

Effects of complement and serum IgG on rituximab-dependent natural killer cell-mediated cytotoxicity against Raji cells

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Abstract. Accumulating evidence indicates that the anti-CD20 monoclonal antibody rituximab significantly improves the clinical prognosis of patients with non-Hodgkin lymphoma and chronic lymphocytic leukemia. However, a number of patients relapse or fail to respond to rituximab. To further understand the cause of this, polymorphisms of FcγRIIIa were initially detected in healthy volunteers. Subsequently, the rituximab-dependent natural killer (NK) cell-mediated cytotoxicity of different FcγRIIIa genotypes was assessed by a cytotoxicity assay *in vitro*. Ultimately, the effect of human serum immunoglobulin (Ig) G and complement on rituximab-dependent NK cell-mediated cytotoxicity was evaluated *in vitro*. It was revealed that FcγRIIIa polymorphisms were associated with the antibody-dependent cell-mediated cytotoxicity (ADCC) of NK cells. In addition, the ADCC of NK cells with FcγRIIIa-158 V/V was increased compared with that of FcγRIIIa-158 V/F. The serum IgG and rituximab Fc segment was able to bind competitively with NK cell FcγRIIIa. It was observed that serum IgG inhibited, whereas complement enhanced rituximab-induced NK-cell mediated ADCC. Therefore, various agents administered synchronously with rituximab may modulate the efficacy of this agent and ultimately its toxicity against tumor cells.

Introduction

The therapeutic use of monoclonal antibodies (mAbs), such as rituximab and trastuzumab, has evolved into a promising approach to the treatment of cancer over the last several years (1,2). However, the use of mAbs is effective in only a proportion of the patients; thus, we must investigate the mechanism of action of these immunoactive mAbs. Rituximab is a chimeric anti-CD20 immunoglobulin G1 (IgG1) mAb that has demonstrated significant therapeutic activity in B-non-Hodgkin lymphoma (B-NHL) and chronic lymphocytic leukemia (CLL) (3-5). Rituximab induces B-lymphoma cell lysis, which is considered to occur mainly through immune-mediated mechanisms: Antibody-dependent cell-mediated cytotoxicity (ADCC) (6), complement-dependent cytotoxicity (CDC) (7), induction of apoptosis (8) and/or antibody-dependent phagocytosis (ADCP) by macrophages/monocytes (9). Clinical studies have demonstrated that ADCC is one of the most important mechanisms enhancing the therapeutic efficacy of mAbs (10-13).

Natural killer (NK) cells are the most important effector cells for ADCC. Different isotypes of mAbs bind to FcγRIIIa (expressed by NK cells) with a highly variable affinity, which appears to be a determining factor for the efficacy of NK cell-mediated ADCC. Previously, Ravetch *et al* (14) described a polymorphism in the membrane-proximal domain of FcγRIIIa. A nucleotide substitution at position 559 (G to T) of FcγRIIIa indicates either a valine or a phenylalanine at amino acid position 158 of FcγRIIIa, which results in three genotypes of FcγRIIIa: FcγRIIIa-158 F/F, FcγRIIIa-158 V/V and FcγRIIIa-158 V/F. Polymorphisms (VV/VF/FF) of FcγRIIIa markedly affect the binding affinity of mAbs, particularly the IgG1 isotype, and has been observed in multiple malignancies by its association with the degree of lysis of tumor cell targets *in vitro* (15-17). Normal serum levels of IgG may effectively compete with IgG1 for binding to low-affinity FcγRIII (CD16) *in vivo*, resulting in low ADCC of the IgG1 antibody (18). Rimawi MF (19) found that physiological levels of IgG in human serum strongly inhibited the ADCC of trastuzumab, and this inhibition could be fully reversed by adding trastuzumab. The competition of serum IgG with therapeutic

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IgG1 for binding to NK cells may be an explanation as to why high antibody doses are required when treating cancer by ADCC-based mechanisms. Some studies (20,21) also suggested that adding fresh frozen plasma (FFP) to rituximab may provide an effective therapeutic option for patients with advanced CLL who are resistant to chemotherapy.

As the specific mechanism underlying the effect of human serum IgG and complement on the anti-tumor efficacy of mAbs has not been fully elucidated, in this study, we used nested polymerase chain reaction (PCR) to detect Fc γ RIIIa polymorphisms in healthy volunteers, we investigated how the different Fc γ RIIIa genotypes affect the response of rituximab-dependent NK cell-mediated cytotoxicity by a cytotoxicity assay, and we evaluated the effect of human serum IgG and complement on rituximab-dependent NK cell-mediated cytotoxicity against tumor cells *in vitro*.

