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IVIG induces apoptotic cell death in CD56^{dim} NK cells resulting in inhibition of ADCC effector activity of human PBMC

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ABSTRACT

The mechanism of the efficacy of Intravenous immunoglobulins (IVIG) in autoimmune and inflammatory diseases is not well understood. This study aimed at understanding mechanisms of IVIG-mediated suppression of effector cell activities of peripheral blood mononuclear cells (PBMC) in antibody-dependent cellular cytotoxicity (ADCC). We were particularly interested in CD56^{dim} NK cells, the main ADCC effector cells in PBMC. Exposure of PBMC to IVIG for at least 48 h induced a caspase-3-dependent apoptotic cell death of CD56^{dim} NK cells without affecting CD56^{bright} NK cells. Induction of apoptosis in CD56^{dim} NK cells and concomitant suppression of ADCC effector activities of PBMC was associated with the monomer fraction of IVIG. Moreover, it was independent of IgG sialyation, did not depend on engagement of FcγRIII and could not be mimicked by IVIG (Fab')$_2$ or IVIG Fc preparations. The described effect could contribute to the reduction of peripheral NK cells observed during IVIG therapy in patients.

1. Introduction

Intravenous immunoglobulins (IVIGs) are therapeutic preparations of polyclonal IgG antibodies prepared from pooled human plasma obtained from thousands of plasma donors. Initially developed for treating primary and secondary immune deficiencies, IVIGs have become an important therapeutic option for a variety of autoimmune and inflammatory diseases including idiopathic thrombocytopenic purpura, Kawasaki disease, chronic inflammatory demyelinating polyneuropathy, multifocal motor neuropathy, Guillain-Barre syndrome and myasthenia gravis [1]. The therapeutic efficacy of IVIG in these diseases has been attributed to its ability to modulate humoral and cellular immune responses. Different modes of action have been proposed in this context, e.g. modulating antibody responses by anti-idiotypic antibodies; shortening the half-life of autoantibodies by saturating the FcRn; blocking immune-complex mediated activation of FcγRs; scavenging cytokines, chemokines or activated complement factors; up-regulating the inhibitory FcγRIIb; inhibiting antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis; modulating the maturation and function of dendritic cells. However, the relevant mode of action in each of the different autoimmune and inflammatory diseases remains to be established [1].

Recent studies in humans highlighted the modulatory effects of IVIG on natural killer (NK) cells. IVIG and cytomegalovirus (CMV)-hyperimmune IVIG (CMVIg), respectively, were reported to induce a marked decline in the number and function of circulating NK cells in patients with neuroinflammatory disorders [2], idiopathic thrombocytopenic purpura or primary CMV infections [3,4]. Furthermore, IVIG treatment was shown to increase the rate of live births in a proportion of female patients with recurrent reproductive failures. The efficacy of IVIG in these pathologies was associated with a decline in numbers of circulating NK cells [5–7].

NK cells play an important role in the host defense against viral infections and malignancies. They can elicit potent effector functions, such as direct cytotoxicity, resulting in the killing of infected or aberrant host cells, or the release of cytokines and chemokines [8]. Although different NK cell subsets have been described, human NK cells comprise two major subsets: CD16^{+} CD56^{dim} NK cells, which are preferentially found in the circulation and are equipped with potent cytotoxic activities, and CD56^{bright} NK cells, which mainly home to

Abbreviations: IVIG, Intravenous immunoglobulin; ADCC, antibody-dependent cellular cytotoxicity; PBMC, peripheral blood mononuclear cells; NK, natural killer

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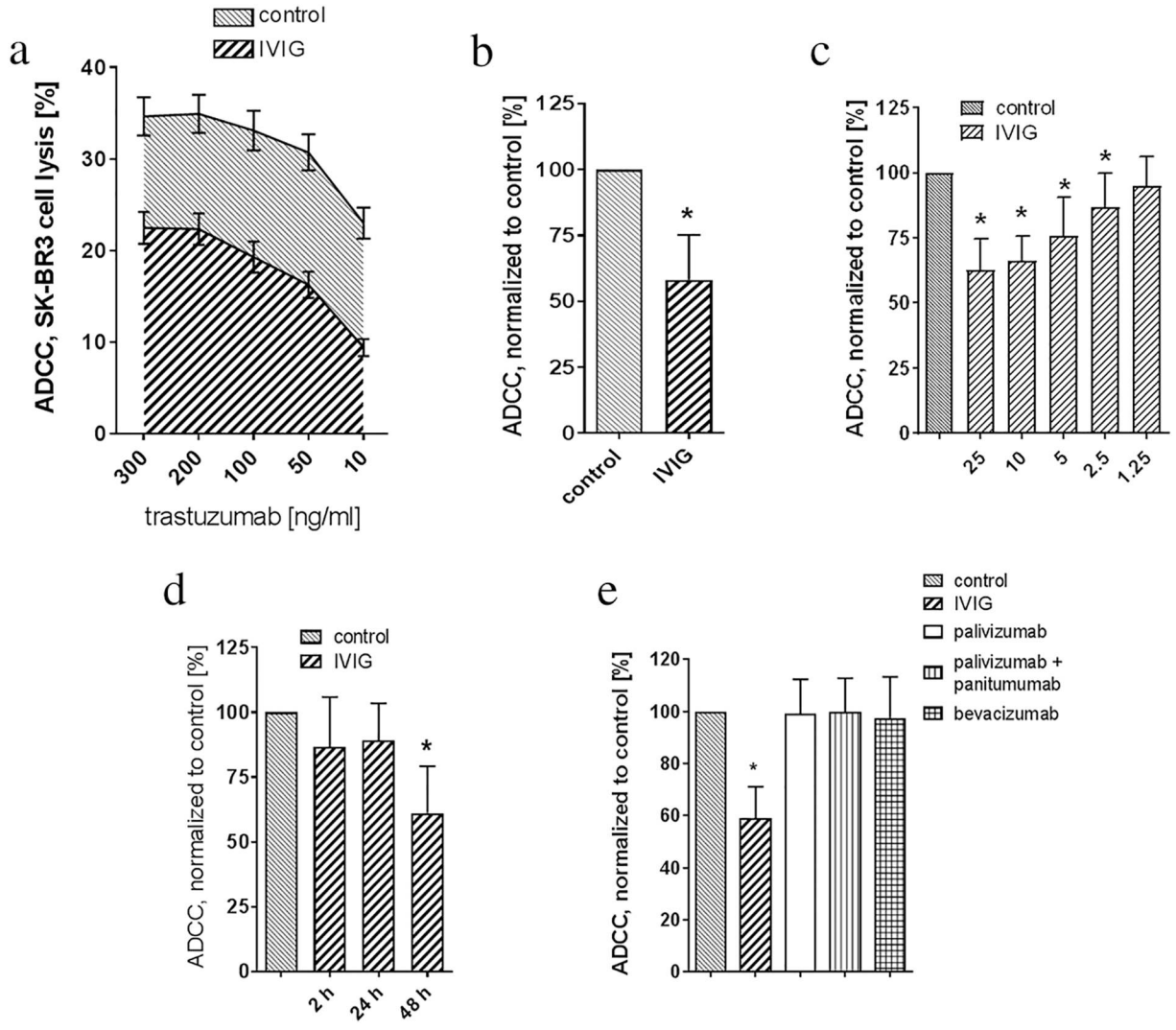

Fig. 1. IVIG inhibits ADCC effector activities of human PBMC

(a) and (b) Human PBMC were incubated with RPMI medium (control) or with 10 mg/ml IVIG (IVIG) for 48 h and then washed. PBMC-mediated ADCC against trastuzumab-opsonized SK-BR3 cells was analyzed. ADCC is either presented as (a) percentage of SK-BR3 cell lysis (mean ± SE) or (b) calculated as the area under the cytotoxicity curve (mean ± SD) after normalization to the control. $n = 47$ healthy subjects; paired Student t-test, $^*P < .01$.

