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Tumour necrosis factor-α mediates neutrophil migration to the knee synovial cavity during immune inflammation☆

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Abstract

Tumour necrosis factor (TNF)-α, interleukin-1β, interleukin-8 and leukotriene B_4 have an important role on neutrophil recruitment during immune-inflammation. Here we evaluated the participation of several inflammatory mediators on ovalbumin-induced neutrophil recruitment in the knee articular space of immunized rats. Ovalbumin administration in immunized, but not in control, rats induced a dose- and time-dependent neutrophil accumulation, which was inhibited by dexamethasone, pentoxifylline or thalidomide, but not by selective inhibitors of nitric oxide (nitro-L-arginine), platelet-activating factor (BN50730 or UK74505), prostaglandins (indomethacin), histamine (meclisine) or leukotriene B_4 (MK 886 and CP105,696). Anti-TNF-α antiserum, but not anti-interleukin-1β or anti-CINC-1 (cytokine-induced neutrophil chemoattractant 1) antisera, impaired ovalbumin-induced neutrophil accumulation. High amounts of TNF-α were detected in the exudates, which was inhibited by dexamethasone, pentoxifylline and thalidomide. These results suggest a specific role for TNF-α in this model, and the ability of pentoxifylline and thalidomide to inhibit both neutrophil influx and TNF-α release may have therapeutic implications in arthritis.

Keywords: TNF-α; Arthritis; Neutrophil; Immune inflammation; Pentoxifylline; Thalidomide

1. Introduction

Rheumatoid arthritis is a chronic inflammatory autoimmune disease associated with destruction of articular cartilage and underlying bone (Wei, 2001). The inflamed rheumatoid synovial tissue displays an extensive infiltration of lymphocytes and macrophages, sometimes with giant cell formation. Polymorphonuclear neutrophils are also presented, mainly during acute bouts of arthritis, particularly in the joint fluid (Harris, 1990). Considerable evidence supports a crucial role for pro-inflammatory mediators such as cytokines (tumour necrosis factor (TNF-α), interleukin-1β and interleukin-18), chemokines (interleukin-8, monocyte chemoattractant protein-1, macrophage inflammatory protein 1α and epithelial cell-derived neutrophil-activating peptide-78) and eicosanoids (leukotrienes and prostaglandins) in rheumatoid arthritis pathogenesis (Arend and Dayer, 1995; Davidson et al., 1983; Feldmann et al., 1996; Koch et al., 1992, 1994; Wei et al., 2001). Furthermore, the tissue damage observed in this disease, as well as in several other immune-inflammatory states, such as glomerulonephritis, immune vasculitis and inflammatory bowel disease, is at least in part due to the release of neutrophil products, such as proteolytic enzymes and oxygen- and nitrogen-derived free radicals (Haynes, 1992; Holdsworth and Bellomo, 1984; Wandall, 1985; Weissmann and Korchak, 1984). The recruitment of neutrophils to inflammatory sites in rheumatoid arthritis is mediated by chemoattractants such as chemokines, C5a and leukotriene B_4. However, few studies have investigated the mechanisms whereby these chemoattractants elicit neutrophil migration. Canetti et al.

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(2001) demonstrated that neutrophil migration during immune peritonitis in mice required CD_4^+ T-cell-derived TNF-α.The TNF-α promoted neutrophil recruitment through a leukotriene B_4-dependent mechanism.

The importance of TNF-α in some human disorders has been greatly emphasized by the successful administration of TNF-α blockers in rheumatoid arthritis (Moreland et al., 1999) and, more recently, in ankylosing spondylitis (Braun et al., 2002). This has been achieved either by means of the administration of a soluble TNF receptor (etanercept) (Moreland et al., 1999), a chimeric mouse–human anti-TNF monoclonal antibody (infliximab) (Maini et al., 1999) or a fully humanized monoclonal anti-TNF antibody (adalimumab) (Weinblatt et al., 2003). These compounds have been collectively designated ‘‘biologic agents’’ to treat rheumatoid arthritis. Interestingly, as it occurs with the so-called disease modifying antirheumatic drugs used in rheumatoid arthritis therapy, the efficacy of these TNF blockers was closely associated with a decrease in neutrophil trafficking into the inflamed joints (Culy and Keating, 2002).

Pharmacological studies also support an important role for TNF-α in neutrophil migration to sites of active rheumatoid arthritis. Thalidomide, for example, has been shown to have anti-inflammatory properties, which are associated with suppression of cytokine expression, and result from suppression of nuclear factor-κB activation. Thalidomide was found to inhibit nuclear factor-κB through a mechanism that involves the inhibition of activity of inhibitory-κB kinase (Keifer et al., 2001). In addition, it was demonstrated that the anti-inflammatory actions of the alkylxanthine pentoxifylline were able to reduce the production of inflammatory cytokines, especially TNF-α (Mandell, 1995).

When attempting to characterize the interactions of inflammatory mediators, it is imperative to recognize that important differences may exist among various species and different models of inflammation. In the present study, we employed pharmacological and immunological approaches to determine whether TNF-α is involved in ovalbumin-induced neutrophil recruitment in immunized rats, in a manner similar to what we described in the setting of peritonitis in ovalbumin-immunized mice (Canetti et al., 2001). In addition, the individual contributions of the interleukin-1β, cytokine-induced neutrophil chemoattractant 1 (rat interleukin-8 related chemokine; Watanabe et al., 1991), eicosanoids (prostaglandins, leukotrienes and platelet-activating factor), histamine, and nitric oxide on the neutrophil recruitment were also investigated.