Subjects and methods

Ethics statement. Blood samples were obtained from 10 healthy volunteers (50% of whom were female), 5 for Fc γ RIIIa-158 V/V and 5 for Fc γ RIIIa-158 V/F. Written informed consent was obtained from all the subjects. All the volunteers were aged 25-30 years. The study protocol was approved by the Ethics Committee of Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University.

Nucleic acid extraction and allele-specific PCR for Fc γ RIIIa genotyping. Genomic DNA was purified from peripheral blood leukocytes using the Universal Genomic DNA Extraction kit v.3.0 (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. The Fc γ RIIIa-158 V/F polymorphism was identified by an allele-specific oligo-hybridization of a nested PCR amplification of genomic DNA (22). A gene-specific 1.2-kb fragment was amplified using the following sense and antisense primers (synthesized by Sangon Biotech, Shanghai, China), 5'-TGGCAAAGGCAGGAAGTATT-3' and 5'-GCGTGTAAGAATCAGGAATCTC-3' in a 25- μ l reaction with 3 μ l of genomic DNA, 2 pmol of each primer, 0.5 μ l of 10 mmol/l dNTP, 2.5 μ l of 10X PCR buffer and 1.5 U Taq polymerase. The PCR conditions were as follows: 5 min of initiation denaturation at 95°C, followed by 35 cycles at 95°C for 45 sec, 64°C for 45 sec, and 72°C for 30 sec. One μ l of this reaction was transferred to a separate microfuge tube for nested PCR in a total volume of 25 μ l, which contained 0.25 pmol of sense and antisense primers, 5'-ATCAGATTCGATCCTACTTCTGCAGGGGGCAT-3' and 5'-ACGTGCTGAGCTTGAGTGATGGTGATGTTTAC-3', 0.5 μ l of 10 mmol/l dNTP, 2.5 μ l 10X PCR buffer and 1.5 U Taq polymerase. The PCR conditions for the second amplification consisted of 35 cycles at 95°C for 45 sec, 65°C for 45 sec, and 72°C for 30 sec. The PCR products were visualized on a 3% agarose gel. A total of 8 μ l of the final PCR reaction was digested by *la*III restriction enzyme at 37°C overnight, and the products were visualized on 15% PAGE gel by silver staining.

NK cell preparation. The blood samples were layered onto Ficoll-Hypaque, and the peripheral blood mononuclear cells (PBMNCs) were isolated by gradient centrifugation (23,24).

These cells were washed twice in phosphate-buffered saline (PBS) and re-suspended at a concentration of 5x10⁶/ml for the NK cell ADCC. The cell surface markers CD3, CD56 and Fc γ RIIIa (CD16a) were analyzed by flow cytometry prior to ADCC. Fc γ RIIIa-positive PBMNCs may be an alternative to Fc γ RIIIa-positive NK cells as effector cells, which has been widely used in the immunology and clinical fields; thus, mononuclear cells were used as the ADCC effector cells in the present study (25-27).

Culture of Raji cells. Raji cells (Human Burkitt's lymphoma cell line), which highly express the CD20 antigen on their surface, were provided by the Shanghai Institute of Cell Bank. Raji cells were grown and maintained in RPMI 1640 (Gino Biomedical Technology, Hangzhou, China) supplemented with 10% fetal calf serum (FCS; Gino Biomedical Technology) at 37°C, 5% CO₂. The cell surface marker CD20 was analyzed by flow cytometry prior to ADCC.

Determination of the optimal concentration of rituximab for blocking CD20 on Raji cells in vitro. Raji cells were harvested and plated in 96-well culture plates at a defined density (1x10⁶/ml) and the regular medium was exchanged to medium containing 10, 20, 30, 40 and 50 μ g/ml rituximab (Roche, Shanghai, China). CD20 was tested after 30 min at 37°C by flow cytometry, and the optimal rituximab concentration was determined as that able to completely block CD20 in Raji cells.

Complement. As previously reported by Nechansky *et al* (28), we collected 5 ml of peripheral blood from healthy volunteers, and coagulation was allowed for 20 min followed by centrifugation of the collection tubes. Immediately after centrifugation, serum was aliquoted in 1.5-ml polypropylene tubes and frozen at -20°C until use. When processing, serum was defrosted and diluted in 1:1 with RPMI 1640, producing a medium with 50% human serum (containing complement).

Serum IgG. Serum was obtained by centrifugation of peripheral blood. Complement in the serum was inactivated in a 56°C water bath incubator for 30 min (28). The inactivated serum was mixed with RPMI 1640 at a ratio of 2:3, achieving a medium of 40% human serum (containing serum IgG).

Serum IgG and Fc γ RIIIa binding assays in the absence of mAb. The binding of serum IgG to Fc γ RIIIa on NK cells was studied by flow cytometry. Briefly, 0.1 ml of 5x10⁶/ml PBMNCs were incubated in the absence or presence of 4.8 mg/ml human serum IgG for 30 mins at 37°C in a 5% CO₂ incubator, washed twice with PBS, followed by flow cytometric analysis of cell-bound Fc γ RIIIa.

