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Adherent endotoxin on dental implant surfaces: a reappraisal

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Abstract:	Osteoimmunology, i.e. the cross-talk between cells from the immuno and skeletal systems, suggests a role of pro-inflammatory cytokines in the stimulation of osteoclasts activity. Endotoxin or bacterial challenges to inflammatory cells are directly relevant to dental implants pathologies involving bone resorption, such as osteointegration failure and periimplantitis. While the endotoxin amount on implant devices is regulated by standards, it is not known whether commercially available dental implants elicit different levels of adherent-endotoxin stimulated cytokines. The objective of this work is to develop a model system and to evaluate endotoxin-induced expression of pro-inflammatory cytokines genes relevant to osteoclasts activation on commercially available dental implants. Murine J774-A1 macrophages were cultured on Ti disks with different level of Lipopolysaccharide (LPS) contamination, to define the time-course of the inflammatory response to endotoxin, as evaluated by RT-PCR analysis. The developed protocol was then used to measure adherent endotoxin on commercially available dental implants, packaged, sterile, that is in the "as-implanted" condition. Results show that tested dental implants induce variable expression of endotoxin-stimulated genes, sometime over the level expected to promote bone resorption in vivo. Results are not affected by the specific surface treatment, rather they likely reflect cares in cleaning and packaging protocols. In conclusion, expression of genes that enhance osteoclasts activity through endotoxins stimulation of inflammatory cells is widely different on commercially available dental implants. A reappraisal of the clinical impact of adherent endotoxins on dental (and bone) implant devices is required on the light of increasing knowledge on crosstalk between cells from the immuno and skeletal systems

Adherent endotoxin on dental implant surfaces: a reappraisal

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Abstract

Osteoimmunology, i.e. the cross-talk between cells from the immuno and skeletal systems, suggests a role of pro-inflammatory cytokines in the stimulation of osteoclasts activity. Endotoxin or bacterial challenges to inflammatory cells are directly relevant to dental implants pathologies involving bone resorption, such as osteointegration failure and periimplantitis. While the endotoxin amount on implant devices is regulated by standards, it is not known whether commercially available dental implants elicit different levels of adherent-endotoxin stimulated cytokines. The objective of this work is to develop a model system and to evaluate endotoxin-induced expression of pro-inflammatory cytokines genes relevant to osteoclasts activation on commercially available dental implants. Murine J774-A1 macrophages were cultured on Ti disks with different level of Lipopolysaccharide (LPS) contamination, to define the time-course of the inflammatory response to endotoxin, as evaluated by RT-PCR analysis. The developed protocol was then used to measure adherent endotoxin on commercially available dental implants, packaged, sterile, that is in the "as-implanted" condition. Results show that tested dental implants induce variable expression of endotoxin-stimulated genes, sometime over the level expected to promote bone resorption in vivo. Results are not affected by the specific surface treatment, rather they likely reflect cares in cleaning and packaging protocols. In conclusion, expression of genes that enhance osteoclasts activity through endotoxins stimulation of inflammatory cells is widely different on commercially available dental implants. A reappraisal of the clinical impact of adherent endotoxins on dental (and bone) implant devices is required on the light of increasing knowledge on crosstalk between cells from the immuno and skeletal systems

Key Words: Dental Implants, Implant surface, endotoxin, osteoimmunology, inflammatory response

Introduction

Bone homeostasis and remodeling occur throughout life in organisms that possess a skeleton. Bone homeostasis is often regulated by immune responses, particularly when the immune system has been activated or becomes pathologic[1]. The name osteoimmunology was coined to identify studies related to interactions between the bone, hematopoietic, and immune systems[2-4]. Crosstalk between activated lymphocytes and bone cells occurs throughout life, because all mammals are constantly challenged by a variety of infectious agents, which produce some level of constant low grade immune system activation.

A particular instance of immune system activation involves implant devices. Beside immunological response to surgery, and ensuing onset of the inflammatory cascade and of wound healing mechanisms, interrogation of the implanted device by pertinent cells from the immune systems occurs at the implant site[5]. Cytokines and chemokines produced by inflammatory cells on contact with the implant surface contribute to the peri-implant biochemical environment and to the overall host response. This topic has been widely investigated both in terms of material surface chemistry[6,7] and surface topography [8-11], providing support to concepts such as enhancement of healing through the "programmed" release of pro-healing cytokines by macrophages on properly engineered implant surfaces[12].

Beside physico-chemical properties of surfaces, it has been shown that endotoxin, the "uninvited guest"[13], significantly affects inflammatory cells response to implant materials and hence may confound any effect of the material itself. Greenfield and coworkers have published an interesting series of papers[14,15], showing that endotoxin adherent to implant surfaces is largely responsible for inducing osteoclast differentiation through production of interleukin-1β (IL-1β), IL-6, and tumor necrosis factor a (TNF-α) on inflammatory cell-material contact. Adherent endotoxin was found at significant levels on the commonly used preparation of commercially pure titanium particles as well as on orthopedic titanium implant surfaces. Removal of adherent endotoxin[14] almost completely inhibited the responses to titanium (Ti) particles by both murine marrow cells and human peripheral blood monocytes. *In vivo* experiments showed that endotoxin removal reduced particle-induced osteolysis by 50–70%, while addition of lipopolysaccharide (LPS) to the "endotoxin-free" particles restored their ability to induce cytokine production and osteoclast differentiation *in vitro*. Thus, adherent endotoxin and not inflammatory response to particle and materials debris could be at the basis of the widely investigated "aseptic loosening" of orthopedic prosthesis.

