Tetraspan microdomains distinct from lipid rafts enrich select peptide–MHC class II complexes

H. Kropshofer[1,2], S. Spindeldreher[1], T. A. Röhn[1], N. Platania[1], C. Grygar[1], N. Daniel[1], A. Wölpl[3], H. Langen[4], V. Horejsi[5] and A. B. Vogt[1,2]

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Complexes of peptide and major histocompatibility complex (MHC) class II are expressed on the surface of antigen-presenting cells but their molecular organization is unknown. Here we show that subsets of MHC class II molecules localize to membrane microdomains together with tetraspan proteins, the peptide editor HLA-DM and the costimulator CD86. Tetraspan microdomains differ from other membrane areas such as lipid rafts, as they enrich MHC class II molecules carrying a selected set of peptide antigens. Antigen-presenting cells deficient in tetraspan microdomains have a reduced capacity to activate CD4$^+$ T cells. Thus, the organization of uniformly loaded peptide–MHC class II complexes in tetraspan domains may be a very early event that determines both the composition of the immunological synapse and the quality of the subsequent T helper cell response.

The density of major histocompatibility complex (MHC) class II on the surface of antigen-presenting cells (APCs) is a crucial parameter in activating T helper (T_H) lymphocytes. The local density of MHC class II loaded with cognate peptide determines the avidity of APC–T_H cell interactions, which are thought to occur through formation of an intercellular junction called the immunological synapse[1]. This synapse has been described as a cluster of T cell receptors (TCRs) surrounded by adhesion molecules on the T_H cell that engage a complementary cluster of MHC class II, adhesion and costimulatory molecules on the APC[2,3].

Until recently, clustering of appropriate peptide–MHC class II complexes was thought to be induced by supramolecular assemblies of monospecific TCRs, and thus driven by T cell interactions[1–3]. But evidence now suggests that professional APCs can regulate the lateral distribution of their surface MHC class II molecules before contact with T_H cells and formation of the immunological synapse. The dimerization of MHC class II molecules has been visualized[4] and shown to have a role in T cell responses, especially in the context of low-affinity antigens[4–7]. In addition, a subset of di- or oligomerized human MHC class II molecules—HLA-DR (DR) and HLA-DP (DP)—localizes to particular membrane micodomains[8–10]. These microdomains are composed of transmembrane proteins such as CD82, CD81 and CD9, which are members of the tetraspan superfamily[11–14]. Clusters of MHC class II molecules located in these tetraspan microdomains are characterized by the CDw78 determinant, which is a marker of activation on B cells[8].

An alternative mode of clustering MHC class II on B cells has been described in which most surface MHC class II molecules segregate constitutively into detergent-resistant cholesterol- and glycosphingolipid-enriched plasma membrane microdomains[15] called lipid rafts[16–17]. Proteins present in lipid rafts are prevented from free lateral diffusion[18]. As MHC class II molecules are expected to be concentrated in these microdomains, lipid rafts may facilitate antigen presentation at low doses of antigen[15]. But other APCs lack constitutive expression of MHC class II molecules in lipid rafts[19,20], raising the possibility that APCs may contain structures distinct from rafts that can organize peptide–MHC class II complexes locally[21].

To increase our understanding of the organization of MHC class II molecules in the plasma membrane, we have examined microdomains populated by tetraspanins. In dendritic cells (DCs) and B cells, we found considerable amounts of MHC class II in tetraspan microdomains, but very low amounts in membrane lipid rafts. Whereas the few MHC molecules found in lipid rafts displayed a complex set of peptides representative of the whole repertoire, those in tetraspan microdomains were enriched for specific peptide–MHC class II complexes, which colocalized with the MHC class II editor HLA-DM (DM) and the costimulatory molecule CD86.

Results

CDw78 enriches specific peptide–MHC class II

We used the monoclonal antibody (mAb) FN1, which reacts specifically with CDw78 microdomains, to investigate the peptide repertoire of MHC class II–tetraspan clusters. Flow-cytometric staining of the Epstein-Barr virus (EBV)-transformed B cell line WT-100 with FN1 showed that there were large amounts of surface CDw78 molecules (**Fig. 1a**), reminiscent of other activated B cells[8–10]. We used FN1 to

[1]*Basel Institute for Immunology, 4005 Basel, Switzerland.* [2]*Roche Center for Medical Genomics, 4070 Basel, Switzerland.* [3]*German Red Cross, Blood Transfusion Service, 89081 Ulm, Germany.* [4]*F. Hoffmann-La Roche Ltd., Pharmaceutical Research, 4070 Basel, Switzerland.* [5]*Institute of Molecular Genetics, AS CR, 14220 Prague, Czech Republic. Correspondence should be addressed to H. K. (harald.kropshofer@roche.com).*

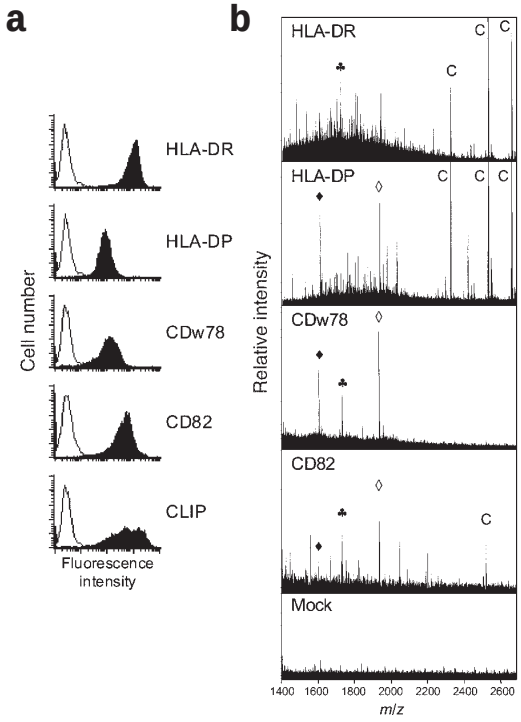

Figure 1. Specific peptide–MHC class II complexes accumulate in CDw78 microdomains. (**a**) Flow-cytometric analysis of the B cell line WT-100 with mAbs to DR (L243), DP (B7/21), CDw78 (FN1), the tetraspanin CD82 (50F11) and CLIP–MHC class II complexes (Cer.CLIP). (**b**) MALDI-MS analysis of self-peptides derived from DR, DP, CDw78 clusters and CD82–MHC class II complexes of WT-100 B cells. MHC class II molecules were precipitated in 1% *n*-octylglucoside with sepharose beads conjugated to mAb L243 (total DR-peptide pool), mAb B7/21 (total DP-peptide pool), mAb FN1 (CDw78 clusters), C33 (CD82–MHC class II complexes) or Tris beads (mock control). Peptides apparently corresponding to the three dominant peptides in the CDw78 profile are marked by symbols. Length variants of CLIP (C) were identified through the specificity of the mAb Cer.CLIP and their unique masses (81–102, *m/z*=2333.4; 81–103, *m/z*=2543.4; 81–104, *m/z*=2674.5) as described[24,46].

precipitate the CDw78 subset of MHC class II molecules. Because CDw78 precipitates contain MHC class II DR and DP molecules in a ratio of roughly 3:1 (H. Kropshofer. *et al.*, unpublished data), we also precipitated DR and DP molecules with pan-DR mAb L243 and pan-DP mAb B7/21, respectively. We eluted self-peptides from these precipitates and subjected them to matrix-assisted laser desorption–ionization mass spectrometry (MALDI-MS).

