Transcriptional Control of Cytokine Release from Monocytes of the Newborn: Effects of Endogenous and Exogenous Interleukin-10 versus Dexamethasone

Lina A. Chusid\textsuperscript{b} Lucy Pereira-Argenziano\textsuperscript{b} Veronika Miskolci\textsuperscript{a, b} Ivana Vancurova\textsuperscript{b, c} Dennis Davidson\textsuperscript{a, b}

\textsuperscript{a}Center for Immunology and Inflammation, The Feinstein Institute for Medical Research, Manhasset, N.Y., \textsuperscript{b}Division of Neonatal-Perinatal Medicine, Schneider Children’s Hospital, New Hyde Park, N.Y., and \textsuperscript{c}Department of Biological Sciences, St. John’s University, New York, N.Y., USA

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Abstract

Background: Monocytes play an important role in the fetal and neonatal inflammatory response syndrome. They are also the precursors of alveolar macrophages, microglial and Kupffer cells. Monocytes have pro-inflammatory (PI) and anti-inflammatory (AI) functions. Interleukin (IL)-10 is a potent AI cytokine released by monocytes. Objective: We determined the effects of endogenous and exogenous IL-10 versus equimolar levels of dexamethasone (DEX) on PI and AI cytokine release, as well as transcription factor DNA-binding activity, in endotoxin (lipopolysaccharide, LPS)-stimulated monocytes of the newborn. Methods: Monocytes were isolated into culture media from cord blood. ELISAs, electrophoretic mobility shift assays and Western blots were employed. Results: LPS-stimulated monocyte release of PI cytokines, tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), IL-1\(\beta\) and IL-8, over 18 h was significantly augmented by addition of an IL-10 monoclonal antibody. Exogenous IL-10 at 10\(^{-8}\) M inhibited PI cytokine release by 89–97%, while DEX at an equimolar level had no effect. DNA-binding activities of the PI transcription factors nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) and activator protein-1 (AP-1), and the AI transcription factor signal transducer and activator of transcription 3 (STAT3) were induced over 18 h. DEX at 10\(^{-8}\) M had no effect on any transcription factor DNA binding, but exogenous IL-10 at 10\(^{-8}\) M produced a 60% inhibition of AP-1 DNA binding and enhanced phosphorylation of nuclear STAT3 for 18 h. Conclusion: At therapeutic levels of DEX, monocyte release of PI cytokine was insensitive to DEX in comparison to IL-10. IL-10 or its mechanism of action could lead to new therapy for inflammatory disorders in the perinatal period. Copyright © 2009 S. Karger AG, Basel

Introduction

Control of inflammation plays an important role in serious perinatal disorders such as the fetal inflammatory response syndrome, preterm delivery, bronchopulmonary dysplasia (BPD) and white matter injury [1, 2].
The innate inflammatory response is generally characterized by polymorphonuclear leukocytes (PMNs) followed by monocyte recruitment, as exemplified by sequential examination of airway fluid in newborns developing BPD [3, 4]. During this process, pro-inflammatory (PI) cytokines, such as interleukin (IL)-8, tumor necrosis factor-α (TNF-α) and IL-1β, are released from inflammatory cells [1, 2, 5, 6]. For the PMNs of the newborn, recent work has demonstrated that exogenous IL-10, an anti-inflammatory (AI) cytokine, is equipotent to dexamethasone (DEX) on a molar basis with regard to inhibition of PI cytokine release [7]. There is now increasing interest in the monocytes [8, 9] of the newborn, which are precursors for alveolar macrophages, Kupffer cells and microglial cells [10]. Monocytes from adults, in comparison to PMNs from adults, have the ability to produce greater amounts of PI and AI cytokines, and therefore may play a pivotal role in the development and resolution of inflammation [11]. However, little is known about the control of PI and AI cytokine release from monocytes of the newborn.

Postnatal corticosteroids, such as DEX, reduce the incidence of BPD, but must be used cautiously owing to potentially serious acute and long-term side effects [12, 13]. Fortunately, therapeutic levels of DEX have been measured in the plasma of newborns treated for BPD [14, 15]. These levels can serve as a benchmark for the comparison of DEX versus other potentially useful agents, such as IL-10, in cell or molecular studies. AI cytokine therapy may have potential therapeutic uses for perinatal inflammatory conditions.

