Sequential Modifications in Class II Transactivator Isoform 1 Induced by Lipopolysaccharide Stimulate Major Histocompatibility Complex Class II Transcription in Macrophages

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By presenting antigenic peptides on major histocompatibility complex class (MHC) II determinants to CD4+ T cells, macrophages help to direct the establishment of adaptive immunity. We found that in these cells, lipopolysaccharide stimulates the expression of MHC II genes via the activation of Erk1/2, which is mediated by Toll-like receptor 4. Erk1/2 then phosphorylates the serine at position 357, which is located in a degron of CIITA isoform 1 that leads to its monoubiquitylation. Thus modified, CIITA isoform 1 binds P-TEFb, which mediates the elongation of RNA polymerase II and co-transcriptional processing of nascent transcripts. This induction leads to the expression of MHC II genes. Subsequent polyubiquitylation results in the degradation of CIITA isoform 1. Thus, the signaling cascade from Toll-like receptor 4 to CIITA isoform 1 represents one connection between innate and adaptive immunity in macrophages.

The immune system is composed of innate immunity, which performs the function of immune surveillance, and adaptive immunity, which eliminates non-self-antigens and creates the immune memory. Constituents of the former are antigen-presenting cells and of the latter are B and T cells. Because the establishment of adaptive immunity is dependent on innate immunity, appropriate interactions between them are indispensable for the normal function of the immune system.

Macrophages are antigen-presenting cells that remove and digest invading pathogens as well as present antigenic peptides, thus directing the immune response against them. They sense the presence of these pathogens via their pathogen-associated molecular patterns, such as lipopolysaccharide (LPS), flagellin, etc., which is mediated by Toll-like receptors (TLRs) (1). For example, the binding between LPS and TLR4 triggers a signaling cascade that results in the activation of p38 mitogen-activated protein kinase (p38 MAPK), extracellular regulated kinase 1/2 (Erk1/2), and Jun kinases (JNK), as well as of nuclear factor κB (NF-κB) (2). This signaling leads to the presentation of antigenic peptides in the groove of major histocompatibility complex class II (MHC II) molecules to CD4+ T cells and subsequently to the establishment of adaptive immunity (1). Because MHC II determinants are involved directly in the establishment of adaptive immunity, it is not surprising that their expression is tightly regulated. One of the levels of this regulation is at transcription and is mediated by the class II transactivator (CIITA).

Transcription of CIITA can be initiated from three distinct promoters called PI, PIII, and PIV, which direct the synthesis of three isoforms (IF) of CIITA: IF1, IF3 and IF4 (3). Different isoforms are expressed following distinct stimuli in different cells. Whereas CIITA IF1 is expressed in macrophages and myeloid dendritic cells, CIITA IF3 is expressed in B cells and plasmacytoid dendritic cells (4). Only one study has been performed on CIITA IF1 (5). In contrast, CIITA IF3 has been studied in great detail (6–12). CIITA isoforms do not bind to DNA. Rather they bind to the preformed enhanceosome on MHC II promoters, where they recruit general transcription factors (13–17) as well as coactivators (11, 18–21) and function as transcriptional integrators.

Transcription of eukaryotic genes is a highly coordinated process and is often regulated by post-translational modifications of activators. The most studied are post-translational modifications of proteins containing class II B or acidic activation domains (AADs). The phosphorylation of proteolytic signaling elements, called degrons, which are located in or near these AADs, leads to their monoubiquitylation and higher transcriptional activity. Subsequent polyubiquitylation finally results in their degradation (22). Indeed, by binding the positive

kinase; CIITA, class II transactivator; IF, isoform; AAD, acidic activation domain; P-TEFb, positive transcription elongation factor b; CycT1, cyclin T1; MEK, MAPK/Erk kinase; CAT, chloramphenicol acetyltransferase; ChIP, chromatin immunoprecipitation; RT, reverse transcription; ALLN, N-acetyl-leucyl-leucyl-L-norleucinal.
transcription elongation factor b (P-TEFB), which is composed of cyclin T1 (CycT1) and cyclin-dependent kinase 9 (Cdk9), the monoubiquitlated VP16 protein increases the rates of elongation rather than initiation of transcription (23). Of note, cyclin-dependent kinase 9 phosphorylates the C-terminal domain of RNA polymerase II and the negative transcription elongation factor, which contains 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-sensitivity inducing factor and negative elongation factor (24). These changes lead additionally to co-transcriptional processing of nascent mRNA species.

LPS increases the levels of MHC II molecules on macrophages via an unknown mechanism (25, 26). In this study, we found that TLR4, which binds LPS, directs several post-translational modifications of CIITA IF1. These modifications include its phosphorylation and monoubiquitylation. They lead to the binding between CIITA IF1 and P-TEFb, which increases the transcriptional activity of CIITA IF1 and thus the expression of MHC II genes. Subsequent polyubiquitylation results in a rapid degradation of CIITA IF1. Thus, these findings connect the innate and adaptive arms of the immune response.

