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B-lymphoma cells escape rituximab-triggered elimination by NK cells through increased HLA class I expression

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Objective. **Antibody-dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells is a major effector mechanism of the monoclonal anti-CD20 antibody rituximab in eliminating B-cell lymphomas. Resistance to this treatment occurs, although CD20 antigen is expressed on the tumor cells.**

Materials and Methods. **A model of ADCC was established by stimulating human bulk NK cells and inhibitory killer immunoglobulin receptor (KIR)−defined NK cells from human leukocyte antigen (HLA)−typed donors. NK-cell activation was triggered via stimulation of the Fc receptor with immunoglobulin G aggregates, rituximab-labeled HLA-defined CD20-positive B-lymphoblast cell lines or CD20-positive B-lymphoma cell lines. The effect of KIR ligation by anti-KIR antibodies and HLA, the HLA expression density and rituximab concentrations on the efficacy of ADCC were analyzed in granzyme B ELISPOT measuring NK-cell activation and fluorescein-activated cell sorting cytotoxicity assay.**

Results. **HLA, but not CD20 expression density correlated with NK-cell activity against rituximab-labeled targets. ADCC was increased or decreased following HLA shielding or KIR activation by anti-KIR antibodies, respectively. Herein we show that rituximab-induced ADCC is attenuated upon ligation of KIR by HLA molecules expressed on human B-lymphoma target cells. Moreover, anti-KIR antibodies do not only block KIR/HLA interactions, but display agonistic effects at the KIR, which has to be considered for therapeutical applications.**

Conclusion. **KIR activation and HLA expression density are critical determinants for the efficacy of rituximab treatment. An explanation for the failure of rituximab treatment may be the protection of the tumor cells from ADCC by inhibiting NK-cell function with their surface HLA.** **© 2010 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

The introduction of rituximab, a chimeric monoclonal anti-CD20 antibody comprising human immunoglobulin (Ig) G1 and κ-constant regions greatly improved the treatment of human CD20-positive B-cell lymphomas [1–3]. Three major mechanisms were proposed to exert the anti-tumoral effect; first, direct cytotoxicity by induction of apoptosis [4]; second, complement-dependent B-cell destruction [5]; and third, natural killer (NK) cell−mediated antibody-dependent cellular cytotoxicity (ADCC) [6].

Despite striking results in the majority of rituximab-treated patients, some B-cell lymphomas are refractory or relapse. Resistance to rituximab treatment was independent of CD20 expression levels [7], but correlated with the activation of B-cell survival pathways [8] and the expression of complement inhibitors CD55 and CD59 [9]. The findings that NK-cell levels [10] and Fcγ-receptor polymorphisms [11–13] influence the efficiency of rituximab treatment implicate the NK-cell activation status of lymphoma patients as an important determinant and ADCC as the dominant in vivo effect of rituximab-mediated tumor eradication.

ADCC is mediated by recruiting NK cells to antibody-coated target cells through an interaction of the antibody Fc domain with the low-affinity Fcγ receptor IIIa (FcγRIIIa, CD16) on the NK-cell surface [14–16]. NK cells discriminate

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between self and nonself by monitoring the expression of HLA class I molecules, and natural cytotoxicity is initiated by the absence of inhibitory signals on virus-infected and transformed cells with altered or lost human leukocyte antigen (HLA) [17]. Activated NK cells release granules containing pore-forming perforin and the cytotoxic serine proteases granzymes A and B leading to lysis of the target cell.

Known inhibitory receptors expressed on the surface of NK cells are the lectin-like receptor CD94/NKG2A [18] and long-tailed isoforms of killer immunoglobulin-like receptors (KIR) [19]. Ligands of CD94/NKG2A on NK target cells are nonclassical HLA-E molecules [20] and for inhibitory KIR subgroups of classical HLA class I proteins: KIR2DL2 and 2DL3 ligand is HLA C group 1 (HLA C^{Asn80}, C1, e.g., HLA Cw1, -3, -7, -8). HLA C group 2 (HLA C^{Lys80}, C2, e.g., HLA Cw2, -4, -5, -6) is ligand to KIR2DL1. KIR2DL4 ligands are HLA G, -A3, and –B46. KIR3DL1 recognizes HLA-Bw4 and KIR3DL2 ligands are HLA A3 and -A11 [21].

Engagement of HLA molecules with these inhibitory receptors balance natural cytotoxicity of NK cells [22–24], but it is unclear whether ADCC-mediated NK-cell action is also under the control of inhibitory receptor signals. The analysis of NK-cell effects is hampered by the lack of representative NK-cell lines and the heterogeneity of primary NK cells. Whereas the Fcγ receptor CD16 seems to be expressed on all NK cells, the expression patterns of inhibitory receptors are diverse and clonally distributed [19]. The human NK-cell repertoire also contains subpopulations expressing only one of the known inhibitory receptor classes (monoKIR NK cells). These NK cells are thus inhibited by a defined group of HLA proteins expressed on the target cell surface.