2. Materials and methods

2.1. Animals and procedures for active sensitization

All experiments were conducted in accordance with the guidelines on the welfare of experimental animals of the School of Medicine of Ribeirão Preto, University of São Paulo. Male Wistar rats, 3 days before the experiments, were obtained from the animals facilities of School of Medicine of Ribeirão Preto, and housed in the Department of Pharmacology, in a temperature-controlled room with access to water and food ad libitum.

Ovalbumin was dissolved in phosphate-buffered saline (PBS; 2 mg ml^{-1}) and mixed with an equal volume of Complete Freund’s Adjuvant. Rats weighing 100 g were subcutaneous (s.c.) injected at two different sites, with a total dose of 200 μg of ovalbumin. Control rats were injected in the same way with an emulsion containing equal volumes of PBS and Complete Freund’s Adjuvant, without ovalbumin. After 28 days, the rats were challenged intra-articularly (i.a.) into a single knee joint with ovalbumin or Keyhole Limpet Hemocyanin (KLH), in 60 μl of PBS.

2.2. Leukocyte migration

Ovalbumin, KLH or PBS were injected i.a. into the knee joint of ovalbumin-sensitized or control rats with a 26 G 1/2 needle. Ovalbumin was injected at doses of 3.3, 10, 30 and 90 μg $cavity^{-1}$ diluted in 60 μl of PBS and leukocyte migration was evaluated at 0.5, 4, 12, 24 and 48 h post-injection as follows. At the indicated times, animals were killed and the cells present within the knee articular space were harvested by washing the cavity with 50 μl of PBS containing 4 mM EDTA. The volumes recovered were similar in all experimental groups with approximately 95% of the injected volume recovered. Total cell counts were performed in a cell counter (COULTER® A^C T; Coulter; Miami, FL, USA) and differential cell counts (200 cells total) were carried out on cytocentrifuge (Cytospin 3®; Shandon Lipshaw; Pittisburg, PA, USA) slides stained with the modified hematoxylin/eosin stain Rosenfeld. The results are presented as the number of neutrophils per joint cavity. Samples of the harvested exudes were centrifuged and the supernatants immediately frozen at −70 °C for subsequent quantification of cytokines by enzyme-linked immunosorbent assay.

2.3. Anti-inflammatory drugs

Sensitized animals were treated 1 h before ovalbumin challenge (30 μg $cavity^{-1}$) with a glucocorticoid dexamethasone (1 mg kg^{-1}; s.c.); the TNF-α synthesis inhibitor thalidomide (45 mg kg^{-1}; s.c.); the phosphodiesterase inhibitor pentoxifylline (100 mg kg^{-1}; s.c.); the 5-lipoxygenase activating protein inhibitor MK 886 (3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid; 1 mg kg^{-1}; p.o.); or 30 min before ovalbumin challenge with the leukotriene B_4 receptor antagonist CP 105,696 ((+)-1-(3*S*,4*R*)-[3-(4-phenyl-benzyl)-4-hydroxy-chroman-7-yl] cyclopentane carboxylic acid; 3 mg kg^{-1}; i.p.); platelet-activating factor

receptor antagonists (UK 74505: 4-(2-chlorophenyl)-1,4-dihydro-3-(ethoxycarbonyl)-6-methyl-2-[4-(2-methylimidazo[4,5-*c*]pyrid-1-yl)phenyl]-5-[*N*-(2-pyridyl)carbamoyl]-pyridine; 0.5 mg kg^{-1}; i.p.; BN 50730: [tetrahydro-4, 7,8,10 methyl-1(chloro-2 phenyl)-6 (methoxy-4 phenyl-carbamoyl)-9 pyrido [4′,3′-4,5] thieno [3,2-*f*] triazolo-1,2,4 [4,3-alpha] diazepine-1,4]; 10 mg kg^{-1}; s.c.); the cyclooxygenase inhibitor indomethacin (5 mg kg^{-1}; s.c.); the selective histamine 1 receptor antagonist meclizine (20 mg kg^{-1}; s.c.); or the nitric oxide synthesis inhibitor *N*-ω-nitro-L-arginine (50 mg kg^{-1}; s.c.). Dexamethasone, pentoxifylline and *N*-ω-nitro-L-arginine were dissolved in PBS; indomethacin in 0.1 M Tris, pH 8; MK 886 was diluted in 0.1% of methyl cellulose in water. Meclizine was first dissolved in Cremofor EL (Basf), no more than 10% of the final volume, and then the volume was completed with PBS; CP 105,696, BN 50730, thalidomide were similarly dissolved in dimethyl sulfoxide and UK 74505, in 0.1 M HCl. Neutrophil migration was assessed 4 h after the challenge.

2.4. Effect of antisera treatment on ovalbumin-induced neutrophil migration

The antisera against rat-TNF-α, interleukin-1β or cytokine-induced neutrophil chemoattractant 1 were injected concomitantly with ovalbumin into the knee joint. The animals received 60 μl of a solution containing 30 μl of anti-cytokine serum plus 30 μg of ovalbumin diluted in 30 μl of PBS. The amount of the antisera used inhibited neutrophil migration induced by i.p. administration of the respective recombinant cytokines by more than 85% (data not shown). Neutrophil migration was evaluated 4 h later.

