Oxymetazoline modulates proinflammatory cytokines and the T-cell stimulatory capacity of dendritic cells

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Abstract: The nasal decongestant oxymetazoline (OMZ) is frequently used in the topical treatment of rhinitis/sinusitis. As proinflammatory cytokines play a critical role in the development and maintenance of local inflammation, the aim of this study was to investigate the influence of OMZ on immune cells in order to diminish the mucosal infiltration of the nose. Peripheral blood mononuclear cells (PBMC) from buffy coats of healthy volunteers were isolated and stimulated in the presence or absence of OMZ. In addition, monocyte-derived dendritic cells (DC) were generated and different concentrations of OMZ were added. DC phenotype and their T-cell stimulatory properties were analysed. The vasoactive substance OMZ showed a concentration dependent inhibitory effect on T-cell activation as well as a dominant effect on T-cell stimulatory properties of DC. Low concentrations of OMZ inhibited the proliferation of polyclonally activated T cells. In addition, secretion of proinflammatory mediators such as the cytokines interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-6 and IL-8 were inhibited in the presence of physiological doses of OMZ. Interestingly, the addition of IL-6 to DC-T-cell coculture was able to completely restore T-cell proliferation. In conclusion, these findings indicate that the anti-inflammatory properties of OMZ are partially mediated by the inhibition of proinflammatory cytokines as well as reduced T-cell stimulatory capacity of DC resulting in a repressed stimulation of T cells. Therefore, the therapeutic benefit of OMZ can be explained in part by its immunomodulating effects in the topical treatment of nasal inflammation.

Key words: dendritic cells – immunomodulation – oxymetazoline – proinflammatory cytokines – rhinitis

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Introduction

The nasal mucosa is the most commonly infected tissue in the body. Most adults are afflicted by two to four symptomatic upper respiratory tract infections (URTIs) every year. In children, these symptomatic infections are even more frequent. About 80% of acute rhinitis infections are caused by viruses, the majority being rhinoviruses (1). Other species frequently causing cold symptoms are coronaviruses, (para)influenza viruses, respirators syncytial virus and adenoviruses (2,3). Despite its frequent occurrence, little is known about the pathogenesis of URTIs. It is currently believed that the symptoms of an URTI are the result of the host’s inflammatory immune response to the virus rather than a direct viral cytotoxic effect (4,5). This concept is supported by various observations in vitro and in vivo demonstrating that nasal secretions during rhinovirus infection become enriched in albumin, kinins, immunoglobulin-G (IgG) and proinflammatory cytokines. This is usually accompanied by a predominantly neutrophilic cellular infiltrate (4–6). The virus-induced strong inflammatory response is mediated by specific proinflammatory cytokines released by, e.g. respiratory syncytial virus-infected human pulmonary epithelial cells (7). Subsequently, proinflammatory cytokines activate various cell populations and strongly induce adhesion molecule expression which facilitates transendothelial migration of inflammatory cells. As regulators of chemotaxis, proliferation and the activity of inflammatory cells, these mediators play a central role in the pathogenesis of URTIs. This assumption has been supported by a clinical study (8) showing that proinflammatory mediators including bradykinin, interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α) and myeloperoxidase rose significantly with the onset of rhinitis symptoms and reached a plateau level over the ensuing symptomatic days. Based on such findings, it is reasonable to assume that
epithelial cell-derived proinflammatory cytokines initiate an adhesion cascade and then activate the recruited inflammatory cells. These activated cells again secrete large amounts of cytokines, resulting in an amplification of the inflammatory process.

