2. MATERIALS AND METHODS

2.1 Mice

Genotypes of GM-CSF- (36), IL-3- (54), GM-CSF/IL-3- (55), IFN-γ- (26), GM-CSF/IFN-γ- (8), and GM-CSF/IL-3/IFN-γ- (8) deficient mice were confirmed by polymerase chain reactions (PCR) using a thermocycler (MJ Research). Primers used for genotyping GM-CSF-deficient mice were: GM-CSF forward 5'-AGG-CCA-CTT-GTG-TAG-CGC-CAA-GT-3', GM-CSF reverse 5'-TCG-TCT-CTA-ACG-AGT-TCT-CCT-TCA-3' and GM-CSF neo reverse 5'-TGC-TCG-AAT-ATC-TTC-AGG-3' (PCR conditions: 35 cycles, annealing: 56ºC for 30 sec, elongation: 72ºC for 1 min). Primers used for genotyping IL-3-deficient mice were: IL-3 forward 5'-GAA-CCT-GAA-CTC-AAA-ACT-GAT-3', IL-3 reverse 5'-CTG-AAG-ATT-GGA-CTT-GAT-AAC-3', and IL-3 neo reverse 5'-TGA-TCT-GGA-CGA-AGA-GCA-TCA-3' (PCR conditions: 35 cycles, annealing: 54ºC for 30 sec, elongation: 72ºC for 1 min). Primers used for genotyping IFN-γ-deficient mice were: IFN-γ forward 5'-AGA-AGT-AAG-TGG-AAG-GGC-CCA-GAA-G-3', IFN-γ reverse 5'-AGG-GAA-ACT-GGG-AGA-GGA-GAA-ATA-T-3', neo IFN-γ forward 5'-TCA-GCG-CAG-GGG-CGC-CGG-GTT-CTT-T-3', and reverse 5'-ATC-GAC-AAG-ACC-GGC-TTC-CAT-CCG-A-3' (PCR conditions: 35 cycles, annealing: 52ºC for 45 sec, elongation: 72ºC for 1 min). GM-CSF/IL-3-deficient mice were genotyped earlier by Southern blotting, using neo and hygro probes as described (55).

Cohorts of 12 age-matched female and male GM-CSF/IL-3/IFN-γ-deficient mice were maintained from birth on enrofloxacin (Baytril®; Bayer), dissolved in chlorinated drinking water (85 mg/liter, corresponding to a dose of 50 mg/kg). Other cohorts of 12 age-matched female and male GM-CSF/IL-3/IFN-γ-deficient mice were maintained from birth on sulindac (Sulindac; Geneva Pharmaceuticals), dissolved in drinking water with 0.5 x PBS (85 mg/liter sulindac, corresponding to a dose of 50 mg/kg) (8, 120). At the

same time, cohorts of 12 untreated, age-matched female and male GM-CSF/IL-3/IFN-γ-deficient mice were maintained as controls.

2.2 Pathological Procedures

Organ and tumor tissues were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin. They were then sectioned at 4 μm thickness, mounted on silanized microscope slides (Fisher Scientific), and stained with hematoxylin and eosin (H & E), or mucicarmine as described (121).

Organ and tumor tissues were also snap frozen in liquid nitrogen. Immunohistochemistry was performed using standard techniques with monoclonal antibodies to CD4, CD8, B-220 and Ig-κ (BD PharMingen) (8). Briefly, tissues were sectioned at 5 μm thickness using a cryo-microtome (Microm) and mounted on silanized microscope slides. The sections were then left in a humidity chamber (1 x PBS, 10% fetal calf serum (FCS)) at 4ºC over night. After air-drying, the mounted tissues were fixed in ice-cold acetone for 10 min. The slides were then dried again and blocked with 1 x PBS/5% FCS for 1 hour at room temperature. After drying with the tip of a paper towel, HRP-labeled antibody was added (1:100 diluted in 1 x PBS/5% FCS) and incubated at 4ºC over night. The slides were then washed 3 x with PBS/0.05% Tween-20®, developed using a HRP substrate kit (Amersham), and shortly dried. They were then covered with a mounting medium (Vector), and, finally, with a cover glass that was fixed with nail polish.

