Maturation of Dendritic Cells Is Accompanied by Rapid Transcriptional Silencing of Class II Transactivator (CIITA) Expression

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Abstract

Cell surface expression of major histocompatibility complex class II (MHCII) molecules is increased during the maturation of dendritic cells (DCs). This enhances their ability to present antigen and activate naive $CD4^+$ T cells. In contrast to increased cell surface MHCII expression, de novo biosynthesis of MHCII mRNA is turned off during DC maturation. We show here that this is due to a remarkably rapid reduction in the synthesis of class II transactivator (CIITA) mRNA and protein. This reduction in CIITA expression occurs in human monocyte-derived DCs and mouse bone marrow–derived DCs, and is triggered by a variety of different maturation stimuli, including lipopolysaccharide, tumor necrosis factor α, CD40 ligand, interferon α, and infection with *Salmonella typhimurium* or Sendai virus. It is also observed in vivo in splenic DCs in acute myelin oligodendrocyte glycoprotein induced experimental autoimmune encephalitis. The arrest in CIITA expression is the result of a transcriptional inactivation of the *MHC2TA* gene. This is mediated by a global repression mechanism implicating histone deacetylation over a large domain spanning the entire *MHC2TA* regulatory region.

Key words: MHC class II • class II transactivator • experimental autoimmune encephalitis • bare lymphocyte syndrome • histone deacetylation

Introduction

Dendritic cells (DCs)* are specialized for the initiation of primary immune responses because they are the most potent APCs for the activation of naive T cells. Moreover, they provide a key link between the innate and adaptive immune systems (1, 2). Two distinct stages of DC differentiation are recognized. Immature DCs are found in the periphery at strategically important sites where they act as surveillance cells. These immature DCs are characterized by a high capacity for antigen uptake but are rather poor at activating T cells. Inflammatory stimuli and exposure to infectious agents triggers an irreversible differentiation into mature DCs. During maturation, endocytotic activity is lost (3), and internalized antigens are processed and presented by MHC molecules at the cell surface (4, 5) cytokines (IL-12) are released (6, 7), costimulatory molecules (CD80, CD86, CD40) are upregulated (3, 8), and the expression of specific chemokine receptors (CCR7) is induced (9). These changes ensure that the DCs migrate to the T cell areas of the secondary lymphoid organs and are efficient at activating antigen-specific T lymphocytes.

Changes in the intracellular localization and cell surface expression of MHC class II (MHCII) molecules are intimately associated with the function and maturation of DCs

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**Abbreviations used in this paper:* BMDC, bone marrow–derived DC; CIITA, class II transactivator; DC, dendritic cell; EAE, experimental autoimmune encephalitis; MOG, myelin oligodendrocyte glycoprotein; RPA, RNase protection assay; SeV^M, Sendai virus strain M; TSA, trichostatin A.

(4, 5, 10, 11). In immature DCs, MHCII molecules are largely retained in intracellular compartments. Upon maturation, MHCII–peptide complexes are assembled and transported to the cell surface. Although these changes at the level of MHCII protein distribution have been addressed in considerable detail, relatively little is known about the mechanisms that regulate alterations in the actual de novo biosynthesis of MHCII molecules during DC maturation (4, 5).

MHCII expression is regulated primarily at the level of transcription (12–16). Several key regulatory factors controlling the transcription of MHCII genes have been identified thanks to the elucidation of the molecular defects underlying MHCII deficiency, a severe hereditary immunodeficiency syndrome resulting from the lack of MHCII expression (17–21). One of the factors identified by these studies—the MHCII transactivator CIITA—is a transcriptional coactivator that functions as a key regulatory factor for MHCII expression (22, 23). The expression pattern of CIITA dictates most qualitative and quantitative aspects of MHCII gene expression. The cell type specificity, induction, and level of MHCII expression are in the majority of situations determined by the expression of CIITA (24–27). The gene encoding CIITA (*MHC2TA*) is controlled by three distinct and independent promoters referred to as pI, pIII, and pIV. These promoters are spread out over a large (>12 kb) regulatory region and exhibit different cell type specificities (28). pIII is used mainly in B cells (28, 29). IFN-γ–induced CIITA expression is mediated by pIV (28, 30–32). Expression of the *MHC2TA* gene in DCs has previously been shown to be controlled primarily by pI, and to a lesser degree by pIII (28).

In the work reported here we have studied the mechanisms controlling de novo MHCII biosynthesis during DC maturation induced by stimuli such as LPS, TNF-α, CD40 ligand (CD40L), IFN-α, infection with bacteria or viruses, and the induction of experimental autoimmune encephalitis (EAE). Although cell surface MHCII expression is increased, de novo synthesis of MHC mRNA is actually reduced. We show that this is due to a rapid downregulation in the abundance of CIITA mRNA and protein. This loss of CIITA expression is the consequence of a simultaneous transcriptional shut down of promoters pI and pIII of the *MHC2TA* gene. The fact that these promoters are situated far apart, and are regulated independently of each other in other cell types, implies that a global silencing mechanism affecting a large region of the *MHC2TA* gene is likely to be involved. In accordance with this, we show that histones along the entire regulatory region of the *MHC2TA* gene are deacetylated during DC maturation, indicating that chromatin remodeling over a large domain is involved in the transcriptional silencing mechanism.

Materials and Methods

DCs. Human monocyte-derived DCs were prepared essentially as described (33). Briefly, PBMCs were prepared from buffy coat fractions (Blood Transfusion Centre) by isolation over Ficoll-Paque (Amersham Pharmacia Biotech). Monocytes were obtained by adhesion in RPMI 2% FCS at 37°C for 1 h and depleted of $CD19^+$ cells using magnetic beads (Dynal). The monocytes, >90% pure as assessed by flow cytometry, were cultured in IMDM 10% FCS supplemented with 2-mercaptoethanol (50 μM), GM-CSF (600 U/ml, Leukomax; Essex Chemie AG), and IL-4 (750 U/ml; R&D Systems). Every 2 d, half of the medium was replaced by fresh medium containing a twofold concentration of cytokines. After 6 d, the cells exhibited an immature DC phenotype ($CD14^-CD1a^+MHCII^{low}CD86^{low}CD40^{low}CD80^-CD83^-$). Unless indicated otherwise, maturation was induced by stimulating the immature DCs with LPS for 24 h. Mature DCs can be identified by high levels of surface MHCII, costimulatory molecules (CD86, CD80, and CD40), and the DC-restricted marker CD83. To induce maturation, immature DCs were plated at 0.5–1 × 10^6 cells/ml in IMDM 10% FCS supplemented with either LPS (from *Salmonella abortus equi*, 10 ng/ml; Sigma-Aldrich), TNF-α (75 ng/ml; R&D systems), IFN-α (1,000 U/ml, provided by C. Weissmann, University of Zürich, Zürich, Switzerland), or IFN-γ (1,000 U/ml; Life Technologies). For stimulation with CD40L, immature DCs were cocultured at a ratio of 1:5 with CD40L-expressing J558L cells (a gift from E. Padovan, University Hospital of Basel, Basel, Switzerland). For infection with *Salmonella typhimurium* (strain 14028 phoQ24), 5 × 10^6 cfu were added per 2.5 × 10^5 immature DCs and the mixture was incubated for 30 min at 37°C. Extracellular bacteria were then killed by the addition of Gentamycin (50 μg/ml; Sigma-Aldrich). For infection with Sendai (strain M, SeV^M), the virus was added to immature DCs at a multiplicity of infection of 20. Trichostatin A (TSA; Sigma-Aldrich) was coadministered with LPS at 100 ng/ml.

Mouse bone marrow–derived DCs (BMDCs) were generated according to M.B. Lutz et al. (34). Bone marrow cells were isolated from femurs and tibiae of 8-wk-old female C57BL/6 mice (RCC) and cultivated in RPMI 10% FCS. mGM-CSF (200 U/ml; PeproTech) was added at day 0, 3, 6, 8, and 10. Cells were then collected and induced to maturate with LPS (10 μg/ml) for 24 h. Maturation was monitored by FACS® analysis of surface expression of MHCII, CD86, CD83, and CD40 and by quantification of CCR1 and CCR7 mRNA expression.

Cell Lines. The Burkitt's lymphoma cell line Raji and the CIITA-deficient cell line RJ2.2.5 were grown in RPMI 10% FCS. RJ2.2.5 was transfected with an episomal expression vector containing a CIITA type I cDNA under the control of the SRα promoter. Transfected cells were selected with hygromycin for 10 d and checked for cell surface MHCII expression by FACS®.