With a focus on the endotoxin (lipopolysaccharide, LPS)-stimulated monocytes of the term, healthy human newborn, our study aims were to: (1) describe the temporal and qualitative release of PI and AI cytokines; (2) assess whether endogenous IL-10 release, as a counter-regulatory cytokine, has any effect on PI cytokine release; (3) compare the effect of equimolar levels of DEX versus exogenous IL-10 on PI cytokine release; (4) determine interrelated transcription factor activity [16, 17]. Under our experimental conditions, we studied the PI transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), as well as the AI transcription factor signal transducer and activator of transcription 3 (STAT3). Fetal inflammatory response syndrome and the neonatal inflammatory response, as well as the regulation of monocyte functions may be important to clarify for both preterm and term infants. Developmental differences were not addressed by this study.

Materials and Methods

Subjects

Cord blood (approximately 60 ml) was obtained from placentas after elective, term, caesarean section deliveries. One of the investigators attended each of the deliveries to exclude deliveries associated with labor, rupture of membranes, meconium-stained fluid, clinical evidence of chorioamnionitis, maternal disorders and maternal medications. Blood was collected in heparinized preservative-free tubes for transport to the laboratory, followed by immediate cell isolation. A total of 21 cord blood samples were used. The study was approved by the Internal Review Board of the North Shore-Long Island Jewish Health System.

Cell Isolation and Culture

Monocytes were isolated from cord blood under pyrogen-free conditions at room temperature. Whole blood was layered over Ficoll-Paque PLUS (Amersham-GE Healthcare, Piscataway, N.J., USA) and centrifuged at 400 g for 40 min. Peripheral blood mononuclear cells (PBMC) layer was collected from the interface and washed with phosphate-buffered saline (PBS; Gibco-Invitrogen, Grand Island, N.Y., USA). Residual erythrocytes were lysed by hypotonic buffer. PBMCs were washed with PBS 3 times and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum (RPMI++; Gibco-Invitrogen) at 5 × 10^6 cells/ml. Monocytes were purified using Percoll (Amersham-GE Healthcare) solution as described previously [18]. PBMCs were layered over hyperosmotic Percoll solution and centrifuged at 580 g for 15 min. The monocyte-enriched fraction was collected at the interface and washed 3 times with PBS. Monocytes were further purified by depletion of nonmonocytes using MACS monocyte isolation kit II supplemented with CD15 microbeads (Miltenyi Biotec, Auburn, Calif., USA). Supplementing with CD15 microbeads helped reduce granulocyte contamination and increase final monocyte purity. Final monocyte purity was ≥90% as determined by flow cytometry. Monocytes were then resuspended at 1 × 10^6 cells/ml in RPMI+ and aliquoted at 1 ml into 48-well culture plates. For the dose response, monocytes were pre-incubated with PBS (vehicle control) and serial doses of IL-10 (R&D Systems, Minneapolis, Minn., USA) or DEX (Abraxis Pharmaceutical Products, Schaumburg, Ill., USA) for 1 h at 37°C and 5% CO2, then stimulated with LPS (10 ng/ml; L4391, from Escherichia coli 0111:B4; Sigma-Aldrich, St. Louis, Mo., USA) for 18 h. For the time course experiments, monocytes were pre-incubated with PBS, IL-10 monoclonal antibody (10 μg/ml), immunoglobulin (IgG) control (MAB2171, MAB004; R&D Systems) and equimolar concentration (10^{-8} M) of IL-10 or DEX for 1 h, then stimulated with LPS (10 ng/ml) for up to 18 h. The IL-10 monoclonal antibody concentration used in our experimental conditions was derived from an antibody titration assay using IL-8 release as an endpoint (data not shown). Transcription factor activity was assayed at 0.5, 4 and 18 h, and cytokines were assayed at 4 and 18 h of LPS stimulation. PMNs were isolated as described previously [7] and resuspended at 1 × 10^6 cells/ml in RPMI+. PMNs were exposed to serial doses of DEX for 1 h and then stimulated with LPS (10 ng/ml) for 18 h as above, serving as a positive control.