**EXPERIMENTAL PROCEDURES**

Animals, Cells, and Cell Culture—C57BL/10ScN mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in our colony. HeLa, COS, and RAW 264.7 cells were maintained in Dulbecco’s modified Eagle’s medium, 5% fetal calf serum, and antibiotics. Bone marrow-derived macrophages were prepared and maintained as described previously (27). LPS Re595 was obtained from Sigma. MEK1/2 inhibitor U0 126 was obtained from Promega (Madison, WI), and proteasome inhibitor ALLN was purchased from Calbiochem (La Jolla, CA). A-Phosphatase was obtained from Sigma.

Plasmid DNAs—Reporter plasmid pDRASCAT was described previously (12). Plasmid m:Ub was described previously (23). To construct a plasmid m:CycT1, the coding sequence of CycT1 was cloned into the expression vector pEF-Myc between EcoRI and XbaI restriction sites. Plasmids f:CIITA1 and h:CIITA1 were gift from Dr. Ting (University of North Carolina) and Dr. Chang (Indiana University School of Medicine). To construct a plasmid coding for f:CIITA1(S357A), the f:CIITA1 plasmid was subjected to site-directed mutagenesis with the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. To construct plasmids coding for the h:Ub.CIITA1 and h:Ub(K48,63R).CIITA1, the Ub and Ub(K48,63R) plasmids were digested with EcoRI and cloned into h:CIITA1. Murine TLR4 was cloned into the pMX-pie bicistronic retroviral vector, as described previously (Onishi 1996). All of the plasmids were verified by DNA sequencing.

Immunoreagents—The monoclonal anti-CIITA (sc-13556), anti-Ub (sc-8017), and anti-Myc (9E10) antibodies, and the polyclonal anti-CycT1 (sc-8127), anti-CIITA (sc-9870 and sc-9869), and anti-Erk1/2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal anti-phospho-Erk1/2 antibody was purchased from Cell Signaling Technology (Beverly, MA). The monoclonal anti-I-A α (KH 118) antibody was obtained from Becton Dickinson (San Diego, CA). The monoclonal anti-FLAG M2 antibody and the anti-FLAG M2 beads were purchased from Sigma.

Viral Production and Infection—Retroviruses were produced by triple transfection of HEK293T cells with retroviral constructs along with gag-pol and vesicular stomatitis virus G glycoprotein expression constructs (28). Bone marrow-derived macrophages were infected as described previously (27).

Transient Transfection and CAT Assay—The cells were seeded into 100-mm-diameter Petri dishes ~12 h prior to transfection. The cells were transfected with FuGene 6 reagent according to the manufacturer’s instructions (Invitrogen). CAT enzymatic assays were performed as described (29). Fold transactivation represents the ratio between the CIITA-activated transcription and the activity of the reporter plasmid alone.

Immunoprecipitation Assay and Western Blot Analysis—The cells were transfected with 2 μg of indicated plasmid vectors. About 18 h after transfection, the immunoprecipitations were performed as described previously (12). Precipitated proteins were resolved on SDS-PAGE and analyzed by immunoblotting with the appropriate antibody followed by horseradish peroxidase-conjugated secondary antibody. The blots were developed by chemiluminescence assay from PerkinElmer Life Sciences.

Chromatin Immunoprecipitation (ChIP) Assays, RT-PCR, and Quantitative Real-time PCR—ChIP assays were performed as described previously (30). RNA was extracted from RAW 264.7 cells using the TRizol protocol from Invitrogen. RT-PCR was described previously (31). cDNAs were then amplified by primers described previously (32). Quantitative PCR was performed by Stratagene Mx3005P quantitative real-time PCR system, according to the manufacturer’s protocol.

In Vitro Transcription and Translation and in Vitro Kinase Assay—f:CIITA1 and f:CIITA1(S357A) were expressed in vitro by using coupled rabbit reticulocyte lysate transcription and translation system from Promega (Madison, WI). In vitro kinase assay with Erk1/2 was performed according to the manufacturer’s instructions (Upstate Biotechnology Inc., Lake Placid, NY).

Pulse-Chase Analysis—18 h after transfection, COS cells were starved for 2 h in medium without cysteine and methionine. Radiolabeling with [35S]cysteine and [35S]methionine was performed for 40 min. After the labeling, the cells were washed three times in phosphate-buffered saline and maintained in Dulbecco’s modified Eagle’s medium for indicated time periods. The cells were lysed for 45 min at 4 °C. The cell lysates were then subjected to immunoprecipitation. Precipitated proteins were resolved on SDS-PAGE and analyzed by radiography.

RESULTS

LPS Increases the Expression of MHC II Determinants via TLR4 in Mouse Macrophages—Upon stimulation with LPS, macrophages increase the surface levels of MHC II molecules (1). Via TLR4, LPS also activates Erk1/2, JNK, and p38 MAPKs (2). Thus, we asked two questions. First, does the expression of MHC II determinants depend on TLR4? Second, does this induction depend on any of these kinases?

