

## Fc $\gamma$ RIIIa Expression Is Not Increased on Natural Killer Cells Expressing the Fc $\gamma$ RIIIa-158V Allotype

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### Abstract

The presence of a valine (V) versus a phenylalanine (F) at position 158 of Fc $\gamma$ RIIIa/CD16a improves the affinity for IgG and is associated with higher therapeutic response to rituximab. Increased CD16 expression on natural killer (NK) cells from donors with the VV or VF versus FF genotype has recently been reported. We indeed observed higher binding of the anti-CD16 monoclonal antibody (mAb) 3G8 on NK cells from V carriers (VV = VF > FF). However, the binding of two other anti-CD16 mAbs, LNK16 and DJ130c, decreased with the number of V allele (VV < VF < FF). CD16 transcript levels were independent on the genotype. Rituximab binding to NK cells from V carriers was higher than its binding to FF NK cells at low concentrations (10 and 100  $\mu$ g/mL). However, the difference was nearly completely abolished at saturating concentrations ( $\geq$ 1,000  $\mu$ g/mL). Finally, nearly 100% of CD16-expressing NK cells displayed a complete down-modulation of the receptor after optimal engagement by plate-bound 3G8, whatever the genotype. By contrast, the percentages of NK cells down-modulating CD16 after competitive engagement of the receptor by plate-bound rituximab increased with the number of V allele (FF, 18.2  $\pm$  8.6%; VF, 32.0  $\pm$  4.9%; and VV, 42.4  $\pm$  9.9%). These results are in discrepancy with the expected increased competition that would result from an increased expression of CD16 on VV and VF NK cells. We conclude that increased binding and functional and clinical responses associated with the high-affinity Fc $\gamma$ RIIIa-158V are unrelated to an increased expression of this allotype. [Cancer Res 2008;68(4):976–80]

### Introduction

Fc $\gamma$ RIIIa/CD16a, one of the low-affinity receptors for IgG Fc, is involved in antibody-dependent cell-mediated cytotoxicity (ADCC). It links IgG-sensitized target cells to Fc $\gamma$ RIIIa/CD16a-bearing cytotoxic cells, such as CD56<sup>dim</sup> natural killer (NK) cells, and activates them. The *FCGR3A* gene, which encodes Fc $\gamma$ RIIIa, displays a functional allelic dimorphism generating allotypes with either a phenylalanine (F) or a valine (V) at amino acid position 158 (1, 2). This residue directly interacts with the lower hinge region of IgG1 (3, 4). Accordingly, NK cells from donors homozygous for Fc $\gamma$ RIIIa-158V (VV) bound more human IgG1 and IgG3 than did NK cells from donors homozygous for Fc $\gamma$ RIIIa-158F (FF; refs. 1, 2). Rituximab (Mabthera, Rituxan) is a chimeric anti-CD20 IgG1

monoclonal antibody (mAb) used to treat patients with B-cell lymphomas and various autoimmune disorders. Among the most convincing evidence that ADCC plays a role in mediating the clinically relevant antitumor response of rituximab is the demonstration by our group that the *FCGR3A* gene polymorphism is associated with the rituximab response in non-Hodgkin's lymphoma patients (5, 6). Similar results were obtained in Waldenström's disease patients (7) and in lupus patients (8). All these studies confirm that the Fc $\gamma$ RIIIa-158V allotype is associated with the best therapeutic response. Several studies have shown the higher affinity of both the soluble and membrane forms of the Fc $\gamma$ RIIIa-158V allotype (9, 10). We have previously shown that VV and FF NK cells killed CD20<sup>+</sup> Daudi cells similarly after Fc $\gamma$ RIIIa engagement by saturating concentrations of rituximab. However, the rituximab concentration resulting in 50% lysis (EC<sub>50</sub>) observed with VV NK cells was lower than that observed with FF NK cells (9). We concluded that the higher rituximab-mediated ADCC of VV NK cells resulted from the higher affinity of the Fc $\gamma$ RIIIa-158V allotype. In accordance, several recombinant mAbs with modified Fc fragment and higher affinity for the Fc $\gamma$ RIIIa display higher ADCC *in vitro* (10–13). By contrast, Hatjiharissi et al. (14) have recently reported higher rituximab-dependent ADCC of VV and VF NK cells at very high rituximab concentrations. They concluded, in accordance with a previous study (15), that the number of Fc $\gamma$ RIIIa receptors accounts for the difference in IgG binding and that a higher membrane expression of CD16 on VV and VF NK cells may also contribute to augmented rituximab-mediated ADCC, in addition to possible differences in binding affinity.