Dendritic cells (DC) are professional antigen-presenting cells essential for the induction of strong T-cell responses (9,10). They are located in an immature form in non-lymphoid tissues and are specialized to capture and process antigen. However, release of inflammatory factors during local infections leads to a functional maturation of DC resulting in an increased migratory and T-cell stimulatory capacity (11,12). Mature DC actively migrate into the regional lymph nodes where they prime and boost antigen-specific T cells (13,14). Therefore, DC play a major role in the process of inflammation. In addition, DC activated through bacterial or viral components release large amounts of factors that again boost the inflammation (15). Considering these facts, our attempt was to investigate whether oxymetazoline hydrochloride (OMZ) influences the inflammatory potential of human immune cells, as both DC and T cells are known to express alpha-adrenergic receptors on the cell surface (16,17), and to determine its possible anti-inflammatory/immunomodulating activities. OMZ is a sympathomimetic imidazoline derivative commonly used for the symptomatic treatment of acute (viral) rhinitis, allergic rhinitis, vasomotor rhinitis, sinusitis and syringitis. Topical application to the nasal mucosa results in a vasoconstriction lasting up to 12 h causing decongestion, reduced rhinitis symptoms and a shortage of the duration of the cold (18).

We demonstrate that OMZ significantly reduces the inflammatory potential of mononuclear cells of the peripheral blood resulting in a decreased proliferation capacity of T cells. Furthermore, non-toxic concentrations of OMZ are able to inhibit the immunostimulatory capacity of human DC including their cytokine production without influencing their phenotype. This functional inhibition of DC through OMZ may explain in part the anti-inflammatory effects of OMZ seen in vivo.

Materials and methods

Proliferation assays

Peripheral blood mononuclear cells (PBMC) were isolated fromuffy coats of healthy volunteers (n = 4) as described before (19) and cultured under serum-free conditions (2 × 10^5/well) in X-VIVO-15 (Cambrex, Verviers, Belgium) in 96-well plates (Corning/Costar, Bodenheim, Germany) in the presence of increasing concentrations of OMZ (kindly provided by Merck Selbstmedikation GmbH, Darmstadt, Germany; range: 0.00001–0.2%). For stimulation of T cells 1 μg/ml PHA – a specific polyclonal T-cell activator – was added (Sigma-Aldrich, Taukirchen, Germany). Alternatively, cells were stimulated using 1 μg/ml anti-CD3 (OKT-3) and 2 μg/ml anti-CD28 (CD28.2; Pharmingen, San Diego, CA, USA) mAb. PBMC were cultured for 4 days (triplicates for each condition) plus an additional 16 h in the presence of 37 kBq/well [3H]-thymidine (Amersham, Braunschweig, Germany).

To analyse the immunostimulatory capacity of DC, CD4+ T lymphocytes were separated immunomagnetically (19,20) from PBMC using limited amounts of CD4-Microbeads (Miltenyi, Bergisch-Gladbach, Germany) according to the manufacturer’s protocol. Purity of CD4+ T cells was >98% as determined by fluorescence activated cell sorting (FACS) analysis (data not shown). freshly isolated CD4+ T lymphocytes (1 × 10^5) were stimulated with allogeneic OMZ-treated or untreated DC (1 × 10^5) in 96-well plates for 4 days plus 16 h in the presence of 37 kBq/well [3H]-thymidine (triplicates for each condition). In a second step, CD4+ T cells were stimulated with allogeneic untreated DC at a DC:T-cell ratio of 1:10 and OMZ-treated DC were added in increasing concentrations (OMZ-DC:DC ratio of 1:1000, 1:100, 1:10, 1:1). Transwell experiments were performed in 24-well plates as described previously (19). Briefly, freshly isolated CD4+ T cells (1 × 10^5) were stimulated with allogeneic untreated DC (1 × 10^5). Additionally OMZ-treated DC (1 × 10^5) were placed in transwell chambers (Millicell, 0.4 μm; Millipore; Schwalbach, Germany) in the same well. After 4 days of culture, 3 × 200 μl per well of each culture were transferred to three individual wells of 96-well plates. Proliferation was measured after an additional 16 h pulse with [3H]-thymidine using a liquid scintillation counter.