2.5. Enzyme-linked immunosorbent assay

The concentrations of TNF-α, interleukin-1β and cytokine-induced neutrophil chemoattractant 1 in synovial exudates obtained 2 h after ovalbumin challenge of control and sensitized rats were measured by enzyme-linked immunosorbent assay based upon a previously protocol (Taktak et al., 1991). Briefly, 96-well microtiter plates (NUNC-Immuno™ Plate) were coated overnight at 4 °C with immunoaffinity-purified polyclonal antibodies against the respective cytokines. After blocking the plates (albumin 1% for 1 h), concentration cytokines and samples were loaded in duplicate for 2 h (22 °C). Rabbit biotinylated immunoaffinity-purified antibody was added, followed by incubation for 1 h (22 °C). Finally, 100 μl of avidin-horseradish peroxidase (1:5000 dilution; DAKO, Denmark) was added to each well, after 30 min the plates were washed and the color reagent o-phenylenediamine (40 μg $well^{-1}$) was added. After 15 min, the reaction was stopped with 1 M H_2SO_4 and the O.D. was measured at 490 nm. Cytokine concentration was expressed as pg ml^{-1} or ng ml^{-1}.

2.6. Materials

Ovalbumin (grade V), Complete Freund's Adjuvant, dexamethasone, meclizine, *N*-ω-nitro-L-arginine and pentoxifylline were purchased from Sigma, USA. MK 886 and indomethacin were obtained from Merck Sharp and Dohme, New Jersey, USA. BN 50730 was a gift from Institute Henri Beaufour. Recombinant rat-TNF-α, recombinant rat-interleukin-1β, biotinylated antibodies, antisera against rat-TNF-α and antisera against rat-interleukin-1β were gifts from Dr. S. Poole (National Institute for Biological Standards and Control, NIBSC, London, UK). Recombinant rat-cytokine-induced neutrophil chemoattractant 1, antisera against rat-cytokine-induced neutrophil chemoattractant 1 were obtained from Preprotec (USA). Thalidomide was obtained from Champion Bioquímico (São Paulo, Brazil). CP 105,696 and UK 74505 were gifts from Professor Mauro Teixeira (Federal University of Minas Gerais, Minas Gerais, Brazil).

2.7. Statistical analysis

The data are reported as mean ± S.E.M. and are representative of two different experiments. The means from different treatments were compared by one-way analysis of variance. When significant differences were identified, individual comparisons were subsequently made with Bonferroni test for unpaired values. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Neutrophil migration induced by ovalbumin in sensitized rats

The i.a. injection of ovalbumin into knee of ovalbumin-sensitized rats induced a dose-dependent neutrophil migration (Fig. 1A), which peaked 4 h after challenge and returned to control levels after 12 h (Fig. 1B). The administration of an unrelated antigen (KLH; 30 μg $cavity^{-1}$) in sensitized rats, or ovalbumin (30 μg $cavity^{-1}$) in non-sensitized (control) rats were unable to produce significant neutrophil recruitment (Fig. 1A). Furthermore, the i.a. administration of ovalbumin in sensitized (but not control) rats also induced the accumulation of eosinophils and mononuclear cells at 24 and 48 h following challenge (data not shown). Immunized rats had a high titer of serum IgG against ovalbumin when compared with those found in the sera of control animals (three-fold more; data not shown).

3.2. Dexamethasone, pentoxifylline and thalidomide inhibit neutrophil migration induced by ovalbumin

Treatment of sensitized rats with dexamethasone (1 mg kg^{-1}), pentoxifylline (100 mg kg^{-1}) or thalidomide (45

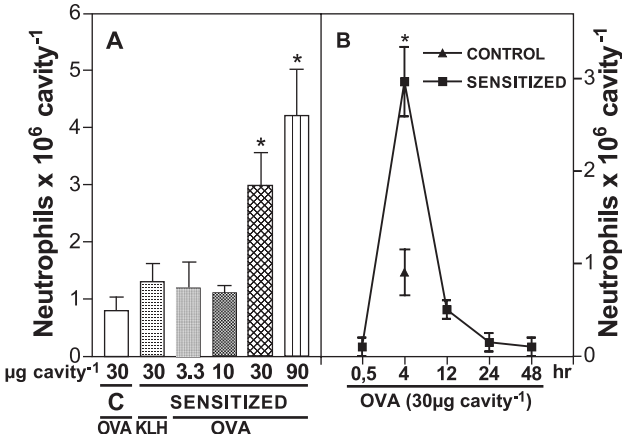

Fig. 1. Dose-dependence and time-course of neutrophil migration induced by ovalbumin (OVA). (A) Ovalbumin was injected at the indicated doses into the knee joint of control (C) or sensitized rats and neutrophil migration was determined 4 h later. Neutrophil migration was also evaluated when 30 μg of KLH was injected in sensitized rats. (B) Time-course of the neutrophil migration induced by ovalbumin (30 μg cavity^{-1}) in control (triangles) or sensitized (squares) rats. Results are expressed as mean ± S.E.M. and are representative of two separate experiments with five rats per group in each experiment. *$P<0.05$ compared to the respective controls (analysis of variance followed by Bonferroni *t*-test).