In both the DR and DP MALDI-MS profiles, we could distinguish about 100 peptides of which length variants of the MHC class II–associated invariant chain peptide (CLIP) were the most prominent (**Fig. 1b**). These CLIP–MHC class II complexes were also strongly expressed on the cell surface (**Fig. 1a**). In the CDw78 MALDI-MS profile, however, only a few peptides were discernible, of which three were dominant (**Fig. 1b**). These peptides were also found in the DR and in the DP profile, as indicated by their unique masses. In support of a limited peptide complexity in tetraspan-CDw78 microdomains, only a few peptides were present in the profile obtained from CD82–MHC class II coprecipitations (**Fig. 1b**). Peptide signals present in the CD82 but not the CDw78 profile might be derived from a subset of CD82–MHC class II complexes located outside CDw78 clusters (H. Kropshofer *et al.*, unpublished data). Thus, certain peptide–MHC class II complexes seem to be enriched in tetraspan-CDw78 microdomains, whereas most other self-peptides—including abundant peptides such as CLIP—are absent.

CDw78 microdomains are distinct from lipid rafts

Tetraspanins, which are major constituents of CDw78 clusters, distribute partially to cholesterol- and glycosphingolipid-enriched lipid rafts[22]. We therefore determined whether MHC class II molecules with the CDw78 determinant localized to lipid rafts[16,17]. Owing to their resistance to detergent solubilization with Triton X-100 (TX-100)[16–18], rafts can be separated from surrounding membrane areas by fractionation on sucrose density gradients. We applied this technique to WT-100 B cells lysed in TX-100 and showed by immunoblot analysis that, quantitatively, the intercellular adhesion molecule 1 (ICAM-1) remained in soluble fractions at the bottom of the density gradient, whereas the Src family kinase Lyn—which is present in TX-100–resistant rafts in B cells[23]—accumulated in light-density fractions on top of the gradient (**Fig. 2a**).

In relation to the total pool of DR molecules, only 1–3% of DR molecules were found in raft fractions, as determined by quantitative immunoblot analysis (**Fig. 2b**). In other B cell lines and DCs, similar amounts of MHC class II (<5%) were observed to fractionate with lipid rafts (H. Kropshofer and A. B. Vogt, unpublished data). Concomitant analysis of the CDw78 subset of DR molecules showed that small amounts (<3%) were located in raft fractions (**Fig. 2c**). The disparate nature of lipid rafts and CDw78 microdomains was strengthened by MALDI-MS analysis: unlike DR molecules in the tetraspan microdomains (**Fig. 1b**), DR molecules in rafts showed a self-peptide repertoire that was highly diverse and very similar to the peptide repertoire of DR molecules derived from nonraft fractions (**Fig. 2d**). Thus, CDw78 microdomains are distinct from lipid rafts in both buoyant density and the diversity of their MHC-associated peptide repertoire.

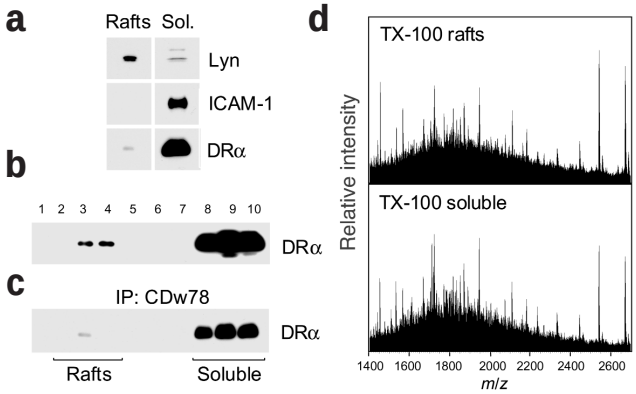

Figure 2. CDw78 microdomains are distinct from TX-100 insoluble lipid rafts. (**a**) Sucrose gradient fractionation of WT-100 B cells (1×10^7) lysed in 1% TX-100. Light-density fractions (2–4) containing TX-100–insoluble lipid rafts and high-density fractions (8–10) containing TX-100–soluble proteins were pooled and probed for the raft marker protein Lyn, the adhesion molecule ICAM-1 and α-chain of DR (DRα) by immunoblotting. (**b**) Each of the ten fractions of the sucrose gradient were analyzed for DRα. Quantitative immunoblotting showed that DR present in rafts comprises 1–3% of total DR. (**c**) In parallel, each fraction was probed for CDw78 clusters by precipitation with mAb FN1 and then stained for DRα. Less than 3% of the DR present in CDw78 clusters localizes to rafts, as determined by quantitative immunoblotting. (**d**) MALDI-MS profiles of DR-associated self-peptides derived from TX-100–insoluble rafts (upper panel) and TX-100-soluble fractions (lower panel) after precipitation with anti-DR (L243).

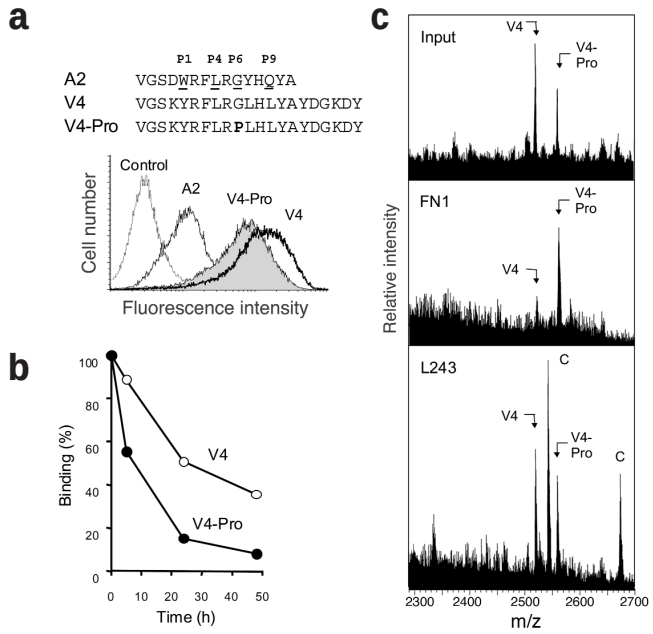

Figure 3. Peptide sequence determines segregation of peptide–MHC class II complexes into CDw78 microdomains. (**a**) Staining of WT-100 B cells with anti–A2-DR1 mAb 5A1 after a 1-h pulse with A2 peptide and the designed peptides V4 and V4-Pro (10 μM each), as determined by flow cytometry. Peptide sequences are aligned according to the DR1 peptide ligand motif[24,29]. Anchor residues W (pocket P1), L (P4), G (P6) and Q (P9) of the A2 peptide are underlined. Bold indicates the mutated proline residue at the P6 anchor position of V4-Pro. (**b**) Kinetics of disappearance of V4-DR1 and V4-Pro-DR1 complexes from the surface of WT-100 B cells after a 1-h pulse with V4 and V4-Pro (10 μM each), respectively, as determined by flow cytometry with the mAb 5A1. (**c**) MALDI-MS analysis of DR1-associated peptides after pulsing WT-100 B cells with a mixture of V4 and V4-Pro (10 μM each) for 1 h (Input). Peptides were acid eluted after precipitation with anti-CDw78 (FN1) and anti-DR (L243). Length variants of CLIP (C; *m/z*=2543.4, *m/z*=2674.5) are also indicated.