In a paper published on this journal in 2001, Wataha and coworkers investigated the effect of LPS contamination on the attachement of osteoblast-like cells on titanium *in vitro*[16], building on the clinical evidence that failing implants with loss of alveolar bone are associated with gram-negative bacteria that carry LPS in the bacterial cell wall. Their study failed to detect evidences on the effect of LPS on the attachment of osteoblasts cells to titanium surfaces *in vitro*, concluding that "Further research is needed to define the clinical liabilities of LPS during implant placement and maintenance". The role of cross-talk between cells from the immuno and the muscoskeletal systems, at that time not yet fully developed, today provides the missing link that can shed more light on this topic.

The *in vivo* effect of adherent endotoxin on osteointegration of titanium implants has recently been discussed by Omar and coworkers[17]. LPS was first adsorbed, through incubation from aqueous solution, on machined and anodized dental implants, that were then implanted in pig femoral diaphyses. Hystological analysis showed, after 2 weeks, large areas of inflammatory infiltrates with active bone resorption, both around the neck as well as around the middle and lower parts of LPS-adsorbed implants, independently from the nature of the implant surface. After 6 weeks, LPS incubated implants demonstrated comparable bone morphology and amount in contact with the implant surfaces as implants not incubated in LPS. The quoted paper nicely describes the interplay between classically activated macrophage and osteogenic cells, that ultimately leads to positive solution of the endotoxin challenge in the periimplant area. However, it is clear that the detected short time (2 weeks) bone resorption and ensuing lack of stability around LPS-adsorbed implants can be highly relevant from a clinical point of view, especially on the light of the increasing demand for immediate or early loading. Thus the amount of adherent endotoxin could rightfully be considered a further clinically relevant variable of dental implant surfaces. Since the eighties, many papers describe chemical contamination of clinically available implant surfaces[18-20], and the interplay between biological stimulation and surface parameters[21-24], but no published comparison exists on the level of adherent endotoxin. It is here worthy to mention that the endotoxin amount on implant devices is regulated by standards and routinely checked by producers. However, measurements are performed on aqueous extracts, and not directly on the implant surface[25].

The aim of the present paper is to evaluate the potential for endotoxin-stimulated pro-inflammatory response of several commercially available dental implants, as follows:

first, we validate a detection method of adherent endotoxin, by developing an *in vitro* simple model that measures endotoxin activity directly on implant surfaces. In particular, gene expression of pro-inflammatory cytokines on implant surfaces can be routinely measured by Real-Time PCR. We first follow the time course of inflammatory transcripts in a model system, involving the continuous murine macrophage cell line J774A-1 and purposely LPS-contaminated titanium disks. We then show that the level of LPS contamination, independently from surface topography, controls the short time (4 h) ILs expression by the selected cell line. Finally, we perform the same measurement on commercially available, sterile and sealed (i.e. in the "as-implanted" condition) dental implants from different producers, to evaluate if and how much they elicit pro-inflammatory activity.

Methods and materials

Samples preparation

For the validation of the method, tests were performed on grade 4 Ti disks, 8 mm diameter and on 3.75 x 13 grade 4 Ti dental implants; all samples were produced by the same supplier, all surfaces were simply turned when received in our laboratory. All subsequent steps were performed according to our ISO 9001:2008 and ISO13485:2004 quality standards and protocols, the following samples were prepared:

- to evaluate the time course and the dose-dependence of the response, tests were performed on endotoxin-free titanium disks, and on LPS-contaminated Ti disks. The endotoxin-free sample (negative control, coded Ctrl in the rest of the paper) was obtained by subjecting Ti disks to a dedicated proprietary cleaning treatment, involving both solvent and plasma (glow discharge) cleaning cycles, using a Plasma Finish microwave reactor placed inside a ISO7 clean room. The absence of detectable endotoxin on the endotoxin-free Ctrl sample was confirmed by LAL tests performed by an external lab, the sensitivity of the test was 0.125 EU/mL. LPS-incubated samples were obtained by overnight incubation of formerly endotoxin-free samples in 1 μg/mL, 5 μg/mL and 10 μg/mL LPS (Sigma) in phosphate buffered saline. After incubation, disks were washed three times in MilliQ water pyrogen-free (MilliQ Synthesis A-10) and dried under a laminary flow hood. With reference to the LPS concentration of the incubation solution, these samples will be coded LPS1, LPS5 and LPS10 in the rest of the paper.

- to evaluate the contribution of surface topography to expression of genes involved in macrophage response to adherent endotoxin, titanium implants were subjected to the following treatments in our lab:

sandblasting using titanium oxides, 250-400 mesh, coded Tiblasted (Sa 1.83 μm, as measured by StereoSEM in a 130 x 120 μm area)

double acid etching treatment, coded DAE (Sa 0.91 μm, as measured by StereoSEM in a 130 x 120 μm area)

Tiblasted and DAE samples, together with machined implants (coded Mach, Sa 0.47 μm, measured as above), were tested both as prepared, that is after a solvent cleaning cycle involving nitric acid passivation, neutralization, DI water and solvent cleaning, and after the complete cleaning cycle discussed above. More details will be provided in the Results section.

Beside samples used for process validation, a second set of samples was tested, for actual measurements through the developed method. This set was made by 22 commercially available dental implants, from different worldwide producers. All samples were received sterile, sealed in their original package, all of them well before their relevant expiry date. All of them were made from commercially pure titanium (cpTi).

Gene expression measurement through RT-PCR was performed to evaluate the amount of adherent endotoxin. Test were performed through the evaluation of the expression by J774A-1 macrophages of a few key-genes involved in the inflammatory response to endotoxin: Interleukin 1 (IL-1), interleukin 6 (IL-6), Tumor Necrosis Factor alfa (TNFα), MCP-1, COX-2 and MCSF.

