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Mice lacking the transcription factor CIITA—a second look

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Abstract

We have generated a second line of mice lacking a transcription factor thought to be a critical regulator of MHC class II gene expression, CIITA (for class II transactivator). Our and the previously published lines differ in the deletion that was engineered and by the fact that we removed the neomycin-resistance promoter and structural gene via the cre–loxP recombination system. Characterization of our line led to two new findings. First, a substantial number of cells can express class II molecules in the absence of CIITA, albeit at 5-fold reduced levels, most notably dendritic cells in s.c. lymph nodes; therefore, the CIITA gene cannot be an absolute 'master gene' controlling the expression of class II molecules, as had been thought. Second, in contrast to recent results on human cell lines, CIITA is not critically involved in the IFN-γ-induced up-regulation of MHC class I genes.

Introduction

MHC class II molecules are crucial for the development and function of the immune system. Although this point was established through a long series of experiments, it is perhaps best illustrated by the phenotype of class II-deficient mice—few $CD4^+$ T cells, impaired T and B cell responses (1,2). Similarly, there is a group of genetic disorders in humans, traced to class II deficiency, that result in severe immunodeficiency and eventually death (3,4).

The regulation of MHC class II genes has been studied extensively. Their expression is restricted to certain cell types: B cells, macrophages, dendritic cells (DC) and thymic epithelial cells. Much of the control is exerted at the level of transcription (5–7).

A critical regulator of MHC class II gene expression is CIITA, first identified by its ability to reverse the defect in class II molecule expression in a B cell line derived from a patient with class II deficiency (8). Complementation cloning and sequencing of the CIITA gene revealed several mutations within a single exon and altered splice sites on both alleles. The element actually responsible for the lack of class II gene expression in the mutant B cell line was confined to a 70 bp deletion in the mutated exon (8). Transfection studies established that the expression of class II molecules relied on the presence of functional CIITA.

The importance of CIITA for MHC class II gene expression was confirmed by transfection of several MHC class II^- cell lines with CIITA cDNA (9–12) which showed that the transactivation potential of CIITA extended to several cell types, demonstrating that control of class II gene expression lies primarily within the CIITA molecule because transfection of class II^- cell types with CIITA cDNA sufficed to induce expression of class II molecules. Treatment of the transfected cells with IFN-γ resulted in an increase in class II molecule expression, prompting the conclusion that this cytokine influences the class II promoter via CIITA (10,13,14).

CIITA has also been shown to influence the expression of several genes whose products are involved in antigen processing, i.e. invariant chain (Ii) and HLA-DM (10,15). Both HLA-DM and Ii were detectable after transfection of class II^- cell lines with CIITA cDNA.

Recently, CIITA was reported to mediate the IFN-γ-induced up-regulation of MHC class I molecules as well (16,17). Previous studies had suggested that the class I and class II pathways were linked together in the response to IFN-γ through IFN response factor (IRF)-1 (18), but there existed no evidence implicating CIITA in class I gene expression (13). Two more recent studies employing *in vitro* assays on human

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cell lines found that CIITA acts through the α site in the class I gene promoter to mediate its response to IFN-γ (16,17).

Collectively, these studies have argued for a pivotal role for CIITA in coordinating the expression of MHC and other molecules involved in the antigen-processing/presentation pathway. However, just how it exerts its transactivation function is still unknown. Sequence analysis of the CIITA cDNA did not reveal any obviously recognizable DNA binding domain (8). On the other hand, a transcriptional transactivation domain was assigned to the acidic N-terminal domain of the CIITA molecule (19,20), and interactions between CIITA and two different transcription factors, RFX-5 and TAFII32, have been demonstrated (21–23). This has led to two proposed models of CIITA function: one where CIITA alters the quaternary structure of the transcription machinery (4); the second where CIITA displaces a negative regulatory protein from the transcription complex, which allows transcription to occur (24).

A mouse line deficient in CIITA expression has recently been reported (25). As a prelude to engineering cell-type-specific 'knockouts' of this transcription factor, we generated an independent CIITA-deficient line via the cre–loxP system. We found a surprising number of cells expressing class II molecules in these animals and have characterized them in some detail.

Methods

Generation of CIITA^o mice

To generate the targeting construct, we screened a genomic library made from the D3 embryonic stem (ES) cell line, derived from a strain 129 mouse, with a CIITA cDNA fragment spanning the first three exons (positions 94–330) (26). A 16 kb clone was obtained, from which an 8 kb *Bam*HI fragment was subcloned into pBS (Fig. 1A2). An oligonucleotide containing a loxP site was inserted into the 5′ *Sac*I site. Next, a cassette containing a thymidine kinase–neomycin fusion gene (*neo^r*) driven by the phosphoglycerol kinase promoter (27) was cloned into the *Sal*I site. Lastly, to increase the 5′ flanking genomic DNA homology, we cloned an additional 2 kb of 5′ genomic DNA into the 5′ *Bam*HI site. The loxP sites were sequenced to verify proper insertion and direction, and correct cloning. The targeting vector was removed from the pBS polylinker by digestion with *Not*I and *Cla*I, and gel-purified using 'Geneclean' (BIO 101, La Jolla, CA).

This DNA was electroporated into the D3 ES cell line (1) and G418-resistant clones screened for the correct targeting event as follows. To verify the presence of the *neo^r* cassette, we digested genomic ES cell DNA with *Kpn*I and analyzed with probe 1 (Fig. 1A3). A correctly targeted cassette will introduce a new *Kpn*I site, yielding a 12 kb band with this probe. The presence of the upstream loxP site was determined by PCR, using the primers pictured in Fig. 1(A3), the additional loxP site making the PCR product 45 bp longer. Of 184 *neo^r* clones, seven had the cassette correctly targeted, but only three of these had the upstream loxP site integrated. Correctly targeted ES cells were injected into B6 blastocysts and chimeric males obtained. These chimeras were crossed to C57Bl/6 (B6) and CMV/cre mice in which the cre recombinase is expressed in essentially all cell types under the dictates of the cytomegalovirus promoter (28); two of six chimeric males gave germline transmission of the cassette. Offspring of these crosses were analyzed by Southern blotting of tail DNA for transmission of the targeted allele and Cre-mediated deletion of the DNA between the loxP sites. Mice carrying the deleted locus were then interbred to generate homozygous knockout mice.

