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SHORT COMMUNICATION

At diagnosis, diffuse large B-cell lymphoma patients show impaired rituximab-mediated NK-cell cytotoxicity

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Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin's lymphoma in adults. It is generally treated by a combination of chemotherapy and CD20-specific mAbs, such as rituximab, which act, at least partially, by activating antibody-dependent cell-mediated cytotoxicity (ADCC). ADCC involves NK cells, particularly the CD56^{dim} NK-cell subset expressing CD16, the low affinity Fcγ receptor. Here, we show that CD16 expression levels are decreased in a cohort of 36 newly diagnosed DLBCL patients compared with those in 20 healthy controls (HCs). CD137, a co-stimulatory molecule expressed on activated NK cells, was also expressed at lower levels in patients compared with controls. Cells sampled from our cohort also showed severely reduced degranulation activity when challenged with rituximab-coated tumor cells, which could not be corrected by stimulation with high doses of IL-2. These results suggest that rituximab-induced NK-cell ADCC could be defective in some DLBCL patients at diagnosis. These patients should be closely monitored and attempts made to improve their NK-cell function.

Keywords: CD137 · CD16 · diffuse large B-cell lymphoma · innate immunity · NK cell

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Introduction

Among non-Hodgkin's lymphoma subtypes in adults, diffuse large B-cell lymphoma (DLBCL) is the most common. Treatment of DLBCL combines specific antibodies (e.g. rituximab) with chemotherapy (R-CHOP) [1]. Rituximab is a humanized IgG1 mAb specific for CD20, which is expressed on healthy B cells and over-expressed on malignant B cells. Rituximab induces several mechanisms including direct signaling, complement-mediated cytotoxicity, and antibody-dependent cell-mediated cytotoxicity (ADCC) [2]. Rituximab-mediated ADCC mainly triggers innate effector cells, such as NK cells. Few studies have specifically

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focused on NK-cell activity in the context of B-cell lymphoma [3–5], even though many studies indicate that patients with enhanced antitumor immune responses may have better clinical outcomes [6–8]. In DLBCL patients, a lower absolute NK-cell count at diagnosis has been linked to poor survival [9]. This may be related to cytokine imbalances as a phase I study [10] showed that in vivo IL-2 stimulation of NK cells enhances the efficacy of rituximab when treating B-cell lymphomas.

As a part of the innate immune system, NK cells monitor for and eliminate infected or abnormal cells. To do this, they express an array of activating and inhibitory receptors [11]. Regulatory receptors control how malignant cells are eliminated, and several activating receptors, including NKp30, NKp46, NKG2D, DNAM-1, and the inducible co-stimulatory molecule CD137 (4–1BB), have been linked to antitumor immunity [12–14]. Efficient tumor cell elimination generally requires collaboration between these receptors, as described for myeloma or melanoma [12, 13].

In addition to regulatory receptors, NK cells also express CD16 (FcγRIIIA, gene *FCGR3A*), a low-affinity Fcγ receptor that can detect antibodies bound to tumor cells. CD16 is strongly and constitutively expressed on the highly cytotoxic $CD56^{dim}$ NK-cell subset, which represents about 90% of peripheral NK cells [15]. The clinical response to rituximab is known to be affected by *FCGR3A* polymorphisms [16–18], which may be a reflection of the role of CD16 in NK-cell-mediated ADCC with this treatment. This study has directly assessed NK-cell phenotype and CD16 expression levels in DLBCL patients at diagnosis while also studying ADCC function and CD137 upregulation.

