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TGF- β 2 induces maturation of immature human intestinal epithelial cells and inhibits inflammatory cytokine responses induced via the NF- κ B pathway

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Abstract

Objectives—Breast milk transforming growth factor (TGF)- β 2 is associated with healthy immune maturation and reduced risk of immune-mediated disease in infants. We sought to investigate whether conditioning with TGF- β 2 might result in a more mature immune responder phenotype in immature human intestinal epithelial cells (IECs).

Methods—Primary human fetal IECs (hFIECs) and the human fetal small intestinal epithelial cell line (H4 cells) were conditioned with breast milk levels of TGF- β 2 and an inflammatory response was subsequently induced. Inflammatory cytokine secretion and mRNA expression were measured by ELISA and qRT-PCR, respectively. Alterations in activation of inflammatory signaling pathways were detected from IECs by immunoblotting and immunofluorescence. The effects of TGF- β 2 conditioning on gene expression patterns in hFIECs were assessed by cDNA microarray analysis and qPCR.

Results—Conditioning with TGF- β 2 significantly attenuated subsequent IL-1 β , TNF- α and poly I:C-induced IL-8 and IL-6 responses in immature human IECs. Conditioning with TGF- β 2 inhibited IL-1 β -induced I κ B- α degradation and NF- κ B p65 nuclear translocation, which may partially result from TGF- β 2-induced changes in the expression of genes in the NF- κ B signaling pathway detected by cDNA microarray and qPCR.

Conclusions—Conditioning with TGF- β 2 attenuates the subsequent inflammatory cytokine response in immature human IECs by inhibiting signaling in the NF- κ B pathway. The immunomodulatory potential of breast milk may in part be mediated by TGF- β 2, which might provide a novel means of supporting intestinal immune maturation in neonates.

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Conflict of interest No conflicts of interest exist.

Keywords

TGF- β ; immature intestinal epithelium; modulation of immune response

INTRODUCTION

Breast milk provides the newborn infant nutrients for growth and development in close to optimal quantities. It is well-established that breastfeeding confers protection against infectious diseases, particularly those of the gastrointestinal tract, *via* antimicrobial molecules such as immunoglobulins, lysozyme, lactoferrin, defensins and oligosaccharides.¹ Accumulating evidence suggests that in addition to this passive immunoprotection, bioactive molecules in breast milk modulate the infant's mucosal and systemic immune responses and may thereby promote adequate and appropriate immune responsiveness against both potentially pathogenic and indigenous microbes as well as harmless environmental and dietary antigens.² Intriguingly, data from well-conducted epidemiological studies suggest that breastfeeding may also have long-term immunological effects by reducing the risk of immune-mediated diseases such as celiac disease³ or atopic disorders⁴ in later life. However, the mechanisms of this immune conditioning by breast milk are poorly understood.

Transforming growth factor (TGF)- β is an immunomodulatory cytokine, which is secreted in breastmilk in significant quantities. Of the three human TGF- β isoforms (TGF- β 1, 2 and 3), TGF- β 2 is most abundant in breast milk. There are experimental data to suggest that breast milk TGF- β 2 may be an important source of TGF- β during the neonatal period when endogenous production of TGF- β in the gut is still inadequate.⁵⁻⁷ A recent report indicates that intestinal expression of TGF- β 2 is decreased in premature infants and especially in those suffering from necrotizing enterocolitis (NEC) as compared to term infants.⁵ Intestinal maturation results in an increase in TGF- β 2 expression in the gut.⁵ Moreover, breast milk TGF- β 2 may induce immune maturation in the immature intestine since epidemiological studies have demonstrated an association between breast milk TGF- β and both maturational changes in immune function and reduced risk of developing immune-mediated disease in infants and children.² High concentrations of both TGF- β 1 and TGF- β 2 in colostrum have been reported to correlate with serum IgA concentrations and reduced the risk of developing atopic eczema during exclusive breastfeeding in high-risk infants.⁸

We have recently demonstrated that TGF- β 2 administered at a concentration corresponding to that found in breast milk simultaneously with a pro-inflammatory stimulus attenuates inflammatory immune responses in the immature human intestinal epithelium.⁹ Given the potential of breast milk to induce long-term immune effects and the association between breast milk TGF- β 2 and infant immune responder phenotype discussed above, we suggest that breast milk TGF- β 2 may provide a maturational stimulus to the immature intestinal epithelium and support an anti-inflammatory tone necessary for withholding from potentially detrimental inflammatory responses against colonizing microbes after birth. We specifically hypothesize that conditioning the neonatal gut with TGF- β 2 might induce maturational changes in the immature intestinal epithelial cell inflammatory responses upon subsequent pro-inflammatory insult.

