

Anti-CD20 Therapy Acts via Fc γ RIIIA to Diminish Responsiveness of Human Natural Killer Cells

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Abstract

Natural killer (NK) immune cells mediate antibody-dependent cellular cytotoxicity (ADCC) by aggregating Fc γ RIIIA/CD16, contributing significantly to the therapeutic effect of CD20 monoclonal antibodies (mAb). In this study, we show that CD16 ligation on primary human NK cells by the anti-CD20 mAb rituximab or ofatumumab stably impairs the spontaneous cytotoxic response attributable to cross-tolerance of several unrelated NK-activating receptors (including NKG2D, DNAM-1, NKp46, and 2B4). Similar effects were obtained from NK cells isolated from patients with chronic lymphocytic leukemia in an autologous setting. NK cells rendered hyporesponsive in this manner were deficient in the ability of these cross-tolerized receptors to phosphorylate effector

signaling molecules critical for NK cytotoxicity, including SLP-76, PLC γ 2, and Vav1. These effects were associated with long-lasting recruitment of the tyrosine phosphatase SHP-1 to the CD16 receptor complex. Notably, pharmacologic inhibition of SHP-1 with sodium stibogluconate counteracted CD20 mAb-induced NK hyporesponsiveness, unveiling an unrecognized role for CD16 as a bifunctional receptor capable of engendering long-lasting NK cell inhibitory signals. Our work defines a novel mechanism of immune exhaustion induced by CD20 mAb in human NK cells, with potentially negative implications in CD20 mAb-treated patients where NK cells are partly responsible for clinical efficacy. *Cancer Res*; 75(19); 1–12. ©2015 AACR.

Introduction

Anti-CD20–based therapies represent a breakthrough in the treatment of B-cell malignancies and autoimmune diseases. The chimeric rituximab is considered the reference molecule for the comparison of new-generation anti-CD20 mAbs designed to reach enhanced clinical activity; among them, the fully human ofatumumab, currently approved for relapsed or refractory chronic lymphocytic leukemia (CLL), binds to a CD20 epitope different from the rituximab-binding site (1, 2). Both rituximab and ofatumumab belong to class I mAbs on the basis of their capability to redistribute CD20 molecules into lipid rafts (2).

Class I mAbs may act through multiple mechanisms, including the activation of complement-dependent cytotoxicity and the recruitment of Fc γ R⁺ effector cells for antibody-dependent phagocytosis and natural killer (NK) cell–mediated antibody-dependent cellular cytotoxicity (ADCC; refs. 1, 3–5).

Despite widespread treatment successes, the development of resistance to anti-CD20 mAbs, leading to a substantial decrease in

the immunotherapeutic efficacy and to high relapse rate, is a major problem in patients with B-cell lymphomas (6). One possible mechanism thought to contribute to a reduced therapeutic efficacy is the decreased ability to execute ADCC, mainly attributed to CD16 downregulation upon recognition of anti-CD20–coated targets (7–11).

NK cell–mediated ADCC is based on the recognition of IgG-opsonized targets by the low-affinity receptor for IgG, Fc γ RIIIA/CD16 (12). In human NK cells, CD16 has 2 extracellular Ig domains, a short cytoplasmic tail, and a transmembrane domain that enables its association with immune receptor tyrosine-based activation motif (ITAM)-containing TCR- ζ and Fc ϵ RI- γ chains (13). Besides CD16, the prototype of NK-activating receptors, NK cells express a variety of activating receptors cooperating in driving the natural cytotoxic response (14). Indeed, several activating receptors, including the lectin-like receptor NKG2D, the signaling lymphocyte activation molecule (SLAM) family member, 2B4, the Ig-like receptor DNAM-1 and the natural cytotoxicity receptor (NCR), NKp46, recognize a multitude of ligands expressed on infected and tumor cells, playing an important role in antitumor response and immunosurveillance (15).

In the last few years, numerous evidences have revealed that the reactivity of NK cells is very plastic and support the idea that mature NK cells undergo continuous re-education (also called licensing or tuning). With a mechanism that complements education via inhibitory receptors, the sustained engagement of selected activating receptors, including NKG2D, NKp46, and KIR2DS1, has been shown to downregulate NK cell responsiveness (16–19).

Here we have addressed whether engagement of CD16 by exposure to anti-CD20 mAbs may lead to NK cell exhaustion.

We show that CD16 ligation on primary human NK cells by rituximab- or ofatumumab-opsonized targets results in a marked and persistent impairment of the spontaneous

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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cytotoxic response due to the cross-tolerance of several unrelated NK-activating receptors, without affecting the ability to secrete IFN γ . Importantly, analogous observations were obtained in NK cells from patients with CLL in an autologous setting. We also provide evidences that in hyporesponsive NK cells, stimulation of the cross-inhibited receptors resulted in the defective tyrosine phosphorylation of signaling elements critical for NK cytotoxicity such as SLP-76, PLC γ 2, and Vav1, likely imputable to the long-lasting recruitment of SHP-1 to CD16 receptor complex.

Materials and Methods

Patients and healthy donors

Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors of Transfusion Center of Sapienza University (Rome, Italy) or from patients with CLL of Hematology Unit, S. Maria Goretti Hospital (Latina, Italy). The study was conducted according to protocols approved by our local institutional review board and in accordance with Declaration of Helsinki. Written informed consent was obtained from all patients. The diagnosis of CLL was based on criteria recommended by the International Workshop on Chronic Lymphocytic Leukemia; the stage of disease was assessed according to the Rai staging system (20). In all specimens, the percentage of CD5⁺CD19⁺ CLL cells was more than 80%. From each patient, a part of PBMC was used to obtain primary cultured NK cells (see below) and a portion was cryopreserved and stored at -160°C . The day before the experiment, samples were thawed, cultured overnight in complete medium, and then checked for cell viability by trypan blue staining. Only samples with viability more than 90% were used.

Cell systems

Primary cultured human NK cells were obtained from healthy donors or from CLL patients as previously described (21). The experiments were performed on NK cells (CD3⁻CD56⁺) more than 80% pure. When specified, NK cells were pretreated for 48 hours with recombinant human IL2 (200 U/mL; R&D Systems).

The following cell lines were used: the human CD20⁺ lymphoblastoid, Raji, provided by Dr. F.D. Batista (Cancer Research UK, London, UK); the thymic lymphocytic leukemia, HPB-ALL, obtained from Dr. G. Scala (University "Magna Graecia" of Catanzaro, Catanzaro, Italy; ref. 22); the murine thymoma, BW5147, obtained from A. Moretta (University of Genoa, Genoa, Italy; ref. 23); human lymphoblastoid Daudi and 721.221, T-cell leukemia MOLT-4, erythroleukemia K562, and colon carcinoma CaCo2 were all obtained from ATCC. NK-resistant targets murine pro-B-cell line Ba/F3 and Ba/F3-MICA (stably expressing MICA*0019) cells were provided by Dr. L.L. Lanier (University of San Francisco, San Francisco, CA; ref. 24); Ba/F3-PVR, Ba/F3-CD48 transfectants were kindly provided by Dr. A. Soriani and Dr. A. Zingoni (Sapienza University). Cells were authenticated (last testing January–May 2014) by morphology, growth, and immunophenotypic characteristics, biologic behavior according to the provider recommendations, and tested for mycoplasma contamination by EZ-PCR Mycoplasma Test Kit (Biological Industries). All cell lines were kept in culture for less than two consecutive months.

