HLA-DO is an intracellular class II molecule with distinctive thymic expression

Daniel C. Douek and Daniel M. Altmann

Transplantation Biology Group, MRC Clinical Sciences Centre, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK

Keywords: HLA class II, HLA-DNA, HLA-DOB, thymus

Abstract

The status of the HLA-DNA and -DOB genes in the HLA class II region has remained unclear for several years. Weak mRNA transcripts from each locus can be detected in B cells and some other cell types, but the issues of whether proteins are generated and, if so, whether they acquire partner chains to form class II heterodimers have not been resolved. The products of the homologous murine genes pair with each other to form the H-2O heterodimer. We now report that the products of HLA-DNA and -DOB are expressed as a heterodimer in humans, HLA-DO. It is expressed in various class II-positive cells including dendritic cells. The heterodimer associates with invariant chain and has a relatively short half-life. Its subcellular distribution differs from HLA-DR such that it is not expressed at the cell surface although it is found in some DR-containing compartments, suggesting a role at the intracellular stage of class II function. In the thymus, subpopulations of cells in both cortex and medulla, including HLA-DR⁺ and DR⁻ cells, are HLA-DO⁺. Hassall’s corpuscles, a medullary site of thymocyte death, are ringed by HLA-DO⁺ epithelium. This unusual pattern of expression may suggest a specialized role for HLA-DO in the thymus.

Introduction

The HLA class II region encompasses genes encoding not only classical antigen-presenting molecules but also proteins involved in related functions such as TAP1, TAP2, LMP2, LMP7 and HLA-DM (1). HLA class II sequences thus encode proteins targeted to the endoplasmic reticulum, to the cytoplasm, to the endosomal pathway or to the cell surface. During the mid-1980s, efforts to isolate new HLA class II genes led to the cloning of two cDNA sequences, HLA-DNA and -DOB. Weak mRNA transcripts were detected in various cell types including B cells, fibroblasts and T cells (2–6). The two genes are distantly separated in the HLA region, straddling the TAP, LMP and DM genes. The question of whether HLA-DNA and -DOB are expressed and, if so, what are their respective partner chains has not been resolved, although there has been characterization of an in vitro translated product of the HLA-DNA gene (7).

HLA-DNA is inducible by IFN-γ, while HLA-DO was believed not to be (3), suggesting the products were unlikely to form a pair. However, subsequent work showed that HLA-DOB is inducible by IFN-γ but that the mRNA has an unusually short half-life (6). Further evidence of differential control comes from the observation that the CIITA (class II trans-activator) mutant, RJ2.2.5, expresses residual DOB mRNA despite having lost expression of all other class II transcripts (3). However, both genes have an intact X-box, Y-box, W-box, CRE motif and TATA box. Despite conservation of generic class II proximal 5’ regulatory sequences, HLA-DOB and -DNA mRNA are detected in B cell lines at about 5–10% of DR mRNA (2,3). The sequences of HLA-DP, -DO and -DR are as similar to each other as they are to the respective sequences of -DNA and -DOB. The evolutionary conservation of HLA-DNA and -DOB has been taken as evidence of a significant functional role (1). Furthermore, expression of mRNA has been detected in all of the species where it has been examined (2–11).

The products of the homologous genes in the mouse pair to form a heterodimer which has been termed H-2O (12,13). It is expressed at the cell surface of mouse peripheral B cells and in thymic medulla on a subpopulation of H-2A⁻ epithelium (14). We set out to determine whether HLA-DNA and -DOB are expressed and, if so, whether they form a heterodimer. We also examined their localization within the thymus since little is known about human thymic HLA expression and how this may affect T cell repertoire development. The transcript...
of the HLA-DNA locus was originally termed DZα; in the interest of consistency we have referred to the product of this locus as DNα; by analogy to the mouse we refer to the DNα/DQβ heterodimer as HLA-DO.

**Methods**

**Northern analysis**

Total RNA was prepared from PGF and RJ2.2.5 cell lines following denaturation of cells in RNAzol B (Biogenesis, UK). Total RNA (20 µg) was separated by electrophoresis on a denaturing agarose gel, blotted onto nylon filters and probed with an HLA-DRβ cDNA probe, or full-length DOβ and DNα cDNA probes which were supplied by Dr J. Trowsdale (Imperial Cancer Research Fund, London, UK).

**Cell and tissues**

The B lymphoblastoid cell line, PGF, HLA class II null mutant cell line, RJ2.2.5 (15), and HLA class II+ melanoma, DX3, were all maintained by passage in RPMI 1640 medium with 10% FCS (RPMI 10). RJ2.2.5 transfected with HLA-DR1 (R/ R1H) or HLA-DQw5 (Q/Q1H) were maintained by passage in RPMI 10 with 1 mg/ml Hygromycin B (Calbiochem, UK). Fresh human dendritic cells (a gift of M. Londei, Kennedy Institute, London, UK) had been cultured in granulocyte macrophage colony stimulating factor for 4 days, and were characterized by up-regulation of CD1 and HLA class II and down-regulation of CD14.