Cytofluorometry. DCs were blocked with mouse IgG (2 mg/ml) before staining. Anti–human antibodies used were FITC-conjugated anti–HLA-DR (clone G46–6; BD PharMingen), anti-CD80 (clone BB1; BD PharMingen), and anti-CD40 (clone 5C3; BD PharMingen); PE-conjugated anti-CD1a (clone BL6; Immunotech), anti-CD83 (clone HB15a; Immunotech), and anti-CD86 (clone IT2.2; BD PharMingen); and biotinylated anti-CD14 (clone UCHM1; Ancell) followed by allophycocyanin-conjugated streptavidin (BD PharMingen). Dead cells were excluded by loading with 7-aminoactinomycin D (Sigma-Aldrich). Staining with isotype-matched antibodies was performed in parallel.

EAE. 7-wk-old C57BL/6 mice (RCC) were immunized as described (35) with myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 in CFA and treated with pertussis toxin intraperitoneally. On day 2 the animals were again treated with pertussis toxin and on day 7 the animals were boosted with MOG peptide.

Animals with acute EAE (scores 2–3) were killed on day 20 or 21. For isolation of ex vivo DCs, eight spleens were pooled, low density cells were enriched over an OptiPrep™ density gradient (Nycomed Pharma), and $CD11c^+MHCII^{high}$ DCs were sorted by FACS®. Total mRNA was isolated from these cells or from total spleen and analyzed by real time PCR.

RNA Preparation. Total RNA was prepared with Trizol (Life Technologies) according to the manufacturer's instructions.

RNase Protection Assay. RNase protection assays (RPAs) were performed with total RNA as described (36). The RPA probes for mRNAs of GAPDH, DRA, total CIITA, and the specific forms of CIITA have been described previously (27, 28, 35, 37). The probes for human CIITA mRNA are depicted schematically in Fig. 3 A. Results were quantified by PhosphorImager analysis using the ImageQuant program.

Real Time PCR. cDNA was synthesized from total RNA using random hexamers and Superscript II reverse transcriptase (Life Technologies). cDNA from 40 ng of total RNA (supplemented with 0.8 μg yeast tRNA) was used per PCR reaction. Real time PCR was performed with the TaqMan sequence detection system (Applied Biosystems). The PrimerExpress software was used to design the primers and TaqMan probes for detection of human mRNAs and nonspliced nascent transcripts (see Fig. 3 A). The sequences of the primers and probes are as follows. Total CIITA mRNA: forward 5′-CCTGCTGTTCGGGACCTAAA-3′, reverse 5′-GGATCCGCACCAGTTTGG-3′, probe 5′-AGGGCCCAGCGCAAACTCCAGT-3′. Spliced type I CIITA mRNA: forward 5′-CTAGAGAAAGGAGACCTGGATTTG-3′, reverse 5′-TCATAGAAGTGGTAGAGGCACAGG-3′, probe 5′-CTGGAGCTTCTTAACAGCGATGCTGACC-3′. Spliced type III CIITA mRNA: forward 5′-TGGGATTCCTACA-CAATGCGT-3′, reverse 5′-GGGTCAGCATCGCTGTTAA-GA-3′, probe 5′-CAGAGCCCCAAGGCAGCTCACAGT-3′. Nonspliced type I CIITA transcripts: forward 5′-GCCCCGGC-CACAGTG-3′, reverse 5′-TCCCATTGACTTCCCTTTC-AGA-3′, probe 5′-TGATTAAAAGTGATGCCAACTTAC-CACCATGG-3′. Nonspliced type III CIITA transcripts: forward 5′-TGCTGGGTCCTACCTGTCAGA-3′, reverse 5′-CAG-GACCAGCTGAGACTGCAC-3′, probe 5′-CTTTCCCGGC-CTTTTTACCTTGGGG-3′. HLA-DRA mRNA: forward 5′-GCCAACCTGGAAATCATGACA-3′, reverse 5′-AGGGCT-GTTCGTGAGCACA-3′, probe 5′-CAACTATACTCCGAT-CACCAATGTACCTCCAGAG-3′. A primer-probe combination for GAPDH mRNA was used as internal control (Applied Biosystems). The primers and probe for mouse mRNAs are as follows. mCIITA I: forward 5′-CAGGGACCATGGAGAC-CATAGT-3′, reverse 5′-CAGGTAGCTGCCCTCTGGAG-3′ and probe 5′-TGTGTGCCACCATGGATCTGGGA-3′. mCI-ITA III: forward 5′-GGTTCCTGGCCCTTCTGG-3′, reverse 5′-ATCCATGGTGGCACACAGACT-3′, and probe 5′-TCT-TACCTGCCGGAGTTGCAAGACCA-3′. mCIITA IV: forward 5′-CAGCACTCAGAAGCAACGGG-3′, reverse 5′-ATCCA-TGGTGGCACACAGACT-3′, and probe 5′-CACAGCCAC-AGCCGCGACCA-3′. mCCR7: forward 5′-ACAGCCCCCA-GAGCACC-3′, reverse 5′-GAGCCACCACCAGCACGT-3′, and probe 5′-TTTCCTGGGTTTCCCTGGGTCCA-3′. An mCCR1 primer-probe combination was purchased from Applied Biosystems. As endogenous control, a set of primers and probe specific for mouse 18S ribosomal RNA (Applied Biosystems) was used. Samples were quantified using relative standard curves for each amplification. All results are normalized with respect to the internal control, and are expressed relative to the levels found in immature DCs.

Measurement of CIITA mRNA Stability. Immature DCs or DCs stimulated with LPS for 2.5 h were supplemented with 50 μM 5,6-dichloro-1-β-ribofuranosyl benzamidazole (DRB; Sigma-Aldrich) for 0, 1, 2, 3, and 4 h. Total RNA was then prepared and the remaining CIITA mRNA was quantified by real time PCR.

Isolation of Nascent RNAs. Nascent transcripts and free nuclear RNAs were isolated from 10×10^6 cells as described (28, 38). Nascent transcripts and free nuclear RNAs were treated with RNase-free DNaseI (Roche). Nascent transcripts and free nuclear RNAs, from 1.2×10^5 and 3×10^4 cells respectively, were analyzed by real time PCR.

In Vivo Genomic Footprinting. In vivo genomic footprinting was done by ligation-mediated PCR (LM-PCR) as described (39) using Vent DNA polymerase (New England Biolabs, Inc.) for all steps. pI was analyzed between bp +80 and −164 using primer set A for the lower strand (A1, 5′-ATTGGCTCCAACA-GAAGGCTG-3′; A2, 5′-CAGAAGGCTGTGGGCTTCTC-TG-3′; A3, 5′-TGGGCTTCTCTGGCACATGCACCTG-3′) and primer set B for the upper strand (B1, 5′-CTGGCCAGT-GCCTGGAATC-3′; B2, 5′-GTGCCTGGAATCTCCGCT-CAC-3′; B3, 5′-TCCGCTCACCCAGCATGCAGCATC-3′). pIII was analyzed between bp −20 to −220 using primer set C for the upper strand (C1, 5′-AGAAGCACACAGCCTCAT-CACTA-3′; C2, 5′-CACTAGCCTCATCACTAACCAGT-CA-3′; C3, 5′-TAACCAGTCACCAGTTGGGAGCCCG-3′).

Immunoprecipitation and Immunoblotting. Antibodies specific for the NH_2 terminus (anti–CIITA-N) or COOH terminus (anti–CIITA-C) of CIITA have been described (40). Anti–CIITA-C antibodies were covalently coupled with dimethyl pimelinediimidate (DMP; Fluka) to protein-A Sepharose beads according to standard protocols (41). Preparation of protein extracts and immunoprecipitation using 5×10^6 cells per sample were done as described (40). SDS-PAGE, immunoblotting with anti–CIITA-N antibodies, and quantification were performed according to standard protocols.

