# PULMONARY MACROPHAGE RELEASE OF INFLAMMATORY CYTOKINES AFTER MULTI-DAY NITRIC ACID VAPOR AND OZONE EXPOSURE

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## ABSTRACT

This study measured *ex-vivo* production of pro-inflammatory cytokines by pulmonary macrophages. The macrophages were isolated from bronchoalveolar lavage fluid (BALF) obtained from human subjects who had under gone controlled exposures to ozone ($O_3$), nitric acid ($HNO_3$), or both together. The study was based on the premise that pulmonary macrophages are activated by exposure to $O_3$ and $HNO_3$ vapor, and that they will continue to secrete cytokines after removal from BALF. The cytokines of interest included tumor necrosis factor-alpha (TNF-$\alpha$), interleukin-1-beta (IL-1-$\beta$), interleukin-6 (IL-6), interleukin-8 (IL-8), and macrophage inflammatory protein-1-alpha (MIP-1-$\alpha$). Cells from BALF were suspended in culture medium, and were plated in microwells. After one hour of incubation at 37°C in 95% air and 5% CO2, non-adherent cells were removed, and fresh cell culture media was added to the microwells. Cell culture supernatant was removed after 3, 24 and 48 hours of incubation, and was frozen for subsequent analysis by an enzyme-linked immunosorbent (ELISA) method. The results showed that the concentration of all five cytokines was greater at 24 hours than 3 hours of incubation. Only IL-8 and MIP-1-$\alpha$ were increased at 48 hours of incubation, compared to 24 hours. Since both IL-8 and MIP-1-$\alpha$ production are induced by TNF-$\alpha$ and IL-1-$\beta$, it makes sense that their peak values would occur after those for TNF-$\alpha$ and IL-1-$\beta$. There were no significant differences between production of any cytokine following a single four hour exposure to 0.20 parts per million (PPM) $O_3$ compared to equivalent exposures on four consecutive days. There were few significant differences in cytokine production between the exposures to $O_3$ alone, $HNO_3$ alone and $O_3$+$HNO_3$. After 3 hours of incubation, only TNF-$\alpha$ varied by protocol, with the lowest synthesis following the combination exposure, and the largest following the $HNO_3$ exposure. A similar statistically significant pattern was present for MIP-1-$\alpha$ after 48 hours of incubation. This suggests that $O_3$ may attenuate the effect of $HNO_3$, possibly by reduction in TNF-$\alpha$ secretion. Since TNF-$\alpha$ is one of the first inflammatory cytokines secreted, and since TNF-$\alpha$ induces secretion of other cytokines, reduction in production of TNF-$\alpha$ may lead to reduction in secretion of other inflammatory cytokines, and thereby to reduction of inflammation with the combined exposure.

## EXECUTIVE SUMMARY

BACKGROUND: Pulmonary macrophages comprise the largest number of immunocompetent cells in bronchoalveolar lavage fluid. When activated, they release a number of cytokines that regulate other lung cells, as well as participate in various inflammatory and immune reactions in the lungs. The cytokines of interest in this study included tumor necrosis factor-alpha (TNF-$\alpha$), interleukin-1-beta (IL-1-$\beta$), interleukin-6 (IL-6), interleukin-8 (IL-8) and macrophage inflammatory protein-1-alpha (MIP-1-$\alpha$). The goal of this project was to measure *ex-vivo* production of pro-inflammatory cytokines by pulmonary macrophages isolated from bronchoalveolar lavage fluid (BALF) obtained from human subjects who had undergone controlled inhalation exposures to ozone, nitric acid, or both together. The study is based on the premise that pulmonary macrophages are activated by exposure to ozone and nitric acid vapor, and that they will continue to secrete cytokines after removal from BALF.

METHODS: This study was an adjunct component to Contract 93-303 (PI: John Balmes, MD). Briefly, volunteer human subjects were exposed to one or more of four exposure conditions: 1) a single exposure to 0.2 PPM ozone alone for four hours, 2) 0.2 PPM ozone alone for four hours on each of four consecutive days, 3) 0.2 PPM ozone and 100 $\mu g/m^3$ nitric acid vapor for four hours on each of four consecutive days, and 4) 100 $\mu g/m^3$ nitric acid alone for four hours on each of four consecutive days. Pulmonary alveolar macrophages were obtained by bronchoscopy with bronchoalveolar lavage (BAL) 18 hours after completion of the final exposure of the protocol. After removal of an aliquot of bronchoalveolar lavage fluid (BALF) for other analyses, including cell counts and differentials, BALF was centrifuged. Cells were plated in microwells at a concentration of $10^6$/ml and maintained in a humidified incubator at 37°C in 95% air and 5% $CO_2$. After one hour, non-adherent cells were removed and fresh media was added to the microwells. After incubation periods of 3, 24 and 48 hours, cell culture supernatant was removed and frozen at -70°C for subsequent analysis by an enzyme-linked immunosorbent assay method (ELISA).

RESULTS: Comparisons between cytokine release at 3, 24 and 48 hours post-exposure showed that TNF-$\alpha$, MIP-1-$\alpha$, IL-1-$\beta$, IL-6 and IL-8 release was significantly greater after 24 hours of incubation compared to following 3 hours of incubation. Only IL-8 and MIP-1-$\alpha$ were significantly greater after 48 hours of incubation than after 24 hours. There were no statistically significant differences in release of any cytokine at any time point between the 1-day and 4 day $O_3$ exposure protocols. TNF-$\alpha$ after 3 hours of incubation, IL-1-$\alpha$ after 24 hours of incubation, and MIP-1-$\alpha$ after 48 hours of incubation were similar following the 1 day and 4 day $O_3$ exposures. Based on this analysis, the 1-day and 4 day ozone exposure samples were combined and treated as an ozone alone protocol in all subsequent analyses. Comparison of the three pollutant protocols ($O_3$, $O_3$ + $HNO_3$, and $HNO_3$) showed that after 3 hours of incubation only TNF-$\alpha$ varied by exposure protocol. Synthesis of TNF-$\alpha$ was lowest following the $O_3$ + $HNO_3$

exposure, and highest following the $HNO_3$ exposure. A similar pattern was present for MIP-1-$\alpha$ at 48 hours post-exposure. The study relates to the ARB's programs investigating adverse health effects of exposure of human subjects to ambient air pollutants. It is important to be able to characterize adverse effects. The development of assays that allow measurement of known inflammatory mediators produced by lung cells allows evaluation and quantification of lung injury.

CONCLUSIONS: Measurement of *ex-vivo* cytokine production appears to be a valid and consistent tool for assessment of biological signals related to inflammation pulmonary macrophages isolated from BALF. Pro-inflammatory cytokines were released in high concentrations by pulmonary macrophages maintained in culture for up to 48 hours. Production of TNF-$\alpha$, IL-1-$\beta$, and IL-6 peaked by 24 hours post-exposure. IL-8 and MIP-1-$\alpha$ increased up to the end of testing at 48 hours. There was no evidence of a step-up in cytokine release after 4 days of $O_3$ exposure (4 hours exposure per day at 0.2 ppm $O_3$) compared to a single exposure. There was evidence of a nitric acid-related attenuation of cytokine release with exposure to $O_3$ + $HNO_3$. Since TNF-$\alpha$ is an early mediator of pro-inflammatory cytokine networking, reduced production of TNF-$\alpha$ could lead to reduced inflammation.

## INTRODUCTION

This report summarizes the results from ARB Contract 93-331, entitled "Pulmonary Macrophage Release of Inflammatory Cytokines after Multi-Day Nitric Acid Vapor and Ozone Exposure". The study was performed between July 26, 1994 and June 26, 1997 (including a no-cost extension).

The study was designed to exploit the availability of pulmonary macrophages already collected through bronchoscopy in a related ARB-supported study (ARB Contract #93-303, JR Balmes, PI) of multi-day nitric acid vapor and ozone exposure. The results of the present study should be interpreted in conjunction with those of Balmes, et al.

The goal of this project was to measure *ex-vivo* production of pro-inflammatory cytokines by pulmonary macrophages isolated from bronchoalveolar lavage fluid (BALF) obtained from human subjects who had undergone controlled inhalation exposures to ozone, nitric acid, or both together. The study is based on the premise that pulmonary macrophages are activated by exposure to ozone and nitric acid vapor, and that they will continue to secrete cytokines after removal from BALF.

Pulmonary macrophages comprise the largest number of immunocompetent cells in bronchoalveolar lavage fluid. When activated, pulmonary macrophages release a number of cytokines that regulate other lung cells, as well as participate in various inflammatory and immune reactions in the lungs. The cytokines of interest in this study included tumor necrosis factor-alpha (TNF-$\alpha$), interleukin-1-beta (IL-1-$\beta$), interleukin-6 (IL-6), interleukin-8 (IL-8) and macrophage inflammatory protein-1-alpha (MIP-1-$\alpha$).

Cytokines are a diverse group of proteins, although there are certain characteristics common to all of them. They are produced during the effector stage of the immune response, and mediate and regulate immune and inflammatory responses. Cytokines are typically secreted in short bursts, and are produced by multiple cell types. They act on a variety of cells, and may have more than one effect on a given target cell type. Some cytokines appear to have redundant actions, although one typically cannot replace another. They often influence synthesis and/or action of other cytokines. They are often secreted in sequential patterns. The cytokines studied in this project are mediators of natural immunity; with inhalation of irritant gases that induce acute cell damage being the initiating factor in macrophage activation (1).

Tumor necrosis factor-alpha (TNF-$\alpha$) is derived from activated macrophages. It is an important link between specific immune responses and acute inflammation. At low concentrations, TNF-$\alpha$ acts locally as a paracrine and autocrine regulator of leukocytes and endothelial cells. Among other things, TNF-$\alpha$ activates neutrophils, and stimulates macrophages. The latter leads to macrophage

release of more TNF-α, as well as IL-1-β, IL-6, and chemokines such as IL-8 (1,3).

Interleukin-1-beta (IL-1-β) is principally a mediator of host inflammatory response in natural immunity. It is produced by many cell types, including epithelial and endothelial cells, in addition to activated macrophages. At low concentrations it is a mediator of local inflammation. It acts on macrophages and vascular endothelial cells to increase synthesis of more IL-1-β, and to induce synthesis of IL-6. It shares many of TNF-α's inflammatory properties, although it does not directly activate neutrophils and other inflammatory leukocytes. Rather, it induces endothelial cells and macrophages to secrete chemokines, such as IL-8, that do activate other leukocytes. It has a number of properties redundant of TNF-α, but can not replace it (1,3).

Interleukin-6 (IL-6) is synthesized by macrophages, vascular endothelial cells, fibroblasts, and other cells in response to stimulation by IL-1-β, and to a lesser degree TNF-α. IL-6 is involved in mediating inflammatory and immune responses to injury and infection. It is a mediator in the synthesis of plasma proteins, and in the growth and differentiation of T- and B-lymphocytes (1).