2.3 ELISAs

Anti-ds DNA antibodies were measured by adding sera to a 96 well MaxiSorp™ ELISA plate (Nunc), coated with S1 nuclease-treated and sonicated calf thymus DNA as described (122). Briefly, Nunc MaxiSorp plates were coated with 50 µl of poly-L-lysine (50 µg/ml) per well and incubated at 4ºC over night. 1 ml of 500 mg/ml grade I calf thymus DNA was sonicated with a Virtis sonicator (Virsonic) with 2 pulses of 10 sec duration each. After verifying the efficiency of the sonication on an agarose gel, the DNA was digested with S1 nuclease (2500 U) (Invitrogen) for 30 min at 37ºC, in presence of 0.3 M NaCl to prevent dsDNA digestion. 50 µl of 1:10 diluted S1 nuclease digests were added to the poly-L-lysine coated plates, after removal of the liquid, and incubated over night at 4ºC. Plates were then washed 3 x with PBS/0.1% Tween-20® and blocked with PBS/5% milk powder for 1 hour at room temperature, followed by 3 washes with PBS. Afterwards, 50 µl of 1:100 diluted serum from mouse blood were added to each well and the plates were incubated over night at 4ºC. As a reference antibody, I used serial dilutions of a mouse IgG anti-dsDNA antibody (Chemicon, Inc.). After washing, the plates were incubated with an Alkaline Phosphatase (AP)-conjugated goat anti-mouse IgG (1:250 diluted in PBS/0.1% Tween-20®; Jackson ImmunoResearch Laboratories) for 1 hour at room temperature, washed 5 x with PBS/0.1% Tween-20® and developed with p-nitro-phenyl phosphate in diethanolamine buffer (Bio-Rad). The absorbances were measured at 405 nm in a microplate reader (Spectramax 190, Molecular Devices).

Rheumatoid factors were determined by adding serum to ELISA plates coated with normal goat Ig (Jackson ImmunoResearch Laboratories, Inc.) (98). Briefly, Nunc MaxiSorp ELISA plates were coated with a solution of 10 µg/ml normal goat Ig in 50 mM sodium bicarbonate buffer, pH 9.6, and incubated over night at 4ºC. Plates were washed 3 x with PBS/0.1% Tween-20®, and blocked with PBS/5% milk powder for 1 hour. After washing, 50 µl of 1:50 diluted mouse serum was added to each well and incubated over night at 4ºC. Detection of mouse isotypes in this assay was performed using AP-labeled goat anti-mouse IgA, IgM, IgG2a, IgG2b, IgG1, and IgG3, as well as purified goat anti-mouse IgA, IgM, IgG2a, IgG2b, IgG1, and IgG3 for standard curves

(all antibodies from Southern Biotechnology Associates, Inc.). The procedure was performed as described above.

Anti-C1q reactivity was measured by adding sera (50 µl of 1:50 diluted in PBS/1M NaCl; the presence of a high salt concentration is supposed to lower the binding affinity of immune-complexes, and to raise the binding affinity of autoantibodies against C1q (123)), to an ELISA plate coated with human C1q (Quidel Corp.) as described (124). Mouse Igs in the immune-complexes were detected using 100 µl/well of a 1 µg/ml solution of an AP-labeled goat anti-mouse Ig (Southern Biotechnology Associates, Inc.) as described above.

ELISAs for detection of low concentrated cytokines (3-10 pg/ml) were developed using Time Resolved Fluorescence Assays (TRFA) (125). Briefly, Europium-labeled streptavidin was added to ELISA reactions already coated with a biotinylated secondary antibody (BD PharMingen), followed by an incubation for 1 hour at room temperature. The plate was then washed 6 x with PBS buffer. DELFIA™ enhancement solution (Perkin Elmer Life Sciences) was added and incubated 10 min at room temperature. Europium-labeled counts were read in a Wallac Victor^2 1420 Multilabel Counter (Perkin Elmer Life Sciences).