(c) and (d) Human PBMC were incubated with RPMI medium (control) or with IVIG at the indicated concentrations in mg/ml (25, 10, 5, 2.5, 1.25) for 48 h (c) or with RPMI (control) and with 10 mg/ml IVIG for 2, 24 or 48 h (2 h, 24 h, 48 h) (d). PBMC-mediated ADCC against trastuzumab-opsonized SK-BR3 cells was measured and is shown as mean ± SD after normalization to the control. 1c: n = 11 healthy subjects; 1d: n = 13 healthy subjects; repeated ANOVA and Tukey's post hoc test, *P < .01.

(e) Human PBMC were incubated with RPMI medium (control), with 10 mg/ml IVIG (IVIG), with 10 mg/ml of the monoclonal IgG1 antibodies palivizumab or bevacizumab or with a mixture of 4 mg/ml of the monoclonal IgG2 antibody panitumumab and 6 mg/ml palivizumab for 48 h and then washed. PBMC-mediated ADCC against trastuzumab-opsonized SK-BR3 cells was measured and is shown as mean ± SD after normalization to the control. n = 11 healthy subjects; repeated ANOVA and Tukey's post hoc test, *P < .01 vs. control

secondary lymphoid organs and efficiently produce cytokines and chemokines [8].

NK cells are the major effector cells in ADCC which represents a set of mechanisms that target cells coated with IgG antibodies for cytolysis executed by immune cells expressing FcγRIII (CD16). ADCC effector cells not only include $CD16^+$ $CD56^{dim}$ NK cells but also other $CD16^+$ cells such as certain monocyte and macrophage subsets, natural killer T (NKT) cells or gamma/delta T cells [9,10]. ADCC is initiated by the binding of the FcγRIII (CD16) receptor to the Fc region of IgG antibodies bound to target cells, which subsequently activates the spatially oriented exocytosis of cytotoxic granules releasing perforins and granzymes, and the paracrine production of IFNγ to attain ADCC [10]. Several lines of evidence have indicated that ADCC reactions are of major importance for the control of bacterial and viral infections, the killing of tumor cells during antibody therapies, and the destruction of healthy tissues during autoimmune diseases such as systemic lupus erythematosus, autoimmune hemolytic anemia, autoimmune thrombocytopenia, and rheumatoid arthritis [9,11,12]. $CD16^+CD56^{dim}$ NK cells can also express direct cytotoxic activities. This process is tightly regulated by the integration of signals from inhibitory and activating surface receptors recognizing MHC-class I proteins, non-MHC self-molecules and other ligands expressed on the target cell [8], [13–15].

In this study, we aimed at understanding the mechanisms of IVIG-mediated suppression of ADCC effector activities in peripheral mononuclear blood cells (PBMC). In particular, we were interested in the effect of IVIG on the ADCC effector activities of $CD16^+CD56^{dim}$ NK cells, the major ADCC effector cells in PBMC. For this purpose, we established an in vitro model of ADCC which allowed us to study the underlying mechanisms of the IVIG-mediated modulation of ADCC effector activities.

2. Methods

2.1. Incubation of human PBMC with IVIG and control monoclonal IgG antibodies and human IgG preparations

PBMC were isolated from fresh heparinized blood from healthy donors by density gradient centrifugation (Lymphoprep™, Axis-Shield). Freshly isolated PBMC were seeded in 12-well cell culture plates with UpCell surface (Nunc) at a density of 4–5 × 10^6 cells per ml in RPMI medium (Gibco) supplemented with 20% autologous serum. PBMC were incubated with IVIG, control monoclonal IgG antibodies or other IgG preparations in a final volume of 1 ml. The IgG1 antibodies bevacizumab (Roche) and palivizumab (Abbott) and the IgG2 antibody panitumumab (Amgen) were used for control purposes. Before the PBMC were incubated, the IVIG (Gammagard Liquid from Baxter AG, 100 mg/ml final container in 0.25 M glycine buffer) preparations and the monoclonal antibodies were dialyzed against RPMI medium using Slide-A-Lyzer dialysis cassettes (Pierce) and assessed for endotoxin impurities using a LAL assay (Endosafe®-PTS™, Charles River Laboratories). The endotoxin concentrations in the final test samples were below the limit of detection of 0.3 EU/ml. IVIG was applied to PBMC at a concentration ranging from 1.25 mg/ml to 25 mg/ml for 2 h, 24 h or 48 h. If not indicated otherwise, IVIG was incubated for 48 h at a concentration of 10 mg/ml corresponding to the serum IgG concentration [16].

FcγR-mediated activities were blocked by adding 10 μg/ml mouse monoclonal blocking antibodies against FcγRI (anti-CD64, clone 10.1, eBioscience), FcγRII (anti-CD32, clone AT10, Abcam) or FcγRIII (anti-CD16, clone 3G8, BD Pharmingen) while caspase-dependent activities were blocked by adding 10 μM of the pan caspase inhibitor zVAD-FMK (BD Bioscience) to PBMC 30 min before the IVIG was added to PBMC. NK cell-depleted PBMC were used for some experiments. NK cells were depleted from PBMC with anti-CD56-conjugated magnetic beads using large depletion (LD) columns (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.2. Assessment of antibody-dependent cellular cytotoxicity (ADCC)

SK-BR3 cells, human breast cancer cells expressing the Her2 antigen (HTB-30 from ATCC), were opsonized with different concentrations of the anti-Her2 antibody trastuzumab (10, 50, 100, 200, 300 ng/ml, Roche) for 50 min at 4 °C and 10 min at 37 °C. PBMC pre-incubated with different IgG preparations were washed and added to these opsonized cells at a ratio of 20:1 and subsequently incubated for 4 h at 37 °C. The lactate dehydrogenase in the cell-free supernatants was measured using a LDH cytotoxicity detection kit (Roche) according to the manufacturer's instructions. ADCC against SK-BR3 cells was calculated as follows: $ADCC = 100 \times (ExpTzm_{10–300} - ExpTzm_0) / (S_{max}–S_{spo})$. $ExpTzm_{10–300}$ and $ExpTzm_0$ represent the experimental LDH release in the presence and absence of trastuzumab, respectively. S_{max} represents the maximum LDH release of SK-BR3 cells lysed with 2% Triton X-114 and S_{spo} represents the spontaneous LDH release in the absence of trastuzumab. For data illustration, ADCC is either presented as the percentage of the SK-BR3 cell lysis (Fig. 1a) or calculated as the area under the cytotoxicity curve followed by normalization to the medium control (Fig. 1b).

