MHC class II antigen presentation pathway in murine tumours: tumour evasion from immunosurveillance?

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Summary Qualitative differences in the MHC class II antigen processing and presentation pathway may be instrumental in shaping the CD4+ T cell response directed against tumour cells. Efficient loading of many MHC class II alleles with peptides requires the assistance of H2-M, a heterodimeric MHC class II-like molecule. In contrast to the HLA-DM region in humans, the β-chain locus is duplicated in mouse, with the H2-Mb1 (Mbβ1)-chain distal to H2-Mb2 (Mbβ2) and the H2-Ma (Maα-chain gene). Here, we show that murine MHC class II and H2-M genes are coordinately regulated in murine tumour cell lines by T helper cell 1 (IFN-γ) and T helper cell 2 (IL-4 or IL-10) cytokines in the presence of the MHC class II-specific transactivator CIITA as determined by mRNA expression and Western blot analysis. Furthermore, Mbβ1 and Mbβ2 heterodimers are differentially expressed in murine tumour cell lines of different histology. Both H2-M isomers promote equally processing and presentation of native protein antigens to H2-Aα- and H2-Eα-restricted CD4+ T cells. Murine tumour cell lines could be divided into three groups: constitutive MHC class II and CIITA expression; inducible MHC class II and CIITA expression upon IFN-γ-treatment; and lack of constitutive and IFN-γ-inducible MHC class II and CIITA expression. These differences may impact on CD4+ T cell recognition of cancer cells in murine tumour models. © 2000 Cancer Research Campaign

Keywords: MHC; gene regulation; transcription factors; antigen processing

Most of the tumour antigens defined by murine or human T cells represent targets for major histocompatibility complex (MHC) class I restricted CD8+ T cells. Nevertheless, recent data (reviewed in Pardoll and Topalian, 1998) rekindled interest in CD4+ T cells mediating anti-tumour-directed immune responses. Both, CD4 and CD8 T cell epitopes may have to be incorporated in a tumour vaccine in order to induce a strong and effective cellular immune response. Tumour-specific T cells may directly recognize MHC class II-positive tumour cells (Mongini et al, 1996; Armstrong et al, 1997), or they may confer protective immunity on cancer cells in the absence of MHC class II molecules (Ossendorp et al, 1998). Thus, efficient MHC class II restricted presentation of tumour-associated antigens, either by tumour cells themselves, or alternatively by professional antigen-presenting cells, appears instrumental in initiating CD4+ T cell responses.

Major histocompatibility complex (MHC) class II molecules are heterodimeric (αβ) cell surface glycoproteins which present peptides derived from self or foreign antigens to CD4+ T cells. Newly synthesized MHC class II α and β chains assemble in the endoplasmic reticulum (ER) with a third glycoprotein, the invariant chain (Ii), to nonameric complexes (Cresswell, 1996). These nonamers are targeted by signals within the cytoplasmic domain of the Ii (Bakke and Dobberstein, 1990) to specialized compartments of the endocytic pathway termed MHC class II compartments, where peptide loading occurs (Kleijmeer et al, 1997). During transport, Ii is stepwise proteolytically cleaved (Cresswell, 1998) yielding a nested set of Ii derived peptides, termed CLIP, for MHC class II-associated invariant peptides, which occupy the peptide-binding groove of class II αβ dimers (Riberdy et al, 1992). CLIP is subsequently exchanged for tightly bound antigenic peptides derived from internalized antigens or endogenous proteins (Wolf and Ploegh, 1995).

Although exchange of CLIP for antigenic peptides might occur spontaneously in MHCII at lysosomal-like pH (Urban et al, 1994), H2-M/DM is required for that final step of peptide loading by many MHC class II alleles (Denzin et al, 1994; Miyazaki et al, 1996; Wolf et al, 1998). H2-M/DM has been shown to interact with MHC class II in MIICs (Sanderson et al, 1996) and to catalyze the exchange of CLIP for cognate peptides by facilitating the removal of CLIP and stabilizing the transiently peptide-free state of MHC class II molecules following CLIP release (Denzin et al, 1996). Simultaneously, H2M/DM functions as a peptide editor, which serves to positively select peptides that can stably bind to MHC class II molecules (Van Ham et al, 1996). Thus, expression of H2-M/DM molecules may critically affect the peptide repertoire displayed to the T cell compartment. In contrast to the HLA-DM loci in humans, the murine H2-M region encoded within the MHC contains one Ma gene, but two Mb genes, termed Mb1 and Mb2 (Cho et al, 1991; Kelly et al, 1991). Regulation of MHC class II genes occurs primarily at the transcriptional level (Mach et al, 1996) and is controlled by a non-DNA-binding cofactor, the class II trans-activator CIITA (Mach et al, 1996). Until this end, the potential role of a differential Mb1 or Mb2 expression in professional or non-professional APCs (i.e. tumour cells) has not been addressed.

Here, we investigated the regulation of H2-M, MHC class II and CIITA gene expression in murine tumour cells lines by Th1 (IFN-γ)- and Th2 (IL-4 or IL-10) cytokines. In addition, we examined whether Mb1 and Mb2, and by consequence Mbβ1 and Mbβ2 heterodimers, are differentially expressed in different tumour
types and if they are able to effectively present surrogate target antigens to MHC class II-restricted T cell lines.