Anti-CD20-mediated NK cell stimulation

Raji cells or primary CLL were loaded with 10 $\mu\text{g}/\text{mL}$ of EZ-Link Sulfo-NHS-SS-Biotin (Thermo Fisher Scientific) for 30 minutes at room temperature. Cells were then opsonized with rituximab

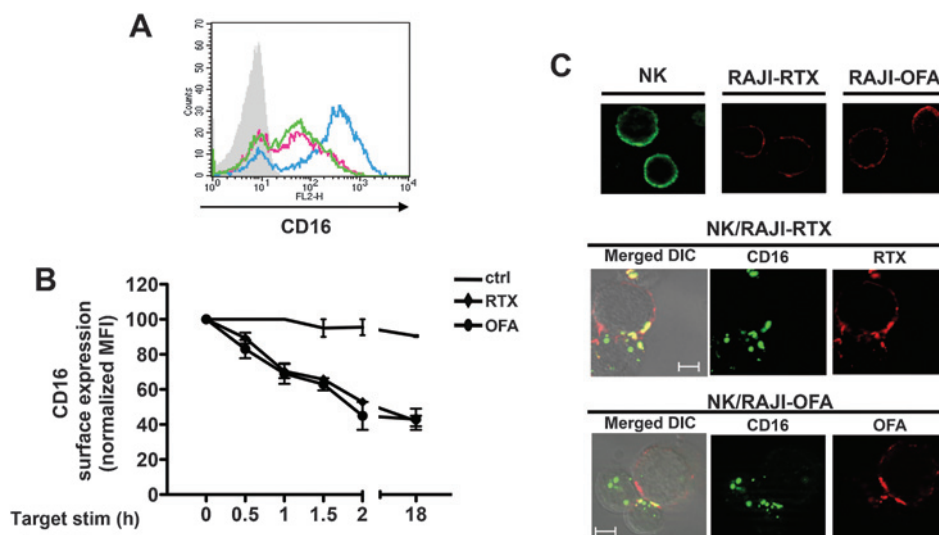


Figure 1.

Rituximab (RTX)- or ofatumumab (OFA)-opsonized target interaction induces CD16 downmodulation at comparable levels. A, IL2-activated NK cells were cocultured for 90 minutes with rituximab (red)- or ofatumumab (green)-opsonized or not opsonized targets (blue) and CD16 surface expression was evaluated by immunofluorescence and FACS analysis gating on CD56⁺ population. One representative of five independent experiments is shown. Gray histogram represents isotype control mAb. B, IL2-activated NK cells were cocultured for the indicated times with rituximab or ofatumumab-opsonized or not opsonized targets (ctrl), stained, and analyzed as in A. CD16 expression was calculated as follows: [mean fluorescence intensity (MFI) of the stimulated samples/MFI of not stimulated sample] \times 100. Data from three independent experiments (mean \pm SEM) are shown. C, IL2-activated NK cells were cocultured for 60 minutes with rituximab (NK/RAJI-RTX)- or ofatumumab (NK/RAJI-OFA)-opsonized targets. Fixed and permeabilized cells were stained with GAH and anti-CD16 mAb. A representative image of conjugates ($n = 55$) or isolated NK or Raji cells is shown. The overlap of merged and differential interference contrast (DIC) images of conjugates is shown. Bar, 5 μm .

(Roche) or ofatumumab (GlaxoSmithKline) by incubating for 20 minutes at room temperature with saturating anti-CD20 concentration ($1 \mu\text{g}/10^6$).

Primary cultured NK cells were mixed [effector:target (E:T) = 2:1] with targets, briefly pelleted, and cocultured for 90 minutes or different times, where indicated. NK cells were then recovered by negative selection on streptavidin-coated Dynabeads (Invitrogen, Life Technologies). When specified, immunomagnetically isolated NK populations were replated in culture medium, supplemented or not with IL2 (200 U/mL).

Evaluation of cytotoxic activity and IFN γ release

To analyze cytotoxic activity, rituximab- or ofatumumab-experienced NK populations were assessed in a 4-hour ^{51}Cr release assay. IFN γ was analyzed by commercial ELISA kit (Thermo Scientific; for details, see Supplementary Data).

Statistical and densitometric analysis

Statistical significance between two groups was determined by 2-tailed, paired Student *t* test. Differences among multiple groups were analyzed with one-way ANOVA using Prism 5 software (GraphPad). Where indicated, values were expressed as mean \pm SEM and differences were considered to be statistically significant when $P < 0.05$. Quantification of specific bands (pixel intensity) was performed with ImageJ1.41o software (NIH, Bethesda, MD).

Additional methods can be found in Supplementary Methods.

Results

NK cell interaction with anti-CD20-opsonized targets induces CD16 internalization

To compare the kinetics of CD16 downmodulation induced by two class I anti-CD20 mAbs, we evaluated CD16 levels in primary human NK cells upon interaction with rituximab- or ofatumumab-opsonized CD20 $^+$ targets. Hereafter, in all experiments, we used NK cells stimulated with not opsonized target as control population. Our data (Fig. 1A and B) show a progressive CD16 downmodulation that was superimposable in NK cells interacting with rituximab- or ofatumumab-opsonized targets. CD16 expression reached minimum levels ($\sim 40\%$ of control) in 2 hours of interaction, keeping a plateau of downmodulation until 18-hour stimulation (Fig. 1B).

Because both internalization and/or receptor shedding may contribute to anti-CD20-induced CD16 reduction (25), we evaluated CD16 dynamics by fluorescence microscopy (Fig. 1C). As compared with the ring pattern of unconjugated cells, CD16 distributed in intracellular dots in NK cells interacting with rituximab- or ofatumumab-opsonized targets, thus indicating an internalization process. No evidence of anti-CD20 internalization was observed; instead, it appeared capped at cell membrane, as reported by others (26), in some extent colocalizing with residual surface CD16. Furthermore, by comparing CD16 levels in fixed and fixed/permeabilized NK cells, we observed that almost 50% of downmodulated CD16 was the result of internalization occurring during opsonized target interaction (Supplementary Fig. S1).

These data demonstrate that the interaction with rituximab- or ofatumumab-opsonized targets similarly induces surface CD16 downmodulation, which is partially attributable to receptor internalization.

NK cell interaction with anti-CD20-opsonized targets results in a marked defect of cytotoxic responses

We investigated the impact of the NK cell interaction with opsonized targets on their cytolytic potential. Hereafter, "experienced NK cells" define primary cultured NK cells immunomagnetically isolated upon 90 minutes of coculture with rituximab- or ofatumumab-opsonized targets. Cytotoxic activity of anti-CD20 experienced NK cells was assayed against rituximab-opsonized or -sensitive targets to test ADCC or spontaneous cytotoxicity, respectively. As expected, experienced NK cells exhibit a reduced ability to execute ADCC (Fig. 2A), but, more interestingly, we observed a marked impairment of the cytotoxic activity toward a panel of sensitive targets, that was comparable in rituximab- or ofatumumab-experienced NK cells (Fig. 2A).

Because in our experiments the impact of rituximab or ofatumumab on NK cell responsiveness was largely superimposable, we focused our analysis on rituximab.

