4. METHODS

4.1. Cell culture

4.1.1. Isolation and cultivation of primary cells

After sacrificing the mice by CO_2 inhalation, organs were removed, shortly washed with PBS and placed in PBS on ice.

Bone marrow cells were isolated from femur and tibia. After removing hind legs, muscles were cleaned from the bones with cotton tissue. Cleaned bones were cut open on both sides with sharp scissors and bone marrow was flushed with 1ml syringe and 24G needle. Single-cell suspensions were obtained by resuspending the clumps through 26G needle 3-4 times. Isolated cells were centrifuged 5 minutes on 560xg.

The cell suspension from organs (spleen, liver, lymph nodes, thymus) were obtained by pressing the small pieces of isolated organs through a stainless steel sieve in cold PBS. Cells were further resuspended by careful pipeting several times. Single cell suspensions were centrifuged 5 minutes on 560xg.

Peripheral blood leukocytes were isolated from full blood obtained from the tail vein. After lysing the erythrocytes in the hypotonic erythrocyte lysis buffer (BD Pharmlyse buffer, see Materials), leukocytes were pelleted by 5 minutes centrifugation on 560xg, washed once with PBS and pelleted again.

Peritoneal exudate cells were obtained by rinsing the peritoneal cavity with 5ml cold PBS. Cells were pelleted by 5 minutes centrifugation on 560xg.

Peritoneal monocytes and macrophages were obtained as adherent cell fraction after plating total peritoneal exudate cells on Falcon tissue culture plates in macrophage medium (see Materials). After 1h incubation on 37˚C, non-adherent cells were discarded. Adherent cells (monocytes and macrophages) were either used immediately, or cultured in macrophage medium supplemented with 20% L-medium.

Bone marrow macrophages were grown from total bone marrow cell suspension plated in macrophage medium (with 20% L-medium) on 10cm cell culture dishes (Falcon). After 3-4 days, the medium was changed (non-adherent cells were spun down and plated back in the culture). After 7 days, medium was changed again (non-adherent cell fraction was discarded). Adherent cells consist almost 100% of macrophages.

Peritoneal eosinophils were isolated from the peritoneal exudate of mice previously injected (i.p.) with 2ml of 3% thioglycolate solution in PBS. After 72h peritoneal cells were lavaged by 5ml cold PBS. Pooled cells from 5 $Icsbp^{+/+}$ mice and 20 $Icsbp^{-/-}$ mice were resuspended in DMEM supplemented with 10% FCS, 2mM glutamine, 0.1 mM 2-mercaptoethanol and 2% penicilline/streptomycine. In order to deplete monocytes and macrophages from total peritoneal exudate, cells were plated on 10cm cell culture dishes (Corning) and incubated on 37°C. After letting

monocytes and macrophages adhere for 1h, non-adherent fraction of cells was harvested and incubated with FITC conjugated anti-F4/80 antibody, PE conjugated anti-Ccr3 antibody and APC conjugated anti-Cd11b antibody for 30 minutes on 4°C. After labeling, cells were washed 2 times with PBS and the $Ccr3^{+}F4/80^{low}Cd11b^{low}$ fraction was sorted by fluorescence activated cell sorting (FACS) (performed at the Flow Cytometry and Cell Sorting Core Facility of DRFZ, Campus Charite Mitte, Berlin, Germany). Microscope analysis of control cytospins made from sorted fractions and stained with May-Grunewald-Giemsa confirmed the purity of sorted eosinophils to be >98%. Purified eosinophils were either used immediately, or cultured in eosinophil medium supplemented with 50ng/ml mIl-5.

4.1.2. Cultivation of cell lines

NIH 3T3 cells were cultured in DMEM, supplemented with 10% FCS, 2mM glutamine and 1% penicilline/streptomycine. Cells were subcultured two times a week.

Retrovirus packaging cell line (Phoenix-gp cells) were obtained from American Type Culture Collection (ATCC, Manassas, VA). This cell line is based on the 293T cells (human embryonic kidney cell line) transformed with adenovirus E1a and carrying a temperature sensitive T antigen co-selected with neomycin. It is further engineered to stably express gag-pol helper construct. Phoenix-gp cells were cultured in Phoenix medium (Section 3.7.2) on 10cm Corning cell culture plates. Cells were subcultured two times a week.

4.2. Molecular biology methods

4.2.1. Genomic DNA isolation from cells and organs

Genomic DNA from cells and tissues was isolated with Dneasy™ Tissue Kit (Qiagen, Hilden, Germany) or E.Z.N.A™ Tissue DNA Mini Kit (Peqlab, Erlangen, Germany) according to the manufacturers protocol.

4.2.2. Plasmid DNA isolation from bacteria

For the plasmid DNA isolation from competent bacteria QIAGEN™Plasmid Maxi kit (Qiagen, Hilden, Germany) was used, according to the manufacturers protocol.

4.2.3. Electrophoretic separation of DNA fragments

For the electrophoretic separation of DNA fragments, 1% agarose gels were used. The gels were prepared by boiling the agarose in TAE-buffer in the microwave oven, and then cooling to approximately 60°C. For the visualization of DNA fragments, ethidium bromide is added in final concentration of 10ng/ml. The mixture is poured in horizontal casting chambers and allowed to

polimeraze. The samples are prepared for loading by mixing with 0.1 volume 10x loading buffer. The fragment separation is performed in TAE buffer, under 70-100V.

4.2.4. Total RNA isolation from cells

Note about the RNAse-free work: in all steps involving RNA handling, the RNAse-free working conditions were maintained, including use of heat-sterilized glassware, single-use pipeting tips and clean gloves. DEPC water was used as solvent at all steps. Surfaces and equipment used for working (gel casting chamber, magnetic stirrers, measuring cups and beakers) were rinsed with DEPC water before use.

