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Pulmonary surfactant inhibits activation of human monocytes by recombinant interferon-γ

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SUMMARY

During an inflammatory reaction in the alveoli, the functional activities of monocytes, macrophages and granulocytes are regulated by a complex network of inflammatory mediators. The primary cytokine involved in activation of these phagocytes is interferon-γ (IFN-γ). The possible influence of local factors, such as pulmonary surfactant, on the activation process has not been studied until now. The aim of the present study was to investigate the effects of surfactant on the activation of monocytes by recombinant (r)IFN-γ. The results revealed that human surfactant significantly inhibited both the increase in the expression of the high-affinity receptor for IgG, i.e. FcγRI, and the production of H_2O_2 by rIFN-γ-activated monocytes. Since our surfactant preparation stimulated the basal production of tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) by monocytes, the effect of Survanta®, a surfactant extract, on the rIFN-γ-induced production of these cytokines by monocytes was studied. The results revealed that Survanta® caused 80–90% inhibition of the rIFN-γ-induced production of TNF-α and IL-1β by these cells. Together, these results could mean that surfactant is involved in the protection of the alveolar epithelium against injury caused by reactive oxygen intermediates (ROI) and TNF-α, and in the down-regulation of the production of inflammatory mediators. In view of these considerations, surfactant therapy may not only improve lung compliance and gas exchange but may also be beneficial in reducing the inflammatory reaction in the lungs.

INTRODUCTION

Interposed between the host and its potential hostile environment, the alveoli constitute two essential functions, i.e. support of gas exchange and mediation of host defence. An efficacious response to various inflammatory stimuli is critical for host survival, yet the structural integrity of the alveoli must be maintained for optimal gas exchange. The alveoli are lined with surfactant, the main function of which is to reduce surface tension.[1] In addition, surfactant can impair various functions of alveolar and peritoneal macrophages and monocytes, including intracellular killing of micro-organisms,[2–5] production of reactive oxygen intermediates (ROI),[2,6] expression of complement and Fc receptors,[7] and production and release of cytokines.[8,9] Furthermore, surfactant inhibits the proliferation of lymphocytes.[10,11]

During inflammation, the functional activities of phagocytes, i.e. granulocytes, monocytes and macrophages, are regulated by a complex network of cytokines comprising proinflammatory cytokines, e.g. tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1), and anti-inflammatory cytokines, e.g. IL-4, transforming growth factor-β (TGF-β) and IL-10.[12–16] Interferon-γ (IFN-γ), produced by $CD4^+$ and $CD8^+$ T lymphocytes[17] and natural killer (NK) cells,[18] is the primary cytokine involved in the activation of these phagocytes (reviewed in ref. 19). Monocytes incubated *in vitro* with recombinant (r)IFN-γ become activated for various functional activities, i.e. intracellular killing of micro-organisms,[20] suppression of the intracellular proliferation of protozoa,[21] production of ROI,[20–22] release of cytokines such as TNF-α,[23] and IL-1,[24] and antibody-dependent cell-mediated cytotoxicity.[25] Phenotypically, activation of monocytes by IFN-γ is characterized by enhanced surface expression of the high-affinity receptor for IgG, i.e. FcγRI (CD64)[26,27] and major histocompatibility complex (MHC) class II antigens.[25]

Many factors, including cytokines,[16,21,22,28,29] eicosanoids such as prostaglandin E_2,[29,30] and glucocorticosteroids,[29,31] can inhibit the activation of monocytes and macrophages by rIFN-γ. However, the possible influence of local factors, such

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Abbreviations: AU, arbitrary units; BAL, bronchoalveolar lavage; FcγRI, high-affinity receptor for IgG; Hydron, poly(2-hydroxyethyl-methacrylate); IL-1β, interleukin-1β; MFI, mean fluorescence intensity; mAb, monoclonal antibody; PE–GAM, phycoerythrin-conjugated goat anti-mouse Ig; ROI, reactive oxygen intermediates; TNF-α, tumour necrosis factor-α.