A suspension of 1.05±0.13 x 105 J774A-1 cells, cultured in DMEM containing L-glutamine (Gibco, INVITROGEN S.r.l), and 20% Fetal Bovine Serum (FBS Gibco, INVITROGEN S.r.l), penicillin and streptomycin was introduced into sterile 12-well polystyrene culture plates (12-well multiwell plates, Cell Star, Greiner One™) containing the samples. Analysis of gene expression was carried out using real time reverse transcription PCR (qRT-PCR). Total RNA was extracted after 1, 4 and 24 h, using the MagMax Total RNA Isolation Kit (Applied Biosystems). The quality of the RNA was assessed by checking that the A260/A280 absorbance ratio was between 1.6 and 2.0. The extracted RNA was subsequently reverse transcribed to give cDNA using the Applied Biosystems High Capacity cDNA Reverse Transcription kit.

Relative quantification of the genes was obtained using Taq Man probes specific for each gene under test and GAPDH as the reference gene. The amplification reactions were carried out in a StepOne thermocycler (Applied Biosystems) in accordance with the manufacturer's instructions. To obtain the gene expression graphs, data were normalised using the StepOne software in accordance with the ΔCt standard method.

Measurements were performed in triplicate in the case of samples involved in the validation step. Measurements on the 22 test samples were obviously performed on a single specimen for each tested implant, to check intra-experiment accuracy amplification reactions were performed in triplicate aliquots of cDNA.

Results

As a first step, expression of IL-1, IL-6, TNFα, MCP-1, COX-2 and MCSF by J774A-1 macrophages was measured on Ctrl and LPS adsorbed Ti disks as a function of time. As expected, all quoted genes were significantly overexpressed on LPS-contaminated samples, reflecting the onset of defence mechanisms against endotoxin challenge by macrophages. Among tested genes, both IL-1 and IL-6 showed very significant upregulation as a function of LPS concentration. Fig. 1 and 2 show respectively fold-expression of IL-1 and IL-6 by J774A-1 macrophages adhering to the Ti disks as a function of time and concentration of LPS in the adsorption solution. Data are presented as fold-expression over the value obtained on the Ctrl sample at 1 h. The figure suggests that peak expression occurs at 4 h (at least among the three time points investigated) and that there is a clear dose-dependent response of gene expression. Interestingly, while the LPS-free control sample shows an increasing trend of gene expression, likely plateauing in the 4-24 h time frame, macrophages on LPS-contaminated disks yield a burst response, that is turned off, or at least attenuated, in the same 4-24 h timespan. On the light of providing an analytical method for the detection of LPS contamination on Ti surfaces, Fig. 1 and 2 suggest that the measurement of IL-1 or IL-6 expression by J774A-1 macrophages at 4 h is a suitable approach, since it provides both sensitivity and dose-dependence, at least within the tested range. Thus the experimental protocol for adherent endotoxin measurement would include evaluation of ILs expression at 4 h on test samples using Ctrl sample as a reference. When this is performed on the present data, that is when 4 h values of LPS1, LPS5 and LPS10 samples are expressed as fold expression over the 4 h Ctrl value, the bargraph shown in Fig. 3 is obtained. This is the "mastercurve" of the present adherent-endotoxin detection method: it shows that expression of both ILs genes is dependent on the amount of LPS in the adsorption solution and therefore on the amount of surface-adsorbed LPS, and that fold expression spans a significant analytical range, from slightly more than ten to more than one hundred, in the tested conditions.

A further required step for the qualification of the present approach as a test method is the demonstration of selectivity. In particular, while the just presented data were obtained on machined disks, it has been reported that surface topography affects macrophages response[10-12]. Hence, it would not be possible to meaningfully compare macrophages gene expression obtained on surfaces with different topographies. Given the huge variety of existing approaches to the control of surface roughness of dental implants[26], this would be a serious limitation of the method. To check this point, we measured 4 h ILs expression by J774A-1 macrophages on Ti implants showing different topographies: Mach, Tiblasted and DAE, as defined in the Methods and Materials section. Measurements were performed after surface treatment and simple solvent cleaning; and after surface treatment followed by the complete endotoxin-removal cycle described above. Obtained results are shown in Fig. 4. Interestingly, the solvent cleaned Mach is more proinflammatory than any other sample. Both sandblasting and acid etching, which destroy the pristine, environment-exposed surface, show some effectiveness in decreasing the endotoxin response to Ti surfaces. In addition, the graph shows that both ILs expression is very significantly dampened by the full endotoxin removal cycle as compared to simple solvent cleaning, and most importantly, it shows that after endotoxin removal, macrophages on the tested surfaces express the same level of ILs transcripts irrespective of the significant variation of surface roughness, as encoded by the Sa value and by the specific topography. These data show that the method provides the required selectivity, in that the short time (4 h) ILs expression by macrophages is not a function of physical parameters such as surface roughness but it is solely controlled by the defence response to endotoxin.

Having shown that the test method provides sensitivity, dose-response and selectivity, we moved to its actual application to clinically relevant samples. In particular, macrophages were cultured on 22 titanium dental implants, from different worldwide producers. All samples were sterile and sealed in their package, that is in the "as sold" condition. Samples encompassed most of the presently adopted approaches to surface roughening: some were sandblasted, some acid etched and some subjected to electrochemical treatment[27]. No sample had a machined surface.

