LPS and Taxol Activate *Lyn* Kinase Autophosphorylation in Lps^n, but Not in Lps^d, Macrophages

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ABSTRACT

Background: The anti-tumor agent, Taxol, has been shown in murine macrophages to stimulate tumor necrosis factor (TNF), modulate TNF receptors, induce a large panel of immediate-early genes, and induce protein tyrosine phosphorylation indistinguishably from LPS. These data, coupled with the finding that lipid A antagonists block Taxol-induced stimulation, support the hypothesis that these two structurally unrelated compounds activate a common, receptor-associated signaling apparatus. A very early event in LPS signaling of human monocytes is activation of *lyn* kinase activity. We therefore sought to evaluate the activation of *lyn* kinase by LPS and Taxol in LPS-responsive (Lps^n) and LPS-hyporesponsive (Lps^d) macrophages.

Materials and Methods: C3H/OuJ (Lps^n) and C3H/HeJ (Lps^d) macrophages were stimulated by LPS or Taxol. Cell lysates were subjected to immunoprecipitation with anti-*lyn* antibody, gel electrophoresis, and in vitro kinase assays. Autoradiography and PhosphorImager analysis were carried out to detect incorporation of ^{32}P into *lyn* protein.

Results: Within seconds of stimulation, LPS and Taxol induce in Lps^n macrophages a depression of autophosphorylation, followed within minutes by autophosphorylation of both p53 and p56 *lyn* species. Lps^d macrophages respond to LPS and Taxol with the initial decrease in activity, but fail to respond to LPS with autophosphorylation, and respond only to a limited extent upon Taxol stimulation. Tyrosine phosphatase inhibitors exerted inhibitory effects on LPS stimulation of *lyn* autophosphorylation.

Conclusions: Decreased *lyn* kinase activity within seconds and autophosphorylation within minutes of LPS or Taxol stimulation in Lps^n macrophages strongly supports the hypothesis that LPS and Taxol share a common signaling pathway. The finding that C3H/HeJ macrophages respond to LPS and Taxol with a normal depression of *lyn* activity, but fail to autophosphorylate *lyn* normally in response to LPS or Taxol, suggests that the Lps^d defect is distal to LPS-receptor interaction. Finally, the inhibitory effect of tyrosine phosphatase inhibitors on LPS-induced *lyn* autophosphorylation suggests that tyrosine phosphatase(s) may participate in the regulation of *lyn* kinase activity.

INTRODUCTION

The chemotherapeutic diterpene, Taxol (paclitaxel), originally isolated from the bark of the Pacific yew *Taxus brevifolia*, is now produced commercially by biochemical semisynthesis from related taxanes. Taxol has been shown to be clinically effective in 30% of ovarian cancer patients (1) and 25% of metastatic breast cancer patients (2). Though the main mechanism of Taxol's chemotherapeutic activity is known to be inhibition of cell proliferation by stabilization of α/β tubulin depolymerization (3), recent studies have shown that Taxol has other intracellular

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effects that may contribute to its efficacy, particularly in macrophages. In murine macrophages, Taxol has been shown to mimic the activity of gram-negative bacterial endotoxin. Both lipopolysaccharide (LPS), the most toxic fraction of endotoxin, and Taxol induce TNF secretion (4) and the rapid involution of TNF receptors (4), and stimulate indistinguishable patterns of protein-tyrosine phosphorylation and immediate-early gene expression (5–7). Moreover, like LPS, Taxol provides a second signal to interferon-γ-primed macrophages to induce tumoricidal activity in vitro (8). Although LPS and Taxol are structurally unrelated, induction of early inflammatory response genes and tyrosine phosphorylation induced by Taxol can be blocked by antagonists that are structurally related to LPS (7). The response of murine macrophages to Taxol has been linked to the gene that controls LPS responsiveness (*Lps*^{n}) (4). Thus, the LPS-mimetic effects of Taxol are only observed in macrophages derived from LPS-responsive (*Lps*^{n}) mouse strains, and not in the LPS-hyporesponsive (*Lps*^{d}) C3H/HeJ strain (9). Taken collectively, the data suggest that Taxol and LPS share a common receptor and/or signal transducing elements.