Peptide sequence determines CDw78 localization

To identify the structural characteristics of CDw78-associated peptides, we monitored a specific peptide–MHC class II complex in APCs. We used mAb UL-5A1 (5A1)[24], which reacts specifically with a complex comprised of DR1 and the associated self-peptide A2, which consists of amino acids 103–117 of the MHC class I molecule HLA-A2[25]. In DR1-expressing WT-100 B cells that were pulsed with exogenous A2 or variants of A2, the surface expression of A2 was increased at least tenfold by COOH-terminal elongation of A2 and replacing suboptimal anchor residues to form peptide V4 (**Fig. 3a**). When the P6 anchor was replaced by a proline residue, which induces conformational changes in other MHC class II molecules[26–27], the resulting mutant peptide V4-Pro bound less well than V4 to DR1, as shown by flow cytometry (**Fig. 3a**). This reduced binding is most probably due to a faster off-rate and/or an enhanced internalization of DR1–V4-Pro complexes ($t_{1/2}$≈8 h, **Fig. 3b**) in relation to DR1-V4 complexes ($t_{1/2}$≈24 h, **Fig. 3b**).

The DR1-expressing WT-100 B cells were pulsed with a peptide mixture containing equimolar amounts of V4 and V4-Pro (**Fig. 3c**, upper panel) and analyzed by MALDI-MS. Similar amounts of V4 and V4-Pro were recovered with the anti-DR mAb L243 (**Fig. 3c**, lower); however, V4-Pro was enriched in CDw78 clusters precipitated with anti-CDw78 mAb FN1 (**Fig. 3c**, middle panel). Thus, the sequence of the peptide has a strong impact on the extent to which peptide–MHC class II complexes are associated with CDw78 microdomains.

DM is located in CDw78 microdomains

Because MHC class II in CDw78 clusters formed complexes with a select set of peptides, we thought that a peptide editor might be involved in the process. Our prime candidate was the MHC class II molecule DM, which engages in complexes with tetraspanins CD82 and CD63[28], and functions as a peptide editor in internal loading compartments[29–31] as well as on the cell surface[32]. To test this idea, we lysed WT-100 B cells in the mild detergent *n*-octylglucoside to maintain the CDw78 clusters. The clusters remained intact because tetraspanins CD82, CD81 and CD9 were coprecipitated with anti-CDw78 (**Fig. 4**), in agreement with previous data[14]. As a control, we precipitated DR molecules with the pan-DR antibodies L243 or DA6.147, and found only small amounts of coprecipitating tetraspanins (**Fig. 4**).

Staining the precipitates with the anti-DM mAb DM.K8 verified the anti-CDw78–specific coprecipitation of DM (**Fig. 4**), which supported the unique composition of CDw78 clusters. The engagement of DM in CDw78 clusters was a general phenomenon, as DM clustered with tetraspanins and DR molecules not only in B cells but also in monocyte-derived DCs and in lymphoid tissue derived from spleen, tonsil and lymph node (**Table 1**). These data suggest that segregation of the peptide editor DM into CDw78 microdomains is a common process.

CDw78 microdomains present a dominant self-peptide

We extended our analysis to naturally processed peptides using the EBV-transformed B cell line Jutthom, which expresses endogenous A2 bound to DR1[25]. Precipitating the A2-DR1 complexes with mAb 5A1 was accompanied by coprecipitation of DM and the tetraspanin CD82 when Jutthom B cells were lysed in the mild detergent CHAPS, but not when they were lysed in the more stringent detergent NP-40 (**Fig. 5a**). In contrast to the TX-100–NP-40 resistance of lipid rafts (**Fig. 2a**), the NP-40 sensitivity and CHAPS resistance of DM-DR and tetraspan-DR complexes is typical for CDw78 microdomains (**Figs. 2c** and **4**).

We verified the presence of endogenous A2-DR1 complexes in CDw78 microdomains by MALDI-MS: the CDw78 (mAb FN1) peptide profile showed three main peaks corresponding to length variants of A2 (**Fig. 5b**, upper panel), as evident from the A2-DR1 (mAb 5A1) peptide profile (**Fig. 5b**, lower panel), which was identical to the CDw78 (FN1) spectrum in most instances. The spectrum obtained with pan-DR mAb L243 showed that Jutthom B cells carry other very prominent DR-associated peptides, such as CLIP (**Fig. 5b**, middle), which are excluded from CDw78 clusters as in WT-100 cells.

We confirmed these results by subjecting Jutthom cell lysates to sequential immunoprecipitations: CDw78-associated DR1 molecules precipitated by mAb FN1 (**Fig. 5c**, middle lane) were quantitatively removed by predepletion with anti–A2-DR1 mAb 5A1 (**Fig. 5d**,

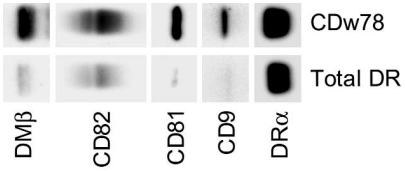

Figure 4. The peptide editor DM is localized in CDw78 microdomains. Immunoblot analysis of CDw78 clusters and total DR in WT-100 B cells, lysed in 1% *n*-octylglucoside and precipitated with anti-CDw78 mAb FN1 and anti-DR mAbs L243 or DA6.147 (total DR pool). Immunoblots were stained for DRα (1B5), CD9 (MEM192), CD81 (M38), CD82 (C33) and DMβ (DM.K8).

