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Presence of HLA-DR Molecules and *HLA-DRB1* mRNA in Circulating CD4⁺ T Cells

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Abstract

The human major histocompatibility complex class II isotype HLA-DR is currently used as an activation marker for T cells. However, whether an endogenous protein expression or a molecular acquisition accounts for the presence of HLA-DR on T cells remains undetermined and still controversial. To further characterize this phenomenon, we compared several aspects of the presence of the HLA-DR protein to the presence of associated mRNA (*HLA-DRB1*), focusing on human T cells from peripheral blood of healthy individuals. Using a flow cytometric approach, we determined that the HLA-DR observed on CD4⁺ T cells was almost exclusively cell surface-associated, while for autologous CD19⁺ B cells, the protein could be located in the plasma membrane as well as in the cytoplasm. Moreover, negligible expression levels of *HLA-DRB1* were found in CD4⁺ T cells, using an *HLA-DRB1* allele-specific qPCR assay. Finally, the presence of HLA-DR was not confined to activated CD4⁺ and CD8⁺ T cells, as evaluated by the co-expression of CD25. The functional role of the HLA-DR molecule on T cells remains enigmatic; however, this study presents evidence of fundamental differences for the presence of HLA-DR on T cells from HLA-DR in the context of antigen-presenting cells, which is a well-known phenomenon. Although an inducible endogenous protein expression cannot be excluded for the T cells, our findings suggest that a re-evaluation of the HLA-DR as a T cells activation marker is warranted.

Introduction

It has been observed for more than four decades that T cells in peripheral blood can present MHC class II (MHCII) molecules on their outer surface and that the number of MHCII⁺ T cells increases upon activation [1–4]. In line with this, one of the three human MHCII isotypes, called HLA-DR, is frequently used as a T cell activation marker along with other molecules, such as CD69 and CD25 [5–8]. Nonetheless, the functional role and significance of HLA-DR on human T cells is not fully determined and the unequivocal confirmation of an endogenous expression or a protein acquisition from other immune cells is absent. The constitutive expression of HLA-DR is limited to a few cell types, collectively called antigen-presenting cells (APCs) [9, 10]. These cells use HLA-DR to present antigenic peptides to CD4⁺ T cells, and consequently, HLA-DR is involved in many facets of immune homeostasis such as T cell activation, peripheral tolerance and induction of apoptosis [11].

Since the first observations of HLA-DR on T cells in the late 1970s, a number of studies have investigated aspects of

the HLA-DR molecule in this particular context. Although still a subject of discussion, the general understanding is that the presence of this protein complex can be explained by either of two scenarios. The first involves an inducible *de novo*, endogenous protein synthesis, while the other encompasses a molecular acquisition from other immune cell types by currently unverified mechanisms. The studies supporting the former explanation have investigated several of the components involved in HLA-DR expression, transport and function, including lysosomal proteases [12], antigen presentation [13, 14] and engagement of CIITA [10, 12, 15], which is reported as the master regulator of *HLA-DR* expression [16–19]. Mouse T cells do not produce MHCII, which has been explained by a lack of CIITA expression [20]. However, even when CIITA is absent, MHCII expression is still observed for several cell types [21, 22]. The second phenomenon explaining HLA-DR on T cells is investigated in several other studies, demonstrating a transfer of a fully functional protein complex from APCs [23, 24]. In accordance with this, the MHCII observed on mouse T cells is a result of an acquisition from APCs [25–27]. In addition, a number of

studies have found that activated rat T cells can both synthesize and absorb MHCII molecules from adjacent cells [28–30]. The transfer of HLA-DR to human T cells has also been reported [31, 32], but a possible link between this acquisition and a potential endogenous expression of HLA-DR remains to be investigated.

Our study aimed to further explain the phenomenon of HLA-DR on human T cells in peripheral blood of healthy individuals. We addressed several aspects of the presence of the protein on T cells and correlated this with a transcript analysis of the corresponding mRNA. Moreover, the validity of HLA-DR as a T cell activation marker was investigated. The study was carried out to elucidate the physiological details of HLA-DR on T cells as well as to obtain novel information about a possible endogenous expression. Firstly, our results indicate that HLA-DR on CD4⁺ T cells in peripheral blood could not be explained by a concurrent presence of an active protein synthesis. This points to important differences between the HLA-DR in the context of T cells as compared to APCs, which may be important, when deciphering the possible functions of HLA-DR-presenting T cells. In addition, we demonstrated that HLA-DR was not confined to activated T cells, thus suggesting to reassess the use of HLA-DR as an activation marker for T cells without any accompanying information.

Methods

Antibodies. The following conjugated antibodies were obtained from BD Biosciences (Mountain View, CA, USA): anti-CD3-PE (SK7), anti-CD3-PerCP (SP34-2), anti-CD4-APC-H7 (SK3), anti-CD4-PE (RPA-T4), anti-CD8-PerCP (SK1), anti-CD8-PerCP-Cy5.5 (SK1), anti-CD14-PE-Cy7 (M5E2), anti-CD19-APC (HIB19), anti-CD19-PE (4G7), anti-CD19-PerCP-Cy5.5 (SJ25C1), anti-CD25-FITC (2A3), anti-HLA-DR-FITC (G46-6), mouse IgG1-APC-H7 (X40), mouse IgG1-FITC (MOPC-21), mouse IgG2a-FITC (27-35), mouse IgG1-PE (MOPC-21), mouse IgG2a-PE (MOPC-173), mouse IgG1-PerCP (MOPC-21), mouse IgG1-PerCP-Cy5.5 (MOPC-21) and mouse IgG1-PE-Cy7 (MOPC-21). From BD Biosciences, the unconjugated anti-CD3 (HIT3a) was also acquired. Anti-CD45-FITC (T29/33), anti-HLA-DR-PE (AB3) and mouse IgG1-APC (DAK-GO1) were purchased from Dako A/S (Glostrup, Denmark). Anti-HLA-DR-Alexa Fluor (AF) 488 (L243), anti-HLA-DR-AF647 (L243), mouse IgG2a-AF488 (MOPC-173) and mouse IgG2a-AF647 (MOPC-173) were purchased from BioLegend (San Diego, CA, USA). Mouse IgG1-PerCP-Cy5.5 (P3.6.2.8.1) was obtained from eBioscience (San Diego CA, USA). The goat anti-mouse IgG-AF555 was purchased from Invitrogen (Life Technologies, Carlsbad, CA, USA).

