

# Anti-inflammatory effects of the GABA<sub>B</sub> receptor agonist baclofen in allergic contact dermatitis

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**Abstract:** The gamma amino butyric acid B (GABA<sub>B</sub>) receptor is a G protein-coupled receptor (GPCR) involved in synaptic transmission. Recent data indicate it to be also expressed on immune cells, along with chemokine receptors, which are also GPCRs. As GPCRs can undergo heterologous desensitization, we have examined the ability of baclofen, a GABA<sub>B</sub> receptor selective agonist, to interfere with the function of pro-inflammatory chemokine receptors known to be upregulated in cutaneous inflammation. *In vitro*, baclofen reduces chemotaxis of human peripheral blood mononuclear cells towards CCL2, CCL5, CXCL10, CXCL2 and CX3CL1 in a dose-dependant manner. Protein kinase C inhibitors calphostin C and G0 6976 could reverse this effect,

pointing towards the involvement of both calcium-dependent and -independent protein kinase C in baclofen-induced inhibition of chemokine receptors. In an *in vivo* model of contact hypersensitivity in C57BL/6 mice, intraperitoneal injection of baclofen markedly alleviated signs of inflammation as well as recruitment of neutrophils, monocytes and lymphocytes into the skin. This study demonstrates a new role for the GABA<sub>B</sub> receptor in inflammation, making it a potential new therapeutic target to treat inflammatory skin diseases.

**Key words:** allergic contact dermatitis – G protein-coupled receptor – gamma amino butyric acid receptor – protein kinase C

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## Introduction

Chemokines and their receptors are known to promote leucocyte migration, recruitment and activation (1). Dysregulation of the chemokine system has been incriminated in a wide spectrum of inflammatory diseases including metabolic as well as autoimmune disorders. Interfering with the chemokine receptor system appears to be an attractive strategy to prevent inflammatory diseases (2). Several clinical trials based on small chemokine peptide antagonists, modified chemokine peptides or chemokine receptor blocking antibodies, are currently being performed.

Chemokine receptors belong to the G protein-coupled receptor superfamily (GPCRs). Like several GPCRs, their activity is tightly regulated at different points of the signalling pathways. At the receptor level, prolonged activation of a chemokine receptor by its agonist results in downregulation of receptor signalling through phosphorylation of the C-terminal tail and intra-cellular loop, a process called 'homologous desensitization' that prevents

potentially damaging effects of continuing inflammation. Chemokine receptor activation can also be impaired after stimulation of another GPCR, a mechanism termed 'heterologous desensitization' which appears to have significant consequences in pathophysiological situations (3,4). For example, heterologous desensitization of chemokine receptors by met-enkephalin and morphine, agonists of opioid receptors also belonging to the GPCR superfamily, was reported to account for the defects in host defense seen with opiate abuse (5). This mechanism has been shown to substantially interfere with the function of B (6) and T lymphocytes (7).

Like chemokine receptors, the gamma amino butyric acid (GABA) B receptor belongs to the GPCR family. It is activated by GABA, known as one of the most important inhibitory neurotransmitters in synaptic transmission. The GABA<sub>B</sub> receptor is widely expressed and distributed in the central nervous system where it has been extensively studied this last decade (8,9). In recent years, expression of GABA receptors on cells of the immune system and a

possible link to inflammatory responses have increasingly been recognized (10,11).

In this study, we analysed the cross-talks between the GABA<sub>B</sub> receptor and chemokine receptors known to be upregulated in cutaneous inflammation (12,13). As a clinically relevant GABA<sub>B</sub> receptor agonist, we used baclofen, which is currently approved for the treatment of *spasticity* in patients with spinal cord injury, cerebral palsy, traumatic brain injury, multiple sclerosis and other disorders.

## Methods

### Reagents

Chemokines were obtained from PeproTech (London, UK). Baclofen as well as the protein kinases A and C (PKC) inhibitors Calphostin C and Go6976 were purchased from Sigma (Steinheim, Germany). The following antibodies were used: a polyclonal rabbit anti-CD45 antibody (DakoCytomation, Denmark), clone 7/4 to stain neutrophils (Serotec, Oxford, Great Britain), MOMA-2 against monocytes/macrophages (Serotec) and LO-DNP-11 as isotype control (Serotec). Corresponding fluorescein isothiocyanate (FITC-) or phycoerythrin (PE)-labelled secondary antibodies were purchased from Pharmingen (BD Biosciences, Heidelberg, Germany). Detection of cytokines was performed by using the cytokine detection kit from Qiagen (Hilden, Germany), with quantification through flow cytometry according to the company's specification; this assay is based on flow cytometric analyses of labelled beads. Flow cytometric analyses were performed using a BD FACScan Cytometer (Becton Dickinson, Mountain View, CA, USA).

