

Analysis of Tlr4-Mediated LPS Signal Transduction in Macrophages by Mutational Modification of the Receptor

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ABSTRACT: In mouse macrophages (RAW 264.7 cells), toll-like receptor 4 (Tlr4) is a limiting factor in lipopolysaccharide (LPS) signal transduction. The expression of only $1-2 \times 10^4$ copies of recombinant Tlr4 per cell enhances sensitivity to LPS, shifting the EC50 by 30-fold to the left. Expression of the Tlr4^{Lps-d} isoform of Tlr4 (found in C3H/HeJ mice) shifts the EC50 2600-fold to the right, essentially abolishing LPS responses. A truncated form of Tlr4, lacking a cytoplasmic domain, exerts only a weak inhibitory effect on signal transduction. Similarly, the normal or Tlr4^{Lps-d} forms of protein lacking a cytoplasmic domain, cause modest inhibition of LPS signaling. Manipulations of Tlr4 structure and expression cause changes in LPS sensitivity that range over 3 to 4 orders of magnitude. These findings support the view that Tlr4 is an integral component of a solitary pathway for LPS signal transduction in macrophages and permit inferences related to the mechanism of signaling and its blockade.

Keywords: toll-like receptor 4, macrophage, endotoxin, receptor, mutagenesis

INTRODUCTION

Homozygosity for mutations of the Toll-like receptor 4 gene (*Tlr4*) can completely prevent LPS signal transduction in mice. Hence, we have proposed that Tlr4 is the signaling component of an unduplicated pathway for endotoxin responses (1). To the contrary, transfection-based analyses, carried out in non-macrophage cell lines and relying upon activation of NF- κ B-driven reporter genes as an endpoint, have suggested that Tlr4 does not transduce the LPS signal (2,3). For many reasons, functional data developed from studies of a mutation *in vivo* are usually more robust than data developed through cell transfection *in vitro*. Nonetheless, if Tlr4 does convey the LPS signal, it should be possible to demonstrate this by transfection, and to do so in cells that are known to be capable of LPS responses.

It has long been known that the lethal effect of lipopolysaccharide (LPS) is conferred by cells of hematopoietic origin (4), and in particular, by macrophages (5). Lethality results from sudden, excessive production of pro-inflammatory cytokines (notably tumor necrosis factor; TNF (6,7)). Most somatic cells are incapable of responding to LPS in

this manner; hence, direct measurement of LPS signal transduction is best carried out in macrophages rather than other cell types, and TNF production is a preferred endpoint in such analyses, because of its proven relevance to the LPS response (6), and the existence of multiple, well-established LPS-dependent signaling events leading to the induction of TNF protein synthesis (8,9). Moreover, a certain advantage results from the study of an endogenous gene lying in its normal context rather than a surrogate reporter (e.g., an NF- κ B responsive luciferase construct), which may, by virtue of truncation and positional effects, respond to many signals irrelevant to the one under study, or be minimally reflective of the process that one has in mind. A rigorous approach to determining LPS sensitivity also requires the measurement of the EC50 for LPS, rather than measurement of the magnitude of response at individual concentrations of LPS. Finally, insofar as the expression of unphysiologically large numbers of any receptor may yield signal transduction that is not biologically significant, determination of number of copies of recombinant Tlr4 at the cell surface must be made in order to permit conclusions concerning the function of the protein.

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The mammalian LPS sensor is formed by a complex array of proteins, some of which may as yet be unknown. Complexes of LPS and LBP are initially engaged at the cell surface by CD14, a glycosphosphatidylinositol-linked protein with no cytoplasmic domain (10). The biological relevance of CD14 in LPS signaling--originally deduced from binding and transfection studies (10,11)--is supported by gene knockout data, which that revealed that CD14 expression is required for normal sensitivity to LPS (12). However, it was assumed that a second, membrane-spanning protein must ultimately transduce the LPS signal, since CD14 lacks a cytoplasmic domain.

The protein that likely fulfills this role was identified through studies of mice, wherein mutations of a single gene (*Lps*) have long been known to abolish LPS signal transduction (13,14). The *Lps^d* allele, represented in C3H/HeJ mice, is codominant in the sense that *Lps^d/Lpsⁿ* heterozygotes exhibit intermediate sensitivity to LPS (15), and their macrophages display intermediate levels of TNF production in response to LPS. The innominate non-responder allele represented in C57BL/10ScCr mice is recessive to the wild-type allele, in that heterozygotes display normal responses to LPS (16). Using positional methods, we determined that *Lps* encodes the toll-like receptor 4 (Tlr4), a single-spanning transmembrane protein with a leucine-rich ectodomain and a "Toll-like" cytoplasmic domain (1). The *Lps^d* allele bears a missense mutation (2342 C→A; P712H) which lies within the cytoplasmic domain of the polypeptide chain. The mutation in C57BL/10ScCr mice is a null allele. Relying on these data (17), other workers confirmed the presence of the mutations (18). Moreover, Hoshino and colleagues demonstrated that a *Tlr4* knockout produces an excellent phenocopy of the naturally occurring *Tlr4* mutations (19).