Interleukin-8 (IL-8) is a chemokine, meaning that it can stimulate leukocyte movement (chemokinesis) and directed movement of cells (chemotaxis). It is mainly produced by activated macrophages, and also by endothelial cells and fibroblasts, among other cell types. The predominant action of IL-8 is to act on neutrophils as a mediator of acute inflammatory responses (1,2).

Macrophage inflammatory protein-1-alpha (MIP-1-α) is also a chemokine. Among other things, it is involved in localized inflammatory reactions, activation of neutrophils and monocytes, and stimulation of the cytotoxic activity of several types of immune cells (1,2).

The principal rationale for studying macrophages ex-vivo is that it allows for a potential confirmatory measure of inflammatory mediators that may also be present in the bronchoalveolar lavage (BAL) supernate but at much lower concentrations due to the inherent dilution of the BAL technique. Ex-vivo study also provides a measure of the inflammatory stimulus from a cell type highly relevant to lower respiratory tract injury.

There are some limitations to the statistical analysis, particularly in terms of confidence intervals about estimates given small sample sizes. The number of samples in the various groups differed, providing another source of variability. Because some the data could be treated in paired comparisons, whereas other comparisons could only be carried out as unpaired analyses, study power was great for some tests (paired v. unpaired) even with the same number of samples. Nonetheless, small sample size would not have accounted for the statistically significant differences that were observed. Moreover, for other comparisons, the

means were so similar that the differences would be unlikely to be biologically relevant even if large samples had sufficient study power to achieve statistical significance in those cases (see Figures).

## METHODS

The exposure protocol from which the alveolar macrophages for this project were obtained will be detailed separately in a final report on Contract 93-303 (PI: John Balmes, MD) to which this project was an adjunct component. Briefly, volunteer human subjects were exposed to one or more of four exposure conditions: 1) 0.2 PPM ozone alone for four hours, 2) 0.2 PPM ozone alone for four hours on each of four consecutive days, 3) 0.2 PPM ozone and 100 $\mu g/m^3$ nitric acid vapor for four hours on each of four consecutive days, and 4) 100 $\mu g/m^3$ nitric acid alone for four hours on each of four consecutive days.

Pulmonary alveolar macrophages were obtained by bronchoscopy with bronchoalveolar lavage (BAL) 18 hours after completion of the final four hours of the given exposure protocol. In all cases, the same BAL protocol was employed by Dr. Balmes's laboratory. After removal of an aliquot of bronchoalveolar lavage fluid (BALF) for other analyses, including cell counts and differentials, BALF was centrifuged.

The cell pellet was then resuspended in RPMI-1640 containing 0.3g/L L-glutamine (University of California, San Francisco Cell Culture Facility, San Francisco, CA) with 10% heat-inactivated fetal bovine serum (GIBCO, Grand Island, NY). Cells were plated in microwells at a concentration of $10^6$/ml and maintained in a humidified incubator at 37°C in 95% air and 5% $CO_2$. After one hour, non-adherent cells were removed and fresh media was added to the microwells. After incubation periods of 3, 24 and 48 hours, cell culture supernatant was removed and frozen at -70°C for subsequent analysis.

The human exposure protocol (see Contract 93-303, J. Balmes, MD, PI) enrolled subjects for more than one arm of the study protocol when possible but was not designed as a paired analysis. Ten subjects participated in more than one exposure. Two participated in all four exposures, three in three exposures, and six in two exposures. Nine others were studied only once. In total, BAL cell pellets were available for at least initial culture from 38 exposure studies in which 20 different subjects participated.

TNF-$\alpha$, MIP-1-$\alpha$, IL-1-$\beta$, IL-6 and IL-8 in cell culture supernatant were quantified by enzyme-linked immunosorbent assay (ELISA: R & D Systems, Minneapolis, MN). The ELISA sensitivities for the cytokines were as follows: TNF-$\alpha$, 4.4 pg/ml; MIP-1-$\alpha$, 2.0 pg/ml; IL-1-$\beta$, 0.3 pg/ml; IL-6, 0.7 pg/ml; and IL-8, 3.0 pg/ml.

The assays utilized a quantitative immunometric "sandwich" enzyme technique. Ninety-six well plastic microtiter plates were coated with monoclonal antibody specific to the cytokine of interest. Standards with known amounts of cytokine and samples were added to wells. After unbound proteins were washed away, an enzyme-linked antibody specific to the cytokine was added. Unbound enzyme-linked antibody was then washed away. Optical density readings were

made on an ELISA reader. The quantity of cytokine in culture supernatants was determined by comparison with a standard curve of known concentrations. Non-detectable observations were assigned a value of one-half of the lower detection limit of the assay for the purposes of statistical analysis.

IL-1-β was not detectable in four samples (each from the 3 hours post-exposure harvest). MIP-1-α was not detectable in 2 samples (both from the 3 hours post-exposure). One to four samples were missing at most time periods, primarily due to the small volume of supernatant available for each experiment.

Available samples for analysis, by exposure protocol, are summarized as follows:

Table 1: Study Sample N by Protocol and Assay

| Cytokine | Time | 1-D $O_3$ | 4-D $O_3$ | 4-D $O_3$+$HNO_3$ | 4-D $HNO_3$ | Total N |
|---|---|---|---|---|---|---|
| TNF-α | 3 HR | 13 | 11 | 9 | 5 | 38 |
| IL-1-β | 3 HR | 13 | 11 | 9 | 5 | 38 |
| IL-6 | 3 HR | 13 | 10 | 9 | 4 | 36 |
| IL-8 | 3 HR | 13 | 11 | 9 | 5 | 38 |
| MIP-1-α | 3 HR | 13 | 11 | 9 | 4 | 37 |
| TNF-α | 24 HR | 11 | 11 | 9 | 5 | 35 |
| IL-1-β | 24 HR | 11 | 11 | 9 | 5 | 35 |
| IL-6 | 24 HR | 11 | 10 | 9 | 4 | 34 |
| IL-8 | 24 HR | 11 | 11 | 9 | 5 | 35 |
| MIP-1-α | 24 HR | 11 | 11 | 8 | 4 | 34 |
| TNF-α | 48 HR | 11 | 11 | 8 | 5 | 35 |
| IL-1-β | 48 HR | 11 | 11 | 8 | 5 | 35 |
| IL-6 | 48 HR | 11 | 10 | 8 | 4 | 34 |
| IL-8 | 48 HR | 11 | 11 | 8 | 5 | 35 |
| MIP-1-α | 48 HR | 10 | 11 | 8 | 5 | 34 |

Data were analyzed using a standard computerized statistical package (SAS). Differences between cytokine concentrations analyzed by post-exposure time period (24 hr vs. 3 hr and 48 hr vs. 24 hr) were tested non-parametrically with the paired-signed rank test. Cytokine concentrations following 1 day and 4 day ozone exposures were compared with the non-paired t-test. Differences in cytokines among the $O_3$ alone, $O_3$ + $HNO_3$ and $HNO_3$ alone exposures were tested using analysis of variance (ANOVA), taking into account intra-subject variability given the number of subjects who participated in multiple exposures.

There are some limitations to the statistical analysis, particularly in terms of confidence intervals about estimates given small sample sizes. The number of samples in the various groups differed, providing another source of variability.

Because some the data could be treated in paired comparisons, whereas other comparisons could only be carried out as unpaired analyses, study power was great for some tests (paired v. unpaired) even with the same number of samples. Nonetheless, small sample size would not have accounted for the statistically significant differences that were observed. Moreover, for other comparisons, the means were so similar that the differences would be unlikely to be biologically relevant even if large samples had sufficient study power to achieve statistical significance in those cases (see Figures).

## RESULTS

The first step in the analysis was to assess the validity (magnitude of signal and kinetics) and internal consistency (intercorrelation) of the data, independent of exposure status. We detected the pro-inflammatory cytokines of interest at a strong signal to noise ratio. Furthermore, the kinetics of cytokine release over time was consistent with established parameters. Figures 1a through 1e show the results of the comparisons between cytokine release at 3, 24 and 48 hours post-exposure. In all cases, TNF-$\alpha$, MIP-1-$\alpha$, IL-1-$\beta$, IL-6 and IL-8 release was significantly greater at 24 hours post-exposure compared to 3 hours post-exposure ($P<0.01$, paired signed rank test). However, only IL-8 ($P<0.0001$) and MIP-1-$\alpha$ ($P<0.006$) were significantly greater at 48 hours post-exposure than at 24 hours post-exposure.

The absolute concentration of cytokine released varied widely. The mean concentration of IL-1-$\beta$ at 48 hours post-exposure was 432 pg/ml compared to 239,000 pg/ml for IL-8, for example. Despite this wide range, there was a strong inter-correlation for any given macrophage culture and harvest time. At 3 hour harvest, the concentrations of all cytokines were statistically correlated with one another r range 0.41 – 0.86; $P<0.05$) except for IL-8, which was poorly correlated with any other cytokine (r range –0.04 – 0.21; $P>0.2$ in all cases). By 24 hours post exposure, all of the cytokines were statistically inter-correlated; only the correlation between MIP-1-$\alpha$ and IL-1-$\alpha$ was of borderline statistical significance ($r=0.34$; $P=0.05$). At 48 hours post-exposure the inter-correlation between IL-1-$\alpha$ and IL-8 ($r=0.33$; $P=0.05$). Excluding IL-8 at 3 hours post-exposure, TNF-$\alpha$ was the one cytokine most strongly correlated with all others ($r>0.60$ in all analyses).

The second step in the analysis was to compare the data for I day ozone exposure with 4 day ozone exposure. The results from these comparisons are shown in Figures 2a through 2e. There were no statistically significant differences in release of any cytokine at any time point. In terms of certain key results to follow, it is particularly noteworthy that for TNF-$\alpha$ at 3 hours post-exposure, IL-1-$\alpha$ at 24 hours post-exposure, and MIP-1-$\alpha$ at 48 hours post-exposure, there was little difference between cytokine expression with the 1 day and 4 day exposures. Based on this analysis, the 1 day and 4 day ozone exposure samples were combined and treated as an ozone alone protocol in all subsequent analyses.

The third step in the data analysis was to compare the three protocols: $O_3$, $O_3$ + $HNO_3$, and $HNO_3$. The data are illustrated in Figures 3a through 3e. The statistical analysis took into account both inter-protocol variance and inter-subject variance, since as noted above, many subjects participated in more than one exposure. At 3 hours post-exposure only TNF-$\alpha$ varied by exposure protocol. The pattern of response was such that synthesis was lowest following the $O_3$ + $HNO_3$ exposure, and highest following the $HNO_3$ exposure.