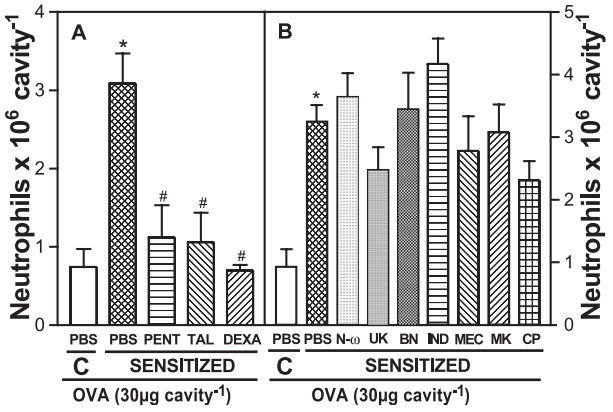

Fig. 2. Effect of anti-inflammatory drugs on ovalbumin (OVA)-induced neutrophil migration. Sensitized rats were treated with PBS (0.5 ml, s.c.), pentoxifylline (PTX; 100 mg kg^{-1}, s.c.), thalidomide (TAL; 45 mg kg^{-1}, s.c.), dexamethasone (DEX; 1 mg kg^{-1}, s.c.) and MK 886 (MK; 1 mg kg^{-1}, p.o.) 1 h before ovalbumin (30 μg cavity^{-1}) challenge. Indomethacin (IND; 5 mg kg^{-1}, s.c.), *N*-ω-nitro-L-arginine (*N*-ω; 50 mg kg^{-1}, s.c.), BN 50730 (BN; 10 mg kg^{-1}, s.c.), meclizine (MEC; 20 mg kg^{-1}, s.c.), UK 74505 (UK; 0.5 mg kg^{-1}, s.c.) and CP 105,696 (CP; 3 mg kg^{-1}, s.c.) were injected 30 min before ovalbumin (30 μg cavity^{-1}) challenge. The first bar represents the neutrophil migration induced by ovalbumin injected into the knee joint in control (C) rats. Neutrophil migration was evaluated 4 h after ovalbumin challenge. Values are expressed as mean ± S.E.M. and are representative of two separate experiments with five rats per group in each experiment. *$P<0.05$ compared to control group and $^{\#}P<0.05$ compared to PBS-treated ovalbumin-sensitized group (analysis of variance followed by Bonferroni *t*-test).

mg kg^{-1}) inhibited neutrophil migration triggered by ovalbumin injection into the knee joint. The number of neutrophils present in the knee joint of treated rats after ovalbumin challenge was similar to the number of neutrophils observed in immunized rats treated with PBS (Fig. 2A). However, treatment of the animals with *N*-ω-nitro-L-arginine (50 mg kg^{-1}), BN 50730 (10 mg kg^{-1}), indomethacin (5 mg kg^{-1}), meclizine (20 mg kg^{-1}), CP 105,696 (3 mg kg^{-1}) and MK 886 (1 mg kg^{-1}) were all ineffective in modifying neutrophil migration induced by i.a. ovalbumin administration (Fig. 2B). Another platelet-activating factor receptor antagonist tested, UK 74505 (0.5 mg kg^{-1}), was also unable to inhibit ovalbumin-induced neutrophil migration (data not shown). Taken together, these results suggest a role for TNF-α, and rule out the participation of nitric oxide, histamine and eicosanoids (platelet-activating factor, prostaglandins and leukotrienes) on ovalbumin-induced neutrophil migration in sensitized rats. The concentrations of anti-inflammatory drugs used were as described in the literature (Bocca et al., 1998; Castro-Faria-neto et al., 1991; Jancar et al., 1991) and their effectiveness has been previously confirmed in our laboratory (data not shown).

3.3. Inhibition of neutrophil migration by antiserum against TNF-α

In order to confirm the participation of TNF-α on neutrophil migration induced by ovalbumin in sensitized mice, we injected an anti-TNF-α antiserum intra-articularly. As can be observed in Fig. 3, concomitant administration of serum against TNF-α with ovalbumin completely abolished

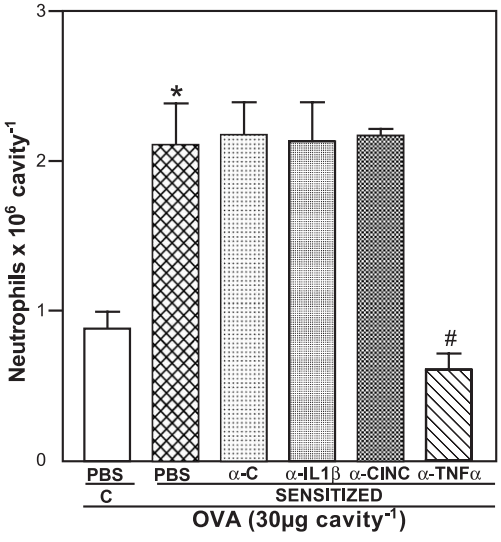

Fig. 3. Inhibition of ovalbumin (OVA)-induced neutrophil migration by anti-TNF-α serum treatment. Sensitized rats were injected with PBS (30 μl cavity^{-1}), control serum (α-C; 30 μg cavity^{-1}) or 30 μl of anti-rat interleukin-1β (α-IL1β), anti-cytokine-induced neutrophil chemoattractant 1 (α-CINC) or anti-TNF-α (α-TNFα) sera concomitantly with ovalbimin (30 μg cavity^{-1}). The first bar represents the neutrophil migration induced by ovalbumin injection into the knee joint of control (C) rats. Neutrophil migration was evaluated 4 h after ovalbumin challenge. Results are expressed as mean ± S.E.M. and are representative of two separate experiments with five rats per group. *$P<0.05$ compared to control group. $^{\#}P<0.05$ compared to α-C treated ovalbumin-sensitized group (analysis of variance followed by Bonferroni *t*-test).

neutrophil accumulation. In contrast, no effect on ovalbumin-induced neutrophil migration was observed after the intra-articular injection of sera against IL-1β or cytokine-induced neutrophil chemoattractant 1. The treatment of the rats with non-immune serum did not modify ovalbumin-induced neutrophil migration (Fig. 3).

