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Defective class II transactivator expression in a B lymphoma cell line

T Prod'homme[1], B Drénou[2], C De Ruyffelaere[1], G Barbieri[1,5], W Wiszniewski[3], C Bastard[4], D Charron[1] and C Alcaide-Loridan[1]

[1]INSERM U396, Centre de Recherches Biomédicales des Cordeliers, Paris, France; [2]CHU, Service d'Hématologie-Immunologie, Rennes, France; [3]Department of Medical Genetics, Institute of Mother and Child, Warsaw, Poland; [4]Laboratoire de Génétique Oncologique, Centre Henri-Becquerel, Rouen, France

Loss of MHC class II expression in B-cell lymphoma has been associated with a higher tumorigenicity resulting from lower titers of tumor-infiltrating lymphocytes. This report aims towards the identification of the molecular mechanism leading to defective MHC class II expression in a B-cell lymphoma cell line, Rec-1. We evidenced a coordinated alteration of *HLA-D* gene transcription, reminiscent of B lymphoblastoid cell lines from patients with MHC class II deficiency. Genetic complementation performed between these cell lines and the lymphoma cells indicated that Rec-1 is altered in the *MHC2TA* gene. *MHC2TA* encodes the class II transactivator (CIITA), the master regulator of *HLA-D* gene expression. However, the coding sequence of the Rec-1 CIITA transcript did not reveal any mutation that could hamper the activity of the encoded protein. In agreement with the genetic complementation analysis, we evidenced a highly residual CIITA protein expression in the Rec-1 cell line resulting from a transcriptional defect affecting *MHC2TA* expression. Anti-HLA-DR monoclonal antibody treatment has proved efficient in the destruction of B lymphoma cells. Our data indicate that the appearance of variants losing CIITA, and thereby HLA-DR, expression will require a thorough monitoring during such immunotherapy protocols.
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Introduction

Major histocompatibility complex (MHC) class II molecules play a central role in the immune response through the presentation of antigens to CD4 + T lymphocytes.[1] Constitutive expression of MHC class II molecules is restricted to professional antigen-presenting cells (APCs), including B lymphocytes, dendritic cells and macrophages. In most other cell types, interferon-gamma (IFN-γ) can, however, induce MHC class II expression.[2]

HLA-D gene expression is mainly regulated at the transcriptional level by four conserved *cis*-acting promoter elements termed W, X1, X2 and Y boxes.[2,3] These DNA motifs are the binding sites for the transcription factors *r*egulatory *f*actor binding to the *X* box (RFX), nuclear factor Y and cyclic-AMP response element-binding protein (CREB).[4,5] An additional protein, the Class II Trans-Activator (CIITA),[6] is essential to the formation of a functional transcriptional complex. Indeed, CIITA creates a scaffold through its interaction with the above-mentioned transcription factors,[7] with histone transacetylases like p300/CREB-binding protein (CBP) and pCAF,[8,9] and with components of the transcription initiation and elongation machinery.[10,11]

In addition, CIITA controls the tissue-specific expression of *HLA-D* genes[12,13] through the alternative usage of at least three distinct promoters present in its encoding gene, *MHC2TA*. Promoter I regulates the constitutive expression of *MHC2TA* in dendritic cells, promoter III is used in B lymphocytes and promoter IV responds to IFN-γ in the other cell types.[14,15] This tissue-specific usage of promoters leads to the generation of three isoforms of CIITA differing in their amino-terminal extremities.[16,17]

MHC class II deficiency is a hereditary disease leading to a severe immunodeficiency, currently named the bare lymphocyte syndrome (BLS).[18] Genetic complementation analysis carried out with patient-derived BLS cell lines has defined four complementation groups (groups A–D).[19] Cell lines from complementation groups B, C and D display mutations in RFXANK,[20] RFX5[21] and RFXAP,[22] respectively, the three subunits of the RFX heterotrimer. Group A patients present mutations in CIITA.[6,23]

Defects in MHC class II expression have also been observed in various tumor cell lines. Indeed, complete or partial loss of cell-surface expression of these molecules was evidenced in small-cell lung cancers,[24] non-small-cell lung cancers, neuroblastoma,[25] cervical carcinoma and retinoblastoma cell lines,[26] pancreatic tumors,[27] fibrosarcoma[28] or in choriocarcinoma.[29] Interestingly in all these cell lines or tumor cells, MHC class II defects result from an altered transcription of *MHC2TA*.

The consequences of MHC class II loss are not clear when considering tumor cells originating from non-APCs. However the disappearance of MHC class II expression in tumors cells from APCs, like B-cell lymphoma, is considered as an immune escape mechanism.[30] Indeed, lymphoma lacking HLA-DR expression are less immunogenic and consequently more tumorigenic.[31,32] It is therefore essential to delineate the molecular events leading to the loss of MHC class II expression in these tumor cells.

We report here about a human B lymphoma cell line, Rec-1, with a defective MHC class II expression. Like cell lines from patients with MHC class II deficiency, this lymphoma cell line displays an altered *HLA-D* gene transcription. Genetic complementation with BLS cell lines further demonstrated that the Rec-1 cell line belongs to group A, thereby suggesting a defect in *MHC2TA*. Accordingly, CIITA transcript and protein amounts are highly residual in this cell line. This analysis therefore shows that like all non-APC-originating tumors lacking MHC class II expression, the Rec-1 cell line is defective for *MHC2TA* transcription. However, this is the first evidence of a defect in the CIITA-encoding gene affecting its expression in a tumor cell line.

