2. Animals, Materials and Methods

2.1 Animals and animal housing

Male Wistar rats (225 - 300 g, Charles River Breeding Laboratories) as well as male wild type (WT) and END knock out ($END^{-/-}$) mice of the genetic background C57BL/6j (kindly supplied by M. Low, Oregon Health Sciences University, Portland, USA) were housed in cages lined with ground corncob bedding. Standard laboratory rodent chow and tap water were available *ad libitum*. Room temperature was maintained at 22°C and at a relative humidity between 40% and 60%. A 12/12 hr (8 a.m./8 p.m.) light/dark cycle was used. Experiments were approved by the animal care committee of the Senate of Berlin and strictly followed the guidelines of the International Association for the Study of Pain (Zimmermann 1983).

2.2 Materials

Materials are listed in alphabetical order and the distributors' names are given, for more details on distributors (city, country) see Appendix A.

2.2.1. Bacterium

EZ Competent Cells (*E. coli*, genotype: (F'::Tn10(Tc^r) $proA^+B^+$ $lac^qZ\Delta M15$) recA1 end A1hsdR17 ($r_{K12}^-m_{K12}^+$) lac glnV44 thi-1 gyrA96 relA1)	QIAGEN

2.2.2. Enzymes

50× Advantage 2 Polymerase Mix	Clontech Laboratories
Avian Myeloblastosis Virus Reverse transcriptase (AMV)	Roche
DNAse	QIAGEN
RNAse inhibitor	Roche
Superscript II reverse transcriptase (Moloney-Mouse-Leukaemia-Virus ; MMLV)	Invitrogen
T4 DNA ligase	QIAGEN
Thermus aquaticus (Taq) Polymerase	Roche

2.2.3. Radioactive labeled peptide

(^{125}I)rat β-endorphin	Peninsula Laboratories

2.2.4. Antibodies

Table 2.1 Listing of antibodies used for the following applications: IF = immunofluorescence, IHC = immunohistochemistry, F = flow cytometry, MACS = magnetic cell separation, RIA = radioimmunoassay. Other abbreviations: FITC = fluorescein isothiocyanate-conjugated, PE = phycoerythrin-conjugated, PE-CyC = PE-Cy-Chrome conjugated.

Antibody	**Application**	**Company**
Anti-rabbit IgG, peroxidase-conjugated	IHC	Vector Laboratories
Donkey anti-mouse IgG, FITC	IF	Vector Laboratories
Goat anti-rabbit IgG, biotinylated	IHC	Vector Laboratories
Goat anti-rabbit, texas red-conjugated	IF	Vector Laboratories
Mouse 3E7 (pan opioid)	F	Gramsch Laboratories
Mouse anti-FITC micro beads	MACS	Miltenyi Biotec
Mouse anti-human POMC	IF	Biogenesis
Mouse anti-rat CD3 (G4.18) PE/FITC	F	Pharmingen
Mouse anti-rat CD4 (Ox-35) PE/FITC	F	Pharmingen
Mouse anti-rat CD4 micro beads	MACS	Miltenyi Biotec
Mouse anti-rat CD45 (OX-1) PE-Cy-Chrome	F	Pharmingen
Mouse anti-rat CD45R micro beads	MACS	Miltenyi Biotec
Mouse anti-rat CD45RA (OX-33) FITC	F	Pharmingen
Mouse anti-rat CD68 (ED-1) FITC	F	Pharmingen
Mouse anti-rat CD8 G4.18 FITC	F	Pharmingen
Mouse anti-rat kappa chain FITC	F	Serotec
Mouse anti-rat PanTcell micro beads	MACS	Miltenyi Biotec
Mouse IgG 2a	F	Pharmingen
Rabbit anti-END	IHC	ProGen Biotechnik
Rabbit anti-mouse IgG 2a,b (RAM) PE	F	Santa Cruz Biotechnology
Rabbit anti-rat END	RIA	Peninsula Laboratories
Rat anti-mouse IgG 2a κ (Isotype control) PE/FITC	F	Pharmingen
Rat anti-mouse IgG1 κ (Isotype control) FITC	F	Pharmingen
Rat anti-mouse IgG2a (Isotype control) FITC	F	Serotec
Rat anti-mouse IgG3 κ (Isotype control) PE/FITC	F	Pharmingen

2.2.5. Chemicals, Kits and Media

β-Mercaptoethanol	C. Roth GmbH
3-(N)-Morpholino)propansulfonic acid (MOPS)	Sigma-Aldrich
3´,3´-diaminobenzidine tetrahydrochloride (DAB)	Sigma-Aldrich
Agarose LE	Roche
Ampicillin	E. Merck AG
Aprotinin	Sigma-Aldrich
Bestatin	Sigma-Aldrich

Boric acid	Sigma-Aldrich
Bovine serum albumin (BSA)	Sigma-Aldrich
Bromphenol blue	Sigma-Aldrich
Cloning Kit	QIAGEN
Complete Freund´s Adjuvant (CFA)	Calbiochem
Deoxy-nucleotide triphosphate mix (dNTP)	Roche
Dextran 500	Amersham Pharmacia Biotech
Dithiothreitol (DTT)	Sigma-Aldrich
DNA molecular weight markers (IX, 72 - 1350 bp; XIV, 100 bp)	Roche
DPX	E. Merck AG
Ethanol (99%)	C. Roth GmbH
Ethidium Bromide	Sigma-Aldrich
Ethylendiaminetetraacetic acid (EDTA)	Sigma-Aldrich
Ethyleneglycoltetraacetic acid (EGTA)	Sigma-Aldrich
Fast Start DNA Master SYBR Green I Kit	Roche
Fetal bovine serum (FBS)	Biochrom AG
Formamide	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Glycine	Sigma-Aldrich
HistoGreen Peroxidase-Substrate Kit	Linaris
Horse serum	Biochrom
Imidazol	Sigma-Aldrich
Interleukin 1α and 1β (IL-1α and IL-1β, rat, recombinant)	R & D Systems
Isofluran Curamed ((1-Chlor-2,2,2-trifluorethyl)difluormethylether)	Rhodia Organic Fine Limited
Kanamycin	E. Merck AG
Luria Bertani (LB) Agar	GIBCO Invitrogen Corporation
Luria Bertani (LB) medium	GIBCO Invitrogen Corporation
Maxiprep system	QIAGEN
Methanol	C. Roth GmbH
Miniprep System	QIAGEN
Normal rabbit IgG	Sigma-Aldrich
Nutridoma-SP	Roche
Paraformaldehyde (PFA)	Sigma-Aldrich
Penicillin/Streptomycin (10,000U/10,000 µg/ml)	Biochrom
Phosphate buffered saline (PBS, sterile, 0.1 M, pH 7.4)	GIBCO Invitrogen Corporation
Picric acid	Sigma-Aldrich
Primer dT for cDNA synthesis (oligo dT)	Roche
Qiaquick Gel Extraction Kit	QIAGEN
rat-END Kit	Peninsula Laboratories
RNAse-free DNAse (1500 U/µl)	QIAGEN
RNeasy Mini Kit	QIAGEN

