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FcγRIIa Is Expressed on Natural IFN-α-Producing Cells (Plasmacytoid Dendritic Cells) and Is Required for the IFN-α Production Induced by Apoptotic Cells Combined with Lupus IgG[1]

Ullvi Båve,[2]* Mattias Magnusson,[†] Maija-Leena Eloranta,[†] Anders Perers,[†] Gunnar V. Alm,[†] and Lars Rönnblom*

An ongoing production of IFN-α may be of etiopathogenic significance in systemic lupus erythematosus (SLE). It may be due to the natural IFN-producing cells (NIPC), also termed plasmacytoid dendritic cells (PDC), activated by immune complexes that contain nucleic acids derived from apoptotic cells. We here examined the role of FcγR in the IFN-α production in vitro by PBMC induced by the combination of apoptotic U937 cells and autoantibody-containing IgG from SLE patients (SLE-IgG). The Fc portion of the SLE-IgG was essential to induce IFN-α production, because Fab fragments or F(ab′)$_2$ were ineffective. Normal, especially heat-aggregated, IgG inhibited the IFN-α production, suggesting a role for FcγR on PBMC. Using blocking anti-FcγR Abs, the FcγRIIa,c (CD32) but not FcγRI or FcγRIII were shown to be involved in the IFN-α induction by apoptotic cells combined with SLE-IgG, but not by HSV or CpG DNA. In contrast, the action of all of these inducers was inhibited by the anti-FcγRIIa,b,c mAb AT10 or heat-aggregated IgG. Flow cytometric analysis revealed that ~50% of the BDCA-2-positive PBMC, i.e., NIPC/PDC, expressed low but significant levels of FcγRII, as did most of the actual IFN-α producers activated by HSV. RT-PCR applied to NIPC/PDC purified by FACS demonstrated expression of FcγRIIa, but not of FcγRIIb or FcγRIIc. We conclude that FcγRIIa on NIPC/PDC is involved in the activation of IFN-α production by interferogenic immune complexes, but may also mediate inhibitory signals. The FcγRIIa could therefore have a key function in NIPC/PDC and be a potential therapeutic target in SLE. ***The Journal of Immunology,*** **2003, 171: 3296–3302.**

Systemic lupus erythematosus (SLE)[3] is regarded as a classic immune complex-mediated autoimmune disease. Immune complexes (IC) are formed in circulation or in situ as a result of produced autoantibodies against nucleic acid and associated proteins, such as dsDNA, ribonucleoprotein, and histone. Such IC may cause inflammation with disease-characteristic clinical symptoms such as nephritis, arthritis, skin rashes, and vasculitis (1). The causes of this autoimmune process are largely unknown, but both genetic and environmental factors are of importance (1). Typical findings in SLE patients are deficient B and T cell regulation, increased amounts of circulating unscavenged cell material, and increased serum levels of cytokines that contribute to the inflammation (2, 3). One of the first cytokine abnormalities described in SLE patients was increased serum levels of IFN-α (4–6) that correlate to disease activity and severity as well as to several markers of immune activation (7, 8). The IFN-α produced in SLE may be of importance for the autoimmune process, because IFN-α therapy of patients with nonautoimmune disorders frequently results in development of anti-nuclear Abs, anti-dsDNA Abs, and occasionally SLE (9–12). The observed adverse effects of IFN-α treatment are perhaps not unexpected considering that IFN-α has important immunomodulatory functions, such as activation and maturation of DC, stimulation of T cells, and enhanced IgG production by B cells (13, 14) which could initiate and sustain an autoimmune process (15, 16).

The ongoing IFN-α production in SLE patients suggested the occurrence of factors triggering IFN-α synthesis and such an IFN-α-inducing factor in sera of SLE patients (SLE-IIF) was identified by us and consisted of autoantibodies and DNA in complex (17, 18). Although the origin of the DNA in SLE-IIF is unknown, apoptotic cells could be a source due to an increased rate of apoptosis (19–21) and decreased clearance of apoptotic cells (22) have been demonstrated in SLE patients. In fact, we recently showed that apoptotic cells when combined with autoantibody-containing IgG from SLE patients (SLE-IgG) caused production of IFN-α by PBMC and, among these, the natural IFN-α-inducing cells (NIPC) were identified as the actual IFN-α producers (23, 24).

The NIPC are the major IFN-α producers that constitute ~1/1000 of all PBMC and upon stimulation produce large amounts of IFN-α (1–2 U/cell). The NIPC can be activated by a variety of inducers, such as viruses (25), bacteria (26), and CpG-rich DNA (17, 27, 28). The phenotype is that of an immature dendritic cell (DC) (29), identical to type 2 DC precursors (pDC2) (30), or

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[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; DC, dendritic cell; DELFIA, dissociation-enhanced lanthanide fluoroimmunoassay; IC, immune complex; IIF, IFN-α-inducing factor; NIPC, natural IFN-α-producing cell; ODN, oligodeoxynucleotide; PDC, plasmacytoid dendritic cell; TLR, Toll-like receptor; SHIP, Src homology 2 domain-containing inositol phosphatase; IVIG, i.v. Ig.