Obtained results are summarized in Fig. 5, that shows 4 h fold-expression of IL-6 by J774A-1 cells over that measured on a Ctrl sample. As a reference, horizontal lines in the figure show the fold expression obtained on purposely LPS-contaminated LPS1, LPS5 and LPS10 samples, as reported in Fig. 3. In

considering these data, it is important to remember that the reference, that is the Ctrl sample, was not the very same one for validation of the method (samples LPS1, LPS5 and LPS10) and for the testing of the actual implants. Thus, the direct comparison of the data rests on the underlying assumption that the cleaning cycle adopted to prepare the different Ctrl samples yields the same level of adherent endotoxin, hence the same macrophage response. While this assumption should be taken in due consideration, it is unquestionable that data of Fig. 5 indicates that clinically available dental implants show wide variation of adherent endotoxin and this evokes significantly different device-induced macrophage activation. Seven out of the 22 tested samples show endotoxin-induced IL-6 expression higher than that promoted by a titanium surface incubated overnight in a 1 μg/mL LPS solution; among these seven samples, three show endotoxin-induced IL-6 expression higher than that promoted by a titanium surface incubated overnight in a 5 μg/mL LPS solution, one of them higher than that promoted by a titanium surface incubated overnight in a 10 μg/mL LPS solution. A few implant surfaces show the lack of any response to adherent endotoxin, suggesting an almost perfect control of surface contamination in the production and packaging steps. Considerations stemming from these data are reported in the following section.

Discussion

The growing field of osteoimmunology underlines the role of inflammatory stimuli in triggering bone-loss pathologies. Cross-talk between cells from the immuno and skeletal systems is of particular relevance in clinical dentistry, that features remodelling bone tissue in close proximity to the thriving oral bacterial population[28,29]. Beside response to bacteria, the present work focused on activation of inflammation by adherent endotoxin on implant surfaces. The classical bacterial endotoxin is lipopolysaccharide (LPS), the primary outer cell wall component of Gram-negative bacteria. However, Gram-positive bacteria also produce molecules such as lipoteichoic acid (LTA) and peptidoglycan with very similar biological effects[30]. Thus, a number of possible sources of bacterial endotoxins (LPS, LTA, peptidoglycan, etc.) exist that might lead to high levels of adherent endotoxin on implant devices.

The adopted approach exploits the direct response of a model continuous cell line, robust and reproducible to endotoxin stimuli. By using a set of purposely LPS-contaminated samples we showed that expression of key inflammatory genes is directly related to the amount of adherent endotoxin, and that the 4

h-response timepoint provides the required sensitivity, dose-response dependence and selectivity. More sophisticated and clinically relevant (e.g. human monocytes) cell lines are not required for the present scope[31], since the role of well-behaved J774A-1 murine macrophages in the present approach is to act as a sensitive "probe", that provides the required answer.

The developed test method was used to evaluate the amount of adherent endotoxin on commercially available implant surfaces. Obtained data show a huge variation of adherent endotoxin. A few of the tested samples are virtually endotoxin-free, while in some instance the response is similar to that obtained after overnight incubation in endotoxin solution. These differences likely reflect the quality of procedures adopted in the production and packaging steps, and cares adopted to remove and prevent endotoxin contamination.

From a basic point of view, present data show that the amount of adherent endotoxin is a further variable that affects cell response on dental implant surfaces. While the latter are often discussed in terms of chemico-physical variables, such as surface chemistry and topography[32,33], present data show that cell response (at least in the case of the tested cell line) is overwhelmingly dominated by "biological cleanliness", or the amount of adherent endotoxin. This is obviously true in the case of tests involving inflammatory cells, such as the J774A-1 macrophages adopted in the present work; however, implications are far ranging, because it is known that most cells, including fibroblasts, endothelial and osteoblasts, respond to endotoxin stimuli. Thus, every study on cell response to implant (and, in general, material) surfaces should include, among surface properties that require proper characterization and definition, the evaluation of adherent endotoxin[14]. This is particularly true in the case of samples prepared in common lab environments, that often lack facilities available to producers of medical devices.

A further consideration involves the clinical implications of present findings. In a recent interesting paper, Omar and coworkers showed that activated human monocytes communicate pro-osteogenic signals to human Mesenchimal Cells (hMSCs)[17]. The signals involve regulation of autologous BMP-2 in the hMSCs, and the contribution by LPS stimulation of monocytes overwhelmed the effect of the surface properties. The quoted paper shows, by an *in-vivo* pig model, that purposely contaminated titanium implants (1h LPS adsorption from a 10 μg/mL solution) resulted in excessive resorption/remodeling activity at the early 2 weeks of implantation. However, after 6 week, LPS-induced resorption areas were replaced with higher percentage of bone contact, suggesting a possible major role for osteoclastic feedback on the process of bone formation at

the interface. Thus, early upregulation of both bone resorption and bone formation genes could be associated with significant increase in implant stability. The just quoted data indicate the outcome of long term exposure of bone tissue to LPS-contaminated implants, and underline once again the complicated and fascinating mechanisms that, within our bodies, control healing and new tissue generation. In clinical practice, especially on the light of the present trend towards immediate or early loading, the detection of very significant (see the impressive fig. 11 of the paper by Omar et al., reference 17) bone resorption and lack of bone tissue around LPS contaminated implants underline the relevance of our present findings. A few of the clinically available implants we tested show endotoxin response in the range of that obtained on LPS contaminated samples, using a similar concentration to that adopted in the quoted reference and able to induce the just described profound *in vivo* effects (note also that our protocol involves overnight LPS adsorption, while in the quoted paper adsorption lasts just one hour and likely results in a lower adsorbed amount, yet deeply effective *in vivo*, as compared to our LPS-contaminated reference samples). Thus, it is possible that early loading of the most contaminated samples we tested could find a not properly regenerated bone tissue; and, more in general, data of Omar et al. show that the amount of adherent endotoxin modulates time and amount of periimplant bone regeneration. This confirms also at the *in vivo* level our previous suggestion that the amount of adherent endotoxin is a further variable required for proper characterization of dental implant surfaces, whose contribution can overwhelm that of "classical" chemico-physical parameter, and whose evaluation is highly required for a correct understanding of periimplant bone regeneration.

