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Regulation of Class II MHC Expression in APCs: Roles of Types I, III, and IV Class II Transactivator

Rish K. Pai,* David Askew,* W. Henry Boom,† and Clifford V. Harding‡*

Class II transactivator (CIITA) is necessary for expression of class II MHC (MHC-II) molecules. In mice, CIITA expression is regulated by three promoters (pI, pIII, and pIV), producing types I, III, and IV CIITA. The relative roles of different CIITA types remain unclear. Unstimulated bone marrow-derived macrophages expressed low levels of CIITA mRNA; type I CIITA was nine times more abundant than type IV (type III CIITA was barely detected). Exposure to IFN-γ (6 h) dramatically increased types I and IV CIITA mRNA to similar absolute levels. Type IV CIITA declined over time, but type I was stable for over 72 h. Thus, the dominant form of CIITA evolved with time during activation by IFN-γ, and type I CIITA explained prolonged expression of MHC-II by macrophages. mRNA half-life was shorter for type I than type IV CIITA, suggesting that sustained transcription contributed to stable expression of type I CIITA induced by IFN-γ. Splenic B cells expressed mRNA for type III CIITA but very little for types I or IV. Treatment with IL-4 increased surface expression of MHC-II protein, but mRNA for MHC-II and CIITA (total, I, III, and IV) remained unchanged, suggesting posttranslational regulation. Splenic dendritic cells expressed type I CIITA but little type III or IV; CpG DNA induced their maturation and decreased types I and III CIITA, consistent with decreased MHC-II protein synthesis. CIITA types differ in regulation in various APCs under different stimuli, and the predominant type of CIITA varies at different stages of APC activation. The Journal of Immunology, 2002, 169: 1326–1333.

binding of TCRs to peptide-class II MHC (MHC-II) complexes expressed by professional APCs is required for CD4+ T cell responses. The nature and consequences of a CD4+ T cell response depends on the APCs involved. Dendritic cells prime naive T cells and initiate immune responses. Macrophages are recruited to sites of infection and activate already primed T cells. B cell presentation to T cells provides T cell help to enhance Ab production.

MHC-II expression in professional APCs is tightly regulated. In the presence of inflammatory cytokines such as IFN-γ, macrophages increase MHC-II expression, and their ability to process Ags to CD4+ T cells is markedly improved. B cell maturation into plasma cells is accompanied by a decrease in MHC-II molecule expression as the focus of the cell shifts from Ag capture and presentation to Ab production (1). Upon maturation, dendritic cell expression of MHC-II molecules increases, but MHC-II mRNA decreases (2, 3); increased MHC-II expression is due to increased half-life of peptide:MHC-II molecules (2, 4, 5).

MHC-II Ag processing requires expression of MHC-II, invariant chain, and H2-DM, which are all controlled by the class II transactivator (CIITA) (6–8). CIITA−/− mice have virtually no MHC-II molecules and thus low numbers of CD4+ T cells, indicating that CIITA is the master regulator of MHC-II expression (9, 10). CIITA does not directly bind to the promoter region of the genes it regulates; rather, it coordinates the action of numerous transcription factors such as members of the regulatory factor X (RFX) family, NF-Y and CREB (11–14). Transcriptional activation of the CIITA gene occurs through the action of three promoters in mice (pI, pIII, and pIV) (15). Immediately downstream of each promoter exists a unique exon 1 that is spliced with the remaining shared exons to form three different types of CIITA (I, III, and IV) (Fig. 1). Translation of CIITA mRNA can begin in exon 2; however, types I and III CIITA mRNA both contain translational start sites in exon 1 (giving rise to CIITA molecules with different N-terminal protein sequences) (16).

When these promoters were first discovered, pI was shown to be active in dendritic cells, pIII in B cells, and pIV in IFN-γ-treated macrophages (15). Recently it has become clear that the restriction of these promoters to different cell types is not so simple (Fig. 1). Type III CIITA has been found in human dendritic cells and monocytes (17), and can be induced by IFN-γ (18–22). In addition, a recent study showed that type I CIITA is critical in regulating MHC-II transcription in murine macrophages (23). In this study, we systematically analyzed expression of different types of CIITA in professional APCs (macrophages, B cells, and dendritic cells) under conditions that include maturation or activation. The primary goal was to solve uncertainties about the relative roles of different types of CIITA at different stages of macrophage activation, but we also tested the roles of different types of CIITA during activation or maturation of B cells and dendritic cells. Because MHC-II expression is modulated by multiple stimuli that vary with different APCs, different types of CIITA may play distinct roles in different stages of response to particular stimuli. A greater understanding of the relative roles of different CIITA types will contribute to our understanding of the control of MHC-II expression under various pathophysiologic conditions.
FIGURE 1. Diagram of the promoter region upstream of the murine CIITA gene. Upstream of the murine CIITA gene exist three independent promoters that give rise to three different CIITA mRNA species (types I, II, and III). The three types of CIITA are formed by splicing alternative first exons with exon 2. Types I and III CIITA mRNA contain translational start sites in exon 1 producing 132- and 124-kDa CIITA molecules, respectively. Type IV CIITA uses a translational start site in exon 2, leading to a 121-kDa form of CIITA. Cell types that express each form are indicated (parentheses indicate lower expression or variation between different conditions or reports).