While it has been shown that overexpression of Tlr4 can enhance the activation of NF-κB that occurs in CD14-transfected CHO cells following LPS stimulation (20), it is also true that transfection of CHO cells with Tlr2 confers LPS sensitivity;

hence, it is not clear that these data are in any way relevant to the LPS response of macrophages. We therefore considered that it would be more informative to study the effect of Tlr4 expression in an authentic macrophage line, utilizing a more specific endpoint than NF-κB translocation, and also making use of various mutant forms of Tlr4.

MATERIALS AND METHODS

Constructs

The Tlr4^{Lps-n} (C3H/HeN) and Tlr4^{Lps-d} (C3H/HeJ) isoforms of the mouse Tlr4 cDNA were cloned in this laboratory as previously described. The primers (5'→3')

CATCGATACCAGGAAGCTTGAATCCCT

and

ATAGGTACCTCAGGTCCAAGTTGCCGTTTC

were used to generate the full-length amplified products, which were cloned into the vector pFLAG-CMV-1 (Sigma) using ClaI and KpnI sites. The native signal peptide was thus removed, and an alternative signal peptide, followed by the flag sequence, was provided by the vector. The ectodomain construct was produced using the downstream primer (5'→3')

CAG GGT ACC TCA CAG GTG AAA ATA GAA
GTG GTA T,

whereas the two cytoplasmic domain constructs were produced using the upstream primer (5'→3'):

GCC GAA TTC AAT GTA CAA GAC AAT CAT
CAG T.

The latter two constructs were cloned into pFLAG-CMV-1 using EcoRI and KpnI sites. All constructs were verified by DNA sequencing on both strands. All expression constructs were shown to yield products of anticipated size in COS cells,

after Western blot detection with M2 monoclonal antibody (not shown). The extent of each protein predicted by the sequence is listed in Table 1.

Measurement of Expression

Expression of each construct at the protein level was ascertained by saturation binding analysis, using monoclonal antibody M2. M2 antibody was labeled to a specific activity of 3.0×10^6 cpm/ μ g using 125 I, by means of the iodogen technique (21). Labeled antibody (concentration range 50 ng/ml to 20 μ g/ml) was added in a volume of 1.0 ml to monolayers of 2.0×10^6 cells in Hank's balanced salt solution, supplemented with 10% fetal bovine serum and buffered with 50 mM HEPES, pH 7.4. The cells were maintained at 0 C for a period of 4 hours. Monolayers were then rapidly washed through successive tanks of normal saline, buffered with 20 mM tris, pH 7.4, and supplemented with 2 mM CaCl_2 and 2 mM MgCl_2 . The cells were then dissolved with 1 ml of 1 M NaOH, and the wells rinsed with an additional 1 ml of 1% SDS. Both solutions were counted in a gamma counter.

Cells

RAW 264.7 cells and L-929 cells were both obtained from the ATCC. They were maintained in DMEM supplemented with 10% FBS, as well as penicillin and streptomycin.

Transfection

RAW 264.7 cells were transfected with each construct using Fugene transfection reagent (Boehringer Mannheim), and maintained under selection with G418 (1 mg/ml) throughout the

experiments. Individual colonies were isolated approximately ten days after transfection, and many clones (between 7 and 10 for each construct) were analyzed in order to remove all chance that deviations from the control level of response were attributable to clonality. Control RAW 264.7 cells were transfected with the empty pFLAG-CMV-1 vector.

Induction by LPS and Assay of Secreted TNF

TNF synthesis was induced in transfected RAW 264.7 cell cultures over a wide range of LPS concentrations, so as to permit determination of the LPS EC50 for each clone. Cells were plated in 24-well plates, and covered with 1.0 ml of medium. LPS was added at the stated concentration, and cells were allowed to incubate for 18 hours, at which time the medium was harvested, chilled, and diluted for determination of TNF concentration through bioassay.

Bioassay of TNF concentration was performed in L-929 cell cultures. The cytotoxicity assay was carried out by plating the cells in 96-well format at a density of 50,000 cells per well. After adherence had occurred, cycloheximide was added to a final concentration of 0.1 mg/ml, followed immediately by the addition of macrophage conditioned medium, suitably diluted for assay. A standard curve was also performed with all assays, to permit translation cytotoxicity into linear units of TNF concentration; in addition, maximal cytotoxicity was induced by the addition of SDS to a series of wells at a concentration of 1%.