Materials and methods

Clinical case

A 61-year-old man presented with fatigue, dysphagia and enlarged lymph nodes. Physical examination showed bilateral cervical and left inguinal lymphadenopathy. The right tonsil was

Correspondence: C Alcaide-Loridan, Unité d'Immunogénétique Humaine, INSERM U396, Centre de Recherches Biomédicales des Cordeliers, 15 rue de l'Ecole de Médecine, Paris 75006, France; Fax: +33 1 43 29 96 44; E-mail: Catherine.Alcaide@bhdc.jussieu.fr
[5]Current address: Istituto di Biomedicina ed Immunologia Molecolare, CNR, Palermo, Italy
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markedly enlarged. A chest tomography (CT) scan disclosed moderate para-aortic and iliac lymphadenopathy. Biopsy of the cervical mass revealed a diffuse lymphoma of B-cell lineage, which was classified as mantle cell lymphoma, blastoid variant. The karyotype of the tumor cells was complex: 45,xy, del(1)(p21p31), t(2;16)(q24;q24), der(8)t(8;11)(p11;q13), add(9)(q32), add(9)(p21), der(12)t(8;12)(q11;p12), del(13)(q32), der(14)t(11;14)(q13;q32), del(17)(q22), i(18q), del(18)(q21),−22 [cp18]. Bone marrow aspiration and biopsy were normal. The LDH level was 265 U/ml. A chemotherapy according to the GELA LNH-84[33] regimen was administered and a complete remission was obtained after the induction phase. After 1 month, as the consolidation phase begun, new cervical lymphadenopathy and a splenomegaly appeared. Biopsy of the cervical lymph node showed a diffuse large B-cell lymphoma. A salvage treatment failed to obtain a response and lymphoma blastoid cells disclosing a nearly identical karyotype appeared in the peripheral blood. The patient died a few weeks later. The Rec-1 cell line spontaneously originate from this blood sample. The presence of a t(11;14)(q13;q32) confirming the mantle cell origin of the cell line was later confirmed using molecular methods: the cell line discloses a BCL-1–JH MTC (major translocation cluster)[34] translocation and over-expresses cyclin D1.

Cell lines

The B-cell lymphoma Raji cell line, expressing MHC class II molecules, is used here as a positive control. RJ2.2.5 is a variant of the Raji cell line obtained by mutagenesis and defective for *MHC2TA*.[35] The COM cell line is a B lymphocyte cell line (B-LCL) immortalized with Epstein-Barr virus (EBV) established from a MHC class II-expressing controls. The BCH, ZAL and THF cell lines are B-LCLs established by EBV transformation from patients with MHC class II deficiency. They belong to groups A,[6] B[36] and C,[21] respectively. BCH and ZAL cells were kindly provided by B Lisowska-Grospierre (INSERM U429, Necker Hospital, Paris), and the THF cell line was a kind gift from P van den Elsen (Leiden University Medical Center, The Netherlands). The RS4,11 cell line is from a pre-B leukemia. The cervical carcinoma HeLa cell line is from the ATCC.

The Raji, HeLa, COM, RJ2.2.5 and RS4,11 cell lines were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS), antibiotics and 2 mM glutamine under standard conditions. For the BCH, ZAL, THF and Rec-1 cell lines, FCS was replaced by 10% Myoclone serum (Invitrogen Life Technologies, Cergy Pontoise, France).

Flow cytometric analyses

Indirect immunofluorescence assays were carried out with a FACScan (Becton Dickinson Biosciences, Mountain View, CA, USA) using the Cell Quest program. The primary monoclonal antibodies (mAbs) were L243 (Becton-Dickinson), B7/21[37] and SPV-L3 (Immunotech, Marseille, France), respectively, directed against membrane HLA-DR, DP and DQ antigens. Human membrane MHC class I antigens were detected with the W6/32 mAb (Serotec, Oxford, UK). Cell labeling was then performed with an anti-mouse Ig FITC-coupled (Biosys, Compiègne, France). Phenotyping of the Rec-1 cell line (Table 1) was performed as described previously.[31]

Table 1 Cell-surface expression of different markers assayed by flow cytofluorometry on the Rec-1 cell line

Antibody	*Percentage of labeled cells*
IgM	100
IgD	78
IgA	0
IgG	0
Lambda light chain	0
Kappa light chain	99
CD19	99
CD20	98
CD3	0
CD4	0
CD8	0
CD5	10
CD10	0
CD22	98
CD23	0
CD37	96
CD138	0

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from subconfluent cultures with RNeasy (Qiagen, Valencia, CA, USA) following the instructions of the manufacturer. cDNA preparation was performed on 1 μg of total RNA with 100 ng poly-dT (Roche, Meylan, France) and 2.5 U of Omniscript reverse transcriptase (Qiagen). PCR was performed with the *Taq* polymerase (Qiagen) following the instructions of the manufacturer. Quantification of cDNA concentrations was further assessed with *GAPDH*-specific primers (sense (S): 5′-GTCGTATTGGGCGCCTGGTCAC-3′; anti-sense (AS): 5′-CACGACGTACT CAGCGCCAGCA-3′) through a 19-cycle PCR in a PTC 200 thermocycler (MJ Research, San Francisco, CA, USA).

