Effect of L-N^6-(1-Iminoethyl)-lysine, an inducible nitric oxide synthase inhibitor, on murine splenic immune response induced by *Actinobacillus actinomycetemcomitans* lipopolysaccharide

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Short Title: L-NIL enhances Th1-like immune response by murine spleen cells stimulated with *A. actinomycetemcomitans* LPS

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THE RESULTS OF THE THIRD YEAR EXPERIMENTS

Abstract

The aim of the present study was to determine the effect of treatment with L-N^6-(1-Iminoethyl)-lysine (L-NIL) on the induction of murine splenic immune response to *Actinobacillus actinomycetemcomitans* lipopolysaccharide (LPS) in vitro. BALB/c mice were sham-immunized and *A. actinomycetemcomitans* LPS immunized. Spleen cells were obtained, cultured and stimulated with *A. actinomycetemcomitans* LPS with or without the presence of L-NIL. Nitric oxide (NO) levels, inducible nitric oxide synthase (iNOS) activity, specific-IgG isotype levels, both IFN-γ and IL-4 levels and cell proliferation in the cell cultures were assessed. The results showed that L-NIL suppresses both NO production and iNOS activity but enhances specific-IgG2a levels, IFN-γ levels and cell proliferation by *A. actinomycetemcomitans* LPS-stimulated cells. Therefore, the results of the present study suggest that reduction of iNOS activity by L-NIL may skew the *A. actinomycetemcomitans* LPS-stimulated murine splenic immune response to a Th-1-like immunity in vitro.

Introduction

Nitric oxide synthase (NOS) catalizes L-arginine to produce nitric oxide (NO), a gaseous molecule, which plays a crucial role in the vascular, neural, endocrinal and immune system. Three NOS isoforms, i.e., the neural form (nNOS), endothelial form (eNOS) and inducible form (iNOS), are known (1). Whilst nNOS and eNOS activity are a Ca^{++}-dependent mechanism and constitutive, iNOS activity is a Ca^{++}-independent mechanism and induced by the pro-inflammatory stimuli to produce large amount of NO (1,2). Inhibition of iNOS activity by L-N^6-(1-Iminoethyl)-lysine (L-NIL) has been shown to deplete NO production and exacerbate inflammatory diseases such as leishmaniasis (3). but reduce renal disease in mice (4).

The immunopathogenesis of periodontal disease may be regulated by NO as seen by the fact that increased number of $iNOS^+$cells could be observed in inflamed gingival tissue (5-9). In the animal models, failure of iNOS-deficient and mercaptoethylguanidine-treated mice to develop *Porphyromonas gingivalis*- and ligature-induced alveolar bone loss, respectively, suggest that iNOS activity may be associated with the destructive periodontal disease (10,11). Previously, we and others have shown that *Actinobacillus actinomycetemcomitans* lipopolysaccharide (LPS) stimulates murine macrophage cell lines and spleen cells to produce NO (12-14). Since the iNOS activity is known to regulate the immune response (1,2), the aim of the present study was, therefore, to determine the effect of L-NIL, a selective iNOS inhibitor, on the murine splenic immune response induced by *A. actinomycetemcomitans* LPS.

Materials and Methods

Preparation of *A. actinomycetemcomitans* lipopolysaccharide

A. actinomycetemcomitans Y4 (serotype b) was grown in Todd-Hewitt broth supplemented with 1% (w/v) yeast extract (Difco Laboratories, Detroit, MI) in an atmosphere of 37°C, 10% O_2, 10% CO_2 and 80% N_2. LPS from this periodontopathic bacterium was isolated using a hot phenol-water, treated with nuclease (Sigma, St. Louis, MO), and then lyophilized as previously described (14).

