$PAFR^{-/-}$ mice respond similarly to wild-type mice (*71*). These findings show that PAF plays major roles in type I (and/or III) allergic anaphylaxis and that it enhances the severity of endotoxin shock. PAFR-Tg mice show bronchial hyper-responsiveness to methacholine as well as PAF (*70*). PAFR-Tg mice are significantly sensitive to PAF injection in terms of bronchial constriction, and these effects seem to be indirectly mediated thromboxane A2 and leukotriene D4 (*70*). $PAFR^{-/-}$ mice are also more resistant to hydrochloric acid aspiration–induced lung edema (a model of aspiration pneumonia) than wild type mice (*72*).

Apparently contradictory to previous pharmacological studies (*69*), $PAFR^{-/-}$ mice exhibited normal LTP and showed no obvious abnormality in excitatory synaptic transmission in the hippocampal CA1 region (*73*). These discrepancies might suggest the existence of PAF receptors other than the cloned one, or that PAF antagonists and/or methylcarbamoyl PAF exert effects *via* a different pathway than PAFR, including PAF acetylhydrolase inhibition.

Conclusion

As the first lipid autacoid receptor to be cloned, the cloned PAFR has furnished information on the inflammatory and non-inflammatory actions of PAF and the signaling mechanisms of GPCRs. The accumulated information suggests that PAFR mediates fine modifications of a variety of biological functions in co-operation with other GPCRs such as chemokine and eicosanoid receptors.

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Host response of platelet-activating factor receptor-deficient mice during pulmonary tuberculosis

SEBASTIAAN WEIJER,*† JAKLIEN C. LEEMANS,* SANDRINE FLORQUIN,‡ TAKAO SHIMIZU,§ SATOSHI ISHII§ & TOM VAN DER POLL*† **Laboratory of Experimental Internal Medicine, †Department of Infectious Diseases, Tropical Medicine and AIDS, ‡Department of Pathology, Academic Medical Centre, University of Amsterdam, the Netherlands. §Department of Biochemistry and Molecular Biology, Faculty of Medicine, The University of Tokyo, and CREST of Japan Science and Technology Corporation, Tokyo, Japan*

SUMMARY

Platelet-activating factor (PAF) is a phospholipid with potent, diverse actions, which has been implicated as an important mediator in host defence against several intracellular pathogens. To determine the role of PAF in host defence in pulmonary tuberculosis, PAF receptor-deficient (PAFR−/−) and wild-type (PAFR+/+) mice were infected intranasally with a virulent strain of *Mycobacterium tuberculosis*. Mycobacterial outgrowth in lungs and liver did not differ significantly between PAFR−/− and PAFR+/+ mice at 2 or 6 weeks postinfection. After 28 weeks, 86% of PAFR−/− mice and 79% of PAFR+/+ mice had died (non-significant). In addition, both mouse strains were indistinguishable with respect to histopathology, the recruitment and activation of lymphocytes, and cytokine concentrations in the lung. These data suggest that PAF is not involved in the protective immune response to tuberculosis.

INTRODUCTION

Tuberculosis (TB) is a re-emerging disease, affecting patients in both developing and industrialized countries.[1] The increasing incidence of antibiotic resistance, together with synergism between human immunodeficiency virus (HIV) and TB, has increased our interest in this important infectious disease and in mechanisms contributing to antimicrobial host defence. Resistance to mycobacterial infections is mediated mainly by macrophages and T cells and requires the formation of granulomas, characterized by lymphocytes, macrophages and granulocytes.[2] Their interaction is dependent on the interplay of cytokines and chemokines produced by different inflammatory cells.[2]

Platelet-activating factor (PAF) is a potent phospholipid mediator that plays an important role in inflammatory and immune responses.[3] PAF is produced by a large number of cells, including platelets, endothelial cells, stromal cells, lymphoid tissue and neutrophils.[4] The biological activity of PAF is mediated through a specific G-protein-coupled receptor (PAFR) on the membrane of responsive cells, which has been identified on many haemopoietic cells, including neutrophils, dendritic cells, macrophages and monocytes.[4,5] Recent studies have suggested that endogenous PAF may play an important role in stimulating an adequate immune response to intracellular microorganisms, such as *Leishmania amazonensis and Trypanosoma cruzi*. Indeed, treatment with PAF antagonists was found to increase the outgrowth of microorganisms and mortality in murine models of these infections.[6,7] In accordance, PAF reduced the intracellular growth of *Leishmania* and *Trypanosoma* in macrophages.[6,7] Notably, *M. tuberculosis* is an intracellular microorganism that uses macrophages as its natural environment in the host, and many of the host defence mechanisms known to be important for the protection against *M. tuberculosis* are also involved in the protective immune response to other intracellular pathogens, including *Leishmania* and *Trypanosoma*.[2,8–10] These findings led us to hypothesize that PAF may also be important for host defence against *M. tuberculosis*. Therefore, in the present study we sought to determine the role of PAF in the immunopathology of TB.

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Correspondence: Sebastiaan Weijer, MD, Academic Medical Centre, Laboratory of Experimental Internal Medicine Room G2-108, Meibergdreef 9, 1105 AZ Amsterdam, the Netherlands. E-mail: s.weijer@amc.uva.nl

MATERIALS AND METHODS

Mice

PAFR gene-deficient (PAFR−/−) mice were generated as described previously.[11] For the experiments described here, female PAFR−/− mice, backcrossed seven times to a

C57BL/6 background, and female wild-type C57BL/6 (PAFR+/+) mice (Harlan Sprague Dawley Inc., Horst, the Netherlands), were used at 6–8 weeks of age. The Animal Care and Use Committee of the University of Amsterdam (Amsterdam, the Netherlands) approved all experiments.

Experimental infection

Pulmonary TB was induced exactly as described previously.[12–14] Briefly, a virulent laboratory strain of *M. tuberculosis* H37Rv was grown for 4 days in liquid Dubois medium containing 0·01% Tween-80. A replicate culture was incubated at 37°, harvested at mid-log phase, and stored in aliquots at −70°. For each experiment, a vial was thawed and washed twice with sterile 0·9% NaCl. Mice were anaesthetized by inhalation of isoflurane (Abbott Laboratories, Kent, UK) and infected with 1×10^5 live bacilli in 50 μl of saline, as determined by viable counts on 7H11 Middlebrook agar plates. Bacterial administration was performed intranasally, as described previously.[12–14] Survival was monitored for 200 days in 14 PAFR−/− and 14 PAFR+/+ mice. In addition, groups of eight mice per time-point were killed 2 or 6 weeks postinfection, and the lungs and one lobus of the liver were removed aseptically. Organs were homogenized using a tissue homogeniser (Biospec Products, Bartlesville, OK), in 5 volumes of sterile 0·9% NaCl, and 10-fold serial dilutions were plated on Middlebrook 7H11 agar plates to determine bacterial loads. Colonies were counted after 21 days of incubation at 37°. Numbers of colony-forming units (CFU) are provided as total in the lungs or as total/g of liver tissue. For cytokine measurements, lung homogenates were diluted 1 : 1 in lysis buffer (150 mM NaCl, 15 mM Tris, 1 mM $MgCl.H_2O$, 1 mM $CaCl_2$, 1% Triton-X-100, 100 μg/ml pepstatin A, leupeptin, and aprotinin), and incubated on ice for 30 min. Supernatants were sterilized using a 0·22-μm filter (Corning, Corning, NY) and frozen at −20° until required.

Histological analysis

The right lungs of six PAFR−/− and six wild-type PAFR+/+ mice were removed 2 or 6 weeks after intranasal inoculation with *M. tuberculosis* and then fixed for 24 hr in 4% paraformaldehyde in phosphate-buffered saline (PBS). After embedding in paraffin wax, 4-μm-thick sections were stained with haematoxylin & eosin or the Ziehl–Neelsen (ZN) stain for acid-fast bacilli. All slides were coded and semiquantitatively scored for the total area of inflammation (percentage of surface of the slide) and granuloma format by a pathologist. In separate experiments, organs of six uninfected PAFR−/− and wild-type PAFR+/+ were harvested and examined as described above.

Fluorescence-activated cell sorter (FACS) analysis

For FACS analysis, pulmonary cell suspensions were obtained using an automated disaggregation device (Medimachine System; Dako, Glostrup, Denmark) and processed as described previously.[13] Cells from two mice per group ($n = 10$) were pooled for each time-point (yielding five samples per group for FACS analysis) and then adjusted to a concentration of 4×10^6 cells/ml of FACS buffer (PBS supplemented with 0·5% bovine serum albumin, 0·01% NaN_3, and 100 mM EDTA). Immunostaining for cell-surface molecules was performed for 30 min at 4° using antibodies (Abs) directly labelled against CD3 [anti-CD3 phycoerythrin (PE)], CD4 (anti-CD4 CyChrome), CD8 [anti-CD8 fluorescein isothiocyanate (FITC); anti-CD8 peridinin chlorophyll protein (PerCP)], CD25 (anti-CD25 FITC) and CD69 (anti-CD69 FITC). All Abs were used at concentrations recommended by the manufacturer (PharMingen, San Diego, CA). To correct for non-specific staining, an appropriate control Ab (rat IgG2; PharMingen) was used. The number of positive cells was obtained by setting a quadrant marker for non-specific staining.

Cytokine measurements

Interferon-γ (IFN-γ) and interleukin (IL)-4 concentrations were measured using commercially available enzyme-linked immunosorbent assay (ELISA) reagents, according to the instructions of the manufacturer (R & D Systems, Abingdon, UK).

Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM). Comparisons were performed using Mann–Whitney *U*-tests. For comparison of survival curves, Kaplan-Meier analysis with a log rank test was used. *P*-Values of $\leq 0{\cdot}05$ were considered statistically significant.

RESULTS

Survival

PAFR−/− and PAFR+/+ mice were inoculated intranasally with 10^5 live *M. tuberculosis* bacilli and their survival was monitored for a time-period of 200 days (Fig. 1). Although PAFR−/− mice tended to succumb to TB earlier than PAFR+/+ mice, the difference between the two strains was not significant. Overall, the survival rate was 14% for PAFR−/− mice and 21% for PAFR+/+ mice (not significant).