An early signaling event known to occur within minutes in human macrophages in response to LPS is the CD14-associated activation of *lyn* kinase (10). To assess the possibility that *lyn* kinase is a common signaling molecule in LPS- and Taxol-induced effects, we compared LPS and Taxol for the ability to activate *lyn* kinase in both LPS-responsive *Lps*^{n} and LPS-hyporesponsive *Lps*^{d} murine macrophages.

MATERIALS AND METHODS

Macrophage Culture and Reagents

Five- to six-week-old C3H/OuJ (*Lps*^{n}) or C3H/HeJ (*Lps*^{d}) mice (Jackson Laboratory, Bar Harbor, ME, U.S.A.) were used as the source of macrophages for all studies. Mice were housed in laminar flow hoods and fed autoclaved mouse chow and acidified water ad libitum until use. Thioglycollate-elicited, peritoneal exudate macrophages were cultured in RPMI 1640 containing 2% fetal calf serum (Hyclone, Logan, UT, U.S.A.) at 20–30 × 10^6 cells per well in 100-mm culture plates (Falcon Plastics, Oxnard, CA, U.S.A.) and incubated at 37°C and 6% CO_2 overnight, as described in detail elsewhere (11). After 20 h incubation, nonadherent cells were removed by washing with fresh medium. Cells were then incubated with medium only or with medium containing the indicated concentrations of phenol/water-extracted *Escherichia coli* K235 LPS (12) or Taxol (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A.) for the indicated time course. Taxol was stored before use as a 20 mM solution in DMSO at −70°C. Macrophage cultures were maintained at 37°C with 6% CO_2 during experimental treatment. At the indicated times, media was aspirated and cells were lysed on ice in 500 μl ice-cold TNE buffer composed of 50 mM Tris Cl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40 (Fluka Chemical Corp, Ronkonkoma, NY), 0.1% DOC (Sigma Chemical Co., St. Louis, MO, U.S.A.), and 0.1% Tween 20. TNE lysis buffer was adjusted to the indicated final concentrations with the following protease inhibitors: 10 μg/ml aprotinin (Boehringer-Mannheim, Indianapolis, IN, U.S.A.), 10 μg/ml leupeptin (Boehringer-Mannheim), 5 mM NaF (Sigma), 4 mM PMSF (Sigma), 400 mM TLPCK (Sigma), and 4 mM sodium orthovanadate (Sigma). Macrophages were pretreated for 10 min with the tyrosine phosphatase inhibitor, phenylarsine oxide (PAO, Sigma), at a final concentration of 10 μM.

Kinase Assay on Cell Lysates

The in vitro kinase assay was performed essentially as described elsewhere (13). Cells were scraped vigorously from the plates with sterile cell scrapers to ensure quantitative isolation and preservation of protein components. Lysates were vortexed after sitting on ice for 10 min, were transferred to 1.5-ml screw cap tubes and centrifuged at 12,000 × *g* for 3 min to sediment insoluble cell fractions (pellets). For the kinase assay, lysates were immunoprecipitated at 4°C with optimized amounts of polyclonal rabbit anti-*lyn* antibody overnight on a serological rotator, followed by incubation with 100 μl per tube of Pansorbin *Staphylococcus aureus* cells (Calbiochem, San Diego, CA, U.S.A.) for 4–6 hr. Control immunoprecipitates were prepared using affinity purified rabbit anti-mouse IgG (Cappel/OrganonTeknika, Durham, NC). Lysates were also submitted to Western blot analysis for detection of relative amounts of *lyn* protein in each sample. To measure the ability of the isolated *lyn* kinase protein to autophosphorylate as measured by incorporation of ^{32}P, *lyn* immuno-