Thymuses were obtained from normal per-operative human paediatric subjects. Tissue was immediately embedded in OCT compound and snap frozen in isopentane in liquid nitrogen. Blocks were stored at −70°C until cut at 5 µm sections in a cryostat.

**Antisera against HLA-DNα and HLA-DOβ and mAb**

Rabbit antisera were raised against keyhole limpet haemocyanin (KLH)-conjugated peptides corresponding to the cytoplasmic tails of HLA-DNα (single-letter amino acid code MGTYVSSVPR) and HLA-DOβ (SRAVLLPQSC). Peptides were conjugated to KLH (Sigma, Poole, UK) with glutaraldehyde. Rabbits were immunized with peptide conjugate in adjuvant and serum was tested for antibody titre and peptide specificity by ELISA. Pre-immune and immune antisera were purified on a Protein G-Sepharose Cl-4B column (Pharmacia, St Albans, UK) and these preparations were used in all experiments except Fig. 1. The following mAb were used: TAL 14.1 (anti-DRβ), L243 (anti-DRα), TAL 1B5 (anti-DRα), L2 (anti-DQα) (all donated by J. Bodmer, Imperial Cancer Research Fund, London, UK), MR6 (anti-IL-4 receptor-associated molecule, cortical epithelium), 4B (anti-thymulin, medullary epithelium) (both donated by M. Ritter, Royal Postgraduate Medical School, London, UK), VICY1 (anti-CD74, II) (donated by L. Lightstone, Royal Postgraduate Medical School, London, UK) and FITC-conjugated anti-CD74 (II) (PharMingen, San Diego, CA). Second layer reagents were horseradish peroxidase (HRP)-conjugated mouse-adsorbed F(ab')₂-anti-rabbit Ig, HRP-conjugated rabbit-adsorbed F(ab')₂-anti-rabbit Ig, FITC-conjugated F(ab')₂-anti-rabbit Ig, lissamine rhodamine (LRSC)-conjugated mouse-adsorbed rabbit-adsorbed F(ab')₂-anti-mouse Ig (Jackson ImmunoResearch, West Grove, PA), streptavidin-Texas Red (Vector, UK) and streptavidin-Cy5 (Cambridge BioScience, Cambridge, UK).

**Biosynthetic labelling**

Cells (10⁶) were washed in methionine- and cysteine-free RPMI (MFR) (Gibco, Paisley, UK), resuspended in MFR supplemented with 10% dialyzed FCS (MFR 10) at 10⁷/ml, and incubated at 37°C for 2 h. Cells were spun and resuspended in MFR 10 at 4 × 10⁶/ml and were pulse-labelled with 2 mCi [³⁵S]methionine and cysteine (Pro-Mix; Amersham, Amersham, UK)/ml for the times specified in the figure legends. Aliquots were then either immediately pelleted on ice or washed, resuspended in RPMI 10 at 10⁶ cells/ml and chased for defined periods.

**Immunoprecipitation**

Cells were lysed in lysis buffer [50 mM Tris, pH 7.4 150 mM NaCl, 1% NP-40, 10 µg/ml mix of peptatin, leupeptin, aprotinin and chemostatin, 1 µg/ml phenylmethylsulphonyl fluoride (Sigma)] and the nuclear material was pelleted. The solubilized membrane fractions were pre-cleared twice for 2 h with 100 µl of Protein A-Sepharose (Pharmacia). While the lysates were pre-clearing 10 µl of purified mAb or 25 µl of purified antisera was added to 25 µl of Protein A-Sepharose and mixed at 4°C for 4 h in 0.5 ml of lysis buffer. To inhibit immunoprecipitation, specific or irrelevant peptide was added at 10 mg/ml to the relevant anti-peptide antisera and added to 25 µl of Protein A-Sepharose and mixed at 4°C overnight in 0.5 ml of lysis buffer. If Western blotting was to be performed, antibody covalently coupled to Protein A-Sepharose with dimethylpimelimidate (Sigma) was used for the immunoprecipitation (see above). Lysates were added to the washed Protein A-Sepharose–antibody and mixed at 4°C for 2 h. Then, 25 µl of sample buffer with or without 5% 1 M dithiothreitol, as indicated in the figure legends, was added to the washed immunoprecipitates and the samples were heated to 95°C for 5 min before loading on to 12% SDS-PAGE gels. Gels were dried and exposed to BioMax MR film (Kodak, Cambridge, UK) or blotted onto membranes.

