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Tolerance Induced by the Lipopeptide Pam₃Cys Is Due to Ablation of IL-1R-Associated Kinase-1

Maciej Siedlar,∗† Marion Frankenberger,‡ Elke Benkhart,∗† Terje Espevik,§ Martina Quirling,¶ Korbinian Brand,‖ Marek Zembala,† and Loems Ziegler-Heitbrock‡∥

Stimulation of the human monocytic cell line Mono Mac 6 with the synthetic lipopeptide (S)-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser(S)-Lys₄-OH, trihydrochloride (Pam₃Cys) at 10 μg/ml induces a rapid expression of the TNF gene in a TLR2-dependent fashion. Preculture of the cells with Pam₃Cys at 1 μg/ml leads to a reduced response after subsequent stimulation with Pam₃Cys at 10 μg/ml, indicating that the cells have become tolerant to Pam₃Cys. The CD14 and TLR2 expression is not decreased on the surface of the tolerant cells, but rather up-regulated. Analysis of the NF-κB binding in Pam₃Cys-tolerant cells shows a failure to mobilize NF-κB-p50p65 heterodimers, while NF-κB-p50p50 homodimers remain unchanged. Pam₃Cys-tolerant cells showed neither IkBα-Ser³² phosphorylation nor IkBα degradation but MyD88 protein was unaltered. However, IRAK-1 protein was absent in Pam₃Cys-induced tolerance, while IRAK-1 mRNA was still detectable at 30% compared with untreated cells. In contrast, in LPS-tolerized cells, p50p50 homodimers were induced, IRAK-1 protein level was only partially decreased, and p50p65 mobilization remained intact. It is concluded that in Mono Mac 6 monocytic cells, inhibition of IRAK-1 expression at the mRNA and protein levels is the main TLR-2-dependent mechanism responsible for Pam₃Cys-induced tolerance, but not for TLR-4-dependent LPS-induced tolerance. The Journal of Immunology, 2004, 173: 2736–2745.

Inflammation may be triggered when specific receptors, known as TLRs, recognize specific, nonself patterns of molecules derived from microbes. Following recognition of (S)-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser(S)-Lys₄-OH, trihydrochloride (Pam₃Cys) by TLR2/TLR1/CD14 and LPS by TLR4/MD-2/CD14, the TLRs bind to the intracellular adaptor protein, MyD88. MyD88 recruits IL-1R-associated kinase-1 (IRAK-1) to this receptor-signaling complex. IRAK-1 is then phosphorylated and activated. Activated IRAK-1 leaves the TLR/MyD88 complex, associates with TNFR-associated factor 6, and activates downstream signaling pathways such as MAPK and (via TGF-β-activated kinase 1 and NF-κB-inducing kinase) the transcription factor NF-κB. TGF-β-activated kinase 1 and NF-κB-inducing kinase are thought to phosphorylate IkB kinase α and β, which in turn leads to the phosphorylation of specific serine residues of IkB (Ser³² and Ser³⁶ of IkBα, or Ser¹⁹ and Ser²³ of IkBβ). This is followed by ubiquitination of IkB, its degradation, and release of active NF-κB complex (NF-κB-p50p65 or NF-κB-p50p50 dimers). NF-κB is then translocated to the nucleus and activates transcription of the major proinflammatory cytokine TNF and a whole range of other proinflammatory factors (1, 2).

Although a single ligation of TLRs induces responses such as TNF production, repeated ligation will lead to a loss of response, i.e., the cells become tolerant. Tolerance to self and also to nonself is a general phenomenon preventing or diminishing inflammatory responses of the immune system, and involves either deletion of responder cells, down-regulation of the respective receptors, blockade of signal transduction, or induction of suppressive cytokines. In monocytes/macrophages, tolerance to bacterial LPS does not appear to involve deletion nor receptor down-regulation because CD14 receptor expression is up-regulated, as reported in several studies (3–5). Blockade of signal transduction appears to be the major mechanism of LPS-induced tolerance in that the interruption of the MAPK and NF-κB pathways has been described (6–9). With respect to the NF-κB pathway, we have described a unique mechanism that involves induction of NF-κB-p50p50 homodimers (3, 10). The p50 protein has no trans activating domain, and therefore the p50p50 homodimers cannot trans activate, but they can bind to DNA in the promotor regions of various genes, including TNF. By binding to the cognate DNA sequences, p50p50 can prevent the classical NF-κB (p50p65) heterodimer from binding and trans activating, thus blocking the expression of genes such as TNF (11).

Although Wysocka et al. (12) were still able to induce LPS tolerance in p50 knockout mice, the NF-κB (p50p50) homodimer mechanism of LPS tolerance has been confirmed by many others (13–16). Also, Bohuslav et al. (17) demonstrated LPS tolerance to be absent in NF-κB p50−/− mice. Finally, Adib-Conquy et al. (18) recently demonstrated that up-regulation of p50p50 homodimers may occur in blood monocytes of patients with severe sepsis.