MATERIALS AND METHODS

Reagents

DMEM/F12 medium, Opti-MEM I medium, penicillin and streptomycin, Hepes buffer and Trypsin-EDTA were obtained from Gibco-Invitrogen (Carlsbad, CA, USA). Collagenase

type IV, protease inhibitor cocktail, phosphatase inhibitor cocktail I and II were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). Recombinant human insulin (Novolin R) was obtained from Novo Nordisk A/S (Bagsvaerd, Denmark). Extracellular matrix ECL was obtained from Upstate Biotechnology (Lake Placid, NY). The cytokines IL-1 β , TNF- α and TGF- β 2 were obtained from R&D Systems (Minneapolis, MN, USA). Rabbit anti-human NF- κ B (p65) polyclonal antibody was obtained from Calbiochem (Gibbstown, NJ, USA). CyTM 3-conjugated F(ab')² fragment goat anti-rabbit IgG was obtained from Jackson ImmunoResearch (West Grove, PA, USA). The BCA Protein Assay kit was obtained from Thermo Scientific (Rockford, IL, USA). The LDH Cytotoxicity Detecton Kit was obtained from Roche (Mannheim, Germany). Human recombinant epidermal growth factor (EGF), Trizol and SuperScript III Platinum SYBR Green One-Step qRT-PCR kits were obtained from Invitrogen (Carlsbad, CA, USA). All other reagents were of analytical or molecular biology grade and from Sigma-Aldrich.

Human intestinal epithelial cell culture

The study was conducted according to the NIH guidelines and Partners Human Study Committee approval (IRB# 1999p003833). Fetal intestinal tissue obtained from therapeutic abortions was used for isolation of primary human fetal intestinal epithelial cells (hFIECs) using a procedure modified from that reported by Quaroni¹⁰ as described previously.⁹ The cells were incubated for 3 hours and rinsed vigorously with PBS. Adherent cells were maintained in tissue culture for 3–8 passages before use in experiments. Immunostaining for epithelial markers including E-cadherin, cytokeratin 18, mucin and ZO-1 was performed to ensure the cells were epithelial cells (data not shown). The hFIEC culture medium consisted of OptiMEM supplemented with 20 ng/mL human EGF, 150 nM hydrocortisone 21-hemisuccinate sodium salt, 0.2 U/mL human recombinant insulin and 4% FBS. In addition, the nontransformed primary human fetal intestinal epithelial cell line H4¹¹ was used in these studies. The H4 culture medium consisted of DMEM supplemented with 5% heat-inactivated foetal bovine serum (FBS), 5% heat-inactivated neonatal bovine serum, 1% glutamine, 1% sodium pyruvate, 1% non-essential amino acids, 1% HEPES, 0.2 U/mL insulin, 50 U/mL penicillin and 50 μ g/mL streptomycin. To determine whether TGF- β 2 modulates inflammatory responses in mature IECs, the adult IEC lines T84 and NCM460 were used in these studies. Both T84 and NCM460 cells are derived from the colon, which should be taken into consideration when interpreting the results. T84 culture medium consisted of DMEM supplemented with 5% heat-inactivated foetal bovine serum, 1% glutamine, 2.5 % HEPES, 50 U/mL penicillin and 50 μ g/mL streptomycin. NCM-460 culture medium consisted of M3D medium supplemented with 10 % FBS and 50 U/mL penicillin and 50 μ g/mL streptomycin. The cells were cultured in culture dishes at 37°C with 95% O₂ and 5% CO₂ atmosphere saturated with water vapor.