Chromatin Immunoprecipitation and Quantification. Chromatin immunoprecipitations were done as described previously (40). Antibodies specific for acetylated histones H3 and H4 were purchased from Upstate Biotechnology. The immunoprecipitated promoter fragments were amplified by PCR and analyzed on 4% agarose gels or quantified with the SYBR green real time PCR method (Applied Biosystems). The primers for each amplicon were as follows. A, 5′-CCCCAGCTGAGAGATGGTAATC-3′ and 5′-GCACAAAACAGAGGATTTGCATAG-3′; B, 5′-AAA-AGCCAATATCCATCCGTTC-3′ and 5′-GCATCCAAAAC-ATGAAGTGAAAAC-3′; C, 5′-CCAGGCTGCTTGCGAAC-3′ and 5′-TGCATTTCAGAAGGAGATGGAAT-3′; D, 5′-TGG-GGCCCCAGACAATATC-3′ and 5′-GCCCATGTGCCAGT-TCAAC-3′; E, 5′-TCCTGGCTGGGTACCACTG-3′ and 5′-CTCTGAGCAGAGCAAGTGACATC-3′; F, 5′-AGAAACA-GAAATCTGACCGCTTG-3′ and 5′-TCATCACTAACCA-GTCACCAGTTG-3′; G, 5′-CCACTGTGAGGAACCGAC-TG-3′ and 5′-TGGAGCAACCAAGCACCTAC-3′; GAPDH, 5′-CCCGGCTACTAGCGGTTTTAC-3′ and 5′-CTGCGGG-CTCAATTTATAGAAAC-3′.

Results

Maturation of DC Is Accompanied by a Reduction in MHCII mRNA Expression. To gain insight into the regulation of MHCII expression during DC maturation, we analyzed

transcription of the *HLA-DRA* gene in immature and mature DCs. Immature DCs were generated from human peripheral blood monocytes by incubation with GM-CSF and IL-4 for 6 d, and were induced to mature with LPS. The $CD1a^+CD14^-$ phenotype of the cells was determined by FACS® analysis and the absence of B lymphocytes was confirmed by the lack of $CD19^+$ cells (data not shown). Upregulation of cell surface CD83, CD86, CD80, and CD40 was examined to control for maturation (Fig. 1, A and B). As described previously (33), the mature DCs are characterized by a markedly increased level of cell surface MHCII expression (Fig. 1, A and B).

To study changes in steady-state MHCII mRNA levels we first performed RPAs with total RNA from immature and LPS-stimulated DCs (Fig. 1 C). Immature DCs and DCs treated with LPS for 3 h expressed high levels of DRA mRNA. However, after 24 h of stimulation a marked reduction in DRA mRNA is evident. DRA mRNA levels were next quantified using a real time PCR approach (Fig. 1 D). A transient 40% increase in DRA mRNA is observed at early time points (3 h). However, after 24 and 48 h of stimulation with LPS the level of DRA mRNA is reduced to 35 and 23% of the initial level. Thus, although cell surface MHCII expression is increased, steady-state MHCII

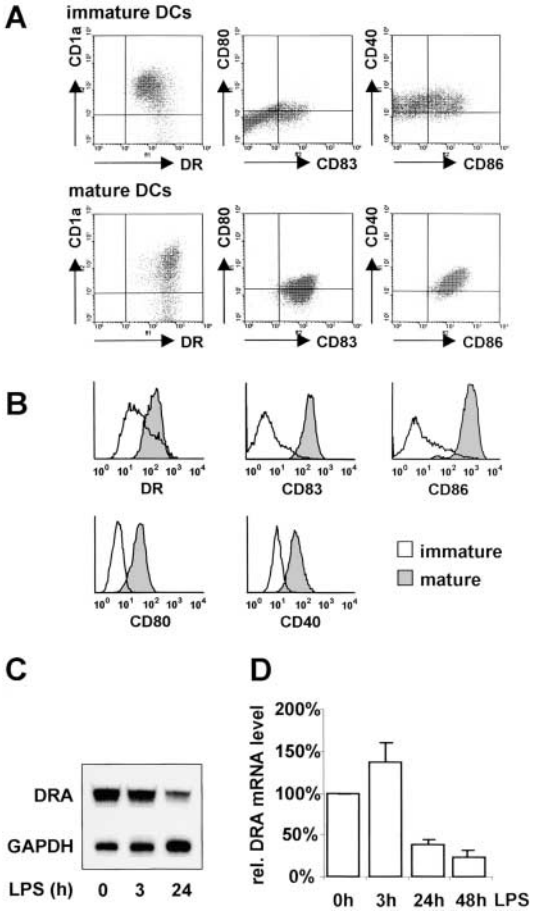

Figure 1. Downregulation of MHCII mRNA expression during the maturation of human monocyte-derived DCs. (A) Cell surface expression of CD1a, HLA-DR, CD83, CD80, CD86, and CD40 was examined by two-color FACS® analysis of immature DCs and DCs matured with LPS for 24 h. (B) Histogram plots for cell surface expression of HLA-DR, CD83, CD86, CD80, and CD40 on immature and mature DCs. The data in panels A and B represent two independent experiments. (C) Steady-state levels of HLA-DRA mRNA were studied by RPA in immature DCs and DCs stimulated for 3 and 24 h with LPS. GAPDH mRNA was measured as an internal control. (D) HLA-DRA mRNA was quantified by real time PCR in immature DCs and DCs stimulated with LPS for 3, 24, and 48 h. Values for DRA mRNA are normalized with respect to GAPDH mRNA, and are provided as the percentage of the level found in immature DCs. The mean and SEM of three independent experiments are shown. rel., relative.

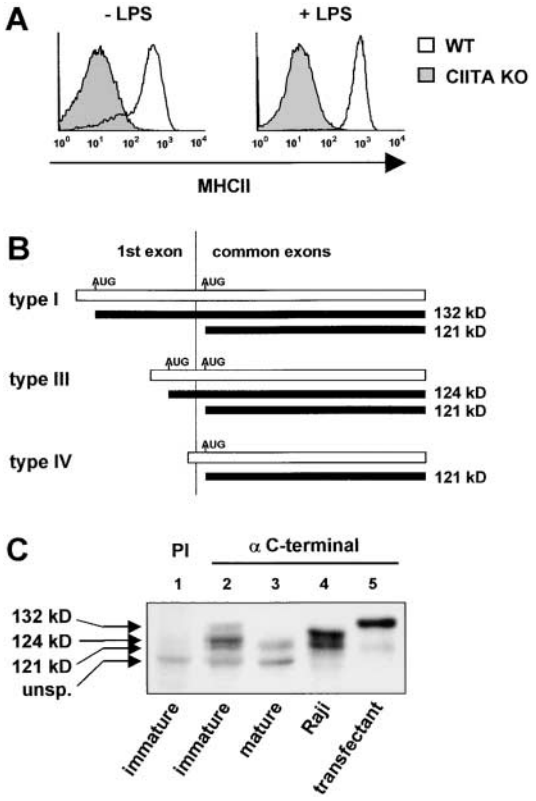

Figure 2. Downregulation of CIITA protein expression during maturation of DCs. (A) CIITA is required for expression of MHCII genes in DCs. MHCII cell surface expression on BMDCs from wild-type (WT) and CIITA knockout (KO) mice. The cells were either left untreated or stimulated with LPS for 24 h. (B) The three types of CIITA mRNA (types I, III, and IV) encode three different protein isoforms (121, 124, and 132 kD). These proteins differ only at their N-terminal end. White bars represent the CIITA mRNAs and black bars represent the proteins encoded by these mRNAs. The boundary between the alternative first exons and the shared downstream exons is indicated by a vertical line. The positions of translation initiation codons are indicated. The scheme is not drawn to scale. (C) CIITA proteins were immunoprecipitated with the anti–CIITA-C antibody from extracts derived from immature (lane 2) and mature (lane 3) DCs, and were revealed by immunoblotting using the anti–CIITA-N antibody. Control immunoprecipitations were done with extracts from Raji cells and a transfectant expressing CIITA type I mRNA. Raji cells express only the type III CIITA mRNA and thus synthesize high levels of the 124-kD isoform and a lower level of the 121-kD isoform (lane 4). The transfectant expresses only type I CIITA mRNA and thus synthesizes high levels of the 132-kD isoform and a low amount of the 121-kD isoform (lane 5). An immunoprecipitation performed with preimmune serum (PI) and the extract from immature DCs was used as negative control (lane 1). A contaminant that is immunoprecipitated nonspecifically from the DC extracts is indicated (unsp.).

mRNA levels are reduced four- to fivefold. This finding is consistent with earlier experiments demonstrating that DC maturation is accompanied by a decrease in de novo synthesis of MHCII proteins (4, 42).