Results and discussion

Reduced CD16 surface expression on NK cells from DLBCL patients

Blood samples were taken from DLBCL patients at diagnosis. Flow cytometric analysis was used to assess levels of activating NK-cell receptors involved in antitumor function. The results for 36 DLBCL patients and 20 healthy donors (HCs) are shown in Figure 1 and Table 1. Of all the markers analyzed, only DNAM-1, CD137, and intracellular perforin were significantly reduced in the overall NK-cell population in DLBCL patients compared to HCs. The $CD56^{dim}/CD56^{bright}$ ratio for PBMC from patients (median: 4.8 (SEM = 1.2; range: 0.4–35.5)) was comparable to that for HCs (7.5 (SEM = 1.5; range: 1.3–28.4), ns), ruling out an indirect effect on activation markers due to an imbalance of NK-cell subsets. In the $CD56^{bright}$ NK-cell subset, cells expressing NKp46 and DNAM-1 – both of which stimulate NK-cell functions – were significantly less abundant in patients ($p < 0.0001$ and $p = 0.0085$, respectively). This suggests that NK cells in DLBCL patients have a reduced activation potential. More importantly, expression of the FcγRIIIa receptor (CD16) on the $CD56^{dim}CD16^{+}$ subset was markedly reduced in patients at diagnosis (CD16 median fluorescence intensity (MFI): 4761) compared to HCs (MFI: 7957; $p = 0.004$; Fig. 1C). In patients with solid tumors, such as kidney carcinomas, no similar reduction in CD16 levels was observed (data not shown). The effects on receptor expression in DLBCL patients were not related to a soluble compound in the serum as incubating PBMCs from healthy donors with serum from DLBCL patients did

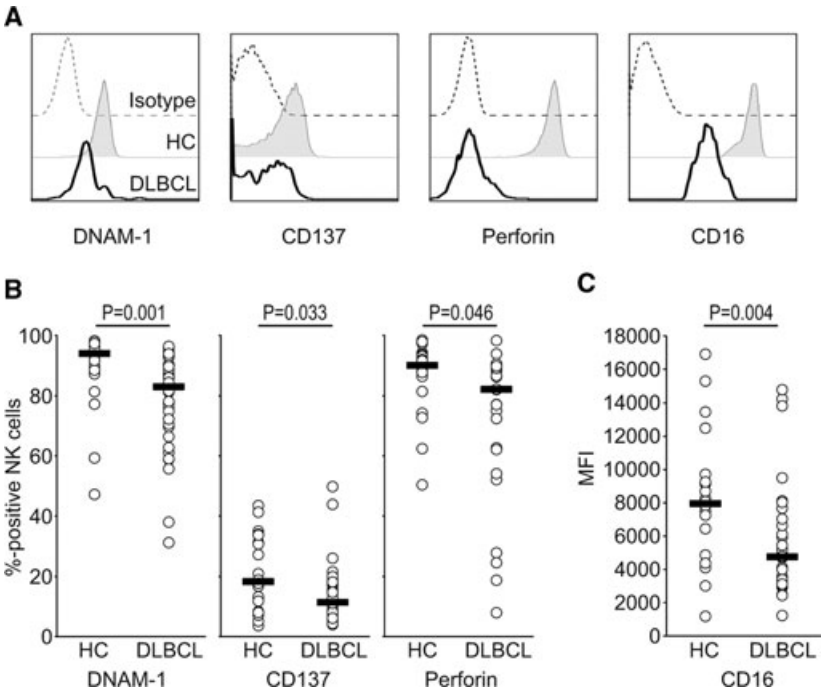


Figure 1. NK-cell phenotype in healthy controls (HCs) and diffuse large B-cell lymphoma (DLBCL) patients at diagnosis. (A) Representative flow cytometry histograms for DNAM-1, CD137, intracellular perforin, and CD16 expression on $CD3^{-}CD56^{+}$ NK cells are shown. Dashed line histogram: isotype controls; filled gray histogram: HC sample; solid line histogram: DLBCL sample. (B–C) Data from 36 DLBCL patients and 20 HCs are shown. (B) Percent DNAM-1, CD137, and perforin positive cells in the overall NK-cell population. (C) CD16 expression (MFI) on the $CD56^{dim}$ NK-cell subset is shown. Individual values are shown as open circles; the black bar marks the median value for each group. Data shown are pooled from 20 experiments performed. *p*-values indicated above plots were calculated using a Mann–Whitney test.