Effect of conditioning with TGF- β 2 on subsequent inflammatory cytokine secretion

Cells were grown to 70% confluence and treated with 3 ng/mL TGF- β 2 for the indicated times before stimulation with 1 ng/mL IL-1 β , 10 ng/mL TNF- α or 50 ng/mL poly I:C. These proinflammatory stimulants were chosen after preliminary experiments showed they induce consistent and significant inflammatory cytokine secretion in the cell culture models. They also provide a representative selection of proinflammatory insults experienced by the infant gut. TNF- α and IL-1 β are powerful endogenous inflammatory mediators implicated e.g. in the pathogenesis of NEC.¹² Poly I:C, on the other hand, is a synthetic ligand for Toll-like receptor (TLR)3, which binds viral antigens whereas IL-1 β , albeit an endogenous molecule, shares an intracellular signaling pathway with TLRs recognizing bacterial molecular structures.¹³ Medium alone served as control. In some experiments, the cells were grown in medium for 2–24 hours after exposure to TGF- β 2 prior to stimulation with IL-1 β (“washout

period”) as indicated. All experiments were performed in triplicate or quadruplicate. After 18 hours, the culture medium was collected, stored and subjected to IL-8 and IL-6 measurement by ELISA as described previously.⁹ IL-8 and IL-6 secretion in intestinal epithelial cell culture experiments was normalized to total cellular protein content measured as described previously.⁹ LDH Cytotoxicity Detection Kit was used to assess cell viability among groups according to manufacturer’s instructions.

Effect of conditioning with TGF- β 2 on subsequent inflammatory cytokine gene expression

hFIECs cells and H4 cells were grown to 70% confluence and treated with TGF- β 2 for 48 hours before stimulation with IL-1 β . Untreated cells served as control. All experiments were performed in triplicate. After 6 hours, total cellular RNA was extracted by the Trizol-chloroform extraction method as described previously.¹⁴ This time point was chosen based on a preliminary time-course experiment (data not shown). mRNA for IL-8 and IL-6 was measured in duplicate for each sample by quantitative real-time polymerase chain reaction (qRT-PCR) using the SuperScript III Platinum SYBR Green One-Step qRT-PCR kit with MJ Opticon 2 DNA engine (MJ Research Inc., Waltham, MA, USA) according to manufacturer’s instructions. OpticonMONITOR analysis software version 2.01 (MJ Research Inc.) was used to normalize the levels of IL-8 and IL-6 mRNA to the standard GAPDH level for each sample.

Effect of conditioning with TGF- β 2 on subsequent IL-1 β -induced NF- κ B p65 nuclear translocation

H4 cells were grown to 70% confluency on cover slips and conditioned with TGF- β 2 for 48 hours before stimulation with IL-1 β for 10 minutes. Medium alone served as control. The cells were fixed in 4% paraformaldehyde for 20 min on ice. Once permeabilized with methanol (10 min on ice) and blocked with 10% goat serum in Tris-buffered saline (TBS) containing 0.25% BSA. The cells were incubated overnight with a rabbit anti-human NF- κ B p65 polyclonal antibody (1:2000) in TBS with 0.25% BSA. After washing, the cells were incubated with a CyTM 3-conjugated goat anti-rabbit IgG (F(ab')₂ (1:2000) in TBS with 0.25% BSA and examined using a Zeiss Axiophot photomicroscope (Germany) and a digital image was obtained. Purified rabbit IgG was used as a control.

Involvement of intracellular signaling pathways

Immunoblotting—H4 cells were grown to 70% confluency and conditioned with TGF- β 2 for 48 hours before stimulation with IL-1 β for 5, 15, 30, 60, 90 and 120 minutes. Untreated cells were used to determine baseline. The changing levels of activated kinases were determined by immunoblotting. Equal amounts of total cellular protein were fractionated by electrophoresis using NuPAGE 4–12% Bis-Tris Gels with protein standards. Fractionated proteins were transferred onto a PDVF membrane and blocked with 5% nonfat dry milk in TBS with 0.05% Tween 20 (TBST) before incubation overnight with the primary antibody at recommended concentration. The membrane was then washed with 5% milk in TBST and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. The amount of specific protein was visualized by enhanced chemiluminescence using the SuperSignal West Femto Maximum Sensitivity Substrate. To confirm equal loading of the lanes, the membranes were stripped and reprobed with anti-GAPDH antibody.

