# 4. Discussion

The major findings of this study are that (1) lymphocytes express low levels of POMC exon 2-3 spanning mRNA and higher amounts of truncated POMC mRNA; (2) in contrast to truncated POMC transcripts the expression of signal sequence-encoding POMC mRNA was selectively upregulated in inflamed LN; (3) signal sequence-encoding POMC mRNA was expressed in both T- and B-cells from LN draining normal and inflamed paws; (4) T-cells from LN draining normal and inflamed paws express PC2 but not PC1/3 while B-cells express both prohormone convertases during inflammation; (5) END contents are elevated in inflamed LN cells after CFA-inoculation of the paw.

## 4.1 Expression of POMC mRNA in lymphocytes

### 4.1.1 Lymphocytes express full-length POMC mRNA during inflammation

The aim of the present study was to prove that lymphocytes express full-length POMC mRNA leading to enhanced END production within these cells in a model of CFA-induced hindpaw inflammation. First, the expression of full-length POMC mRNA comprising all three exons was investigated in LN draining normal and inflamed paws using RT-PCR. Exon 1-3 spanning transcripts of 853 bp were detectable at very early (2 and 6 h) and later (12, 24, and 96 h) stages of CFA-induced inflammation but not in control LN. The sequence analysis revealed that such amplicons were similar to pituitary transcripts and contained all mRNA parts downstream from the 5`-end cap of exon 1 to the stop codon that terminates END in exon 3. Since this transcript did not comprise the part of exon 3 located downstream of END (~120 bp) or the entire poly A-tail (~200 bp), it did not display the full size of rat full-length POMC mRNA (~1.1 kb). The presence of full-length POMC mRNA in inflamed LN was confirmed by the amplification of a shorter transcript spanning POMC Exon 1-3 of 637 bp by using a different primer pair. Previous studies that provided evidence for full-length POMC mRNA expression in lymphocytes under pathological conditions investigated cells that were infected with pathogens *in vitro*. One example is the study of Buzzetti and colleagues (1989) who investigated HIV-infected T lymphoma cells by using a DNA probe containing 780 bp of exon 3 and 313 bp of 3′-flanking DNA for Northern Blot analysis. These authors showed full-length POMC mRNA expression in these

lymphoma cells while noninfected human peripheral blood mononuclear cells expressed the truncated 800 bp POMC mRNA only. Another group investigated the expression of full-length POMC mRNA in Newcastle disease virus-infected mouse splenic lymphocytes (Westly et al. 1986). These authors used a plasmid probe of 1026 bp containing the entire coding sequence for murine POMC and demonstrated the expression of full-length POMC mRNA in infected cells by Northern Blotting. In concert with this finding Smith and Blalock have shown that human peripheral blood lymphocytes infected with Newcastle disease virus contain γ-endorphin (Smith and Blalock 1981) which is a cleavage product of POMC-derived END. Contrary to that, noninfected lymphocytes did not stain with γ-endorphin antiserum. In accordance with the absence of the peptide in noninfected lymphocytes, Westly and co-workers (1986) could not detect full-length POMC mRNA in normal lymphocytes. To date there were no studies that have confirmed the impact of a pathological situation on the expression of full-length POMC mRNA and END in lymphocytes *in vivo*. The present study now shows that lymphocytes express full-length POMC mRNA under inflammatory conditions which corroborates the theory of enhanced full-length POMC transcription under pathological situations.

To further confirm our data and to exclude primer contamination several other amplicons were generated from lymphocytic RNA. Smaller PCR products spanning POMC exon 2-3 were easily detectable in inflamed LN. In addition, such transcripts were already detectable in lymphocytes from normal animals but PCR products were below detection limit in 50 - 75% of samples. Mechanick and colleagues (1992) previously reported that they had similar inconsistencies in detecting POMC mRNA in normal rat splenocytes. In their study three out of five spleens were POMC mRNA-positive. In the present study POMC exon 2-3 transcripts were identified using three different primer pairs. All three amplicons contained at least parts of the signal sequence and revealed sequence identity with pituitary POMC mRNA. Thus, the origin of these exon 2-3 POMC transcripts most likely is the 1.1 kb full-length POMC mRNA. These findings refine our previous data showing exon 2 spanning POMC mRNA in LN lymphocytes draining normal and CFA-infected tissue by ribonuclease protection assay using an antisense 470-bp-cDNA probe that corresponded to 30 bp of intron A, 150 bp of exon 2, and 270 bp of intron B (Cabot et al. 1997). The present data moreover showed that POMC exon 2-3 mRNA fragments were expressed in T- and B-cell fractions of control and inflamed LN. These findings are contrary to the

