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Induction of Type I IFN Is a Physiological Immune Reaction to Apoptotic Cell-Derived Membrane Microparticles

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Membrane microparticles (MMP) released from apoptotic cells deliver signals that secure the anti-inflammatory response beyond the nearest proximity of the apoptotic cell. Plasmacytoid dendritic cells (pDC) are sentinels prepared to detect cellular processes that endanger the organism. They play a key role in the regulation of both pro- and anti-inflammatory immune responses. Based on the assumption that pDC could participate in the initiation of the anti-inflammatory response to apoptotic cells, we investigated the effects of apoptotic cell-derived MMP on human pDC. The results obtained in our experiments confirmed that MMP released from apoptotic cells trigger IFN-α secretion from human pDC. They further suggest that pDC activation results from sensing of DNA contained in MMP. MMP-DNA displays a particularly strong stimulatory activity compared with MMP-RNA and other sources of DNA. Inhibition of MMP-induced IFN-α secretion by cytochalasin D, chloroquine, and an inhibitory G-rich oligodeoxynucleotide identify TLR9 as the receptor for MMP-DNA. In marked contrast to the pDC response in autoimmune patients, in healthy subjects MMP-mediated stimulation of pDC-derived IFN-α was found to be independent of FcγRIIA (CD32A). Based on our findings, we conclude that induction of pDC-derived IFN-α by MMP is a physiological event; future investigations are necessary to elucidate whether pDC activation promotes inflammation or propagates tolerance in the context of apoptotic cell clearance.


The release of high quantities of membrane microparticles (MMP) is a hallmark of apoptosis (1–3). These subcellular vesicles consist of an envelope of phospho- and glycolipids that carries self-Ags, such as integrins, complement components, histones, and nucleic acids, derived from the parental cell (4, 5). This molecular composition, their small size, and their quantity designate them to serve as versatile cellular messengers spreading the signals required for the induction of an anti-inflammatory response reaching beyond the close proximity of the apoptotic cell. But, it is only recently that this key role of MMP and other subcellular membrane vesicles released from activated or apoptotic cells as mediators of cell-to-cell communication and modulators of the immune response has been recognized (6, 7) and that an adjuvant role for microparticles is discussed in the pathogenesis of autoimmune diseases, as well as in cancer progression (7, 8).

Plasmacytoid dendritic cells (pDC) are ubiquitously present in the mammalian organism. They are well-known for their ability to produce large amounts of type I IFNs, an important effector function that is facilitated by high expression of the nucleic acid-sensing TLR7 and -TLR9 and constitutive activation of IRF-7 (9, 10). Despite this, only a few studies have investigated their actual role in the immune response. These studies defined pDC as vital cellular components in the initiation of NK and T cell responses to viral Ags (11, 12), effectors in the memory response to microbial Ags, where Ag-specific IgG is a prerequisite for pDC activation (13, 14), important mediators of transplant tolerance (15–18), and inducers of T regulatory cells (19, 20).

Because of its IFN-producing capacity, this particular leukocyte subset is further thought to play a crucial role in the manifestation of systemic lupus erythematosus, as well as other autoimmune diseases that coincide with elevated systemic levels of type I IFN (21, 22). Activation of TLR7 and -9 by nucleic acid-containing autoimmune complexes triggers the release of IFN-α (23, 24). The released amounts can reach concentrations sufficient for systemic activity and are thought to represent a perpetuating factor for autoimmunity that is continuously nourished by a positive autoamplification loop (25–28): first, IFN-I–dependent gene expression primes innate immune cells for a stronger and more rapid response to exogenous and endogenous danger signals (29); second, IFN-I support a Th1 response and terminal differentiation of autoantibody-secreting B cells and class switch recombination (30–33). This pluripotency of type I IFNs turns pDC into global regulators of the immune response.
The conclusions drawn from the observations made in the autoimmune context suggest that pDC activation by nucleic acids released from apoptotic cells counteracts the tolerogenic immune response to apoptotic cell debris. However, little is known about the role of pDC in the context of apoptotic cell clearance in healthy subjects (i.e., in the absence of autoantibodies and accumulation of extracellular DNA or RNA). Despite their expression of scavenger receptors and integrins thought to be involved in the recognition and clearance of apoptotic cells (34–36), it is not known whether and how pDC react to apoptotic stimuli. Therefore, the present study was designed to investigate the physiological pDC response to MPO derived from apoptotic cells.