Materials and Methods
Reagents
Murine IFN-γ, IL-4, and GM-CSF were purchased from R&D Systems (Minneapolis, MN). Phosphorothioate-modified CpG oligodeoxynucleotide (ODN) 1826 (TCCATGACCTGCCTGACGTT) (CpG motif are underlined) was provided by Coley Pharmaceutical Group (Wellesley, MA) and was dissolved in TE buffer (10 mM Tris, 1 mM EDTA).

Cell culture
Unless otherwise specified, all experiments were performed at 37°C in 5% CO₂ atmosphere using cells derived from C57BL/6J-H-2b female retired breeders (The Jackson Laboratory, Bar Harbor, ME) housed under specific pathogen-free conditions. Macrophages and B cells were cultured in standard medium composed of DMEM (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated FCS (Hyclone Laboratories, Logan, UT), 50 μM 2-ME, 1 mM sodium pyruvate, 10 mM HEPES buffer, and antibiotics. Dendritic cells were cultured in RPMI 1640 (Life Technologies) supplemented with 5% FCS, 50 μM 2-ME, 25 mM HEPES, β-glutamine, 20 μg/ml gentamicin, and 4 ng/ml GM-CSF.

Generation and treatment of bone marrow-derived macrophages (BMM) and thyglocollate-elicited peritoneal macrophages
BMM were derived from bone marrow precursors that were harvested from femur marrow and differentiated in bacterial grade dishes for 7 days in standard medium supplemented with 20% LADMAC cell conditioned medium (24). The resultant cells were used during the following week. The cells were adherent with macrophage-like morphology, and >98% of the cells were CD11b+ positive by flow cytometry. BMM were removed from Petri dishes with trypsin plus 0.02% EDTA (Life Technologies). For flow cytometry, BMM were plated at 2–3 × 10⁵ cells/60 mm Petri dish and cultured with or without 2 ng/ml of IFN-γ for the indicated time period. BMM were removed with trypsin plus 0.02% EDTA, counted, and stained for I-Aβ expression. For RT-PCR analysis, BMM were plated at 4 × 10⁶ cells/60 mm Petri dish and allowed to rest overnight. The following day BMM were stimulated with or without the indicated concentrations of IFN-γ and removed from the plate with trypsin plus 0.02% EDTA. RNA was isolated, and RT-PCR analysis was performed. Thyglocollate-elicited peritoneal exudate cells were harvested 5 days after injection of 1 ml of sterile 3% thyglocollate. Peritoneal exudate cells were plated in bacterial grade Petri dishes overnight, and nonadherent cells were washed away. Adherent macrophages were harvested, stimulated, and analyzed as described above. For mRNA stability studies, BMM were stimulated with IFN-γ for 6 h. After 6 h of stimulation, 50 μM 5,6-dichloro-1-β-D-ribosyl-sulfanilamidine (DRB) (Sigma-Aldrich, St. Louis, MO) was added to stop de novo mRNA synthesis. RNA was isolated at 3, 6, 9, and 12 h after DRB addition.

Isolation and stimulation of splenic B cells
Naïve splenic B cells were purified from whole splenocytes by negative selection with anti-CD43 microbeads (Miltenyi Biotec, Auburn, CA). Briefly, spleens were pressed through a 70-μm cell strainer (BD Labware, Franklin Lakes, NJ) using the plunger of a 5-ml syringe. The cell strainer was washed with DMEM and splenocytes were pelleted at 300 × g. To lys RBC, the pellet was resuspended in 5 ml of ACK buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.2) per spleen and incubated for 5 min at room temperature. Standard medium was added, and the cells were pelleted. Resultant splenocytes were treated with anti-CD43 magnetic microbeads, and CD43-negative cells (naïve B cells) were collected according to the manufacturer’s protocol. Flow cytometry showed that CD43-negative cells expressed little or no TCR β-chain or Mac-1, but >95% of the cells expressed the B cell marker, B220. For analysis of I-Aβ expression, 4 × 10⁶ B cells were plated in a 60-mm Petri dish and incubated with or without 10 ng/ml IL-4 for 24 h. Cells were harvested by gentle scraping and stained for I-Aβ. For RT-PCR analysis, freshly purified B cells were plated at 1 × 10⁷ cells/100-mm Petri dish, stimulated with IL-4 for 12 h, harvested as above, and used to isolate RNA.