**Cell surface protein biotinylation and immunoprecipitation**

After biosynthetic labelling, intact cells were incubated with 2.5 mg N-hydroxysuccinimide-SS-biotin (Pierce and Warriner, Chester, UK) per 4 × 10⁶ cells in PBS for 5 min at 4°C. Cells were washed twice in PBS and the reaction was quenched with 50 mM NH₄Cl for 10 min on ice. Cells were washed once in PBS and lysed as above. Proteins were immunoprecipitated with antibody–Protein A–Sepharose, washed as above and boiled for 5 min in 100 µl 2% SDS in PBS. A 25 µl aliquot of supernatant was taken from each sample and added to 25 µl of sample buffer (representing total labelled protein). The remaining supernatant was resuspended in 500 µl of wash buffer and incubated with streptavidin–Sepharose beads (Pierce and Warriner) overnight at 4°C to precipitate biotinylated cell surface molecules. Washed beads were resuspended in sample buffer with 5% 1 M dithiothreitol and loaded onto the gels as above.
Expression of HLA-DO

Fig. 1. HLA-DO expression in the B-lymphoblastoid cell line, PGF, and class II null mutant, RJ2.2.5. (A) HLA-DNA and -DOB mRNA was analysed in PGF and RJ2.2.5. PGF and RJ2.2.5 RNA was loaded in alternate tracks starting with PGF in track 1. Northern filters were hybridized to probes for HLA-DOβ (tracks 1 and 2), DRβ (tracks 3 and 4) and Dνα (tracks 5 and 6). The lower panel shows re-hybridization with the 7B6 cell-cycle-independent probe as a loading control. HLA-DR and -DO protein expression was examined in cytopsins of PGF (B, D and F) and RJ2.2.5 (C, E and G). Both the anti-DRα mAb TAL 1B5 (B) and the DOβ antiserum (D) stain PGF. Neither TAL 1B5 (C) nor DOβ antiserum (E) stains RJ2.2.5. Pre-immune serum stained neither PGF (F) nor RJ2.2.5 (G).
Expression of HLA-DO

Cell surface iodination

Cells (5 × 10^6) were washed and resuspended in 0.5 ml PBS. Then, 50 µl lactoperoxidase (Sigma; diluted 1:10 in PBS) and 0.5 mCi Na^2^125I (Amersham) were added to the cells and gently mixed on ice. Then, 5 µl of 0.003% (1 mM) hydrogen peroxide (1:10,000 dilution in PBS of 30% stock) was added and the mixture gently swirled on ice. At 1 min intervals a further 5 µl H_2O_2 was added four times. Cells were washed three times thoroughly in PBS and lysed, and proteins were immunoprecipitated as above.

Western blotting

PGF cells (5 × 10^6) were lysed, immunoprecipitated and run on 12% SDS–PAGE gels as described above. Gels were electroblotted onto Immobilon P membranes (Millipore, Bedford, MA) which were probed with the antibodies indicated in the figure legends. Membranes were incubated with the appropriate HRP-conjugated second layer antibody (Jackson ImmunoResearch) and visualized using enhanced chemiluminescence (Amersham).

Cell staining

PGF or RJ2.2.5 cells were spun onto glass slides and air-dried. They were fixed and permeabilized for 10 min in ice-cold acetone and incubated for 1 h with TAL 1B5 or unpurified anti-DNα or anti-DOβ antisera at a dilution of 1:50 in Tris-buffered saline. Slides were washed in Tris-buffered saline and incubated with the appropriate HRP-conjugated second layer antibody and developed with diaminobenzidine (Fast DAB; Sigma).

Confocal fluorescence immunocytochemistry

DX3 cells were grown on glass slides, fixed and permeabilized in ice-cold methanol, and incubated for 1 h with FITC-conjugated anti-CD74, anti-DNα and biotinylated L243 in TBS. Slides were washed in TBS and incubated with LRSC-conjugated mouse-adsorbed F(ab')_2-anti-rabbit Ig and streptavidin–Cy5 for 30 min in TBS containing 1% mouse serum. Stained cells were imaged by confocal microscopy (Leica, Heidelberg, Germany). DC were dried onto glass slides, fixed in methanol, and incubated with anti-DNα and biotinylated L243. Second layers were FITC-conjugated mouse-adsorbed F(ab')_2-anti-rabbit Ig and streptavidin–Texas Red. Slides were visualized as above.

