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Expression of CD1d and presence of invariant NKT cells in classical Hodgkin lymphoma

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3 **We studied CD1d-restricted invariant natural killer T (iNKT) cells in classical Hodgkin**
4 **lymphoma (cHL). Tumor cells stained positive for CD1d in 21/44 cHL cases, whereas in**
5 **non-Hodgkin lymphoma (NHL) only 9/31 stained positive. In contrast, CD1c expression**
6 **was more common in NHL. The percentage of iNKT cells in cHL cell suspensions was**
7 **similar to the percentage in reactive lymph nodes and was not related to the CD1d**
8 **expression status of the tumor cells. In conclusion, we found expression of CD1d in**
9 **HRS cells in half of the cHL cases, and also observed a substantial population of iNKT**
10 **cells in cHL cell suspensions.**

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20 Classical Hodgkin lymphoma (cHL) is a B-cell neoplasm characterized by a minority of
21 neoplastic cells, the Hodgkin and Reed-Sternberg (HRS) cells, which are located within an
22 extensive infiltrate of reactive cells, such as T cells, B cells, plasma cells, stromal cells,
23 eosinophils and macrophages. The HRS cells shape their microenvironment by attracting
24 specific favorable T cell subsets, like regulatory T (Treg) and T helper 2 (TH-2) cells and
25 producing factors such as CC-chemokine ligand 17 (CCL17 or TARC), transforming growth
26 factor (TGF)- β and interleukin (IL)-10 [1]. Additionally, HLA class I is downregulated in HRS
27 cells in 65% of the cHL cases and HLA class II in 40%, possibly as a strategy to escape from
28 an effective immune surveillance [2]. All together, these factors may suppress the
29 development of an effective anti-tumor response and provide a microenvironment that is
30 favorable for the HRS cells.

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44 Recent studies have suggested that T cells restricted to non-classical MHC class I-like CD1
45 molecules may be involved in immune surveillance of hematological malignancies [3-6]. CD1
46 molecules are non-classical MHC class I-like molecules that present lipid antigens to T cells,
47 triggering a specific immune response. Of the five CD1 isoforms (CD1a, CD1b, CD1c, CD1d
48 and CD1e) expressed in human tissue, only CD1c and CD1d are expressed in B cells [7].
49 The T cell receptors (TCRs) of T cells that recognize CD1c are indistinguishable from those
50 that recognize MHC class I or II complexes. Most CD1c-restricted T cells appear to be TH-1-
51 like cells that provide adaptive immunity to microbial-lipid antigens [8]. In contrast, CD1d
52 presents lipid antigens to natural killer T (NKT) cells. The best characterized CD1d-restricted

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3 NKT cells in humans are the invariant NKT (iNKT) cells, also known as type I NKT cells.
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5 These cells are characterized by the expression of an invariant V α 24Ja18 chain paired to a
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7 semi-invariant V β 11 chain [9]. Two distinct functional subsets of human iNKT cells are
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9 recognized in terms of cytokines production and cytotoxic activation. CD4⁻ iNKT cells are
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11 more likely to be cytotoxic and produce TH-1 type cytokines, whereas CD4⁺ iNKT cells are
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13 more likely to produce both TH-1 and TH-2 type cytokines after stimulation [10]. Several
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15 studies have shown the importance of iNKT cells in B cell malignancies [4-6]. In chronic
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17 lymphocytic leukemia (CLL) CD1d⁺ tumor cells loaded with α GalCer can activate iNKT cells
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19 and in turn induce cell death of tumor cells [4]. Malignant multiple myeloma is characterized
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21 by a reversible functional defect in iNKT cells in comparison with nonprogressive myeloma
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23 and premalignant myeloma [5]. Furthermore, iNKT cells were shown to induce effective anti-
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25 lymphoma responses and were essential for the survival of mice in murine lymphoma models
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27 [6]. The role of iNKT cells in the immune surveillance in cHL is unknown. Here, we examined
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29 the expression of CD1c and CD1d in HRS cells and tumor infiltrating cells in 44 cHL cases,
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31 as well as in four cHL cell lines. Furthermore, we studied the presence of iNKT cells in cell
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33 suspensions of ten cHL cases.