Cytotoxicity assay. The cytotoxicity assay was divided into two groups (Fc γ RIIIa V/V and Fc γ RIIIa V/F) according to the Fc γ RIIIa genotypes of NK cells, and each group was further subdivided into four groups: Negative control, ADCC, ADCC+CDC and serum IgG groups. Raji cells were labeled with DIO (Beyotime Biotechnology, Jiangsu, China) at 37°C for 30 min and washed three times with PBS to remove unreacted and unbound DIO. A total of 3 μ l of 0.1 μ g/ μ l rituximab

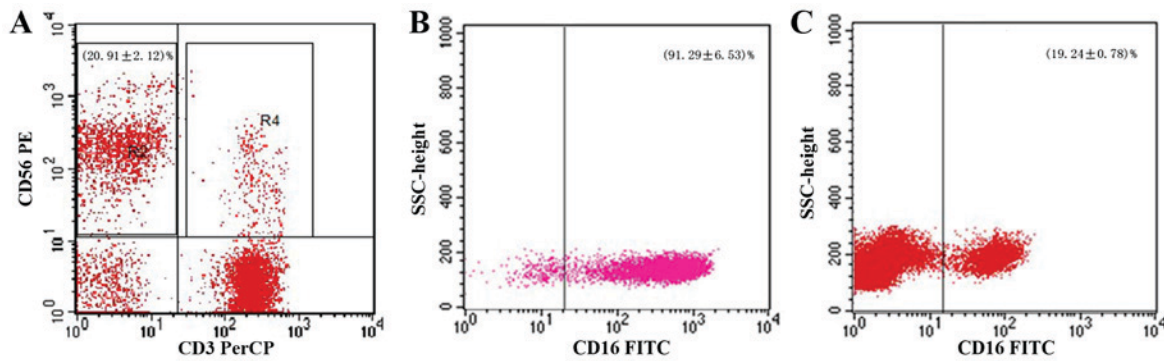


Figure 1. Surface markers of NK cells, PBMNCs, CD3, CD56 and Fc γ RIIIa (CD16a) were analyzed by flow cytometry. (A) The results indicate that 20.91 \pm 2.12% of PBMNCs were NK cells with CD3-CD56+. (B) The expression level of Fc γ RIII on NK cells was 91.29 \pm 6.53% and (C) 19.24 \pm 0.78% of PBMNCs express Fc γ RIII. NK, natural killer; CD, cluster of differentiation; PBMNC, peripheral blood mononuclear cell.

was added to the ADCC, ADCC+CDC and serum IgG groups, and serum was added to the serum IgG group at the same time. Each group was incubated for 4 h at 37°C in a 5% CO₂ incubator, and then the labeled target cells were re-suspended in RPMI 1640 containing 10% FCS (only the ADCC+CDC group was re-suspended in RPMI 1640 containing 50% human serum) and mixed with PBMNCs at an effector/target ratio (E/T) of 5:1. All the cells were incubated at 37°C for 4 h and washed twice with PBS, followed by the addition of 5 μ l propidium iodide (PI) (Beyotime Biotechnology). Finally, cells were analyzed by flow cytometry after a 30-min incubation in the dark. The negative control did not contain PBMNCs. The percentage of killed cells was calculated as follows: (% of living cells in negative control-% of living cells in sample)/% of living cells in negative control.

Statistical analysis. The results are expressed as the mean \pm standard error of the mean, and the data were analyzed by SPSS 16.0 statistical software. An independent samples t-test was used to evaluate the difference between Fc γ RIII-positive PBMNC and Fc γ RIII-positive NK cells in PBMNCs. The expression levels of Fc γ RIIIa in NK cells before and after adding serum in the absence of mAb *in vitro* were also analyzed using an independent samples t-test. The comparison of cytotoxic index between the groups with multivariate analysis of variance, after the equal check of variance, and the two-two comparisons among the means were performed using the Student-Newman-Keuls method. P<0.05 was considered to indicate a statistically significant difference.

Results

Human PBMNCs may be an alternative to NK cells as the effector cells. In this study, the results demonstrated that 20.91 \pm 2.12% of PBMNCs were CD3⁺CD56⁺ NK cells (Fig. 1A), and the expression level of Fc γ RIII on NK cells was 91.29 \pm 6.53% (Fig. 1B). A total of 19.24 \pm 0.78% of PBMNCs expressed Fc γ RIII (Fig. 1C), and NK cells expressing Fc γ RIII accounted for 19.02 \pm 0.57% of PBMNCs (Table I and Fig. 2); thus, NK cells were the main Fc γ RIII-positive cells in PBMNCs. Therefore, Fc γ RIII-positive PBMNCs may be an alternative to Fc γ RIII-positive NK cells as effector

cells in our experiment, which was also confirmed by other studies (25-27).