Materials and Methods

Cell isolation and culture

The use of human leukocytes was approved by the local ethics committee. For isolation of PBMC, heparinized blood was drawn from healthy volunteers, and PBMC were isolated by density-gradient centrifugation. pDC were isolated from PBMC using anti-BDCA4 MicroBeads on an autoMACS device (both from Miltenyi Biotec, Bergisch Gladbach, Germany). pDC purity was analyzed by flow cytometry on a FACS Canto (Becton Dickinson, Heidelberg, Germany) using FITC-labeled anti-BDCA2 Abs (Miltenyi Biotec). For many experiments, pDC were only enriched (iso-}

pDC stimulation

pDC were stimulated directly after isolation. Synthetic TLR7 and TLR9 agonists were used as controls: these included R848 (1 μg/ml; InvivoGen, San Diego, CA), 2′-C-DMTX (5 μM; St. Louis, MO), and cyclic phosphorothioate modification; 1 μM) and CpG 2216 (5′-ggGGGACGATCGTCgggggG-3′; small letters indicate phosphorothioate modification; 1 μM) and CpG 2006 (5′-tctgttggttgttggttgttgg-3′; 1 μM) (both from MWG Biotech, Munich, Germany). Newcastle disease virus (NDV) (provided by R. Zawatzky, German Cancer Research Center, Heidelberg, Germany) was prepared as described (37); for stimulation, the NDV preparation (1.6 × 10^6 HAU) was diluted in 1/500. One microgram of DNA purified from Staphylococcus aureus SA 113 WT complexed with Lipofectamine 2000 (Invitrogen) was isolated and used as previously described (13). pDC were preincubated with inhibitory reagents for 60 min prior to stimulation: FZS (5′-CTCTATGGGTTTTGGTATCT-3′; 1 μM (MWG Biotech), cytochalasin D (dissolved in DMSO, Alexis Biochemicals), and chloroquine (dissolved in water; Sigma) were used at the concentration indicated. Anti-human CD32 mAb (GeneTex GTX74628) or anti-human CD36 (Cayman Chemicals, Hamburg, Germany) were used at 15 μg/ml.

Generation of viable, apoptotic, and necrotic lymphoblasts

To generate lymphoblasts, freshly isolated PBMC were resuspended in RPMI 1640 medium containing 10% FCS (PAA Laboratories) and activated with 1 μg/ml PHA (Sigma) and 10 U/ml IL-2 (Roche, Mannheim, Germany) for 5 d. For additional expansion, cells were resuspended in 10 U/ml IL-2 for 2 more days. Before further treatment, lymphoblasts were washed extensively. Apoptosis was induced by UV-B irradiation (90 mJ/cm^2, 312 nm, TFX-35.MC MS Laboratory Instruments, Heidelberg, Germany). For induction of necrosis, cells were heated at 56°C for 30 min. To quantify the rate of apoptosis, cells were analyzed by flow cytometry after staining with FITC-conjugated Annexin V (400 ng/ml; Roche) in Ringer’s solution (Braun) with propidium iodide (1 μg/ml) for 30 min at 4°C, as described (38). Flow cytometric analysis was performed using an EPICS XL flow cytometer (Coulter, Hialeah, FL), by counting ≥20,000 events.

Preparation of MPO from T cell blasts

For preparation of MPO, cell culture supernatant was collected, and two centrifugation steps (500 × g for 5 min) were performed to remove residual cells. The supernatant was passed through a 1.2-μm nonpyrogenic, hydrophilic syringe filter. After centrifugation at 100,000 × g for 40 min, the pellet containing the MPO was harvested and used directly for pDC stimulation. Homogeneity of all MPO preparations was controlled with respect to size and granularity by flow cytometry (Fig. 1A). DNase (Sigma) digestion with 6 μg/ml was carried out for 18 h at 37°C. For all experiments, the MPO suspension was adjusted to an OD260nm of 0.09 on an Eppendorf Bio Photometer. Directly after MPO preparation, pDC stimulation was performed with 25 μM MPO suspension, unless otherwise indicated. Because initial experiments comparing allogenic and autologous MPO did not result in qualitative or quantitative differences in the pDC response, the majority of experiments were performed with allogeneic MPO suspensions or nucleic acid preparations.