Isolation and stimulation of dendritic cells
Splenocytes were generated by digesting spleen fragments with 150 U/ml collagenase (Worthington Biochemical, Lakewood, NJ) and 30 U/ml DNase (Sigma-Aldrich) for 1 h at 37°C and passing the digest through a 70-μm cell strainer (BD Labware). RBC were lysed with ACK lysis buffer (as above). Cells were layered over Ficoll-Paque (Amersham Pharmacia, Piscataway, NJ) and centrifuged to isolate viable cells. CD11c+ cells were then purified using anti-CD11c microbeads (Miltenyi Biotec) according to the manufacturer’s protocol. Flow cytometry showed that >95% of the resulting cells expressed high levels of CD11c. Immediately after purification, some splenic dendritic cells were harvested for RNA preparation. Other dendritic cells were incubated for 18 h in 100-mm Petri dishes (9 × 10⁵ cells/dish) with 4 ng/ml GM-CSF (R&D Systems) with or without 1 μg/ml of CpG ODN 1826. Cells were collected by gentle scraping, and RNA was isolated. Bone marrow-derived dendritic cells were prepared as described (25), except that cells were harvested after 5 days of culture with GM-CSF (4 ng/ml) and dendritic cells were then purified by positive selection with anti-CD11c magnetic beads, as described above for splenic dendritic cells. Greater than 90% of the purified dendritic cells expressed high levels of CD11c by flow cytometry.

Detection of I-Aβ by flow cytometry
Cells (2 × 10⁷) were incubated with 10 μg/ml FCBlock (anti-CD16/CD32; BD Pharmingen, San Diego, CA) in PBS containing 1% FBS and 10% normal mouse serum. Cells were then stained with biotinylated 10.3.6-2 anti-I-Aβ or biotinylated IgG2a isotype control Ab (BD Pharmingen) at 5 μg/ml. Cells were washed, incubated with streptavidin-Chrome (1:100; BD Pharmingen), washed, and resuspended in 2% paraformaldehyde. Stained cells were analyzed with a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA).

RNA purification, cDNA synthesis, and real-time quantitative PCR
Cells were lysed with a QiaShredder (Qiagen, Valencia, CA), and total cellular RNA was purified from lysates using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. Residual genomic DNA was removed during the purification process by incubation with RNaseA-free DNase (Qiagen). RNA was stored in RNase free water (Qiagen) at −80°C. RNA (1 μg) was converted to cDNA using the SuperScript preamplification system (Life Technologies) for first-strand cDNA synthesis. The cDNA mixture was diluted 1/5 with PCR grade water. Ten percent (10 μl) of the cDNA product was used per reaction for real-time quantitative PCR using a high-speed thermal cycler (LightCycler; Roche Diagnostics, Indianapolis, IN), and the product was detected by FastStart Master SYBR Green I (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s instructions. The amplification cycle was 95°C for 15 s, 50°C for 5 s (57°C for GAPDH primers), and 72°C for 20 s. The CIITA and MHC-II primers were designed using OLOG v6.4 (Molecular Biology Insights, Cascade, CO). The GAPDH primer sequences were as published (25). The sequences for primers are as follows for the 18S: sense 5'-AACGACCCCTTCATTGAC-3' and antisense 5'-TCCACGCATACCATGCAC-3' (predicted size = 191 bp). MHC-II (I-Aβ chain): sense 5'-GGCACGTTGGGCGGAGTACC-3' and antisense 5'-CTATTCGGAACAGCGCCA-3' (predicted size = 276 bp). Total CIITA mRNA: sense 5'-AAGCTTTTCTGCTGAGTATTG-3' and antisense 5'-CTAACCGGACTCCGGAAG-3' (predicted size = 342 bp). Antisense primer for types I, III, and IV CIITA: 5'-GGTCCGCACTATGTTAAAGGA-3'. Sense primer type I CIITA: 5'-AAAGACTGCTGCTCTACGGGAT-3' (predicted size = 264 bp). Sense primer type III CIITA: 5'-TCTTACGTCCGGC