### Cell isolation and culture conditions

Human peripheral blood monocyctic cells (PBMCs) were prepared by Ficoll-Hypaque density gradient sedimentation (Pharmacia, Uppsala, Sweden) from buffy coats obtained from the Department for Transfusion Medicine, Frankfurt, and adjusted to  $1 \times 10^6$  cells/ml in AIM-V cell culture medium, supplemented with 10% foetal calf serum (Life Technologies, Paisley, UK) and activated with phytohemagglutinin at 1 µg/ml when indicated.

### In vitro chemotaxis assay

Chemotaxis was assayed in a 96-well chemotaxis chamber (NeuroProbe, Cabin John, MD, USA). Briefly, the lower compartment of the chamber was loaded with the chemo-attractant diluted in AIM-V medium without serum. The upper compartment of the chamber was loaded with human PBMCs (500 000 cells/well), and the two compartments were separated by a 5-µm pore-size filter (NeuroProbes, Gaithersburg, MD, USA). Cells were allowed to

migrate for 2 h at 37°C. After brief centrifugation of the plate (1200 rpm), the filter was removed, and cells present in the lower well compartment were collected and counted by flow cytometry. Results are expressed as migration index (number of cells in the lower well after incubation with the indicated compounds divided by the number of cells in the lower well in the negative controls, i.e. untreated cells) and are the result of six independent experiments. For experiments using the PKC inhibitors, cells were pre-incubated for 10 min with baclofen  $10^{-5}$  M and then exposed to either Calphostin C at 200 nM or Go 6976 at 10 nM, and subsequently seeded on the upper level of the chemotaxis chamber.

### Induction of allergic contact dermatitis in C57BL/6 mice

Animal experiments were approved by the local authority (Regierungspräsidium Darmstadt, F79/38). Female C57BL/6 mice, aged 6–10 weeks, were purchased from Charles River (Sulzfeld, Germany) and housed with food and water *ad libitum*. Mice were sensitized by applying 75 µl of 0.5% 2,4-dinitrofluorobenzene (DNFB) (Sigma, Seelze, Germany) solution (4 vol acetone/1 vol olive oil) on the back at day 1. At day 5, 20 µl of 0.25% DNFB was applied on the left ear, and the vehicle as negative control on the right ear. At day 6, the resulting ear swelling as a result of the developing contact dermatitis was quantified using a spring-loaded micrometer (Mitutoyo, Neuss, Germany) in a blinded fashion. Ear swelling responses of DNFB-challenged ears were compared to the response of the vehicle-treated ear in sensitized animals and were expressed as centimetres  $\times 10^{-3}$  (mean  $\pm$  SD). Data were generated from four independent experiments, using six animals per group each; a total of 96 mice were used for these experiments.

### Evaluation of the inflammatory infiltrate in the murine allergic contact dermatitis reaction by immunohistochemistry

Five-micrometre tissue sections from DNFB- and vehicle-treated ears were stained with H&E. In parallel, the inflammatory infiltrate was analysed by immunohistochemistry, using cryostat sections of the same ears. In brief, cryostat sections were stained after fixation and permeabilization with primary antibodies specific for either CD45, neutrophils or monocytes/macrophages, at a dilution of 1:100. Staining was visualized by addition of appropriate secondary antibodies (goat-anti rat-FITC or -PE) at a dilution of 1:1000. Specimen stained with appropriate isotype control antibodies served as negative controls. Quantification of stained cells within the examined tissues was performed with Image J<sup>®</sup> program and is the result of five to six representative samples.

## Statistics

All results are expressed as mean  $\pm$  SEM of replicate samples. Significance was evaluated using SigmaStat® (SPSS, Chicago, Illinois). *P*-values of  $<0.05$  were considered as statistically significant.