precipitates were first washed once each with low-salt TNE buffer containing 150 mM NaCl and, high-salt TNE buffer containing 1 M NaCl, and low-salt TNE buffer again, prior to resuspension of the Pansorbin cells in TPK buffer (20 mM MOPS, pH 7.0, and 5 mM $MnCl_2$). A master mix of 20 μl TPK buffer, 2.5 μl 100 μM ATP (Sigma), and 2.5 μl ATP-γ ^{32}P (10–25 mCi/mmol, ICN, Costa Mesa, CA, U.S.A.) per sample was prepared, and 25 μl of the mix was added to each pellet of Pansorbin cells. Samples were shaken vigorously in an Eppendorf thermomixer at 25°C for 10 min, prior to stopping the incorporation reaction with the addition of 25 μl 4 X protein loading buffer (20 ml glycerol, 4 g SDS, 25 ml 0.5 M Tris HCl containing 0.4% SDS [pH 6.8], 3.1 g dithiothreitol, 1 mg bromphenol blue, H_2O to 50 ml).

Electrophoresis by SDS PAGE and Quantitation by Autoradiography

Proteins were eluted from the Pansorbin in 4 X loading buffer at room temperature for 30 min. Samples were vortexed vigorously and boiled for 5 min prior to centrifugation at 12,000 × *g* in a microfuge for 3 min to sediment the Staphylococci. Samples of 50 μl each were loaded onto 9% SDS PAGE gels with 4% stacking gels and run at 45 V at constant current overnight. Bands were tightened with brief electrophoresis at 200 V. Gels were fixed and unincorporated label was eluted by soaking the gel in 2 changes for 1 hr each of a fixative composed of methanol:glacial acetic acid:water in the ratio of 3:1:6. Bands were visualized by 4-hr exposure of the dried gels to Kodak XAR5 film and label incorporation was quantified by PhosphorImager technology essentially as described elsewhere (11). Briefly, after measurement of each band by PhosphorImager analysis, the relative level of *lyn* autophosphorylation for each treatment was normalized to the level of autophosphorylation in medium-treated cells. Nonspecific PhosphorImager background signal due to nonspecific incorporation of ^{32}P from control samples immunoprecipitated with polyclonal rabbit anti-mouse antibody averaged 7.6% of the specific signal detected in medium-treated cells for all experiments (n = 17). For multiple experiments, the geometric mean of the relative autophosphorylation level demonstrated by identical treatment groups is presented.

Western Blot Analysis

Equivalent volumes of cell lysates or pellets were dissolved in 4 X protein loading buffer and 10–15 μl samples electrophoresed through 10% SDS PAGE, pH 8.35 with 4% stacking gels, pH 6.8 in a BioRad mini-Protean II at 100 V at constant current. Western transfers of proteins to Immobilon (Amersham Corp., Arlington Heights, IL, U.S.A.) membranes were prepared by electrophoretic transfer at 100 V for 1 hr at 4°C in Tris-Glycine-Methanol transfer buffer. Membranes were stained by incubation on a serological rotator in 1 μg/ml of the appropriate primary antibody diluted in TBS + 0.1% Tween 20 for 40 min at room temperature after blocking for 45 min or storage in blocking buffer containing 1% gelatin and 5% nonfat milk in TBS. After blocking and between primary and secondary antibody treatments, membranes were washed for optimized times in changes of TBS + 0.1% Tween 20. BiRad Affinity purified horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG antibody (BioRad) was diluted 1:2500 in TBS + 0.1% Tween 20 and used for secondary antibody incubations of 50 min at room temperature. Bands on the Western transfer were visualized by means of ECL (Amersham) chemiluminescent reagents and autoradiography on Kodak XAR5 film as described elsewhere (7).

RESULTS

LPS and Taxol Activation of *Lyn* Autophosphorylation

Autophosphorylation of *src*-related *lyn* kinase is activated by both LPS and Taxol in *Lps*^{n} mouse macrophages. Figure 1 is a representative autoradiogram of in vitro *lyn* kinase autophosphorylation induced by LPS. Figure 2 illustrates the pooled results of replicate experiments (n = 7 for LPS and n = 4 for Taxol), quantified by PhosphorImager analyses of the digitized images. The concentrations of LPS and Taxol used in these studies were chosen based on concentrations found previously to result in maximum signaling leading to gene induction and tyrosine phosphorylation (6–8). In contrast to human macrophages (10), murine macrophages exhibit a significant constitutive level of *lyn* activity (Fig. 1, Lane 2), as evidenced by two discrete bands of *lyn* kinase activity that correspond to p56 and p53 forms of *lyn*. When C3H/OuJ (*Lps*^{n}) macro-