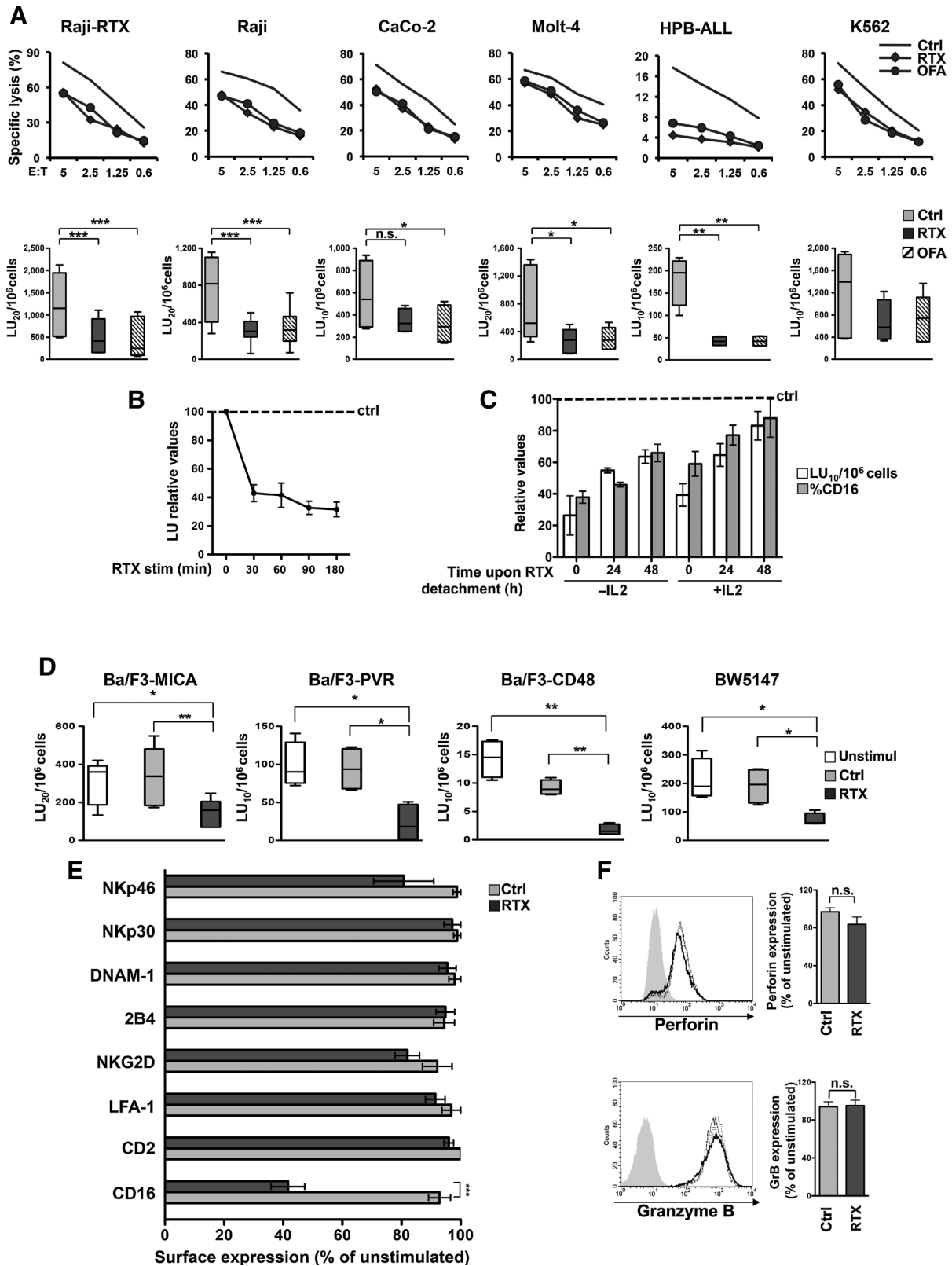
The analysis of the kinetics of anti-CD20-dependent hyporesponsiveness revealed that it was already evident after 30 minutes and reached a plateau between 90 and 180 minutes of interaction with opsonized targets (Fig. 2B). We then addressed the persistence of the hyporesponsiveness by testing the cytotoxic activity of NK cells maintained in culture for different lengths of time upon opsonized target detachment. Albeit a partial rescue was observed, a clear reduction of the spontaneous cytotoxicity persisted until 48 hours. Notably, when the same effector population was preactivated and cultured in the presence of IL2, a progressive recovery of the cytolytic function that paralleled CD16 re-expression was evident, being almost complete after 48 hours (Fig. 2C).

These experiments demonstrate that CD16 downregulation induced by rituximab- or ofatumumab-opsonized target interaction is associated with a persistent defect of the cytotoxic responses.

NK hyporesponsiveness involves the cross-tolerance of different activating receptor systems

With the aim to investigate, at individual receptor level, whether anti-CD20-induced NK cell hyporesponsiveness could regard selected activating receptors, we tested the ability of rituximab-experienced NK cells to kill resistant targets (Supplementary Fig. S2A) bearing individual receptor ligand, that is, MHC-I-related chain A (MIC-A), poliovirus receptor (PVR), or CD48, to explore NKG2D-, DNAM-1-, or 2B4-dependent lysis, respectively; moreover, we took advantage of BW5147 thymoma cell line whose killing is mostly due to Nkp46 triggering (Supplementary Fig. S2B). Our findings show a significant defect of the cytotoxic activity triggered by the different receptors analyzed demonstrating that rituximab-mediated CD16 aggregation cross-inhibits multiple and unrelated activating receptor families (Fig. 2D, Supplementary Fig. S2C).

To assess whether the defect of spontaneous cytotoxicity could be related to the downmodulation of activating receptors occurring during target interaction, we analyzed the expression levels of a panel of activating and adhesion receptors. Unlike CD16 expression, we did not observe significant differences in rituximab-experienced NK cells with respect to control population or to the NK cells that did not interact with target cells (unstimulated; Fig. 2E). When we evaluated perforin and granzyme B, we observed that cytolytic mediator levels in



rituximab-experienced NK cells was similar to that of control populations, leading us to exclude that the cytotoxic defect depends on the depletion of lytic mediators (Fig. 2F). Analogously, the same rituximab-induced hyporesponsive phenotype was also observed in the absence of IL2 stimulation (Supplementary Fig. S3).

Rituximab-opsonized primary B-CLL cells tune-down CD16 expression and cytolytic potential in autologous NK cells

To extend our observations to NK cells of patients potentially candidate to rituximab treatment, we analyzed 6 untreated patients with CLL (Supplementary Table S1). Primary CLL cells were opsonized with rituximab and allowed to interact with autologous primary NK cells. Rituximab-experienced NK cells were tested for CD16 expression and for spontaneous cytotoxicity. Our findings show that NK cell interaction with autologous-opsonized leukemia cells induces a marked downmodulation of CD16 receptor (Fig. 3A) that is associated with a relevant defect of spontaneous cytotoxic activity against Raji cells and PVR-expressing target cells (Fig. 3B and C).

Lytic granule secretion but not IFN γ production is impaired in rituximab-experienced NK cells

We sought to investigate whether the defective cytotoxic activity could selectively involve different steps of the lytic event (27, 28). Our data show that neither the ability to form conjugates with targets nor the polarization of lytic granules at cytolytic synapse (Fig. 4A and B) resulted impaired. However, when we measured the ability to secrete lytic granules in response to target stimulation by assessing CD107a surface levels, we observed a significant defect of lytic granule exocytosis in rituximab-experienced NK cells with respect to unstimulated cells. Such impairment was observed in both CD16⁺ and CD16 downregulated populations (Supplementary Fig. S4). In line with our evidences of a normal lytic granule content in hyporesponsive cells, degranulation induced by phorbol 12-myristate 13-acetate (PMA) plus ionomycin resulted unaffected (Fig. 4C).