The primers used to study gene expression of *HLA-D* and *DHLAG*, encoding the invariant chain, were as follows: HLA-DRA (S: 5′-TGGGAGTTTGATGTCCCAAG-3′; AS: 5′-AACATCATCACCTCCATGTG-3′), HLA-DRB (S: 5′-CAGCATTRAAGTCAGGTGGTTCC-3′; AS: 5′-CTCAGCATCTTGCTCTGTGCAG-3′), HLA-DQA (S: 5′-GTGCTGCAGGTGTAAACTTGTACCAG-3′; AS: 5′-CACGGATCCGGTAGCAGCGGTAGAGTTG-3′), HLA-DQB (S: 5′-CGAGTACTGGAAYAGCCAGAAGG-3′; AS: 5′-GGAGTCATTTCCAGCATCACCAGG-3′); HLA-DPA (S: 5′-GGAGACCGTCTGGCATCTGGA-3′; AS: 5′-CTCTCAGCGACACCCTCAGT-3′); HLA-DPB (S: 5′-GGGACACAGCGCTTCCTGGAG-3′; AS: 5′-CAAGCAGGTTGTGGTGCTGCA-3′); HLA-DMA (S: 5′-GGTTGGCTGGGTTGGTAGC-3′; AS: 5′-GCTGGCATCAAACTCTGGT-3′); HLA-DMB (S: 5′-ATCTTTACAGAGCAGAGCAT-3′; AS: 5′-CCTTCTCACTTGGAGTGGA-3′); HLA-DNA (S: 5′-ACCAGATGCCATGGAGACCC-3′; AS: 5′-CCTGCCATGAATACTGGGGCC-3′); HLA-DOB (S: 5′-GGCTGACTGTTACTTCACCA-3′; AS: 5′-GCTGGTGCAGGAGTGGGGAC-3′); DHLAG (S: 5′-GGATGACCAGCGCGACCTT-3′; AS: 5′-CCAGATCCTGCTTGGTCAC-3′).

Analysis of total CIITA transcript expression was performed with 28-cycle amplifications at a 59°C annealing temperature (S: 5′-TTTCTGGGCACCCGCCTCAC-3′; AS: 5′-CTGGGGGAAGGTGGCTGAGA-3′). Promoter III-specific transcripts of CIITA were amplified using the following primers: S, 5′-GGAATTCCA GACTCCGGGAGCTGCTGC-3′; AS, 5′-TGCTGAACTGGTCGCAGTTGATGG-3′. Primers amplifying promoter IV-specific

transcripts of CIITA were: S, 5′-GGAATTCCCAGAGCTGGCGGGAGGGA-3′; AS, 5′-TGCTGAACTGGTCGCAGTTGATGG-3′. Stability of the CIITA transcript was assayed using 100 μM of the transcription inhibitor DRB (5,6-dichlorobenzimidazole 1-β-D-ribofuronoside) from Sigma-Aldrich (Saint-Quentin Fallavier, France).

The study of genes regulated by CIITA in lymphoma was performed with the following primers: YARS, S: 5′-CAACCAGATGAGGAGCTCAAGC-3′; AS: CAGGAAATGGAGCCCAGCTTG (30-cycle amplification); EIF3S2, S: 5′-GCAACATCA TCATGTTCTCCAC-3′, AS: 5′-CAGGACCACATGGTCATAGTTG-3′ (26-cycle amplification).

Cell fusions

At 2 days before the fusion, BLS cell lines were depleted from L243-positive cells (caused either by reversion of the phenotype or nonspecific binding of the antibody) through cell sorting using anti-mouse Ig coupled to magnetic beads (Dynal, Great Neck, NY, USA) as described previously.[38] Cell fusions were performed with 10^7 cells of each fusion partner using PEG 4000 (Merck, Strasbourg, France). As a control, homokaryons were prepared with each fusion partner in order to assess the complete lack of MHC class II-positive cells. After fusion, cells were cultured in RPMI 1640 supplemented with 10% Myoclone serum during 48 h. Indirect immunofluorescence analysis was then performed on cells incubated with the anti-HLA-DR L243 mAb. Cells were analyzed under a fluorescence microscope without any permeabilization step of the cells.

Immunoprecipitation and western blot of CIITA

Immunoprecipitation was performed with a polyclonal rabbit anti-human CIITA directed against a peptide (^{726}GEIKDKELPQYLALTR^{741}) described by Zhou *et al*.[39] Western blots were performed with the mAb anti-human CIITA clone 7-1 H (R&D Systems, Lille, France). Cells were lyzed in high salt buffer (10 mM HEPES pH 7.9, 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 5% glycerol) containing 0.5% Nonidet P-40 (NP-40; Sigma). Lysates were cleared for 2 h with protein-G–Sepharose (Amersham Biosciences), and CIITA proteins were next immunoprecipitated overnight with 2.5 μg of affinity-purified polyclonal anti-CIITA antibodies per sample. Immune complexes were recovered by binding for 30 min to protein-G–Sepharose, resolved on a 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and adsorbed onto a nitrocellulose membrane (Hybond ECL, Amersham Biosciences). Blocking was performed overnight in PBS-Tween buffer containing 2.5% low-fat dry milk. Incubation with 1.5 μg/ml of mAb anti-CIITA was carried out for 1 h at 4°C in PBS-Tween buffer containing 0.25% low-fat dry milk. Immunoreactive bands were visualized with the ECL-Plus Western blotting system (Amersham-Pharmacia Biotech).

Sequencing

Amplification of the 5′ (nt. 107–2188) part of the *MHC2TA* cDNA was performed with the sense 20S primer (5′-TCCTACACAATGCGTTGCCTG-3′) and the antisense TAR3 primer (5′-CTAAGCCTTTGGCCATCGCC-3′). The 3′ part of the cDNA (nt. 2142–3591) was amplified with the sense primer TAF4 (5′-AGGAGGACCAGTTCCCATCC-3′) and the antisense TAR5 (5′-GAGCTGTGTCCACAAGTACC-3′). Sequencing was next carried out on both strands using primers (whose sequences are available on request) covering the whole coding sequence by the Big Dye Terminator cycle sequencing (Perkin-Elmer Applied Biosystem, Norwalk, CT, USA) using an ABI 377 automatic sequencer (Perkin-Elmer).