LPS acts via CD14 and the associated TLR4 (19–21). In the present study, we have investigated which mechanisms operate in...
tolerance induced by TLR2 by the synthetic lipopeptide Pam 3 Cys. Using this pathway of monocyte activation, an entirely different mechanism appears to operate, because mobilization of NF-κB homodimers is completely blocked. The failure to mobilize classical NF-κB-p50p65 heterodimers is shown to be due to a blockade at the level of IRAK-1 protein in Pam 3 Cys-tolerant Mono Mac 6 cells. We also confirm that LPS tolerance operates via induction of NF-κB-p50p50 homodimers and that highly purified (lipoprotein-free) LPS only partially inhibits IRAK-1 protein expression in LPS-tolerant cells.

Hence, in monocytes, different mechanisms of tolerance appear to coexist. This indicates that it is important for the host to provide more than one mechanism to ensure down-regulation of TNF and to prevent the detrimental effects of excessive amounts of proinflammatory cytokines during bacterial infection.

Materials and Methods

Cell culture and tolerance induction
Mono Mac 6 cells (22) were cultured in 24-well plates (Costar, Cambridge, MA) (3 x 10^5/well) in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 2 mM L-glutamine (043-05030 H; Invitrogen Life Technologies, Gaithersburg, MD), 200 U/ml penicillin, 200 μg/ml streptomycin (043-05140 H; Invitrogen Life Technologies), 1–2% nonessential amino acids (043-01140 H; Invitrogen Life Technologies), and OPI supplement (contains oxalacetic acid, sodium pyruvate, and insulin; O-5003; Sigma-Aldrich, Munich, Germany). The culture medium, as well as all other buffers, were passed through a Gamber U-2000 ultrafiltration column (Gambro Medizintechnik, Planegg-Martinsried, Germany) to deplete inadvertent LPS contamination, and this was followed by addition of low-LPS 10% FCS (Biochrom). All medium were checked for LPS contamination using Limulus Amebocyte Lysate assay (AE 01; Charles River Laboratories, Sulzfeld, Germany; sensitivity, 0.06 EU/ml). The cells were controlled for absence of Mycoplasma sp. on a weekly basis (for detailed method, see www.mycocytes.de) (23) and using Mycoplasma PCR ELISA (1663925; Roche, Mannheim, Germany) for tolerance induction. Mono Mac 6 cells were precultured in complete culture medium in 24-well plates for 42 h with either 1 μg/ml Pam 3 Cys (L2000; EMS Microcollections, Tübingen, Germany, www.microcollections.de) or 20 ng/ml LPS from Salmonella minnesota (L6261; protein content <3%; Sigma-Aldrich). For Western blotting experiments, the highly purified LPS from Salmonella abortus equi (kind gift from C. Galanos (Freiburg, Germany) (24)) and from Salmonella typhimurium (25) were used. The culture medium, Mono Mac 6 cells were washed and stimulated for 10 min (cytoplasmic protein extracts), 1 h (nuclear extracts and RT-PCR), 4 h (transfection experiments), or 5 h (intracellular cytokine staining) with 10 μg/ml Pam 3 Cys, or with 1 μg/ml LPS.

Immunofluorescence analysis

For detection of cell surface Ags, Mono Mac 6 cells (2 x 10^5/sample) were stained for 30 min on ice with 10 μg/ml of either anti-CD14 FITC mAbs (clone MY-4; 6603531; Coulter, Krefeld, Germany) or anti-TLR2 Alexa 488 mAbs (clone TL2.1) (25) vs IgG2b FITC (6603034; Coulter) or IgG2a Alexa 488 isotype controls, respectively. For intracellular TNF staining, Mono Mac 6 cells were stimulated (or not) for 5 h with appropriate doses of Pam 3 Cys in the presence of 10 μg/ml brefeldin A (B-7651; Sigma-Aldrich), then collected, washed in PBS/FCS, fixed (5 x 10^5 cells/sample) for 30 min on ice in 200 μl of Cytofix/Cytoperm (2090KK; BD Pharmingen, Heidelberg, Germany), washed twice, and incubated on ice for 10 min with 50 μl of Perm/Wash buffer (2091KK; BD Pharmingen) together with 10 μg/ml anti-TNF PE mAbs (clone MAb11: 18645A; BD Pharmingen) or mouse IgG1 PE (20605A; BD Pharmingen). Samples were then washed and resuspended in 200 μl of PBS/0.1% FCS, and cells were acquired immediately and then analyzed using a FACScan flow cytometer equipped with CellQuest software (version 3.1; BD Biosciences, Mountain View, CA). The specificity of TNF staining was determined by subtracting the median fluorescence of the isotype control from the median fluorescence of the anti-TNF mAbs. Percentage of positive cells was determined by subtraction of the negative control histogram from the positive staining histogram.

