Abstract  Blood monocyte-derived macrophages invading the alveolus encounter pulmonary surfactant, a phospholipidoprotein complex that changes composition during lung development. We tested the hypothesis that characteristic phosphatidylcholine (PC) components differentially influence macrophage phenotype and function, as determined by phagocytosis of green fluorescent protein-labeled *Escherichia coli* and αCD3-induced T cell proliferation. Human macrophages were exposed to surfactant (Curosurf®), to two of its characteristic phosphatidylcholines (PC) components (dipalmitoyl-PC and palmitoylmyristoyl-PC), and to a ubiquitous PC (palmitoyloleoyl-PC) as control. Interaction of Curosurf and PC species with macrophages was assessed using Lissamine™-dihexadecanoyl-phosphoethanolamine-labeled liposomes. Curosurf and both saturated surfactant PC species downregulated CD14 expression and upregulated CD206. HLA-DR and CD80 were upregulated by Curosurf and palmitoylmyristoyl-PC, whereas dipalmitoyl-PC showed no effect. The latter upregulated TLR2 and TLR4 expression, whereas Curosurf and palmitoylmyristoyl-PC had no effect. PC species tested were incorporated in comparable amounts by macrophages. Curosurf and PC species inhibited phagocytosis of *E. coli*. Scavenger receptor CD36, CD68, SR-A, and LOX-1 mRNA expression was upregulated by Curosurf, whereas PC species only upregulated SR-A. Curosurf and palmitoylmyristoyl-PC inhibited αCD3-induced T cell proliferation by 50%, whereas dipalmitoyl-PC and palmitoyloleoyl-PC showed no effect. These data identify individual surfactant PC species as modifiers of macrophage differentiation and suggest differential effects on innate and adaptive immune functions.—Gille, C., B. Spring, W. Bernhard, C. Gebhard, D. Basile, K. Lauber, C. F. Poets, and T. W. Orlikowsky. Differential effect of surfactant and its saturated phosphatidylcholines on human blood macrophages. *J. Lipid Res.* 2007. 48: 307–317.

Supplementary key words  phospholipids • molecular species • PC16:0/14:0 • PC16:0/16:0 • costimulation • HLA-DR

Pulmonary surfactant is a phospholipoprotein complex synthesized by type II pneumocytes and plays an essential role in reducing surface tension in terminal air spaces. Phospholipids comprise 80–85% of the mass of mammalian surfactant, together with 10% neutral lipids and 5–10% surfactant proteins (SPs) A to D. The phospholipids comprise 80–85% phosphatidylcholine (PC), with an enrichment in dipalmitoyl-PC (PC16:0/16:0) and palmitoylmyristoyl-PC (PC16:0/14:0) (1, 2). Surfactant composition changes characteristically during development, with increasing concentrations of disaturated PC species such as PC16:0/14:0 and PC16:0/16:0 at the expense of ubiquitous components such as palmitoyloleoyl-PC (PC16:0/18:1) and, specifically, a relative preponderance of PC16:0/14:0 in term neonates compared with adult organisms (2, 3). Together with increased concentrations of hydrophobic surfactant proteins SP-B and SP-C, these molecular changes improve the surface tension-lowering properties of surfactant around birth (4) and correlate to physiologic parameters (2). Recently, significant decreases in PC16:0/14:0 were found in response to inflammatory processes affecting structural development or homeostasis of the lungs like bronchopulmonary dysplasia (BPD) or lung emphysema (5).

In addition to its function in reducing surface tension in the terminal air spaces, surfactant is part of the local pulmonary host defense. Both innate immune functions, such as induction of respiratory burst, as well as adaptive tasks, are influenced by surfactant (as reviewed in Ref. 5). Macrophages as targets for surfactant were studied primarily in context with hydrophilic surfactant proteins SP-A and SP-D, which are C-type lectins (collectins) and mediate binding, agglutination, phagocytosis of pathogens, and

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**Abbreviations:** CSFE, 5-carboxyfluorescein diacetate succinimidyl ester; DAPI, 4',6-diamidino-2-phenylindol; DHPE, 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine; FSC, forward scatter; GFP, green fluorescent protein; MFI, mean fluorescence intensity; PBMCN, peripheral blood mononuclear cell; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, phosphatidylinositol; SP, surfactant protein; SSC, sideward scatter.

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production of reactive oxygen species, and inhibit T cell proliferation (as reviewed in Ref. 6). By contrast, the hydrophobic surfactant components (phospholipids and SP-B/C) were found to inhibit the respiratory burst of alveolar macrophages and the proliferative T cell responses after challenge with mitogens, allergenic cells, or antigens (5). Predominantly, mixtures of natural or synthetic PC, phosphatidylglycerol (PG), and phosphatidylethanolamine (PE) were used, and were shown to be of either inhibitory or stimulatory effect on proliferative lymphocyte responses, or absent an effect, depending on the concentration and composition of the phospholipid classes and their molecular species as well as on the experimental setup (7, 8). For instance, alterations in lipid composition caused by interstitial lung diseases, such as sarcoidosis, hypersensitivity pneumonitis, and idiopathic pulmonary fibrosis, led to changes in immunomodulatory properties of surfactants (9, 10).