2.4 Generating Apoptotic Thymocytes

Thymocytes from 3-5-weeks-old GM-CSF-deficient mice were exposed to 1 µM dexamethasone in RPMI/10% FCS for 6 hours to induce a 60% annexin V positive and 95% propidium iodide negative population, as confirmed by fluorescence activated cell sorting (FACS®).

2.5 *In Vivo* Phagocytosis of Apoptotic Thymocytes

1 x 10^7 apoptotic thymocytes were injected into the peritoneal cavities of 3-4-months-old WT, GM-CSF-, or GM-CSF/IL-3-deficient mice. 30 min later, the animals were killed and the peritoneal cavities were lavaged with 3 ml of ice-cold PBS/0.3% BSA/0.03% EDTA/0.15% NaN_3 buffer. 100 μl of the eluate (~1 x 10^6 cells) were loaded on a cytospin funnel with a siliconized glass slide, following a centrifugation at 500 rpm for 5 min in a cytospin centrifuge (Statspin 800). The slides were then dried and stained with Diff-Quik® (Dade Behring AG). FACS® analysis of phagocytes was performed by labeling the treated thymocytes (5 μl/10^8 cells), before injection, with 5- (and 6-) carboxytetramethylrhodamine ester (5[6]-TAMRA, SE, Molecular Probes) as described (8, 126). Macrophages were detected with a fluorescein-isothyocyanate (FITC)-conjugated anti-Mac-1 Ab (BD PharMingen).

2.6 B Cell Purification

Splenocytes were processed to single cells by smashing spleens between two frosted microscope slides (Fisher) in ice cold RPMI buffer containing 10% FCS. Cells were depleted of erythrocytes by resuspending them in 150 mM NH_4Cl plus 10 mM $NaHCO_3$ for 5 min, followed by 1 wash with PBS and filtration through a cell strainer® (70 μm; BD Falcon). Cells were then counted with a hematocytometer. 1.0 x 10^8 cells were diluted in 1.5 ml PBS + 2% FCS. 'Cell reagent' was added and cells were loaded onto B cell columns according to manufacturers recommendations (Cedarlane, Ontario). These columns remove, by a process of negative selection, virtually all $CD4^+$ and $CD8^+$ lymphocytes and $Mac1^+$ monocytes/macrophages leaving enriched mouse B cells (> 95%). Enrichment was checked by FACS® using a FITC-labeled anti-B220 Ab (BD PharMingen).

2.7 Genomic DNA Isolation from Tissue

DNA from mouse tissue was isolated according to an adapted method of Laird (127). Briefly, tissue in a microtube was covered with DNA isolation buffer (Laird) and incubated at 50ºC for 6 hours. Then, the solution was boiled for 10 min and 10 x diluted with H_2O. 1 µl of the diluted solution was used for PCRs. For Southern blots the DNA was precipitated with ethanol in presence of NH_4Ac, dried, and resuspended in TE buffer. The DNA concentration was determined by measuring the absorbance at 260 nm with a spectrophotometer.

2.8 Isolation of Total RNA

Isolation of total RNA from purified B cells was performed using RNeasy columns (Qiagen). Briefly, 1 x 10^7 B cells were resuspended in 600 µl RLT buffer and homogenized using a QIAshredder spin column. After adding of 1 volume of 70% ethanol to the homogenized lysate, the lysate was loaded onto a RNeasy column and centrifuged for 15 sec. The column was washed with RW1 buffer, followed by 2 washes with RPE buffer/ethanol. Purified total RNA was obtained after elution with RNase-free H_2O. The RNA concentration was measured at 260 nm.