A similar, and statistically significant, pattern was present for MIP-1-$\alpha$ at 48 hours post-exposure. Of note, the correlation among all subjects for TNF-$\alpha$ at 3 hours post-exposure and MIP-1-$\alpha$ at 48 hours post-exposure was $r = 0.8$ ($P<0.0001$). The only other cytokine which approached a statistically significant difference in expression was IL-1-$\alpha$ at 24 hours post-exposure ($0.05<P<0.10$).

## DISCUSSION AND CONCLUSIONS

1. *Ex-vivo* cytokine measurement appears to be a valid and consistent tool for assessment of biological signals related to inflammation. Pro-inflammatory cytokines were released in high concentrations by pulmonary macrophages maintained in culture for up to 48 hours. Production of TNF-$\alpha$, IL-1-$\beta$, and IL-6 peaked by 24 hours post-exposure. IL-8 and MIP-1-$\alpha$ increased up to the end of testing at 48 hours. As a group, release of these cytokines was strongly inter-correlated.

2. There was no evidence of a consistent step-up in cytokine release after 4 days of $O_3$ exposure (4 hours exposure per day at 0.2 ppm $O_3$) compared to a single exposure.

3. Based on our findings there is no evidence for any ozone-related potentiation of cytokine release with exposure to $O_3$ + $HNO_3$. Indeed, there is evidence to support a nitric acid-related attenuation of cytokine release with exposure to $O_3$ + $HNO_3$. This is important because TNF-$\alpha$, along with IL-1-$\alpha$, is an early mediator of pro-inflammatory cytokine networking. Its release typically begins 3 hours after a pro-inflammatory stimulus (e.g. after controlled exposure to lipopolysaccharide).

4. This analysis was intentionally carried out <u>blinded</u> to the cell and cytokine findings from Balmes's separate analysis of the BALF samples. The present study, therefore, should be interpreted in conjunction with the related BALF analysis from ARB Contract 93-303.

5. There are other pro-inflammatory markers not studied here that could be of potential interest, ie. granulocyte–macrophage colony stimulating factor (GMC-SF), IL-10, and interferon. Unfortunately, the fact that there was less than 1 ml of culture supernatant per sample limited the number of analyses, which could be performed. There was already insufficient quantity to perform all analyses for some of the samples.

6. The sample size was small, particularly for the nitric acid alone group. This may have prevented meaningful differences from being identified statistically ($\beta$ error), but it should not have accounted for the statistically significant observations that were made.

7. There was no air alone (sham) control group. However, the study question was not "Is $O_3$ exposure worse that air exposure?" which is already established, but rather, "Is there a multiplicative effect of nitric acid together with $O_3$"? The data analysis presented here is appropriate to that question, and suggests that it is not.

## REFERENCES


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## GLOSSARY OF TERMS, ABBREVIATIONS, AND SYMBOLS

1-D: single 4 hour ozone exposure

4-D: 4 hour ozone exposure on four consecutive days

ANOVA: analysis of variance

ARB: Air Resources Board

BAL: bronchoalveolar lavage

BALF: bronchoalveolar lavage fluid

ELISA: enzyme-linked immunosorbent assay

Ex-vivo: removed from a living body

$HNO_3$: nitric acid

IL-1-$\beta$: Interleukin 1-beta

IL-6: interleukin-6

IL-8: interleukin-8

MIP-1-$\alpha$: macrophage inflammatory protein-1-alpha

$O_3$: ozone

PI: Principal Investigator

PPM: parts per million

TNF-$\alpha$: tumor necrosis factor-alpha

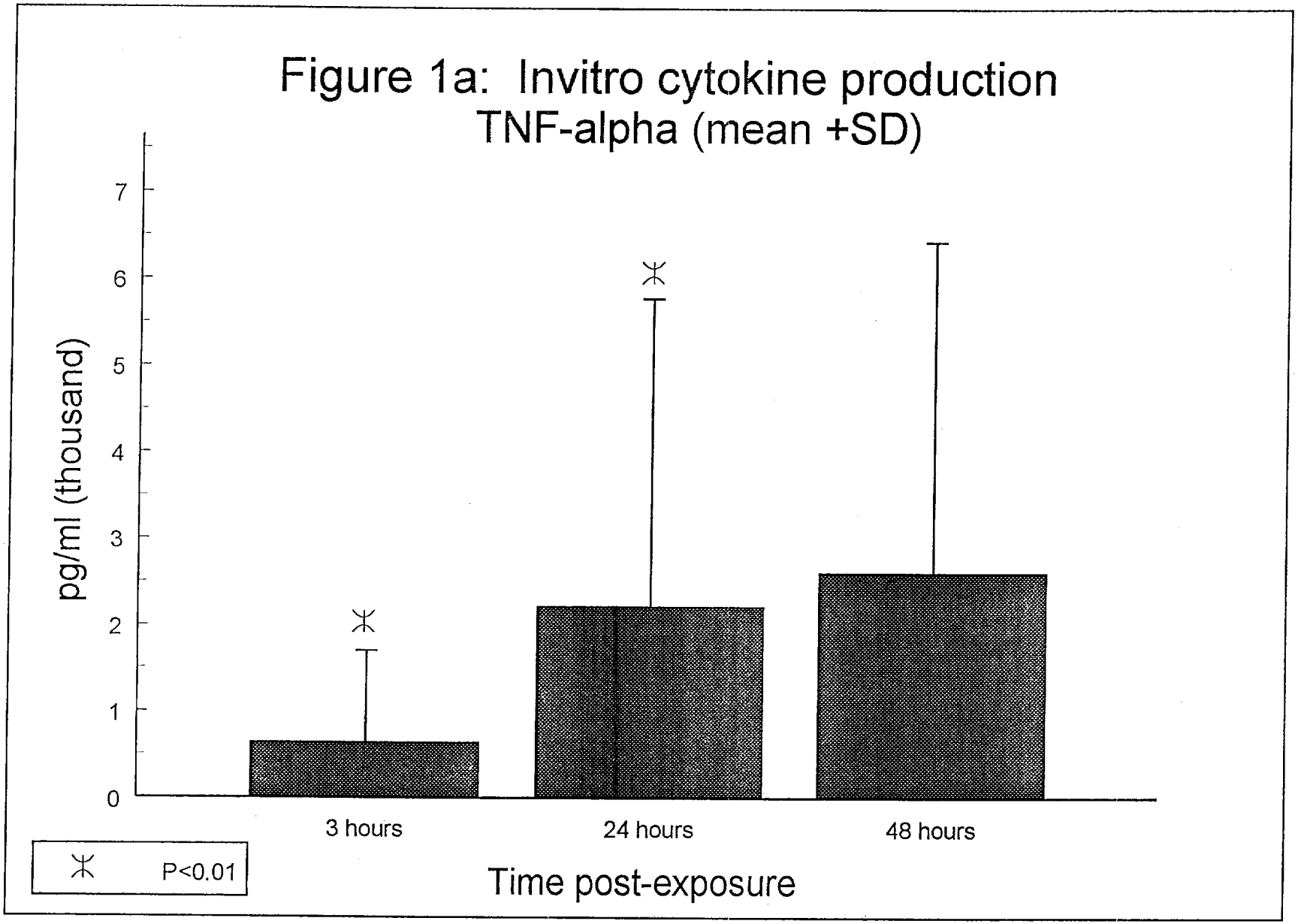
Figure 1a: Invitro cytokine production
TNF-alpha (mean +SD)
pg/ml (thousand)
0
1
2
3
4
5
6
7
3 hours
24 hours
48 hours
Time post-exposure
P<0.01

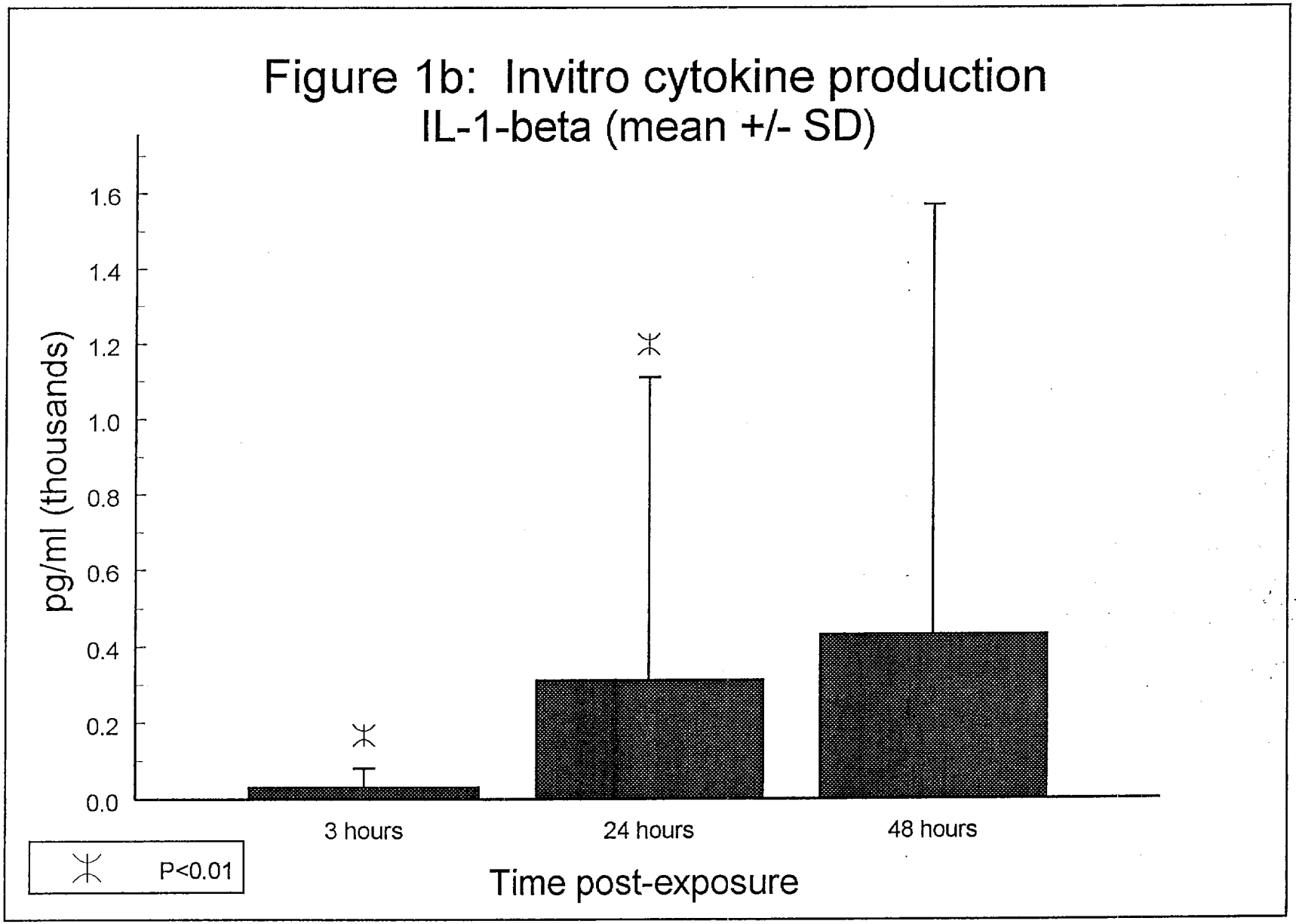
Figure 1b: Invitro cytokine production
IL-1-beta (mean +/- SD)
pg/ml (thousands)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
3 hours
24 hours
48 hours
Time post-exposure
P<0.01