2.3. Preparation of Fc and F(ab`)$_2$ fragments of IVIG, separation of IVIG multimers and monomers, preparation of sialic-acid-enriched IVIG and sialic-acid-depleted IVIG

Fc fragments of IVIG were generated as described previously using papain digestion and subsequent purification with size exclusion chromatography (SEC) and protein A affinity chromatography [17]. F(ab`)$_2$ fragments of IVIG were generated by digestion of IVIG with pepsin (200 mg/g IgG; Sigma Aldrich) in 0.1 M glycine buffer at pH 4.0 at 37 °C. Digestion was stopped after 2 days by adjusting the pH to 7.0 using 0.5 M NaOH. F(ab´)$_2$ fragments were purified by SEC using an Agilent 1260 HPLC system, followed by an ultrafiltration/diafiltration step. The Agilent 1260 HPLC system was equipped with a diode array detector. The signals were monitored at an absorbance of 280 nm with a reference wavelength of 550 nm. Moreover, the HPLC system was equipped with a TSK SWXL 3000 size exclusion column preceded by a SWXL guard column (Tosoh Bioscience). The samples were run at a flow rate of 0.5 ml/min for a total of 25 min in a mobile phase consisting of 400 mM NaCl, 50 mM Tris, 5 mM $CaCl_2$, 0.05% NaN_3, at pH 7. Purified samples we subjected to an ultrafiltration/diafiltration step using a 30-kDa cut-off membrane. IVIG monomer and multimer fractions were separated by the same method using the Agilent 1260 HPLC system. The fractions were further enriched by repetition of the separation cycles.

Sialic-acid-enriched IVIG was prepared by affinity chromatography using *Sambucus nigra* agglutinin (SNA) according to a published protocol [17,18]. Sialic-acid-depleted IVIG was prepared by treating IVIG with 6× His-tagged neuraminidase for 48 h at 37 °C. Residual neuraminidase was removed using Ni-NTA resin. A control IVIG preparation was treated under similar conditions in the absence of neuraminidase.

2.4. Flow cytometric analysis

All samples incubated with or without IVIG or control antibodies were washed and potential binding sites of IVIG including FcγR binding sites were saturated with IVIG for 30 min at 4 °C to equalize all samples for the potential interference of IVIG with detection antibody binding. Washed cells were stained with LIVE/DEAD® Fixable Yellow Dye (Invitrogen) followed by a washing step in PBS for 30 min at 4 °C to assess cell viability. Subsequently, cells were labelled with the following flurochrome-conjugated surface markers for 60 min at 4 °C: CD56-PE (clone NCAM16.2, BD Biosciences), CD3-APC or CD3-AlexaFluor700 (clone UCHT1), CD14-Pacific Blue (clone M5E2), CD64-APC-H7 (clone 10.1), CD32-FITC or CD32-APC (clone FLI8.26) (all from BD Pharmingen), CD16-PE-Texas Red or CD16-APC (clone 3G8, Invitrogen), CD107a-PerCp/Cy5.5 (clone H4A3, Biolegend) or CD107a-AlexFluor488 (clone H4A3, eBioscience). NK cells were identified as $CD3^-/CD56^{bright}$ or $CD3^-/CD56^{dim}$ populations, T cells were identified as $CD3^+/CD56^-$ cells, NKT cells as $CD3^+/CD56^+$ cells and monocytes as $CD14^+$ cells. Intracellular staining was done using the Cytofix/Cytoperm kit (BD Pharmingen) according to manufacturer's instructions for active caspase-3-FITC (clone C92–605, BD Pharmingen), perforin-PerCP/Cy5.5 (Clone dG9, Biolegend) and granzyme B-AlexaFluor700 (Clone GB11, Biolegend). 50,000 PBMC were analyzed on a flow cytometer (FACS Aria, BD Biosciences) and data were analyzed using FlowJo software (Tree Star Inc.).

2.5. Statistical analysis

Statistical analysis and Pearson's correlation of data were done using Prism5 software (GraphPad software). Significant differences were analyzed with repeated measures ANOVA followed by Tukey's post-hoc test or with the paired *t*-test. An asterisk (*) indicates *P*-values of < 0.01 and hash mark ($^{\#}$) differences to the medium control and between other groups. All data are presented as mean ± SD, until otherwise indicated.

3. Results

3.1. IVIG but not monoclonal IgG suppress the ADCC effector activities of PBMC

We established an in vitro ADCC platform to study IVIG-mediated modulation of ADCC effector activities mediated by human PBMC. ADCC effector activity was calculated based on the area under the

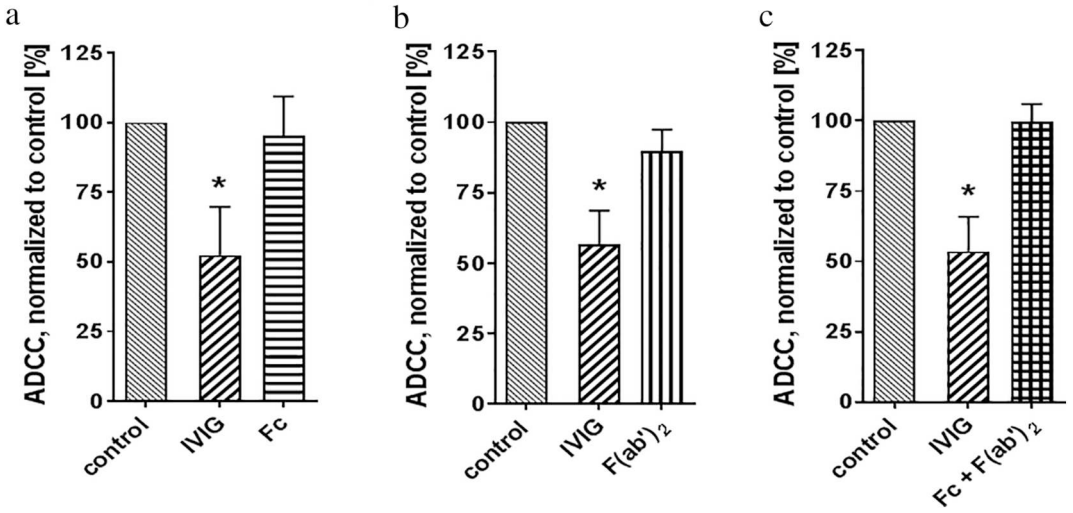

Fig. 2. IVIG-mediated inhibition of ADCC cannot be mimicked by purified Fc or $F(ab`)_2$ fragments or a mixture of Fc and $F(ab`)_2$ fragments of IVIG.
Human PBMC were incubated with RPMI medium (control), with 10 mg/ml IVIG (IVIG), with 3.3 mg/ml IVIG Fc fragments (2a: Fc), with 7.3 mg/ml IVIG $F(ab)_2$ fragments (2b: $F(ab`)_2$) or with a mixture of Fc and $F(ab`)_2$ fragments (2c: Fc + $F(ab`)_2$) for 48 h and then washed. PBMC mediated ADCC against trastuzumab-opsonized SK-BR3 cells was analyzed and is presented as results (mean ± SD) normalized to the control. 2a: $n = 10$ healthy subjects; 2b: $n = 7$ healthy subjects; 2c: $n = 8$ healthy subjects; repeated ANOVA and Tukey's post hoc test, $^*P < .01$ vs. control.

