

B Lymphocytes Express *Pomc* mRNA, Processing Enzymes and β -Endorphin in Painful Inflammation

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Abstract Immune cell-derived beta-endorphin (END) and other opioid peptides elicit potent and clinically relevant inhibition of pain (analgesia) in inflamed tissue by activation of peripheral opioid receptors. Pro-opiomelanocortin (POMC) is the polypeptide precursor of END and is processed by prohormone convertases (PCs). This study aims to decipher the processing of POMC in lymphocyte subsets in a rat model of unilateral painful hindpaw inflammation. Lymphocytes, isolated from popliteal lymph nodes, were separated into B-cells, T-cells, T-helper cells and cytotoxic T-cells using magnetic cell sorting, and were examined by polymerase chain reaction, immunofluorescence and radioimmunoassay. At 2 h of inflammation, POMC exon 2–3 mRNA was mostly expressed in B- but not in T-cells. Prohormone convertase 1 (PC1) mRNA and protein were upregulated in B-cells and T-helper cells. Prohormone convertase 2 (PC2) was expressed in T- and B-cells, both in inflamed and non-inflamed lymph nodes. END was expressed in B- but not in T-cells. We conclude that POMC, its processing enzymes and END are predominantly expressed in B-lymphocytes at 2 h of paw inflammation.

Keywords Inflammation · Lymphocytes · Pain · Opioid · Endorphin · Proopiomelanocortin · Prohormone convertase

Abbreviations

CFA	Complete Freund's adjuvant
POMC	Proopiomelanocortin
qRT-PCR	Quantitative real-time-polymerase chain reaction
RIA	Radioimmunoassay
RT	Reverse transcriptase
ACTH	Adrenocorticotrophic hormone
PC1	Prohormone convertase 1
PC2	Prohormone convertase 2
END	Beta-endorphin
MACS	Magnetic cell sorting
LN	Lymph nodes

Introduction

Pro-opiomelanocortin (POMC) is the precursor of adrenocorticotrophic hormone (ACTH), melanocyte-stimulating hormones, and β -endorphin (END) (Nakanishi et al. 1979). POMC is mainly produced in the pituitary gland (Seidah et al. 1993; Bennett et al. 1992). The *Pomc* gene comprises three exons that are transcribed into full-length POMC mRNA. Translation of exons 2 and 3 gives rise to the prepropeptide including the signal sequence required to enter the regulated secretory pathway. The subsequent formation of POMC-derived active peptides involves extensive proteolytic cleavage (Tanaka 2003), mediated by prohormone convertases PC1 and PC2 (Seidah et al. 1993). Several non-pituitary tissues also express POMC and END (Maddila 2015). In particular, we and others have shown that END is

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produced and released by immune cells, and can elicit potent and clinically relevant inhibition of inflammatory pain (analgesia) via activation of opioid receptors expressed in peripheral sensory neurons (Sehgal et al. 2011; Busch-Dienstfertig et al. 2012; Rittner et al. 2006). In our previous studies *Pomc* exon 2–3 expression was stimulated by IL-4 in mixed lymphocyte populations in vitro (Busch-Dienstfertig et al. 2012), and it was upregulated in draining lymph nodes (LNs) after induction of painful paw inflammation in vivo (Cabot et al. 1997; Sitte et al. 2007). To further delineate the regulatory pathways in lymphocyte subpopulations, we now investigated *Pomc* exon 2–3 expression, processing enzymes and END production in separated T- and B-cell subpopulations.

Methods

Animals and Induction of Inflammation Experiments were carried out in male Wistar rats (Janvier labs, Saint-Berthevin Cedex, France) (200–250 g). Unilateral hindpaw inflammation was induced by intraplantar (i.pl.) injection of complete Freund's adjuvant (CFA) as described (Busch-Dienstfertig et al. 2012). Experiments were approved by the animal care committee of the State of Berlin, Germany, and strictly followed the guidelines of the International Association for the Study of Pain (Zimmermann 1983).