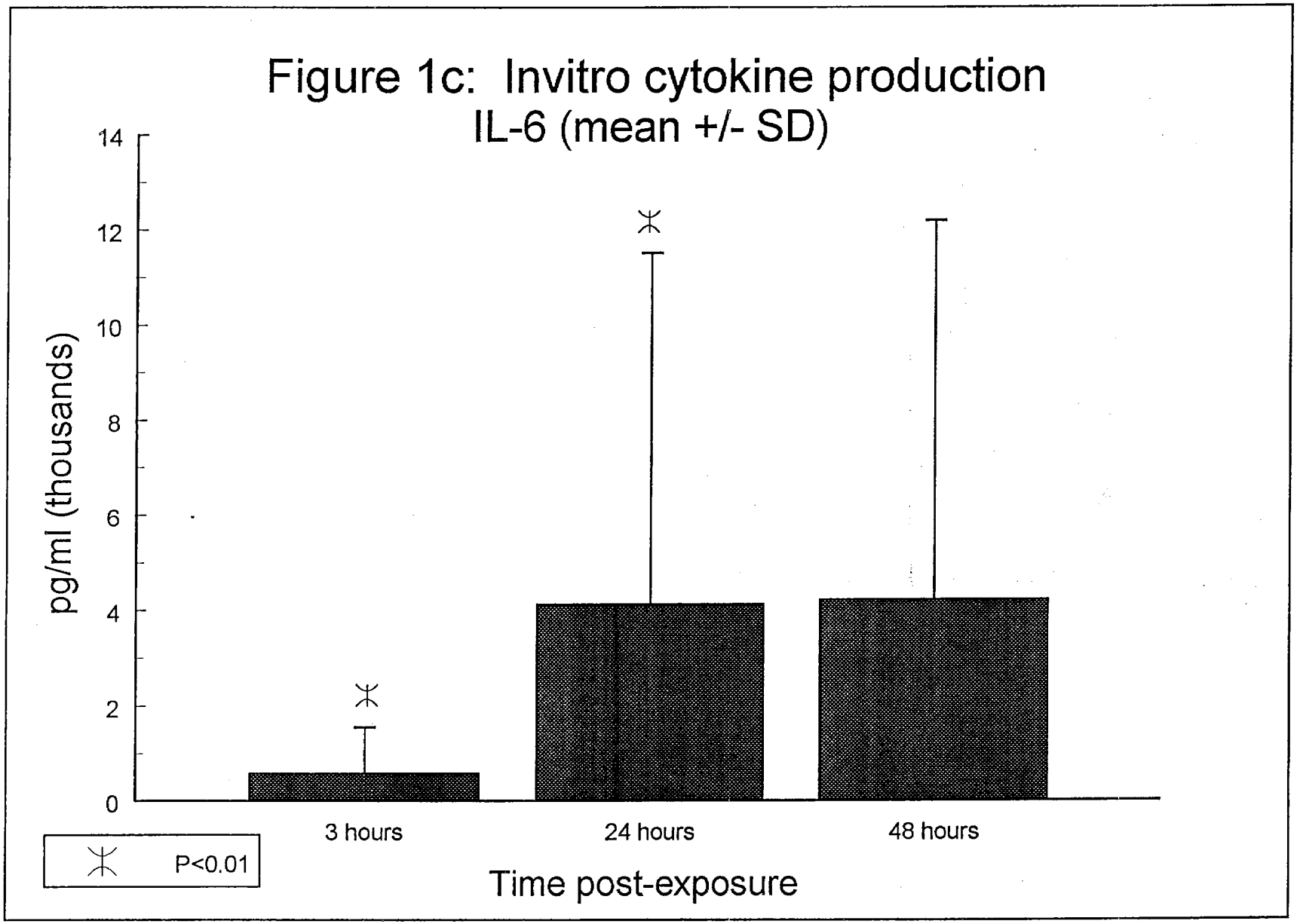
Figure 1c: Invitro cytokine production
IL-6 (mean +/- SD)
pg/ml (thousands)
14
12
10
8
6
4
2
0
3 hours
24 hours
48 hours
Time post-exposure
P<0.01

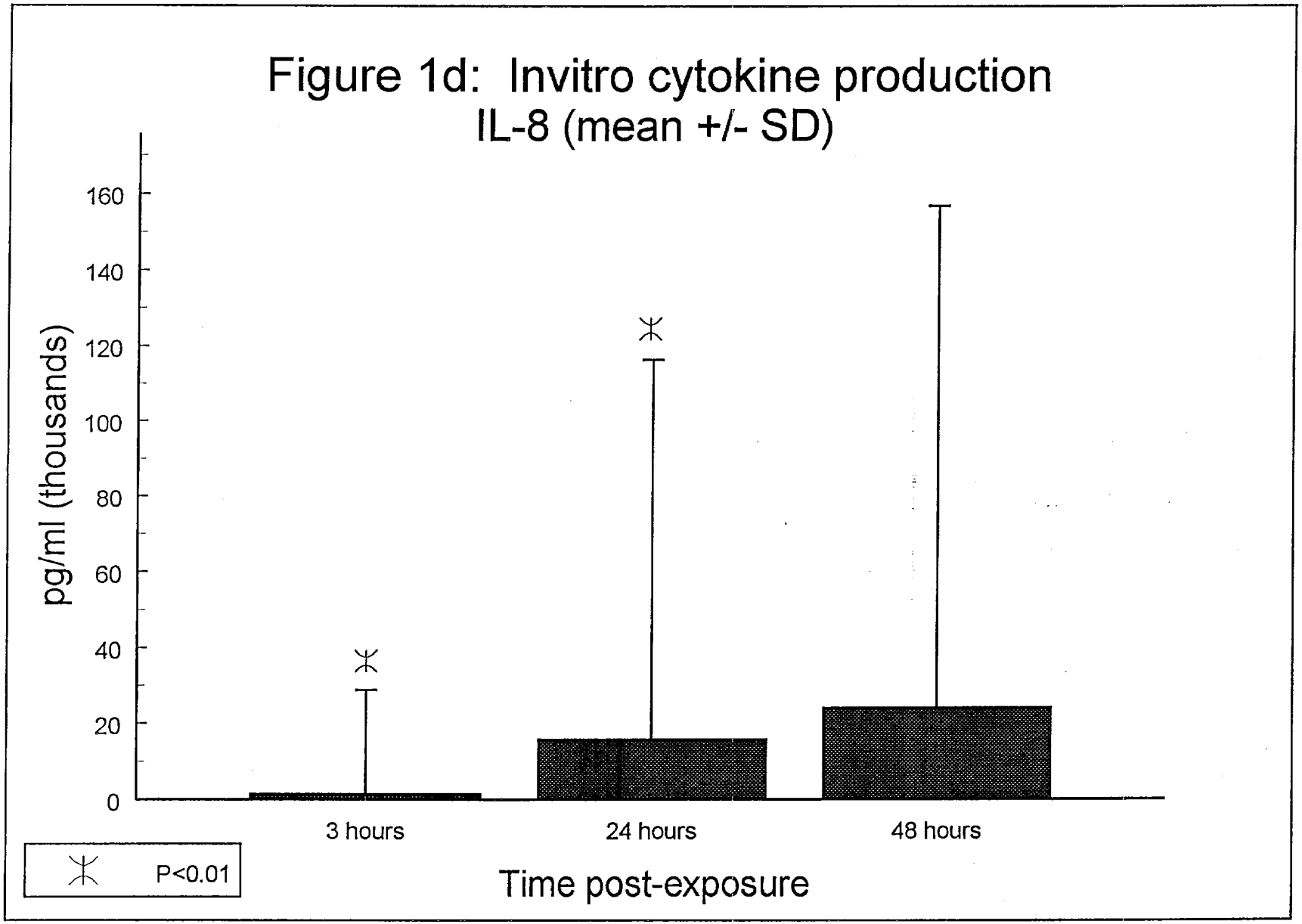
Figure 1d: Invitro cytokine production
IL-8 (mean +/- SD)
pg/ml (thousands)
160
140
120
100
80
60
40
20
0
3 hours
24 hours
48 hours
Time post-exposure
P<0.01