cytotoxicity curve. We observed a marked suppression of the ADCC effector activities of PBMC upon exposure to therapeutically relevant IVIG concentrations for 48 h (Fig. 1a and b). The mean suppression rate of 10 mg/ml IVIG was 42% (Fig. 1b) and ranged between 15% and 74% when we tested 47 healthy individuals. While suppression of ADCC effector activities was observed at different IVIG concentrations ranging from 2.5 to 25 mg/ml (Fig. 1c), the suppressive effect was not detectable until 48 h of exposure to IVIG and was not seen after 2 or 24 h (Fig. 1d). Importantly, suppression of ADCC effector activities was specifically associated with IVIG and could not be mimicked with control monoclonal IgG antibodies, as the humanized monoclonal IgG1 antibodies palivizumab or bevacizumab alone or in combination with the human monoclonal IgG2 antibody panitumumab did not suppress ADCC effector activities (Fig. 1e and Supplementary Fig. 1). We next examined whether the potential of IVIG to modulate ADCC effector activities of PBMC is mediated via the Fc region or the $(Fab`)_2$ region of IgGs contained in IVIG. Our data show that purified preparations of Fc and $F(ab`)_2$ fragments of IVIG do not have any suppressive effects on ADCC effector activities of PBMC, neither when both preparations are added individually nor when they are added together (Figs. 2 a-c), indicating that intact IgG molecules are required for the IVIG-mediated suppression observed in ADCC effector activities of human PBMC.

3.2. IVIG-mediated suppression of ADCC effector activities correlates with the induction of apoptotic cell death in $CD56^{dim}$ NK cells

To further investigate the mechanisms underlying the suppression of ADCC effector activities of PBMC by IVIG, we used flow cytometry to measure the viability of PBMC cell populations including NKT cells, T cells, $CD56^{bright}$ NK cells as well as $CD56^{dim}$ NK cells and monocytes, the two main ADCC-mediating cell types in PBMC. After pre-incubation of PBMC with IVIG for 48 h, the proportion of viable cells was unaltered among NKT cells, T cells, $CD56^{bright}$ NK cells and monocytes but was significantly decreased in $CD56^{dim}$ NK cells (Fig. 3a). This effect was specific for IVIG as the viability of $CD56^{dim}$ NK cells was unaltered after incubation with the control monoclonal IgG1 antibody palivizumab (data not shown).

We compared ADCC effector activities of total PBMC and NK cell-depleted PBMC to confirm that the suppression of ADCC effector activities in IVIG-exposed PBMC can be attributed to the effect of IVIG on $CD56^{dim}$ NK cells. Our data demonstrate that the IVIG-induced suppression in ADCC effector activities of PBMC is dependent on the presence of NK cells. NK cell-depleted PBMC did not show reduced ADCC effector activities after exposure to IVIG (Fig. 3b). Moreover, we observed a significant inverse correlation between the level of IVIG-mediated suppression in ADCC effector activities and the viability of $CD56^{dim}$ NK cells when we tested the PBMC of 47 healthy individuals (Fig. 3c). These data support the hypothesis that increased cell death of $CD56^{dim}$ NK cells is responsible for the suppressive effect of IVIG on ADCC effector activities of PBMC. In line with this data set, we found an increased proportion of apoptotic $CD56^{dim}$ NK cells in IVIG-exposed PBMC but not in monoclonal IgG-exposed PBMC (Fig. 3d), as indicated by increased staining of active caspase-3 used as a marker for apoptosis. Next, we incubated PBMC with the pan caspase inhibitor zVAD-FMK prior to exposure with IVIG for 48 h to confirm the induction of apoptosis in $CD56^{dim}$ NK cells. As shown in Fig. 3e, the addition of zVAD-FMK prevented IVIG-induced cell death in $CD56^{dim}$ NK cells, which supports a major role of apoptotic cell death of $CD56^{dim}$ NK cells in IVIG-mediated suppression of ADCC effector activities of PBMC.

3.3. IVIG-induced apoptosis of $CD56^{dim}$ NK cells in PBMC is not related to engagement of FcγRIII

It is well established that NK-cell-mediated ADCC requires engagement of FcγRIII and the subsequent release of cytotoxic granzymes and perforins [8]. Therefore, we asked if FcγRIII engagement is also involved in IVIG-induced apoptosis of $CD56^{dim}$ NK cells in PBMC. We exposed PBMC to IVIG or control monoclonal antibodies and analyzed intracellular levels of granzyme B and perforin and the levels of surface expression of FcγRIII in $CD56^{dim}$ NK cells. The results indicate that IVIG exposure of PBMC for 48 h results in asignificant reduction in intracellular levels of granzyme B and perforin and a reduction of FcγRIII surface expression in $CD56^{dim}$ NK cells, while control monoclonal antibodies do not induce any alteration in expression levels (Fig. 4a, Supplemental Figs. 2a, 2b). However, the reduction in the FcγRIII surface expression of $CD56^{dim}$ NK cells did not correlate with the levels of IVIG-induced suppression of ADCC effector activities when we tested the effect in PBMC from 47 healthy donors (Fig. 4b). Next, we asked if a blockade of FcγRIII prevents IVIG-induced apoptosis in $CD56^{dim}$ NK cells contained in PBMC. For this purpose, we incubated PBMC with FcγR-blocking antibodies prior to a 48-h exposure to IVIG. Blocking antibodies against FcγRIII significantly reduced the degranulation of