Isolation of polymorphonuclear cells and generation of apoptotic PMN-derived MPO

After isolation of PBMC by density-gradient centrifugation, the bottom fraction is rich in erythrocytes and polymorphonuclear cells (PMN). The neutrophil layer of the bottom fraction was subjected to hypotonic lysis for removal of residual erythrocytes (30 s, two times). The remaining PMN were resuspended in RPMI 1640 medium containing 10% FCS (PAA Laboratories). To generate and isolate MPO derived from apoptotic PMN, we induced apoptosis by UV-B irradiation (90 mJ/cm^2). The rate of apoptosis was quantified by flow cytometry after staining with FITC-conjugated Annexin V (400 ng/ml; Roche) in Ringer’s solution (Braun) with propidium iodide (1 μg/ml). Apoptotic PMN-derived MPO were then isolated by ultracentrifugation and filtration, as described above.

Detection of IFN-α and cytokine ELISA

Cytokine concentrations were quantified in pDC supernatants harvested 24 h after stimulation. The following ELISA kits were used according to the manufacturer’s protocols: human IFN-α (BenderMedSystems, Vienna, Austria) and human TNF-α, IL-6, and IL-1β (OptEIA; Becton Dickinson). IFN-α secretion assay (Miltenyi Biotec): enriched pDC (2 × 10^5/well) were stimulated for 18 h at 37°C, washed, and treated with IFN-α Catch Reagent (20 min at 37°C) and IFN-α Detection Ab (PE), as suggested by the manufacturer. For detection of pDC, cells were stained with anti-CD123 (PerCP-Cy5.5), anti-HLA-DR (allophycocyanin), and anti-CD14 (FITC) (all from Becton Dickinson). Cells were subsequently analyzed on a FACS Canto device (Becton Dickinson).

Isolation of mitochondria

Mitochondria were isolated from PBMC using magnetic cell separation (MACS; Miltenyi Biotec) with the human mitochondria isolation kit (Miltenyi Biotec). Briefly, 10^9 PBMC were lysed and homogenized with a syringe needle. Subsequently, the lysate was incubated in separation buffer with anti-TOM22 Microbeads for 1 h, washed on an LS column, and eluted. The mitochondrial preparations used in the experiments shown in Fig. 5A were heated at 95°C for 10 min prior to DNA isolation.

DNA and RNA isolation

Total RNA isolation from MPO was performed using the High Pure RNA Isolation Kit (Roche), according to the manufacturer’s instruction. RNA isolation from T cell blasts was performed following the standard TRIzol protocol for RNA isolation (Invitrogen). We used the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) to isolate DNA from PBMC, MPO, platelets, and mitochondria. Platelet concentrates were provided by the Institute for Clinical Transfusion Medicine and Cell Therapy of the University Hospital of Heidelberg, DNA from T cell blasts was either isolated by continuing the TRIzol protocol after RNA separation or with the QIAamp DNA mini kit.

PCR

PCR was performed with DreamTaq (Fermentas, St. Leon-Roth, Germany). PCR conditions were denaturation at 94°C, annealing at 58°C for 1 min, extension at 72°C for 1 min, 35 cycles. The following primers, published previously (39), were used: β-actin: forward: 5′-AGAGCTACGAGCTGCCT-3′, reverse: 5′-AGCACTGTGTTGGCGTACAG-3′ (184 bp); mitochondrial encoded NADH dehydrogenase-4 (ND4): forward: 5′-TTTGAATCAACACAAACCAACCCACGC-3′, reverse: 5′-CGGCAAATCTAATFTGGACCCAGCAGATG-3′ (630 bp); and cytochrome B: forward: 5′-ACATCCACCCACCCACACCATCAGGATTTGTTCAC-3′, reverse: 5′-GGTTACTCGTTGTTGCCTCGGATCAGG-3′ (532 bp).

Statistics

Results are depicted as mean ± SEM. Statistically significant differences were calculated with the paired two-tailed Student t test using Microsoft Excel software (*p ≤ 0.05 and **p ≤ 0.005).
Results

MMP derived from apoptotic T cell blasts induce IFN-α secretion in human pDC

To investigate whether MMP derived from apoptotic cells stimulate human pDC, MMP were prepared from apoptotic PHA-induced T cell blasts. As previously described, the activation process is used to sensitize the lymphocytes for apoptosis, which significantly increases the yield in apoptotic cell-derived MMP upon induction of apoptosis (3, 40). For better comparability of experiments, the homogeneity of all MMP preparations was controlled with respect to size and granularity by flow cytometry as shown in Fig. 1, and the suspension was photometrically adjusted to an OD_{260nm} of 0.09.