3.4. Detection of TNF-α in the exudates harvested from the knee joint of the ovalbumin sensitized rats: effect of dexamethasone, thalidomide and pentoxifylline

Following i.a. ovalbumin challenge, the inflammatory knee joint exudates were harvested and TNF-α levels determined by enzyme-linked immunosorbent. As show in Fig. 4, the administration of the same dose of ovalbumin that caused neutrophil migration into the knee joint of sensitized rats was also able to promote a significant increase in TNF-α concentration in the synovial lavage fluid, when compared with control rats. Moreover, close association between TNF-α concentrations and neutrophil migration was verified in sensitized rats treated with dexamethasone, thalidomide and pentoxifylline (Fig. 4). Additionally, i.a. ovalbumin injection also significantly increased the levels of interleukin-1β (5.45-fold) and cytokine-induced neutrophil chemoattractant 1 (2.3-fold) compared with control (Fig. 5).

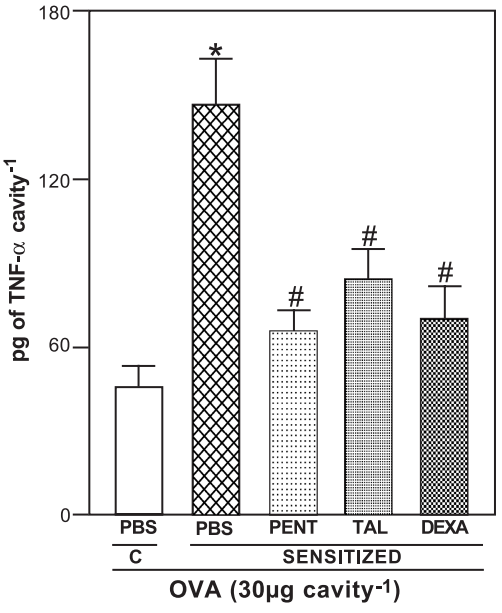

Fig. 4. Effect of pentoxifylline, thalidomide and dexamethasone on ovalbumin (OVA)-induced TNF-α release. Sensitized rats were treated s.c. with PBS (0.5 ml), pentoxifylline (PTX; 100 mg kg^{-1}), thalidomide (TAL; 45 mg kg^{-1}) or dexamethasone (DEX; 1 mg kg^{-1}) s.c., 1 h before to ovalbumin (30 μg $cavity^{-1}$) challenge. The concentration of TNF-α was determined 2 h after the challenge in the synovial lavage fluid. The first bar represents the concentration of TNF-α induced by ovalbumin injected into the knee joint of control (C) rats. Values are expressed as mean ± S.E.M. and are representative of two separate experiments with two rats per group. *$P<0.05$ compared to control group. #$P<0.05$ compared to PBS-treated ovalbumin-sensitized group (analysis of variance followed by Bonferroni *t*-test).

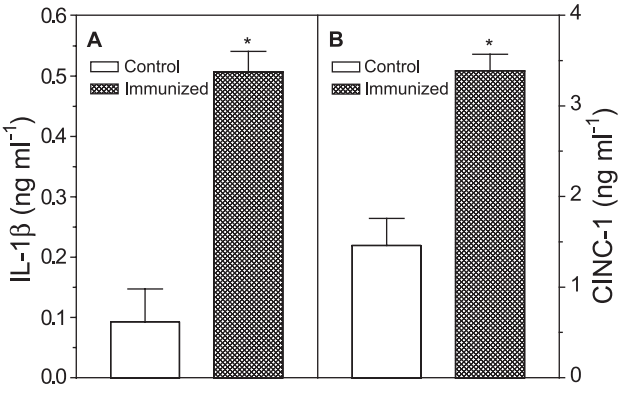

Fig. 5. Concentration of interleukin (IL)-1β and cytokine-induced neutrophil chemoattractant 1 (CINC-1) in knee joint exudates of control and sensitized rats challenged with ovalbumin (OVA). The exudates were harvested 2 h after ovalbumin injection (30 μg $joint^{-1}$). The cytokines were quantified as described in Materials and methods and the data are expressed as mean ± S.E.M. Results are expressed in ng ml^{-1} for interleukin-1β and in ng ml^{-1} for cytokine-induced neutrophil chemoattractant 1. *$P<0.05$ compared to control group (Student's *t*-test).

4. Discussion

In this study, we used a model of aseptic arthritis to explore the role of inflammatory mediators in neutrophil trafficking to sites of acute inflammation. We observed that the injection of ovalbumin into the knee joint of active immunized rats induced a dose- and time-dependent neutrophil accumulation that peaked 4 h after challenge and returned to basal levels 12 h later. The ovalbumin challenge also induced eosinophil and mononuclear cell infiltration within 24 and 48 h. The challenged animals also present synovitis and reduction of knee articular mobility (data not shown) probably due the inflammatory process. Similar kinetics for cell infiltration following antigen challenge in immunized animals have been reported (Abe et al., 1994; Klein et al., 1995; Canetti et al., 2001; Zuany-Amorim et al., 1993, 1995).