Flow cytometry

Mice, 6–8 weeks old, were sacrificed, and lymphocyte suspensions prepared by teasing in DMEM, 5% FCS from thymus, spleen and lymph node. Dendritic cells were prepared in an adaptation of the collagenase release procedure (29), miniaturized for treatment of single lymph nodes (Vremec *et al.*, in preparation); briefly, DC were released by collagenase/DNase/EDTA digestion of lymph nodes and enriched by magnetic depletion of lymphocytes and granulocytes. Cells were stained with the following reagents: anti-CD4–phycoerythrin (PE; Caltag, San Francisco, CA), anti-CD8–FITC (Caltag), anti-CD45RA–FITC (RA3-6B2) (30), anti-H-2D^b–PE (PharMingen, San Diego CA), anti-HSA–FITC (M1/69) (PharMingen), anti-DEC205–FITC (NLDC145) and anti-MHC class II (N22) as directly-conjugated antibodies; biotinylated anti-CD69 (Pharmingen), anti-CD8 (clone H35.27.5) and anti-CD11c (N418); unconjugated mAb IM7 (anti CD44), 3F12, Y3P, M5/114, BP107, 25.3.17 (all anti-MHC class II) (see 31), AF6-88.5.3 (anti-K^b) (32) and KT3 (anti-CD3) (33); used as hybridoma culture supernatants; and revealed by FITC- or Texas Red-conjugated anti-rat IgG, anti-mouse IgG or anti-hamster IgG second-step antibodies (Jackson Immunoresearch, West Grove PA). All antibodies were used at saturating quantities. Antibody dilutions or cell washes were performed in FacsWash (PBS supplemented with 30 mM HEPES, 5% horse serum and 0.1% Na azide). Flow cytometry was performed on either a Coulter Elite or Profile flow cytometer and data stored as list mode for analysis. Analysis of list-mode files was performed on Elite or WIN-MDI software, generating contour pseudo-dot-plots. Live cells were gated on forward scatter versus side scatter profiles.

Immunohistochemistry

Mice, 6–8 weeks old, were sacrificed, and their organs removed and frozen in 'Tissue-Tek' embedding compound at –80°C (Bayer, Puteaux, France). Sections (6 μm thick) were cut on a cryostat, acetone fixed for 15 s and air-dried. Prior to staining, sections were rehydrated in FacsWash/PBS/Tween 20 1:2:0.001 for 5 min then stained for 30 min in a humid chamber with one of the following antibodies: MTS44 (anti-cortical epithelium) (34), ERTR4 (anti-cortical epithelium) (35), MTS10 (anti-medullary epithelium) (34), ERTR5 (anti-medullary) (35), CDR1 (anti-thymic cortex) (36), N418 (anti-DC) (37), M5/114, Y3P, MTS6 or 25.3.17, as supernatant. They were then washed twice for 20 min in FacsWash/PBS/Tween and revealed with FITC- or Texas Red-conjugated anti-mouse IgG or anti-rat IgG (Jackson Immunoresearch). The mAb 95 and 25 (38) were used as purified antibody revealed by Texas Red-conjugated anti-rat IgM. Double-labeling for epithelium was performed using anti-keratin (Dako, Carpinteria, CA), as described (34). Slides were fixed in 1% paraformaldehyde in PBS and fixed in Dako mounting medium (Dako). Sections