Peripheral blood pDC enriched with anti-BDCA4 MicroBeads were stimulated with increasing concentrations of apoptotic MMP or with the TLR7 ligand loxoribine, as indicated in Fig. 1B. After 24 h of incubation, IFN-α released from pDC was quantified in the cellular supernatants. The data obtained showed that apoptotic MMP indeed trigger IFN-α production from healthy human pDC in a concentration-dependent manner. Notably, enrichment of pDC with anti-BDCA4 was necessary, because MMP did not trigger detectable amounts of IFN-α in total PBMC (data not shown). Furthermore, IFN-α concentrations induced by apoptotic cell-derived MMP are generally significantly less (i.e., ∼30-fold) than the levels achieved with synthetic ligands for TLR7 or TLR9 (Fig. 1B). This is also markedly lower than the concentrations measured in experiments using sera or IgG from autoimmune patients (23, 24, 41, 42). Occasionally, IFN-α secretion could not be detected in individual donors, a finding that might be explained by concentrations below the detection threshold of the ELISA (15 ng/ml).

FIGURE 1. Induction of IFN-α by apoptotic cell-derived MMP. (A) Apoptosis of T cell blasts was triggered by UV-B irradiation. Flow cytometric analysis was used to quantify the accumulation of MMP at 0, 5, and 24 h after UV-B exposure. MMP were defined by forward (FSC) and side scatter (SSC) properties. Absolute numbers of MMP at each time point are shown. (B) IFN-α secretion of unstimulated human pDC enriched with anti-BDCA4 MicroBeads was compared with pDC stimulated with MMP derived from apoptotic T cell blasts (black bars) or pDC stimulated with loxoribine (LOXO) (gray bar) as a positive control. MMP were titrated in declining concentrations: 25, 12.5, and 6 μl, respectively. IFN-α was analyzed in 24-h supernatants. The graph shows the summary of four independent experiments (mean ± SEM). (C) pDC were stimulated with MMP obtained from viable T cell blasts (Vital-MMP), apoptotic polymorphonuclear leukocytes (PMN-MMP), PHA (0.1 μg/ml) and IL-2 (1 U/ml), or CpG 2216 (2216) or were left unstimulated. The bars show the mean ± SEM of IFN-α concentrations measured in the 24-h-supernatants (n = 3 independent experiments). (D) IFN-α-secretion assay. Enriched pDC were left unstimulated or stimulated with CpG 2216, apoptotic T cell blast-derived MMP, or loxoribine (Loxo). Eighteen hours after stimulation, IFN-α-secreting cells were labeled with anti-IFN-α and anti-CD123 and analyzed by flow cytometry. The graphs depict the percentage of double-positive cells. The data show the results obtained in two of three experiments.
When comparing cellular supernatants containing MMP, only those from apoptotic cells were stimulatory. Those derived from viable or necrotic cells were always nonstimulatory, a finding explained by the lower absolute number of MMP (apoptotic) or their absence (necrotic), respectively (data not shown). However, concentrated preparations of MMP from viable T cell blasts displayed stimulatory activity (Fig. 1C). Notably, IFN-α induction was also observed when pDC were stimulated with MMP derived from apoptotic PMN (e.g., non-PHA-treated cells) (Fig. 1C), indicating that the stimulatory activity is independent of the cellular source and of PHA treatment. Overall, these findings suggested that MMP purified from apoptotic cells and from viable cells contain specific ligands that induce the secretion of low concentrations of pDC-derived IFN-α.

To investigate whether pDC represent the source for IFN-α in our experimental setting, we measured IFN-α concentrations in the supernatants of human monocytes stimulated with apoptotic cell-derived MMP and performed an IFN-α-secretion assay in enriched pDC fractions. Importantly, IFN-α was not detectable in monocyte cultures (data not shown), but it could be stained on pDC (CD123<sup>high</sup>, HLA-DR<sup>high</sup>, CD14<sup>−</sup>) stimulated with apoptotic cell-derived MMP, CpG oligodeoxynucleotide (ODN), or loxoribine (Fig. 1D). Strikingly, at the time of analysis, only a small subpopulation of pDC was responsive to both MMP and loxoribine. Notably, CpG ODN were not only more potent, they also induced IFN-α secretion in non-pDC. Taken together, these data indicate that pDC represent the major IFN-α source in response to apoptotic cell-derived MMP.