The aim of this study was to further investigate the influence of the *FCGR3A* polymorphism on cell-surface Fc $\gamma$ RIIIa expression, Fc $\gamma$ RIIIa gene transcription, rituximab binding to NK cells, and rituximab- or anti-CD16-induced NK cell CD16 down-modulation. Our results support the conclusion that Fc $\gamma$ RIIIa/CD16a expression is not increased on NK cells expressing the Fc $\gamma$ RIIIa-158V allotype.

### Materials and Methods

**FCGR3A-158V/F genotyping.** Genotyping of the *FCGR3A*-158V/F polymorphism was done as previously described (16).

**NK cell preparation.** NK cells were prepared from peripheral blood mononuclear cells with the NK cell Isolation Kit II MACS (Miltenyi Biotec) as described (9).

**CD16 expression.** Blood samples were incubated with 20  $\mu$ L of FITC-conjugated UCHT1 anti-CD3 mAb (anti-CD3-FITC), phycoerythrin-cyanin 5.1-conjugated NKH-1 anti-CD56 mAb (anti-CD56-PC5), and phycoerythrin-conjugated 3G8 (Beckman Coulter), LNK16 (Abcam), and DJ130c (DakoCytomation) anti-CD16 mAbs during 10 min at 20°C, and then analyzed by flow cytometry.

**Binding of rituximab.** Purified NK cells were incubated with increasing concentrations (from 0.01 to 3,000  $\mu$ g/mL) of FITC-conjugated rituximab during 30 min at 4°C and analyzed by flow cytometry.

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**CD16 down-modulation and CD107 expression on NK cells stimulated by plate-bound 3G8 or rituximab.** Maxisorp microplates (Nunc) were sensitized overnight with 5  $\mu$ g/mL of rituximab or 3G8 at 4°C. Freshly isolated NK cells were then incubated (4 h at 37°C) in the presence or absence of 10  $\mu$ L of anti-CD107-PC5 mAb (BD Biosciences; ref. 17). Cells were then stained with 10  $\mu$ L of FITC-conjugated 3G8 (Beckman Coulter) and analyzed by flow cytometry. Percentages of NK cells with CD16 down-modulation were calculated using the following formula:

$$\% = [(A - B)/A] \times 100$$

where *A* is the percentage of CD16-expressing NK cells after incubation in unsensitized plates and *B* is the percentage of CD16-expressing NK cells after incubation in sensitized plates.

**ELISA assay.** Binding of 3G8 mAb and rituximab on a culture plate were checked by ELISA using peroxidase-conjugated antimurine and antihuman IgG (Sigma), respectively. Absorbances at 492 and 620 nm were obtained with an iEMS reader MF spectrometer (Labsystems).