data of several other groups that could not detect POMC mRNA comprising both the signal sequence of exon 2 and the peptide sequence of exon 3 in normal lymphocytes. In one study, van Woudenberg and colleagues (1993) investigated POMC gene expression in human peripheral blood mononuclear cells by Northern Blotting. They used a probe directed against the third exon of human POMC but could neither detect the truncated 800 bp POMC transcript nor the 1200 bp full-length POMC mRNA. Others like Mechanick and co-workers or Lyons and Blalock investigated full-length POMC mRNA expression in normal rat spleen. The first group used cDNA probes against POMC exon 1 or POMC exon 1-3 for *in situ* hybridization (Mechanick et al. 1992). Both probes confirmed the expression of full-length POMC mRNA in macrophages located in the red pulp of the spleen, but no hybridization was detectable within the white pulp (composed of lymphocytes). Lyons and Blalock (1997) used an [$\gamma^{32}$P]ATP-labeled antisense primer complementary to the 3'-region of POMC exon 3 for primer extension analysis of normal rat splenocytes. The POMC exon 1-3 spanning fragment of 816 bp that they found in unseparated splenocytes was undetectable in macrophage-depleted splenocytes containing T and B lymphocytes. There was only one previous study that demonstrated full-length POMC mRNA expression in normal lymphocytes. This study was performed by Stephanou and colleagues (1991) who investigated resting human peripheral blood lymphocytes using Northern Blot analysis. They used a probe directed against POMC exon 1 and its 5′-end flanking region and amongst larger POMC transcripts of 9.5 and 1.5 kb the 1.2 kb full-length and 0.8 kb truncated mRNA were detected. These authors have used larger amounts of poly A$^+$ RNA (15 µg) than used in studies that did not detect full-length POMC mRNA: van Woudenberg and colleagues used 5 or 10 µg poly A$^+$ RNA (van Woudenberg et al. 1993), Lyons and Blalock used 7 µg poly A$^+$ RNA (Lyons and Blalock 1997), and Westly and colleagues used 6.5 or 10 µg poly A$^+$ RNA (Westly et al. 1986). Together, these findings indicate that signal sequence-encoding POMC mRNA seems to be expressed at very low levels in normal lymphocytes and that this expression is enhanced during inflammation. Accordingly, POMC exon 2-3 mRNA fragments could be detected in T- and B-cell fractions of control LN only by enhancing the sensitivity using semi-nested PCR while amplicons were detectable in inflamed LN by single-round PCR.

The difficulties to amplify POMC mRNA may also be due to the high GC-content of the POMC sequence (~62% on average). GC-rich double-strands tend to separate

at higher temperatures than AT-rich sequences, because of three *vs.* two hydrogen-bonds between these base pairs, respectively. GC-rich sequences were found to increase the possibility of PCR failure (Benita et al. 2003). Gardiner-Garden and Frommer have investigated the distribution of GC-rich sequences (CpG-islands) of the human POMC gene (Gardiner-Garden and Frommer 1994). CpG-islands were reported to be pronounced in the first and third exon. If a high GC-content would influence PCR success for POMC amplification, then this should also be the case during POMC exon 3 amplification. In contrast, POMC exon 3 was amplified without problems in the present and previous studies (Galin et al. 1991; Maier and Blalock 1994). Still, the detection of POMC exon 2-3 or exon 1-3 spanning amplicons was more likely, if the annealing temperature during cycling was set between 59 and 66°C, while lower temperatures seemed to increase failure of the PCR. However, the low level of expression is probably the main reason for the difficulties to detect POMC mRNA in lymphocytes. In the present study the amplification was performed by running 40 PCR cycles, but such high cycle numbers increase the risk of amplifying non-specific template. To identify contamination or false product generation, exon-intron spanning primer pairs were used and non-reverse transcribed RNA was always analyzed in parallel. There are only two other groups that have tried to amplify exon 1-3 or 2-3 spanning POMC mRNA fragments from immune cells. Blalock and colleagues investigated the expression of exon 1-3 or 2-3 spanning POMC mRNA in murine splenocytes after 30 cycles of amplification at an annealing temperature of 58°C (Galin et al. 1991; Maier and Blalock 1994). This cycle number was apparently insufficient and the annealing temperature may have been too low. In the other study, exon 2-3 spanning primers were used to generate POMC amplicons from human peripheral blood mononuclear cells after 35 cycles of amplification at an annealing temperature of 60°C (van Woudenberg et al., 1993). Although no PCR product was detected after electrophoresis, Southern Blot analysis of these PCR products revealed the expected product band. These findings demonstrate that van Woudenberg and co-workers have applied an appropriate annealing temperature which lead to the amplification of POMC exon 2-3, but they apparently did not run enough amplification cycles to get a visible PCR product.