The last comment involves the observation that, among tested implants, a few of them are virtually free from adherent endotoxin, even when probed by sensitive and surface-specific tests like the present one. This result underlines that methods and techniques exist to produce and market actual "implant devices", as opposed to "titanium fixtures", designed and produced with a proper understanding and control of cell interfacial biology highly relevant for the intended clinical use.

Conclusions

In conclusion, the present data show that short time (4 h) expression of proinflammatory genes, in particular IL-1 and IL-6, by J774A-1 macrophages is directly and selectively related to the amount of adherent endotoxin and it is largely independent from surface topography. The application of this approach

to several clinically available dental implants shows significant heterogeneity among tested products, some of them are virtually free from adherent endotoxin, a few show ILs upregulation similar to that detected after overnight incubation in LPS solutions. Given the ascertained *in vivo* effect on periimplant bone regeneration provided by LPS contamination, evaluation of adherent endotoxin should be reappraised and ranked among relevant surface properties required for proper understanding of interfacial tissue response to dental implants.

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Captions for the figures

Fig. 1 Expression of IL-1 gene by J774A-1 macrophages after 1, 4 and 24 h culturing on endotoxin-free (Ctrl) and LPS-contaminated Ti samples. Data are expressed as fold expression over the value of the Ctrl sample at 1 h; a) full y-axis scale; b) reduced y-axis scale

Fig. 2 Expression of IL-6 gene by J774A-1 macrophages after 1, 4 and 24 h culturing on endotoxin-free (Ctrl) and LPS-contaminated Ti samples. Data are expressed as fold expression over the value of the Ctrl sample at 1 h; a) full y-axis scale; b) reduced y-axis scale

Fig. 3 Dependence of the expression of IL-1 and IL-6 genes by J774A-1 macrophages after 4 h culturing on the concentration of LPS in the solution used to prepare LPS-contaminated Ti samples. Data are expressed as fold expression over expression of the Ctrl sample

Fig. 4 Expression of IL-6 and IL-1 genes by J774A-1 macrophages after 4 culturing on solvent-cleaned Mach, Tiblasted and DAE implants and on the same implants after a complete endotoxin removal cycle (shown by +). Data are expressed as fold expression over the value of the Mach +; a) full y-axis scale; b) reduced y-axis scale

Fig. 5 Expression of IL-6 gene by J774A-1 macrophages after 4 culturing on 22 different commercially available dental implants. Data are expressed as fold expression over the value of an endotoxin-free control dental implant. The horizontal LPS1, LPS5 and LPS10 lines show the reference values of IL-6 fold expression obtained on purposely contaminated samples already shown in Fig. 3; a) full y-axis scale; b) reduced y-axis scale. Measurements performed on a single specimen for each sample, the error bar shows intra-experiment accuracy through the standard deviation obtained from triplicate aliquots of cDNA

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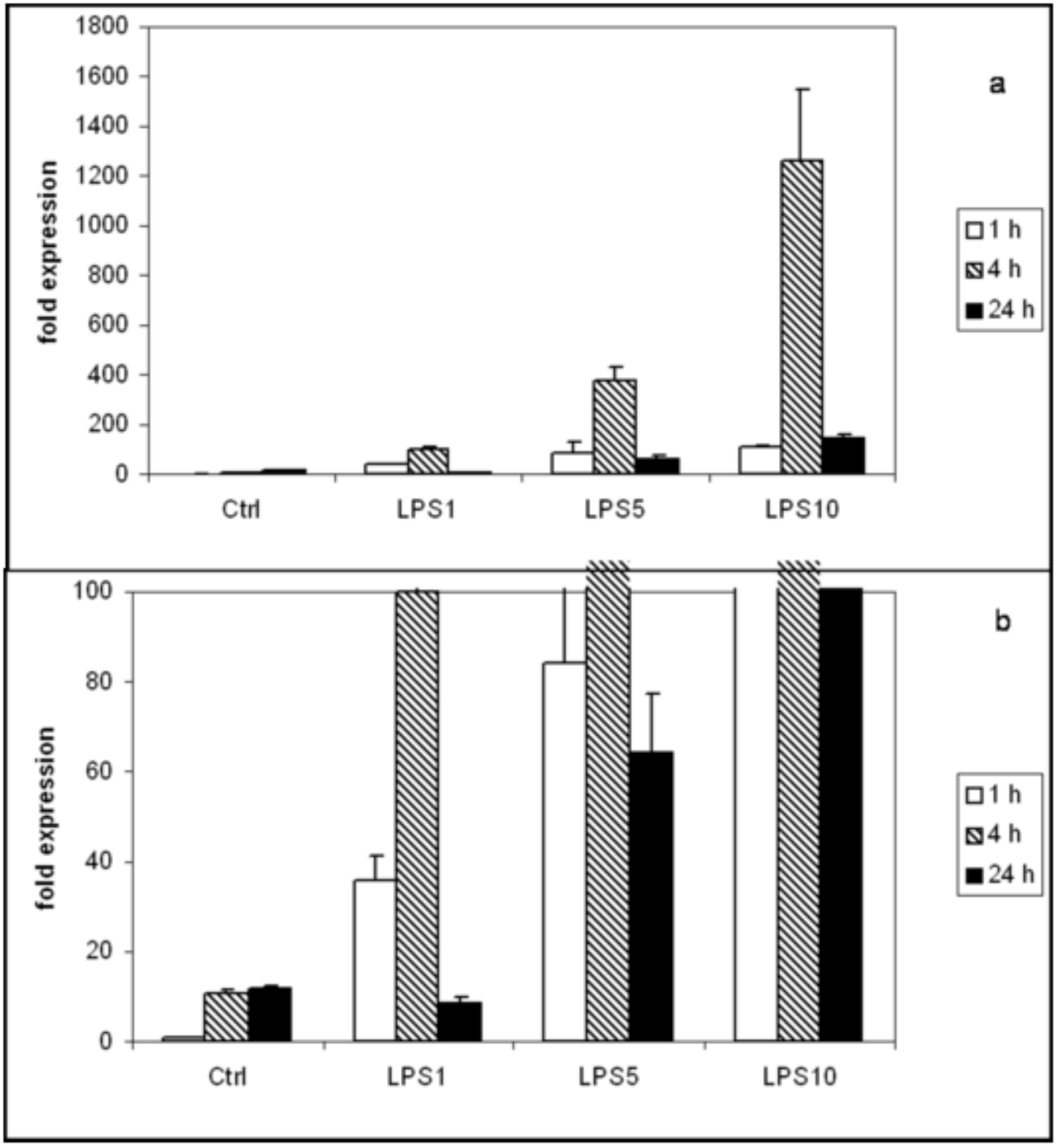