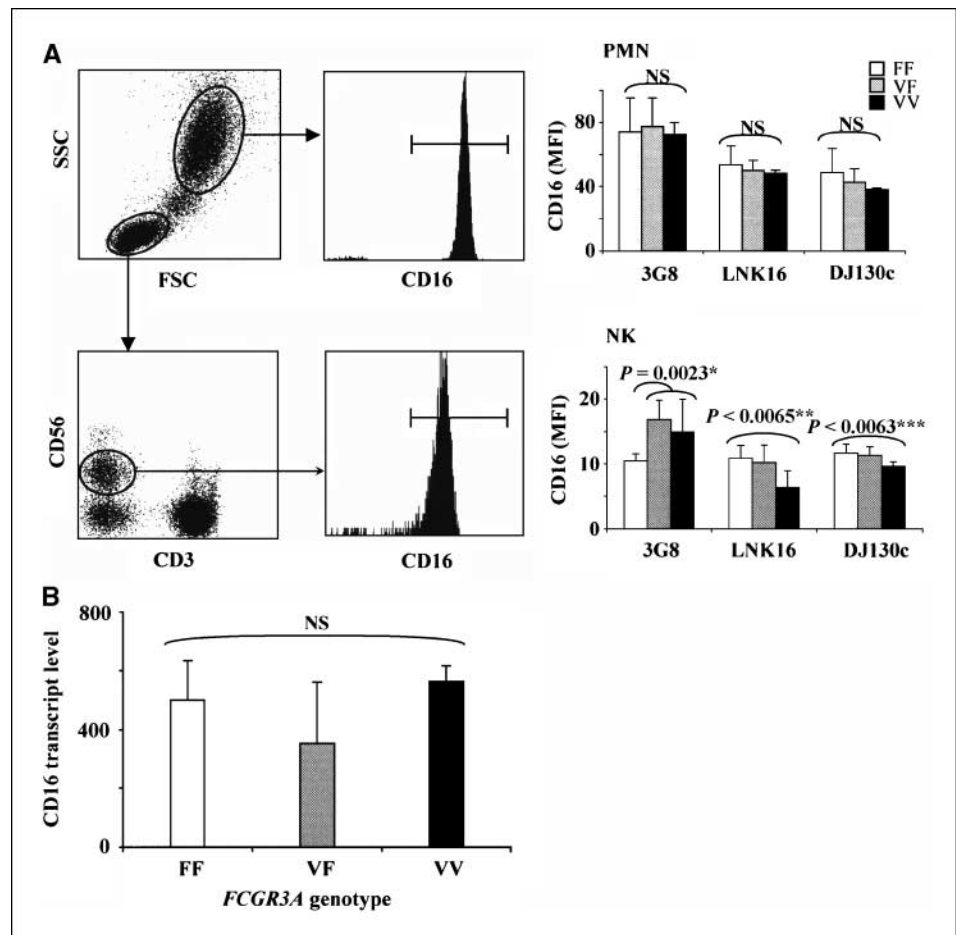
**Reverse transcription-PCR.** Fc $\gamma$ RIIIa gene expression was determined by quantitative real-time PCR (iCycler, Bio-Rad). Two hundred nanograms of RNA extracted from NK-cells were reverse transcribed with the RevertAid MMuV reverse transcriptase (Fermentas) according to the manufacturer's recommendations. The quantitative PCR reaction was conducted with 1  $\mu$ L of cDNA in a 25- $\mu$ L final volume mixture containing Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and *FCGR3A* primers (sense, 5'-ATGTGCTCTCAGAGACTGTGAAC-3'; antisense, 5'-TTTATGGTCCTTCAGTCTCTTG-3'; Eurogentec). The quantity of Fc $\gamma$ RIIIa mRNA in each sample was normalized to the relative quantity of glyceraldehyde-3-phosphate dehydrogenase.

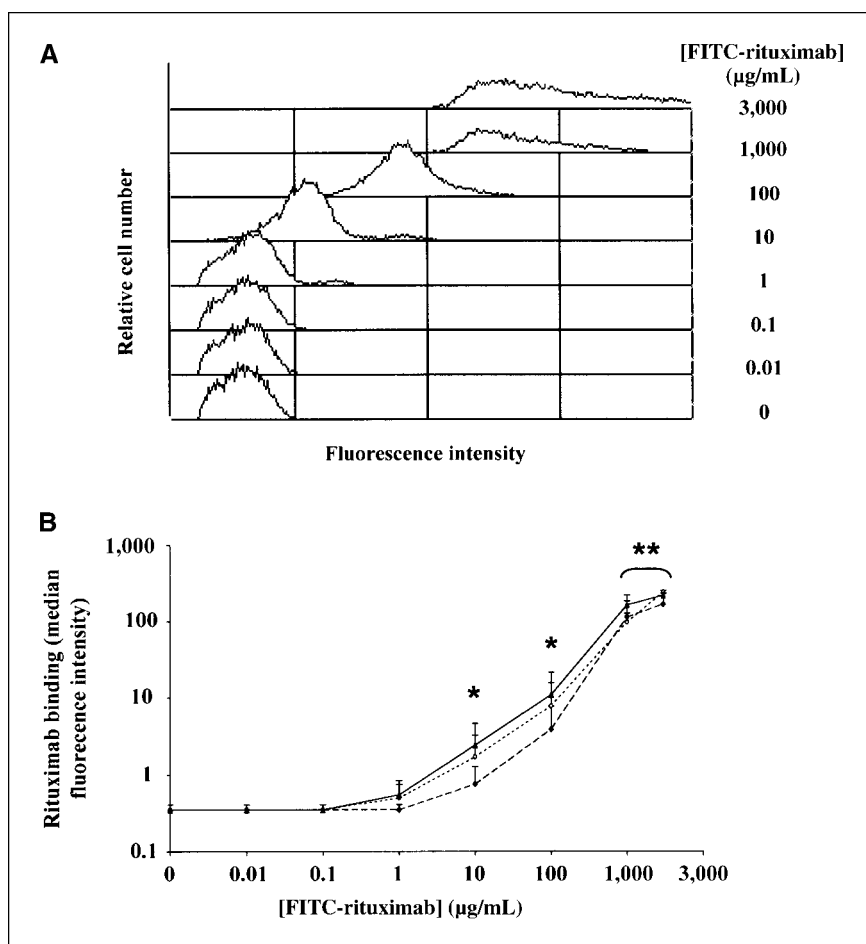
**Statistics.** Differences among polymorphic groups were analyzed using the Kruskal-Wallis and Wilcoxon tests. The influence of the V allele number was analyzed by linear regression and significance of the slope was calculated with the Wald test.