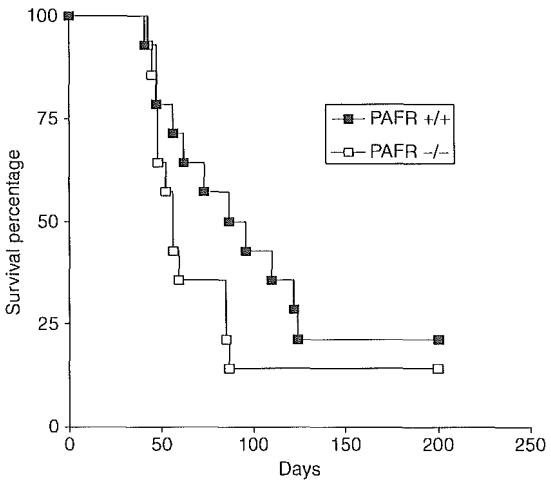

Figure 1. Platelet-activating factor (PAF) receptor deficiency does not influence survival during murine lung tuberculosis. Survival of PAF receptor-deficient (PAFR−/−) and wild-type (PAFR+/+) mice infected intranasally with 10^5 *Mycobacterium tuberculosis* colony-forming units (CFU) ($n = 14$ per group). No significant difference was found in lethality between the two strains of mice.

Mycobacterial outgrowth

Next, the numbers of *M. tuberculosis* CFU were determined in lungs and livers of PAFR−/− and PAFR+/+ mice at 2 and 6 weeks after intranasal infection. Both organs contained a similar number of *M. tuberculosis* CFU in PAFR−/− and PAFR+/+ mice at each time-point (Fig. 2).

Cellular recruitment to lungs

The histology of parenchymatous organs of 8–10-week-old PAFR−/− and PAFR+/+ mice, without *M. tuberculosis* infection, was similar and displayed no signs of abnormalities (data not shown). Histopathological examination of lungs from PAFR−/− and PAFR+/+ mice at 2 and 6 weeks after intranasal infection with *M. tuberculosis* revealed no differences between the two mouse strains. Figure 3 shows representative slides of lungs from mice killed 6 weeks after intranasal infection. At this time-point, dense and diffuse infiltrates were found in the lungs of both mouse strains; the percentage of inflamed parenchyma was similar in both groups (data not shown). To obtain

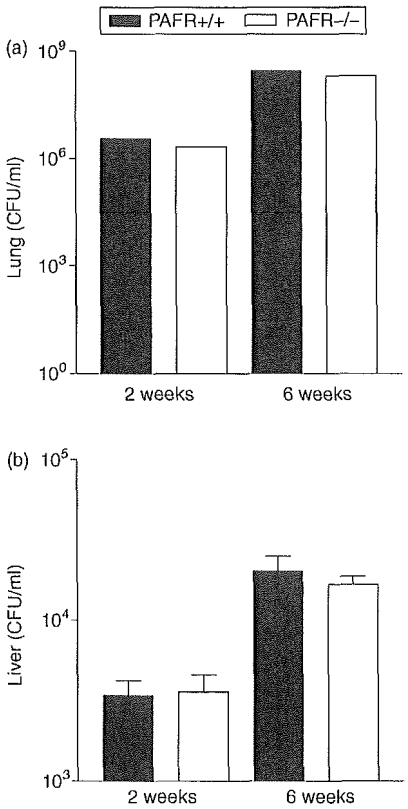

Figure 2. Platelet-activating factor (PAF) receptor deficiency does not influence mycobacterial outgrowth in lungs or liver during murine tuberculosis. Bacterial outgrowth is represented, in colony-forming units (CFU)/ml of organ, in PAF receptor-deficient (PAFR−/−) and wild-type (PAFR+/+) mice in lungs (a) and livers (b) at 2 and 6 weeks after intranasal infection with 10^5 *Mycobacterium tuberculosis* CFU. Data represent mean values ± standard error (SE) (n = 8/group). SE values in Fig. 2(a) were too small to show on the figure. No significant differences were found in mycobacterial outgrowth.

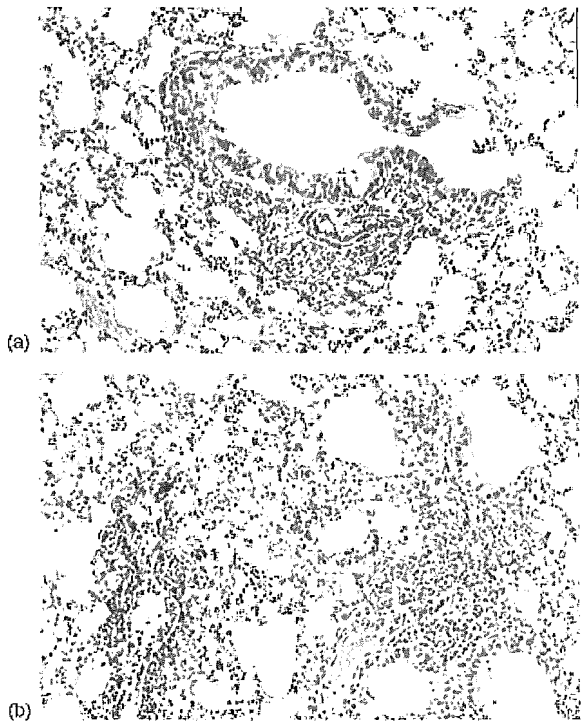

Figure 3. No differences in histopathology were observed between platelet-activating factor (PAF) receptor-deficient (PAFR−/−) and wild-type (PAFR+/+) mice. (a) Representative slides of lung tissue of PAFR+/+ mice, 6 weeks after intranasal infection with 10^5 *Mycobacterium tuberculosis* colony-forming units (CFU), showed a diffuse inflammatory infiltrate which was almost confluent. Macrophages were the most predominant cell type observed, together with small number of lymphocytes (haematoxylin & eosin staining; original magnification × 25). A comparable picture was observed in PAFR−/− mice (b) 6 weeks postinfection (haematoxylin & eosin staining, original magnification × 25). Slides are representative for six mice per strain.

further insight into the cellular composition of the pulmonary infiltrates, we analysed whole lung cell suspension by FACS analysis. The percentages of $CD4^+$ and $CD8^+$ lymphocytes did not differ significantly between PAFR−/− and PAFR+/+ mice; furthermore, the surface expression of CD25 and CD69 on T cells was similar in both mouse strains (shown for the 6-week postinfection time-point in Table 1).

Lung IFN-γ and IL-4 concentrations

Cytokine concentrations in lung homogenates of 8–10-week-old PAFR−/− and PAFR+/+ mice, without *M. tuberculosis* infection, were either low or undetectable, with no differences between groups. IFN-γ and IL-4 concentrations in lung homogenates obtained at 2 and 6 weeks postinfection were similar in PAFR−/− and PAFR+/+ mice (shown for the 6-week postinfection time-point in Table 1).

DISCUSSION

PAF has been implicated as a protective mediator in the host response to several intracellular pathogens. The data presented

Table 1. Cellular composition and cytokine concentrations in lungs

Cells (10^4/ml)	PAFR+/+	PAFR−/−
Total cells	290·0 ± 31·1	278·0 ± 45
Cell subsets (percentage of total)		
$CD4^+$	67·2 ± 0·8	71·3 ± 1·2
$CD8^+$	27·1 ± 1·1	22·0 ± 0·9
$CD4^+/CD69^+$	9·9 ± 0·9	11·5 ± 2·4
$CD4^+/CD25^+$	8·6 ± 0·7	11·1 ± 1·6
$CD8^+/CD69^+$	16·7 ± 2·2	15·2 ± 0·6
$CD8^+/CD25^+$	1·7 ± 0·2	2·0 ± 0·4
Cytokines (ng/ml)		
IFN-γ	7·75 ± 0·64	7·68 ± 0·64
IL-4	5·96 ± 0·75	6·67 ± 0·64

Total cell counts and lymphocyte typing were performed on pulmonary cell suspensions 6 weeks postinfection, as described in the Materials and methods. Fluorescence-activated cell sorter (FACS) analysis was performed on pooled cells from two mice for each analysis from a total of 10 mice per group (i.e. yielding five samples per mouse strain). FACS results are expressed as the percentage of $CD4^+$, $CD8^+$, $CD25^+$ and $CD69^+$ within the $CD3^+$ population [i.e. for each of the five samples per mouse strain the percentage of positive cells relative to the total number of $CD3^+$ cells was determined, and from these data means ± standard error (SE) were calculated]. Cytokine data were obtained from eight mice per group and data are expressed as mean ± SE.

PAFR−/−, platelet-activating factor receptor gene-deficient mice; PAFR+/+, wild-type platelet-activating factor mice.

here argue against such a protective role of PAF in pulmonary TB. Indeed, intranasal infection with live *M. tuberculosis* was associated with similar mortality rates in PAFR−/− and PAFR+/+ mice, and the mycobacterial loads in lungs and liver, determined during the early phase of the infection when all animals were still alive, did not differ significantly between the two mouse strains.

Host defence against TB, at least in part, relies on $CD4^+$ and $CD8^+$ T cells.[2] We therefore determined the number of T cells in whole-lung cell suspensions and, in addition, obtained insight into their activation state by measuring the surface expression of CD25 and CD69. Theoretically, PAF can inhibit certain lymphocyte functions. Indeed, PAF has been found to reduce proliferation of $CD4^+$ T cells induced by phytohaemagglutinin, which is associated with a reduced expression of CD25.[15] PAF also suppresses the mitogen-stimulated production of IL-2 by human lymphocytes.[16] However, to our knowledge little, if anything, is known about the effects of PAF on lymphocyte activation *in vivo*. We here demonstrate that deficiency of the PAFR does not influence the recruitment or activation of $CD4^+$ and $CD8^+$ lymphocytes during pulmonary TB.

The clinical outcome of pulmonary TB is considered to be dependent on a type 1-mediated host response.[2] We therefore determined whether PAFR deficiency influences the type 1/type 2 balance by measuring the concentration of the type 1 cytokine IFN-γ and the type 2 cytokine, IL-4, in lung homogenates of infected PAFR−/− and PAFR+/+ mice. However, no differences in the pulmonary concentrations of these cytokines were found between these two strains.

Our assumption, that PAF could be involved in the protective immune response to TB, was primarily based on its reported protective role in experimental infections of mice with *L. amazonensis*, *T. cruzi* and *Candida albicans*.[6,7,17] From the present study it remains unclear why PAF does not contribute to protective immunity in TB. PAFR−/− mice are capable of producing PAF, yet PAF cannot exert any biological effect owing to the absence of its receptor.[4] Knowledge of the production of PAF in TB, either experimentally induced or in patients, is, to the best of our knowledge, not available. In this respect it is important to realize that PAF measurements do not necessarily provide insight into the production of this lipid mediator, as PAF that is synthesized remains predominantly in cell-associated form.[18] Clearly, further research is warranted to dissect the distinct molecular mechanisms that contribute to an adequate immune response to different intracellular pathogens.