Cell cultures

Female BALB/c mice (6 to 8 weeks old) were divided into 2 groups, each consisting of 5 mice. Mice were injected intraperitoneally with 100 μL of phosphate buffered saline (PBS) alone (sham-immunized mice) or with 100 μL of PBS containing 100μg of *A. actinomycetemcomitans* LPS (immunized mice) weekly for two weeks. One week after the last immunization, all animals were sacrificed by CO_2 asphyxiation and spleens were removed aseptically. Single cell suspension was prepared by teasing through sterile stainless steel grids and suspended in RPMI-1640 medium (Sigma) containing 10% fetal calf serum (Sigma) and 1% penicillin-streptomycin (Sigma). L-NIL (Sigma) was diluted in PBS and filtered sterile. One million cells in 1 ml of culture medium were incubated with different concentration of L-NIL for 1 hour in room temperature. After washing, the cells were

resuspended in 1 ml of the culture medium and cultured in 24-well plates (Nunc, Roskilde, Denmark), stimulated with 10 μg of *A. actinomycetemcomitans* LPS and incubated for 4 days in an incubator in 5% CO_2 at 37°C (13). All cultures were in triplicates. The experimental procedures were approved by the Ethical Committee of Universiti Sains Malaysia, Malaysia and Gadjah Mada University, Yogyakarta, Indonesia.

Nitric oxide assay.

The levels of nitric oxide were determined from the culture supernatant by the Griess reagent as previously described (14). Briefly, 100 μl of samples were mixed with equal volume of the Griess reagent (1% sulfanilamide, 0.1% naphthlethylenediamine dihydrochloride in 2.5 %phosphoric acid) and read in an automated reader μQuant spectrophotometer (Biotek-Instrument Inc., Vermont, USA) at 540 nm. A standard curve was prepared with sodium nitrite. All materials were purchased from Sigma.

iNOS activity assay

The culture supernatants were discarded carefully. Subsequently, the cells were washed three times in RPMI-1640 medium lysed by incubating with 100 μl of 0.1% Triton X-100 and shaken for 30 min. The protein concentration of the cell lysates was measured using Bradford protein assay (Bio-Rad, Hercules, CA). The iNOS activity was assessed as previously described (15). Briefly, 50 μg of cell lysate were incubated for 120 min at 37°C in 100 μl of

20 mM Tris-HCl (pH 7.9) containing 4 μM BH_4, 4 μM FAD, 3 mM dithiothreitol, 2 mM NADPH and 2 mM L-arginine. The reaction was then stopped by adding lactate dehydrogenase (20 U/ml) and the levels of nitrite were measured by the Griess reagent as described above. Otherwise stated, all materials were purchased from Sigma.

ELISA

The levels of specific IgG isotype antibody were assessed by ELISA as previously described (16). Otherwise stated, all materials were purchased from Sigma. Briefly, 96-well plates (Nunc, Roskilde, Denmark) were coated with 50 μl of PBS containing 0.2 μg/ml of *A. actinomycetemcomitans* LPS and incubated overnight at 4°C. Non-specific binding sites were blocked by 1% dry milk powder in PBS-0.05% Tween. After washing, 100 μl of diluted samples were added and incubated for 1 hour at room temperature. After further washing, 100 μl of biotin-conjugated goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 antibodies diluted in 1:5000 were added, incubated for 1 hour and washed. One hundred microliters of horseradish peroxidase diluted in 1:10.000 were added and incubated for 30 minutes. Following washing, color was developed by adding TMB substrate and incubated for 10 minutes. The color reaction was stopped by adding HCL and read at an absorbance of 450 nm on an automatic reader (Biotek-Instrument Inc.). PBS only and serum from *A. actinomycetemcomitans* LPS -hyperimmunized mice were used as the

negative and positive control, respectively, on every plate. The results were expressed as absorbance unit

Cytokine assay

The levels of IFN-γ and IL-4 were assessed from the culture supernatants by ELISA kits (R&D Systems, Minneapolis, USA) as described by the manufacturer.

Proliferation assay

The cell proliferation was assessed using an MTT assay as previous described (17). Briefly, 50 μl of MTT stock solution were added into all wells. The plates were incubated for 4 hours and then centrifuged. After removing the culture supernatants, 150 μl of solution containing a mixture of equal volume of DMSO and ethanol were added. The plates were shaken for 20 minutes and read in an automated reader (Biotek-Instrument Inc.) at 590 nm. The results were expressed as absorbance unit. All materials were purchased from Sigma.

