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Staphylococcus aureus-Induced Plasmacytoid Dendritic Cell Activation Is Based on an IgG-Mediated Memory Response

Marijo Parcina,* Constanze Wendt,* Friedrich Goetz,† Rainer Zawatzky,‡ Ulrich Zähringer,§ Klaus Heeg,* and Isabelle Bekeredjian-Ding2*

Type I IFNs represent a major antimicrobial defense mechanism due to their property of enhancing immune responses by priming both innate and adaptive immune cells. Plasmacytoid dendritic cells (pDC) are the major source of type I IFN in the human body and represent innate immune cells involved in first-line defense against invading pathogens. Although pDC activation has been extensively studied upon stimulation with synthetic TLR ligands, viruses, and intracellular bacteria, there is only scarce information on extracellular bacteria. In this study we show that the triggering of human pDC-derived IFN-α secretion by Staphylococcus aureus is independent of the bacterial virulence factors protein A and α-toxin but is mediated by Ag-specific IgG and CD32. S. aureus-induced pDC activation can be blocked by inhibitory DNA oligonucleotides and chloroquine, suggesting that engagement of TLR7/9 by bacterial nucleic acids after CD32-mediated uptake of these compounds may play a central role in this process. Altogether, we propose that in marked contrast to nonselective TLR2-dependent activation of most innate immune cells, pDC activation by S. aureus represents an Ag-specific memory response since it requires the presence of class-switched immunoglobulins. The Journal of Immunology, 2008, 181: 3823–3833.
and specific for coagulase-positive staphylococci. This specificity is mediated by preformed anti-staphylococcal IgG and FcγRIIA (CD32) engagement. Based on our findings, we propose that pDC activation is a hallmark of an Ag-specific memory response rather than an early event in a primary immune response.

Materials and Methods

Cell isolation and culture

The use of PBMC from healthy donors was approved by the local ethics committee. PBMC were isolated from heparinized blood by density gradient centrifugation. Since IFN-α secretion was used as an indicator of pDC activation, peripheral blood-derived pDC were enriched from PBMC by positive selection with anti-BDCA4 microbeads (Miltenyi Biotec) and automated magnetically activated cell sorting (autoMACS, Miltenyi) to enhance IFN-α concentrations. pDC enrichment achieved 54 ± 15% purity. To exclude effects mediated by accessory co-purified leukocytes, impurity was confirmed using highly purified pDC (98.5% purity). To exclude effects mediated by accessory co-purified leukocytes, impurity was confirmed using highly purified pDC (98.5% purity). To exclude effects mediated by accessory co-purified leukocytes, impurity was confirmed using highly purified pDC (98.5% purity).

Cell stimulation

All stimuli were added in 100 μl RPMI 1640 without supplements. After 24 h, cellular supernatants were taken off and stored at −20°C until further use. All reagents were tested for endotoxin contamination. The following synthetic stimuli were used: CpG DNA ODN 2216 (CpG), loxoribine (LOXO), and Pam3CSK4 (Pam3). IFN-α secretion in the supernatants was determined after a 24-h stimulation. The graph summarizes the results: filled circles indicate the individual values obtained in each experiment; gray lines give the mean value from n = 5 experiments. **, p = 0.004 for loxoribine compared with Pam3CSK4.

Bacteria and isolation of bacterial nucleic acids and cell wall components

Reference S. aureus strains Cowan I (SAC) and Wood 46 were purchased from DSMZ (DSM 20372, and 20491 respectively); S. epidermidis (S. epid) and S. saprophyticus (S. saps) were kindly provided by T. Hartung (Konstanz, Germany). Stimulation of cells with bacteria was performed at a ratio of 1:5 cells/bacteria. This ratio was based on extensive titration studies and gave the best reproducibility of results for all bacteria with regard to variability of IFN-α levels and donor-to-donor variations. The inhibitory DNA ODN PZ3 (CTC GTA TTG GGG GTT TCC TAT, G-rich ODN) (41) was used at 1 μM and purchased from MWG Biotech. Recombinant α-toxin (titrated from 50 to 1000 ng/ml) and protein A (titrated from 5 to 20 μg/ml) were dissolved in PBS and purchased from Sigma-Aldrich and Amersham Biosciences, respectively. The anti-protein A mAb (clone SPA-27, murine IgG1, Sigma-Aldrich) was used at 5 μg/ml for neutralization of CD32, pDC were preincubated with anti-human CD32 mAb (GeneTex GTX74628) or murine IgG1 (BD Biosciences) at 15 μg/ml for 30 min before stimulation. Cytochalasin D (dissolved in DMSO, Alexis Biochemicals) and chloroquine (dissolved in water, Sigma-Aldrich) were used as indicated in the diagrams. Newcastle disease virus (NDV) was used as previously described (42). The NDV preparation had a titer of 1600 hemagglutinating units and was diluted at 1/500 for stimulation.