To analyse the influence of reduced cytokine production by OMZ-DC on T cell proliferation, CD4+ T cells were stimulated using allogeneic OMZ-treated DC (DC:T-cell ratio 1:10). IL-6 (100 pg/ml), TNF-α (100 pg/ml) and IL-8 (500 pg/ml) were added to the co-cultures. Proliferation was measured after 4 days plus an additional 16 h pulse with [3H]-thymidine.

Viability of PBMC and CD4+ T cells in the presence of OMZ was determined by trypan blue exclusion (data not shown).

Generation of monocyte-derived DC

Dendritic cells were generated as described previously (20,21). Briefly, adherent monocytes were cultured in X-VIVO-15, 800 U/ml GM-CSF and 1000 U/ml IL4 (both from ImmunoTools, Friesoythe, Germany). Cells were fed on day 3 (±1 ml) with X-VIVO-15, 1600 U/ml GM-CSF and 1000 U/ml IL-4. On day 6, immature DC were transferred to fresh culture plates and stimulated with 10 ng/ml IL-1β, 10 ng/ml TNF-α, 1000 U/ml IL-6 (all from
Flow cytometric analysis
The following antibodies (mAb) were used for immuno-staining: mouse IgG: CD80 (MAb104), CD86 (IT2.2), CD83 (HB15A) (Coulter/Immunotech, Hamburg, Germany), rat IgG: anti-HLA-DR (YE2/36HLK) (Sero tec/Camon, Wiesbaden, Germany), mouse and rat-specific iso-types (Coulter/Immunotech). Conjugated secondary reagents included fluoresceinthiocyanat (FITC)-conjugated goat-anti-mouse-IgG and phycoerythrin (PE)-conjugated goat-anti-rat IgG (Jackson Immunoresearch, West Grove, PA, USA).

For phenotyping of DC, cells were washed in cold PBS + 0.5% human serum albumin (HSA) and incubated for 20 min at 4°C with each mAb (5 μg/ml). After washing with cold PBS/HSA the cells were incubated with FITC- and PE-conjugated second-step mAb for 20 min. at 4°C, washed three times and analysed by flow cytometry (FACScalibur, Cellquest software; Becton Dickinson, Mountain View, CA, USA), data being collected on 5000–10 000 viable cells.

Annexin-V-FITC staining was performed according to the manufacturer’s protocol (Annexin-V-FITC staining kit; BD Pharmingen, Hamburg, Germany). Cells (1 × 10⁶) were washed in cold binding buffer and incubated with Annexin-V-FITC for 15 min at room temperature in the dark. After incubation cells were diluted using binding buffer, stained subsequently with propidium iodide solution and analysed by flow cytometry, data being collected on 100 000 cells.

Cytokine ELISA
Commercially available ELISA specific for the human IL-6, IL-1β, TNF-α, IL-8, IL-12 and PGE₂ (all from Diac lone, Besancon, France) were used as indicated by the manufacturer.

Statistical analysis
Statistical analysis comparing two different groups was performed using the Student’s t-test.

Results
Oxymetazoline suppresses the proliferation and cytokine production of peripheral blood mononuclear cells in a dose-dependent manner
To analyse the effect of OMZ on PBMC, cells were stimulated with the polyclonal stimulus PHA (1 μg/ml). Briefly, besides its mitogenic function on T cells, PHA is known to activate antigen-presenting cells such as monocytes. In addition to PHA, different concentrations of OMZ were added simultaneously to the cultures. At concentrations above 0.002%, OMZ showed toxic effects on PBMC. However, as shown in Fig. 1, non-toxic concentrations of OMZ inhibited the polyclonal activation of T cells in a dose-dependent manner. Similar results were obtained after stimulation with anti-CD3/anti-CD28 mAb (Fig. 2) demonstrating that reduced T-cell proliferation is not only due to an indirect effect of OMZ on T cells through modulation of proinflammatory cytokines produced by activated PBMC but that OMZ also directly acts on T-cell proliferation.