Blockade of Pam 3 Cys stimulation with Abs

Fifteen minutes before Pam 3 Cys stimulation, purified mAbs anti-CD14 (clone MY-4; 6602622; Coulter) and anti-TLR2 (clones TL2.1 and TL2.3), or relevant purified isotype controls IgG2b (6603001; Coulter) and IgG2a (clone UPC 10; M-9144; Sigma-Aldrich) were added (single or in combinations) to the cells (each of mAbs and isotype controls at a final concentration of 10 μg/ml). Mono Mac 6 cells were then stimulated for 5 h with 1 μg/ml Pam 3 Cys in the presence of brefeldin A. Thereafter, the cells were stained with either PE-conjugated isotype control, or PE-conjugated anti-TNF mAbs, as described above.

LightCylder RT-PCR for TNF mRNA

Quantitative PCR was performed using the LightCycler system (Roche), according to the manufacturer’s instructions, using the primer pairs, as noted below. In brief, mRNA was isolated and reverse transcribed as for conventional RT-PCR using oligo(dT) as primer and Moloney murine leukemia virus reverse transcriptase (all Applied Biosystems, Heidelberg, Germany). A total of 3 μl of cDNA was used for amplification in the SYBR Green format using the LightCycler-FastStart DNA Master SYBR Green 1 Kit from Roche (2 239 264). Amplification was performed with 5 mM MgCl₂ and 40 cycles with 1 s, 95°C, 10 s, 60°C, and 25 s, 72°C. Fluorescent signals generated during the informative log-linear phase are used to calculate the relative amount of template DNA.

The following primers were used: TNF, 5'-CAC AGG GAA GAG TTC CCC AG and 3'-CCT TGG TCT GGT AGG AGA CG (325 bp); IRAK-1, 5'-AAA GGA GGC CTC CTA TGA CC and 3'-ATG ATG CAG TGC TGG CAA G (441 bp); and IRAK-2, 5'-CTG AGG ATG ATT AAC AGG AGG and 3'-CCA CCA GCA GTA AGA CAT TGG (453 bp).

The mRNA expression was shown as fold difference compared with untreated controls.

Plasmids and transfection

The pTNF-1064 luci β-β reporter plasmid containing the human TNF 5' region was obtained by exchanging the mouse β-globin promoter of the pJPTATA luci-β reporter plasmid for the HindIII/BglII fragment from the TNF-5' region pvp2 luciferase construct (3). The resultant pJPTATA luci-β reporter plasmid contains 3' to the luciferase gene the rabbit β-globin intron (26).

Mono Mac 6 cells were transfected with this construct (5 μg of DNA/ 10^5 cells), according to Shakhov et al. (27), using DEAE-dextran (62.5 μg/ml; E1210; Promega, Madison, WI), as described in detail previously (10). 24 h after transfection, the culture medium, Mono Mac 6 cells were washed and stimulated for 10 min (cytoplasmic protein extracts), 1 h (nuclear extracts and RT-PCR), 4 h (transfection experiments), or 5 h (intracellular cytokine staining) with 10 μg/ml Pam 3 Cys, or with 1 μg/ml LPS.

Nuclear extracts were isolated, according to Dignam et al. (28), in the presence of a protease inhibitors' mixture and admixed with a β̅-labeled double-stranded oligonucleotide representing the −605 NF-κB motif of the human TNF promoter, as described previously (29). After 15 min of incubation at 21°C, samples were electrophoresed on nondenaturing polyacrylamide gels in 0.25 TBE buffer (22.5 mM Tris borate, 0.5 mM EDTA, pH 8.5). Gels were dried and exposed for 24–48 h at ~80°C to x-ray films.

Western blotting

Cytoplasmic protein extracts (20 μg/lane) were separated after 10 min, as previously described (10), on 4–12% Tris-glycine gels (EC63085; Invitrogen Life Technologies), followed by electroblotting using Xcell SureLock MiniCell & Xcell II Blot Module (E10002; Invitrogen Life Technologies). Blots were then reacted with Abs specific for: IRAK-1 (sc-5287), MyD88 (sc-6546), actin (sc-8432) (all from Santa Cruz Biotechnology, Santa Cruz, California), IκBα (9243), and IκBα-5β′ (9241) (both from New England Biolabs, Frankfurt/Main, Germany), followed by appropriate goat anti-rabbit/mouse peroxidase-conjugated Abs (Sigma-Aldrich), development with ECL reagent (RPN2106; Amersham, Braunschweig, Germany), and exposure to Hyperfilm (RPN3103; Amersham). The specificity of stainings was corroborated, using as a positive control appropriate cytoplasmic protein.
extracts provided by the manufacturer. Blots were scanned and then analyzed using the analyzer program (Soft Imaging System, Münster, Germany).