Total RNA from the cells (including messenger RNA, transport RNA, ribosomal RNA and small nuclear RNA) was isolated by phenol-chlorophorm method, using peqGOLD TriFast™ reagent (Peqlab Biotechnology, Eralngen, Germany). According to this method, up to 10^7 cells were homogenized and at the same time lyzed in 1ml peqGOLD TriFast™ reagent. After complete lysis of cells (5 minutes on RT), 200µl chlorophorm was added. The mixture was vigorously shaken for 15 seconds and then incubated another 5 minutes on RT in order to allow the phases to separate. After centrifugation step (15 minutes, 12000g, 4˚C), upper water phase, which contains RNA, was carefully transferred in a new tube. RNA was then precipitated by the addition of 500ml isopropanol and centrifugation 10 minutes on 12000g on 4˚C. Supernatant was removed and RNA-containing pellet was washed with 75% ethanol in DEPC water. After centrifugation (15 minutes on 12000g, 4˚C), ethanol was removed, pellet was shortly air-dried and dissolved in 10-30µl DEPC water. Isolated RNA was kept on -80˚C. RNA concentration was photometrically determined shortly before use.

4.2.5. Photometric determination of the DNA and RNA concentration

In order to determine the concentration of isolated nucleic acids, the samples were diluted in 1:20 to 1:100 ratio and their light absorption on 260nm and 280nm is measured. The nucleic acid concentration is automatically calculated by the photometer, based on the measured extinction (E), dilution factor and optical density factor of DNA (50) or RNA (40). The purity of the isolated nucleic acids is estimated by the E_{260}/E_{280} ratio of the sample (greater than 1.8).

4.2.6. Reverse transcription from the RNA

First strand cDNA synthesis was performed with PowerScript® Reverse Transcriptase kit (BD Biosciences Clontech, Mountain View, CA, USA) according to the manufacturers protocol.

4.2.7. Polymerase Chain Reaction (PCR)

4.2.7.1. Qualitative and semiquantitative PCR amplification

The cDNA samples, prepared as described in Section 4.2.6, were used as templates with sequence-specific primers listed in Section 3.9.1. The sequential 1:10 and 1:100 dilutions of template cDNA were used for semiquantitative analysis. All PCR amplifications were performed in 25µl volume reactions, containing 1xPCR buffer (Bioline, Luckenwalde, Germany), 1.5mM $MgCl_2$, 0.2mM dNTP mix (MBI Fermentas, St. Leon Rot, Germany), 0.3mM each primer and 0.1U/µl of Taq DNA Polymerase (Bioline, Luckenwalde, Germany). The following program was used for amplification:

initial denaturation	95°C----5min	
denaturation step	95°C----30sec	** cycles
annealing step	**°C----30sec	
elongation step	72°C----30sec	
final elongation	72°C----7min	

**The optimal annealing temperature and number of amplification cycles are listed in Table 3.

Sequence amplified	Annealing temperature	Product length:	N°. of amplification cycles
Klf4	58°C	515 bp	35
Icsbp	59°C	652 bp	35
p21^{Waf1}	60°C	517 bp	35-40
p53	60°C	495 bp	35
Gapdh	59°C	459 bp	23-25
Il-5Rα	58°C	389 bp	35-40
Gata1	61°C	451 bp	35-40

Table 3*:* Optimal annealing temperatures, product lengths and number of amplification cycles used for PCR reactions.

4.2.7.2. Real-time quantitative PCR

The cDNA samples prepared as described in Section 4.2.6 were used as templates for the real-time PCR amplification on the LightCycler™ System (Roche Diagnostics, Mannheim, Germany) using LightCycler FastStart DNA Master^{PLUS} SYBR Green1 kit and primers listed in Section 3.9.2. Reactions were carried out in 20µl volume, with the following program:

initial denaturation	95°C----10 min	
denaturation step	95°C----10 sec	40 cycles
annealing step	**°C----5 sec	
elongation step	72°C----8 sec	
fluorescence reading	**°C	
final elongation	72°C----7 min	
melting curve analysis	55°C - 98°C	

The fluorescent signal was monitored in each cycle (single reading at the end of the elongation step), at temperatures indicated in the Table 4. The program was completed with a melting curve analysis. The crossing points (cycle number at which the fluorescence exceeds the background limit) for each reaction were determined by the Second Derivative Maximum algorithm with the Arithmetic

baseline adjustment. For quantification, the normalized target/reference transcript amounts were calculated without the correction for PCR efficiency according to the formula:

Relative Gene Amount = $2^{(Cp(target, wt) - Cp(reference, wt)) - (Cp(target, ho) - Cp(reference, ho))}$.

All PCR reactions were performed in duplicate. The size of acquired products was confirmed by agarose gel electrophoresis and EtBr staining.

Sequence amplified	Annealing temperature	Product length:	Melting temperature
Mxd1	61°C	331 bp	90°C
Marco	61°C	219 bp	90°C
Ceruloplasmin	61°C	302 bp	85°C
Cd36	60°C	321 bp	85°C
Cd177	61°C	339 bp	90°C
Gapdh	61°C	162 bp	90°C

Table 4: Optimal annealing temperatures, product lengths and product melting temperatures used for Real-time PCR reactions.

4.2.8. Northern blotting

4.2.8.1. The preparation of the gel and the probes

For the Northern blotting, 5-10µg of isolated RNA probes were diluted up to 13µl with DEPC-water and mixed with 39µl of RNA-Premix, 10µl RNA loading buffer and 1µl ethidium bromide (10ng/ml). After the short centrifugation, the probes were denatured on 55°C for 15 minutes, briefly cooled on ice and loaded on the denaturing agarose gel. The denaturing agarose gel was prepared by boiling 4.68g agarose in 340ml DEPC water (1.2% gel). After cooling to 60°C in the water bath, 39ml of 10xMOPS buffer and 11.6ml of filtered 37% formaldehyde were added and the mixture was casted in a horizontal gel chamber.

4.2.8.2. The electrophoresis and blotting procedure

The electrophoretic separation of RNA fractions was performed in 1xMOPS buffer, under 70V for 3.5-4 hours.