Based on the fact that T-cell proliferation was markedly reduced in the presence of OMZ, we next examined the production of cytokines. Therefore, supernatants of activated PBMC were collected 48 h after stimulation and the concentration of proinflammatory cytokines was analysed. Cytokine production was significantly reduced by OMZ in a dose-dependent manner. The prominent proinflammatory cytokine IL-6 produced after PHA stimulation was inhibited even at very low concentrations of OMZ (0.0005%). Similar results were obtained for TNF-α and...
IL-1β (Fig. 3). Activation of PBMC with PHA also resulted in the production of high amounts of IL-8. Addition of OMZ at concentrations >0.0002% inhibited IL-8 production of PBMC significantly.

To rule out that low doses of OMZ exerted their immunosuppressive effects by being subtoxic to T cells exposed for a prolonged period of time, apoptosis of T cells was analysed by Annexin-V-FITC and propidium iodide staining after 7 days of culture. However, no increase in apoptosis induced by low doses of OMZ (>0.0002%) could be observed in these cultures (data not shown).

These data demonstrate that proliferation of T cells and production of proinflammatory cytokines by activated PBMC are significantly inhibited by non-toxic concentrations of OMZ.

**Modulation of DC maturation and function in the presence of OMZ**

As professional antigen-presenting cells DC play a major role in the induction of inflammatory T-cell responses. To investigate the immunomodulatory effects described above in more detail, we analysed the influence of OMZ on DC maturation. Therefore, different concentrations of OMZ were added to cultures of monocyte-derived DC during terminal maturation. As presented in Fig. 4, OMZ in non-toxic doses (0.001%) did not show any influence on the well-known upregulation of costimulatory molecules such as CD80 and CD86 or the differentiation marker CD83 (11) when compared with untreated DC, showing that the phenotype of mature DC is not altered in general by the presence of OMZ during terminal differentiation. However, DC matured in the presence of OMZ showed a remarkably reduced T-cell stimulatory capacity detectable in an allogeneic mixed lymphocyte reaction (MLR) (Fig. 5a) when compared with untreated DC. Even addition of 0.00005%
OMZ to maturing DC suppressed the T-cell stimulatory properties of DC significantly. To rule out that the observed inhibition of T-cell proliferation resulted from contaminating OMZ in the MLR, transwell experiments were performed. After physical separation in transwell chambers (Fig. 5b), OMZ-treated DC could not suppress the proliferation of alloreactive T cells stimulated by untreated DC showing that the observed effect was not mediated by an OMZ contamination or other soluble factors induced by OMZ. However, direct addition of OMZ-treated DC to the cultures markedly inhibited the MLR in a dose-dependent manner (Fig. 5c) showing again that OMZ suppresses the stimulatory properties of DC significantly.

In a next step, the cytokine production of DC matured in the presence of non-toxic concentrations of OMZ was analysed. Therefore, DC were generated as described above. On day 7 (1 day after the addition of the maturation cocktail), cells were washed intensively and recultured in medium alone without any cytokines. The supernatants were collected after 24 h and proinflammatory cytokines were detected by ELISA. As shown in Fig. 6a, production of IL-8 was significantly reduced in the presence of OMZ (0.001%) compared with the IL-8 production of untreated DC. In addition, the synthesis of TNF-α and IL-6 by DC was inhibited in the presence of OMZ. In contrast, production of IL-1β, IL-12 and PGE2 was not altered (data not shown).

In order to analyse whether the suppressed cytokine production of OMZ-treated DC is relevant for their reduced immunostimulatory capacity we added the above-mentioned cytokines to DC-T-cell co-cultures. Whereas the addition of TNF-α and IL-8 did not show any effect (data not shown), addition of IL-6 completely restored T-cell proliferation (Fig. 6b).