Cells and isolation. Venous peripheral blood was obtained from healthy donors with known HLA types. Each blood donor had signed a written consent form, allowing for the use

of his or her blood for research purposes. The procedure was approved by local ethics legislation. The blood was collected in heparinized tubes (Vacuette[®], Lithium Heparin; Greiner Bio One, Frickenhausen, DE). Isolation of Peripheral blood mononuclear cells (PBMCs) was accomplished using Lymphoprep[™] gradient centrifugation (Axis-Shield, Oslo, Norway). The PBMCs were either used directly after the isolation or stored at –140 °C in a storage medium [RPMI 1640 (Gibco, Life Technologies), 40% heat-inactivated foetal calf serum (FCS; Gibco), 10% DMSO (Merck Millipore, Darmstadt, Germany), 100 U/ml penicillin/10 µg/ml streptomycin (Ampliqon, Odense, Denmark)]. The CD4⁺ T cells were isolated from PBMCs using the Dynabeads[®] CD4 Positive Selection kit (Invitrogen) according to the manufacturer's guidelines. As an additional purification step, any residual B cells were removed from the isolated CD4⁺ T cells by positive selection, using Dynabeads[®] CD19 Pan B (Invitrogen). The purity of the isolated CD4⁺ T cells was evaluated by staining with fluorochrome-conjugated antibodies with subsequent flow cytometric analysis. From the CD4⁺ depleted PBMCs, CD19⁺ B cells were isolated using the Dynabeads[®] Untouched[™] Human B Cells kit (Invitrogen) according to the manufacturer's guidelines.

Detection of cell surface-associated and intracellular markers by flow cytometry. For the detection of cell surface-associated and intracellular HLA-DR, 1×10^6 PBMCs were stained with antibodies against CD3, CD19, HLA-DR (L243-AF488) or the corresponding isotype control antibodies (30 min, room temperature (RT)). Subsequently, 100 µl of fixation medium (reagent A) from FIX & PERM[®] Cell Fixation and Permeabilization Kit (Invitrogen) was added (15 min, RT). Following one wash with PBS (Gibco), a second antibody against HLA-DR (L243-AF647), or the corresponding isotype antibody, and 100 µl of permeabilization medium (reagent B) were added to the cells (20 min, RT). Finally, the cells were washed once with PBS prior to flow cytometric analysis. For the detection of cell surface-associated HLA-DR on CD4⁺ and CD8⁺ T cells, the procedure only included the primary surface staining, using antibodies against CD3, CD4, CD8, CD25 and HLA-DR (L243-AF647).

The flow cytometric analysis of stained cells was performed on a FACSCanto A using FACSDIVA[™] software (version 6.1.3; BD Biosciences). Calibration and compensation settings for the cytometer were obtained each day using the 7-Color Setup Beads (BD Biosciences) and once a week with the FACSDIVA[™] CS&T Research Beads (BD Biosciences). The analysis of the data was carried out with the FLOWJO software (version 10.0.7; FlowJo LLC, Ashland, OR, USA). Negative isotype controls were utilized to identify the positive events. The controls were either combined in a single tube or part of a 'fluorescence minus one' (FMO) tube [33]. The median fluorescence intensity (MFI) was the statistical value of choice.

Detection of cell surface-associated and intracellular HLA-DR by confocal microscopy. For all experiments, 1.5×10^5 isolated CD4⁺ T cells or CD19⁺ B cells were left to adhere to poly-L-lysine-coated slides (Sigma-Aldrich, St. Louis, MO, USA; 20 min, RT). The T cells were initially stained with anti-CD3 (HIT3a; 1:250; 45 min, RT), followed by incubation with secondary antibody (goat anti-mouse IgG-AF555; 1:200; 45 min, RT). Slides were washed three times in PBS and cells were fixed in 2% paraformaldehyde (PFA) (Sigma-Aldrich) (15 min, RT). Both T and B cells were then stained with anti-HLA-DR-AF488 (L243) (1:100; 45 min, RT). Slides were washed three times in PBS and cells were fixed in 2% PFA (15 min, RT) followed by another round of washing and permeabilization with permeabilization buffer [1% bovine serum albumin (BSA; Sigma-Aldrich), 0.1% saponin (Sigma-Aldrich), PBS; 8 min, RT]. To visualize cytosolic HLA-DR, cells were subsequently stained with anti-HLA-DR-AF647 (L243) (1:100 in permeabilization buffer; 45 min, RT). Slides were washed four times in PBS and mounted with mounting medium (Sigma-Aldrich). Images were captured with a Leica TCS SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany) and a 100 \times oil immersion objective using the LAS AF software (Leica). All images were slightly adjusted for background fluorescence and signal intensity using IMAGEJ (version 1.48; NIH, Bethesda, MD, USA).

Total RNA extraction and cDNA synthesis. Total RNA was extracted from the isolated CD4⁺ T cells and CD19⁺ B cells using the TRIzol extraction method (Invitrogen). The A260/A280 ratio was determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Total cDNA for the quantitative PCR (qPCR) was generated from 1 μ g total RNA from all samples using a mixture of poly-T and random primers with SuperScript VILO cDNA Synthesis Kit (Invitrogen) at a temperature profile of 25 °C for 10 min, 42 °C for 60 min and 85 °C for 5 min according to the manufacturer's guidelines.

Quantitative PCR. PCR primers for amplification of 57–136-base pair gene-specific PCR products were used from conserved regions of the four studied genes (*HLA-DRB1*03*, *CD19*, *B2M*, and *GUSB*). Primers and FAM-labelled minor groove binder (MGB) TaqMan probes were used to detect the RNA expression level of the reference genes *B2M* and *GUSB* [34] and the target gene *HLA-DRB1*03* for which the *HLA-DRB1*03:01:01:01* mRNA sequence from the IMGT/HLA database was used as target sequence. Expression levels of *CD19* were detected using 20 \times probe assays mix (Life Technologies). The information about primer and probe sequences is given in Table 1. The qPCR assay was applied to evaluate the gene expression profiles of the four genes, and the data were analysed using the QuantStudio 12K Flex system (Life Technologies). The real-time PCR reactions were performed in a final volume of 25 μ l, containing 1 μ l

cDNA solution, 12 μ l Master Mix (TaqMan Universal PCR Master Mix; ABI, Darmstadt, Germany), 0.05 μ M probe and 0.9 μ M of forward and reverse primers. The PCR reaction for TaqMan gene expression assays contained 1 μ l cDNA solution, 12 μ l Master Mix and 1.25 μ l TaqMan gene expression assay. Reactions were made up to a final volume of 25 μ l with sterile water. All experiments were performed in triplicate and the real-time PCR protocol was as following: denaturation by a hot start at 95 °C for 10 min, followed by 50 cycles of a two-step program (denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min). For all the included gene targets, only the threshold cycle (Ct) values below 40 were included in the subsequent data analysis. Gene expression of the target gene was normalized to the mean Ct of *B2M* and *GUSB* applying the formula $2^{-\Delta C_t}$, where $\Delta C_t = C_t \text{ target gene} - C_t \text{ reference genes}$. The normalization, using the mean of the two reference genes, could be applied as target and reference genes were amplified with comparable efficiencies (data not shown).