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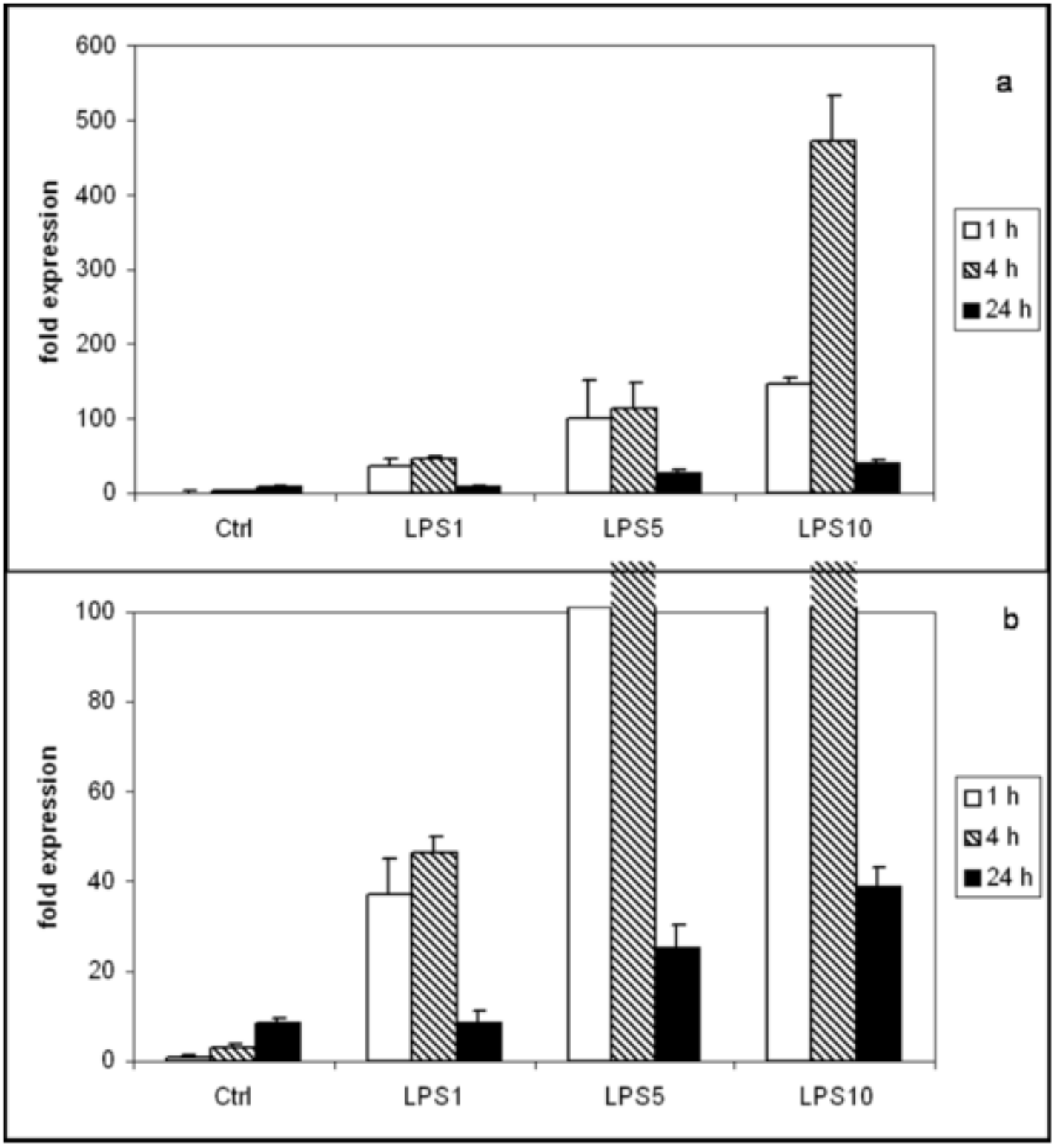