Inhibition of the ERK pathway—To determine the role of ERK activation in IL-1 β -induced secretion of IL-8 and IL-6, the specific MEK1/2 inhibitor PD 98059 was used. H4 cells were grown to 70% confluence and treated with 100 μ M PD 98059 1 hour prior to stimulation with IL-1 β . Untreated cells served as control.

cDNA microarray

hFIECs were grown to 50% confluence and conditioned with TGF- β 2 for 48 hours. Untreated cells served as control. The experiment was performed in quadruplicate. Total cellular RNA was extracted by the Trizol-chloroform extraction method as described previously¹⁴ and purified using RNA extraction columns obtained from Qiagen as per the manufacturer's instructions. Affymetrix cDNA microarray analysis was carried out by the genomic core facility at Harvard Medical School using Human Genome U133 Plus 2.0 Array. Differences in gene expression between hFIECs exposed and not exposed to TGF- β 2 were analyzed using GeneGo Pathway Maps analysis software with a false discovery rate filter significance level $p < 0.01$.

Statistical analyses

Secretion of IL-8 and IL-6 in cell culture experiments was expressed as means $\pm$ standard error (SE); comparisons between groups were performed using two-tailed Student's t test. Gene expression data obtained by qRT-PCR are expressed as geometric means with SE after logarithmic transformation; comparisons between groups were performed using Student's two-tailed t test after logarithmic transformation. A p value < 0.05 was considered statistically significant.

RESULTS

Conditioning with TGF- β 2 attenuates subsequent inflammatory cytokine secretion in immature human intestinal epithelial cells

To investigate whether exposure to TGF- β 2 modulates subsequent inflammatory responses in immature human IECs, H4 cells were conditioned 48 hours with and without 3 ng/mL TGF- β 2 and then stimulated with IL-1 β , and the inflammatory response was assessed by measuring secretion of IL-8 (Figure 1A) and IL-6 (Figure 1B). IL-1 β increased IL-8 secretion from 0 ± 0 ng/mg protein to 3784 ± 41 ng/mg protein ($p < 0.0001$). Conditioning with TGF- β 2 for 48 hours significantly attenuated subsequent IL-1 β -induced IL-8 secretion to 1294 ± 106 ng/mg protein ($p = 0.00012$). In parallel, IL-1 β increased IL-6 secretion from 100 ± 4 ng/mg protein to 3184 ± 145 ng/mg protein ($p < 0.0001$) and this response was attenuated to 631 ± 52 ng/mg protein ($p = 0.00091$) after conditioning with TGF- β 2. The anti-inflammatory effect of TGF- β 2 was not confined to IL-1 β -induced responses, since a similar reduction in TNF- α -induced IL-8 (Figure 1C) and poly I:C-induced IL-8 (Figure 1D) and IL-6 (Figure 1E) secretion as a consequence of TGF- β 2 conditioning was observed in H4 cells. As previously reported⁹, TGF- β 2 administered simultaneously with IL-1 β resulted in a reduction of IL-8 and IL-6 secretion. A similar modest reduction was observed in TNF- α -induced inflammatory cytokine secretion (Figure 1C) whereas TGF- β 2 administered simultaneously with poly I:C had no effect on inflammatory cytokine secretion (Figure 1D and 1E). The anti-inflammatory effect on IL-1 β or TNF- α -induced cytokine secretion was significantly more pronounced after conditioning with TGF- β 2 for 48 hours. In addition, a significant reduction in poly I:C-induced IL-6 secretion was observed after 48 h conditioning (Figure 1E).