2.9 Protein Isolation and Protein Concentration Measurement

Protein preparations of whole cells, and nuclei, were performed using the Nuclear Extract Kit (Active Motif, Carlsbad, CA). Briefly, for preparation of whole cell extract, 1 x 10^7 cells were washed with 5 ml ice cold PBS with phosphatase inhibitors (50 mM NaF, 10 mM sodium pyrophosphate, 2 mM EGTA, 1 mM sodium orthovanadate) according to

the manufacturer's recommendations. After centrifugation at 500 rpm for 5 min in a refrigerated centrifuge, the supernatant was discarded and the cells were resuspended in 300 µl of complete lysis solution (containing lysis buffer, protease inhibitors, and DTT). The suspension was incubated on ice for 10 min and vortexed at highest setting in 2 min intervals. After the incubation, the suspension was vortexed for 30 sec at highest setting and centrifuged at 14 000 g in a cooled microcentrifuge. The supernatant was collected and protein concentrations were determined according to the Bradford method. Briefly, 160 µl of 1:50 diluted protein extracts, and albumin standards with known concentrations, were added to microtiter plates filled with 40 µl of Bradford solution (Bio-Rad). Absorbances at 595 nm were measured, and protein concentrations were calculated.

For nuclear extracts, cells were resuspended in 100 µl of hypotonic buffer and incubated for 15 min on ice. After adding 5 µl of detergent, the cells were vortexed for 10 sec at the highest setting. The cytoplasmic fraction was separated by centrifugation for 30 sec at 14 000 rpm. The pellet was then resuspended in 50 µl lysis buffer (see above) and incubated for 30 min on ice followed by short vortexing at the highest setting in 10 min intervals. After 30 min the suspension was vortexed for 30 sec at the highest setting followed by a centrifugation for 10 min at 14 000 g. Protein concentrations were measured as describe above.

2.10 FACS® Analysis

FACS® analysis with single cells from spleens, thymi, and lymphomas were performed. Briefly, 1 x 10^6 of filtered cells (70 µm cell strainer®) in a volume of 100 µl PBS/2% FCS were stained with FITC- or phycoerythin (PE)-conjugated monoclonal antibodies to: CD4, CD8α, CD3ε, CD25, Mac-1, Gr-1, Ig-M, NK1.1, NK1.1-T, CD80, CD86, CD11c, CD11d, CD14, fasR (CD95), fasL (CD95L), I-A^b, and B220 (all BD PharMingen), in the presence of 1 µg of a blocking antibody against FcγIII/II receptor

(BD PharMingen). Stained cells were washed 1 x with PBS before they were analyzed in a total volume of 500 µl of PBS + 2% FCS on a Beckmann Coulter FACS® (Beckmann Instruments).

2.11 Anti-CTLA-4 Antibody Purification and Injection Into Mice

Supernatants from a hybridoma cell line producing anti-mouse CTLA-4 antibody 9H10 (gift from Dr. J. P. Allison, UC Berkeley) were collected and purified on a Hi-Trap™ protein A sepharose column (Amersham Bioscience) (71). Antibodies were eluted with 0.1 M citric acid, pH 3.0, and flow through was collected in 1 ml aliquots and rapidly neutralized with 100 µl 1M Tris-Cl, pH 8.0. Protein concentrations of the fractions were measured by the Bradford method. Aliquots with the highest protein concentrations were collected and dialyzed against 1 x PBS over night at 4ºC using dialysis tubes (10000 MW cut off, Spectrum). Antibody concentrations were determined by coating a Nunc MaxiSorp™ ELISA plate (Nunc) with 1:50 diluted pooled aliquots, following an incubation at 4ºC over night. Mouse IgG (Jackson ImmunoResearch) was used for standardization. After washing and blocking, a secondary AP-labeled antibody against mouse Ig (Southern Biotech, Inc.) was added and treated as described above (section 2.3). Absorbances were measured at 405 nm.

100 µg of purified anti-CTLA-4 were injected into the peritoneal cavities of the mice in a volume of 200 µl PBS three times over a three days interval. IgG isotype-matched antibodies (NA/LE formulation, BD PharMingen) were applied in the same way and served as negative controls. Blood sugar measurements started at day 10 after the first injection.