Taken together, these data indicate that OMZ has modulatory effects on the stimulatory properties of DC by the selective inhibition of proinflammatory cytokines, especially the inhibition of IL-6, resulting in a reduced T-cell stimulatory capacity of OMZ-modulated DC.

**Discussion**

Upper respiratory tract infection is the most frequent infectious disease in man characterized by strongly elevated levels of proinflammatory cytokines in nasal lavages during acute infection (6,7). In the current study we demonstrate that OMZ, commonly used for the symptomatic treatment of acute rhinitis, significantly reduces the production of proinflammatory cytokines by mononuclear blood cells as well as the proliferative capacity of T cells. In addition, OMZ in non-toxic concentrations is able to inhibit the immunostimulatory capacity of human DC without influencing their mature phenotype.

Several studies demonstrated a significant rise in proinflammatory cytokines in the nasal fluid during the infectious episode with high but stable levels during the symptomatic days (6,7,22,23). These proinflammatory

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**Figure 5.** Reduced stimulatory capacity of oxymetazoline-treated dendritic cells. (a) Freshly isolated CD4+ T lymphocytes (1 x 10^5) were stimulated with allogeneic OMZ-treated (0.001%) human DC (1 x 10^4) in 96-well plates for 4 days plus 16 h in the presence of [3H]-thymidine (37 kBq/well). Untreated mature and immature DC served as controls. The results of four independent experiments are shown. (b) CD4+ T cells (1 x 10^5) were stimulated with allogeneic untreated DC (1 x 10^4) in 24-well plates. OMZ-treated (0.001%) DC (1 x 10^4) were placed in transwell chambers in the same well. After 4 days of culture, 3 x 200 μl per well of each culture were transferred to three individual wells of 96-well plates. Proliferation was measured after an additional 16 h pulse with [3H]-thymidine. CD4+ T cells stimulated with untreated mature and immature DC and OMZ-DC (0.001%) served as control. The results of three independent experiments are shown. (c) CD4+ T cells were stimulated with allogeneic untreated DC at a DC:T-cell ratio of 1:10 and OMZ-treated (0.001%) DC were added in increasing concentrations. Results of three independent experiments are shown.
Cytokines are secreted at least in part by the respiratory epithelial cells and are known to induce and enhance the expression of adhesion molecules on endothelial cells. Adhesion molecules induce the recruitment and transendothelial migration of granulocytes and lymphocytes to the site of inflammation by interacting with their cognate receptors (7,24). Proinflammatory cytokines released during viral infections also stimulate the migration of monocytes to the site of infection and their subsequent activation and proliferation. Upon activation, monocytes secrete cytokines such as IL-1β and TNF-α that have antiviral and proinflammatory effects. Especially, IL-1β was found to cause a marked upregulation of proinflammatory cytokines including TNF-α, IL-6 and IL-8 (12,25). Thus, monocytes may also sustain and amplify the host inflammatory response, leading to increased cold symptoms. These data demonstrate the importance of proinflammatory cytokines in the pathogenesis of a naturally acquired URTI and in the amplification of the inflammatory response induced by viral infection.

In our study we demonstrate that OMZ is able to reduce the production of cytokines by activated mononuclear cells of the peripheral blood significantly resulting in a decreased activation and proliferation of T cells. This may explain the anti-inflammatory effects of OMZ seen in vivo as T cells are in part responsible for the strong inflammatory process during viral infection of the upper respiratory tract. Whether cytokine levels (e.g. TNF-α or IL-6) in the nasal fluid of OMZ-treated rhinitis patients are reduced in comparison with untreated patients and thus our findings are of biological relevance is to be clarified in future clinical studies. It will be of great interest to profile induced changes in the (sub)cutaneous cytokine environment to analyse OMZ effects in vivo (25). Nevertheless, one study already demonstrated that the total protein content of the nasal fluid of OMZ-treated rhinitis patients was significantly reduced (26).