Immunohistochemistry of thymic sections

Cryosections (5 µm) were fixed in acetone and incubated for 1 h with DNα or DOβ antisera, washed in TBS, incubated with FITC-conjugated mouse-adsorbed F(ab')_2-anti-rabbit Ig and then incubated with either biotinylated L243, un conjugated MR6 or 4β containing 1% mouse serum. Stained sections were then incubated with either streptavidin–Texas Red or LRSC-conjugated rabbit-adsorbed F(ab')_2-anti-mouse Ig accordingly and visualized by indirect fluorescence microscopy.

Results

HLA-DO antisera stain human B cells

Antisera were raised to peptides representing the predicted cytoplasmic tail sequences of HLA-DNA and -DOB. These were initially characterized by comparative staining of a B-lymphoblastoid cell line, PGF, and the class II null mutant, RJ2.2.5. It has previously been shown that HLA-DOβ transcripts are made by RJ2.2.5 cells (3); we found that whereas PGF produces weak mRNA transcripts for both HLA-DNα and -DOβ, RJ2.2.5 produces no DNα transcript and little DOβ mRNA (Fig. 1A). Despite the marginal amount of DOβ transcript, staining of stable β chain would not be predicted in the absence of a partner α chain. Antiserum to DOβ stains PGF but not RJ2.2.5 (Fig. 1D and E). Staining of PGF cells could be inhibited by pre-incubation of the antiserum with specific peptide (data not shown). The antiserum does not stain RJ2.2.5 transfected cells expressing a high level of HLA-DR or -DQ (17) (data not shown).

HLA-DO antisera precipitate a heterodimer

PGF cells were biosynthetically labelled with [35S]methionine (1 h pulse) and lysates immunoprecipitated with Protein G-affinity purified anti-DNα, anti-DOβ or the mAb TAL 14.1 (anti-DRβ) before separation on SDS–PAGE (Fig. 2a). TAL 14.1 precipitated the expected HLA-DRα chain and β chains from PGF B cells (lane 4), while the pre-immune rabbit serum precipitates no similar sized product (lane 1). Pre-bled sera from rabbits prior to immunization with DNα or DOβ cytoplasmic tail peptide had, like hyperimmune anti-DNα and -DOβ antisera, been subjected to Protein G-affinity purification. Both DNα (lanes 2 and 5) and DOβ (lanes 3 and 8) antisera precipitated species of mol. wt of ~33–35 and 30 kDa. The α chain bands comprise species at different stages of glycosylation, due to the long pulse time of 1 h, and are therefore less discrete than the β chains which only possess a single glycosylation site. Five-fold less TAL 14.1 immunoprecipitate was loaded on to the SDS–PAGE gel than anti-DNα or anti-DOβ immunoprecipitate to prevent relative over-exposure in the HLA-DR track.

To control for specificity of the antisera, anti-DNα and anti DOβ were pre-incubated overnight with 10 mg/ml of specific cytoplasmic tail peptide (anti-DNα with DNα peptide and anti-DOβ with DOβ peptide). As a negative control samples were also pre-incubated with the peptide sequence from the other chain. Precipitation of the class II heterodimer by DNα antisem is abolished by preincubation with DNα peptide (lane 6) but not DOβ peptide (lane 7). Similarly, HLA class II precipitation by anti-DOβ is abrogated by DOβ peptide (lane 9) but not by DNα peptide (lane 10). Precipitation of the HLA DO α and β chain products was thus specific, depending on the specific sequences in the cytoplasmic tails of the two molecules.

However, since it was possible that immunoprecipitation resulted from cross-reactive recognition of the cytoplasmic tails of classical class II molecules expressed by PGF, the antisera were tested for the ability to precipitate HLA class II heterodimer from RJ2.2.5 null mutants transfected to express only HLA-DR or HLA-DQ (17). Figure 2(b) shows HLA class II immunoprecipitation from the HLA-DR transfectant, R/R1H, and the HLA-DQ transfectant Q/Q1H. The HLA-DR mAb TAL 1BS immunoprecipitates HLA-DR heterodimer from R/R1H (lane 3), no class II product being seen lanes precipitated by anti-DNα (lane 1), anti-DOβ (lane 2) or anti-DQα (lane 4). HLA-DQ is immunoprecipitated from Q/Q1H only by the DQ
were probed with anti-DNα immune rabbit serum (control), TAL 1B5 or L2 as indicated at the top of the panel and non-reducing SDS–PAGE was performed. Western blots covalently conjugated to Protein A–Sepharose beads. Proteins Figure 3(a) shows [35S]methionine biosynthetic labelling of α and β) or L2 (anti-DQα, lanes 4 and 8). (c) Western blots of PGF lysates. PGF lysate was immunoprecipitated with anti-DNα, anti-DQß, pre-immune rabbit serum (control). TAL 1B5 or L2 as indicated at the top of the panel and non-reducing SDS–PAGE was performed. Western blots were probed with anti-DNα, anti-DQß, a mixture of TAL 1B5 and TAL 14.1 (anti-DRß), a mixture of L2 and Leu 10 (anti-DQß) or pre-immune rabbit serum (control) as indicated at the side of the panel. Class II α chains and β chains and mol wt are indicated by arrows.