**Results and Discussion**

We first analyzed the CD16 expression on leucocytes from *FCGR3A*-genotyped donors, with a combination of anti-CD16, anti-CD56, and anti-CD3 mAbs, followed by flow cytometry analysis (Fig. 1). Three different phycoerythrin-conjugated anti-CD16 mAbs were used: 3G8, LNK16, and DJ130c. They recognize an epitope within the FG loop of the membrane-proximal domain (EC2; ref. 18), within the C'  $\beta$  sheet of the EC2 and within the membrane-distal (EC1) domain, respectively. As expected, the binding of the three anti-CD16 mAbs to polymorphonuclear cells, which express Fc $\gamma$ RIIIb/CD16b (V at position 158), was independent of the *FCGR3A* genotype (Fig. 1A). We observed a significantly higher binding of the 3G8 mAb to CD3<sup>+</sup>CD56<sup>dim</sup> NK cells (Fig. 1A) from VV and VF donors compared with FF donors in accordance with previous reports (1, 15) including ours (9) and with the results obtained with an unspecified mAb by Hatjiharissi et al. (14). Conversely, the binding of the LNK16 mAb and, to a lesser extent, that of the DJ130c mAb to these NK cells decreased with the number of V allele (VV<VF<FF). The opposite results obtained with the 3G8 and LNK16 mAbs strongly suggest that the F→V substitution at position 158 increases the affinity of the 3G8 for

**Figure 1.** Influence of the V158F polymorphism on CD16 membrane expression and on Fc $\gamma$ RIIIa transcript level. **A**, blood samples from *FCGR3A* genotyped donors were labeled with anti-CD3-FITC, anti-CD56-PC5, and anti-CD16-phycoerythrin mAbs (clone 3G8, LNK16, or DJ130c). Cells were then analyzed by flow cytometry. A gate was set on polymorphonuclear cells (PMN; top histogram) and on CD3<sup>+</sup>CD56<sup>dim</sup> lymphocytes (bottom histogram). The mean fluorescence intensity (MFI) of CD16 staining obtained with each mAb is expressed according to the *FCGR3A* genotype [white columns, FF (*n* = 6); gray columns, VF (*n* = 6); black columns, VV (*n* = 6)]. **B**, Fc $\gamma$ RIIIa gene expression was determined by quantitative real-time PCR. RNA was extracted from NK cells from 12 of the 18 donors evaluated above (*n* = 4 for each genotype). The quantity of Fc $\gamma$ RIIIa mRNA in each sample was normalized to the relative quantity of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Columns, mean; bars, SD. \*, *P* = 0.0023, 3G8 binding on NK cells from FF donors compared with NK cells from V carriers. \*\*, *P* = 0.0065, influence of the V allele number on LNK16 binding. \*\*\*, *P* = 0.0063, influence of the V allele number on DJ130c binding. NS, not significant.