We then explored the ability of rituximab-experienced NK cells to secrete IFN γ . We observed that NK cell stimulation with target cells, with mAbs specific for the cross-inhibited receptors or with

IL2, induced a comparable amount of IFN γ with respect to control populations (Fig. 4D).

These data demonstrate that the hyporesponsive status involves a selective defect of lytic granule exocytosis but not the ability to produce IFN γ .

Defect of SLP-76, PLC γ 2, and Vav1 tyrosine phosphorylation in rituximab-experienced NK cells

Assuming that CD16 downregulation may subtract dose-limiting signaling elements (29), we analyzed the phosphorylation events downstream to the cross-inhibited receptors. On the basis of our observation of a defective lytic granule exocytosis, we focused our analysis on signaling pathways controlling granule secretion at the cytolytic synapse. Our data demonstrate a marked defect on tyrosine phosphorylation of SLP-76 at Y128 residue, induced by CD16 itself or by NKp46, NKG2D, or DNAM-1 stimulation in rituximab-experienced NK cells. Concomitantly, we also observed reduced levels of PLC γ 2 tyrosine phosphorylation at Y1217 residue. Similarly, receptor-induced Vav1 tyrosine phosphorylation at Y160 residue was also reduced, although some basal levels of tyrosine phosphorylation were evident in rituximab-experienced cells (Fig. 5A and B). Conversely, in hyporesponsive cells, Akt and Erk phosphorylation downstream cross-inhibited receptors resulted unaffected (Fig. 5C).

These results demonstrate that in rituximab-experienced NK cells tyrosine phosphorylation of SLP-76, PLC γ 2, and Vav1, downstream to activating receptors is impaired.

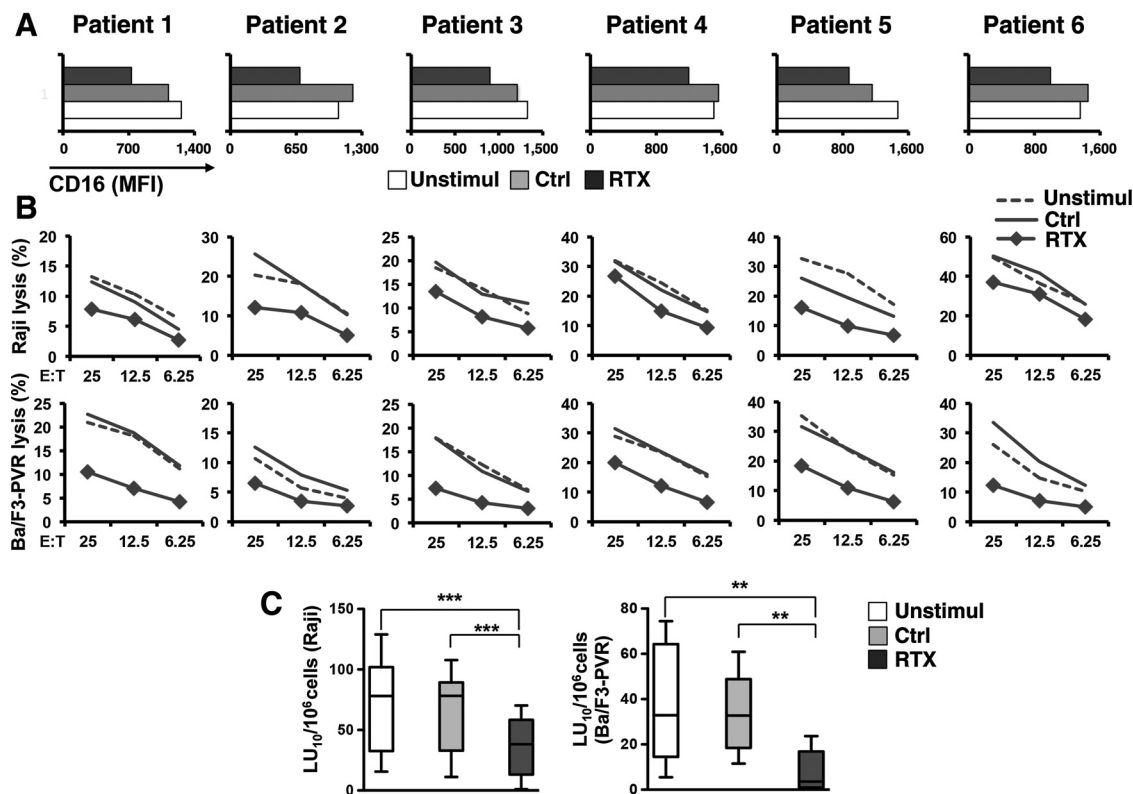
Recruitment of SHP-1 tyrosine phosphatase to CD16 receptor complex upon rituximab stimulation

The defective tyrosine phosphorylation of SLP-76, PLC γ 2, and Vav1 led us to hypothesize the involvement of a tyrosine phosphatase coupled to CD16. We sought to analyze whether CD16 would be coupled to SHP-1/2 upon rituximab stimulation.

Immunoblot analysis of CD16 ζ chain immunoprecipitates revealed that rituximab stimulation induces a delayed and long-lasting association of SHP-1 to phosphorylated CD16 ζ chain reaching maximal levels after 60 minutes of stimulation. Notably, the ability of ζ chain to recruit SHP-1 was inversely proportional to its tyrosine phosphorylation levels: indeed, SHP-1 was not detectable at 5-minute stimulation, when ζ chain

Figure 2.

Anti-CD20-experienced NK cells have a reduced cytolytic potential involving the cross-tolerance of different activating receptors. A, top, IL2-activated NK cells were cocultured for 90 minutes with biotinylated rituximab (RTX)- or ofatumumab (OFA)-opsonized or not opsonized (ctrl) targets and immunomagnetically isolated. Specific lysis from a single representative experiment of five performed is shown. Bottom, lytic units (LU) from five independent experiments performed as in top are shown. Bars represent median and 10–90 percentile. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0005$, repeated-measures ANOVA with Tukey post-test correction. B, IL2-activated NK cells were cocultured with biotinylated rituximab-opsonized or not opsonized (ctrl) Raji cells. For each time of stimulation, NK cells were immunomagnetically isolated and tested toward Raji cells. Data are presented as relative reduction of LU with respect to ctrl from three independent experiments (mean \pm SEM). C, untreated or IL2-activated NK cells were cocultured for 90 minutes with biotinylated rituximab-opsonized or not opsonized (ctrl) Raji cells, and immunomagnetically isolated. NK cells were then cultured for the indicated times with or without IL2 and assayed either for cytotoxic activity toward Raji cells or for CD16 surface expression. Data are presented as relative value of LU and CD16 levels with respect to control from three independent experiments (mean \pm SEM). D, IL2-activated NK cells were left unstimulated or cocultured for 90 minutes with biotinylated rituximab-opsonized or not opsonized (ctrl) targets, immunomagnetically isolated, and tested toward the indicated targets. LU from five independent experiments are shown. Bars, median and 10th to 90th percentile. *, $P < 0.05$; **, $P < 0.01$, repeated-measure ANOVA with Tukey post-test correction. E, NK cells as in D were stained with receptor-specific mAbs. Assuming as 100% the mean fluorescence intensity (MFI) of unstimulated samples, receptor expression was calculated as follows: (MFI of the stimulated samples/MFI of unstimulated samples) \times 100. Data from five independent experiments (mean \pm SEM) are shown. ***, $P < 0.0005$, repeated-measures ANOVA with Tukey post-test correction. F, NK cells as in D were fixed, permeabilized, and stained as indicated. Left, histograms relative to a representative experiment are showed. Unstimulated, dashed line; control, dotted line; rituximab, unbroken line. Gray histogram represents isotype control mAbs. Right, expression levels were calculated as for E. Data from three independent experiments (mean \pm SEM) are shown. No significant (n.s.) differences were observed, Friedman with Duns' post-test correction.