## Results

### Baclofen induces heterologous desensitization of chemokine receptors

To investigate possible cross-talks between the GABA<sub>B</sub> receptor and pro-inflammatory chemokine receptors, we performed chemotaxis assays with human peripheral blood mononuclear cells (PBMCs) and analysed the impact of baclofen on the migration induced through chemokine receptors CCR2, CCR5, CCR10, CXCR3 and CXCR4. As expected, the respective ligands used in our assays, namely CCL2, CCL5, CX3CL1, CXCL9, CXCL10 and CXCL12 induced chemotaxis in a dose-dependant manner (Fig. 1). We observed a similar effect with the GABA<sub>B</sub> receptor agonist baclofen, thus confirming a previous report (11). However, when PBMCs were pretreated with baclofen at  $10^{-5}$  M, migration of cells towards the above-mentioned chemokines was significantly reduced by up to 70% (Fig. 1). Interestingly, the inhibitory effect of baclofen was less pronounced in the case of CCL5 which induces coupling to both G $\alpha$ i but also G $\alpha$ s protein, whereas the other chemokines mainly activate G $\alpha$ i protein (14). This may point towards the inhibitory effect of baclofen at least preferentially involving the G $\alpha$ i signalling pathway.

The chemotaxis blocking effect could not be attributed to a possible toxicity of baclofen as no difference in cell

viability was observed up to 72 h after baclofen exposition at  $10^{-5}$  M (data not shown).

The observed blocking effect of baclofen could also be explained by chemokine receptor internalization. We therefore performed flow cytometric analyses on leucocytes incubated with different concentrations of baclofen. However, we could not detect any changes in chemokine receptor surface expression on these cells; in contrast, incubation with chemokines did result in receptor internalization after 30 min (data not shown).

Taken together, our results suggest that baclofen interferes with the function of pro-inflammatory chemokine receptors linked to the G $\alpha$ i signalling pathway through heterologous desensitization, without affecting chemokine receptor internalization.

### Baclofen-mediated heterologous desensitization of chemokine receptors involves calcium-dependent PKC isoenzymes

Heterologous chemokine receptor desensitization has previously been reported to involve PKC. To clarify the mechanism through which baclofen causes heterologous desensitization of chemokine receptors, we first repeated the chemotaxis experiments in the presence of the general PKC inhibitor Calphostatin C. As this agent was able to almost completely reverse the baclofen-induced inhibitory effect at 200 nM (Fig. 2), we repeated the experiments using Go 6976, a calcium-dependent classic PKC inhibitor, and obtained similar results (Fig. 2). These data suggest that calcium-dependent PKC isozymes are involved in GABA<sub>B</sub> receptor-mediated heterologous desensitization of chemokine receptors.

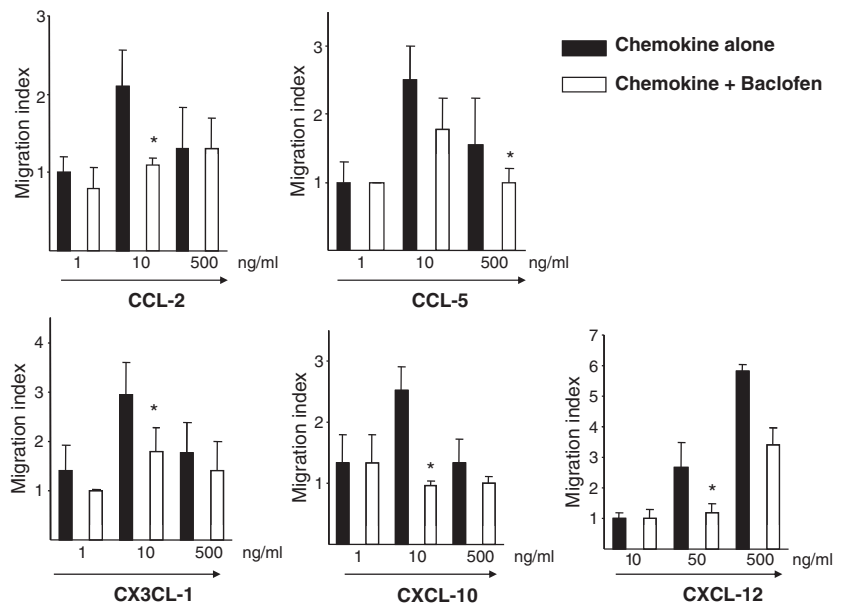


**Figure 1.** Gamma amino butyric acid B receptor agonist induces heterologous desensitization of chemokine receptors. Baclofen inhibits chemokine-induced chemotaxis of peripheral blood mononuclear cells (PBMCs) in a dose-dependent manner. Data represent the result of six independent experiments and are expressed as migration index  $\pm$  SEM. \*Indicates statistical significance ( $P < 0.05$ ).