Untranslated 5′ sequences of the CIITA cDNA were amplified with the pIII-CIITA-sense primer (5′-GGAATTCCAGACTCCGGGAGCTGCTGC-3′) (nt +92–119 from the transcription initiation site[14]) and the antisense CM20 primer (5′-TGCTGAACTGGTCGCAGTTGATGG-3′), which hybridizes to nt. 330–353 following the numbering from Genbank (accession number: X74301). Nucleotide sequencing was performed using the same primers.

In vitro *mutagenesis and transfection*

Site-specific mutagenesis was performed on a wild-type CIITA cDNA, subcloned in the IRES-Neo vector (Clontech Laboratories, Palo Alto, CA, USA). It was performed with the Quick Change Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) following the instructions of the manufacturer, and using primers encompassing nt. 2440–2476 and carrying the C2457 T and T2460C substitutions. Nucleotide sequencing assessed that the substitutions were introduced in the cDNA. HeLa cells were transfected by electroporation using 20 μg of plasmid construct with 975 μF, 300 V and 200 Ω conditions, using an ECM 630 electroporator (BTX-Genetronics, San Diego, CA, USA). G418-resistant transfected cells were selected prior to the analysis of HLA-DR cell-surface expression.

Results

B-cell lymphoma cell line Rec-1 is defective for cell-surface expression of MHC class II isotypes

Indirect immunofluorescence analysis of the Rec-1 cell line revealed that none of the MHC II isotypes (HLA-DR, -DP and -DQ) are expressed at its cell surface (Figure 1). A similar observation was made with the RJ2.2.5 B lymphoma cell line presenting a defect in *MHC2TA*.[6] In contrast, the B lymphoma cell line Raji used as a positive control expresses high levels of cell-surface MHC class II molecules. As observed in several BLS and lymphoma cell lines,[40] Rec-1 cells additionally present a reduced expression of the HLA-A, -B and -C antigens compared to the positive control.

Phenotyping of the Rec-1 cell line (Table 1) evidenced the expression of CD19, CD20 and cell-surface IgM and IgD. These data therefore assess that the lack of MHC class II expression in this cell line is neither related to a pro-B state of differentiation, where *HLA-D* genes are not expressed[2] nor to a terminal plasma phenotype where *HLA-D* gene expression is extinguished.[41] Characterization with additional cell-surface markers (Table 1), and the detection of a t(11;14)(q13;q32) translocation (data not shown) further indicate that the Rec-1 lymphoma cells originate from mature B lymphocytes and have a mantle origin.

Rec-1 cell line is defective for the transcription of HLA-D genes

To identify the molecular causes of the absence of the MHC class II molecules in the Rec-1 cell line, *HLA-D* gene

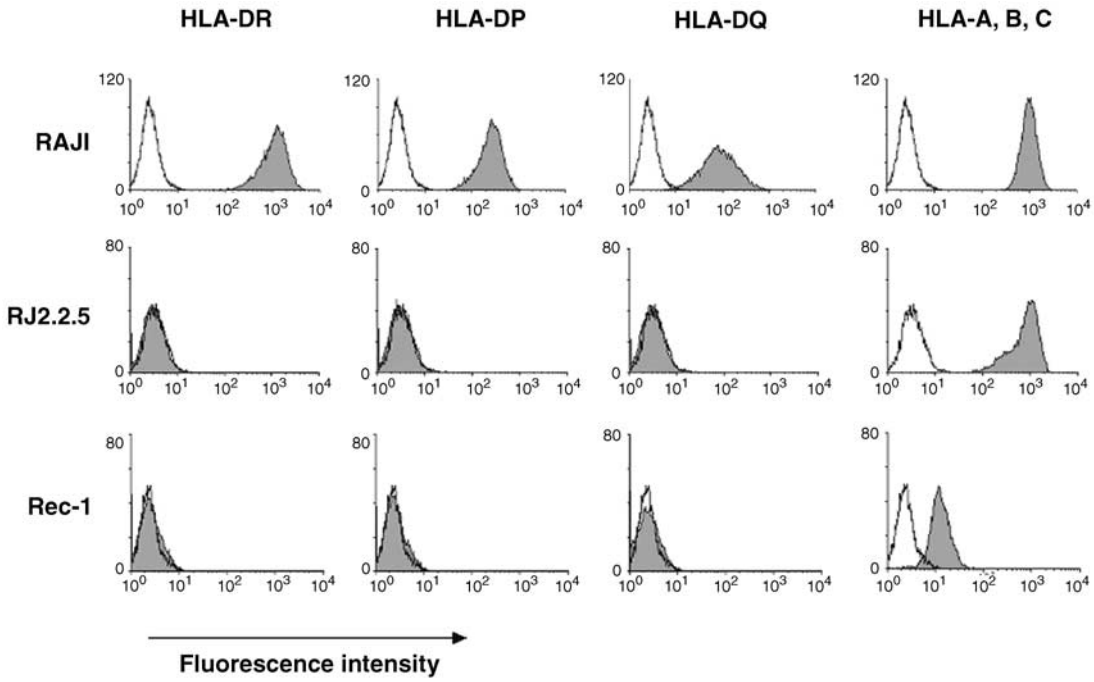

Figure 1 Flow cytometric analysis of cell-surface expression of MHC class I and class II molecules in the Rec-1 cell line. The Raji and the *MHC2TA*-defective RJ2.2.5 B lymphoma cell lines were used, respectively, as positive and negative controls for the expression of the HLA-DR, -DP and -DQ isotypes, respectively, detected with the L243, B7/21 and SPV-L3 mAbs (gray curves). MHC class I expression was analyzed with the W6/32 mAb that recognizes all three HLA-A, -B and -C isotypes. White curves represent cells incubated with the secondary FITC-labeled antibody.

