CTTCATG**CTCGAG**GAGCTCTCAAGCTGTGGCAGGGAAACC 3'

## 3.10. Equipment

| | |
|---|---|
| Automated cell counter CASY | Scharfe System, Reutlingen, Germany |
| Automated veterinary hematological counter ABC | SCIL GmbH, Viernheim, Germany |
| Flow cytometer FACSCalibur, with CellQuest software | BD Biosciences, Heidelberg, Germany |
| Fluorescent microscope | LEICA, Solms, Germany |
| Inverse microscope | LEICA, Solms, Germany |
| Stereomicroscope | LEICA, Solms, Germany |
| PCR machines: | |
| Mastercycler®Gradient | Eppendorf, Hamburg, Germany; |
| GenAmp®PCR System 9700 | Perkin Elmer, Wellesley, USA |
| LightCycler™ System | Roche Diagnostics, Mannheim, Germany |
| Mini-gel system for vertical electrophoresis (PAGE) | Hoefer, San Francisco, USA |
| Chambers for horizontal electrophoresis | Bio Rad, USA |
| TransBlot SD | IKA Labortechnik, Staufen, Germany |
| IMAGE Reader | Fujifilm, Düsseldorf, Germany |
| Phosphoimager | Amersham Bioscience, Buckinghamshire, UK |
| UV Stratalinker® 2400 | Stratagene, CA, USA |
| Photometer | Eppendorf, Hamburg, Germany |
| Gased incubator | Heraeus, Hanau, Germany |
| Laminar flow benches | Heraeus, Hanau, Germany |
| Cytospine 4 | Thermo Shandon, Cheshire, UK |
| Centrifuges | Beckman Coulter, Krefeld, Germany; Eppendorf, Hamburg, Germany; Hettich, Tuttlingen, Germany |

## 3.11. Mice

Icsbp-deficient mice (on C57/BL6 or C57/BL6 - 129/Ola mixed background) were generated as described (Holtschke et al., 1996).

Mice with loxP-flanked *Klf4* allele (*Klf4*$^{fl/fl}$ mice) were generated as described (Katz et al., 2002) and kindly provided by KH Kästner, University of Pennsylvania, USA. *Klf4*$^{fl/fl}$ mice were cross-bred with transgenic mice carrying *Cre* recombinase under *Mx* promoter control (*Mx-Cre*$^{+/-}$ mice, mixed background). Activation of *Mx* promoter is achieved in all cells responsive to interferon α/β. In particular, this system enables efficient deletion in liver, spleen and bone marrow (Kuhn et al, 1995). In order to induce the Klf4 deletion, mice between 4 and 6 weeks of age were injected i.p. with 250µg poly(I)poly(C), synthetic double stranded RNA molecule, which stimulates endogenous production of interferon α/β. Three injections in 48h intervals were performed.

All experiments described in this work were performed with 6-10 weeks old mice housed under specific pathogen-free conditions in accordance with the German animal protection law.