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immature plasmacytoid DC (PDC) (31). The NIPC/PDC can be identified by their unique expression of the two novel markers BDCA-2 and BDCA-4 (32). Furthermore, NIPC/PDC express several Toll-like receptors (TLR), of which TLR9 has been suggested to be important in the induction of IFN-α production by unmethylated CpG-rich DNA (33). Such receptors could well be involved in the activation of the NIPC/PDC by interferogenic IC. In addition, FcR may also be important because the IFN-α production induced by SLE-IIF and by virus-containing IC could be blocked by anti-FcγRII (CD32) Abs (34, 35). The FcRs, such as FcγRII could therefore be essential for the IFN-α production induced by the combination of apoptotic cells and SLE-IgG. Although expression of FcγRII has not been clearly demonstrated on NIPC/PDC, it cannot be excluded that they and other FcR may be present in low but functionally important concentrations on these cells.

In the present study, we first examined the role of the Fc portion of IgG and FcγR in the IFN-α production induced by the combination of apoptotic cells and SLE-IgG and found that both were important. Using mAbs blocking FcγRI, II, or III, we obtained evidence for the involvement of FcγRII and further directly demonstrated its presence on NIPC/PDC by flow cytometry. We furthermore only detected expression of mRNA for FcγRIIa, but not FcγRIIb or FcγRIIc, by means of RT-PCR applied to purified NIPC/PDC. Consequently, interferogenic IC appear to require the FcγRIIa to trigger IFN-α production in NIPC/PDC. However, we also obtained evidence that the same receptors could down-regulate the IFN-α production under certain conditions.

Materials and Methods

Preparations of IgG

IgG was prepared from plasma obtained by plasmapheresis of two SLE patients with active disease (24). Briefly, the first patient was a 16-year-old female with a disease duration of 2 years, and the second patient was a 66-year-old male with a disease duration of 3 years, both with an American College of Rheumatology index of 6. The IgG prepared from the patients was designated SLE-IgG 1 and SLE-IgG 2, respectively. The study protocol was approved by the Committee of Ethics, Faculty of Medicine, Uppsala University.

The citrated plasma was converted to serum by addition of 1 M $CaCl_2$ and IgG was prepared as described before (23). In short, the serum was passed through filters, 0.45 μm (Acrodisc; Gelman Sciences, Ann Arbor, MI), and endogenous DNA was eliminated by treatment with DNase I (2000 U/ml; Boehringer Mannheim, Mannheim, Germany) in 100 mM Tris-HCl (pH 7.5) and 10 mM $MnCl_2$ for 1 h at 37°C. The IgG was then purified on protein G-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden). The eluted IgG was dialyzed against RPMI 1640 medium (ICN Biomedical, Costa Mesa, CA) supplemented with penicillin (60 μg/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and HEPES (20 mM) and used at a concentration of 1 mg/ml in the PBMC cultures.

Human Ig (Gammagard; Baxter, Deerfield, IL) was either used untreated or aggregated by heating in a water bath at 63°C for 60 min at a concentration of 50 mg/ml.

Preparation of Fab and F(ab′)$_2$

Purified SLE-IgG were treated with immobilized papain or pepsin (ImmunoPure Fab and F(ab′)$_2$ Preparation kits; Pierce, Rockford, IL) according to the manufacturer's description. The Fc parts and whole IgG were removed using an immobilized protein A column and the samples were finally dialyzed in RPMI 1640 supplemented as described above. The efficiency of the enzymatic cleavage was verified by SDS-PAGE.

Culture conditions and treatment of U937 cells

Human monocytic U937 cells were cultured at 37°C in 7% CO_2 using RPMI 1640 medium supplemented as described above and with 5% FCS (Myoclone; Life Technologies, Paisley, U.K.). *Mycoplasma* could not be detected in the U937 cells by cultivation and staining with bis-benzimide (Hoechst no. 33258; Sigma-Aldrich, St. Louis, MO). The U937 cells were treated at a concentration of 1×10^6 cells/ml by UV light (60 mJ, 254 nm) and cultured for 4 h before use as IFN-α inducers (see below). Apoptosis in U937 cells was regularly checked by staining with FITC-annexin V and propidium iodide as described previously (24).

Preparation of PBMC and IFN-α induction cultures

The PBMCs were prepared from normal blood donors by Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation of buffy coats. The PBMC for flow cytometry were used immediately, otherwise cells were frozen and stored at −80°C in FCS containing 10% DMSO. The PBMC used for IFN-α induction cultures were from donors that had high IFN-α production in response to HSV and SLE sera. Frozen PBMC were thawed rapidly, washed twice, and cultured for 24 h at 5×10^6 cells/ml in RPMI 1640 medium supplemented as above, with further addition of costimulatory IFN-α2b (500 U/ml; Schering-Plough, Bloomfield, NJ). All cultures were in triplicates using final volumes of 0.1 ml/well in 96-well round-bottom plates (Nunclone; Nunc, Roskilde, Denmark). As IFN-α inducers, HSV or the combination of UV-irradiated U937 cells (0.5×10^6 cells/ml) and SLE-IgG (1 mg/ml) were used. The HSV was prepared and UV inactivated as described before (18) and used at a final concentration corresponding to 2×10^7 PFU/ml.

The oligodeoxynucleotide (ODN) 2216 (36), with the sequence 5′-ggGGGA**CG**AT**CG**TCgggggG-3′ (Cybergene, Huddinge, Sweden), was used at a final concentration of 3 μg/ml. Small letters denote nucleotides with phosphorothioate backbone, capital letters denote nucleotides with phosphodiester backbone, and bold letters denote CpG dinucleotides.