Statistical analysis

All data were analyzed by One way-analysis of variance followed by the Fischer's least square differences using a statistical package (SPSS Inc, Chicago).

Results

NO levels and iNOS activity

Stimulation with *A. actinomycetemcomitans* LPS resulted in the production of NO by both sham-immunized and immunized spleen cells ($P<0.05$) (Fig. 1). The levels of NO in the cultures of immunized spleen cells were higher than those in the cultures of unimmunized cells ($P<0.05$). Interestingly, L-NIL at 10 μM and 100 μM did inhibit the production by both sham-immunized and immunized cells stimulated with *A. actinomycetemcomitans* LPS ($P<0.05$).

When both sham-immmunized and immunized spleen cells were stimulated with *A. actinomycetemcomitans* LPS, the iNOS activity was elevated as compared with the cells without stimulation ($P<0.05$) (Fig. 2). However, the activity of this enzyme was significantly suppressed by the cells stimulated with *A. actinomycetemcomitans* LPS in the presence of L-NIL in a dose dependent mechanism ($P<0.05$).

The levels of specific IgG isotypes

Detectable anti-*A. actinomycetemcomitans* LPS IgG1, IgG2a, IgG2b and IgG3 isotype in the immunized but not sham-immunized spleen cells were spontaneously produced without stimulation with *A. actinomycetemcomitans* LPS ($P<0.05$) (Table 1). However, following stimulation with *A. actinomycetemcomitans* LPS, the levels of all IgG isotypes in the cultures of the immunized cells were significantly higher than those in the cultures of sham-immunized mice ($P<0.05$). Of interest, L-NIL did augment the

production of specific IgG2a, but not IgG1, IgG2b and IgG3, isotype by both sham-immunized and immunized cells stimulated with *A. actinomycetemcomitans* LPS ($P<0.05$). Increased specific IgG2a isotype by *A. actinomycetemcomitans* LPS-stimulated cells was much apparent when 100 μM of L-NIL were added ($P<0.05$).

The levels of cyokines

A. actinomycetemcomitans LPS stimulated the production of IFN-γ and IL-4 by spleen cells, but the levels of these cytokine produced by the immunized cells were higher than those by the sham-immunized cells ($P<0.05$) (Fig. 3). The presence of L-NIL at 10 μM and 100 μM in the cell cultures stimulated with *A. actinomycetemcomitans* LPS enhanced the production of IFN-γ, but not IL-4 ($P<0.05$).

Cell Proliferation

A. actinomycetemcomitans LPS induced the proliferation of both sham-immunized and immunized cells, but the proliferation of the former cells were higher than those by the latter cells ($P<0.05$) (Fig. 4). Of interest, L-NIL particularly at 100 μM did enhance the proliferation of both sham-immunized and immunized cells stimulated with *A. actinomycetemcomitans* LPS as compared with that of the cells stimulated with this antigen only ($P<0.05$).

Discussion

The present study was carried out to assess the effect of L-NIL, a selective iNOS inhibitor on the immune response induced by *A. actinomycetemcomitans* LPS. L-NIL is an L-arginine analogue which binds onto iNOS 30-fold higher than eNOS and blocks NO production from iNOS activity (18). Therefore, the results of the present study showing that L-NIL did inhibit the production of NO and the activity of iNOS activity by spleen cells from *A. actinomycetemcomitans* LPS-immunized or sham–immunized mice were not unexpected. These results are comparable with a previous report showing that the production of NO by murine spleen cells treated with *Staphylococcus aureus* and aminoguanidine, an iNOS inhibitor, was inhibited (19). Furthermore, Stenger and colleagues demonstrated that L-NIL suppressed the production of NO and iNOS activity by *Escherichia coli* LPS-stimulated murine macrophages (15). Indeed, our previous results showed that splenic macrophages were the main source of the production of NO by *A. actinomycetemcomitans* LPS-stimulated murine spleen cells (13). Therefore, one may speculate that L-NIL may bind to splenic macrophage-derived iNOS which may in turn inhibit the production of NO and the activity of this enzyme by *A. actinomycetemcomitans* LPS-stimulated murine spleen cells as seen in the present study.