**Figure 2.** Influence of the V158F polymorphism on rituximab binding to NK cells. NK cells from *FCGR3A* genotyped donors were incubated with FITC-conjugated rituximab and analyzed by flow cytometry. **A**, rituximab binding to NK cells from a given donor incubated with increasing concentrations of FITC-conjugated rituximab. **B**, rituximab binding observed on NK cells from four VV (solid line), four VF (dotted line), and four FF (broken line) donors. Points, mean fluorescence intensities; bars, SD. \*, rituximab binding to VV NK was  $\approx 285\%$  that to FF NK cells. \*\*, rituximab binding to VV NK was 145% and 130% that to FF NK cells at 1,000 and 3,000  $\mu\text{g/mL}$ , respectively.

its epitope while decreasing that of the LNK16 for its own. Indeed, the 3G8 epitope includes the polymorphic site and the LNK16 epitope is very close to it (18). This conclusion is in line with previous observations showing that the binding of the MEM154 anti-CD16 is nearly restricted to the V allotype, whereas that of other mAbs (CLBFcGran1, B73-1) is independent of the V158F polymorphism (1). Thus, depending on the mAb used, it can be concluded that the V allotype is overexpressed (3G8), similarly expressed (CLBFcGran1, B73-1), or underexpressed (LNK16, Dj130c) on NK cells as compared with the F allotype. These results point out the important limitations in the use of mAbs and flow cytometry to conclude on the levels of expression of a polymorphic antigen such as CD16a. Indeed, this approach absolutely requires that the epitope recognized by the mAb(s) is invariant in the different allotypes to avoid the intrinsic difference in affinity. This condition is rarely fulfilled in the case of CD16. Indeed, even the EC1 domain recognized by the Dj130c, which is distant from the 158 polymorphic site, includes another polymorphic site at position 48, which is in linkage disequilibrium with the former (1, 7). Hatjiharissi et al. (14) have also reported higher Fc $\gamma$ R11a transcript levels in NK cells from VV donors compared with those from F carriers. However, the sequence that was amplified included the polymorphic site. To avoid a possible allele-dependent variability of the reverse transcription-PCR (RT-PCR) efficiency, we analyzed the transcript levels in NK cells by carrying out real-time RT-PCR analysis with primers designed to amplify a conserved sequence of both alleles. These levels were not different according to the V158F

polymorphism (Fig. 1B). Obviously, they were not correlated with CD16 expression evaluated with any of the three mAbs. Thus, our results show that evaluation of CD16 expression by flow cytometry using different mAbs leads to conflicting results and that it is unrelated to the transcription level. Therefore, they do not validate the assumption that the CD16 expression is increased on NK cells expressing the V allotype.