To answer the first question, we infected primary bone marrow-derived macrophages from TLR4−/− mice with TLR4 (Fig. 1A, lanes 3 and 4) or an empty vector as the control (Fig.
no effect on the induction of MHC II determinants by LPS (data not presented). UO 126 is a specific MEK1/2 inhibitor that prevents the phosphorylation and subsequent activation of Erk1/2. These findings were confirmed by Western blotting with anti-phospho-Erk1/2 antibodies (20 min; Fig. 1B, bottom panel, compare lanes 2 and 3). Moreover, as presented in supplemental Fig. S1 (top panel, lanes 1–6), the activation of Erk1/2 was detectable by 10 min and disappeared by 2 h upon the addition of LPS in primary cells. We conclude that the activation of Erk1/2 precedes and is required for the induction of MHC II determinants by LPS.

To determine effects of LPS on MHC II transcription, we stimulated RAW 264.7 cells with LPS and measured levels of I-Aα transcripts starting at the zero time point. After 2 h, the cells were washed, and the medium was changed. We performed RT-PCR at the indicated time points (Fig. 1C, top panel). Initially, we did not detect MHC II transcripts in unstimulated cells (Fig. 1C, top panel, lane 1). They appeared 1 h and peaked 2 h after the addition of LPS (Fig. 1C, top panel, compare lanes 3 and 4). 4 h later, MHC II transcripts disappeared almost completely (Fig. 1C, top panel, lane 5). Importantly, the levels of actin mRNA, which we used as the internal control, were equivalent in all samples (Fig. 1C, bottom panel). Thus, LPS activates transiently Erk1/2, which is followed by the induction of MHC II transcription.

Next, we wanted to determine whether the expression of MHC II genes depended on the de novo synthesis of CIITA. Because different isoforms of CIITA had been described in RAW 264.7 cells (33, 34), we investigated first CIITA IF1, IF3, and IF4 transcripts by RT-PCR in the presence and absence of LPS in these cells. However, as presented in supplemental Fig. S2, only CIITA IF1 mRNA encodes a functional CIITA protein in RAW 264.7 cells. Moreover, CIITA IF1 was the only isoform that we detected by immunoprecipitation and subsequent Western blotting in these cells (see Fig. 3D, bottom panel). Next, we performed quantitative real time RT-PCR to quantify mRNA levels of CIITA IF1 in stimulated and unstimulated cells (Fig. 1D). Time points 0, 2, and 4 h correspond to samples from Fig. 1C (lanes 1, 4, and 5). CIITA IF1 mRNA levels were determined by quantitative real time RT-PCR. The graph presents the quantified values in arbitrary units. The error bars give standard errors of the mean for two experiments performed in duplicate. WB, Western blot.

FIGURE 1. LPS increases the expression of MHC II determinants via TLR4 in mouse macrophages. A, LPS induces the expression of MHC II determinants via TLR4. Primary bone marrow derived macrophages from TLR4−/− mice were infected with TLR4 (lanes 2 and 4) or an empty vector as the control (lanes 1 and 2). Two days after the infection, the cells were treated with LPS (lanes 2 and 3) or left untreated (lanes 1 and 4). The arrow on the right indicates the presence of MHC II determinant I-Aα (top panel). The middle and bottom panels present phosphorylated and total levels of Erk1/2 proteins, as indicated by the arrows on the right. B, LPS increases the expression of MHC II determinants via activation of Erk1/2. RAW 264.7 cells were treated with LPS or not (lanes 1 and 2) or with LPS in the presence of UO126 (lane 3). The bottom panel shows the presence of phosphorylated Erk1/2. C, LPS induces the transcription of MHC II genes. RAW 264.7 cells were treated with LPS (denoted by WB above the time points). The arrow on the left indicates the levels of MHC II determinant I-Aα mRNA, determined by RT-PCR at indicated time points (top panel). The bottom panel presents the levels of actin mRNA, as indicated by the arrow on the left. D, LPS decreases levels of CIITA IF1 transcripts. RAW 264.7 cells were treated with LPS as in Fig. 1C. Time points 0, 2, and 4 h correspond to samples from Fig. 1C (lanes 1, 4, and 5). CIITA IF1 mRNA levels were determined by quantitative real time RT-PCR. The graph presents the quantified values in arbitrary units. The error bars give standard errors of the mean for two experiments performed in duplicate. WB, Western blot.

1A, lanes 1 and 2). Two days later, we stimulated these macrophages with LPS for 2 h (Fig. 1A, lanes 2 and 3) or left them untreated (Fig. 1A, lanes 1 and 4). To exclude a possible translocation of MHC II molecules from the cytoplasm to the cell surface, we examined their levels by Western blotting rather than by fluorescence-activated cell sorter. Indeed, LPS induced the expression of MHC II determinants only in macrophages that expressed TLR4 (12 h; Fig. 1A, top panel, compare lane 3 with lanes 1, 2, and 4), which correlated with the activation of Erk1/2 by 20 min (Fig. 1A, middle panel, compare lanes 1–4). Levels of Erk1/2 were comparable in all samples (Fig. 1A, bottom panel). These results indicate that LPS activates Erk1/2 and increases the expression of MHC II determinants in primary macrophages via TLR4.