Sequential immunoprecipitation and Western blotting of PGF lysate was conducted to confirm the products of HLA-DNα and -DOB pair to form a heterodimer, and that they do not associate with HLA-DR or -DQ chains (Fig. 2c). PGF lysate was immunoprecipitated with pre-immune serum, anti-DNα, anti-DQß, TAL 14.1 (anti-DRß) or L2 (anti-DQα), all covalently conjugated to Protein A-Sepharose beads. Proteins were separated by non-reducing SDS–PAGE and Western blotted. Five-fold less TAL 14.1 and L2 immunoprecipitate was loaded on to the SDS–PAGE gel than anti-DNα or anti-DQß immunoprecipitate. The membranes were probed with pre-immune serum, anti-DNα, anti-DQß, a mixture of TAL 14.1 (anti-DRß) and TAL 1B5 (anti-DRα) or a mixture of L2 (anti-DQα) and Leu 10 (anti-DQß). Immunoprecipitation with either anti-DNα or anti-DQß produced a band at ~33–35kDa when probed with the anti-DNα antisera. Immunoprecipitation with either anti-DNα or anti-DQß produced a band at ~30 kDa when probed with the anti-DQß antisera. Protein immunoprecipitated with either anti-DNα or anti-DQß produced no bands when probed with either a mixture of TAL 14.1 and TAL 1B5, a mixture of L2 and Leu 10 or pre-immune serum. Protein immunoprecipitated with TAL 14.1 or L2 was recognized by neither anti-DNα, anti-DQß nor pre-immune serum but showed α and β bands when probed with a mixture of TAL 14.1 and TAL 1B5 or a mixture of L2 and Leu 10 respectively. Protein immunoprecipitated with pre-immune serum gave no bands on probing with any antibody. These data confirm that the proteins precipitated by anti-DNα and anti-DQß pair to form a heterodimer with an apparent mol. wt of ~33 and 30 kDa respectively, and that they do not associate with the α or β chains of HLA-DR or -DQ.

HLA-DO has a short half-life and associates with invariant chain

Expression of HLA-DO

mAb (lane 8) and not by the anti-DO or anti-DR antibodies (lanes 5–7). Therefore the anti-DO antiserum precipitate a heterodimer which is not present in the class II null mutant RJJ2.2.5 and which is neither HLA-DR nor -DQ.

$\text{Expression of HLA-DO}$
anti-li shows specific li products of 33 and 35 kDa (lane 7) as well as a non-specific Ig light chain signal picked up by the second layer and also detected in the mouse Ig and pre-immune serum tracks (lanes 2 and 3). The anti-li mAb detects the same 33 and 35 kDa products in lysate precipitated by anti-DOβ (lane 4). Lane 8 shows the converse experiment in which lysate immunoprecipitated by anti-li was probed with anti-DNα confirming the association between li and HLA-DO.

The observation that HLA-DO has a short half-life and does not mature into an li-free form suggested that it may not reach the cell surface. This was investigated by immunoprecipitation from cell surface biotinylated, biosynthetically labelled PGF cells (Fig. 3c). At a chase time of 2 h biotinylated HLA-DR could be immunoprecipitated from the cell surface (lane 3) but no precipitate could be detected with anti-DNα or -DOβ (lanes 1 and 2). HLA-DO should be detectable under these conditions if present at the cell surface since the HLA-DO antisera show precipitation of DOα and β from total cell lysate (lanes 4 and 5) as does TAL 14.1 (anti-DRβ) of DR (lane 6). Since the amount of expressed HLA-DO protein is low compared to HLA-DR it is impossible to rule out that the sensitivity of the assay is too low to detect very small amounts of surface protein. However, the results do indicate that little if any HLA-DO is expressed at the cell surface. The possible lack of surface expression was confirmed by [125I]lactoperoxidase cell surface labelling (Fig. 3d).

Subcellular distribution of HLA-DO

Subcellular localization of HLA-DO was examined in the class II-expressing human melanoma cell line, DX3, since it is a large and adherent cell type allowing good morphological resolution. Confocal microscopy of these cells confirms that HLA-DO is intracellular and not at the cell surface (Fig 4a).