After the separation, the RNA on the gel was visualized with the UV-light and photographed. The blotting of the RNA to the positively charged membrane (nylon membrane) was performed by passive downward elution in the 20xSSC buffer over night. After completing the RNA transfer, the membrane was rinsed in 2xSSC buffer and the RNA was fixed to the membrane by UV irradiation (auto-crosslink option on the UV Stratalinker® 2400).

4.2.8.3. The preparation of the radioactive labeled probe

The specific probes were made by PCR amplification (as described in Section 4.2.7.1) using the primers listed in Section 3.9.1 and the cDNA from wild type macrophages as template. The amplified fragments were purified by NucleoSpin® ExtractII kit (Macherey-Nagel, Düren, Germany) according to the manufacturers instruction. For the radioactive labeling of the probe, the kit

Rediprime™II (Amersham Biosciences) was used according to the manufacturers instruction. Shortly, 2-20ng of the probe was diluted with TE buffer up to 40µl, denatured for 5 minutes on 95°C and cooled on ice for 5 minutes. The denatured probe was added to the Rediprime-mix together with 5µl of [α-32P]dCTP and incubated 10 minutes on 37°C. During this step, the enzyme Klenow-polymerase synthesizes the second cDNA strand using the randome priming principle and builds in the radioactive dCTPs in the nucleotide strands. The unbound radioactive dCTPs are removed by another purification step with the NucleoSpin® ExtractII kit. The purified radioactive probe was denatured again on 95°C, cooled on ice for 5 minutes and used immediately for hybridization.

4.2.8.4. The hybridization, washing and exposing procedure

The pre-hybridization (blocking) of the membrane was performed in 5ml of the ExpressHyb® solution (BD Biosciences, Clontech) on 68°C for 30 minutes. After the pre-hybridization, the radioactively labeled probe was added to the solution and incubation was continued on 68°C for at least 4 hours. In order to remove the unspecifically bound radioactive probe, the membrane was washed first with the Wash solution I for 20 minutes on 55°C (low stringency wash buffer) and then two more times with the Wash solution II (higher stringency wash buffer), each time 20 minutes on 55°C. Washed membrane was placed on the phosphoimager screen and exposed 24-72 hours. Finally, the screen was scanned with the Phosphoimager.

4.2.8.5. The stripping and reprobing procedure

In order to prepare the membrane for the new hybridization, the remaining old probe was "stripped" by boiling 0.5% SDS solution. The hybridization procedure was repeated with the new probe in the same way as described above, starting with the pre-hybridization step.

4.3. The retroviral constructs

4.3.1. Vector

The pMIEG3 plasmid was obtained from D.A. Williams (Children's Hospital Medical Center, Cincinnaty, USA). MIEG3 is a bicistronic murine stem cell virus (MSCV) based retroviral vector, containing an encephalomyocarditis virus IRES element (internal ribosome entry site) immediately preceding the gene encoding eGFP (enhanced green fluorescent protein). The coexpression of eGFP with the gene of interest enables detection of infected cells by flow cytometry or fluorescent microscopy. Multiple unique cloning site permits convenient insertion of target genes in front of the IRES. The expression of a gene inserted in the MIEG3 multiple cloning site is driven by a strong MSCV promoter, which enables high expression levels in the target cell. The MIEG3 plasmid also harbors ampicillin resistance gene as a selection marker.

4.3.2. Cloning of the retroviral constructs

4.3.2.1. PCR amplification of desired cDNA sequences (*Icsbp, Klf4, $p21^{Waf1}$*)

Unless otherwise stated, the cDNA obtained from wild type bone marrow macrophages was used as a template for the PCR amplification of all sequences described. In each case, the PCR primers were designed to add common restriction sites at the sequence ends in order to enable fragment insertion into the multiple cloning site of the MIEG3 vector (listed in section 3.9.3).

The PCR primers amplifying the *Icsbp*, *Klf4* and $p21^{Waf1}$ sequences for constitutive expression constructs were designed to include the Kosak sequence in front of the start codon and to flank the PCR product with *EcoRI* site at the 5´end and *XhoI* at the 3´end.

The PCR primers amplifying *Icsbp, Klf4* and $p21^{Waf1}$ sequences for inducible expression constructs (chimeric constructs with ER^{T2} sequence fused on their C-terminus) were designed to include the Kozak sequence in front of the start codon, and to flank the PCR product with *EcoRI* site at the 5´end and *XhoI* at the 3´ end. Stop codon in the antisense primer was removed in order to allow the fusion of the ER^{T2} sequence at the C-terminus.

Modified estrogen receptor sequence (ER^{T2}) fused to C-terminus of *Cre* recombinase was a kind donation from Daniel Metzger, INSERM, Strasbourg, France (Feil et al., 1997). *Cre-*ER^{T2} sequence was released by *EcoRI* digestion and subcloned in the *EcoRI* site of the MIEG3 vector.

ER^{T2} fragment was obtained from Cre-ER^{T2}/MIEG3 plasmid by PCR amplification (10ng of plasmid was used as template for the PCR reaction). PCR primers were designed to add *NotI* site on 5' terminus and *XhoI* site on 3' terminus (behind the stop codon). This sequence does not contain a start codon, therefore it can readily be fused with other sequences at its N-terminus.

Deletion mutants of *Klf4* were cloned by using *Klf4*/MIEG3 plasmid as template (10ng per PCR reaction).

The primers for amplification of the $Klf4^{delZn}$ sequence (zinc-finger region in the C-terminus of Klf4 removed), were designed to include amino acids 1-401 of the Klf4 protein. The 5' primer contains *EcoRI* restriction site, Kozak sequence and start codon. The 3' primer contains the *NotI* restriction site and for the constitutive expression construct, stop codon was added.

The $Klf4^{delN}$ construct contains amino acids 402-483 (the zinc-finger region). The 3´primer has the stop codon removed and *NotI* site added. The 5´primer was placed within the *Klf4* sequence in order to obtain the region starting with the amino acid 402 (the zinc-finger region). The start codon was added in front of this region, as well as the Kozak sequence and *EcoRI* restriction site.