To answer the second question, we duplicated these experiments in RAW 264.7 cells, which are a mouse macrophage cell line. At identical time points, we determined levels of MHC II determinants (Fig. 1B, top panel, lanes 1 and 2). Indeed, LPS induced the expression of MHC II genes in RAW 264.7 cells equivalently to primary macrophages (Fig. 1B, top panel, compare lanes 1 and 2). In contrast, when these cells were treated with the MEK inhibitor UO 126 prior to the addition of LPS, the expression of MHC II determinants was abolished (Fig. 1B, top panel, compare lanes 2 and 3). These experiments were repeated with inhibitors of JNK and p38 MAPKs, but they had
double band, we wanted to determine whether CIITA IF1 is also modified by phosphorylation. Thus, we expressed the FLAG epitope-tagged CIITA IF1 protein (f:CIITA1) (Fig. 2A, lane 1) in COS cells. In these cells, Erk1/2 is active constitutively. Thus, they do not need the stimulation by LPS. An aliquot of cell lysates was incubated with λ-phosphatase, which removes phosphates from serines and threonines (Fig. 2A, lane 2). As expected, the upper f:CIITA1 band disappeared completely (Fig. 2A, compare lanes 1 and 2). We conclude that CIITA IF1 is also phosphorylated in cells.

Because in Fig. 1 we showed that the increased expression of MHC II determinants after LPS stimulation depended on the activation of Erk1/2, we searched for putative Erk1/2 phosphorylation sites in CIITA IF1. Erk1/2 is a proline-directed kinase with the consensus phosphorylation sequence PX(S/T)P, where the serine or threonine is phosphorylated. Indeed, there is only one consensus Erk1/2 phosphorylation site in CIITA IF1, which contains a serine at position 357 (S357) (Fig. 2B).

To determine whether this site is phosphorylated, we created the mutant f:CIITA1 protein, where the serine at position 357 was changed to alanine (f:CIITA1(S357A)). Next, we expressed both the wild type f:CIITA1 and the mutant f:CIITA1(S357A) proteins in COS cells (Fig. 2C). We made two observations. First, expression levels of the mutant f:CIITA1(S357A) protein were 4-fold higher than those of the f:CIITA1 protein (Fig. 2C, compare lanes 1 and 2). Second, the mutant f:CIITA1(S357A) protein migrated as a single lower band (Fig. 2C, compare lanes 1 and 2), which represents the nonphosphorylated form of CIITA IF1. Thus, we conclude that CIITA IF1 is phosphorylated on Ser357 in cells.

Next, we wanted to determine whether Erk1/2 is involved in the phosphorylation of CIITA IF1 and whether the upper band of CIITA IF1 would diminish under the influence of UO 126. Again we expressed f:CIITA1 (Fig. 2D, lanes 1 and 2) and treated COS cells with UO 126 (Fig. 2D, lane 2) or the solvent as the control (Fig. 2D, lane 1). As expected, in the presence of UO 126, the upper band of f:CIITA1 disappeared (Fig. 2D, top panel, compare lanes 1 and 2), which correlated with the absence of phosphorylated Erk1/2 (Fig. 2D, bottom panel, compare lanes 1 and 2). This finding suggests that Erk1/2 is involved in the phosphorylation of CIITA IF1.

To extend our findings, we determined whether Erk1/2 could phosphorylate CIITA IF1 in vitro and whether this phosphorylation depended on Ser357. Thus, we performed in vitro kinase assays with Erk1/2. Wild type f:CIITA1 and mutant f:CIITA1(S357A) proteins were transcribed and translated using the rabbit reticulocyte lysate in vitro (Fig. 2E). Aliquots of the reaction were incubated with Erk1/2 (Fig. 2E, lanes 2 and 4) or with the reaction buffer as the control (Fig. 2E, lanes 1 and 3). Indeed, as indicated by the appearance of the upper band, Erk1/2 phosphorylated the wild type f:CIITA1 protein (Fig. 2E, compare lanes 1 and 2). In contrast, Erk1/2 did not phosphorylate the mutant f:CIITA1(S357A) protein, which was confirmed by the absence of the upper band (Fig. 2E, compare lanes 3 and 4 with lane 2). Overall, we conclude that Erk1/2 phosphorylates Ser357 in CIITA IF1 in vitro and in vivo.

CIITA IF1 Is Ubiquitylated in Cells—Thus far, we determined not only that Erk1/2 phosphorylates Ser357 in f:CIITA1, but we also observed that this phosphorylation affects the stability of f:CIITA1. Namely, expression levels of the mutant f:CIITA1(S357A) protein were higher than those of f:CIITA1 (Fig. 2C, compare lanes 1 and 2). These findings led us to hypothesize that the phosphorylation and degradation of CIITA IF1 could be connected via ubiquitin.

To address this notion, we determined first whether different expression levels of f:CIITA1 and mutant f:CIITA1(S357A) proteins were due to differences in their rates of degradation. We expressed f:CIITA1 (Fig. 3A, top panel) and the mutant f:CIITA1(S357A) proteins (Fig. 3A, bottom panel) and performed pulse-chase analyses in COS cells. Indeed, the mutant f:CIITA1(S357A) protein had a half-life of ~2 h, which was 4 times longer than that of f:CIITA1 (Fig. 3A, compare top and bottom panels). We conclude that the phosphorylation of CIITA IF1 leads to its degradation.