No signal was obtained when antisera were used to stain class II null cells or when pre-immune sera were used to stain DX3 cells (data not shown). In DX3 cells, HLA-DO is present in the endoplasmic reticulum and Golgi, and co-localizes with DR and li, although its distribution extends neither to the more mature HLA-DR-containing vesicles (presumably MIIC) nor to the cell surface. Staining for HLA-DR can clearly be seen to light up the cell membrane. Without additional compartment-specific markers it is not possible to define precisely the extent of HLA-DO egress beyond the Golgi. In support of restriction to very early stages of the class II pathway is the observation that HLA-DO molecules, while clearly glycosylated, are too short-lived for acquisition of endoglycosidase-H resistance to be detectable (data not shown). Staining of dendritic cells showed that HLA-DO is expressed with a similar distribution pattern in professional antigen presenting cells (Fig. 4b).

Thymic distribution of HLA-DO

An unusual aspect of the murine homologue H-2O is its restricted distribution: it is expressed on B cells and on a H-2A+ subset of thymic medullary cells but is absent from thymic cortical epithelium (12,14). This has led to speculation about a possible novel role in immune function. The distribution of the human homologue is different. In human thymus DO is expressed by a minority of cells in both cortex and medulla (Fig. 5): the DOβ antiserum co-localizes with subpopulations of MR6+ cortical epithelium (18) and 4B4+ medullary epithelium (19). DOβ and DNα antisera display identical patterns of staining. Co-localization with the anti-DNα mAb L243 reveals that the medullary DO+ cells include both DR+ and DR− subsets. The keratinized whorls of epithelium which constitute Hassall’s corpuscles are negative for DR, but their boundaries are demarcated by a ring of strongly DO+ cells. Transcriptional regulation of class II genes in Hassall’s corpuscles is unusual in other regards since it is the only tissue so far described in

![Image](44x482 to 288x735)

Fig. 3. (a) Pulse–chase and SDS–PAGE of PGF cells. Cells were biosynthetically labelled for 30 min and chased as indicated (0–8 h). Lysates were immunoprecipitated with TAL 14.1 (anti-DRβ) or anti-DNα. (b) Western blot of PGF lysates. PGF cells were lysed and immunoprecipitated with mouse IgG (lane 2), pre-immune rabbit serum (lane 3), anti-DOβ (lane 4), Protein A–Sepharose beads alone (lane 5), TAL 1B5 (lane 6) and VICY1 (anti-li, lane 7); lane 1 contains mol. wt markers. Reducing SDS–PAGE was performed and Western blots were probed with VICY1 (lanes 1–7). The top and bottom panels are different exposure times of the same blot. Two bands corresponding to the p33 and p35 forms of li (indicated with asterisks) are present in the anti-DOβ, TAL 1B5 and VICY1 lanes. The non-specific band seen <30 kDa is also present in the mouse IgG and pre-immune serum lanes, and is likely to be Ig light chain. Lane 8 shows the converse experiment: lysate was immunoprecipitated with anti-li and probed with anti-DNα. (c) Immunoprecipitates of cell surface biotinylated proteins (lanes 1–3) or of total cell lysate (lanes 4–6) after biosynthetic labelling for 30 min and a 2 h chase. PGF lysates were precipitated with anti-DOβ (lanes 1 and 4), anti-DNα (lanes 2 and 5) or TAL 14.1 (lanes 3 and 6). There is little or no DOβ or DNα at the cell surface (lanes 1 and 2) in contrast to abundant DR (lane 3). Class II α chains and β chains, li, and mol. wt are indicated by arrows. (d) Immunoprecipitates of 125I-labelled cell surface proteins. PGF cells were surface labelled with 125I using lactoperoxidase and lysates precipitated with TAL 14.1 (anti-DRβ, lane 1), anti-DOβ (lane 2), anti-DNα (lane 3) or pre-immune serum (lane 4). There is little or no DOβ or DNα at the cell surface (lanes 2 and 3) in contrast to abundant DR (lane 1). Class II α chains and β chains and mol wt are indicated by arrows.
Fig. 4. Confocal scanning laser microscopy of DX3 human melanoma cells (a) and granulocyte macrophage colony stimulating factor cultured human dendritic cells (b). DX3 cells were triple stained with anti-II mAb in green, DNα antiserum in red and L243 in blue. Dendritic cells were double stained with DNα antiserum in green and L243 in red. Composite images are at the bottom of each panel to show co-localization.
which HLA-DQ can be expressed in the absence of DR (D. C. Douek et al., unpublished observations).