Little is known about the effects on immune responses of surfactant phospholipid components preferentially secreted into the alveolar spaces and characteristically regulated during normal lung development, namely PC16:0/16:0 and PC16:0/14:0 (2–4, 11). The concentrations of these saturated components are regulated di-
ametrically in neonatal and adult mammalian lungs and comprise different biophysical behavior with respect to phase transition temperature and stability upon lateral compression of air-liquid interfaces (12). We therefore tested the hypothesis that PC16:0/16:0 and PC16:0/14:0 differentially influence the macrophage phenotype, its phagocytic capacity, and macrophage-mediated T cell proliferation in comparison to lipid extract surfactant (Curosurf) and the ubiquitous component PC16:0/18:1.

MATERIALS AND METHODS

Reagents

Therapeutic surfactant (Curosurf®) was provided by Nycomed (Unterschleißheim, Germany). PC16:0/16:0, PC16:0/14:0, and PC16:0/18:1 were from Avanti Polar Lipids (Abalaster, AL), Lissamine™ rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (Lissamine-DHPE) was from Invitrogen/Molecular Probes (Eugene, OR), and anti-CD3 monoclonal antibody (OKT3) was from Ortho Diagnostics (Raritan, NJ). Antibodies to CD3 (SK7), CD14 (macrophage P9), CD80 (L307.4), CD86 (2331 FUN-1), HLA-DR (L243), CD16 (NKP15), HLA-ABC (G56-2.6), CD83 (HB15e), CD206 (19.9) and Ig-matched controls (IgG1, IgG2) were from BD Biosciences (Heidelberg, Germany), CD86 (2331 FUN-1), HLA-DR (L243), CD16 (NKP15), and Ig-matched controls (IgG1, IgG2) were from BD Biosciences (Heidelberg, Germany), and TLR2 (TLR2.1) and TLR4 (HTA125) were from eBiosciences (San Diego, CA). 4,6-Diamidino-2-phenylindol (DAPI) was from Merck (Darmstadt, Germany). Propidium iodide was from Sigma (St. Louis, MO). Chloroform (HPLC grade) was from Baker (De-
venter, Netherlands), whereas methanol and water (both LiChrosolv® grade) were from Merck. All other materials were of analytical grade and were from various commercial sources.

Preparation of blood cells and purification of macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque (Biochrom AG; Berlin, Germany) density gradient sedimentation as previously described (13). Cells were placed at 2 × 10^6 cells/ml in flat-bottom 24-well cell culture plates (Costar; Bodenheim, Germany) in VLE-RPMI 1640 medium (Biochrom), containing 10% heat-inactivated fetal calf serum (Sigma). For mRNA detection, macrophages were further separated by negative selection using magnetic cell sorting (MACS) monocyte isolation kit II (Miltenyi Biotec; Bergisch Gladbach, Germany) according to the manufacturer’s instructions. The purity of the resulting population was >92% CD14-positive cells as detected by fluorescence activated cell sorting.

Preparation of PC species

PC16:0/16:0, PC16:0/14:0, and PC16:0/18:1 were extracted with chloroform-methanol according to Bligh and Dyer (14) in a sterile glass vial, and purity was checked by HPLC (2). Organic phases were evaporated under a stream of nitrogen. Dried materials were resuspended above their gel-sol phase transition temperatures (room temperature: PC16:0/14:0 and PC16:0/18:1; 45°C: PC16:0/16:0) in phosphate-buffered saline (PBS, Biochrom) by vigorous shaking for 3 min in the presence of sterile glass beads, followed by ultrasound homogenization. Phospholipid concentrations were checked (15), and the preparations diluted to give final concentrations of 11 μmol/ml in PBS. The phospholipid concentration was determined for Curosurf, and the material was adjusted to 11 μmol/ml. Curosurf comprised 37 ± 2% PC16:0/16:0, 6 ± 1% PC16:0/14:0, and 11 ± 1% PC16:0/18:1 relative to total phospholipids as described, while all other individual molecular species were below these values (16). Suspensions were aliquotted to 100 μl and stored at −70°C until use. Before use, surfactant and PC suspensions were warmed to 37°C and mixed for 1 min at 1,400 rpm in an Eppendorf Thermomixer comfort (Eppendorf, Germany). Substances were used at a final concentration of 1 μmol/ml in 1 ml culture medium.

Preparation of Lissamine-DHPE-labeled liposomes and assessment of interaction with macrophages

Lissamine-DHPE was purified as described above. The organic phase was mixed with Curosurf or PC species in a molar ratio of 5:95 (17) and dried under a stream of nitrogen. Lissamine-DHPE-labeled liposomes were analyzed by FACS. Size, as determined by forward scatter (FSC) and granularity (sideward scatter, SSC) of labeled liposomes, did not differ from corresponding unlabeled liposomes. The mean fluorescence intensity (MFI) was 202 ± 164, 374 ± 151, 822 ± 115, and 817 ± 138 above background for Curosurf, PC16:0/16:0, PC16:0/14:0, and PC16:0/18:1, respectively. Cell cultures were incubated in the presence of 1 μmol/ml Lissamine-DHPE-labeled liposomes of PC species or Curosurf for 48 h. To quantify liposome uptake, the MFI of Lissamine-DHPE-carrying macrophages was determined and adjusted to the MFI values of the respective Lissamine-DHPE-labeled liposomes. For confocal microscopy, 2 × 10^6 cells/ml were seeded onto coverslips and incubated with Lissamine-DHPE-labeled liposomes for 48 h. Cells were washed twice with PBS, stained with anti-CD14-phycocyanin or anti-CD3-phycocerythrin (BD Biosciences) and DAPI (Merck) or isotype-specific controls for 20 min in the dark, washed again, and mounted in Fluoprep mounting medium (bioMérieux; Marcy Etoile, France). Stained samples were analyzed with a Leica DM IRE 2 confocal laser-scanning microscope (Leica; Bensheim, Germany). Fluorescence images were acquired sequentially to avoid nonspecific channel interference. Images were digitally processed with Photoshop 7.0 (Adobe Systems, Mountain View, CA).
Phenotypic analysis