**Endocytotic uptake of MMP is a prerequisite for pDC activation**

We subsequently asked whether cellular uptake of apoptotic MMP is necessary for pDC activation. To investigate this question, we pretreated human pDC with cytochalasin D, an inhibitor of actin polymerization frequently designated as an inhibitor of endocytosis (43). The experiments performed showed that MMP-triggered IFN-α secretion is completely blocked by pDC pretreatment with cytochalasin D but not by the solvent control (Fig. 2A). In contrast, induction of IFN-α by NDV is only partially inhibited by cytochalasin D (Fig. 2B), thereby proving the preserved capacity of pDC to secrete IFN-α in the presence of cytochalasin D.

Having defined the requirement for endocytotic uptake of apoptotic MMP for stimulation of human pDC, we reasoned that pDC activation could be driven by nucleic acid-sensing TLR that are located in the endosome. Previously, endosomal acidification was found to be an important prerequisite for activity of these TLR (44). Therefore, human pDC was stimulated or not with MMP after pretreatment with chloroquine or the respective solvent control. The results showed that MMP-triggered IFN-α secretion was abolished in the presence of chloroquine (Fig. 2A), although NDV-induced IFN-α production was, again, only partially affected (Fig. 2B). This indicated that MMP most likely stimulated pDC in a TLR7/9-
dependent manner, whereas NDV, an RNA virus, triggered IFN induction by TLR7-dependent and -independent mechanisms (45).

To further confirm the participation of TLR7/9 in apoptotic MMP-mediated pDC activation, MMP stimulation was carried out in the presence of the G-rich inhibitory ODN (iODN) PZ3, which was previously shown to interfere with TLR9 activity (46, 47). The results obtained showed that PZ3 blocks MMP-induced IFN-α production, although IFN induction with the synthetic TLR7 ligand R848 was unaffected (Fig. 2C). Taken together, these results showed that MMP may use TLR9-dependent DNA recognition to initialize pDC activation.

**Apoptotic MMP contain DNA and RNA at a ratio of 10:1**

Because our data suggested a role for TLR9 in pDC recognition of apoptotic cell-derived MMP, we decided to study the nucleic acid content contained within the MMP lipid bilayer. To this end, DNA and RNA were isolated from MMP preparations. Interestingly, photometric quantification of RNA and DNA at OD 260nm revealed that the DNA/RNA ratio was 10:1, with little donor-to-donor variation (Fig. 3A). This finding was in accordance with the concept that DNA recognition via TLR9 could represent the major stimulus driving pDC activation by MMP. Furthermore, DNA agarose gel electrophoresis with ethidium bromide revealed that MMP DNA is dsDNA, an important prerequisite for the immunostimulatory activity of genomic DNA (gDNA) (48). Interestingly, MMP DNA was confined to ~3000 ± 1000 bp compared with gDNA isolated from apoptotic cells (Fig. 3B).

**DNA accounts for the stimulatory action of MMP**

To investigate whether DNA is responsible for the IFN-inducing capacity displayed by MMP, MMP was pretreated or not with DNase. The results indicated that DNase treatment significantly reduces IFN-α secretion levels (Fig. 3C). Preserved IFN-α secretion, despite DNase digestion, most likely results from incomplete accessibility of MMP DNA within the phospholipid membrane.

**Immune stimulatory activity of MMP DNA is superior to cellular DNA**

Next, we sought to compare the stimulatory activity of MMP DNA with gDNA isolated from viable, necrotic, and apoptotic T cell blasts. The stage of cellular viability was confirmed using Annexin V/propidium iodide staining, as described in Materials and Methods (data not shown). Purified DNA was complexed with cationic lipids (Lipofectamine 2000), and pDC was stimulated with “lipoplexes” containing equivalent amounts of DNA of distinct origins. Analysis of IFN-α induction showed that DNA only displayed stimulatory activity when transfected within a liposomal complex and that the IFN-inducing capacity of MMP DNA was clearly superior to DNA isolated from other cellular sources (Fig. 4A). These experiments highlighted the immune stimulatory potential of DNA contained in apoptotic cell-derived MMP.