**Discussion**

The HLA-DO heterodimer is closely related to the classical class II molecules, with the same potential glycosylation sites and the same potential for disulphide bonding of cysteine residues (2,3,20). The relative conservation of DO sequences and genomic organisation in the species so far analysed in detail (rabbit, rat, sheep, marsupial, mouse and human) has been regarded as evidence of a conserved functional role (1,8–13). Certainly, HLA-DO shows more extensive conservation than, for example, HLA-DP. Neither DNA nor DOB show extensive polymorphism: DNA has 10 conservative nucleotide substitutions, only one non-conservative polymorphism in the transmembrane region by sequence analysis and two alleles by restriction fragment length polymorphism (RFLP) analysis (21–24). As yet only two polymorphisms have been identified in DOB by RFLP (20) and one amino acid substitution in the signal sequence (21). This limited degree of polymorphism is reminiscent of other intracellular HLA class II products such as HLA-DM and the TAP proteins.

The promoter sequence of HLA-DOB differs slightly in sequence and organization from that of classical class II molecules (20), and it was initially suggested that DOB was not inducible by IFN-γ (3), although this was later found not to be the case for all cell types examined (6). These observations of differential regulation of HLA-DOB and -DNA were thought to rule out the possibility that their products could form an expressed heterodimer (3); however, the murine homologue H-2O is expressed as a heterodimer and yet H-2Ob has an upstream promoter region remarkably similar to that of HLA-DOB (21,25) and is not induced by IFN-γ in macrophages. The thymus appears to be an important site of HLA-DO expression and it may prove that other cytokines other than IFN-γ are of importance for the thymic regulation of HLA class II expression.

In the present study a number of differences are seen from previous analysis of H-2O in the mouse (12,14). Whereas H-2O was detectable at the cell surface, HLA-DO is intracellular and the human heterodimer is present in thymic cortex whilst the mouse homologue was not. Furthermore, the expression of either H-2A or H-2O on murine thymic medullary epithelial cells was mutually exclusive, while in human thymus, dual positive cells are observed. Furthermore, the medullary epithelium demarcating the edges of Hassall’s corpuscles are strongly DO-+. The failure to detect strong staining around Hassall’s corpuscles in the mouse is not surprising since these bodies are small and difficult to detect in murine thymus. Without having conducted detailed studies of H-2O it is difficult to ascertain which of the discrepancies are species differences and which simply attributable to differences in sensitivity of detection.

The observation that the HLA-DO heterodimer is not found at the cell surface argues that it has a function other than conventional presentation of exogenous peptides to the TCR. What could be the function of a class II heterodimer which is very highly conserved, shows limited polymorphism, is restricted to the earlier part of the class II biosynthetic pathway and associates with invariant chain? Any model must also account for the fact that its tissue distribution, while not as limited as originally thought from the murine studies, does not extend to all class II antigen-presenting cell types. Like HLA-DM, it is likely to function in an earlier, intracellular stage of peptide processing or loading. Clues to the function of HLA-DO may be gained by comparison of sequence motifs in the β chain of class II heterodimers. One of the key sequences for the class II antigen-presenting molecules, HLA-DR, -DQ and -DP, is the CD4 binding site which comprises residues 134–138 of the β2 domain (26). This sequence in DOB contains five amino acid substitutions with respect to DRβ making functional interaction with CD4 unlikely, as would be predicted from the intracellular localization of HLA-DO. Residues 80–83 of the DRβ chain have been identified by Chervonsky and co-workers as an endocytic compartment targeting sequence (27), although in classical antigen-presenting cells this targeting function is fulfilled by li (for review see 28). In DOB this sequence is identical to that of DRβ (in contrast to the divergent sequence in DMβ) and this may indicate a role for HLA-DO in the binding of exogenous processed peptides or invariant chain peptides. HLA-DMβ contains a targeting sequence in its cytoplasmic tail, YTPL, which targets the molecule to the MHc class II compartments of the lysosomal pathway (29,30). This sequence conforms to the consensus tyrosine-based targeting motif (single-letter amino acid code), YXXZ, where Z is preferably an hydrophobic residue. Many proteins which are targeted directly from the trans-Golgi network or by rapid internalization from the plasma membrane to endosomes and lysosomes use this motif or another motif based on the dipeptide LZ (for review see 31). Furthermore, it has been reported that targeting of the LgpA protein to lysosomes is dependant on the motif GYRTV present in its cytoplasmic tail and that the proximity of the glycine residue to the plasma membrane enhances this targeting (32). The cytoplasmic tail of HLA-DOβ is unusual in that it is considerably longer than those of the other class II molecules. Its full sequence is GYVRTQMSGNEVSRAVL of HLA class II expression.

In the present study a number of differences are seen from previous analysis of H-2O in the mouse (12,14). Whereas H-2O was detectable at the cell surface, HLA-DO is intracellular and the human heterodimer is present in thymic cortex whilst the mouse homologue was not. Furthermore, the expression of either H-2A or H-2O on murine thymic medullary epithelial cells was mutually exclusive, while in human thymus, dual positive cells are observed. Furthermore, the medullary epithelium demarcating the edges of Hassall’s corpuscles are strongly DO-+. The failure to detect strong staining around Hassall’s corpuscles in the mouse is not surprising since these bodies are small and difficult to detect in murine thymus. Without having conducted detailed studies of H-2O it is difficult to ascertain which of the discrepancies are species differences and which simply attributable to differences in sensitivity of detection.