4.1.2 Upregulation of signal sequence-encoding POMC mRNA

The next step was to analyze the kinetics of POMC mRNA expression during the development of inflammation. Due to the better results obtained during RT-PCR using POMC exon 2-3 spanning primer pairs compared to exon 1-3 spanning primers, the qRT-PCR analysis were performed using the former. This quantification of signal sequence-encoding POMC mRNA transcripts by qRT-PCR revealed that lymphocytes contain 0.01% of the amount present in pituitary cells. Other groups, by using different methods, also found immunocytic POMC mRNA levels to be much lower than in pituitary (Mechanick et al. 1992; van Woudenberg et al. 1993; Lyons and Blalock 1997). In line with the present findings Lyons and Blalock showed that full-length POMC mRNA in non-stimulated mononuclear cells was approximately 0.01% of that in pituitary corticotroph cells determined by autoradiographic exposure. Mechanick and colleagues (1992) used quantitative solution hybridization to compare the amount of POMC mRNA in the spleen and intermediate pituitary lobe. In that study the spleen contained 0.005% of the amounts measured in the intermediate lobe.

The present kinetic analysis revealed a fast but transient 6-fold upregulation of POMC exon 2-3 mRNA transcripts in LN at 2 h after CFA-injection *in vivo*. There are no comparable studies on the kinetics of signal sequence-encoding POMC exon 2-3 mRNA expression during *in vivo* stimulation. However, the present study extends our previous findings showing elevated POMC exon 2 mRNA levels in 96 h inflamed LN (Cabot et al. 1997). Other groups have only reported on the upregulation of signal sequence-encoding POMC mRNA in lymphocytes following pathogenic or mitogenic stimulation *in vitro*. For example Westly and colleagues (1986) demonstrated a strong induction of full-length POMC mRNA expression in murine splenocytes at 18 h after Newcastle disease virus-infection. However, this Northern Blot analysis was not quantitative. The autoradiographic exposure of primer extension products investigated by Lyons and Blalock (1997) showed that the treatment of rat splenic lymphocytes with concanavalin A for 21 h enhanced full-length POMC mRNA expression about 4-fold.

The data obtained in the present study by single-round qRT-PCR were usually at the level of detection limit. To increase the sensitivity a semi-nested qRT-PCR strategy was applied. Nested real-time PCR was successfully used in another study to detect rarely expressed tyrosinase transcripts in melanoma cells (Max et al. 2002).

The amplification of POMC exon 2-3 spanning transcripts using this strategy reproduced the data obtained by single-round qRT-PCR: the relative expression levels of POMC exon 2-3 mRNA in lymphocytes increased significantly 8- and 6-fold at 2 and 6 h post CFA-injection, respectively. This clearly shows that lymphocytic signal sequence-encoding POMC mRNA is upregulated *in vivo* under pathological conditions such as inflammation. Moreover, this finding provides an important missing link in the classical processing of POMC into biologically active peptides in immune cells by showing that lymphocytes do express signal sequence-encoding POMC mRNA during inflammation. Such POMC mRNA allows the translation of pre-POMC and this molecule can enter the ER to undergo further processing along the regulated secretory pathway. This complements our previous findings that POMC protein and END co-localized with POMC processing enzymes in immune cells from inflamed paw tissue and that END was located within vesicular structures (Mousa et al. 2004). Moreover, the present results corroborate our functional data that END was released from LN cells in a calcium dependent manner and this release could be mimicked by potassium (Cabot et al. 1997), which indicated a vesicular release of POMC peptides as previously described in neuronal cells (Cool and Loh 1994).

Interestingly, the upregulation of signal sequence-encoding POMC mRNA presented here paralleled the time course of the development of inflammation and mechanical hyperalgesia. Rats showed decreased paw pressure thresholds (hyperalgesia) and increased paw swelling already 2 h after CFA-injections (Sitte et al. 2007). At 6 h both parameters reached their maximum. These findings raise the question whether inflammatory mediators or neurogenic factors could be involved in the regulation of signal sequence-encoding POMC mRNA expression in lymphocytes.

Previous analysis in polyarthritic rats demonstrated that IL-1$\beta$ mRNA upregulation peaked before that of truncated POMC mRNA in the spleen of polyarthritic rats (Stephanou et al. 1992) indicating a relation between IL-1$\beta$ and POMC expression in lymphocytes. In the present study inflamed LN cells were stimulated *ex vivo* to investigate whether IL-1$\beta$ enhances POMC exon 2-3 mRNA expression with similar kinetics as observed *in vivo* during inflammation. The concentration (5 ng/ml) was chosen based on pilot experiments that revealed about 2-fold increases of POMC exon 2-3 mRNA expression over control values after 24 h of stimulation with 3 and 10 ng IL-1$\beta$/ml while 30 ng/ml showed no effect. When LN