RPMI-1640 medium (+Glutamax I)	GIBCO Invitrogen Corporation
Saponin from Quillaja bark	Sigma-Aldrich
SMART RACE cDNA Amplification Kit	Clontech Laboratories
SOC medium	QIAGEN
Sodium azide (NaN_3)	Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich
Tissue-Tek compound	OCT Miles
Trishydroxymethylaminomethane (Tris)	Sigma-Aldrich
Triton X-100 (t-Octylphenoxy-poly-ethoxyethanol)	Sigma-Aldrich
Trypan blue Stain (0.4%),	GIBCO Invitrogen Corporation
Trypsin/EDTA	GIBCO Invitrogen Corporation
Vectastain avidin-biotin peroxidase complex (ABC)	Vector Laboratories
Vectastain Elite Kit	Vector Laboratories
Vectashield	Vector Laboratories
Xylene cyanole FF	Sigma-Aldrich

2.2.6. Oligodeoxynucleotides

Oligonuclotides purchased from TIBMOLBIOL are listed in Table 2.2.

Table 2.2 Oligonuclotides purchased from TIBMOLBIOL. Primer names, sequences, melting temperature (Tm), NIH acession numbers, and positions are given. Names indicate the target gene.

Name	**Sequency (5`- to -3`)**	**bp**	**Tm (°C)**	**Primer type**	**Accession number**	**Position**
1POMCex2se	ttctgctacagtcgctcaggg	21	62.8	se	NM_139326	131-151
1POMCex3as	cggggattttcagtcaaggg	20	63.8	as	NM_139326	345-326
1POMCex3ENDas	cgttcttgatgatggcgttct	21	62.3	as	J00759	644-624
2POMCex2se	ccctcctgcttcagacctcca	21	66.2	se	NM_139326	165-185
2POMCex3as	tctcttcctccgcacgcctct	21	68.1	as	J00759	254–274
2POMCex3se	ggcgccctgtgaaggtgta	19	64.4	se	NM_139326	540-558
2POMex3ENDas	cttgatgatggcgttcttga	20	59.6	as	NM_139326	805-786
3POMCex3as	ctcactggcccttcttgtgc	20	63.2	as	J00759	646-665
3POMCex3as+C	cctcactggcccttcttgtgc	21	66.6	as	J00759	66-646
3POMCex3se	cccaatgtcgccgagaac	18	62.5	se	J00759	394-412
3POMGSP	ttgactgaaaatccccggaagtacgtc	28	70.1	se	NM_139326	328-355
3POMGSPN	cttcggcccgagaaacag	18	61.7	se	J00759	214-231
4POMCex2se	agccagtgccaggacctcacc	21	68.4	se	NM_139326	215-235
4POMCex3as	gccttccagctccctcttgaa	21	65.2	as	NM_139326	625-605
5POMCex2se	agtgccaggacctcaccacg	20	65.9	se	NM_139326	219-238
5POMCex3as	gttctcggcgacattggggta	21	66.5	as	NM_139326	577-557
5POM-GSP	ccagcggaagtgctccatggagtag	25	71.0	as	NM_139326	517-493
5POMGSPN	gtctcttcctccgcacgc	18	62.0	as	NM_139326	441-424
6POMCex2se	ctcaccacggaaagcaacctg	21	65.2	se	NM_139326	230-250

Table 2.2 continued

Name	Sequency (5`- to -3`)	bp	Tm (°C)	Primer type	Accession number	Position
6POMCex3as	tctcggcgacattggggta	19	64.8	as	NM_139326	575-557
7B2- forward	aatccagcattcgcttatag	20	50.7	se	M63901	94-113
7B2- reverse	taggaatattgtcgccagtc	20	50.9	as	M63901	324-343
pDrive cloning vector M13 forward	gtaaaacgacggccagt	17	60.8	se	Qiagen cloning kit manual	431-447
pDrive cloning vector M13 reverse	aacagctatgaccatg	16	49.5	as	Qiagen cloning kit manual	209-224
Oligo 1 (POMC)	atgccgagattctgctacagt	21	58.7	se	J00759	68–88
Oligo2se (POMC)	tgccgagattctgctacagtcgc	21	61.5	se	NM_139326	123-145
PC1/3- forward	aatcctgtaggcacctggac	20	55.8	se	M76705	1905-1924
PC1/3- reverse	ggagtttttgggtaccagga	20	55.6	as	M76705	2140-2159
PC2- forward	gagaggagacctgaacatca	20	50.4	se	NM_012746	1870-1889
PC2- reverse	gcaagcccttctgtggtgca	20	63.5	as	NM_012746	2050-2069
POMC long aS	cgtctatggaggtctgaagc	20	57.2	as	NM_139326	191-172
POMC long S	cagtgactaagagaggccac	20	54.6	se	NM_139326	43-62
POMC rat aS	tgacccatgacgtacttccg	20	61.8	as	NM_139326	363-344
POMC rat S	gagattctgctacagtcgctc	21	56.6	se	NM_139326	127-147
POMC2ex2se	tggccctcctgcttcagac	19	63.8	se	NM_139326	162-180
POMC2ex2seACC	acctggaagatgccgagaggt	21	64.5	se	K01878	59-76
POMCex1se	cgacggaggagaaaagaggt	20	60.6	se	NM_139326	16-35
POMCex3as	cttgctccaagccatcaggc	20	65.5	as	J00759	465-484
POMCex3se	tacgtcatgggtcacttccg	20	61.8	se	J00759	185-204
preSIGex2seB	ctccaatcttgtttgcctc	19	55.4	se	K01878	25-43
preSTGex2seA	agagcctcagccacctggaag	21	65.4	se	NM_139326	101-121
rat-RPL19b-as	tgctccatgagaatccgcttg	21	65.8	as	X82202	3254-3274
rat-RPL19b-se	aatcgccaatgccaactctcg	21	66.7	se	X82202	1521-1540
Very3`Ex3POMCas	tacaggtaactctaagaggctggaggt	27	63.0	as	J00759	746-720

The following oligonuclotides were purchased from **Clontech Laboratories**; primer names and sequences are given:

10 × Universal Primer Mix (UPM): 1 µM of 5`-ctaatacgactcactatagggc-3` (22 mer) and 0.2 µM of 5`-ctaatacgactcactatagggcaagcagtggtaacaacgcagagtacgcggg-3` (45 mer)

5´-RACE cDNA Synthesis Primer (5´-CDS), 5`- $(T)_{25}N_{-1}N$-3`, N = A, C, G or T and N_{-1} = A, G or C