Optimal concentration of rituximab for blocking CD20 on Raji cells. In order to establish the peak dose of rituximab for saturating CD20 on Raji cells, rituximab was added to the medium with Raji cells (1 \times 10⁶/ml) at sequential concentrations of 10, 20, 30, 40 and 50 μ g/ml in different groups. After incubating for 30 min at 37°C, the levels of CD20 on Raji cells decreased in a dose-dependent manner to 6.16, 3.60, 0.38, 0.29 and 0.27%, respectively. CD20 downregulation was first noted at a rituximab concentration of 10 μ g/ml, reaching a plateau at concentration of 30 μ g/ml. There was no significant difference among the last three groups (P>0.05); thus, 30 μ g/ml was selected as the optimal concentration of rituximab for blocking CD20 in subsequent experiments.

Normal human serum does not affect the expression of Fc γ RIIIa in NK cells. To clarify whether normal human serum affects the expression of Fc γ RIIIa in NK cells *in vitro*, we examined the expression of Fc γ RIIIa in NK cells before and after the addition of serum in the absence of mAb *in vitro*, and it was found to be 91.16% (Fig. 3), compared with that before adding normal human serum; the difference was not statistically significant.

Complement enhances NK cell cytotoxicity induced by rituximab-coated Raji cells. We performed an *in vitro* ADCC assay with the Human Burkitt's lymphoma cell line Raji as target cells, human PBMC as effector cells and rituximab as human IgG1 mAb. In the ADCC group, with the absence of human serum, NK cells with different Fc γ RIIIa genotypes exerted various cytotoxic effects on Raji cells. The cytotoxic indices of NK cells with the Fc γ RIIIa-158 V/V and Fc γ RIIIa-158 V/F genotype were 69.05 \pm 2.38 and 39.63 \pm 3.86%, respectively (Table II, Figs. 4 and 5), with the cytotoxic index of NK cells with the Fc γ RIIIa-158 V/V genotype being significantly higher compared with that of the Fc γ RIIIa-158 V/F genotype (n=5, P<0.05 in all cases).

Irrespective of the group, the cytotoxic index in NK cells with the Fc γ RIIIa-158 V/V genotype was significantly higher compared with that of the Fc γ RIIIa-158 V/F genotype (n=5, P<0.05 in all cases). Irrespective of the Fc γ RIIIa-158 genotype,

Table I. Fc γ RIII-positive PBMNC and Fc γ RIII-positive NK cells in PBMNCs (n=5).

Location	Fc γ RIII-positive PBMNCs (%)	Fc γ RIII-positive NK cells (%)
PBMNCs	19.24 \pm 0.78	19.02 \pm 0.57

Data are expressed as the mean \pm standard error of the mean. NK, natural killer; PBMNC, peripheral blood mononuclear cell.

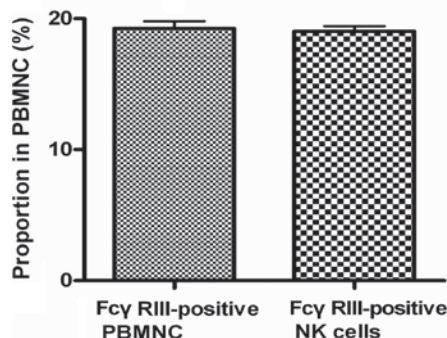


Figure 2. Fc γ RIII-positive PBMNCs and Fc γ RIII-positive NK cells in PBMNC. Fc γ RIII expression of PBMNCs was 19.24 \pm 0.78% and Fc γ RIII expression of NK cells in PBMNCs was 19.02 \pm 0.57% as NK cells were the primary Fc γ RIII-positive cells in the PBMNCs. The Fc γ RIII-positive PBMNCs may be an alternative to the Fc γ RIII-positive NK cells as effector cells in the experiment. The difference in Fc γ RIII expression in PBMNC and NK cells was not statistically significant. NK, natural killer; PBMNC, peripheral blood mononuclear cell.

the cytotoxic index in the ADCC+CDC group was significantly higher compared with that in the ADCC group, and the cytotoxic index in the ADCC group was higher compared with that in the serum IgG group (n=5, P<0.05 in all cases).

In group ADCC+CDC, replacement of 50% of the assay buffer with human serum containing complement strongly enhanced ADCC by rituximab, and the cytotoxic index in the ADCC+CDC group was significantly higher compared with that in the ADCC group (P<0.05), irrespective of the Fc γ RIIIa-158 genotype (Table II, Figs. 4 and 5).