FIGURE 1. Impact of TLR2 activity and staphylococcal species on pDC activation. A, Enriched human BDCA4+ pDC were stimulated with synthetic TLR ligands: CpG DNA ODN 2216 (CpG), loxoribine (LOXO), and Pam3CSK4 (Pam3). IFN-α secretion in the supernatants was determined after a 24-h stimulation. The graph summarizes the results: filled circles indicate the individual values obtained in each experiment; gray lines give the mean value from n = 5 experiments. **, p = 0.004 for loxoribine compared with Pam3CSK4. B, Enriched pDC were stimulated with SA113 WT (WT) and SA113 Δlgt (Δlgt). TLR7 agonist loxoribine (LOXO) was used as a positive control for IFN-α induction measured after 24 h. The diagram shows the detection of a phosphodiester linkage 3'-H11042 and the gray line indicates the average (gray line) obtained in n = 9 experiments. C, IFN-α secretion was confirmed in pure pDC stimulated with loxoribine or S. aureus reference strains Cowan I (SAC) or SA113 WT (WT). IFN-α secretion levels were quantified after 24 h. The diagram summarizes the results of n = 4 experiments. D, IFN-α concentrations in the supernatants of enriched human pDC were measured after 24 h in unstimulated and stimulated conditions. Cells were stimulated with loxoribine (LOXO) as a positive control or with bacteria (S. aureus Cowan strain I (SAC), S. hominis (S. hom), S. saprophyticus (S. saps), S. epidermidis (S. epid)). The diagram shows the single values obtained in n = 9 experiments (● and the average values (gray lines). *, p = 0.02 for SAC compared with S. epidermidis.

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and RNA preparations were treated with RNase-free DNase I (Roche) at 37°C overnight. Subsequently, DNA was reparated using the DNA tissue kit and RNA was purified using the RNaseasy kit (both from Qiagen). 1 μg of DNA or RNA was complexed with 2 μl of Lipofectamine (Invitrogen) in 50 μl antibiotic-free medium (OptiMEM, Invitrogen) for 20 min before pDC stimulation.

For UV inactivation, bacterial cells were adjusted to a density of 0.5 McFarland. During UV light exposure (1 h, 1 J/cm²), the 24-well plate was incubated on ice to avoid excessive heating. Successful inactivation was confirmed by comparing this fraction to whole serum by immunoblot analysis.

Depletion and isolation of serum IgG and Fc Fragments

All procedures were performed according to the manufacturer’s instructions for the Fab preparation kit (Pierce). In brief, for depletion of serum IgG from normal human donor serum (whole serum), serum was diluted 1/1 with 0.9% saline and 4 ml were applied twice to a protein A column and was concentrated using a 50K Amicon BioSeparation Ultrafree-CL nominal molecular mass device (Millipore). For the generation of Fc fragments, isolated IgG were digested with immobilized papain overnight at 37°C. The supernatant was then applied to the protein A column and the Fc fragments were eluted and subsequently concentrated on a 10K Amicon BioSeparation Ultrafree-CL device. IgG and Fc fragment concentrations were measured at a wavelength of 280 nm. For stimulation, pDC were resuspended in IgG-depleted human serum (allogeneic) and isolated (allogeneic) IgG (50 μg/ml) and Fc fragments (25 μg/ml) were both added back. Additionally, human Fc fragments were purchased from Jackson ImmunoResearch Laboratories and used at 20 μg/ml (data not shown).

Cytokine detection

Cytokine concentrations after 24 h of stimulation in cell-free supernatants were quantified by ELISA. The following kits were used according to manufacturers’ instructions: human IFN-α (Bender MedSystems); human IL-8 (Opteia, BD Biosciences).

Flow cytometry

Cells were stained in PBS/1% FCS according to standard procedures. The following mAbs were used: anti-human BDCA-2 (FITC) and anti-human CD36 (PE) (BD Biosciences). Propidium iodide (Bender MedSystems) was used to determine cell viability. Data were acquired using a FACSCanto and analyzed using FACSDiv software (BD Biosciences).

Assessment of TLR2 activity

HEK293 cells were transfected with TLR2 plasmid as previously described (44) and stimulated for 24 h as indicated. CpG DNA ODN 2216 and/or PamCSK4 were used as negative controls (data not shown); Pam,CSK4 was used as a positive control (100 ng/ml). Cellular supernatants were analyzed for IL-8 secretion.