For flow cytometric analysis of IFN-α-producing cells, PBMC were stimulated by HSV or by medium only (control) for 9 h in flat-bottom 24-well plates (Nunc) using 2-ml volumes and 2×10^6 cells/ml. The culture medium was supplemented with IFN-α2b, as described above, and with 200 ng/ml GM-CSF (Leucomax; Schering-Plough). Brefeldin A (10 μg/ml; Sigma-Aldrich) was added 2 h before harvest of the cultures to increase intracellular concentrations of IFN-α.

Immunoassay for IFN-α

The concentration of IFN-α in culture medium was estimated by dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) with modifications as described before (37). In brief, microtiter plates were coated with the anti-IFN-α mAb LT27:293, detecting the majority of IFN-α subtypes but not the IFN-α2b. The europium-labeled LT27:297 anti-IFN-α mAb was incubated in the plates with samples or IFN-α standard for 1 h at 37°C. The standard was calibrated using the National Institutes of Health reference leukocyte IFN-α GA-23-902-530. The detection level was 2 U/ml.

Anti-FcR Abs

Abs specific for the FcγRIII/CD16 (clone 3G8), FcγRII/CD32 (clone IV.3), and FcγRI/CD64 (clone 32.2) were obtained from Medarex (Annandale, NJ). The anti-CD32 IV.3 mAb was also used as Fab fragment. The anti-CD32 mAb AT10 was purchased from BioSource International (Camarillo, CA). The Abs were dialyzed and diluted in RPMI 1640 medium supplemented as described above. The different Abs, at indicated final concentration, were added to the PBMC cultures immediately before the IFN-α inducers.

Flow cytometry

PBMC from eight healthy individuals were double stained using FITC-labeled anti-BDCA-2 mAb (7.5 μg/ml, IgG1, clone AC144; Miltenyi Biotec, Bergisch Gladbach, Germany) and PE-labeled anti-CD32 mAb (0.5 μg/ml, IgG2b, clone FLI8.26; BD PharMingen, San Diego, CA). The isotype controls were a FITC-labeled IgG1 (7.5 μg/ml, clone MOPC-31C; BD PharMingen) and PE-labeled IgG2b (0.5 μg/ml, clone DAK-GO9; DAKO, Glostrup, Denmark). The PBMC were coincubated with Abs to BDCA-2 and either anti-CD32 Abs or the IgG2b isotype control for 30 min on ice. The anti-BDCA-2-positive cells were defined by staining PBMC with its IgG1 isotype control. In addition, PBMC from four healthy individuals were double stained using unconjugated anti-CD32 (clone IV.3; Medarex) or the mouse IgG2b isotype control (DAKO), followed by PE-labeled rabbit anti-mouse IgG (DAKO). Subsequently, the cells were incubated with normal mouse serum followed by the addition of FITC-labeled anti-BDCA-2 mAb (Miltenyi Biotec).

Furthermore, PBMC from four normal blood donors were stimulated by HSV (see above) and stained by using FITC-labeled anti-CD32 mAb (0.5 μg/ml, IgG2b, clone FLI8.26; BD PharMingen) or FITC-labeled isotype control IgG2b (0.5 μg/ml, clone 27-35; BD PharMingen). In addition, HSV-stimulated PBMC from three donors were stained with the unconjugated anti-CD32 mAb IV.3, (Medarex) or the mouse IgG2b isotype control (DAKO), followed by blocking with normal mouse serum, and addition of FITC-labeled goat anti-mouse IgG (DAKO). The cells were subsequently

fixed in 2% paraformaldehyde for 30 min and then stained for intracellular IFN-α using biotin-labeled LT27:295 mAb (0.5 μg/ml; produced in our laboratory) and PE-labeled streptavidin, as described before (29). The isotype control for the IFN-α staining was biotinylated IgG1 (0.5 μg/ml, clone DAK-GO1; DAKO). The cells were acquired and analyzed using a FACScan flow cytometer and CellQuest software (BD Biosciences, San Jose, CA).

FACS purification of BDCA-2-positive cells

NIPC/PDC were purified on the basis of their expression of the markers BDCA-2 and BDCA-4 (32, 38). PBMC were first enriched for BDCA-4-positive cells using the BDCA-4 cell isolation kit (Miltenyi Biotec) according to the manufacturer's description. The BDCA-4-enriched PBMC were then stained with FITC-conjugated anti-BDCA-2 mAb (7.5 μg/ml; Miltenyi Biotec) and subsequently sorted by flow cytometry according to the forward light scatter characteristics and the BDCA-2 expression using a FACStar^{Plus} (BD Biosciences). Both BDCA-2-positive and -negative cells were sorted and collected using PBS as sheath fluid. All samples were handled and stored on ice until further processing.