Statistical analysis. The statistical analysis of data from both qPCR and flow cytometry experiments was performed using SIGMAPLOT (version 11; Systat Software Inc., San Jose, CA, USA). A paired or unpaired *t*-test was applied to test for differences between the groups of cells from the same individual or between two individuals, respectively. For non-normally distributed data, the corresponding nonparametric analysis was applied. Differences between groups were considered statistically significant, when $P < 0.05$. Unless otherwise specified, the data are presented as mean \pm SEM.

Results

Identification and separation of HLA-DR on the cells surface and in the intracellular compartment of CD3⁺ T cells

We initially wanted to investigate the cellular distribution of the HLA-DR antigen both on the cell surface/plasma membrane (HLA-DR mem) and in the intracellular space (HLA-DR IC) of CD3⁺ T cells using flow cytometry. A two-step strategy was developed, including an initial staining of HLA-DR mem was followed by fixation, permeabilization of the plasma membrane and a secondary staining of HLA-DR IC. To separate the two separate HLA-DR localizations, two anti-HLA-DR antibodies of the same clone, but with different fluorophores conjugations, were applied (anti-HLA-DR mem AF488; anti-HLA-DR IC AF647). The distribution of HLA-DR mem and HLA-DR IC in CD3⁺ T cells was compared to the distribution in CD19⁺ B cells, as the HLA-DR expression pattern in this APC is well characterized. Our flow cytometric assay necessitated a number of analytical and experimental controls to ensure the validity of such an approach.

Table 1 Primer and probe sequences, assay id and amplicon size for the genes analysed by qPCR.

Gene	Primers	FAM-labelled MBG probe	Amplicon size	Exon boundary
<i>HLA-DRB1*03</i>	F: 5'-CACCTATTGCAGACACAA-3' R: 5'-AACCACTCACAGAACAGA-3'	5'-ACCTTAGGATGGACTCGC-3'	136	2–3
<i>B2M</i>	F: 5'-GAGTATGCCTGCCGTGTG-3' R: 5'-AATCCAAATGCGGCATCT-3'	5'-CCTCCATGATGCTGCTTACATGTCTC-3'	109	3
<i>GUSB</i>	F: 5'-GAAATATGTGGTTGGAGAGCTCATT-3' R: 5'-CCGAGTGAAGATCCCCTTTTA-3'	5'-CCAGCACTCTCGTCGGTGACTGTTCA-3'	100	11–12
<i>CD19</i>	Hs01047410_g1		57	1–2

F, forward; R, reverse.

The specificity of the *HLA-DRB1*03* assay is located in the forward primer. For *B2M*, the forward primer is located in exon 2 and reverse primer in exon 4.

For the analysis, the gating control called the FMO [33] was applied to identify the HLA-DR mem and HLA-DR IC-positive events (Fig. 1). This approach is very useful for resolving weakly stained cells in multicolour panels in flow cytometry analyses [35, 36]. It also aids in placing a gate for a marker, which displays a continuous expression pattern, as is the case for HLA-DR on T cells. The applied FMO included a tube containing all markers and replacing the HLA-DR antibody of interest with an isotype control antibody. In this way, the HLA-DR single and double positive events could be determined, as exemplified in Fig. 1.

To verify the experimental setup, in which the plasma membrane-associated HLA-DR mem could be separated from the intracellular HLA-DR IC, it was essential to demonstrate and validate that a saturation of HLA-DR mem was obtained with the initial staining step. Hence, a saturation control was included in which all antibodies and staining conditions were applied, only omitting the permeabilization agent. Consequently, the HLA-DR IC signal should be absent in this sample if both the HLA-DR mem staining had saturated all cell surface-associated HLA-DR and no HLA-DR IC antibody entered the cell (Fig. 2). Initially, the optimal amount of applied anti-HLA-DR mem (L243-AF488) was determined with an 8-point titration experiment (0.025–4 µg; data not shown). The optimal amount (1 µg) was applied for staining with both anti-HLA-DR antibodies. For the saturation control experiments, it was observed as a general feature that a small HLA-DR IC signal could be detected in the saturation control for both the CD3⁺ HLA-DR mem⁺ T cells and CD19⁺ HLA-DR mem⁺ B cells (Fig. 2B). However, for the CD3⁺ T cells, this signal never surpassed the HLA-DR IC signal from the fully stained sample. Similar observations were made for the CD19⁺ B cells. A doubling of the applied amount of both HLA-DR antibodies did not increase or decrease the recorded intensities and cellular distributions of these markers (data not shown).

As a final investigation, the antibody staining procedure applied in the flow cytometric analysis was tested in a

confocal microscopy setting. The purpose of this qualitative analysis was to visualize the separation of the fluorescence signals obtained, when targeting HLA-DR mem and HLA-DR IC with two different anti-HLA-DR antibodies. In contrast to the flow cytometric analysis, in which CD3⁺ T cells and CD19⁺ B cells from peripheral blood were the target cells, isolated CD4⁺ T cells and CD19⁺ B cells were used for the confocal microscopy investigation. With these cells, it was shown that CD4⁺ T cells presenting HLA-DR mem could be identified (Figure S1A, top panel). In contrast, CD4⁺ T cells presenting both HLA-DR mem and HLA-DR IC were almost absent (Figure S1B). To ensure that the observed HLA-DR signals in the CD4⁺ T cell population were not due to contaminating APCs, such as monocytes, the identification of these T cells included a CD3 antibody. For the CD19⁺ B cells, the HLA-DR proteins were present in both the plasma membrane and intracellular compartment of the entire cell population (Figure S1A, bottom panel). The purity of the isolated CD4⁺ T cells and CD19⁺ B cells was $97.6 \pm 2.2\%$ and $96.6 \pm 0.4\%$, respectively, as determined by flow cytometry ($n = 2$; data not shown).