Genomic PCR

Genomic DNA from bacteria was isolated using InstaGene Matrix solution (Bio-Rad). PCR was performed using the AmpliTaq DNA polymerase (Applied Biosystems). Amplifications for hlyA gene (α-toxin) and hlyD (δ-toxin) were performed with the following program: 5 min at 94°C, 30 cycles of 40 s 94°C, 60 s 57°C, 90 s 72°C; final extension 10 min at 72°C.
assessed by measuring IFN-α in supernatants of enriched pDC fractions of n = 3 (A) and n = 4 (B) experiments as filled diamonds. The mean values are shown as gray bars. The diagrams in the upper panels summarize the IFN-α concentrations measured in unstimulated and CpG DNA ODN 2216-stimulated pDC controls; the diagrams in the lower panels depict the IFN-α concentrations determined after stimulation with Cowan strain I (SAC), Wood 46 (W46), or SA113 WT (WT) S. aureus reference strains as indicated. A, IFN-α induction in pDC incubated with 4% heat-inactivated human serum in RPMI 1640 culture medium was compared with that in pDC cultured in serum-free X-Vivo 15 medium. B, Before stimulation, pDC were resuspended in heat-inactivated human normal donor serum (whole serum) or IgG-depleted human serum with or without add-back of previously depleted IgG (add-back + IgG) or Fc fragments thereof (generated by papain digestion) (add-back + Fc). * p = 0.014 for W46 IgG-depleted compared with W46 with IgG; ** p = 0.014 for W46 IgG-depleted compared with W46 with IgG.

Statistics
Statistical significance was determined using the paired two-tailed Student’s t test with n of ≥8 or the Wilcoxon-Mann-Whitney U test with n of ≥8. All tests were performed using GraphPad Prism software.

Results
pDC activation by staphylococci is independent of TLR2-active lipopeptides
S. aureus has repeatedly been mentioned as an extracellular bacterium triggering IFN-α secretion in human PBMC (38–40). One characteristic of this Gram-positive bacterium is its very potent TLR2 activity that is mediated by membrane-anchored LP and can be easily detected in TLR2-transfected HEK293 cells (data not shown and Ref. 46). TLR2-active LP have, in fact, been proposed to represent its major immune stimulatory component (46–48). The results showed that synthetic TLR2 ligands induced IFN-α secretion in human pDC (Fig. 1A and data not shown). Also, neither soluble nor insoluble peptidoglycan or lipoteichoic acid from S. aureus stimulated IFN-α secretion (data not shown).

Next, we wanted to demonstrate that TLR2-active LP are negligible. pDC were stimulated with synthetic TLR2 ligands (Pam3CSK4, a triacylated LP, Pam2CSK4, a diacylated LP, Pam2CSK4, an inactive monoacylated LP, and FSL-1-R, a MALP-2 analog, and FSL-1-S, its inactive S-enantiomer), as well as with LP fractions chemically isolated from S. aureus cell walls. The results obtained suggested that human pDC-derived IFN-α secretion is not induced by TLR2-active LP since all LP reagents failed to trigger IFN-α secretion (Fig. 1A and data not shown).

To test this hypothesis, human pDC were enriched from whole PBMC and stimulated with TLR2 agonists. pDC activation was assessed by measuring IFN-α secretion in the supernatants since IFN-α production by immune cell types other than pDC is negligible. pDC were stimulated with synthetic TLR2 ligands (Pam3CSK4, a triacylated LP, Pam2CSK4, a diacylated LP, Pam2CSK4, an inactive monoacylated LP, and FSL-1-R, a MALP-2 analog, and FSL-1-S, its inactive S-enantiomer), as well as with LP fractions chemically isolated from S. aureus cell walls. The results obtained suggested that human pDC-derived IFN-α secretion is not induced by TLR2-active LP since all LP reagents failed to trigger IFN-α secretion (Fig. 1A and data not shown).

To confirm that the IFN-α detected originates from pDC, we repeated the experiments using highly purified pDC preparations. Indeed, pDC-derived IFN-α production was triggered in response to S. aureus stimulation (Fig. 1C). Moreover, IFN-α levels

FIGURE 3. Role of serum and serum-IgG in S. aureus-mediated stimulation of pDC-derived IFN-α secretion. The diagrams show the single values measured for IFN-α in supernatants of enriched pDC fractions of n = 3 (A) and n = 4 (B) experiments as filled diamonds. The mean values are shown as gray bars. The diagrams in the upper panels summarize the IFN-α concentrations measured in unstimulated and CpG DNA ODN 2216-stimulated pDC controls; the diagrams in the lower panels depict the IFN-α concentrations determined after stimulation with Cowan strain I (SAC), Wood 46 (W46), or SA113 WT (WT) S. aureus reference strains as indicated. A, IFN-α induction in pDC incubated with 4% heat-inactivated human serum in RPMI 1640 culture medium was compared with that in pDC cultured in serum-free X-Vivo 15 medium. B, Before stimulation, pDC were resuspended in heat-inactivated human normal donor serum (whole serum) or IgG-depleted human serum with or without add-back of previously depleted IgG (add-back + IgG) or Fc fragments thereof (generated by papain digestion) (add-back + Fc). * p = 0.014 for W46 IgG-depleted compared with W46 with IgG; ** p = 0.014 for W46 IgG-depleted compared with W46 with IgG.