**MATERIALS AND METHODS**

**Animals**

C57BL/6 mice (H2d) and New Zealand White rabbits were purchased from Charles River Laboratories (Sulzfeld, Germany). TCR DO11.10 transgenic mice expressing a TCR-α/β specific for peptides 323–339 from ovalbumin presented by H2-Aβ (Murphy et al, 1990) and mice expressing a TCR-α/β specific for peptides 111–119 from influenza haemagglutinin (HA) presented by H2-Eβ (Kirberg et al, 1994) were generous gifts from Dr D Loh and Dr J Kirberg at the Basel Institute for Immunology (Basel, Switzerland).

**Cell lines and culture conditions**

Murine cell lines included: Renca (H2d) renal adenocarcinoma kindly provided by Dr B Seliger (University of Mainz, Germany), P815 (H2d) mastocytoma, TS/A (H2d) mammary adenocarcinoma, MC-38 (H2d) colon adenocarcinoma, MCA-102 (H2d) fibrosarcoma, L929 (H2b) fibroblasts, B16 (H2b) melanoma, RMA (H2d) T cell lymphoma, RMAs (H2b) T cell lymphoma, EL-4 (H2d) thymoma, YAC-1 (H2b) T cell leukaemia, P388D1 (H2b) macrophages, IC-21 (H2d) macrophages, A20 (H2b) B cell lymphoma (kindly provided by Dr G Hämmerling, DKFZ, Heidelberg, Germany) and LB27.4 (H2b) B cell lymphoma. Cell lines were maintained in RPMI-1640 (Gibco, Ebersberg, Germany) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamate, 100 IU ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin (all from Gibco) and 50 µM 2-mercaptoethanol (Sigma, Deisenhofen, Germany), termed complete medium (CM).

In experiments using rIL-4 (a generous gift from Shering Ploegh, Kenilworth NJ, USA) rIL-10 or rIFN-γ (both from PharMingen, Hamburg, Germany), culture medium was changed before experiment and IL-4 (250 U ml⁻¹), IL-10 (100 ng ml⁻¹) or IFN-γ (150 U ml⁻¹) were added for 72 h.

**Preparation of CD4+ T cells**

Isolation of CD4+ Mel-14⁺ naive T cells from spleens of TCR transgenic mice and generation of CD4+ Th cells that express a Th1 cytokine pattern after specific activation with antigen, were performed as recently described in detail elsewhere (Lingnau et al, 1998).

**Antibodies**

The anti-class II mAbs FITC-conjugated 39–10–8 (anti-H2-Aβ) and PE-conjugated 14–4–4S (anti-H2-Eβ) were obtained from PharMingen; FITC-conjugated secondary staining reagents: goat anti-hamster IgG, rabbit anti-rat IgG, goat antimouse IgG and goat anti-rabbit IgG were purchased from Dianova (Hamburg, Germany) and unlabelled, FITC- or PE-conjugated isotype-matched control antibodies were from Coulter-Immunotech (Hamburg, Germany). The rabbit antisera R.Mtx-C.69.3, R.Mtx-β12.C.71.3 and R.hCLIP73.11 were prepared by immunizing rabbits with carboxy-terminal peptides from H2-Mtx (aa 238–248) or H2-Mβ1/2 (aa 228–243) coupled by an added amino terminal cysteine to diphtheria toxoid-MCS (Chiron Micromotopes, Victoria, Australia). Antisera were affinity purified using columns of NHS-activated Fast Flow Sepharose 4 (Pharmacia, Freiburg, Germany) cross-linked to the respective Mtx, Mβ or CLIP peptide according to the manufacturer’s instructions. Individual fractions of affinity purified antisera were screened for specific antibody titres by ELISA and Western blot analysis using T2 cell lines transfected either with Ma, Mβ1 or Mβ2 genes (Walter et al, in press).

**Template cDNA preparation and competitive reverse transcription PCR**

Total RNA isolation and cDNA synthesis have been previously described (Walter et al, 1996). Briefly, PCR amplification was performed in an amplification mix adjusted to 50 µl containing 50–100 ng cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01 (w/v) gelatin, 1 mM each dNTP, 25 pmol each primer and 2.5 U of Ampli Taq Gold Polymerase (Perkin-Elmer, Weiterstadt, Germany). The RT-PCR amplification profile involved an initial denaturation step at 94°C for 10 min followed by 35 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 1 min; the last extension was for 10 min at 72°C. The following primers were used for PCR: β-actin sense 5'-TGGAAATCCT-GTGGCAGCATCATGAAAC-3'; β-actin antisense 5'-TAAAACGCAGCTCATAGAAC-3'; Ma sense 5'-AAGTGATGG-GAGCATGAGCAAGTTG-3'; Ma antisense 5'-GATCAGTCAC-TCGAGGAGGTGCTG-3'; two sets of primers were used to amplify CIITA; sense 5'-CCACTCTCGGCAGAGCAGGT-3'; antisense 5'-TGTTCAAGGCTTCTTGACTC-3'; and a set previously identified to amplify both human and mouse CIITA (Chang et al, 1996). Competitive RT-PCR analysis has been performed as previously described (Bouaboula et al, 1992). After reverse transcription, cDNA concentrations were normalized by co-amplifying constant amounts of cDNA (50 ng) and known concentrations of the serially diluted β-actin competitor plasmid pMCQ (kindly provided by Dr Blankenstein, University of Berlin, Germany). PCR products generated by the competitor plasmid pMCQ (248 bp) and the endogenous β-actin mRNA (348 bp) were resolved on ethidium-bromide-stained agarose gels and band intensities were quantified as OD₅₉₅ units by means of the Gelprint 2000i densitometer (MWG Biotech, Ebersberg, Germany). The pMCQ concentration which was required to achieve equal band intensities for both fragments was determined by linear regression analysis and the number of copies of β-actin mRNA molecules per µg of total RNA for each cDNA sample calculated (Bouaboula et al, 1992). For semiquantitative analysis of Ma mRNA expression, a heterologous MA competitor fragment (termed Ma cf) was generated as described according to MIMIC PCR technology (Haberhausen et al, 1998). Following co-amplification, Ma cf yields a 742 bp fragment as compared to the 794 bp PCR product derived from endogenous Ma mRNA. Equivalent to β-actin mRNA analysis, the number of copies of Ma mRNA molecules per µg of total RNA were determined for each sample by titration of known amounts of the serially diluted Ma cf against constant amounts of cDNA (100 ng). In order to compare differences in Ma mRNA expression levels, samples of each cell line were normalized for cDNA contents based on the number of β-actin mRNA molecules. Expression of Ma transcripts in cytokine-treated samples of each
individual cell line was calculated as Ma mRNA in cytokine-treated cells/Ma mRNA expression (± 1) in non-treated cells.