**Statistical analysis**

Data were analyzed with the software program SchoolStat (version 1.0.7; WhiteAnt Occasional Publishing, Newton, MA) or GraphPad InStat (GraphPad, San Diego, CA). Significance analysis was performed using a paired Student’s t test, and two-sided p values <0.05 were considered significant.

**Results**

*Induction of TNF by Pam<sub>3</sub>Cys*

When the CD14-positive monocytic cell line Mono Mac 6 is stimulated with Pam<sub>3</sub>Cys at 10 μg/ml, then TNF protein is rapidly expressed within 5 h. This can be detected within the cytoplasm of cells stimulated in the presence of the protein transport blocker, brefeldin A. As shown in Fig. 1 (right panel), intracellular staining with anti-TNF mAbs gives a clear signal in flow cytometry analysis as compared with staining with an isotype control. When the anti-TNF staining is performed in the presence of a 10-fold molar excess of rTNF, then the signal is reduced almost to the level of the isotype control, thereby demonstrating the specificity of the staining. Only a negligible signal is observed in unstimulated Mono Mac 6 cells (Fig. 1, left panel).

In dose-response analysis (Fig. 2), Pam<sub>3</sub>Cys induces a weak, but clear response at 1 μg/ml (average median fluorescence intensity of 3.2 channels) with a maximum expression at 100 μg/ml (20.3 channels).

**Blockade of TNF induction by Pam<sub>3</sub>Cys using anti-receptor Abs**

We then analyzed the type of receptors involved in Pam<sub>3</sub>Cys stimulation of Mono Mac 6 cells. In these studies, a set of two anti-TLR2 mAbs (clones TL2.1 and TL2.3) reduced TNF protein expression by ~50%, as did the anti-CD14 mAb, MY-4 (Fig. 3). The combination of the anti-TLR2 mAbs with the anti-CD14 mAb reduced Pam<sub>3</sub>Cys-induced TNF expression to background level, while the addition of isotype controls, in the same final concentration as anti-TLR2 and anti-CD14 mAbs, had no effect. These data indicate that Pam<sub>3</sub>Cys does use the CD14-TLR2 receptor complex for signal transduction.

**Induction of tolerance by Pam<sub>3</sub>Cys**

We have next asked the question as to whether Pam<sub>3</sub>Cys can induce a state of tolerance in Mono Mac 6 cells. For this purpose, the cells were first precultured for 42 h in the presence of 1 μg/ml Pam<sub>3</sub>Cys, and after washing were stimulated (in the presence of brefeldin A) for 5 h with 10 μg/ml Pam<sub>3</sub>Cys. As expected, Mono Mac 6 cells that were precultured without Pam<sub>3</sub>Cys (naive cells) gave a strong intracellular TNF signal after Pam<sub>3</sub>Cys stimulation (Fig. 4, second panel). By contrast, when the cells were precultured in the presence of the low Pam<sub>3</sub>Cys dose, followed by

![FIGURE 1. Induction of TNF protein by Pam<sub>3</sub>Cys in Mono Mac 6 cells. Mono Mac 6 cells were either untreated (left panel) or stimulated with Pam<sub>3</sub>Cys at 10 μg/ml (right panel) for 5 h in the presence of brefeldin A. Cells were permeabilized and stained with either PE-conjugated isotype control or PE-conjugated anti-TNF mAbs admixed with a 10-fold molar excess of rTNF. One representative example of five is shown.]

![FIGURE 2. Dose-response analysis for Pam<sub>3</sub>Cys-induced TNF production. Mono Mac 6 cells were cultured for 5 h with various doses of Pam<sub>3</sub>Cys in the presence of brefeldin A. Cells were permeabilized and stained with either PE-conjugated isotype control or PE-conjugated anti-TNF mAbs. Fluorescence intensity for each dose of Pam<sub>3</sub>Cys is expressed as specific median (in channels). The average of five experiments ± SD is given. * p < 0.05 as compared with the unstimulated cells.]

![FIGURE 3. Blockade of Pam<sub>3</sub>Cys-induced TNF production in Mono Mac 6 cells by anti-receptor Abs. Fifteen minutes before Pam<sub>3</sub>Cys stimulation, mAbs or their combinations (each mAb at final concentration of 10 μg/ml) and relevant isotype controls were added to the cells. Mono Mac 6 cells were then stimulated for 5 h with 1 μg/ml Pam<sub>3</sub>Cys in the presence of brefeldin A. Thereafter, the cells were permeabilized and stained with either PE-conjugated isotype control or PE-conjugated anti-TNF mAbs. Fluorescence intensity is expressed as specific median and shown as percentage of Pam<sub>3</sub>Cys stimulation (second column). The average of three experiments ± SD is given. * p < 0.05 as compared with the culture with isotype controls (sixth column).]
PamCys (10 μg/ml) stimulation for 5 h, then there was no intracellular TNF expression observed (Fig. 4, fourth panel). This demonstrates that the cells have become tolerant to PamCys stimulation. In an average of three experiments, the PamCys-induced TNF expression was 8.5 ± 0.9 channels in the naive cells, and it was reduced to 0.1 ± 0.7 channels in tolerant cells (p < 0.05).