Cell and Tissue Preparation Rats were killed by an overdose of isoflurane anesthesia. The pituitary gland and popliteal LNs draining the paws were dissected. Single cell suspensions were prepared as described (Maddila 2015). Cell viability was examined by the trypan blue exclusion method. Cell suspensions were then subjected to magnetic cell separation (MACS), immunofluorescence, radioimmunoassay (RIA) or immediately stored at -80°C ; cDNA was prepared for subsequent polymerase chain reaction (PCR) analyses as previously described (Busch-Dienstfertig et al. 2012).

Magnetic Cell Sorting The single cell suspensions of lymphocytes were resuspended in 1 ml of RPMI medium-1640 with Glutamax I (GIBCO Life technologies, Darmstadt, Germany) and were incubated with magnetic microbeads coated with either anti-rat CD45RA (against B-cells; #130-090-494), anti-rat MHC class II (against antigen presenting non-T-cells; #130-090-759), anti-rat CD8 (against cytotoxic T-cells; #130-090-318), anti-rat CD4 (against T-helper cells; #130-090-319) (all Miltenyi Biotec, Bergisch-Gladbach, Germany), and were separated as described (Sitte et al. 2007). Cells incubated with anti-rat CD45RA (B-cells) were retained as positive fraction and used for further experiments. The negative fraction (non B-cells; CD45RA-) was incubated with anti-rat MHC class II (against antigen presenting non-T-

cells). The positive fraction was discarded, while the flow-through negative fraction (enriched untouched T-cells) was used for further experiments. Cells incubated with anti-rat CD8 (cytotoxic T-cells) and with anti-rat CD4 (T-helper cells) were retained as positive fractions for further experiments. The cell suspensions were then subjected to gene/protein expression analysis (PCR, RIA, immunofluorescence).

Cytospins and Immunofluorescence 1.5×10^6 cells were loaded into a cuvette followed by spinning at 300 rpm and plating the cells onto slides as described (Maddila 2015). Primary antibodies were anti-PC1 and anti-PC2 (dilution 1:1500, kindly donated by Dr. Donald Steiner, University of Chicago), secondary antibodies were goat anti-rabbit, Texas red-conjugated (dilution 1:2000, Vector Laboratories, Cambridgeshire, UK). We used a fluorescence microscope and the AxioVision program (Zeiss Axioskop 2). Image analysis was performed using Image J (Version 1.47 developed by NIH) as follows: Fluorescence intensity was measured in the sample incubated with the secondary antibody to determine the background threshold. This threshold was subtracted from the fluorescence of all other images. The number of particles above threshold, their area, and their mean fluorescence was determined using the option 'analyse particles' in Image J. The size of single cells was measured using the circle tool and values of 30 cells were used to obtain a mean area. This mean was used to calculate the cell numbers from the total area above the threshold determined with the particle analyser. The mean fluorescence was plotted on graphs. Although the specificity of the antibodies was previously shown (Ugleholdt et al. 2006), we verified specificities for our methods and our cell types using preabsorption with antigen, and western blots of pituitary lysates from PC1 and PC2 knockout animals (data not shown).

Radioimmunoassay (RIA) Cellular content of END was determined by measuring immunoreactive (ir) END in cell lysates as previously described (Binder et al. 2004; Sitte et al. 2007; Busch-Dienstfertig et al. 2012). Briefly, lymphocytes were lysed by sonication (15 s, 1 impulse/sec) at a concentration of approximately 3×10^6 cells per 100 μl assay buffer and ir-END was determined in pg/100 μl of lysates in duplicates.