Normal human serum inhibits NK cell cytotoxicity induced by rituximab-coated Raji cells. In order to identify the effect of human serum on ADCC, 40% of the assay buffer was replaced with human serum (containing human IgG; complement was inactive) in the serum IgG group. The result revealed that the cytotoxic index in the serum IgG group was lower compared with that in the ADCC group (P<0.05). The inhibitory effect of human serum on ADCC by rituximab was obvious, irrespective of the Fc γ RIIIa-158 genotype (Table II, Figs. 4 and 5).

Discussion

Fc γ Rs are the key link between humoral and cellular immune responses. They are important in immune regulation and mediate ADCC, endocytosis, phagocytosis, release of inflammatory cytokines and antigen presentation (29). There are three

Table II. Complement and serum IgG on rituximab-dependent NK cell-mediated cytotoxicity against Raji cells (n=5).

Genotype	ADCC group (%)	ADCC+CDC group (%)	Serum IgG group (%)
V/V	69.05 \pm 2.38	94.25 \pm 1.79 ^{a,c}	59.79 \pm 0.66 ^{a,b}
V/F	39.63 \pm 3.86	66.71 \pm 5.57 ^{a,c}	18.13 \pm 2.99 ^{a,b}

Data are expressed as the mean \pm standard error of the mean. ^aP<0.05 vs. the ADCC group; ^bP<0.05 vs. the ADCC+CDC group; and ^cP<0.05 vs. serum IgG group. In NK cells with Fc γ RIIIa-158V/V, the cytotoxic indices of ADCC group, ADCC+CDC group and serum IgG group were (69.05 \pm 2.38)%, (94.25 \pm 1.79)% and (59.79 \pm 0.66)% respectively, thus significantly different between groups. Similarly, the group of NK cell with Fc γ RIIIa-158V/F shows the same result. Ig, immunoglobulin; NK, natural killer; ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity.

classes of Fc γ Rs: Fc γ RI (CD64) Fc γ RII (CD32) and Fc γ RIII (CD16), with Fc γ RIII further subdivided into Fc γ RIIIa and Fc γ RIIIb (30). Fc γ RIIIa is a transmembrane glycoprotein, which is expressed on monocytes, macrophages, NK cells and some T cells, while Fc γ RIIIb is only expressed on neutrophils and IFN-induced eosinophils (31). In the present study, we found that the Fc γ RIII on NK cells was all Fc γ RIIIa. The expression of Fc γ RIII on PBMNCs was 19.24 \pm 0.78%, and that on NK cells was 19.02 \pm 0.57%; therefore, the Fc γ RIII-positive PBMNCs were essentially Fc γ RIII-positive NK cells and are referred to as effector cells in our experiment (29-31).

Previous studies (10,11,32-35) demonstrated that polymorphisms of Fc γ RIIIa on NK cells result in variable affinity for mAbs, subsequently affecting the ADCC. The therapeutic efficacy of mAbs in patients with the Fc γ RIIIa-158 V/V genotype is superior to that in patients with the Fc γ RIIIa-158 V/F and Fc γ RIIIa-158 F/F genotypes. Hatjiharissi *et al* (36), found that patients with NHL expressing at least one valine at Fc γ RIIIa-158 may, in part, exhibit a better therapeutic response to mAb treatment. In the present study, we determined the effect of Fc γ RIIIa polymorphism on ADCC activity of rituximab-dependent NK cells, and our experimental results demonstrated that the ADCC activity of NK cells with Fc γ RIIIa-158 V/V was higher compared with that of Fc γ RIIIa-158 V/F, in accordance with the abovementioned reports.

A nucleotide substitution at position 559 of Fc γ RIIIa predicts either a valine or a phenylalanine at amino acid position 158 of Fc γ RIIIa, and results in three genotypes of Fc γ RIIIa, namely Fc γ RIIIa-158 F/F, Fc γ RIIIa-158 V/V and Fc γ RIIIa-158 V/F (14). Dall'Ozzo *et al* (16) detected circulating CD16⁺ mononuclear cells in these three Fc γ RIIIa genotype subsets, demonstrated that the expression of CD16 on NK cells did not change significantly, and Fc γ RIIIa-158 V exhibited a higher affinity to rituximab compared with Fc γ RIIIa-158 F, as the former can bind more strongly to the mAb Fc segments, resulting in enhancement of rituximab-mediated ADCC. Hatjiharissi *et al* (36) also demonstrated that Fc γ RIIIa-158 V/V may increase rituximab binding, which was confirmed by Dall'Ozzo *et al* (16). Therefore, according to the results of