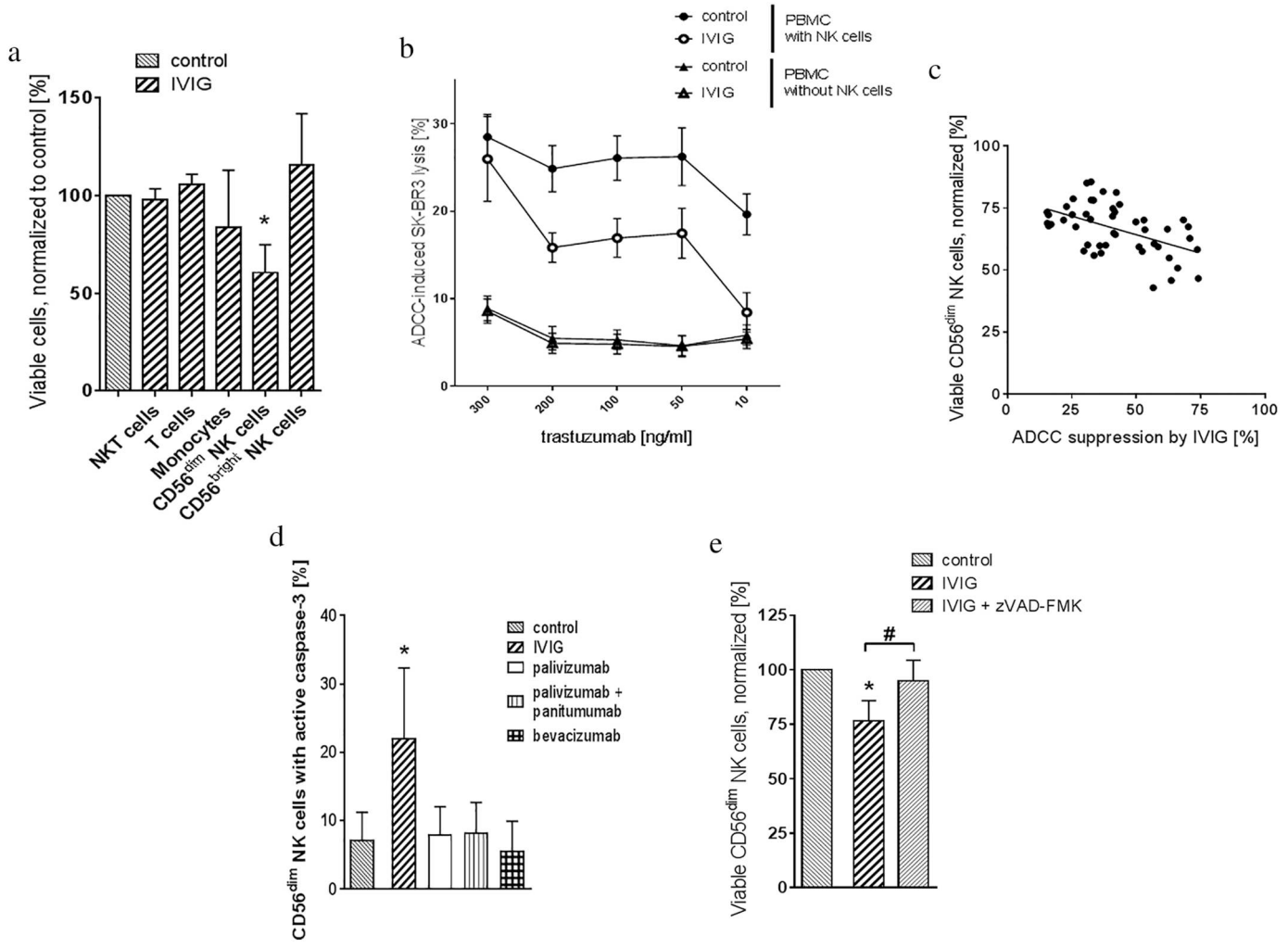

Fig. 3. IVIG induces apoptosis of CD56^{dim} NK cells without affecting other cell populations in human PBMC

(a) Human PBMC were incubated with RPMI medium (control) or 10 mg/ml IVIG (IVIG) for 48 h. Viability of NKT cells, T cells, monocytes, CD56^{dim} NK cells and CD56^{bright} NK cells was assessed by flow cytometry and is expressed as percentage of viable cells (mean ± SD) normalized to the control (n = 11 healthy subjects; repeated ANOVA and Tukey's post hoc test, $^{*}P < .01$ vs. control).

(b) Human PBMC with or without NK cells were incubated with RPMI medium (control) or 10 mg/ml IVIG (IVIG) for 48 h and ADCC was analyzed as described before. ADCC against trastuzumab-opsonized SK-BR3 cells is presented as percentage of SK-BR3 cell lysis (n = 18 healthy subjects, mean ± SE).

(c) Human PBMC were incubated with RPMI medium or 10 mg/ml IVIG for 48 h. The viability of CD56^{dim} NK cells as well as the ADCC activity of PBMC against trastuzumab-opsonized SK-BR3 cells was assessed. Pearson's correlation analysis between CD56^{dim} NK cell viability normalized to RPMI control and IVIG-mediated suppression of ADCC is shown (n = 47 healthy subjects, P = .0002, r^2 = 0.26).

(d) Human PBMC were incubated with RPMI (control), 10 mg/ml IVIG (IVIG), 10 mg/ml of the control monoclonal IgG1 antibodies palivizumab or bevacizumab or a mixture of 6 mg/ml of the IgG1 antibody palivizumab and 4 mg/ml of the monoclonal IgG2 antibody panitumumab for 48 h. The percentage of CD56^{dim} NK cells positive for intracellular activated caspase-3 was assessed by flow cytometry and the results of 11 healthy subjects (mean ± SD) from three different experiments are shown (repeated ANOVA and Tukey's post hoc test, $^{*}P < .01$ vs. control).

(e) Human PBMC were incubated with RPMI medium (control), 10 mg/ml IVIG (IVIG) or with 10 mg/ml IVIG together with 10 μM of the pan caspase inhibitor zVAD-FMK (IVIG + zVAD-FMK) for 48 h. Presented is the viability of CD56^{dim} NK cells from 9 healthy subjects (mean ± SD) after normalization to the control (mean ± SD, repeated ANOVA and Tukey's post hoc test, $^{*}P < .01$ vs. control, $^{\#}P < .01$).

CD56^{dim} NK cells (Fig. 4c) observed in IVIG-exposed PBMC, in line with a previous study [2]. However, the blocking of FcγRIII could not prevent IVIG-induced cell death of CD56^{dim} NK cells (Fig. 4d). Our findings suggest that cell death of CD56^{dim} NK cells in response to IVIG is not related to engagement of FcγRIII.

3.4. IVIG-induced apoptosis of CD56^{dim} NK cells in PBMC is caused by IVIG monomers rather than multimers and is independent of IgG sialylation

IVIG mainly consists of monomeric IgG and contains < 15% and 1% of dimeric and multimeric IgG, respectively [19]. To further explain the mechanism of IVIG-induced apoptosis of CD56^{dim} NK cells contained in PBMC, we asked if the monomeric or the multimeric fraction of IVIG is responsible for the observed effects.

We purified IVIG monomers (97.7% purity) and IVIG multimers (57.2% purity) by SEC and incubated these purified fractions with human PBMC at concentrations corresponding to their natural occurrence in IVIG. IVIG monomers induced both suppression of ADCC effector activities of PBMC (Fig. 5a) and enhanced cell death of CD56^{dim} NK cells in PBMC (Fig. 5b) with a similar potential as unfractionated IVIG, suggesting that inhibition of ADCC effector activity and enhanced cell death of CD56^{dim} NK cells by IVIG is not dependent on IVIG multimers. Data shown in Fig. 5a and b indicate that the multimeric fraction of IVIG also induces a certain reduction of PBMC effector activities and cell death of CD56^{dim} NK cells. However, the multimeric fraction of IVIG was only 57.2% pure and still contained monomeric IVIG.