The results of the present study showed that following stimulation with *A. actinomycetemcomitans* LPS, L-NIL enhanced preferentially the production of specific IgG2a, but not IgG1, IgG2b and IgG3, antibodies by

spleen cells from both sham-immunized and *A. actinomycetemcomitans* LPS-immunized mice. These results suggest that reduced iNOS activity may up-regulate the production of specific IgG2a antibody by activated spleen cells. Increased serum IgG2a response in autoimmune interstitial nephritis rats pretreated with L-NIL has also been reported (20). However, the exact mechanism by which splenic *A. actinomycetemcomitans* LPS –specific IgG2a antibodies were increased by L-NIL as seen in the present study is unknown. IFN-γ appears to selectively stimulate IgG2a production (21), while there is no evidence to suggest that NO may directly or indirectly involves in immunoglobulin class switching. Indeed, the present study also indicated that L-NIL augments the production of IFN-γ, but not IL-4, by spleen cells stimulated with *A. actinomycetemcomitans* LPS. Increased IFN-γ, but not IL-4, production by murine spleen cells derived from mice lacking iNOS and stimulated with mitogen and antigens in experimental autoimmune encephalomyelitis (EAE) supports the results of the present study (22). Therefore, it suggests that L-NIL-reduced iNOS activity in the spleen cells activated by *A. actinomycetemcomitans* LPS may lead to increase the production of IFN-γ which in turn may stimulate preferentially specific IgG2a antibody production. This notion is further supported by a recent report demonstrating that IFN-γ is prerequisite for the production of IgG2 antibodies by human mononuclear cells stimulated with *A. actinomycetemcomitans* (23). Furthermore, increased production of IFN-γ and specific IgG2a antibody by *A. actinomycetemcomitans* LPS-activated murine spleen cells seen in the

present study seem to indicate that treatment with this iNOS inhibitor may skew the immune response toward a Th1-like response, a similar phenomenon observed by a previous report (20, 22).

The present study also showed that L-NIL augments the proliferation of murine spleen cells after stimulation with *A. actinomycetemcomitans* LPS, suggesting that reduction of iNOS activity may promote *A. actinomycetemcomitans* LPS-activated spleen cell proliferation. A previous study showing that in experimental autoimmune encephalomyelitis, the ex vivo proliferation of spleen cells derived from mice lacking iNOS were significantly higher than that from wildtype mice (22) highlights the results of the present study. It seems plausible that increased cell proliferation by *A. actinomycetemcomitans* LPS-activated murine spleen cells in the presence of L-NIL may represent a Th1 cell response in vitro as also previously suggested (22).

The extrapolation of the results of the present study in the immunopathogenesis of periodontal diseases needs to be further investigated. Increased $iNOS^+$ cells in the inflamed gingival tissues in humans (5-9) may indicate that increased activity of this enzyme may ameliorate the course of periodontal disease. Furthermore, our previous studies indicated that increased Th1-response may be protective in periodontopathic-induced lesion in mice (24-25). One may speculate, therefore, that reduction of iNOS activity by its specific inhibitor may enhance Th1-like immune response in the gingival tissue and hence,

decreased the severity of periodontal disease. Reduced expression of iNOS in the gingival tissues in periodontal diseased patients following periodontal treatments (26) may support this notion.

In conclusion, the results of the present study showed that L-NIL suppresses NO production and iNOS activity but enhance the production of specific-IgG2a antibody and IFN-γ and cell proliferation by *A. actinomycetemcomitans* LPS-activated murine spleen cells. These results suggest, therefore, that reduction of iNOS activity and NO production by L-NIL may stimulated preferentially a Th1-like immune response by *A. actinomycetemcomitans* LPS-activated murine spleen cells.