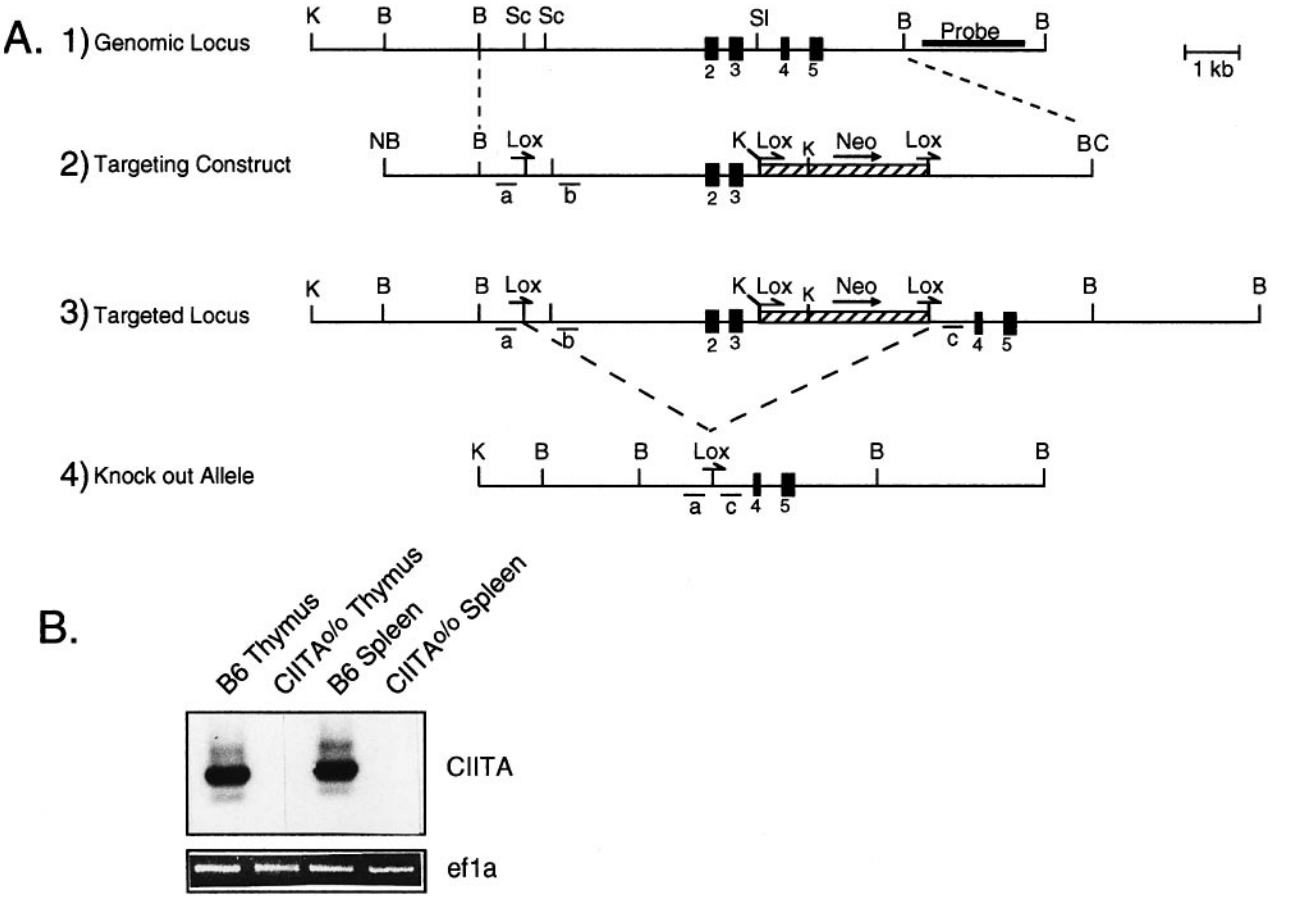

Fig. 1. Targeting of the CIITA gene. (A) Maps. (1) Map of the 5′ region of the murine CIITA gene containing exons 2–5. (2) Targeting construct used to introduce the loxP sites. (3) Targeted locus. (4) Deletion mutation induced by cre-mediated rearrangement of the loxP sites. Exons 2–5 are shown as filled boxes, whose width is not intended to be to scale. The 3′ probe used to screen ES cells is represented as a filled box above the genomic clone. The positions of primers used to screen for the 5′ loxP (Lox) site (a and b) and to confirm the deletion (a and c) are given. Restriction enzyme sites used in constructing the targeting vector are shown as: B, *Bam*HI; K, *Kpn*I; Sc, *Sac*I; Sl, *Sal*I, C, *Cla*I. The *neo* gene used for selecting ES cell clones is shown as a hatched box and the arrow above indicates the direction of transcription. (B) Abrogation of synthesis. CIITA (upper panel) and control *ef1a* (lower panel) RT-PCR of thymus and spleen RNA. One-fifth of the CIITA RT-PCR reaction was run on an agarose gel, transferred to a membrane, hybridized with a ^{32}P-labeled CIITA-specific oligonucleotide and exposed to X-ray film to confirm its identity. One-fifth of the *ef1a* RT-PCR reaction was run on an agarose gel and a photograph of the ethidium bromide-stained gel is shown.

were subsequently examined and photographed with a Zeiss Axiophot fluorescence microscope or on a BioRad confocal (MRC1024) fitted on a Nikon E600 microscope.

Induction and culture of peritoneal macrophages

Mice were injected i.p. with 100 μl of thioglycolate medium (Gibco/BRL, Paisley, UK). Four days later the peritoneal cavity was rinsed with 5 ml of PBS, peritoneal cells were washed in PBS, and were then cultured at a concentration of 10^6 cells/ml in DMEM supplemented with 10% FCS, 2 μM β-mercaptoethanol and antibiotics. Recombinant IFN-γ (Pharmingen) was added at 100 U/ml. After 48 h of culture the cells were harvested and analyzed by flow cytometry.

Results

Generation of CIITA$^{o/o}$ mice

In order to assess their roles in various cell types and throughout an immune response, we set out to generate mouse lines permitting conditional extinction of MHC class II molecules. By virtue of its reported ability to control expression of the set of class II genes, as well as functionally related genes (10,39), the CIITA locus seemed an attractive candidate to target, as its nullification should eliminate all class II molecule expression with a single targeting event.

Our knockout strategy entailed using the cre–loxP system (40) to generate a deletion mutation. A genomic fragment was cloned, which contains the second to fifth exons found in all known CIITA transcripts—in the course of these analyses, we mapped introns after positions 145, 292, 388, 451 and 533 of the coding sequence (numbering as per ref. 26). The alternative first exons found in different tissues were not found in this fragment. A single loxP site was introduced 5′, and a thymidine kinase–neomycin fusion gene (27) flanked by loxP sites 3′, of exons 2 and 3 (Fig. 1A2). Should these loxP sites be recombined by the cre recombinase, the intervening sequences would be deleted, creating the desired deletion