cells were then stimulated for 2 h a weak but significant upregulation (1.5-fold over unstimulated controls) of exon 2-3 spanning POMC mRNA expression was found. Others have similarly shown that full-length POMC mRNA expression peaked at 18 - 24 h in resting human peripheral blood leukocytes stimulated with IL-1$\beta$ *in vitro* (Stephanou et al. 1991). An early effect of IL-1$\beta$ on POMC mRNA expression was previously described in other cell systems. One group investigated the effect of low IL-1$\beta$ doses (0.1 and 1 ng/ml) on the POMC exon 2 mRNA expression in human dermal endothelial cells (Scholzen et al. 2000). These authors showed that IL-1$\beta$ can rapidly (within 1 - 5 h) enhance the transcription of POMC mRNA by 2-fold over control levels as determined by semi-quantitative RT-PCR. Similarly, IL-1$\beta$ stimulated the POMC promoter activity in AtT-20 cells by about 1.5-fold after short term stimulation (2 - 3 h) (Katahira et al. 1998). Taken together these findings indicate that IL-1$\beta$ can rapidly enhance the expression of signal sequence-encoding POMC mRNA in pituitary and non-pituitary cells. Thus, IL-1$\beta$ could be involved in the early upregulation of POMC transcription in the draining LN at 2 h after CFA-inoculation of the paw. IL-1$\beta$ may be transported from inflamed tissue to the LN via the efferent lymph as has been shown in rheumatoid arthritis patients (Olszewski et al. 2001). However, the small effect of IL-1$\beta$ indicates that this cytokine is not the sole factor inducing POMC exon 2-3 mRNA expression in lymphocytes.

## 4.1.3 Relevance of truncated POMC mRNA

Several studies have detected truncated POMC mRNA in lymphocytes using Northern Blotting (Bardin et al. 1987; Jeannotte et al. 1987; Lacaze-Masmonteil et al. 1987; Oates et al. 1988; Stephanou et al. 1991; van Woudenberg et al. 1993) or by RT-PCR (Smith et al. 1990; Galin et al. 1991). It remained unclear, whether these truncated POMC transcripts are products from the degradation of full-length POMC mRNA or results of alternative transcription initiation. By analyzing the 5´-ends of LN-derived RACE-PCR transcripts, the present study shows that POMC exon 3 was often flanked by intron B-related sequences while full-length POMC transcripts were rarely detected. The length of these intron B fragments differed by 100 - 200 bp which accounted for the variable length of the truncated POMC mRNAs detected. Truncated POMC mRNAs were previously analyzed by different groups (Jeannotte et al. 1987; Lacaze-Masmonteil et al. 1987). In contrast to the present findings the protected mRNA fragments detected were devoid of intron sequences in both of

these studies. Lacaze-Masmonteil and colleagues (1987) investigated POMC mRNA transcripts obtained from human thymus and testis by S1 nuclease mapping and primer extension analysis. They found six truncated POMC mRNAs that corresponded to a variable 5'-end starting 41, 110, 126, 128, 137, and 162 bp downstream from the 5'-end of exon 3. These 5'-end map into the 5'-flanking region encoding for γ-MSH (41), into the γ-MSH sequence (110, 126, 128), and into the 5'-flanking region encoding for JP (137 and 162). The other group demonstrated five to six truncated POMC mRNA transcripts in the rat testis (Jeannotte et al. 1987). By using primer extension analysis and S1 nuclease mapping this study could map the heterogeneous 5'-end into the region of exon 3 encoding for γ-MSH. Since no TATA box sequence is present in the 5'-end region of exon 3, Jeannotte and colleagues speculated that the heterogeneous truncated POMC mRNAs may originate from RNA intermediates. Lacaze-Masmonteil and colleagues suggested the use of alternative transcription initiation sites like GC-boxes located within POMC intron B. However, the presence of truncated POMC mRNA flanked by intron B-related sequences shown here applies to both explanations since intronic sequences could derive from alternative transcription initiation or from heteronuclear RNA molecules. However, the question remained whether truncated POMC mRNA expression was upregulated by *in vivo* stimulation. To this end primers spanning POMC exon 3 only were used to amplify 'truncated' POMC mRNA by qRT-PCR. In LN draining both normal and inflamed paw tissue the amount of POMC exon 3 mRNA was 10 times higher than that of signal sequence-encoding transcripts. This difference in the expression levels did not seem to be due to different amplification rates of the primer pairs, because POMC exon 3 mRNA levels obtained from pituitary exceeded POMC exon 2-3 levels only by 2-fold under the same PCR conditions as in LN. The kinetic analysis showed no changes between POMC exon 3 mRNA levels of control and inflamed LN, indicating that truncated POMC mRNAs are not regulated during local inflammation. In contrast, truncated POMC mRNA levels were reported to be upregulated during systemic inflammation in the spleen. Stephanou and co-workers (1992) investigated POMC mRNA expression in splenic lymphocytes and pituitary of polyarthritic rats injected intradermally with CFA at the tail base which leads to polyarthritis. In this model full-length POMC mRNA expression increased 7 and 14 days after CFA-injections in the pituitary which was determined by Northern Blot analysis using a probe directed to the third exon of rat POMC. In splenocytes, the

amount of truncated POMC mRNA (800 bp) was reported to increase with a similar time course like pituitary full-length POMC mRNA. In the present study pituitary POMC exon 3 and exon 2-3 transcripts were not elevated during the observation period of four days CFA which confirms that the local injection of CFA into the paw did not produce a systemic inflammation as suggested by Stein et al. (1988).