Surviving cells were stained with crystal violet dissolved in 50% methanol after exposure to TNF for 16 hours, washed, drained, and solubilized with 50% acetic acid. Optical density at 595 nm was

Table 1

Construct	Residues expressed	Copies/cell	\pm SD	n
<i>Tlr4</i> ^{Lps-n}	22 - 835	2.1×10^4	4.1×10^3	10
<i>Tlr4</i> ^{Lps-d}	22 - 835 (P712H)	2.0×10^4	1.4×10^4	10
<i>Tlr4</i> ^{Lps-n} Cyt. Dom.	630 - 835	1.2×10^4	3.8×10^3	7
<i>Tlr4</i> ^{Lps-d} Cyt. Dom.	630 - 835 (P712H)	9.9×10^3	4.0×10^3	8
Ectodomain	22 - 660	1.4×10^4	5.1×10^3	9

measured using a plate reader, and the % cytotoxicity was calculated for each well using the formula

$$\% \text{ cytotoxicity} = \left\{ \frac{(\text{OD}_{595}[\text{control}] - \text{OD}_{595} [\text{unknown}])}{(\text{OD}_{595}[\text{control}] - \text{OD}_{595} [\text{SDS}])} \right\} \times 100,$$

where $\text{OD}_{595}[\text{control}]$ represented the optical density of a well to which no TNF had been added. The standard curve was plotted using the program Prism 3.0 (Graphpad Software, Inc.), assuming sigmoidicity of a curve relating % cytotoxicity to $\log[\text{TNF}]$ (in pg/ml). All unknown TNF concentrations were mathematically derived from the nonlinear regression curve. All measurements were performed in quadruplicate. The standard curve for each assay was such that standard deviation from the mean measurement at each TNF concentration was less than 5% of the measurement, and departures from the idealized curve were smaller still.

Computation of Results

The quantity of TNF produced in response to varying LPS concentrations was plotted using Prism 3.0 (Graphpad Software, Inc.), assuming a sigmoidal relationship between TNF produced and the $\log[\text{LPS}]$ (in pg/ml), with variable slope. Two methods of calculation of EC50 shift were used. First, the composite EC50 was calculated by pooling all data from a set of clones transfected with a given construct. This method permitted an "average" curve to be deduced, and allowed in the

immediate mathematical interpretation of variation EC50. A second, more conservative approach entailed the determination of individual EC50 values for each clone. These were then plotted on a logarithmic scale, and the significance of differences between responses conferred by different constructs was assessed using a t-test with Welch's correction for unequal variance.

Miscellaneous Reagents

Fetal bovine serum was obtained from GIBCO-BRL. *E. coli* LPS, strain 0127:B8, pFLAG-CMV-1 and monoclonal antibody M2 were obtained from Sigma. All restriction enzymes were obtained from Pharmacia; PfuTurbo polymerase was obtained from Stratagene. Iodo-Gen reagent was obtained from Pierce. Carrier-free Na^{125}I was obtained from Amersham.

RESULTS AND DISCUSSION

In order to directly examine the role of Tlr4 as a transducer of the LPS signal in macrophages, we expressed the normal mouse protein- and various mutant forms- in RAW 264.7 macrophages (Figure 1; Table 1). These cells of murine origin are highly responsive to LPS, and are known to express the Tlr4 mRNA (1). Unlike cell lines of non-myeloid origin (i.e., 293 cells or CHO cells), they express all proteins required for the elicitation of a biologically relevant response to LPS (e.g., TNF production). As such, they may be used to determine whether Tlr4 is a limiting factor in the

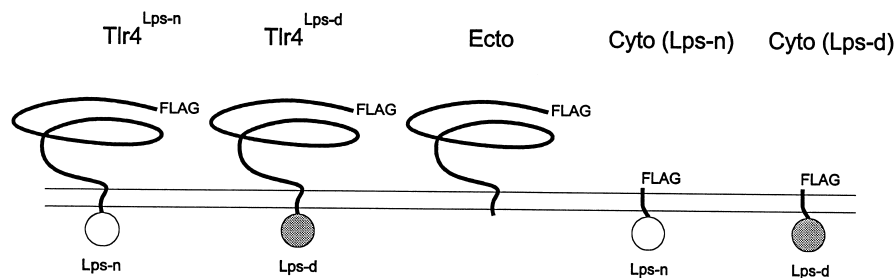


Figure 1. Schematic illustration of recombinant proteins expressed in RAW 264.7 cells. Constructs were made by PCR, using cDNA derived from C3H/HeJ and C3H/HeN mice.