Analysis of CD32 expression by RT-PCR

The mRNA was prepared from 1×10^5 purified BDCA-2-positive cells or PBMC with 20 μl of oligo(dT) beads per cDNA reaction using a Dynabeads mRNA Direct micro kit (Dynal, Oslo, Norway) according to the manufacturer's instructions. Reverse transcription was performed using Superscript II (Life Technologies, Paisley, U.K.) in a total volume of 40 μl following the manufacturer's instructions. The cDNA from each cell population was stored at −20°C until used in PCR. To analyze the three different forms (a,b,c) of FcγRII, the following primer pairs were used: FcγRIIa, 5′ primer: 5′-CAGCATGGGCAGCTCTTC-3′ and 3′ primer: 5′-CACATGGCATAACGTTAC-3′ (39); FcγRIIb, 5′ primer: 5′-GGAATCC TGTCATTCTTACCTGTC-3′ and 3′ primer: 5′-CCCAACTTTGTCAGC CTCATC-3′ (40); and FcγRIIc, 5′ primer: 5′-CTCCCAGCTCTTCACC GA-3′ and 3′ primer: 5′-CACATGGCATAACGTTAC-3′ (39). Note that the same downstream primer is used to detect FcγRIIa and c. The amplification efficiency of primer pairs was verified by using serial dilutions of plasmids encoding human FcγRIIa, FcγRIIb2 (kindly provided by J. van de Winkel, Utrecht University, The Netherlands), or FcγRIIc (kindly provided by P. Morel, Pittsburgh Cancer Institute, Pittsburgh, PA) as a template. Primers used to amplify β-actin were: 5′ primer, 5′-GATTCCTATGTGGGCGAGGAG-3′ and 3′ primer, 5′-GAGGGCATACCCCTCGTAGATG-3′. Each PCR was conducted in a total volume of 15 μl with 1–2 μl of cDNA on beads, 6 pmol of each primer, 0.25 mM of each dNTP, 2 mM $MgCl_2$, 2 U AmpliTaq DNA polymerase, and 1× PCR buffer (Roche Molecular Systems, Branchburg, NJ). The following cycles were used: An initial denaturing step of 5 min at 94°C, followed by 35–40 cycles with denaturation for 30 s at 94°C, annealing for 30 s at 59°C (β-actin), 56.4°C (FcγRIIa), 65.5°C (FcγRIIb), 56.6°C (FcγRIIc), elongation for 30 s (β-actin and FcγRIIa,c) or 50 s (FcγRIIb) at 72°C, and a final extension for 7 min at 72°C. The expected size of PCR products are 362 bp for β-actin, 352 bp for FcγRIIa, 852 bp for FcγRIIb1, 795 bp for FcγRIIb2, 347 bp for FcγRIIc1, 361 bp for FcγRIIc2, and 309 bp for FcγRIIc3 and FcγRIIc4. PCR products were visualized with ethidium bromide in agarose gel electrophoresis.

Results

The Fc portion of SLE-IgG is required for IFN-α induction in PBMC induced by apoptotic U937 cells and SLE-IgG

We have previously shown that the combination of SLE-IgG and apoptotic U937 cells induces IFN-α production in NIPC/PDC (24). To elucidate the role of the Fc portion of the Abs in the IFN-α response, Fab fragments and $F(ab')_2$ were prepared by papain and pepsin treatment of SLE-IgG. Replacing the SLE-IgG with Fab fragment or $F(ab')_2$ from the same SLE patient almost abolished the IFN-α production when PBMC were cultured with apoptotic cells as an IFN-α inducer (Fig. 1*A*). Thus, the Fc portion of SLE-IgG seems to be of major importance for IFN-α production induced by IC consisting of SLE Abs and apoptotic material. No inhibitory effects of the Fab fragments or $F(ab')_2$ were seen on the IFN-α production induced by HSV in PBMC cultures (Fig. 1*B*).

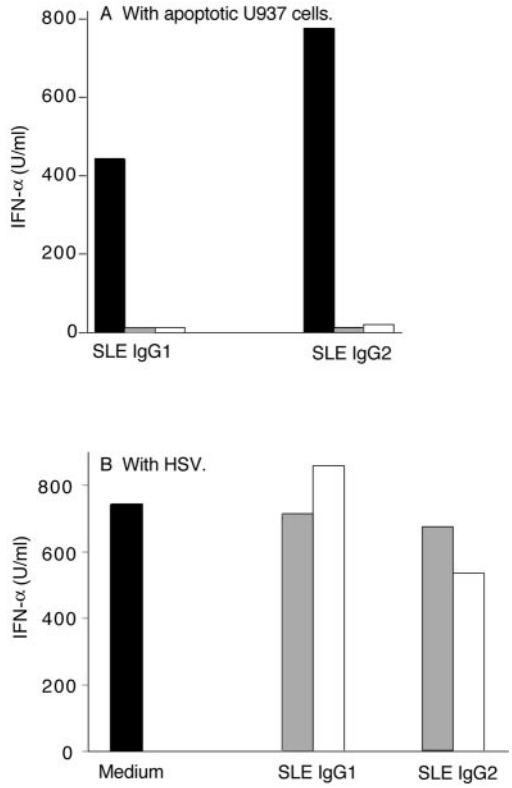

FIGURE 1. Intact SLE-IgG, but not Fab and $F(ab')_2$, combined with apoptotic U937 cells can induce IFN-α production by PBMC in vitro. The PBMC were cultured with UV-treated U937 cells, along with intact SLE-IgG (■) or either Fab (▨) or $F(ab')_2$ (□) prepared by papain or pepsin treatment, respectively (*A*). As a control, the Fab (▨) and $F(ab')_2$ (□) or medium (■) were added to cultures stimulated by HSV (*B*). The Fab, $F(ab')_2$, and the intact SLE-IgG were used at a concentration of 0.25 mg/ml. After 24 h, the IFN-α levels (units per milliliter) in the culture medium were measured by DELFIA. Representative results from one of three experiments.

Heat-aggregated or untreated normal IgG inhibits the IFN-α production induced by apoptotic U937 cells and SLE-IgG or by CpG ODN

Because the Fc portion of the SLE-IgG appeared to be crucial for the IFN-α induction, we investigated whether multimeric IgG made by heat aggregation of IgG from normal individuals could interfere with the IFN-α response induced by UV-treated U937 cells and SLE-IgG. Heat-aggregated IgG complexes have previously been described to have both positive and negative effects on cytokine production (41, 42).