Primers: hlyA, forward 5'-GCC TTT ATT ATT GCG TAC GA; reverse, 5'-CCA TAT ACC GGG TTC CAA GA; product size 176 bp; hlyD, forward 5'-TTT TTA GTG AAT TTG TTC ACT GTG T; reverse 5'-TAA TTA AGG AAG GAG TGA TTT CAA T.

Statistics
Statistical significance was determined using the paired two-tailed Student’s t test with n of ≥8 or the Wilcoxon-Mann-Whitney U test with n of ≥8. All tests were performed using GraphPad Prism software.
were below the detection limit when whole PBMC ($4 \times 10^7$/well) were stimulated with *S. aureus* (data not shown). IFN-α was only detected upon enrichment of BDCA4+ cells. We concluded that *S. aureus*-mediated IFN-α induction results from pDC activation.

**IFN-α induction is characteristic for *S. aureus* and is not a constant finding with coagulase-negative staphylococci**

Because TLR2 activity could be excluded as a major stimulatory factor in *S. aureus*-triggered pDC activation, and because type I IFN induction has only been described for *S. aureus* while other staphylococcal species are never mentioned in this context, we wanted to know whether coagulase-negative staphylococci would trigger comparable IFN-α levels as seen with *S. aureus* cells. We therefore compared the IFN-α inductory potential of a *S. aureus* reference strain (Cowan I) to different typed clinical isolates of coagulase-negative staphylococci such as *S. epidermidis*, *S. hominis*, *S. saprophyticus*, *S. haemolyticus* (Fig. 1D), *S. lugdunensis*, *S. xylosus* and *S. hyicus* (data not shown) in several donors. Surprisingly, all staphylococcal species except for *S. aureus* failed to induce high levels (>500 pg/ml) of IFN-α (Fig. 1D), with the exception of high reactivity toward *S. epidermidis* in a few individuals (Fig. 1D). Based on these findings, we concluded that factors specific for *S. aureus* must be responsible for the selectivity of the pDC response.

**S. aureus-induced IFN-α secretion is not dependent on protein A expression**

Since the expression of staphylococcal protein A (SpA) represents one of the major differences between coagulase-negative staphylococci and coagulase-positive *S. aureus*, and it has previously been published that protein A is responsible for the induction of type I IFN in human PBMC (40), we wanted to assess the role of SpA in pDC-derived IFN-α secretion. To do so, we chose different approaches. First, we compared two well-described *S. aureus* reference strains: Cowan I (SAC), a high producer of SpA, and Wood 46, a strain deficient in SpA protein expression due to a sigB deficiency (46, 49, 50). Both strains were comparable in regards to their IFN-α triggering potential (Fig. 2A). Next, we compared a mutant *S. aureus* strain lacking the spa gene (SA113 Δspa) with its wild-type counterpart (SA113 WT) (Fig. 2B). Lastly, we stimulated pDC with recombinant SpA and did not detect IFN-α in the supernatants despite titrating SpA over a wide range of concentrations (1–10 µg/ml) (data not shown).

The induction of pDC-derived IFN-α requires the viability of *S. aureus* cells but is independent of α-toxin secretion

Another important observation was that pretreatment of bacteria with antibiotics or UV inactivation (Fig. 2C) resulted in lower or even absent IFN-α secretion. Furthermore, IFN-α secretion was not detectable after stimulation of pDC with crude cell wall preparations or peptidoglycan (data not shown). Based on these observations we concluded that triggering of IFN-α may require the active secretion of *S. aureus*-derived stimulatory factors.

Since α-toxin is an important *S. aureus*-specific protein that is secreted into the extracellular space and has previously been shown to exert immune stimulatory effects at low concentrations (51–53), we hypothesized that α-toxin may be responsible for the species-specific induction of IFN-α in human pDC. We therefore typed a series of *S. aureus* clinical isolates for the α-toxin gene *hlyA* (Fig. 2D) and tested α-toxin-positive and -negative strains for their reactivity in our system. The data obtained in these experiments indicated that IFN-α induction from pDC is independent of α-toxin (Fig. 2D). These data were further corroborated by the finding that recombinant α-toxin failed to induce IFN-α secretion despite testing a wide range of α-toxin concentrations (data not shown). We drew the conclusion that factors other than α-toxin and SpA but specific for coagulase-positive staphylococci must be responsible for pDC activation.