A FACSscan flow cytometer (BD Biosciences) calibrated daily was used to perform phenotypic analysis and to assay phagocytic activity and T cell proliferation. For phenotypic analysis, cells were washed out of the culture plates and separated from extracellular surfactant aggregates by gently placing them onto a Histopaque-PBS (1:2) cushion (Biochrom), followed by centrifugation at 400 g for 10 min. The resulting pellets were washed twice and resuspended in 100 µl PBS. The remaining cell suspension was free from extracellular surfactant particles as assessed by FSC versus SSC. To prevent nonspecific binding, cells were incubated with 10% human serum on ice for 10 min before staining with FITC- or phycoerythrin-labeled monoclonal antibodies or isotype-specific controls for 20 min over ice in the dark. Macrophages were gated by FSC versus SSC and CD14. Viability was analyzed by propidium iodide exclusion (5 µg/ml, 5 min; Sigma).

Quantitative real-time RT-PCR analysis

The detection of scavenger receptor mRNA levels was performed by SYBR green quantitative real-time RT-PCR analysis using the ABI Prism 7000 Sequence Detection System (Applied Biosystems; Foster City, CA) and qPCR Mastermix Plus (Eurogentec; Seraing, Belgium). Primers were designed to span intron-exon junctions in order to avoid genomic DNA amplification and were synthesized by MWG Biotech (Ebersberg, Germany). The following primers were used: CD36 forward 5'GAA AGT CAC TGC GAC ATG ATT AAT G3', CD36 reverse 5'ACT GCA ATA CCT GGC TGC TTT TCT CA3', CD68 forward 5'TGC TTC TCT CAT TCC CCT ATG G3', CD68 reverse 5'CCA TGT AGC TCA GGT CCA GAA ACG TGG AAG 3', SR-A forward 5'AAA TTT GAT CCT GGC TCA ATG AC3', SR-A reverse 5'GAC GAG GTA AAG GCC AAT 3', ALAS-1 forward 5'TCC ACT GCA GTA CAC TAC CA, and ALAS-1 reverse 5'ACG GAA GCT GTG TGC CAT CT3'.

Total RNA from 2–5 x 10⁶ isolated macrophages was extracted according to the manufacturer's instructions with the Nucleospin® RNA II-Kit (Macherey and Nagel; Düren, Germany). Then 0.5 µg of total RNA was reverse transcribed with 200 U Superscript RT II™ reverse transcriptase (Invitrogen Life Technologies; Karlsruhe, Germany) in the presence of 50 µM random hexamers (Amersham Biosciences; Freiburg, Germany), 400 µM dNTPs, and 1.6 U/ml RNAsIn™ (Invitrogen Life Technologies) in a final volume of 25 µl. Forty nanograms of the resulting cDNA were applied to the following qRT-PCR analyses (20 µl final volume) with 300 nM primers in 1 x qPCR Mastermix Plus (Eurogentec) and amplified with the standard temperature profile [2 min at 50°C, 10 min at 95°C, 40 x (15 s at 95°C, 1 min at 60°C)]. Relative quantification was performed employing the 2-ΔΔCT method. The results for target gene expression were normalized on ALAS-1 as endogenous control, and the untreated cell population was used as calibrator. Mean values ± standard deviation of three independent experiments (for Curosurf, n = 2) are shown.

Bacterial culture

Escherichia coli DH5α, carrying the green fluorescent protein (GFP)-mut2 encoding plasmid pCD355 (E. coli-GFP), which expresses a prokaryotic variant of GFP under the control of a lactac promotor (18), was freshly grown on agar plates supplemented with kanamycin (50 µg/ml; Sigma) and isopropyl-β-D-thiogalactopyranoside (1 mmol/l; Sigma) for GFP induction. After 24 h, a single colony was picked and grown in Lennox I Broth medium (Invitrogen Life Technologies) until early logarithmic growth phase (optical density, OD₆₀₀ = 0.4–0.5). Bacteria were washed, resuspended in PBS, and used immediately.

Phagocytosis assay for bacteria

Cell cultures were inoculated with 1 x 10⁸ E. coli-GFP and suspended in 20 µl PBS to achieve a multiplicity of infection of 1:50 at 37°C in 5% CO₂ for 45 min as previously described (19). Cells were washed, laid over a Histopaque-PBS (1:2) cushion (Biochrom), and centrifuged for 10 min at 400 g at 4°C. The cell pellet did not contain free bacteria or extracellular surfactant aggregates as detected by flow cytometry. Cells were fixed in 2% paraformaldehyde (Sigma) for 10 min at room temperature, washed, and stained. Phagocytosis index (CD14+ GFP+ macrophages:CD14+ macrophages) and phagocytic capacity (MFI on CD14+ macrophages) were analyzed with Cellquest 3.3 software for Apple Macintosh (BD Biosciences). One representative experiment is shown in Fig. 3A. To estimate extracellular binding of bacteria, actin-dependent phagocytosis was blocked by cytochalasin D (Sigma; 10 µg/ml, 30 min), resulting in a decrease of phagocytosis index to less than 5% in all groups (data not shown). In some experiments, polyclonal IgG (Polyglobin 10%; Bayer, Leverkusen, Germany) was added to a final concentration of 1 mg/ml to cell cultures simultaneously with bacteria.