PCR Primers and Quantitative Real-Time PCR (qRT-PCR) Primers were designed to amplify β -Actin, *Pomc* (exon 2–3), *PC1* and *PC2* transcripts from LNs and pituitary cDNAs as described (Sitte et al. 2007). Primers and their PCR parameters are shown in Table 1. qRT PCR was performed as detailed in (Sitte et al. 2007; Busch-Dienstfertig et al. 2012). Pituitary (PT) cDNA was used as positive control and double-distilled (dd) H_2O was used as negative control (NC). Results were evaluated by the delta-delta crossing point

Table 1 PCR primer pairs and PCR parameters

Gene	sense primer: 5' - 3'	antisense primer: 5' - 3'	AT	Elongation	TM	Product Size (bp)	Cycles (n)
<i>Pomc</i> exon 2-3	TGGCCCTCCTGCTTCAGAC	CTCACTGGCCCTTCTTGTGC	63	72 °C / 27	89	668	40
<i>PC1</i>	AATCCTGTAGGCACCTGGAC	GGAGTTTTTGGGTACCAGGA	55	72 °C / 10	82	254	40
<i>PC2</i>	GGGCCACAGACAACGGGA	GCCGTCGTTGATGGCCGAGT	69	72 °C / 10	87	207	40
<i>β-Actin</i>	ACCCTAAGGCCAACCCTGAA	TGGCATGAGGGAGCGCGTAACC	68	72 °C / 10	85	191	35

AT Annealing temperature, TM Melting temperature, BP Base pairs

(CP) method as described (Pfaffl 2001; Busch-Dienstfertig et al. 2012).

Conventional PCR (cPCR) 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 MgCl₂, 0.8 mM dNTPs, 1 U AMV polymerase, and either 2 µl template or RT⁻ control. Positive controls contained 2 µl PT cDNA; NC were supplemented with 2 µl ddH₂O. Each assay started with an initial denaturing cycle of 5 min at 95 °C, continued for 40 amplification cycles and was terminated with an extension cycle of 10 min at 72 °C. Each cycle included 45 s at 94 °C to denature the template followed by 45–60 s at 55–68 °C for primer annealing, and 45–60 s at 72 °C for primer extension. To confirm successful cDNA preparation, *β-Actin* mRNA was amplified for 35 cycles including 30 s at 94 °C, 30 s at 68 °C, and 30 s at 72 °C.

Agarose Gel Electrophoresis Electrophoresis was done as described (Maddila 2015). A 100 bp DNA ladder (Roche, Germany) was run in parallel to estimate sizes of PCR products. PCR products were imaged with a Biorad documentation system.

Statistical Analysis Data were analyzed with GraphPad Prism Version 5.01 for Windows (GraphPad Software, USA). Statistical analysis was performed on normalized CP = CP_{PCs}-CP_{β-Actin} in case of qRT-PCR data; ir-END values (cellular content as well as amount in the supernatant in pg) were normalized to 1 × 10⁶ cells. Statistical significance with respect to qRT-PCR data was analysed by One-Way ANOVA. In case of nonparametric data, Kruskal-Wallis or Wilcoxon signed rank tests were applied. Multiple comparisons of qRT-PCR and RIA data were performed using the non-parametric Friedman Test; post-hoc comparisons were performed by Dunn's test. Statistical significance was considered if *P* < 0.05. Data are shown as means ± standard error of the mean (SEM).