**Figure 3.**

NK cell interaction with rituximab (RTX)-opsonized autologous CLL cells induces CD16 downregulation and NK hyporesponsiveness. NK cells from six patients with CLL were left unstimulated or cocultured for 90 minutes with biotinylated rituximab-opsonized or not opsonized (ctrl) primary CLL cells and immunomagnetically isolated. NK cells were stained with anti-CD16 mAb (A) or tested in a 4-hour ⁵¹Cr release assay (B). C, lytic units (LU) from the same patients are shown in A and B. Bars represent median and 10–90 percentile. ***, $P < 0.0005$; **, $P < 0.01$, repeated-measure ANOVA with Tukey post-test correction.

was maximally phosphorylated, but appeared upon 30 minutes, further increasing at 60 minutes of rituximab stimulation (Fig. 6A). In contrast, we did not observe any evidences of SHP-2 recruitment. Intriguingly, the aggregation of CD16 obtained with anti-CD16 (B73.1) mAb did not induce a detectable SHP-1 recruitment. Of note, in such aggregation conditions, although a very efficient CD16 downmodulation occurred (Fig. 6B), NK cells preserved their cytolytic potential (Fig. 6C).

These experiments show that rituximab-mediated but not anti-CD16 mAb-mediated stimulation induces the recruitment of SHP-1 to CD16 receptor complex.

Pharmacologic inhibition of SHP-1 activity counteracts rituximab-induced NK hyporesponsiveness

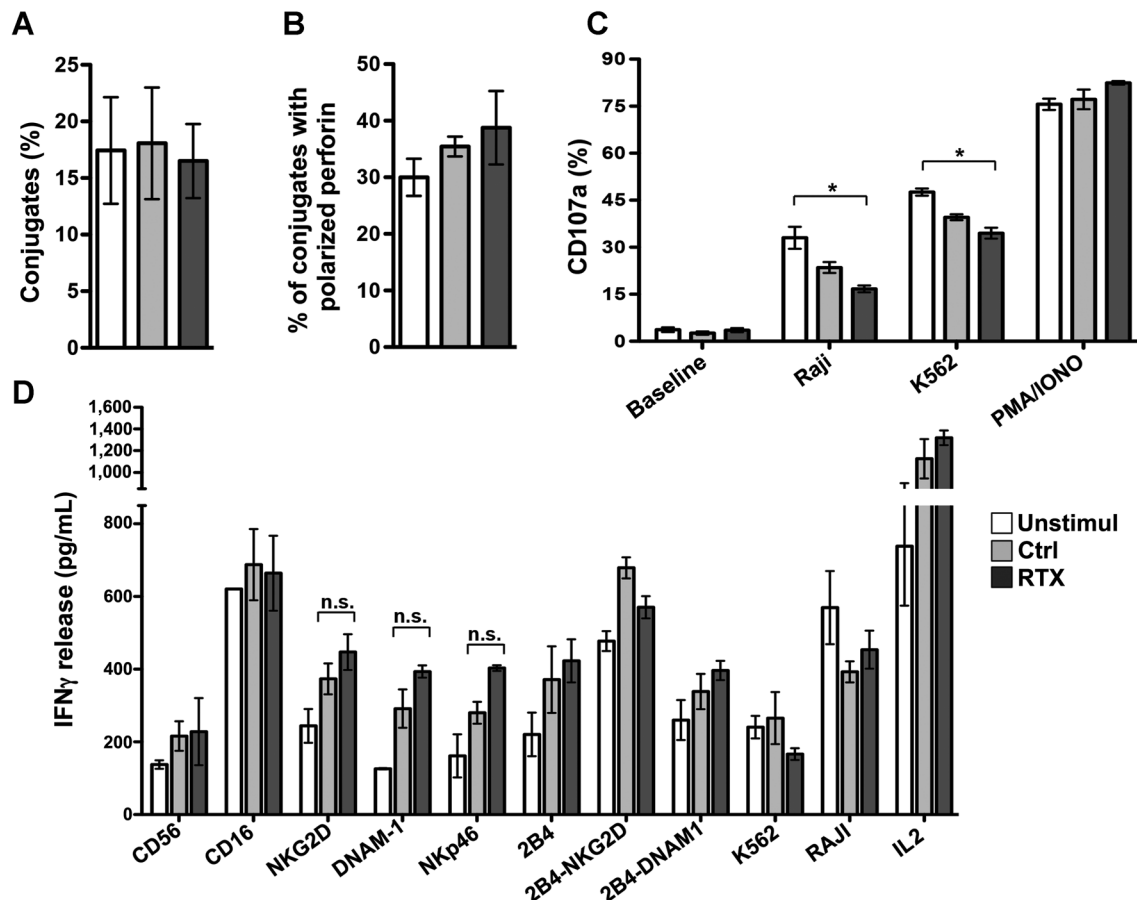
The hypothesis that SHP-1 phosphatase activity may be responsible for the functional impairment was tested by inhibiting SHP-1 with SSG, which has been shown to be the most sensitive tyrosine phosphatase (30, 31). Because SHP-1 activity classically mediates the inhibitory signals downstream MHC1 inhibitory NK receptors in co-aggregation conditions (32), we avoided that their engagement would occur during target recognition by using MHC1-negative Daudi cell line. Our data show that in SSG-pretreated NK cells, the defect of spontaneous cytotoxicity in rituximab-experienced NK cells was partially but significantly prevented. This effect was observed at variable degree in all donors tested (Fig. 6D), indicating that SHP-1

acts in limiting the cytotoxic responses in rituximab-experienced NK cells.

Discussion

The central observation of our report is that preligation of CD16 by anti-CD20 mAbs reduces the ability of NK cells to kill target cells; indeed, we demonstrate that the outcome of NK cell interaction with rituximab- or ofatumumab-opsonized targets is a comprehensive reduction of both Ab-dependent and -independent cytotoxic potential. Such NK hyporesponsive status is almost fully established upon 30 minutes of CD16 ligation and persists at least until 48 hours of stimulation. Our data highlight that the hyporesponsiveness does not involve the ability to secrete IFN γ in response to the cross-inhibited receptors. This observation is not really surprising, taking into account the uncoupling of the signaling pathways and molecular machinery leading to the activation of the cytolytic program or to the production of cytokines, including IFN γ (33).

The impairment of ADCC response can easily be explained by CD16 downmodulation induced by the stimulation of NK cells with anti-CD20-opsonized targets, which is only partially attributable to receptor internalization; such finding complements recent observations demonstrating that rituximab stimulation, through activation of metalloprotease-17 (ADAM17), promotes CD16 shedding (25).