We show in this study the impact of inhibitory influences on NK cells via HLA/KIR interaction on the efficacy of rituximab-induced ADCC. We have now quantified rituximab-induced ADCC of monoKIR and bulk NK cells against HLA-matched and HLA-mismatched CD20-positive B-cell lines. This approach shows that NK-cell−mediated ADCC is negatively regulated by simultaneous KIR ligation. Therefore, our data provide a potential tumor escape mechanism from immunotherapy.

Material and methods

NK-cell purification and cell lines

From peripheral blood of serologically HLA-typed voluntary donors, bulk NK cells were immunomagnetically selected (NK cell isolation kit II; Miltenyi Biotec, Bergisch Gladbach, Germany) with a purity of >95% for $CD56^{+}CD3^{-}$ NK cells (data not shown) and interleukin-2−stimulated with 500 IE/mL interleukin-2 (Proleukin; Novartis, Nuremberg, Germany) to increase cytotoxic activity. To obtain monoKIR NK cells, bulk NK cells were further depleted by anti-phycoerythrin (PE) beads (Miltenyi Biotec) using the following monoclonal antibody, except the desired one: CD158a-PE (clone HP-3E4, mouse IgMκ), NKAT2-PE (clone DX27, mouse IgG2a, κ), NKB1-PE (clone DX9, mouse IgG1) (all Becton Dickinson, Heidelberg, Germany), and NKG2A-PE (clone Z199, mouse IgG2b; Beckman Coulter, Krefeld, Germany). Procedure was followed by CD56-positive selection of NK cells (CD56 MicroBeads, Miltenyi Biotec; anti-human CD56 antibodies, mouse isotype IgG1) as described previously [25]. MonoKIR NK cells with matching ligands in the donor were chosen to assure full reactivity.

As cell lines are heterogeneous in their stimulation capacity of NK cells, B-lymphoid cell lines (BLCL) were generated from healthy donors that differed with respect to the expression of the KIR ligands HLA C group 1 and 2 for testing the influence of KIR on ADCC. These were generated by Epstein-Barr-Virus-transformation (B95-8 strain) of B cells from serologically HLA-typed three voluntary donors resulting in three cell lines expressing a defined HLA C, serving as ligand for KIR: either one KIR ligand or both ligands for KIR2DL1 and KIR2DL2/3: BLCL HLA C group 1 (HLA-C^{Asn80}) expressed the specific HLA ligand for KIR2DL2/3, BLCL HLA-C group2 (HLA-C^{Lys80}) expressed the ligand for KIR2DL1, and BLCL HLA group 1+2 expressed ligand for both KIR2DL1 and KIR2DL32/3. Ligands for KIR3DL1 and KIR3DL2 were not expressed. These BLCL and CD20-positive B-lymphoid cell lines SUDHL-4, Karpas-422, Balm-3, Granta-519, Raji, and Ramos (DSMZ, Braunschweig, Germany) served as target cells.

HLA class I and CD20 expression densities were determined by fluorescein-activated cell sorting (FACS) using pan-HLA class I monoclonal antibody (clone w6/32; Beckman Coulter) and CD20-PE (clone L27, BD Pharmingen), respectively.

ADCC assays and antibody reagents

Bulk and monoKIR NK-cell activity was monitored by granzyme B enzyme-linked immunospot (ELISPOT) assay as described previously [26,27]. Polyvinylidine difluoride membrane 96-well plates (ImmunoSpot M200; Cellular Technologies Ltd., Cleveland, OH, USA) were coated with anti−granzyme B capture antibody (50 μL per well, 10 μg/mL in phosphate-buffered saline [PBS]; Becton Dickinson) for 16 hours at 4°C. Plates were blocked with 1% bovine serum albumin (Gibco-BRL, Karlsruhe, Germany) in PBS for 1 hour at 18°C. After washing with complete RPMI, assays were performed by adding 1.5×10^4 bulk NK cells or 2.5×10^4 monoKIR NK cells with 4×10^4 target B cells. Alternatively, immune aggregates consisting of 100 μg/mL heat-aggregated human IgG served as cell-free stimulant of Fcγ receptor on NK cells. These were prepared by heating human IgG (Octagam; Octapharma, Dessau, Germany), 50 mg/mL, to 63°C for 20 minutes, and diluted in PBS to the desired concentration immediately prior to cell culture [28].

As indicated antibodies were added: Rituximab (Roche, Hertfordshire, UK) and following antibodies at 1 μg/mL: KIR2DL/S1 (clone HP-3E4, mouse IgMκ; BD Pharmingen), KIR2DL2/3 (clone DX27, mouse IgG2aκ) and NKG2A (clone Z199, mouseIgG2b, both Beckman Coulter). Positive control was NK-cell activity against K562 resulting in reasonable NK-cell response and negative controls were media alone. Background spots per well were determined by incubating identical number of effector cells without stimulator cells. Cells with and without antibodies were 4 hours coincubated at 37°C and 5% CO_2. After washing plates with PBS containing 0.05% Tween (Sigma,