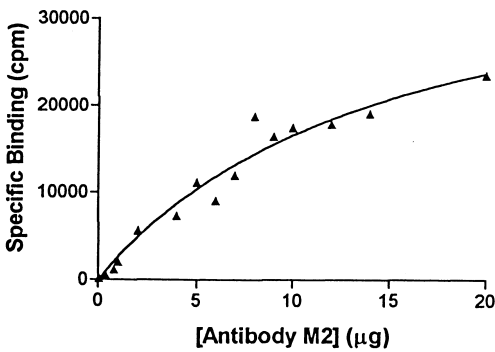


Figure 2a. Saturation isotherm of monoclonal antibody M2 binding to a single clone of RAW 264.7 cells transfected with an expression vector encoding $Tlr4^{Lps-d}$. 56.4% saturation of the surface receptor was achieved at equilibrium using the highest concentration of antibody. Nonlinear regression analysis, based on the assumption of hyperbolic binding kinetics, suggested a K_d of $1.0 \times 10^{-7} \pm 3.0 \times 10^{-8}$ M and the presence of $2.81 \times 10^4 \pm 4.99 \times 10^3$ binding sites per cell. R^2 for the analysis was 0.9576. Controls, performed for all plates, included the addition of the flag peptide to the system at a 10 μ M concentration in order to block specific binding, and only specific binding (in general, approximately 80% of total binding) is presented here. Mathematical analysis was performed using the program Prism 3.0 (GraphPad Software Inc).

initiation of an LPS signal, and to examine the mechanism by which the $Tlr4^{Lps-d}$ allele exerts its dominant inhibitory effect on signaling. This was determined by performing more than 3,000 assays of TNF production, induced over an extensive range of LPS concentrations in numerous stable clones, since individual clones show considerable background variability in LPS responses.

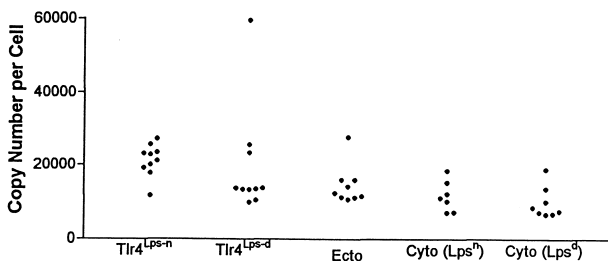


Figure 2b. The expression level of each construct (copy number per cell) was determined by direct measurement of equilibrium M2 monoclonal antibody binding at a fixed concentration, with reference to the nonlinear regression analysis of saturation isotherms presented in Figure 2a. Each point represents the result of duplicate determinations of specific binding. Cells transfected with vector alone had zero specific binding (not shown).

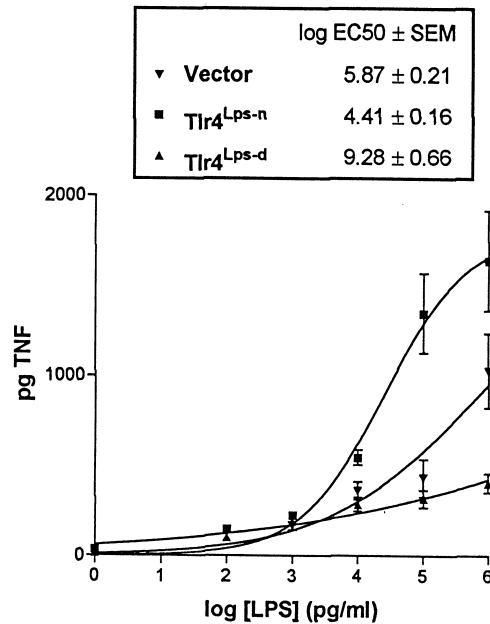


Figure 2c. Composite analysis of the shift in EC50 caused by expression of $Tlr4^{Lps-n}$ and $Tlr4^{Lps-d}$ in RAW 264.7 cells. Each point represents an EC50 determination performed on ten independent clones at the stated LPS concentration. For each clone, four replicate assays were performed at each concentration. Hence, each curve represents the composite analysis of 240 assays. Linear estimates of TNF concentration in unknown samples were based on non-linear interpolation from the standard curve (also performed using Prism 3.0). The curves shown were generated assuming sigmoidicity of response with variable slope for each curve, and further assigning a maximum response value of 1760 pg TNF, which yielded an optimal fit for the most responsive curve. Error bars indicate standard deviation among clones at each LPS concentration. Log EC50 values and standard error values are shown in the inset table.

Moreover, because culture conditions can affect the sensitivity of the TNF assay and production of TNF by LPS-stimulated cells, all transfected and control clones were induced and assayed in parallel. Statistical analyses of shifts in the EC50 values determined for individual clones were then applied in order to determine the influence of recombinant protein expression.