Results

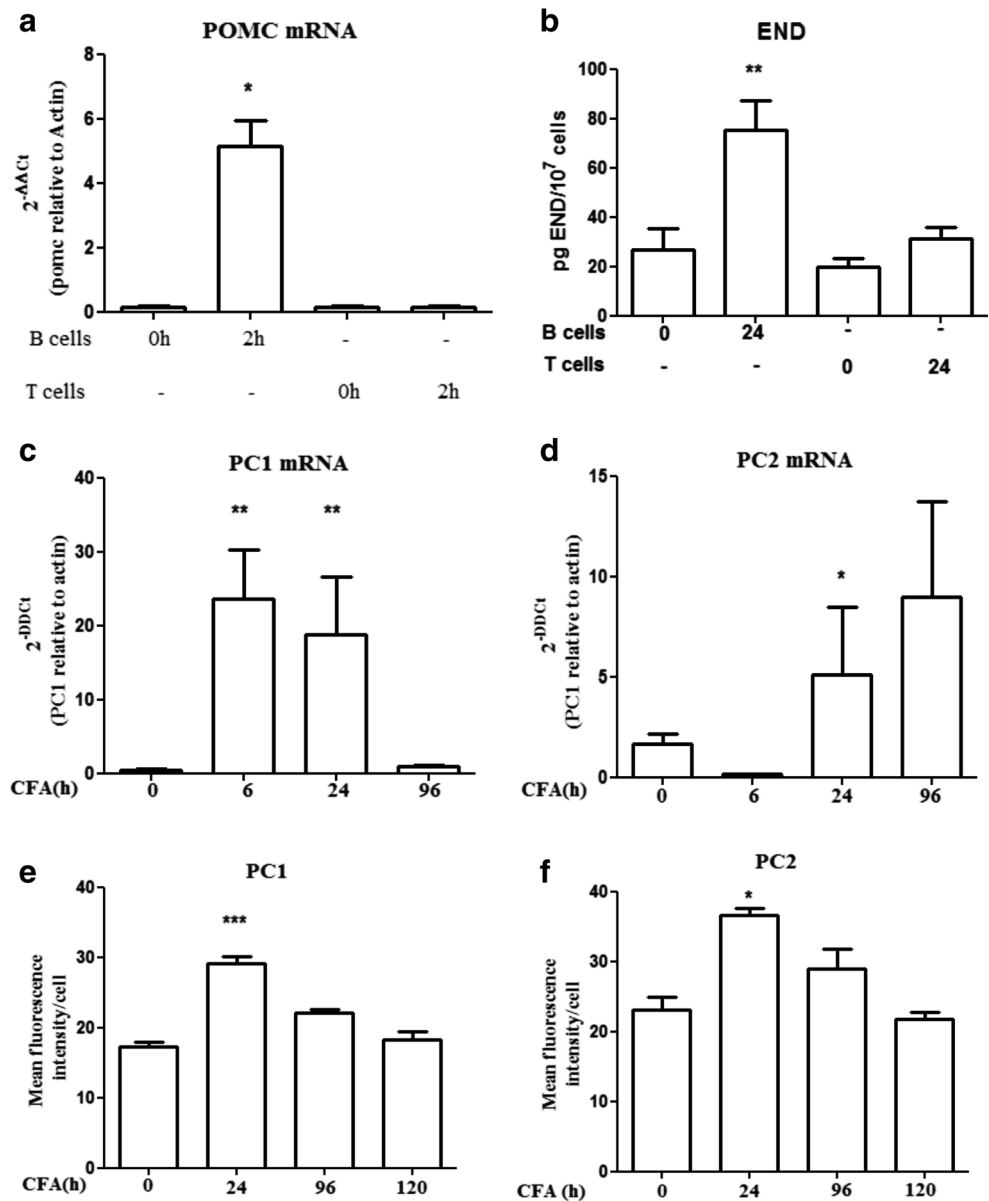
Expression of *Pomc* exon 2–3 mRNA Previous experiments in our group had shown that *Pomc* exon 2–3 mRNA was detectable in inflamed (but not in noninflamed) LNs with a maximum at 2 h after i.pl. CFA (Sitte et al. 2007). We now found that *Pomc* exon 2–3 mRNA transcripts were present mostly in B- but not in T-cells at this time point (Fig. 1a).

Expression of Prohormone Convertase 1 (PC1) In unseparated LN cells, *PC1* mRNA was upregulated within 6 h after i.pl. CFA and declined thereafter (Fig. 1c). Immunofluorescence analysis showed that PC1 protein was significantly increased within 24 h after the onset of paw inflammation and declined thereafter (Figs. 1e and 3a). At the time point of maximum PC1 expression (24 h, Fig. 1e) *PC1* mRNA was below detection limit in B- and T-cells from normal LNs but was observed in both B- and T-cells of inflamed LNs (Fig. 2a upper panel). PC1 protein was expressed both in B- and T- cells and was elevated in cells from inflamed LNs (Figs. 2c, 3c and d left panel). *PC1* mRNA was detected in T-helper cells, but not in cytotoxic T-cells from inflamed LNs (Fig. 2e upper panel) and was undetectable in all cell fractions of noninflamed LNs. PC1 protein was elevated in T-helper cells (Figs. 2g, 3e and f left panel).