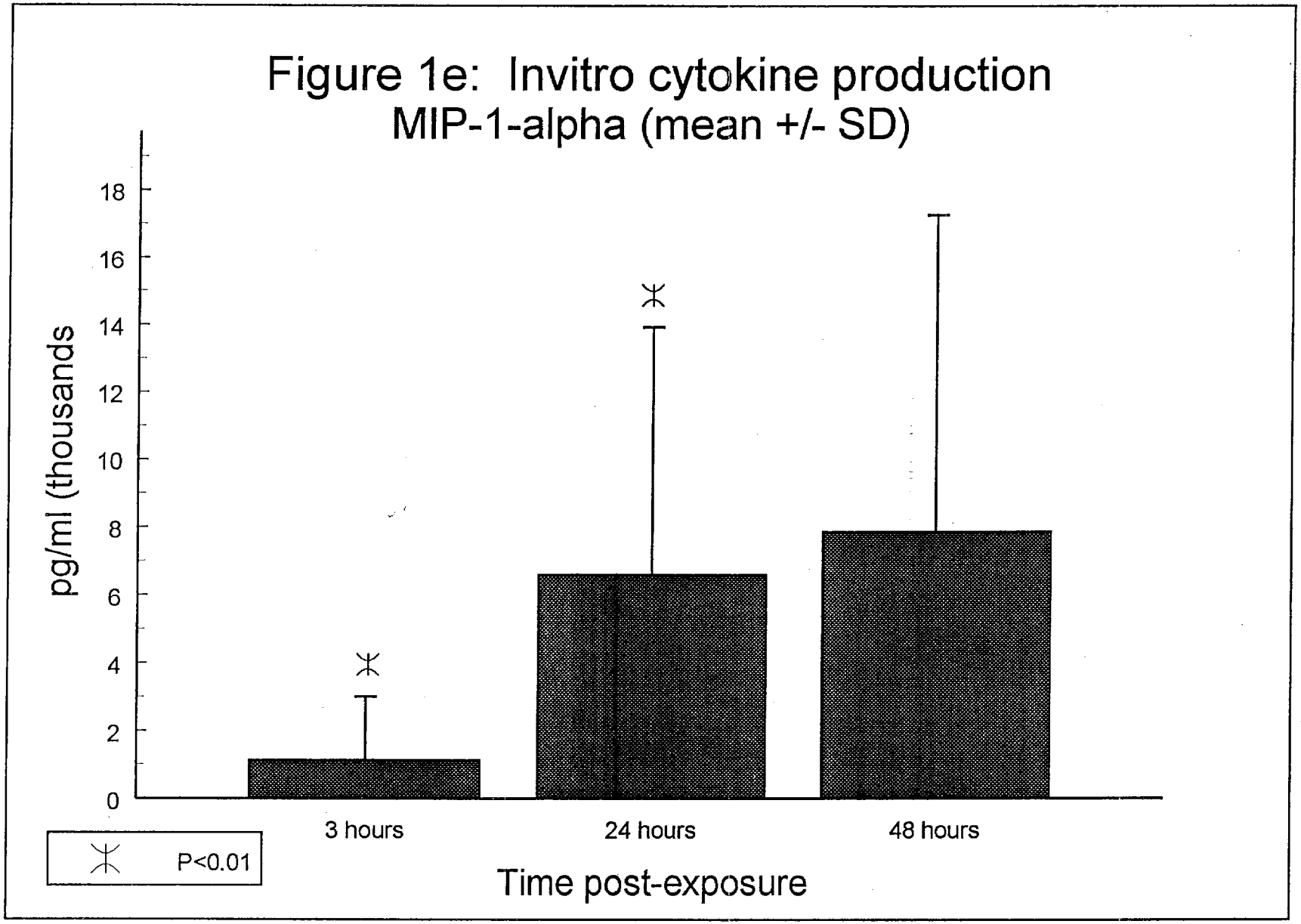
Figure 1e: Invitro cytokine production
MIP-1-alpha (mean +/- SD)
pg/ml (thousands
0
2
4
6
8
10
12
14
16
18
3 hours
24 hours
48 hours
Time post-exposure
P<0.01

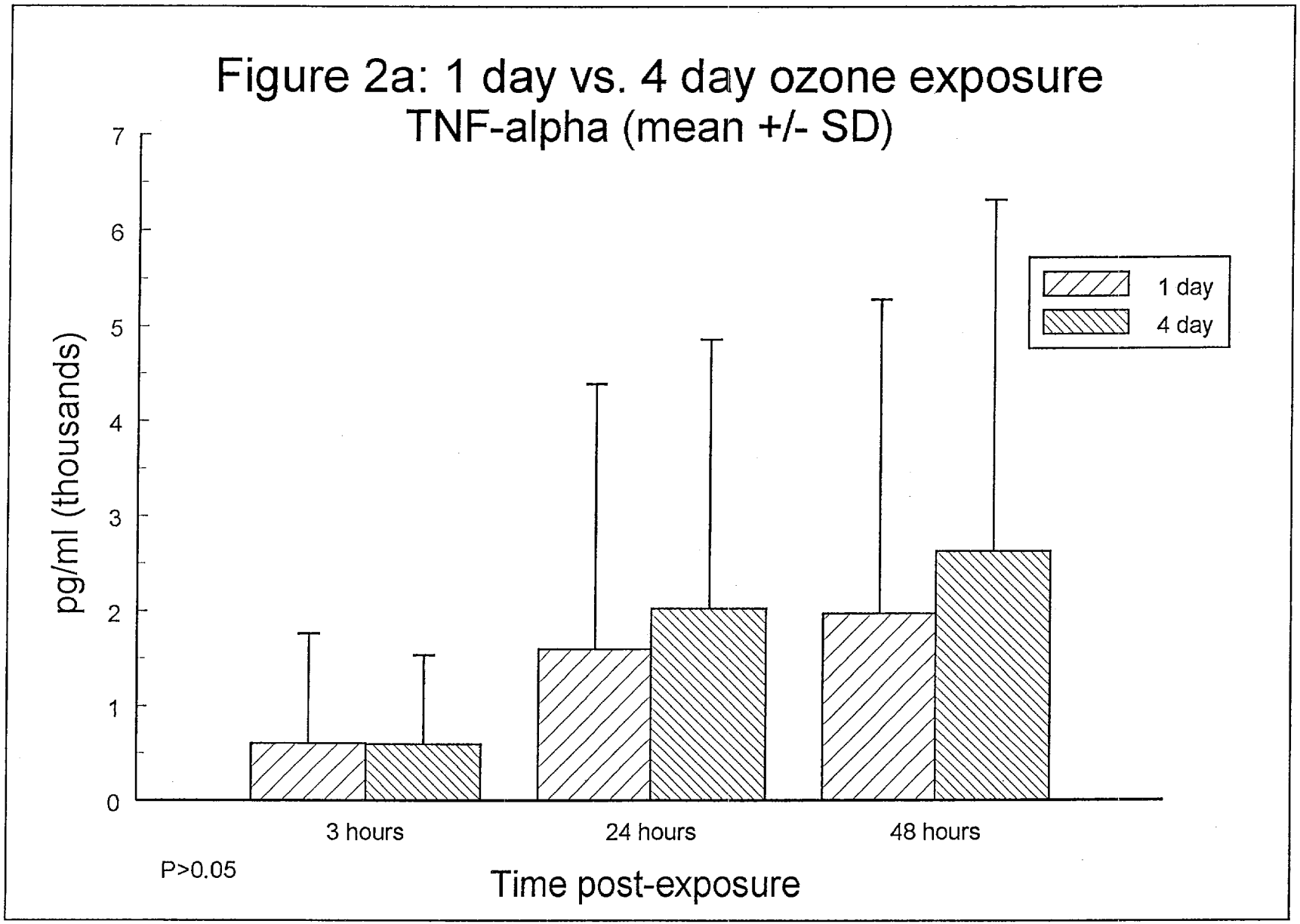
Figure 2a: 1 day vs. 4 day ozone exposure
TNF-alpha (mean +/- SD)
pg/ml (thousands)
0
1
2
3
4
5
6
7
1 day
4 day
3 hours
24 hours
48 hours
P>0.05
Time post-exposure

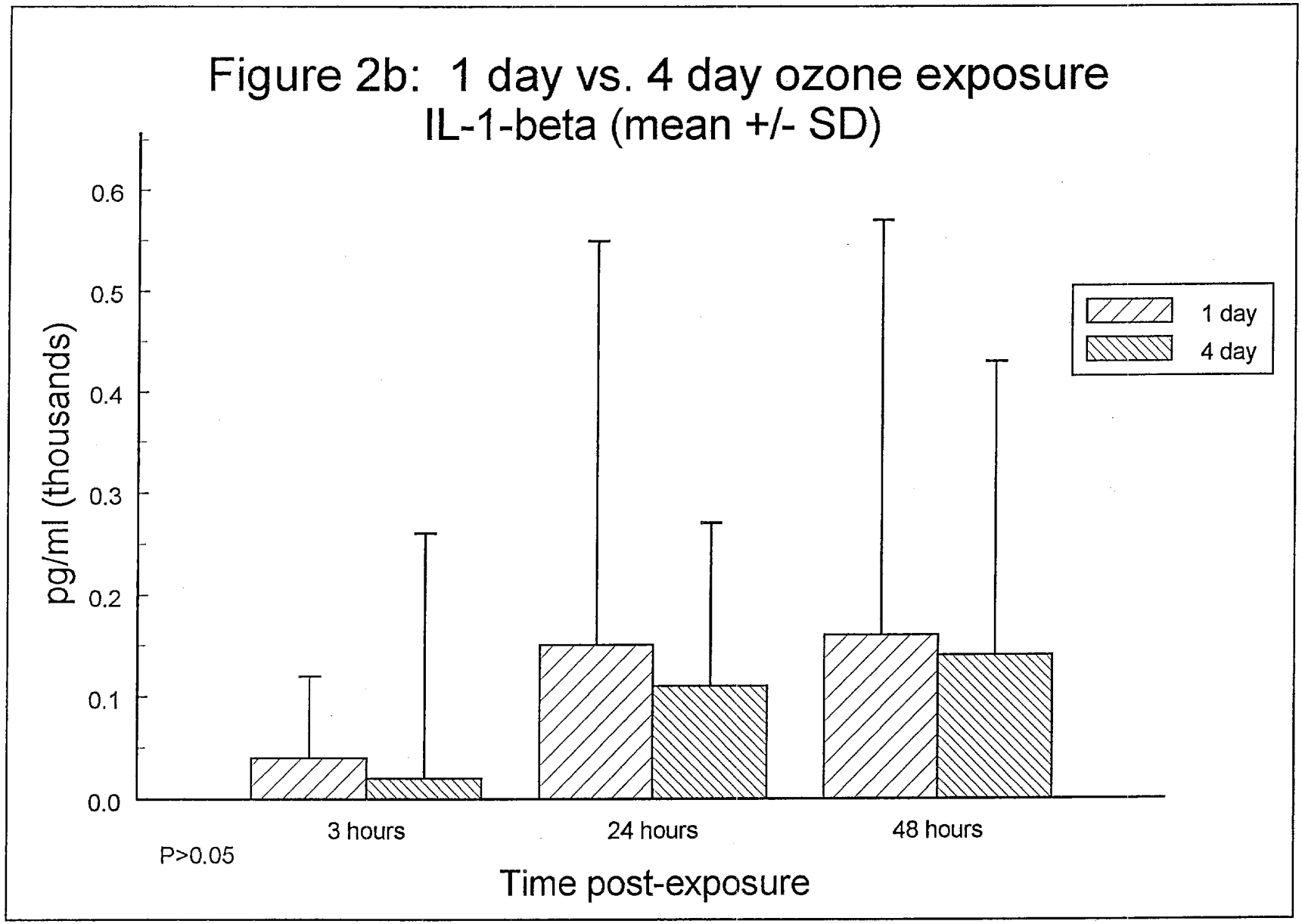
Figure 2b: 1 day vs. 4 day ozone exposure
IL-1-beta (mean +/- SD)
pg/ml (thousands)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
1 day
4 day
3 hours
24 hours
48 hours
P>0.05
Time post-exposure