**Figure 4.**

Lytic granule exocytosis but not IFN γ production is impaired in rituximab (RTX)-experienced NK cells. NK cells were left unstimulated or were cocultured for 90 minutes with biotinylated rituximab-opsonized or not opsonized (ctrl) targets and immunomagnetically isolated. A, CFSE $^{+}$ NK cells were allowed to bind to PKH26 $^{+}$ Raji for 30 minutes. The percentage of conjugates was determined by flow cytometry. Data are presented as mean \pm SEM of three independent experiments. B, NK cells were mixed to CMAC-labeled Raji cells for 15 minutes. Cell conjugates were fixed, permeabilized, and stained with anti-perforin mAb. The percentage of conjugates containing polarized granules was analyzed on randomly acquired fields of three independent experiments (mean \pm SEM; $n = 55$ conjugates). C, NK cells were stimulated with the indicated targets or with PMA plus ionomycin. The percentage of CD107a $^{+}$ cells was analyzed by flow cytometric gating on CD56 $^{+}$ cells. Data are presented as mean \pm SEM of three independent experiments. *, $P < 0.05$, paired Student t test. D, NK cells were stimulated with the indicated targets or plastic-immobilized mAbs or with IL2. After 18 hours, supernatants were collected and assessed for IFN γ levels. Data are presented as mean \pm SEM of three independent experiments.

Less expected is the finding that anti-CD20-mediated CD16 stimulation also promotes a marked reduction of the ability of NK cells to kill sensitive targets attributable to the cross-inhibition of multiple unrelated activating receptors, including NKG2D, 2B4, NKp46, and DNAM-1, which are coupled to both ITAM- and non-ITAM-containing signaling adaptors (14, 15). The observation that preligation of CD16 induces NK cell hyporesponsiveness is strengthened by the finding that the exposure of primary NK cells from patients with CLL to rituximab-opsonized autologous leukemia cells induces a significant reduction of natural killing potential. Importantly, our observations might account for the marked defect of spontaneous cytotoxicity in *ex vivo* isolated NK cells from patients with diffuse large B-cell lymphoma treated with rituximab (34). Furthermore, the cooperation of activating receptors, stimulated by novel therapeutic mAbs or by tumor ligands, in determining the overall response to tumor targeting mAbs have been recently highlighted (35–37); their cross-toler-

ance induced by rituximab treatment may negatively affect the clinical outcome.

We also provide evidences that the cytotoxic defect is not attributable to an altered phenotype or to depletion of cytolytic mediators: indeed, the levels of activating and adhesion receptors as well as of perforin and granzyme B are largely unaffected.

We were much intrigued in understanding the molecular basis of NK hyporesponsiveness induced by CD16 preligation. In natural killing, a mechanistic role of CD16 has been defined through the demonstration that CD16-dependent proximal signaling elements may be exploited by other activating receptors (29). We explored the possibility that the persistent engagement of CD16 may lead to the inhibition of critical molecules acting in signal integration downstream to activating receptors. On this regard, the adaptor protein SLP-76 has been shown to orchestrate the formation of a molecular platform allowing the tyrosine phosphorylation of

critical signaling elements such Vav1 and PLC γ 2 (14, 38). Our data show in hyporesponsive NK cells a marked defect in SLP-76, PLC γ 2, and Vav1 tyrosine phosphorylation induced by the stimulation of cross-inhibited receptors or by CD16 itself. Furthermore, we observed that the activation of PI3K and MAPK-dependent signals resulted unaffected. These data, in the context of our observation of a selective defect of

lytic granule exocytosis with normal granule polarization in rituximab-experienced NK cells, recapitulate the dichotomy of molecular signals governing polarization versus degranulation: the pathway sequentially involving PI3K/Rac1/Pak1/MEK/ERK1/2 has been referred as pivotal for granule polarization, whereas PLC γ /calcium-dependent signals are the hallmark for their secretion (27, 28, 39).