Nested Universal Primer (NUP): 5`- aagcagtggtaacaacgcagagtacgcggg-3`, 23 mer

SMART II Oligonucleotide: 5`-aagcagtggtaacaacgcagagtacgcggg-3`

2.2.7. Plasmid vector

pDrive cloning vector (linearized, U overhangs, 3.85 kb) QIAGEN

2.2.8. Technical and other materials

Bacterial incubator (Heraeus Function Line)	Kendro
Bacterial Shaker	GFL
Cell counting chamber	Glaswarenfabrik Karl Hecht
Cell scraper	Sigma-Aldrich
Cell strainer (70 µm mesh)	BD Biosciences Discovery Labware
Cryostat (Microm HM560)	MICROM
Electrophoresis apparatus (Power Pac 300)	Bio-Rad Laboratories
Flow cytometry apparatus (FACS Calibur)	Becton-Dickinson
Fluorescence microscope	Carl Zeiss Mikroskopie
Gamma-Counter, Wallac Wizard 1470	Wallac
Heraeus incubator	Kendro
Kryo tubes	Nunc
Laminar Flow (Hera Safe)	Kendro
Mikroskope (Axiovert 25)	Carl Zeiss Mikroskopie
Photometer (Gene Quant II RNA/DNA calculator)	Pharmacia Biotech
Separation columns (mean size)	Miltenyi Biotec
Separator (VarioMACS™)	Miltenyi Biotec
Sterile filters	Millipore
Thermocycler (Gene Amp 9700)	Applied Biosystems
Thermocycler (LightCycler 1.5 Instrument)	Roche
Thermocycler (Mastercycler personal)	Eppendorf
Tubes (15 and 50 ml)	Falcon
Ultrapure Water Systems (Direct-Q™ 5)	Millipore
UV-Light (Macro Vue UV=25)	Hoefer

2.3 Methods

2.3.1. Induction of inflammation

Rats received an intraplantar (i.pl.) injection of 0.15 ml of Complete Freund's adjuvant (CFA) into the right hindpaw under brief isoflurane anesthesia. This inflammatory model is routinely used in our own as well as in other research groups (Stein et al., 1988; Barber and Gottschlich 1992). Control animals remained either untreated or received i.pl. injections of 0.15 ml NaCl into the right hind paw. From 2 - 96 hours after CFA-injection, paw tissue was swelling, hyperemic and animals showed significantly increased pain sensitivity (hyperalgesia). During the observation period (2 – 96 h) the inflammation remained confined to the right paw and no

significant differences in feeding behavior, body weight, body temperature, and general activity range occurred compared to untreated animals (Stein et al., 1988).

2.3.2. Tissue preparation

Unless otherwise stated, untreated, NaCl-, and CFA-treated rats were sacrificed at different time intervals after injections by CO_2 inhalation.

2.3.2.1 Preparation of lymph nodes

Popliteal and inguinal lymph nodes (LN) of the NaCl- or CFA-treated side or in case of untreated animals from both sides were dissected. LN dissected from CFA-treated limbs will be referred to as 'inflamed LN'. Embedding fat tissue was thoroughly removed using scalpels. For RNA-isolation LN tissue was immediately frozen and stored at –80°C. For magnetic cell sorting, flow cytometry, *ex vivo* stimulation, or radioimmunoassay the tissue was homogenized using sterile plastic pestles. Cells were dissociated from homogenates using 70 µm mesh cell strainer and suspended in sterile-filtered 1x phosphate buffered saline (PBS). In parallel to cell counting the cell viability was examined by the trypan blue exclusion method using Fuchs-Rosenthal cell counting chambers. Finally, cell suspensions were either directly transferred to magnetic cell sorting, flow cytometry and *ex vivo* stimulation or were pelleted for storage at –80°C for subsequent radioimmunoassay or polymerase chain reaction analysis.

2.3.2.2 Preparation of peripheral blood leukocytes

The thorax was opened by a mid-line incision. By direct parasternal cardiac puncture an average of 8 ml venous blood per rat was withdrawn using heparin-coated syringes. Dextran solution (0.9% NaCl, 3% dextran) was overlaid by an equal volume of blood and left for 45 min at room temperature for sedimentation. The upper phase containing white blood cells was then transferred to a new tube and centrifuged for 10 min at 350 × *g* at room temperature. Remaining erythrocytes contaminating the white blood cell fraction (peripheral blood leukocytes, PBL) were lysed by hypotonic shock as previously described (Vindrola et al. 1994). Briefly, cell pellets were suspended in ice-cold 0.2% NaCl for 30 sec, and then the reaction was stopped by adding 1.6% NaCl. Suspensions were then centrifuged for 10 min at 350 × *g*. Cell numbers and viability were determined as described above after

reconstitution of the pellets in PBS. Finally, samples were centrifuged again (10 min, 4°C, 350 × *g*) and pellets were stored at –80°C prior to RIA or PCR analysis.

2.3.3. Flow cytometry

All centrifugations of cell suspensions were run for 10 min at 4°C and 450 × *g* using a swinging bucket rotor unless otherwise stated. Pellets were reconstituted in PBS containing 0.5% bovine serum albumin (BSA), cell viability as determined by the trypan blue exclusion method usually was 90 - 95%. Antibodies used were fluorescein isothiocyanate (FITC) - or phycoerythrin (PE)-conjugated. The antibodies were used in single or 2-color staining. A total of 10,000-20,000 events were measured per LN in a FACS Calibur apparatus.

2.3.3.1 Cell surface staining

Staining of cell surface markers was performed as previously described (Rittner et al. 2001). Cells were suspended in FACS buffer (1× PBS containing 0.5% BSA and 2 mM EDTA, pH 7.25) supplemented with 0.5% whole rat serum and centrifuged. Supernatants were softly decanted to leave 50 - 100 µl of buffer per sample. Then monoclonal antibodies (1 µg/10^6 cells) were added and mixtures were incubated at room temperature for 20 min without exposure to light. Antibodies used were: mouse anti-rat CD3 PE/FITC to identify T lymphocytes, mouse anti-rat CD4 PE /FITC to identify monocytes and helper T-cells, and mouse anti-rat CD8 FITC for the recognition of cytotoxic T-cells. B lymphocytes were usually identified by incubation with anti-rat kappa chain FITC but this antibody did not bind after PFA-fixation of the cells. Already fixed cells were therefore stained with mouse anti-rat CD45RA or B-cells were identified as CD3-negative. Unbound antibody was removed by washing with PBS three times. Unfixed cells were then fixed for 30 min in 0.1% PFA. After removal of PFA by centrifugation, the cells were resuspended in PBS and kept at 4°C prior to measurements.

The specificity of stains was verified by incubation with appropriate isotype-matched controls. For surface stains 1 µg/10^6 cells of rat anti-mouse IgG3 κ PE/FITC (CD3 and CD8 isotype), rat anti-mouse IgG2a FITC (IgG kappa Chain isotype), rat anti-mouse IgG1 κ FITC (CD45RA isotype), or rat anti-mouse IgG 2a κ PE/FITC (CD4 isotype) were used. Cells were further processed as described above and kept at 4°C in PBS prior to measurements.