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GAGTT-3' (predicted size = 100 bp). Sense primer type IV CIITA: 5'-\text{GAGACTGCATGACGCAGA}-3' (predicted size = 129 bp). All ODN were purchased from Life Technologies. Specific cDNA was quantified with a standard curve based on known amounts of product. Standards were generated by purification of amplified cDNA product from an agarose gel using a QiAQuick gel extraction kit (Qiagen). Melting curve analysis confirmed that only one product was amplified. Further confirmation of specificity was conducted by amplification of cDNA from whole splenocytes using the primers listed above, centrifugation of capillaries to obtain PCR products, electrophoresis of products through a 1.5% agarose gel, and staining with ethidium bromide. In all cases, there was only one product observed with each primer set, and the observed product had an amplicon size that matched the size predicted from published cDNA sequences.

**Calculations**

Normalized copy number = (copy number of mRNA of interest/copy number of GAPDH) × 1000. Fold induction = (normalized copy number of treated cells)/(normalized copy number of untreated cells). The half-lives of types I and IV CIITA were calculated using linear least squares to determine the best-fit line of \( \log_2(\text{normalized CIITA copy number}) \) plotted vs time after DRB addition. The slope (m) was used to calculate the half-life of types I and IV CIITA mRNA expression after addition of DRB (\( t_{1/2} = -\log_2(2)/m = -1/m \)).

**Results**

**IFN-γ induces expression of MHC-II and CIITA in BMM**

MHC-II expression by macrophages is not constitutive and depends on activating stimuli, e.g. IFN-γ. Because macrophage activation is complex and affected by multiple factors or stimuli, the relative roles of different CIITA types in MHC-II induction may vary with different stimuli or stages of activation. To assess these issues, BMM were stimulated with IFN-γ, and I-A\(^{\alpha}\) expression was determined by flow cytometry. Unstimulated BMM expressed little MHC-II protein, but after IFN-γ treatment for 24 h, cell surface expression of MHC-II increased dramatically (Fig. 2A). MHC-II expression was monitored over time in IFN-γ-treated cells; MHC-II expression was near maximum by 48 h (Fig. 2B). The induction of mRNA for MHC-II and CIITA in BMM treated with IFN-γ was analyzed by real-time quantitative RT-PCR (Fig. 2C). Detection of CIITA (any measurement of CIITA refers to mRNA levels as CIITA protein was not analyzed) was consistently observed after 6 h of IFN-γ treatment and remained relatively constant for 24 h. As expected, kinetic studies showed that induction of CIITA preceded induction of MHC-II mRNA. Induction of MHC-II mRNA was evident at 12 h, and levels increased during the next 12 h.

**FIGURE 2.** IFN-γ induces MHC-II and CIITA in macrophages. A, BMM were incubated with or without 2 ng/ml of IFN-γ for 24 h, and I-A\(^{\alpha}\) protein expression was measured by flow cytometry. Background staining with isotype-matched negative control Ab is shown in the solid gray curve. B, I-A\(^{\alpha}\) protein expression was measured at various time points of culture with or without 2 ng/ml IFN-γ. C, BMM were stimulated with medium or 2 ng/ml IFN-γ for 6, 12, or 24 h. Expression of mRNA for CIITA and MHC-II was determined by real-time quantitative RT-PCR. The results were standardized with respect to GAPDH values and are represented as fold induction (see Materials and Methods). For each panel, the experiment was performed at least three times.

All three types of CIITA are induced in BMM by IFN-γ, but types I and IV are the most abundant

Different type-specific primer pairs were used to detect types I, III, and IV CIITA by real-time RT-PCR. A common antisense primer located in exon 2 was used in conjunction with type-specific sense primers located in different first exons of types I, III, and IV CIITA (Fig. 3A). To confirm the specificity and sensitivity of this approach, types I, III, and IV CIITA were amplified from splenocyte cDNA; in all cases, the size of the amplicon corresponded to the size predicted from the mRNA sequence (Fig. 3B).