ELISA

TNF-α, IL-8, IL-1β, IL-10, IL-4 and IL-1 receptor antagonist (IL-1ra) were measured in cell culture supernatants using com-
commerially available ELISA kits (R&D Systems). The assays were performed according to the manufacturer’s instructions. The volume of samples ranged from 50 to 200 μl. The minimal detectable doses for TNF-α, IL-8, IL-1β, IL-10, IL-4 and IL-1ra were 1.6, 3.5, 1, 3.9, 10 and 14 pg/ml, respectively.

**Electrophoretic Mobility Shift Assay**

The nuclear extracts (1 x 10⁶ cells) were prepared and electrophoretic mobility shift assay (EMSA) assays were performed as described previously [19, 20]. Additional protease inhibitors used were 1x Complete (Roche Applied Science, Indianapolis, Ind., USA), 2 mM diisopropylfluorophosphate (Sigma) and 200 μM MG132 (Biomol International, Plymouth Meeting, Pa., USA). The oligonucleotides used as probes were 42-base pair double-stranded custom construct 5'-TTGTTACAAGGGACCTTGCTG-GGGCTTTCCCAGGGAGGC-3' (Invitrogen) for NF-κB (tandem repeats of NF-κB-binding sites underlined), and consensus oligos for AP-1 and STAT3 (sc-2501, sc-2571; Santa Cruz Biotechnology, Santa Cruz, Calif., USA). The oligonucleotides used for competition assays were mutant construct 5'-TTGTTACAAGGGACCTTGCTG-GGGCTTTCCCCAGGGAGGC-3' for NF-κB, and mutant oligos for AP-1 and STAT3 (sc-2514, sc-2572; Santa Cruz).

Western Blotting

Cytoplasmic extracts were prepared from 1 x 10⁶ cells as described previously, including the additional inhibitors listed above [19, 20]. Cytoplasmic extracts and washed nuclear pellets were boiled with sample buffer (SDS reducing buffer; Bio-Rad Laboratories, Hercules, Calif., USA) to denature proteins. Denatured proteins were separated on 10% denaturing polyacrylamide gel and transferred to nitrocellulose membrane (Hybond-ECL; Amersham-GE Healthcare). The detection of phosphorylated STAT3 (pSTAT3), membranes were blocked overnight with 5% (w/v) bovine serum albumin (Sigma) in 10 mM Tris-Cl (pH 7.5), 140 mM NaCl, 1.5 mM MgCl₂ and 0.1% Tween 20 solution (TBST-BSA) before incubating with primary antibody against pSTAT3 (sc-8059; Santa Cruz) for 2 h at room temperature, diluted 1:200 in TBST-BSA. After washing with TBST, membranes were incubated for 1 h with horseradish peroxidase-labeled secondary antibody, diluted 1:20,000 in TBST-BSA. For blocking and incubations with antibodies, TBST-BSA was supplemented with 0.01% (v/v) of each phosphatase inhibitor cocktails A and B (Santa Cruz). Digestions with antibodies, TBST-BSA was supplemented with 0.01% (v/v) of each phosphatase inhibitor cocktails A and B (Santa Cruz). For the detection of total STAT3, membranes were blocked with TBST containing 5% (w/v) nonfat dry milk, before incubating with primary antibodies against STAT3 (sc-8019; Santa Cruz) for 2 h at room temperature, diluted 1:200. After washing, membranes were incubated with secondary antibodies as above, diluted 1:2,000. Labeled proteins were detected using enhanced chemiluminescence reagents as described by the manufacturer (ECL PLUS, Amersham-GE Healthcare).

**Data Analysis**

The EMSA images were scanned and analyzed by densitometry using image analysis software UN-SCAN-IT gel v. 5.1 (Silk Scientific, Orem, Utah, USA). Data were expressed as means ± SEM. Statistical analyses were performed using one-way repeated measures ANOVA and Bonferroni corrections for multiple comparisons by GraphPad InStat version 3.0 (GraphPad Software, San Diego, Calif., USA).