Our results (Fig. 2*A*) show that heat-aggregated IgG caused a clear dose-dependent decrease in the IFN-α production induced by the combination of apoptotic U937 cells and SLE-IgG in cultures of PBMC, prominent even at very low concentrations. Also the untreated IgG caused an inhibition of the IFN-α production, but at higher concentrations. In addition, heat-aggregated, DNase-treated SLE-IgG inhibited the IFN-α production to the same extent as the heat-aggregated commercial IgG and did not induce IFN-α in itself (results not shown). Furthermore, both heat-aggregated and untreated IgG also inhibited the IFN-α production induced by ODN 2216 (Fig. 2*B*), known to be a potent type I IFN inducer selectively activating natural IFN-α-producing cells (NIPC)/plasmacytoid DC (PDC) (28). The IFN-α production induced by HSV was also decreased by heat-aggregated and untreated IgG (results not shown).

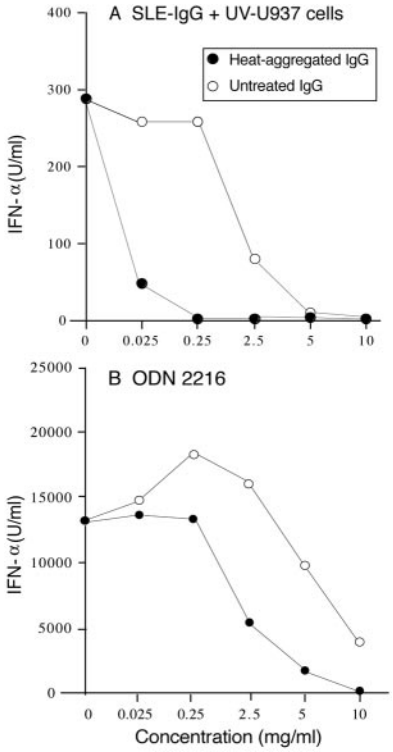

FIGURE 2. Normal IgG inhibits the IFN-α production by PBMC induced by the combination of UV-treated U937 cells and SLE-IgG or by ODN 2216. The PBMC were cultured with SLE-IgG (1 mg/ml) and UV-treated U937 cells (*A*) or with ODN 2216 (*B*) in the presence of indicated concentrations of either heat-aggregated (●) or untreated (○) normal human IgG. After 24 h, the IFN-α levels in the culture medium were measured by DELFIA. Representative results from one of three experiments.

These results suggest that FcR binding can inhibit the IFN-α production stimulated by principally different IFN-α inducers.

Abs to FcγRII inhibit IFN-α production induced by apoptotic U937 cells and SLE-IgG

The inhibition of the IFN-α production by heat-aggregated IgG could be due to blocking of FcR or activation of inhibitory FcR, such as FcγRIIb. We therefore examined whether mAbs to different FcR were inhibitory in this experimental system. Initially, we used mAbs to FcγRIII (CD16), FcγRII (CD32), and FcγRI (CD64) that were tested at different concentrations in the PBMC cultures. As seen in Fig. 3*A*, the IFN-α production was clearly reduced when the IV.3 mAb to CD32 or the Fab fragment of this mAb were added to PBMC cultures with apoptotic U937 cells and SLE-IgG as an IFN-α inducer. In contrast, the mAbs to CD16 or CD64 did not alter the IFN-α production (Fig. 3*A*), nor did the control mAb to CD19 (results not shown). Importantly, none of these anti-FcR mAbs inhibited the IFN-α production induced by HSV or by ODN 2216 in PBMC (Fig. 3, *B* and *C*).

The anti-CD32 Ab IV.3 binds to FcγRIIa and c, but not to b. We therefore also examined the effects of the anti-CD32 Ab clone AT10, which binds to all three forms of FcγRII (a, b, and c). We found that the AT10 Ab inhibited the IFN-α production induced by apoptotic U937 cells and SLE-IgG, but unlike the IV.3 Ab, also inhibited the IFN-α response induced by HSV (Fig. 3*B*) and ODN 2216 (Fig. 3*C*). These results indicate that the FcγRII is of importance for the IFN-α induction by the combination of apoptotic cells and SLE-IgG, but because the AT10 mAb inhibited the IFN-α production induced by HSV and ODN 2216, the FcγRII may also mediate a general inhibition of the IFN-α response, perhaps via FcγRIIb.