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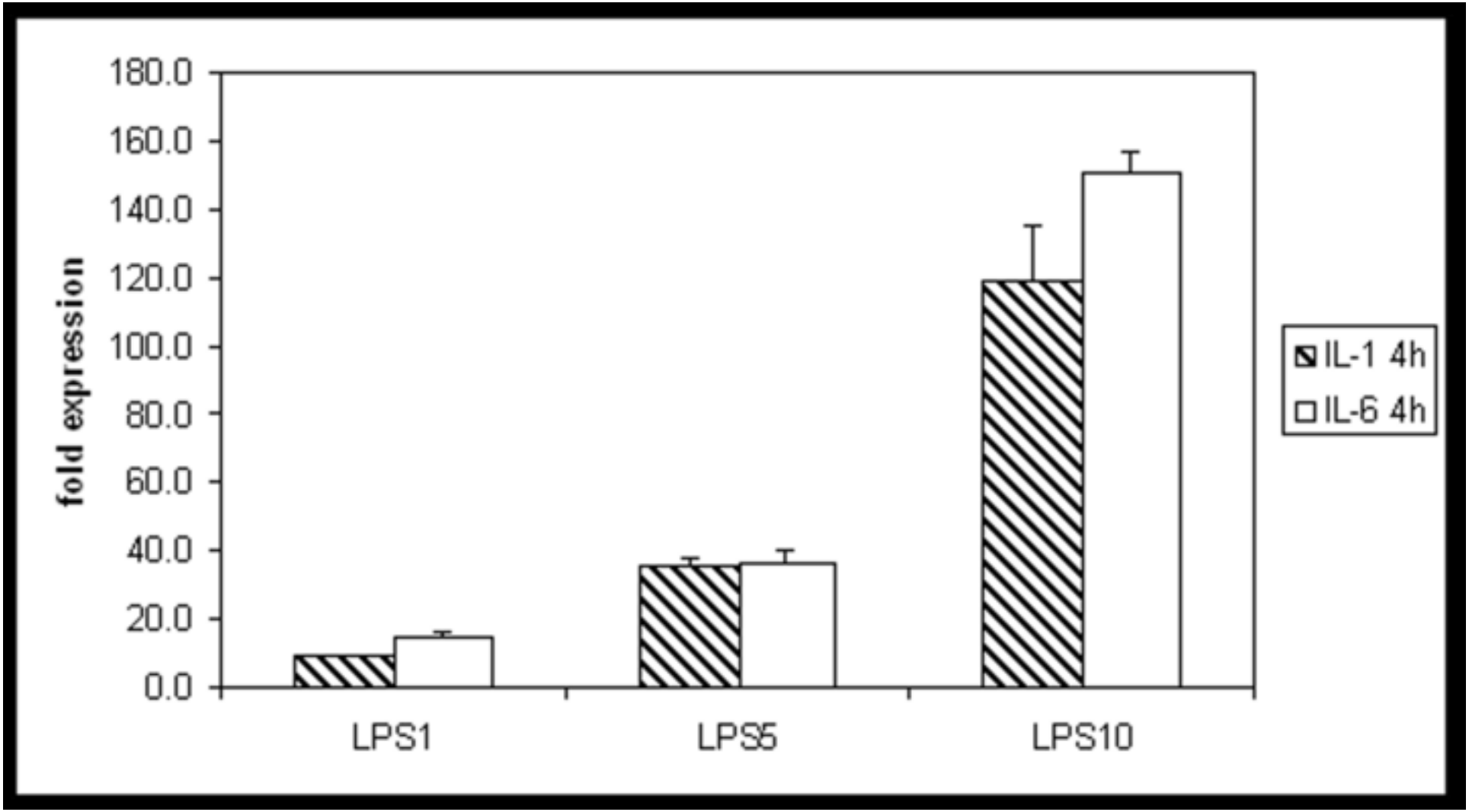

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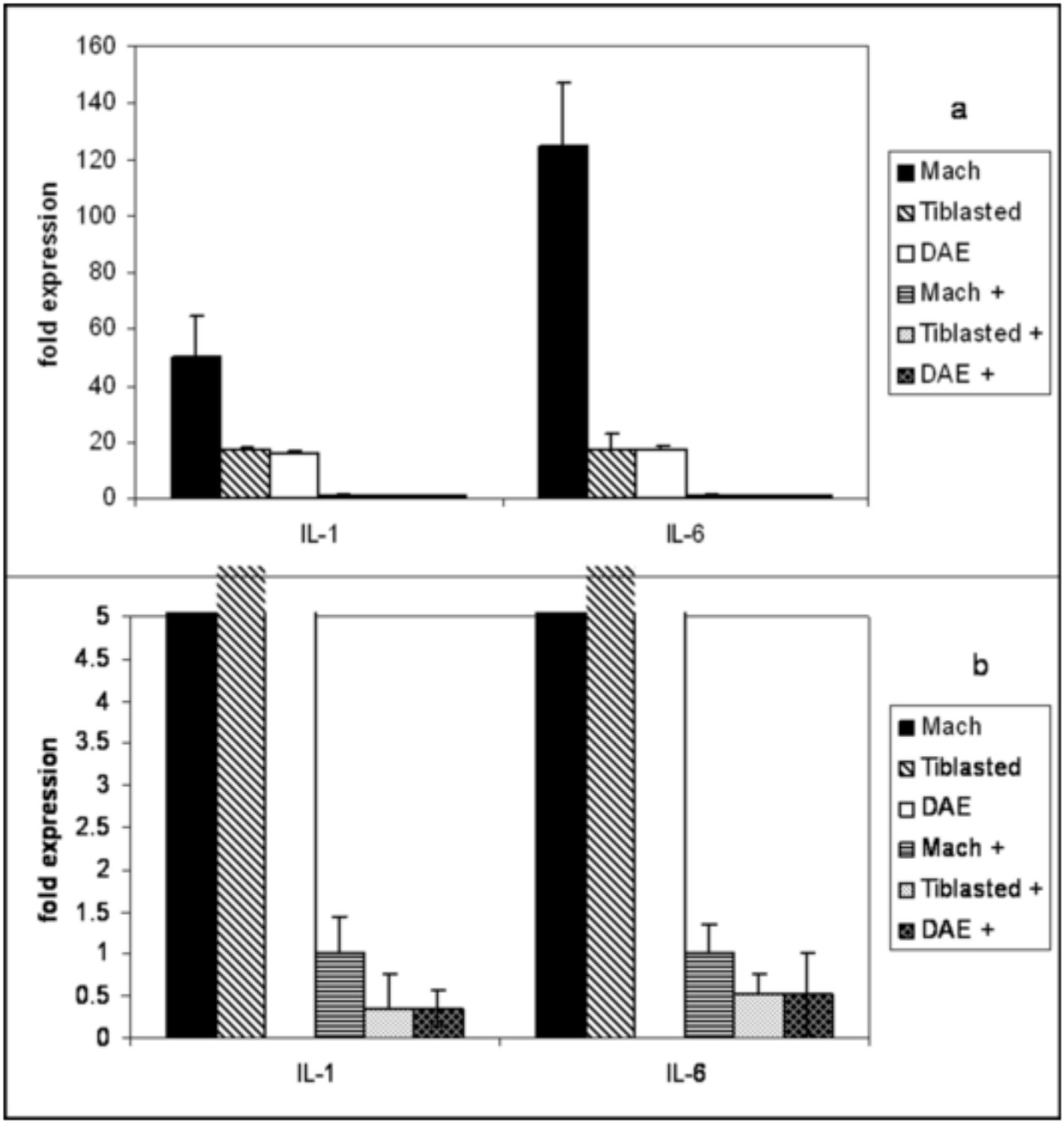