RAW 264.7 cells were first cotransfected with cDNAs derived from the $Tlr4^{Lps-n}$ and $Tlr4^{Lps-d}$ alleles and with a vector encoding *neo*. The amino terminus of each protein was flag-tagged to permit measurement of expression at the cell surface using the monoclonal antibody M2 (obtained from Sigma). After G418 selection, stable clones

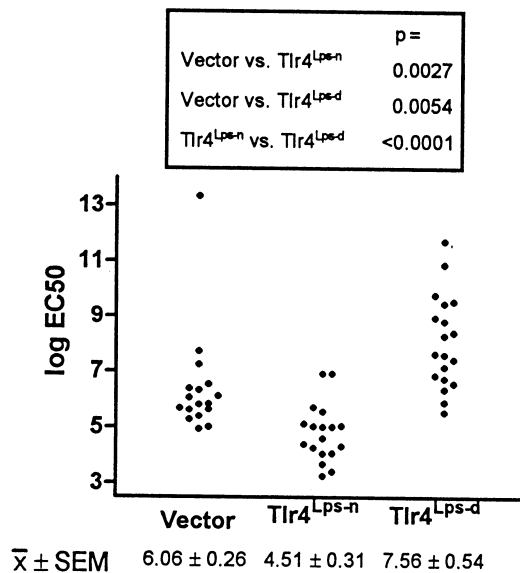


Figure 2d. Shifts of the log EC50, determined for individual clones. A more conservative approach, in which log EC50 values were determined for individual clones transfected with vector alone, or with the Tlr4^{Lps-n} or Tlr4^{Lps-d} constructs. The log EC50 data were then represented as a scatter plot, in which each point represents the log EC50 determination of a single clone (confidence limits not shown for the sake of simplicity), and is derived from 24 separate TNF assays performed on samples stimulated over a 10⁴-fold range of LPS concentration (100 pg/ml to 1 μg/ml, as well as an unstimulated control). In most instances, two determinations of log EC50 were made independently for each clone. The mean and standard error of each cluster is shown in the inset, together with p values defining the likelihood that the EC50 displacements are attributable to chance. Data were analyzed by means of a one-tailed t test, using Welch's correction for unequal variance.

expressing each Tlr4 isoform or transfected with vector alone were examined for LPS signal transduction. The binding isotherm from one such clone, transfected with the Tlr4^{Lps-n} construct, is displayed in Figure 2a, and the flag copy number for clones bearing each construct is displayed in Figure 2b. Despite the use of a strong promoter, the mean copy number rarely exceeded 3×10^4 per cell, and the range of expression among all clones spanned less than an order of magnitude. The relatively low copy number achieved is consistent with the possibility that surface expression may be limited by the level of co-expression of other proteins (e.g. MD-2 (22)), and the lower copy number in clones bearing truncated constructs as compared with full-length constructs may reflect diminished stability.

Composite EC50 analysis revealed strong augmentation of the LPS response (a 30-fold leftward shift of the curve) resulting from modest overexpression of the normal protein; even stronger suppression (a 2600-fold shift to the right) was observed with expression of the mutant isoform. Hence, with respect to the overexpression of Tlr4^{Lps-n} and Tlr4^{Lps-d} proteins, a 74,000-fold difference in the mean response is apparent at the EC50 point (Figure 2c). A more conservative approach, based on measurement of the LPS EC50 for each individual clone, also revealed that the Tlr4^{Lps-n} isoform strongly enhances LPS sensitivity, while the Tlr4^{Lps-d} isoform strongly suppresses it (Figure 2d). According to this method of estimation, the composite range of the responsiveness (mean EC50 of Tlr4^{Lps-d} transfected clones/mean EC50 of Tlr4^{Lps-n} transfected clones) was 1120-fold. Taking the dimension of receptor number into account (Figure 2e), and excluding the statistical contribution of the clones transfected with vector alone, no significant correlation was observed between LPS signal intensity and the

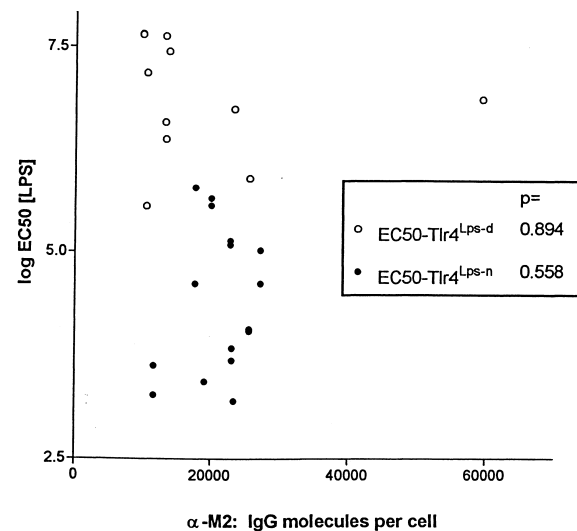


Figure 2e. Lack of correlation between level of recombinant receptor expression and the magnitude of effect on EC50. Plotting the EC50 of Tlr4^{Lps-n} transfected cells (nine clones; duplicate assays) and Tlr4^{Lps-d} transfected cells (eight clones; duplicate assays) vs. the receptor number measured for each clone (shown separately in Figures 2b and 2d), it is apparent that no correlation exists over the range of receptor number surveyed. Inset: p values calculated to assess the significance of departure of the slope (determined by linear regression for each set of points) from zero.