2.3.4. Magnetic cell sorting (MACS)

Freshly isolated cells were centrifuged for 10 min at 450 × *g* and room temperature. Cells (1×10^7) were resuspended in 80 µl MACS buffer (1 × PBS containing 0.5% BSA and 2 mM EDTA, pH 7.25). Then, 30 µl/10^7 cells of rat PanTCell (anti-CD3) or rat CD45R (anti-CD45R) micro beads were added to isolate T- or B-cells from the cell suspension, respectively. Samples were incubated for 15 min at 6°C sitting on a slow rotor socket. Finally, cells were washed twice in MACS buffer to remove unbound beads. Labeled cell suspensions were loaded onto separation columns placed in a magnetic field according to the company's instructions. Unlabelled cells passing the magnetic field were collected as negative fractions ($CD3^-$ or $CD45R^-$). After washing of the column with MACS buffer, it was removed from the magnetic field. Labeled cells were rinsed off the column with MACS buffer under high pressure and collected as positive fractions ($CD3^+$ or $CD45R^+$). Fractions were then centrifuged for 5 min at 4°C and 350 × *g*, supernatants were removed, and cell pellets were resuspended in PBS for cell counting.

To isolate $CD4^+$ T-cells, a two-step magnetic separation was used: cells were first incubated with 10 µl mouse anti-rat CD68 FITC and unbound antibody was removed by washing. Then, anti-FITC micro beads (30 µl micro beads/10^7 cells) were added and cells were further processed as detailed above to retain $CD68^+$ macrophages/monocytes on the column. $CD68^-$ cells were subsequently incubated with 30 µl anti-CD4 micro beads/10^7 cells. Finally, MACS revealed $CD4^+$ T-cells and macrophage-depleted $CD4^-$ cells ($CD8^-$ T-cells and B-cells). Separation efficiency was verified by flow cytometry analysis (see 2.3.3.1) comparing nonseparated cells, positive and negative cell fractions.

2.3.5. Total RNA preparations

Total RNA was isolated using QIAGEN RNeasy Mini Kits following the manufacturer's protocol. Fresh or frozen cells or tissues (pituitary and LN) were homogenized in 4 M guanidine thiocyanate buffer containing 10 µl β-mercaptoethanol per ml. Homogenates were loaded onto shredder columns to remove cell debris by spinning samples for 2 min at 8000 × *g* and room temperature. After adding 70% ethanol to the flow-through, the samples were immediately mixed and then transferred to silica membranes to bind DNA and RNA. To remove DNA, membranes were treated with RNase-free DNase for 15 min. Subsequently, membranes were

washed with 70% ethanol and briefly dried by centrifugation. Total RNA was dissolved in 30 - 40 µl RNase free double-distilled H_2O and stored at -80°C. RNA was isolated from LN or pituitary tissue on separate days to avoid contamination.

2.3.6. RNA electrophoresis

Ribosomal bands were identified by gel electrophoresis of total RNA to verify the RNA integrity. Gels contained 1.2% agarose, 0.001% ethidium bromide, 10× FA gel buffer (200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA, NaOH), RNAse-free H_2O and 12.3 M formaldehyde. RNA was mixed with 5× RNA loading buffer (500 mM EDTA, 12.3 M formaldehyde, 0.25% bromphenolblue, 100% glycerol, formamide, 10× FA gel buffer, RNase free H_2O), incubated for 5 min at 65°C, chilled on ice and loaded onto the gel. Gels were run at 5 - 7 V/cm in 1× FA gel running buffer (10× FA gel buffer, 12.3 M, formaldehyde, RNase free H_2O).

2.3.7. RNA/DNA quantification and quality

The amount of single-stranded RNA or double-stranded DNA was measured photometrical at a wavelength of λ = 260 nm. An $A_{260\ nm}$ = 1 corresponds to a concentration of 40 µg RNA/ml or 50 µg DNA/ml. The quality of RNA and purity of DNA was verified by $A_{260\ nm}/A_{280\ nm}$ ratios. This ratio lies between 1.5 and 1.9 for intact RNA and between 1.75 and 2.0 for DNA.

2.3.8. PCR Primers

Oligodeoxynucleotides were designed applying the OLIGO Primer Analysis Software Version 5.0 for Windows (Wojciech Rychlik, National Biosciences, Inc., Plymouth, MN). Several primer pairs were used to amplify and sequence different single exon or exon-intron spanning POMC fragments from LN and pituitary cDNAs as detailed in Table 2.3. Another exon-intron spanning primer pair was designed to amplify the ribosomal protein L19 (rpL19) which was used as housekeeping gene for different PCR strategies (for details see Table 2.3). Primer pairs designed for the amplification of PC1, PC2, and 7B2 were exon-intron spanning (Table 2.3).

Table 2.3 Primer pairs used for the following applications: PCR amplification (RT-PCR, qRT-PCR, RACE-PCR), nested PCR (nRT-PCR, nqRT-PCR, nRACE-PCR), and sequencing (SEQ). The annealing temperatures (AT) and time, as well as the expected PCR product sizes are given.