CIITA Expression during DC Maturation. Multiple lines of evidence indicate that expression of the transactivator CIITA is a key requirement for expression of MHCII genes in a wide variety of cell types, including DCs. Epidermal DCs in a patient suffering from MHCII deficiency due to a mutation in the CIITA gene were found to be completely devoid of MHCII expression (43). In addition, fresh DCs from the spleen of CIITA knockout mice were found to be negative for MHCII expression (44, 45). MHCII expression is also strongly reduced in DCs isolated from lymph nodes of CIITA-deficient mice, although a residual (20% of normal) expression level is retained in these cells (46). Here we provide further evidence that cell surface MHCII is strongly reduced (100-fold) on BMDCs from CIITA knockout mice, both in the presence and absence of LPS (Fig. 2 A). We therefore investigated whether the decrease in MHCII mRNA observed in mature DCs could be accounted for by a reduction in the level of CIITA protein. CIITA is a rare protein that is not abundant enough in DCs to be detected directly by immunoblotting. We therefore performed immunoprecipitations followed by immunoblotting using two different anti-CIITA antibodies to increase sensitivity and specificity (Fig. 2 C). Three different CIITA protein isoforms can be synthesized from the three types of CIITA mRNA derived from promoters pI, pIII, and pIV (Fig. 2 B). These three CIITA isoforms have predicted molecular weights of 121, 124, and 132 kD. The shortest 121-kD isoform can be synthesized from all three types of CIITA mRNA by initiation at the AUG codon situated in the shared second exon. The 132-kD and the 124-kD isoforms are produced by initiation at the upstream AUG codons present in the specific first exons of the type I and type III CIITA mRNAs. All three CIITA isoforms are detected in immature DCs (Fig. 2 C, lane 2). After maturation there is an eightfold reduction in CIITA protein. The remaining CIITA is almost exclusively (95%) of the 121-kD isoform.

CIITA mRNA Reduction during DC Maturation. We next examined the steady-state levels of the different types of CIITA mRNA in immature and LPS-stimulated DCs. Two different quantitative approaches, RPA and real time PCR, were used. The probes and primers used for RPA and real time PCR are depicted schematically in Fig. 3 A. Types I and III CIITA mRNAs are abundant in immature DCs (Fig. 3 B). This is consistent with the finding that these cells express 132 and 124 kD CIITA proteins (see Fig. 2). On the other hand, type IV CIITA mRNA is scarcely detectable (Fig. 3 B). This implies that the 121-kD isoform observed in the immature DCs is likely to be derived from initiation at the translation initiation codon situated in the second exon of the type I and type III mRNAs (see Fig. 2). The CIITA mRNA profile observed in the immature DCs contrasts with the one observed for the monocytes from which they are derived. The latter express much lower levels of CIITA mRNA and essentially only type III is detected (Fig. 3 B). This is consistent with previous work indicating that the type I mRNA is specific for DCs while the type III mRNA is detected in DCs as well as in other cell types (28).

After LPS-induced maturation, a rapid reduction in CIITA mRNA is observed. This is evident both when using RPA and real time PCR to measure the CIITA mRNA levels (Fig. 3, B–D). Expression of CIITA mRNA is reduced within a matter of hours. After 3 h of stimulation the level of CIITA mRNA is already reduced by 80–90% and is even lower after 24 h. Interestingly, this strong reduction concerns both type I and type III CIITA mRNA even though pI and pIII have not been shown previously to be regulated coordinately. The reduction in CIITA mRNA expression is considerably more rapid than the reduction observed for MHCII mRNA (compare Figs. 1 and 3). This difference in kinetics is due, at least in part, to the fact that the half life of MHCII mRNA in DCs is very long (over 24 h; data not shown) with respect to the half life of CIITA mRNA (1 h; see Fig. 6).

To delineate more precisely the kinetics and extent of the decrease in CIITA mRNA during LPS-induced maturation we performed detailed time course experiments using real time PCR to measure mRNA levels (Fig. 3 E). Expression levels of both type I and type III CIITA mRNAs start to fall significantly within the first hour and basal levels are reached after 3–5 h. This loss in expression is essentially complete for CIITA type I mRNA, whereas the minimum attained by type III represents only ~20% of the initial level.

To determine whether our findings with human monocyte-derived DCs could be extended to other DC preparations we examined CIITA expression during the maturation of mouse BMDCs. The predominant form of CIITA mRNA in mouse BMDCs is of type I (data not shown; reference 35). The type III and type IV mRNAs are expressed at lower levels. After exposure to LPS, the mature BMDCs express high levels of cell surface MHCII, CD86, CD80, and CD40 (data not shown; reference 47). Furthermore, maturation of DCs is associated with the upregulation of CCR7 and reduction of CCR1 mRNA expression (48; Fig. 4 A). In these LPS-matured BMDCs, the expression of all three types of CIITA mRNA is reduced (Fig. 4 B). As observed in human monocyte-derived DCs, the reduction is strongest for type I CIITA mRNA. The decrease is less marked for types III and IV. However, final absolute levels of the three forms are likely to be similar because types III and IV are expressed at lower levels before maturation.

Extinction of CIITA Expression Is a General Consequence of DC Maturation. To determine whether the decrease in CIITA expression was a specific response to stimulation with LPS or a more general consequence of DC maturation, we measured CIITA mRNA levels in human monocyte-derived DCs exposed to a variety of different maturation signals. These included stimulation with LPS, TNF-α, CD40L, or IFN-α, and infection with *S. typhimurium* or Sendai (SeV^M). Real time PCR analysis revealed that all