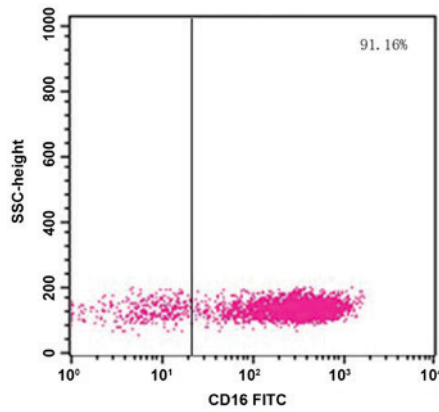


Figure 3. Expression of FcγRIIIa in natural killer cells after adding serum in the absence of monoclonal antibodies *in vitro*. FcγRIIIa expression was 91.16% following the addition of serum. There is no significant difference between them.

previous studies (16,36) and our findings, NK cells with the FcγRIIIa-158 V allele have a higher affinity for rituximab, suggesting a more effective rituximab-mediated ADCC compared with those having the FcγRIIIa-158 F allele; these findings may help improve the elimination of tumor cells to achieve a better therapeutic effect.

The complement system is composed of >30 soluble plasma proteins and membrane proteins that can trigger three distinct protease cascades known as the classical, mannose-binding lectin (MBL), and alternative pathways (37). When the complement system is activated, the membrane attack complex (MAC) is inserted into the cell membrane and directly induces the lysis of targeted cells through subsequent influx of ions and water, leading to lethal colloid-osmotic edema (CDC). CDC and ADCC are both suggested as being important for rituximab-mediated tumor cell eradication; however, the determinant mechanisms remain unknown. Van Meerten *et al* (38), established an experimental system in which the CD20 molecule is the only variable factor, and the results suggested that the CDC activity of rituximab depends on the level of CD20 expression. Other studies (39,40) also reported a linear correlation between CD20 and CDC, proving that rituximab-mediated CDC depends on the expression level of CD20.

In the present study, we investigated the changes in rituximab-dependent NK cell-mediated cytotoxicity in Raji cells after adding serum complement *in vitro*, in order to elucidate the role of serum complement in mAb-dependent NK cell-mediated cytotoxicity against tumor cells. Our results demonstrated that rituximab-mediated cytotoxicity in the presence of complement was significantly enhanced. Other studies (38,40) demonstrated that, as the C1q and FcγRIIIa binding sites were in close proximity, following rituximab binding to CD20, the C1q of complement binds to the CH2 domain of IgG1, thereby triggering the classic complement pathway that finally activates CDC, which supports the results of our study. Anaphylatoxin C5a, the by-product of CDC, interacting with the C5a receptor, can upregulate the expression of FcγRIIIa on effector cells, downregulate the expression of inhibitory receptors FcγRIIb, activate effector cells and enhance the mAb-mediated ADCC antitumor

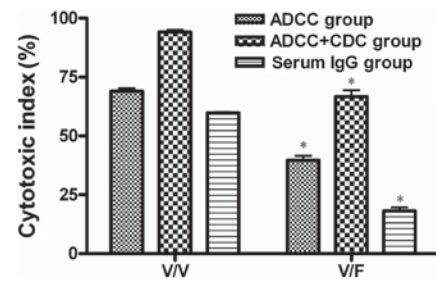


Figure 4. Complement and serum IgG on rituximab-dependent NK cell-mediated cytotoxicity against Raji cells. The cytotoxic index in NK cells of the FcγRIIIa-158 V/V genotype was significantly increased compared with the respective FcγRIIIa-158 V/F genotype individuals in all groups. *P<0.05 vs. the corresponding V/V group. Ig, immunoglobulin; NK, natural killer; ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity.

effect (18,41-44). In the presence of mAb, the activity of CDC depends on the CD20 expression level. When CDC is activated, ADCC is synchronously promoted. CDC and ADCC interact in a complementary manner and enhance mAb-dependent NK cell-mediated cytotoxicity *in vitro*. Plasma contains complement, which can effectively activate the classic complement pathway to trigger CDC, promote ADCC and mediate cytotoxicity targeted against tumor cells. Our findings indicate that CDC plays a key role in tumor cell elimination, and complement blocks rituximab-induced NK-cell mediated ADCC.

As is well-known, human IgG1 mAb, which is commonly used in cancer treatment, requires a higher blood concentration; however, the reason as to why the strong ADCC of anticancer mAb *in vitro* is contrasted by its weakness *in vivo* has not yet been fully elucidated. Some researchers (18,45) observed that physiological levels of IgG in human serum strongly inhibited the ADCC of the IgG1 antibody, which may be one of the reasons explaining why ADCC of mAb is sometimes suppressed *in vivo*. In our study, we detected the changes in rituximab-dependent NK cell-mediated cytotoxicity against Raji cells by adding serum IgG *in vitro*, aiming to investigate the effect of serum IgG on mAb-dependent NK cell-mediated cytotoxicity. Our results demonstrated that rituximab-mediated cytotoxicity in the group with the addition of serum was significantly reduced compared with that in the group without serum addition, which is consistent with the results of other studies (18,45-48). Preithner *et al* (18), demonstrated that removal of serum IgG abolished the inhibitory effect on ADCC, while this could be fully reversed by adding exogenous serum IgG, indicating that physiological levels of human serum IgG could inhibit the ADCC of mAb *in vitro*. Nechansky *et al* (28) reported that mAb diluted in 40% of normal human serum (NHS), containing high amounts of endogenous IgG (~4.8 mg/ml), resulted in a significantly lower ADCC activity compared with that diluted in 10% FCS, which contains less IgG (~10 μg/ml), indicating that the high IgG content in NHS can significantly decrease the ADCC activity mediated by mAbs. Further research demonstrated that physiological level of human serum IgG can also impair the ADCC of the therapeutic mAb *in vivo* by competitively binding to FcγRIIIa on the effector cells (18,46-48), thus resulting in inhibition of mAb-mediated ADCC. Our results also indicated that serum IgG competes with rituximab Fc