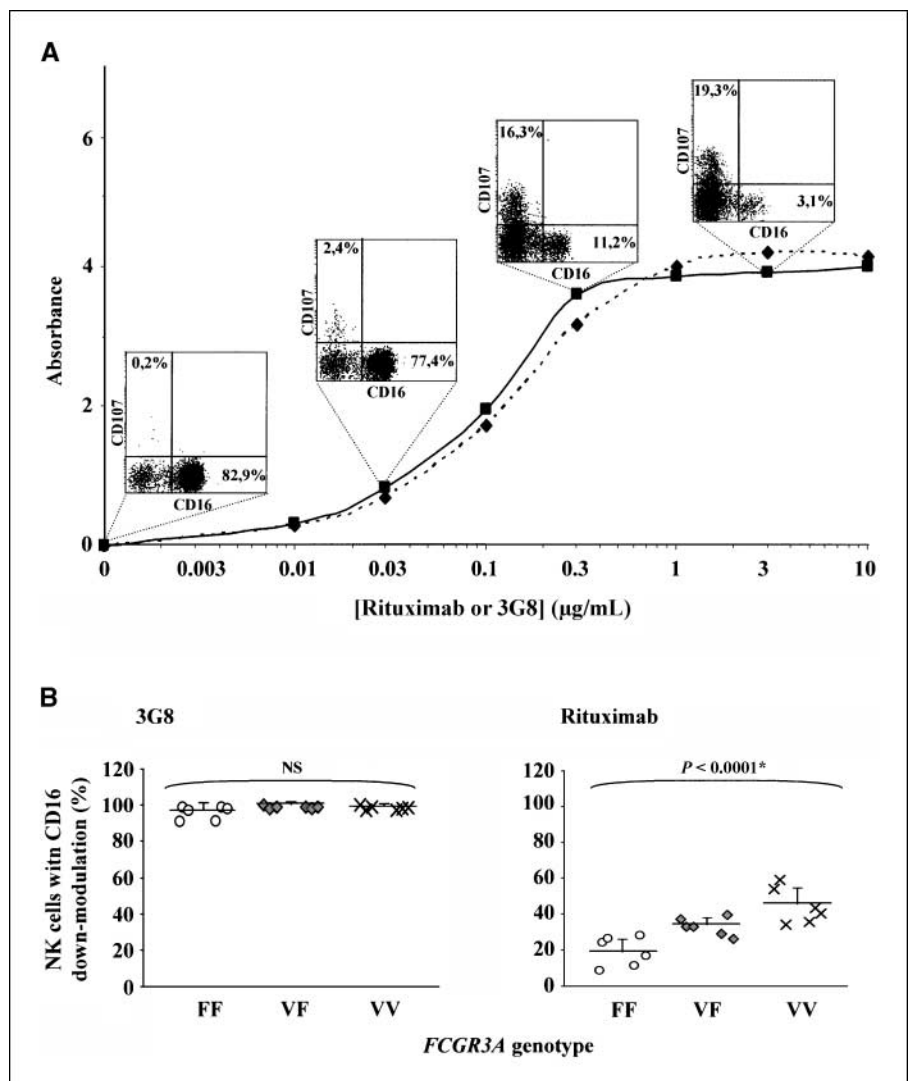
An increased expression of CD16 on NK cells expressing the V allotype should translate in a higher binding of rituximab at both low and high concentrations, whereas a higher affinity of the V allotype should lead to a higher binding restricted to low concentrations and erasing at saturation. Therefore, the binding of increasing concentrations of FITC-conjugated rituximab to purified NK cells from *FCGR3A*-genotyped donors was then evaluated. As expected, rituximab binding increased with increasing concentrations and reached a plateau at high concentrations ( $\geq 1,000 \mu\text{g/mL}$ ), whatever the genotype (Fig. 2A and B). Rituximab binding observed at 10 and 100  $\mu\text{g/mL}$  to NK cells from VV donors was indeed around three times higher than that to FF NK cells, in accordance with Hatjiharissi et al. (14). However, the difference was reduced to 1.45 and 1.30 times at 1,000 and 3,000  $\mu\text{g/mL}$ , respectively. Thus, it was nearly completely abolished at the saturating concentrations in discrepancy with the assumption of an increased expression of CD16 on NK cells expressing the V allotype.

We finally questioned on what may be the influence of a putative increased expression of CD16 on the responses of NK cells induced

by its engagement. Therefore, NK cells were incubated in microplates sensitized overnight with increasing concentrations of 3G8 or with rituximab to study their responses to the sole Fc $\gamma$ RIIIa engagement. The adsorption of 3G8 and rituximab on the plates was compared by an ELISA assay. They were very similar (Fig. 3A), reaching a plateau at 1  $\mu$ g/mL. It has been shown that activation of NK cells by K562 cells (19), anti-CD16 mAb (20), or rituximab (13) leads to CD16 down-modulation by a metalloprotease-dependent pathway (19, 20). We indeed observed a dramatic decrease of CD16 staining on purified NK cells stimulated by increasing concentrations of plate-bound 3G8 (Fig. 3A). This decrease was unrelated to a putative masking effect due to the detachment of the stimulating anti-CD16 mAb from the microplate. Indeed, we observed a simultaneous CD107 expression on a fraction of NK cells (Fig. 3A) showing that Fc $\gamma$ RIIIa/CD16 engagement induced NK cell activation and degranulation. Furthermore, CD16 down-modulation was inhibited in the presence of the metalloprotease inhibitor 1,10-phenanthroline (not shown). CD16 down-modulation was evaluated on NK cells from five donors of each genotype incubated in microplates ( $10^5$  cells per well) sensitized with a saturating concentration (5  $\mu$ g/mL) of 3G8 or rituximab. Nearly 100% of the NK cells that initially expressed CD16, displayed a