T cell proliferation assay

T cell proliferation was assessed by Vybrant CFDA SE Cell Tracer Kit (Molecular Probes, Eugene, OR). In brief, 5-carboxyfluorescein diacetate succinimidyl ester (CFSE) diffuses into cytoplasm and, cleaved by esterases, becomes fluorescent and membrane-impermeable, and is thus trapped intracellularly. During cell division, CFSE is transmitted to filial cells in equal parts, exhibiting lower fluorescence. Staining was performed according to the manufacturer's protocol. Briefly, to prepare CFSE staining solution, component B was dissolved in 90 µl component A and diluted with PBS to 1.6 µmol/l. CFSE staining solution (500 µl) was mixed with 2.5 x 10⁷ PBMCN (ratio monocytes:T cells about 1:3) to a final volume of 1.5 ml and incubated for 30 min at 37°C. Staining reaction was stopped by adding 4 ml fetal calf serum (Sigma). After 2 min at room temperature, cells were washed and seeded in 24-well plates at a final concentration of 2 x 10⁶ cells/ml. Stained cells were pre-incubated with surfactant components for 24 h before OKT3 (5 µg/ml) was added for another 48 h. Proliferating T cells were analyzed in histogram plots (Fig. 4A) with the help of Cellquest 3.3 software for Apple Macintosh (BD Biosciences).

Data display and statistical analysis

Results are expressed as mean ± SD. MFIs were determined, and nonspecific background staining was subtracted. Statistical analysis was performed using the decadic logarithm of the values of CD14, CD80, CD86, HLA-DR, TLR2, TLR4, HLA-ABC, CD16, GFP, and CFSE for a Student's t-test (Sigmaplott 2000 software for Windows; SPSS, Chicago, IL). Values of P < 0.05 (adjusted according to Bonferroni-Holm for multiple group comparisons) were considered significant. Comparisons between means of phagocytosis index across levels of Curosurf, PC16:0/18:1, and immunoglobulin. Dunnett's test was used for pairwise posthoc analysis using experiments without surfactant as reference. Analyses were done with statistical software (Statistical Package for the Social Sciences, release 12.0 for Windows; SPSS). When not otherwise stated, cell culture experiments were repeated at least three times, and numbers are indicated in the results section.
RESULTS

Effect of Curosurf and PC species on macrophage phenotype

Macrophages were characterized phenotypically after 48 h (n = 8). One representative experiment for CD14, HLA-DR, and CD80 is shown in Fig. 1A. Untreated macrophages expressed CD14 (239 ± 104 MFI), CD206 (48 ± 9 MFI), HLA-DR (116 ± 89 MFI), TLR2 (17 ± 3 MFI), and TLR4 (14 ± 3 MFI). CD14 macrophages (28 ± 10%) expressed CD80 (Fig. 1B–G).

Curosurf, PC16:0/16:0, and PC16:0/14:0 downregulated CD14 expression by 49, 40, and 57%, respectively (Fig. 1B; all P < 0.05 vs. control). Mannose receptor CD206 was upregulated by 72, 168, and 80%, respectively (all P < 0.05 vs. control; Fig. 1C). Ubiquitous PC16:0/
18:1, which is present but not specifically enriched in mammalian surfactant, showed no effect on CD14 or CD206.

Curosurf upregulated HLA-DR on macrophages by 97% (Fig. 1D; P < 0.05) and the percentage of CD80 expressing macrophages by 93% (Fig. 1E, P < 0.05). PC16:0/14:0 upregulated HLA-DR by 188% and increased the percentage of CD80 expressing macrophages by 65% (both P < 0.05 vs. control). By contrast, PC16:0/16:0 did not influence HLA-DR expression (P = 0.52 vs. control) or the percentage of CD80-positive macrophages (P = 0.27 vs. control; Fig. 1D, E), whereas TLR2 and TLR4 expression was upregulated by 130% and 98%, respectively (both P < 0.05 vs. control; Fig. 1F, G). In contrast to their effects on HLA-DR and CD80, Curosurf and PC16:0/14:0 did not affect TLR2 or TLR4 expression. Again, PC16:0/18:1 had no effect on either HLA-DR, CD80, TLR2, or TLR4 expression. These phenotypic changes were concentration dependent for the substances tested, with decreased or no effect at 0.1 μmol/ml or 0.01 μmol/ml, respectively.

HLA-DR and CD80 upregulation and CD14 down-regulation upon Curosurf challenge was also seen on macrophages preincubated for 48 h without surfactant followed by exposure to surfactant for a subsequent 48 h. A second challenge with Curosurf for another 48 h after 48 h of incubation showed no additional effect with respect to the above-mentioned receptors (data not shown).

The percentage of CD16+/CD14+ macrophages was reduced by PC16:0/16:0 (21 ± 5% vs. 39 ± 10%; P < 0.05 vs. control), whereas the other substances had no effect. Macrophage survival, as detected by propidium iodide, and cell size, as well as receptor densities of CD83, CD86, and HLA-ABC were not affected by any substance tested (data not shown).

Interaction of Curosurf and PC species with macrophages

PBMNCs were incubated with Lissamine-DHPE-labeled Curosurf or PC species at 37°C or 4°C for 48 h and analyzed by confocal microscopy. At 37°C, liposomes were localized intracellularly only in macrophages; CD3-expressing T cells did not take up liposomes (Fig. 2A).