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Role of PAF receptors during intestinal ischemia and reperfusion injury. A comparative study between PAF receptor-deficient mice and PAF receptor antagonist treatment

[1]Danielle G. Souza, [1]Vanessa Pinho, [1]Adriana C. Soares, [2,3]Takao Shimizu, [2,3]Satoshi Ishii & *,[1]Mauro M. Teixeira

[1]Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil; [2]Department of Biochemistry and Molecular Biology, Faculty of Medicine, The University of Tokyo, Japan and [3]CREST of Japan Science and Technology Corporation, Tokyo, Japan

1 The reperfusion of ischemic tissues may be associated with local and systemic inflammation that prevents the full benefit of blood flow restoration. The present study aimed to confirm a role for platelet-activating factor receptor(s) (PAFR) during ischemia and reperfusion injury by using genetically modified mice deficient in the PAFR ($PAFR^{-/-}$ mice) and to evaluate comparatively the effectiveness of pharmacological treatment using the PAFR antagonist UK-74,505 (modipafant).

2 The reperfusion of the ischemic superior mesenteric artery (SMA) induced marked local (intestine) and remote (lungs) tissue injury, as assessed by the increase in vascular permeability, neutrophil influx and intestinal hemorrhage and in the production of TNF-α. There was also a systemic inflammatory response, as shown by the increase in serum TNF-α concentrations and marked reperfusion-associated lethality.

3 After reperfusion of the ischemic SMA, $PAFR^{-/-}$ mice had little tissue or systemic inflammation and lethality was delayed, but not prevented, in these mice. Interestingly, the reperfusion-associated increases in tissue concentrations of IL-10 were significantly greater in $PAFR^{-/-}$ than wild-type mice.

4 Pretreatment with PAFR antagonist UK-74,505 ($1\,mg\,kg^{-1}$) markedly prevented tissue injury, as assessed by the increase in vascular permeability, neutrophil accumulation, hemorrhage and TNF-α concentrations in the intestine and lungs. In contrast, UK-74,505 failed to affect reperfusion-associated lethality and increases in serum TNF-α when used at $1\,mg\,kg^{-1}$.

5 Reperfusion-associated lethality and increase in serum TNF-α were only affected when a supra-maximal dose of the antagonist was used ($10\,mg\,kg^{-1}$). At this dose, UK-74,505 also induced a marked enhancement of reperfusion-associated increases in tissue concentrations of IL-10. However, at the same dose, UK-74,505 failed to prevent reperfusion-associated lethality in $PAFR^{-/-}$ mice any further.

6 The present studies using genetically modified animals and a receptor antagonist firmly establish a role of PAFR activation for the local, remote and systemic inflammatory injury and lethality which follows reperfusion of the ischemic SMA in mice. Moreover, it is suggested that high doses of PAFR antagonists need to be used if the real efficacy of these compounds is to be tested clinically.

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Keywords: PAF receptor; lung injury; inflammation; TNF-α;IL-10

Abbreviations: I/R, ischemia and reperfusion; MPO, myeloperoxidase; PAF, platelet-activating factor; PAFR, platelet-activating factor receptor(s); $PAFR^{-/-}$ mice, platelet-activating factor receptor-deficient mice; SMA, superior mesenteric artery

Introduction

A major goal in the treatment of ischemia of a vascular territory is to restore blood flow to normal values, that is, to 'reperfuse' the ischemic vascular bed (Carden & Granger, 2000). However, reperfusion of ischemic tissues is associated with local and systemic leukocyte activation and trafficking, endothelial barrier dysfunction in postcapillary venules, enhanced production of inflammatory mediators and great lethality (Lefer & Lefer, 1996; Granger, 1999; Carden & Granger, 2000). For example, after intestinal ischemia and reperfusion (I/R), there is marked intestinal and pulmonary injury that may also be accompanied by a systemic inflammatory response syndrome and significant lethality (Souza *et al.*, 2000b; 2001; 2002). Among the mediators of the inflammatory cascade released and thought to be important for the reperfusion-associated injury is platelet-activation factor (PAF) (Kubes *et al.*, 1990a, b; Montrucchio *et al.*, 2000; Souza *et al.*, 2000b).

PAF is a natural phospholipid that under normal physiological conditions is minimally expressed. However, during acute inflammation or under conditions of oxidative stress, as occurs during I/R injury, PAF is released by neutrophils and/

*Author for correspondence at: Departamento de Bioquímica e Imunologia, Instituto de Ciencias Biologicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627 Pampulha, 31270-901 Belo Horizonte MG, Brazil; E-mail: mmtex@icb.ufmg.br

or monocytes and expressed at the outer leaflet of endothelial cells (Montrucchio *et al.*, 2000). Once released, activation of PAF receptors (PAFR) results in diverse biological activities associated with acute inflammation, including neutrophil activation and chemotaxis, alterations in vascular permeability and platelet activation, all of which may contribute to the clinical manifestation of I/R injury (Montrucchio *et al.*, 2000).

We have recently described the effects of the treatment with the PAFR antagonists UK-74,505 and WEB-2086 in models of neutrophil-dependent mild and severe I/R injury in rats (Souza *et al.*, 2000b). Our results demonstrated that treatment with the PAFR antagonists prevented the increases in vascular permeability, neutrophil recruitment and TNF-α production (Souza *et al.*, 2000b). Furthermore, activation of PAFR has been shown to play an important pathophysiological role in models of I/R injury in several other vascular territories, including the heart, gut, kidney and lung (Canale *et al.*, 1994; Carter *et al.*, 1996; Riera *et al.*, 1997; Qayumi *et al.*, 1998; Morgan *et al.*, 1999; Kecskemeti & Balogh, 2000; Kim *et al.*, 2000; Sun *et al.*, 2001; 2002). The objectives of the present study were two-fold: (i) to confirm a role for PAFR during I/R injury by using genetically modified mice deficient in the PAFR ($PAFR^{-/-}$ mice); and (ii) to evaluate comparatively the effectiveness of pharmacological treatment using the PAFR antagonist UK-74,505 and the genetic PAFR deficiency.

Methods

Animals

Male C57BL/6 mice (8–10 weeks) obtained from the Bioscience unit of Instituto de Ciências Biológicas were housed under standard conditions and had free access to commercial chow and water. All procedures described here had prior approval from the local animal ethics committee. $PAFR^{-/-}$ mice were generated as previously described (Ishii *et al.*, 1998) and intercrossed for at least seven generations to establish the C57BL/6 strain.

Ischemia and reperfusion

Mice were anesthetized with urethane (140 mg kg^{-1}, i.p.) and laparotomy was performed. The superior mesenteric artery (SMA) was isolated and ischemia was induced by totally occluding the SMA for 60 min. For measuring the percentage of surviving mice, reperfusion was re-established, and mice were monitored for indicated time periods. For the other parameters, reperfusion was allowed to occur for 30 min (I60R30) when mice were killed. This time of reperfusion (30 min) was chosen based on the presence of significant tissue injury without unduly high mortality rates. Sham-operated animals were used as controls. The treatment with UK-74,505 or vehicle was administrated (i.v.) 10 min before reperfusion.

Evaluation of changes in vascular permeability

The extravasation of Evans blue dye into the tissue was used as an index of increased vascular permeability, as previously described (Saria & Lundberg, 1983; Souza *et al.*, 2000a). Evans blue (20 mg kg^{-1}) was administered i.v. (1 ml kg^{-1}) *via* a tail vein 2 min prior to reperfusion of the ischemic artery. At 30 min after reperfusion, a segment of the duodenum (approximately 3 cm) was cut open and allowed to dry in a Petri dish for 24 h at 37°C. The dry weight of the tissue was calculated and Evans blue extracted using 1 ml of formamide (24 h at room temperature). The amount of Evans blue in the tissue was obtained by comparing the extracted absorbance with that of a standard Evans blue curve read at 620 nm in an ELISA plate reader. Results are presented as the amount of Evans blue per μg per 100 mg of tissue. The right ventricle was flushed with 10 ml of phosphate-buffered saline (PBS) to wash the intravascular Evans blue in the lungs. The left lung was then excised and used for Evans blue extraction. The right lung was used for the determination of myeloperoxidase as described below.

Myeloperoxidase (MPO) concentrations

The extent of neutrophil accumulation in the intestine and right lung tissue was measured by assaying myeloperoxidase (MPO) activity, as previously described (Kuebler *et al.*, 1996; Souza *et al.*, 2002). Briefly, a portion of duodenum and the flushed right lungs of animals that had undergone I/R injury were removed and snap frozen in liquid nitrogen. Upon thawing and processing, the tissue was assayed for MPO activity by measuring the change in optical density (OD) at 450 nm using tetramethylbenzidine. Results were expressed as the neutrophil infiltration. An index unit denotes the MPO activity present in 10^5 casein-elicited murine peritoneal neutrophils processed in the same way.

Measurement of hemoglobin concentrations

The determination of hemoglobin concentrations in tissue was used as an index of tissue hemorrhage. After washing the intestines to remove excess blood, a sample of approximately 100 mg of duodenum was removed and homogenized in Drabkin's color reagent according to the instructions of the manufacturer (Analisa, Belo Horizonte, Brazil). The suspension was centrifuged for 15 min at 3000 $\times g$ and filtered using 0.2 μm filters. The resulting solution was read using an ELISA plate reader at 520 nm and compared against a standard curve of hemoglobin.

Measurement of cytokine/chemokine concentrations in serum, intestine and lungs

The concentration of TNF-α and IL-10 in samples was measured in serum and tissue of animals using commercially available antibodies and according to the procedures supplied by the manufacturer (R&D Systems, Minneapolis). Serum was obtained from coagulated blood (15 min at 37°C, then 30 min at 4°C) and stored at −20°C until further analysis. Serum samples were analyzed at a 1 : 3 dilution in PBS. Duodenum (100 mg) or lung of sham-operated and reperfused animals were homogenized in 1 ml of PBS (0.4 M NaCl and 10 mM de $NaPO_4$) containing antiproteases (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 KI aprotinin A) and 0.05% Tween 20. The samples were then centrifuged for 10 min at 3000 $\times g$ and the supernatant immediately used for ELISA assays at a 1 : 3 dilution in PBS.

Drugs and reagents

The following drugs were obtained from Sigma (U.S.A.): urethane, Evans blue, hexadecyltrimethylammonium bromide. The PAF receptor antagonist UK-74,505 (modipafant) was a gift of Dr J. Parry (Pfizer, Sandwich, U.K.) (Alabaster *et al.*, 1991). UK-74,505 was dissolved in 0.1 M HCl and further diluted 10-fold in saline just prior to use.