complete down-modulation after stimulation by plate-bound 3G8 (Fig. 3A and B), whatever the genotype. This result indicates that both allotypes are similarly susceptible to the activity of the membrane metalloprotease and that the level of Fc $\gamma$ RIIIa/CD16 engagement was sufficient on each NK cell to induce its down-modulation in these experimental conditions. By contrast, CD16 down-modulation was restricted to a fraction of NK cells stimulated by plate-bound rituximab (Fig. 3B). The higher levels of CD16 down-modulation observed with 3G8 likely result from the ability of 3G8 to simultaneously engage two Fc $\gamma$ RIIIa (by its two Fabs) and from its higher affinity for Fc $\gamma$ RIIIa compared with that of the rituximab Fc. The level of Fc $\gamma$ RIIIa/CD16 engagement per cell was insufficient on more than 50% of rituximab-stimulated NK cells to induce its down-modulation (Fig. 3B), indicating that NK cell Fc $\gamma$ RIIIa/CD16 engagement by rituximab was strongly competitive in these conditions (as expected, the percentages of NK cells down-modulating CD16 after rituximab stimulation increased when the number of cells per well decreased; data not shown). In such competitive conditions, the level of Fc $\gamma$ RIIIa/CD16 engagement may depend on both the affinity of the receptor for the Fc and on the level of CD16 expression. If the V158F polymorphism affects primarily the latter, one would expect that NK cells

**Figure 3.** Influence of the V158F polymorphism on NK cell responses to plate-bound 3G8 and rituximab. **A**, culture plates were sensitized overnight with increasing concentrations of anti-CD16 3G8 mAb or rituximab. The binding of 3G8 and rituximab was evaluated by ELISA with peroxidase-conjugated antimurine and antihuman IgG, respectively. Absorbance values (Y axis) according to concentrations of 3G8 (solid line) or rituximab (broken line) used to sensitize the plate (X axis). NK cells were incubated in sensitized plates in the presence of anti-CD107-PC5 mAb. Cells were then stained with anti-CD16-FITC mAb 3G8 and analyzed by flow cytometry. CD16/CD107 cytograms obtained after incubation of NK cells in wells sensitized with 0, 0.03, 0.3, and 3  $\mu$ g/mL of 3G8 mAb are shown. **B**, percent of NK cells that have down-modulated their CD16 after incubation in microplates sensitized with 5  $\mu$ g/mL of 3G8 (left) or rituximab (right) according to their *FCGR3A* genotype (white circle, FF; gray diamonds, VF; black crosses, FF). Black columns, mean; bars, SD. \*,  $P < 0.0001$ , influence of the V allele number on CD16 down-modulation. NS, not significant.



expressing high levels of CD16 (VV = VF>FF, according to ref. 14) will be able to engage individually more rituximab Fc, resulting in an increased competition between cells. Therefore, the proportion of NK cells down-modulating CD16 will tend to be decreased in NK cells from V carriers. Conversely, if the V158F polymorphism does not affect the level of CD16 expression per cell, one would expect that the more NK cells express the high-affinity receptor (VV > VF > FF), the more they will interact efficiently with the available rituximab Fc. Therefore, the proportion of NK cells down-modulating CD16 will tend to increase with the number of V allele. Our results substantiate the latter assumption. Indeed, we found that the percentages of NK cells from FF, VF, and VV donors down-modulating CD16 after stimulation by plate-bound rituximab were  $18.2 \pm 8.6\%$ ,  $32.0 \pm 4.9\%$ , and  $42.4 \pm 9.9\%$ , respectively. These results are in line with those showing that VV NK cells respond by a higher increase in  $\text{Ca}^{2+}$  than FF NK cells to aggregated human IgG but that both respond similarly to 3G8 (2). They are also consistent with our previous finding showing the higher lysis of Daudi cells by NK cells from VV donors compared with NK cells from FF donors was restricted to rituximab concentrations weakly sensitizing

CD20, whereas VV and FF NK cells killed Daudi cells similarly after optimal FcγRIIIa engagement by saturating concentrations of rituximab or 3G8 (9).

In summary, our results support the conclusion that the level of CD16 expression is not influenced by the V158F polymorphism and that it is not involved in the higher binding and functional responses of NK cells expressing the V allotype. They strongly suggest that the higher clinical response associated with the latter results from its better affinity for IgG1.

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