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Lipopolysaccharide- and Lipoteichoic Acid-Induced Tolerance and Cross-Tolerance: Distinct Alterations in IL-1 Receptor-Associated Kinase

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Human Toll-like receptor (TLR) 4 and TLR2 receptors recognize LPS or lipoteichoic acid (LTA), respectively. Prolonged exposure of human macrophages/monocytes to bacterial LPS induces a state of adaptation/tolerance to subsequent LPS challenge. Inflammatory gene expressions such as IL-1β and TNF-α are selectively repressed, while certain anti-inflammatory genes such as secretory IL-1R antagonist are still induced in LPS-adapted/tolerant cells. In this report, we demonstrate that LPS-tolerized human promonocytic THP-1 cells develop cross-tolerance and no longer respond to LTA-induced IL-1β/TNF-α production, indicating that disruption of common intracellular signaling is responsible for the decreased IL-1β/TNF-α production. We observe that down-regulation of IL-1R-associated kinase (IRAK) protein level and kinase activity closely correlates with the development of cross-tolerance. IRAK protein levels and kinase activities in LPS-tolerized cells remain low and hyporesponsive to subsequent LPS or LTA challenges. We also demonstrate that THP-1 cells with prolonged LTA treatment develop LTA tolerance and do not express IL-1β/TNF-α upon further LTA challenge. Strikingly, cells tolerant with LTA are only refractory to subsequent LTA challenge and can still respond to LPS stimulation. Correspondingly, stimulation of TLR2 by LTA, although activating IRAK, does not cause IRAK degradation. IRAK from LTA-tolerized cells can be subsequently activated and degraded by further LPS challenge, but not LTA treatment. Our studies reveal that LTA-induced tolerance is distinct compared with that of LPS tolerance, and is likely due to disruption of unique TLR2 signaling components upstream of MyD88/IRAK. The Journal of Immunology, 2002, 168: 6136–6141.

During infection, components of microbial pathogens (lipoteichoic acid (LTA), bacterial RNAs, LPS, bacterial flagellin FliC protein, and bacterial DNAs, etc.) activate the cellular innate immune response by binding to distinct Toll-like receptor proteins (TLR2, 3, 4, 5, and 9, respectively). Others and we have observed that early signaling via TLR4, 5, and 9 all involves activation of the IL-1R-associated kinase (IRAK) (1–3). TLR-mediated intracellular signaling subsequently induces expression of potentially autotoxic proinflammatory genes, such as IL-1β and TNF-α.

Recurrent multiple exposures to microbial products such as LPS during infection result in an adaptive immune response, which is reflected by repression of proinflammatory genes when blood leukocytes are stimulated ex vivo with bacterial endotoxin (4). This phenomenon is often referred to as endotoxin tolerance. Repression of transcription and rapid degradation of proinflammatory cytokine mRNAs contribute to decreased proinflammatory cytokine protein production in LPS-tolerant cells as well as septic blood leukocytes (4, 5). However, tolerant cells are not totally unresponsive to further LPS challenge. In contrast, LPS-tolerized cells continue to produce several anti-inflammatory proteins such as secretory IL-1R antagonist (sIL-1RA) (6). We have observed that efficient translation of the stable sIL-1RA message contributes to the prolonged induction of sIL-1RA protein in both septic blood leukocytes and tolerant THP-1 cells (5). Therefore, selective and specific disruption of certain TLR4 signaling component(s) is likely to contribute to the altered LPS response in tolerant cells. It has been reported that TLR4 receptor level decreases following LPS treatment in mouse peritoneal macrophages, suggesting that TLR4 down-regulation may disrupt the LPS signaling. However, the decrease of TLR4 receptor level may not be the only cause of LPS signaling disruption. First, it is observed that LPS and IL-1β can induce a state of cross-tolerance against each other (7). IL-1β-pretreated leukocytes do not respond to LPS treatment and no longer express cytokine genes. Second, induction of tolerance to LPS and mycobacterial components in Chinese hamster ovary/CD14 cells is not affected by overexpression of either TLR2 or TLR4 (8). Third, LPS-tolerized cells can still respond to an additional dose of LPS challenge and produce several anti-inflammatory proteins such as IL-1RA (6). These studies suggest that repression of IL-1β-like inflammatory genes in leukocytes is due to impaired function of intracellular signaling intermediate(s) of the TLR/IL-1 signaling pathway rather than defect or down-regulation of the TLR receptor. Using the model human promonocytic THP-1 cells, we have first observed that the protein level of IRAK, one of the proximal TLR signaling components, is greatly decreased in LPS-tolerant cells. In addition, IRAK kinase activity is no longer responsive to further LPS challenge in tolerant cells.