Using these primer sets, the induction of types I, III, and IV CIITA was analyzed after 6 h of stimulation with increasing doses of IFN-γ (Fig. 4). All three types of CIITA were induced by IFN-γ, but type IV CIITA showed the greatest relative enhancement (Fig. 4A). Type IV CIITA was consistently induced 60- to 80-fold with 2 ng/ml IFN-γ, whereas types I and III CIITA were induced only 6- to 8-fold. Although type IV was increased by a greater factor than type I CIITA by 6 h of IFN-γ treatment, the absolute levels of the two types were similar at 2–10 ng/ml IFN-γ, due to the higher baseline level of type I CIITA (without IFN-γ). The absolute level of type I CIITA was nine times greater than type IV; Fig. 4B). Even though type III CIITA was induced by IFN-γ, it was present in such low amounts that it did not contribute substantially to total CIITA expression (Fig. 4, A and B). The absolute levels of type III CIITA were so low that the bars are not visible above the x-axis in Fig. 4B). In summary, unstimulated BMM expressed low levels of type I CIITA and almost undetectable levels of type IV CIITA, but stimulation with IFN-γ for 6 h increased both types I and IV CIITA to high levels.

Types I and IV CIITA were monitored over time after induction with IFN-γ (Fig. 4C). After 6 h of IFN-γ treatment the amounts of types I and IV CIITA were similar. From 6 to 24 h, type I CIITA remained constant or continued to increase over time, whereas type IV CIITA declined slightly. We conclude that types I and IV CIITA were both increased in BMM by IFN-γ. The suggestion that type I CIITA was expressed more stably was pursued in additional kinetic experiments.

**FIGURE 3.** Detection of type I, III, and IV CIITA by real-time quantitative RT-PCR. A, A common antisense primer located in exon 2 was used in combination with type-specific sense primers located in exon 1 to generate primers able to detect spliced types I, III, and IV CIITA mRNA. The size of the amplicon predicted from the cDNA sequence is shown. B, To test detection of CIITA by these primers, splenic cDNA was amplified by PCR and the products were electrophoresed through a 1.5% agarose gel containing ethidium bromide.
Waldburger et al. (23) demonstrated that thioglycollate-elicited macrophages from pIV\^{+/−}H11002 and pIV\^{−/−}H11002 mice had equivalent surface expression of MHC-II after 72 h of IFN-γ treatment. Additionally, they showed that after 72 h of IFN-γ treatment, both pIV\^{+/−} and pIV\^{−/−} macrophages expressed mostly type I CIITA; even pIV\^{−/−} macrophages expressed little type IV CIITA. The results obtained by Waldburger et al. (23) differ from our observation that types I and IV CIITA are both induced by IFN-γ, but their experiments examined different cells (peritoneal macrophages) at a different time point (72 h). To resolve this difference, we examined responses of both BMM and thioglycollate-elicited peritoneal macrophages to IFN-γ at 6–72 h. Expression of MHC-II protein was induced similarly in both BMM and peritoneal macrophages to IFN-γ at 6–72 h. Expression of MHC-II protein was induced similarly in both BMM and peritoneal macrophages to IFN-γ at 6–72 h. RT-PCR analysis showed differences in the kinetics of type I vs type IV CIITA expression induced by IFN-γ. At 6 h, IFN-γ induced similar levels of types I and IV CIITA in both BMM and peritoneal macrophages (Fig. 5C). However, after 72 h of exposure to IFN-γ, type IV CIITA declined in both cell populations while type I CIITA remained constant (Fig. 5C). We conclude that IFN-γ induces prolonged expression of type I CIITA but only transient expression of type IV CIITA.

**Kinetic differences in expression of types I and IV CIITA**

A difference in mRNA stability could explain the persistence of mRNA for type I CIITA and the disappearance of type IV CIITA over a 72-h period (i.e., mRNA for type I CIITA could have a longer half-life than mRNA for type IV CIITA). To address this hypothesis, BMM were stimulated with IFN-γ for 6 h, at which time mRNA synthesis was inhibited by the addition of 50 μM DRB. The stability of existing mRNA for types I and IV CIITA was determined by real-time RT-PCR of samples taken at various time points after addition of DRB (Fig. 6). Type I CIITA (t_{1/2} = 3.5 h) actually had a shorter half-life than type IV CIITA (t_{1/2} = 7.8 h), contrary to the above hypothesis. Thus, the high amount of type I CIITA mRNA observed after treatment with IFN-γ for 72 h cannot be explained by a long half-life, suggesting that continued transcription of mRNA for type I CIITA contributes to its sustained expression.
B cell induction of MHC-II expression in response to IL-4 is not due to increased transcription of MHC-II or types I, III, or IV CIITA