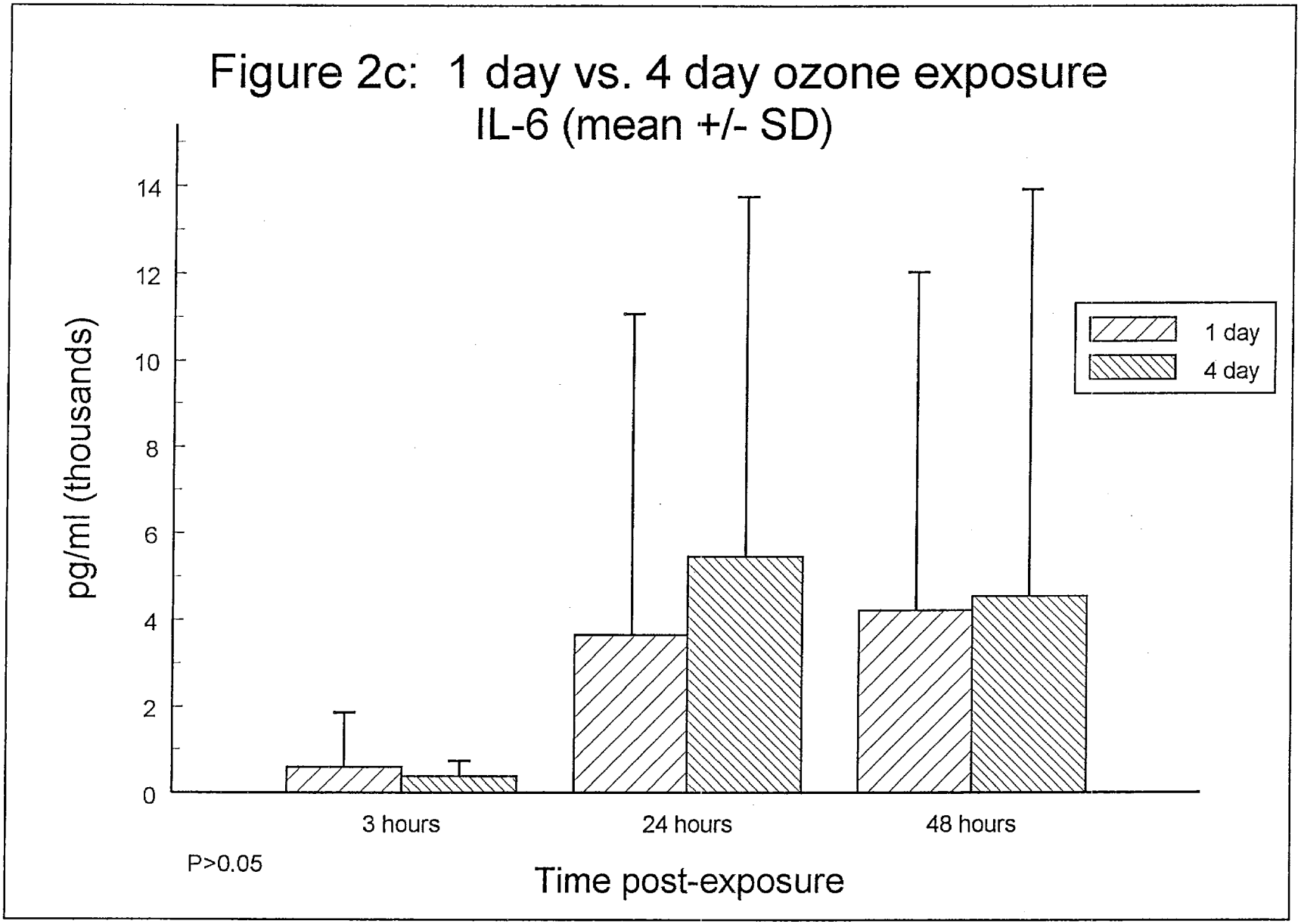
Figure 2c: 1 day vs. 4 day ozone exposure
IL-6 (mean +/- SD)
pg/ml (thousands)
0
2
4
6
8
10
12
14
1 day
4 day
3 hours
24 hours
48 hours
P>0.05
Time post-exposure

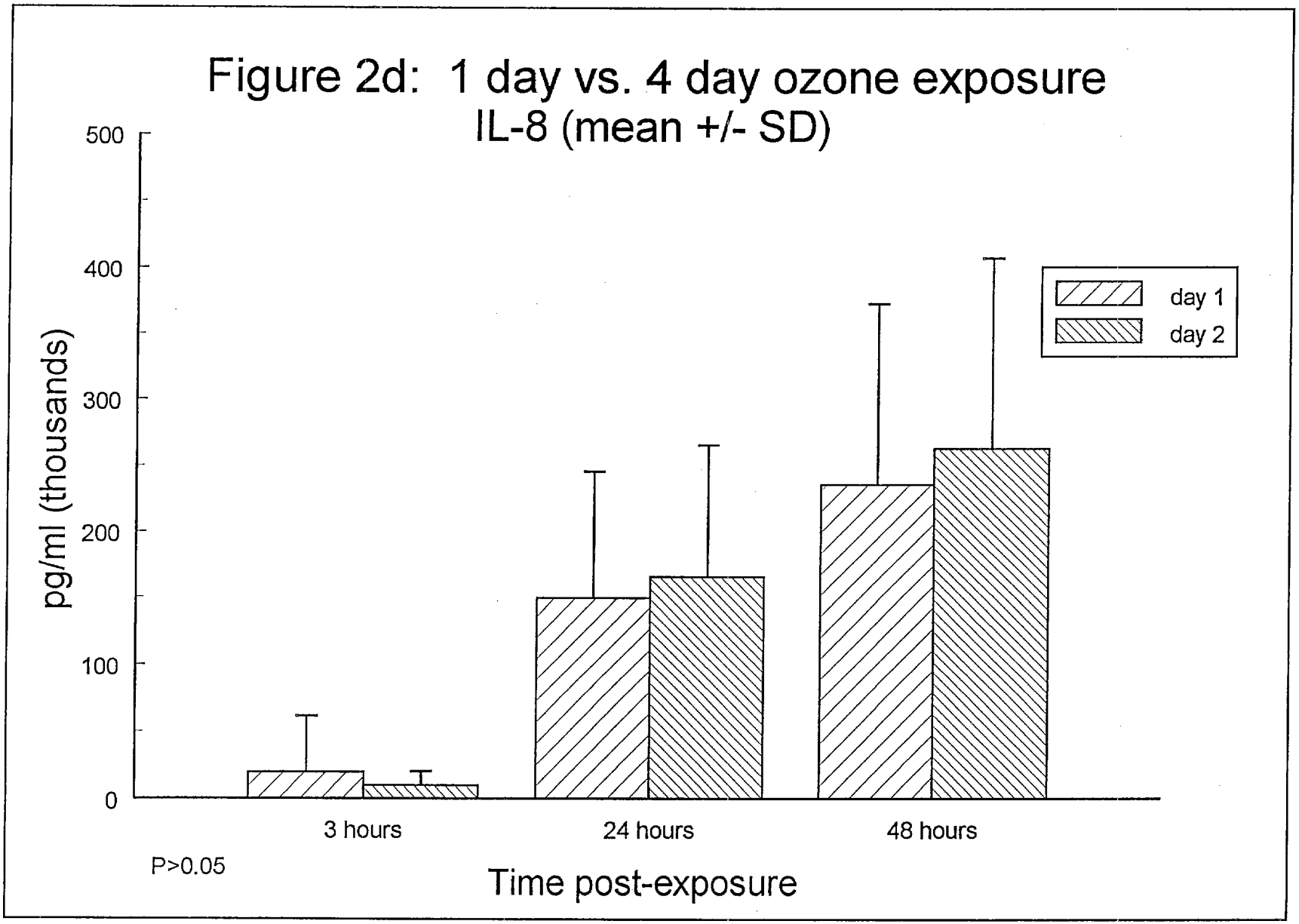
Figure 2d: 1 day vs. 4 day ozone exposure
IL-8 (mean +/- SD)
500
400
300
200
100
0
pg/ml (thousands)
day 1
day 2
3 hours
24 hours
48 hours
P>0.05
Time post-exposure

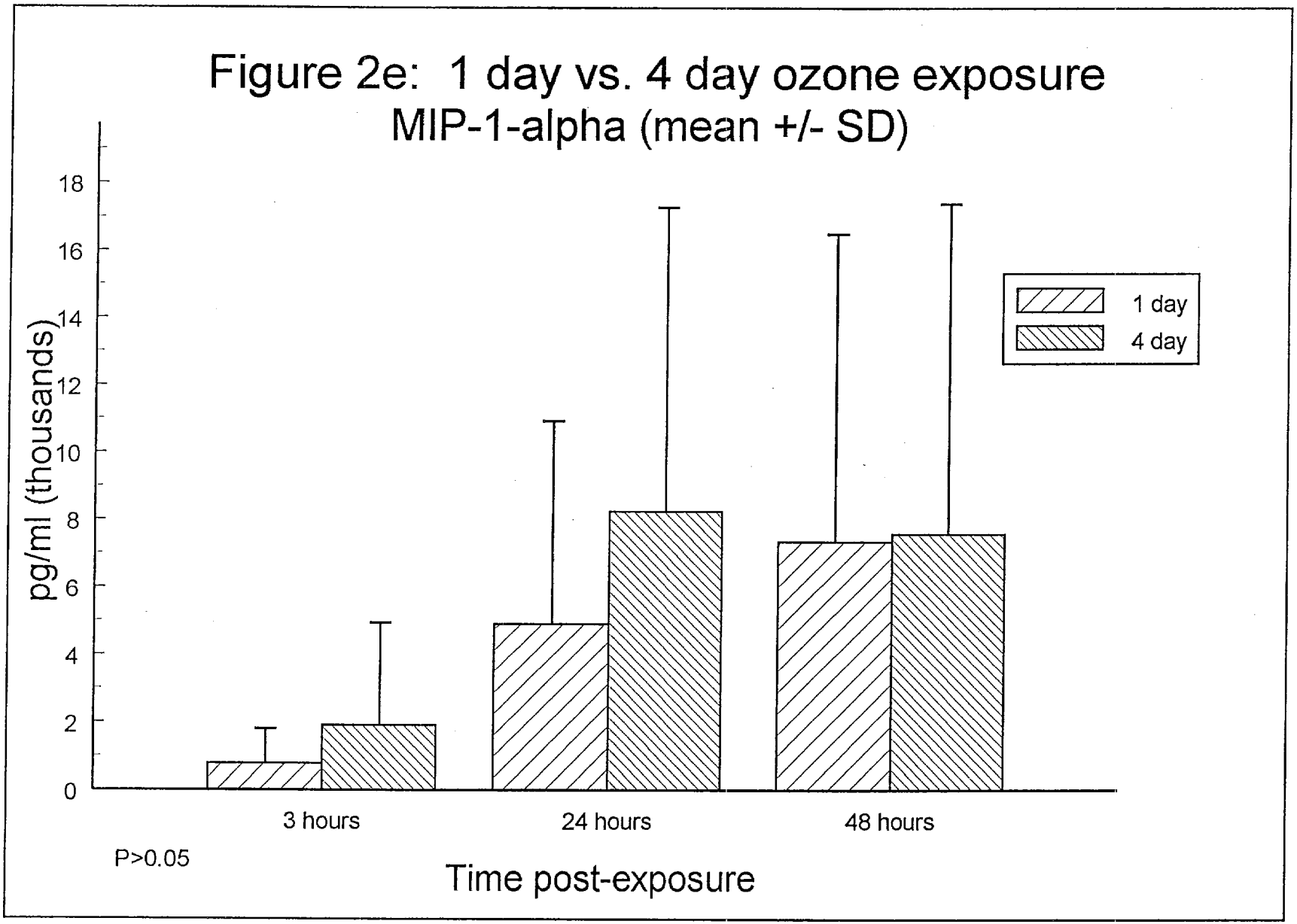
Figure 2e: 1 day vs. 4 day ozone exposure
MIP-1-alpha (mean +/- SD)
pg/ml (thousands)
0
2
4
6
8
10
12
14
16
18
1 day
4 day
3 hours
24 hours
48 hours
P>0.05
Time post-exposure