Next, we wanted to determine whether CIITA IF1 was degraded via the proteasome. We expressed f:CIITA1 (Fig. 3B, lanes 1 and 2) and the mutant f:CIITA1(S357A) (Fig. 3B, lanes 3 and 4) proteins in COS cells. Transfected cells were treated with the proteasomal inhibitor ALLN for 6 h (Fig. 3B, lanes 2 and 4) and the solvent as the control (Fig. 3B, lanes 1 and 3). ALLN
was dependent on the phosphorylation of Ser357. Thus, we per-

Because the mutant f:CIITA1(S357A) protein was more stable than f:CIITA1, we assumed that the ubiquitylation of f:CIITA1 because the mutant f:CIITA1(S357A) proteins were expressed in COS cells. The cells were starved for cysteine and methionine and then incubated in medium containing [35S]cysteine and [35S]methionine. The samples were collected at the time points indicated above the top panel. The arrows on the right indicate the presence of wild type f:CIITA1 and the mutant f:CIITA1(S357A) proteins. B, proteasomal inhibitor ALLN increases levels of the wild type f:CIITA1 (top panel) but not mutant f:CIITA1(S357A) (bottom panel) proteins. Wild type f:CIITA1 (lanes 1 and 2) and mutant f:CIITA1(S357A) proteins (lanes 3 and 4) were expressed in COS cells. Before lysis, the cells were treated with ALLN for 6 h (lanes 2 and 4) or with the solvent as the control (lanes 1 and 3). The arrows on the right indicate the presence of phosphorylated and nonphosphorylated f:CIITA1 proteins, respectively. C, in vivo ubiquitylation assay of the wild type f:CIITA1 and mutant f:CIITA1(S357A) proteins. f:CIITA1 and mutant f:CIITA1(S357A) proteins were expressed in COS cells in the presence of m:Ub (lanes 2 and 4). As a control, f:CIITA1 and mutant f:CIITA1(S357A) proteins were co-expressed with the empty plasmid vector (lanes 1 and 3). Conjugates between f:CIITA1 proteins and ubiquitin ([Ub]n Conj.) are bracketed (top panel). The numbers on the left indicate relative molecular mass markers in kDa. The bottom panel presents input of f:CIITA1 proteins, as indicated by the arrow on the right. D, the endogenous CIITA IF1 is ubiquitylated in RAW 264.7 cells. RAW 264.7 cells were treated with LPS (lane 1) or LPS and ALLN (lane 2). Conjugates between CIITA IF1 proteins and ubiquitin ([Ub]n Conj.) are bracketed (top panel). The numbers on the left indicate relative molecular mass markers in kDa. The bottom panel presents phosphorylated and unphosphorylated CIITA IF1 proteins (23, 36). Therefore, we constructed the CIITA IF1 and ubiquitin (h:Ub.CIITA1). We expressed f:CIITA1 (Fig. 4A, lane 1) and the h:Ub.CIITA1 fusion proteins (Fig. 4A, lane 2) in COS cells. Of interest, the steady state levels of h:Ub.CIITA1 chimera were 10-fold lower than those of f:CIITA1 (Fig. 4A, compare lanes 1 and 2). This suggested that the h:Ub.CIITA1 fusion protein was extremely unstable. Indeed, pulse-chase analyses revealed that the half-life of the h:Ub.CIITA1 chimera was less than 10 min (Fig. 4B). As expected, the presence of ALLN for 6 h increased levels of