Expression of Prohormone Convertase 2 (PC2) *PC2* mRNA was significantly upregulated in unseparated LN cells at 24 and 96 h after i.pl. CFA (Fig. 1d). PC2 protein was significantly increased at 24 h and declined thereafter (Figs. 1f and 3b). *PC2* mRNA and protein were seen both in B- and T-cells from naïve and inflamed LNs (Figs. 2b upper panel, d; 3c and d right panel). After cell separation, *PC2* mRNA and protein were detected in both T-helper cells and in cytotoxic T-cells from inflamed and noninflamed LNs (Figs. 2f upper panel; 2h; 3e and f right panel). *β-Actin* transcripts confirmed successful cDNA preparation and PCR in all experiments (lower panels in Fig. 2a, b, e, f).

Measurement of END The amount of END was significantly higher in B- than in T-cells at 24 h (Fig. 1b). These data complement our previous studies in total LNs (Sitte et al. 2007).

Fig. 1 **a** Expression of *Pomc* exon 2–3 mRNA in T- and B-cells from inflamed LNs. Transcripts were amplified by qRT-PCR. Data were calculated using the delta-delta CP method and represent mean \pm SEM fold change of mRNA in cells from inflamed LNs (2 h) over levels in naïve LNs relative to β -Actin expression. Kruskal-Wallis and Dunn's tests. $*P < 0.05$ ($n = 6$ animals per time point). **b** END production in lymph node cell subsets. Cells were isolated from inflamed (24 h) or noninflamed (0 h) LNs. In cell lysates ir-END was determined by RIA; means \pm SEM; Kruskal-Wallis and Dunn's tests. $**P < 0.01$ ($n = 6$ animals per time point). **c** and **d** PC1 and PC2 mRNA in unseparated LN cells. Transcripts were amplified by qRT-PCR. Data were calculated using the delta-delta CP method and represent mean \pm SEM fold change of mRNA in cells from inflamed LNs (6, 24 and 96 h) over levels in naïve LNs relative to β -Actin expression. Kruskal-Wallis and Dunn's tests. $**P < 0.01$; $*P < 0.05$ ($n = 12$ per group). **e** and **f** PC1 and PC2 protein expression in unseparated LN cells. PC1 and PC2 staining of cytospin preparations; mean fluorescence intensity per cell \pm SEM; Kruskal-Wallis and Dunn's tests. $*P < 0.05$; $***P < 0.001$ ($n = 6$ animals per time point)