Dimorphic distribution of HLA-DR in the plasma membrane and intracellular compartment of CD3⁺ T cells and CD19⁺ B cells

Based on the abovementioned staining and gating strategy and experimental approach, we identified the fraction of CD3⁺ T cells from peripheral blood in which HLA-DR could be found in either the plasma membrane or the intracellular compartment or in both. An identical procedure was carried out for autologous CD19⁺ B cells, to ensure a positive reference. The presence of HLA-DR mem on CD3⁺ T cells and CD19⁺ B cells differed significantly in terms of the percent-wise distribution. For the T cells, $18.2 \pm 4.2\%$ CD3⁺ HLA-DR mem⁺ cells were identified, while this number was 98.8 ± 0.5 for the B cells ($n = 10$). The associated *P*-value was 0.002. Moreover, the corresponding median fluorescence intensity (MFI) values were also markedly different with a nearly 14 times greater MFI

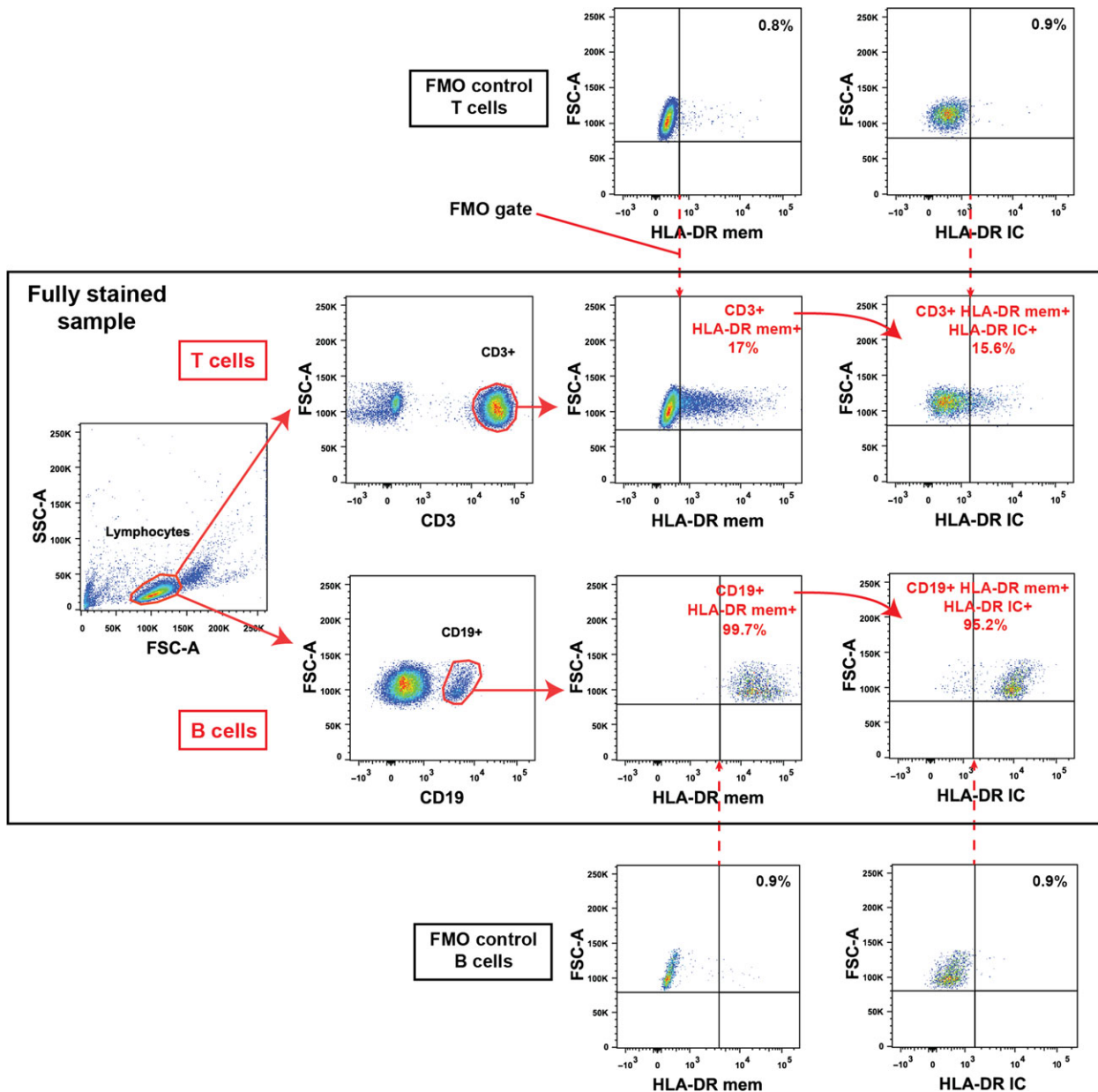


Figure 1 Improved flow cytometric gating strategy identifies HLA-DR in the plasma membrane and intracellular compartment of CD3⁺ T cells and CD19⁺ B cells. Initially, the lymphocyte population was gated in a forward scatter/side scatter plot. From the lymphocytes, the CD3⁺ T cells and CD19⁺ B cells were subsequently gated. Subsequently, the CD3⁺ HLA-DR mem⁺ (17%) and CD19⁺ HLA-DR mem⁺ (99.7%) populations were identified with pre-defined gates set using the fluorescence minus one (FMO) control for HLA-DR mem. In the last step, it was determined, which HLA-DR mem⁺ cells also carried the HLA-DR protein intracellularly. For this purpose, gates set with the FMO controls were used, identifying the double positive CD3⁺ HLA-DR mem⁺ HLA-DR IC⁺ (15.6%) and CD19⁺ HLA-DR mem⁺ HLA-DR IC⁺ (95.2%) populations. For all the FMO controls, the threshold was set so that a maximum of 1% of the events fell into the double positive quadrant. The plots are from one donor as a representative example of the 10 included donors.

value detected for the CD19⁺ B cells ($23,047 \pm 2059$) than the equivalent value for the CD3⁺ T cells (1662 ± 138). For the CD3⁺ T cells, $17.9 \pm 2.5\%$ of the CD3⁺ HLA-DR mem⁺ cells also contained HLA-DR IC. This was significantly higher for the CD19⁺ B cells (96.9 ± 0.9 ; $P < 0.001$). Here, the MFI for HLA-DR IC in the CD19⁺ B cells (9227 ± 596) was approximately 5.5-fold higher compared to the MFI for the CD3⁺ T cells