Statistical analysis

Results are shown as means ± s.e.m. Percent inhibition was calculated by subtracting the background values obtained in sham-operated animals. Differences were compared by using analysis of variance (ANOVA) followed by Student–Newman–Keuls *post hoc* analysis. Results with $P<0.05$ were considered significant.

Results

Intestinal I/R injury and lethality in wild-type and PAFR$^{-/-}$ mice

The reperfusion of the ischemic SMA artery induced marked local (intestine) and remote (lungs) tissue injury, as assessed by the increase in vascular permeability, neutrophil influx and intestinal hemorrhage observed in wild-type mice (Figure 1). In contrast, there was a marked and almost complete inhibition of tissue injury in PAFR$^{-/-}$ mice submitted to the same experimental conditions (Figure 1).

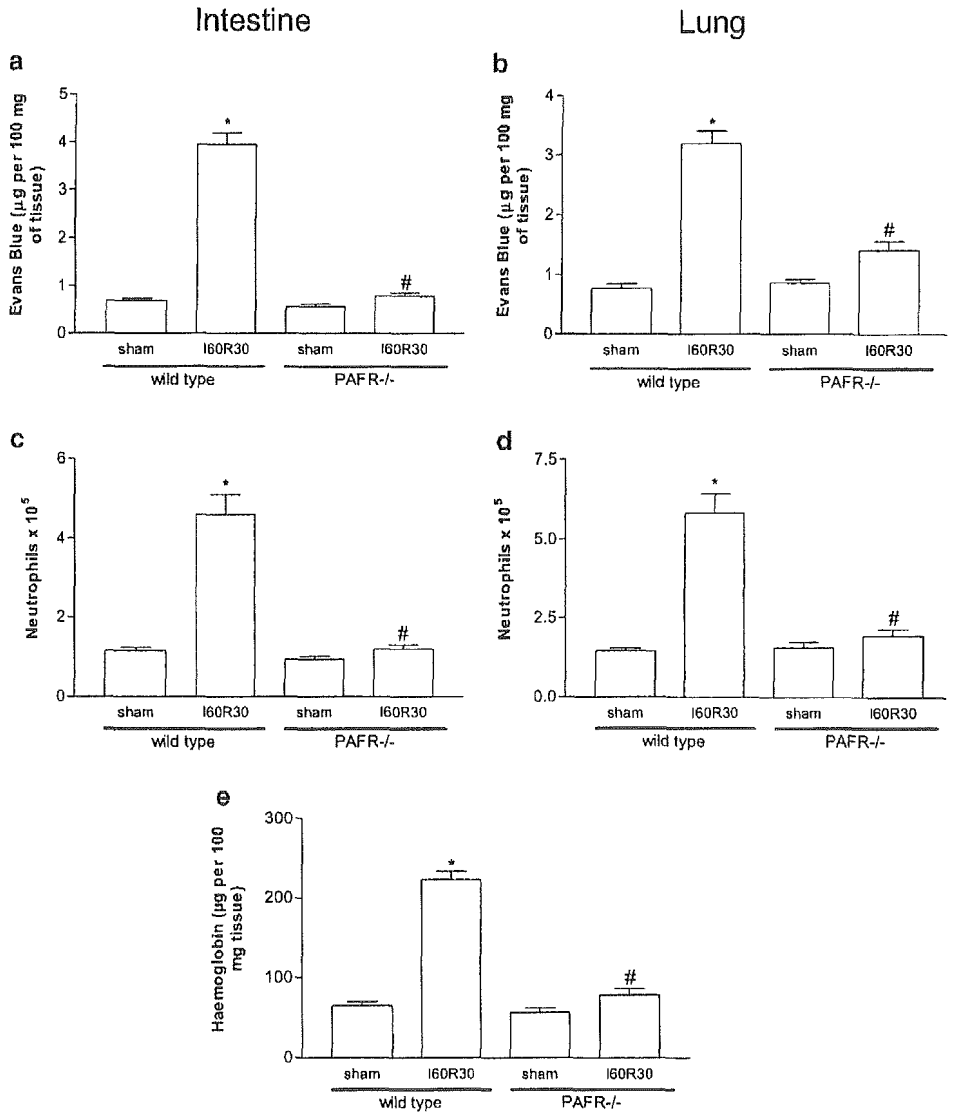

Figure 1 Tissue injury in wild-type (WT) and in PAFR-deficient mice (PAFR$^{-/-}$) submitted to ischemia and reperfusion of the SMA. WT or PAFR$^{-/-}$ were sham-operated or submitted to 60 min of ischemia and 30 min of reperfusion of the SMA. Changes in vascular permeability in the intestine (a) and lungs (b) were evaluated by measuring the extravasation of Evans Blue (µg per 100 mg of tissue). Neutrophil infiltration was determined by measurement of intestinal (c) and pulmonary (d) MPO activity. Hemorrhage in the intestine (e) was evaluated by measuring the concentration of hemoglobin in 100 mg of tissue. Data are shown as the mean ± s.e.m. of five to six mice in each group. *$P<0.01$ when compared with the sham-operated group and $^{\#}P<0.01$ when compared with WT mice submitted to I/R.

In addition to the local and remote inflammatory changes described above, there was a marked increase in the concentrations of TNF-α in the intestine, lungs and serum of wild-type reperfused mice (Figure 2). Again, the increases in the concentration of this cytokine were markedly suppressed in PAFR$^{-/-}$ mice submitted to intestinal I/R (Figure 2). Following reperfusion of the ischemic SMA, the concentrations of the anti-inflammatory cytokine IL-10 were much greater in the intestine and lungs of reperfused PAFR$^{-/-}$ mice than in the tissues of their wild-type controls (Figure 3).

In addition to the observed suppression of tissue and systemic inflammation, there was a significant delay in reperfusion-induced lethality in PAFR$^{-/-}$ (Figure 4). However, delay in lethality was not accompanied by prevention of lethality, as all animals were dead after 90 min of reperfusion (Figure 4).

Effects of the treatment with UK-74,505 on intestinal ischemia and reperfusion injury and lethality

As tissue and systemic inflammation was suppressed and lethality delayed in PAFR$^{-/-}$ mice, it was of interest to examine whether similar effects could be observed after treatment with a PAF receptor antagonist. To this end, UK-74,505, a selective and potent PAFR antagonist, was used at a

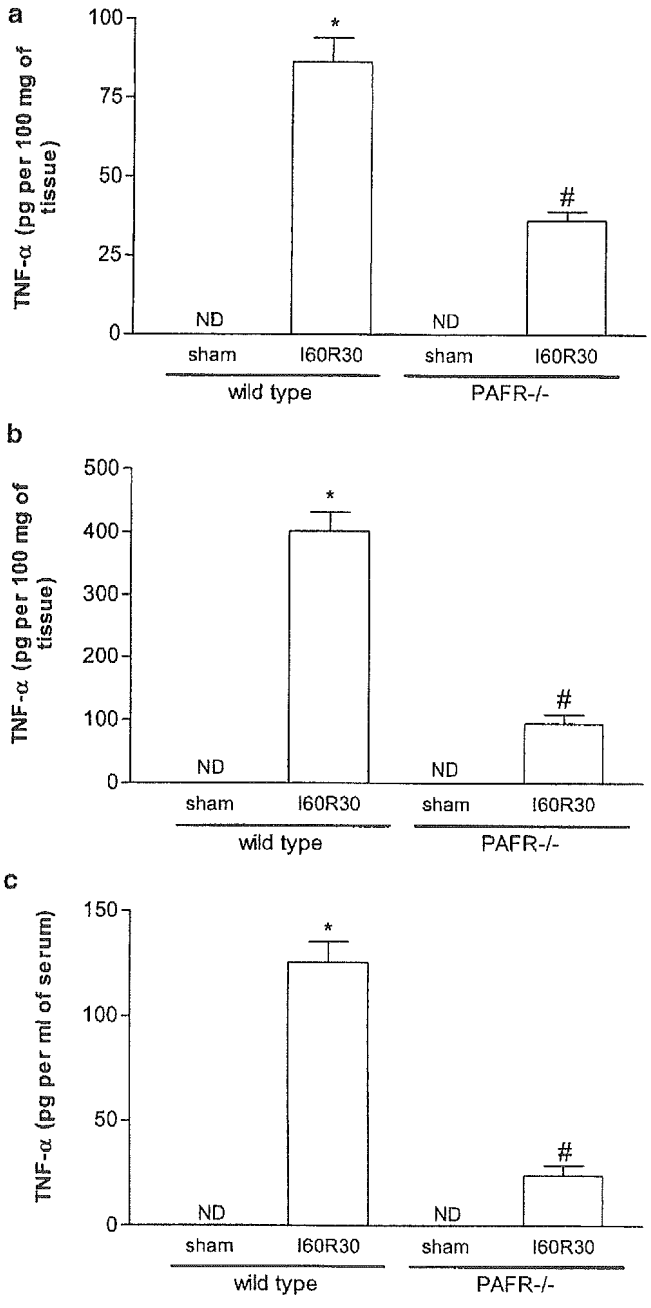

Figure 2 Concentration of TNF-α in the intestine, lungs and serum of WT and in PAFR$^{-/-}$ submitted to ischemia and reperfusion of the SMA. WT or PAFR$^{-/-}$ were sham-operated or submitted to 60 min of ischemia and 30 min of reperfusion of the SMA. The concentrations of TNF-α in intestine (a), lungs (b) and serum (c) were measured by ELISA. Results are shown as picogram of TNF-α per 100 mg of tissue or as picogram TNF-α per milliliter of serum and represent the mean±s.e.m. of five to six mice in each group. $^{*}P<0.01$ when compared with the sham-operated group and $^{\#}P<0.01$ when compared with WT mice submitted to I/R.