### Results

**PI Cytokine Release**

Figure 1 depicts the release of 3 PI cytokines (TNF-α, IL-1β and IL-8) from LPS-stimulated monocytes of the newborn at 4 and 18 h. TNF-α levels from cells stimulated with LPS alone were higher at 4 compared to 18 h, while the reverse was observed for IL-1β and IL-8. IL-8 levels were greatly increased compared to TNF-α and IL-1β when these cytokines were measured from medium with the same number of cells. On a molar basis, IL-8 increased 134- and 116-fold at 18 h over TNF-α and IL-1β, respectively. For all 3 PI cytokines, exogenous IL-10 significantly reduced release compared to LPS alone; inhibition was 89.4, 91.4 and 92.3% at 4 h and 92.8, 97.4 and 97.4% at 18 h for TNF-α, IL-1β and IL-8, respectively. There was no reduction in PI cytokine release by an equimolar (10⁻⁸ M) level of DEX at 4 and 18 h of LPS stimulation. Release of all PI cytokines 18 h after stimulation with LPS was significantly increased by a monoclonal antibody against IL-10, specifically 233, 173 and 150%, for TNF-α, IL-1β and IL-8, respectively. The specificity of this antibody for IL-10 was supported by the results that the control pre-immune IgG exhibited the same release of PI cytokines at 18 h as LPS alone. Interestingly, the release of PI cytokines 4 h after stimulation was not enhanced by the IL-10 monoclonal antibody, suggesting that the endogenous IL-10 is released from LPS-stimulated monocytes at later time points.

**AI Cytokine Release**

Figure 2 depicts the release of 2 AI cytokines, IL-1ra and IL-10, from LPS-stimulated monocytes of the newborn. IL-4 was not detectable under the same study conditions and therefore the data is not shown. Figure 2a shows that IL-1ra release is detectable at 4 h and to a greater extent at 18 h of LPS stimulation. The molar concentration of IL-1ra at 18 h was 2 x 10⁻⁹ M compared to 2.8 x 10⁻¹⁰ M for the PI cytokine IL-1β. IL-1ra release was unaffected by DEX (10⁻⁸ M), IL-10 monoclonal antibody or the IgG control. However, exogenous IL-10 (10⁻⁸ M) significantly increased IL-1ra release by 4 h. Figure 2b demonstrates that LPS stimulated the release of IL-10 to a level of approximately 900 pg/ml/10⁶ monocytes by 18 h; this is a concentration of 5 x 10⁻¹¹ M for IL-10. DEX had no effect on IL-10 release at 4 and 18 h.

**Effect of Exogenous IL-10 and DEX on IL-8 Release: Dose Responses**

Figure 3 demonstrates the dose response to DEX and IL-10 on an equimolar basis, with regard to the inhibition
Fig. 1. PI cytokine release from endotoxin (LPS)-stimulated monocytes (n = 5) of the newborn at 4 h (□) and 18 h (■): effects of monoclonal antibody (mAb) to endogenous IL-10, IgG control for the monoclonal antibody, exogenous IL-10 and DEX. TNF-α (a), IL-1β (b) and IL-8 (c) release are shown. * p < 0.01 versus LPS alone at the same time point.

Fig. 2. AI cytokine release from endotoxin (LPS)-stimulated monocytes (n = 5) of the newborn at 4 h (□) and 18 h (■): effects of monoclonal antibody (mAb) to endogenous IL-10, IgG control for the monoclonal antibody, exogenous IL-10 and DEX. IL-1ra (a) and IL-10 (b) are shown. * p < 0.01 versus LPS alone at the same time point.
**Fig. 3.** IL-8 release from endotoxin (LPS)-stimulated monocytes (n = 5) of the newborn at 18 h: dose response to DEX (a) and IL-10 (b) on a molar basis. *p < 0.05 versus LPS alone.

**Fig. 4.** Representative EMSAs and densitometry from all experiments (n = 5) for NF-κB, AP-1 and STAT3. EMSA was analyzed in nuclear extracts of monocytes stimulated by endotoxin (LPS) at 0.5, 4 and 18 h. At each time point the effects of monoclonal antibody to endogenous IL-10 (mAb IL-10), IgG control for the monoclonal antibody, exogenous IL-10 and DEX are shown. Statistical analyses of densitometric values were compared to LPS alone at each time point, which was considered 100%. Wild-type oligonucleotides (oligo) and mutant oligonucleotides were used to demonstrate DNA-binding specificity.
of IL-8 release from LPS-stimulated monocytes over 18 h. IL-10 significantly reduced IL-8 release by 92.2 and 91.2% at $10^{-9}$ and $10^{-8}$ M, respectively, compared to LPS alone. On the other hand, no inhibition by DEX was observed below $10^{-6}$ M. DEX inhibited IL-8 release by 45 and 80% for $10^{-6}$ and $10^{-5}$ M, respectively. As a positive control for the effect of DEX ($10^{-8}$ M) on monocytes, we simultaneously measured IL-8 release from PMNs of the newborn stimulated by LPS for 18 h. As previously shown [5], DEX inhibited IL-8 release in PMNs by 83% (data not shown).