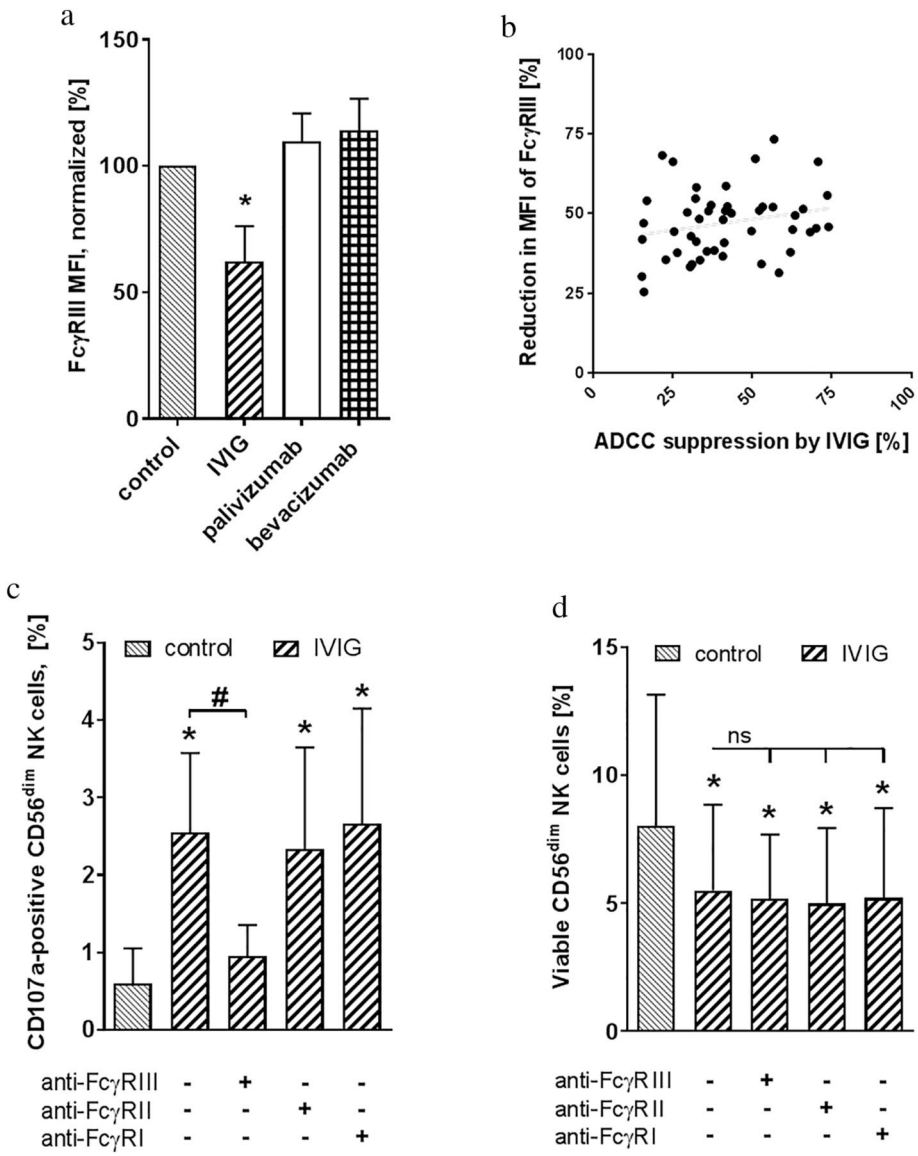

Fig. 4. IVIG-induced reduction of FcγRIII expression does not correlate with IVIG-induced apoptosis in CD56^{dim} NK cells

(a) Human PBMC were incubated with RPMI medium (control), 10 mg/ml IVIG (IVIG) or 10 mg/ml of the control monoclonal IgG1 antibodies palivizumab or bevacizumab for 48 h. CD56^{dim} NK cells were assessed for surface FcγRIII expression by flow cytometry. Results are shown as mean fluorescence intensity (MFI) ± SD after normalization to the control ($n = 10$ healthy subjects; repeated ANOVA and Tukey's post hoc test, $^{*}P < .01$ vs. control).

(b) Human PBMC were incubated with RPMI medium or 10 mg/ml IVIG for 48 h. Surface expression of FcγRIII on CD56^{dim} NK cells (MFI) as well as the ADCC activity of PBMC against trastuzumab-opsonized SK-BR3 cells was assessed. Pearson's correlation analysis between IVIG-mediated reduction in FcγRIII expression on CD56^{dim} NK cells and IVIG-mediated suppression of ADCC is shown (n = 47 healthy subjects, $P = .133$, $r^2 = 0.04$).

(c) and (d) Human PBMC were incubated with RPMI medium (control) or 10 mg/ml IVIG (IVIG) for 48 h in the absence or presence of blocking antibodies against FcγRIII, FcγRII or FcγRI. The percentage of (c) degranulation marker CD107a-positive CD56^{dim} NK cells and (d) viable CD56^{dim} NK cells was measured by flow cytometry. Data represent mean ± SD ($n = 9$ healthy subjects; repeated ANOVA and Tukey's post hoc test, $^{*}P < .01$, ns indicates non-significant, $^{\#}P < .01$).

Therefore, we cannot exclude that the modulatory effect of the multimeric IVIG fraction on ADCC effector activities of PBMC and on NK cell viability was mediated by IVIG monomers contained in the IVIG multimer fraction.

Kaneko et al. previously reported an increase in anti-inflammatory efficacy for sialic-acid-enriched IVIG which was obtained by SNA affinity chromatography [18]. Based on these data, we asked if sialic acid enrichment of IVIG could also alter its potential to modulate ADCC effector activity of human PBMC. When we used SNA affinity chromatography for the preparation of sialic-acid-enriched IVIG, we observed an increase in Fab sialylation but not in Fc sialylation in the SNA-binding fraction of IVIG (SNA^{+}), which confirms previously published reports [17,20]. The SNA^{+} fraction contained higher levels of mono- and disialylated glycans related to total IgG contained in IVIG (Supplemental Fig. 3a) but not related to the Fc region of IgG contained in IVIG (Supplemental Fig. 3b). When testing SNA^{+} and SNA^{-} IVIG fractions, we did not observe differences in their potential to suppress ADCC effector activities of PBMC (Fig. 5c). In a second approach, we treated IVIG with neuraminidase to remove terminal sialic acid residues from Fc and Fab glycans (Supplemental Fig. 3c). Neuraminidase treatment resulted in a marked decrease in mono- and disialylated glycans derived from total IgG contained in IVIG and from the Fc region of IgG contained in IVIG (Supplemental Figs. 3a and 3b). The enzymatic removal of sialic acid did not alter the potential of IVIG to suppress ADCC effector activity of PBMC (Fig. 5d), which indicates that sialic acid enrichment of IVIG could not alter its potential to modulate ADCC effector activities of human PBMC.