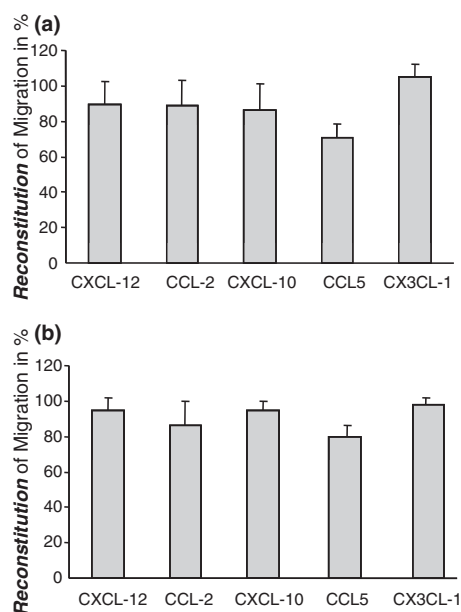


**Figure 2.** Role of protein kinases A and C (PKC) in baclofen-mediated heterologous desensitization of chemokine receptors. Calphostin C (200 nM), a general PKC inhibitor, reconstitutes baclofen-induced inhibition of chemotaxis towards numerous chemokines (50 ng/ml, a). Go 6976, a calcium-sensitive PKC inhibitor, has similar effects (b). Data represent the result of three independent experiments  $\pm$  SEM.

### Baclofen reduces production of tumor necrosis factor alpha (TNF- $\alpha$ ) in activated PBMCs

As cytokines are essential effector molecules in inflammation, we also analysed the effects of baclofen on secretion of pro-inflammatory cytokines by PBMCs. Flow cytometric measurements of supernatants from mitogen-stimulated PBMC cultures showed no significant changes of secreted interferon (IFN)- $\gamma$ , interleukin (IL)-2, IL-4, IL-5, IL-6 and IL-10 (Fig. 3). In contrast, TNF- $\alpha$  secretion was reduced by about 40% after baclofen treatment (Fig. 3).

### Anti-inflammatory efficacy of baclofen in a murine model for allergic contact dermatitis

So far, our data document that baclofen interferes with leucocyte chemotaxis through heterologous desensitization of several chemokine receptors and secretion of TNF- $\alpha$  from activated PBMCs *in-vitro*, thus targeting key aspects of inflammation. To investigate baclofen's anti-inflammatory efficacy *in vivo*, we used it in a murine model for allergic contact dermatitis. Female C57BL/6 mice were sensitized with the contact allergen DNFB and challenged 5 days later on the right ear. The ear swelling response after 24 h serves as a quantitative measure for the resulting allergic contact dermatitis, with the left ear (challenged with the vehicle) as a negative control.

In this model, mice sensitized with DNFB developed a profound inflammatory response upon challenge with

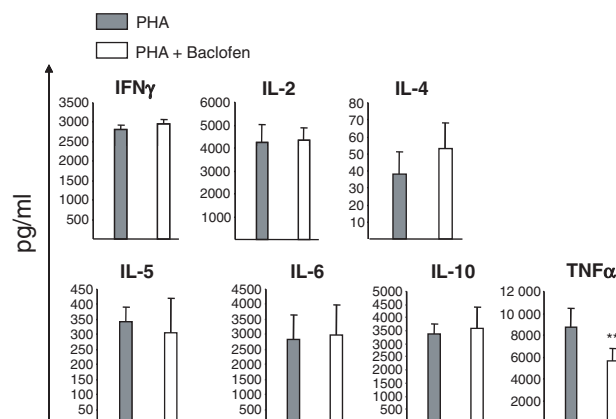


**Figure 3.** Effect of baclofen on cytokine production. Freshly isolated human peripheral blood mononuclear cells (PBMCs) previously activated with PHA (1  $\mu$ g/ml) were exposed to baclofen at  $10^{-5}$  M for 48 h. Among the cytokines measured, only tumor necrosis factor alpha secretion was reduced significantly (\*\* $P < 0.01$ ). Cytokines produced in the culture medium were evaluated by CBA assay; data are expressed in ng/ml  $\pm$  SEM.

DNFB compared to vehicle challenged ears. In contrast, mice receiving a single intraperitoneal injection of baclofen at doses of 0.01–1 mg/kg body weight exhibited a substantially weaker allergic contact dermatitis, reflected by a significantly reduced ear swelling response ( $P < 0.001$ ) (Fig. 4). Immunohistochemical analyses of ear tissue revealed that this effect was paralleled by a substantial reduction of infiltrating neutrophils, monocytes and CD45<sup>+</sup> lymphocytes in baclofen-treated versus control mice (Fig. 5). Taken together, these findings document that baclofen is a potent anti-inflammatory compound *in vivo* capable of preventing the manifestation of allergic contact dermatitis in a mouse model. This effect is at least in part due to interference with leucocyte recruitment to the site of inflammation, strengthening our *in-vitro* studies.