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Our results indicate that down-regulation of IRAK is at least in part responsible for the development of LPS tolerance in which IL-1β-like gene expression is repressed. Besides LPS, other Gram-positive bacterial toxins such as LTA can also activate innate immune response and induce a wide array of gene expressions. Resembling the effect of LPS, murine peritoneal macrophage cells pretreated with LTA no longer express IL-1β or TNF-α upon a second dose of LTA stimulation (9). The molecular mechanism of LTA-induced tolerance is not clear.

In this report, we intend to examine in detail LPS- and LTA-induced homologous and heterologous tolerance phenotype in terms of repressed IL-1β and TNF-α production. Using human promonocytic THP-1 cells, we observed that LTA-tolerized cells no longer produce IL-1β or TNF-α protein upon subsequent LTA challenge, while still maintaining normal response to LPS stimulation. In contrast, LPS-tolerized THP-1 cells show severe hyporesponsiveness to subsequent LPS or LTA challenge as reflected by decreased IL-1β and TNF-α production. Correspondingly, we show that only LPS treatment causes IRAK level decrease, while LTA treatment can activate IRAK kinase activity, but cannot cause a decrease in IRAK protein level. Our result indicates that the mechanism for LTA-induced tolerance is distinct compared with that of LPS tolerance and likely involves disruption of signaling component(s) upstream of IRAK.

Materials and Methods

Cell culture and stimulation

THP-1 cells, an undifferentiated human promonocytic cell line, were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium (Life Technologies, Rockville, MD) supplemented with 10 U/ml penicillin G, 10 μg/ml streptomycin, 2 mM L-glutamine, and 10% FBS (HyClone Laboratories, Logan, UT) at 37°C and 5% CO2 in a humidified incubator. We chose to use the undifferentiated THP-1 cells to study the tolerance phenotype due to its resemblance to the human blood cells (5, 6). Log-phase cells were used in all experiments. Normal THP-1 cells were suspended in media at 1 × 10^7 cells/ml and stimulated with either 1.0 μg/ml LPS (Escherichia coli 011:B4; Sigma-Aldrich, St. Louis, MO) or 1.0 μg/ml LTA (highly purified through butanol extraction as described previously (10)) for 10 min, lysed on ice for 20 min in 800 μl lysis buffer (50 mM HEPES [pH 7.6], 20 mM MgCl2, 20 mM β-glycerophosphate, 20 mM p-nitrophenylphosphatase, 1 mM EDTA, 1 mM sodium orthovanadate, and 1 mM benzamidine). Each sample was incubated at 37°C for 30 min in 50 μl kinase buffer supplemented with 5 μM ATP, 2 μg myelin basic protein (MBP), and 1 μM [γ-32P]ATP. SDS sample buffer was added after incubation, and the samples were subjected to SDS-PAGE (15% acrylamide) analysis. To quantify IRAK kinase activity, the gel was dried and exposed to a Molecular Dynamics PhosphorImager 425 SI (Sunnyvale, CA). Band intensity was quantified using the ImageQuant 4.1 software (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis

A mean constitutive activity or fold induction was determined for each experiment. Data are presented as the mean ± SEM. Statistics were performed using either two-tailed paired or nonpaired t tests to determine significant changes in activities. Data were analyzed using Microsoft Excel 97 software (Microsoft, Seattle, WA).