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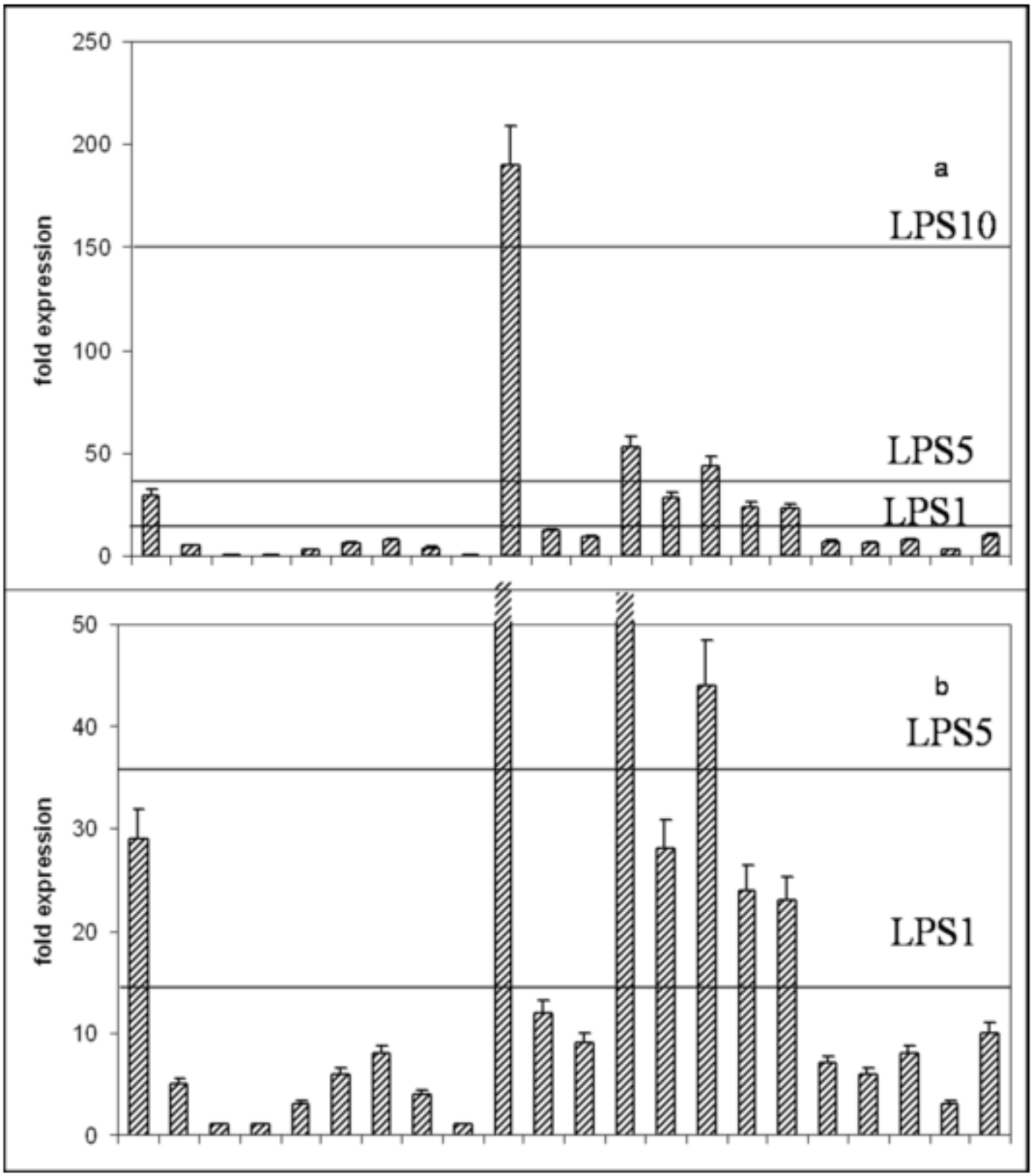

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