Table 1. NK-cell phenotype in healthy donors and DLBCL patients at diagnosis

	DLBCL patients		Healthy donors		*p*-value
	Median[a)]	Range[a)]	Median[a)]	Range[a)]	
Global $CD56^{+}CD3^{neg}$ NK population					
DNAM-1	83.1	31.3–96.5	94.1	47.3–98.3	0.0014
NKp30	48.5	10.7–94.0	58.0	3.4–89.3	ns[b)]
NKp46	67.8	7.7–95.4	70.0	28.1–95.8	ns
NKG2D	92.5	28.1–98.5	93.2	22.2–98.9	ns
HLA-DR	18.9	4.3–75.8	15.2	4.3–88.5	ns
CD137	11.5	4.1–49.9	18.4	3.7–43.6	0.0333
CD94	69.8	39.6–95.9	68.6	34.6–91.5	ns
NKG2A	50.5	22.8–88.3	42.4	18.6–74.4	ns
NKG2C	10.7	0.4–44.3	4.8	0.3–68.0	ns
Perforin	82.2	8.0–98.4	90.2	50.5–98.6	0.0462
$CD56^{dim}CD16^{+}$ NK-subset					
DNAM-1	85.6	29.5–97.9	95.2	53.6–99.0	0.0052
NKp30	46.4	8.6–95.2	52.8	3.3–93.8	ns
NKp46	66.7	6.7–96.6	68.4	21.8–96.1	ns
NKG2D	92.7	26.4–98.7	94.1	18.9–99.2	ns
HLA-DR	14.4	2.8–71.1	8.9	1.1–87.6	ns
CD137	12.8	4.4–50.2	19.6	3.8–48.4	0.0394
CD94	69.1	34.4–89.2	64.5	25.8–91.4	ns
NKG2A	49.0	13.9–86.2	38.6	15.6–71.0	0.0292
NKG2C	9.2	0.3–49.2	3.9	0.3–69.4	ns
Perforin	91.9	10.2–99.7	97.8	73.9–99.8	0.0114
CD16 (MFI)[a)]	4761	1229–14809	7957	1183–16947	0.0041
$CD56^{bright}CD16^{neg}$ NK-subset					
DNAM-1	77.4	24.4–94.8	88.9	31.3–95.3	0.0085
NKp30	49.6	11.1–77.7	55.1	7.0–80.2	ns
NKp46	68.7	19.7–96.2	88.8	65.5–95.8	<0.0001
NKG2D	88.8	32.3–98.1	82.0	33.1–97.1	ns
HLA-DR	37.6	9.9–84.6	48.7	16.1–96.0	0.0135
CD137	8.6	1.8–45.2	8.6	1.2–20.9	ns
CD94	82.5	16.7–98.7	89.5	61.6–95.6	0.0436
NKG2A	71.2	10.0–95.1	74.6	52.4–87.1	ns
NKG2C	10.8	0.0–39.4	8.4	0.7–44.3	ns
Perforin	34.0	1.0–90.0	37.2	9.1–78.2	ns