(1748 ± 152). Finally, the analysis identified $3.0 \pm 0.7\%$ CD3⁺ HLA-DR IC⁺ and these were all HLA-DR mem⁺ (Fig. 3A). The corresponding CD19⁺ HLA-DR IC⁺ B cells amounted to $95.9 \pm 1.2\%$ and these were also all HLA-DR mem⁺. To investigate whether the T cells, which presented cell surface-associated HLA-DR to the greatest extent, also carried the largest amount of HLA-DR IC, a correlation for HLA-DR in the two compartments was

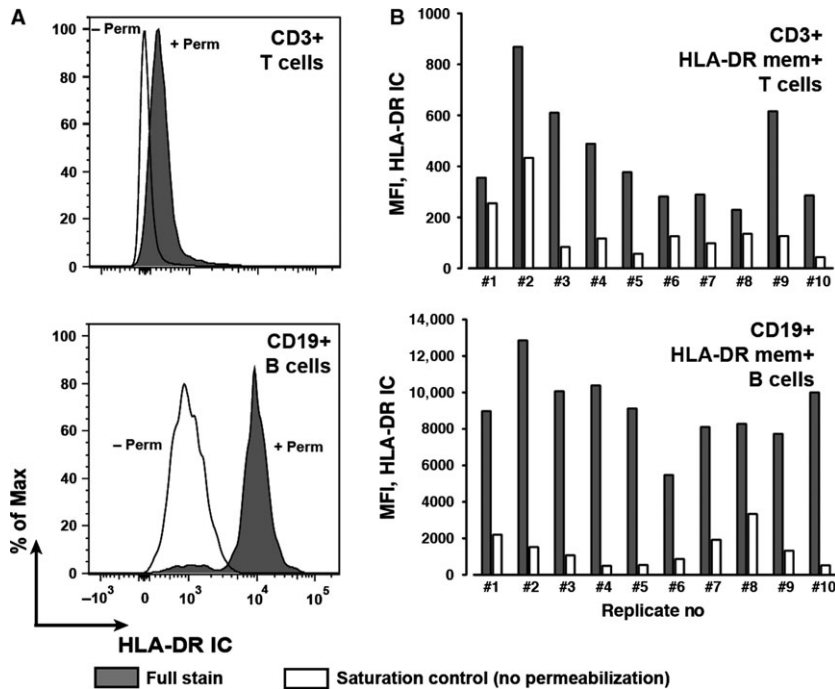


Figure 2 Comparison of HLA-DR IC signal in CD3⁺ T and CD19⁺ B cells with/without permeabilization of the outer plasma membrane. To test for saturation of cell surface HLA-DR (HLA-DR mem) with the initially applied anti-HLA-DR antibody, a saturation control was made for all the included donors. For the control, the entire staining setup was applied, only omitting the permeabilization agent. Subsequently, the gating strategy displayed in Fig. 1 was applied to identify the CD3⁺ HLA-DR mem⁺ T cells and CD19⁺ HLA-DR mem⁺ B cells. (A) The histograms show the HLA-DR IC signals for the HLA-DR mem⁺ cells for the fully stained sample and the saturation control. The histograms are a representative example of the 10 donors included in the study. (B) For all the 10 donors, there was a detectable HLA-DR IC signal present in the saturation control for both the CD3⁺ T cells and the CD19⁺ B cells. However, this signal was always lower than the specific HLA-DR IC signal detected in the fully stained sample.

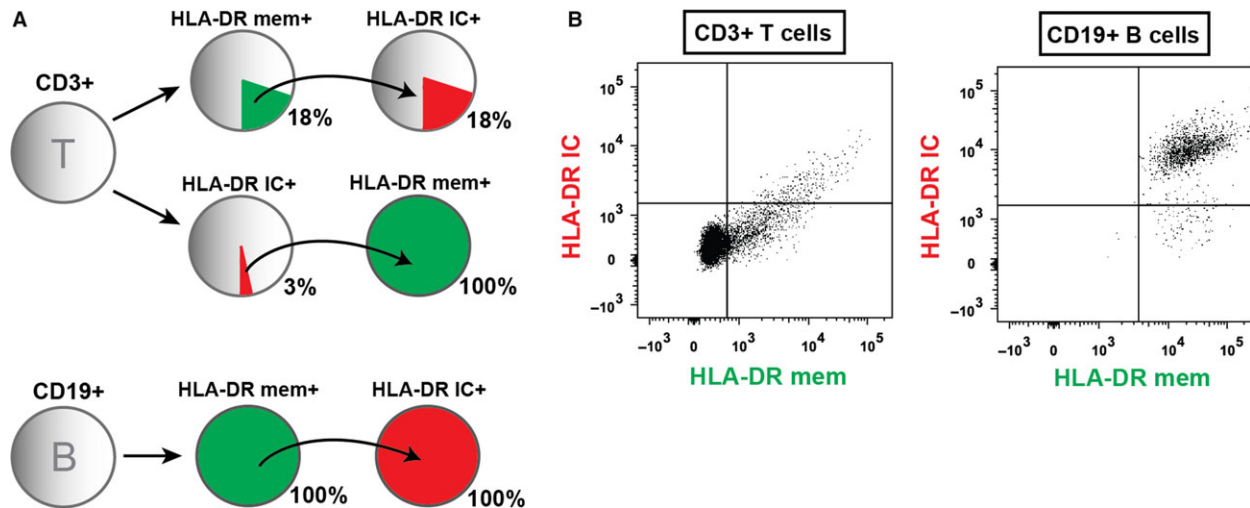


Figure 3 Correlation between HLA-DR mem and HLA-DR IC in CD3⁺ T cells and CD19⁺ B cells. (A) The distribution of HLA-DR mem and HLA-DR IC determined by flow cytometry for CD3⁺ T cells and CD19⁺ B cells. The circle charts visualize the obtained results ($n = 10$). (B) The gating strategy presented in Fig. 1 was employed to identify the HLA-DR mem⁺ and HLA-DR IC⁺ CD3⁺ T cells and CD19⁺ B cells in the presented dot plots. These plots were made to visualize a possible correlation between HLA-DR in the two cellular compartments. Both dot plots are representative examples of the included donors ($n = 10$).

made. This revealed that a positive correlation between these two proteins existed, both for CD3⁺ T cells and CD19⁺ B cells (Fig. 3B).