All PCR reactions were carried out in the 50µl volume, in the same reaction mix described for the qualitative PCR (Section 4.2.7.1), with the following program:

initial denaturation	95°C----5min	
denaturation step	95°C----30sec	
annealing step	58°C----30sec	28 cycles
elongation step	72°C----90sec	(for p21 30 sec).
final elongation	72°C----7min	

PCR products were purified by using NucleoSpin® ExtractII kit (Macherey-Nagel, Düren, Germany) according to the manufacturers instruction (elution volume 10µl) and used for cloning.

4.3.2.2. Digestion

Purified PCR products were double-digested with *EcoRI*/*NotI*, i.e. with *NotI*/*XhoI* enzymes (MBI Fermentas, St. Leon Rot, Germany) under following conditions:

EcoRI	0,5µl (5 Weiss)
XhoI (*NotI*)	0,5µl (5 Weiss)
O buffer (10x)	2µl
PCR product	10µl (0.5-1µg DNA)
H_2O	up to 20µl

Incubation was carried out over night on 37°C.

In parallel, 1µg of MIEG3 vector was digested under same conditions with *EcoRI*/*XhoI* enzymes. At the end of the digestion, vector was dephosphorylated by the addition of 2µl of CIAP (MBI Fermentas; St. Leon Rot, Germany) and 15 minute incubation on 37°C.

After digestion, restriction enzymes were inactivated 10 minutes on 65°C. Digested vector and PCR fragments were purified from enzymes and small DNA fragments by electrophoretic separation on an 1% agarose gel (in TAE buffer, on 70-100V). The corresponding bands were visualised by EtBr staining, cut out of the gel and the DNA was extracted from the gel slices by QIAexII®Gel extraction kit (Qiagen, Hilden, Germany), according to the manufacturer instruction (elution volume 10µl). In order to estimate the concentration of purified fragments, 2µl of each fragment was applied on new 1% agarose gel along with quantitative λ HIND III markers and separated by electrophoresis (70-100V). The concentration was estimated by visually comparing the intensity of sample and marker bands.

4.3.2.3. Ligation

Molar ratio 1:3 (vector:construct) was used for ligation. In each case, 100ng of vector was used and quantity of the insert was calculated according to the formula:

$$(ng_{\text{vector}} \times kb_{\text{insert}}) / (kb_{\text{vector}} \times \text{desired molar ratio}_{\text{insert/vector}}) = ng_{\text{insert}}$$

For creating the fusion constructs the ER^{T2} , *NotI*/*XhoI* flanked fragment was used with *Icsbp*, *Klf4* (full length), $Klf4^{delZn}$, $Klf4^{delN}$ or $p21^{Waf1}$ *EcoRI*/*NotI* flanked fragments and were cloned into

EcoRI/*XhoI* site of MIEG3 in one-step ligation reaction. Ligation resulted in in-frame fusion of ER^{T2} to the C-terminus of the specific sequence (*Icsbp, Klf4,* $Klf4^{delZn}$, $Klf4^{delN}$ or $p21^{Waf1}$).

Ligation was carried out under following conditions:

- calculated amounts of vector and insert(s)
- 1µl T4 ligase, 5U/µl (MBI Fermentas)
- 2µl T4 ligase buffer 10x (MBI Fermentas)
- up to 20µl water

For the vector religation control the reaction was set without the insert.

Ligation mix was incubated over night on 16°C and the product was transformed in DH5-α *E.coli* bacteria.

4.3.2.4. Retransformation

For retransformation, 2µl of ligation product was mixed with 100µl of DH5-α suspension and incubated on ice for 30 minutes. Heat shock was performed by 60 second incubation on 42°C and the DNA up-take by immediate placing the bacteria on ice for another 90 seconds. Transformed bacteria were preincubated in LB-medium without antibiotics for 30 minutes at 37°C (in loose-cap tubes, with shacking). Finally, bacteria were plated out on LB-agar plates supplemented with 100ng/ml ampicilline and incubated over night on 37°C.

4.3.2.5. The colony screening

Ampicilline resistant colonies were picked the next day with sterile pipeting tips and incubated in 2ml LB-medium (with 100ng/ml ampicilline) over night. Bacterial cells were pelleted by 5 minutes centrifugation on 6000xg, on 4°C. The pellets were resuspended in 100µl of resuspention buffer P1 (Qiagen), lysed by 200µl of lysis buffer P2 (Qiagen) and neutralized by 150µl of neutralization buffer P3 (Qiagen). Proteins and genomic DNA were pelleted by 15 minutes centrifugation on 20.000xg, on 4°C. The clarified supernatants, containing plasmid DNA were separated and the DNA was precipitated by the isopropanol addition (0.7 volume) and 15 minutes centrifugation on 20.000xg, on 4°C. Pellets were washed once with 70% ethanol and centrifugated 15 minutes on 20.000xg. Precipitated plasmid DNA was dissolved in 20µl of 10µM Tris buffer (pH 8,0) and enzymatic digestions were used for screening of clones. The successful clones were used for retransformation and preparation of plasmid DNA by using QIAGEN™Plasmid Maxi kit, according to the manufacturers protocol.

All constructs cloned were confirmed by sequencing (sequencing service by Agowa, Berlin, Germany and analysis by SeqMan DNA Star software).

The expression of the constructs is confirmed by Western blotting of protein samples obtained from stable transfected NIH 3T3 cells (see Section 4.3.4) and probed with antibody against estrogen receptor (Euromedex, France), which detects the ER^{T2} sequence on the C-terminus of the chimerical protein (Figure 4).