FACS analysis revealed no difference in the uptake of labeled PC species, whereas Curosurf was better internalized (P < 0.05 vs. PC species; Fig. 2B). This process was time and concentration dependent, starting after 1 h (data not shown). Incubation at 4°C showed nearly no liposome uptake (Fig. 2B), making passive diffusion or attachment rather unlikely. Lissamine-DHPE-labeled Curosurf and PC species induced the same phenotypic changes with regard to HLA-DR, CD80, and CD14 expression as seen with unlabeled liposomes (data not shown).

Effect of Curosurf and PC species on phagocytic activity of macrophages

PBMNCs were incubated for 48 h with Curosurf or PC species prior to challenge with E. coli-GFP. One representative experiment for PC16:0/14:0 is shown in Fig. 3A. Curosurf diminished the phagocytosis index from 51 ± 13% to 29 ± 12% (P < 0.05; Fig. 3B). PC16:0/16:0 and PC16:0/14:0 nearly abrogated phagocytosis (11 ± 9% and 17 ± 7%; P < 0.005 of control values), whereas PC16:0/18:1 exhibited no effect. In the same experiments, mean bacterial load (phagocytic capacity) of phagocytosing macrophages was assessed. Here PC16:0/16:0 as well as PC16:0/14:0 decreased the phagocytic capacity by 44% and 45%, respectively (P < 0.05 vs. control), whereas Curosurf or PC16:0/18:1 had no effect (Fig. 3D).

Separate experiments were performed by adding substances directly prior to bacterial exposure. Here PC16:0/16:0 and PC16:0/14:0 reduced the phagocytosis index to 18 ± 11% and 13 ± 6% vs. 45 ± 7% for the control (both P < 0.05; Fig. 3C), and phagocytic capacity by 45% and 48% (both P < 0.05 vs. control; Fig. 3E), whereas Curosurf had no effect. In this setting, PC16:0/18:1 also reduced the phagocytosis index (21 ± 8%), whereas phagocytosis capacity again was unaffected. Removing Curosurf or PC species by centrifugation before the addition of bacteria did not influence the results (data not shown). All these inhibitory effects on phagocytosis exerted by surfactant or individual PC species were blunted, however, by opsonization of bacteria with immunoglobulin (Fig. 3F).

Effect of Curosurf and PC species on scavenger receptor mRNA expression

Incubation of purified macrophages with either Curosurf or PC species for 48 h resulted in upregulation of mRNA levels of scavenger receptors CD36, CD68, SR-A, and LOX-1 (Fig. 4). The most pronounced effects were seen for SR-A: Curosurf and all PC species tested led to an induction of mRNA transcription by more than 3-fold. Curosurf induced increased CD36 and CD68 mRNA levels (3.47-fold and 2.75-fold, respectively). PC species led to an upregulation of less than 2-fold.

Effect of Curosurf and PC species on macrophage-dependent T cell proliferation

Macrophage-dependent T cell proliferation was analyzed after stimulation with αCD3 monoclonal antibody (MAb) for 48 h. One representative experiment for the effect of PC16:0/14:0 is depicted in Fig. 5A. We found no proliferation of T cells in the absence of either anti-CD3 MAb or macrophages (Fig. 5A). In the absence of Curosurf and PC species, 68 ± 13% of T cells in coculture readily proliferated, with at least two filial generations. Preincubation with Curosurf or PC16:0/14:0 for 24 h decreased αCD3-mediated T cell proliferation to 40 ± 16% and 49 ± 9%, respectively (each P < 0.05 vs. αCD3 MAb only), with only one filial generation detectable (Fig. 5A, B). By contrast, neither PC16:0/16:0 nor PC16:0/18:1 inhibited T cell proliferation. Viability of lymphocytes was not affected, as determined by propidium iodide staining.

DISCUSSION

Surfactant comprises many individual phospholipid components, together with neutral lipids and specific proteins SP-A to -D. Recent data suggest that along with the classical component PC16:0/16:0, which is rigid at body
temperature, other fluidic PC species with short fatty acyl chains are effectively released into the air spaces of mammalian lungs (2, 3). Among these, disaturated PC16:0/14:0 raised clinical interest, because its concentrations increase during alveolar development, and are specifically decreased in inflammatory lung diseases affecting alveolar development or homoeostasis like bronchopulmonary dysplasia and emphysema (2, 3). It is principally absent from surfactant in nonalveolar bird lungs; its specific functions in the mammalian surfactant complex are still hypothetical and, so far, related to alveolar curvature and air-liquid interface dynamics (3, 11). Our results support the view that PC16:0/14:0 exerts additional functions that connect the lipidomic features of surfactant with the regulation of immune functions of terminal lung tissue.