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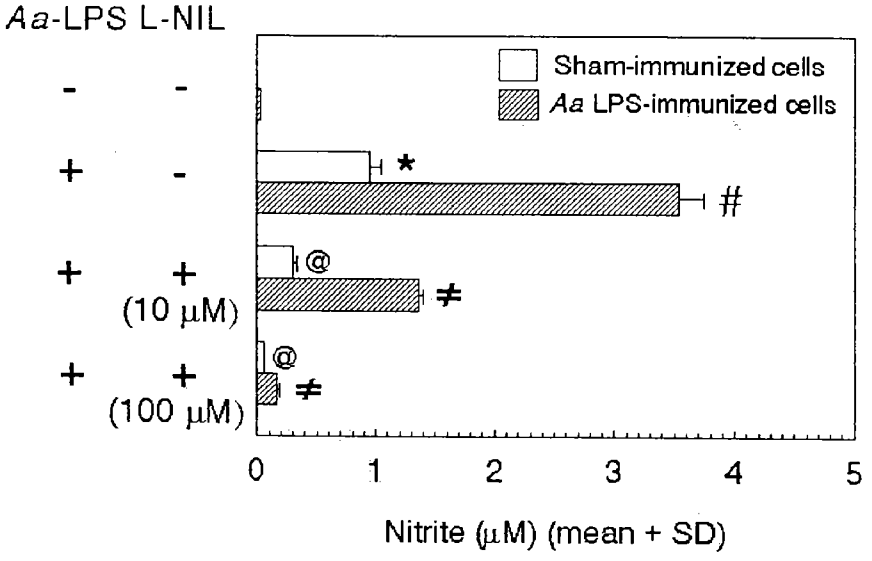
Legend of figures

Fig. 1. The effect of L-NIL on the production of NO by spleen cells-stimulated with *A. actinomycetemcomitans* LPS. Spleen cells were obtained from sham-immunized and *A. actinomycetemcomitans* LPS-immunized mice, cultured and stimulated with 10 μg of *A. actinomycetemcomitans* LPS (*Aa* LPS) with without the presence of L-NIL. (*) and (#) = significant difference to the unstimulated cells at $P<0.05$. (@) = significant difference to the *A. actinomycetemcomitans* LPS-stimulated cells obtained from sham-immunized mice at $P<0.05$. (≠) = significant difference to the *A. actinomycetemcomitans* LPS-stimulated cells obtained from *A. actinomycetemcomitans* LPS (*Aa* LPS)-immunized mice at $P<0.05$.

Fig. 2. The effect of L-NIL on the iNOS activity by spleen cells-stimulated with *A. actinomycetemcomitans* LPS. Notes are similar to Fig. 1.

Fig. 3. The effect of L-NIL on the production of IFN-γ and IL-4 by spleen cells-stimulated with *A. actinomycetemcomitans* LPS. Notes are similar to Fig. 1.

Fig. 4. The effect of L-NIL on the proliferation of spleen cells stimulated with *A. actinomycetemcomitans* LPS. Notes are similar to Fig. 1.


Aa-LPS L-NIL
- -
+ -
+ +
(10 μM)
+ +
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Sham-immunized cells
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1
2
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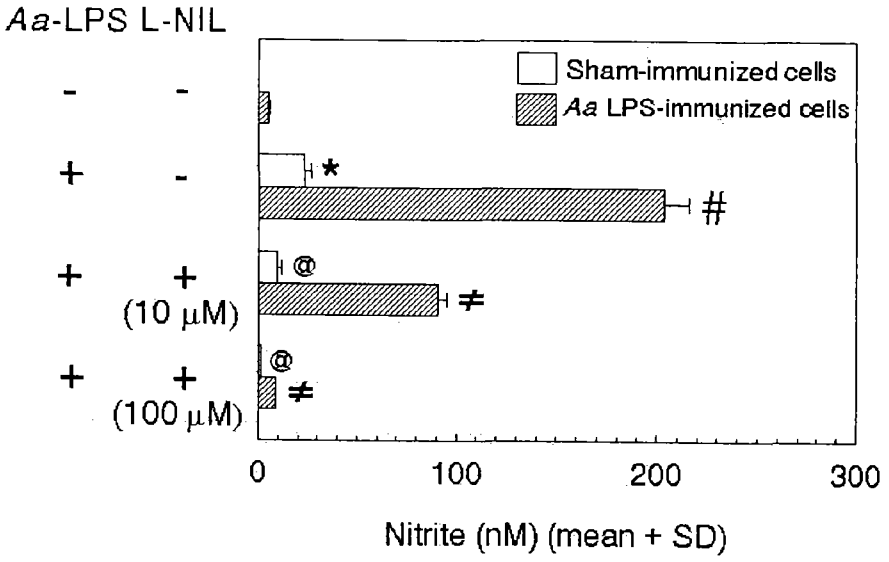
4
5
Nitrite (μM) (mean + SD)