Oxymetazoline is a sympathomimetic imidazoline derivative commonly used for the symptomatic treatment of acute (viral) rhinitis. Its alpha-adrenergic activity results in a vasoconstriction lasting up to 12 h, if applied topically to the nasal mucosa, causing decongestion. As nasal decongestants including OMZ effectively reduce rhinitis symptoms, a few studies already dealt with their possible anti-inflammatory/immunomodulating activities besides their vasoconstrictive function. The influence of OMZ on pro-, anti-inflammatory and oxidative stress responses through modulation of mediators of the arachidonic acid system such as lipooxygenase and leukotriene could be demonstrated in cell-free systems as well as in the canine macrophage system (27). In addition, it has been demonstrated that OMZ inhibits human neutrophil functions including actin polymerization, phagocytosis and oxidative

![Figure 6. Suppressed cytokine production of dendritic cells matured in the presence of oxymetazoline. (a) At day 6, immature DC were stimulated using a cocktail of proinflammatory cytokines, simultaneously 0.001% OMZ was added to the culture. Mature and immature DC cultured without OMZ served as controls. Twenty-four hours after stimulation, DC were washed intensively and cultured in medium without any cytokines; 48 h after stimulation, the supernatants were collected and analysed by ELISA. The results of three independent experiments are shown. (b) To analyse the influence of reduced cytokine production by OMZ-DC on T cell proliferation, CD4+ T cells were stimulated using allogeneic OMZ-treated (0.001%) DC (DC:T-cell ratio 1:10). IL-6 was added to the co-culture. Proliferation was measured after 4 days plus an additional 16 h pulse with [3H]-thymidine. *P < 0.05, statistical analysis was performed by Student’s t-test.](image)
burst (28). In contrast to our study, these inhibitory effects were most pronounced for benzalkonium chloride, which is frequently used as a nose drop preservative. However, benzalkonium can be excluded as a reason for our results as the OMZ solution used in this study did not contain any preservative. The concrete mechanism of action of OMZ still is to be analysed in detail. It is known that OMZ acts via stimulation of alpha-adrenergic receptors as mentioned above. Most immune cells, including DC and T cells, express these receptors on their cell surface (16,17) allowing a direct effect of OMZ on these cells. In prior studies it was shown that in mice, modulation of immune cells through alpha-adrenergic receptor stimulation was not mediated through inhibition of NFκB but through the MAP38K pathway (29,30). Whether this holds true for the human system is to be examined.

An interesting aspect is the effect of OMZ on the T-cell stimulatory capacity of human DC. As many studies in the last decade showed that in vitro generated immature and mature DC show phenotypic and functional properties that are comparable to DC in vivo (9–11), our results allow to draw conclusions on the in vivo situation. We show that OMZ is able to inhibit the immunostimulatory capacity of human DC without influencing their phenotype in general. Particularly, upregulation of surface expression of CD80, CD86 and the induced maturation marker CD83 was not affected. Nevertheless, the stimulatory capacity of DC was clearly inhibited by OMZ, most likely through the blockade of cytokine production. Herein, the markedly reduced secretion of IL-6 seems to play an important role for the reduced secretion of IL-8, well known to be a potent chemotactic cytokine necessary for the recruitment and activation of inflammatory cells (5), plays an important role as well needs to be examined in further studies. As DC are antigen-presenting cells capable of inducing and activating T-cell responses in the local draining lymph nodes, the functional inhibition of DC through OMZ at the site of inflammation may result in a reduced recruitment of immune cells from the local lymph nodes into the skin and mucosa contributing in part to the effect of decongestion seen after application of OMZ in vivo.

In conclusion, we demonstrate that OMZ significantly reduces the production of proinflammatory cytokines by activated mononuclear cells of the peripheral blood. This results in a decreased proliferation capacity of T cells. In addition, OMZ is able to inhibit the immunostimulatory capacity of human DC without influencing their phenotype. These findings may explain the anti-inflammatory and immunomodulatory effects of OMZ in acute viral rhinitis seen in vivo.

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