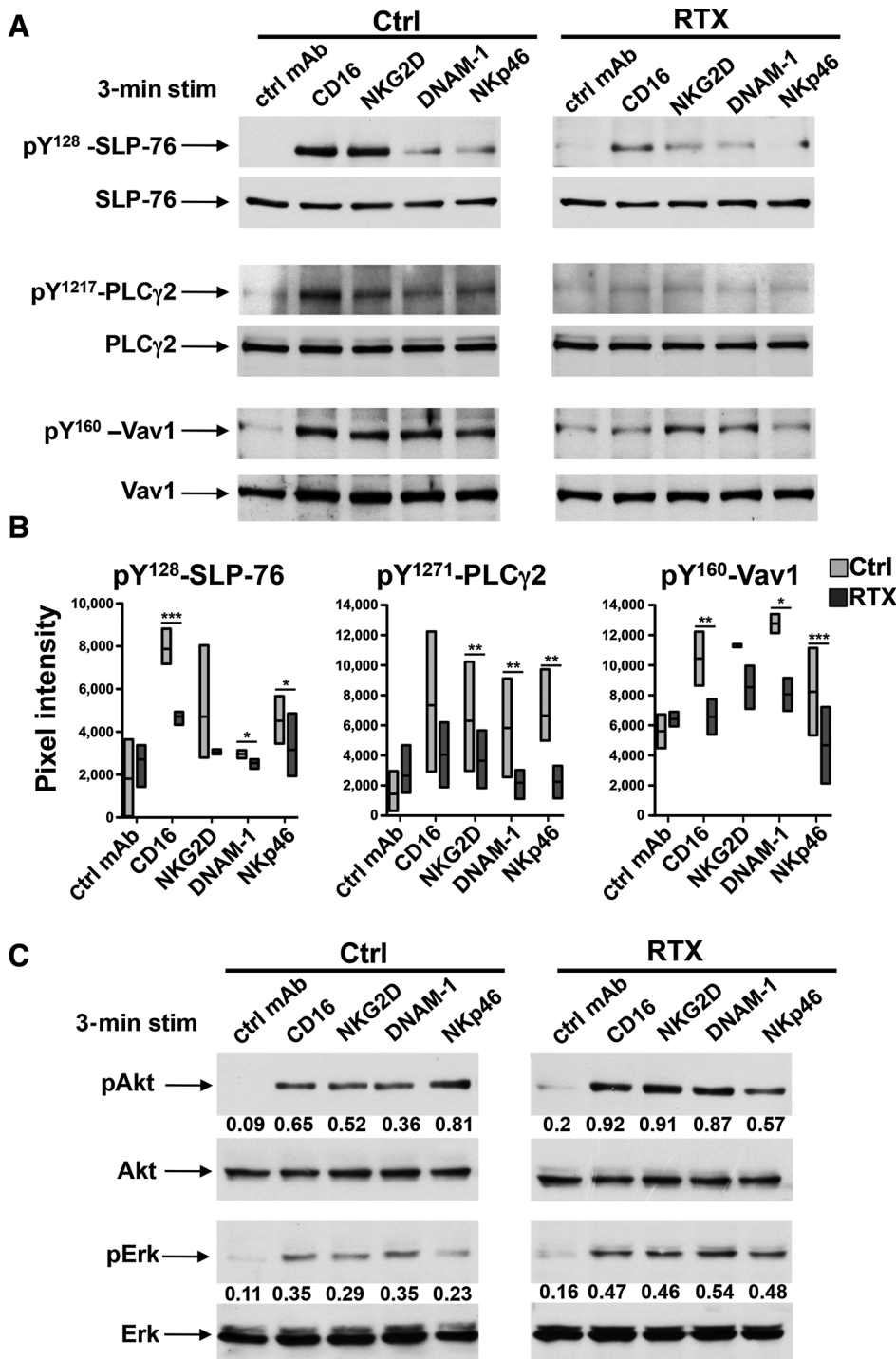
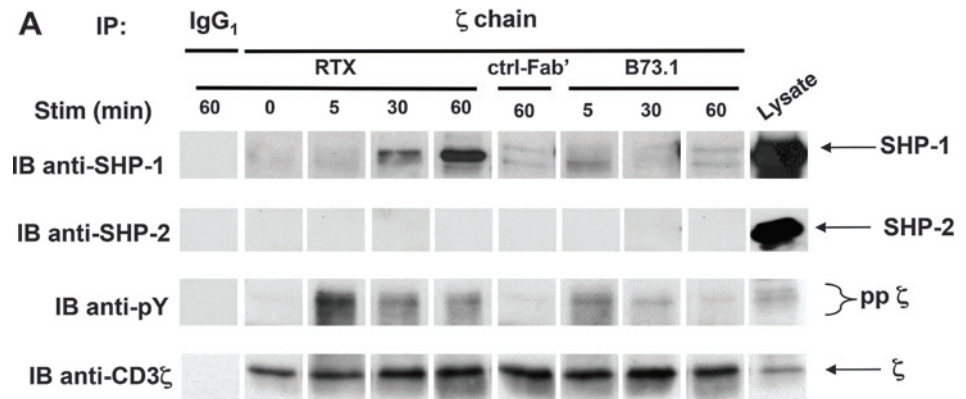
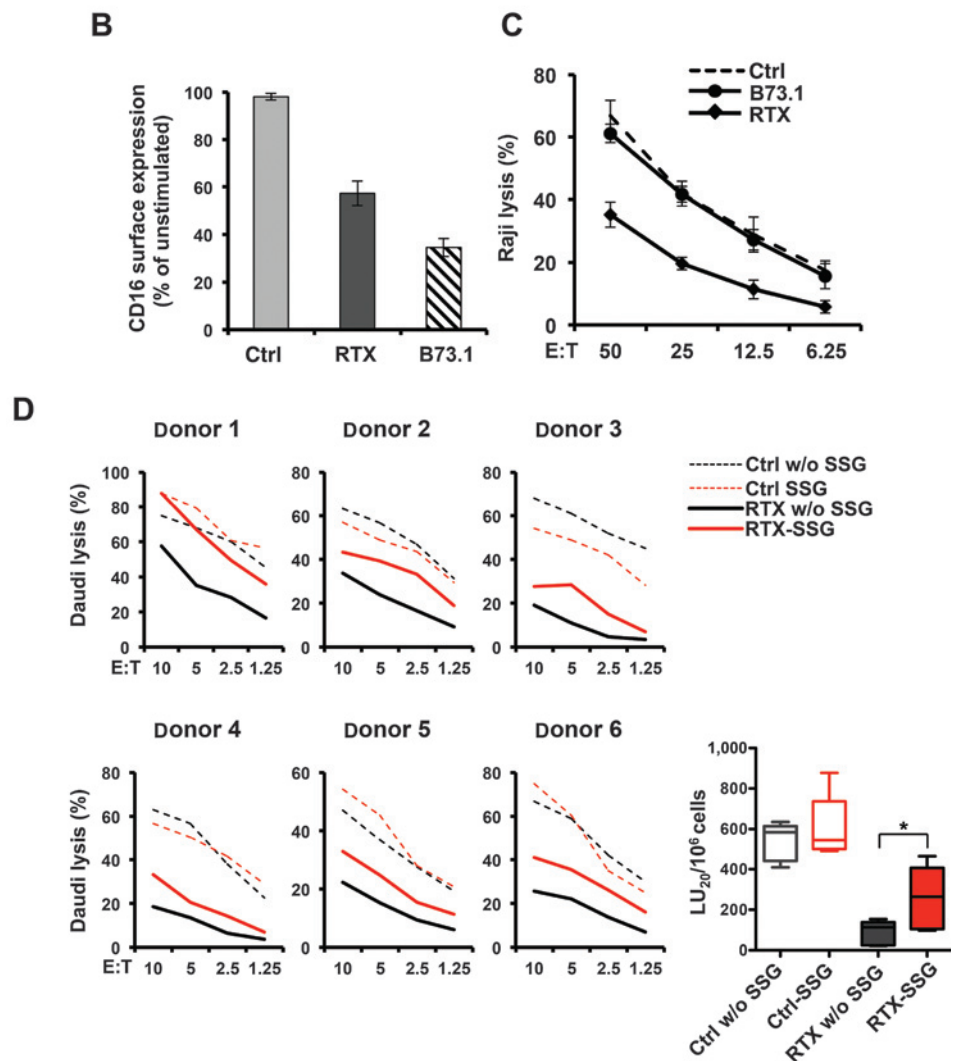


Figure 5. Defect of SLP-76, PLC γ 2, and Vav1 tyrosine phosphorylation in rituximab (RTX)-experienced NK cells. NK cells were cocultured for 90 minutes with biotinylated rituximab-opsonized or not opsonized (ctrl) targets and immunomagnetically isolated. Recovered NK cells were stimulated with anti-CD16, anti-NKG2D, anti-DNAM-1, anti-NKp46, or isotype-matched anti-CD56 mAb (ctrl mAb) followed by GAM F(ab')₂. An equal amount of proteins was immunoblotted with Abs anti-phosphorylated proteins of the indicated specificity. The same membranes were reprobbed as indicated for sample normalization. For each phosphorylated protein, control and rituximab membranes were derived from the same film. One representative experiment of three performed is shown. B, data from three independent experiments as in A are presented as min-to-max bar graphs with average mean lines. ***, $P < 0.0005$; **, $P < 0.01$; *, $P < 0.05$, paired Student t test. C, NK cell lysates were immunoblotted with the indicated Abs. The numbers between lanes represent phosphorylation levels of the indicated proteins after normalization with the relative total levels.

**Figure 6.**

Rituximab (RTX) stimulation induces SHP-1 recruitment to CD16 ζ chain. SHP-1 inhibition counteracts rituximab-dependent NK hyporesponsiveness. A, NK cells were left unstimulated (0) or stimulated with rituximab- or anti-CD56 F(ab')₂ (ctrl-Fab')-coated polystyrene beads, or anti-CD16 mAb followed by GAM F(ab')₂ for the indicated times. Whole lysate of unstimulated sample (lysate) or IgG1 or ζ chain immunoprecipitates were run on 8% (top) and 15% (middle and bottom) SDS-PAGE and immunoblotted with the indicated mAbs. All lanes were from the same experiment but were noncontiguous. B, NK cells were left unstimulated or were cocultured for 90 minutes with biotinylated rituximab-opsonized or not opsonized (ctrl) targets and immunomagnetically isolated. Alternatively, NK cells were stimulated with anti-CD16, B73.1 mAb for 90 minutes. NK cells were stained with anti-CD16 mAb (ctrl and rituximab populations) or with phycoerythrin-conjugated GAM (B73.1 population). CD16 expression was calculated as in Fig. 2E. Data are presented as mean \pm SD of three independent experiments. C, specific lysis is presented as mean \pm SD of three independent experiments. D, NK cells were left untreated (w/o SSG) or treated for 4 days with SSG (50 μ g/mL). Cells were cocultured for 90 minutes with biotinylated rituximab-opsonized or not opsonized (ctrl) targets and immunomagnetically isolated. Specific lysis of six individual donors and lytic units (LU) of the same donors are shown. Bars represent median and 10–90 percentile. *, $P < 0.05$, paired Student t test.