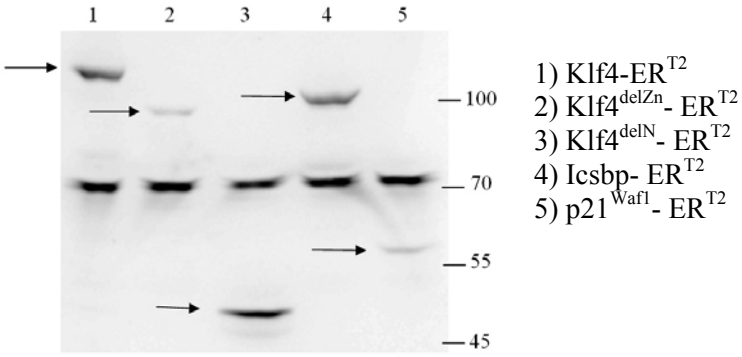

Figure 4: The expression of fusion constructs in NIH 3T3 cells
NIH 3T3 cells were transduced with indicated constructs in MIEG3 viral vector (as described in Section 4.3.4) and sorted for eGFP expression. Total cell protein lysates were obtained, separated by SDS-PAGE on 5-15% gradient gel and analysed with anti-estrogen receptor antibody (raised against F-region of the human estrogen receptor, Euromedex, France). The signal specificity is confirmed by reprobing the blot with anti-Icsbp, anti-Klf4 and anti-p21^{Waf1} antibodies (not shown). The unspecific band at approximately 70kD is used as loading control.

4.3.3. Production of retrovirus containing supernatants

The Phoenix-gp packaging cell line was used for the production of retroviral particles. This cell line is engineered to stably express retroviral helper plasmid coding the gag-pol proteins. The expression of envelope proteins is achieved by transient cell transfection with the plasmid coding for either ecotropic or amphotropic envelope proteins (K73 or M75 plasmids respectivelly). In the experiments described in this work, only ecotropic constructs were used. The *gag-pol* and envelope constructs are driven by non-Moloney promoters, which minimizes the recombination events and replication-competent virus formation.

For the virus production, 3-5x10^6 Phoenix-gp cells were seeded in 9cm culture dish (Corning) in 10 ml Phoenix medium one day prior to transfection. The next day, cells were co-transfected with plasmids carrying *gag-pol* (M57), ecotropic envelope (K73) and desired MIEG3 construct using the calcium-phosphate method. 10µg of M57, 2µg of K73 and 5µg of MIEG3 plasmid were diluted in 450µl H_2O and mixed thoroughly with 50µl of 2M $CaCl_2$. The mixture was dropwise added to 500µl of Hebs buffer with constant vortexing. Just prior to transfection, the cell medium was removed and replaced with fresh medium containing 25mM chloroquine (in order to prevent the lysosomal degradation of inserted plasmid DNAs). Plasmid mixture with $CaCl_2$ and precipitation buffer was added to the cells. After 6-12h of incubation, the medium was replaced. Medium containing retrovirus particles was collected 30h, 48h, 56h and 72h after the transfection and stored on –80°C.

4.3.4. Determining the titer of the retrovirus containing supernatans

The standard for determining the activity of harvested retroviral supernatants is the infection of NIH 3T3 fibroblasts with produced virus supernatants. The NIH 3T3 cells were seeded in the 6-well plates at the density of 5x10^4 cells per well (in 2ml of 3T3 medium). The next day, the polycationic compound polybrene was added to the medium (8ng/ml) in order to enhance the transfection

efficiency. Virus supernatants were added to the medium in 1:10 and 1:100 dilutions and cells were incubated for 24h. After that period, the polybrene containing medium was replaced with fresh 3T3 medium and the cells were incubated for another 24-48h in order to allow integration of retroviruses into the host genome and the expression of the eGFP marker. The efficiency of infection was determined by analyzing the percentage of eGFP positive cells by flow cytometry.

4.3.5. Infection of bone marrow cells by retrovirus constructs

The progenitor-enriched bone marrow cells (Section 4.4.1 and 4.4.2) were plated in the stimulation medium (IMDM, supplemented with 10% FCS, 2mM glutamine, 0.1 mM 2-mercaptoethanol, 2% pen/strep. and recombinant cytokines: rrSCF (50ng/ml), rhFlt-3 ligand (100ng/ml), rhTPO (100ng/ml) and rmIl-3 (5ng/ml) (Peprotech, NY, USA). This cytokine combination stimulates the proliferation of immature hematopoietic progenitors and enables their infection with retroviral particles, which require active cell division for effective integration in the host genom. After 48h stimulation, cells were loaded on 6-well plates (non-cell culture treated plates, Falcon, Germany) coated with fibronectin (Retronectin™, Takara, Japan) as follows: 40µg of Retronectin™ in 2ml of cold PBS were pipeted pro well and incubated on 4°C over night. After coating, the Retronectin™ solution was removed and cytokine-stimulated cells were loaded on wells. The cells were incubated 1 hour on 37°C in order to allow the VLA-4 and VLA-5 expressing cells (immature progenitors) to adhere to the corresponding Retronectin™ domains, CS1 (Connecting Site 1) and central cell binding domain (Moritz et al., 1994). After cell loading, the medium with non-adhered cells was removed and virus preloading was performed by adding 2ml of undiluted virus supernatant. During 30 minutes incubation at 37°C the virus particles bind to the Retronectin™ through the heparin binding domain II (Hanenberg et al., 1996). Thus achieved colocalization of the virus particles and target cells was shown to greatly enhance the efficiency of the transfection procedure (Moritz et al., 1996.; Hanenberg et al., 1996; Chono et al., 2001). After virus preloading, the virus supernatants were removed and replaced with 2ml of fresh supernatant diluted with 2ml of stimulation medium and incubated over night. Next day, the virus-containing medium was replaced with fresh stimulation medium, and cells were incubated for 6h. In the evening, the infection procedure was repeated as described (cell-loading step omitted). After the second infection, cells were incubated for another 48h in the stimulation medium, and then detached from the Retronectin™ by using cold Cell Dissociation buffer (PAA Laboratories, Pasching, Germany). Detached cells were plated once more in fresh stimulation medium, incubated over night and than sorted for eGFP expression (Section 4.6.3).