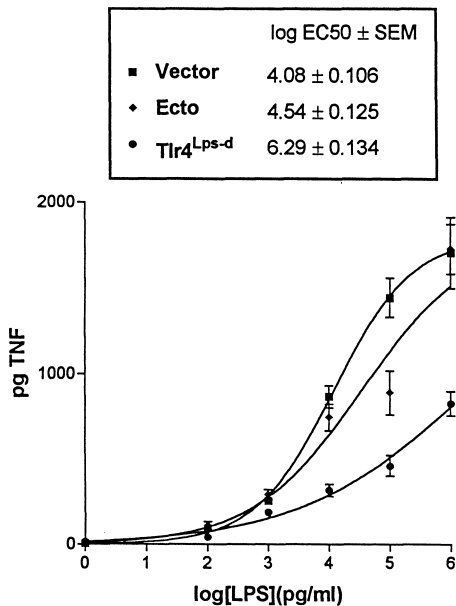


Figure 3a. Overexpression of the Tlr4 ectodomain fails to inhibit LPS signaling. Composite analysis of the shift in EC50 caused by expression of the Tlr4 ectodomain (no cytoplasmic domain) or the full-length Tlr4^{Lps-d} protein in RAW 264.7 cells. Control cells were transfected with the empty vector. Analysis was carried out in a manner identical to that described in Figure 2c. Inset: log EC50 for each curve, and standard error.

absolute number of recombinant Tlr4 molecules on the cell surface. This suggests that the number of artificial receptors expressed (usually $\sim 1-2 \times 10^4$) is well in excess of the number of native receptors, and that maximum augmentation of LPS response is achieved in each clone transfected with Tlr4^{Lps-n}, with variation attributable to other factors. It is interesting in this regard that, on a linear scale, the inhibitory effect of Tlr4^{Lps-d} overexpression vastly exceeds the augmenting effect of Tlr4^{Lps-n} expression. In fact, most of the macrophage clones were rendered virtually unresponsive to LPS through overexpression of Tlr4^{Lps-d} (LPS EC50 > 10 μ g/ml).

Several conclusions can be drawn from these results. First, the dominant effect of the *Tlr4*^{Lps-d} allele may be directly demonstrated through transfection-based expression of the protein at moderate levels in an LPS-responsive macrophage cell line. Second, since overexpression of Tlr4^{Lps-n}

augments the LPS response, the intensity of the LPS signal is normally limited by the quantity of Tlr4 protein on the macrophage membrane. Third, since there appears to be little correlation between the absolute number of recombinant receptors expressed and the magnitude of augmentation or inhibition achieved, it may be inferred that saturation of another component of the signaling cascade, either proximal or distal to Tlr4, occurs when the level of Tlr4 expression exceeds a certain threshold: perhaps in the range of several thousand copies per cell. By the same token, the level of endogenous Tlr4 expression is probably very low: perhaps lower than 10^3 copies per cell (a finding consistent with the fact that Tlr4 mRNA is of very low abundance (1), but nonetheless remarkable in view of the global effects wrought by activation of the receptor). Fourth, and perhaps most important of all, since rather modest changes in the level of normal Tlr4 protein expression or the expression of a dominant negative Tlr4 isoform can shift LPS sensitivity over a range that spans three to four

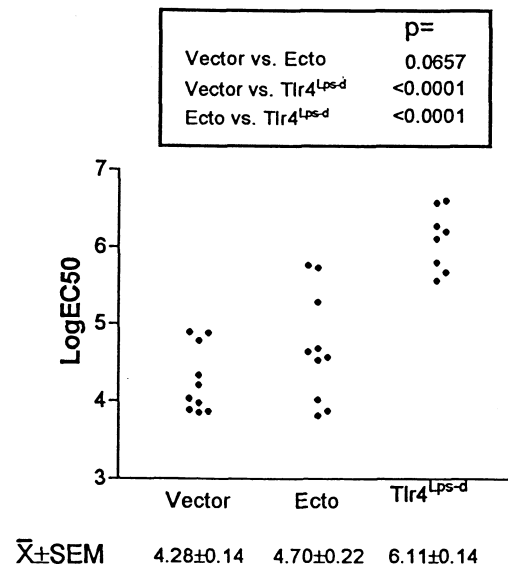


Figure 3b. Distribution of individual log EC50 data for clones expressing the Tlr4 ectodomain, the full-length Tlr4^{Lps-d} protein, or no recombinant receptor (transfected with empty vector). Inset: the mean and standard error of each cluster, together with p values defining the likelihood that the EC50 displacements are attributable to chance. Data were analyzed by means of a t test, using Welch's correction for unequal variance.