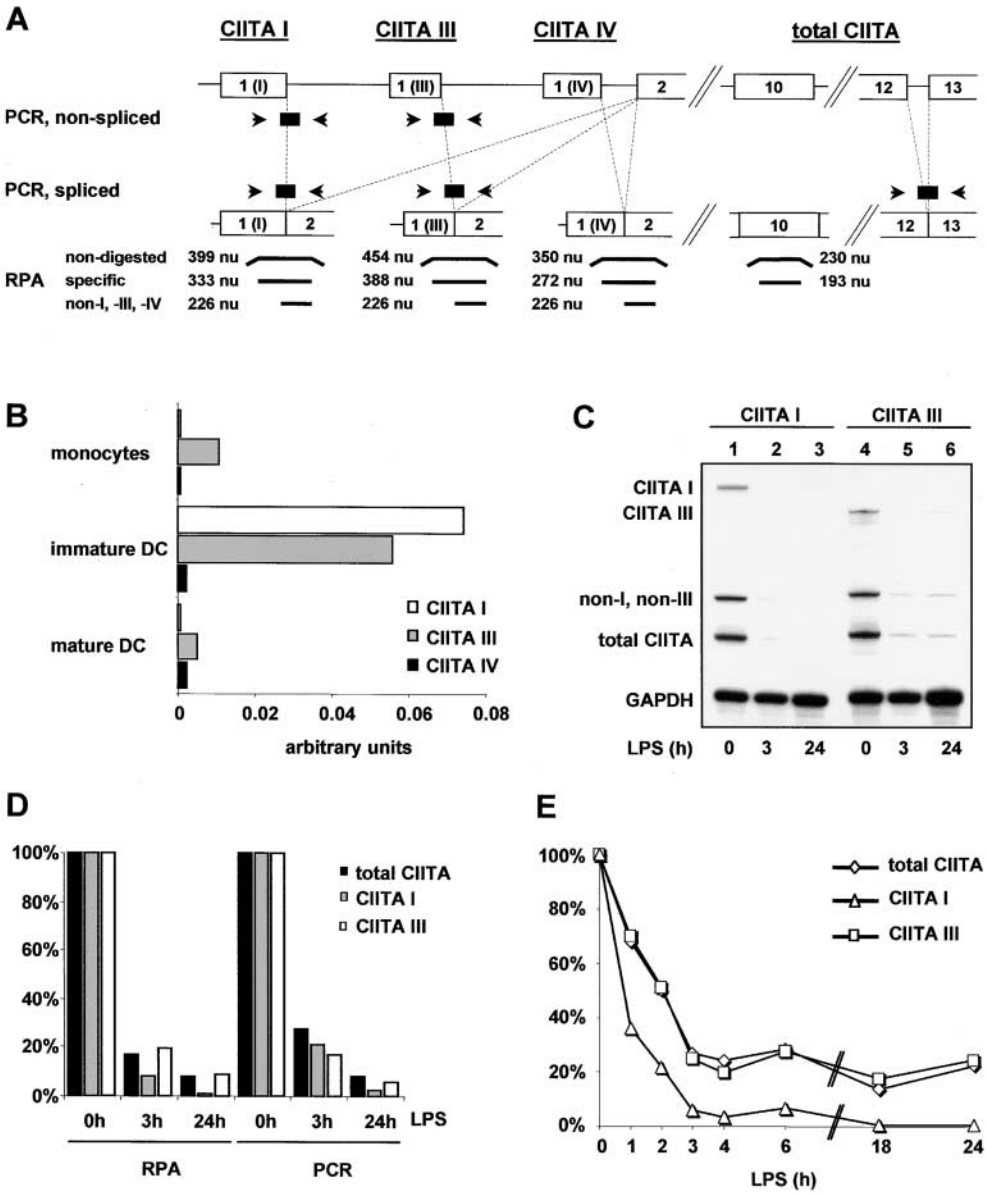

Figure 3. A strong and rapid downregulation of CIITA mRNA expression is induced during maturation of DCs. (A) Strategy for detecting the different spliced and unspliced types of CIITA mRNA by RPA and real time PCR. The structure of the *MHC2TA* gene (top) and the different CIITA mRNAs (bottom) are indicated schematically. Dashed lines indicate the pattern of splicing. White boxes represent exons; thin black lines represent introns. RPA probes specific for total CIITA mRNA and CIITA mRNAs of types I, III, and IV are indicated below. For each probe the sizes in nucleotides (nu) are indicated for the nondigested probe, the protected fragment specific for the corresponding type of CIITA mRNA (specific), and the protected fragment corresponding to the other types of CIITA mRNA (non-I, non-III, non-IV). Sets of primers (arrows) and probes (black rectangles) used in real time PCR to quantify total CIITA mRNA, and spliced and unspliced CIITA transcripts of type I and type III are shown in the middle. (B) Quantification of the different types of CIITA mRNA present in monocytes, immature, and mature DCs as measured by RPA. The results are standardized with respect to endogenous GAPDH mRNA. (C) Expression of type I and type III CIITA mRNA was analyzed by RPA in immature DCs (lanes 1 and 4) and DCs stimulated with LPS for 3 h (lanes 2 and 5) and 24 h (lanes 3 and 6). A probe specific for total CIITA was included in addition to the probes specific for CIITA type I or type III. A probe for GAPDH mRNA was included as internal control. (D) Results form the RPA analysis (C) were quantified by PhosphorImager and compared with data obtained by real time PCR on the same RNAs. The values are normalized with respect to GAPDH mRNA and are given as the percent relative to the levels found in immature DCs. (E) The kinetics of the reduction in type I, type III, and total CIITA mRNA was determined by real time PCR on RNA prepared from DCs stimulated with LPS for various periods of time. The values are normalized and presented as in D.

stimuli elicited a striking downregulation of total CIITA mRNA expression (Fig. 5). As observed for LPS, CIITA mRNA type I was affected more strongly than type III by the other maturation stimuli (data not shown). *S. typhimurium*, LPS, and IFN-α provoked a downregulation of total CIITA mRNA to 20–30% of initial levels within 4 h, whereas the reduction induced by SeV^M, TNF-α, and CD40 liter reached these levels after only 24 h. Despite this variability in kinetics, we can conclude that the decrease in CIITA expression is an integral feature of DC maturation rather than a response restricted to stimulation with LPS.

Further confirmation of the reduction in CIITA mRNA expression in DCs was obtained in an in vivo system (35). To study changes in CIITA mRNA levels in DCs in an inflammatory disease, we examined expression of the DC-specific type I CIITA mRNA in the spleen of mice with acute EAE. In mice with MOG-induced EAE we found a marked decrease in CIITA type I mRNA in total spleen (Fig. 4 C), suggesting that a decrease in CIITA expression also occurs in DCs in vivo. To confirm that this is indeed the case we compared CIITA mRNA levels in purified DCs isolated from the spleen of control mice and mice with EAE (Fig. 4 D). In the latter, the expression of all three forms of CIITA mRNA is strongly reduced. A strong downregulation of CIITA expression is thus observed in mouse DCs in vivo during an inflammatory process.

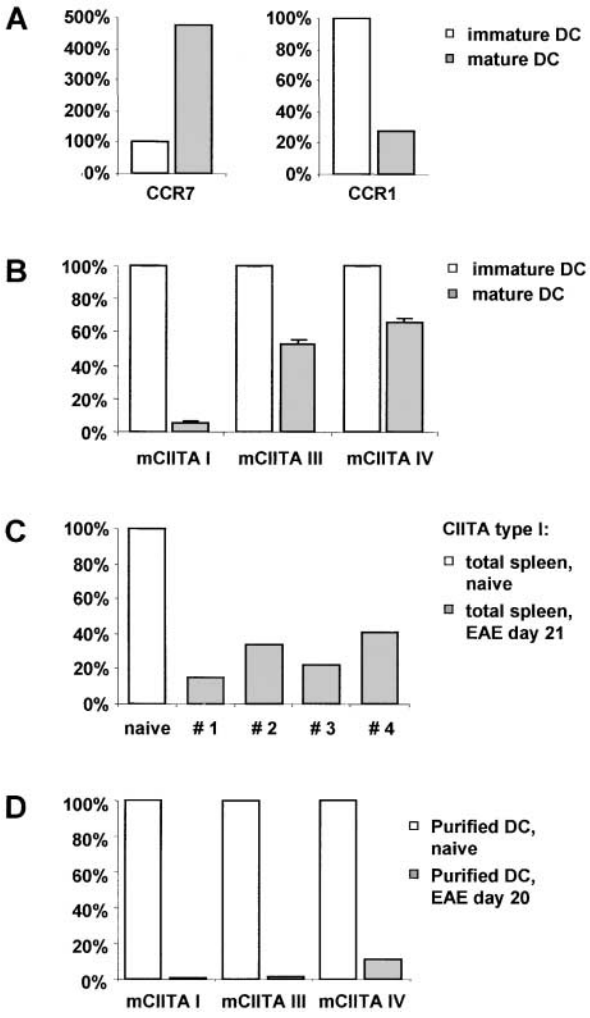

Figure 4. The reduction in CIITA mRNA expression is also observed in mouse DCs. (A) Mouse BMDCs were induced to maturate by stimulation with LPS for 24 h. CCR1 and CCR7 mRNA expression levels were determined by real time PCR to control for DC maturation. (B) The expression levels of the three types of CIITA mRNA were compared in the immature and mature BMDCs using real time PCR. The results are normalized with respect to 18S ribosomal RNA and are given as the percent relative to the levels found in immature BMDCs. The mean and SEM of three independent experiments are shown. (C) CIITA type I mRNA expression in total spleen was determined by RPA for control mice and mice with MOG-induced EAE. The values are normalized with respect to expression levels in naive animals. Four mice from three independent experiments are shown (#1 to #4). Types III and IV CIITA mRNAs were not considered here because they are expressed at high levels in B cells and IFN-γ induced cells, which would obscure the analysis. (D) Splenic DCs were purified from mice with MOG-induced EAE and total RNA was isolated. CIITA type I, type III, and type IV mRNAs were quantified by real time PCR. The results were normalized with respect to 18S rRNA. Expression levels in naive animals were set at 100%.