FIGURE 3. CIITA IF1 is ubiquitylated in cells. A, f:CIITA1 is degraded faster than the mutant f:CIITA1(S357A) protein. Wild type f:CIITA1 and mutant f:CIITA1(S357A) proteins were expressed in COS cells. The cells were starved for cysteine and methionine and then incubated in medium containing [35S]cysteine and [35S]methionine. The samples were collected at the time points indicated above the top panel. The arrows on the right indicate the presence of wild type f:CIITA1 and the mutant f:CIITA1(S357A) proteins. B, proteasomal inhibitor ALLN increases levels of the wild type f:CIITA1 (top panel) but not mutant f:CIITA1(S357A) (bottom panel) proteins. Wild type f:CIITA1 (lanes 1 and 2) and mutant f:CIITA1(S357A) proteins (lanes 3 and 4) were expressed in COS cells. Before lysis, the cells were treated with ALLN for 6 h (lanes 2 and 4) or with the solvent as the control (lanes 1 and 3). The arrows on the right indicate the presence of phosphorylated and nonphosphorylated f:CIITA1 proteins, respectively. C, in vivo ubiquitylation assay of the wild type f:CIITA1 and mutant f:CIITA1(S357A) proteins. f:CIITA1 and mutant f:CIITA1(S357A) proteins were expressed in COS cells in the presence of m:Ub (lanes 2 and 4). As a control, f:CIITA1 and mutant f:CIITA1(S357A) proteins were co-expressed with the empty plasmid vector (lanes 1 and 3). Conjugates between f:CIITA1 proteins and ubiquitin ([Ub]n Conj.) are bracketed (top panel). The numbers on the left indicate relative molecular mass markers in kDa. The bottom panel presents input of f:CIITA1 proteins, as indicated by the arrow on the right. D, the endogenous CIITA IF1 is ubiquitylated in RAW 264.7 cells. RAW 264.7 cells were treated with LPS (lane 1) or LPS and ALLN (lane 2). Conjugates between CIITA IF1 proteins and ubiquitin ([Ub]n Conj.) are bracketed (top panel). The numbers on the left indicate relative molecular mass markers in kDa. The bottom panel presents phosphorylated and unphosphorylated CIITA IF1 proteins (23, 36). Therefore, we constructed the CIITA IF1 and ubiquitin (h:Ub.CIITA1). We expressed f:CIITA1 (Fig. 4A, lane 1) and the h:Ub.CIITA1 fusion proteins (Fig. 4A, lane 2) in COS cells. Of interest, the steady state levels of h:Ub.CIITA1 chimera were 10-fold lower than those of f:CIITA1 (Fig. 4A, compare lanes 1 and 2). This suggested that the h:Ub.CIITA1 fusion protein was extremely unstable. Indeed, pulse-chase analyses revealed that the half-life of the h:Ub.CIITA1 chimera was less than 10 min (Fig. 4B). As expected, the presence of ALLN for 6 h increased levels of

increased levels of only the phosphorylated form of f:CIITA1 ~4-fold (Fig. 3B, lanes 1 and 2, compare top and bottom bands). It had almost no effect on levels of the mutant f:CIITA1(S357A) protein (Fig. 3B, compare lanes 3 and 4). Thus, we conclude that the phosphorylated CIITA IF1 is also degraded via the proteasome.

To determine whether CIITA IF1 is ubiquitylated, we performed ubiquitylation assays in vivo. In COS cells, we co-expressed f:CIITA1 with the Myc epitope-tagged ubiquitin (m:Ub) (Fig. 3C, lane 2) or an empty plasmid vector as the control (Fig. 3C, lane 1). Indeed, in the presence of f:CIITA1 and m:Ub, there was a strong ubiquitylation ladder (Fig. 3C, lane 2). Because the mutant f:CIITA1(S357A) protein was more stable than f:CIITA1, we assumed that the ubiquitylation of f:CIITA1 was dependent on the phosphorylation of Ser357. Thus, we performed the ubiquitylation assay in vivo with the mutant f:CIITA1(S357A) protein (Fig. 3C, lanes 3 and 4). Indeed, by densitometry we determined that the mutant f:CIITA1(S357A) protein was ubiquitylated 4-fold less than f:CIITA1 (Fig. 3C, compare lanes 2 and 4). We conclude that CIITA IF1 is ubiquitylated, which is promoted by the phosphorylation of Ser357 in CIITA IF1.

Finally, we asked whether the endogenous CIITA IF1 is also ubiquitylated in mouse macrophages. To address this question, we repeated ubiquitylation assays in RAW 264.7 cells (Fig. 3D). Because of low levels of expression or accelerated degradation, we were not able to detect CIITA IF1 or its ubiquitylation in untreated cells (Fig. 3D, lane 1) or cells treated with LPS and UO 126, respectively (data not presented). However, we were able to observe the heavily ubiquitylated CIITA IF1 only after treatment with LPS and ALLN (Fig. 3D, lane 2). We treated cells with ALLN for 2 h prior to 40 min of treatment with LPS. Afterward, the cells were incubated with ALLN for an additional 2 h. Interestingly, CIITA IF1 was present in phosphorylated and unphosphorylated forms (Fig. 3D, bottom panel, lane 2), which can be explained by transient activation of Erk1/2 by LPS (Fig. 1D). Thus, we conclude that the endogenous CIITA IF1 is phosphorylated and ubiquitylated in RAW 264.7 cells.

In CIITA IF1, there is a predicted PEST sequence from positions 360 to 385 (Fig. 3E). The phosphorylation of these sequences decreases the stability of proteins (22, 35). Because the phosphorylation of Ser357, which is flanked by a proline, a serine, and an acidic residue, led to the degradation of CIITA IF1, we surmise that the actual PEST sequence in CIITA IF1 is extended on its N terminus. Thus, Ser357 becomes a part of the functional degron, which is located from positions 355 to 385 in CIITA IF1 (Fig. 3E). Overall, we conclude that the phosphorylation of Ser357 in the degron precedes the ubiquitylation of CIITA IF1 that subsequently leads to its degradation.

Ubiquitylated CIITA IF1 Protein Is More Active Than Its Counterpart That Is Not Ubiquitylated—Thus far, we connected two post-translational modifications in CIITA IF1, namely phosphorylation and ubiquitylation, with the stability of CIITA IF1. It is known that ubiquitylation strongly influences the function of activators (23, 35–38). Of note, monoubiquitylation increases the activity of transcription factors, whereas polyubiquitylation leads to their degradation. For example, by recruiting P-TEFb better, the monoubiquitylation of VP16 increases its effects on transcriptional elongation (23). Moreover, CIITA IF1 binds CycT1 (17). Thus, we hypothesized that the monoubiquitylated CIITA IF1 protein also binds P-TEFb better and is therefore more active than its nonubiquitylated counterpart.