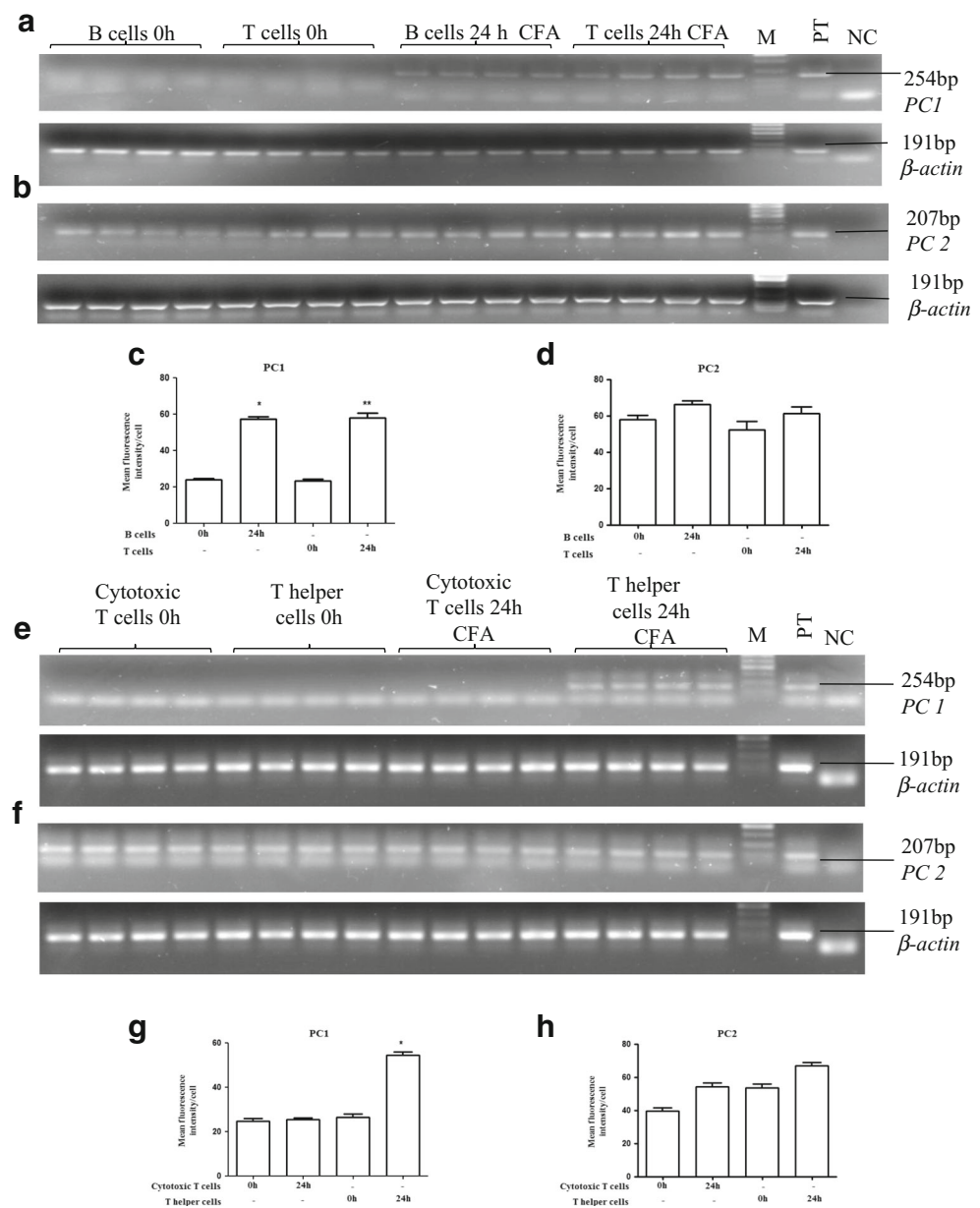
Discussion

We previously found an upregulation of *Pomc* exon 2–3 mRNA in unseparated LN cells in the same model at 2 h of painful inflammation (Sitte et al. 2007). As the earlier study was performed in a mixed cell population, the present study aimed to investigate subsets of LN cells. *Pomc* exon 2–3 mRNA expression was mainly found in B-cells, consistent with our data on END production (see below). However, this does not exclude the presence of *Pomc* mRNA in other cell types at different stages of the inflammatory process or in other models. For example, previous studies detected truncated *Pomc* transcripts in naïve lymphocytes (Buzzetti et al. 1989; Cabot et al. 1997; Lacaze-Masmonteil et al. 1987;

Oates et al. 1988; Przewlocki et al. 1992; Busch-Dienstfertg et al. 2012) or in T-cells (Sitte et al. 2007).

Because processing enzymes are necessary for peptide production, we examined whether lymphocytes express PC1 and PC2. *PC1* mRNA transcripts and protein were detectable in B- and T-cells of inflamed but not of noninflamed LNs. T-helper cells but not cytotoxic T-cells expressed *PC1* mRNA and protein. This could be a quantitative issue, as previously shown in T-helper cells (O’Garra et al. 2011; Buettner et al. 2015), or it might be specific for this particular stage of paw inflammation (24 h). *PC2* transcripts and protein were expressed in all cell subsets of both noninflamed and inflamed LNs. Thus, *PC2* was expressed at similar levels with or without inflammation, while *PC1* was upregulated in inflamed LN. The present data