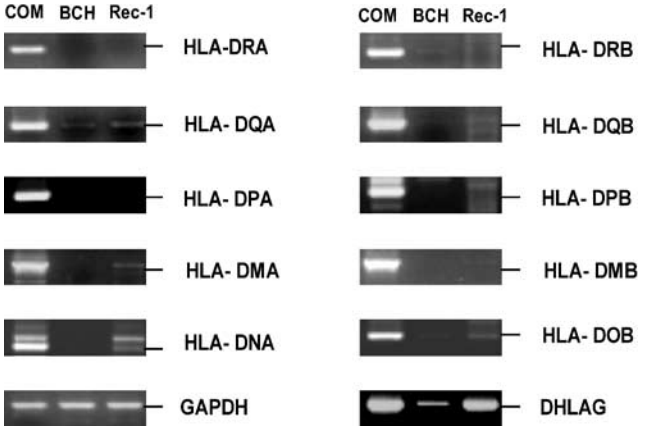

Figure 2 Analysis of *HLA-D* and *DHLAG* (encoding the invariant chain) gene transcription assayed by RT-PCR in the Rec-1 cell line. The COM and BCH B-LCLs are used here as positive and negative controls, respectively. Normalization of cDNA amount was performed through a 19-cycle PCR with *GAPDH*-specific primers prior to the experiment.

transcription was studied by RT-PCR. The BCH cells are an EBV-established B-LCL derived from a BLS patient defective for *MHC2TA*. The positive control, COM, is an unrelated B-LCL expressing MHC class II molecules. As seen in Figure 2, *HLA-D* gene transcription in the Rec-1 cell line is strongly impaired. The residual expressions of *HLA-DRA, -DRB, -DQA, -DMA, -DOA* and *-DOB* detected in the Rec-1 cells further assess that the *HLA-D* locus is not deleted. These data evidence a transcriptional defect of the *HLA-D* genes in the Rec-1 cell line, thereby resembling cell lines from patients with MHC class II deficiency.

Rec-1 cell line belongs to the BLS group A

Genetic complementation was performed by cell fusion of the Rec-1 cell line with BLS cells from groups A (BCH), B (ZAL) and C (THF), respectively, mutated in *MHC2TA*, *RFXANK* and *RFX5*. Heterokaryons were analyzed by indirect immunofluorescence for the cell-surface expression of HLA-DR molecules. As a control, homokaryons were prepared with each fusion partner in order to assess the lack of residual HLA-DR expression or nonspecific binding of the antibody.

The data displayed in Table 2 summarize the results from at least three independent experiments. The fusion between the Rec-1 cell line and BLS cell lines from groups B and C leads to heterokaryons expressing HLA-DR, thereby showing that Rec-1 is not defective for *RFXANK* or *RFX5*. This additionally shows that this cell line does not express a dominant repressor of *HLA-D* gene expression. In contrast, heterokaryons generated by the fusion of Rec-1 and BCH cell lines do not display any HLA-DR cell-surface expression. This demonstrates that the Rec-1 lymphoma cell line belongs to BLS group A, thereby suggesting (a) mutation(s) in both alleles of *MHC2TA*.

Nucleotide sequencing of the Rec-1 CIITA cDNA

Nucleotide sequencing of the CIITA cDNA isolated from the Rec-1 cell line was performed. Homozygous G2446 T (S777) and G2680A (A855), in addition to heterozygous C3286 T (C1057) silent substitutions, were observed. A heterozygous

conservative substitution (A500G) results from a C1614G mutation. The insertion of a TAG codon at nt. 472 was additionally detected. However, this insertion has been previously described in unrelated cell lines and was shown not to affect CIITA activity.[42] Finally, two homozygous mutations were found at positions C2457 T and T2460C, leading to S781L and V782A substitutions. To assay the consequences of their presence in the cDNA, we reproduced both substitutions by site-directed mutagenesis. Mutant and wild-type cDNAs were next stably transfected in the HeLa cell line, and cell-surface HLA-DR expression was assayed on the G418-resistant transfected cells. However, flow cytometric analysis did not reveal any major difference between the wild-type and double mutant constructs in their capacity to restore MHC class II expression (Figure 3).

In addition, sequencing of the region upstream the translation initiation site of the CIITA cDNA did not reveal any mutation. Based on these data, we can conclude that both alleles of *MHC2TA* are expressed in the Rec-1 cell line, as heterozygous mutations were observed. We additionally demonstrate here that the Rec-1 cell line, although defective for *MHC2TA* as assessed by genetic complementation, does not display any mutation in the *MHC2TA* coding sequence that could alter its activity.

Table 2 Complementation analysis performed by cell fusion

Fusion partners	*HLA-DR expression*
Rec-1	−
BCH	−
ZAL	−
THF	−
BCH × ZAL	+
BCH × THF	+
THF × ZAL	+
Rec-1 × BCH	−
Rec-1 × ZAL	+
Rec-1 × THF	+

Cell-surface expression of HLA-DR molecules on the various homokaryons and heterokaryons was detected by immunofluorescence. The BCH, ZAL and THF cell lines are B-LCLs, respectively, defective for CIITA, RFXANK and RFX5.

CIITA protein expression is strongly reduced in the Rec-1 cell line

We next analyzed the expression of the CIITA protein in the Raji, RJ2.2.5 and Rec-1 cells. Protein extracts (3.6 mg) were submitted to immunoprecipitation with a rabbit polyclonal Ab directed against CIITA and revealed by immunoblotting with an anti-CIITA mAb (Figure 4a). As previously reported by our group, CIITA is expressed in the Raji cell line as two isoforms, B-CIITA and F-CIITA, where B-CIITA is 24 amino acids longer in its N-terminal extremity due to an alternative usage of translation initiation codons.[16] The RJ2.2.5 cell line presents defects in both alleles of *MHC2TA*.[35] Accordingly, wild-type CIITA is not expressed in this cell line, indicating that the bands observed in Raji are specific for CIITA. In the Rec-1 cells, CIITA is detected, although it is present at very low levels compared to Raji (Figure 4a).