We found that ovalbumin-induced neutrophil recruitment was mediated by TNF-α, since neutrophil accumulation was inhibited by multiple anti-TNF-α treatments: pentoxifylline, thalidomide and dexamethasone, and also by anti-TNF-α antibody. Moreover, our pharmacological data ruled out the participation of arachidonic acid metabolites (leukotriene B_4, prostaglandins and platelet-activating factor), nitric oxide or histamine in this model, since compounds that either selectively inhibit their synthesis or actions (receptors antagonists) were ineffective in modifying neutrophil accumulation induced by antigen challenge. Despite the fact that ovalbumin-challenge also induced an increase in cytokine-induced neutrophil chemoattractant 1 and interleukin-1β levels, our results fail to support a role

for these mediators in neutrophil migration, as evidenced by the fact that anti-interleukin-1β and anti-cytokine-induced neutrophil chemoattractant 1 sera treatment had no effect on neutrophil migration into the knee joints of ovalbumin-challenged sensitized animals. Importantly, the anti-cytokine sera (anti-TNF-α, anti-interleukin-1β and anti-cytokine-induced neutrophil chemoattractant 1) we used were active, as evidenced by their ability to inhibit neutrophil accumulation induced by the respective i.p. injections of rat recombinant cytokines by more than 85% (data not shown).

The participation of TNF-α in human diseases where neutrophil migration is prominent can be illustrated by the detection of increased levels of this cytokine in the joints of rheumatoid arthritis patients as well as in the intestinal lesions in Chron's disease (Harris, 1990; Cuzzocrea, 2003). Moreover, the administration of TNF blockers to these patients resulted in a great amelioration of signs and symptoms of the disease and, at least for rheumatoid arthritis, led to the reduction of the structural joint damage, evidenced by reduced radiographic lesions (Culy and Keating, 2002). TNF-α is a pivotal player in arthritis among several animal species, including mice and rabbits and models. In several studies, TNF-α actions are associated with leukocyte recruitment, activation and articular destruction (Williams et al., 1992; Maini et al., 1993; Idogawa et al., 1997; Podolin et al., 2002). It has also been shown that the antigen-induced neutrophil recruitment into the lung is inhibited by anti-TNF-α antiserum (Zuany-Amorim et al., 1993, 1995). Interestingly, we previously demonstrated that neutrophil migration induced by ovalbumin in immunized mice is also dependent on TNF-α (Canetti et al., 2001). However, in this animal species, as opposed to rats, that were used in the present study, leukotriene B_4 was also involved in the process. We have shown that the release of this chemotactic mediator by ovalbumin immunized mice depends on TNF-α (Canetti et al., 2001).

During an inflammatory response, leukocytes traverse the endothelial barrier into the tissue space. Extravasation of leukocytes is a multistep process involving rolling, tethering, firm adhesion to the endothelium, and finally, transendothelial migration. Normally, the endothelium is not adhesive and is impermeable to leukocytes, but under inflammatory conditions, intracellular signals are generated enhancing adhesiveness and leading to endothelial permeability. In this study, we did not pursue the mechanisms by which TNF-α modulation affects endothelium activation, due to the difficulty of the experimental design in vivo. Recently, it was demonstrated that TNF-α enhances beta2-integrin CD11/CD18 phosphorylation, a process dependent on protein kinase Czeta (Javaid et al., 2003), as well as activates endothelial cells though a mechanism dependent on extracellular signal-regulated kinase (Stein et al., 2003).

Pentoxifylline and thalidomide have been used since 1980s in small clinical trials to the treatment of inflammatory disorders. Thalidomide is currently used for the treatment of the reactional state secondary to the treatment of leprosy (Laffitte and Revuz, 2004). It has also been shown to be effective in some lupus cutaneous lesions (Laffitte and Revuz, 2004). In patients with rheumatoid arthritis, thalidomide was shown to provide partial relief of symptoms (Agle et al., 2003) However, the fact that women in the childbearing age are the most important group of patients affected by rheumatoid arthritis has limited the studies on the use of this compound. Our study reveals a strong and apparently specific association between neutrophil accumulation and increased TNF-α levels within the synovial fluid of ovalbumin-injected sensitized rats. Moreover, the inhibition of neutrophil migration in ovalbumin sensitized rats was closely associated with the ability of pentoxifylline, thalidomide, and dexamethasone in lowering the intra-articular levels of TNF-α.

The levels of interleukin-1β and cytokine-induced neutrophil chemoattractant 1 were significantly increased in the joint exudates of ovalbumin-sensitized rats. Since the anti-IL-1β treatment did not modify ovalbumin-induced neutrophil recruitment, we believe that IL-1β might be involved in cell activation rather then neutrophil influx. We suggest that these cytokines may be involved in the activation of the recruited neutrophils or resident cells, but further studies are necessary to prove this hypothesis. In our previously reports using the ovalbumin-induced peritonitis model in rats and mice (Klein et al., 1995; Canetti et al., 2001), we also noted the lack of participation of interleukin-8 family molecules in the neutrophil migration process. The fact that in the current model, cytokine-induced neutrophil chemoattractant 1 is not participating in neutrophil influx, despite of the fact that higher levels of the protein were observed, reinforces that in our experimental condition, during antigen-induced neutrophil influx in immunized animals, the interleukin-8 family molecules are not relevant.

It should be stressed that the introduction of the anti-TNF agents in the rheumatology clinical practice has provided substantial pain relief and appears to significantly alter disease outcome. However, though current data suggest a relatively safety administration, that are some concerns about an increased incidence of opportunistic infections and neoplasias with these compounds (Furst et al., 2003). Additionally, due to their immune nature, the appearance of neutralizing antibodies to some of these agents may limit their effectiveness in the long term. Therefore, it could well be that, at least in some patients, alternative strategies to block TNF, perhaps associated with the so-called "biologic agents", may be beneficial in the clinical practice either by enhancing their effectiveness or tolerability or also by reducing their high costs.