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as pulmonary surfactant, on the activation process has not been studied until now. The aim of the present study was to investigate the effects of pulmonary surfactant on the activation of monocytes by rIFN-γ.

MATERIALS AND METHODS

Stimuli

Recombinant IFN-γ was a generous gift from Dr P.H. van der Meide (Department of Virology, TNO, Rijswijk, the Netherlands). Lipopolysaccharide (LPS) derived from *Salmonella typhimurium* was purchased from Sigma Chemical Company (St Louis, MO).

Antibodies

The hybridoma cell line producing monoclonal antibody (mAb) 32·2 (anti-human FcγRI, murine IgG1) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Monoclonal antibody PdV 5·2 (anti-human MHC class II antigens, murine IgG2) was a kind gift from Dr F. Koning (Department of Immunohematology and Bloodbank, University Hospital, Leiden, the Netherlands). Phycoerythrin-conjugated goat anti-mouse Ig (PE-GAM) was purchased from Southern Biotechnology Associates Inc. (Birmingham, AL).

Surfactant

Human surfactant was prepared from bronchoalveolar lavage (BAL) fluid derived from lung segments with no underlying pathological condition, collected after informed consent from patients who underwent BAL for diagnostic reasons. BAL fluid was centrifuged at 750 *g* for 20 min to remove cells and debris; the supernatant was collected and centrifuged at 21,000 *g* for 2 hr, and the resulting pellet was resuspended in a small volume of saline and stored at −20°. The concentration of surfactant was expressed in mM organic phosphate, which was determined according to the method of Bartlett.[32] Since surfactant consists of almost 90% phospholipids, the concentration of phospholipids is representative of the concentration of surfactant. The surfactant preparations contained approximately 1–5 ng LPS/mM phospholipid, as quantified by Limulus assay. Survanta®, a kind gift of Abbott NV Diagnostics (Amstelveen, the Netherlands), is an organic solvent extract of minced bovine lungs comprising phospholipids, neutral lipids, fatty acids, and surfactant protein B (SP-B) and SP-C, to which dipalmitoylphosphatidylcholine (DPPC) is added to mimic the surface tension-lowering properties of natural lung surfactant.[33] Survanta® contained less than 5 pg LPS/mM phospholipid.

Monocytes

Buffy coats from blood of healthy donors were diluted in phospate-buffered saline PBS and subjected to differential centrifugation at 400 *g* for 20 min on a Ficoll–Amidotrizoate gradient (ρ = 1·077 g/ml; Pharmacia, Uppsala, Sweden).[34] Cells of the monocyte–lymphocyte layer were washed four times with PBS containing 0·5 U heparin/ml (PBS–hep). Differential analysis of Giemsa-stained cytocentrifuge preparations of these cells revealed that this preparation consisted of about 32% monocytes, 66% lymphocytes and fewer than 2% granulocytes. Viability of the monocytes was more than 95%, as determined by trypan blue exclusion.

Incubation of monocytes with rIFN-γ

Monocytes (2×10^6/ml) were suspended in RPMI-1640 (Flow Laboratories, Rockville, MD) supplemented with 100 U penicillin G/ml, 50 μg streptomycin/ml and 10% heat-inactivated newborn calf serum (NBCS) (culture medium). This medium contained about 300 pg LPS/ml, as quantified by Limulus assay. Next, the cells were incubated in the presence of rIFN-γ or saline, the diluent of rIFN-γ, for 18 hr at 37° in 24-well tissue culture plates (Costar, Cambridge, U.K.) coated with 2·4% poly(2-hydroxyethyl-methacrylate) (Hydron; Interferon Sciences Inc., New Brunswick, N.J.) to prevent adherence of the cells[35,36] Viability of monocytes incubated with rIFN-γ exceeded 90%, as measured by trypan blue exclusion.

Incubation of monocytes with surfactant

Monocytes (2×10^6/ml) were suspended in culture medium and incubated in the presence of various concentrations of surfactant for 18 hr at 37° in 24-well tissue culture plates coated with Hydron. Viability of monocytes incubated with surfactant exceeded 90%, as determined by trypan blue exclusion.