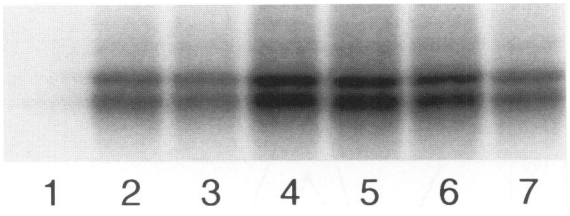

FIG. 1. Activation of p56 (top band) and p53 (bottom band) *lyn* kinase autophosphorylation by LPS

C3H/OuJ (Lps^n) macrophages were treated with medium only (resting cell kinase level), or stimulated with 100 ng/ml LPS. Lane 1, unstimulated cells immunoprecipitated with an irrelevant antibody; Lanes 2–6 were immunoprecipitated with anti-*lyn* antibody. Lane 2, unstimulated cells (resting cell kinase level); lane 3, 0.083 min; Lane 4, 5 min; Lane 5, 10 min; Lane 6, 15 min; and Lane 7, 30 min after LPS stimulation. *Lyn* kinase autophosphorylation was measured in the in vitro kinase assay as described in Materials and Methods by incorporation of ^{32}P. This autoradiogram is derived from a single representative experiment.

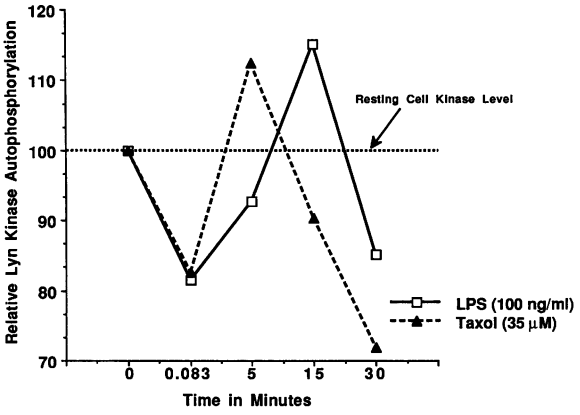

FIG. 2. Activation of *lyn* kinase autophosphorylation by LPS and Taxol in Lps^n macrophages

C3H/OuJ macrophages were treated as indicated with medium only, 100 ng/ml LPS, or 35 μM Taxol. Results of the kinase assay were quantified by PhosphorImager analysis and relative autophosphorylation levels represent the geometric means of seven identical experiments for LPS and four identical experiments for Taxol. Dashed horizontal line indicates the resting cell kinase expression level found in untreated cells. The standard error of the mean for LPS-stimulated C3H/OuJ macrophages was ≤4.5% of the geometric mean for each timepoint. The standard error of the mean for Taxol-stimulated C3H/OuJ macrophages was ≤5.8% of the geometric mean for each timepoint.

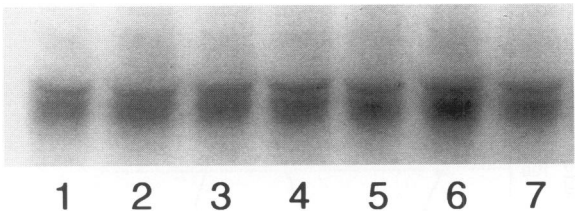

FIG. 3. Anti-*lyn* Western blot analysis of p56 and p53 *lyn* protein in cell lysates used for immunoprecipitation in the kinase assay shown in Fig. 1

Equivalent volumes of sample lysates were processed for Western blot analysis as described in Materials and Methods. Lanes 1 and 2, unstimulated cells; Lane 3, 0.083 min; Lane 4, 5 min; Lane 5, 10 min; Lane 6, 15 min; and Lane 7, 30 min after LPS stimulation. Molecular weights were calculated based on prestained protein standards.