Results

LPS induces homologous tolerance and cross-tolerance to LTA, while LTA only induces homologous tolerance to itself

Through distinct TLRs, diverse bacterial products trigger human leukocyte innate immunity response and induce IL-1β as well as TNF-α production. As shown in Fig. 1, fresh THP-1 cells treated with LPS or LTA express IL-1β and TNF-α proteins as measured by ELISA. We then examined the ability of LPS to induce innate immunity tolerance. Undifferentiated THP-1 cells were pretreated with various concentrations of LPS for 16 h. Pretreated THP-1 cells were washed with LPS-free RPMI 1640 medium and subjected to another LPS or LTA challenge for 6 h. The IL-1β and TNF-α protein levels were subsequently assayed using ELISA. We observed that THP-1 cells with overnight LPS treatment develop cross-tolerance to subsequent LPS or LTA challenge. Production of TNF-α and IL-1β in response to a second dose of LPS or LTA was decreased in a dose-dependent fashion when THP-1 cells were pretreated with various concentrations of LPS for 16 h before re-stimulation. (Fig. 1, A and C).

We next examined the ability of LTA in inducing tolerance and cross-tolerance. Fresh THP-1 cells were treated with various concentrations of LTA for 16 h followed by washing with fresh RPMI 1640 medium. LTA-pretreated THP-1 cells were then subjected to further LTA or LPS challenge for 6 h. As shown in Fig. 1, B and D, pretreatment with 1 μg/ml LTA induced a statistically significant hyporesponsive state to further LTA challenge as measured by decreased IL-1β and TNF-α production. Interestingly, unlike LPS pretreatment, THP-1 cells pretreated with various concentrations of LTA can still respond to further LPS challenge and express normal amounts of IL-1β and TNF-α comparable to fresh
THP-1 cells (Fig. 1, B and D). These results indicate that LTA can only induce tolerance to itself, while LPS can induce cross-tolerance to LPS and LTA in human THP-1 cells.

**Both LPS and LTA activate IRAK in normal THP-1 cells**

Only LPS, not LTA stimulation, results in IRAK degradation. The activation of a common TLR intracellular signaling pathway contributes to the innate immunity stimulation upon various microbial products. IRAK is one of the proximal TLR intracellular signaling components. We previously showed that LPS and bacterial flagellin protein FliC can both induce endogenous IRAK protein kinase activation through TLR4 and TLR5 (1, 2). To examine whether LTA could similarly activate IRAK, we examined the IRAK protein kinase activity and protein levels in THP-1 cells upon LTA challenge. Undifferentiated THP-1 cells were subjected to either LPS or LTA treatment for various time periods. IRAK protein levels were subsequently determined by Western blot analysis using total protein extracts. To examine LPS- and LTA-induced IRAK kinase activities, IRAK protein was immuno-precipitated from total protein extracts and subjected to in vitro kinase assays as described in Materials and Methods. As shown in Fig. 2B, IRAK kinase activity was maximally induced following an 80-min stimulation with either LPS or LTA. Interestingly, LPS stimulation resulted in a 2- to 3-fold greater induction of IRAK kinase activity compared with LTA stimulation.

**LTA stimulation does not induce apparent IRAK phosphorylation and degradation**. IRAK remained as a single band and its intensity was not altered at various times of LTA challenge (Fig. 2A). We performed three independent experiments and obtained consistent results. Our data indicate that LTA stimulation does activate IRAK kinase in fresh nontolerant cells and suggest that LTA/TLR2 also utilize IRAK in mediating innate immunity activation and inflammatory gene expression. However, there are distinct differences regarding IRAK protein regulation upon LPS/TLR4 and LTA/TLR2 stimulation.