It had been demonstrated previously that transcription factors fused to ubiquitin behave as endogenously ubiquitylated proteins (23, 36). Therefore, we constructed the hemagglutinin epitope-tagged fusion protein between CIITA IF1 and ubiquitin (h:Ub.CIITA1). We expressed f:CIITA1 (Fig. 4A, lane 1) and the h:Ub.CIITA1 fusion proteins (Fig. 4A, lane 2) in COS cells. Of interest, the steady state levels of h:Ub.CIITA1 chimera were 10-fold lower than those of f:CIITA1 (Fig. 4A, compare lanes 1 and 2). This suggested that the h:Ub.CIITA1 fusion protein was extremely unstable. Indeed, pulse-chase analyses revealed that the half-life of the h:Ub.CIITA1 chimera was less than 10 min (Fig. 4B). As expected, the presence of ALLN for 6 h increased levels of

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the h:Ub.CIITA1 chimera substantially (Fig. 4C, compare lanes 1 and 2). This finding is in agreement with data presented in Fig. 3D, where the endogenous CIITA IF1 protein was detectable only after the addition of ALLN in RAW 264.7 cells. Thus, we conclude that h:Ub.CIITA1 chimera is rapidly degraded via the proteasome.

To compare activities of the nonubiquitylated and ubiquitylated CIITA IF1 proteins, we performed transcriptional assays in cells. The mutant f:CIITA1(S357A) protein represented the nonubiquitylated CIITA IF1 protein, and the h:Ub.CIITA1 fusion protein represented the ubiquitylated CIITA IF1 protein. They were co-expressed with pDRASCAT reporter plasmid in COS cells. Prior to performing CAT assays, we equalized the levels of both proteins (Fig. 4D). Results revealed that the ubiquitylated CIITA IF1 protein stimulated transcription ~6-fold higher than its nonubiquitylated counterpart. We conclude that the ubiquitylation of CIITA IF1 increases its transcriptional activity.

Next, we wanted to determine whether the monoubiquitylated CIITA IF1 protein behaves similarly to the monoubiquitylated VP16 protein (23). Thus, we linked the mutant Ub(K48,63R) protein, where lysines at positions 48 and 63 in ubiquitin were changed to arginines and no longer support polyubiquitylation, to CIITA IF1 (mutant h:Ub(K48,63R).CIITA1 chimera). The Myc epitope-tagged CycT1 protein (m:CycT1) was co-expressed with the mutant h:Ub(K48,63R).CIITA1 chimera (Fig. 5A, lane 1) and the mutant f:CIITA1(S357A) protein (Fig. 5A, lane 2) in COS cells. We immunoprecipitated CIITA1 proteins with the anti-CycT1 antibody and blotted the membrane with the anti-Myc antibody (Fig. 5A, top panel). Whereas the mutant h:Ub(K48,63R).CIITA1 fusion protein clearly bound m:CycT1, we could not detect the binding between the mutant f:CIITA1(S357A) and m:CycT1 (Fig. 5A, top panel, compare lanes 1 and 2). The inputs of m:CycT1 and CIITA1 proteins were comparable (Fig. 5A, middle and bottom panels). Thus, we conclude that the monoubiquitylation of CIITA IF1 precedes its binding to CycT1.

To extend our findings, we performed ChIP assays in a mouse macrophage cell line with endogenous CIITA IF1 and CycT1 proteins (Fig. 5B). As presented in Fig. 1B, I-Aα mRNA was detectable 1 h after the stimulation with LPS (Fig. 1B, top panel, lane 3). To determine whether P-TEFb is on MHC II promoters before the appearance of MHC II mRNA species, we performed ChIP prior to this time point. We stimulated RAW 264.7 cells with LPS alone (Fig. 5B, lane 2) or with UO126 (Fig. 5B, lane 3) for 40 min, or left them untreated (Fig. 5B, lane 1). After cross-linking and sonication, we performed immunoprecipitations with anti-CIITA and anti-CycT1 antibodies. Immunoprecipitated DNA was amplified by PCR with primers complementary to the I-Aα promoter. When immunoprecipitated with anti-CycT1 antibodies, PCR products were present only in cells, which were stimulated with LPS (Fig. 5B, top panel, lane 2). In contrast, when immunoprecipitated with anti-CIITA antibodies, PCR products were detected only in cells treated with UO126 (Fig. 5B, middle panel, lane 3). The treatment with UO126 prevented the activation and binding of CIITA IF1 to CycT1 but led to its stabilization and subsequent accumulation on MHC II promoters (Fig. 5B, middle panel, lane 3). As presented in supplemental Fig. S3, only monoubiquitylated CIITA IF1 bound CycT1. Overall, these results suggest that the phosphorylated, ubiquitylated CIITA IF1 recruits...
P-TEFb to MHC II promoters. P-TEFb then directs the expression of MHC II genes in cells.