Fig. 2 **a** and **b** PC mRNA expression in B- and T-cells. Cells were isolated from inflamed (24 h) or noninflamed (0 h) LNs. *PC1*, *PC2* and β -Actin mRNA analyzed by cPCR. Pituitary (PT) cDNA served as positive control ($n = 4$ animals per time point). **c** and **d** PC protein expression in B- and T-cells. Anti-PC1 and anti-PC2 staining of cytospin preparations of T- and B-cells. Mean fluorescence intensity per cell \pm SEM; Kruskal-Wallis and Dunn's tests. $**P < 0.01$; $*P < 0.05$ ($n = 6$ animals per time point). **e** and **f** PC mRNA expression in cytotoxic T-cells and T-helper cells. *PC1*, *PC2* and β -Actin mRNA analyzed by cPCR ($n = 4$ animals per time point). **g** and **h** PC protein expression in cytotoxic T-cells and T-helper cells. Anti-PC1 and anti-PC2 staining of cytospin preparations of cytotoxic T-cells and T-helper cells. Mean fluorescence intensity per cell \pm SEM; Kruskal-Wallis and Dunn's tests. $*P < 0.05$ ($n = 6$ animals per time point)

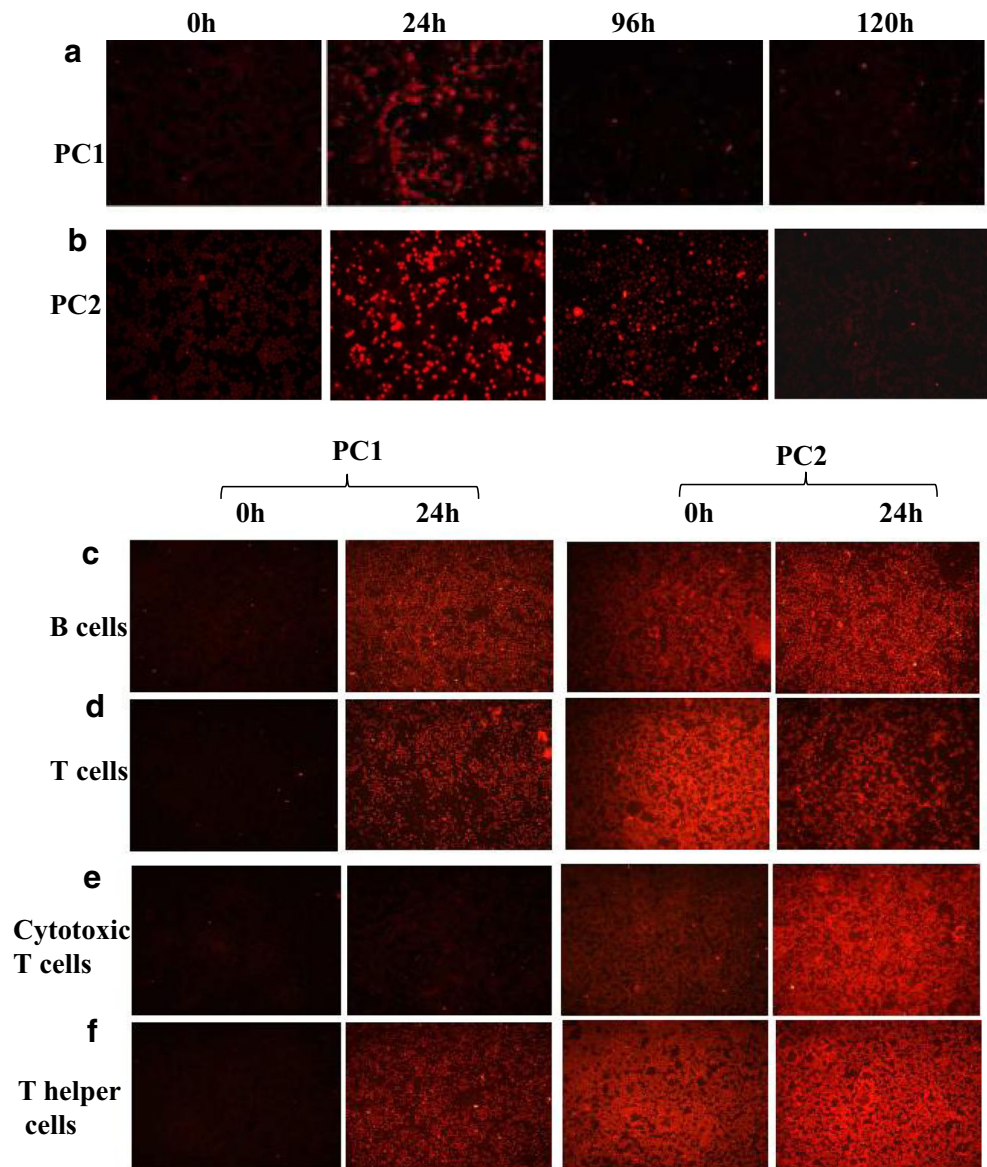


extend our previous findings in circulating leukocytes and inflammatory cells in paw tissue (Mousa et al. 2004) and data on the differential expression of *PC1* and *PC2* mRNA in human pituitary (Tateno et al. 2007). The expression of the two PCs in lymphocytes under inflammatory conditions is largely in line with previous findings (Lansac et al. 2006). Another group investigated the expression of PCs in the spleen of diabetic rats (Nakashima et al. 2001). Their analysis showed basal PC1 in both white (T-lymphocytes) and red (monocytes and macrophages) pulp and demonstrated an increase after streptozotocin-induced diabetes in white pulp, which is in line with our present observations. Novel findings in our study are *Pomc* gene expression, the presence of processing enzymes and of END in LN-derived B-cells at 24 h of paw

inflammation. mRNA expression of *PC1* and *PC2* coincided with the respective protein expression, in line with findings of (Sanchez et al. 2004). Again, the expression of these proteins in B-cells might be specific to the stage of the inflammatory process (24 h) and does not rule out different expression patterns in other models or stages of inflammation.