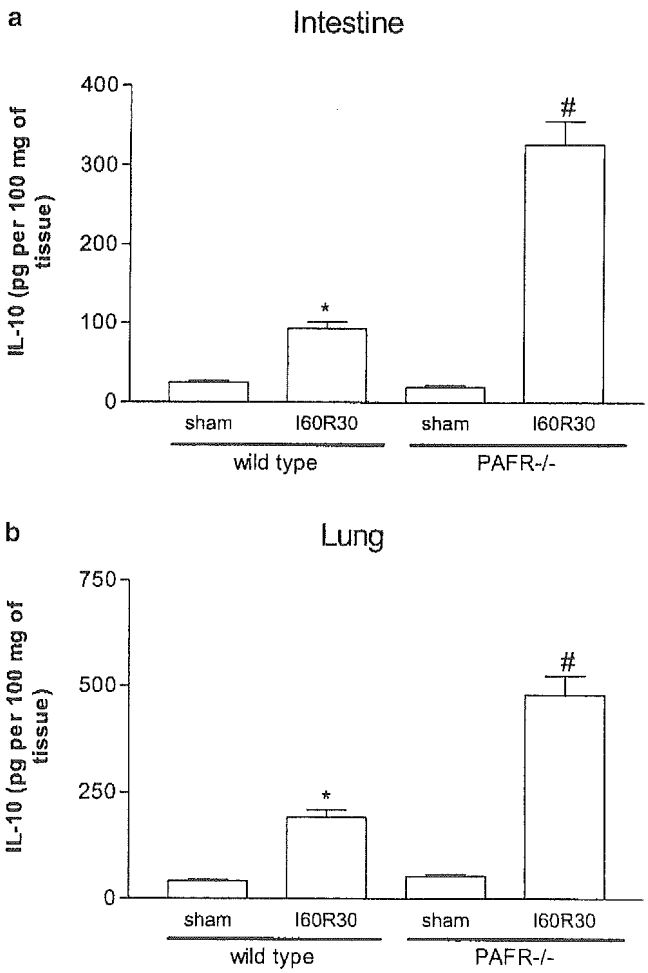

Figure 3 Concentration of IL-10 in the intestine and lungs WT and in PAFR$^{-/-}$ submitted to ischemia and reperfusion of the SMA. WT or PAFR$^{-/-}$ were sham-operated or submitted to 60 min of ischemia and 30 min of reperfusion of the SMA. The concentrations of IL-10 in intestine (a) and lungs (b) were measured by ELISA. Results are shown as picogram of IL-10 per 100 mg of tissue and represent the mean±s.e.m. of five to six mice in each group. $^{*}P<0.01$ when compared with the sham-operated group and $^{\#}P<0.01$ when compared with wild-type mice submitted to I/R.

dose of 1 mg kg^{-1} that has been previously shown to block PAFR effectively in the mouse or rat (Miotla *et al.*, 1998; Borges *et al.*, 2000; Souza *et al.*, 2000b; Klein *et al.*, 2002). The treatment with UK-74,505 10 min prior to the reperfusion virtually abolished the increases in vascular permeability and influx of neutrophils in the intestine and lungs following intestinal I/R (Table 1). The reperfusion-induced intestinal hemorrhage, as assessed by extravasation of hemoglobin, was abrogated in UK-74,505-treated animals (Table 1).

Interestingly, although the increase in reperfusion-induced tissue concentrations of TNF-α was markedly inhibited by UK-74,505 used at 1 mg kg^{-1}, this dose of the PAFR antagonist had no significant effect on serum concentrations of TNF-α (Figure 5b). Note that the concentrations of TNF-α in the intestine and lungs of UK-74,505-treated mice were equivalent to the concentrations found in reperfused PAFR$^{-/-}$ mice (compare Figure 2 and Table 1). At this dose, UK-74,505 also failed to enhance significantly the increases in IL-10 production in the lungs and intestine following reperfusion of the ischemic SMA (Figure 6). Our previous studies have shown a strong correlation between serum, but not tissue, concentrations of TNF-α and lethality (Souza *et al.*, 2001; 2002). Consistently with these results, treatment of mice with 1 mg kg^{-1} of UK-74,505 had no effect on the lethality that followed reperfusion of the ischemic mesenteric artery (Figure 5a).

The lack of effects of UK-74,505 on reperfusion-associated increase in serum concentrations of TNF-α and lethality was unexpected in the face of the results obtained in PAFR$^{-/-}$ mice. Although the dose of UK-74,505 used has been previously to block PAFR effectively in several *in vivo* systems (Miotla *et al.*, 1998; Borges *et al.*, 2000; Souza *et al.*, 2000b; Klein *et al.*, 2002) and effectively blocked tissue injury in our model, we carried out a series of experiments using a supramaximal dose of the antagonist (10 mg kg^{-1}) reasoning that maximal occupation of the receptor by the antagonist might be necessary to prevent reperfusion-associated lethality. At the higher dose of UK-74,505 used, there was a marked suppression of the reperfusion-induced increase in the serum concentrations of TNF-α (Figure 5b). More importantly, the inhibition of TNF-α was associated with a delay and partial prevention of reperfusion-associated lethality (Figure 5a). Akin to the results observed in PAFR$^{-/-}$ mice, treatment with the higher dose of UK-74,505 markedly enhanced the increases in IL-10 production in the intestine and lung of reperfused mice (Figure 6). To exclude an action of UK-74,505 (10 mg kg^{-1}) outside its effects on the PAFR, PAFR$^{-/-}$ mice were treated with the drug prior to experiments evaluating reperfusion-associated lethality. As seen in Figure 4, the treatment of PAFR$^{-/-}$ with UK-74505 (10 mg kg^{-1}) had no further effect on lethality in addition to that of the PAFR$^{-/-}$ phenotype.

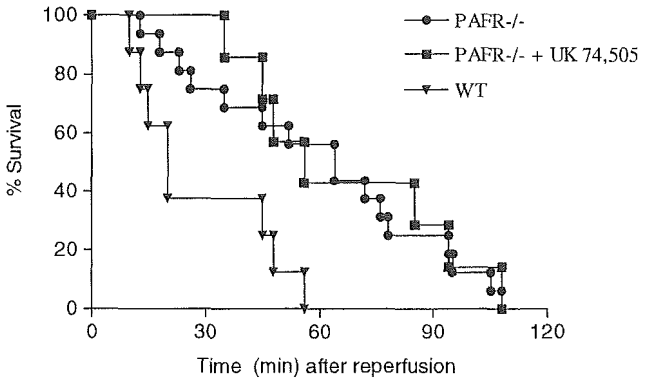

Figure 4 Survival curves of WT and in PAFR$^{-/-}$ submitted to ischemia and reperfusion of the SMA. Mice ($n=10$ in each group) were anesthetized and submitted to ischemia of the SMA for 60 min. Vehicle or UK-74,505 (10 mg kg^{-1}) was administered i.v. 10 min prior to reperfusion. Tissue perfusion was then re-established and survival was monitored. The survival curve of PAFR$^{-/-}$ mice was significantly ($P<0.05$) different from that of WT mice.

Discussion

The restoration of blood flow to an ischemic vascular bed, that is, reperfusion, is a major therapeutic objective after ischemia of an organ or tissue. However, reperfusion may be accompanied by significant local and systemic inflammatory injury, limiting the potential benefits of blood flow restoration. Thus, understanding the pathophysiology of the inflammation that occurs after reperfusion may be useful in the development of novel therapeutic strategies that limit the injury caused by the reperfusion process. Here, the role of PAFR in I/R injury was investigated using mice with a targeted deletion of the PAF receptor gene (Ishii *et al.*, 1998) and the PAFR antagonist UK-74,505 (Alabaster *et al.*, 1991).

Table 1 Effects of the postischemic treatment with the PAF receptor antagonist, UK-74,505 (1 mg kg^{-1}) on the tissue injury in the intestine and lungs of mice submitted to ischemia and reperfusion of the superior mesenteric artery (SMA)

	Intestine			*Lung*		
	Sham	*Vehicle*	*UK*	*Sham*	*Vehicle*	*UK*
Evans Blue	0.7±0.1	4.0±0.2	1.3±0.1	0.8±0.1	3.2±0.2	1.1±0.1
MPO	1.2±0.1	4.6±0.5	1.6±0.1	1.5±0.1	5.8±0.6	2.1±0.2
Hemoglobin	64.6±5.3	223±10.5	79.6±6.7	—	—	—
TNF	ND	86.2±8.0	38.7±4.0	ND	401.3±31	162.5±18.3

Changes in vascular permeability were evaluated by measuring the extravasation of Evans Blue (μg per 100 mg of tissue), neutrophil infiltration was determined by measurement of tissue myeloperoxidase activity, hemorrhage by measuring the concentration of hemoglobin in 100 mg of tissue and concentrations of TNF-α in intestine and lungs by ELISA. UK-74,505 (1 mg kg^{-1}) was given i.v. 5 min prior to reperfusion and control animals received vehicle. Results are shown as picogram of TNF-α per 100 mg of tissue and represent the mean±s.e.m. of six mice in each group. $^{*}P<0.01$ when comparing to the sham-operated group and $^{\#}P<0.01$ when comparing to vehicle-treated mice submitted to I/R.

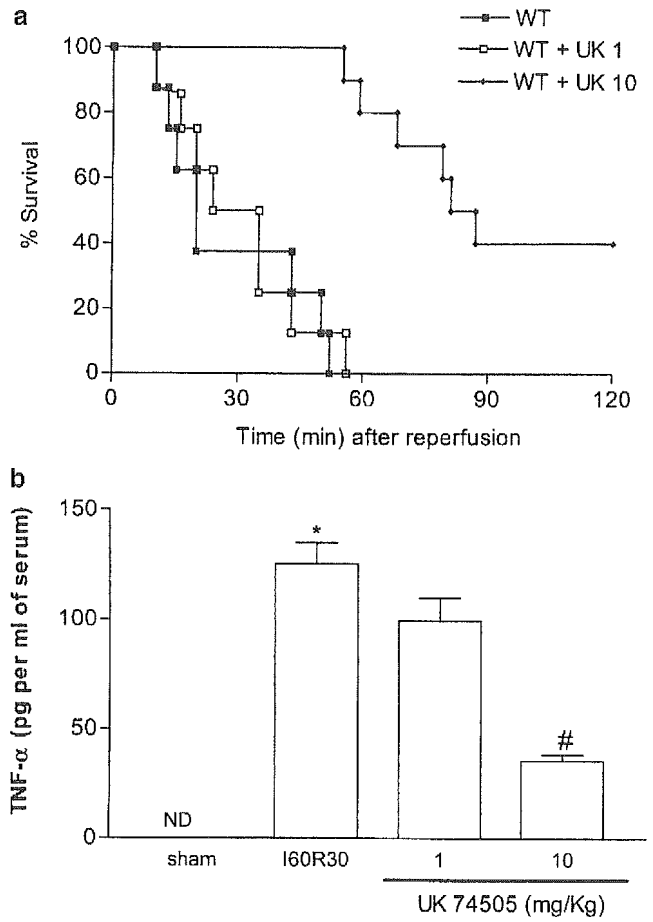

Figure 5 Effects of postischemic treatment with the PAF receptor antagonist UK-74,505 on the (a), survival and (b) serum concentration of TNF-α in mice submitted to ischemia and reperfusion of the SMA. In (a) mice treated with UK-74,505 (1 or 10 mg kg^{-1}) or vehicle ($n=10$ in each group) were anesthetized and submitted to ischemia of the SMA for 60 min. Tissue perfusion was then re-established and survival was monitored. The survival curve of UK-74,505-treated mice (10 mg kg^{-1}) was significantly ($P<0.05$) different from that of vehicle-treated mice. In (b), mice were sham-operated or submitted to 60 min of ischemia of the SMA and reperfusion was allowed for 30 min. Concentrations of TNF-α serum were measured by ELISA. Results are shown as picogram of TNF-α per milliliter of serum and represent the mean ± s.e.m. of six mice in each group. $^{*}P<0.01$ when compared with the sham-operated group and $^{\#}P<0.01$ when compared with vehicle-treated mice submitted to I/R.