Our study of a panel of different B-LCLs and lymphoma cell lines had revealed that the RS4,11 cell line, although expressing cell-surface MHC class II molecules, contains very low amounts of CIITA protein (data not shown). As seen in Figure 4b, where 2 mg of protein extracts were used for the immunoprecipitation, CIITA protein is barely detectable in the Rec-1 protein extracts compared to those of the RS4,11 cells. These data thereby confirm a defect in the expression of CIITA in the Rec-1 cell line, in agreement with its classification in BLS group A. However, the weak expression of the protein is in agreement with the residual transcription of certain *HLA-D* genes mentioned above (Figure 2).

Altered expression of MHC2TA transcript in the Rec-1 cell line

A defect in *MHC2TA* transcription might explain the highly residual expression of the CIITA protein. We then studied the expression of this gene in the MHC class II-defective Rec-1 and RJ2.2.5, compared to the MHC class II-expressing Raji and RS4,11 cell lines through RT-PCR using CIITA-specific primers (Figure 4c). This experiment showed that the level of amplification products is lower in the Rec-1 cell line than in the Raji and RS4,11 cell lines. Similar data were observed with several sets of primers hybridizing to different regions of the CIITA cDNA (data not shown). It must be noted that an alternatively spliced messenger was observed more frequently in the Rec-1 cell line

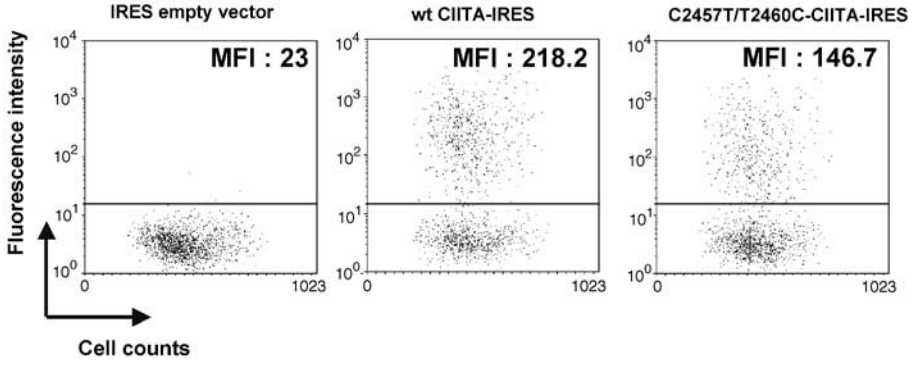

Figure 3 Functional complementation of the HeLa cell line with the *in vitro*-mutagenized CIITA cDNA construct containing the C2457 T and T2460C substitutions. HeLa cells were transfected with either the empty IRES-Neo expression vector, with the wild-type CIITA cDNA subcloned in IRES-Neo (wt CIITA-IRES) or with the double C2457T/T2460C mutant. G-418-resistant cells were analyzed by flow cytometry for cell-surface expression of HLA-DR with the L243 mAb. MFI: medium intensity of fluorescence.

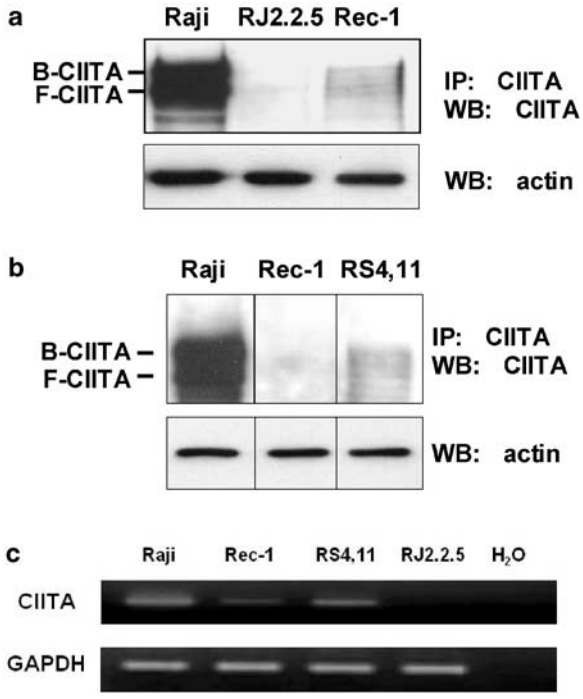

Figure 4 Expression of CIITA transcript and protein in the Rec-1 cells. (a) Expression of the CIITA protein in the Rec-1, Raji and RJ2.2.5 cell lines. Cell extracts (3.6 mg) were immunoprecipitated with a rabbit polyclonal antibody directed against CIITA. Immunoblotting was next performed with the 7-1 H mAb. (b) Expression of the CIITA protein in the Rec-1, Raji and RS4,11 cell lines. This analysis was performed as above, except that 2 mg of protein extracts were used. (c) *MHC2TA* transcript expression in the Raji, RS4,11, RJ2.2.5 and Rec-1 cell lines was assayed by a 28-cycle RT-PCR using primers amplifying all types of CIITA transcripts. Equal loading of cDNAs was assessed using GAPDH primers.