In contrast to macrophages, B cells constitutively express MHC-II even without stimulation, but the level of MHC-II protein expressed by B cells can be modulated by certain stimuli. IL-4 increases expression of MHC-II protein on B cells, but the mechanism by which this occurs is unclear (26, 27). We purified B cells from whole splenocytes by negative selection with anti-CD43 magnetic microbeads. Greater than 95% of CD43-negative cells expressed B220, a marker for B cells, and there was little or no staining for CD11b (Mac-1) and TCR β-chain. B cells were incubated with or without IL-4 for 24 h, and I-A^k expression was measured by flow cytometry. B cells expressed MHC-II protein in the absence of any stimulation, but treatment with IL-4 led to a 4-fold increase in MHC-II protein expression (Fig. 7A). We tested whether IL-4 increased MHC-II expression by increasing CIITA mRNA levels in B cells. Purified B cells were treated with or without IL 4, and quantitative real-time RT-PCR was used to measure mRNA for MHC-II, total CIITA, and types I, III, and IV CIITA. B cells constitutively expressed large quantities of type III CIITA, a small amount of type IV and very little type I. Treatment of B cells with IL-4 for 12 h did not affect levels of mRNA for MHC-II, total CIITA, or type I, III, or IV CIITA (Fig. 7A, B, and C). Additional studies examined CIITA and MHC-II expression at other time points; CIITA and MHC-II mRNA levels remained unchanged after stimulation with IL-4 for 6, 12, or 24 h (data not shown). Thus, increased MHC-II protein expression in response to IL-4 was not due to increased CIITA or MHC-II mRNA levels. These results suggest that posttranslational mechanisms contribute to the increase in MHC-II expression induced by IL-4.

Splenic dendritic cells predominantly express type I CIITA, and their maturation after exposure to CpG DNA silences types I and III, but not type IV, CIITA

Recently it was shown that dendritic cells rapidly silence CIITA transcription upon maturation (17). We analyzed expression of the various forms of CIITA by dendritic cells immediately after isolation from spleens or after incubation for 18 h with or without CpG ODN 1826 (which induces dendritic cell maturation (2, 28, 29)). Freshly isolated splenic dendritic cells expressed mostly type I CIITA, but little type III or IV CIITA (Fig. 8). After culture in standard medium containing GM-CSF, levels of total CIITA and types I and III CIITA decreased, indicating that some maturation occurred spontaneously with culture of dendritic cells in standard medium (Fig. 8). However, total CIITA and types I and III CIITA were decreased to a greater degree after culture of dendritic cells with CpG DNA (Fig. 8). Similar results were obtained using a 19-kDa lipoprotein from Mycobacterium tuberculosis (30) to promote maturation of dendritic cells (data not shown). Bone marrow-derived dendritic cells behaved similarly to splenic dendritic cells (data not shown). Expression of type IV CIITA did not change upon treatment with CpG, and type IV CIITA was the only form detected in significant amounts in mature DC (Fig. 8), but it was expressed at a very low level.

Splenic dendritic cell silence CIITA upon treatment with CpG ODN. Splenic dendritic cells were analyzed for total CIITA and types I, III, and IV CIITA directly after purification (ex vivo) or after incubation with or without 1 μg/ml of 1826 CpG ODN for 18 h. These results are representative of three similar experiments.
Discussion

CIITA controls the expression of MHC-II by all types of APCs and thus impacts all CD4+ T cell responses. CIITA transcription is highly regulated (6, 31–34), and posttranslational mechanisms also affect CIITA activity (35, 36). Transcriptional regulation of CIITA occurs through the actions of three independent promoters (pI, pIII, pIV) in mice. These promoters lead to three CIITA mRNA species in which a unique exon 1 downstream of each promoter is spliced with the remaining exons 2–19 (Fig. 1). In this study, we analyzed the relative expression of types I, III, and IV CIITA in professional APCs after stimulation with cytokines or bacterial products that modulate the expression of MHC-II molecules. The primary aim was to address uncertainties about the relative expression of different types of CIITA during different stages of macrophage activation, but expression patterns of different CIITA types also were assessed in B cells and dendritic cells.

Several studies have analyzed the use of different CIITA promoters in various cell types and the effect of IFN-γ on CIITA transcription (15, 18, 19, 22, 37–41). Ribonuclease protection assays showed that THP-1 cells (a human macrophage cell line) treated with IFN-γ express high amounts of type IV CIITA (15). Using reporter constructs in which CIITA promoter sequences were linked to a luciferase gene, it was shown that both types IV and III CIITA could be induced by IFN-γ (induction of type I CIITA was not analyzed) (18, 19). Recently, pIV−/− mice were generated and found to have loss of MHC-II expression on non-hematopoietic cells, but preserved MHC-II expression on professional APCs (23). This finding indicates that type IV CIITA is essential for IFN-γ induction of MHC-II on non-hematopoietic cells, but not on macrophages. Macrophages in pIV−/− mice were found to express type I CIITA in response stimulation with IFN-γ for 72 h.