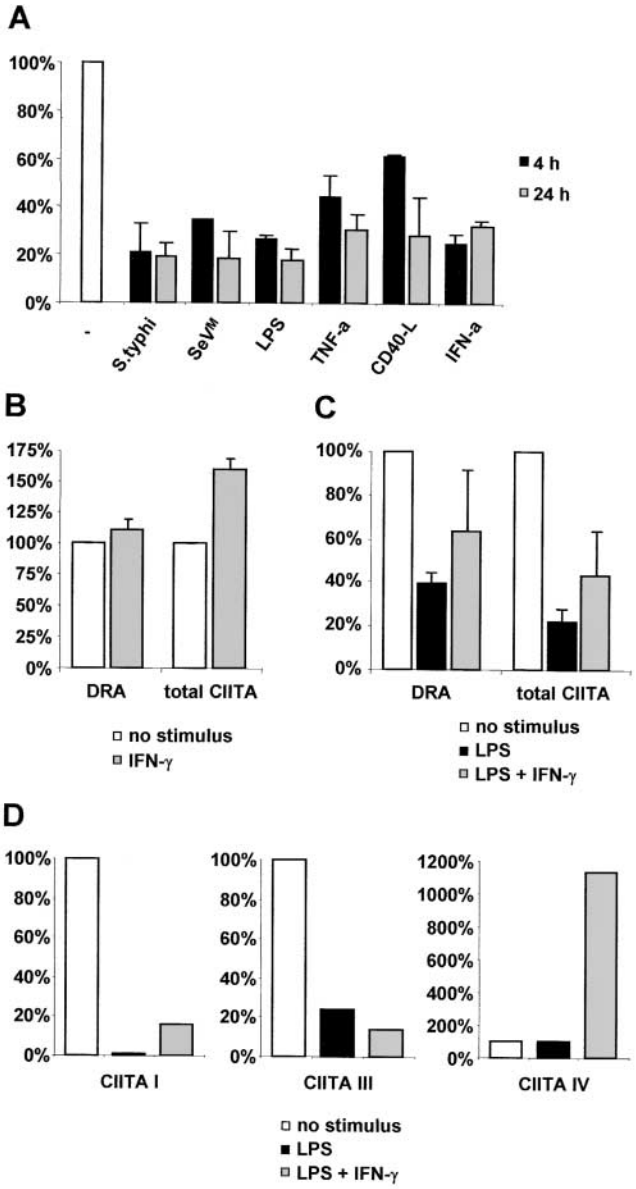

Figure 5. Downregulation of CIITA expression in DCs exposed to various different stimuli. (A) Immature DCs were exposed to the indicated maturation stimuli for 4 and 24 h and total CIITA mRNA was quantified. (B) DRA and total CIITA mRNA levels were quantified in immature DCs stimulated with IFN-γ for 24 h. (C) DRA and total CIITA mRNA levels were quantified in immature DCs stimulated with LPS for 24 h in the presence or absence of IFN-γ. (D) CIITA type I, type III, and type IV mRNA levels were analyzed in the samples from panel C. Real time PCR was performed for the quantification of mRNAs in all panels. The values are normalized and represented as in Fig. 3 D. For each stimulation (A–C), the mean and SEM of two to four independent experiments are shown.

In addition to known maturation stimuli, we also examined the effect on DCs of IFN-γ, a stimulus known to modulate CIITA and MHCII expression in many cell types. Treatment of immature DCs with IFN-γ does not lead to maturation (data not shown) and does thus not result in a reduction of MHCII and CIITA mRNA expression (Fig. 5 B). If anything, a slight increase in MHCII and total CIITA mRNA expression is observed (Fig. 5 B). This IFN-γ induced increase is very mild compared with the induction seen in other cell types, probably because the basal level in DCs is already very high.

We also examined the effect of including IFN-γ during

DC maturation induced by LPS. The presence of IFN-γ partially prevented the decrease in MHCII and total CIITA mRNA during DC maturation (Fig. 5 C). This is not due to an inhibition by IFN-γ of the decrease in types I and III CIITA mRNA (Fig. 5 D). Instead, it results from the fact that the decrease in types I and III mRNA is compensated for partially by activation of CIITA type IV mRNA expression (Fig. 5 D).

The Decrease in CIITA mRNA Expression Is Due to an Arrest in Transcription. To determine whether the decrease in steady-state levels of CIITA mRNA could be due to mRNA destabilization, we compared the stability of the CIITA transcripts in immature and mature DCs (Fig. 6 A). The cells were treated with DRB to block de novo transcription and CIITA mRNA levels were then determined at various time points. The half-life of CIITA mRNA was found to be very short (~1 h) in both immature and mature DCs. No statistically significant difference in half-life was evident between the two mRNA decay curves. These results show that the decline in CIITA mRNA abundance during DC maturation is not due to a change in mRNA stability.

The fact that the CIITA mRNA decay rate is not modified during DC maturation implies that the reduction in CIITA mRNA expression must be due to an arrest in transcription. To confirm this interpretation we measured the transcription rate of the CIITA gene. A method relying on the quantification by real time PCR of chromatin-bound nascent transcripts was employed. This type of approach represents a sensitive and reliable alternative to the classical run-on assay (38). In immature DCs, spliced and unspliced nascent CIITA transcripts derived from pI and pIII are readily detected (Fig. 6 B). After LPS-induced maturation, the synthesis of these nascent transcripts is strongly reduced. Consistently with the steady-state CIITA mRNA measurements (Fig. 3), the reduction is stronger for pI (10- to 20-fold) than for pIII (5- to 10-fold) (Fig. 6 B). The same arrest in transcription is also revealed by measurements of the abundance of spliced type I and type III CIITA transcripts present in the free nuclear RNA fraction (Fig. 6 B). Unspliced CIITA transcripts were as expected present at only very low levels in the free nuclear RNA fraction (data not shown). This is consistent with the fact that splicing of precursor transcripts is known to be a cotranscriptional process that is largely completed before release from the chromatin template. In conclusion, our findings indicate that the decrease in CIITA expression during DC maturation is controlled by transcriptional silencing of the CIITA gene. The arrest in transcription concerns both pI and pIII of the *MHC2TA* gene.

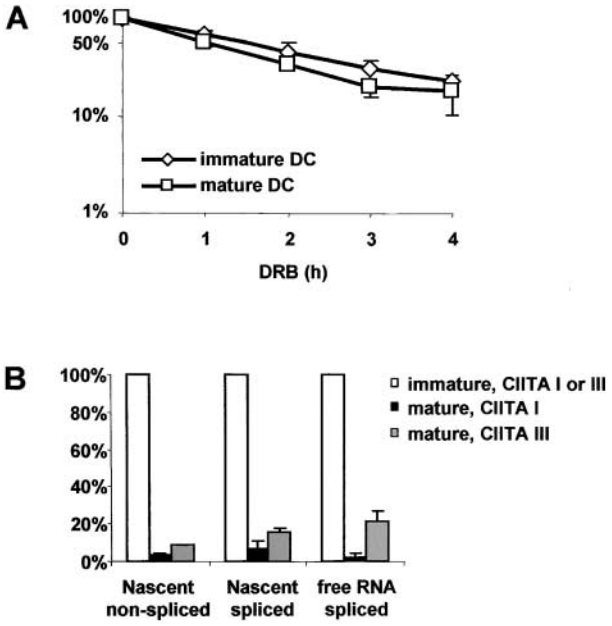

Figure 6. The reduction in CIITA expression during DC maturation is regulated at the level of transcription rather than mRNA stability. (A) Immature DCs and DCs stimulated with LPS for 2.5 h were treated with DRB for 0, 1, 2, 3, and 4 h. CIITA mRNA abundance was then quantified by real time PCR. The mean and SEM of three independent experiments are shown. (B) Nascent chromatin-bound transcripts and free nuclear RNAs were isolated from immature and mature DCs. Spliced and nonspliced forms of CIITA type I and type III transcripts were quantified by real time PCR. Levels of type I and III CIITA transcripts in immature DCs were set at 100% and are represented by a single bar. The mean and SEM of two independent experiments are shown.

Silencing of the MHC2TA Gene Is Not Accompanied by a Change in Promoter Occupation. Changes in transcriptional activity are frequently reflected by alterations in promoter occupation that can be visualized in living cells by means of in vivo genomic footprint experiments (39). We used this technique to study whether silencing of the *MHC2TA* gene during DC maturation is accompanied by modifications in the occupation of pI and pIII. The region situated immediately upstream of the transcription initiation site of pI contains a short 120-bp sequence that is highly homologous between the human and mouse genes. In immature DCs, the occupation of this proximal region of pI is revealed by the presence of five enhanced guanine residues on the lower strand and five protected guanine residues on the upper strand (Fig. 7 A). These enhanced and protected residues fall within or near sequence motifs representing potential binding sites for known transcription factors (28; Fig. 7 A). Surprisingly, no clear changes in this footprint pattern are detected following the silencing of pI in mature DC (Fig. 7 A). The proximal region of pI thus remains occupied despite the reduction in transcription.