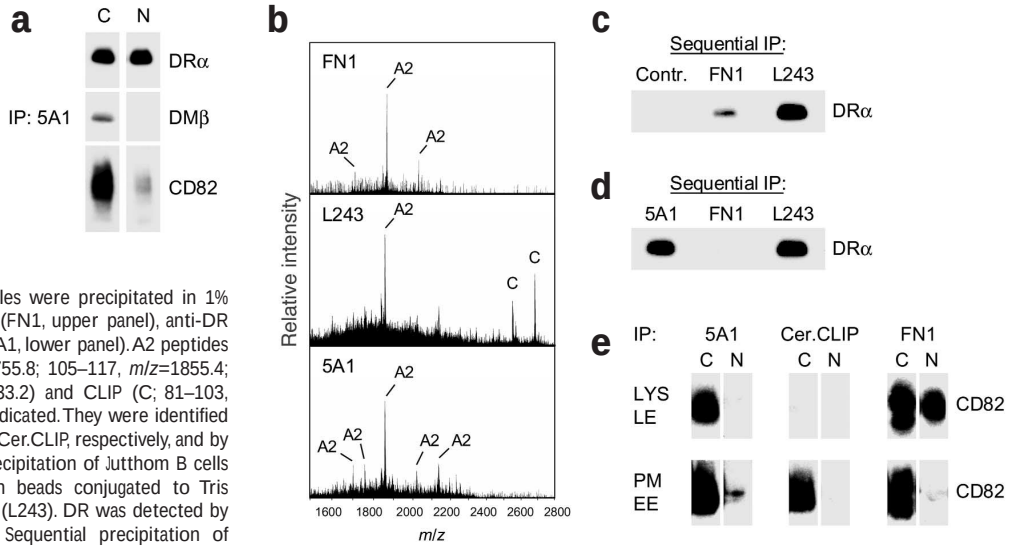

Figure 5. CDw78 microdomains of Jutthom B cells comprise A2-DR1 complexes localized in internal compartments and on the cell surface. (**a**) Jutthom B cells were lysed in 1% (w/v) CHAPS (C) and in 1% NP-40 (N). Lysates were precipitated with the mAb anti–A2-DR1 (5A1) and probed by immunoblotting for DRα (1B5), DMβ (DM.K8) and tetraspanin CD82 (C33). (**b**) MALDI-MS profile of DR1-associated self-peptides from Jutthom B cells. DR1 molecules were precipitated in 1% (w/v) *n*-octylglucoside with anti-CDw78 (FN1, upper panel), anti-DR (L243, middle panel) and anti–A2-DR1 (5A1, lower panel). A2 peptides (105–117, *m/z*=1698.6; 104–117, *m/z*=1755.8; 105–117, *m/z*=1855.4; 103–118, *m/z*=2018.6; 103–119, *m/z*=2133.2) and CLIP (C; 81–103, *m/z*=2543.4; 81–104, *m/z*=2674.54) are indicated. They were identified through the specificity of mAbs 5A1 and Cer.CLIP, respectively, and by their unique masses[24,48]. (**c**) Sequential precipitation of Jutthom B cells lysed in 1% (w/v) *n*-octylglucoside with beads conjugated to Tris (Contr.), anti-CDw78 (FN1) and anti-DR (L243). DR was detected by immunoblot with anti-DRα (1B5). (**d**) Sequential precipitation of Jutthom B cells lysed in 1% (w/v) *n*-octylglucoside with beads conjugated to anti–A2-DR1 (5A1), anti-CDw78 (FN1) and anti-DR (L243). DR was detected by immunoblot with anti-DRα (1B5). (**e**) Percoll gradient fractionation of Jutthom B cells. Heavy-density fractions positive for lysosomal (LYS) and late endosomal (LE) markers, and light-density fractions positive for plasma membrane (PM) and early endosomal (EE) markers, were pooled and lysed in 1% CHAPS (C) or 1% NP-40 (N). Lysates were precipitated with anti–A2-DR1 (5A1), anti-CLIP–MHC class II (Cer.CLIP) and anti-CDw78 (FN1), and the precipitates were analyzed by immunoblotting. All precipitates were normalized for equal amounts of DR by staining with anti-DRα (1B5) and then analyzed for the tetraspanin CD82 with mAb C33.

middle lane); however, DR1 molecules excluded from CDw78 clusters could be recovered in a successive precipitation step with anti-DR mAb L243 (**Fig. 5d**, right lane). Together, these findings show that CDw78 microdomains of Jutthom B cells comprise mainly A2-DR1 complexes.

CDw78 in intracellular loading compartments

To investigate the cellular distribution of A2-DR1 complexes in CDw78 microdomains, we fractionated Jutthom homogenates by Percoll density gradients[32,33]. High-density fractions, corresponding to lysosomes and late endosomes, and low-density fractions, corresponding to plasma membrane and early endosomes, were analyzed by immunoblotting for various subsets of DR1-peptide complexes. The CDw78 clusters were precipitated with mAb FN1 in fractions containing plasma membrane and in fractions of endosomal-lysosomal origin, as indicated by anti-CD82 staining (**Fig. 5e**).

Coprecipitation of CD82 and DR was also shown for the A2-DR1 subset (**Fig. 5d**), consistent with the above finding that CDw78 microdomains comprised mainly A2-DR1 complexes (**Fig. 5a,b**). In contrast, CLIP–MHC class II complexes were not associated with CD82 in endosomal-lysosomal fractions, but were found on the cell surface (**Fig. 5e**). Because CLIP was not found in the peptide profiles of CDw78 microdomains (**Figs. 1b** and **5b**), this cell-surface fraction might have been due to a small subset of CD82–MHC class II complexes that has been found outside CDw78 clusters in B cells (H. Kropshofer *et al.*, unpublished data). These data indicate that selected peptide–MHC class II complexes, such as A2-DR1, are incorporated into CDw78 clusters not only on the cell surface but also in internal compartments where loading of processed antigen occurs.

Developing DCs up-regulate CDw78 microdomains

Precipitations with mAb FN1 showed that CDw78 microdomains consisting of DR and associated tetraspan molecules were present in monocyte-derived DCs (**Table 1**). Flow-cytometry data showed that CDw78 microdomains were mostly absent from the surface of immature DCs (**Fig. 6a**). These two findings together implied that CDw78 clusters were located in internal compartments in immature DCs. Treating DCs with lipopolysaccharide (LPS) for 24 h led to their maturation, as indicated by a 4–5-fold up-regulation of MHC class II molecules DR and DP and about a 100-fold up-regulation of the costimulatory molecule CD86 (**Fig. 6a**). Likewise, the surface expression of CDw78 clusters increased roughly 30-fold on maturation (**Fig. 6a**).

Table 1. Protein content of CDw78 microdomains

	Percentage of total DR in CDw78[a]	Protein content[b]					
		CD9	CD63	CD82	CD81	DR	DM
B cell line WT-100	5–12	+	+	++	+	++	+
B cell line Jutthom	6–10	–	–	++	–	++	+
DCs	2–4	++	++	–	–	+	++
DCs (+ LPS)[c]	9–12	+++	++	+	–	+++	++
Spleen	4–5	+	+++	++	–	+	+
Tonsil	15–17	++	++	+++	++	++	+
Lymph node	8–12	++	+++	+++	+++	+++	+++
Thymus	15–17	++	+	+	+	++	–

[a]The DR content was determined by lysis of the respective cells or tissues in 1% *n*-octyl-glucoside, precipitation of CDw78 microdomains with mAb FN1, and comparison of CDw78-associated DR molecules in relation to total DR in the respective lysates by quantitative immunoblotting. [b]Anti-CDw78 precipitates were blotted and stained for the indicated molecules, as described in Fig. 4. For each protein, the strongest signal intensity was set to 100% and the other values were ranked according to the categories +++, 100–60%; ++, 59.9–20%; +, 19.9–1%; –, <1%. [c]DCs after treatment with LPS for 24 h.