**Human serum is required for bacterial stimulation of IFN-α production**

Next, we wanted to assess which factors define the species specificity of the IFN-α response upon stimulation of pDC with coagulase-positive and coagulase-negative staphylococcal strains. Since our experimental system was based on the culture of pDC in autologous serum, we decided to perform experiments that would allow the distinction between pDC-related factors and serum components. Our first approach consisted in comparing a serum-free medium (X-Vivo 15) to our standard approach using autologous

![FIGURE 4. Role of Ag-specific IgG in pDC recognition of *S. aureus*.](http://www.jimmunol.org/)

Enriched pDC were cultured in heat-inactivated chicken serum. A, pDC were stimulated with Cpg DNA ODN 2216 (*upper panel*) or Wood 46 (*W46* (*lower panel*)) in the presence or absence of isolated human IgG (Chicken serum + IgG) or Fc fragments (Chicken serum + Fc). The values from *n* = 4 experiments are shown, *p* = 0.01 for W46 without IgG compared with W46 with IgG. B, pDC were stimulated with SA113 WT or SA113 Δspa in the presence or absence of a murine anti-SpA mAb. The diagram summarizes the results obtained in *n* = 5 independent experiments. *p* = 0.01 for WT without anti-SpA compared with WT with anti-SpA.
human serum. The data obtained showed that bacterial stimulation of human pDC-derived IFN-α was absent under serum-free conditions (Fig. 3A, lower panel). In marked contrast, stimulation with the CpG DNA ODN 2216 was preserved in the X-Vivo 15 condition despite a lower pDC survival rate in the absence of serum (Fig. 3A, upper panel). pDC survival in X-Vivo 15 medium was reduced to ~60% of serum-containing medium (data not shown).

Importantly, pDC-derived IFN-α secretion was not dependent on the serum source (autologous vs allogeneic) (data not shown).

IFN-α induction requires the presence of IgG

Since the specificity for S. aureus was mediated by serum components, we hypothesized that pDC activation by staphylococci may occur in an antibody-dependent manner. Since FcyRIIA (CD32A) is expressed on pDC, and previous reports have shown that Fcy receptor-mediated uptake of immune complexes containing stimulatory nucleic acids can trigger dendritic cell stimulation by engaging TLR7 or TLR9 (54–59), we postulated that recognition of extracellular bacteria by the human pDC requires preformed S. aureus-specific IgG. We therefore depleted human serum from IgG and collected the IgG fraction. Additionally, IgG was treated with papain to generate Fc fragments. Thereafter, human pDC were stimulated with S. aureus in the presence of normal human serum (whole serum) or IgG-depleted serum from an unrelated donor with or without reconstitution of the nonautologous whole IgG or the Fc fragments (Fig. 3B, lower panel). The results showed that IFN-α secretion was strongly reduced when using IgG-depleted human serum (Fig. 3B, lower panel), which was compatible with the presence of low amounts of residual IgG. Add-back of isolated IgG potently enhanced IFN-α secretion, while Fc fragments did not trigger additional IFN-α secretion. On the other hand, the stimulatory potential of CpG DNA ODN 2216 and loxoribine was not affected by the presence or absence of human IgG (Fig. 3B, upper panel).

Since avian IgG structurally differs from human and bovine IgG and cannot engage human Fcγ receptors (60), as an alternate approach pDC were resuspended in culture medium containing chicken instead of human serum or FCS, which was used in other studies. pDC were then stimulated with S. aureus in the presence or absence of human IgG or Fc fragments (Fig. 4A, lower panel). As expected, IFN-α was not detectable when the stimulation with S. aureus was performed in chicken serum (Fig. 4A, lower panel). Reconstitution of human IgG restored full pDC reactivity toward S. aureus (Fig. 4A, lower panel). Furthermore, IFN-α secretion was only restored by whole IgG and not by Fc fragments (Fig. 4A, lower panel). Again, the stimulatory activity of CpG DNA ODN 2216 and loxoribine was not influenced by the presence or absence of human IgG (Fig. 4A, upper panel). Taken together, these experiments provided evidence that pDC recognition of S. aureus depends on the presence of human IgG. Note also that titration experiments showed that in the experimental settings chosen the strength of the IFN-α response was determined by the concentration of total IgG rather than the bacteria-to-cell ratio (data not shown).