**pDC stimulation with MMP DNA is more potent than MMP-derived RNA**

Next, we assessed the stimulatory potential of MMP-derived RNA. pDC were stimulated with MMP DNA, MMP RNA, and RNA derived from viable, necrotic, and apoptotic T cells. Again, MMP DNA displayed potent stimulatory activity when complexed with Lipofectamine, and it was clearly more potent than MMP RNA or cellular RNA used at the same concentration (Fig. 4A). However, it should be noted that RNA isolated from apoptotic T cell blasts was significantly more active than was RNA purified from necrotic cells or apoptotic MMP (Fig. 4A). Nevertheless, because of the quantity present in MMP and its stimulatory potency, the DNA moiety most likely accounts for the IFN induction of apoptotic MMP.

**MMP DNA-mediated stimulation of human pDC is inhibited by cytochalasin D, chloroquine, and a G-rich inhibitory oligonucleotide**

Having identified DNA as the major pDC stimulus contained in apoptotic MMP, we asked whether stimulation of human pDC with “lipoplexes” containing MMP DNA would be similarly affected by inhibition of endocytosis and endosomal maturation using cytochalasin D or chloroquine, respectively. Indeed, cytochalasin D inhibited IFN-α production in pDC stimulated with MMP DNA lipoplexes in a concentration-dependent manner (Fig. 4B), thus indicating that endocytosis of the “lipoplexes” is a precondition for pDC activation. Moreover, pDC stimulation with MMP DNA “lipoplexes” was blocked by chloroquine in a concentration-dependent manner and by preincubation with the iODN PZ3. These results supported our hypothesis that endosomal sensing of DNA via TLR9 drives pDC activation by MMP (Fig. 4B). Additionally, MMP DNA-containing “lipoplexes” can be used to in-

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**FIGURE 3.** Quantification of nucleic acid content in MMP. (A) DNA or RNA were isolated from preparations of apoptotic cell-derived MMP (n = 6). Data are mean ± SEM. Total amount of purified DNA (in μg) compared with the total amount of purified RNA (in μg) (left panel). Absolute amounts of DNA and RNA were normalized to DNA = 100% (right panel). The calculated DNA/RNA ratio is 11.9 ± 3. (B) DNA preparations from four MMP samples (#1–4, left) and from the corresponding apoptotic T cell blasts (#1–3, right) were compared on agarose gel electrophoresis. MMP DNA size is ~3000 ± 1000 bp. (C) Enriched pDC were stimulated by MMP treated or not with DNase. CpG 2216 (2216) and CpG 2006 (2006) were used as positive controls, and DNase alone was used as a negative control; data are mean ± SEM (n = 4 independent experiments).
vestigate DNA-mediated effects exerted by intact apoptotic cell-derived MMP.

Apoptotic MMP contain mitochondrial DNA as well as nuclear DNA

Because of their bacterial origin, mitochondria have recently been discussed as a source of immunostimulatory DNA (49–51). Therefore, we decided to assess whether the DNA sorted into the apoptotic cell-derived MMP could contain an increased quantity of mitochondrial DNA (mtDNA) compared with gDNA preparations. To this end, we established a set of PCR that enabled us to distinguish mtDNA from nuclear DNA (nDNA). Amplification of mitochondrial encoded NADH dehydrogenase-4 (ND4) was used as marker for mtDNA, a PCR for human β-actin served to detect nDNA. Primers specific for cytochrome B amplified DNA from both sources and, therefore, were used as control. The specificity of the PCR was confirmed using mtDNA isolated from platelets (that naturally lack the nucleus and nDNA) or from mitochondria purified from human PBMC by magnetic cell sorting via the mitochondrial import receptor TOM22 and with gDNA isolated from human PBMC. The results obtained showed that ND4 was detectable in mtDNA and in some, but not all, gDNA preparations (Fig. 5A, 5B), β-actin was absent in the mtDNA preparation and detectable in gDNA (Fig. 5A, 5B), and cytochrome B was detectable in all preparations (Fig. 5A, 5B). Importantly, PCR products for all three genes could be detected in MMP DNA (Fig. 5A).

Based on these results, we reasoned that an increased proportion of mtDNA in MMP DNA could be responsible for pDC activation. To investigate this possibility we used liposome-complexed platelet-derived mtDNA (Fig. 5B, 5C) to stimulate pDC. In contrast to bacterial DNA isolated from S. aureus, purified mtDNA was inactive with regard to IFN-α induction (Fig. 5C). We concluded that the mtDNA content in MMP DNA is probably not responsible for pDC activation.