**Ratio reverse transcriptase PCR analysis**

The ratio reverse transcriptase PCR (ratio-RT-PCR) assay performed in this study was based on the simultaneous amplification of Mb1 and Mb2 transcripts using primers (Mb1/2 sense 5′-GGACCAGTGGCTGAGTCTG-3′, Mb1/2 antisense 5′-GCATCACGGGCTCCCTTGTG-3′) annealing within conserved regions (exon 1 and 3) of both Mb messages (Walter et al, 1996). Equal amplification efficiency of both Mb transcripts was assured by comparative cycle kinetic and linear regression analyses (Bouaboula et al, 1992) using cloned Mb1 and Mb2 full-length cDNA (Walter et al, 1996). Discrimination between co-amplified Mb transcripts was performed utilizing Mb1- and Mb2-specific restriction sites within exon 2. Based on the polymorphism of Mb1 and Mb2 genes (Walter et al, 1996), we took advantage of the restriction enzymes EoeI and XbaI for discrimination of Mb1a and Mb2a transcripts, respectively. BanHI, which cleaves at different sites within both Mb1b targets, was implemented to discriminate between Mb1b and Mb2b messages. PCR was performed using Ampli Taq Gold Polymerase and standard PCR conditions. After 25 cycles, the amplified mixture was diluted 25-fold in fresh PCR amplification mixture containing 25 nCi μl⁻¹ (α-32P)dCTP (ICN, Eschwege, Germany) and then two additional cycles were performed. The labelled Mb PCR products, or their respective restriction fragments, were separated on 6% polyacrylamide gels. For quantification of individual Mb fragments, gels were subjected autoradiographically. Corresponding bands were excised from the gel and radioactivity was measured by means of a β-counter (LS6000TA, Beckman, München, Germany) using a Cerenkov program. In order to calculate the ratio of Mb1 and Mb2 expression levels, their respective restriction fragments were corrected for length and cytokine and guanine (GC) content, since exclusively dCTP was radioactively labelled in the assay.

**Western blot analysis**

Cells were lysed at 1 × 10⁷ cells ml⁻¹ in 20 mM Tris-HCl (pH 7.4) buffer containing 1% NP40 (Sigma), 5 mM MgCl₂, 5 μg ml⁻¹ chymostatin, 2.5 μg ml⁻¹ leupeptin, 5 μg ml⁻¹ pepstatin A, 200 μM PMSF (all protease inhibitors were from Boehringer) for 30 min at 4°C. Nuclei and insoluble debris were removed by centrifugation at 14 000 rpm for 30 min. Aliquots of 1–5 × 10⁶ cell equivalents of a cleared lysate were mixed with Laemmli buffer, boiled for 5 min, separated on 10% polyacrylamide SDS-gels, and then transferred onto Immobilon PVDF membranes (Millipore, Eschborn, Germany). Membranes were blocked overnight in blocking reagent (Boehringer). Antibody binding was detected by incubation with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Dianova) followed by enhanced chemiluminescence using Super-Signal-Ultra (Pierce, Rockford IL, USA).

**Flow cytometry**

Cells (5 × 10⁶ per sample) were washed in PBS supplemented with 1% BSA (PBS/BSA) and incubated on ice with FITC- or PE-conjugated or unlabelled primary mAb for 30 min. After washing in PBS/BSA, cells were either fixed in 1% formaldehyde for flow cytometry, or, for unlabelled primary mAb, a secondary staining reagent, FITC-goat anti-hamster IgG (FITC-GAH) or FITC-goat anti-mouse IgG (FITC-GAM) or FITC-rabbit anti-rat IgG (FITC-RAR) or FITC-goat anti-rabbit IgG (FITC-GAR) was applied for 30 min at 4°C. Background fluorescence was evaluated using either FITC- or PE-coupled irrelevant matched isotypes, or unlabelled matched isotypes and FITC-GAH, FITC-GAM, FITC-RAR or FITC-GAR. Cell surface fluorescent labelling was visualized on an EPICS®-PROFILE II flow cytometer (Coulter Immunotec Co, Hamburg, Germany) and data analysis was performed using EPICS®ELITE 3.0 software.