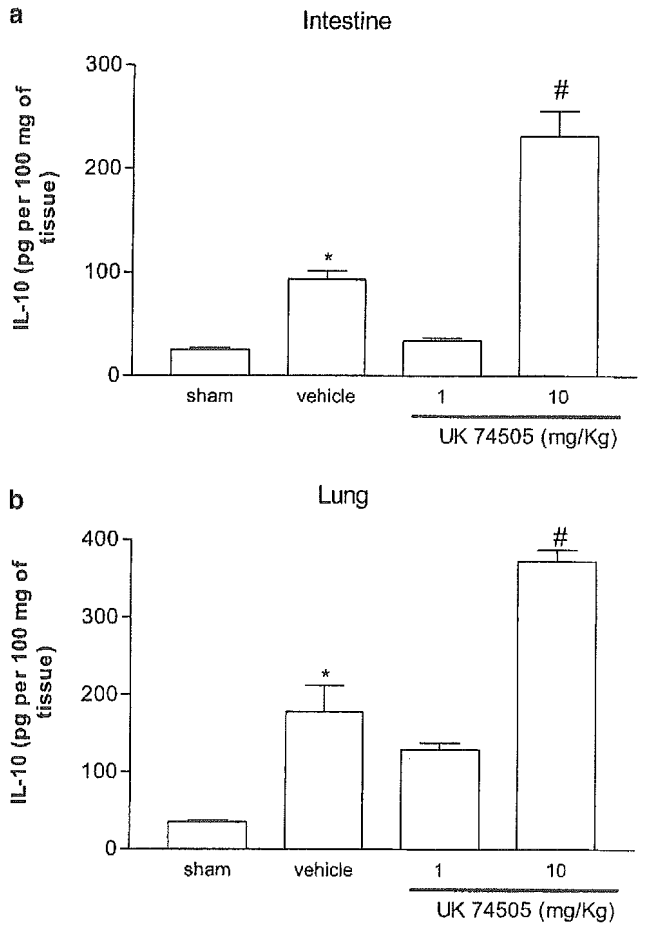

Figure 6 Effects of postischemic treatment with the PAF receptor antagonist UK-74,505 on the concentrations of IL-10 (a) intestine and (b) lungs in mice submitted to ischemia and reperfusion of the SMA. Mice were sham-operated or submitted to 60 min of ischemia of the SMA and reperfusion was allowed for 30 min. UK-74,505 (1 or 10 mg kg^{-1}) was given i.v. 5 min prior to reperfusion. Control animal received vehicle. Concentrations of IL-10 in the intestine (a) and lungs (b) were measured by ELISA. Results are shown as pg of IL-10 per 100 mg of tissue and represent the mean ± s.e.m. of six mice in each group. $^{*}P<0.01$ when comparing to the sham-operated group and $^{\#}P<0.01$ when compared with vehicle-treated mice submitted to I/R.

PAFR$^{-/-}$ mice have been shown to have a normal response to LPS administration, but were extremely resistant to antigen-induced systemic anaphylaxis (Ishii *et al.*, 1998) and to hydrochloric acid aspiration-induced lung injury (Nagase *et al.*, 1999). In addition to playing a role during inflammatory tissue injury caused by different stimuli, we have recently shown that PAFR$^{-/-}$ mice were more susceptible to *Klebsiella pneumoniae* pulmonary infection (Soares *et al.*, 2002) and to *Trypanosoma cruzi* infection (Talvani *et al.*, 2003). Of interest, the protective role of PAFR during infection appeared to be largely related to the relevance of this receptor in mediating phagocytosis of the bacteria or the parasites (Soares *et al.*, 2002; Talvani *et al.*, 2003). In the present study, we show that the inflammatory injury that follows intestinal I/R is markedly inhibited in PAFR$^{-/-}$ mice when compared to their wild-type controls. Thus, there was no increase in vascular permeability, neutrophil accumulation and hemorrhage in the intestine and lung of reperfused PAFR$^{-/-}$ mice. In PAFR$^{-/-}$ mice, the reperfusion-associated increases in serum concentration of TNF-α was significantly suppressed and this was associated with an increase in the concentrations of IL-10. Additionally, there was a significant delay in reperfusion-associated lethality in PAFR$^{-/-}$ mice. These results strongly corroborate the role of PAFR during I/R tissue injury and, to the best of our knowledge, are the first demonstration that the PAFR plays a relevant role for reperfusion-associated lethality.

Several studies, including one of our own, have shown that blockade of PAFR with receptor antagonists blocks the inflammatory injuries that occur following I/R of several vascular beds (Canale *et al.*, 1994; Carter *et al.*, 1996; Riera *et al.*, 1997; Qayumi *et al.*, 1998; Morgan *et al.*, 1999; Kecskemeti & Balogh, 2000; Kim *et al.*, 2000; Souza *et al.*, 2000b; Sun *et al.*, 2001; 2002). It was, thus, of interest to

examine whether pharmacological antagonism of PAFR would prevent tissue injury and lethality, as observed in PAFR$^{-/-}$ mice. To this end, we used the long-acting and selective PAF receptor antagonist, UK-74,505 (Alabaster *et al.*, 1991). In rats, UK-74,505 markedly blocked the severe injuries that followed prolonged I/R of the SMA (Souza *et al.*, 2000b). Maximal inhibition occurred at the dose of 1 mg kg^{-1}. Similar to our previous studies in rats, postischemic treatment with UK-74,505 suppressed the increase in vascular permeability, neutrophil influx and hemorrhage induced by reperfusion of the ischemic SMA. In addition to inhibiting the above-mentioned parameters, UK-74,505 effectively suppressed reperfusion-induced increases in the concentration of TNF-α in tissues. Overall, these results are in agreement with other studies demonstrating a role for PAFR during I/R injury (Canale *et al.*, 1994; Carter *et al.*, 1996; Riera *et al.*, 1997; Qayumi *et al.*, 1998; Morgan *et al.*, 1999; Kecskemeti & Balogh, 2000; Kim *et al.*, 2000; Sun *et al.*, 2001; 2002). In contrast, at the lower dose used, UK-74,505 failed to affect reperfusion-induced increase in serum concentrations of TNF-α and lethality. As neutrophil influx is essential for tissue production of TNF-α (Souza *et al.*, 2000b; 2001), the inhibitory effects of UK-74,505 on tissue TNF-α concentrations may be secondary to its ability to suppress neutrophil influx. However, the inability of UK-74,505 to affect serum TNF-α concentrations and lethality was surprising, as we used a previously shown effective dose of the drug (in the present experiments, tissue injury was abolished) and PAFR$^{-/-}$ mice had diminished amounts of serum TNF-α and delayed lethality.

Although tissue injury was abolished by the lower dose of UK-74,505, it was possible that the mechanisms underlying serum TNF-α production and ensuing lethality relied on PAFR not totally inhibited by the lower dose of the drug. In support of this possibility, intracellular PAFR that are relevant for proinflammatory cytokine production have been recently demonstrated on neutrophils (Marrache *et al.*, 2002). Thus, it is feasible that higher doses (and hence intracellular concentrations) of UK-74,505 are necessary to block this intracellular receptor. Alternatively and akin to previous studies demonstrating the effects of selectin inhibition during I/R injury, it is possible that a virtually complete blockade of PAFR is necessary if inhibition of systemic TNF-α and lethality is to occur (Kubes *et al.*, 1995). Regardless of the underlying explanation for our results, they clearly showed that the use of a higher dose (10 mg kg^{-1}) of UK-74,505 was associated with effective inhibition of serum TNF-α release and delay and prevention of lethality. Interestingly and similar to results seen in PAFR$^{-/-}$ mice, the higher dose of UK-74,505 enhanced the reperfusion-associated increase in tissue concentrations of IL-10. Overall, these results demonstrate that a high dose of PAFR antagonists is needed if we are to delay and/or suppress lethality, in addition to suppressing tissue injury. Whether the need for the use of a high dose of UK-74,505 reflects inhibition of an intracellular PAFR is not known at present, but clearly deserves further investigation. Another relevant observation was that the high dose of UK-74,505 was more effective in preventing lethality than the genetic strategy, suggesting that compensatory mechanisms may be operative in PAFR$^{-/-}$ mice. One alternative explanation to our results was the possibility that UK-74,505 was having an effect distinct from its action on the PAFR. To address this possibility, we administered UK-74,505 (10 mg kg^{-1}) to PAFR$^{-/-}$ mice and evaluated reperfusion-induced lethality. There was no prevention of lethality in addition to that observed in PAFR$^{-/-}$ mice, suggesting that the effects of high-dose UK-74,505 were still related to the ability of the drug to block the PAFR and not because of nonspecific effects.

Finally, the inhibition of reperfusion-associated increases in serum TNF-α concentration and lethality was associated with an enhanced production of IL-10. Other studies have shown that the endogenous IL-10 produced in response to I/R injury may protect the tissue from excess injury (e.g. Frangogiannis *et al.*, 2000; Zingarelli *et al.*, 2001). Whether the IL-10 produced in response to PAFR blockade or in PAFR$^{-/-}$ mice is relevant for the suppressive effects observed is not known. Moreover, the observation that the enhancement of IL-10 production is only observed when a high dose of UK-74,505 or PAFR$^{-/-}$ mice are used is intriguing and may suggest that the activation of intracellular PAFR may play a role in controlling not only proinflammatory, but also anti-inflammatory, cytokines (Marrache *et al.*, 2002). This possibility needs further investigation.

In conclusion, our studies using genetically modified animals and receptor antagonists firmly establish a role of PAFR activation for the local, remote and systemic inflammatory injury and lethality, which follows reperfusion of the ischemic SMA in mice. Whereas tissue injury was inhibited by a lower dose of the PAFR antagonist UK-74,505, lethality was only suppressed when a higher dose of the compound was used. These results suggest that high doses of PAFR antagonists need to be used if the real efficacy of these compounds is to be tested clinically.