4. Discussion

The studies presented in this paper were designed to unravel the mechanisms of IVIG-induced suppression of the ADCC effector activities of human PBMC. The results of the studies presented indicate that exposure of human PBMC to IVIG for at least 48 h induces cell death of CD56^{dim} NK cells without affecting the viability of other mononuclear leukocytes such as NKT cells, T cells, CD56^{bright} NK cells and monocytes. When testing the PBMC of 47 healthy donors, we observed a significant correlation between the levels of IVIG-induced suppression of ADCC effector activities and the decrease in viability of CD56^{dim} NK cells contained in PBMC. We did not detect a suppression of ADCC effector activities by IVIG in NK cell-depleted PBMC which still expressed residual ADCC effector activity, presumably mediated by other FcγRIII (CD16) expressing cells types such as monocytes or gamma/delta T cells. Our data indicate that IVIG-induced cell death of CD56^{dim} NK cells is due to apoptosis, as indicated by the accumulation of active caspase-3 and further supported by the ability to prevent IVIG-induced

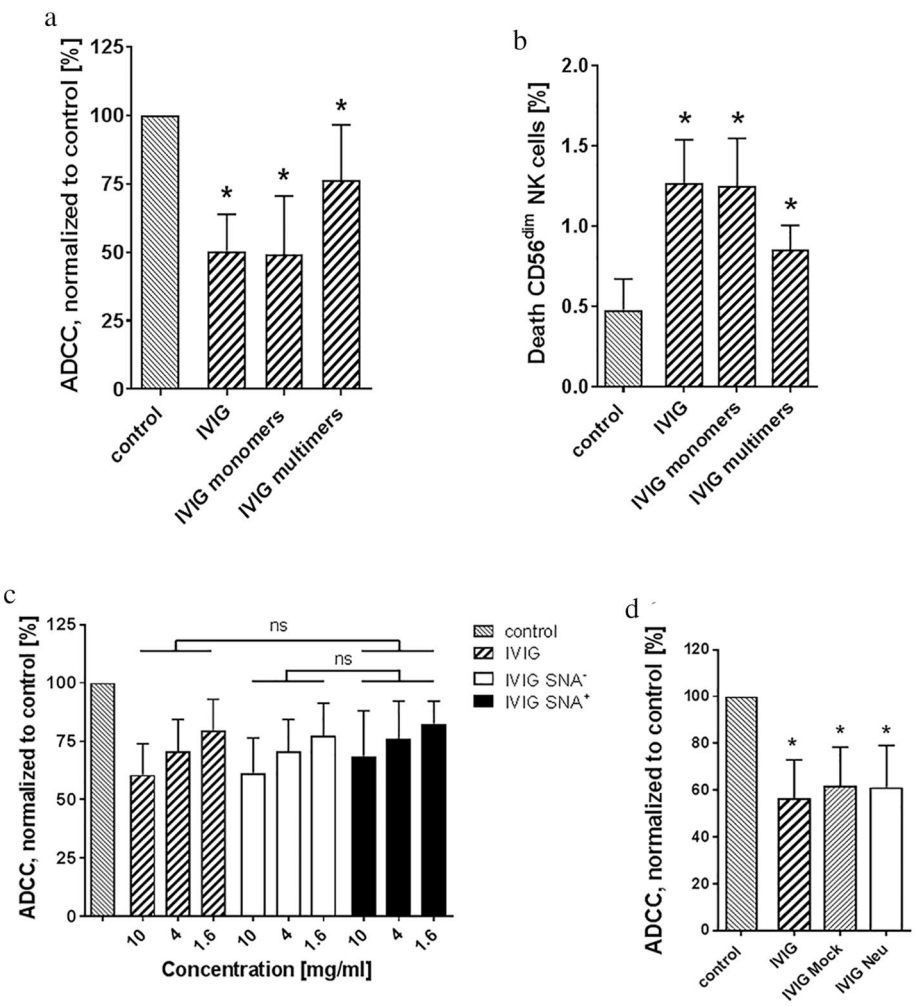

Fig. 5. IVIG-mediated inhibition of ADCC is associated with the monomer fraction of IVIG and is not influenced by sialylation of IVIG

(a) and (b) Human PBMC were incubated with RPMI medium (control), 10 mg/ml IVIG (IVIG), 8 mg/ml IVIG monomers (IVIG monomers) or 2 mg/ml IVIG multimers (IVIG multimers) for 48 h. The concentration of IVIG monomers and IVIG multimers corresponds to their calculated concentration in 10 mg/ml IVIG. (a) PBMC mediated ADCC against trastuzumab-opsonized SK-BR3 cells was measured and normalized to the control (mean ± SD; $n = 7$ healthy subjects; repeated ANOVA and Tukey's post hoc test, $^*P < .01$). (b) The percentage of dead $CD56^{dim}$ NK cells was assessed by flow cytometry and is presented as mean ± SD ($n = 6$ healthy subjects; repeated ANOVA and Tukey's post hoc test, $^*P < .01$ vs. control).

(c) and (d) Human PBMC were incubated as indicated for 48 h and then washed. PBMC-mediated ADCC against trastuzumab-opsonized SK-BR3 cells is given as the percentage lysis of SK-BR3 cells normalized to the medium control. The results are expressed as mean ± SD ($n = 8$ healthy donors; repeated ANOVA and Tukey's post hoc test, $^*P < .01$ vs. control, ns indicates non-significant). (c) Human PBMC were incubated with RPMI medium (control) or with different concentrations (10, 4 or 1.6 mg/ml) of IVIG (IVIG), of the IVIG fraction which does not bind to SNA (IVIG SNA^-) or of the IVIG fraction which binds to SNA (IVIG SNA^+). (d) Human PBMC were incubated with RPMI medium (control), 10 mg/ml untreated IVIG (IVIG), 10 mg/ml mock-treated IVIG (IVIG Mock) or 10 mg/ml IVIG pretreated with neuraminidase (IVIG Neu).

cell death in the presence of the pan caspase inhibitor zVAD-FMK. IVIG-induced apoptosis of $CD56^{dim}$ NK cells could provide an explanation for the decreased numbers of circulating NK cells and the concomitant decrease in NK-cell cytotoxicity observed in patients undergoing IVIG therapy [2,13,21,22].

A previous report [3] described the induction of apoptosis in human PBMC incubated with CMV-hyperimmune IVIG (CMVIg). Moreover, the authors demonstrated apoptosis in highly purified $CD8^+$ T cells and NK cells but not in $CD4^+$ T cells or B cells after incubation with CMVIg. However, they only used annexin V staining for the detection of apoptosis [3]. Annexin V binds phosphatidylserine, which is exposed at the cell surface following the loss of membrane phospholipid asymmetry in the early stages of apoptosis [23]. While the externalization of phosphatidylserine has been linked to early apoptosis and is frequently used as a marker of apoptotic cell death, several recent studies have demonstrated that phosphatidylserine is also exposed at the cell surface in several caspase-independent and/or non-apoptotic cellular pathways [24]. This could explain why our studies demonstrated the induction of apoptotic cell death only in $CD56^{dim}$ NK cells and not in T cells when human PBMC were exposed to IVIG. Another report described the induction of apoptosis by IVIG in $CD19^+$ B cells when PBMC from two unrelated healthy humans were mixed 1:1 in a mixed lymphocyte reaction and cultured for 3 days with 10 mg/ml IVIG [25]. The authors also observed minor effects on T cells and monocytes but these were not consistent. Similar to our studies, the induction of apoptosis required the whole IgG molecule as $F(ab`)_2$ fragments showed a minimal if any effect. However, the authors did not analyze the effect of IVIG on NK cells contained in PBMC, which precludes a direct comparison with our data.