**Antigen presentation assay**

Presentation assays were performed by incubating A20 B cells or IFN-γ-stimulated (150 U ml⁻¹ for 48 h) P388D1 macrophages as APCs (5 × 10⁴ cells per well), T cells specific for OVA₁₂₅₋₁₃₀ presented by H2-Aa or T cells specific for HA₁₁₉₋₁₁₉ presented by H2-Eb (both at 2 × 10⁵ cells per well), and varying concentrations of antigen in flat-bottomed 96-well plates in a final volume of 200 μl per well. Supernatants were collected after 24 h and assayed for IFN-γ by ELISA as recently described (Lingnau et al, 1998). Alternatively, A20 and P388D1 cells were fixed in 1% paraformaldehyde at room temperature for 20 min and washed extensively with CM prior to assay.

**RESULTS**

**Differential expression of MHC class II and CIITA in IL-4, IL-10 or IFN-γ-stimulated tumour cell lines**

In order to examine the regulation of genes involved in the MHC class II antigen processing and presentation pathway, we defined the status of MHC class II cell surface (H-2A and H-2E) and CIITA mRNA expression in a panel of murine tumour cell lines prior to and after stimulation with IL-4, IL-10 or IFN-γ. Table 1 summarizes the mean values of fluorescence obtained for H-2A and H-2E expression correlated with specific CIITA transcripts in IL-4, IL-10 or IFN-γ-treated cells. Tumour cell lines could be divided into three groups based on MHC class II cell surface expression. The first group is defined by constitutive MHC class II and CIITA expression and includes the B cell lymphoma cell lines A20 and LB27.4, as well as the macrophage cell line P388D1. Different cytokines were found to enhance H-2A and H-2E cell-surface expression dependent on the cell type: IL-4 represents the most potent stimulus for the B-cell lymphoma cell lines and IFN-γ for P388D1 macrophages. Remarkably, stimulation of P388D1 cells with IL-10 did not affect steady state H-2A cell surface molecules as compared to P388D1 cells cultured in medium without cytokine addition. The second group exhibits no constitutive, but inducible expression of MHC class II and CIITA genes upon IFN-γ-treatment, represented by the macrophage-derived cell line APC IC-21, the melanoma cell line B16, L929 fibroblasts and the renal adenocarcinoma cell line Renca. Of note, although IFN-γ-treatment of Renca cells did not induce appreciable H-2A or H-2E surface expression, RT-PCR analysis of H₂-Aa, -Ab, -Eb and -Eb mRNA expression demonstrated the presence of specific mRNA for all components of the MHC class II antigen presentation pathway in concert with CIITA mRNA after
Defects in the MHC class II pathway in murine tumours

Table 1 Expression of cell surface MHC class II Ags and CIITA mRNA in cytokine-treated professional and nonprofessional APCs

| Cell-line   | Histology               | H2-A surface expression | H2-E surface expression | CIITA mRNA expression |
|-------------|-------------------------|-------------------------|-------------------------|-----------------------|
|             |                         | Nil IL-4 IL-10 IFN-γ    | Nil IL-4 IL-10 IFN-γ    | Nil IL-4 IL-10 IFN-γ  |
| A20         | B cell lymphoma         | 25.54 57.61 41.28 31.73 | 99.75 165.41 161.71 128.68 | + + + +               |
| LB27.4      | B cell lymphoma         | 16.31 24.18 19.11 21.45 | 26.77 45.32 39.58 42.89 | + + + +               |
| P388D1      | macrophage              | 1.34 3.78 1.36 14.42   | 3.76 11.67 2.72 51.93   | + + + +               |
| IC-21       | macrophage              | 0.59 0.74 0.56 2.62    | n.d. n.d. n.d. n.d.    | – – – –               |
| B16         | melanoma                | 0.23 0.51 0.97 8.57    | n.d. n.d. n.d. n.d.    | – – – –               |
| L929        | fibroblast              | 0.61 0.65 0.58 2.89    | n.d. n.d. n.d. n.d.    | – – – –               |
| Renca       | renal cell adenocarcinoma | 0.60 0.73 0.89 0.75   | 0.82 0.91 0.78 0.87    | – – + +               |
| P815        | mastocytoma             | 0.88 0.96 0.77 0.72    | 0.87 0.92 0.83 0.74    | – – – –               |
| TS/A        | mammary adenocarcinoma  | 0.22 0.29 0.23 0.26    | 0.47 0.51 0.55 0.49    | – – – –               |
| MC-38       | colon adenocarcinoma    | 0.45 0.42 0.57 0.59    | n.d. n.d. n.d. n.d.    | – – – –               |
| MCA-102     | fibroblast              | 0.34 0.29 0.39 0.37    | n.d. n.d. n.d. n.d.    | – – – –               |
| EL-4        | thymoma                 | 0.12 0.07 0.08 0.10    | n.d. n.d. n.d. n.d.    | – – – –               |
| RMA         | T cell lymphoma         | 0.32 0.39 0.47 0.44    | n.d. n.d. n.d. n.d.    | – – – –               |
| RMAa        | T cell lymphoma         | 0.46 0.40 0.53 0.41    | n.d. n.d. n.d. n.d.    | – – – –               |
| YAC-1       | T cell lymphoma         | 0.17 0.19 0.19 0.20    | n.d. n.d. n.d. n.d.    | – – – –               |

Cells were stained with FITC- or PE-conjugated isotype-matched irrelevant antibody (negative control), FITC-labelled mAb 39-10-8 (anti-H2-Aβ), or PE-conjugated mAb 14-4-4S (anti-H2-Ek,d) and analysed by flow cytometry. Results are expressed as mean channel fluorescence. Expression of CIITA mRNA was determined by RT-PCR as described in Material and Methods. Cells were cultured in medium (Nil) or in medium supplemented with 250 U ml⁻¹ IL-4, 100 ng ml⁻¹ IL-10 or 150 U ml⁻¹ IFN-γ for 48 h.