In our studies, BMM treated with IFN-γ for a short period, 6 h, expressed high levels of both types I and IV CIITA. Type IV CIITA was induced rapidly, but its mRNA levels declined over time. Type I CIITA also was induced rapidly, and high levels were maintained over a 72-h period. These experiments establish that IFN-γ induces both types I and IV CIITA in macrophages, but the two types are maintained with different kinetics. After stimulation with IFN-γ, both types I and IV CIITA are expressed at early time points and may both contribute to early induction of MHC-II by IFN-γ. At late time points, however, type IV CIITA declines and CIITA expression is dominated by type I CIITA, which remains elevated for long periods of time and may contribute to maintenance of MHC-II expression on macrophages for longer periods.

Our studies provide new information that helps to reconcile differences between previous reports regarding the regulation of CIITA expression in macrophages by IFN-γ. When the existence of different CIITA promoters was discovered, type IV CIITA was reported to control MHC-II expression in macrophages (15), and subsequent studies addressed the mechanism by which IFN-γ regulates expression of type IV CIITA (22, 37, 40, 41). In contrast, the study of Waldburger et al. (23) reported that type I CIITA was induced by 72 h of IFN-γ stimulation, and type IV CIITA was not expressed to a substantial degree at that time point. Waldburger et al. concluded that type IV CIITA is not absolutely required for MHC-II expression in macrophages. Our data do not conflict with those of Waldburger et al., because we also found that type I CIITA was the predominant form expressed in macrophages after 72 h of stimulation with IFN-γ. However, our results also suggest that type IV CIITA may contribute substantially to the synthesis of MHC-II shortly after the initiation of IFN-γ stimulation (e.g. within 24 h), consistent with other reports (15, 18, 19, 22, 37, 40, 41). Thus, our studies reconcile the different results of previous reports and demonstrate that both type I and type IV CIITA are responsive to IFN-γ and may both contribute to MHC-II expression at different stages of macrophage activation.

The transcriptional machinery necessary for induction of type IV CIITA has been analyzed in detail and involves three cis-acting sequences, a γ-activated site, an IFN regulatory factor (IRF) binding site, and an E box (40). Stimulation of the IFN-γ receptor leads to occupation of the γ-activated site and IRF sites by activated STAT1 and IRF-1 respectively. The E box is bound by the constitutively expressed transcription factor upstream stimulator factor-1 (40). The transcription factors that govern expression of type I CIITA are less clear, and no known IFN-γ-responsive elements have been found upstream of pI (15). Types I and IV CIITA were expressed with different kinetics following stimulation of BMM with IFN-γ (Fig. 5), and this difference was not explained by differing mRNA half-lives (Fig. 6). This finding suggests differences in transcriptional control of types I and IV CIITA, e.g. the hypothesis that different transcription factors may be involved in the induction of types I and IV CIITA by IFN-γ.

Different studies have reported varying results regarding the induction of type III CIITA by IFN-γ. Type III CIITA is expressed constitutively by B cells (15) and human melanoma cells (20), and it is induced by IFN-γ in some human tumor cells (e.g. fibrosarcoma and malignant glioma cells) (19, 21). Nikcevich et al. (22) found that IFN-γ induced expression of a luciferase reporter construct driven by human CIITA pIII in transfected murine RAW264.7 cells. In contrast, we observed that IFN-γ induced only extremely low levels of type III CIITA in murine macrophages (Fig. 4, A and B), and the amount of type III CIITA present after IFN-γ stimulation was insignificant when compared with types I and IV CIITA (Fig. 4B). Thus, type III CIITA appears to play little or no significant role in IFN-γ induction of MHC-II in the primary murine macrophage systems that we have studied. To reconcile these various findings, it is important to note that the studies illustrating induction of type III CIITA by IFN-γ were performed in transformed human or murine cells that were transfected with human type III CIITA promoter constructs, whereas our studies were performed with primary murine macrophages. Thus, the variation in results and lack of substantial IFN-γ-induced type III CIITA expression in our studies may reflect differences between transformed and primary cells or different properties of murine and human type III CIITA promoters.