The results of our studies also indicate that exposure of human PBMC to IVIG causes alterations in functional characteristics of $CD56^{dim}$ NK cells such as reduced surface expression of FcγRIII (CD16) and reduced intracellular levels of perforin and granzyme B. Interestingly, the reduction in surface expression of FcγRIII did not correlate with the levels of IVIG-mediated ADCC suppression when we analyzed the effects of IVIG exposure on PBMC from 47 healthy donors. Albeit that our data cannot fully rule out that a reduced FcγRIII expression contributes to IVIG-induced ADCC suppression in human PBMC, this contribution would be minor. We assume that upon IVIG exposure the residual density of surface FcγRIII expressed on $CD56^{dim}$ NK cells is still sufficiently high to fully engage these cells for ADCC in our in vitro model.

The possibility that reduced FcγRIII surface expression of IVIG-exposed $CD56^{dim}$ NK cells is an analytical artefact caused by receptor blockade can be excluded because the effect of IVIG could not be mimicked with different monoclonal IgG1 antibodies containing non-modified Fc domains. In summary, our findings support the hypothesis that decreased viability of $CD56^{dim}$ NK cells is the main cause for IVIG-mediated suppression of ADCC effector activities in human PBMC.

The question arises as to which mechanisms trigger IVIG-induced apoptosis of $CD56^{dim}$ NK cells in human PBMC. Our data indicate that ADCC suppression by IVIG cannot be mimicked with monoclonal IgG1 or IgG2 antibodies. Moreover, neither $(Fab)_2$ or Fc fragments of IVIG nor a mixture of both types of fragments induced the suppression of ADCC effector activities observed with intact IVIG. Therefore, we conclude that the mechanisms underlying the induction of $CD56^{dim}$ NK

cell apoptosis require intact IgG molecules in IVIG and involve both Fc and $(Fab`)_2$ domains. Importantly, IVIG-induced cell death of $CD56^{dim}$ NK cells was independent of FcγRIII engagement, suggesting that the previously reported ADCC-like activation of $CD56^{dim}$ NK cells in response to IVIG [2] did not cause death of these cells.

Anti-idiotypic activities of the $(Fab`)_2$ domains of IgG contained in IVIG were described to be involved in the formation of IgG dimers and multimers which were found to be essential for some of the immune modulatory effects described for IVIG [19,26,27]. Therefore, we asked if similar mechanisms could explain the IVIG-mediated effects observed in our studies. However, our studies indicate that IVIG dimers or multimers were not required for the IVIG-induced apoptosis of $CD56^{dim}$ NK cells in PBMC. The IVIG-mediated cell death in $CD56^{dim}$ NK cells and the related suppression of ADCC effector activities of PBMC was mainly triggered by the monomeric fraction of IVIG.

Several reports have highlighted the anti-inflammatory role of IgG antibodies with Fc glycans terminating in α2,6-linked sialic acid, albeit these findings have been debated [28,29]. The data obtained in our studies indicate that the decreased ADCC effector activities of human PBMC in response to IVIG exposure were not dependent on the level of Fc or Fab sialylation. *Sambucus nigra* agglutinin-fractionated IVIG with increased Fab sialylation and unaltered Fc sialylation as well as enzymatically desialylated IVIG induced a similar level of suppression of ADCC effector activities of human PBMC as non-modified control IVIG preparations.

Previous studies have suggested that IVIG contains agonistic antibodies against cell surface proteins of the death receptor family such as Fas (CD95) receptor [30,31]. It is possible, that antibodies targeting Fas receptors are involved in IVIG-mediated cell death of $CD56^{dim}$ NK cells. However, the IVIG-induced reduction in ADCC effector activities of human PBMC could not be mimicked with IVIG $(Fab`)_2$ fragments, which suggests that sole binding of anti-FAS antibodies contained in IVIG to Fas receptors expressed on $CD56^{dim}$ NK cells is insufficient to induce cell death. Therefore, we hypothesize that the Fc domains of anti-Fas antibodies contained in IVIG are required to promote clustering of Fas receptor-binding antibodies to express full efficacy in our model. Such a mechanism might be particularly important for the induction of apoptosis in $CD56^{dim}$ NK cells which express low levels of Fas receptor. The question arises which IgG-binding receptor expressed on peripheral blood cells could mediate such a clustering. Our data indicate that blocking FcγRI, FcγRII or FcγRIII did not affect the reduction in viability of CD56dim NK cells induced by IVIG. Therefore, we speculate that non-classic Fcγ-binding receptors such as e.g. FcR-like molecules [32,33] might be involved in such clustering. Future studies are required to address this potential mechanism.

It is well established that IVIG treatment of patients with certain autoimmune and autoinflammatory conditions such as Kawasaki disease or chronic inflammatory demyelinating polyneuropathy is highly effective in a subgroup of patients only. Other patients show no or only suboptimal treatment responses. This situation emphasizes the clinical need for immune biomarkers which can predict the efficacy of IVIG treatment in individual patients. Considering the substantial heterogeneity in the amplitude of the IVIG effect on ADCC effector activities of PBMC and on $CD56^{dim}$ NK-cell viability using blood cells from the humans included in our study, it is tempting to speculate that in vitro responses of human PBMC to IVIG predict IVIG treatment outcomes in patients with certain autoimmune diseases. Clearly, this topic will require clinical studies.

5. Conclusion

The studies presented in this paper were designed to unravel mechanisms of IVIG-induced suppression of ADCC effector activities of human PBMC. The results of the studies indicate that exposure of human PBMC to IVIG induces apoptotic cell death of $CD56^{dim}$ NK cells without affecting the viability of other mononuclear leukocytes such as NKT cells, T cells, $CD56^{bright}$ NK cells and monocytes. The levels of IVIG-induced suppression of ADCC effector activities of PBMC correlated with the decrease in viability of $CD56^{dim}$ NK cells when the PBMC of 47 healthy individuals were studied.

Although the mechanism for IVIG-induced apoptosis of $CD56^{dim}$ NK cells contained in PBMC cannot be fully explained, the observed effect is associated with the monomeric fraction of IVIG and requires intact IgG molecules but does not require FcγR engagement. Therefore, we hypothesize that the IVIG-induced apoptosis of $CD56^{dim}$ NK cells involves alternative mechanisms such as agonistic antibodies against proteins of the death receptor family.

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Competing interests

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Appendix A. Supplementary data

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