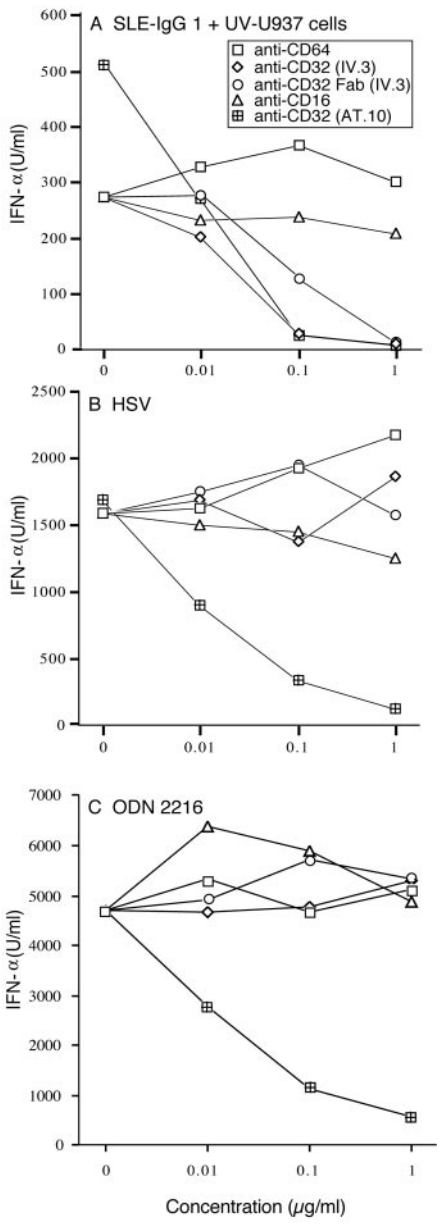

FIGURE 3. Abs to FcγRII inhibit the IFN-α production by PBMC induced by the combination of UV-treated U937 cells and SLE-IgG. The PBMC were cultured together with the combination SLE-IgG and UV-treated U937 cells (*A*), HSV (*B*), or ODN 2216 (*C*), and the indicated concentrations of mAbs directed against FcγRI (CD64; mAb 32.2), FcγRIIa,c (CD32; mAb IV.3 or IV.3-Fab), FcγRIIa,b,c (CD32; mAb AT10), or FcγRIII (CD16; mAb 3G8). The results with the anti-FcγRIIa,b,c mAb AT10 were obtained in a separate experiment. After 24 h, the IFN-α levels (units per milliliter) in the culture medium were measured in the supernatants by DELFIA. Representative results from one of three experiments.

FcγRII/CD32 is expressed on NIPC/PDC

Findings in previous studies suggested that NIPC/PDC did not express surface FcγRII/CD32 (29, 32). This, along with our results above, i.e., that anti-FcγRII Abs or heat-aggregated IgG had inhibitory effects on the IFN-α production by NIPC/PDC, motivated a re-examination of the FcγRII/CD32 expression on these cells. This was conducted by flow cytometry using staining of PBMC

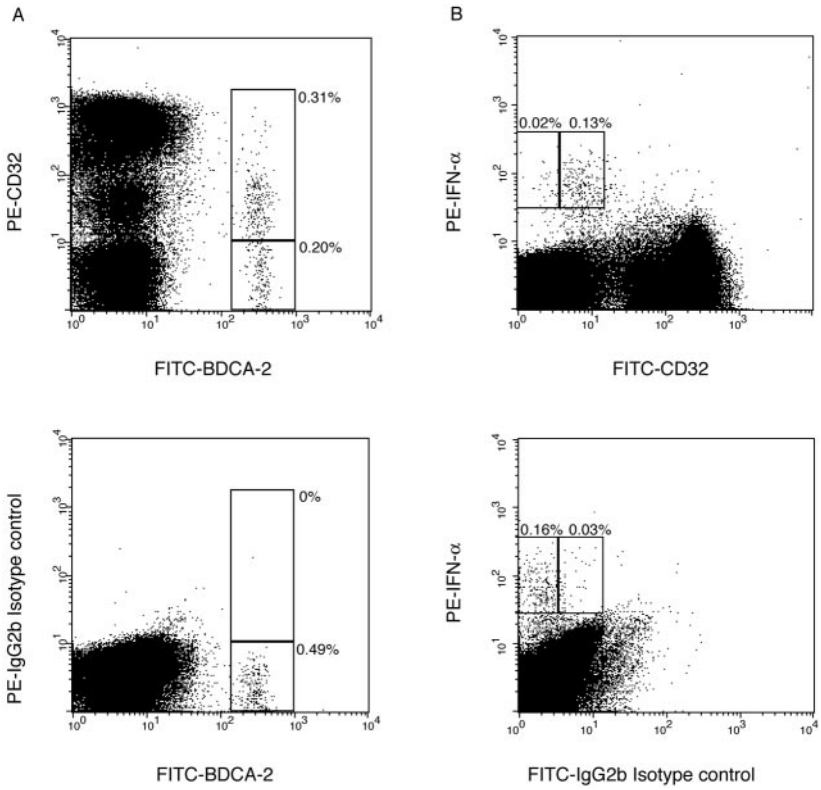

FIGURE 4. Expression of FcγRII on BDCA-2-positive PBMC and IFN-α-producing cells. *A*, Unstimulated PBMC were double stained with FITC-labeled anti-BDCA-2 Abs and PE-labeled FLI8.26 anti-CD32 Abs (*upper dot plot*) using a PE-labeled IgG2b isotype control (*lower dot plot*). *B*, The PBMC were also stimulated by HSV for 9 h in vitro, stained with FITC-labeled anti-CD32 mAb (*upper dot plot*) or FITC-labeled isotype control IgG2b (*lower dot plot*) and after permeablization with biotin-labeled anti-IFN-α mAb and PE-labeled streptavidin to detect intracellular IFN-α. Flow cytometric analysis was performed and indicated rectangular gates were used to define BDCA-2-positive PBMC (i.e., NIPC/PDC) that were either considered negative or positive for CD32. The percentage figures in the gates represent the proportion of cells in each gate of all analyzed PBMC. Representative results from the analysis of a total of eight (*A*) or four (*B*) different PBMC donors.

with an Ab to the NIPC/PDC marker BDCA-2 (32, 38) and with the anti-CD32 mAb FLI8.26 that binds to all three forms of FcγRII (a, b, and c). We found that 0.52 ± 0.16% of PBMC were BDCA-2 positive and that 49.7 ± 16.3% of the BDCA-2-positive cells expressed FcγRII/CD32, although at relatively low levels (Fig. 4*A*). Among the eight PBMC donors, a large individual variation was noted in the proportion of PBMC positive for both BDCA-2 and FcγRII/CD32, which varied between 0.09 and 0.45% (mean, 0.26%).