**Discordate vs coordinate expression of H2-M and MHC class II genes in tumour cells**

To address the question whether H2-M and MHC class II genes are coordinately regulated in IL-4, IL-10 or IFN-γ-stimulated tumour cells, we analysed Mu and Mb mRNA expression by RT-PCR. Low levels of Mu and Mb mRNA could be detected in each cell line independent of MHC class II and CIITA expression (data not shown). In view of this and based on the observation that the human DNA and DMB genes appear to be co-regulated (Westheide et al., 1997), we examined the regulation of H2-M genes by analysing Mu mRNA expression in untreated, IL-4-, IL-10- or IFN-γ-stimulated tumour cells utilizing a semiquantitative RT-PCR approach. In a first step, the amount of cDNA of each individual sample was evaluated using β-actin as a standard (Figure 1A–C), followed by determination of the Mu mRNA expression level (Figure 1D–F). For both steps, constant amounts of cDNA and serially diluted competitor fragments were co-amplified (Figure 1A and 1D). The concentration of the competitor required to achieve equal band intensities for both fragments was determined by linear regression analysis (Figure 1B and 1E) and the relative mRNA levels (molecules μg⁻¹ of total RNA) were calculated (Figure 1C and 1F, white bars). In order to determine differences in Mu mRNA expression levels, the relative amount of β-actin mRNA molecules was used to standardize the cDNA contents of each sample (Figure 1F, black bars). The results of the competitive RT-PCR analysis for each cell line are summarized in Table 2. Coordinate regulation of MHC class II and H2-M genes could be detected in the CIITA-positive B cell lines A20 and LB27.4. Like MHC class II, IL-4 followed by IFN-γ and IL-10 represented the most potent upregulator of constitutive Mu mRNA expression (Figure 1F and Table 2). Similar to its effect on MHC class II, IFN-γ induced a substantial increase of basal Mu mRNA expression in CIITA-positive tumour cells, including P388D1 and IC-21 macrophages, L929 fibroblasts, B16 (melanoma) and Renca (renal adenocarcinoma) cells. Enhanced Mu mRNA expression could also be detected in IL-4-treated P388D1 and IC21 macrophages, while IL-4 did not impact on Ma mRNA levels in IL-4-stimulated L929, B16 and Renca tumour cells, which lack CIITA and MHC class II. Stimulation of P388D1, IC21, B16, L929 and Renca cells with IL-10 did not result in significant increase or decrease of Ma mRNA expression as compared to untreated control cells. Most interestingly, neither IL-4, IL-10 nor IFN-γ was able to modulate Ma mRNA expression in CIITA- and MHC class II-negative cell lines including the mammary adenocarcinoma TS/A, the colon adenocarcinoma MC-38, the hepatic fibrosarcoma MCA-102 and the T cell-derived cell lines RMA, RMAAs, EL-4 and YAC-1.