Incubation of adherent monocytes for determination of TNF-α and IL-1β release

Monocytes (1×10^6/ml) were suspended in culture medium that contained about 80 pg LPS/ml (LPS-low culture medium). Monocytes were allowed to adhere to non-coated 24-well tissue culture plates for 1·5 hr at 37°, the non-adherent cells removed by washing, and the adherent cells incubated in LPS-low culture medium in the presence of stimuli, with or without 1 mM Survanta®, for 18 hr at 37°. After incubation, viability of the cells exceeded 90%. Next, the suspension was centrifuged for 10 min at 1500 *g* and the resulting supernatant collected and filtered (Millipore, Costar, Cambridge, MA) to remove cells and debris. The amount of TNF-α and IL-1β in the supernatants was quantified by ELISA, according to the manufacturers' instructions (Amersham, Amersham, U.K.). Spiking of control supernatants with 1 mM Survanta® did not affect the detection of TNF-α in these samples (data not shown).

Indirect immunofluorescence assay

Monocytes were washed twice with PBS and then incubated with optimal concentrations of mAb in PBS supplemented with 1% bovine serum albumin (PBS–BSA) for 30 min at 4°, washed with cold PBS–BSA, and then stained with PE–GAM in PBS–BSA supplemented with 5% human serum for 30 min at 4°. After washing, the mean fluorescence intensity (MFI) of the cells was quantified by FACScan (Becton Dickinson, Mountain View, CA) equipped with an argon–ion laser (excitation wavelength at 488 nm, laser power 300 mW) and a band pass filter of 585 nm (width 44 nm). Monocytes were gated by forward and sideward angle scatter; in each sample 1×10^4 monocytes were analysed. Control cells incubated with PE–GAM alone were used to determine background fluorescence. These values were used to correct the observed MFI for non-specific staining. The results are expressed as MFI in arbitrary units (AU).

Measurement of H_2O_2 production

H_2O_2 production by resting monocytes and cells upon stimulation with 25 ng phorbol myristate acetate (PMA)/ml

(Sigma) was assayed by the horseradish peroxidase-mediated H_2O_2-dependent oxidation of homovanillic acid.[37]

Statistics

All results are means and standard deviations of at least three experiments. The significance of differences was determined with the Mann–Whitney *U*-test.

RESULTS

Effect of surfactant on the rIFN-γ-stimulated expression of FcγRI by monocytes

Since rIFN-γ markedly enhances the expression of FcγRI by monocytes,[26,27] this parameter was used as a marker to optimize the conditions for the incubation of monocytes with rIFN-γ and surfactant. Monocytes incubated with rIFN-γ for 18 hr displayed enhanced expression of FcγRI dependent on the concentration of this cytokine; a plateau was reached at a concentration of 100 U rIFN-γ/ml (Fig. 1). Longer incubation periods of monocytes with 100 U rIFN-γ/ml did not result in higher expression of FcγRI (data not shown). On the basis of these data, monocytes were incubated with 100 U rIFN-γ/ml for 18 hr in all subsequent experiments.

When monocytes were preincubated with rIFN-γ for 30 min and subsequently incubated with rIFN-γ and various concentrations of surfactant for 17·5 hr, the rIFN-γ-induced expression of FcγRI by these cells was inhibited; maximal inhibition was reached with 0·4 mM surfactant (Fig. 2). The basal expression of FcγRI by monocytes was not affected by incubation of these cells with 0·04 mM or 0·1 mM surfactant (data not shown) and was slightly reduced after incubation with 0·4 mM surfactant (Fig. 3a). Similar results were found when monocytes were first incubated with surfactant for 30 min and subsequently incubated with both surfactant and rIFN-γ for 17·5 hr (Fig. 3b). Since *in vivo* monocytes migrating to the alveoli arrive in a surfactant-rich environment, where they are exposed to IFN-γ, in all further experiments monocytes were incubated with surfactant for 30 min prior to the addition of rIFN-γ; subsequent incubation was continued in the presence of surfactant and rIFN-γ, unless otherwise stated. Surfactant added during the last 30 min of the 18-hr incubation of