IFN-α induction requires the presence of S. aureus-specific IgG

To demonstrate that the IgG has to be Ag-specific for induction of IFN-α, we stimulated pDC in chicken serum using SA113 wild type S. aureus (WT) or an SpA-deficient mutant derived from this strain (ΔSpA) in the presence or absence of a murine monoclonal IgG Ab directed against SpA. In these experiments IFN-α was only
detectable in the presence of both the anti-SpA Ab and the SpA-expressing wild type S. aureus (Fig. 4B). In marked contrast, the Δspa mutant failed to induce IFN-α with or without the anti-SpA Ab, thus proving both the requirement for immune complex formation and the Ag specificity of the Ab. In summary, these results provided evidence that stimulation of human pDC by S. aureus depends on Ag-specific IgG.

pDC stimulation by S. aureus occurs via a CD32-dependent mechanism

To confirm that stimulation occurs via CD32 engagement by S. aureus-specific anti-staphylococcal IgG-complexes containing bacterial components, human pDC were stimulated with S. aureus in the presence or absence of an anti-human CD32 neutralizing Ab or an isotype control Ab, respectively (Fig. 5, upper panel). The results obtained were well compatible with our hypothesis since IFN-α production was abrogated in the presence of the anti-CD32-neutralizing Ab but was not affected by murine IgG1 (Fig. 5). Notably, the pDC response by control stimuli TLR9 agonist CpG DNA ODN 2216 and TLR7 agonist loxoribine was not affected by CD32 neutralization (Fig. 5, lower panel). These data supported the finding that S. aureus-triggered pDC activation is selectively induced via IgG-mediated bacterial recognition and ruled out a significant role for other Ig isotypes such as IgM. Consequently, S. aureus-induced pDC-derived IFN-α secretion requires IgG-dependent species-specific recognition of the bacterium and the interaction of the immune complex with the Fcγ receptor IIA (CD32A).

S. aureus-triggered pDC activation is most likely mediated by bacterial nucleic acid recognition

To date, the best described stimuli for human pDC activation are microbial nucleic acids that are recognized via TLR7 and TLR9. We therefore assumed that bacterial recognition by human pDC may also be mediated by these pattern recognition receptors. To assess whether bacterial RNA or DNA is responsible for the stimulatory effect, we isolated RNA and DNA from S. aureus and from different coagulase-negative staphylococci. As shown in Fig. 6A, our DNA preparations were significantly more active than the RNA preparations when used at the same concentration (1μg/well) and complexed with cationic lipids to ensure cellular uptake. In spite of this, both types of nucleic acids were capable of triggering IFN-α secretion from human pDC. Most importantly, in all donors the IFN-α levels induced were comparable with both S. aureus strains and coagulase-negative staphylococci. The results shown using S. epidermidis DNA and RNA preparations are representative for all other preparations from coagulase-negative staphylococci tested (Fig. 6A and data not shown).

Since TLR-deficient human pDC are not available and murine pDC do not express CD32A (61), we blocked TLR7- and TLR9-mediated human pDC activation with inhibitory ODN to assess whether bacterial stimulation of pDC could be exerted by TLR-dependent nucleic acid recognition. Human pDC were preincubated with the inhibitory ODN PZ3 (41) and subsequently stimulated with S. aureus. Indeed, the inhibitory ODN suppressed pDC...
Triggering of IFN-α production by S. aureus involves endocytosis and engagement of endosomal TLR

The engagement of CD32A by bacterial immune complexes triggers endocytic uptake of bacteria or bacterial components that may thereby gain access to endosomally located TLR. To prove the involvement of endocytosis, we analyzed pDC activation in the presence or absence of cytochalasin D that inhibits actin polymerization (63). Chloroquine, a substance thought to selectively inhibit TLR7 and TLR9 activation by preventing endosomal acidification (64), was used in parallel.

The data obtained in these experiments are summarized in Fig. 7. They show that cytochalasin D-mediated inhibition of pDC-derived IFN-α production occurs in a concentration-dependent manner. Interestingly, IFN-α induction by S. aureus and by CpG DNA was blocked with low concentrations of cytochalasin D. In contrast, inhibition of Lipofectamine-mediated uptake of staphylococcal DNA and infection with NDV were only partially blocked at low concentrations. Similarly, low concentrations of chloroquine suppressed CpG DNA- and S. aureus-mediated pDC stimulation, albeit staphylococcal DNA was only partially affected and a negligible effect was found on NDV stimulation.

NDV induces type I IFNs via cytoplasmic RNA recognition receptor activation (65). Since both inhibitors failed to suppress its activity, NDV served as a control for pDC viability in the presence of these reagents. S. aureus-mediated pDC activation closely resembled pDC activation by CpG DNA. Thus, both depend on endocytotic uptake and endosomal acidification. Therefore, S. aureus-induced type I IFN secretion may, indeed, be induced via endosomal nucleic acid-sensing TLR.