4.4. Progenitor enrichment of bone marrow cells

4.4.1. 5-fluorouracil (5-FU) treatment of mice

72 hours prior to harvest, mice were treated with 5-fluorouracil, injected i.p. in the dose of 0.15mg/g body weight (for Icsbp$^{-/-}$ mice 0.075mg/g body weight). After 72h, animals were sacrificed, femurs and tibias were collected and bone marrow cells were flushed out with cold PBS. Erythrocytes in the bone marrow cell suspension were lysed with BD PharmLyse® buffer (BD Biosciences, Heidelberg, Germany) for 5 minutes on room temperature. After erythrocyte lysis, cells were washed two times with cold PBS and plated in IMDM medium (10%FCS, 1%pen/strep) containing the pre-stimulation cytokine combination (50ng/ml rSCF, 100ng/ml TPO, 100ng/ml Flt3L, 5ng/ml mIL-3).

4.4.2. Depletion of mature cells by MACS

Total bone marrow cell suspension was obtained by rinsing femora and tibia with cold PBS. Freshly prepared cells were washed once with IMDM (5% FCS, 1% penicillin/streptamycin), centrifuged and pellets were resuspended in combination of ready-to-use biotinolated antibodies against: Cd3e, Cd11b (Mac-1), Cd45R/B220, Ly-6G (Gr-1) and Ter-119 (Mouse Lineage Panel, BD Biosciences Pharmingen). 2µl of each antibody were used per 1x10^6 cells. After incubating the suspension for 20 minutes on 4°C, cells were washed 2 times with IMDM (5% FCS, 1% penicillin/streptamycin). In the second labeling step, cell pellets were resuspended with streptavidin-coated magnetic beads (Milteniy Biotec) (1µl of bead suspension per 1x10^6 cells, diluted 1:9 with cold PBS). Suspensions were incubated with gentle shaking for 20 minutes on 4°C, then washed once and resuspended in IMDM (5% FCS, 1% pen/strep) to final concentration of 5x10^7 cells/ml.

For separation of lineage negative cells, LS columns (Miltenyi Biotech) were placed on magnetic holder, and pre-conditioned by runing 3ml cold IMDM (5% FCS, 1% pen/strep) through them (flow through discarded). 2ml of labeled cell suspension was applied on the column through 30µm filters (Miltenyi Biotech). Flow-through fraction is highly enriched for lineage negative cells, since cells expressing lineage markers are coupled with magnetic beads and retained in the column placed in magnetic filed. Columns were rinsed with 3x2ml cold IMDM (5% FCS, 1% pen/strep) and flow-through fractions were collected and pooled.

After rinsing lineage negative cells, columns were removed from magnetic holders and rinsed again with cold IMDM (5% FCS, 1% pen/strep). Under these conditions lineage positive cells are released from the column and collected in the flow-through.

A sample from both lineage positive and lineage negative cell fraction was taken for checking the separation efficiency by FACS. Cells were incubated with APC conjugated streptavidin, washed once with PBS and analyzed by FACS. The purity of lineage negative cell fraction was routinely greater than 90%.

4.4.3. Mononuclear cell enrichment by Ficoll gradient centrifugation

The isolated bone marrow cells (up to $5x10^7$) were resuspended in 3ml IMDM (10% FCS, 1% pen/strep). 2ml of Biocoll solution (density 0,090 on 20°C) was slowly added underneath the cell suspension, taking care that the layers don't mix. After 25 minutes centrifugation on 2.500rpm and 20°C, the mononuclear cells were separated as a layer floating between the cell medium and Biocoll solution, while erythrocytes and polymorphonuclear cells pellet on the bottom of the tube. The mononuclear cells were collected, washed once in PBS and plated in the culturing medium.

4.5. The Colony Forming Unit (CFU) assay

The sorted GFP^+ cells were plated in the methyl-cellulose based medium, containing 1% methylcellulose (Methocult H3100, Stem Cell Technologies, Vancouver, Canada), 30% FCS (Biochrom, Berlin, Germany), 2mM glutamine, 0.1 mM 2-mercaptoethanol and 2% pen/strep. in IMDM. In order to differentiate the precursor cells into different cell types, the following cytokines were added: rmM-CSF 100ng/ml for macrophages, rmGM-CSF for macrophages and granulocytes, combination of rrSCF 50 ng/ml, rhTPO 100ng/ml and rhG-CSF 100ng/ml for granulocytes and rmIl-5 50ng/ml for eosinophils. For the Colony Forming Unit assay (CFU assay), $1x10^4$ cells/ml were plated in 3cm dishes (Falcon, Germany) and grown in humidified atmosphere gassed with 7% CO_2. Colonies were scored after 6-8 days using an inverted microscope. Colonies were classified in three types (M-CFU, GM-CFU and G-CFU) according to the standard morphologic criteria (Nakahata et al., 1982). In order to confirm the colony types, cells were rinsed out of methylcellulose medium with warm PBS, spun onto microscope slides, stained according to May-Gruenewald-Giemsa and inspected under the microscope.

In parallel, cells were grown in liquid culture (IMDM, 10%FCS, 1% glutamine, 1% pen/strep) with the same cytokine combinations as in the CFU assay.

4.6. Fluorescence Assisted Cell Sorting (FACS)

4.6.1. Cell labeling for FACS

Cell samples of $1\text{-}5x10^5$ cells were resuspended in 100µl FACS buffer with 0.2µl of desired fluorchrome-coupled or biotinylated antibodies (maximum of 3 different fluorochromes was used) and incubated for 20 minutes on room temperature, protected from light. After incubation, surplus antibody was removed by washing the cells two times with FACS buffer. In case biotinilated antibodies were used, streptavidin coupled with fluorochrome was used in the second staining step (0.2µl of fluorochrome coupled streptavidine in 100µl FACS buffer) and incubated 20 minutes on room temperature, protected from light. After labeling, cells were washed once, resuspended in 150µl FACS buffer and analyzed.

Fluorochrome coupled Annexin V was used to label apoptotic cells: 5µl of annexin V was used per $1x10^5$ cells, resuspended in 100µl of annexinV staining buffer. In parallel, dead cells were labeled by adding vital dye propidium iodide to the staining mixture (5µl of 0.1mg/ml solution per probe). Samples were incubated for 20 minutes on 4°C and measured within 1 hour directly in the staining solution.