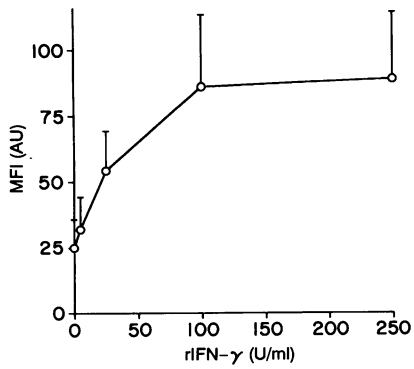

Figure 1. Effect of rIFN-γ on the expression of FcγRI by human monocytes. Monocytes were incubated with various concentrations of rIFN-γ for 18 hr at 37°. Expression of FcγRI was detected by immunofluorescence and quantified by FACS analysis ($n = 3$).

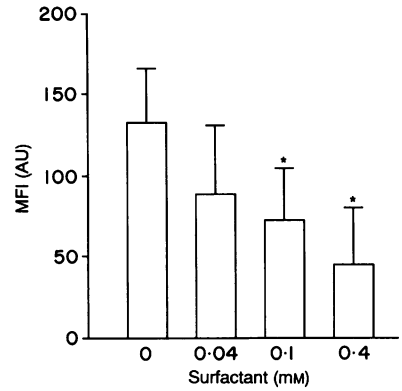

Figure 2. Effect of surfactant on the rIFN-γ-induced expression of FcγRI by monocytes. Monocytes were incubated with rIFN-γ for 30 min prior to the addition of surfactant and then in the presence of surfactant and IFN-γ for 17·5 hr at 37°. Expression of FcγRI by monocytes was detected by immunofluorescence and quantified by FACS analysis ($n = 7$). The MFI for monocytes incubated in the absence of rIFN-γ and surfactant amounted to 39 ± 19 AU ($n = 7$). *$P < 0·05$ for monocytes incubated in the presence and absence of surfactant.

monocytes with rIFN-γ did not reduce ($P = 0·77$) the expression of FcγRI by these cells (Fig. 3c), indicating that surfactant does not affect the detection of FcγRI. The inhibitory effect of surfactant on FcγRI-expression by monocytes was not due to LPS contamination, since incubation of monocytes with a concentration of LPS similar to that present in 0·4 mM surfactant, i.e. 1–2 ng/ml, did not reduce the rIFN-γ-stimulated increase in the expression of FcγRI by these cells. The MFI for monocytes incubated with rIFN-γ or with rIFN-γ and LPS amounted to 170 ± 45 AU and 175 ± 43 AU, respectively (n = 3).

Effect of surfactant on the rIFN-γ-stimulated expression of MHC class II antigens by monocytes

Monocytes incubated with rIFN-γ displayed enhanced ($P < 0·05$) expression of MHC class II antigens; the MFI for rIFN-γ-incubated cells and control cells amounted to 1276 ± 395 and 815 ± 277 AU, respectively ($n = 4$). Surfactant did not affect either the rIFN-γ-stimulated expression or the basal expression of MHC class II antigens by monocytes, the respective values being 1410 ± 475 AU and 969 ± 308 AU ($n = 4$).

Effect of surfactant on the rIFN-γ-stimulated production of H_2O_2 by monocytes

Since rIFN-γ enhances production of H_2O_2 by monocytes,[20–22] and surfactant inhibits the production of H_2O_2 by monocytes,[2] we next investigated the effect of surfactant on the rIFN-γ-stimulated production of H_2O_2 by monocytes. The results revealed that in the presence of surfactant the PMA-induced production of H_2O_2 by rIFN-γ-stimulated monocytes was reduced by 66 ± 24%, and that produced by control monocytes without rIFN-γ by 35 ± 11% (Fig. 4). Incubation of monocytes with rIFN-γ for 30 min prior to the addition of surfactant yielded similar results (data not shown).