However, it is possible that the expression of truncated POMC mRNA is enhanced in lymphocytes with progressing CFA-inflammation. In line with the present data POMC exon 3 mRNA was not upregulated in immune cells during earlier stages of inflammation in another animal model (Chadzinska et al. 2005). In this study peritoneal leukocytes of mice were analyzed for their POMC exon 3 mRNA expression at 4 and 24 h after intraperitoneal injections of zymosan, an insoluble carbohydrate from the cell wall of yeast. Previous findings already indicated that signal sequence-encoding POMC mRNA seemed important for the synthesis of POMC peptides in immune cells when the effect of POMC exon 2 antisense oligonucleotides on the production of POMC peptides was investigated in rat splenocytes (Fulford et al. 2000). These authors found that blockade of the signal sequence significantly reduced the levels of ACTH and END production (by about 50% of controls) in concanavalin A-stimulated cells. This indicated that lymphocytes preferentially translated full-length and not truncated POMC mRNA upon mitogenic stimulation. The present study could now show that lymphocytes upregulate signal sequence-encoding but not truncated POMC mRNA after *in vivo* stimulation.

## 4.2 Distinct expression of prohomone convertases in lymphocyte subsets

Proteolytic cleavage of the POMC prohormone within the trans-Golgi network and within secretory vesicles involves the two prohormone convertases PC1/3 and PC2, and the chaperone 7B2 (Benjannet et al. 1991; Benjannet et al. 1995). Studies in the pituitary have shown that corticotroph and melanotroph cells exert cell specific expression pattern of the prohormone convertases (Day et al. 1992). The present study investigated whether normal T and B lymphocytes also show a differential PC1/3 and PC2 expression and whether these enzymes are upregulated during inflammation. PC1/3 was not expressed in T- or B-cell-enriched fractions of controls nor of the contralateral LN at 24 h post CFA-injection. But in inflamed LN PC1/3 mRNA expression was prominent in B-cell-enriched fractions. PC2 mRNA expression was detected in T-cell fractions of both noninflamed and inflamed LN but only in B-

cell fractions from inflamed LN. The mRNA of the co-factor 7B2 was detected in all samples. Thus, T and B lymphocytes show differential PC1/3 and PC2 expression under both normal and inflammatory conditions. These findings refine our previous data that have shown PC1/3, PC2, and 7B2 within circulating lymphocytes without differentiating between the cell subsets (Mousa et al. 2004). Other groups have analyzed these prohormone convertases after *in vivo* stimulation in the spleen but not in the LN. Basal expression of PC1/3 was seen in the macrophage-rich red pulp/marginal zone areas of the spleen and was inducible within germinal centres (B lymphocytes) after *in vivo* LPS-treatment (Lansac et al. 2006). This is in line with the present finding showing that PC1/3 expression was induced in LN-derived B-cells after *in vivo* CFA-treatment. While normal LN-derived T lymphocytes expressed PC2 in the present study, Lansac and co-workers did not detect PC2 in normal splenic lymphocytes. This discrepancy may be due to the different cell types that predominate in the two lymphoid tissues: the spleen accommodates mainly B-cells and macrophages, whereas the LN contains predominantly T-cells. However, similar to the present study, PC2 expression was inducible in splenic B-cells by *in vivo* stimulation (Lansac et al. 2006).

The prohormone convertase expression in stimulated T and B lymphocytes does not resemble corticotroph or melanotroph cells (corticotrophs express only PC1 and melanotrophs contain predominantly PC2 and to a much lesser extent PC1/3) (Day et al. 1992). However, according to a study of Benjannett and colleagues (1991), PC2 alone is able to produce END by the cleavage of POMC. These authors have analyzed the production of POMC-derived peptides in AtT-20 cells and two other cell lines that do not express PC1/3 and PC2 endogenously (the African green monkey kidney epithelial cell line BSC-40 and the rat pheochromocytoma cell line PC12). After infection of the cells by vaccinia virus recombinants encoding PC1, PC2 or POMC mRNAs, the major cleavage product of PC2 was END, while that of PC1/3 was ACTH. Taken together, these findings indicate that under normal and inflammatory conditions PC2-expressing T-cells in the LN should be able to process POMC proteolytically into END. In B-cells PC2 and PC1/3 expression is induced under inflammatory conditions allowing posttranslational processing of POMC into END and ACTH, respectively. T-cells do not seem to express PC1/3 which indicates that these cells cannot cleave POMC into ACTH and LPH in models that induce inflammation *in vivo*. However, other studies have shown ir-ACTH in both T and B-

cells *in vitro*. For example Lyons and Blalock analyzed rat splenocytes by immunohistochemistry and found ACTH expression in concanavalin A or LPS stimulated cytotoxic T-cells, T helper cells and B-cells (Lyons and Blalock 1995). ACTH production was also observed by immunofluorescence and Western blotting in cultured T lymphocytes (H9 cell line) infected with HIV-1 (Hashemi et al. 1998). Thus, it seems that the expression pattern of prohormone convertases in T-cells differs between *in vivo* and *in vitro* stimulation or may be dependent on the stimulus.