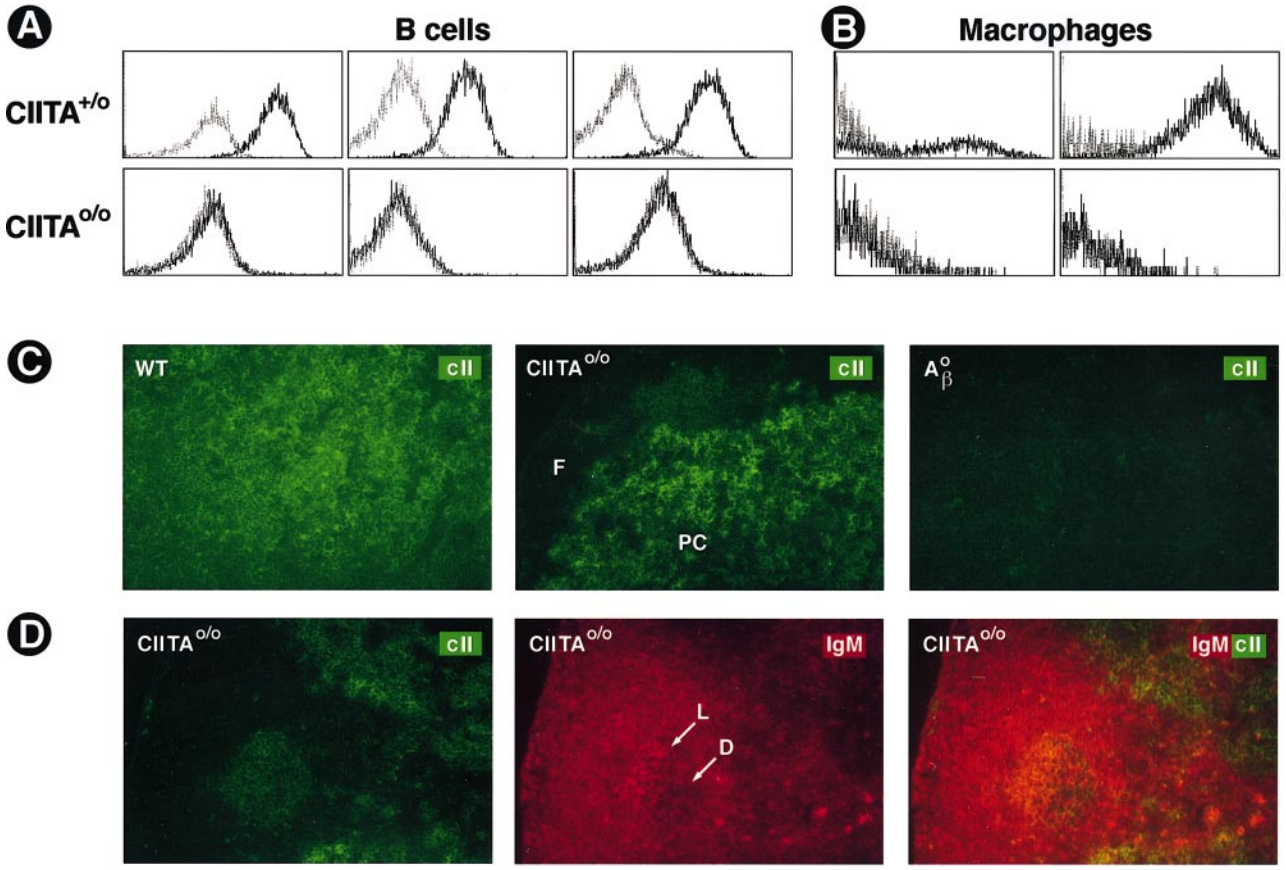

Fig. 2. Expression of MHC class II molecules in the periphery. (A) Flow cytometry profiles of anti-class II staining on gated B220$^+$ cells in CIITA$^{+/o}$ (upper row) and CIITA$^{o/o}$ (lower row) mice. Shown are stainings using 25.9.17, Y3P, and M5/114 from left to right. Dotted lines represent control staining with an irrelevant primary antibody. (B) Profiles of anti-class II staining on thioglycolate-induced peritoneal macrophages from CIITA$^{+/o}$ or CIITA$^{o/o}$ mice after culture with or without IFN-γ. Histograms are gated on Mac-1$^+$ cells. Dotted lines represent control staining with an irrelevant primary antibody. (C) Single-color anti-class II staining of thin sections of inguinal lymph nodes from CIITA$^{+/o}$, CIITA$^{o/o}$ and A$_\beta^o$ mice. F and PC mark the B cell follicle and paracortex. (D) Two-color immunofluorescent staining of inguinal lymph nodes from CIITA$^{+/o}$, CIITA$^{o/o}$ and A$_\beta^o$ mice. Red fluorescence depicts anti-IgM and green fluorescence anti-class II. The same field of view is shown in all three images. L and D mark the light and dark areas of the GC.

(Fig. 1A4). The deletion removes nucleotides 146–388 (amino acids 18–79) of the cDNA sequence, thus eliminating the major part of the essential acidic transactivation domain (19,20). This strategy has the additional advantage of removing the drug-resistance marker (*neo*^{r}) used to select ES cells. This is important as the inserted drug-resistance promoter elements could drive expression of an altered gene product which, if functional, could lead to an artificial phenotype (41,42).

The targeting construct was electroporated into ES cells, and the G418-resistant cells were screened for homologous recombination by Southern blotting and PCR analysis, testing for a correctly integrated *neo* gene and for the presence of the 5′ loxP site (Fig. 1A). Correctly targeted clones were obtained at a frequency of 1/60. (Note that half of the clones with proper recombination of the *neo*^{r} cassette did not carry the 5′ loxP site owing to intragenic recombination.) One of the ES cell clones was injected into blastocysts and the chimeras bred to B6 mice. Two of the chimeric males bred to B6 mice had agouti-colored offspring with germline transmission of the 'floxed' allele. These males were also bred to female mice carrying a cre transgene under the control of the CMV promoter (28). Tail DNA from these offspring was analyzed for transmission of the targeted allele and for cre-mediated deletion; mice bearing the deletion were interbred and the offspring analyzed.

The CIITA deletion we generated removes the first four common exons (2–5) generating a drastic mutation in the protein. To determine if there was residual expression of a truncated CIITA mRNA, we performed RT-PCR on spleen and thymus RNA from heterozygous and homozygous-null mice, as illustrated in Fig. 1(B). Using primers that amplify a fragment in the downstream region of the gene (position 2908–3082), we were unable to detect any CIITA transcripts in CIITA$^{o/o}$ mice. Primers that amplify *ef1a* transcripts were used as a positive control for RNA integrity and for the RT-PCR.

Expression of MHC class II molecules in CIITA$^{o/o}$ mice

The expression of MHC class II molecules on a variety of cell types was compared for CIITA$^{o/o}$ and wild-type littermates.

To analyze expression on B cells, we stained splenocyte suspensions with anti-B220, anti-IgM and a panel of anti-class II antibodies, and analyzed them by flow cytometry. All CIITA$^{o/o}$ mice had normal numbers of B cells, indicating that the mutation did not compromise B cell differentiation (not shown). As illustrated in Fig. 2(A), display of class II molecules on B220$^+$ IgM$^+$ cells was drastically reduced by the CIITA mutation. We did occasionally detected low-level staining with some antibodies (amounting to 1–3% of wild-type levels), suggesting trace-level expression of class II molecules by B cells in the absence of CIITA. In contrast to class II, class I molecules were present at normal levels (data not shown). Mice heterozygous for the mutation expressed class II molecules at levels indistinguishable from wild-type B6 mice (not shown).

To evaluate expression on macrophages, we stained thioglycolate-induced peritoneal macrophages with anti-Mac-1 and anti-MHC class II antibodies, either directly or after culture *in vitro* in the presence of IFN-γ and analyzed them by flow cytometry. In both cases, the macrophages from CIITA$^{o/o}$ mice failed to display class II molecules (Fig. 2B).

The expression of class II molecules was also examined by immunofluorescent staining of frozen sections of lymph node and spleen. Figure 2(C) shows inguinal lymph node sections stained with M5-114, which detects determinants on $A_\beta{}^b$ molecules. Staining was clearly present in the lymph nodes from mutant mice (Fig. 2C, central panel), above the background labeling of nodes from the $A_\beta{}^o$ control mouse (Fig. 2C, right panel) or labeling with irrelevant antibodies (not shown), but still distinctly weaker than the staining of control littermate nodes (Fig. 2C, left panel). While not formally quantitated, estimation from film densities indicated a staining intensity 3- to 5-fold lower than wild-type. Staining was restricted to the paracortex and the vast majority of B cells in the follicles were negative, in agreement with the cytofluorimetric analysis. Their location suggests that the class II-positive cells are DC. This was confirmed in a three-color cytofluorimetric analysis: gated CD11c$^+$DEC205$^+$ DC from inguinal lymph nodes exhibited a clear staining with the anti-A^b reagent N22, 5-fold reduced from normal levels (Fig. 3). Clear staining of DC was also observed on sections and by cytofluorimetry in mutant mice crossed onto the H-2^s background (not shown). Interestingly, most DC in the corresponding T cell areas of the spleen (the peri-arteriolar lymphoid sheath) expressed very few class II molecules in the same CIITA$^{o/o}$ mice (not shown).

Clausen *et al*. (43) have suggested that the leakiness of CIITA mutants primarily concerns the $A_\beta{}^b$ chain, detected as isolated chains by the same M5-114 reagent we used here. Yet the staining we observe in lymph node paracortex was also clearly detected with the conformation-specific mAb Y3P (albeit with perhaps slightly lower intensity), indicating that at least some of this staining corresponds to fully assembled A^b molecules. Further, we have been able to detect A^b molecules at the surface of lymph node DC by flow cytometry (Fig. 3), again indicating that these molecules must be properly paired for transport.