**Mb1 and Mb2 are differentially expressed in IL-4-, IL-10- or IFN-γ-stimulated tumour cells**

We investigated whether Mb1 and Mb2 genes are differentially expressed in IL-4, IL-10 or IFN-γ-treated cells by ratio RT-PCR analysis. Discrimination between co-amplified Mb1 and Mb2 isoforms was performed by restriction enzyme analysis using specific restriction sites for each Mb type in the amplified DNA (see Materials and Methods section). In summary, a divergent expression pattern of both Mb genes could be observed (Table 3). Mb2 was predominantly expressed in the transformed B cell lines A20 and LB27.4. As shown in Figure 2A, the majority of the Mbα1 amplons from untreated, IL-4-, IL-10- or IFN-γ-stimulated A20 B cells was cleaved by the Mbα2-specific restriction endonuclease Xbal (Figure 2A, lane X). This result is confirmed by complementary digestion
with EaeI, that specifically cleaves Mb1 (Figure 2A, lane E). As an additional control, complete cleavage was obtained by a combination of XbaI and EaeI (Figure 2A, lane E and X), demonstrating that the Mb1 and Mb2 expression pattern was not due to heterodimer formation, which may occur between closely related sequences during RT-PCR (Becker Andre and Hahlbrock, 1989). In comparison, both Mb genes were found to be expressed in the P815 masto-
cytoma cell line (Table 3). However, stimulation of P815 cells with IL-10 augmented Mb2 expression, while IL-4 or IFN-γ did not exert any effect on the Mb1 or Mb2 mRNA expression pattern.
was the prominent transcript in P388D1.2.4 72.3 106) 4.1 (4.9 0.3 11.3 correlated with expression as compared to untreated, IL-4- or IL-10-stimulated and 104) 1 heterodimers exist in tumour cells of or an antiserum which recognizes both M 105) 2.7 (5.9 104) 1.4 (7.3 104) 1.2 (6.3 104) 6.5 (3.3 104) Renca 1.0 (4.3 104) 1.1 (4.6 104) 1.1 (4.7 104) 3.0 (1.3 104) P815 1.0 (8.5 104) 3.0 (2.6 104) 3.8 (3.2 104) 1.3 (1.1 104) TSA 1.0 (8.6 104) 1.0 (8.8 104) 1.4 (1.2 104) 1.2 (1.1 104) MC-38 1.0 (2.5 104) 1.0 (2.4 104) 1.4 (3.5 104) 1.0 (2.6 104) MCA-102 1.0 (1.3 104) 1.2 (1.5 104) 1.0 (1.2 104) 1.2 (1.6 104) EL-4 1.0 (3.9 104) 1.2 (4.6 104) 0.9 (3.6 104) 1.0 (4.0 104) RMA 1.0 (1.1 104) 0.9 (9.8 104) 0.9 (1.0 104) 1.1 (1.2 104) RMA 1.0 (4.3 104) 1.3 (5.5 104) 1.0 (4.4 104) 1.1 (4.8 104) YAC-1 1.0 (3.6 104) 1.3 (4.7 104) 1.1 (4.0 104) 0.9 (3.2 104) Cells were cultured in medium (Nil) or medium supplemented with 250 U ml⁻¹ IL-4, 100 ng ml⁻¹ IL-10 or 150 U ml⁻¹ IFN-γ for 48 h prior to RNA isolation and cDNA synthesis. Expression of Ma mRNA was determined by competitive RT-PCR. β-actin mRNA expression was used to normalize each individual sample. Relative Ma mRNA expression in cytokine-treated samples of each cell line is expressed as the ratio: Ma mRNA in cytokine treated cells / Ma mRNA expression (ratio = 1.0) in untreated cells. The number of Ma mRNA molecules µg⁻¹ total RNA for each sample are shown in brackets.

| Cells | Nil (µg) | IL-4 (µg) | IL-10 (µg) | IFN-γ (µg) |
|-------|---------|-----------|------------|------------|
| A20   | 4.3 ± 0.5 | 95.7 ± 0.4 | 5.9 ± 1.3 | 94.1 ± 0.9 |
| LB27.4| 16.8 ± 2.5 | 83.2 ± 4.2 | 15.9 ± 1.6 | 84.1 ± 2.0 |
| P815  | 43.6 ± 1.7 | 56.4 ± 2.4 | 39.3 ± 2.1 | 60.7 ± 2.0 |
| P388D1| 88.7 ± 0.3 | 11.3 ± 0.7 | 83.8 ± 1.3 | 16.2 ± 2.4 |
| TSA   | 64.5 ± 1.3 | 35.5 ± 1.4 | 63.6 ± 2.0 | 36.4 ± 1.5 |
| Renca | 82.7 ± 2.5 | 17.3 ± 4.2 | 84.8 ± 3.9 | 15.2 ± 4.5 |
| MC-38 | 100       | 100       | 0          | 0          |
| IC-21  | 100      | 100      | 0          | 0          |
| B16   | 100       | 100      | 0          | 0          |
| L929  | 100       | 100      | 0          | 0          |
| MCA-102 | 100   | 100      | 0          | 0          |
| EL-4  | 100       | 100      | 0          | 0          |
| RMA   | 100       | 100      | 0          | 0          |
| RMAa  | 100       | 100      | 0          | 0          |
| YAC-1 | 100       | 100      | 0          | 0          |

Expression of Mb1 and Mb2 mRNA was assayed by ratio-RT-PCR analysis. Cells were cultured in medium (Nil) or medium supplemented with 250 U ml⁻¹ IL-4, 100 ng ml⁻¹ IL-10 or 150 U ml⁻¹ IFN-γ for 48 h prior to assay. Values indicate the relative expression levels of alternative Mb isoforms in a percentage of total Mb mRNA expression. Values are means ± SD for three determinations.

In contrast, Mb1 was the prominent transcript in P388D1 macrophages and in tumour cells of epithelial origin including the renal adenocarcinoma Renca and the mammary adenocarcinoma TS/A. IFN-γ-treatment of P388D1, Renca and TSA cells enhanced Mb1 expression as compared to untreated, IL-4- or IL-10-stimulated cells. Moreover, Mb1 was found to be exclusively expressed in IC-21 macrophages, L929 fibroblasts, the hepatic fibrosarcoma MCA 102, the colon adenocarcinoma MC-38 and the B16 melanoma, as well as in the T cell-derived cell lines RMA, RMAs, EL-4 and YAC-1 (Table 3). As shown in Figure 2B, BamHI digestion of Mbα ampli- cons from IL-4-, IL-10- or IFN-γ-treated B16 and L929 cells yielded restriction fragments exclusively corresponding to Mbα transcripts, while fragments corresponding to Mbβ could not be detected as compared to the CS7/BL (H2α) spleen control. The complete cleavage of the Mbα amplification product by BamHI confirmed that heterodimer formation did not occur during RT-PCR amplification.