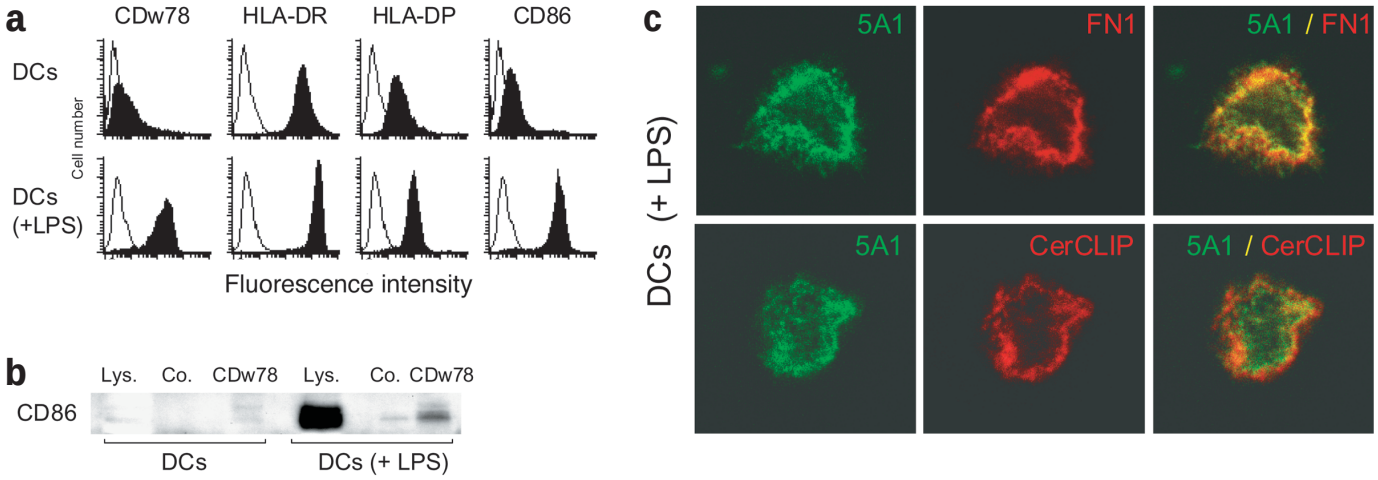

Figure 6. Developing DCs up-regulate CDw78 microdomains that contain the costimulatory molecule CD86. (**a**) Flow-cytometric analysis of DCs with and without treatment with LPS (1 μg/ml) for 24 h. Cells were stained for CDw78, DR, DP and CD86, as in Fig. 1. (**b**) DCs treated with or without LPS (1 μg/ml) for 24 h were lysed in 1% (w/v) *n*-octylglucoside and precipitated with anti-CDw78 or control beads. Together with aliquots of the respective cell lysates, the precipitates were probed for the costimulatory molecule CD86. (**c**) For confocal microscopy, monocyte-derived DR1-positive DCs were first pulsed with recombinant A2 protein (5 μg/ml) in the presence of LPS (1 μg/ml) for 14 h. Cells were adhered to poly-L-lysine-coated coverslips for 30 min at 37 °C and stained at 4 °C with 5A1 (green, left panel) specific for A2-DR1 complexes and FN1 (red, upper middle panel) to visualize CDw78 clusters or Cer.CLIP (red, lower middle panel) to visualize CLIP–MHC class II complexes. Areas of colocalization are indicated in overlay images (yellow, right panel). Cells depicted are representative of a minimum of three cells per field with ten fields observed.

When we probed developing DCs for costimulatory molecules, we found CD86 in the CDw78 microdomains of mature DCs (**Fig. 6b**).

Because surface CD86 is involved in presenting exogenous antigen to T cells, we administered exogenous recombinant A2 protein to immature DR1-expressing DCs and incubated them with LPS for 24 h. When these mature DCs were analyzed by confocal microscopy (**Fig. 6c**, upper panels), anti–A2-DR1 staining (5A1, green) colocalized strongly with anti-CDw78 staining (FN1, red); however, there was little costaining with anti–A2-DR1 (5A1) or the anti-CLIP mAb Cer.CLIP (**Fig. 6c**, lower panels). Thus, the A2 peptides derived from exogenous A2 protein were loaded onto DR1 molecules and moved into CDw78 microdomains before finally appearing on the cell surface of activated

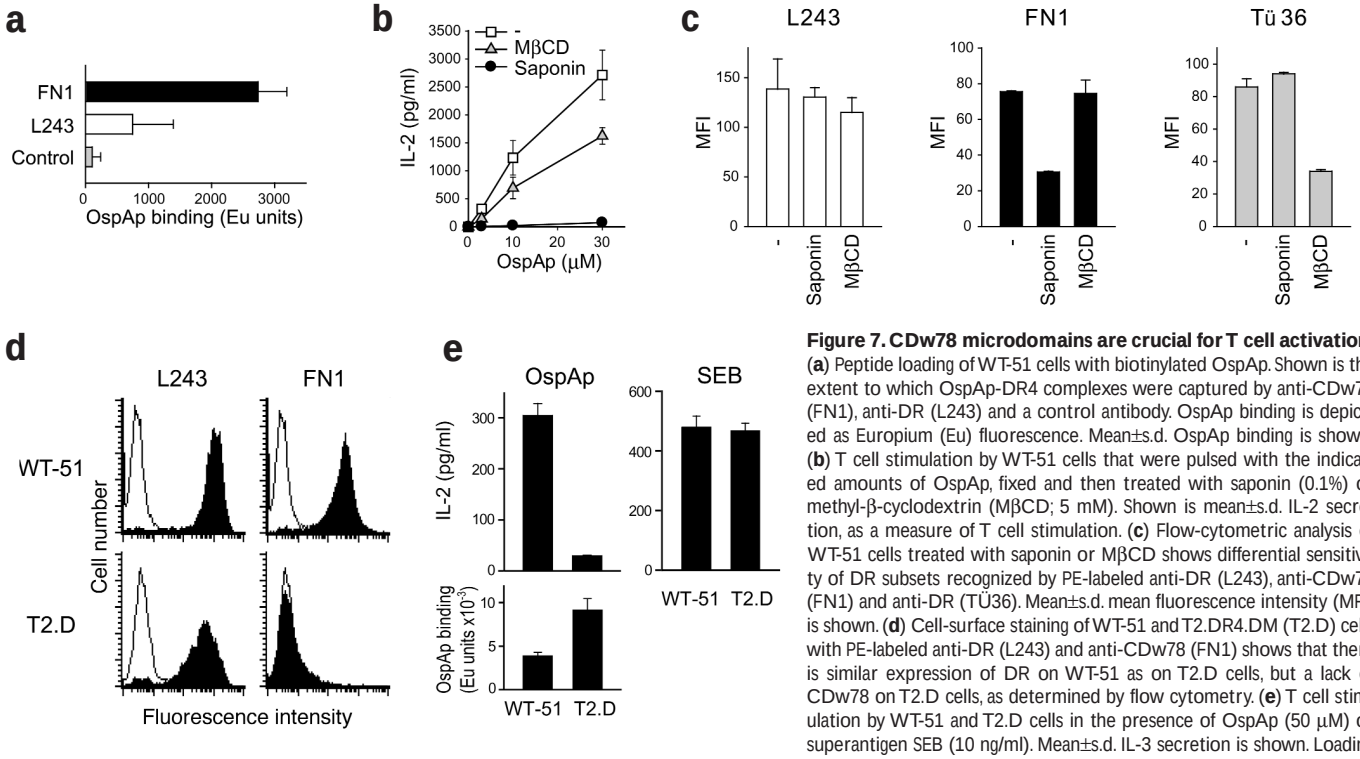

Figure 7. CDw78 microdomains are crucial for T cell activation. (**a**) Peptide loading of WT-51 cells with biotinylated OspAp. Shown is the extent to which OspAp-DR4 complexes were captured by anti-CDw78 (FN1), anti-DR (L243) and a control antibody. OspAp binding is depicted as Europium (Eu) fluorescence. Mean±s.d. OspAp binding is shown. (**b**) T cell stimulation by WT-51 cells that were pulsed with the indicated amounts of OspAp, fixed and then treated with saponin (0.1%) or methyl-β-cyclodextrin (MβCD; 5 mM). Shown is mean±s.d. IL-2 secretion, as a measure of T cell stimulation. (**c**) Flow-cytometric analysis of WT-51 cells treated with saponin or MβCD shows differential sensitivity of DR subsets recognized by PE-labeled anti-DR (L243), anti-CDw78 (FN1) and anti-DR (TÜ36). Mean±s.d. mean fluorescence intensity (MFI) is shown. (**d**) Cell-surface staining of WT-51 and T2.DR4.DM (T2.D) cells with PE-labeled anti-DR (L243) and anti-CDw78 (FN1) shows that there is similar expression of DR on WT-51 as on T2.D cells, but a lack of CDw78 on T2.D cells, as determined by flow cytometry. (**e**) T cell stimulation by WT-51 and T2.D cells in the presence of OspAp (50 μM) or superantigen SEB (10 ng/ml). Mean±s.d. IL-3 secretion is shown. Loading of biotinylated OspAp onto DR4 molecules using equal amounts of WT-51 and T2.D cells is shown below. Mean±s.d. of OspAp binding is shown

DCs. On the basis of these results, it seems possible that CDw78 microdomains may be involved in presenting antigen to T cells.