Gene	Primer pair	Sense primer	Antisense primer	AT (°C/sec)	Fragment size (bp)	Application
POMC	Ex3A	POMCex3se	POMCex3as	62/45 or 66/45	302	RT-PCR
POMC	Ex3B	3POMCex3se	3POMCex3as	67/45	271	RT-, qRT-PCR, SEQ
POMC	Ex2	PreSIGex2se	POMCex2SIGas	61/30	91	RT-PCR
POMC	Ex2-3A	POMC rat S	POMC rat aS	57/30	237	RT-PCR
POMC	Ex2-3B	POMC rat S	POMCex3as	61/45	520	RT-PCR
POMC	Ex2-3C	POMC rat S	3POMCex3as	61/60	702	RT-PCR
POMC	Ex2-3D	POMC2ex2se	POMCex3as	55/45	489	RT-PCR
POMC	Ex2-3E	POMC2ex2se	3POMCex3as	63/60	668	RT-, qRT-PCR, SEQ
POMC	Ex2-3F	2POMCex2se	2POMCex3as	66/30	276	RT-, qRT-PCR, nqRT-PCR, SEQ
POMC	Ex2-3G	Oligo 1	POMCex3as	59/60	529	RT-, qRT-PCR, SEQ
POMC	Ex2-3H	Oligo 1	1POMCex3as	55/30	224	RT-PCR
POMC	Ex2-3J	Oligo 1	3POMCex3as	59/60	708	RT-PCR
POMC	Ex2-3K	Oligo 1	5POMGSPN	55/45	320	RT-PCR
POMC	Ex2-3L	Oligo 1	5POM-GSP	55/45	396	RT-PCR
POMC	Ex2-3M	Oligo2se	POMCex3as	59/45	528	RT-PCR
POMC	Ex2-3N	preSIGA	POMCex3as	55/45	550	RT-PCR
POMC	Ex2-3O	preSIGA	3POMCex3as	55/60	730	RT-PCR
POMC	Ex2-3P	preSIGB	POMCex3as	55/60	572	RT-PCR
POMC	Ex2-3Q	preSIGB	3POMCex3as	55/60	752	RT-PCR
POMC	Ex2-3R	preSIGB	5POM-GSP	55/45	439	RT-PCR
POMC	Ex2-3S	preSIGB	5POMGSPN	55/45	363	RT-PCR
POMC	Ex1-2A	POMC ex1se	POMCex2SIGas	55/30	205	RT-PCR
POMC	Ex1-2B	POMC long S	POMC long aS	55/30	178	RT-PCR
POMC	Ex1-3A	POMC ex1se	POMCex3as	55/60	664	RT-PCR
POMC	Ex1-3B	POMC ex1se	1POMCex3as	55/45	359	RT-PCR
POMC	Ex1-3C	POMC ex1se	2POMCex3as	60/45	454	RT-PCR
POMC	Ex1-3D	POMC ex1se	3POMCex3as	55/60	844	RT-PCR
POMC	Ex1-3E	POMC ex1se	5POMCex3as	55/60	591	RT-PCR
POMC	Ex1-3F	POMC long S	POMCex3as	61/60	637	RT-PCR
POMC	Ex1-3G	POMC long S	1POMCex3as	60/45	332	RT-PCR
POMC	Ex1-3H	POMC long S	2POMCex3as	61/45	427	RT-PCR
POMC	Ex1-3J	POMC long S	3POMCex3as	55/60	708	RT-PCR
POMC	Ex1-3L	POMC long S	4POMCex3as	55/60	612	RT-PCR
POMC	Ex1-3M	POMC long S	5POMCex3as	55/60	564	RT-PCR
POMC	Ex1-3N	POMC long S	6POMCex3as	55/60	562	RT-PCR
POMC	Ex1-3O	1POMCex1cap se	3POMCex3as	63.2/60	853	RT-PCR, SEQ
POMC	5´-RACE 1	UPM	5´GSP	see 3.3.13.2.	>540	RACE-PCR
POMC	5´-N-RACE 1	NUP	5´GSPN	see 3.3.13.2.	>460	nRACE-PCR
POMC	5´-RACE 2	UPM	3POMCex3as	see 3.3.13.2.	>850	RACE-PCR
POMC	5´-N-RACE 2	NUP	3POMCex3as	see 3.3.13.2.	>830	nRACE-PCR
POMC	5´-RACE 3	UPM	POMC rat as	see 3.3.13.2.	>387	RACE-PCR
POMC	5´-N-RACE 3	NUP	1POMCex3as	see 3.3.13.2.	>369	nRACE-PCR, SEQ

Table 2.3 continued

Gene	Primer pair	Sense primer	Antisense primer	AT (°C/sec)	Fragment size (bp)	Application
7B2	7B2	7B2-forward	7B2- reverse	50/45	198	RT-PCR
PC1/3	PC1/3	PC1/3-forward	PC1/3-reverse	50/45	255	RT-PCR
PC2	PC2	PC2-forward	PC2- reverse	50/45	200	RT-PCR
pDrive- vector		M13 forward	M13 reverse	55		RT-PCR, SEQ
Rpl19	rpL19	rat-RPL19b-se	rat-RPL19b-as	68/30	333	RT-, qRT-PCR

2.3.9. Two-step reverse-transcription polymerase chain reaction (RT-PCR)

2.3.9.1 First-strand synthesis and negative controls

Total RNA (1 - 2.5 µg/15.6 µl) was mixed with 0.2 µg/ml primer oligo dT, heated for 3 min at 63°C and subsequently chilled on ice. Then the following reagents were added to a final volume of 30 - 40 µl, giving the final concentrations in the mixtures: 1× RT reaction buffer (50 mM Tris HCl, pH 8.3; 75 mM KCl, 3 mM $MgCl_2$), 10 µM dithiothreitol (DTT), 1 µM deoxynucleotide triphosphate mix (dNTP), 1 U RNAse inhibitor, and 6 U AMV reverse transcriptase (RT). For negative controls, the RT was replaced by RNase-free H_2O (RT^-). Mixtures were incubated for 120 min at 42°C. cDNA was stored at –20°C thereafter.

2.3.9.2 RT-PCR

Amplification of dT-tailed cDNA was performed in thermal cyclers. The final reaction volume of 25 µl contained 25 pM antisense primer; 25 pM sense primer; 1× PCR buffer with 2.0 mM $MgCl_2$, 0.8 mM dNTPs, 1 U AMV polymerase, and either 2 µl template or RT^- control. Positive controls contained 2 µl pituitary cDNA (PT); negative controls (NC) were supplemented with 2 µl of double-distilled H_2O. Each assay started with an initial denaturizing cycle of 5 min at 95°C and was terminated with an extension cycle of 10 min at 72°C. POMC templates were amplified for 40 cycles. Each cycle included 45 sec at 94°C to denaturize the template followed by 45 - 60 sec at 55 - 68°C for primer annealing, and 45 - 60 sec at 72°C for primer extension (for details see Table 3.2). RpL19 was amplified for 35 cycles including 30 sec at 94°C, 30 sec at 68°C, and 30 sec at 72°C. PC1/3, PC2, and 7B2 were amplified for 40 cycles including 30 sec at 94°C, 45 sec at 50°C, and 60 sec at 72°C.

2.3.10. Rapid Amplification of cDNA Ends (RACE-PCR)

First-strand synthesis and RACE PCR were performed using the SMART RACE cDNA Amplification Kit according to the manufacturer’s instructions.

2.3.10.1 Ready-RACE cDNA synthesis

Total RNA was dried using a speed vac apparatus and resuspended in RNase-free H_2O to a final concentration of about 1 µg RNA/3 µl. For synthesis of 5`-ready-RACE cDNA 1 µg RNA was primed in a final volume of 10 µl with 1 µM oligo dT primer (5`-CDS) and 1 µM SMART II Oligonucleotide, the volume was adjusted with sterile H_2O. The mixture was incubated for 2 min at 70°C and subsequently chilled on ice. Then 2 µl of 5× first strand buffer (250 mM Tris.Cl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 1 mM DTT, 0.5 mM dNTP Mix, and 200 U Moloney-Mouse-Leukaemia-Virus (MMLV) RT were added to a final reaction volume of 20 µl. Reaction mixtures were incubated for 90 min at 42°C in a thermocycler. Besides its reverse transcription activity, the MMLV RT exhibits a terminal transferase activity that usually adds 3 - 5 dC-residues to the 3`-end of the first strand cDNA. These dC residues anneal with the SMART II Oligonucleotides which then provides an extended template for the RT. First strand reaction products were diluted with 20 - 100 µl Tricine-EDTA buffer supplied by the company. 5`-ready-RACE cDNA was stored at –20°C.