phages were treated with 100 ng/ml LPS or 35 μM Taxol in 30-min time course experiments, there was an initial average depression of kinase activity (to ~80% of the resting cell kinase level) that was typically detectable at 5 sec (0.083 min) following stimulation. (Note that, in individual experiments, there was some variation as to the timing of this initial depression). Following this depression, *lyn* kinase activity increased (typically between 5 and 15 min) to above the level observed in resting cells, and then fell again to below the resting cell level by 30 min post-treatment with either LPS or Taxol. Based on the pooled data (Fig. 2), the peak of *lyn* activity occurred slightly earlier in Taxol- than in LPS-stimulated macrophages. Western blot analysis of both the lysates used for the immunoprecipitation (Fig. 3) for the presence of *lyn* protein failed to show changes in the amount of *lyn* protein that could account for the observed changes in the in vitro kinase assay. Therefore, the modulation observed in the kinase assay reflects a change in the specific activity of *lyn* rather than a change in the abundance of *lyn*.

Effect of LPS and Taxol on *lyn* Activity in C3H/HeJ (Lps^d) Macrophages

Resting cell levels of *lyn* kinase activity were found to be comparable in Lps^n and Lps^d macrophages. When LPS-hyporesponsive C3H/HeJ macrophages were compared to LPS-responsive C3H/OuJ macrophages for the ability of LPS to modulate *lyn* activity, the initial depression in *lyn* activity was indistinguishable (Fig. 4). However,

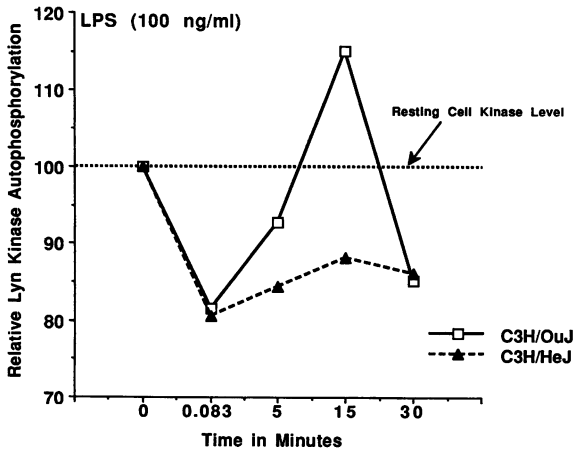

FIG. 4. Activation of *lyn* kinase autophosphorylation by LPS in C3H/OuJ (Lps^n) and C3H/HeJ (Lps^d) macrophages

Macrophages were treated with medium only or 100 ng/ml LPS. Results of the kinase assay were quantitated by PhosphorImager analysis and relative autophosphorylation levels represent the geometric means of seven identical experiments for C3H/OuJ macrophages (from Fig. 2) and five identical experiments for C3H/HeJ macrophages. Dashed horizontal line indicates the resting cell kinase expression level found in untreated cells. The standard error of the mean for LPS-stimulated C3H/HeJ macrophages was ≤2.6% of the geometric mean for each timepoint.

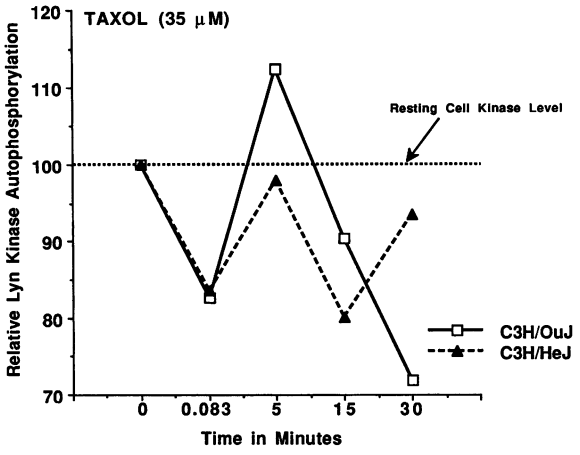

FIG. 5. Activation of *lyn* kinase autophosphorylation by Taxol in C3H/OuJ (Lps^n) and C3H/HeJ (Lps^d) macrophages

Macrophages were treated as indicated in Materials and Methods with medium only or 100 ng/ml LPS. Results of the kinase assay were quantitated by PhosphorImager analysis and relative autophosphorylation levels represent the geometric means of four identical experiments for C3H/OuJ macrophages (from Fig. 2) and five identical experiments for C3H/HeJ macrophages. Dashed horizontal line indicates the resting cell kinase expression level found in untreated cells. The standard error of the mean for Taxol-stimulated C3H/HeJ macrophages was ≤4% of the geometric mean for each timepoint.