The region situated upstream of the transcription initiation site of pIII contains regulatory sequences that are conserved between the human and mouse genes (28). In immature DCs, the occupation of pIII is characterized by a footprint pattern that is similar to the one observed in B cell lines (49; unpublished results). This pattern consists of (a) a cluster of protections lying within a sequence designated ARE-1 (a TEF-2–like binding site), (b) a protection and an enhancement lying within a sequence designated ARE-2, (c) two protections falling within a sequence designated site A (an nuclear factor [NF]-1–like binding site), and (d) a protection in a sequence designated site C (which resembles an IFN regulatory element; Fig. 7 B). As ob-

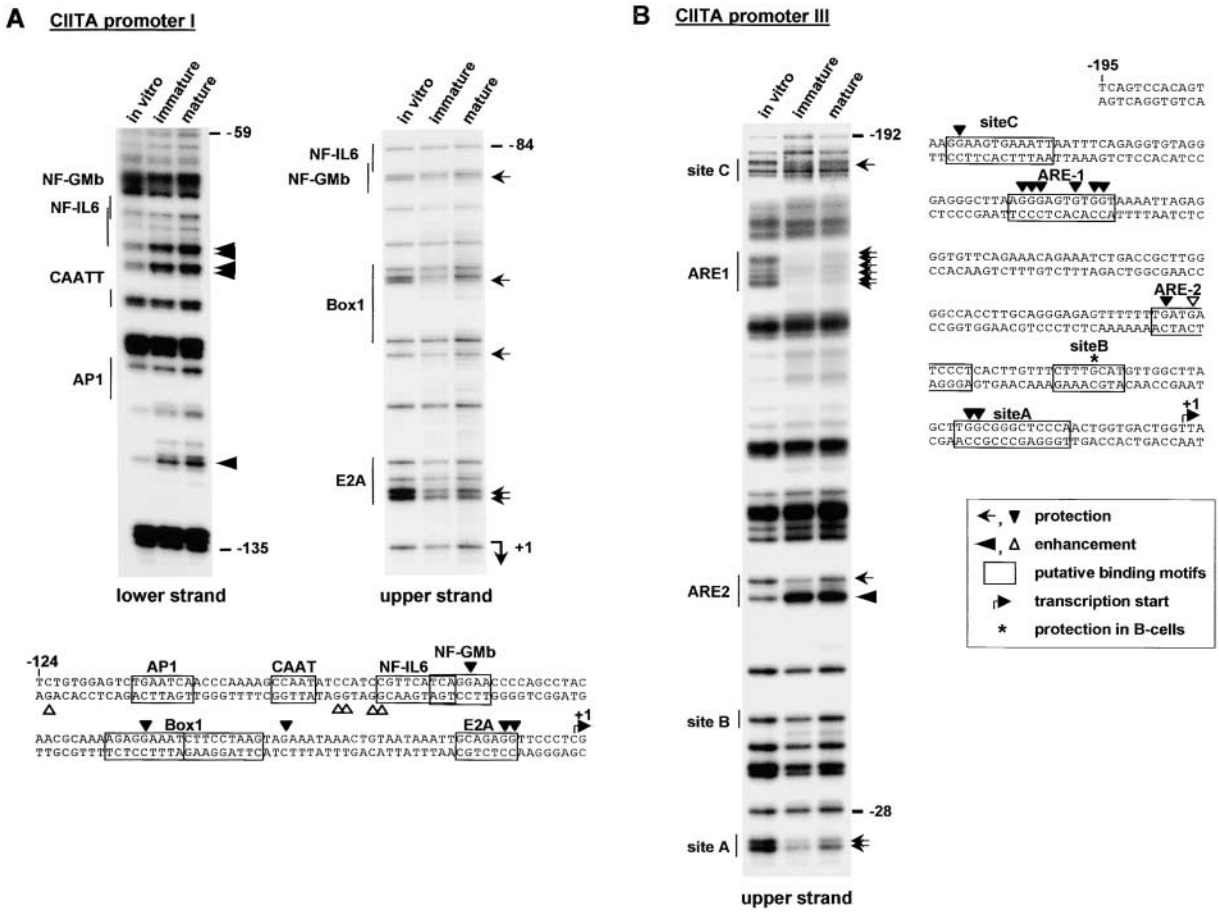

Figure 7. Extinction of *MHC2TA* transcription during DC maturation is not accompanied by a change in the occupation of promoters pI and pIII. The occupation of pI (A) and pIII (B) was analyzed in immature and mature DCs by in vivo genomic footprinting. Results are shown for the upper and lower strands of pI and for the upper strand of pIII. The first lane in each group shows the pattern obtained in vitro with naked control DNA. The second and third lanes in each group show the patterns obtained for immature and mature DCs, respectively. Arrows indicate protected residues. Arrowheads indicate enhancements. The bent arrows indicate the sites of transcription initiation. The putative binding motifs are indicated at the left of the gels. Box 1 is composed of either two NF-IL6 sites or two PEA3 sites. Positions of the protections, enhancements and putative binding sites are indicated on the sequences of the proximal promoter regions of pI and pIII.

served for pI, the footprint pattern of pIII does not change significantly after maturation of the DCs, indicating that silencing of pIII is not accompanied by an obvious change in promoter occupation (Fig. 7 B). The similarity in the observations made for pI and pIII suggests that these two promoters are governed by a common regulatory mechanism during DC maturation.

An additional protection previously identified in B cells could not be detected in DCs (49; Fig. 7 B). This protection falls in a region (site B) representing a putative octamer sequence, which can be bound by the Oct-1 and Oct-2 transcription factors in association with the B cell specific coactivator OBF-1 (50). The lack of occupancy of the octamer site suggests that it is not an essential cis-acting sequence for expression of pIII in DCs.

The Entire Regulatory Region of the CIITA Gene Is Deacetylated during Maturation. Deacetylation of lysine residues of the core histone tails is frequently associated with transcriptional repression. Histone deacetylation is thought to result in a more compact chromatin structure that decreases the accessibility of chromatin and represses transcription (51–54). To determine whether histone deacetylation plays a role in the repression of CIITA transcription during DC maturation, we treated the cells with LPS in the presence of the deacetylase inhibitor TSA. Administration of TSA inhibited LPS-induced downregulation of CIITA mRNA (Fig. 8 A). As expected, this also abolished the downregulation of MHCII mRNA expression (Fig. 8 A). The finding that silencing of the *MHC2TA* gene is relieved by TSA suggests that it is mediated by a mechanism leading to deacetylation of histones associated with its regulatory region. To determine whether this is indeed the case, we performed chromatin immunoprecipitation assays with antibodies specific for acetylated histones. In immature DCs expressing high levels of CIITA, histone H4, and to a lesser extent histone H3, are acetylated over the entire 12-kb regulatory region of the *MHC2TA* gene (Fig. 8 B). This is evident both within and outside of the regions containing pI, pIII, and pIV. After maturation, histone acetylation is lost over the whole regulatory region (Fig. 8, B and C). The mechanism driving si-

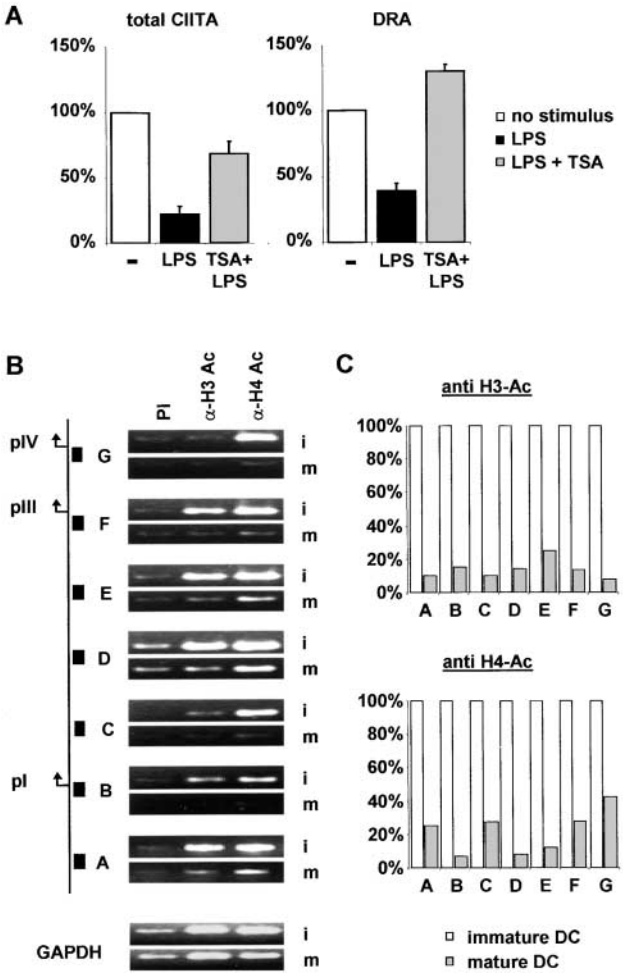

Figure 8. The entire regulatory region of the *MHC2TA* gene is deacetylated during maturation of DCs. (A) Immature DCs were stimulated with LPS in the absence or presence of TSA for 24 h. Total CIITA and DRA mRNAs were quantified by real time PCR. (B) Chromatin immunoprecipitation was performed on immature (i) and mature (m) DCs with antibodies specific for the acetylated histones H3 and H4. Immunoprecipitations with preimmune serum (PI) served as a negative control. The immunoprecipitates were analyzed by PCR for the presence of seven fragments spanning the entire regulatory region of the *MHC2TA* gene (primer pairs A to G). The regulatory region of the MHC2TA gene and the positions of primer pairs A to G are represented on the left. The proximal promoter of the GAPDH gene was used as a control. (C) Real time PCR was performed for quantification of the immunoprecipitated samples from panel B. Results were normalized with respect to the GAPDH promoter. For each region (A to G) the value obtained in immature DCs was set at 100%.