### Discussion

Evidence for a possible cross-talk between the GABA<sub>B</sub> receptor and pro-inflammatory chemokine receptors was initially established by documenting inhibitory effects of the GABA<sub>B</sub> receptor agonist baclofen on chemokine-induced chemotaxis. This effect was most pronounced in the case of chemokines mainly activating G $\alpha$ i protein, but less obvious in the case of CCL5 which induces coupling to both G $\alpha$ i but also G $\alpha$ s protein, whereas the other chemokines mainly activate G $\alpha$ i protein (15), thus pointing towards the inhibitory effect of baclofen at least preferentially involving the G $\alpha$ i signalling pathway. Toxic effects of baclofen as well as chemokine receptor internalization could be ruled out as possible causes for this effect by the

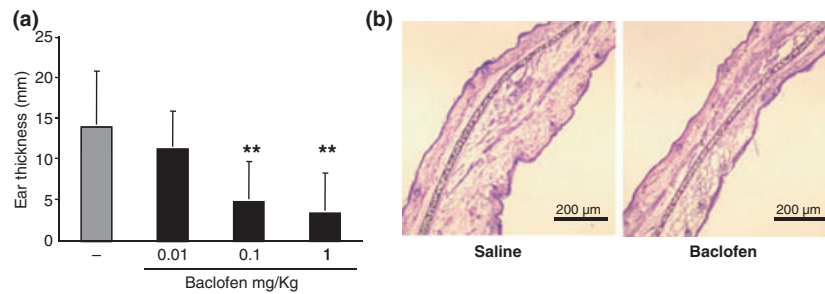
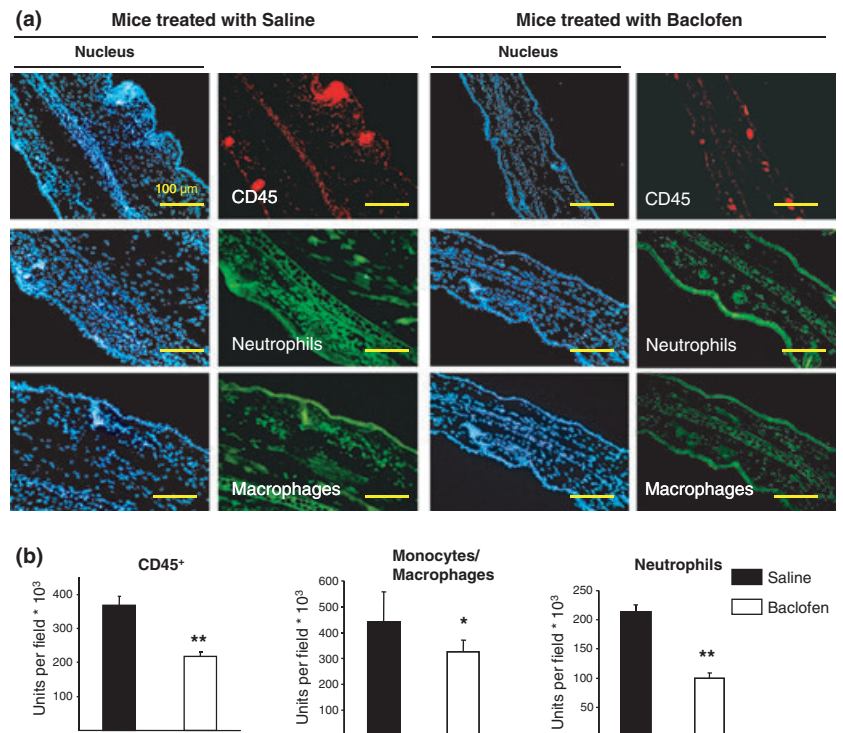


**Figure 4.** Baclofen shows anti-inflammatory efficacy in a murine model for allergic contact dermatitis. (a) Ear swelling response in mice treated with baclofen. C57Bl/6 mice were sensitized with dinitrofluorobenzene (DNFB) on day 1 and challenged with DNFB on day 5 after receiving a single intraperitoneal injection of either baclofen (0.01–1 mg/kg) or saline. Baclofen treatment reduced the ear swelling response in a dose-dependent manner. The results are shown as mean ear swelling at day 6  $\pm$  SEM of a representative experiment with six mice per group. Statistical significance was evaluated using the Student t-test (\*\* $P < 0.01$ ). (b) Histology of allergic contact dermatitis in the mouse ear. The photos show representative H&E-stained histologies of ear tissue from mice challenged with DNFB in the absence (left panel) or presence (right panel) of baclofen. Baclofen-treated mice show substantially less oedema and inflammatory infiltrate.