Mbα1 and Mbα2 heterodimers are expressed in tumour cells

Next, we addressed the question whether differential expression of Ma, Mb1 and Mb2 genes is restricted to mRNA expression, or whether Mbα1 and Mbα2 heterodimers exist in tumour cells of different histology. Western immunoblots of lyses from untreated, IL-4-, IL-10- or IFN-γ-stimulated A20 B cells, P388D1 macrophages, B16 melanoma and EL-4 thymoma cells were stained with a polyclonal rabbit serum raised against the cyto- plasmic domain of Mtx or an antiserum which recognizes both Mb isoforms in order to assess H2-M protein expression (Figure 3). As expected (see Table 2), A20, P388D1, B16 and EL-4 cells did express detectable amounts of Mtx monomers (Figure 3, left panel). Modulation of Mtx monomer expression by IL-4, IL-10 or IFN-γ correlated with Ma mRNA expression levels in cytokine-
gene expression is primarily regulated at or for 48 h before preparation of the cell lysates. Co-amplified (Peleraux et al, 1996).

2) cells were able to process and present native β2M protein expression is differentially regulated in A20 B cells, C.69.3 (anti-MHC class II genes are coordinately expressed in IL-4-, IL-10- or IFN-γ-stimulated P388D1 macrophages, B16 melanoma cells and EL-4 thymoma cells by IL-4, IL-10 or IFN-γ. The rationale of this work was twofold, first to investigate whether H2-M and MHC class II genes might be co-regulated similarly to human DMA and DMB (Peleraux et al, 1996).

stimulated A20, P388D1, B16 and EL-4 cells (Table 2), suggesting that expression of H2-M gene expression is primarily regulated at the transcriptional level. Noteworthy, untreated, IL-4-, IL-10- or IFN-γ-stimulated P388D1, B16 and EL-4 cells expressed MB1 monomers, while A20 cells expressed MB2 (Table 3 and Figure 3, right panel). Comparison of Mα and MB expression in untreated, IL-4-, IL-10- or IFN-γ-stimulated A20, P388D1, B16 and EL-4 cells revealed a similar pattern, implicating that expression of Mα and MB genes might be co-regulated similarly to human DMA and DMB (Peleraux et al, 1996).

Alternative H2-M isofoms display similar functional activities in antigen presentation to H2-Aβ- and H2-Eβ-restricted CD4+ T cells

To assess whether differential expression of H2-M isofoms impacts on the presentation of native protein antigens, we studied the ability of MBβ1-expressing P388D1 macrophages and MBβ2-expressing A20 B cells to effectively process and present surrogate target antigens, ovalbumin (OVA) and influenza haemagglutinin (HA) and the corresponding peptides to H2-Aβ- and H2-Eβ-restricted CD4+ T cells from TCR transgenic mice. To ensure that levels of H2-M and MHC class II expressed in P388D1 macrophages were comparable to those in A20 B cells (Tables 1 and 2), P388D1 cells were stimulated with IFN-γ for 48 h prior to antigen presentation. As shown in Figure 4, P388D1 (MBβ1) as well as A20 (MBβ2) cells were able to process and present native OVA and HA to H2-Aβ- and H2-Eβ-restricted CD4+ T cells, respectively. In contrast, presentation of native antigens by fixed APCs was severely impaired, indicating that the capacity of P388D1 and A20 cells to process and present the OVA323-339 and HA107-122 determinants did not result from extracellular antigen processing or peptide contamination. As expected, native and paraformaldehyde-fixed P388D1 and A20 cells present exogenously supplied OVA323-339 or HA107-122 peptides to CD4+ T cell populations. Overall, these findings implicate equivalent functional capabilities for individual H2-M isofoms in selecting the OVA323-339 or HA107-122 T cell epitopes presented by H2-Aβ and H2-Eβ, respectively.

**DISCUSSION**

H2-M is a MHC class II-like heterodimeric molecule which appears to be required for efficient antigen presentation by many MHC class II alleles (Denzin et al, 1994; Miyazaki et al, 1996; Wolf et al, 1998). The rationale of this work was twofold, first to investigate whether H2-M and MHC class II genes are coordinately regulated by cytokines in tumour cells, and second, whether
the two Mb genes of the H2-M region are differentially expressed and display similar or distinct functional activities in MHC class II antigen presentation.

The present study demonstrates that H2-M and MHC class II gene expression is coordinately regulated by IL-4, IL-10 or IFN-γ in transformed cells derived from professional APCs (i.e. macrophages or B cells) in the presence of CIITA. This is in agreement with previous data which indicated that the 5' proximal promoter region of H2-M and MHC class II genes share conserved cis-acting sequences (Peleraux et al, 1996), particularly the 'X1 box'. which binds the RFX transcription factor complex that appears to be crucial for CIITA interaction (Scholl et al, 1997).

In contrast, our analysis identified low levels of H2-M transcripts in nonprofessional APCs prior to IFN-γ-mediated induction of CIITA expression (Table 2), implicating that basal H2-M expression might occur independently of CIITA. Indeed, CIITA-deficient mice still exhibit a basal level of Mb expression in splenocytes (Chang et al, 1996), and some IFN-γ-responsive human cell lines express detectable amounts of DM transcripts in the absence of CIITA expression (Westerheide et al, 1997; Sartoris