4.6.2. Propidium iodide staining for cell cycle and apoptosis analysis

Cell cycle stage and percentage of apoptotic cells can be determined by analyzing the DNA content of cells. Quiescent cells (in G_0/G_1 phase) have diploid chromosome number (2n), cells in G_2/M phase have tetraploid chromosome number (4n), cells in S-phase have DNA content between 2n and 4n, while apoptotic cells show DNA fragmentation and sub-diploid peak. In order to stain the DNA, samples of approximately $1x10^5$ cells were resuspended in the staining buffer (0.05mg/ml propidium iodide, 0.3% NP-40 and 1mg/ml RNAse A in PBS). Detergent in the staining buffer permeabilizes the membranes and allows access of propidium iodide to DNA. The RNAse A digests released RNA and prevents high background staining. After 30 minutes incubation on 4°C, cells were analyzed directly in the staining buffer.

4.6.3. The cell sorting

The cell samples were labeled with flurochrome coupled antibodies in the same way as the samples used for the analysis. Cells were washed 2 times with PBS, and filtered through 30µm filters to exclude cell aggregates. Fluorescence activated cell sorting (FACS) was performed at Flow Cytometry and Cell Sorting Core Facility of DRFZ (Campus Charité Mitte, Berlin, Germany).

- The peritoneal eosinophiles were sorted from the peritoneal exudate as $Ccr3^+F4/80^{low}Cd11b^{low}$ fraction (see Section 4.1.1).
- The peritoneal macrophages were sorted from the peritoneal exudate as $F4/80^{high}/Cd11b^{high}$ fraction.
- The mature granulocytes were sorted from the bone marrow suspension as $Gr1^{high}/Cd11b^{high}$ fraction.
- The mature B- and T-lymphocytes were sorted from the spleen cell suspension as $B220^+$ and $Cd3^+$ fraction respectively.
- The sorting of the progenitor cell populations from the bone marrow of $Icsbp^{+/+}$ and $Icsbp^{-/-}$ mice was performed by Dr. Grzegorz Terszowski (Department of Immunology, University of Ulm, Germany) as follows: GMP (Lin^- c-kit^+ Sca-1^- $Cd34^+$ $Fc\gamma R^{high}$),
  EoP (Lin^- $Cd34^+$ c-kit^{low} Il-5Rα^+),
  EP (Lin^- c-kit^+ Il3-Rα^- Il-7Rα^- Sca-1^- $Cd41^-$ $Cd71^+$)
- The GFP-expressing cells were sorted without additional labeling.

4.7. Analysis of hematopoietic parameters

4.7.1. Automated blood cell counting

Blood samples were obtained from the tail vein and collected in EDTA coated tubes. The blood cell counts were performed with automated veterinary hematological counter ABC (SCIL GmbH, Viernheim, Germany), with the software optimized for mouse blood parameters.

4.7.2. Analysis of cell distribution in organs

Single cell suspensions from the bone marrow, spleen, lymph node, thymus and peritoneal cavity were obtained as described above. Approximately $1x10^5$ cells from each suspension was used for the FACS analysis. Antibody combinations used are listed in Table 5, and different stainings used for each organ tested are listed in Table 6.

Staining:	FITC conjugated	PE conjugated	APC conjugated
Ly	Cd4	Cd8	B220
My	F4/80	Gr1	Cd11b
D/NK	Cd11c	DX5	Cd11b
B-Akt.	MHC II IAb	Cd80	B220
T-Akt.	Cd4	Cd8	Cd62L
Stem	Sca1	c-kit	Lineage markers (Mouse Lineage Panel, BD)

Table 5: Antibody combinations used for analyzing different immune cell populations

Organ:	Applied staining:					
peripheral blood (PB)	Ly	My				
bone marrow (BM)	Ly	My	Stem			
spleen (Spl)	Ly	My		D/NK	B-Akt.	T-Akt.
lymph node (LN)	Ly	My				
thymus (Thy)	Ly					
peritoneal lavage (PL)	Ly	My		D/NK		

Table 6*:* Staining combinations (listed in Table 4) used to analyze immune cell populations in different organs

4.8. The mouse inflammation models

4.8.1. Thioglycolate induced sterile peritoneal inflammation

The 3% thioglycolate solution in PBS was used for inducing inflammatory reaction. The solution was prepared fresh each time and autoclaved, since autoclaving greatly enhances non-enzymatic formation of advanced glycosylation products (AGPs). 2ml of prewarmed solution were injected intraperitoneally. The animals were sacrificed 72 hours after injection.

4.8.2. *Nippostrogylus brasiliensis* infection

The mouse-addapted strain of rat intestinal parasite *Nippostrongylus brasiliensis* was maintained and passed in Lewis rats at the Infection Research Center, University of Würzburg,

Germany. The collected rat feces was used as the source of L3 larvae, obtained by the incubation of the fecal slurry (performed by Dr. Klaus Erb, Infection Research Center, University of Würzburg, Germany). The mice were infected by s.c. injection of 750 L3 larvae.

4.9. Histopathology

The animals infected with *N. brasiliensis* were sacrificed 14 days after infection. The small intestines were removed and fixed in a solution containing 10% formalin, 70% ethanol and 5% acetic acid. Parafine sections of small intestines were stained with hematoxylin and eosin and the number of goblet cells was determined microscopically by cell counting in randomly chosen areas (performed by Daniela Struck, Institute for Microbiology, Charité, Berlin, Germany).

4.10. Protein biochemistry methods

4.10.1. Total protein extraction from cells

The total protein extracts from cells were obtained by lysis in the RIPA buffer supplemented with the combination of protease inhibitors (Roche). The amount of the lysis buffer was adjusted to contain $2x10^4$ cells/µl. The cells were incubated in the lysis buffer on ice for 30 minutes and then centrifuged for 30 minutes on 20.000xg on 4°C. Clarified lysates were transferred into fresh tubes and the protein concentration was determined by Bradford method (Section 4.10.2). The proteins were denatured by adding 0.25 volume of the 4xSDS denaturing and loading buffer and boiled for 5 minutes on 95°C. After denaturing, the samples were cooled on ice, briefly centrifuged and used immediately for electrophoresis or frozen on -20°C.