**Figure 5.** Baclofen reduces the inflammatory infiltrate in murine allergic contact dermatitis. (a) Immunohistochemical stainings of ear cryosections from dinitrofluorobenzene (DNFB)-sensitized mice receiving either saline (left) or baclofen (right) prior to DNFB challenge. The photos show nuclear staining as well as staining for neutrophils, macrophages/monocytes and CD45<sup>+</sup> lymphocytes, which are more abundant in saline-treated mice. Data are representative of four experiments. (b) Quantification of the inflammatory infiltrate. The density of the infiltrate was quantified, using a computer program (image J®). Baclofen treatment resulted in a significantly reduced inflammatory infiltrate in the allergic contact dermatitis elicited by DNFB. Data are expressed as cells per field (\* $P < 0.05$  and \*\* $P < 0.01$ ). Mean  $\pm$  SEM are indicated, generated from six representative slides of ears from all mice of one representative experiment ( $n = 6$  per group). Scale bars represent 100 µm.



appropriate controls, thus pointing towards heterologous desensitization as the underlying mechanism.

Heterologous chemokine receptor desensitization involves PKC. Calcium flux, a potent activator of PKC, has been considered essential for heterologous desensitization of chemokine receptors in PBMCs (14). Noteworthy, calcium-independent PKCs have been reported to be involved in heterologous desensitization of leucocyte chemokine receptors (16). As both, the general PKC inhibitor Calphostatin C as well as the calcium-dependent classic PKC inhibitor Go 6976 almost completely reversed the baclofen-

induced inhibition, GABA<sub>B</sub> receptor-mediated heterologous desensitization of chemokine receptors may primarily involve calcium-dependent PKC isozymes (14).

When analysing effects of baclofen on the secretion of relevant inflammatory effector cytokines by leucocytes, we observed a selective suppression of TNF- $\alpha$ . As TNF- $\alpha$  is considered a key cytokine in numerous chronic inflammatory diseases (17), and blockade of TNF- $\alpha$  has proven effective in the treatment of several of these (18), the ability of baclofen to inhibit TNF- $\alpha$  secretion of activated PBMCs may contribute to a possible anti-inflammatory efficacy

*in vivo*. We therefore tested this in a murine model for allergic contact dermatitis and were able to document profound and dose-dependent anti-inflammatory effects, including a significantly reduced ear swelling response and a substantial reduction of infiltrating leucocytes in baclofen-treated versus control mice. These findings document that baclofen is a potent anti-inflammatory compound *in vivo*, capable of preventing the manifestation of allergic contact dermatitis in a mouse model. This effect is at least in part due to interference with leucocyte recruitment to the site of inflammation, strengthening our *in vitro* studies.

Interference with leucocyte migration in general and chemokine-mediated chemotaxis in particular has been proven to be an effective means to treat inflammation, namely of the skin (12,19–21). Based on these observations, several compounds are currently under clinical development (1,2,22). In practice, selective blockade of chemokine receptors has proved difficult to inhibit inflammatory diseases, whereas combination of antagonists seems to be giving promising results. Therefore, interference with the chemokine receptor signal transduction pathway may be an attractive alternative as it can target not only one but several chemokine receptors (2). In this context, strategies like heterologous desensitization might become an approach worthwhile to be evaluated not only in pre-clinical models but also in clinical development. Our observation supports the idea that contact dermatitis may be treated by targeting the GABA<sub>B</sub> receptor.

In summary, our studies suggest that baclofen interferes with leucocyte chemotaxis through heterologous desensitization of several chemokine receptors. This mechanism involves calcium-dependent PKC isoenzymes and signalling through G protein  $\alpha_i$ . Additionally, TNF- $\alpha$  secretion by PBMCs is also reduced by baclofen. The former mechanism contributes at least in part to baclofen's anti-inflammatory efficacy in a murine model for allergic contact dermatitis. These observations point towards an important role of the GABA<sub>B</sub> receptor in inflammation, making it a potential target for novel anti-inflammatory strategies.

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