DISCUSSION

This study revealed one of the mechanisms that connect innate and adaptive immunity. First, we found that LPS increases the expression of MHC II genes in macrophages via the activation of Erk1/2, which is mediated by TLR4. Second, Erk1/2 phosphorylates CIITA IF1 on the serine at position 357. Third, this phosphorylation in a degron leads to the monoubiquitylation of CIITA IF1. Fourth, by binding P-TEFb better, these modifications increase the transcriptional activity of CIITA IF1. Fifth, subsequent polyubiquitylation of CIITA IF1 results in its rapid degradation.

One of the most intriguing properties of CIITA is the presence of three different isoforms, all of which support transcription from MHC II genes. In our study, we defined two post-translational modifications of CIITA IF1 that regulate its transcriptional activity. Moreover, we connected them with signaling events that arise in macrophages after they encounter bacteria or LPS. First, we determined that IF1 is the CIITA isoform in RAW 264.7 cells. Of note, CIITA IF1 was the only isoform detected in Fig. 3D. Next, we demonstrated that the upper band of CIITA IF1 represents the phosphorylated form of the protein. This conclusion was obtained by treating CIITA IF1 with λ-phosphatase and by mutating the serine at position 357 to alanine, both of which converted CIITA IF1 to the more quickly migrating form. The phosphorylation of this serine in CIITA IF1 led to its ubiquitylation and degradation. Of note, the mutant CIITA IF1(S357A) protein that was less ubiquitylated had increased stability and lower activity when compared with the wild type CIITA IF1 protein. The latter effect can be explained by interactions between CIITA IF1 and P-TEFb. In contrast to the mutant Ub(K48,63R) CIITA IF1 chimera that binds P-TEFb and represents the monoubiquitylated form of CIITA IF1, the binding between the mutant CIITA IF1(S357A) protein and P-TEFb could not be demonstrated. Indeed, ChIP revealed that only the mutant Ub(K48,63R) CIITA IF1 chimera bound P-TEFb on DNA in HeLa cells. Moreover, the same results were obtained by ChIP in RAW 264.7 cells. Collectively, these findings suggest that the ubiquitylation of CIITA not only stabilizes the MHC II enhanceosome (7) but also enables interactions between CIITA and P-TEFb.

Results of our study extend the findings with the viral activator VP16 (23, 36) to the eukaryotic CIITA protein. Of note, VP16 and CIITA share AADs, which frequently overlap with degrons and whose ubiquitylation plays an important role in the elongation of transcription (22). Moreover, these activators also bind P-TEFb (15, 17, 23). Two hypotheses, which could be complementary, have been proposed to explain the connection between such ubiquitylation and activation of transcription. The first is the recruitment of the proteasome to the transcription complex, which could facilitate the removal of initiation factors and lead to chromatin remodeling (22). The second is the recruitment of P-TEFb, which phosphorylates the C-terminal domain of RNA polymerase II and negative transcription elongation factor that enable the elongation of transcription (23). Notably, when not ubiquitylated, AADs increase the rates of initiation of transcription (23). However, when monoubiquitylated, AADs increase rates of elongation of transcription by recruiting P-TEFb (23). Subsequent polyubiquitylation of AADs leads to their degradation, thus enabling the next rounds of transcription. CIITA is such a eukaryotic activator where sequential post-translational modifications integrate initiation and elongation of transcription.

How do our findings contribute to the understanding of connections between innate and adaptive immune responses? It is well known that LPS increases the levels of MHC II determinants on macrophages (25, 26). However, the mechanism for this observation had not been determined. In this study, we propose that the sequential modifications in CIITA IF1 induced by LPS via TLR4 stimulate MHC II transcription and thus represent one of the connections between innate and adaptive immunity. Moreover, it is tempting to speculate that in macrophages, stimulation with LPS triggers a short burst of antigen processing, which leads to sustained presentation of exogenous antigenic peptides. Several findings support this notion. First, the activation of Erk1/2, JNK, and p38 MAPKs after the stimulation with LPS is brief (27). Second, levels of CIITA IF1 transcripts decrease, implying that little or no synthesis of new CIITA IF1 protein occurs. Third, modifications of CIITA IF1 not only increase its transcriptional activity but also lead to its rapid degradation. Fourth, increased levels of MHC II mRNA are transient. Fifth, in macrophages, the activation of p38 MAPK is needed for the maturation of phagosomes after the engagement of TLR2/4 (39). In this scenario, activated macrophages could still remove the debris from infected tissues without a further deleterious presentation of self-antigens. These observations are in agreement with recent findings in dendritic cells, where LPS also directed endocytosed antigens into appropriate cellular compartments (40). However, in contrast to macrophages, immature dendritic cells already contain abundant intracellular MHC II determinants. Thus, a transient increased expression of MHC II genes by LPS would be less noticeable in these cells. We conclude that via TLR4 and CIITA IF1, LPS directs molecular and cellular mechanisms involved in antigen processing and presentation.

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