## 4.3 END expression in LN lymphocytes during inflammation

### 4.3.1 POMC and END co-localize in T and B lymphocytes

While co-localization of POMC and END was demonstrated within circulating leukocytes and within immune cells of the inflamed paw tissue (Mousa et al. 2004), a detailed analysis of opioid peptide-expressing lymphocytes within the draining LN has not been performed. In the present study the expression of ir-POMC and ir-END in LN sections was analyzed and revealed that precursor and end-product co-localized within cells located in the cortical, paracortical, and marginal zone of control and inflamed LN. The paracortical and marginal zone predominantly comprise T lymphocytes and monocytes/macrophages while B-cells are located within the LN cortex (Janeway et al. 2001). In addition, in inflamed LN POMC- and END-expressing cells could be morphologically identified as lymphocytes and monocytes/macrophages. By flow cytometry analysis of opioid peptide-expressing lymphocyte subsets in inflamed LN these immunohistochemistry data were confirmed. Opioid peptides were detected in approximately 12% of B-cells, about 9% of T helper cells, and in roughly 9% of cytotoxic T-cells. Opioid peptide-containing monocytes/macrophages were below detection limit when analyzed by flow cytometry, although the immunohistochemistry staining seemed stronger in these cells than in lymphocytes. Other studies on the localization of POMC-derived peptides have mainly been performed in spleen. In one of those studies END was found to be expressed only within the red pulp area which contained ED1^{+} and ED2^{+} macrophages (Mechanick et al. 1992). END was not detected within lymphocyte-rich regions of the spleen (white pulp). Lyons and Blalock, who also used immunocytochemistry, showed that freshly isolated splenic macrophages from healthy rats but not lymphocytes expressed ACTH (Lyons and Blalock 1995; 1997).

Thus, the presence of POMC-derived peptides aligns with the distribution of the prohormone convertases within the different cell types of the two lymphoid tissues.

4.3.2 END levels increase in lymphocyte subsets during inflammation

The next step was to investigate the time course of END production in the draining LN during the development of CFA-induced inflammation. For that purpose it was important to choose a reliable reference parameter (such as cell numbers, protein amount, per organ) for the quantification of END. In line with previous observations (Mousa et al. 1996), CFA-inoculation of the paw produced enlargement of the draining LN which was accompanied by increasing cell numbers. This is the result of enhanced retention of trafficking lymphocytes within the LN and subsequent proliferation as has previously been analyzed in an isolated LN model in the sheep (Hall and Morris 1965; Mackay et al. 1992). Thus, when Mackay and colleagues perfused the LN with purified tuberculin protein about 80% of the cells that usually pass the LN during the first 24 h were retained. Hall and Morris investigated the cell proliferation and found proliferative activites at 50 h after node perfusion with antigen. The present study covered an observation period of up to 96 h which would include retention as well as proliferation events in the node. Therefore, the organ was no appropriate reference parameter. The cytosolic protein content per LN cell increased after CFA-injections in our pilot studies (unpublished data). This may be due to the upregulation of chemokine receptors and adhesion molecules that allow antigen-activated lymphocytes to enter inflamed tissues (Tanaka et al. 2004). Thus, the determination of the END contents per cell seemed the most accurate procedure and moreover has been frequently used before (Buzzetti et al. 1989; Buzzetti et al. 1991; Cabot et al. 1997). The present data show that the END concentration was $37 \pm 10$ pg per $10^7$ cells in control LN. Similar amounts of another POMC-derived peptide (ACTH) were previously found in blood-derived lymphocytes from normal volunteers (29 pg per $10^7$ cells) (Buzzetti et al. 1989). To exclude cross-reactivity of our END antibody with other POMC peptides a control experiment using END$^{-/-}$ mice was included in the present study. In contrast to WT mice, the pituitaries of END$^{-/-}$ animals revealed no detectable END which confirms the specificity of the antibody used.

The amount of cellular END was subsequently determined in LN cells and found to be significantly increased 2.5-fold in inflamed LN over control levels at 12 h after CFA-injection, then remained elevated until 48 h, and dropped back to baseline

levels at 96 h. This extends our previous findings, where END levels were compared between normal and 96 h inflamed LN (Cabot et al. 1997). This study showed that the END content was not different between these LN cells at the time points analyzed. It is conceivable that 96 h after induction of paw inflammation by CFA END-containing lymphocytes migrated towards the inflamed tissue leading to decreasing END levels in LN cells at that time. Another possibility is that END was released within the LN.