The results obtained from H4 cells were confirmed by conducting similar experiments using primary hFIECs isolated from an 18-week old fetus (Figure 2). IL-1 β induced a significant increase in IL-8 secretion from 43 ± 4 ng/mg protein to 5731 ± 262 ng/mg protein ($p < 0.0001$). Conditioning with TGF- β 2 for 48 hours attenuated IL-1 β -induced IL-8 secretion to 3788 ± 133 ng/mg protein ($p = 0.0027$, Figure 2A). IL-1 β increased IL-6 secretion from 127 ± 11 ng/mg protein to 5297 ± 306 ng/mg protein ($p < 0.0001$), which was reduced to 1754 ± 21 ng/mg protein by exposure to TGF- β 2 ($p = 0.00032$, Figure 2B). A significant

reduction in TNF- α -induced IL-8 (Figure 2C) and IL-6 (Figure 2D) secretion as a consequence of TGF- β 2 conditioning was detected in hFIECs.

TGF- β 2 had no effect on inflammatory responsiveness in mature human colonic IECs. IL-1 β induced IL-8 secretion in NCM460 cells (Figure 3A) and TNF- α -induced IL-8 secretion in T84 cells (Figure 3B) remained unaltered after conditioning with TGF- β 2.

To investigate whether exposure to TGF- β 2 induces a long-lasting change in IEC immune responder phenotype, H4 cells were conditioned with TGF- β 2 for 24 hours and then stimulated with IL-1 β after a 2–24 hour “washout period” with medium alone (Figure 4). The 24-hour conditioning with TGF- β 2 resulted in a significant attenuation of subsequent IL-1 β -induced IL-8 (Figure 4A) and IL-6 (Figure 4B) secretion. It is of note that presence of TGF- β 2 at the time of stimulation with IL-1 β was not necessary for the inhibitory effect. Moreover, the immunomodulatory effect of TGF- β 2 conditioning remained unaltered after up to 24 hours in medium devoid of TGF- β 2.

Conditioning with TGF- β 2 attenuates subsequent IL-1 β -induced inflammatory cytokine secretion in immature intestinal epithelial cells by reducing mRNA expression

The effect of conditioning with TGF- β 2 on subsequent IL-1 β -induced expression of IL-8 and IL-6 mRNA in H4 cells was assessed using qRT-PCR (Figure 5A and B). Stimulation with IL-1 β resulted in a 6849-fold increase in IL-8 mRNA ($p < 0.0001$) after 6 hours. This response was reduced to 540-fold by prior conditioning with TGF- β 2 ($p = 0.00011$). In a similar fashion, IL-6 mRNA expression was increased 691-fold by IL-1 β ($p < 0.0001$) but the response was attenuated to 39-fold after conditioning with TGF- β 2 ($p < 0.0001$).

Conditioning with TGF- β 2 reduces IL-1 β -induced NF- κ B nuclear translocation by inhibiting I κ B- α degradation

Nuclear translocation of the transcription factor NF- κ B is an important event in IL-1 β -induced transcriptional activation of inflammatory gene expression. Stimulation with IL-1 β was observed to induce nuclear translocation of NF- κ B p65 within 10 minutes in H4 cells (Figure 6A). Conditioning with TGF- β 2 for 48 hours prior to stimulation with IL-1 β partially inhibited IL-1 β -induced nuclear translocation of NF- κ B p65 (Figure 6A).

Degradation of I κ B- α is necessary for the release and subsequent nuclear translocation of NF- κ B p65. In order to assess whether IL-1 β -induced I κ B- α degradation was reduced in H4 cells conditioned with TGF- β 2, the rate of I κ B- α degradation was monitored by immunoblotting. Stimulation with IL-1 β led to rapid degradation of I κ B- α in H4 cells within 15 minutes of stimulation (Figure 6B). Conditioning of H4 cells with TGF- β 2 for 48 hours partially inhibited subsequent IL-1 β -induced degradation of I κ B- α (Figure 6B).