We then analyzed whether the down-regulation of TNF protein in PamCys tolerance is regulated at the transcriptional level. For this purpose, we performed RT-PCR using the LightCycler technology for real-time monitoring of cDNA amplification. In Fig. 5A, the SYBR Green fluorescence, as a measure of dsDNA on the y-axis, is plotted vs cycle number on the x-axis. When analyzed at a fluorescence intensity of 3 U, unstimulated Mono Mac 6 cells reached this level at 25.8 cycles, i.e., they express appreciable amounts of TNF-mRNA. After stimulation with 10 μg/ml PamCys, the same level of fluorescence intensity was reached at 20.1 cycles, reflecting an increase of TNF-mRNA by the factor of 52. Preculture of Mono Mac 6 cells with 1 μg/ml PamCys reduced the constitutive TNF-mRNA (3 U at 27.3 cycles), and the stimulation of the precultured cells with 10 μg/ml PamCys increased the expression only by the factor of 4 (3 U at 25.2 cycles). The level reached by the stimulation of the tolerant cells only minimally exceeded the level seen in unstimulated naive cells (Fig. 5A). The levels for the constitutively expressed α-enolase gene were comparable for all four samples (Fig. 5B).

Tolerance was not restricted to TNF, because when looking at IL-8 mRNA by LightCycler technology expression in PamCys-tolerant cells was 10-fold lower (n = 4).

To demonstrate that down-regulation of TNF-mRNA in PamCys tolerance is due to reduced trans activation of the TNF gene, we transfected Mono Mac 6 cells with the pTEN-1064 luci-β reporter plasmid containing the human TNF promoter sequence up-stream of the luciferase reporter gene. Transfected cells were then precultured and stimulated with PamCys. In these studies, in naive cells trans activation was 16-fold, while the PamCys-tolerant cells showed only a 2-fold trans activation (Fig. 6). These data indicate that PamCys tolerance is regulated at the transcriptional level.

Receptor expression in PamCys-tolerant cells

We therefore asked the question: which mechanism upstream of transcription might be responsible for the trans activation failure in PamCys tolerance? We first analyzed expression of the two cell surface receptors involved in PamCys-monocyte activation. In this study, after 42-h preculture of Mono Mac 6 cells with 1 μg/ml PamCys, CD14 expression increased dramatically. Furthermore, TLR2 cell surface expression showed an increase from an average of 12 channels to an average of 17 channels (Fig. 7). In parallel, in the LightCycler experiments, we found the CD14-mRNA expression to be increased by the factor of 17 and the TLR2-mRNA expression to be unchanged (data not shown).

**Gel-shift analysis in PamCys tolerance**

NF-κB is a crucial transcription factor for the regulation of TNF gene expression. LPS tolerance has been shown to act via NF-κB, either by reduced mobilization or by shifting to p50p50 homodimers. We therefore performed gel-shift analysis using the −605 NF-κB motif from the human TNF promoter (GGGGCT GTGCC). In these studies, we confirmed the intact mobilization of p50p65 heterodimers and the strong induction of p50p50 homodimers in the LPS-tolerant Mono Mac 6 cells (Fig. 8, lane 7). In the case of PamCys tolerance, an entirely different pattern emerged in that there was only a slight increase in p50p50 and a failure to mobilize p50p65 (Fig. 8, lane 4).

This indicates that the signaling cascade leading from the cell surface receptor to the mobilization of the classical NF-κB p50p65 heterodimer is blocked in PamCys tolerance.

**Analysis of the PamCys-triggered signaling cascade**

Of the elements of the NF-κB signaling cascade, the MyD88, IRAK-1, IxBα, and IxBα-Fser 32 proteins were studied by Western blotting. As shown in Fig. 9A, the MyD88 protein expression did not change with PamCys stimulation and during tolerance induction. By contrast, IxBα was degraded upon PamCys stimulation (lane 2), but it remained unchanged in stimulated tolerant cells (lane 4). Serine-phosphorylated IxBα did show an inverse pattern, in that it was only detectable in PamCys-stimulated naive cells (lane 2) and there was no phosphorylation in PamCys-stimulated tolerant cells (lanes 4). These data indicate that elements upstream of IxBα phosphorylation and degradation are blocked in PamCys tolerance.

By contrast, when looking at LPS tolerance in the Mono Mac 6 cells, then IxBα degradation was evident after LPS stimulation both in naive and in tolerant cells (Fig. 9B).