2.3.10.2 RACE-PCR

Amplification of 5`-ready-RACE cDNA was performed by combining a universal primer mix (UPM) that recognizes the SMART II Oligonucleotide sequence with POMC-specific primers detailed in Table 2.3. The reactions were performed in a final volume of 50 µl containing 1× UPM, 0.2 µM POMC-specific antisense primer, Advantage 2 PCR buffer with a final concentration of 2 mM $MgCl_2$, 0.2 mM dNTPs, 1× Advantage 2 Polymerase Mix, and either 5 µl of template or double-distilled H_2O (NC). Assays were run following a touch-down PCR protocol as recommended by the company:

Step	Cycles	Denaturation	Annealing	Extension
1.	1	60 sec/95°C		
2.	5	10 sec/94°C	3 min/72°C	
3.	5	10 sec/94°C	10 sec/70°C	3 min/72°C
4.	25	10 sec/94°C	10 sec/68°C	3 min/72°C
5.	1			5 min/72°C

RACE PCR products (5 µl) were re-amplified using the Nested Universal Primer (NUP) in combination with nested POMC-specific primers as detailed in Table 2.3.

Reactions were performed in a final volume of 50 µl as described above. Amplification was done according to the following touch-down PCR protocol:

Step	Cycles	Denaturation	Annealing	Extension
1.	1	60 sec/95°C		
2.	1	10 sec/94°C	10 sec/68°C	3 min/72°C
3.	1	10 sec/94°C	10 sec/67°C	3 min/72°C
4.	1	10 sec/94°C	10 sec/66°C	3 min/72°C
5.	1	10 sec/94°C	10 sec/65°C	3 min/72°C
6.	1	10 sec/94°C	10 sec/64°C	3 min/72°C
7.	25	10 sec/94°C	10 sec/68°C	3 min/72°C
8.	1			5 min/72°C

2.3.11. Quantitative real-time RT-PCR (qRT-PCR)

2.3.11.1 Single round and nested qRT-PCR

Assays were performed using the Fast start DNA Master SYBR Green I assay according to the instructions of the manufacturer in a LightCycler 1.5 instrument. The final volume of 20 µl contained 31.25 pM antisense primer, 31.25 pM sense primer, 4 µl 5× PCR buffer mix (FastStart Taq DNA polymerase, $MgCl_2$, SYBR Green I dye, dNTP mix with dUTP instead of dTTP) supplemented with 1 U polymerase inhibitor, 2 - 5 µl of oligo dT-cDNA or 10 µl of diluted DNA-standard, and 4 - 12 µl PCR-grade H_2O to adjust the volume. All samples except NC and PT were run in duplicate. Each assay started with an initial cycle of 10 min at 95°C to inactivate the polymerase inhibitor. POMC templates were amplified for 40 cycles (5 sec at 94°C, 5 sec at 68°C, and 11 sec at 72°C). RpL19 templates were amplified for 35 cycles (5 sec at 94°C, 5 sec at 68°C, and 17 sec at 72°C).

Sensitivity for POMC mRNA amplification from LN samples was enhanced using a nested qRT-PCR protocol. First, 2 µl dT-tailed cDNA were pre-amplified to generate exon 2-3 spanning POMC transcripts (Ex2-3G or Ex2-3E, see Table 2.3) for 25 or 30 cycles using RT-PCR as described above. RT-PCR products (5 µl) were then subjected to qRT-PCR to amplify a smaller POMC fragment listed as Ex2-3F in Table 2.3. This re-amplification was performed for 20 cycles in a final volume of 20 µl as described above.

2.3.11.2 Melting curve analysis

Subsequent to amplification, qRT-PCR products were heated from 65°C to 99°C using a temperature transition rate of 0.1°C/sec following the manufacturer's instructions. The level of fluorescence (F) was measured continuously during heating. Changes were relative to the presence or absence of PCR products and relative to the temperature (T). Melting curves - providing information about the product specificity - were automatically calculated by the LightCycler system and plotted as the ratio of changes of fluorescence to temperature changes [$-\Delta F1/\Delta T$] over temperature. Then products were cooled down to 4°C and analyzed for their product sizes using agarose gel electrophoresis if required. Curves obtained from pituitary were used as positive controls to evaluate LN-derived qRT-PCR products.

2.3.12. Agarose gel electrophoresis

Electrophoresis was run at 110 volt constantly for 40 min in 1x TBE buffer (100 mM Tris base, 100 mM boric acid, 2.5 mM EDTA). Therefore, 20 - 25 µl PCR products were mixed with 4 - 5 µl agarose gel sample buffer (0.25% bromphenol blue, 50 mM Tris pH 7.6, 60% glycerol, H_2O) and transferred to 2% agarose gels (1x TBE, 2% agarose, 0.0005% ethidium bromide). A 100 bp or 72 – 1350 bp DNA ladder was run in parallel to estimate sizes of PCR products. Product bands were visualized under UV-light and results were documented using a Polaroid camera.

2.3.13. Purification and ligation of PCR products

After electrophoretic separation, PCR product bands were cut out and DNA was extracted from the gel using the QIAGEN gel extraction kit following the manufacturer's instructions. DNA was eluted in 30 - 40 µl H_2O. Purified PCR products were ligated into linearized pDrive Cloning vectors using T4 DNA ligase by following the QIAGEN cloning kit instructions. The T4 DNA ligase utilizes single deoxyadenosines added to the 3`-ends of PCR products by the Taq polymerase during PCR amplification in a nontemplate-dependent manner. The DNA concentration of PCR products was determined by photometry (see 'RNA/DNA quantification and quality'). The amount of PCR product used for ligation with 50 ng of vector DNA exceeded the molar vector concentration by 5-times following the equation:

$$\text{ng PCR product required} = (50\ \text{ng} \times \text{PCR product size [bp]} \times 5)/3851\ \text{bp}$$

Reactions were performed in a final volume of 10 µl containing 1 µl vector DNA, 5 µl 2× ligation master mix, 1 - 4 µl PCR product, and 0 - 3 µl distilled water. Mixtures were then incubated for 2 h at 6°C and stored at –20°C thereafter.

2.3.14. Transformation of *Escherichia coli (E. coli)*

Amount of vector DNA was amplified by cloning. Therefore chemocompetent *E. coli* were transformed with vector DNA using heat-shock transformation. Vector DNA (1 - 2 µl) was added to chemocompetent cells, mixed, and incubated for 5 min on ice. Subsequently, mixtures were heated for 30 - 45 sec in a 42° heating block and then quickly placed back on ice. After 2 min, room temperatured SOC medium was added to each reaction mixture. Freshly prepared LB agar selection plates supplemented with ampicillin (100 µg/ml) or kanamycin (30 µg/ml) were used to plate transformed bacteria. Plates were incubated for 15 h at 37°C. A selection of 20 clones was picked and transferred to 20 µl SOC medium each. To test which clones contained the vectors, 1 µl each of these mixtures were subjected to RT-PCR. Amplification was performed for 20 cycles (94°/30 sec, 61°/60 sec, 72°/60 sec) and PCR products were analyzed by agarose gel electrophoresis. Positive clones were subsequently plated onto fresh LB agar selection plates and kept for 15 h at 37°C.