By comparing the kinetics of POMC mRNA expression and END production it becomes apparent that the upregulation of POMC exon 2-3 mRNA expression was followed 6 - 10 h later by a 2-fold elevation of the END levels in LN-derived cells. This is consistent with the notion that *de novo* production of END is due to the expression of signal sequence-encoding POMC mRNA. In fact, this time span is similar to that previously reported by Mains and Eipper (1979) for the posttranslational processing of POMC into END in rat intermediate pituitary cells. These authors treated the cells for 15 min with [$^{35}$S] methionine and found that radioactive END was present 6 h later, which was determined by pulse chase experiments. Previous data on the time needed for POMC protein biosynthesis in lymphocytes were obtained from *in vitro* studies. For example, Lyons and Blalock compared ACTH levels in normal and stimulated splenocytes. When they stimulated these cells with concanavalin A or LPS the ACTH levels increased within 18 - 21 h 3 to 8-fold over controls and then decreased again 48 h after both treatments (Lyons and Blalock 1995). Although such *in vitro* data cannot directly be compared to a pathologic *in vivo* situation, it is interesting that POMC-derived peptides seem to be upregulated with quite similar kinetics under both conditions.

Further investigation set out to analyze which lymphocyte subsets contributed to the elevation of the END levels during inflammation. To rule out that – in addition to an enhanced POMC mRNA expression – changes among the cell subsets contributed to the observed increases of the END levels during inflammation proportional shifts of LN cell subsets were assessed by flow cytometry. The proportion of T- and B-cells did not change during the first two days of inflammation. Thus, like in controls, T-cells predominated in the node with a ratio of 4:1 over B-cells for two days. This dominance of T over B lymphocytes may be a consequence of the preferential retainment of naive-type T-cells within LN tissue (Mackay et al. 1992). However, at 96 h after CFA-injection B-cells proportionally increased and the T-cell

population decreased leading to a decreased T/B-cell ratio of 1:1. This finding is reminiscent of previous findings in adjuvant-induced polyarthritis in rats. Within four days the intradermal injection of CFA into the base of rat tails lead to a decreased T/B-cell ratio from 4:1 to 1:1.5 in regional LN (Rodriguez-Palmero et al. 1999). In the present study the percentage of $CD4^+$ LN cells did not change during the observation period while the proportion of $CD8^+$ cells slowly increased leading to decreased $CD4^+/CD8^+$ ratios. Rodriguez-Palmero and colleagues (1999) likewise reported on increasing numbers of $CD8^+$ cells within regional LN four days after induction of adjuvant-induced polyarthritis in rats. Taken together, none of the cell subsets analyzed showed a substantial increase in the population size at 24 and 48 h in the present model, indicating that the elevation of the END contents detected by then were not due to cellular changes.

The END levels of cell subsets were subsequently determined in magnetically separated LN suspensions. Like in unseparated LN cells END levels increased at 24 h after CFA-inoculation in both T- and non-T-cell fractions. This is in line with the present data on enhanced POMC exon 2-3 mRNA expression in separated cells of inflamed LN and confirms the finding of ir-POMC and ir-END in both T- and B-cell zones of this tissue. At the same time, baseline END levels were higher in non-T- than in T-cell fractions. These non-T-cell fractions contained B-cells and monocytes/macrophages. According to previous findings (van Woudenberg et al. 1993) and our own unpublished data macrophages produce more END than lymphocytes. Thus, these cells probably contributed to the high basal END levels found in non-T-cell fractions. In the present study, the END levels in T- and non-T-cell fractions decreased to baseline levels at 48 h. This is in contrast to the findings obtained from nonseparated LN cells where END levels were still elevated at 48 h. It is possible that the magnetic separation procedure eliminated cells that may contain END like DCs. DCs enter lymphoid tissues in increasing numbers with ongoing infection (Jiao et al. 2002).

END levels were further analyzed in $CD4^+$ T-cells and $CD4^-$ cells. $CD4^+$ T-cells displayed a biphasic 3-fold elevation of ir-END at 24 and 96 h. END concentrations in $CD4^+$ T-cells were significantly larger than in nonseparated T-cells ($55 \pm 17$ pg/$10^7$ cells vs. $35 \pm 8$ pg/$10^7$ cells determined at 24 h, respectively). Consistent with this finding END was previously identified by immunohistochemistry mainly in activated memory T-cells of LN draining inflamed tissue (Cabot et al. 1997; Mousa et al. 2001).