Relative contribution of CD4⁺ and CD8⁺ T cells to CD3⁺ HLA-DR mem⁺ T cell subset

In addition to the results about the presence of HLA-DR mem and HLA-DR IC for CD3⁺ T cells, it was

investigated to which extent CD4⁺ and CD8⁺ T cells each contribute to the CD3⁺ HLA-DR mem⁺ subset. Accordingly, CD3⁺ CD4⁺ HLA-DR mem⁺ and CD3⁺ CD8⁺ HLA-DR mem⁺ T cells (hereafter referred to as CD4⁺ HLA-DR mem⁺ and CD8⁺ HLA-DR mem⁺ T cells) were identified in a flow cytometric analysis of PBMCs. The presence of HLA-DR mem was evaluated for these cells (Fig. 4A, top panel) using the FMO approach described in Fig. 1. This resulted in the detection of $8.9 \pm 1.6\%$ CD4⁺

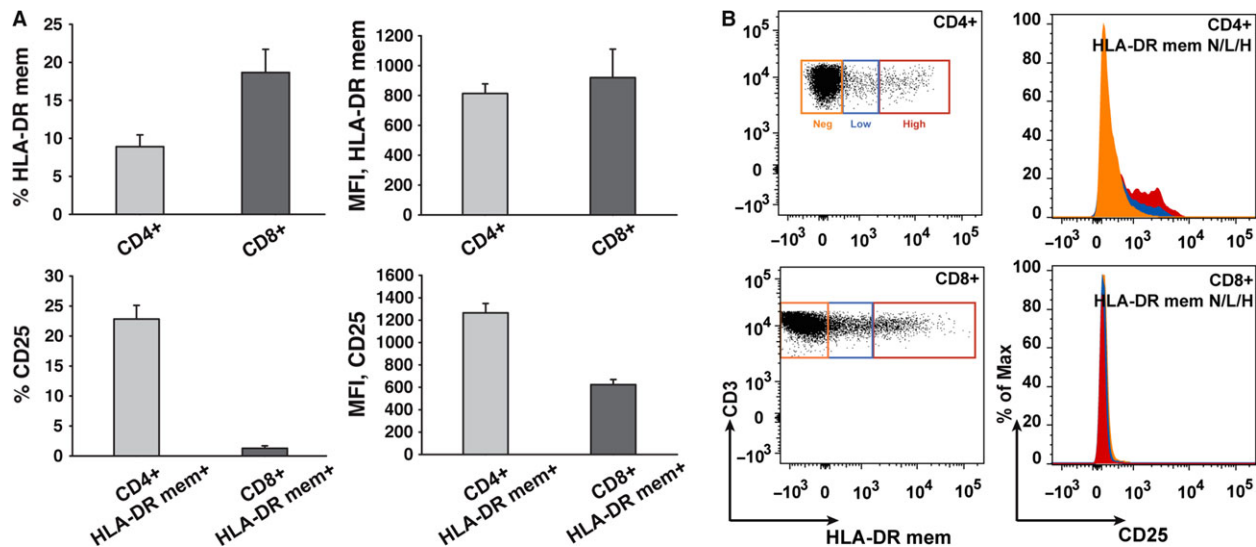


Figure 4 Contribution of CD4⁺ and CD8⁺ T cells to the CD3⁺ HLA-DR mem⁺ T cell population. The relative contribution of CD4⁺ and CD8⁺ T cells to the CD3⁺ HLA-DR mem⁺ T cell population was investigated by staining PBMCs with antibodies against CD3, CD4, CD8, CD25, and HLA-DR mem. (A) Using the gating strategy described in Fig. 1, the HLA-DR mem-presenting CD4⁺ and CD8⁺ T cells were identified (top panel, left). In addition, the corresponding median fluorescence intensity (MFI) values for HLA-DR mem were obtained (top panel, right). Subsequently, the percent-wise co-expression of CD25 was determined for the CD3⁺ CD4⁺ HLA-DR mem⁺ and CD3⁺ CD8⁺ HLA-DR mem⁺ cells (bottom panel, left) along with the corresponding MFI (bottom panel, right). Data are presented as the mean \pm SEM ($n = 6$). (B) The highest degree of CD25 expression was observed for the CD4⁺ T cells, which presented the most HLA-DR mem (top panel). In contrast, CD25 was found to the greatest extent of the CD8⁺ T cells presenting no HLA-DR mem (bottom panel).

HLA-DR mem⁺ T cells and $18.7 \pm 3.1\%$ CD8⁺ HLA-DR mem⁺ T cells ($n = 6$). As the CD4/CD8 ratio in peripheral blood is approximately two for adults [37, 38], the CD4⁺ and CD8⁺ T cells each contributed roughly equally to the entire CD3⁺ HLA-DR mem⁺ T cell population.

To further characterize the HLA-DR mem-presenting CD4⁺ and CD8⁺ T cells, the co-expression of another T cell activation marker, CD25, was also investigated for these cells. Of the CD4⁺ HLA-DR mem⁺, $22.8 \pm 2.3\%$ co-expressed CD25 (Fig. 4A, bottom panel). Additionally, CD25 was enriched in the CD4⁺ HLA-DR mem⁺ population, when compared to the corresponding CD4⁺ HLA-DR mem⁻ population, for which the expression of CD25 was $12.7 \pm 1.2\%$ (Fig. 4B). In contrast, the CD25 expression was greatest in the HLA-DR mem⁻ subset of the CD8⁺ T cells ($3.5 \pm 0.8\%$) as compared to the CD8⁺ HLA-DR mem⁺ T cells ($1.3\% \pm 0.3$).

Contrasting expression profiles of HLA-DRB1 mRNA in CD4⁺ T cells and CD19⁺ B cells

To link the presence of the HLA-DR protein in T cells to its transcription, an investigation of the *HLA-DRB1* mRNA expression by qPCR was performed. The assay targeted a specific allele of the most prevalently expressed beta subunit of the heterodimeric HLA-DR protein, namely the beta 1 (B1) chain. For the specific allele, we chose *HLA-DRB1*03* (Table 1), which is a part of the

DR52 haplotype (*HLA-DRB1*03*, *11, *12, *13 and *14) [39]. The amount of *HLA-DRB1* transcripts in this haplotype is the most abundant, compared to other haplotypes [40], making it a plentiful and advantageous target. To ensure that only the desired target was amplified, the *HLA-DRB1*03*-specific assay was initially tested on a panel of PBMCs from 13 individuals with *HLA-DRB1* alleles representative of the major DR haplotypes [39] (data not shown). Accordingly, the *HLA-DRB1*03*-positive individuals were selected, when they had either the homozygous *HLA-DRB1*03* genotype or the heterozygous *HLA-DRB1*03/*15* genotype. For the *HLA-DRB1*03*-negative samples, individuals with either the homozygous *HLA-DRB1*15* genotype or the heterozygous *HLA-DRB1*01/*07* were selected.