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37 CD1c was undetectable in cHL cell lines L428, KMH2, L1236 and U-HO1 (data not shown).
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39 In tonsil tissue, CD1c stained positive in the mantle zone (MZ) and predominantly negative in
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41 the germinal centers (GC) of secondary lymphoid follicles (Figure 1A) consistent with the
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43 previously reported expression pattern [11]. HRS cells in cHL were consistently CD1c
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45 negative in all cases (Figure 1B), while 14 out of 39 non Hodgkin lymphomas (NHL) were
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47 positive (Table I). The absence of CD1c expression in both GC B cells and HRS cells is
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49 consistent with the current assumption that HRS cells are derived from GC B cells [12]. CD1c
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51 expression has been reported in some other B-cell NHL subtypes, including GC B cell
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53 derived follicular lymphoma [11]. In B-cell NHL an inverse correlation was noticed between
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55 CD1c and the proliferative activity assessed by expression of Ki-67 [11]. This is in line with
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57 the lack of CD1c staining in GC B cells that show a high proliferation index and high CD1c
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59 expression in the MZ B cells that are in a resting state. Ki-67 was shown to be highly
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3 expressed in HRS cells [13] correlating with the lack of CD1c expression. CD1c expression
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5 was found in reactive cells in all cases with a percentage varying from a few to the vast
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7 majority, similar to the CD1c expression pattern observed in interfollicular regions of tonsil
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9 tissue.

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11 In contrast, CD1d was detected in all four cHL cell lines, varying from weakly positive in U-
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13 HO1 to strongly positive in the other three cHL cell lines (Figure 1C-F). By flowcytometry we
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15 validated that CD1d was present on the membrane in cHL cell lines (data not shown). In
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17 normal tonsil tissue CD1d staining was observed mainly in the MZ and showed no positive
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19 staining in the GC (Figure 1G) consistent with the reported results [14]. CD1d was detected in
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21 HRS cells in 21 out of 44 cHL cases (48%) (Figure 1H, Table I, supplementary table I),
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23 showing both cytoplasmic and membranous staining. **No correlation was found with EBV**
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25 **status and CD1d expression (Table I).** CD1d was also positive in the reactive background,
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27 usually in more than half of the reactive cells. Nine of the 39 NHL cases were positive for
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29 CD1d (23%, Table I), i.e. in 3 out of 19 GC B cell derived NHL and in 6 out of 20 non-GC B
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31 cell NHL (Table I, supplementary table I). The CD1d expression in HL is remarkable since
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33 GC B cells and the majority of the GC B cell derived NHL are negative. Positivity of HRS
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35 cells indicates that these cells might have acquired CD1d expression during malignant
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37 transformation. CD1d expression has been detected in some B cell malignancies, such as
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39 CLL [4] and multiple myeloma [5]. In these B cell malignancies, iNKT cells activated by α -
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41 GalCer loaded CD1d⁺ tumor cells resulted in the induction of apoptosis of the tumor cells in
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43 vitro [4,5].
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49 Since half of the cHL cases were positive for CD1d in HRS cells, we further investigated the
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51 iNKT cell population in cell suspensions of cHL and reactive lymph node (RLN). The mean
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53 percentage of iNKT cells was 4% (range 0.8-8%) in cHL and 4% (range 0.4-7%) in RLN
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55 (Table I). iNKT cells were present at similar percentage in either CD1d positive or CD1d
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57 negative cHL cases. Two distinct iNKT subsets have been distinguished as Th1 type (CD4⁻
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59 iNKT) and Th2 type (CD4⁺ iNKT) in terms of cytokine production and cytotoxic activation [10].
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We observed a similar percentage of CD4⁺ iNKT cells, i.e. around 50%, in cHL and RLN

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3 (Table I). Given the high expression of CCL17 in HRS cells of cHL [15] and the high
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5 expression of the CCL17 receptor, CCR4, on CD4+ iNKT cells [16], it might be speculated
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7 that especially the CD4+ iNKT cells are present in the close vicinity of HRS cells and might
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9 favor the survival and growth of HRS cells. Since it is known that the rosetting CD4+CD26- T
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11 cells in HL have several features of anergy [17], the CD1d-restricted iNKT cells might also be
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13 dysfunctional or anergic favoring HRS cells survival by escaping from an effective
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15 immunosurveillance in cHL.
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18 Taken together, we showed expression of CD1d in HRS cells of cHL cell lines and a
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20 significant proportion of cHL cases as well as the presence of a marked population of iNKT
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22 cells in the reactive background of cHL. The CD1d – iNKT cell axis might play a role in the
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24 disturbed immunoregulation in cHL.
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27 28 29 **Methods**