lencing of the *MHC2TA* gene thus involves global histone deacetylation over a large regulatory domain.

Discussion

Precisely orchestrated changes in MHCII expression are crucial for the ability of DCs to function as professional APCs. Immature DCs synthesize MHCII molecules at a high rate but store them in an intracellular compartment rather than displaying them at the cell surface. When maturation is induced by inflammatory stimuli or infections, MHCII–peptide complexes are assembled and transported to the cell surface (4, 5, 10, 11). This increased cell surface MHCII expression reflects the commitment of DCs to a state optimized for the presentation of antigenic peptides to naive $CD4^+$ T lymphocytes. In parallel with the increase in cell surface expression, de novo synthesis of MHCII molecules is actually decreased. This block in synthesis of MHCII molecules ensures that the existing MHCII–peptide complexes are not replaced or diluted by new complexes assembled during the maturation and migration of the DCs. Reduced synthesis of new MHCII molecules in mature DCs is coherent with the fact that these cells also loose their capacity for antigen uptake. We show here that the reduction in de novo MHCII synthesis occurring during maturation of DCs is due to transcriptional silencing of the gene encoding CIITA, a key regulator of MHCII expression. Two independent promoters, pI and pIII, of the *MHC2TA* gene are repressed in a coordinated fashion after exposure to stimuli eliciting DC maturation. This silencing of *MHC2TA* transcription is remarkably rapid. Minimum expression levels are attained after <4 h of stimulation. A wide variety of different maturation stimuli have the same effect. Finally, the relevance of this finding is emphasized by the fact that it is observed in human monocyte-derived DCs, in mouse BMDCs, and in vivo in splenic DCs in acute Th1-mediated autoimmunity (EAE).

During an early phase of DC maturation we observed a transient increase in MHCII mRNA abundance. Elevated MHCII mRNA levels are evident after 3 h of stimulation with LPS, but are then reduced to 20–30% of initial levels by 24–48 h (Fig. 1 C). This initial increase in MHCII mRNA abundance is consistent with previous metabolic labeling experiments showing that de novo biosynthesis of MHCII molecules goes up transiently at early time points during DC maturation (4). Surprisingly, the transient increase in MHCII mRNA expression is not associated with elevated CIITA expression and it is thus unlikely to be due to enhanced transcription of MHCII genes. Instead, it is tempting to speculate that it could be due to an increase in the stability of MHCII mRNA. In this respect it should be mentioned that there is indeed evidence suggesting that MHCII mRNA stabilization may contribute to the regulation of MHCII expression in other cell types (55, 56).

LPS induces a reduction of CIITA and MHCII expression in DCs. This contrasts with the effect of LPS in other types of APCs. For instance, LPS is well known to enhance constitutive MHCII expression in mouse B cell lines and primary B cells (57). Human B cells do not respond to LPS by changing cell surface MHCII expression or DRA mRNA levels, although we have observed a mild reduction in CIITA mRNA expression (data not shown). This reduction is much less rapid and extensive that what is observed in DCs. In human monocytes and macrophages, two opposing effects have been described: LPS alone has been shown to stimulate MHCII expression transiently while it inhibits induction of MHCII expression by IFN-γ

or IL-4 (58, 59). Taken together, these observations indicate that the mechanisms governing modulation of MHCII expression by LPS must differ between DCs and other MHCII-positive cell types.

DC maturation can be induced by a variety of infectious agents and cytokines. We found that downregulation of CIITA expression is induced by all of the maturation stimuli that we tested, indicating that the reduction in CIITA and MHCII expression is a general consequence of DC maturation. Although the extinction of CIITA expression is qualitatively comparable for all stimuli, the efficiency of this response is variable. Variations between the stimuli are not unexpected because they use different signaling pathways for turning on the DC maturation program. It is remarkable that all these signal transduction pathways converge on the molecular machinery regulating transcription of the *MHC2TA* gene.

We show here that the type I and type III CIITA mRNAs give rise to three different CIITA proteins having distinct N-termini. Until now, the existence of three CIITA protein isoforms had been inferred from the structures of the different types of CIITA mRNA, but they had not been formally shown to be synthesized in vivo. Nothing is known to date about the relative translation efficiencies or stabilities of the different CIITA protein isoforms, and it is thus no possible to draw a direct correlation between the relative abundance of the three CIITA protein isoforms and the relative abundance of the three types of CIITA mRNA. In contrast to the rapid disappearance of the 124- and 132-kD CIITA proteins, the 121-kD protein persists for a prolonged period of time after the initiation of DC maturation. Type IV mRNA is not expressed at significant levels in either immature of mature DCs (see Fig. 3) and is thus unlikely to account for the persistence of the 121-kD isoform. Instead, it is likely to be derived from type III CIITA mRNA (Fig. 2). Continued synthesis of some CIITA protein is not unexpected because a basal level of type III CIITA mRNA is retained. However, the explanation for the differential rate of disappearance of the isoforms is not clear. It could be accounted for by differences in the stability of the isoforms. Persistence of the 121-kD isoform is likely to be responsible for the residual (20%) level of MHCII gene transcription that is retained in mature DCs after 24 to 48 h of stimulation.

Our genomic footprint experiments have pinpointed a number of sites in pI and pIII that are occupied in vivo in DCs. This represents a strong indication that these sequences constitute important cis-acting regulatory elements for the activity of pI and pIII in DCs. Confirmation of this, and identification of the relevant transcription factors that bind to these sequences, will require further work. These studies will not be trivial because they are severely hampered by the necessity of working with primary DCs, which are difficult to obtain in large numbers and to transfect. Stable DC cell lines have been established (60–63). However, these cell lines are not suitable because in our hands they either do not express significant levels of CIITA and MHCII, or do not respond to maturation stimuli in a manner that is identical to that observed for primary DCs (unpublished data).

In mature DCs, pI and pIII remain occupied despite the fact that they are transcriptionally silent. This is similar to the situation observed in plasmocytes, in which silencing of CIITA expression is also accompanied by continuous pIII occupation (unpublished data). On the other hand it contrasts with observations made in other cell types in which these promoters are not active. For instance, pI is bare in B cells, and neither pI nor pIII are occupied in IFN-γ stimulated fibroblasts (unpublished data).

The type I and type III CIITA mRNAs are lost coordinately and with similar kinetics in LPS-stimulated human monocyte-derived DCs. This reduction is due to a coordinate silencing of the two promoters, pI and pIII, that drive transcription of the *MHC2TA* gene. The three different forms of CIITA mRNA are also reduced coordinately in mouse BMDCs treated with LPS. These findings are rather surprising because the different CIITA promoters are distributed over more than 12 kb of DNA, do not share any sequence homology, and are known to be regulated independently in cell types other than DCs. Taken together, these results suggest that silencing during DC maturation is mediated by a higher order regulatory mechanism affecting all *MHC2TA* promoters simultaneously. We show here that this mechanism involves global histone deacetylation over a large 12-kb region encompassing the entire regulatory region of the *MHC2TA* gene. Silencing of the *MHC2TA* gene during DC maturation thus appears to be mediated by chromatin remodeling of a large domain rather than by localized repression restricted to individual promoters.

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