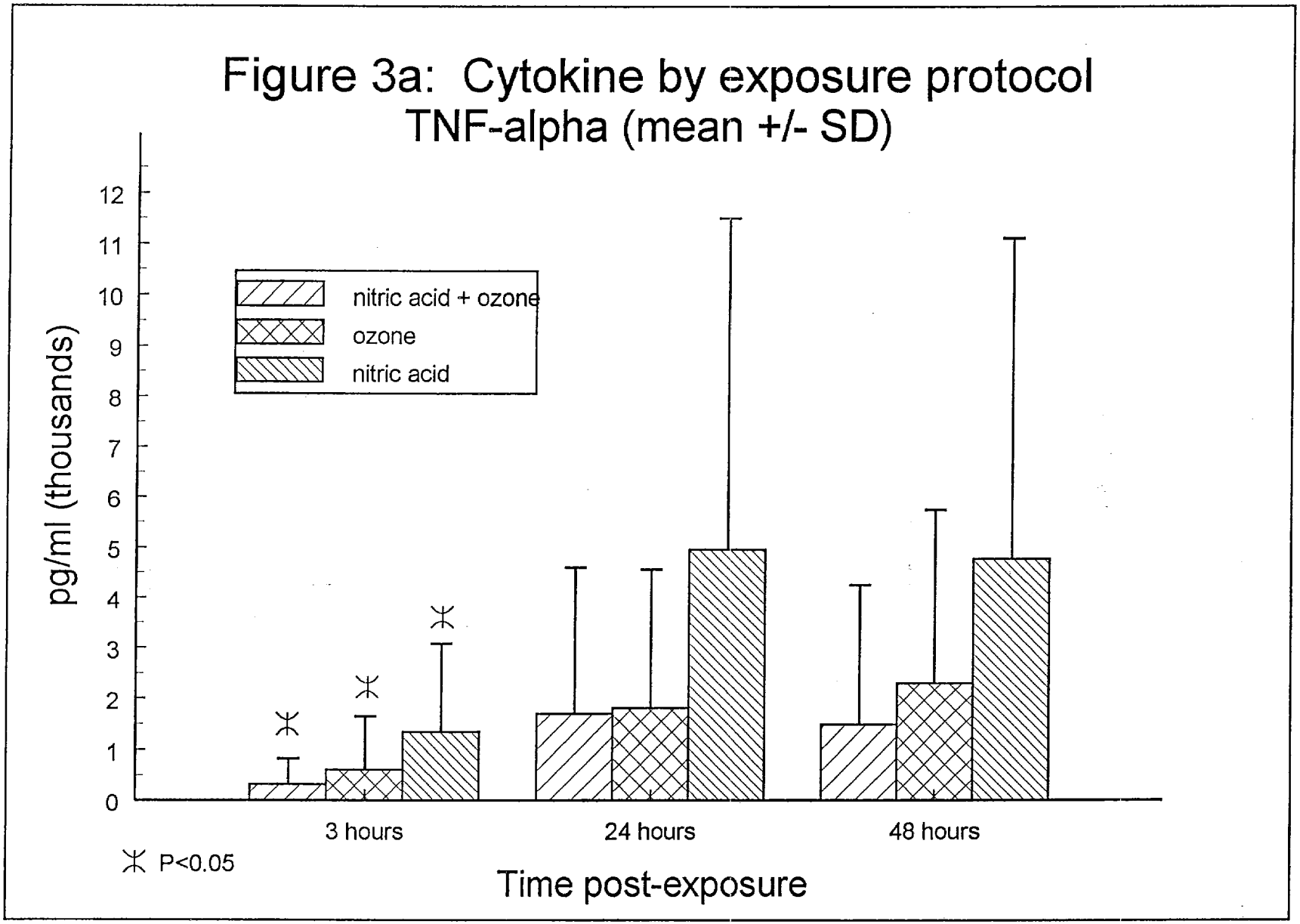
Figure 3a: Cytokine by exposure protocol
TNF-alpha (mean +/- SD)
pg/ml (thousands)
0
1
2
3
4
5
6
7
8
9
10
11
12
nitric acid + ozone
ozone
nitric acid
3 hours
24 hours
48 hours
P<0.05
Time post-exposure

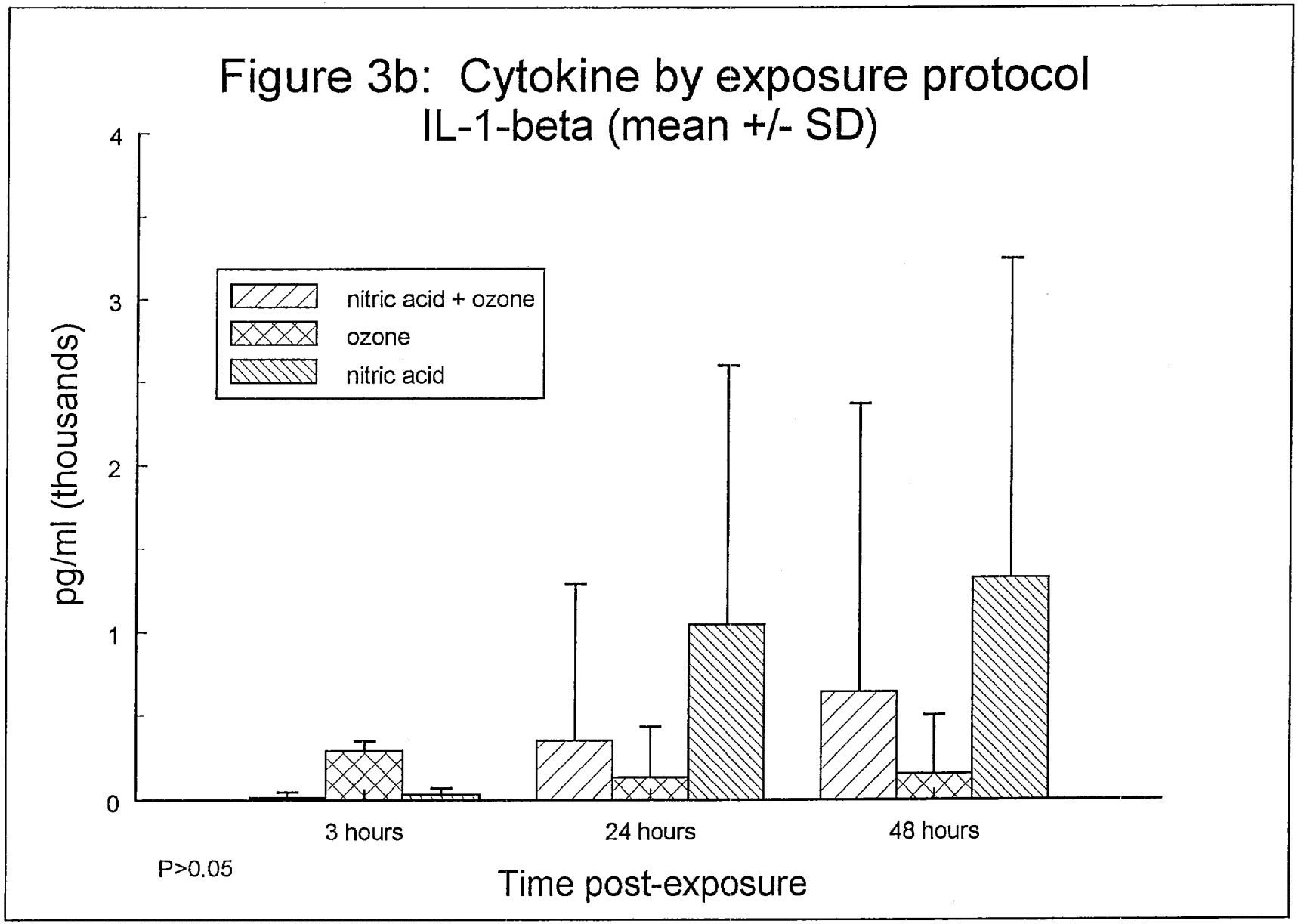
Figure 3b: Cytokine by exposure protocol
IL-1-beta (mean +/- SD)
pg/ml (thousands)
4
3
2
1
0
nitric acid + ozone
ozone
nitric acid
3 hours
24 hours
48 hours
P>0.05
Time post-exposure