To examine whether the actual IFN-α-producing cells express FcγRII/CD32, PBMC from four different donors were stimulated by HSV and stained for intracellular IFN-α and FcγRII/CD32. The results show that most of the IFN-α-producing cells were stained for FcγRII/CD32 (Fig. 4*B*). The staining was weak but seen as a clear shift of anti-CD32-stained cells compared with cells stained with the IgG2b isotype control.

Similar results regarding the CD32 expression were obtained when fresh PBMC or HSV-induced PBMC were stained using the IV.3 mAb instead of the FLI8.26 mAb (results not shown).

Expression of FcγRIIa, but not FcγRIIb,c in NIPC/PDC

Because we could demonstrate an expression of FcγRII on the NIPC/PDC by flow cytometry, we proceeded by determining the presence of the different types of FcγRII, i.e., the activatory FcγRIIa and FcγRIIc as well as the inhibitory FcγRIIb. This was done by enrichment for BDCA-4-positive cells by magnetic separation, followed by purification of BDCA-2-positive cells by FACS, resulting in almost 100% pure BDCA-2$^+$ cells (results not shown). Subsequently, PCR was performed on cDNA prepared from these BDCA-2-positive cells and from unsorted PBMC to detect mRNA expression of the three types of FcγRII. A clear expression of FcγRIIa mRNA, but no FcγRIIb or FcγRIIc mRNA, was detected in NIPC/PDC (Fig. 5*A*). In contrast, all three types of FcγRII mRNA were readily detected in unsorted PBMC. The expression of β-actin confirmed mRNA integrity in all samples tested. All PCR products had the expected molecular size in agarose gel electrophoresis (see *Materials and Methods*), as judged by comparison to molecular mass markers ranging from 100 to 1000

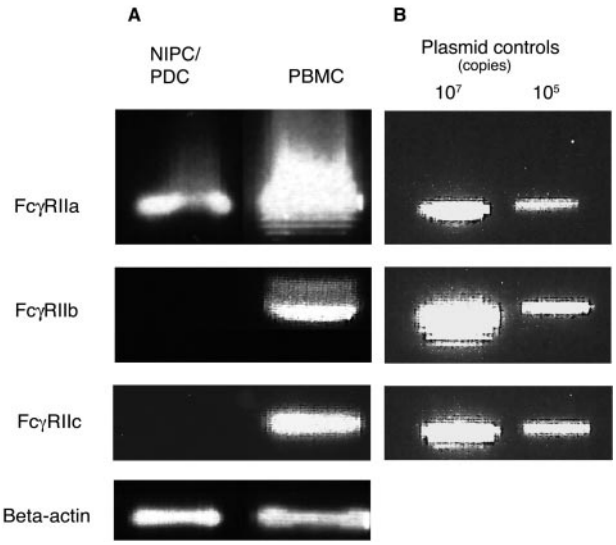

FIGURE 5. Analysis of FcγRIIa, FcγRIIb, and FcγRIIc gene expression in purified NIPC/PDC. The PBMC from six donors were enriched for BDCA-4-positive cells by magnetic bead separation, subsequently stained with FITC-conjugated anti-BDCA-2 Abs, and purified by FACS. RT-PCR was performed on mRNA prepared from the BDCA-2-positive cells (*first lane*) and unsorted PBMC (*second lane*) using specific primer pairs for FcγRIIa, FcγRIIb, FcγRIIc, and β-actin. Note that the primer pair for FcγRIIb detected FcγRIIb1 and FcγRIIb2 in unsorted PBMC, whereas the primer pairs for FcγRIIa or FcγRIIc only resulted in one band, respectively (*A*). The efficiency of the primers detecting the different isoforms of FcγRII was verified using as templates serial dilutions of plasmids encoding each subtype (*B*). For RT-PCR and FACS details, see *Materials and Methods*.

bp (results not shown). A similar amplification efficiency of the different primer pairs was verified by amplifying serial dilutions of plasmids encoding FcγRIIa, FcγRIIb2, or FcγRIIc (Fig. 5*B*). The higher intensity of the FcγRIIb band is most likely explained by the larger size of the PCR product.

Discussion

We have previously proposed that IC, containing autoantibodies and nucleic acids, cause a continuous IFN-α production by NIPC/PDC in SLE that is pivotal in the initiation and maintenance of the autoimmune process (15, 17). Such IC have been identified in the blood of SLE patients (18) and our previous results suggest that the nucleic acid component of IC could be derived from apoptotic cells because the combination of purified IgG from SLE patients and apoptotic U937 cells triggers IFN-α production in NIPC/PDC (23, 24). The results of the present study indicate that the IFN-α inducing activity in the case of apoptotic cells and SLE-IgG was dependent on the Fc portion of the IgG because the Fab fragments or $F(ab')_2$ of SLE-IgG were inactive. Furthermore, the IFN-α production was inhibited by either heat-aggregated normal IgG or by the anti-FcγRII mAb IV.3, known to have a high affinity to FcγRIIa and c, but not to b (43). These results are in line with previous results showing that IFN-α induction by SLE serum, i.e., SLE-IIF, was inhibited by anti-FcγRII mAb (35). Consequently, FcγRIIa and/or c appear essential for the IFN-α production triggered by IC. Recent data indicate that the interferogenic property of the combination of apoptotic cells and SLE-IgG is dependent on DNA and RNA derived from the apoptotic cells (T. Lövgren, M.-L. Eloranta, U. Båve, G. V. Alm, and L. Rönnblom, manuscript in preparation). The nucleic acids may interact with TLR9 and perhaps other TLRs present on NIPC/PDC (33, 44) and may be the actual IFN-α inducers, while the FcγR mediate cellular uptake of IC and/or mediate costimulatory signals. Such interferogenic IC resemble the IC containing chromatin and IgG that in mice were able to stimulate rheumatoid factor-producing B cells via interaction with the Ag receptor and, probably, TLR9 (45).