2.12 RNase Protection Assays

RNase Protection Assays (RPAs) were performed to detect the RNA levels of the following apoptotic proteins: caspase-1, -2, -3, -6, -7, -8, -11, -12, -13, and -14, fas ligand (CD95L), fas receptor (CD95), FADD, FAP, FAF, TRAIL, TNFR, TRADD, RIP, bcl-x, bak, bax, bcl-2, and bad. L32 and GAPDH probes were used as loading controls. Template sets and the transcription kit were obtained from BD Biosciences. 0.5 µg of Qiagen-purified total RNA was used for each of the assays. All procedures were performed according to the manufacturer's recommendations (BD Biosciences). Briefly, RNA probes from the template plasmids were synthesized with T7 RNA polymerase in presence of [α-^{32}P]UTP. Templates were then digested with DNase and the probes were purified using a Sephadex G-50 column (Ambion). After hybridization at 56ºC for 14 hours, single stranded RNA was removed by treatment with RNase A + T1. The dsRNA was purified by ethanol precipitation. The protected probes were heated at 90ºC for 3 min before being loaded on a 0.4 mm thick 4.75% acrylamide sequencing gel (4.75% acrylamide at a final ratio 19:1 acrylamide/bis; Fisher Scientific). The gel was run at 55 W. After the run the gel was dried and exposed to X-ray films (Kodak-XOMAT AR) over night at -80ºC.

2.13 Immunoblots

10-15 µg of protein per slot were separated on 7% SDS-polyacrylamide gels at 100 V. The separated proteins were then blotted at 4ºC over night onto PVDF Immobilon membranes (Millipore) in presence of 15% methanol (Bio-Rad Miniblot apparatus). Membranes were blocked for 1 hour with PBS/5% milk powder. Then, the membranes were incubated over night at 4ºC with rabbit polyclonal primary antibodies (1:1000 diluted) against: FLIP, DAP-kinase, AKT, and phosphorylated AKT (Cell Signaling). Membranes were washed, follwing an incubation with a HRP-conjugated anti-rabbit

secondary antibody (1:2000 diluted; Amersham), for 1 hour at room temperature. After washing, the membranes were incubated with 1 ml of enhancer/peroxide solution (Pierce) and exposed to a superRX film (Fujifilm).

2.14 Statistical Analysis

ELISA results were analyzed using a one-way analysis of variance. The results from the killing assays were analyzed by one-sample t-tests. The Fisher exact test was used to compare tumor formation in treated and untreated cytokine deficient mice. Comparisons of fasting blood sugars were done with the Wilcoxon rank sum test (one sided). The Wilcoxon test was also used to analyze glucose challenge tests.

2.15 Apoptosis Assays and Correction of the Fas Resistance

5×10^6 B cells in a volume of 1 ml DMEM, containing 10% FCS, were stimulated for 48 hours with 1 μg of anti-CD40 antibody (NA/LE formulation; BD PharMingen), or, 50 μg of Lipopolysaccharide (LPS; *Escherichia coli* 0111:B4; Sigma). 1×10^6 aliquots of cells in a volume of 1 ml were then incubated with: 1 μg of anti-fas antibody (Jo2 antibody, NA/LE formulation; BD PharMingen), 100 μg of etoposide (Sigma), 1 μM of staurosporine (Sigma), 2 μg of actinomycin D (Sigma), 1 μg of anti-TNFα (BD PharMingen), or 1 μg of isotype-matched IgG control antibody (BD PharMingen), for 12 hours and cell viability was determined by trypan blue exclusion (8).

To correct the fas resistance, recombinant mouse TNF-α, or recombinant mouse IFN-γ (both BD PharMingen), were added to the supernatants of the B cells from the cytokine-deficient mice in a way that these molecules reached the concentrations measured in WT supernatants.

2.16 Determination of Cell Viability by Trypan Blue Exclusion and by Annexin V/ Propidium Iodide Staining

20 µl of cell suspension was added to 180 µl of trypan blue (Sigma) and thoroughly mixed by pipetting up and down. After an incubation at room temperature for 5 min, trypan blue excluding cells were considered as viable and counted on a haemacytometer (Bright Line).