It was previously shown that END is expressed by peripheral blood B-cells after culture with corticotropin-releasing factor and arginine vasopressin (Kavelaars et al. 1989). These findings suggested that full-length *Pomc* mRNA was expressed in B cells, which is in line with our data. This is also supported by the findings that a mouse B-cell line produced and secreted ACTH (Weigent and Blalock 1987) and that the production of ACTH and END in B-cells was stimulated with

Fig. 3 PC protein expression in cytospin preparations of lymph node cells. Cells were isolated from inflamed (24, 96 or 120 h) or noninflamed (0 h) LNs and stained with anti-PC1 or anti-PC2. Representative anti-PC1 (a) and anti-PC2 (b) staining. c Representative staining of B-cells. d Representative staining of T-cells. e Representative staining of cytotoxic T-cells. f Representative staining of T helper-cells (*n* = 6 animals per time point)



corticotropin-releasing factor and lipopolysaccharide (Harbour et al. 1987, 1991). Our previous findings showed increased cellular END levels in mixed LN cells in vivo (Sitte et al. 2007), and following in vitro stimulation with IL-4 plus conA (Busch-Dienstfertig et al. 2012). We now found a strong upregulation in B-cells of inflamed nodes. These data indicate that B-cells are the predominant subtype expressing *Pomc* at this stage of paw inflammation. Since B-cells expressed PC1 and PC2 alike, it is consistent that we found END elevation in B-cells. Others also investigated the cleavage products of POMC in B-cells (Harbour et al. 1987, 1991). These authors found that stimulating B-cells with corticotropin-releasing hormone or viruses produced ACTH 1–39 and END. Together, these findings suggest that B-cells are a major source of END production at 24 h of paw

inflammation and support that *Pomc* gene expression and precursor processing are independently regulated in lymphocytes. A limitation of the present study is that we did not examine the functional role of B-cell-derived END (e.g. in antinociception) or the expression of *Pomc* in cell subpopulations at later stages of the inflammatory process. This will be the subject of future investigations.

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Compliance with Ethical Standards

Disclosures All authors confirm to have no conflicting financial interests.

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