MMP-mediated pDC activation is not dependent on FcγRIIA engagement

Bearing in mind that MMP-triggered induction of IFN-α in pDC was formerly demonstrated to be dependent on chromatin or RNA-bearing autoimmune complexes, we next asked whether this would also be the case in healthy individuals. To address this question, we stimulated pDC in the presence or absence of neutralizing Abs targeting either the FcγRIIA or the scavenger receptor CD36. Surprisingly, MMP-triggered activation of human
pDC was found to occur independently of both CD36 and FcγRIIA (Fig. 6A), although, using the same Abs, *S. aureus*-induced pDC activation was previously found to depend on FcRγIIA-mediated uptake (13). In marked contrast, MMP-induced IFN-α levels were even found to be significantly increased (≥2-fold) in the presence of FcRγIIA neutralization (Fig. 6A), an effect that was not observed when stimulating with the TLR7 ligand loxoribine. To confirm that MMP stimulate pDC in the absence of human IgG, we performed pDC stimulation with MMP in the absence of human Ig by using chicken serum instead of human serum. This method provides the advantage that avian IgY cannot activate human FcγRII, whereas other serum components, such as complement proteins, are well conserved (52). The results obtained in these experiments confirmed that the absence of human IgG and immune complex formation or the prevention of FcγRIIA engagement did not suppress apoptotic cell-derived MMP-induced pDC-derived IFN-α secretion. Additionally, as observed using blocking Abs, prevention of FcγRIIA engagement and uptake resulted in MMP-triggered pDC-derived IFN-α secretion that was enhanced ~2-fold (Fig. 6A). Notably, after stimulation with loxoribine, pDC-derived IFN-α levels were unchanged when pDC cultured in chicken serum were compared with pDC cultured in human serum (Fig. 6B). Taken together, these results indicate that, in marked contrast to the findings reported in the autoimmune setting, pDC-derived IFN-α secretion in healthy individuals can be triggered by apoptotic cell-derived MMP in an FcγRIIA-independent manner.

**Discussion**

The data presented in this study demonstrate that MMP derived from apoptotic cells provide immunostimulatory DNA (Fig. 4A) as a trigger for release of IFN-α in human pDC (Fig. 1B). Because of the analogies that we observed when comparing apoptotic cell-derived MMP with lipofection of MMP-DNA, we propose that the lipid-based vesicular structure of the MMP protects the encapsulated DNA from DNAses and promotes cellular uptake, two important prerequisites for stimulation of intracellular DNA receptors. Thus, MMP may act as vehicles for extracellular DNA, as recently suggested (4).

Although extracellular DNA released from dying cells was reported to modulate immune cell function (48), strikingly, we found that the IFN-inducing capacity of MMP DNA was superior to that of DNA from other sources, most importantly DNA isolated from apoptotic cells (Fig. 4A). Considering recent reports that mitochondria are expelled in early apoptosis (53) and that mtDNA stimulates either TLR9 in inflammation and transplant rejection (49, 50) or, alternatively, the AIM2 inflammasome and/ or caspase-1 in sepsis (51), we initially argued that the pronounced immunostimulatory activity of MMP DNA could be attributed to selective accumulation of mtDNA in MMP derived from apoptotic cells (Fig. 5A). Although this hypothesis was tempting in view of the endosymbiotic evolution of mitochondria, purified mtDNA transfected with cationic lipids failed to stimulate IFN-α production (Fig. 5C); therefore, we rejected this hypothesis.
MMP-induced pDC activation

FIGURE 6. Impact of FcγRIIA (CD32A) and CD36 neutralization on MMP-induced IFN-α production. Highly purified pDC were resuspended in autologous human serum and pretreated or not with neutralizing anti-human CD32A and anti-human CD36 mAbs (black bars) before stimulation with MMP (A) or loxoribine (LOXO) (B). Alternatively, highly purified pDC were resuspended in chicken serum (gray bars) and stimulated with MMP (A) or loxoribine (LOXO) (B). Data are mean ± SEM (n = 5 independent experiments).