Fig. 1.


Aa-LPS L-NIL
- -
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(10 μM)
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Sham-immunized cells
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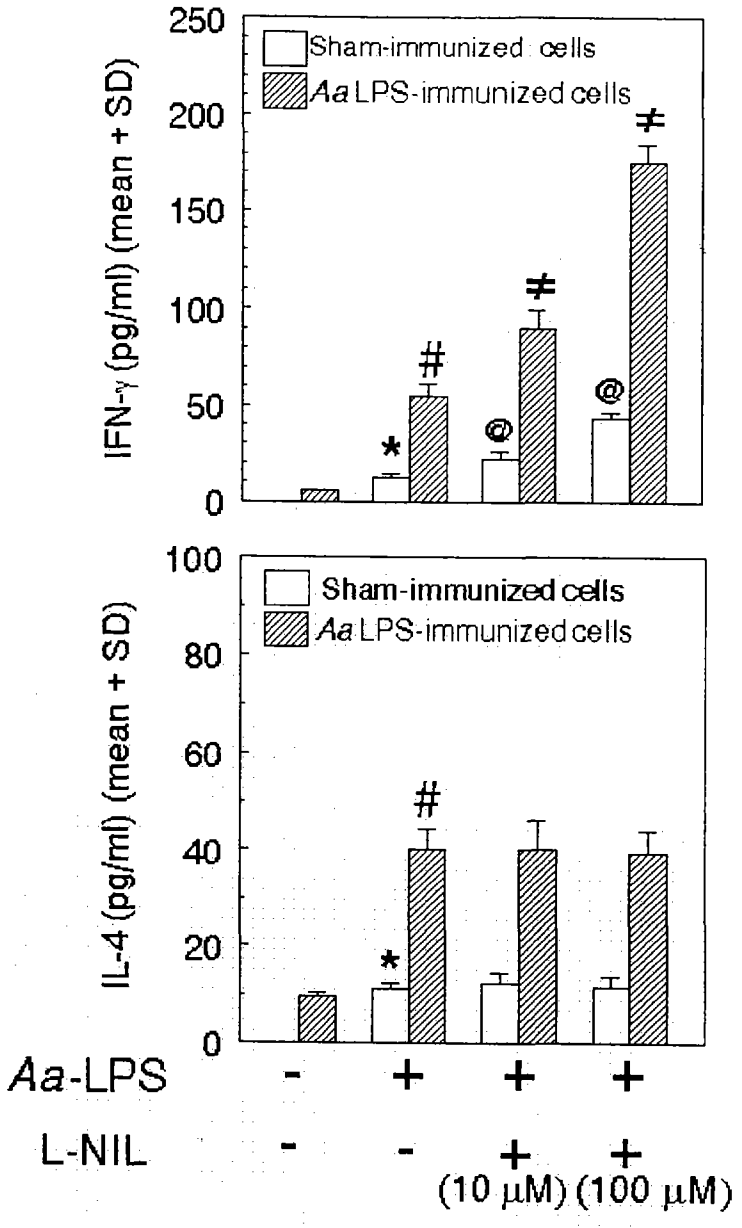
0
100
200
300
Nitrite (nM) (mean + SD)