We then studied IRAK-1 during PamCys tolerance. As shown in Fig. 10, upper panel, IRAK-1 protein was strongly expressed in naive cells. In PamCys-tolerant cells, however, no signal for IRAK-1 was detectable. When analyzing blots by densitometry (Fig. 10, left-hand graph in upper panel), then PamCys-tolerant
cells in an average of six experiments gave only a background signal (<5% of the level in naive cells). In contrast, LPS from *S. minnesota* only partially depleted IRAK-1 protein after preculture (Fig. 10, middle panel). Because this commercial LPS may still contain some lipoprotein, we also looked at a highly purified form of LPS from *S. abortus equi* (kindly provided by C. Galanos). Also, with this type of LPS, IRAK-1 protein was only moderately reduced in tolerance (Fig. 10, lower panel). Densitometry showed that in an average of three experiments the level of IRAK-1 in tolerant cells was 36% for LPS *S. minnesota* and 39% for *S. abortus equi* (+/+ lane in comparison with the naive cells). Thus, in LPS-tolerant cells the IRAK-1 adaptor protein, albeit at lower levels, is still available for signaling.

By contrast, in Pam₃Cys tolerance, IRAK-1 protein is completely ablated such that no signaling through this pathway can occur.

*Time course of IRAK-1 expression in Pam₃Cys-stimulated Mono Mac 6 cells*

Next, we analyzed the time course for IRAK-1 mRNA and protein expression in Mono Mac 6 cells precultured for up to 48 h in the presence of 1 μg/ml Pam₃Cys. As shown in Fig. 11A, the mRNA levels showed some variation over time, but up to the 24-h time
extracts were prepared. They were tested for binding to the
/H11002 regulation during tolerance induction after Pam3 Cys stimulation.

Pam3 Cys to Pam3 Cys does not affect its TNF-inducing capacity (33). Furthermore, in experiments comparing LPS and Pam3 Cys, intracellular TNF protein accumulation could not be induced anymore. The same pattern was evident at the TNF-mRNA level. The action of Pam3 Cys cannot be ascribed to contaminant LPS, because in previous studies we have shown that addition of polymyxin B to Pam3 Cys does not affect its TNF-inducing capacity (33). Furthermore, in experiments comparing LPS and Pam3 Cys in this study, we show that LPS induces p50 homodimers, while Pam3 Cys has little such activity, thus demonstrating that Pam3 Cys cannot contain appreciable amounts of LPS.

The high precision and sensitivity of the LightCycler analysis allowed us to also demonstrate a decrease of the basal level of TNF-mRNA in tolerant cells. Stimulation of these tolerant cells by Pam3 Cys showed some induction of the TNF-mRNA. This induction was, however, substantially lower than that seen after stimulation of naive cells, and it barely exceeded the basal level found in naive Mono Mac 6 cells (Fig. 5A). These data clearly show that Pam3 Cys tolerance occurs at the transcript level. Similar to what has been observed with LPS tolerance (10), trans activation directed by the TNF promoter is also clearly reduced in Pam3 Cys-tolerant cells. This indicates that Pam3 Cys tolerance acts by decreasing transcription of the gene rather than by increasing mRNA decay.

We have therefore analyzed crucial upstream elements that are involved in regulation of TNF gene transcription. The earliest step at which tolerance might occur is at the level of the cell surface receptor. In this study, Wang et al. (34) have shown by Western blots in lipoprotein-tolerant cells a down-regulation of TLR2 in cellular lysates. In the present study, CD14 and TLR2 showed strong and moderate cell surface expression in tolerant cells, respectively. This indicates that Pam3 Cys tolerance does not act by down-regulating these cell surface receptors, but we have not studied expression of TLR1, which is the partner for TLR2 in Pam3 Cys signaling (35). In a study by Nomura et al. (36), murine macrophages tolerant to LPS failed to stain with an Ab that recognizes the complex of TLR4 and MD-2, which suggests that these molecules are down-regulated or that they do not interact anymore. Currently, we cannot exclude the possibility that, in Pam3 Cys tolerance, there is a disruption of the interaction of the TLR1, TLR2, and CD14 receptor components.

Discussion

In the present study, we analyzed whether tolerance induction by Pam3 Cys can be achieved in human monocytes and what mechanisms might be operative. We showed that Pam3 Cys is an efficient inducer of TNF in the human monocytic Mono Mac 6 cell line and that this induction can be blocked completely by a combination of anti-TLR2 and anti-CD14 Abs. This is consistent with earlier studies that reported Pam3Cys to act via TLR2 (30–32).

When preincubating Mono Mac 6 cells with a low dose of Pam3 Cys for 2 days, followed by stimulation with a high dose of Pam3 Cys, intracellular TNF protein accumulation could not be induced anymore. The same pattern was evident at the TNF-mRNA level. The action of Pam3 Cys cannot be ascribed to contaminant LPS, because in previous studies we have shown that addition of polymyxin B to Pam3 Cys does not affect its TNF-inducing capacity (33). Furthermore, in experiments comparing LPS and Pam3 Cys in this study, we show that LPS induces p50 homodimers, while Pam3 Cys has little such activity, thus demonstrating that Pam3 Cys cannot contain appreciable amounts of LPS.