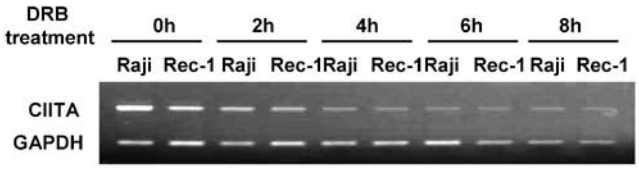

Figure 5 Stability of the CIITA transcript in the Raji and Rec-1 lymphoma cell lines. Cells were treated for various time periods with 100 μM DRB inhibitor prior to RNA extraction. RT-PCR was next performed through coamplification of GAPDH and CIITA. A three-fold higher cDNA loading was used for the Rec-1 cell line in order to visualize more accurately the decay of transcript expression.

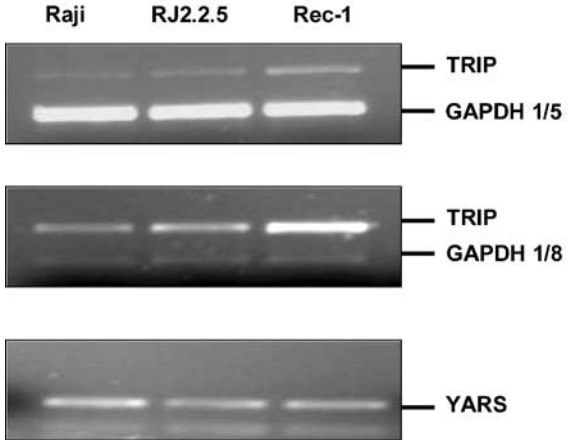

Figure 6 Expression of the TRIP- and YARS-encoding genes analyzed by RT-PCR on the Raji, RJ2.2.5 and Rec-1 B lymphoma cell lines. Equal loading of cDNAs was assessed with GAPDH primers using a 1/5 or 1/8 ratio of GAPDH primers compared to the TRIP-1-specific primers.

when compared to the Raji cell line (data not shown). This transcript, representing about 5–10% of the total amplification products of the Rec-1 cell line, is deleted of one exon (nt. 3263–3349). This exon skipping leads to the production of a truncated CIITA protein that has been shown to lack transactivating activities.[43]

These data were suggestive of either a defect in *MHC2TA* transcription or an instability of the transcript that could hamper its efficient translation. To test this latter hypothesis, synthesis of neotranscripts was blocked using the DRB inhibitor. The Raji and Rec-1 cell lines were then treated with the inhibitor during 0, 2, 4, 6 or 8 h. Expression of the CIITA transcripts was next assayed by RT-PCR with an overloading of the Rec-1 cDNA to gain better detection of the amplification product, and more accurate conclusions when compared to Raji (Figure 5). This experiment demonstrates that the transcripts from Raji and Rec-1 cell lines present similar kinetics of disappearance. These data thereby show that the stability of the *MHC2TA* transcripts is not altered in the Rec-1 cell line and indicate that the low amounts of CIITA transcripts result from a transcriptional defect.

Genes deregulated by CIITA

In addition to *HLA-D* genes, it has been shown that CIITA might affect the expression of other genes in lymphoma. Indeed, using the microarray methodology, transfection of the CIITA-defective RJ2.2.5 cell line with a CIITA cDNA evidenced a deregulation of different genes, including *YARS* and *EIF3S2*.[44] The *YARS* product is a tyrosyl tRNA synthetase that can be proteolyzed and thereby generate a cleaved protein resembling IL-8 and possessing inflammatory capacities.[45] The *EIF3S2* gene encodes the TRIP-1 (TGF-βR-interacting) protein that is involved in the resistance to the antiproliferative properties of TGF-β.[46] As their products might participate in tumor progression, it was interesting to examine whether these genes were repressed in Rec-1 as a consequence of the altered CIITA expression. We then performed this analysis by RT-PCR, and set the amplification conditions to minimize the cycle numbers in order to obtain semiquantitative data. As seen in Figure 6, *EIF3S2* expression is not reduced in the cell lines lacking CIITA compared to the positive control. In contrast, *YARS* expression is lower in both CIITA-defective cell lines Rec-1 and RJ2.2.5 compared to Raji. These data indicate that the alteration of CIITA expression in the Rec-1 cell line leads not only to the complete lack of cell-surface MHC class II molecules but might additionally reduce the expression of (a) factor(s) involved in immune escape of the tumor cells.

Discussion

In this report we show that the lack of MHC class II expression in the Rec-1 B lymphoma cell line (Figure 1) results from an altered

transcription of the *HLA-D* genes (Figure 2). The cell-surface expression of IgM and IgD, in addition to the CD20 and CD19 markers (Table 1), assesses that Rec-1 originates from a mature B lymphocyte. This indicates that the transcriptional defect of the Rec-1 cell line is not a consequence of methylation[47] or extinction[41] of the *MHC2TA* promoters observed in early or late stages of differentiation of B cells, respectively.

Genetic complementation experiments revealed that the Rec-1 cell line belongs to the BLS complementation group A, suggestive of a defect in *MHC2TA* (Table 2). In agreement, the expression of the CIITA protein was shown to be highly residual in the Rec-1 cell line (Figure 4). However, we did not evidence any mutation in the coding sequence of the CIITA cDNA that might explain a lack of activity or an increased instability of CIITA. Indeed, complementation was obtained with a cDNA carrying the two nonconservative S781L and V782A homozygous substitutions found in Rec-1 (Figure 3). These experiments were performed in the HeLa cell line in which we have shown that recombinant CIITA is expressed at levels close to physiological ones.[16] Functional complementation of this cell line is therefore useful as it allows to discriminate fully invalidating defects from leaky mutations.[48] We additionally have examined the level of CIITA protein expression in the transfected clones without evidencing any reduction when compared to cell clones transfected with the wild-type cDNA (data not shown). These data therefore indicate that the S781L and V782A mutations do not inactivate CIITA transactivating properties and do not confer a higher instability to the CIITA protein. In agreement with these data, it has to be noted that these aminoacids are not conserved in the mouse CIITA cDNA (corresponding aminoacids: V729 and A730). This suggests that the C2457T and T2460C variants might represent polymorphisms of the *MHC2TA* gene.