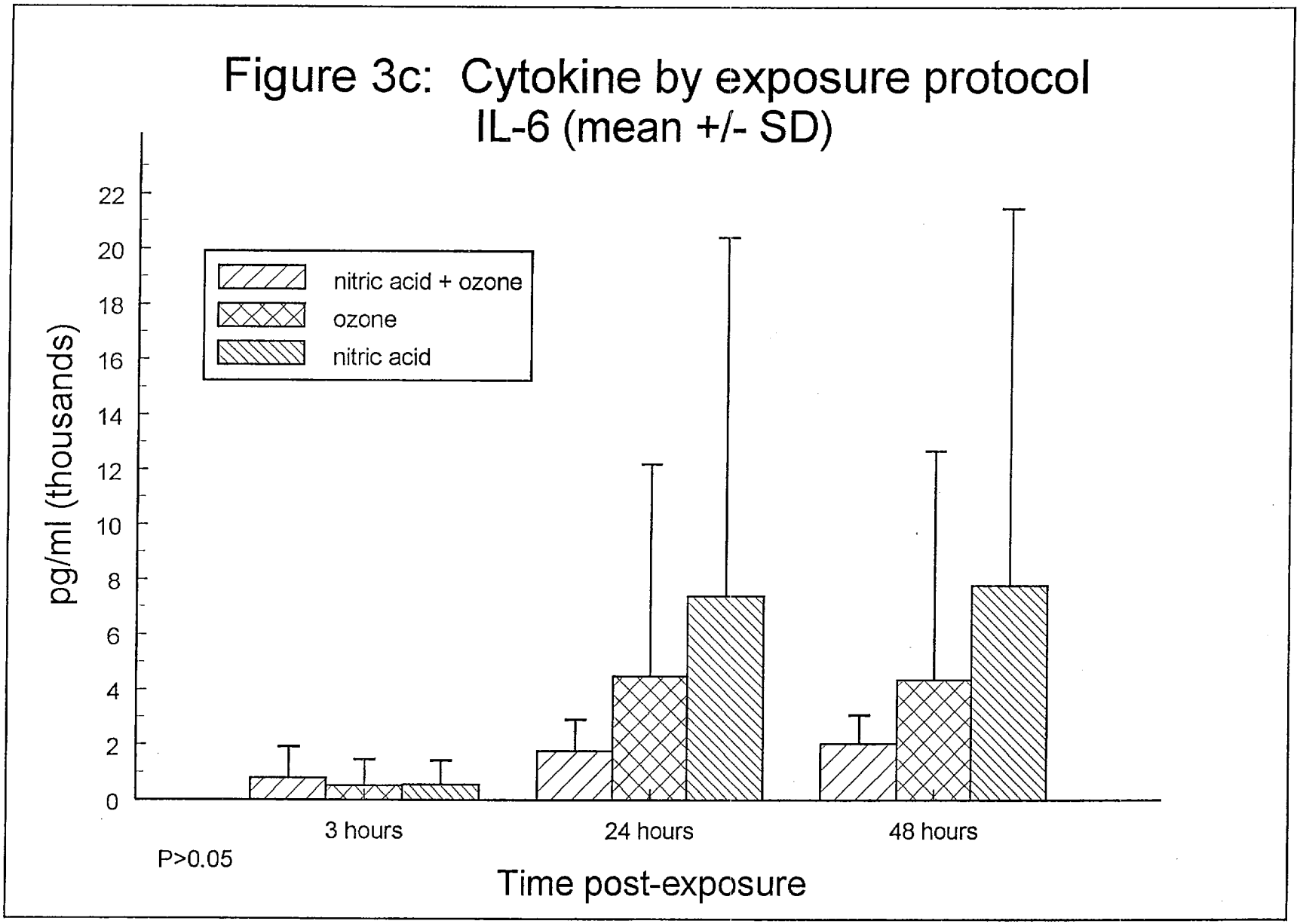
Figure 3c: Cytokine by exposure protocol
IL-6 (mean +/- SD)
pg/ml (thousands)
0
2
4
6
8
10
12
14
16
18
20
22
nitric acid + ozone
ozone
nitric acid
3 hours
24 hours
48 hours
P>0.05
Time post-exposure

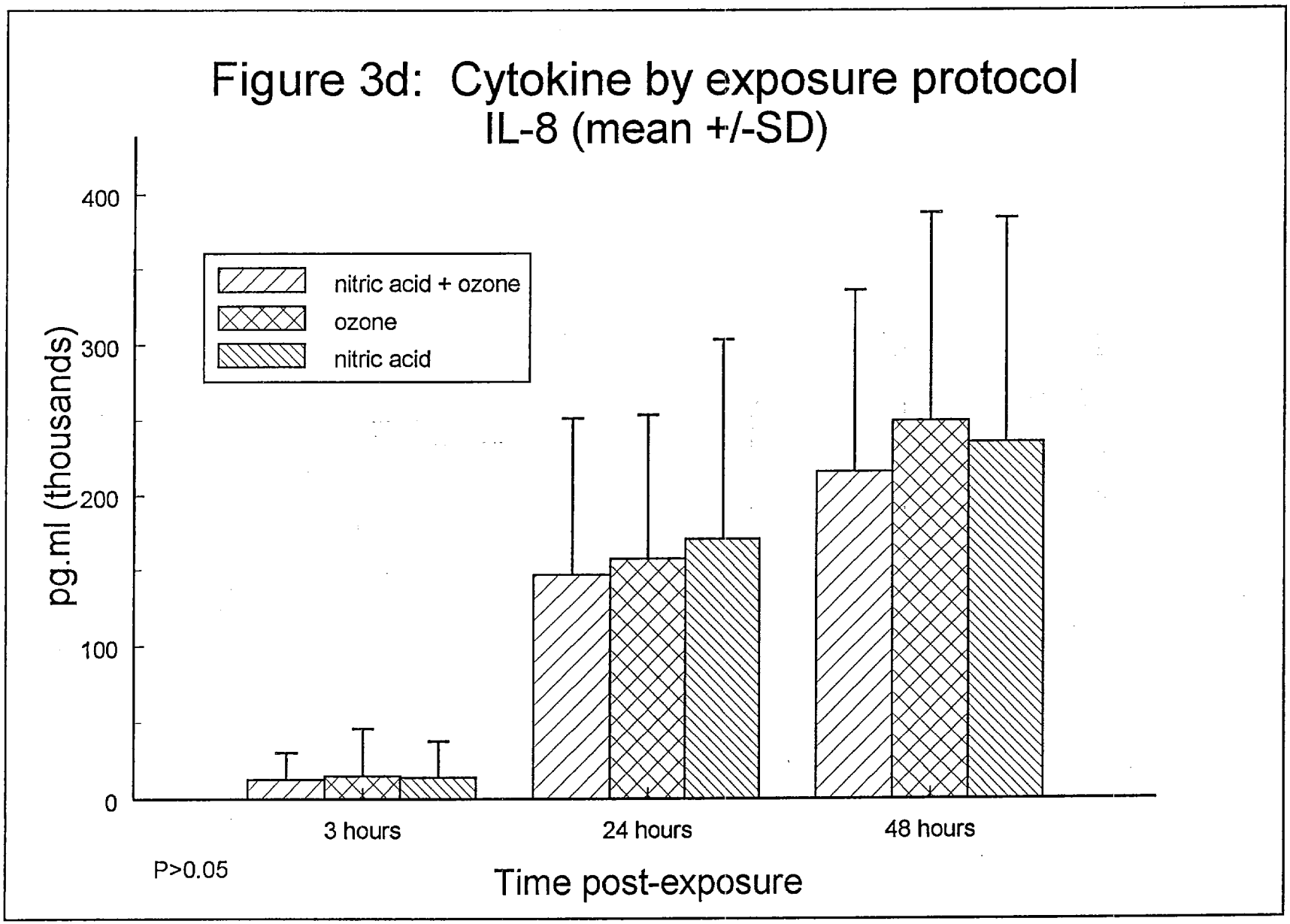
Figure 3d: Cytokine by exposure protocol
IL-8 (mean +/-SD)
pg.ml (thousands)
400
300
200
100
0
nitric acid + ozone
ozone
nitric acid
3 hours
24 hours
48 hours
P>0.05
Time post-exposure

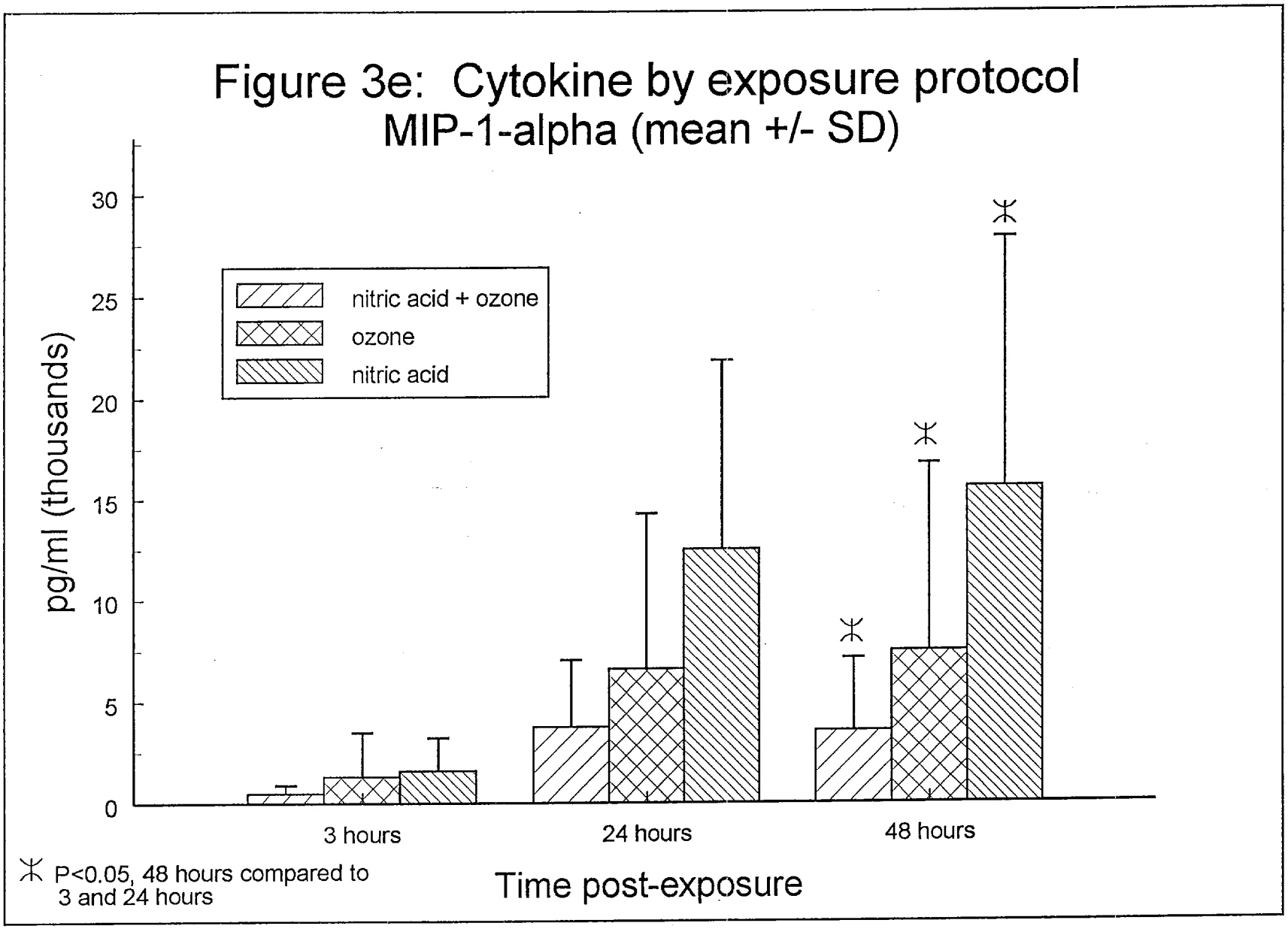
Figure 3e: Cytokine by exposure protocol
MIP-1-alpha (mean +/- SD)
pg/ml (thousands)
30
25
20
15
10
5
0
nitric acid + ozone
ozone
nitric acid
3 hours
24 hours
48 hours
Time post-exposure
* P<0.05, 48 hours compared to 3 and 24 hours