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Original article

Experimental *Trypanosoma cruzi* infection in platelet-activating factor receptor-deficient mice

André Talvani [a], Gilcinea Santana [a], Lucíola S. Barcelos [a], Satoshi Ishii [b,c], Takao Shimizu [b,c], Álvaro J. Romanha [d], João S. Silva [e], Milena B.P. Soares [f], Mauro M. Teixeira [a,d,*]

[a] *Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Avenida Antônio Carlos, 6627 Pampulha, 31270-901 Belo Horizonte, Minas Gerais, Brazil*
[b] *Department of Biochemistry and Molecular Biology, Faculty of Medicine, The University of Tokyo, Japan*
[c] *CREST of Japan Science and Technology Corporation, Tokyo, Japan*
[d] *Centro de Pesquisas René Rachou, FIOCRUZ, Belo Horizonte,Brazil*
[e] *Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil*
[f] *Centro de Pesquisa Gonçalo Moniz, FIOCRUZ, Salvador, Brazil*

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Abstract

The generation of an inflammatory response driven by *Trypanosoma cruzi* or its subproducts appears to be essential for tissue injury and disease pathogenesis. However, this inflammatory response is also relevant in the control of *T. cruzi* replication. The lipid mediator platelet-activating factor (PAF) has been implicated in a number of pathological conditions characterized by tissue inflammation. In the present study, we aimed at evaluating the role of PAF during *T. cruzi* infection by using mice that were genetically deficient in the PAF receptor. We observed that infected hearts of $PAFR^{-/-}$ mice had an increased number of parasite nests, associated with a more intense inflammatory infiltrate. This was associated with greater parasitemia and lethality. When wild-type and $PAFR^{-/-}$ mice were compared, there were no marked changes in the kinetics of the expression of MCP-1, RANTES, IFN-γ and TNF-α in heart tissue of infected animals. Moreover, serum concentrations of TNF-α, nitrate and parasite-specific IgM were similar in both groups of mice. In vitro, macrophages from $PAFR^{-/-}$ animals did not phagocytose trypomastigote forms when activated with PAF, leukotriene B_4 or MCP-1 and produced less nitric oxide when infected and activated with IFN-γ. These results are consistent with the hypothesis that endogenous synthesis of PAF and activation of PAF receptors control *T. cruzi* replication in mice in great part via facilitation of the uptake of the parasite and consequent activation of macrophages.

Keywords: Platelet-activating factor; Protozoan infection; Inflammation; Chemokines; TNF-α; Knockout

1. Introduction

Chagas' disease, a common cause of congestive heart failure and sudden death in South and Central America, is caused by a hemoflagellate parasite, *Trypanosoma cruzi*, which is widely distributed in this geographic area [1,2]. The generation of an inflammatory response driven by *T. cruzi* or its subproducts appears to be essential for tissue injury and disease pathogenesis [3,4]. In addition to parasite-driven inflammation, an autoimmune component may also play a role in the tissue damage during the chronic stages of the disease, especially in cardiac tissues [5–7]. The inflammation observed in infected tissues is characterized by the infiltration of $CD4^+$ and $CD8^+$ T cells and macrophages, which appear to secrete a range of cytokines, chemokines, oxygen reactive products and other inflammatory mediators, such as platelet-activating factor (PAF) and leukotriene B_4 (LTB_4) [3,8–13]. Although inflammation determines tissue damage, the cellular influx and mediators formed in response to parasite invasion appear to be relevant in the control of *T. cruzi* replication. The question of whether a similar set of mediators is responsible for tissue damage and defense against the parasite clearly deserves investigation.

The lipid mediator PAF (1-0-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorocholine), is produced by a diverse number of inflammatory cells, including macrophages, neutrophils, ba-

* Corresponding author.
Tel.: +55-31-3499-2651; fax: +55-31-3441-5963.
E-mail address: mmtex@icb.ufmg.br (M.M. Teixeira).

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sophils, eosinophils, platelets and endothelial cells [14,15]. PAF has been implicated in a number of pathological conditions, including endotoxic shock, thrombosis, allergic reactions and a variety of other inflammatory conditions [14–16]. Studies on immunity to *T. cruzi* infection have focused on the relationship between cytokines/chemokines and production of nitric oxide (NO) and oxygen intermediates [3,4,8,17,18]. The involvement of other mediators, such as those derived from membrane metabolism, in immunity to *T. cruzi* has been largely ignored. More recently, our group demonstrated that PAF and another lipid mediator, LTB_4, were able to induce NO and tumor necrosis factor-α (TNF-α) synthesis in cultured *T. cruzi*-infected peritoneal macrophages and kill the parasite in a NO-dependent manner [12,19]. In vivo, blockade of PAF or LTB_4 receptors was accompanied by higher parasitemia and lower survival of infected animals [12,19].

In the present study, we aimed at evaluating the protective role of PAF during *T. cruzi* infection by using mice that were genetically deficient in the PAF receptor [20]. Moreover, we evaluated several aspects of the inflammatory response and parasitological indices during *T. cruzi* infection in an attempt to identify mechanisms involved in the protective effects of PAF in our model. We observed that infected hearts of $PAFR^{-/-}$ mice had an increased number of parasite nests, associated with a more intense inflammatory infiltrate. This was associated with greater parasitemia and lethality. In vitro, macrophages from $PAFR^{-/-}$ animals did not phagocytose trypomastigote forms when activated with PAF, LTB_4 or MCP-1 and produced less NO when infected and activated with IFN-γ. Together with our previous studies [12,19], our results are consistent with the hypothesis that endogenous synthesis of PAF and activation of PAF receptors control *T. cruzi* replication in mice in great part via facilitation of the phagocytosis of the parasite and consequent activation of macrophages.

2. Materials and methods

2.1. Experimental animals

Female C57BL/6 wild-type and PAF receptor-deficient mice [20] were bred at Centro de Pesquisas Gonçalo Muniz, FIOCRUZ (Salvador, Brazil) and maintained under standard conditions in the animal house of our institution. Animals were used when 8–10 weeks old. All procedures had prior approval from the local animal ethics committee.

2.2. Parasites and experimental infection

The Y strain of *T. cruzi* was used in all experiments. Trypomastigote forms were cultured and purified from the monkey kidney fibroblast cell line LLC-MK2 for experiments in vitro. C57BL/6 mice were infected intraperitoneally with 5×10^3 blood trypomastigote forms, and parasitemia was evaluated daily by counting the number of parasites in 5 μl of blood from a tail vein, as previously described [21].

2.3. Macrophage culture

Inflammatory macrophages were harvested from the peritoneal cavity of mice 3 d after the injection of 1.5 ml of 3% (w/v) sodium thioglycolate (Sigma). Cells (10^6/ml) were plated onto chamber slides in 24-well tissue culture plates and incubated for 2–4 h at 37 °C. The non-adherent cells were removed by exhaustive washing with Hank's medium. Adherent cells were then infected at a parasite-to-cell ratio of 1:1 for 120 min. After incubation with *T. cruzi*, extracellular parasites were removed by six washes with RPMI-1640 and infected macrophages were incubated at 37 °C in 5% CO_2 in the presence or absence of 50 U/ml of recombinant murine IFN-γ (Life Technologies, Bethesda, Md.) for 48 h. Supernatants from these infected cultured peritoneal macrophages were collected to measure NO_2 + NO_3 (NO_x) levels, as described below.

In parallel experiments, the uptake of trypomastigote forms by macrophages was evaluated in vitro [17]. Macrophages (10^6/ml) derived from the peritoneal cavity of wild-type or $PAFR^{-/-}$ mice were plated onto glass slides that fitted 48-well plates. After washing to remove non-adherent cells, macrophages were treated with PAF (10^{-7} M), LTB_4 (10^{-6} M) or MCP-1 (10^{-7} M) for 10 min. Parasites were added in a 2:1 parasite:cell ratio, and 2 h later the extracellular trypomastigote forms were removed by washing with RPMI-1640. The macrophages were fixed with methanol and stained with Giemsa solution (1:20) in order to determine the number of intracellular amastigotes. At least 200 macrophages were analyzed on each slide, and all experiments were carried out in triplicate.

2.4. NO_X measurement in vivo and in vitro

Nitrite levels in supernatants of infected macrophage cultures were determined using the Griess reaction, as an index of the NO produced [22]. To determine the levels of NO_x in vivo, blood was collected on different days before sacrifice of infected wild-type and transgenic animals. Serum was obtained from these samples, and NO_x was measured using the Griess reaction [22] after treatment of samples with nitrate reductase [23].

2.5. TNF-α, IFN-γ, RANTES and MCP-1 measurement

Left and right ventricles from wild-type and $PAFR^{-/-}$ mouse hearts were homogenized in phosphate-buffered saline (PBS) pH 7.4, centrifuged at 1000 rpm at 4 °C and supernatants were stored at –70 °C. The concentrations of TNF-α, IFN-γ, MCP-1 and RANTES in cardiac extracts were evaluated by ELISA using commercially available antibodies and according to the protocol provided by the supplier (duo-set R&D Systems).

2.6. IgM ELISA

Flat-bottom 96-well microtiter plates (Nunc) were coated with 100 μl/well of the *T. cruzi* epimastigote antigen

(10 µl/ml) at 4 °C overnight and then washed with 1× PBS (pH 7.4) containing 0.05% Tween-20 (wash buffer). Non-specific binding sites were blocked 2 h with 200 ml/well of 1% BSA in PBS. Plates were rinsed with wash buffer and 1:100 diluted serum samples were added (100 µl/well), followed by incubation for 1 h at room temperature. Plates were then washed and 100 µl/well of the anti-IgM biotinylated detection antibodies 1:20,000 diluted in wash buffer were added for 1 h at room temperature. After that, plates were newly washed, 1:4000 wash buffer diluted streptavidin-horseadish peroxidase was added (100 µl/well), and the plates were incubated for 30 min at room temperature. Plates were then washed, 100 µl/well of the 0.04% chromogen substrate OPD (*o*-phenylendiamide, Sigma) diluted in citrate buffer (pH 5.0) containing 0.02% 30 v/v H_2O_2 was added, and the plates were incubated in the dark for 30 min at room temperature. The reaction was terminated with 50 µl/well of 1M H_2SO_4 solution. Plates were read at 492 nm in a spectrophotometer (E max–Molecular Devices). All samples were assayed in duplicate.

2.7. Histology

Hearts were removed at necropsy, and sections were immediately fixed in 10% phosphate-buffered formalin. After fixation, sections were prepared by standard methods for paraffin-embedded blocks, and cut sections were stained with hematoxylin and eosin. Cardiac parasitism and inflammation of ventricles were estimated in three different 7-µm-depth sections in each heart, and five different animals were analyzed in each group. Amastigote nests and inflammation were assessed with a Zeiss (Öberkohen, Germany) integrating eyepiece, with 100 hits at a final magnification of 400×. A total of 3000 hits was evaluated in each section of cardiac tissue. The infection index represents the number of hits covered by amastigote nests, and the inflammatory index represents the number of hits in which inflammatory cells were present.