For Annexin-V/propidium iodide staining the Annexin V-FITC apoptosis detection kit (BD PharMingen) was used. Briefly, 1 x 10^6 cells/ml were washed 2 x with ice-cold PBS and then resuspended in 1 x binding buffer. 5 µl Annexin V-FITC and 5 µl propidium iodide solution were added followed by a light-protected incubation at room temperature for 15 min. After adding 400 µl of binding buffer, cells were analyzed by FACS®.

2.17 Cell Cycle Analysis

Cell cycle analysis was performed using the propidium iodide method. Briefly, 1 x 10^6 cells were washed in PBS (without Ca^{+2} or Mg^{+2}). Cells were resuspended in 1 ml of ice cold PBS buffer. 9 ml of 70% ice cold ethanol were dropwise added to the cell suspension. After an incubation at 4ºC for at least 30 min, the cells were centrifuged and washed (all steps performed at 4ºC). The cells were then resuspended in 500 µl propidium iodide/Triton X-100 solution (10 ml 0.1% (v/v) Triton X-100 (Sigma) in PBS with 2 mg DNAse-free RNAse (Sigma) and 0.40 ml of 500 µg/ml propidium iodide (Roche)). After an incubation at 37ºC for 15 min, the tubes were transferred on ice and protected from light. After filtation through a 70 µm cell strainer®, the suspensions were analyzed by FACS® using the Multicycle AV program (Beckman).

2.18 Blood Sugar Measurements and Glucose Tolerance Tests

Age and sex matched mice were starved for 6 hours prior to blood glucose testing. Fasting Blood Sugars (FBS) obtained from tail vein blood were measured with an ExacTech R·S·G·™ testing system (Abott). A FBS concentration between 80 mg/dl and 130 mg/dl was considered to be in the normal range according to FBS measurements with 30 healthy, 8-months-old WT mice on the C57Bl/6 background (Mean 96 mg/dl glucose, SD 14.3 mg/dl glucose).

For glucose tolerance tests, age and sex matched mice were starved as above and intraperitoneally injected with a 3g/kg of body weight glucose solution in a volume of 50 µl 0.9% NaCl solution per 10 g of body weight. Glucose measurements from tail blood were performed at times 0, 15, 30, 60, and 120 min.

2.19 Southern Blot Analysis

For Southern blot hybridization analysis, 15 µg of DNA were digested with *EcoR*I, separated on an 1% agarose gel and transferred to a Hybond-N^+ membrane (Amersham) in alkaline buffer (1 M NaCl, 0.5 M NaOH) (8). After UV crosslinking using the Stratalinker® (Stratagene), membranes were hybridized to probes (in case of GM-CSF/IL-3-/- genotyping: cDNA probe as described in (55); in case of lymphoma analysis: J_κ and J_H probes as described in (128, 129)), labeled with [α-^{32}P]dATP (3000 Ci/nmol, Amersham). Hybridization was performed at 42ºC for 16 hours in the following buffer: 5.5 x sodium chloride citrate (SSC), 13.3% formamide, 0.1 M Tris-HCl (pH 9.0), 0.1% Denhardt's solution, 1% Sodium Dodecyl Sulfate (SDS), 100 µg/ml yeast tRNA (Boehringer), containing 1 x 10^6 cpm/ml of radiolabeled probe. Membranes were washed with 6 x SSC, 0.1 % SDS at room temperature for 5 min, then at 55ºC for 20 min, and were visualized by exposition to a Kodak XOMAT film over night at -80ºC.

2.20 Electromobility Shift Assays

Electromobility Shift Assays (EMSAs) were performed using a dsOligo probe specifically binding Nuclear Factor Kappa B (NFκB): 5'-GGA TCC TCA ACA GAG GGG ACT TTC CGA GGC CA-3' (forward strand). The probe was radioactively labeled with [α-^{32}P]dCTP using Klenow polymerase (Stratagene). Briefly, 1μg/ml ds oligo DNA was incubated with 4 μl 10 x dCTP buffer (Stratagene) and 1 μl Klenow polymerase (10 U) (Stratagene) in a total volume of 20 μl at room temperature for 20 min. The reaction was stopped by the addition of 2 μl 0.5 M NaCl. After dilution with 80 μl H_2O, the probe was purified on a Nu-Clean D25 spin column (Kodak).