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the p50p50 homodimer mechanism and Pam₃Cys via interruption of the NF-κB signaling cascade.

Recent studies indicate that the IRAK-1 protein may be a crucial switch in the induction of tolerance in monocytes/macrophages (9, 37–39). It has been shown that IRAK-1 protein is degraded in LPS-tolerant THP-1 cells, and this can be prevented by pretreatment with GM-CSF and IFN-γ (40). Therefore, the level of IRAK-1 protein expression appears to be a main factor influencing activation of IRAK-1-dependent genes.

In our study, Pam₃Cys-induced, TLR2-dependent tolerance is associated with a complete depletion of IRAK-1 protein expression in the cytoplasm of Mono Mac 6 cells. Time course analyses have shown that pretreatment with Pam₃Cys at 1 μg/ml will gradually deplete IRAK-1 protein over several hours with a significant decrease first seen at 4 h posttreatment. This slow depletion is consistent with studies by Adib-Conquy and Cavaillon (40), who reported on a clear decrease of IRAK-1 protein only after 6 h of treatment when using a low dose of LPS. Higher doses of a stimulus may result in more rapid depletion of the protein, as seen by Li et al. (9) for LPS in THP-1 cells.

Of note, depletion of IRAK-1 protein did not accompany a depletion of the respective mRNA (Fig. 11, A and C). Adib-Conquy and Cavaillon (40) also noted no decrease of IRAK-1 mRNA when the protein expression decreased. These data suggest that IRAK-1 protein is either less efficiently synthesized or more rapidly degraded in cells tolerized with either Pam₃Cys or LPS. Reduced synthesis may be due to translational silencing, as shown for T cell-restricted intracellular Ag-1, which acts on the TNF gene (41). No such silencer has been described for IRAK-1 protein synthesis, as yet. In contrast, given the previous findings of IRAK-1 phosphorylation and ubiquitination (9, 40), which target protein degradation, we assume that degradation is the relevant mechanism. Pulse-chase analysis is, however, required to distinguish between these possibilities.

Although we see a strong depletion of IRAK-1 with induction of Pam₃Cys tolerance, this protein is only moderately reduced during LPS tolerance (Fig. 10) such that NF-κB mobilization can still occur upon stimulation with LPS. This is in contrast to other studies (9, 40, 42), which suggested IRAK-1 degradation to be the relevant mechanism in LPS tolerance. In studies not shown, we have seen no change of IRAK-1 protein in THP-1 cells that were pretreated with LPS from Escherichia coli (Sigma-Aldrich; L4391). Our data in Mono Mac 6 and THP-1 suggest that a complete ablation of IRAK-1 by LPS may depend on the type and purity of LPS. We propose that TLR2-dependent IRAK-1 depletion is the specific mechanism operating during Pam₃Cys-triggered tolerance induction. This depletion is demonstrated in this study in Mono Mac 6 cells, and also in our studies on THP-1 and primary monocytes. In the latter studies, pretreatment with Pam₃Cys led to an 82% decrease of IRAK-1 protein for THP-1 and an 83% decrease for monocytes (data not shown).

Dobrovolskaia et al. (43) demonstrated a reduced IRAK-1 kinase activity in Pam₃Cys tolerance in murine peritoneal macrophages, but IRAK-1 protein levels were reported to be unchanged. This difference to our findings is probably due to differences in species (mouse vs human) and/or cell type (peritoneal cells vs monocytic cells).

Changes in IRAK-1 protein expression and activity after LPS/TLR4-dependent tolerance induction observed in other studies may be partially dependent on the use of commercially available LPS contaminated with other bacterial cell wall compounds, such as lipoproteins (44, 45). For Porphyromonas gingivalis natural
lipid A, it was recently shown that contamination by only trace amount of lipopeptide may induce cell activation via TLR2 (46). These lipoproteins may target TLR2 and thereby induce IRAK-1 degradation. In contrast, differences in cell types and treatment protocols may explain the more pronounced IRAK-1 protein depletion seen with LPS in other studies.

The association of TLR2 with other TLRs, i.e., with TLR1 and TLR6 (35, 47, 48), may be of importance for the mechanism of tolerance induction. For example, pretreatment of cells with TLR2/6-dependent mycoplasma lipopeptide-2 only slightly decreases IRAK-1 protein expression and IRAK-1 phosphorylation (49), while lipopeptide Pam3Cys, which appears to act via TLR2/1, completely depletes IRAK1, as shown in the present study. This depletion can be explained in part by a decrease in IRAK-1-mRNA expression, but with 30% of the mRNA remaining, we assume that additional active protein degradation may substantially contribute to the complete disappearance of IRAK-1 protein in tolerant cells.