**Figure 4** APCs expressing Muβ1 or Muβ2 can efficiently process and present OVA and HA to H2-Aβ and H2-Eα-restricted CD4+ T cells. 2 x 10⁵ Th1 differentiated CD4+ T cells (Lingnau et al, 1998) from TCR transgenic mice either specific for peptides 323–339 derived from OVA and presented by H2-Aβ (Murphy et al, 1990) or specific for peptides 111–119 from HA and presented by H2-Eα (Kirberg et al, 1994) were cultured with 5 x 10⁵ IFN-γ-stimulated P388D1 (Muβ1) or A20 cells (Muβ2) as APCs in the presence of increasing antigen concentrations or synthetic peptides. Supernatants were collected after 24 h and tested for IFN-γ secretion. APCs were fixed with 1% paraformaldehyde prior to assay.
et al, 1998). Furthermore, our data demonstrate that murine tumour cell lines which are deficient in IFN-γ-mediated CIITA expression, such as MCA-102, TS/A, or MC-38 (Tables 1 and 2), do not exhibit increased basal H2-M expression levels, nor induction of MHC class II gene expression following IFN-γ-treatment. Taken together, these observations can be reconciled with a model in which CIITA is a prerequisite in professional and nonprofessional APCs for co-regulation of genes which are instrumental for efficient MHC class II antigen presentation. However, based on data presented in this report, CIITA might be no longer viewed as the exclusive regulator for H2-M expression.

Additionally, suppression of CIITA activity, in addition to the occurrence of tumour antigen loss variants (Kerkmann-Tucek et al, 1998), may provide one potential mechanism for neoplastic cells to escape immune surveillance. Notably not only the lack of inducible CIITA expression (e.g. tumour cells summarized in Table 1), may account for insufficient MHC class II cell surface expression. Other, as yet poorly defined, factors may be responsible for the failure of tumour cells to upregulate MHC class II. For instance, IFNγ-treatment of the renal cell carcinoma Renca results in CIITA mRNA expression (Table 1), enhanced H2-Mu mRNA expressibity, but does not lead to H2-A, or H2-E cell surface expression (Table 1). H2-Ma, -Mb1 and -Mb2 are co-expressed in splenocytes of mice carrying different haplotypes (Walter et al, 1996), indicating that Mbβ1 and Mbβ2 heterodimers might be operational in antigen presentation by many MHC class II alleles/isotypes. As shown in this study, Mb1 is predominantly expressed in transformed macrophages, melanoma cells and in tumour cells of epithelial and mesenchymal origin, while Mb2 is expressed in B cells. Of note, the mastocytoma cell line P815 was found to express both Mb genes at almost equal levels (Table 3). Differential expression of members of a gene family in different cell types or at different developmental stages has been described for many eukaryotic genes (Hardison, 1998). For instance, an inverse relationship between gene activity and levels of CpG dinucleotide methylation has been observed (Hsieh, 1997; Agarwal and Rao, 1998). The 5’-flanking promoter region of both Mb genes contains conserved S, X1, X2 and Y elements (Peleriaux et al, 1996) which is required for constitutive and inducible expression (Mach et al, 1996). Since DNA methylation has been proposed to affect chromatin structure (Hsieh, 1997), methylation of CpG islands may critically influence the binding of transcription factors to cis-acting sequences and which may be able to silence the transcriptional activity of the Mb2 promoter in a cell-type-specific manner. Alternatively, but not mutually exclusive, cellular diversity of Mb1 and Mb2 expression might be controlled by cell-type-specific transcriptional activators selectively interacting with one of the Mb promoters. Alternative CIITA isoforms have been discovered, which selectively control cell-type-specific and inducible MHC class II expression (Muhllehner Mottet et al, 1997). Mb1 and Mb2 differ predominantly within exon 2, where similarity at the nucleotide level drops to 90%, as compared to 97%–100% within the other exons (Walter et al, 1996). The present study addressed the question whether H2-M isoforms differ within their functional capabilities in MHC class II antigen presentation. P388D1 and A20 cells, which selectively express Mbβ1 and Mbβ2, respectively, can process and present the OVAα25-319 and HA143-258 epitopes to H2-Aγ and H2-Eγ-restricted CD4 + T cells, implicating a similar biological activity of both H2-M isoforms in mice carrying two functional MHC class II isotypes. Consistent with these observations, recent crystallographic analysis revealed a comparable overall molecular surface architecture of both H2-M isoforms, suggesting similar functional activities in peptide loading (Fremont et al, 1998).

The apparent lack or need of certain human or murine MHC class II alleles to require DM/H2-M for CLIP removal in vivo (Denzin et al, 1994; Miyazaki et al, 1996; Wolf et al, 1998) has been attributed to the affinities of these MHC class II alleles for CLIP as determined by in vitro binding studies (Sette et al, 1995). Moreover, the apparent lack of Ii or H2-M in tumour cells in the presence of MHC class II cell surface expression may turn out to be beneficial for the host. Tumour cells transfected with syngeneic MHC class II genes without co-expressing Ii or Ii/H2M are highly immunogenic and appear to present rather endogenous as compared to exogenous antigens to tumour-specific T cells (Armstrong et al, 1997).

In summary, Th1- and Th2-associated cytokines coordinate regulation of expression of genes involved in the MHC class II antigen processing and presentation pathway in the presence of CIITA in a cell-type-specific manner. Yet, our findings established for the first time that Mbβ1 and Mbβ2 are selectively expressed in different murine tumour cells and that both isoforms can select for MHC class II/peptide assembly. Future studies may address whether differences in the MHC class II antigen presentation pathway in tumour cells impacts on disease progression and clinical outcome of vaccine strategies targeting tumour-associated antigens displayed by MHC class II molecules.

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