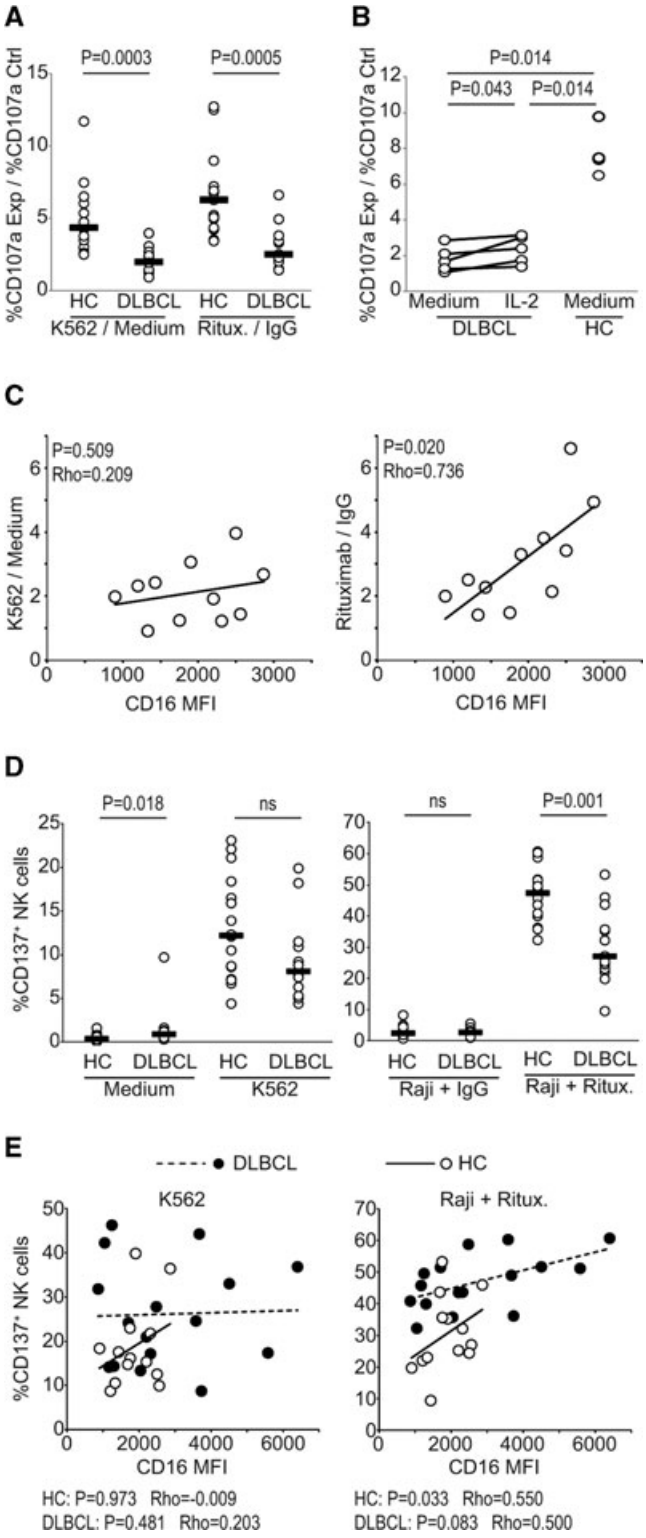
[a)] Values are reported as percent positive cells, except for CD16, which is reported as MFI.
[b)] ns, not significant.

not reduce CD16 expression, or cause any modifications to surface expression of DNAM-1, NKG2D, or CD137 (data not shown).

Defective ADCC activity in NK cells from DLBCL patients

Our phenotypic observations suggest that the cytotoxic function of NK cells from DLBCL patients at diagnosis is reduced compared to healthy subjects. To analyze this, we investigated NK-cell degranulation either as a part of natural cytotoxicity upon stimulation by the NK-sensitive K562 cell line, or in rituximab-mediated ADCC directed against the $CD20^{+}$ lymphoma cell line Raji. During cytotoxic degranulation, CD107a is transferred from cytolytic granules onto the cell surface, surface CD107a labeling can therefore be used as a surrogate marker for cytotoxic activity [19]. We assessed CD107a labeling on NK cells from 11 patients and 15 HCs, NK cells from patients had a strongly reduced capacity to delocalize CD107a after stimulation both with K562 (median experimental/control ratios: 2.0 for patients versus 4.4 for HCs; $p = 0.0003$) and with rituximab + Raji (median experimental/control ratios: 2.5 for patients versus 6.3 for HCs; $p = 0.0005$; Fig. 2A). Stimulating DLBCL NK cells with IL-2 during cytotoxicity assays increased their ADCC capacity slightly (median of experimental/control ratios: 1.7 in medium versus 2.4 with IL-2; $p = 0.043$) but did not fully restore ADCC to the levels measured for HCs (Fig. 2B). The CD107a experimental/control ratio in patients was also found to positively correlate with CD16