Another interesting observation can be made in Fig. 2(C) and was confirmed by the two-color immunofluorescence study of Fig. 2(D), i.e. the presence of class II$^+$ cells in the germinal centers (GC) of CIITA$^{o/o}$ mice. This staining was duller than that of the DC, but specific in that it was never seen with control mAb or Ig. These structures were formally identified as GC by staining with fluorescent peanut agglutinin on serial sections (not shown). Class II molecules occurred on most cells of the GC, encompassing both the 'light' and 'dark' areas, and this is most likely to reflect expression on B cells. Dull staining was also detected by cytofluorimetric analysis, gating on GC B cells by B220/peanut agglutinin counterstaining (not shown). MHC class II positivity was also found in GC in the spleens of CIITA$^{o/o}$ mice.

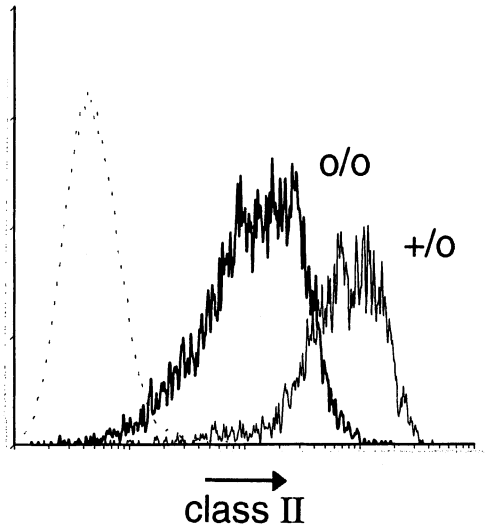

Fig. 3. Expression of class II molecules on DC. DC were enriched from inguinal lymph nodes, and stained with anti-CD11c, anti-DEC205 and anti-class II (mAb N22). Class II expression on gated CD11c$^+$DEC205$^+$ cells is shown here.

We also examined expression of MHC class II molecules in the thymus of CIITA$^{o/o}$ mice by immunohistochemistry on frozen sections. Figure 4(b) illustrates that some class II$^+$ areas were found in the cortex as well as the medulla, duller than in wild-type controls (Fig. 4a) but distinctly different from the class II$^-$ control (Fig. 4c). In the cortex, the staining occurred in patches of connected cells. These cells were morphologically indistinguishable from the epithelial cells identified by staining with ER-TR4 or MTS 44 on serial sections; this assignment was confirmed by two-color staining with anti-class II and anti-keratin (pan-epithelium) antibodies (Fig. 4d–f). Interestingly, the staining intensity with anti-class II and anti-keratin antibodies varied inversely: class II$^+$ cells were characteristically weaker with the anti-keratin antibody. In the medulla, anti-class II staining occurred on isolated cells that did not stain with anti-keratin (Fig. 4g) or with the epithelial cell markers MTS10 (Fig. 4h), 95 or ER-TR5 (not shown). The class II$^+$ cells in the medulla of the CIITA$^{o/o}$ thymus were also negative for the DC marker N418, so that their identity remains a mystery.

T cell differentiation in CIITA$^{o/o}$ mice

As CD4$^+$ T cell differentiation is dependent on the correct expression of MHC class II molecules, we examined the generation of CD4$^+$ T cells by flow cytometry. The CD4/CD8

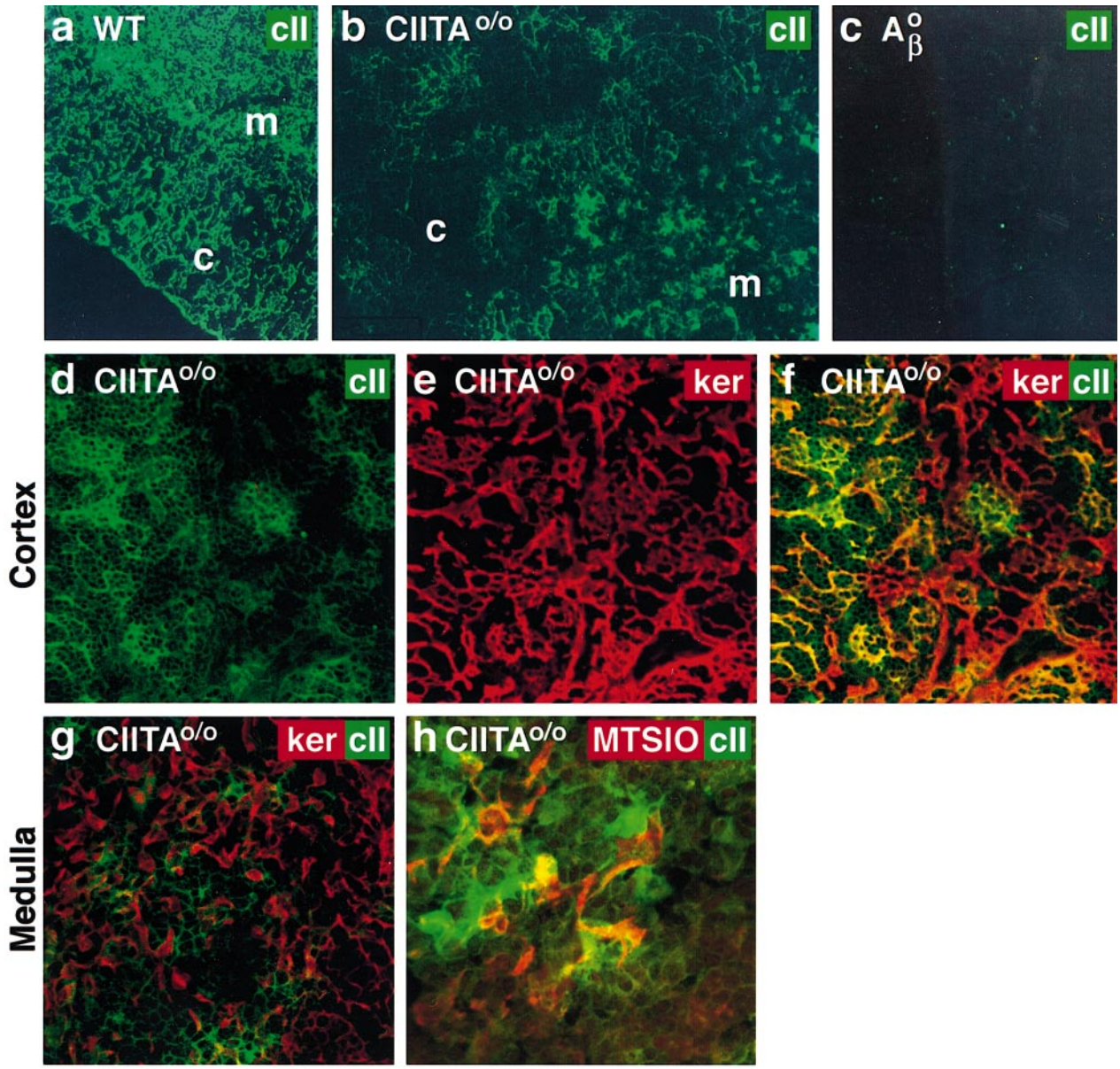

Fig. 4. Expression of class II molecules in the thymus. (a–c) Single-color anti-class II staining of thymic sections of CIITA$^{+/o}$, CIITA$^{o/o}$ and A$_\beta^o$ mice. c and m mark the cortex and medulla. (d–f) Two-color staining with anti-class II, green (d and f), and anti-keratin, red (e and f), of the cortex of a CIITA$^{o/o}$ mouse. The same field of view is shown in all three images. (g) As (f), anti-class II, green, and anti-keratin, of a medullary area of the thymus of a CIITA$^{o/o}$ mouse. (h) Two color staining with anti-class II, green, and MTS10 (anti-thymic medulla), red (h), of the medulla of a CIITA$^{o/o}$ mouse.