In contrast to S. aureus cells, Lipofectamine-complexed staphylococcal DNA was only partially inhibited by chloroquine or low concentrations of cytochalasin D. This could be due to the relatively high amount of DNA utilized when compared with the nucleic acid content of endocyotically bacteria. Nevertheless, it seems likely that both TLR9-dependent and -independent pathways are involved in the response to Lipofectamine-complexed staphylococcal DNA. Taken together, these data highlight the role of CD32 in selectively targeting microbial nucleic acids to endosomally located TLR.

Discussion

In this report we show for the first time that human pDC activation and subsequent IFN-α secretion triggered by extracellular bacteria such as S. aureus is an Ag-specific event requiring preformed species-specific IgG (Fig. 4). We further provide evidence that stimulation of IFN-α secretion requires CD32 (Fig. 5) and is most likely promoted by engagement of endosomal TLR7 and/or TLR9 via staphylococcal nucleic acids (Figs. 6 and 7).

To our knowledge, this study is the first to systematically analyze the mechanisms involved in S. aureus stimulation of human pDC. Excluding cell wall-intrinsic TLR2 activity as a relevant pDC stimulus was very important since cell wall-associated TLR2-active LP represent the major stimulatory molecules triggering the activation of innate immune cells other than pDC. Additionally, this finding provides us with a better understanding of the function of the human pDC in bacterial recognition: the lack of TLR2 responsiveness is the basis for specific recognition of distinct staphylococcal species because TLR2-active LP are unselectively expressed by all Gram-positive bacteria. pDC activation requires more selective triggers. This restriction of the IFN-α response is an important safeguard mechanism to prevent premature and overwhelming immune responses such as the autoimmune phenomena observed in systemic lupus erythematosus and as side effects of type I IFN treatment (66).

Furthermore, our study excludes two representative, well-characterized S. aureus-specific immunostimulatory molecules as IFN-α inducers: the Ig-binding protein SpA, which has recently been shown to stimulate the TNF receptor I (67), and hemolysin A, a pore-forming membrane toxin that provides a rather unspecific stimulus for immune activation (51–53) (Fig. 2). Thus, IFN-α induction by S. aureus is not due to an aberrant immune stimulation caused by virulence factors of S. aureus but represents a targeted physiological host response.

Viral induction of type I IFNs and, specifically, stimulation of pDC have been shown to be triggered by two distinct mechanisms: 1) pDC infection by viruses such as HIV, influenza, and herpes simplex virus that utilize cell surface receptor-mediated endocytosis for their entry (22, 25–28, 68) and mediate pDC activation via endosomal engagement of TLR9 by dsDNA viruses or TLR7 by ssRNA viruses (19, 22, 23, 25, 26), thereby triggering type I IFN production and subsequent up-regulation of antiviral proteins such as PKR (69); and 2) CD32-dependent uptake of antiviral IgG complexed with viral constituents (26, 57, 70–74). Type I IFN levels remain lower in the latter context when compared with pDC infection (71, 75). Our findings indicate that the antibacterial IFN-α response is similarly initiated by IgG-mediated bacterial recognition.

Previous studies have provided evidence that anti-DNA or anti-RNA autoantibody complexes in lupus patient serum can stimulate human pDC via a CD32-dependent mechanism (76–78). DNA or RNA, most likely released from apoptotic cells, is dragged into the pDC via Fc receptor-mediated endocytosis of the anti-DNA autoantibody where it engages TLR7 or TLR9 in the endosome, thus inducing type I IFN synthesis (54, 56, 58, 59). This sequence of events provokes a pathological type I IFN response due to the presence of pathognomonic anti-DNA autoantibodies. Similarly, microbial pDC infection via receptor-mediated endocytosis triggered by viral or bacterial virulence factors and subsequent type I IFN secretion can be viewed as a sign of disease manifestation. In our context, the presence of anti-staphylococcal serum IgG is reminiscent of a previous Ag-specific B cell response and thus represents a physiological mediator of immune activation.

FcγRIIA-mediated uptake of S. aureus or its components has been demonstrated in other cellular systems (79, 80). In our experimental system the stimulatory activity requires both endocytosis of the bacterial cells or compounds and the presence of DNA and/or RNA at concentrations not reached in the cell wall and PG preparations tested (data not shown). Moreover, IFN-α production was absent in experiments using purified tetanus toxoid in anti-toxoid-positive sera as a pDC stimulus (M. Parcina and I. Bekeredjian-Ding, unpublished data). This finding emphasizes the importance of bacterial nucleic acids since the Ag-antibody complex itself is not sufficient to trigger IFN-α production.
In further support of nucleic acid recognition as the trigger for pDC activation, recent studies suggest that the abundance of stimulatory microbial motif reflects a prerequisite for immune cell stimulation (81–84). These studies have proposed that specific enzymes inactivate microbial ligands, thereby defining a threshold for stimulation when concentrations exceed full enzyme saturation. Stimulatory concentrations of PG, for example, were only reached with proliferating bacteria. A similar observation was made by several groups working on viral pDC activation via nucleic acids that seemed to be dependent on viral replication (26, 57, 70). Therefore, ongoing bacterial DNA and RNA synthesis may represent a prerequisite for passing the threshold concentration of nucleic acids necessary for TLR stimulation.