**IRAK from LPS-tolerized cells exhibits no response to either LPS or LTA**

In contrast, IRAK from LTA-tolerized cells fails to respond to further LTA challenge, yet is capable of responding to further LPS challenge. We previously showed that IRAK protein and kinase levels remain persistently low in LPS-tolerized cells and unresponsive to further LPS challenge (1). Given the hypothesis that IRAK is utilized by many TLR receptors in mediating responses to a variety of microbial stimulants, we would predict that IRAK from LPS-tolerized cells would no longer respond to further LTA challenge. To test this, THP-1 cells were pretreated with 1 μg/ml LPS overnight followed by washing with fresh RPMI 1640 medium. Pretreatment with such concentrations of LPS was shown above to induce statistically significant tolerance to subsequent LPS and LTA challenge (Fig. 1). LPS-pretreated cells were subjected to subsequent LPS or LTA challenge for various periods of time. Total protein extracts were prepared and analyzed through SDS-PAGE. IRAK protein levels were detected by Western blot. As shown in Fig. 3, IRAK levels in LPS-pretreated cells remained low
following subsequent LPS or LTA challenge. In vitro IRAK kinase activity toward MBP was subsequently assayed using immunoprecipitated IRAK from the total protein extracts as described in Materials and Methods. As expected, neither LPS nor LTA induced IRAK kinase activation in LPS-pretreated THP-1 cells (Fig. 4). We next studied the IRAK response in LTA-tolerized cells following LPS or LTA stimulation. We chose to use LTA at 1 μg/ml to tolerize the cells since that concentration is shown to induce statistically significant tolerance to subsequent LTA stimulation (Fig. 1). Undifferentiated THP-1 cells were treated with 1 μg/ml LTA overnight followed by washing with fresh RPMI 1640 medium. LTA-pretreated cells were subsequently subjected to further LPS or LTA challenge for various periods of time. IRAK protein levels and kinase activities were subsequently determined as previously described. As shown in Fig. 3, there was no apparent IRAK protein level decrease in LTA-tolerant THP-1 cells challenged by an additional dose of LTA. Despite the presence of high IRAK protein level in LTA-tolerant cells, subsequent LTA challenge did not cause IRAK kinase activation (Fig. 4). This suggests that LTA pretreatment causes disruption of the unique TLR2 signaling component upstream of IRAK. Interestingly, we observed that IRAK protein from LTA-tolerized cells still responded to further LPS challenge and underwent rapid kinase activation as well as protein degradation (Figs. 3 and 4). These results indicate that the hyporesponsiveness of LPS-tolerant cells to subsequent LPS or LTA challenge as seen by the decrease in IL-1β and TNF-α protein production (Fig. 1, A and C) can be attributed to, at least in part, the down-regulation of IRAK protein levels and low IRAK kinase activity. In contrast, LTA pretreatment may cause disruption upstream of IRAK and therefore leaves IRAK unmodified and responsive to further LPS challenge through the TLR4 receptor. LTA-tolerized THP-1 cells still retain some LPS/LTA response such as increased sIL-1RA production

Previous studies reported that the TLR4 receptor is down-regulated in LPS-tolerant cells (13). It is therefore likely that down-regulation of the TLR4 or TLR2 receptor would lead to cellular hyporesponsiveness to LPS or LTA. In discordance to the above hypothesis, others and our studies indicate that LPS-tolerized THP-1 cells and human blood cells still retain some responsiveness to LPS such as elevated production of anti-inflammatory sIL-1RA protein (6, 14). These studies suggest that the TLR4 receptor is still functional in LPS-tolerized cells. The decreased IL-1β and TNF-α production is likely caused by selective disruption of intracellular signaling component. To gain some insight regarding whether the TLR2 receptor is functional in LTA-tolerized cells, we...
IL-1 protein levels were determined by ELISA. Data are expressed as ±SD. Pretreated cells were subjected to further LPS or LTA treatment for 6 h. Total protein extracts were prepared as described in Materials and Methods, and the sIL-1RA protein levels were determined by ELISA. Data are expressed as ±SD.

Discussion

Multiple microbial stimulants including LPS and LTA can induce a wide array of gene expressions through the TLR4 and TLR2 receptors. Activation of TLR4 and TLR2 subsequently lead to common as well as unique downstream signaling events. The common signaling events include MyD88/IRAK kinase and downstream NF-κB activation, which contribute to the induction of IL-1β as well as TNF-α. On the other hand, TLR4, instead of TLR2 signaling may activate unique downstream components (such as STAT1) and induce a subset of genes such as MCP5 and IP10 (15).