CDw78 microdomains are essential for T cell activation

To determine whether CDw78 clusters contribute to T cell activation, we used the DR4-expressing EBV-transformed B cell line WT-51 and a T cell hybridoma specific for the DR4-restricted immunodominant OspA(164–175) peptide (OspAp) from *Borrelia burgdorferi*, which distributed readily into CDw78 clusters (**Fig. 7a**). As a potential drug that might interfere with CDw78 microdomains, we tested the glycoside saponin, which disperses protein clusters by inserting into cholesterol-rich membrane domains[34,35]. We treated fixed WT-51 cells with very low doses of saponin so that the total number of surface DR molecules remained unchanged (**Fig. 7c**, left panel); however, this low dose of saponin was sufficient to severely reduce the quantity of CDw78 microdomains (**Fig. 7c**, middle panel).

Saponin-treated WT-51 B cells loaded with OspAp lost their capacity to stimulate interleukin 2 (IL-2) secretion from OspAp-specific T cells (**Fig. 7b**). This effect was not due to the toxicity of saponin to T cells mediated by saponin-treated WT-51 cells because saponin-treated WT-51 cells did not affect IL-2 secretion in an irrelevant T cell hybridoma, and saponin did not interfere with OspAp binding to DR4 molecules (H. Kropshofer *et al.*, unpublished data). When WT-51 B cells were treated with methyl-β-cyclodextrin (MβCD), a drug that disrupts membrane rafts by cholesterol depletion[16], MβCD led to a reduction in IL-2 secretion, as described[15]. Unlike saponin, however, MβCD left CDw78 microdomains unchanged and diminished the DR subset recognized by anti-DR mAb TÜ36 (**Fig. 7c**, right panel). These findings strengthen the above conclusion that CDw78 microdomains are distinct from raft microdomains and support the hypothesis that CDw78 microdomains are engaged in T cell activation.

One could argue that saponin-mediated interference with T cell activation might not be exclusively due to the disruption of CDw78 microdomains because the function of other crucial molecules might also have been impaired. We obtained independent evidence from studies with the T cell–B cell hybrid cell line T2.DR4.DM (T2.D)[36], which expressed similar amounts of DR4 as did WT-51, but did not contain CDw78 microdomains (**Fig. 7d**). In comparison to WT-51 cells, T2.D cells were compromised severely in their capacity to stimulate OspA-specific T cells (**Fig. 7e**), even though T2.D cells bound more OspAp than WT-51 cells did (**Fig. 7e**). The differential T cell response was specific for OspAp because T2.D and WT-51 cells were equally potent in activating T cells *via* the superantigen staphylococcal enterotoxin B (SEB, **Fig. 7e**). Thus, these data support the idea that CDw78 microdomains are essential for T cell activation.

Discussion

Our study shows that human B cells and DCs are equipped to constitutively organize a subset of MHC class II molecules according to their peptide cargo, so that MHC class II molecules carrying the same antigenic peptide are juxtaposed to each other. Membrane organization is accomplished through microdomains made up by proteins of the tetraspan family, such as CD9, CD81 and CD82, which form two-dimensional networks in certain areas of the plasma membrane[13]. The subset of DR and DP molecules found in these tetraspan domains covers about 10% of surface MHC class II molecules and can be differentiated phenotypically from other MHC class II molecules on behalf of the CDw78 determinant[8–10]. The CDw78 determinant is most probably caused by a specific spatial arrangement of at least two MHC class II molecules, which are embedded in an array of tetraspanins and accessory molecules such as DM and the costimulator CD86.

The number of molecules that constitute a single tetraspan microdomain is estimated to be between 10 and 15 from its relative molecular mobility (M_r) of 500,000–600,000 under conditions of native gel electrophoresis (S. Spindeldreher *et al.*, unpublished data). The tetraspanin CD82 seemed to be one of the main constituents of the microdomains in both internal compartments and on the cell surface, in agreement with previous reports of CD82-DR complexes[14,28]. When B cell lysates were extensively depleted of tetraspan microdomains, however, CD82-DR complexes were still recovered (H. Kropshofer *et al.*, unpublished data), suggesting that APCs contain at least one other type of MHC class II–tetraspan cluster that is unrelated to CDw78 cluster. This might explain why the CD82-associated peptide profile contained CLIP and other peptides that were not detectable in CDw78 profiles, and why CLIP-DR complexes coprecipitated with CD82.

With regard to the coclustering of MHC class II and costimulatory CD86 molecules, tetraspan microdomains are reminiscent of clusters that are located in transport vesicles of murine DCs[37]. *Via* these vesicles, MHC class II–CD86 clusters are transported from internal loading compartments to the cell surface, where they remain in a clustered configuration[37]. We have shown here that MHC class II–tetraspan clusters localize to internal compartments and to the plasma membrane. Because the cytosolic tail of the tetraspanin CD82 and the peptide editor DM contain internalization signals[38], CD82-DM–positive tetraspan microdomains are likely to recycle between the plasma membrane and endosomal-lysosomal peptide-loading compartments. This recycling has been shown for DM[39]. Because CD82 was associated with A2-DR1 and not with CLIP-DR1 complexes in lysosomes and late endosomes, it seems that newly synthesized DR molecules engage in recycling CD82-DM clusters only after CLIP has been removed. Complexes comprising peptide-receptive "empty" DR molecules and DM in endocytic organelles have been described[33,40].

After the uptake of exogenous antigen into endosomes and the generation of antigenic peptides, tetraspan-DR-DM clusters located inside a particular endosomal loading compartment might be exposed to only a few different types of antigenic peptide rather than a highly diverse antigen repertoire. Owing to DM-mediated peptide editing and/or the abundance of certain antigenic peptides, most CDw78–MHC class II molecules could be loaded with the same peptide epitope. Thus, one essential function of tetraspan microdomains might be to prevent uniformly loaded peptide–MHC class II clusters from dissipation by lateral diffusion during and after trafficking to the cell surface. This would give rise to surface domains in which particular peptide antigens are kept locally enriched, thereby increasing the avidity for a putative T cell encounter.