2.8. Statistical analysis

All results are presented as the mean ± S.E.M. Normalized data were analyzed by one-way ANOVA and differences between groups were assessed using the Student–Newman–Keuls post-test. A value of $P < 0.05$ was considered to be significant. All analyses were made using the INSTAT program (Graph PAD Software, Inc. San Diego, California).

3. Results

3.1. Infection indices in T. cruzi-infected PAFR$^{-/-}$ mice

We have previously shown that blockade of PAFR with the PAFR antagonist WEB-2170 [2] or UK-74,505 (our own unpublished data) was associated with increased parasitemia following infection with 5 × 10^3 *T. cruzi* trypomastigotes. Here, parasitemia in PAFR$^{-/-}$ mice was approximately twice that of C57BL/6 wild-type mice at the peak of parasitemia (Fig. 1A). More importantly, the survival of infected PAFR$^{-/-}$ mice was lower than that of infected wild-type mice (Fig. 1B). Indeed, 15 d after infection, none of the PAFR$^{-/-}$ mice, but around 70% of wild-type animals were alive (Fig. 1B).

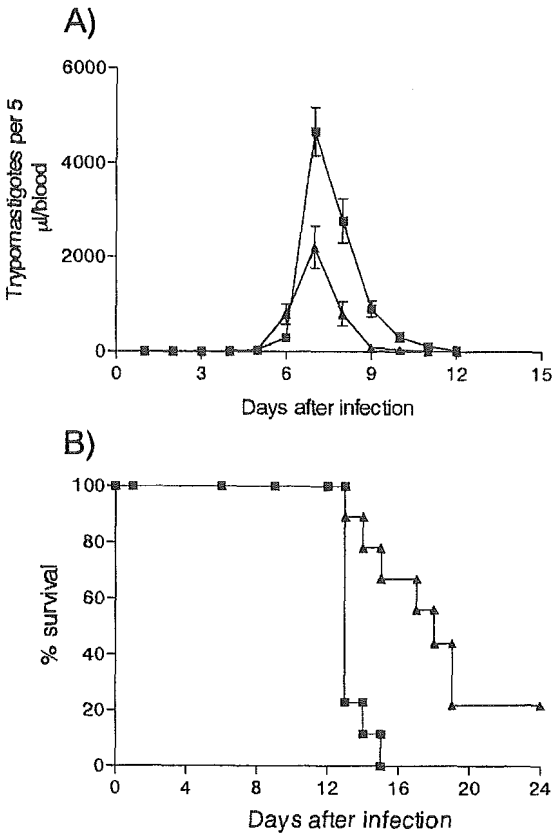

Fig. 1. Blood parasitemia (A) and lethality (B) during experimental *T. cruzi* infection in PAFR$^{-/-}$ and wild-type mice. PAFR$^{-/-}$ (squares) and wild-type (triangles) mice were infected with 5 × 10^3 trypomastigote forms of *T. cruzi* intraperitoneally. Results are shown as the mean ± S.E.M. of 10 mice per group in one of two independent experiments. The parasitemia and lethality curves of PAFR$^{-/-}$ and wild type were significantly different ($P < 0.01$).

3.2. Pathological alterations in T. cruzi-infected PAFR$^{-/-}$ mice

The morphological analysis of cardiac tissue of wild-type mice 10 and 15 d after infection showed few parasite nests scattered throughout the tissue (Fig. 2A). Parasite nests were already noticeable 10 d after infection only in PAFR$^{-/-}$ mice (Fig. 2A). Albeit in small quantities, heart sections of PAFR$^{-/-}$ mice had three-fold more parasite nests than those of wild-type mice 15 d after infection (Fig. 2A). Heart inflammation was characterized by the presence of multiple inflammatory focci composed mainly of mononuclear cells and a diffuse mononuclear cell infiltrate. The inflammatory infiltrate was already present on day 10 after infection but was more marked on day 15 in wild-type mice (Fig. 2B).

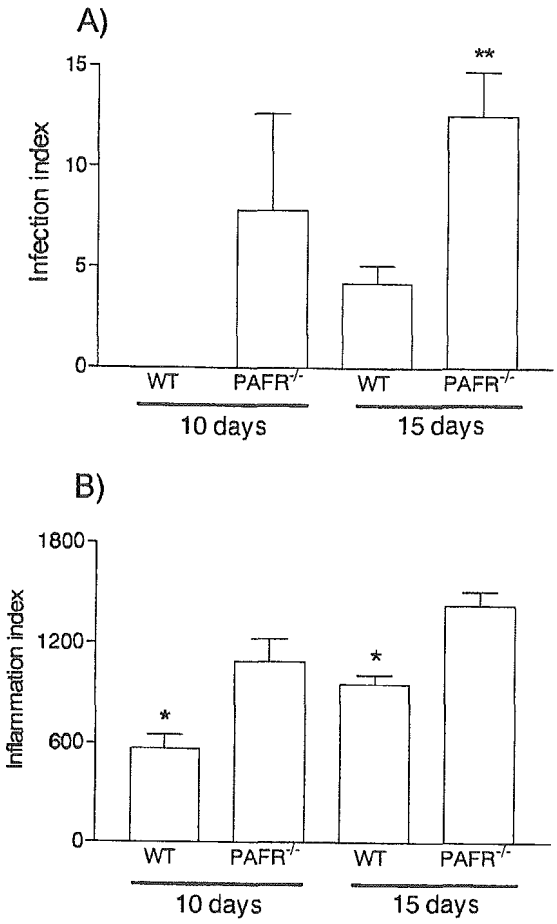

Fig. 2. Quantification of infection (A) and tissue inflammation (B) in $PAFR^{-/-}$ and wild-type mice. $PAFR^{-/-}$ and wild-type (WT) mice were infected with 5×10^3 trypomastigote forms of *T. cruzi* intraperitoneally, and inflammatory and parasitological indices were evaluated 10 and 15 d after infection. Results are shown as the mean ± S.E.M. of five mice per group. * and * * for $P < 0.05$ and 0.01, respectively, when compared with wild-type mice.

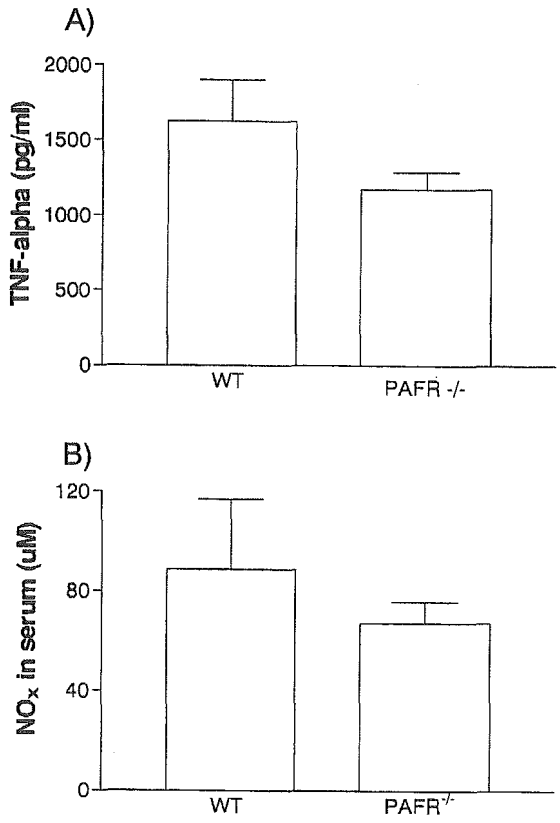

Fig. 3. Serum concentrations of TNF-α (A) and nitrate/nitrite (B) during experimental *T. cruzi* infection in $PAFR^{-/-}$ and wild-type mice. $PAFR^{-/-}$ and wild-type (WT) mice were infected with 5×10^3 trypomastigote forms of *T. cruzi* intraperitoneally, and serum concentrations of TNF-α and nitrate/nitrite (NO_x) were evaluated 10 d after infection. Results are shown as the mean ± S.E.M. of five mice per group.

Tissue inflammation in heart sections of $PAFR^{-/-}$ mice was of greater magnitude than that found in sections of hearts from wild-type mice (Fig. 2B).

3.3. TNF-α, MCP-1, RANTES and IFN-γ synthesis in heart tissue T. cruzi*-infected PAFR$^{-/-}$ mice*

The concentrations of TNF-α in serum of *T. cruzi*-infected wild-type and $PAFR^{-/-}$ mice are shown in Fig. 3A. Concentrations of TNF-α in serum of $PAFR^{-/-}$ mice were 28% lower than those of wild-type mice, but this difference failed to reach statistical significance ($P = 0.06$). TNF-α was not detected in serum of uninfected controls. Similarly, there was no significant difference between the concentration of nitrate and nitrite (NO_x) in serum samples of $PAFR^{-/-}$ and wild type after *T. cruzi* infection (Fig. 3B).

We have previously demonstrated the expression of TNF-α, RANTES, MCP-1 and IFN-γ mRNA in cardiac tissue of mice following *T. cruzi* infection [11]. Here, the concentrations of TNF-α, RANTES, MCP-1 and IFN-γ protein in heart tissues were detected using ELISA. Five days after infection, the concentration of TNF-α in infected mice was already elevated, and dropped to those found in uninfected controls by day 15 (Fig. 4A). The concentration of MCP-1 in infected wild-type mice was significantly greater than uninfected controls from day 5 and remained elevated throughout the 15-day observation period (Fig. 4B). The concentrations of TNF-α and MCP-1 in heart tissue of $PAFR^{-/-}$ mice were not different from those of infected wild-type mice (Fig. 4A,B). In contrast, there were some differences in the concentration of RANTES between the two groups. The RANTES concentration was higher than in uninfected controls only 10 d after infection, decreasing on day 15 (Fig. 4C). Ten days after infection RANTES protein expression was lower in $PAFR^{-/-}$ than wild type. However, there was a 40% increase in the levels of RANTES in $PAFR^{-/-}$ on day 15 (Fig. 4C). Concentrations of IFN-γ in heart tissue of infected wild-type mice were elevated from days 5 through 15 after infection, although there was a tendency for IFN-γ to drop on day 15 (Fig. 4D). The concentrations of IFN-γ in $PAFR^{-/-}$ mice were similar to those of wild-type controls at days 5 and 10 after infection and significantly greater on day 15 (Fig. 4D).