We firstly provide evidences of the involvement of SHP-1 in CD16-dependent cross-tolerance of activating receptors. Indeed, we observe that rituximab stimulation promotes the recruitment of SHP-1 phosphatase, but not SHP-2, to the tyrosine phosphorylated CD16 ζ chain. This recruitment was delayed (30 minutes) and long-lasting (1 hour) with a

time course drastically different from the classical rapid recruitment of SHP-1 by ITIM-containing MHCII inhibitory receptors in condition of co-aggregation with activating receptors (32).

We propose that the recruitment of SHP-1 to targeted CD16 promotes its accumulation at the plasma membrane, reaching a

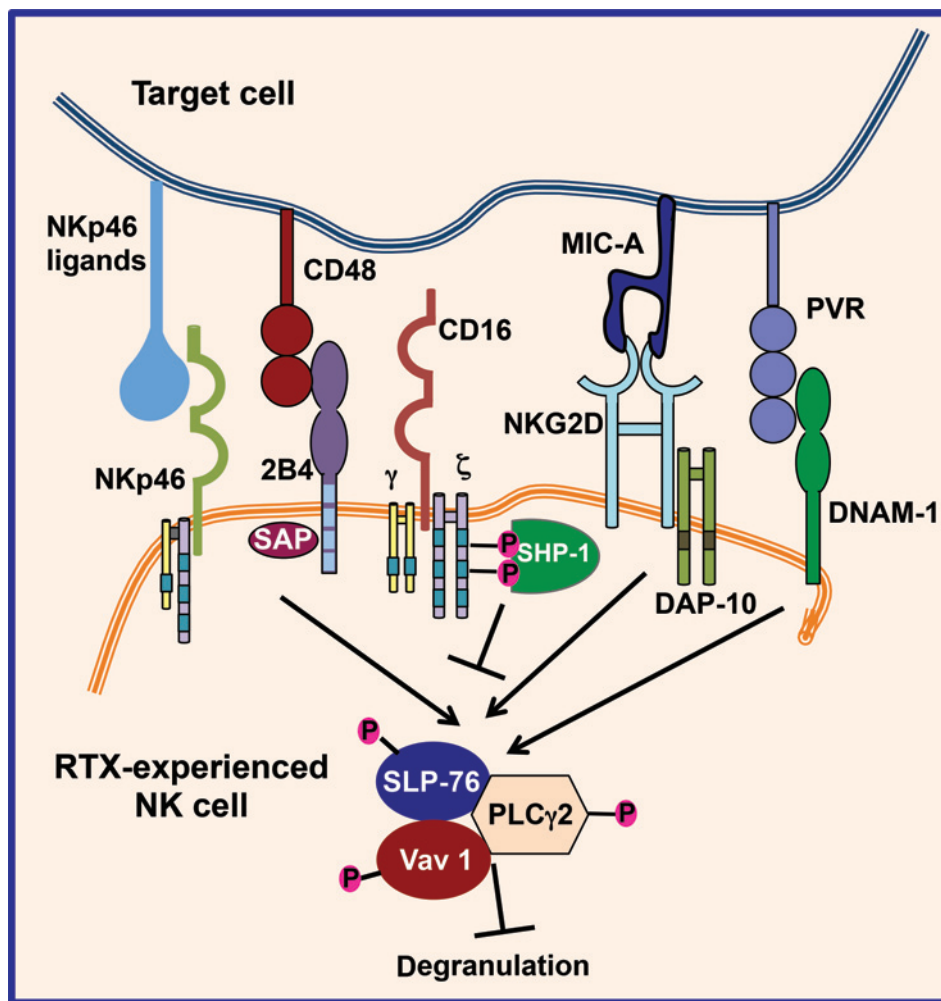


Figure 7. Proposed CD16-dependent inhibitory signal: spatiotemporal integration. CD16 aggregation by anti-CD20-opsinized targets results in a persistent SHP-1 recruitment to phosphorylated ζ chain, which mediates the dephosphorylation of critical signaling elements downstream activating receptors subsequently engaged, leading to the impairment of granule exocytosis.

threshold level that would allow the dephosphorylation of signaling proteins, thus desensitizing activating receptors subsequently stimulated (Figure 7). Indeed, both SLP-76 and Vav1 have been shown to be key targets of SHP-1 triggered by killer inhibitory receptors in NK cells (40, 41). We are currently investigating by imaging approaches the spatial coordination of inhibitory signals; on this purpose, a recent report demonstrated a raft-dependent cosegregation of SHP-1 with signaling effectors into intracellular clusters (called inhibisomes), providing an appropriate scaffold for substrates interaction during inhibitory process (42). A functional role of SHP-1 in mediating CD16-dependent hyporesponsiveness is indicated by our observation that SHP-1 pharmacologic inhibition with SSG, known to inhibit SHP-1 (30), significantly interferes with rituximab-induced inhibitory responses.

On the basis of our data showing a long-lasting NK exhaustion following rituximab stimulation, we cannot exclude that a transcriptional or a posttranscriptional modulation may contribute to NK hyporesponsiveness. Accordingly, the recovery of NK responsiveness driven by IL2 treatment, strictly associated to the restoration of CD16 levels, may indicate a *de novo* gene expression and protein synthesis. Indeed, recent data demonstrated that the calibration of NK cell reactivity is subjected to a genetic-based tuning attributable to transcription factor modulation (19, 43).

Strikingly and in contrast with rituximab, receptor ligation by anti-CD16 mAb was ineffective in promoting SHP-1 recruitment. Notably, in the same stimulation conditions, the ability of NK cells to kill sensitive targets was preserved, indicating a relationship between aggregation conditions and the ability to promote inhibitory signal toward heterologous receptors. It would be interesting to address whether other therapeutic mAbs, acting through the triggering of ADCC, such as trastuzumab and cetuximab or with improved affinity for CD16, as obinutuzumab, may also mediate a CD16-dependent inhibitory signal.

Overall our findings are in line with the emerging paradigm considering that the targeting of some classically activating Fc receptors in myeloid cells with low-avidity ligands, paradoxically transmit inhibitory signals, through the recruitment of SHP-1, leading to the inhibition of a number of innate immune responses (44–46).

We identify here a mechanism of exhaustion of NK cells likely contributing to the resistance and/or to the development of a refractory status to rituximab-containing regimens leading to disease relapse or progression (6). Furthermore, our data may provide insights on the high frequency of viral reactivation in rituximab-treated patients, which may implicate an NK hyporesponsiveness leading to reduced immunosurveillance (47).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C. Capuano, R. Galandrini

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Capuano, M. Romanelli, C. Pighi, G. Cimino, A. Rago, R. Molfetta

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Capuano, R. Paolini, A. Santoni, R. Galandrini

Writing, review, and/or revision of the manuscript: C. Capuano, G. Cimino, R. Paolini, A. Santoni, R. Galandrini

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Anti-CD20 Therapy Acts via Fc γ R11A to Diminish Responsiveness of Human Natural Killer Cells

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