Our results show that blood monocyte-derived macrophages are targets for animal-derived lipid extract surfactant, the most widely used preparation for treatment of neonatal respiratory distress syndrome, and that these cells are differentially influenced by their two principle disaturated PC components of surfactant, namely PC16:0/16:0 and PC16:0/14:0, in phenotype (Fig. 1) as well as in functions of nonspecific and specific immunity (Figs. 3–5). Although the concept of macrophages as targets of surfac-

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**Fig. 2.** Uptake of Lissamine-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (Lissamine-DHPE)-labeled liposomes by macrophages. PBMCs were incubated for 48 h with Lissamine-DHPE-labeled liposomes (1 μmol/ml) at 37°C or 4°C as control. A: A representative image of confocal laser scanning microscopy from Lissamine-DHPE-labeled PC16:0/14:0 incubated PBMCs. Green, anti-CD14-FITC; red, Lissamine-DHPE-labeled PC16:0/14:0; blue, 4',6-diamidino-2-phenylindol. B shows MFI of CD14+ cells incubated as indicated (n = 3); *, P < 0.05 versus Curosurf. Figures give the arithmetic mean, error bars indicate the standard deviation.
tant components is well known, investigation of these PC species, which are subject to changes during lung development (2, 3), is new. Moreover, the use of peripheral blood monocyte-derived macrophages contrasts to other studies on macrophages from lung lavage fluid: besides limited access to the latter, our experimental system offers the advantage of studying “surfactant-naive” cells that are not yet primed within the alveolar environment, and corresponds to inflammatory processes in vivo, when the number of resident alveolar macrophages is reduced and blood monocytes are recruited to the alveolar space (20). Although for physiological conditions, macrophages in the alveolar spaces are subject to regulation by the whole phospholipoprotein complex of surfactant, the isolated action of individual phospholipid molecular species is important to define their role in the whole environmental scenario, and for clinical situations in which patients are only treated with the hydrophobic components of surfactant, namely the phospholipids and SP-B and -C or their synthetic analogs.

Our data provide evidence for a maturating effect of Curosurf and both surfactant-specific PC species for monocytes with regard to the receptor pattern of CD14, CD206, and HLA-DR (Fig. 1B–D), which is found predominantly in more mature macrophages (21). To test whether the substances tested influence the global macrophage maturation and differentiation program, further experiments are needed. Differentiation into a dendritic cell after 72 h is unlikely, however, because CD83 expression remained negligible (22). This would make sense in the context of monocyte differentiation to alveolar macrophages by surfactant components within the alveolar compartment, instead of triggering differentiation to interstitial dendritic cells.

Phenotypic effects could be specific for PC species preferentially incorporated into surfactant, because PC16:0/18:1, a ubiquitous PC species predominantly found in cell membranes and retained in tissue rather than being secreted into the alveolar space (1, 2), did not induce similar phenotypic changes (Fig. 1). Downregulation of CD14 expression after incubation with lipid extract surfactant already had been described, using the monocytic cell line THP-1 (23). Our phenotypic findings (Fig. 1) were also seen on 48-h-preincubated macrophages, which were more differentiated prior to surfactant challenge. With regard to the physiological situation in the alveolar environment, this could mean that surfactant lipids may have an impact on the phenotype of both monocytes migrated directly from the bloodstream into the alveolus and resident interstitial macrophages encountering the alveolus, e.g., during inflammation. Effects on macrophage phenotype could not be boosted by a second surfactant challenge. This is in line with findings by Kramer, Jobe, and Ikegami (24), who saw no effect of exogenous surfactant on resident alveolar macrophages of surfactant-treated mice, cells which are principally not “surfactant-naive.”

Further analysis of surface receptors important for innate (CD14, TLR2, TLR4) and adaptive (HLA-DR, CD80) recognition.

Fig. 3. Effect of surfactant preparations and lipid components on the phagocytosis of E. coli-green fluorescent protein (GFP) by macrophages. A: One representative experiment is depicted, in which PBMNCs were cultured for 48 h in the absence (upper graphs) or presence (lower graphs) of PC16:0/14:0 and exposed to E. coli-GFP for 45 min. Ratio of CD14+ GFP+ to CD14+ macrophages and mean GFP fluorescence intensities are depicted. Macrophages were preincubated for 48 h in the presence (B, D; n = 8) or absence (C, E; n = 8) of substances (1 μmol/ml) indicated and either challenged with bacteria only (B, D) or with bacteria and substances simultaneously (C, E) for 45 min. Cells were harvested and phagocytosis index (B, C) and phagocytic capacity (D, E) were determined. F: Phagocytic index of macrophages incubated with substances for 48 h and then challenged with opsonized bacteria (gray bars) and nonopsonized bacteria (open bars). Data given are mean ± SD. §, P < 0.05 versus bacteria and substances simultaneously; §§, P < 0.005 versus bacteria and substances simultaneously; *, P < 0.05 versus cibitrik, ** P < 0.005 versus control; #, versus bacteria with immunoglobulins.
immune functions revealed both groups to be influenced differently by surfactant-specific PC species. Curosurf promoted the development of macrophages into an HLA-DR\textsuperscript{high} CD80\textsuperscript{+} macrophage phenotype (Fig. 1D, E). This effect was mimicked by PC16:0/14:0, whereas there was no effect on TLR2 and TLR4. In contrast, specifically PC16:0/16:0 upregulated TLR2 and TLR4, whereas HLA-DR and CD80 expression remained unchanged (Fig. 1F, G). Our experiments suggest that differential effects on macrophages are not due to different internalization of PC species (Fig. 2A, B). Although PC species were internalized in equal amounts, Curosurf showed a 10-fold higher ingestion. However, although the effects of PC16:0/14:0 were concentration dependent and mostly required an uptake during 48 h preincubation, the amount of PC16:0/14:0 taken up into macrophages from Curosurf approximated that of the isolated compound given as liposomes. The principle difference in uptake between Curosurf and PC species might be due to lipophilic SP-B and SP-C, anionic PGs, and neutral lipids being present in animal-derived surfactants (17). However, our data demonstrate that internalization of either compound given as liposomes is an active process, because it was blunted at 4°C (Fig. 2B) and by cytochalasin D.