Subsequently, the expression pattern of *HLA-DRB1*03* was determined for human, isolated CD4⁺ T cells and CD19⁺ B cells from peripheral blood. As for the flow cytometric analysis of HLA-DR mem and HLA-DR IC, the CD19⁺ B cells were included as a positive control. The qPCR analysis was performed with cells from individuals with either a positive or a negative *HLA-DRB1*03* genotype, as indicated on the section above. The results of the expression analysis are displayed in Fig. 5A. The difference, which was observed between CD4⁺ T cells and CD19⁺ B cells with an *HLA-DRB1*03*-positive genotype, corresponded to a 240-fold higher transcript quantity in the B cells. No detectable signal was obtained for the

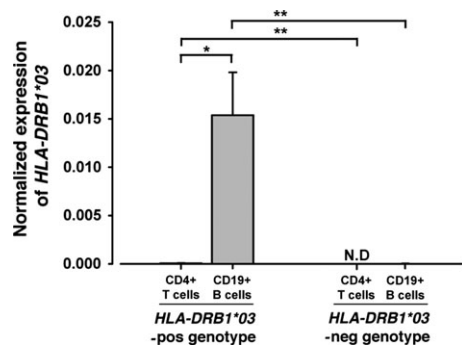


Figure 5 Quantifying mRNA from target genes by qPCR. The expression of HLA-DRB1*03 mRNA was investigated in isolated CD4⁺ T cells and CD19⁺ B cells from donors with an HLA-DRB1*03 positive or negative genotype ($n = 5$ for each genotype). The expression of the target gene was normalized to the mean of two reference genes (B2M and GUSB). Data is presented as mean ± SEM. Statistical significant differences are indicated with p-values; * $p < 0.05$; ** $p < 0.01$.

CD4⁺ T cells with an *HLA-DRB1*03*-negative genotype. In contrast, two of the five corresponding CD19⁺ B cell samples produced a detectable signal, yielding normalized expression values in a similar range as those for the CD4⁺ T cells from the *HLA-DRB1*03*-positive individuals. The corresponding Ct values were 37.18 ± 0.17 ($n = 2$) and 36.20 ± 0.52 ($n = 5$), respectively.

As determined by flow cytometry, the purity of the isolated CD4⁺ T cells was $98.8 \pm 0.37\%$ ($n = 10$; data not shown). Moreover, the presence of any cellular contamination, which could contribute to the minimally detected mRNA signal in the CD4⁺ T cells from the *HLA-DRB1*03*-positive individuals, was also investigated. First, the amount of mRNA for *CD19* was evaluated by qPCR. This B cell differentiation marker was included as B cells constitute the largest *HLA-DRB1*-expressing population in PBMCs, thus encompassing the greatest source of a possible cellular contamination. The *CD19* gene transcripts were approximately 1500 times more abundant in the CD19⁺ B cell samples compared to the CD4⁺ T cell samples, regardless of *HLA-DRB1*03* genotype (data not shown). Secondly, the presence of CD14⁺ monocytes was also assessed by flow cytometry. For the investigated samples, $0.97 \pm 0.10\%$ CD4⁺ CD14⁺ cells could be detected ($n = 8$ of the 10 included for qPCR analysis; all the *HLA-DRB1*01*-positive donors were included).

Discussion

The presence of HLA-DR antigens on T cells has been observed for more than four decades. The expression pattern and functionality of HLA-DR is well described for APCs but in the context of T cells, many unidentified aspects still remain.

With the use of a stringent staining and gating strategy for flow cytometry, this study investigated the distribution

of HLA-DR in the cellular membrane (HLA-DR mem) and intracellular compartment (HLA-DR IC) of human CD3⁺ T cells from peripheral blood. As a positive reference, this distribution was compared to that of autologous CD19⁺ B cells. We hypothesized that if the cell of question contained the functional protein in both the membrane and intracellular compartment, it was likely that an endogenous protein expression existed.

The flow cytometric analysis identified that approximately 18% of CD3⁺ T cells from peripheral blood contained HLA-DR mem (Fig. 3A). Other studies, which investigate HLA-DR on T cells, generally report a smaller amount of HLA-DR mem⁺ T cells. However, these reports range widely from less than 5% [37, 38] to approximately 10% [7], but also above 15% [41, 42]. Using FMO controls [43] to set less user-biased positive gates than previously performed, the estimation of HLA-DR⁺ cells is consequently more accurate. The identification of a uniformly HLA-DR mem-expressing, autologous B cell population validated the applied strategy. For future identification of HLA-DR on T cells, this strategy could therefore prove very useful. Only a few per cent of all CD3⁺ T cells in peripheral blood contained HLA-DR IC (Fig. 3A), and we believe that this fraction of CD3⁺ T cells has not previously been evaluated. Interestingly, a positive correlation between HLA-DR mem and HLA-DR IC was observed for both CD3⁺ T cells and CD19⁺ B cells (Fig. 3B). However, not all CD3⁺ HLA-DR mem⁺ T cells were also HLA-DR IC⁺ (Fig. 3A), which might be expected if an active protein synthesis was present. Regardless, the results obtained from the presented experiments point to significant differences for HLA-DR in the context of T cells and APCs of which the latter comprise a well-documented constitutive expression of this protein.

To further investigate the link between the presence of the HLA-DR protein on T cells to an endogenous expression, a transcriptional analysis of *HLA-DRB1* was performed, using an allele-specific qPCR assay, targeting the *HLA-DRB1*03* allele in isolated CD4⁺ T cells and CD19⁺ B cells. By choosing to target only one allele, it was intended to circumvent the issues associated with the extensive homology of the *HLA-DR* genes, thus providing unambiguous expression results. With the qPCR of *HLA-DRB1*03* presented here, only minimal amounts of transcripts could be detected in the CD4⁺ T cells from *HLA-DRB1*03*-positive individuals, while autologous CD19⁺ B cells clearly possessed an active gene transcription of *HLA-DRB1* (Fig. 5). The marginally positive signals obtained for the T cells were very close to the expected and inevitable non-specificity of the assay. It was not expected that the *HLA-DRB1*03*-negative CD4⁺ T cells could yield a similar non-specific signal, as these cells would not produce B cell-comparable amounts of *HLA-DRB1* transcripts, given the existence of such a production in the CD4⁺ T cells. Moreover, a cellular

contamination with approximately 1% of residual monocytes, known to express both HLA-DR [44] and CD4 [45], could very likely have contributed to the *HLA-DRB1*03* mRNA in the relevant CD4⁺ T cell samples. In total, this suggests that the signal detected in the CD4⁺ T cells with an *HLA-DRB1*03*-positive genotype could be non-specific or ascribed to a possible cellular contamination from APCs.