staining profiles (Fig. 5A) showed a strong reduction in the number of CD4$^+$CD8$^-$ thymocytes in CIITA$^{o/o}$ mice, grossly similar to that in class II-deficient A$_\beta^o$ animals. More refined analysis, however, argued for some degree of positive selection into the CD4$^+$ lineage in CIITA$^{o/o}$ mice (Fig. 5B): when gated on CD3^{hi} cells, the CD4/CD8 profiles showed significant numbers of cells at the position of mature CD4$^+$CD8$^-$ thymocytes, although on average 6-fold lower than in wild-type mice; these numbers were consistently higher than those in A$_\beta^o$ animals. Similarly, when fully mature CD4$^+$CD8$^-$ cells were visualized by counterstaining with peanut agglutinin and anti-CD3 (Fig. 5C), a higher proportion was found in CIITA$^{o/o}$ (11% of wild-type levels) compared with A$_\beta^o$ (4.4% of wild-type levels) mice. These observations indicate that a significant, albeit limited, number of CD4$^+$CD8$^-$ thymocytes in CIITA$^{o/o}$ mice can successfully undergo class II-mediated positive selection. The difference in numbers between the CIITA$^{o/o}$ and A$_\beta^o$ mutants (Fig. 5D) implies that some thymocytes were selected on the low levels class II molecules expressed in the CIITA$^{o/o}$ thymus.

That the positively selected thymocytes in CIITA$^{o/o}$ mutants can be exported to the periphery was demonstrated by the

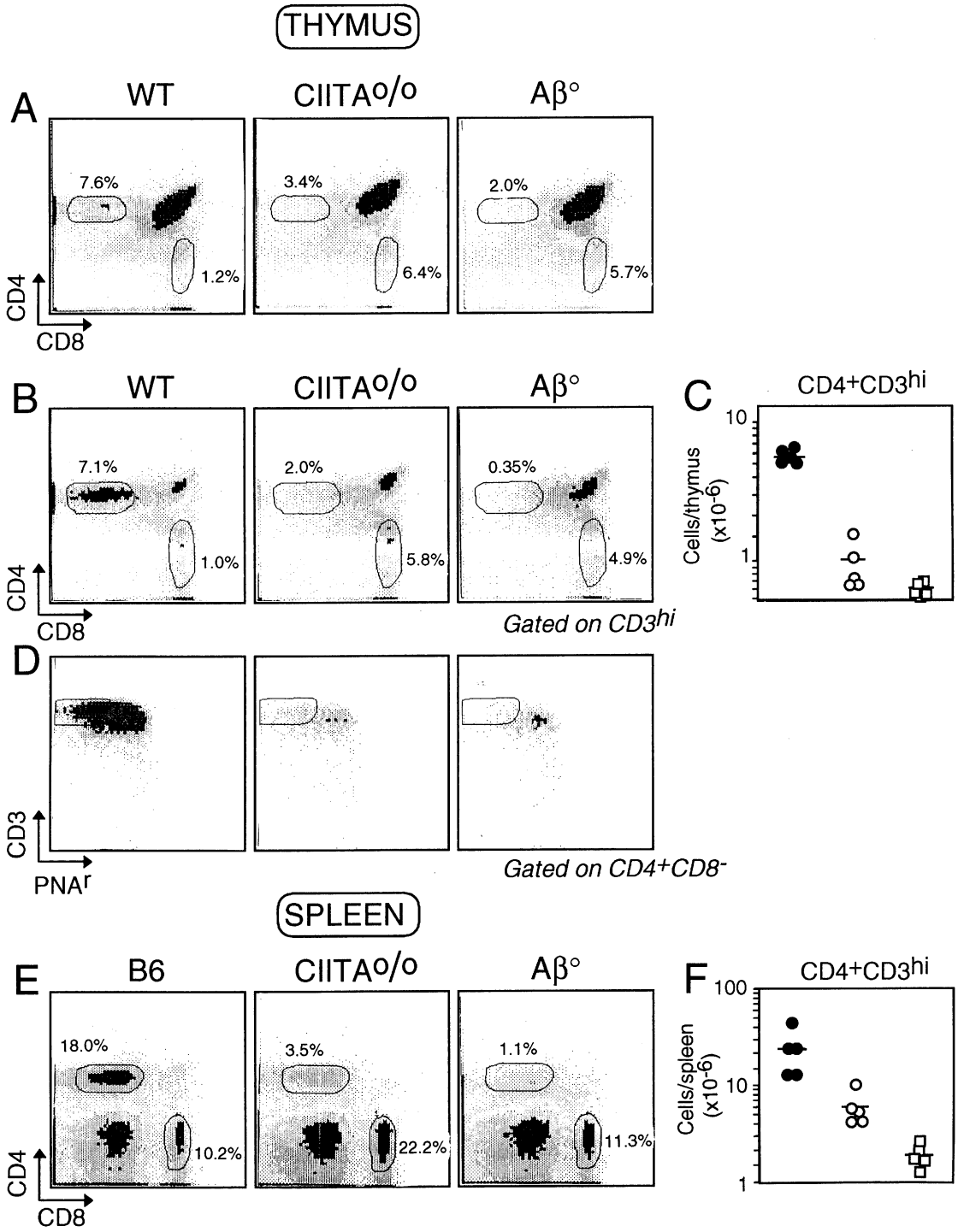

Fig. 5. T cell differentiation. (A) Anti-CD4 and anti-CD8 staining of thymocytes. (B) As (A), gated on $CD3^{hi}$ thymocytes. (C) Profiles of CD3 and peanut agglutinin expression on $CD4^+CD8^-$ thymocytes. (D) Numbers of $CD4^+CD3^{hi}$ thymocytes in $CIITA^{+/o}$ (solid circles), $CIITA^{o/o}$ (open circles) and $A_\beta^{o/o}$ (open squares) mice. Each point represents an individual mouse. Bars represent the arithmetic means of the values. (E) CD4/CD8 profiles of splenocytes from $CIITA^{+/o}$, $CIITA^{o/o}$ or $A_\beta^{o/o}$ mice. (F) Numbers of $CD4^+CD3^{hi}$ splenocytes in $CIITA^{+/o}$ (solid circles), $CIITA^{o/o}$, (open circles) and A_β^{o} (open squares) mice. Each point represents an individual mouse. The bars represent the arithmetic means of the values.