Our results clearly reinforce that TNF-α has a prominent and specific role in neutrophil recruitment in inflammatory arthropathies. The effectiveness of thalidomide and pentox-

ifylline in reducing both neutrophil influx and TNF release should prompt the need for further studies in order to the search for safer and cost-effective alternatives in the treatment of these diseases.

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References

Abe, T., Yoshida, K., Omata, T., Segawa, Y., Matsuda, K., Nagai, H., 1994. Effects of ZCR-2060 on allergic airway inflammation and cell activation in guinea-pigs. J. Pharm. Pharmacol. 46, 876–882.

Agle, L.M., Rosenkranz, M., Lehman, T.J., 2003. Novel therapies for the treatment of juvenile rheumatoid arthritis (juvenile idiopathic arthritis). Expert Opin. Investig. Drugs 12, 19–28.

Arend, W.P., Dayer, J.M., 1995. Inhibition of the production and effects of interleukin-1 and tumor necrosis factor alpha in rheumatoid arthritis. Arthritis Rheum. 38, 151–160.

Bocca, A.L., Hayashi, E.E., Pinheiro, A.G., Furlamento, A.B., Campanelli, A.P., Cunha, F.Q., Figueiro, F., 1998. Treatment of *Paracoccidioides brasiliensis*-infected mice with a nitric oxide inhibitor prevents the failure of cell-mediated immune response. J. Immunol. 161, 3056–3063.

Braun, J., Brandt, J., Listing, J., Zink, A., Alten, R., Golder, W., Gromnica-Ihle, E., Kellner, H., Krause, A., Schneider, M., Sorensen, H., Zeidler, H., Thriene, W., Sieper, J., 2002. Treatment of active ankylosing spondylitis with infliximab: a randomised controlled multicentre trial. Lancet 359, 11187–11193.

Canetti, C., Silva, J.S., Ferreira, S.H., Cunha, F.Q., 2001. Tumour necrosis factor-alpha and leukotriene B_4 mediate the neutrophil migration in immune inflammation. Br. J. Pharmacol. 134, 1619–1628.

Castro-Faria-neto, H.C., Martins, M.A., Bozza, P.T., Perez, S.A., Correa-da-Silva, A.C., Lima, M.C., Cruz, H.N., Cordeiro, R.S., Sousa, M.V., Morhy, L., 1991. Pro-inflammatory activity of enterolobin: a haemolytic protein purified from seeds of the brazilian tree *Enterolobium contortisiliquum*. Toxicon 29, 1143–1150.

Culy, C.R., Keating, G.M., 2002. Etanercept. updated review of its use in rheumatoid arthritis, psoriatic arthritis and juvenile rheumatoid arthritis. Drugs 62, 2493–2537.

Cuzzocrea, S., 2003. Emerging biotherapies for inflammatory bowel disease. Expert Opin. Emerg. Drugs 8, 339–347.

Davidson, E.M., Rae, S.A., Smith, M.J., 1983. Leukotriene B_4, a mediator of inflammation present in synovial fluid in rheumatoid arthritis. Ann. Rheum. Dis. 42, 677–679.

Feldmann, M., Brennan, F.M., Maini, R.N., 1996. Rheumatoid arthritis. Cell 85, 307–310.

Furst, D.E., Breedveld, F.C., Kalden, J.R., Smolen, J.S., Burmester, G.R., Dougados, M., Emery, P., Gibofsky, A., Kavenaugh, A.F., Keystone, E.C., Klareskog, L., Russell, A.S., van de Putte, L.B.A., Weisman, M.H., 2003 (May). Updated consensus statement on biological agents for the treatment of rheumatoid arthritis and other immune mediated inflammatory diseases. Ann. Rheum. Dis. 62, ii2–ii9.

Harris Jr., E.D., 1990. Rheumatoid arthritis. Pathophysiology and implications for therapy. N. Engl. J. Med. 322, 1277–1289.

Haynes, B.F., 1992. Vasculitis: phatogenic mechanisms of vessel damage. In: Gallin, J., Goldstein, I., Snyderman, M. (Eds.), Inflammation: Basic Principles and Clinical Correlates. Raven Press, New York, pp. 921–941.

Holdsworth, S.R., Bellomo, R., 1984. Differential effects of steroids on leukocyte-mediated glomerulonephritis in the rabbit. Kidney Int. 26, 162–169.

Idogawa, H., Imamura, A., Onda, M., Umemura, T., Ohashi, M., 1997. Progression of articular destruction and the production of tumour necrosis factor-alpha in antigen-induced arthritis in rabbits. Scand. J. Immunol. 46, 572–580.

Jancar, S., Riffo-Vasquez, Y., Ferreira, A.P., Macedo, M.S., 1991. PAF antagonists do not modify IgE antibody production in mice. Braz. J. Med. Biol. Res. 24, 619–621.

Javaid, K., Rahman, A., Anwar, K.N., Frey, R.S., Minshall, R.D., Malik, A.B., 2003. Tumor necrosis factor-alpha induces early-onset endothelial adhesivity by protein kinase Czeta-dependent activation of intercellular adhesion molecule-1. Circ. Res. 92, 1089–1097.

Keifer, J.A., Guttridge, D.C., Ashburner, B.P., Baldwin Jr., A.S., 2001. Inhibition of NF-kappa B activity by thalidomide through suppression of IkappaB kinase activity. J. Biol. Chem. 276, 22382–22387.

Klein, A., Cunha, F.Q., Ferreira, S.H., 1995. The role of lynphocytes in the neutrophil migration induced by ovalbumin in immunized rats. Immunology 84, 577–584.