The observation that the HLA-DO heterodimer is not found at the cell surface argues that it has a function other than conventional presentation of exogenous peptides to the TCR. What could be the function of a class II heterodimer which is very highly conserved, shows limited polymorphism, is restricted to the earlier part of the class II biosynthetic pathway and associates with invariant chain? Any model must also account for the fact that its tissue distribution, while not as limited as originally thought from the murine studies, does not extend to all class II antigen-presenting cell types. Like HLA-DM, it is likely to function in an earlier, intracellular stage of peptide processing or loading. Clues to the function of HLA-DO may be gained by comparison of sequence motifs in the β chain of class II heterodimers. One of the key sequences for the class II antigen-presenting molecules, HLA-DR, -DQ and -DP, is the CD4 binding site which comprises residues 134–138 of the β2 domain (26). This sequence in DOβ contains five amino acid substitutions with respect to DRβ making functional interaction with CD4 unlikely, as would be predicted from the intracellular localization of HLA-DO. Residues 80–83 of the DRβ chain have been identified by Chervonsky and co-workers as an endocytic compartment targeting sequence (27), although in classical antigen-presenting cells this targeting function is fulfilled by li (for review see 28). In DOβ this sequence is identical to that of DRβ (in contrast to the divergent sequence in DMβ) and this may indicate a role for HLA-DO in the binding of exogenous processed peptides or invariant chain peptides. HLA-DMβ contains a targeting sequence in its cytoplasmic tail, YTPL, which targets the molecule to the MHc class II compartments of the lysosomal pathway (29,30). This sequence conforms to the consensus tyrosine-based targeting motif (single-letter amino acid code), YXXZ, where Z is preferably an hydrophobic residue. Many proteins which are targeted directly from the trans-Golgi network or by rapid internalization from the plasma membrane to endosomes and lysosomes use this motif or another motif based on the dipeptide LZ (for review see 31). Furthermore, it has been reported that targeting of the LgpA protein to lysosomes is dependant on the motif GYRTV present in its cytoplasmic tail and that the proximity of the glycine residue to the plasma membrane enhances this targeting (32). The cytoplasmic tail of HLA-DOβ is unusual in that it is considerably longer than those of the other class II molecules. Its full sequence is GYVRTQMSGNEVSRAVL of HLA class II expression.
Fig. 5. Indirect immunofluorescence co-localization of HLA-DO with thymic epithelium and HLA-DR. Cryosections of human thymus were double-stained with DOβ antiserum (a, g and j) or DNα antiserum (d) in green and either L243 (anti-DRα) (b and e), MR6 (anti-cortical epithelium) (h) or 4β (anti-medullary epithelium) (k) in red. Each row shows three photomicrographs of the same section with the right-hand column (c, f, i and l) a double exposure to show co-localization of the two antibodies in yellow. Panels (a–c) show thymic medulla stained for DOβ and DRα; (d–f) show a similar pattern of staining and co-localization is seen for DNα and DRα on a contiguous section; (g–i) show staining with DOβ antiserum and mAb MR6. A high-powered view of DO− 4β− medullary epithelial cells is shown in panels (j–l).
Expression of HLA-DO
cells and dendritic cells although expression on thymic cortical epithelium is limited when compared to the high expression of HLA-DR and -DQ. In terms of functional correlates, it is noteworthy that there is strong expression of HLA-DO around Hassall’s corpuscles in the thymus since these are the only site of thymocyte apoptosis in human thymic medulla (34, D. C. Douek et al., in preparation).

Acknowledgements
D. C. D is supported by the Wellcome Trust and Muirhead Trust. The authors would like to thank Professor E. Simpson and Dr J. Elliott for helpful comments, and Professor M. Ritter, Dr J. Bodmer and Dr L. Lightstone for advice and reagents.

Abbreviations
HRP horseradish peroxidase
KLH keyhole limpet haemocyanin
LRSC lissamine rhodamine
RFLP restriction fragment length polymorphism

References
11 Accolla, R. S. 1983. Human B cell variants immunoslected against Hassall’s corpuscles in the thymus since these are the only site of thymocyte apoptosis in human thymic medulla (34, D. C. Douek et al., in preparation).
14 Accolla, R. S. 1983. Human B cell variants immunoslected against a single la antigen subset have lost expression of several la antigen subsets. J. Exp. Med. 157:1053.