Based on these data, we propose that the CIITA protein, although functional in the Rec-1 cell line, is likely in a concentration that is not sufficient to permit cell-surface expression of MHC class II molecules. This is in agreement with reports evidencing a threshold of CIITA required for MHC class II expression.[49,50] We therefore analyzed the possibility that a defect in *MHC2TA* might alter its level of expression in the Rec-1 cell line. Indeed, we have evidenced a strongly reduced amount of total CIITA transcripts in this cell line compared to the Raji and RS4,11 cell lines (Figure 4a). Interestingly promoter III- and promoter IV-initiated CIITA transcripts are reduced in the Rec-1 cell line when compared to the RS4,11 cell line (data not shown). We additionally provide evidence that this reduced amount does not result from an abnormal instability (Figure 5). These data therefore are in agreement with a mutation affecting the transcription of *MHC2TA*. This mutation might be localized in the promoter regions of the gene or in an intronic sequence. Given the length of these regions (about 10 and 30 kb, respectively), and the high degree of polymorphism in both promoters and introns that we had observed during the analysis of BLS group A patients (unpublished data), the identification of this mutation would first necessitate sequencing of *MHC2TA* in nontumoral cells, however unavailable, of the patient from which Rec-1 was established.

Various tumor cell lines, fibrosarcoma, neuroblastoma, retinoblastoma cell lines, SCLC and pancreatic tumors, were shown by us and others to lack MHC class II expression due to defective *MHC2TA* transcription.[24,26–28] One can wonder if this indicates that the alteration of CIITA expression can provide an advantage to the tumor cells. A hypothesis is that the loss of CIITA expression might favor tumor proliferation or resistance to apoptosis. Indeed, it has been shown that CIITA can sequester the transactivators CBP/p300.[8,51] As CBP is now described as an antioncogene involved cell growth, differentiation and apoptosis,[52] it is possible to hypothesize that the loss of CIITA, through the release of CBP, might favor tumor progression.

Alternatively, through transactivator sequestration or yet unknown mechanisms, it has been shown that CIITA regulates, in addition to *HLA-D*, the expression of different genes.[44] As some of them might be involved in tumor progression, we have assayed two of these genes (Figure 6) and found that one of them, *YARS*, was presenting a slightly reduced expression in the Rec-1 and RJ2.2.5 cell lines when compared to Raji. As *YARS* encodes a protein with IL-8-like properties,[45] the lower expression of this gene in the Rec-1 cell line might reduce the inflammatory response. To assess further this point, a large panel of lymphomas should be analyzed to determine if the reduced expression of the *YARS* gene is recurrent for CIITA-defective cells.

In addition to MHC class II, CIITA was shown to enhance MHC class I promoter expression.[53] Its disappearance might then further reduce antitumor cytotoxic CD8+ lymphocyte activity. Indeed, in the Rec-1 cell line, MHC class I expression is strongly reduced (Figure 1). Further quantification of cell-surface MHC class I molecules assessed that this reduction is about 60-fold in the Rec-1 cell line when compared to Raji (data not shown). However, we cannot ascertain here that the CIITA defect is entirely or partially responsible for this phenotype. Indeed, the RJ2.2.5 cell line, although defective for CIITA, does not display reduced MHC class I expression (Figure 1). It is then possible that an additional mutation affects a transcription factor required for MHC class I gene expression in the Rec-1 cell line. Alternatively, as the W6/32 mAb recognizes all three MHC class I isotypes, mutations might have occurred in the genes encoding certain isotypes or alleles of MHC class I molecules.

Having reviewed all nonclassical effects of CIITA, it remains important to consider that the main consequence of CIITA defect in APC-derived tumors is the loss of MHC class II expression. Indeed, these tumors express the B7-1 and B7-2 costimulatory molecules, and therefore might activate tumor-specific T lymphocyte responses. The loss of MHC class II molecule expression has been proposed as an immune escape mechanism through a reduced recognition by helper CD4+ T lymphocytes. Titers of tumor-infiltrating lymphocytes were shown to be reduced with human HLA-DR-defective lymphoma.[30] Accordingly, the loss of MHC class II expression in mouse lymphoma cells was shown to correlate with a higher tumorigenicity resulting from a lower immunogenicity.[32] In addition, although not evidenced to our knowledge in B-cell lymphoma, loss of MHC class II expression might also provide resistance to cytotoxic CD4+ T lymphocytes, as shown in melanoma.[54]

As MHC class II signaling via HLA-DR has been shown to induce cell death in human lymphoma,[55] immunotherapy protocols based on humanized 1D10 and Lym-1 anti-HLA-DR mAbs[56] have been envisaged for B-cell lymphoma treatment.[57] Indeed, antibody-based lymphoma therapy has already shown its high efficiency with Rituximab, a humanized anti-CD20 mAb.[58] However, relapse occurs in about half of Rituximab-treated patients as rituximab-resistant tumor cells arise.[59] Interestingly, these resistant tumor cells are mostly CD20− variants.[59,60]

Spontaneous loss of MHC class II expression in B-cell lymphoma is a rare phenomenon.[31] However, anti-HLA-DR immunotherapy protocols might favor the proliferation of HLA-DR- variants. The results presented here demonstrate that lymphoma cells, like other HLA-DR- tumor cells, can lose MHC class II expression due to a defect in CIITA expression. We

therefore propose that patients should be scrutineously monitored for the appearance of variants losing MHC class II expression and thereby escaping anti-HLA-DR antibody-based therapies.

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