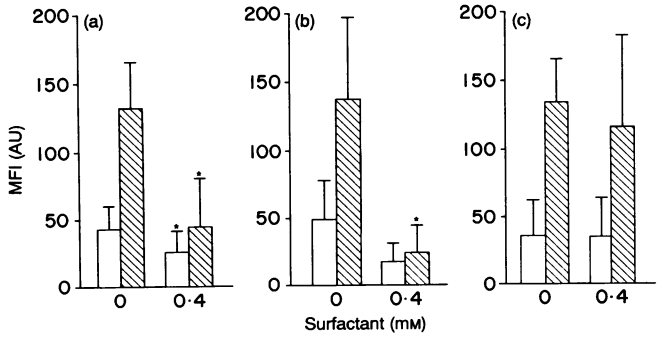

Figure 3. Effect of surfactant on the basal and rIFN-γ-induced expression of FcγRI by monocytes. Monocytes were incubated (a) with rIFN-γ for 30 min prior to the addition of surfactant, (b) with surfactant for 30 min prior to the addition of rIFN-γ, or (c) with rIFN-γ for 17·5 hr prior to the addition of surfactant; subsequently the incubation was continued in the presence of both rIFN-γ and surfactant at 37°. Expression of FcγRI by monocytes was measured after 18 hr by immunofluorescence and FACS analysis. Hatched bars represent cells incubated in the presence, open bars in the absence of rIFN-γ. $*P < 0.05$ for monocytes incubated in the presence and absence of surfactant (a, $n = 7$; b, c, $n = 4$).

Effect of Survanta® on the release of TNF-α and IL-1β by monocytes

Pilot experiments had revealed that surfactant stimulates the release of TNF-α and IL-1β by monocytes (data not shown). To circumvent this difficulty, Survanta®, which does not stimulate the release of TNF-α (Fig. 5) or IL-1β (data not shown) by monocytes, was used to investigate its effect on the production of TNF-α and IL-1β by rIFN-γ-stimulated monocytes. Incubation of monocytes with rIFN-γ resulted in an increase ($P < 0.05$) in the production of TNF-α by these cells, which was inhibited for 88 ± 16% by Survanta® (Fig. 5). This surfactant preparation inhibited the rIFN-γ-stimulated release of TNF-α by monocytes in a dose-dependent fashion; maximal inhibition was obtained with 0·4 mM Survanta® (data not shown). Incubation of monocytes with rIFN-γ together with LPS resulted in a synergistic increase in the production of TNF-α by these cells (Fig. 5). Survanta® yielded 90 ± 7% inhibition of the release of TNF-α by monocytes stimulated with rIFN-γ together with LPS, and 86 ± 12% inhibition for LPS alone (Fig. 5). Similar results were obtained when monocytes were incubated with rIFN-γ and LPS for 30 min prior to addition of Survanta® (data not shown).

Although the basal production of IL-1β by monocytes differed between various donors, rIFN-γ consistently induced an increase in the production of this cytokine by these cells (the fold-increase amounted to 2·9 ± 1·7) ($n = 3$). Survanta® inhibited the rIFN-γ-induced production of IL-1β by monocytes by 75 ± 20% ($n = 3$). The inhibition of the release of IL-1β by monocytes stimulated with rIFN-γ together with LPS, and with LPS alone, by Survanta® was 81 ± 15% and 78 ± 6%, respectively ($n = 3$).