To further elucidate the role of FcγRII for the IFN-α production, it was important to verify the actual expression of FcγRII by NIPC/PDC. The results of previous studies have been contradictory in the sense that NIPC/PDC have either been reported to lack (24, 32) or possibly have a low expression of FcγRII (46). In the present study we did, however, demonstrate a low but clear expression of the FcγRII on PDC, defined as BDCA-2-positive cells. Interestingly, only ~50% of the BDCA-2-expressing cells were positive for FcγRII and it is therefore likely that only these cells or even a small part of them produce IFN-α in response to interferogenic IC such as SLE-IIF or the combination of SLE-IgG and apoptotic cells. In fact, the expression of FcγRII may be a marker of potential IFN-α-producing ability in general, because most IFN-α-producing cells triggered by HSV were positive for FcγRII (see Fig. 5).

The interaction of the anti-FcγRII mAb or heat-aggregated IgG may not solely block the FcγR and thereby prevent generation of signals required for IFN-α production by IC, but could also deliver an inhibitory signal to the NIPC/PDC. Indeed, we found that also the IFN-α production induced by ODN 2216 or HSV, not known to be dependent on FcR, was inhibited by heat-aggregated IgG. Furthermore, both the responses to HSV and ODN 2216 were inhibited by the anti-FcγRIIa,b,c mAb AT10, but not by the anti-FcγRIIa,c mAb IV.3. This general inhibition of the IFN-α production may therefore be related to an activation of the inhibitory FcγRIIb (47, 48). In contrast, IFN-α production triggered by SLE-IgG and apoptotic cells could be dependent on FcγRIIa,c. Accordingly, both the activatory FcγRIIa,c and inhibitory FcγRIIb could be involved in regulating the IFN-α production by NIPC/PDC. To determine the actual expression of FcγRII in NIPC/PDC, the three types of FcγRII were assessed at the mRNA level by RT-PCR. Surprisingly, only FcγRIIa was expressed by these cells, but we could not detect FcγRIIb or FcγRIIc. Although this does not completely exclude that there could be a very low but functionally significant level of FcγRIIb, a more likely explanation is that FcγRIIa may deliver both activating and inhibitory signals for the IFN-α production in NIPC/PDC. The FcγRIIa is usually regarded as an activator of multiple intracellular pathways because of its content of the immunoreceptor tyrosine-based activating motif that mediates activation of tyrosine kinases of the Src family and Syk, as well as phosphatidylinositol 3-kinase, resulting in for example, phagocytosis and production of inflammatory cytokines (reviewed in Ref. 47). In contrast, the FcγRIIb has an inhibitory function because of its immunoreceptor tyrosine-based inhibitory motif that associates to and activates the Src homology 2 domain-containing inositol phosphatase (SHIP). Interestingly, it has recently been shown that also FcγRIIa can bind and mediate activation of SHIP, providing direct evidence that FcγRIIa can have a dual activatory/inhibitory function (49, 50). This may also apply to NIPC/PDC, explaining the results of the present investigation. For instance, the inhibitory effect of heat-aggregated IgG and the anti-FcγRII mAb AT10 on the IFN-α production may be due to strong activation of SHIP through clustering of the FcγRIIa. The reason for the failure of the mAb IV.3, that also binds to FcγRIIa, to inhibit the IFN-α production induced by HSV is at present unclear and deserves further study.

The finding that heat-aggregated IgG, and at higher concentrations also untreated IgG, strongly inhibited the IFN-α production is interesting in the light of the therapeutic effects of i.v. Igs (IVIG) given to SLE patients (reviewed in Ref. 51). Several different mechanisms of action have been proposed to explain the effects of IVIG, including blocking activatory or activating inhibitory Fc receptors, inhibition of maturation, and function of DC, as well as modulation of cytokine production by DC (52, 53). The inhibitory effects of normal IgG on the IFN-α production by NIPC/PDC observed in the present study indicate a novel mechanism whereby IVIG can down-regulate the autoimmune process in SLE and perhaps other autoimmune diseases.

We have in previous publications argued for a pivotal role of IFN-α in the initiation and maintenance of the autoimmune process in SLE (15, 16). A key event in the pathogenesis is the formation of endogenous IFN-α inducers, such as SLE-IIF and the combination of apoptotic cells and autoantibodies, causing a continuous production of IFN-α by NIPC/PDC. Such IFN-α in turn stimulates DC as well as autoimmune T and B cells, resulting in further autoantibody production. Increased apoptosis and decreased clearance along with the autoantibodies result in formation of more IFN-α inducers. In this way a vicious circle is established. Our observation that the IFN-α production by IC is dependent on FcγRIIa on NIPC/PDC suggests that this receptor constitutes a potential therapeutic target in SLE treatment. Interestingly, there is a functionally important polymorphism of FcγRIIa that comprises a significant risk factor for SLE (54, 55), and it is therefore important to further clarify its precise role in the production of IFN-α by NIPC/PDC.

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