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31 *Patient samples and cell lines.* Frozen cHL samples consisted of 39 cases of nodular
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33 sclerosis (NS), four cases of mixed cellularity (MC) and one case of not otherwise specified
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35 cHL (NOS). Frozen samples of 39 NHL cases were used and consisted of 13 chronic
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37 lymphocytic leukemia, 7 mantle cell lymphoma, 9 follicular lymphoma and 10 diffuse large B
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39 cell lymphoma cases. Cell suspensions were available of eight NS and two MC cHL cases.
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41 The cHL cell lines L428, KMH2, L1236 and U-HO1 [18] were cultured in RPMI-1640 medium
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43 (Lonza Walkersville, Walkersville, MD USA) supplemented with ultraglutamine-1, 100 U/ml
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45 penicillin/streptomycin, 10% fetal calf serum (5% for L428) (Lonza Walkersville).
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49 *Immunohistochemistry.* Immunohistochemistry was performed with monoclonal antibodies
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51 against CD1c (L161, Abd Serotec, Oxford, UK) and CD1d (NOR3.2, Abcam, Cambridge, UK)
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53 (both 1:100) on frozen cHL tissue sections and cytopins using standard laboratory protocols
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55 and appropriate positive and negative controls. Cases were defined positive when more than
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57 50% of HRS cells showed a clear staining.
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60 *Flow Cytometry.* 1×10^6 cells were stained simultaneously with PE labeled Mouse Anti-Human
iNKT Cell (clone 6B11, BD Bioscience, San Jose, CA), FITC labeled anti-TCR V β 11 (clone

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3 C21, Beckman Coulter, Fullerton, CA) and CyQ labeled anti-CD4 (clone Edu-2, IQ Products,
4 Groningen, Netherlands). A total of 100,000 lymphocytes was collected. The percentage of
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iNKT cells was determined by positive staining for both 6B11 and V β 11, and the percentage of CD4+ iNKT cells was determined by the percentage of CD4+ cells among the gated iNKT cells by flow cytometer (Calibur, Becton Dickinson, San Jose, CA). We used an iNKT cell clone as a positive control, and samples without primary antibody were included as a negative control. cHL cell lines were stained with PE labeled CD1d (clone 51.1, eBioscience, Hatfield, UK), and a IgG2b isotype control antibody, to show the presence of membrane CD1d.

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For Peer Review

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12 **Legend to the figure**
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16 Figure 1: CD1c and CD1d expression in cHL. Both CD1c and CD1d are positive in the
17 mantle zone area and predominantly negative in the germinal center area in tonsil (A and G).
18 Original image magnification: 200x. CD1c is negative in HRS cells (arrows), but positive in
19 reactive cells (B). CD1d is positive both in HRS cells (arrows) and reactive cells (H). Original
20 magnification 400x. CD1d is weakly positive in U-HO1, and strongly positive in the other
21 three cHL cell lines (C-F). Original image magnification: 400x.
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Table I: Comparison of expression of CD1c and CD1d in cHL and NHL

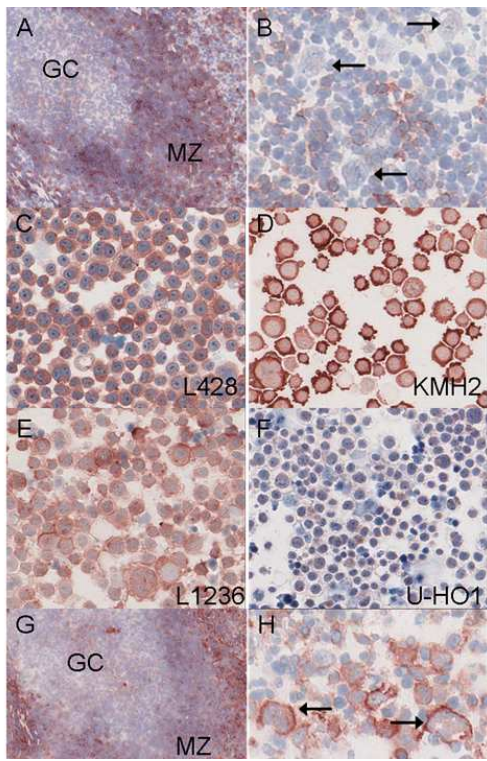
lymphoma		CD1c positive	CD1d positive
cHL (n=44)		0%	48%
	EBV+		42%
	EBV-		57%
NHL (n=39)		36%	23%
	CLL (n=13)	8%	31%
	MCL (n=7)	57%	14%
	FL (n=9)	67%	22%
	DLBCL (n=10)	30%	10%

cHL: classical Hodgkin lymphoma; NHL: non-Hodgkin lymphoma; CLL: chronic lymphocytic leukemia; MCL: mantle cell lymphoma; FL: follicular lymphoma; DLBCL: diffuse large B cell lymphoma

Table II: iNKT cells in cHL and RLN

	cases	Subtype	CD1d in HRS	% iNKT	% CD4+ iNKT
cHL	1	NS	+	5	64
	2	NS	+	2	54
	3	NS	+	2	8
	4	NS	+	2	59
	5	NS	+	1	59
	6	MC	-	8	40
	7	NS	-	6	76
	8	MC	-	5	69
	9	NS	-	4	38
	10	NS	-	0.8	39
RLN	1			7	40
	2			5	50
	3			4	53
	4			4	68
	5			0.6	58
	6			0.4	52

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