2.3.15. Isolation and purification of plasmid DNA

Clones from the selection plates were used to inoculate 6 ml of LB medium plus ampicillin or kanamycin. Cultures were grown overnight at 37°C in an orbital shaker at 225 rpm. Plasmid DNA was isolated using the QIAGEN Miniprep system according the manufacturer's instructions. Purified recombinant pDrive-POMC templates were alkaline-denatured in 0.1 volume 2 M NaOH, 2 mM EDTA at 37°C for 30 min. The mixtures were neutralized with sodium acetate (NaAc, pH 5.5), and DNA was precipitated overnight with 100% ethanol. Plasmid DNA was then centrifuged and supernatants were removed. DNA was resuspended in 40 µl H_2O. Plasmid DNA was stored at –80°C.

2.3.16. Sequencing

Annealing and sequencing reactions were performed by Dr. Hans Krause (Dep. of Urology, Charité Campus Benjamin Franklin). For direct sequencing of POMC RT-PCR products the same gene-specific sense or antisense primers were used as during the RT-PCR assay listed in Table 2.3. For sequencing of pDrive cloning vector

inserts primers M13 forward/reverse were used. Sequences were analyzed using the NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST).

2.3.17. Immunofluorescence

Staining was performed with the help of Dr. S. A. Mousa (Dep. of Anaesthesiology, Charité Campus Benjamin Franklin, Berlin, Germany). The expression of POMC and END in control and inflamed LN (24 h after CFA-inoculation) was analyzed. Under deep isoflurane anesthesia, rats were perfused transcardially with 0.1 M PBS, followed by fixative solution (PBS containing 4% paraformaldehyde, pH 7.4). Popliteal LN were removed, postfixed for 30 min at 4°C in the fixative solution, and cryoprotected overnight at 4°C in PBS containing 10% sucrose. The tissue was embedded in Tissue Tek compound, frozen, cut into 8 µm sections, mounted onto gelatin-coated slides, and processed for immunofluorescence (Mousa et al. 2000). To prevent non-specific binding, sections were incubated for 60 min in blocking solution (PBS containing 0.3% Triton X-100, 1% BSA, 5% goat serum, and 5% donkey serum). Sections were then incubated overnight with mouse monoclonal anti-POMC (10 µg/ml; recognizing the N-terminal amino acids 1 - 50 of POMC, crossreactivities: 100% for N-POMC, < 6% for γ-MSH and β-LPH) in combination with polyclonal rabbit anti-END_{1-31} (1:1000; crossreactivities: 100% for α-END; 60% for γ-END; 0% for ACTH and α-MSH, according to the manufacturer). Then, the tissue sections were washed with PBS and incubated with texas red-conjugated goat anti-rabbit antibody in combination with FITC-conjugated donkey anti-mouse antibody. Unbound antibodies were removed by washing. Nuclei were stained by incubation for 5 min with 4'-6'-Diamidino-2-phenylindole (DAPI; 10 µg/ml). Sections were mounted in vectashield and viewed under a fluorescence microscope with appropriate filters. The following control experiments were included: preabsorption of anti- END with END and omission of either the primary or the secondary antibodies (Mousa et al. 2000).

2.3.18. Immunohistochemistry

Immunohistochemical staining was also performed with the help of Dr. S. A. Mousa. Rats were deeply anesthetized with isoflurane at 96 h after CFA-injection and perfused transcardially with 100 ml 0.1 m PBS (pH 7.4) and 300 ml cold fixation buffer (pH 7.4; 0.1 M PBS, 4% PFA; 0.2% picric acid) for light microscopic analysis. LN were removed and postfixed at 4°C in 1 ml fixation buffer overnight. Tissue was

subsequently cryoprotected by incubation for 24 h at 4°C in PBS containing 10% sucrose. The tissues were washed in PBS, embedded in Tissue-Tek compound and frozen. Consecutive sections of 10 µm thickness were prepared on cryostat and mounted onto gelatine-coated slides. Staining of serial sections was then performed with the ABC system as previously described (Mousa et al. 2001). Unless otherwise stated, all incubations were performed at room temperature and PBS was used for washing (three times for 10 min each time) after each step. The sections were incubated with PBS, 0.3% H_2O_2 and 10% methanol for 45 min to block endogenous peroxidase. Non-specific binding was prevented by incubating the sections for 60 min in blocking solution (0.1 M PBS, 0.3% Triton X-100, 1% BSA, 4% goat serum, 4% horse serum). The sections were then incubated overnight at 4°C with rabbit polyclonal antibodies against END or POMC (1:1000 dilutions). Thereafter, the sections were incubated for 1 h with goat anti-rabbit biotinylated secondary antibody and then with ABC for 45 min. Finally, the sections were washed and stained for 5 min with DAB containing 0.01% H_2O_2 in 0.05 mM Tris-buffered saline (pH 7.6). After the enzymatic reaction, the sections were washed in tap water, counterstained with thionin, then dehydrated in alcohol, cleared in xylene, and mounted in DPX. Control experiments included: preabsorption of anti-END with END and omission of either the primary or the secondary antibodies as previously described (Mousa et al. 2000).

2.3.19. Morphological identification of cell subsets

Cells were identified by morphological characteristics using the light microscope. Tissue macrophages/monocytes have large cell bodies, vacuolated cytoplasm, and irregular-shaped nuclei. Circulating monocytes have large cell bodies and kidney-shaped nuclei. The cell bodies of lymphocytes are small, nuclei show a regular shape surrounded by small amounts of cytoplasm. Polymorphonuclear leukocytes would show large cell bodies and multisegmented nuclei but typically do not occur in LN.

2.3.20. Intracellular staining of LN cells

Staining was performed as previously described (Rittner et al. 2001). Cells were resuspended in 1% paraformaldehyde (PFA) and fixed for 30 min at 4°C after centrifugation. After removal of the PFA by centrifugation, the cells were permeabilized with saponin buffer (1x PBS, 0.5% Saponin, 0.5% BSA, 0.05% NaN_3) and spun down for 10 min at 4°C and 450 × *g*. Supernatant was softly decanted and

4 µg antibody/10^6 cells was added to the persisting saponin buffer. Mixtures were incubated at room temperature for 15 - 30 min without exposure to light. Opioid peptides were stained with unconjugated mouse 3E7 (primary antibody) and rabbit anti-mouse IgG2a PE (RAM PE) as secondary antibody. 3E7 recognizes the pan-opioid sequence Tyr-Gly-Gly-Phe-Met at the N-terminus of all opioid peptides except endomorphins (Gramsch C 1983). Unbound antibody was removed by washing with saponin buffer once and twice with PBS. Cells were resuspended in PBS and kept at 4°C prior to the measurement. 3E7 staining was verified by incubating the cells with 4 µg/10^6 cells of unconjugated mouse IgG 2a in combination with the secondary antibody RAM PE. Cells were further processed as described above and kept at 4°C in PBS prior to flow cytometry analysis (see 2.3.3).