4.10.2. Determining the protein concentration by Bradford method

In order to assure the equal sample loading on the gel, the protein concentration in the probes was measured according to Bradford. For this method, 1µl of each probe was added into 99µl of Bradford solution. The protein binding to the Coomassie Brilliant Blue causes the shift in the dye absorption maximum from 465 to 595nm which linearly correlates to the concentration of the protein. Therefore, the protein content of the probes was determined by measuring the probe extinction on 595nm and calculating the concentration from the standard curve obtained with known BSA dilutions.

4.10.3. The denaturing discontinuous polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

4.10.3.1. The gel preparation

The electrophoretic separation of proteins was performed on discontinuous denaturing SDS-polyacrylamide gels. In order to analyze the proteins of different sizes in one probe (from 20-120kD), the resolving 5-15% gradient gels were used.

Acrylamide concentration (%)	5%	15%
Acrylamide (30%) / Bisacrylamide (0.8%)	2.5ml	7.5ml
Separation buffer	3.75ml	3.75ml
10% APS	6µl	6µl
TEMED	12µl	12µl
H_2O	8.75ml	3.75ml

The gradient gels were casted in vertical molds using the gradient maker chambers (Hoefer, USA). 3.75ml of the higher percentage acrylamide solution (15%) was loaded into the first chamber of the gradient maker, and the same volume of the lower percentage solution in the second (the separator between the chambers was closed at his point). The rubber tube connected to the first chamber was placed in the middle of the casting mold and the gravity flow of the 15% acrylamide solution was allowed. Immediately after starting the flow from the first chamber, the separator between the chambers was opened, allowing the 5% solution to flow into the first chamber and gradually dilute the solution poured into the mold. This method gives gels with the linear concentration gradient. When both chambers were emptied, the rubber tube was removed from the mold and the gel was overlaid with iso-butanol in order to prevent the contact with oxygen, which can interfere with the gel polymerization. Completely polymerized separating gel (after 2-3h on room temperature) was overlaid with a large-pore stacking gel, prepared as follows: 0.65ml Acrylamide (30%)/Bisacrylamide (0.8%), 1.25ml stacking buffer, 3.05ml H_2O, 36µl 10%APS and 6µl TEMED. The comb forming the slots for sample application was inserted into the stacking gel and it was allowed to polymerize completely (couple of hours or over night). The ion gradient formed between the stacking and resolving gel enables focusing (“stacking”) of the proteins into sharp bands which greatly enhances their resolving.

4.10.3.2. The electrophoresis and blotting procedure

The denatured protein samples were loaded on the gel along with adequate size markers and separated in 1x protein running buffer at the constant current (20mA per gel).

After the separation, the proteins from the gel were transferred to the nitrocellulose membrane using the semi-dry blotting method. For that purpose, the gel was placed on the membrane pre wet in the 1x transfer buffer, and both are laid on the electroblotting apparatus between two extra thick Whatman papers soaked in the transfer buffer. The blotting was performed at $0.4V/cm^2$ for 45 minutes.

The protein transfer efficiency was confirmed by Ponceau staining: the membrane was soaked 5 minutes in Ponceau solution, rinsed with water and photographed using the IMAGE Rader™ (Fuji).

4.10.3.3. The specific protein detection

Before probing the membrane with specific antibodies, the non-specific binding was blocked by 1 hour incubation in blocking buffer with gentle shaking on room temperature (alternatively, over night incubation on 4°C). The specific antibodies were used in producer recommended dilutions and buffers and incubated over night on 4°C. After the primary antibody incubation, the membrane was washed 3 times 5 minutes with TBS/T buffer and then incubated 1 hour at room temperature with secondary peroxidase-conjugated antibody (Santa Cruz Biotechnologies) diluted 1:3000 in blocking buffer. The membrane was washed again 3 times with TBS/T buffer and the bound antibody was detected using the ECL™Western blotting detection reagent (Santa Cruz Biotechnologies). The chemiluminescence was visualized with IMAGE Rader™ (Fuji).

4.10.4. The cytokine concentration determination by ELISA

The mesenteric lymph nodes and spleens were removed and homogenized using a mortar and pestle. The cytokine concentration was determined in cell-free supernatants by sandwich ELISA method using paired antibodies against Il-4 (clones 11B11 and BVD6-24G2) and Il-5 (clones TRFK4 and TRFK5). The cytokines were quantified by reference to commercially available recombinant murine standards (Pharmingen). The analysis is performed by Daniela Struck, Institute of Microbiology, Charité, Berlin.

4.11. Microscopy

4.11.1. Preparation of slides

The cytospins were prepared from the primary cells or cell lines resuspended in the medium with high FCS content (IMDM, 30% FCS), approximately 0.5-1x10^5 cells in 200µl. The cell suspension was transferred in the cytospin chambers (placed on microscope slides over a filter for excess liquid) and centrifuged 5 minutes on 560xg on room temperature. The microscope slides were air-dried for at least 1 hour before fixation.

The blood smears were prepared from 2-3µl blood freshly taken into EDTA coated tubes. The smears were air-dried at least 1 hour before fixation.

4.11.2. May-Grünwald-Giemsa staining

The air-dried slides were fixed 10 minutes in daily fresh methanol. The staining was performed by 3 minutes incubation in the May-Grünwald staining solution (Sigma), 20 minutes in 1:20 diluted Giemsa staining solution (prepared fresh) and destained 2 minutes in water.

4.11.3. Fluorescent microscopy

The GFP expression in cells or colonies formed in the CFU assay (Section 4.5) was monitored under the standard fluorescence microscope (Leica) using the filter set to select the 470-490nm excitation beam.