When correlated with the flow cytometric analysis of HLA-DR, the results from the qPCR assay signify that the presence of HLA-DR on the surface of a rather constant-sized population of circulating CD4⁺ T cells in healthy individuals cannot be explained by a concomitant presence of an active gene expression. Even if the specific mRNA may have been partially degraded in the HLA-DR-presenting CD4⁺ T cells, it can be still be reasonably expected that these cells would give rise to a detectable *HLA-DRB1* mRNA signal, if an expression was present, as they constituted roughly 9% of the investigated cells. However, the results do not exclude the possibility that an inducible, endogenous expression of HLA-DR is present in these T cells. They also do not exclude the possibility that mRNA levels and protein expression of HLA-DR do not correlate in the investigated T cells. Nevertheless, this study showed tendencies for circulating HLA-DR-presenting CD4⁺ T cells in healthy individuals, thus describing the physiological details of HLA-DR on T cells. These characteristics may be different for CD4⁺ HLA-DR mem⁺ T cells found in others tissues, such as in the lymph nodes, where elevated levels of HLA-DR-presenting T cells have been observed [41]. To support these theories and to fully appreciate the presence and dynamics of HLA-DR on human T cells, further analyses are required. These could include activated CD4⁺ T cells in lymphoid tissue, such as the palatine tonsils, or in individuals with constantly activated T cells, such as HIV-infected patients [46]. Concomitant analysis of CIITA expression would also further complement the HLA-DR analysis.

In classical terms, HLA-DR has been used to identify activated T cells [5–8]. Our analyses revealed that CD4⁺ and CD8⁺ T cells each account for approximately 50% of the entire CD3⁺ HLA-DR mem⁺ subset in peripheral blood. However, the presence of HLA-DR mem was seemingly not confined to activated CD4⁺ and CD8⁺ T cells, as evaluated by a co-expression CD25 (Fig. 4A). In effect, the expression of CD25 on the CD8⁺ HLA-DR mem⁺ T cells was minimal, while the co-expression of the two proteins was found to correlate to a higher degree for the CD4⁺ T cells (Fig. 4B). Although both CD4⁺ HLA-DR mem⁺ [4, 47] and CD8⁺ HLA-DR mem⁺ T cells [48] found in peripheral blood have been associated with regulatory functions, the difference in the expression of CD25 presented in this study could indicate distinct functionalities of these two cellular subsets. However, further investigations are required to substantiate this hypothesis.

In terms of functionality, HLA-DR on T cells has mainly been associated with immunosuppressive signals, inducing either apoptosis or anergy in activated T cells [4, 49, 50]. However, reports about T cell activation can also be found [26, 32]. Nonetheless, it seems that the function of HLA-DR in APCs cannot simply be extrapolated to T cells, which is based on a number of observations from this and other studies. In the current study, clear differences were demonstrated for the presence of HLA-DR both at the protein and transcript level, when comparing CD4⁺ T cells to CD19⁺ B cells. Others describe how T cell–T cell and T cell–APC interactions have significantly different effects on the responder T cells. Although TCR involvement, and consequently also HLA-DR, and co-stimulatory molecules are present for both types of cellular interaction [50], the resulting cytokine production (IL-2 and IL-4) and Ca²⁺ influx differ [49, 51, 52]. Moreover, most T cell–T cell interactions seemingly result in the induction of anergy, further underlining these differences [50]. In relation to key features of APCs, the mechanism for antigen acquisition remains unidentified for T cells [12], while other studies have investigated other requirements of antigen presentation in these cells, including HLA-DM, CLIP and proteolytic enzymes [12, 50]. Finally, the expression of essential co-stimulatory molecules, including CD80 and CD86, has been investigated. While some report an endogenous expression by T cells [50], others demonstrate an acquisition of functional molecules from APCs [32, 53, 54]. One of these studies demonstrated a simultaneous acquisition of HLA-DR by CD4⁺ T cells in a contact-dependent manner [32], but also a contact-independent transfer of HLA-DR has been shown [27]. It has been recognized that immune cells are particularly prone to engage in such molecular exchange, bestowing new functional phenotypes on the recipient cells [55–61]. This challenges the classical subdivision of immune cells, which is based on such functional phenotypes, and also expands the regulatory mechanisms of the immune system. In relation to HLA-DR, many studies have used allogeneic stimulator cells to activate T cells, as part of a mixed lymphocyte culture, thereby inducing the presence of HLA-DR. However, if acquisition of HLA-DR and other molecules can occur in such a cell culture, it will have a major impact on future study design and interpretation of data and possibly also explain some of the contradictory results reported about HLA-DR in T cells to date [50].

In this study, we further elucidated the presence of HLA-DR on human T cells. To the best of our knowledge, no study has to date investigated the direct *HLA-DRB1* expression in human CD4⁺ T and CD19⁺ B cells from peripheral blood and correlated it to the concurrent presence of HLA-DR antigens in the plasma membrane and intracellular compartment. Using this approach, we were able to document fundamental differences between

HLA-DR in connection with T cells and APCs in healthy individuals, both at the protein and the transcriptional level. Moreover, we demonstrated that HLA-DR is not limited to activated T cells, thus challenging the use of HLA-DR as a T cell activation marker without supplementary information. Collectively, this study adds more layers to the understanding of the presence of HLA-DR on T cells. With a possible connection between HLA-DR and regulatory functions of T cells, questions of a possibly significant role of HLA-DR for T cells emerge. It also underlines the immense complexity of one of the most important molecules for the antigen specificity and function of the adaptive immune system.

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Competing interest

The authors declare that they have no competing interests.

Author contributions

ALSR designed, performed and analysed data from the flow cytometric analyses, qPCR and confocal microscopy experiments and also drafted the manuscript. RS designed and performed the qPCR experiments and edited the manuscript. LHP performed the experimental procedures and the data analysis of the confocal microscopy and edited the manuscript. AS, MMJ and KV contributed to the study design, supervised the study and edited the manuscript. All authors read and approved the final version of the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Confocal microscopy analysis of HLA-DR mem and HLA-DR IC in CD4⁺ T cells and CD19⁺ B cells.