The particularly high END levels in these cells may suggest that END plays a special functional role in such lymphocytes. For example, previous studies have indicated an important role of CD4$^+$ lymphocytes in the inhibition of visceral nociception (Verma-Gandhu et al. 2006). The authors found that normal pain thresholds are preserved in the presence of CD4$^+$ cells but not B lymphocytes. The authors further observed that opioid peptides mediated this antinociceptive effect because somatic pain was increased by naloxone-methiodide treatment. However, in CD4$^-$ cells comprising CD8$^+$ cells and B lymphocytes, END levels were also elevated by 2-fold at 24 and 48 h but decreased at 96 h. The presence of opioid peptides in cytotoxic T- and B-cells was confirmed by the present flow cytometry data. The expression of END in B-cells is also consistent with previous findings (Kavelaars et al. 1989; Przewlocki et al. 1992). Kavelaars and colleagues investigated END release from human peripheral blood B-cells and found increasing END release after 24 h of stimulation with IL-1$\beta$ in a plaque-forming cell assay. To the best of one's knowledge the production of END in CD8$^+$ cells was previously not explicitly described but Lyons and Blalock (1995) showed that ACTH expression can be stimulated with concanavalin A or LPS in cytotoxic T-cells like in other lymphocytes. Moreover, END was previously demonstrated in T and B lymphocytes located in inflamed paw tissue by immunohistochemistry analysis (Przewlocki et al. 1992). Together these findings indicate that T- and non-T-cells produced larger amounts of END within the draining LN during the first 24 h after CFA-injections. Thus, in concert with an enhanced POMC exon 2-3 mRNA expression observed in the node, it seems likely that the draining LN represents an important site of END production. Such END-containing T and B lymphocytes of the LN may subsequently migrate towards inflamed tissue.

It is conceivable that an increased END production is the result of antigen-stimulation of LN lymphocytes. For example DCs that have encountered mycobacterium in the paw, enter the T-cell rich medullar region of the LN where they express antigen/MHC class I or II complexes important for the activation of CD8$^+$ and CD4$^+$ lymphocytes, respectively (Hickman et al. 2002). However, the present study shows that END levels also increased at 24 h in contralateral LN cells where antigen presentation presumably does not take place. Elevated END levels in the contralateral LN were also detected at 96 h post CFA-injection (Cabot et al. 1997). These findings rather point towards a systemic than to an antigen-induced effect. However, END levels in inguinal LN that do not drain the site of inflammation showed

no increases in END levels. In addition, pituitary POMC mRNA expression was not altered in the present study. These findings argue against a systemic modulation of END levels in lymphocytes during paw inflammation. Thus, bilateral elevation of END may point towards an involvement of the nervous system. Others have shown that cytokine expression can be induced bilaterally in nervous tissue after unilateral injury. For example the cytokine gene induction in ipsi- and contralateral nerves was analyzed after unilateral sciatic nerve crush and chronic constriction injury in mice (Kleinschnitz et al. 2005). IL-1β mRNA was significantly increased in both sides within 24 h. Interestingly, contralateral gene induction was restricted to the homonymous opposite sciatic nerve, but spared the femoral nerve. Future studies will have to analyze the stimuli and mechanism leading to enhanced END production in detail.

## 4.4 Future studies

The present data have characterized the distribution and identity of END producing cells in the draining LN. Moreover, this study established the modalities for the quantitative analysis of signal sequence-encoding POMC mRNA expression in lymphocytes and demonstrated that the amount of END is regulated on the transcriptional level early during inflammation. The present cell stimulation experiments indicated an involvement of inflammatory mediators like IL-1β in the upregulation of POMC expression. This is reminiscent of previous findings obtained from pituitary cells. To study the signal transduction pathways and transcription factors involved in the upregulation of POMC mRNA transcription in lymphocytes investigations are planned using micro arrays and protein/antibody arrays (2D gel electrophoresis). Thereby, the expression profiles of genes and proteins, respectively, may be compared in LN-derived cell subsets before and after CFA-treatment.

Previous studies have shown that the cytosines of the POMC promoter and 5'- and 3'-flanking regions are highly methylated in nonexpressing and low level expressing tissues like lymphocytes while POMC is unmethylated in high expressing tissues such as the pituitary (Newell-Price et al. 2001; Newell-Price 2003). Since DNA methylation is associated with condensed chromatin and silencing of gene expression, the upregulation of POMC mRNA expression in lymphocytes described

here, could reflect demethylation. Future experiments may analyze whether the degree of POMC DNA methylation decreases in lymphocytes during inflammation.

In the present model POMC mRNA expression was not enhanced in the pituitary and END was not upregulated in non-draining LN but END was upregulated in the ipsi- and contralateral popliteal LN. These findings indicate that END production is not systemically elevated. The bilateral effect may point towards an involvement of the nervous system and further investigations are planned to clarify whether the 'pain' stimulus affects lymphocyte POMC mRNA expression. Lymphocytic POMC mRNA expression may be investigated by qRT-PCR analysis after nerve blockade with bupivacaine that has previously been shown to inhibit transmission of nociception in the unilateral CFA-model (Schmitt et al. 2003; Puehler et al. 2004).