expression after stimulation by rituximab + Raji (Rho = 0.7; $p = 0.020$; Fig. 2C), but not after exposure to K562 (Rho = 0.2; $p = 0.509$). In our cohort, CD16 expression levels did not significantly associate with *FCGR3A* polymorphism (data not shown). Adding IL-2 to culture medium increased CD16 expression on NK cells from HCs, but this effect was very weak or negligible on DLBCL NK cells (data not shown). Taken together, these results confirmed that the reduced CD16 expression on patients' NK cells was, at least partially, responsible for the impaired rituximab-mediated ADCC measured.



CD16-mediated activation induces lower CD137 levels on DLBCL NK cells

Recently, Levy and colleagues [20] used a xenotransplanted human lymphoma model to show that the antilymphoma activity of rituximab could be enhanced by administering an anti-CD137 agonist mAb. To test whether ADCC-related NK-cell activation led to upregulated CD137 expression in patient samples, we analyzed CD137 expression in 13 DLBCL and 16 HC samples at the end of co-incubation. In vitro unstimulated NK cells express very low levels of CD137 (median in HCs: 0.35% and in patients: 0.9%; Fig. 2D). For both HCs and patients, the frequency of $CD137^+$ cells was moderately increased by stimulation with K562. This increase was dramatic after stimulation with rituximab-coated tumor B cells, while cells coated with an irrelevant antibody induced no increase. However, with rituximab the increase in CD137 expression was significantly greater for HC samples (median $CD137^+$ cells: 47.4%) than for DLBCL samples (median $CD137^+$ cells: 27.1%, $p = 0.001$). After stimulation by rituximab-coated Raji, the frequency of $CD137^+$ NK cells was positively correlated with CD16 expression in HCs, a similar trend was noted for DLBCL patients (Fig. 2E).

Figure 2. NK-cell degranulation. (A) Experimental/control degranulation ratio, calculated based on the percentage of $CD107a^+$ NK cells after stimulation divided by the percentage of $CD107a^+$ NK cells in appropriate control samples. K562/medium samples were exposed to K562 cell lines or medium (control). Ritux./IgG samples were exposed to Raji cells coated with rituximab or an irrelevant IgG (control). Symbols represent individual values; the black bar indicates the median for each group. *p*-values indicated above plots were calculated using a Mann–Whitney test. (B) The experimental/control degranulation ratio was calculated for NK cells co-cultured with rituximab- or irrelevant IgG-coated Raji cells. DLBCL samples ($n = 5$) were stimulated with IL-2 where indicated; HC samples ($n = 4$). Statistical significance was determined using a signed Wilcoxon rank test when comparing with/without IL-2 conditions for patients, and using a Mann–Whitney test when comparing patients and HCs. Data shown are pooled from three experiments performed. (C) CD16 levels (MFI) on NK cells from 11 patients plotted against degranulation ratios for NK cells stimulated by either K562 (left panel) or rituximab-coated Raji cells (right). Individual values and Spearman correlation curves are shown. (D) CD137 expression on NK cells from 16 HCs and 13 DLBCL patients after overnight co-culture of PBMCs with the indicated targets. Symbols represent individual values; the black bars represent the median for each group. *p*-values indicated above plots were calculated using a Mann–Whitney test. (E) Correlation between the percentage of $CD137^+$ NK cells and the MFI for CD16 after NK-cell co-culture either with K562 or rituximab-coated Raji cells is shown. Symbols represent individual values and the Spearman correlation curve for each group. Data shown are pooled from 11 experiments performed.