Peptide epitopes that accumulate in tetraspan microdomains, such as V4-Pro, were enriched about tenfold in relation to their abundance in the total DR population (H. Kropshofer *et al.*; unpublished data). V4-Pro and the other peptides that segregated into tetraspan domains, such as the self-peptide A2 or the foreign antigen OspAp, all have one or two unfavorable anchor residues; consequently, they only attain moderate binding to MHC class II molecules and mediocre binding stability. These characteristics are shared by most disease-associated T_H epitopes. A rationale for the local enrichment of this type of antigen in tetraspan microdomains is that a random distribution of antigen on the cell surface of APCs might not create a high enough avidity to trigger cognate T cells efficiently.

What could be the mechanistic basis for the formation of DM-DR-peptide complexes in a peptide-selective manner? According to the

kinetic proofreading model[31], the relationship of the off-rate of a peptide (k_{pep}) to the off-rate of DM (k_{DM}) determines the extent to which DM-DR-peptide complexes are formed. The DM-DR-peptide complexes dissociate if the peptide has a very low stability ($k_{pep}>k_{DM}$), as in the case of CLIP, resulting in peptide-free DR-DM complexes[31,33]. These complexes also dissociate when a peptide with high stability ($k_{pep}<k_{DM}$) such as V4 binds, leading to the dissociation of DM. This would explain why both CLIP and V4 are mostly excluded from CDw78 microdomains. Thus, peptides of presumably mediocre stability, such as A2 and OspAp, will give rise to long-lasting DM-DR-peptide complexes because the peptide off-rate is similar to the off-rate of DM ($k_{pep}\approx k_{DM}$), and the dissociation of neither DM nor the peptide is promoted.

A study has indicated that in B cells at least half of the surface MHC class II molecules may be constitutively concentrated in lipid rafts, thereby facilitating T cell activation at low densities of peptide-MHC[15]. Another study has shown that MHC class II association with lipid rafts does not occur constitutively but only after antibody-induced ligation of MHC class II[19]. It has been also argued that the number of lipid rafts is likely to exceed the number of MHC class II molecules carrying cognate peptide so that lipid rafts will be unable to concentrate cognate peptide–MHC class II complexes to an extent sufficient for triggering T cells[20,41]. According to our data, less than 3% of the DR molecules of human B cells and DCs are expressed constitutively in membrane rafts. Disrupting lipid rafts by MβCD treatment reduced the degree of T cell activation, but could not block it completely. This suggests that DR molecules outside lipid rafts are involved actively in initiating a T cell response.

This last idea is supported by our finding that low doses of saponin abolished the capacity of B cells to trigger IL-2 secretion of T cells without interfering with MβCD-sensitive DR subsets in lipid rafts. In addition, the hybrid cell line T2.D, which did not contain surface CDw78 microdomains, activated OspA-specific T cells poorly in relation to CDw78-positive WT-51 cells, even though T2.D carried more DR4-OspAp complexes. This indicates that it is not merely the number but the spatial organization of peptide–MHC class II complexes in the plasma membrane that determines the T cell activation potential. That is, the lack of CDw78 microdomains might have prevented T2.D cells from attaining high local surface densities of OspAp-DR4 complexes so that the avidity towards T cells was too low to trigger secretion of IL-2. Presentation of the superantigen SEB, however, showed no dependence on tetraspan microdomains. This may be because SEB associates with many different peptide-DR complexes because its binding site is located outside the peptide-binding groove[42], thereby circumventing the need for preclustering peptide–MHC class II complexes.

In summary, our data indicate that MHC class II molecules may populate the surface of APCs in an organized manner that comprises at least two types of membrane domain: lipid raft microdomains, in which MHC class II molecules are concentrated irrespective of their antigen cargo, and tetraspan-CDw78 microdomains, which are enriched in MHC class II molecules carrying a select set of antigenic peptides. Because both sets of microdomains differ with regard to their antigenic peptide repertoire and the density of particular peptides, it is tempting to propose that they may evoke different types of T cell response; for example, they may determine whether naïve T cells differentiate into T_H type 1 or type 2 cells. Mechanistically, this might be accomplished by regulating the number of tetraspan and lipid raft microdomains that are locally available for building up the immunological synapse between APCs and T cells. Future studies that are based on these ideas may lead to a better understanding of the very early molecular events in initiating TCR signaling and eventually resolve the issue of whether APCs—through the molecular organization of their peptide–MHC class II complexes—control the activation status of T cells to a greater extent than has been anticipated in the past.

Methods

Cells. The EBV-transformed B cell lines WT-100 (DR1$^+$; DRB1*0101), Jutthom (DR1$^+$; DRB1*0101), WT-51 (DR4$^+$; DRB1*0401) and the T cell/B cell hybrid cell line T2.D (DR4$^+$; DRB1*0401) were maintained in RPMI-1640 medium supplemented with 20 mM HEPES, pH 7.2, 1 mM pyruvate, 2 mM glutamine and 10% heat-inactivated fetal calf serum (FCS, Gibco-BRL, Rockville, MD).

Antibodies. We purified DR-specific mAb L243 (recognizing DRαβ dimers)[43], DR-specific mAb 1B5 (recognizing the cytoplasmic tail of DRα)[44], DM-specific mAb DM.K8 (recognizing the cytoplasmic tail of DMβ)[29], anti–A2(103–117)-DR1–specific human mAb 5A1[25] from hybridoma supernatants by affinity chromatography on Protein A (Pharmacia, Uppsala, Sweden). The anti-CDw78 mAb FN1[8] was provided by A.-M. Rasmussen. For immunoprecipitation with beads, mAbs were conjugated to CNBr-activated sepharose CL-4B (Pharmacia) at a concentration of 3 mg of antibody per ml beads. The following antibodies were used in flow-cytometric analysis: anti-CLIP mAb Cer.CLIP (recognizing CLIP bound to DR, DP or DQ molecules, Pharmingen, San Diego, CA), anti-CD82 mAb 50F11 (Pharmingen), DR-specific mAb TÜ36 (recognizing the DRβ chain; Pharmingen), anti-CD86 mAb FUN-1 (Pharmingen), anti-DP mAb B7/21 (Leinco Technologies, Ballwin, MO) and the corresponding isotype controls (Pharmingen). We used the following antibodies for immunoblot analysis: anti-Lyn mAb cl42 (Transduction Laboratories, Lexington, KY), anti–ICAM-1 mAb G5 (Santa-Cruz Biotechnology, Santa Cruz, CA) and anti-CD86 serum H-200 (Santa-Cruz Biotechnology). The anti-CD82 mAb C33[45] was provided by O. Yoshie. The anti-CD9 mAb MEM192 was produced and characterized in the laboratory of V. Horejsi.

Peptides. We synthesized peptides by F-moc chemistry and purified them by reverse-phase high performance liquid chromatography. Some peptides were biotinylated by coupling biotinyl-amino-hexanoic acid at the NH_2-terminus during F-moc synthesis. We checked the purity of peptides routinely by MALDI-MS. Peptide A2(103–117), VGSDWRFLRGYHQYA, is derived from human MHC class I molecule HLA-A2 and has been described as a DR1-associated self-peptide[24]; V4, VGSKYRFLRGLHLYAYDGKDY, is a COOH-terminally elongated version of A2(103–117) that has been optimized for binding to DR1 and recognition by mAb 5A1; V4-Pro, VGSKYRFLRPLHLYAYDGKDY, differs from V4 by a Gly→Pro substitution at the P6 anchor position; OspA(164–175), SYVLEGTLTAEK, is derived from the outer surface protein A (OspA) of *B. burgdorferi* and has been described as a DR4-restricted immunodominant T cell epitope[46].