Unlike macrophages, dendritic cells, and B cells constitutively express MHC-II and CIITA. In B cell populations, MHC-II expression is under strict developmental control. Early pro-B cells do not express MHC-II, expression in pre-B cells is low, mature B cells constitutively express MHC-II, and expression of MHC-II is lost in plasma cells (42). Very little is known about regulation of MHC-II expression in B cells. Recently, expression of MHC-II on mature B cells was linked to constitutive expression of type III CIITA. A relatively small region (600 bp) upstream of pIII is required for constitutive expression, but the transcription factors responsible for pIII induction are not known (18). Reduction in MHC-II expression as B cells mature into plasma cells is due to transcriptional silencing of type III CIITA by B lymphocyte-induced maturation protein-1 (43). However, little additional information is available about the regulation of type III CIITA, and the potential regulation of type III CIITA by IL-4 has not been examined before our current studies. In our studies, primary B cells expressed MHC-II protein, and IL-4 increased cell surface expression of MHC-II protein ~4-fold over 24 h. Surprisingly, IL-4 did not increase mRNA for CIITA (types I, III, and IV) or MHC-II. In other studies, treatment of splenic B cells with IL-4 increased
MHC-II mRNA ∼2-fold or less, whereas MHC-II protein expression was increased 10- to 15-fold (27). Together, these results suggest that posttranscriptional events contribute to the IL-4-induced increase in expression of MHC-II protein on B cells.

It is interesting that IL-4 increased MHC-II protein expression on B cells in the absence of increased levels of CIITA mRNA. We can only speculate as to the exact mechanism by which IL-4 increased expression of MHC-II molecules, but it would appear to be posttranscriptional and probably posttranslational. It is possible that IL-4 increases the stability and half-life of MHC-II molecules on B cells, perhaps by decreasing their endocytosis and degradation, increasing the steady state expression of MHC-II. Alternatively, MHC-II molecules could be mobilized from some intracellular compartment to the cell surface upon IL-4 activation. Although MHC-II molecules are known to reside in intracellular vesicular compartments, there is no precedent for intracellular stores of MHC-II of magnitude sufficient to explain the amount of increase in MHC-II expression that we observed. We are currently in the process of testing these hypotheses.

Immature dendritic cells express low levels of MHC-II and costimulatory molecules. Ligation of Toll-like receptors (TLRs) on dendritic cells leads to their maturation (44), enhancing their ability to present antigenic peptides to naive T cells. Dendritic cell maturation increases expression of MHC-II (2, 4, 5) even though mRNA for MHC-II is decreased (2, 46). Consistent with a recent report (17), we observed that decreased MHC-II mRNA in response to TLR agonists reflects decreased expression of types I and III CIITA. In contrast, type IV CIITA was unaffected by TLR activation (Fig. 8) and was the only form of CIITA expressed at significant levels in splenic dendritic cells after exposure to CpG ODN (>95% of CIITA was type IV), but was expressed at low levels. This is consistent with a previous report identifying a 121-kDa form of CIITA present in mature dendritic cells (17). Thus, type IV CIITA most likely mediates the residual expression of MHC-II mRNA in mature dendritic cells, but the role and significance of this residual expression is not known.

Reduction of CIITA synthesis upon maturation of dendritic cells has important implications. MHC-II synthesis is dramatically reduced, but the stability of peptide:MHC-II complexes on the cell surface is increased (2, 4, 5). In effect, TLR ligation “freezes” the cohort of peptide:MHC-II complexes present on the cell surface; a hypothesis we refer to as the “freeze frame model” (2). Peptide complexes generated in the period near initiation of TLR signaling will be presented preferentially.

Regulation of MHC-II and CIITA expression differs considerably among different types of professional APCs in response to activation or maturation stimuli. IFN-γ-inducible expression of MHC-II on macrophages is due to the presence of both types I and IV CIITA. Our results indicate that type I CIITA is expressed for a longer period than type IV CIITA via a mechanism independent of the relative stabilities of the two mRNA species, suggesting that continued transcription of type I CIITA may contribute to prolonged expression of MHC-II molecules by macrophages. Constitutive expression of MHC-II on B cells correlates with expression of type III CIITA. Although IL-4 increases expression of MHC-II molecules, our studies indicate that IL-4 does not affect expression of mRNA for CIITA or MHC-II, suggesting the existence of posttranscriptional regulatory mechanisms for MHC-II. Splenic dendritic cells primarily express type I CIITA and, upon maturation, silence expression of types I and III CIITA, although a small amount of type IV CIITA is still expressed. An understanding of the roles of different types of CIITA will help clarify how MHC-II expression is regulated under different pathophysiologic conditions.