2.3.21. Radioimmunoassay (RIA)

END-immunoreactive peptides (ir-END) were determined using a rat-END RIA kit. All substances were dissolved in 1× RIA buffer. LN cells and PBL were isolated as described above (see 'Tissue preparation'). Whole cell lysates were prepared from fresh or frozen cell pellets by freeze-and-thaw lysis of 3×10^6 cells/100 µl RIA buffer containing protease inhibitors (4 µg/ml bestatin and 1 µg/ml aprotinin). Freezing-and-thawing was repeated five times: first, samples were kept for 5 min at –80°C in a methanol-dry-ice bath, were then quickly thawed in a normal water bath (22°C) and briefly vortexed. After final 10 minute incubation on ice the cell samples were centrifuged for 10 min at 16,000 × *g* and 4°C. Supernatants (100 µl) were used for measurements thereafter. Samples were prepared in duplicate. Serial diluted END standards containing 1 - 128 pg END/100 µl RIA lysis buffer were prepared in parallel. Then, antiserum (100 µl) was added to all tubes except total count, non-specific binding, and total binding tubes. According to the manufacturer' s specifications the rabbit anti-END used does not cross-react with other POMC-derived antigens like ACTH or MSH as already mentioned (see 'Immunofluorescence'). Tubes were incubated for 16 - 24 h at 4°C, then 100 µl ^{125}I-labeled END with a radioactivity of 11,000 - 15,000 counts per minute (CPM) were added to each tube. Tubes were left for another 16 - 24 h at 4°C. Thereafter, 100 µl each of goat anti-rabbit IgG and normal rabbit serum were added to all samples to precipitate antibody-protein complexes. The reaction was stopped after 90 - 120 min at room temperature by adding 0.5 ml RIA buffer. Finally, tubes were spun at 1700 × *g* for 20 min at 4°C. Supernatants were aspired of all except total count tubes and

bound radioactivity in the pellets was counted as CPM in a gamma-counter. To determine specific binding, the mean CPM of non-specific binding samples was subtracted from all other samples. CPM thereby decreases with an increasing endogenous peptide concentration in the samples or with increasing standard peptide concentrations.

2.3.22. *Ex vivo* LN cell stimulation experiments

Popliteal inflamed LN were dissected 96 h after CFA-inoculation and cells were prepared as described in the 'Tissue preparation' section. Cells (1 - 2 x 10^7 cells/well) were loaded on 6-well-plates and RPMI-1640 medium supplemented with 1% penicillin/streptomycin was added to a final volume of 3 ml/well. Different concentrations of IL-1α, IL-1β or IL-1α+β were added to the culture medium as listed in Table 2.4. Cells were subsequently incubated for 2 - 24 h (see Table 2.4) at 37°C and 5% CO_2. Control cells were incubated without stimulating agent.

Stimulated and control cells were harvested after 2 - 24 h. Cells in suspension were collected in 15 ml tubes on ice. Wells were treated for 3 min with 500 µl trypsin to detach adhered cells, the reaction was stopped with PBS, and cells were added to the appropriate sample tube. Tubes were centrifuged for 10 min at 4°C and 350 $\times$ *g* using a swinging bucket rotor. Aliquots of the culture medium supernatants were collected and stored at –20°C until control measurements using RIA were performed. Cells were washed in PBS, centrifuged, reconstituted in PBS, and counted. After spinning, supernatants were thoroughly removed and pellets were stored at –20°C for further processing (RIA or RNA isolation).

Table 2.4 Concentration of stimulation agents and stimulation times.

Stimulating agent	Concentration (ng/ml culture medium)	Stimulation time (h)
IL-1α	0, 3, 10, 30	24
IL-1α+β	0, 3, 10, 30	24
IL-1β	0, 3, 10, 30, 100, 300, 1000	24 and 48
IL-1β	0, 5	2, 4, 12, and 24

2.4 Data processing

All data were extrapolated using Microsoft Excel 2000 for Windows (Microsoft Corporation) and GraphPad Prism Version 4.01 for Windows (GraphPad Software, Inc.) software. The proportions of reactive lymphocyte subsets measured by flow

cytometry were determined as percentages using CellQuest Pro software (BD Biosciences). RIA measurements of ir-END from single experiments are given in means ± SD. To compare data from more than one experiment raw data were based on control values and percentages are given in means ± SD. Control values were obtained from either control LN or from co-incubated but nonstimulated cells.

Quantitative real-time RT-PCR data were analyzed using the LightCycler software 3.5 (Roche Diagnostics Corporation, Mannheim, Germany). Standard curves were obtained using the 'Fit Points' analysis method by plotting the cycle numbers needed to reach a defined level of fluorescence versus the standard concentrations from serially diluted DNA-standards (mean ± SD; n = 2). PCR efficiencies (E) were subsequently obtained from the equation: $E = 10^{[-1/slope]}$. Amounts of POMC and rpL19 mRNA transcripts were calculated by extrapolation from the standard curves. POMC mRNA copies were divided by the number of rpL19 mRNA copies to obtain normalized ratios.

Relative expression of POMC mRNA was assessed using the 'Second Derivative Maximum' analysis method. Thereby, the excess of background emission by specific amplification was automatically determined as crossing point (CP). The expression of POMC mRNA in inflamed LN was evaluated in comparison to control LN and relative to the expression of rpL19 as housekeeping gene by applying the delta-delta method previously described by PE Applied Biosystems (Perkin Elmer, Foster City, CA). Samples were always analyzed as a set that was amplified together in the same qRT-PCR run (this applies for the amplification of POMC as well as of rpL19 transcripts). The 'Relative Expression Ratio' of POMC mRNA was calculated following the equations previously described by de Longueville and colleagues (2003):

$$\Delta CP = CP_{POMC} - CP_{rpL19}$$

$$\Delta\Delta CP = \Delta CP \text{ inflamed} - \Delta CP \text{ control}$$

$$\text{Relative Expression Ratio} = 2^{-\Delta\Delta CP}$$

2.5 Statistical analysis

GraphPad Prism Version 4.01 for Windows (GraphPad Software, Inc.) software was used for the statistical analysis. Data were analyzed for equal variance and for normal distribution using the D'Agostino & Pearson omnibus normality test. Normally

distributed data were analyzed by Student t-test and nonparametric data by Mann-Whitney-U-Test if only two groups were compared. Multiple measurements of normally distributed data were analyzed by One-Way ANOVA or by Kruskal-Wallis test in case of not normally distributed data. Post-hoc comparisons were performed by Dunnett's Multiple Comparison test for normally distributed data and by Dunn's method for not normally distributed data. For all tests statistical significance was considered if $P < 0.05$.