significant population of $CD4^+$ T cells in the spleens of $CIITA^{o/o}$ mice, 5-fold reduced compared with wild-type spleens, but nevertheless 3-fold enriched over fully class II-deficient A_β^o mice (Fig. 5E and F). Most of these cells had an activated phenotype, similar to the $CD4^+$ T cells found in the A_β^o mouse, i.e. $CD44^+CD69^+$ $CD62L^{lo}$ (data not shown). Given the drastically different abundance of class II^+ DC in the spleen versus lymph nodes of $CIITA^{o/o}$ animals mentioned

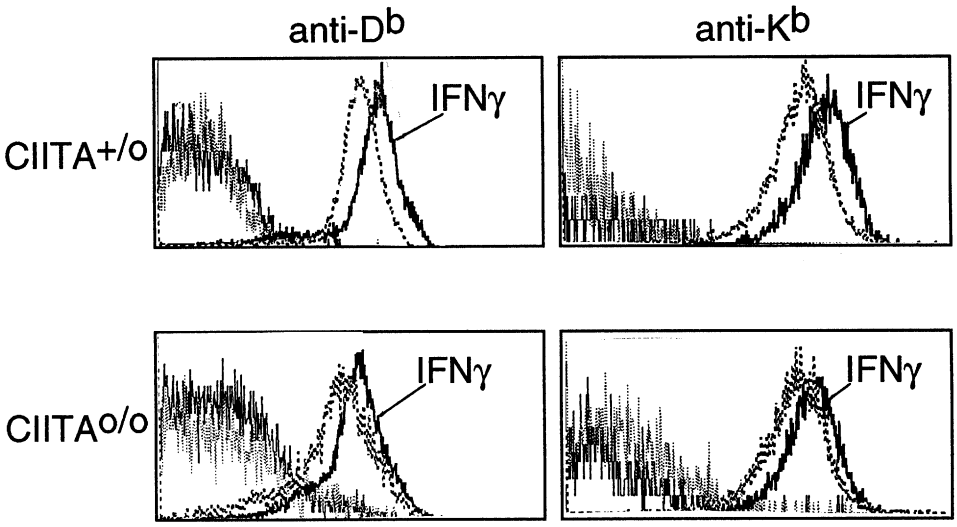

Fig. 6. IFN-γ up-regulation of MHC class I molecules. Cytometry profiles of anti-MHC class I staining on thioglycolate-induced macrophages from CIITA$^{+/o}$ or CIITA$^{o/o}$ mice following culture in the presence or absence of IFN-γ. Histograms are gated on Mac1$^+$ cells. The dotted lines represent control staining in the absence of primary antibody (anti-K^b panels) or control staining of an isotype-matched antibody (anti-D^b panels).

above, one might have predicted that higher numbers of CD4$^+$ T cells would be found in the lymph nodes, with their more frequent DC. However, this was not the case—the frequencies of CD4$^+$ T cells being quite similar in the spleen and lymph nodes of CIITA$^{o/o}$ mice (data not shown).

In summary, the MHC class II molecules detected in the thymus of CIITA$^{o/o}$ mice can select a small but significant number of CD4$^+$ T cells, which are able to colonize the peripheral lymphoid organs.

CIITA and IFN-γ-induced up-regulation of MHC class I genes

It was reported recently that CIITA is also responsible for the induction of MHC class I genes by IFN-γ in human cell lines (16,17). Thus, it seemed of interest to determine whether the IFN-γ-induced up-regulation of surface MHC class I molecule expression was compromised in CIITA-deficient mice. Peritoneal macrophages were elicited with thioglycolate in CIITA$^{o/o}$ or control littermates and were cultured *in vitro* in the presence or absence of IFN-γ. After 48 h, MHC class I molecule expression was analyzed by flow cytometry. As indicated in Fig. 6, macrophages from CIITA$^{o/o}$ mice showed increased expression of both K and D class I molecules in response to IFN-γ, just like those from wild-type mice. This discordance may reflect a mouse/human difference in promoter function or it is possible that it merely reflects a difference in assay systems—the *in vivo* complexes being less dependent on CIITA than those formed in transfection experiments *in vitro*.

Discussion

We have generated CIITA-deficient mice by targeted disruption of the CIITA gene. The strategy for generating our mutant line differed in several respects from that used for the CIITA-deficient line reported recently (25). We employed the cre–loxP system(40), in effect producing two lines: one in which the deletion has not yet taken place, useful for conditional knockout experiments by crossing to transgenic strains expressing the cre recombinase in an inducible and/or tissue-specific manner (44); another, the main focus of the present paper, in which the deletion was effected by crossing to a transgenic strain expressing cre in germline cells. This line's deletion removed several exons of the CIITA gene, far upstream from the segment deleted in the previously reported knockout line (25). It also removed the *neo*^{r} gene and associated promoter, used to select the deletion-bearing ES cells, eliminating the possibility of aberrant CIITA transcription driven from the inserted promoter. Since the previously reported line did show some residual MHC class II expression (25), it was worthwhile to rule out that this might be due to incomplete elimination of CIITA activity owing to an effect of the *neo*^{r} cassette.

Although the immunohistological characterization performed in this study was more detailed than the already published one, the two CIITA-deficient lines appeared to display quite comparable patterns of residual MHC class II molecule expression. In the thymus of both lines, there was weak expression in a subset of epithelial cells characterised by a low level of keratin synthesis, potentially representing a different lineage or maturation stage; there was stronger expression in the medulla, on a rather rare population of cells that so far evades identification in spite of our employing a number of counterstains to identify epithelial, dendritic and macrophage populations. In the periphery, B cells (at least those outside GC) and macrophages expressed little or no class II molecules in both lines. The only apparent discordance between the two concerned DC, described as clearly negative for MHC class II expression in the spleens of the mice of Chang *et al.* (25,43), while we found them to be quite positive in the paracortical T cell areas of s.c. lymph nodes, even when staining with mAb Y3P, specific for fully assembled class II molecules. This discordance can probably be attributed to the different tissues analyzed, as splenic DC were also negative in our CIITA$^{o/o}$ mice and we directly confirmed that

the spleen/lymph node dichotomy could be seen in the line of Chang *et al*. (not shown). Thus, the DC residing in the spleen and s.c. lymph nodes appear to represent distinct subpopulations, differing at the molecular level in their ability to express MHC class II genes independently of CIITA. It will be interesting to see whether the difference we observed here correlates with other functional or phenotypic distinctions.