LPS-hyporesponsive macrophages did not respond with a subsequent peak of *lyn* autophosphorylation above the resting cell level, though levels at 30 min were identical once again for both LPS-responsive and -hyporesponsive macrophages.

When C3H/HeJ macrophages were compared with LPS-responsive C3H/OuJ macrophages for the ability to respond to Taxol (Fig. 5), the initial depression of *lyn* kinase activity was, again, indistinguishable between the two strains. However, in Taxol-stimulated C3H/HeJ cells, a sub-basal, but measurable, increase of *lyn* activity was observed, in contrast to LPS-stimulated C3H/HeJ macrophages. The relative peak of *lyn* autophosphorylation coincided in LPS-responsive and -hyporesponsive macrophages treated with Taxol.

Effects of Phenylarsine Oxide (PAO) on *lyn* Activity

C3H/OuJ macrophages were pretreated for 10 min with PAO, a tyrosine phosphatase inhibitor. Although PAO did not eliminate the LPS-induced depression of activity, it did curtail the increase in autophosphorylation of *lyn* (Fig. 6) to resting cell levels. A 4-hr pretreatment of macrophages with sodium orthovanadate (1 mM), another potent tyrosine phosphatase inhibitor, also inhibited LPS-induced autophosphorylation without affecting the initial depression in kinase activity (data not shown).

DISCUSSION

Taxol has been found to serve as an LPS mimetic for patterns of protein tyrosine phosphorylation, gene induction, TNF release, TNF receptor recycling, the development of tumoricidal effects in murine macrophages (4–8), and NFκB translocation (P.-Y. Perera, unpublished observations). These commonalities imply that part or all of the signaling mechanisms for LPS and Taxol is shared. *Lyn* is a member of the *src* family of nontransmembrane protein tyrosine kinases. *Src* protein kinases are constitutively associated with

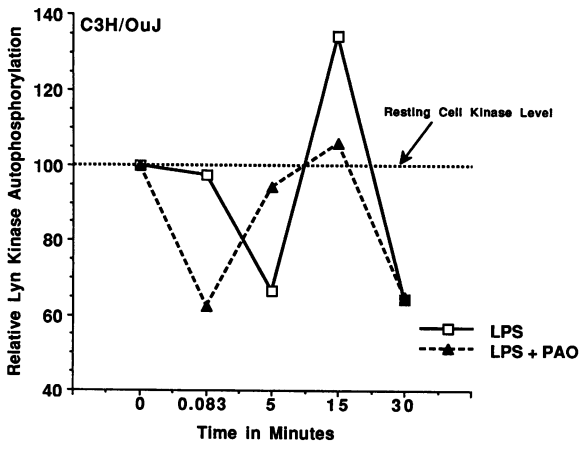

FIG. 6. Effect of phenylarsine oxide (PAO) on activation of *lyn* kinase autophosphorylation by LPS in C3H/OuJ (*Lps*^{n}) macrophages

Macrophages were treated as indicated with either medium only or 10 μM PAO for 10 min prior to incubation with medium only (0 min) or 100 ng/ml LPS for the indicated times. The results are the relative autophosphorylation levels of a single representative experiment (n = 3) quantified by PhosphorImager analysis. Dashed horizontal line indicates the resting cell kinase expression level found in untreated cells.