Since bacterial stimulation of pDC was blocked by inhibitory ODN as well as by chloroquine, our data suggest that TLR7 and TLR9 are the pattern recognition receptors in question. CD32 engagement and subsequent endocytosis of the bacterium or its components are required for pDC activation, and they serve to selectively promote contact of nucleic acids with endosomal TLR. On the other hand, note that our data do not rule out an additional contribution to specific uptake of S. aureus mediated by other serum components such as mannose-binding lectin (85) or other cell-surface molecules, including CD36 (15), RAGE (86), or integrin α5β1 (87).

It also seems noteworthy that in the human immune system only B cells and pDC are prone to respond to TLR7 and TLR9 stimuli. Moreover, these two cell types share a very limited responsiveness toward TLR2 and TLR4 ligands (Fig. 1 and Ref. 46) which, in contrast, is well developed in other innate immune cells such as macrophages. We therefore propose that recognition of the rather abundant TLR2 and TLR4 ligands can be considered as a nonselective innate immune response, while endosomal sensing of microbial nucleic acids via TLR7/9, due to the restricted accessibility of the receptors and, in this case, the necessity of preformed IgM, is reserved as a trigger for Ag-specific innate immune responses.

Moreover, the stimulatory activity of nucleic acid preparations from S. aureus and coagulase-negative staphylococci was comparable in all donors tested, indicating that species-specific differences in base composition do not result in major differences in the potency of pDC activation. Thus, bacterial nucleic acids trigger pDC activation without conferring the specificity of the response to S. aureus over coagulase-negative staphylococci. Therefore, pDC activation occurs in a rather unspecific manner once the microbial acids are introduced into the endosome and can bind the pattern recognition receptor. The selectivity of the response is defined at an earlier stage, for example, by IgG-mediated Ag specificity.

Furthermore, our data indicate that pDC activation only occurs after prior Ab formation and class-switch recombination resulting in Ag-specific recognition of the bacterium by serum IgG-targeting staphylococcal cell-surface Ags. In marked contrast to S. aureus, only few donors responded strongly to S. epidermidis, and the response to other coagulase-negative staphylococci was mostly weak (Fig. 1). Since the IFN response requires preformed Abs, this finding indicates that all donors have previously developed humoral immunity toward S. aureus, a bacterium frequently encountered in purulent cutaneous infections and on mucosal surfaces. In contrast, it is plausible that most individuals have not initiated a comparably strong B cell response against S. epidermidis since it is only part of the cutaneous normal flora, or against other coagulase-negative staphylococci that are even less abundant in human flora. Interestingly, one of the responsive donors when questioned remembered having passed a deep wound infection with cultural evidence of S. epidermidis. Furthermore, titration of clinical-grade polyvalent IgG indicated that the level of IFN-α secretion is determined by the Ag-specific IgG titer (data not shown). Thus, frequency and intensity of exposure and subsequent mounting of a humoral immune response may, indeed, represent a prerequisite for pDC activation by extracellular bacterial pathogens.

Additionally, the necessity for switched Ig isotypes represents another safeguard: species specificity could not be guaranteed by IgM due to the existence of unspecific antibacterial IgM Abs. Ag-specific IgG-mediated pDC activation, in turn, represents a potent regulatory mechanism that in vivo could serve to initiate the secondary Ag-specific (memory) immune response by priming innate immune cells to sense the invading pathogens and by inducing a Th1 response as well as rapid plasma cell formation (6, 88, 89).

Furthermore, our findings may have important clinical implications with regard to prophylaxis and the treatment of bacterial infections. Based on our data, we suggest that eliciting TLR7/9-dependent pDC activation may be essential for the initiation of the secondary immune response. Thus, vaccination against obligate bacterial pathogens should be considered in patients at risk for developing sepsis. pDC activation by preexisting IgG could enable the immune system to respond more rapidly or more efficiently in the case of bacterial infection. Furthermore, the development of therapeutic Abs of IgG subclasses able to introduce bacterial immune complexes into the endosomes of pDC may represent an equally important approach in the treatment of sepsis patients.

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Disclosures

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