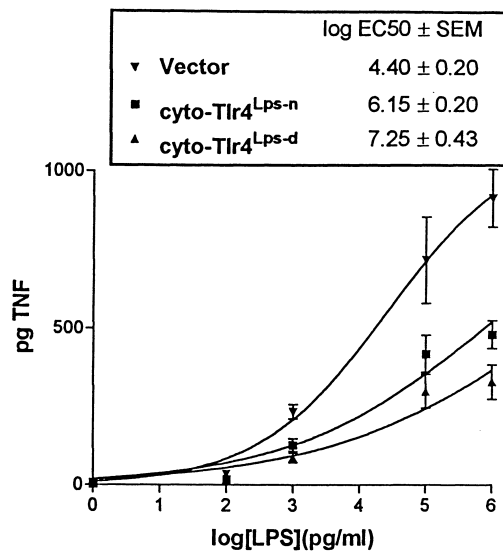


Figure 4a. Overexpression of the Tlr4 cytoplasmic domain, either with or without the Lps-d mutation, impairs signal transduction in RAW 264.7 cells. Composite analysis of the shift in EC50 caused by expression of the Tlr4 ectodomain (no cytoplasmic domain) or the full-length Tlr4^{Lps-d} protein in RAW 264.7 cells. Control cells were transfected with the empty vector. Analysis was carried out in a manner identical to that described in Figure 2c. Inset: log EC50 for each curve, and standard error.

orders of magnitude, one may infer that Tlr4 is of preponderant importance in LPS signal transduction: there is little room for the belief that independent pathways act to transduce the LPS signal as well. This last point is fully supported by the observation that mutation or deletion of the *Tlr4* locus can completely abrogate LPS signaling (1,13,16).

In one model, the dominant suppressive effect of Tlr4^{Lps-d} might be ascribed to the postulated multimeric structure of the Tlr4 protein (23,24), given that unproductive association between normal and abnormal subunits yields inhibition of signaling. If association between subunits is principally dependent upon ectodomain contacts, one would predict that any mutation that disrupts function of the Tlr4 cytoplasmic domain might impede signal transduction in a dominant fashion, just as observed with Tlr4^{Lps-d}. To examine this hypothesis, we expressed a truncated version of the Tlr4 protein, lacking the entire cytoplasmic domain. This protein was well expressed on the cell surface, but had only

a weak inhibitory effect on LPS signaling, which fell short of significance according to the more stringent method of analysis (Figures 3a and 3b). Hence, the Tlr4^{Lps-d} isoform exerts a strong dominant effect whereas deletion of the entire cytoplasmic domain does so weakly at most. We conclude that, if the model of dominant inhibition based on multimeric structure is correct, cytoplasmic domain contacts, in addition to ectodomain contacts, must serve to maintain the holoprotein in a multimeric state. Given free exchange between subunits, mutant Tlr4 proteins lacking a cytoplasmic domain would predictably be excluded from the signaling complex in favor of intact subunits that interact more strongly with one another.

The biological consequence of interactions between normal and mutant cytoplasmic domains and the intact Tlr4 protein were tested directly. Upon expressing membrane-associated versions of the Tlr4 cytoplasmic domain (with either the normal or mutant sequence and an exteriorized flag peptide, but lacking the entire ectodomain), we observed that the all-cytoplasmic Tlr4^{Lps-d} isoform inhibited LPS signaling (rightward EC50 shift of

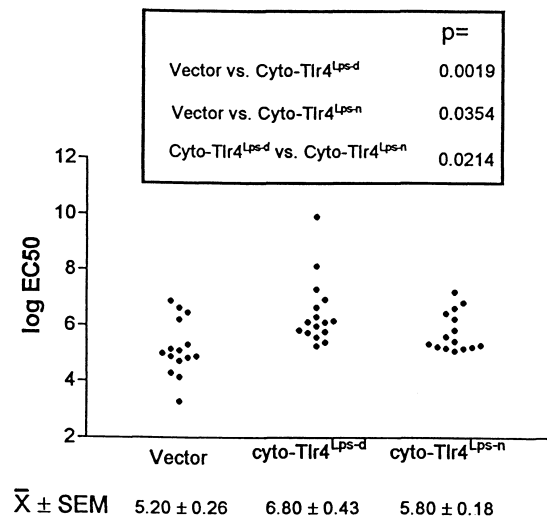


Figure 4b. Distribution of individual log EC50 data for clones expressing the cytoplasmic domain of the Tlr4^{Lps-d} protein, the Tlr4^{Lps-n} protein, or no recombinant protein (transfected with empty vector). Inset: the mean and standard error of each cluster, together with p values defining the likelihood that the EC50 displacements are attributable to chance. Data were analyzed by means of a t test, using Welch's correction for unequal variance.