We assessed phagocytic activity as an innate immune function, for which TLR2 and TLR4 (25) were discussed to be relevant. Although both TLR2 and TLR4 were upregulated by PC16:0/16:0 (Fig. 1F, G), no such effect was seen with either Curosurf or any other PC species, whereas phagocytosis of \textit{E. coli}-GFP was diminished by either saturated PC species and by Curosurf (Fig. 3). Although the latter only reduced the percentage of phagocytosing macrophages (Fig. 3A), PC species also diminished the number of ingested bacteria per macrophage (Fig. 3B) and inhibited phagocytosis when given simultaneously with bacteria, whereas Curosurf in that case had no effect (Fig. 3D, E).

It is conceivable that mixtures of surfactant lipids with surfactant proteins B and C exert more complex effects that are contrary to those of individual PC components, as previously shown for the antagonisms between whole phospholipids and surfactant proteins A and D (6). Hence, our data demonstrate that therapeutic surfactants, particularly those without a physiological phospholipid pattern, absent PC16:0/14:0 or based simply on PC16:0/16:0 with or without hydrophobic SPs, may exert effects on immunological parameters differing from those of natural or lipid extract surfactant.

Data on surfactant lipids and phagocytic activity of macrophages are conflicting. In an experimental setup with lyophilized and FITC-coated \textit{E. coli}, Ding et al. (26) found that Survanta, a surfactant preparation from bovine lungs enriched with PC16:0/16:0, and therefore reduced concentrations in PC16:0/14:0 and impaired surface tension function in vitro, (16) did not affect the phagocytic capacity of the immature monocytic cell line THP-1. These data, however, are not comparable, because we used viable \textit{E. coli}, whose surface was unaffected by the labeling. Morito et al. (27) found a decreased phagocytic activity of alveolar macrophages for Fc receptor-mediated phagocytosis, as determined by IgG-coated erythrocytes after treatment with PC16:0/16:0. Downregulation of CD14 (Fig. 1) may inhibit the uptake of gram-negative bacteria (28). We showed that inhibition of phagocytosis by surfactant and PC species (Fig. 3) was prevented by opsonizing bacteria with polyvalent immunoglobulins (Fig. 3F). Therefore, our data suggest that bacterial uptake via Fc receptors (29) is not likely to be compromised by surfactant or PC species. Because brief incubation with PC species (Fig. 3C, E) exerted limited inhibition of phagocytosis...
Macrophage differentiation by phospholipids

Fig. 5. Effect of surfactant preparations and lipid components on macrophage-dependent T cell proliferation assayed by loss of 5-carboxyfluorescein diacetate succinimidyl ester (CFSE) stain. PBMNCs were loaded with CFSE, incubated for 24 h in the presence or absence of the substances indicated (1 μmol/ml), stimulated with anti-CD3 MAb (5 μg/ml) for 48 h, harvested, and assayed for CFSE fluorescence intensity. A: One representative experiment is depicted as a histogram, which shows CFSE fluorescence intensity in the absence of substances (gray-filled graph) or presence of PC16:0-14:0 (black line). Dotted line shows proliferation in the absence of macrophages. M1 are the proliferating T cells. B: Ratio of proliferating T cells to total T cells was calculated (n = 8).

as well, part of the effect might be due to competition between bacteria and liposomes. While in the alveolar spaces of healthy mature lungs, immunoglobulin levels are low, SP-A and -D as C-type lectins might take charge of bacterial opsonization instead, and blunt inhibitory effects exerted by phospholipids. Hence, lipid effects on phagocytosis may be particularly relevant under conditions of low (immature lungs of preterm infants), absent (knocked-out), or nonfunctioning (mutated) SP-A or -D.

To further characterize the effect on the “phagocytic synapse” (30) after incubation with Curosurf or PC species, we analyzed mRNA expression of members of the scavenger receptor family, namely CD36, CD68, SR-A, and LOX-1 (Fig. 4). This group was originally defined by the ability to bind and internalize lipoproteins (30). Moreover, SR-A (31) and CD36 (32) were shown to be involved in binding and internalization of E. coli or Staphylococcus aureus; the latter also in the uptake of anionic phospholipids like PS, phosphatidylinositol (PI), and oxidized PC (33, 34). SR-A-negative mice and human macrophages, differentiated for 7 days and blocked by a general SR inhibitor, were impaired to phagocyte paraformaldehyde-fixed E. coli (35). Our model was different, using undifferentiated human macrophages cultured for 48 h and challenged with viable GFP-labeled E. coli. On a transcriptional level, we found a more than 2-fold increase of SR-A mRNA for Curosurf and all PC species tested; for CD36 and CD68, only Curosurf had this effect (Fig. 4). Despite the above-described mRNA upregulation, we found a diminished bacterial uptake by Curosurf, PC16:0-16:0, and PC16:0-14:0. PC16:0-18:1 did not affect bacterial phagocytosis (Fig. 3B, D). Whether this suggests that SR-A in phagocytosis of GFP-labeled E. coli is of minor relevance or that the reduced phagocytic activity may be due to the competitive utilization of scavenger receptors by PC species cannot be answered by these experiments.