That significant expression of MHC class II molecules was observed in two independent CIITA-deficient lines provides strong evidence for a CIITA-independent mode of class II gene expression. This would be consistent with reports of the dyscoordinate control of the expression of certain HLA-D isotypes in man, some of which can be synthesized in the absence of CIITA (45). CIITA-independent expression of class II genes in a limited array of cell types may reflect the activity of an as-yet-unidentified CIITA homologue. Alternatively, it may be that these cells exhibit a particular combination of factors binding to the X/X2/Y motif of class II promoters, one that allows them to assemble a functional pre-initiation complex without input from CIITA. Whatever the interpretation, the wide variety of cell types that show CIITA-independent class II gene expression indicates that the CIITA gene cannot be considered as the 'master' control gene for the MHC class II locus, as had been proposed (4,8). This CIITA-independent mode of expression also largely applies to the class II-associated Ii. Widespread expression of Ii was detected in thymus, spleen and lymph node of the present CIITA-deficient mice, on B cells as well as on DC (data not shown), in keeping with results from Chang *et al*., who observed only a modest reduction in RNA levels (25).

The overall pattern of CIITA-independent class II gene expression does not obviously match that observed in mice carrying class II transgenes with promoter-region deletions (46,47). Unlike the clear analogy that can be drawn between the expression of class II molecules in X-box deletion mutants and RF-X-deficient mice (blank cortex, strong expression in the medulla) (43,47), the pattern of expression in CIITA$^{o/o}$ mice did not reproduce that of any of the reported promoter mutants. One might, however, parallel the specific expression in GC B cells observed here with that of the 'Sma' mutants, in which deletion of the far upstream enhancer element of the E_α gene restricted expression in the B cell lineage to those residing in GC (47). It is tempting to speculate that the function of CIITA in the bulk of B cells is to bring together the distant elements of the class II gene enhancer.

Evidence of class II-mediated selection into the CD4$^+$ T cell lineage was found in these mice. CD4$^+$ T cell numbers were well below normal levels but clearly above those of fully class II-deficient $A_\beta{}^o$ mice (Fig. 5). That these cells are functional is indicated by the presence of GC in the spleen, which are quite abundant in CIITA$^{o/o}$ mice but never observed in $A_\beta{}^o$ mice. This CD4$^+$ population was not seen in previous studies (25,43), most likely because the stainings that were performed could not resolve these cells from the background of CD4$^+$ cells abortively selected or selected on non-classical MHC class I, in the absence of class II molecules (1,48,49). The very limited positive selection of CD4$^+$ T cells in CIITA$^{o/o}$ mice, despite clear expression of class II molecules in the thymic cortex, might be thought paradoxical. The explanation may be essentially quantitative: if 15% of those cells potentially capable of selecting express class II molecule at ~25% the normal level, one might expect the efficiency of selection into the CD4$^+$ lineage to be only ~5% the normal levels, which is roughly in the range of observed numbers. In a sense, the CIITA-deficient mice represent an *in vivo* analog of the mixed class II$^+$/class II$^-$ thymuses produced *in vitro* with reaggregation chimeras. Experiments employing the latter showed that differentiating thymocytes are not free to circulate and sample the MHC molecules on the various cells of the cortical epithelium, but rather are fixed in 'niches' (50). Alternatively, the cortical cell(s) that express class II molecules in the CIITA$^{o/o}$ mice may be inefficient at mediating positive selection.

Recently, several groups have shown that a mature CD4$^+$ T cell needs contact with MHC class II molecules in the periphery to survive (51–53). It would appear that CD4$^+$ T cells are receiving at least some survival signals in the periphery of CIITA-deficient mice, as their relative numbers, compared with those in the positive and negative controls, are rather higher in the peripheral lymphoid organs than initially in the thymus (*cf*. Fig. 5C and F). As DC residing in the lymph node paracortex are the main class II$^+$ cell type, these data are consistent with published results that DC are sufficient to mediate survival signals (53). Given the over-representation of class II molecules in the lymph nodes of CIITA$^{o/o}$ mice, we expected the CD4$^+$ T cells to congregate in lymph nodes more than in spleen, assuming that they would prefer class II$^+$ areas. This did not prove to be the case, however. Delivery of survival signals in a particular compartment does not inhibit CD4$^+$ T cells from recirculating into other organs and the presence or absence of class II molecules does not grossly affect retention times in the different compartments.

By generating a second CIITA-deficient mouse line, we have confirmed and extended the observations of Chang *et al*., reinforcing the view that significant class II gene expression can occur in the absence of CIITA. Do CIITA-deficient mice, then, represent a good model for the MHC class II deficiency disease (of complementation group A)? With some differences, yes they do. The reduction in expression levels of MHC class II molecules is variable in human patients (3), something that we have not seen with CIITA$^{o/o}$ mice, nor could we restore expression of MHC class II molecules with IFN-γ, as has been possible in some human patients (54,55). However, the expression of class II molecules on B cells has been found previously in humans (3,56–58). The thymic expression of class II molecules has also been documented, with a thymic expression pattern strikingly similar to that of CIITA$^{o/o}$; strong in the medulla, but weak in the cortex; however, the complementation group of the patient showing this pattern is unknown (58). This thymic expression is also capable of allowing the selection of CD4$^+$ T cells as the presence of CD4$^+$ T cells in human patients has been well documented, although the human patients have higher levels of CD4$^+$ T cells than the CIITA-deficient mice (59–62). However, the absence of IgG2 in these patients, a result found also in the $A_\beta{}^o$ mouse, suggests that, unlike the CIITA$^{o/o}$ mice, the human patients are unable to form GC, as GC formation is critical for the Ig switch to these isotypes (1,2,57,63). Lastly the human disease differs from the

CIITA$^{o/o}$ mice in the expression levels of class I molecules. Human patients often show reduced levels of expression of class I molecules in addition to the absence of class II molecules, something that neither we, nor Chang *et al*. have seen (25,57,64,65; this paper).

It is unfortunately clear, though, that the quite significant levels of residual class II molecules found in CIITA$^{o/o}$ mice make them poor candidates for conditional knockout experiments, as no lymphoid organ is completely class II^{-}, rendering the interpretation of results from controlled or tissue-specific gene activation difficult to interpret.

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Abbreviations

B6	C57Bl/6
CIITA	class II transactivator
DC	dendritic cell
ES	embryonic stem
GC	germinal center
Ii	invariant chain
IRF	IFN- response factor
neo	neomycin

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