Generation of DCs. DCs were differentiated from peripheral blood monocytes as described[47]. Briefly, we isolated monocytes from blood PBMCs by positive sorting with anti-CD14 magnetic beads (Miltenyi Biotech, Auburn, CA) and cultured them in complete RPMI-1640 medium containing granulocyte macrophage–colony stimulating factor (GM-CSF, 50 ng/ml, Leucomax, Novartis, Basel Switzerland) and IL-4 (3 ng/ml, R&D, Minneapolis, MN). We induced maturation on day 5 by adding LPS (1 μg/ml) from *Salmonella abortus equi* (Sigma, St Louis, MO). Maturation of DCs was evaluated routinely for surface expression of CD80, CD83, CD86 and DR by flow-cytometric analysis.

Flow cytometry. We stained B cells and DCs with the appropriate antibodies (5 μg/ml) followed by fluorescein isothiocyanate (FITC)-labeled goat-anti-mouse (Dianova, Hamburg, Germany). Cells were analyzed on a Becton-Dickinson FACScalibur flow cytometer, with the CellQuest software package (Becton-Dickinson, Heidelberg, Germany).

Preparation of lipid rafts. Cells (1×10^9) were lysed in a fivefold volume of MNE lysis buffer (25 mM MES, 150 mM NaCl, 1 mM EDTA, pH 6.5, 1 mM Na_3VO_4, 10 mM NaF, 4 °C) containing 1% TX-100 (Roche Diagnostics, Mannheim, Germany) and protease inhibitors (leupeptin, phenylmethylsulfonylfluoride, chymostatin and pepstatin) for 30 min on ice. The lysate was mixed with an equal volume of 80% sucrose in MNE, placed at the bottom of an ultracentrifugation tube and overlaid with 30% sucrose and 5% sucrose in MNE. Ultracentrifugation was done with 200,000*g* at 4 °C for 16 h. We collected ten fractions starting from the top of the gradient. For immunoblot analysis and immunoprecipitation, we solubilized fractions in 60 mM *n*-octylglucoside. For MALDI-MS analysis, insoluble raft fractions (2–4) were mixed with a fivefold volume of MNE buffer and centrifuged at 100,000*g* for 1.5 h at 4 °C. Raft pellets were solubilized in 60 mM *n*-octylglucoside in MNE, whereas soluble fractions (8–10) were supplemented with 60 mM *n*-octylglucoside. We isolated DR molecules from both fractions by using mAb L243 conjugated to sepharose beads.

Subcellular fractionation. Density gradient fractionation of B cells was done with 27% (v/v) Percoll (Pharmacia) as described[33]

Immunoblot analysis. Samples were separated by SDS-PAGE (12%) and transferred onto Immobilon PVDF membrane (Millipore, Bedford, MA). After blocking with Blocking reagent (Roche Diagnostics), the membrane was stained with the respective primary antibody followed by incubation with horseradish-peroxidase–conjugated, isotype-specific

secondary antibodies (Southern Biotechnology, Birmingham, AL). We visualized staining with Super-Signal Dura West (Pierce, Rockford, IL). For quantitative immunoblotting, calibration standards were loaded onto the gel and the immunoblots analyzed on a LumiImager F1 with the software LumiAnalyst (Roche Diagnostics).

Mass spectrometry. We did MALDI-MS analysis of DR-associated peptides essentially as described[48]. Briefly, 10^6–10^7 WT-100 or Jutthom cells were lysed in 1% *n*-octylglucoside and DR molecules were precipitated by FN1, 5A1 or L243 mAb conjugated to sepharose beads. We used Tris-conjugated sepharose beads as a control. Peptides were eluted with 0.1% trifluoroacetic acid and analyzed on a REFLEX III mass spectrometer (Bruker, Bremen, Germany).

Confocal microscopy. Monocyte-derived DCs were pulsed for 14 h with recombinant A2 protein (5 μg/ml) in the presence of LPS (1 μg/ml). Recombinant A2 protein expressed in insect cells[49] was provided by M. Smith. We allowed the pulsed DCs to adhere to poly-L-lysine–coated coverslips for 60 min at 37 °C and then stained them at 4 °C with biotinylated mAbs 5A1 and FN1, or biotinylated 5A1 and Cer.CLIP. We used Alexa488-conjugated streptavidin and Rhodamin red–conjugated goat anti-mouse Fab fragments (Dianova), respectively, as secondary reagents. We mounted cells in FluorSave (Calbiochem, San Diego, CA) and visualized them with an MRC 1024 confocal microscope (Biorad, Hercules, CA). For dual color analysis cells were excited at 488 and 568 nm.

Saponin and methyl-β-cyclodextrin cell treatment. We washed WT-51 cells in PBS and fixed them with 1% paraformaldehyde for 15 min at 20 °C. Fixation was stopped by adding 100 mM glycine in PBS. After being washed in PBS, cells were treated with 0.1% saponin in PBS containing 2% FCS for 20 min at 4 °C, or with 5 mM MβCD in RPMI for 10 min at 37 °C, or were left untreated in RPMI containing 10% FCS. Cells were washed in RPMI containing 10% FCS, incubated for 20 min at 37 °C, washed, counted and used for T cell assays. In parallel, we checked the surface expression of DR molecules by flow cytometry using PE-labeled L243, FN1, TÜ36 and respective isotype controls.

T cell assay. We incubated 10^5 WT-51 or T2.D cells with 10^5 T cell hybridoma cells specific for OspA(164–175)-DR4 derived from the outer surface protein A of *B. burgdorferi*[46] in RPMI with 10% FCS in the presence of OspA(164–175) peptide or superantigen SEB (Toxin Technologies, Sarasota, FL). After incubation overnight, IL-2 production was quantified by with an IL-2–capture ELISA according to the manufacturer's protocol (Pharmingen). In the experiment with pretreatment of APCs, WT-51 cells were pulsed at a density of 2×10^6 cells/ml for 2 h with OspA(164–175) peptide, fixed and treated with saponin, MβCD or RPMI, as described above. We then incubated 10^5 peptide-pulsed and pretreated WT-51 cells with 10^5 T cell hybridoma cells specific for OspA(164–175)-DR4. After incubation overnight, IL-2 production was quantified with an IL-2–capture ELISA. We assessed the integrity of treated WT-51 cells by flow cytometric analysis before and after overnight incubation with T cells. To rule out any toxic or inhibitory effect of the MβCD- or saponin-treated WT-51 cells on T cell hybridoma cells, MβCD- or saponin-treated WT-51 cells were incubated with an OspA(235–245)-specific T cell hybridoma and untreated WT-51 cells loaded with OspA(235–245).

Peptide-binding assay. We pulsed WT-51 or T2.D cells (10^6 cells/ml) with biotinylated peptide for 1.5 h, and washed and lysed them at 4 °C in 20 mM Tris, 5 mM $MgCl_2$, 1% (w/v) *n*-octylglucoside containing protease inhibitors. Peptide binding was analyzed by an ELISA capture assay, as described[32], by using L243, FN1 mAbs and a control IgG as capture antibodies and streptavidin-Europium as the detection reagent. In parallel, we quantified the amount of DR molecules bound to FN1 or L243 by using biotinylated anti-DR mAb 1B5 to normalize the amount of bound peptide in relation to the amount of MHC class II molecules being recovered.

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