cellular membranes as the result of the first 16 residues of the protein in combination with covalent, cotranslational myristylation of glycine residue 2 (14). *Lyn* can also be further modified by post-translational palmitylation that occurs on cysteine residue 3 (14). Like other *Src* family members, *lyn* is thought to be maintained in an enzymatically repressed (inactive) conformation that is dependent upon the association of a phosphotyrosine residue in the carboxyterminal portion of the molecule with the enzyme's own aminoterminal SH2 domain (15). Thus, in this model, enzymatic activity is associated with a conformational change that exposes the active site of the enzyme, perhaps mediated by the action of a phosphatase that cleaves the carboxyterminal phosphate. Activation of *lyn* kinase is also associated with the autophosphorylation of a second tyrosine residue within the catalytic domain. CD14, a nonsignaling receptor for LPS, and its catalytic serum binding protein, LBP (16,17), have been shown to be associated transiently with *lyn* kinase after LPS stimulation. Since LPS hyporesponsiveness exhibited by C3H/HeJ mice has been attributed to a single gene defect (reviewed in Ref. 9), associated with membrane components (18), and genetically linked to Taxol unresponsiveness (4), we reasoned that analysis of *lyn* activation in LPS-defective macrophages might provide important insights into the nature of the *Lps*^{d} defect.

LPS-responsive C3H/OuJ macrophages responded to Taxol with activation of *lyn* autophosphorylation that is essentially identical to the LPS response, with the exception of timing (Fig. 2). This observation supports previous data that Taxol shares at least some of the LPS-response receptor signaling molecules (7). The average delay of the peak of LPS-induced *lyn* autophosphorylation activity may imply that Taxol interacts more efficiently with a molecule that is slightly upstream in the LPS signaling pathway. LPS-hyporesponsive C3H/HeJ macrophages did not respond to LPS with increased activation of *lyn*, although the initial depression in activity appeared to be C3H/OuJ-like. This common depression may be due to a specific, LPS-induced intracellular phosphatase that dephosphorylates the autophosphorylation site of *lyn*, thus rendering it less activated. Alternatively, inhibition of *lyn* activity could be related to the activation of a specific tyrosine kinase that selectively phosphorylates the carboxyterminal site, thus favoring the repressive conformation. Lack of a subsequent peak of *lyn* activity suggests that the defect in C3H/HeJ macrophages is expressed downstream of CD14 and/or other receptor-LPS interactions that lead to the initial depression in *lyn* activity and prior to the induction of *lyn* autophosphorylation that, in turn, may lead to the activation of downstream Ras, Raf, and MAP kinases (19). The suboptimal autophosphorylation response of Taxol-treated C3H/HeJ macrophages following the initial depression of activity may imply that Taxol is capable of eliciting signaling pathways that are independent of and in addition to those shared with LPS. Coupled with the observation that Taxol-induced autophosphorylation precedes that induced by LPS, this finding provides a second example of subtle cell signaling differences between Taxol and LPS stimulation of macrophages.

Signal transduction by a variety of different types of surface receptors requires the proper balance between the activities of protein tyrosine kinases and protein tyrosine phosphatases (20). PAO has been shown to be an effective inhibitor of protein tyrosine phosphatases and thereby may shift this balance towards the accumulation of protein tyrosine phosphorylation. It is thus possible that PAO treatment of *Lps*^{n} macrophages

causes *lyn* to undergo autophosphorylation at the catalytic site in the cell, thereby reducing the capacity of the enzyme to autophosphorylate in immune complex kinase assays. Alternatively, PAO may inhibit the action of an LPS-induced tyrosine phosphatase that acts normally to remove the carboxyterminal phosphate, thus rendering the active site of the enzyme exposed. It is possible that the failure of LPS to increase *lyn* activity in C3H/HeJ macrophages may represent a phosphatase dysregulation that is analogous to the effect of PAO pretreatment of C3H/OuJ macrophages.

In summary, *lyn* kinase is a common signaling element in response of macrophages to both Taxol and LPS. Modulation of *lyn* activity is initiated normally in C3H/HeJ macrophages, yet these cells fail to increase the level of *lyn* autophosphorylation, in contrast to C3H/OuJ macrophages. Taxol- and LPS-induced *lyn* activation is potentially under positive control by tyrosine phosphatase(s) since tyrosine phosphatase inhibitors exerted negative effects on LPS-induced *lyn* activity. Further search for intracellular proteins that physically interact with Taxol are likely to identify proteins shared in the LPS signal transduction mechanism.

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