Fig. 2


IFN-γ (pg/ml) (mean + SD)
250
200
150
100
50
0
Sham-immunized cells
Aa LPS-immunized cells
*
#
@
≠
IL-4 (pg/ml) (mean + SD)
100
80
60
40
20
0
Sham-immunized cells
Aa LPS-immunized cells
Aa-LPS
L-NIL
- + + +
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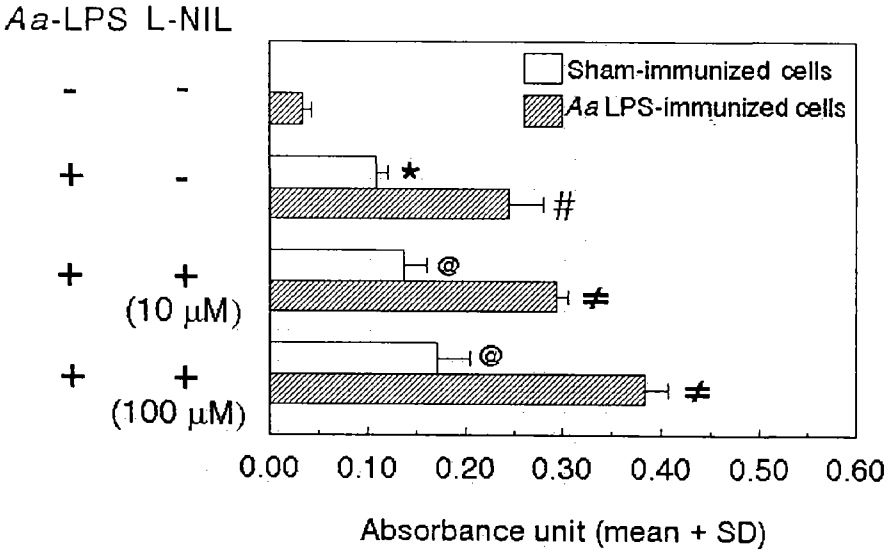
(10 μM) (100 μM)

Fig. 3


Aa-LPS L-NIL
- -
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Sham-immunized cells
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0.00 0.10 0.20 0.30 0.40 0.50 0.60
Absorbance unit (mean + SD)

Fig. 4.

Table 1. The effect of L-NIL on the production of the specific IgG isotypes produced by murine spleen cells stimulated with *A. actinomycetemcomitans*-LPS

Cells	Treatments		Specific IgG isotypes (absorbance unit) Mean (SD)			
	Aa-LPS	L-NIL	IgG1	IgG2a	IgG2b	IgG3
Sham-immunized	-	-	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)
	+	-	0.099 (0.024) (*)	0.091 (0.018) (*)	0.102 (0.027) (*)	0.105 (0.026) (*)
	+	+ (10 μM)	0.102 (0.027)	0.128 (0.023) (#)	0.102 (0.019)	0.106 (0.021)
	+	+ (100 μM)	0.103 (0.037)	0.156 (0.011) (#)	0.099 (0.034)	0.106 (0.030)
Aa-LPS-immunized	-	-	0.081 (0.004)	0.087 (0.009)	0.090 (0.017)	0.088 (0.014)
	+	-	0.216 (0.021) (*)	0.189 (0.038) (*)	0.179 (0.025) (*)	0.201 (0.027) (*)
	+	+ (10 μM)	0.213 (0.031)	0.268 (0.030) (#)	0.181 (0.028)	0.202 (0.024)
	+	+ (100 μM)	0.213 (0.046)	0.317 (0.032) (#)	0.181 (0.038)	0.204 (0.033)

Note: Spleen cells derived from sham-immunized or *A. actinomycetemcomitans*-LPS (*Aa* LPS)-immunized mice were stimulated with or without *A. actinomycetemcomitans*-LPS (10 μg) and with or without the presence of L-NIL . *Aa*-LPS = *A. actinomycetemcomitans*-LPS; (*) = significant difference to the

unstimulated cells at $P < 0.05$; (#) = significant different to the *A. actinomycetemcomitans*-LPS-stimulated cells at $P < 0.05$.