We assessed macrophage-dependent T cell proliferation as an adaptive immune function. In our model, the latter depends upon the amount and activation status of professional antigen-presenting cells, e.g., macrophages (36, 37). T cell proliferation was diminished by Curosurf and PC16:0-14:0, whereas PC16:0-16:0 and PC16:0-18:1 did not affect bacterial phagocytosis (Fig. 5B). The inhibitory effect of Curosurf and PC16:0-14:0 could not be explained by injurious effects of lipid extract surfactants or PC species on T cell vitality. As shown previously (37), T cell proliferation was macrophage dependent, because their depletion resulted in abolished proliferation (Fig. 4A). To efficiently fulfill their broad spectrum of tasks, blood monocyte-derived macrophages differentiate into subpopulations (as reviewed in Ref. 29). We have previously characterized macrophage subsets with overlapping but distinct phenotypes and functions. One, referred to as helper macrophages (38), is characterized by high expression of HLA-DR and costimulatory molecules CD80 and CD86, which facilitate T cell stimulation (39, 40). The other, referred to as cytotoxic macrophages (38), lacks expression of CD80 and CD86 but expresses the Fc-gamma III receptor (CD16) in high density and acts as a negative immune regulator (41). In light of this, our results suggest that lipid extract surfactants, and in particular their component PC16:0-14:0, influence costimulatory receptors on macrophages. Surprisingly, although phenotypic data on Curosurf- and PC16:0-14:0-incubated cells hint at an Mh subtype, T cell proliferation was diminished by these components. It is not yet clear whether these results are a consequence of direct lipid binding to T cell plasma membranes, inasmuch as T cells do not ingest Curosurf or PC components (Fig. 2), or are instead based on a change of balance between CD80 and CD86 on the macrophage surface. Although both CD80 and CD86 can act as costimulators, evidence exists that CD80 (more than CD86) is also a ligand for inhibitory receptors on T cells, like CD152, and may negatively influence T cell proliferation (42).
This effect was shown to be mediated by reverse signaling into the macrophage and induction of indoleamine 2,3-dioxygenase, which suppresses T cell proliferation (43). Because CD80 but not CD86 expression was enhanced by Curosurf and PC16:0/14:0, phenotypic changes of macrophages may lead to an inhibitory signal for T cells, irrespective of its promoting effects on monocyte differentiation to HLA-DR\textsuperscript{high} macrophage. Preliminary data on corresponding receptors for CD80 on T cells show that CD28 expression is downregulated upon exposure to lipid extract surfactants, which might explain reduced T cell proliferation (unpublished observations).

The underlying mechanisms of surfactant lipids on macrophage phenotype (Fig. 1), macrophage phagocytic activity (Fig. 3), and macrophage-dependent T cell proliferation (Fig. 5) are unclear, as are the biochemical properties that account for their differential effects. One difference between PC16:0/16:0 and PC16:0/14:0 is their phase transition temperature: whereas the former is rigid at physiological conditions (37°C), the latter is fluidic (12). However, this is also true for PC16:0/18:1, suggesting that such biophysical characteristics alone are less likely to explain the specific actions of PC16:0/14:0 on macrophage phenotype or function. Instead, geometrical properties (short, straight acyl chain for 14:0 vs. long, angled structure for 18:1) together with low phase transition temperature may have substantial impact on the interaction of PC16:0/14:0 with membrane structures and the imbedded proteins of macrophages and T cells and for their regulation. While this may specifically condition the alveolar macrophage to its environmental functions, surfactant from lungs with no alveolar but only interstitial macrophages, like those from birds, does not possess PC16:0/14:0, whereas the latter comprises up to 20% of surfactant PC, at the expense of PC16:0/16:0, during alveolar formation in rats, and is also increased in neonatal pigs and humans (2, 4, 44). This supports the view that PC16:0/14:0 and its balance with PC16:0/16:0 are important for the differentiation and function of macrophages in mammalian lungs, particularly during alveolar formation (2–4, 11).

Phospholipids with different headgroups differentially influence T cell proliferation, with PE and sphingomyelin augmenting and PC, PG, and PI suppressing T cell proliferation, as detected in an experimental setup with phytohemagglutinin-stimulated T cells (7). In contrast to our system, PC16:0/16:0 suppressed T cell proliferation at a concentration comparable to that used in our experiments (7). This may indicate that the impact of phospholipids on T cell proliferation in vitro is dependent on the stimulus used. Moreover, although PC possesses a zwitterionic and PG and PI an anionic headgroup, our data on differential effects of PC molecular species underscore the relative importance of fatty acyl chain composition compared with the charge of the headgroup.

In conclusion, surfactant phospholipid molecular species differentially influence macrophage phenotype and function, and this may be important for the design of synthetic therapeutic surfactants. In particular, the effects of surfactants like those on HLA-DR and CD80 expression of macrophages and inhibition of T cell proliferation can be mimicked by PC16:0/14:0, but not by other components. On the contrary, other effects, such as those of pure PC16:0/16:0 on TLR2 and TLR4 expression, are not exerted by whole surfactant or other components, suggesting differential and both physiological and artificial effects on macrophages, depending on the lipidomic profile of therapeutic surfactants. The differential effect of individual PC species on macrophage phenotype and macrophage-dependent T cell proliferation may be of importance because PC16:0/16:0 and PC16:0/14:0 are selectively secreted into terminal air spaces and are modulated diametrically during ante- and postnatal changes in surfactant composition (2, 3). In view of this, our in vitro results suggest that inflammatory reactions may be modulated differently in the terminal air space of preterm infants compared with term neonates and adults because of changes in PC16:0/14:0 content of surfactant. Further investigation of the impact of the lipidomic profile of surfactants will be necessary, particularly with regard to their role in macrophage-triggered inflammatory lung reactions.\textsuperscript{AP}

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