Koch, K.C., Ye, K., Clark, B.D., Dinarello, C.A., 1992. Interleukin 4 (IL) 4 up-regulates gene and surface IL 1 receptor type I in murine T helper type 2 cells. Eur. J. Immunol. 22, 153–157.

Koch, K., Melvin Jr., L.S., Reiter, L.A., Biggers, M.S., Showell, H.J., Pettipher, E.R., Cheng, J.B., Milici, A.J., Breslow, R., et al., 1994. (+)-1-(3*S*,4*R*)-[3-(4-phenylbenzyl)-4-hydroxychroman-7-yl]cyclopentane carboxylic acid, a highly potent, selective leukotriene B_4 antagonist with oral activity in the murine collagen-induced arthritis model. J. Med. Chem. 37, 3197–3199.

Laffitte, E., Revuz, J., 2004. Thalidomide: an old drug with new clinical applications. Expert Opin. Drug Saf. 3, 47–56.

Maini, R.N., Brennan, F.M., Williams, R., Chu, C.Q., Cope, A.P., Gibbons, D., Elliott, M., Feldmann, M., 1993. TNF-alpha in rheumatoid arthritis and prospects of anti-TNF therapy. Clin. Exp. Rheumatol. 8, S173–S175.

Maini, R., St Clair, E.W., Breedveld, F., Furst, D., Kalden, J., Weisman, M., Smolen, J., Emery, P., Harriman, G., Feldmann, M., Lipsky, P., 1999. Infliximab (chimeric anti-tumour necrosis factor alpha monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomised phase III trial. ATTRACT Study Group. Lancet 354, 1932–1939.

Mandell, G.L., 1995. Cytokines, phagocytes, and pentoxifylline. J. Cardiovasc. Pharmacol. 25, S20–S22.

Moreland, L.W., Schiff, M.H., Baumgartner, S.W., Tindall, E.A., Fleischmann, R.M., Bulpitt, K.J., Weaver, A.L., Keystone, E.C., Furst, D.E., Mease, P.J., Ruderman, E.M., Horwitz, D.A., Arkfeld, D.G., Garrison, L., Burge, D.J., Blosch, C.M., Lange, M.L., McDonnell, N.D., Weinblatt, M.E., 1999. Etanercept therapy in rheumatoid arthritis. A randomized, controlled trial. Ann. Intern. Med. 130, 478–486.

Podolin, P.L., Bolognese, B.J., Foley, J.J., Schmidt, D.B., Buckley, P.T., Widdowson, K.L., Jin, Q., White, J.R., Lee, J.M., Goodman, R.B., Hagen, T.R., Kajikawa, O., Marshall, L.A., Hay, D.W., Sarau, H.M., 2002. A potent and selective nonpeptide antagonist of CXCR2 inhibits acute and chronic models of arthritis in the rabbit. J. Immunol. 169, 6435–6444.

Stein, B.N., Gamble, J.R., Pitson, S.M., Vadas, M.A., Khew-Goodall, Y., 2003. Activation of endothelial extracellular signal-regulated kinase is essential for neutrophil transmigration: potential involvement of a soluble neutrophil factor in endothelial activation. J. Immunol. 171, 6097–6104.

Taktak, Y.S., Selkirk, S., Bristow, A.F., Carpenter, A., Ball, C., Rafferty, B., Poole, S., 1991. Assay of pyrogens by interleukin-6 release from monocytic cell lines. J. Pharmacol. 43, 578–582.

Wandall, J.H., 1985. Function of exudative neutrophilic granulocytes in patients with Crohn's disease or ulcerative colitis. Scand. J. Gastroenterol. 20, 1151–1156.

Watanabe, K., Koizumi, F., Kurashige, Y., Tsurufiji, S., Nakagawa, H.,

1991. Rat CINC, a member of the IL-8 family, is a neutrophil-specific chemoattractant in vivo. Exp. Mol. Pathol. 55, 30–37.

Wei, X., Leung, B.P., Arthur, H.M.L., McInnes, I.B., Liew, F.Y., 2001. Reduced incidence and severity of collagen-induced arthritis in mice lacking IL-18. J. Immunol. 166, 517–521.

Weinblatt, M.E., Keystone, E., Furst, D.E., Moreland, L.W., Weisman, M.H., Birbara, C.A., Teoh, L.A., Fischkoff, S.A., Chartash, E.K., 2003. Adalimumab, a fully human anti-tumor necrosis factor alpha monoclonal antibody, for the treatment of rheumatoid arthritis in patients taking concomitant methotrexate. The ARMADA trial. Arthritis Rheum. 48, 35–45.

Weissmann, G., Korchak, H., 1984. Rheumatoid arthritis: the role of neutrophil activation. Inflammation 8, S3–S14.

Williams, R.O., Feldmann, M., Maini, R.N., 1992. Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. Proc. Natl. Acad. Sci. U. S. A. 89, 9784–9788.

Zuany-Amorim, C., Leduc, D., Vargaftig, B.B., Pretolani, M., 1993. Characterization and pharmacological modulation of antigen-induced peritonitis in actively sensitized mice. Br. J. Pharmacol. 110, 917–924.

Zuany-Amorim, C., Hailé, S., Leduc, D., Dumarey, C., Huerre, M., Vargaftig, B.B., Pretolani, M., 1995. Interleukin-10 inhibits antigen-induced cellular recruitment into the airways of sensitized mice. J. Clin. Invest. 95, 2644–2651.