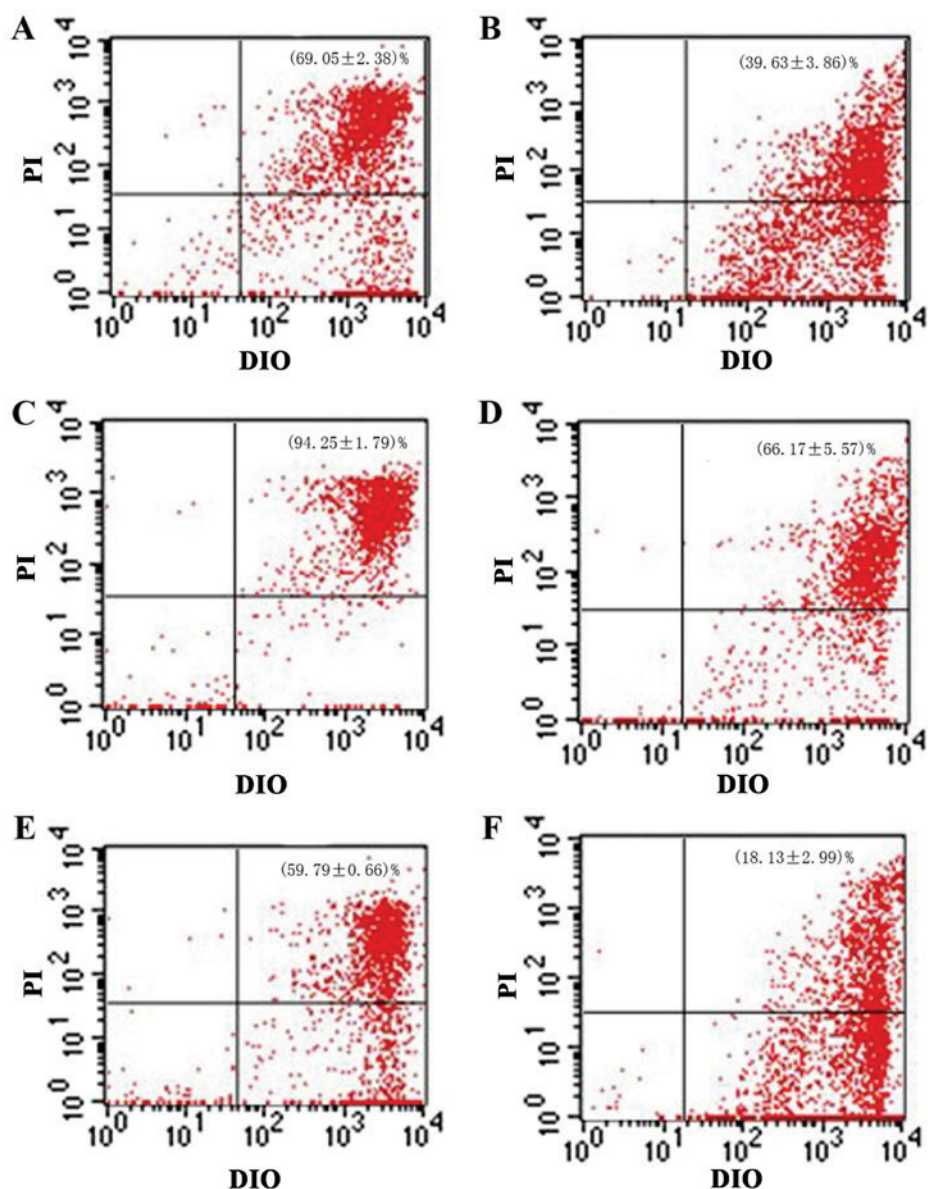


Figure 5. Rituximab-dependent NK cell-mediated cytotoxicity against Raji cells prior to and following the addition of complement and serum IgG. (A) The cytotoxic index of ADCC group in Fc γ RIIIa-158 V/V. (B) The cytotoxic index of ADCC group in Fc γ RIIIa-158 V/F. (C) The cytotoxic index of ADCC+CDC group in Fc γ RIIIa-158 V/V. (D) The cytotoxic index of ADCC+CDC group in Fc γ RIIIa-158 V/F. (E) The cytotoxic index of serum IgG group in Fc γ RIIIa-158 V/V. (F) The cytotoxic index of serum IgG group in Fc γ RIIIa-158 V/F. The results indicated that the cytotoxic index in the serum IgG group was significantly reduced compared with the ADCC group ($P < 0.05$). The inhibitory effect of human serum on ADCC by rituximab was clear, irrespective of Fc γ RIIIa-158 V/V or Fc γ RIIIa-158 V/F genotype. Ig, immunoglobulin; NK, natural killer; ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity.

segments for binding to the Fc γ RIIIa, thus interfering with the antitumor effect of rituximab.

Furthermore, we examined the ratio of Fc γ RIIIa expression on NK cells before and after adding serum in the absence of mAb, which was 95.91 and 91.16%, respectively; interestingly, the difference was not statistically significant. Although we demonstrated that serum did not significantly affect the blockade of Fc γ RIIIa on NK cells, it did ultimately inhibit ADCC of rituximab. This may be explained by the following hypotheses: ADCC is usually investigated in the absence of human serum through the quantification of target cell lysis by the addition of effector cells to the mixture of mAbs and target cells. Previous studies have demonstrated that the

primary activating Fc γ R on NK cells is the low-affinity type III receptor (CD16) (48,49). NK cell-bound endogenous IgG may be rapidly displaced by the mAb IgG1 due to an avidity effect when it forms a polyvalent CD16-binding matrix on the surface of its target cell (18); therefore, if the serum IgG does not combine with epitopes on the target cells in advance, the affinity of the free serum IgG to NK cells may be low and the serum appears to exert no significant effect on the blockade of Fc γ RIIIa on NK cells. However, in our experiment, the serum IgG concentration (0.5-0.9 mg/ml (18)) was ~17-30 times that of rituximab (30 μ g/ml) in the serum IgG group; this high difference in concentration and resulting competitive effect may at least in part account for the serum IgG combining

with Fc γ RIIIa more effectively and inhibiting the ADCC of rituximab. We observed that replacement of 50% of the assay buffer with human serum containing complement strongly enhanced ADCC by rituximab. This enhanced ADCC was not in line with previous results (50) and the specific underlying mechanism is not clear. The observed enhanced killing of tumor cells may also be the consequence of simultaneous induction of ADCC and CDC, and CDC may participate in blocking this reaction, while allowing opsonization with complement components. Although these are possible explanations, further investigation is required to fully elucidate the specific underlying mechanisms.