39-fold; $p = 0.0019$). The Tlr4^{Lps-n} isoform was a significantly weaker inhibitor than the Tlr4^{Lps-d} isoform ($p = 0.0214$), though it also blocked signaling when overexpressed (rightward EC50 shift of 4-fold; $p = 0.035$). These results suggest that the Tlr4^{Lps-d} mutation not only permits interaction between normal and mutant subunits, but actually enhances the interaction, or actively engages downstream signaling molecules, thus blocking the LPS response (Figures 4a and 4b).

The primacy of Tlr4 in LPS signal transduction was originally suggested by positional cloning data (1,25). Direct confirmation of Tlr4's function as the transmembrane signaling component of the LPS receptor complex has lagged, due largely to the difficulties inherent in transiently transfecting macrophage lines with high efficiency. Rather, there has been a tendency to utilize non-macrophage lines (e.g., HEK 293 cells) in transfection-based studies of LPS signaling. However, these cells do not replicate the biological response phenotype of authentic macrophages. In particular, they do not produce TNF in response to LPS; hence, a surrogate endpoint of response (nuclear translocation of NF- κ B, measured using a transcriptional reporter) has been adopted in most such studies. However, it is quite clear that NF- κ B translocation cannot be equated with an LPS response, since many stimuli that elicit NF- κ B translocation yield effects that have little or nothing in common with the LPS response in other respects. Moreover, it has recently been shown that mutational inactivation of MyD88, which is known to engage Tlr4, leads to a state of profound LPS unresponsiveness, though permitting NF- κ B translocation (26). Finally, given that a response of any kind is observed in non-macrophage cell lines, there exists no standard for comparison. It has never been clear, for example, that the magnitude of the NF- κ B response approaches that witnessed in a normal macrophage over an identical range of LPS concentrations, nor is it known what effect this might have in a macrophage. These technical issues have, to date, confounded interpretation of which molecule actually does transduce the LPS signal, a

role previously ascribed to Tlr2 (2,3) but now clearly attributable to Tlr4.

The present data reveal that Tlr4 is the limiting factor in LPS signal transduction in LPS responsive macrophages. Overexpression of Tlr4 in cells that already express it augments the LPS response, by about 30-fold on average. The relationship between the level of Tlr4 expression and biological response indicates that, although other proteins fulfill indispensable functions in LPS signal transduction both upstream (10) and downstream (26-28) from Tlr4, the quantity of Tlr4 expressed is an important limiting factor in the intensity of the signal that is evoked. Hence, sensitivity to LPS is likely controlled through modulation of Tlr4 biosynthesis or activity. Priming by interferon- γ (29-31) or by treatment with facultative intracellular pathogens (32-34) can greatly enhance sensitivity to LPS, while corticosteroids create a state of LPS resistance (35). Such modulation may be achieved through alteration of Tlr4 structure or expression, or alternatively, through changes in sensitivity to the signal that Tlr4 initiates, or changes in the intensity of the signal that Tlr4 receives.

The overexpression of a membrane-anchored Tlr4 ectodomain (lacking any of the wild-type cytoplasmic domain) inhibited the LPS response only weakly, if at all. This failure of the overexpressed ectodomain to block signaling by a competitive mechanism implies that upstream components of the signal transduction pathway must either exist in excess with respect to Tlr4, or must interact with Tlr4 at very low affinity. There is now good reason to doubt the proposal (36) that the expression of soluble Tlr4 might prove an effective means of interdicting the LPS signal *in vivo*, particularly in view of the fact that a membrane-anchored form of the protein would be sterically positioned to exert such an effect with maximum efficiency, while a soluble form would not be. It is, for example, possible to calculate the local concentration of Tlr4 ectodomain achieved through overexpression of a membrane-associated version of the protein. Assuming that the Tlr4 ectodomain resides within a space that is 100 Å "deep" from its

most apical point to the surface of the membrane, and further assuming that the macrophage is a spherical body with a 15 μM radius, the expression of 2×10^4 receptors per cell corresponds to a protein concentration of 1.2×10^{-6} M, or 840 μg of ectodomain per ml. While it might be possible to achieve such concentrations of soluble ectodomain *in vivo*, it would not be easy to do so, and at that, little or no attenuation of the LPS signal would be anticipated. On the other hand, interventions that inhibit contact between Tlr4 subunits would be likely to have a pronounced impact on signal transduction.

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