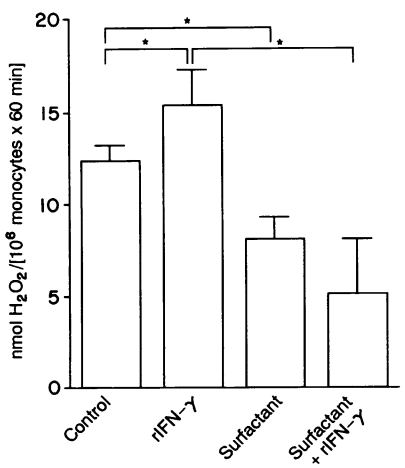

Figure 4. Effect of surfactant on the rIFN-γ-induced production of H_2O_2 by monocytes. Monocytes were incubated with 0·4 mM surfactant for 30 min prior to the addition of IFN-γ and then incubated in the presence of surfactant and IFN-γ for 17·5 hr at 37°. After three washes, the production of H_2O_2 by these cells upon stimulation with PMA was measured ($n = 4$). $*P < 0.05$.

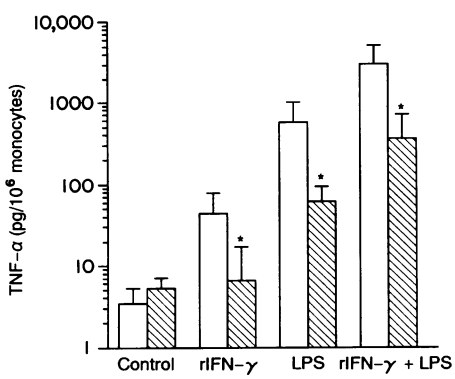

Figure 5. Effect of Survanta® on the rIFN-γ-induced production of TNF-α by monocytes. Monocytes were incubated in the presence (hatched bars) or absence (open bars) of 1 mM Survanta® for 30 min prior to the addition of rIFN-γ and/or LPS and then incubated for 17·5 hr at 37°. Next, the suspension was centrifuged and the supernatants were collected, filtered, and assayed for TNF-α by ELISA ($n = 3$). $*P < 0.05$ for monocytes incubated with Survanta® and the respective control cells.

DISCUSSION

Two main conclusions can be drawn from the present study. Firstly, human surfactant inhibits the increase in the expression of FcγRI and the production of H_2O_2 by monocytes stimulated with rIFN-γ. This latter finding extends our previous observation that surfactant inhibits H_2O_2 production by normal monocytes.[2] Secondly, Survanta® inhibits the production of TNF-α and IL-1β by monocytes stimulated with rIFN-γ or the combination rIFN-γ and LPS. This study is the first to demonstrate that the rIFN-γ-mediated production of cytokines is impaired by Survanta®, and extends previous studies on the effect of other surfactant preparations on the LPS-stimulated release of cytokines by phagocytes.[8,9] Curosurf®, a porcine surfactant preparation, suppressed the release of TNF-α by LPS-stimulated monocytes,[9] and Exosurf®, a synthetic surfactant preparation, decreased the release of TNF-α, IL-1β, IL-6 and IL-8 by LPS-stimulated human alveolar macrophages.[8]

Since surfactant, but not Survanta®, stimulated the production of TNF-α and IL-1β by monocytes, Survanta® was used to measure its effect on the rIFN-γ-stimulated production of these cytokines by monocytes. The stimulatory effect of surfactant on the production of TNF-α and IL-1β by monocytes was not due to LPS contamination, since Survanta® efficiently inhibited the production of TNF-α and IL-1β by monocytes upon stimulation with LPS in a concentration similar to that present in the human surfactant preparation. Since surfactant protein A (SP-A) can induce the production of TNF-α by monocytes (unpublished observations) and rat alveolar macrophages,[38] this protein, which is present in human surfactant but not Survanta®, may cause the stimulatory effect of human surfactant on the production of TNF-α and IL-1β by monocytes. It should be considered that some components of surfactant, e.g. phospholipids which are present in Survanta®, can impair, and others, e.g. SP-A, can stimulate the production of cytokines by phagocytes.