For the EMSA assay, 1 μl nuclear extract and 15 000 cpm [^{32}P]-labeled probe were used and incubated at room temperature for 20 min, together with 4 μl 5 x gel shift buffer (50 mM Hepes pH 7.9; 250 mM KCl; 0.5 mM EDTA; 2.5 mM DTT; 10% Glycerol; 0.05% NP-40) and H_2O to a total volume of 20 μl. Samples were loaded on a 4% non-denaturating acrylamide gel (1 x TBE; 37.5:1 acrylamide/bis acrylamide (30%); 30% acrylamide; 8% glycerol; 0.25% (v/v) TEMED and 0.1% (v/v) Ammonium-Peroxy-Sulfate (APS)). After a pre-run at 250 V for 10 min 2 μl loading buffer (250 mM Tris Cl pH 7.5; 40% glycerol; 0.2% bromophenol blue) were added to the samples and they were loaded onto the gel and run at 200 V in 0.5 x TBE buffer.

2.21 Spectral Karyotype Analysis

B cell lymphoma tissue was processed to single cells by smashing spleens between two frosted microscope slides (see section 2.6) in ice cold RPMI buffer containing 10% FCS. Single cell lymphoma solutions were stimulated *in vitro* with anti-CD40 antibody (see section 2.15). Metaphase spreads were collected and visualized by spectral karyotype (SKY) analysis (130). This technique is based on spectral imaging to measure chromosome-specific spectra after Fluorescence In Situ Hybridization (FISH)

with differentially labeled mouse chromosome painting probes (131). Utilizing a combination of Fourier spectroscopy and conventional optical microscopy, spectral imaging allows the simultaneous measurement of the fluorescence emission spectrum at all sample points. A spectrum-based classification algorithm had been adapted to karyotype mouse chromosomes and was used in our analysis.

2.22 Purification of DNA by Precipitation

DNA was extracted with phenol/chloroform and then precipitated with EtOH (1 volume) in presence of 2M NH_4Ac. After a centrifugation at maximum speed in an microcentrifuge (Eppendorf) for 10 min, the pellet was 1 x washed with 70% EtOH and resuspended in the appropriate buffer.

2.23 First Strand cDNA Synthesis from RNA and Real Time PCR

The first strand DNA synthesis from total RNA, isolated from splenic or lymphoma tissue, was performed using the SuperScript™ First-Strand Synthesis System (Invitrogen). Briefly, 0.15 µg of total RNA was prepared for each reaction (all reactions were performed in triplicates). Random hexamer primers and dNTPs were added according to the manufacturer's recommendations and the mixture was heated at 65ºC for 5 min. After adding RNase inhibitor, 1 µl of the SuperScript enzyme was added, following an incubation at 42ºC for 50 min. The enzyme was finally heat-inactivated and the remaining RNA was digested with RNase H. The mixures were 10 x diluted and 10 µl were used for each reaction (each reaction performed in triplicate) in a 96 well optical plate (Applied Biosystems). 12.5 µl of SYBR green® PCR solution (Applied Biosystems) was added, together with 10 nM of forward and backward primers. The total reaction volume was 25 µl. Sequences for cyclooxygenase 2 (COX-2) primers were as follows:

forward primer 5'-TGG TGC CTG GTC TGA TGA TG-3', reverse primer 5'-GTG GTA ACC GCT CAG GTG TTG-3'. Reactions were normalized to the constitutively expressed cyclophilin (cyclophilin forward primer: 5'-ATG GTC AAC CCC ACC GTG T-3'; reverse primer: 5'-TTC TTG CTG TCT TTG GAA CTT TGT C-3'). Reactions were performed in a thermo light cycler ABI PRISM™ 7700 Sequence Detector (Applied Biosystems) and analyzed as described (132).