We have recently reported that, when administered simultaneously with IL-1 β , TGF- β 2 inhibits IL-1 β -induced phosphorylation of ERK in H4 cells and this inhibitory effect is causally related to attenuation of IL-1 β -induced IL-8 and IL-6 secretion by TGF- β 2.⁹ However, in H4 cells conditioned with TGF- β 2 48 hours before stimulation with IL-1 β , a modest reduction in ERK phosphorylation not exceeding that resulting from exposure to TGF- β 2 simultaneously with IL-1 β was observed (Figure 7A). In line with this observation, conditioning with TGF- β 2 induced a significant further reduction in IL-1 β -induced IL-8 (Figure 7B) and IL-6 (Figure 7C) secretion when ERK signaling was inhibited by the specific ERK kinase inhibitor PD 98059. Taken together, these data suggest that modulation of ERK signaling is not the primary mechanism by which conditioning with TGF- β 2 attenuates IEC inflammatory responses.

Effect of TGF- β 2 conditioning on expression of genes in the IL-1 β signaling pathway

Systematic analyses of functional gene groups and pathways contained within the well-annotated MetaCore database canonical gene pathway maps (MA) using our gene expression data obtained by cDNA microarray from hFIECs identified enrichment of genes involved in several signaling pathways or cellular processes. Our data indicate that conditioning with TGF- β 2 for 48 hours had a significant impact on the expression of genes in a number of physiological processes including development and immune responses. The ten most significantly affected pathways by conditioning with TGF- β 2 for 48 hours are presented in Table 1. Of particular interest, the IL-1 signaling pathway was amongst the most significantly modulated pathways which allowed further investigation into gene expression patterns within this pathway (Table 2). Among the down-regulated genes, several proteins that are known to be positive regulators of IL-1 signaling and among the up-regulated genes, a number of proteins that are known to be negative regulators were identified (Table 2). Results obtained from cDNA microarray analyses were confirmed using qRT-PCR for selected key genes in the IL-1 signaling pathway. According to the qRT-PCR analyses, conditioning with TGF- β 2 for 48 hours increased the expression of I κ B- α 1.55 fold ($p=0.047$) and decreased that of MyD88 2.70 fold ($p=0.058$). These results correspond to those obtained by cDNA microarray analysis.

DISCUSSION

Our studies demonstrate that conditioning immature human IECs with TGF- β 2 at a concentration corresponding to that in breast milk modulates their response to subsequent pro-inflammatory stimulation. Secretion of the inflammatory cytokines IL-8 and IL-6 in response to IL-1 β , TNF- α and the TLR3 ligand poly I:C was significantly reduced in human fetal IECs conditioned with TGF- β 2 for 48 hours prior to the pro-inflammatory insult. Importantly, the reduction in IEC inflammatory cytokine secretion after conditioning with TGF- β 2 was more pronounced than that previously reported⁹ when IECs were treated with TGF- β 2 simultaneously with pro-inflammatory stimulation and this anti-inflammatory effect was not dependent on TGF- β 2 being present during stimulation with IL-1 β . It is also of note that conditioning with TGF- β 2 is necessary for significant attenuation of IL-8 secretion in response to poly I:C not seen with simultaneous stimulation. Taken together, these findings suggest that the anti-inflammatory effect of breast milk TGF- β 2 on immature human IECs spans inflammatory responses elicited towards a wide range of endogenous and microbial signals and is mediated by several distinct mechanisms.

We have recently reported that TGF- β 2 inhibits IL-1 β -induced activation of the ERK signaling pathway in immature IECs if administered simultaneously with the pro-inflammatory stimulus and that intact ERK signaling is necessary for an optimal inflammatory cytokine mRNA expression and protein secretion.⁹ However, conditioning with TGF- β 2 48 hours prior to stimulation did not result in a greater inhibition of ERK activation as compared to when TGF- β 2 was introduced simultaneously with IL-1 β whilst the reduction in IL-1 β -induced IL-8 and IL-6 response achieved by conditioning with TGF- β 2 was greater than that observed after blocking ERK signaling by the specific inhibitor PD 98059 (Figure 7). These data demonstrate that, in contrast to the immediate effect of TGF- β 2 on immature IEC inflammatory responsiveness, inhibition of the ERK signaling pathway is not the primary mechanism by which conditioning with TGF- β 2 attenuates subsequent IL-1 β -induced inflammatory cytokine secretion in immature human IECs.