The rituximab-mediated ADCC may be inhibited by the presence of serum IgG; thus, serum IgG may interfere with the efficacy of rituximab. Due to the presence of serum, we hypothesized that the ADCC efficacy of IgG1 antibodies *in vivo* would be low, despite it being high *in vitro*, so that antitumor efficacy in the clinical setting usually requires large doses of mAb to maintain a high serum level. Our findings demonstrated that serum IgG plays a negative role in tumor cell elimination.

In summary, the present study suggests that Fc γ RIIIa polymorphism in NK cells may modulate the ADCC activity of rituximab, and the ADCC activity of NK cells with Fc γ RIIIa-158 V/V is higher compared with that with Fc γ RIIIa-158 V/F. Additionally, serum IgG inhibits rituximab-mediated ADCC, and complement can effectively enhance ADCC, both of which are involved in mediating the cytotoxicity of rituximab in opposite ways. Although either complement or serum IgG can also affect the cytotoxic ability against non-CD20-expressing tumor cells, a large volume of evidence shows that loss of CD20 expression results in inhibition of anti-CD20 mAb (rituximab) binding, and a CD20-negative phenotypic change after treatment with rituximab can be found, with relapse or progression in a number of CD20-positive B-cell lymphoma patients, indicating a correlation between the CD20 expression and rituximab sensitivity (51-53). Therefore, in this experiment we did not select CD20-negative cells as the target cells. Due to time and funding limitations, in this experiment we set the polymorphism of Fc γ RIIIa on NK cells as the only variable, and set the optimal dosage (according to the conclusion of the preliminary experiment or reference) of complement or serum IgG as a constant, so there are no data on the association between the concentration of complement or serum IgG and cytotoxicity. To better elucidate this issue, selecting more tumor cell lines with or without expression of CD20 may help provide a better explanation of the underlying mechanism, as further data accumulation is required.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YL was responsible for the experiment and wrote the manuscript. KH performed the design of experiment and collection of the samples. LL and YQ analyzed the experiment data. YW, YH and JW were accountable for performing data interpretation and revising the manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University and written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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