Concluding remarks

The data presented in this paper show that NK-cell-mediated ADCC may be impaired in DLBCL patients at diagnosis. How this affects individual responses to R-CHOP therapy remains to be determined. It will be necessary to monitor the NK-cell compartment in large patient cohorts to determine how NK-cell phenotype and function are affected. Nevertheless, it seems likely that improving NK-cell function in these patients could enhance the efficiency of lymphoma-specific antibodies [21]. Given the correlation between ADCC activity and CD137 expression, CD16 and CD137 might be potential candidates to optimize mAb-mediated therapies. Next generation anti-CD20 antibodies, such as GA101 [22–25] and LFB-R603 [26], have already been engineered to optimize CD16-mediated ADCC. Furthermore, the advantages of combining a tumor-specific mAb with an anti-CD137 agonistic mAb have recently been described in xenotransplant models of lymphoma [20] and breast cancer [27]. However, given the reduced CD137 expression on patient's NK cells before and after activation shown here, a similar strategy might be unable to enhance NK-cell–mediated ADCC in DLBCL patients.

Materials and methods

Study cohort

Patients were diagnosed with DLBCL without circulating tumor cells in the Hematology department of the Saint-Louis Hospital, Paris, France, between 2007 and 2009 (Supporting Information Table 1) [28]. The study cohort included 49 newly diagnosed DLBCL patients and 31 HCs. All participants gave their informed consent to participate in this study, which was approved by the Saint-Louis Hospital review board. Samples from 36 patients and 20 HCs were used for phenotype analysis experiments; ADCC was studied in 16 patients and 19 HCs (of which three patients and eight HCs were also used for NK-cell phenotyping).

PBMCs from each individual was isolated by density gradient centrifugation. Cells were stored in liquid nitrogen before analysis. All phenotype and functional analyses were performed on thawed PBMCs for which viability was greater than 50%.

Flow cytometry analysis

NK-cell phenotypes were determined as previously described [29] using the following mAbs from Becton Dickinson, Beckman Coulter or R&D Systems: FITC–DNAM-1 (DX11), FITC–HLA-DR (L243), FITC–CD94 (HP-3D9), FITC-Perforin (δG9), PE–NKp30 (Z25), PE–NKp46 (BAB281), PE–NKG2C (134591), allophycocyanin–NKG2D (ON72), allophycocyanin–CD137 (4B4–1), allophycocyanin–NKG2A (Z199), allophycocyanin/H7–CD16 (3G8), V450–CD3 (UCHT1), and PE/Alexa750–CD56 (B159). Flow cytometry data were acquired on a BD Biosciences Canto II (Becton Dickinson) and analyzed using FACSDiva software (Becton Dickinson). To ensure consistency across flow cytometry acquisitions, patient samples were always paired with HC samples. For CD16 expression levels, the data shown are the mean of four individual MFI quantifications per sample. MFI for negative controls were identical for patients and HCs.

CD107a degranulation assay

PBMCs were cultured overnight in one of the following four conditions: alone; with the chronic myelogenous leukemia cell line K562; with rituximab-coated (5 μg/mL) $CD20^+$ Burkitt's lymphoma cell line Raji; or with irrelevant IgG1 isotype control-coated Raji cells. A PE–CD107a-specific mAb (H4A3, Becton Dickinson) was added to cell cultures. Where indicated, IL-2 (1000 UI/mL) was added to the cell cultures the day before the experiment. After overnight culture, cells were labeled with allophycocyanin/H7–CD16, allophycocyanin–CD137, PerCP–CD3 and PE/cyanin7–CD56, and analyzed as described above. To ensure consistency across experiments, patient samples were always paired with HC samples.

Statistical analysis

Statistical analyses were performed using Statview software (v5.0). The statistical tests used were: Mann–Whitney test, signed Wilcoxon rank test, and Spearman correlation test. In all cases, $p \leq 0.05$ was taken as the significance threshold.

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Abbreviations: **ADCC**: antibody-dependent cell-mediated cytotoxicity · **DLBCL**: diffuse large B-cell lymphoma · **HC**: healthy control · **MFI**: median fluorescence intensity · **R-CHOP**: rituximab-cyclophosphamide/hydroxydaunorubicin/oncovin/prednisone

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