Three explanations can be offered for the inhibitory effect of surfactant on the activation of monocytes by rIFN-γ. Firstly, surfactant may prevent the binding of IFN-γ to its receptor. This possibility is not very likely, since the addition of surfactant to monocytes that had already been incubated with rIFN-γ for 30 min also resulted in inhibition of activation. The second possibility is that surfactant modulates downstream components of the signal transduction pathways from IFN-γ receptors. It should be noted that intracellular signalling in monocytes and macrophages from IFN-γ receptors occurs within seconds to minutes after exposure of these cells to rIFN-γ.[19,39] It is not very likely that surfactant affects early signal transduction events from these receptors, since addition of this material to monocytes that had been incubated with rIFN-γ for 30 min also inhibited the activation of these cells. We previously reported that inhibition of monocyte bactericidal functions by surfactant can be explained partially by a sustained surfactant-induced increase in the intracellular cAMP concentration in these cells.[40] Therefore, one of the mechanisms for the surfactant-mediated inhibition of the activation of monocytes by rIFN-γ may be an increase in the intracellular cAMP concentration. Consistent with these arguments, rat peritoneal macrophages incubated with cAMP-inducing agents display reduced release of TNF-α,[30] and db-cAMP, a membrane-permeable cAMP analogue, inhibits the rIFN-γ-mediated increase in expression of FcγRI by U937 cells[41] and monocytes (unpublished observations). Since preliminary experiments showed that surfactant enhances the plasma membrane fluidity of monocytes (data not shown), a third possibility is that surfactant reduces rIFN-γ-induced expression of FcγRI by monocytes by altering the membrane fluidity of these cells. Membrane fluidity is thought to determine the effect of rIFN-γ on the expression of FcγRI by U937 cells.[42] Assuming that membrane fluidity is also important for the rIFN-γ-stimulated expression of MHC class II antigens by monocytes, this possibility is not very likely since surfactant does not affect the rIFN-γ-induced expression of these antigens by monocytes. The absence of an effect of surfactant on the rIFN-γ-induced expression of MHC class II antigens is not yet understood. There are some indications that regulation of the rIFN-γ-induced expression of FcγRI and MHC class II antigens is different, i.e. the rate of formation of mRNA and the sensitivity to cycloheximide is dissimilar.[39,43] Possibly, surfactant inhibits an intracellular pathway in monocytes that is not involved in the IFN-γ-mediated expression of MHC class II antigens.

Excessive production of ROI, TNF-α and IL-1 by monocytes may be deleterious for tissue integrity, because they can cause epithelial damage,[44] and TNF-α and IL-1 can stimulate enhanced production of inflammatory mediators, such as monocyte chemotactic protein-1 (MCP-1),[45] IL-8[46] and granulocyte–monocyte colony-stimulating factor (GM-CSF), by epithelial cells.[47] Our results could mean that the outcome of inflammatory reactions in the lower airways depends not only on the balance between proinflammatory and anti-inflammatory mediators but also on the presence of local factors, such as surfactant which can inhibit the inflammatory reaction. The reverse, i.e. cytokines affecting the production of surfactant, is also possible. In this respect, IFN-γ stimulates the production of SP-A by alveolar epithelial cells of fetal lung and human pulmonary adenocarcinoma cell lines,[48] and rTNF-α inhibits the expression of SP-A and also SP-B by these cell lines.[49]

Various pulmonary diseases are characterized by altered cytokine levels in BAL fluid and changes in surfactant composition. In patients with adult respiratory distress syndrome (ARDS), enhanced levels of TNF-α, IL-1 and IL-8 have been found in BAL fluid;[50] moreover, the relative quantities of various phospholipids are altered and the amounts of SP-A and SP-B decreased.[51] In asthmatics, the production and release of IFN-γ, TNF-α, and IL-1β by leucocytes isolated from BAL are increased[52,53] and the concentration of SP-A in surfactant is reduced.[54] These data could indicate that surfactant plays a role in the regulation of the inflammatory process, although it is of course not clear whether altered cytokine levels are the cause or the consequence of altered surfactant composition. In view of the above, it would appear feasible that surfactant replacement therapy, which has already been administered to a limited group of patients with ARDS to improve lung compliance and gas exchange,[51] may also be beneficial in reducing pulmonary inflammation.

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