**Transcription Factor Activity**

Figure 4 demonstrates DNA-binding activity from nuclear extracts of LPS-stimulated monocytes at 0.5, 4 and 18 h under different experimental conditions. Below each representative EMSA image, the densitometric analyses of 5 experiments are shown. Densitometric values were statistically analyzed by comparing experimental conditions to the LPS-stimulated sample within each time point. Specificity of the EMSA for the respective transcription factor was analyzed by using unlabeled oligonucleotides (right panels). The top EMSA images demonstrate persistent, but waning NF-κB DNA-binding activity over 18 h of LPS stimulation. Neither the EMSA images nor the densitometry demonstrate any effects of IL-10 ($10^{-8}$ M), DEX ($10^{-8}$ M) or IL-10 monoclonal antibody on NF-κB DNA-binding activity. The middle EMSA image demonstrates increasing AP-1 DNA binding dur-
revealed no effect of IL-10 (10^{-8} \text{ M}), DEX (10^{-8} \text{ M}) or IL-10 monoclonal antibody on the STAT3 DNA-binding activity in LPS-stimulated monocytes.

\textit{pSTAT3}

Figure 5a illustrates a representative Western blot analysis (n = 5) of pSTAT3 protein levels in cytoplasmic and nuclear extracts of monocytes stimulated by LPS. pSTAT3 was not detected in the cytoplasm under any condition or time point. pSTAT3 was detected in nuclear extracts of LPS stimulated monocytes at 4 and 18 h after stimulation. This STAT3 phosphorylation at 4 and 18 h was markedly reduced by IL-10 antibody. Conversely, exogenous IL-10 increased the STAT3 phosphorylation at the 0.5, 4 and 18 h time points. DEX had no effect on pSTAT3 levels. Figure 5b illustrates the densitometric evaluation of the above Western blot analyses of pSTAT3/total STAT3 levels measured in the nuclear extracts (n = 5).

**Discussion**

The overall aim of this study was to determine mechanisms of cytokine regulation by the monocyte of the newborn that may lead to new AI therapy for serious perinatal disorders in term and preterm infants [1, 13, 21]. The present in vitro studies used a highly purified, cord blood monocyte preparation with LPS as a stimulant for cytokine release and transcription factor activity studies. Cord blood monocytes were purposely isolated from the placenta of healthy term infants, to avoid the confounding influences of prenantal steroids, clinical chorioamnionitis, maternal conditions leading to an indicated delivery and maternal medications. Accordingly, the results of our studies may not be accurately extrapolated to very premature infants. Although a subset of newborns that develop inflammatory conditions, such as BPD, may have an associated exposure to microbial toxins, other factors such as oxidative stress and ventilator-induced lung injury need to be considered.

In the present study, LPS-stimulated monocytes released TNF-\(\alpha\), with highest levels at 4 h, compared with IL-1\(\beta\) and IL-8 with highest levels at 18 h. Corrected for cell number, the monocytes of the newborn released a much greater amount of these PI cytokines compared to the PMN of the newborn [7]. LPS-stimulated monocytes released the AI cytokines IL-1ra and IL-10, with highest levels at 18 h, as opposed to 4 h. IL-4, an AI cytokine, was not detectable after LPS stimulation. Endogenous IL-10 release was in the range of 10^{-10} to 10^{-11} \text{ M}. We then examined whether endogenous IL-10 release by monocytes had a negative feedback effect on PI cytokine release. This was verified by adding an IL-10 monoclonal antibody to LPS-stimulated monocytes, resulting in significantly elevated PI cytokine release by 18 h for all 3 PI cytokines assayed. Previous work from adults has also demonstrated this regulatory role of IL-10 in macrophages stimulated by pathogens or their products [22].

Surprisingly, when monocytes were pre-incubated with therapeutic levels of DEX for BPD (10^{-8} \text{ M}), there was no inhibition of PI cytokine release. In fact, no significant effect was observed until 10^{-6} \text{ M}. Marked inhibition of IL-8 by DEX from neutrophils served as a positive control. DEX also had no effect on AI cytokine release. On the other hand, IL-10 at 10^{-8} \text{ M} markedly inhibited monocyte release of all 3 PI cytokines.