According to a report by Choi and colleagues,¹⁵ TGF- β 1 inhibits IL-1 β -induced nuclear translocation of the transcription factor NF- κ B by reducing I κ B- α degradation. In line with this observation, we detected a reduction in IL-1 β -induced I κ B- α degradation in H4 cells conditioned with TGF- β 2 for 48 hours (Figure 6B). Furthermore, the same treatment

appeared to partially inhibit nuclear translocation of the p65 subunit of NF- κ B as visualized by immunofluorescence, (Figure 6A) which may at least partially explain the reduction in IL-1 β -induced IL-8 and IL-6 mRNA expression and IL-8 and IL-6 protein secretion observed in immature IECs conditioned with TGF- β 2 (Figure 5).

Significant changes in the expression of genes involved in the IL-1 signaling pathway were detected in immature human IECs conditioned with TGF- β 2 for 48 hours according to a preliminary cDNA microarray analysis (Table 2). These data suggest that the observed decrease in IL-1 β -induced I κ B- α degradation and subsequent inflammatory cytokine gene expression and cytokine secretion in immature IECs conditioned with TGF- β 2 may in part be explained by increased expression of mRNA for I κ B- α and reduced expression of mRNA for MyD88 and IKK- β , the function of which is to degrade I κ B- α when activated in by e.g. IL-1 β . Our laboratory has previously reported that the expression of I κ B- α is developmentally underexpressed in immature IECs¹⁶ and our present data may therefore be interpreted to suggest that breast milk TGF- β 2 induces a maturational upregulation of this gene and thus promotes a more mature, anti-inflammatory tone in the intestinal epithelium. The changes in the expression of genes relevant to the present investigation induced by TGF- β 2 were not confined to these intracellular signaling molecules. The expression of IL-1 receptor mRNA was significantly decreased in IECs conditioned with TGF- β 2, which might suggest that responsiveness to IL-1 β in these cells may be diminished as a consequence of reduced expression of the receptor on the cell surface. Assessing cell surface protein expression and more detailed analyses of the microarray data are beyond the scope of the present report. Nonetheless, these expression data highlight the potential clinical importance of breast milk TGF- β 2 in the modulation of expression of genes regulating innate immunity and inflammation.

It is well-established that TGF- β is involved in maturation processes in immune cells.² TGF- β induces IgA class switch in B cells¹⁷ and alters antigen-presenting cell function¹⁸ and thus modulates T cell maturation. In particular, TGF- β favors generation of regulatory T cells which are essential in tolerance towards e.g. dietary antigens and indigenous intestinal microbes.¹⁹ Excessive expression of inflammatory cytokines in immature human intestinal macrophages has recently been shown to be suppressed as a result of maturation upon exposure to TGF- β 2.⁵ It is intriguing to speculate that TGF- β 2 might also have a maturational effect on neonatal human IECs, which are in direct contact with breast milk TGF- β 2. Based on our cDNA microarray data, conditioning with TGF- β 2 induces changes in the expression of genes involved in cytoskeleton remodeling and development (Table 1), which may be interpreted to support the notion of TGF- β 2-induced generalized maturation in immature IECs. Importantly, according to our present observations and in contrast to our previous report⁹, conditioning with TGF- β 2 induces functional changes in IEC inflammatory responsiveness to IL-1 β , which are not dependent on TGF- β 2 being present at the time of the pro-inflammatory insult and remain unaltered after up to a 24-hour period devoid of TGF- β 2 (Figure 4). Unfortunately, the cell culture models used in the present series of experiments did not allow longer exposure or washout times to further assess the matter. Nonetheless, together with the fact that mature IEC responses were not affected by exposure to TGF- β 2 (Figure 3), we interpret our data to suggest that neonatal gut immune responder may be modified by exposure to TGF- β 2 and that breast milk TGF- β 2 may act as an important maturational signal to the developing intestine. This notion is consistent with data from experimental animal studies demonstrating that TGF- β in breast milk is necessary for healthy immune maturation as indicated by defective formation of immune tolerance to inhaled²⁰ or dietary antigens²¹ in newborn animals deprived of breast milk TGF- β function, as well as epidemiological data suggesting an inverse correlation between breast milk TGF- β 2 and subsequent development of immune-mediated disease in infants and children.⁸