Nevertheless, gel electrophoresis showed that MMP-derived DNA is double stranded, a known prerequisite for the stimulatory activity of gDNA (48), and size restricted, as opposed to gDNA purified from apoptotic T cell blasts (Fig. 3B). This raised the question whether the immune stimulatory properties could be associated with DNA size. In this regard, it was proposed that a minimal length is required for cellular uptake and that stimulatory activity is gained by increased fragment length (500 bp) (54, 55). By contrast, other investigators provided evidence that stimulation with DNA is sequence dependent and largely independent of the fragment size (56, 57). Nevertheless, future work is needed to address this issue and define the physico-chemical parameters characteristic of MMP DNA, including fragment length, base composition, cytosine–phosphate–guanine site methylation status, and histone modifications.

The data presented in this study further highlight a possible contribution of TLR9 to MMP-mediated induction of pDC-derived IFN-α secretion: MMP DNA was identified as a potent stimulus for pDC activation, endocytosis and endosomal maturation were both required for MMP-induced IFN secretion, and stimulation with apoptotic cell-derived MMP was blocked by the iODN PZ3. This was not surprising, because release of IFN-α in pDC is thought to be exclusively mediated via TLR7 and TLR9 activation and IRF-7 signaling (58).

Endocytosis upon plasma membrane contact of “lipoplexes” and escape to the cytoplasm are hallmarks of DNA delivery by lipofection (59–61). Because MMP lipids could mediate comparable effects, we reasoned that MMP DNA, which we showed to be double stranded (Fig. 3B), may also exert its effects by engaging cytosolic pattern recognition receptors, such as the dsDNA receptors DAI and AIM2 (55, 56). Moreover, the stimulatory capacity of NDV was not abolished by cytochalasin D, chloroquine, or PZ3 pretreatment (Fig. 2), indicating that pDC can also be activated in a TLR-independent manner. These findings are compatible with prior reports that demonstrate that NDV induces IFN-I via engagement of the cytosolic RNA sensor RIG-I (45). However, IL-1β secretion could not be detected in response to pDC stimulation with apoptotic cell-derived MMP (data not shown), which supported the conclusion that AIM2 and NALP3 inflammasomes are not involved in this context.

However, neutralization of FcγRIIA resulted in an increase (not the expected loss) of IFN-α secretion triggered by apoptotic cell-derived MMP (Fig. 6A). This was surprising, because blocking of FcγRIIA in similar experiments with bacterial immune complexes and chromatin autoimmune complexes abolishes IFN-α release from human pDC (13, 23, 41). These data suggested that, in the absence of immune complex formation, interaction of serum IgG with the FcγRIIA exerts a negative regulatory effect on MMP-mediated stimulation of human pDC. Thus, our results stand in marked contrast to the observations made using sera or IgG from systemic lupus erythematosus patients (24, 62–64). Our findings indicate that, in the healthy donor (i.e., in the absence of relevant autoantibody titers), interference with the IgG/FcγRIIA interaction unleashes an ill-defined alternate FcγRIIA-independent uptake mechanism or pDC-activation pathway, leading to IFN-α production that is used by the apoptotic cell-derived MMP. Additional studies are needed to identify the mechanisms involved and to clarify whether complement components, such as C1q and/or natural IgM Abs, are involved in this process, as previously proposed (65). It will be left to future studies to define the molecular basis for the discrepancies between healthy subjects and patients with autoimmune disease.

Type I IFNs have been considered major players in the breaking of tolerance in autoimmune disease (66). However, it should be noted that this view has been challenged by a variety of studies. As already pointed out for pDC function, the effects of type I IFNs include suppression of inflammatory responses through interference with TNF and IL-1β action (67, 68), as well as promotion of tolerance by induction of Tr1 T regulatory cells (69), although this is less known. Both pro- and anti-inflammatory properties of MMP have been reported and are currently debated (7, 70–72). Taking into consideration that apoptotic cell debris induces TLR9-dependent IL-10 production in B lymphocytes (73) and that it was earlier demonstrated that pDC augment B cell-derived TLR9-dependent IL-10 production (32), we speculate that pDC activation by apoptotic cell-derived MMP might reveal an as-yet unappreciated role in establishing tolerogenicity in response to apoptotic cells. However, our data do not provide insight into the immunological consequences of MMP-induced pDC activation. Future work will address the role of pDC and/or IFN-α in the context of apoptosis, along with the in vivo relevance of our findings.

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Disclosures
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References