Similar to the action of Pam3Cys, triggering via TLR7 with imidazoquinoline R-848 substantially reduces IRAK-1 protein in peritoneal macrophages, although some protein remains (49). Taken together, tolerance induced by triggering different TLRs (TLR2, TLR7, and to some extent TLR4) may target IRAK-1 degradation. IRAK-1 may also be crucial to tolerance without being degraded, e.g., release of IRAK-1 from the TLR5 complex was shown to be blocked in flagellin-induced tolerance (38). Of note, we have recently shown that tolerance induction in monocytes by incubation with tumor cells will also decrease IRAK-1 protein (50), suggesting that the IRAK-1 mechanism may not be restricted to tolerance induced by microbial products.

Upstream of IRAK-1 the MyD88 adaptor may determine tolerance. Medvedev et al. (8) have shown that after LPS tolerance induction there is a failure of MyD88 to be recruited to TLR4. There is the possibility that following LPS stimulation, MyD88 could be alternatively spliced such that IRAK-1 phosphorylation and/or autophosphorylation are inhibited (51, 52). In our experiments, we did not observe a change in MyD88 protein expression, but the splice variant may not readily show in Western blots.

In Pam3Cys tolerance, with the complete disappearance of IRAK-1 protein, it is clear that no signaling can occur through this kinase. It is, however, unclear whether the depletion of IRAK-1 in Pam3Cys tolerant cells is sufficient to explain the degree of blockade of TNF gene expression. It appears that LPS signaling can still occur in IRAK-deficient mouse cells (53). There may be a role for IRAK-4 and IRAK-M in tolerance after repeated TLR stimulation, as IRAK-4 may be deficient or functionally inactive (52, 54–56) or IRAK-M may be overexpressed (57, 58). In fact, IRAK-M has been shown to be up-regulated in the RAW264.7 mouse macrophage cell line made tolerant by high doses of peptidoglycan (59).

In our studies, we also observed an up-regulation by 20% of IRAK-2 mRNA in tolerant Mono Mac 6 cells (data not shown). Recently, Ruckdeschel et al. (60) described that Yersinia enterocolitica-induced apoptosis involves a proapoptotic signal delivered through MyD88 and IRAK-2, which potentially targets the Fas-associated death domain protein/caspase-8 apoptotic pathway, whereas IRAK-1 and TNFR-associated factor 6 counteract the bacteria-induced cytotoxic response by signaling macrophage survival. Hence, it is possible that in tolerant monocytes, an imbalance of IRAK-2 over IRAK-1 may lead to apoptosis. Therefore, apoptosis mediated by changes of the IRAK proteins could be another mechanism of tolerance induction in monocytes/macrophages.

In addition to the classical mechanism of tolerance that involves NF-κB p50p50 homodimers and the mechanisms targeting IRAK-1, recent studies have shown a role for suppressor of cytokine signaling 1 which potentially targets the classical NF-κB pathway in TNF gene expression, but suppressor of cytokine signaling 1 might also act by binding to IRAK-1 (61).

**FIGURE 11.** Time course of IRAK-1 expression in Pam3Cys-stimulated Mono Mac 6 cells. Mono Mac 6 cells were stimulated for indicated (0.5–48 h) periods of time with 1 μg/ml Pam3Cys. A, Real-time monitoring of cDNA amplification using the LightCycler technology was applied. Isolated RNA was reverse transcribed and amplified for IRAK-1 and α-enolase as a control. Results were normalized for α-enolase expression. Reduction of IRAK-1 mRNA expression in cells pre cultured for 48 h was down to 33.8 ± 8.6% in comparison with 0-h, untreated cells. Average of IRAK-1 mRNA expression, in comparison with unstimulated Mono Mac 6 cells ± SD (in relative units) from five independent experiments is shown; *, p < 0.01 as compared with naive cells. B, One representative time course of a Western blot for IRAK-1 protein expression in Pam3Cys-precultured Mono Mac 6 cells is shown. C, Western blots were analyzed by densitometry and normalized for actin content, and the average results from four independent experiments ± SD (in gray relative units), in comparison with 0-h cells, are given. Reduction of IRAK-1 protein content in stimulated cells was down to 48.8 ± 26.5% after 4 h, 37 ± 19.9% after 6 h, 2.5 ± 1.3% after 24 h, and 2.6 ± 1.6% after 48 h of Pam3Cys stimulation; *, p < 0.05 as compared with naive cells; **, p < 0.01 vs naive cells.
Taken together, our findings demonstrate that in the same type of cell two different mechanisms of tolerance can coexist: 1) induction of p50 homodimers for LPS/TLR4 signals, and 2) deple tion of IRAK-1 protein for Pam3CSK4/TLR2 signals. This indicates that it may be of importance to the host to ensure down-regulation of TNF and to prevent the detrimental effects of excessive amounts of this cytokine after repeated stimulation, i.e., during a persistent bacterial infection that will involve engagement of more than one type of TLR.

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References


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