Dose-response studies demonstrated that IL-10 at 10^{-9} \text{ M} almost completely inhibited IL-8, which served as marker for PI cytokines. Therefore, the results of the present study indicate that endogenous IL-10 release in the 10^{-10} to 10^{-11} \text{ M} range has a modulating effect on monocyte PI cytokine release, and approximately a 100-fold increase above endogenous levels would be needed to markedly inhibit PI cytokine release and have a therapeutic AI effect. Endogenous IL-10 is either undetectable or detectable at very low levels in the airways of preterm and term newborns at risk for BPD [23–26]. The present study with monocytes and the previous study with identical conditions for PMNs [7] suggest that exogenous IL-10 at 10^{-8} \text{ M} could have therapeutic potential for BPD [7, 27]. The relative resistance of the LPS-stimulated monocyte of the newborn to DEX compared to exogenous IL-10 at known therapeutic levels of DEX may suggest a developmental cause or a glucocorticoid receptor resistance to DEX as opposed to other corticosteroids [28].

IL-10 has been used in the treatment of inflammatory bowel disease and psoriasis in adults [29]. IL-10 is generally considered an AI cytokine. However, elevated levels of IL-10 have been detected in a minority of preterm infants during early development of BPD [26], sepsis [30] and acute respiratory distress syndrome [31]. It is unclear if the elevated IL-10 levels measured under these conditions are only counterregulatory for excessive PI cytokine release.

The transcription factor STAT3 is required for IL-10 AI actions. Possible mechanisms of STAT3 activity include the posttranslational modification of PI transcription factor structure, modification of chromatin attachment to transcription factors, or sequestration of tran-
scription factors away from their PI promoters [22]. In monocytic cells, NF-κB is found in the cytoplasm as an inactive NF-κB-inhibitor-κB (IκB) complex. A stimulus such as LPS, via its Toll-like receptor and signal transduction pathway, activates IκB kinase to phosphorylate IκB, freeing NF-κB to translocate to the nucleus and initiate transcription on a persistent basis [32]. AP-1 is a group of transcription factors composed of dimeric proteins activated by the extracellular signal-regulated kinase subgroup of mitogen-activated protein kinases [33]. NF-κB and AP-1 modulate the activity of each other [34]. IL-10 and STAT3, like glucocorticoids and the glucocorticoid receptor, may also have a mechanism of action involving a complex interaction between multiple transcription factors [16].

In the present study, nuclear extracts from LPS-stimulated monocytes exhibited a waning but persistent DNA-binding activity of NF-κB, increasing activity of AP-1 and a persistent activity of STAT3 at the 0.5, 4 and 18 h time points, using EMSAs. There was no effect of DEX (10⁻⁸ M), which is in concordance with the lack of DEX inhibition of PI cytokine release. IL-10 did not inhibit NF-κB DNA binding in contrast to work in PBMCs from adults [35]. PBMCs are a mixture of lymphocytes predominantly along with monocytes, making comparisons between studies invalid. However, in monocytes of the newborn, exogenous IL-10 caused a striking reduction in AP-1 activity over 18 h of LPS stimulation. At the same time, this was associated with increased levels of nuclear pSTAT3. Part of this increase in pSTAT3 was related to endogenous IL-10. When IL-10 monoclonal antibody was added to LPS-stimulated monocytes, a reduction in nuclear pSTAT3 was observed.

In summary, with a focus on the LPS-stimulated monocytes of the healthy term newborn, we demonstrated that endogenous IL-10 provides a negative feedback mechanism for PI cytokine release and exogenous IL-10 is a potent inhibitor of PI cytokine release. The mechanism of the AI action of IL-10 involves an increase in nuclear pSTAT3 levels associated with a reduction in AP-1 DNA binding. We speculate that the observed DEX insensitivity of the monocyte may partly explain the variable response to DEX in the treatment of BPD [12, 13]. It would be of interest to determine whether the insensitivity to DEX in cells derived from monocytes (that is, macrophages, microglial cells and Kupffer cells) also occurs. Exploring the AI pathways of IL-10 and corticosteroids at the molecular level could lead to new therapy for serious inflammatory disorders with collateral healthy tissue damage in the newborn.

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