With regard to the common signaling pathway that leads to IL-1β and TNF-α production, it was shown that prolonged treatment with LPS or LTA could induce a state of tolerance and decreased IL-1β and TNF-α production upon subsequent challenge (4, 9). The mechanism of LPS-induced tolerance is complex and probably involves down-regulation of multiple TLR signaling components, including down-regulation of the TLR4 receptor, disruption and degradation of IRAK kinase, as well as reduced activation of mitogen-activated protein kinases (13, 16). However, total disruption of the TLR4 receptor is unlikely during LPS tolerance, since LPS-tolerant human macrophages/microphages can still respond to further LPS challenge and express many other genes and proteins (6). Furthermore, induction of tolerance to LPS in Chinese hamster ovary/CD14 cells is not affected by overexpression of TLR4 (8). We and others reported that LPS treatment induces rapid degradation and inactivation of the intracellular signaling molecule IRAK (1, 17). The timing of IRAK disruption correlates well with the development of LPS tolerance (within 3 h of LPS treatment). Our present study further confirms our conclusion regarding the IRAK response upon LPS challenge and indicates that disruption of the IRAK response may also be responsible for the development of LPS-induced cross-tolerance (Fig. 6).

Murine monocytes/macrophages following prolonged LTA treatment can similarly develop hyporesponsiveness to subsequent LTA-induced IL-1β/TNF-α production (9). Although the TLR signaling pathway between mouse and human bear similarities, there are also striking differences (18, 19). To date, there has been no report whether human macrophages can develop LTA tolerance. In this report, we studied the innate immune response of human monocytic THP-1 cells upon LTA challenge. We observed that, like LPS stimulation, LTA stimulation similarly induces IRAK kinase activation and subsequent IL-1β and TNF-α protein production. Furthermore, we first observed that THP-1 cells treated with LTA acquire hyporesponsiveness and decreased IL-1β/TNF-α production upon subsequent LTA treatment. Correspondingly, LTA no longer induces IRAK kinase activation nor IL-1β/TNF-α protein production in LTA-tolerized cells.

Strikingly, we observed that LTA-tolerized THP-1 cells can still respond normally to further LPS challenge and produce IL-1β as well as TNF-α proteins. This suggests that LTA-induced tolerance is caused by disruption of the unique TLR2 signaling component not shared by LPS/TLR4 signaling, and likely resides upstream of IRAK (Fig. 6). This would enable LTA-tolerized cells to still maintain functional TLR intracellular signaling response shared by other TLRs. In accordance with this, we observed that the IRAK protein level is not decreased following prolonged LTA treatment. Furthermore, we show that IRAK in LTA-tolerized cells can still respond normally to subsequent LPS challenge and exhibit kinase activation as well as protein degradation. In sharp contrast, LPS-tolerized THP-1 cells exhibit severe hyporesponsiveness to both LPS and LTA challenge.

The disruption of TLR2 signaling leading to decreased IL-1β and TNF-α production in LTA-tolerant cells is not due to inactivation of the TLR2 receptor. LTA-tolerized THP-1 cells are not totally unresponsive to subsequent LTA challenge. Instead, LTA-tolerized cells can still respond to further LTA treatment and selectively express sIL-1RA protein (Fig. 5). This indicates that the TLR2 receptor is still functional in LTA-tolerant cells. The decreased expression of inflammatory proteins such as IL-1β and TNF-α is likely due to disruption of intracellular signaling components downstream of the TLR2 receptor and upstream of IRAK. The candidate protein(s) include adapter protein MyD88, Tollip, TIRAP/MAL, as well as other enzymes such as phosphatidylinositol 3-kinase (PI3). The LPS/TLR4 pathway has been shown to utilize all of these adapter proteins as well as PI3 in the downstream signaling (1, 20, 15, 6). However, the LTR2 pathway was shown to use only the MyD88 and PI3 (15, 21). One likely scenario is that the LPS/TLR4 signaling can recruit the specific adapter protein(s) that leads to IRAK phosphorylation and subsequent degradation, whereas LTA/TLR2 signaling recruited adapter(s) could only lead to IRAK activation, but not phosphorylation/degradation. Indeed, we observed that LTA cannot induce IRAK phosphorylation (Fig. 2). It was previously reported that IRAK phosphorylation is a prerequisite for subsequent degradation. The fact that LTA can induce IRAK kinase activation indicates that...
IRAK activation and phosphorylation is two independent events and may be controlled by distinct upstream regulatory steps.

Taken together, our study reveals that cellular innate immunity tolerance toward distinct microbial toxins may involve alteration of distinct TLR signaling components. However, down-regulation of the common TLR intracellular signaling factor IRAK will lead to a state of cross-tolerance and decreased IL-1β and TNF-α production upon subsequent challenge with multiple microbial toxins.

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