We conclude that our data demonstrate that conditioning with TGF- β 2 at a concentration comparable to that found in breast milk profoundly affects immature human IEC immune responsiveness. We hypothesize that breast milk TGF- β 2 might function as a maturational stimulus to the infant's developing intestine and have a long-term impact on immune responsiveness. It is conceivable that supplementing premature infants with TGF- β 2 may provide a novel means to support gut maturation and reduce the risk of disorders resulting from intestinal immaturity such as NEC. Further investigation into immune conditioning by breast milk TGF- β 2 in more complex *in vivo* models including experimental animals and eventually clinical studies are warranted.

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Abbreviations

ELISA	Enzyme-linked immunosorbent assay
IκB-α	inhibitor of κ B
IL	Interleukin
NF-κB	Nuclear factor κ B
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
TGF-β	Transforming growth factor- β

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A. IL-1 β -induced IL-8 secretion in H4 cells.

*** $p < 0.001$ compared to IL-1 β -induced IL-8 secretion

B. IL-1 β -induced IL-6 secretion in H4 cells.

*** $p < 0.001$ compared to IL-1 β -induced IL-6 secretion

C. TNF- α -induced IL-8 secretion in H4 cells.

** p<0.01 compared to TNF- α -induced IL-8 secretion

*** p<0.001 compared to TNF- α -induced IL-8 secretion

D. poly I:C-induced IL-8 secretion in H4 cells.

E. poly I:C-induced IL-6 secretion in H4 cells.

Figure 1. Conditioning with TGF-β2 attenuates subsequent IL-1β, TNF-α and poly I:C-induced inflammatory cytokine secretion in H4 cells

IL-1β induced a significant increase in IL-8 (A) and IL-6 (B) secretion in H4 cells. The IL-1β-induced IL-8 and IL-6 responses were moderately reduced by TGF-β2. A more profound reduction in IL-8 (A) and IL-6 (B) secretion was observed in cells conditioned with TGF-β2 48 hours before stimulation with IL-1β. In a similar fashion, TNF-α induced a significant IL-8 response, which was attenuated by conditioning with TGF-β2 (C). Stimulation with poly I:C led to a significant IL-8 (D) and IL-6 (E) response. TGF-β2 administered simultaneously with poly I:C had no effect on the inflammatory response. However, 48-hour conditioning with TGF-β2 decreased poly I:C-induced inflammatory cytokine secretion.

A. IL-1 β -induced IL-8 secretion in hFIECs.

** p<0.01 compared to IL-1 β -induced IL-8 secretion

B. IL-1 β -induced IL-6 secretion in hFIECs.

** p<0.01 compared to IL-1 β -induced IL-6 secretion

*** p<0.001 compared to IL-1 β -induced IL-6 secretion

C. TNF- α -induced IL-8 secretion in hFIECs.

D. TNF- α -induced IL-6 secretion in hFIECs.

* $p < 0.05$ compared to TNF- α -induced IL-6 secretion

Figure 2. Conditioning with TGF- β 2 attenuates subsequent IL-1 β and TNF- α -induced inflammatory cytokine secretion in primary human fetal intestinal epithelial cells (hFIECs) obtained from an 18-week old fetus

IL-1 β induced a significant increase in IL-8 (A) and IL-6 (B) secretion in hFIECs. The IL-1 β -induced IL-8 and IL-6 responses were moderately reduced by TGF- β 2. A more profound reduction in IL-8 (A) and IL-6 (B) secretion was observed in cells conditioned with TGF- β 2 48 hours before stimulation with IL-1 β . In a similar fashion, TNF- α induced significant IL-8 (C) and IL-6 (D) responses, which were attenuated by conditioning with TGF- β 2.

A. IL-1 β -induced IL-8 secretion in NCM460 cells.

B. TNF- α -induced IL-8 secretion in T84 cells.

Figure 3. Conditioning with TGF- β 2 has no effect on subsequent IL-1 β or TNF- α -induced inflammatory cytokine secretion in mature human intestinal epithelial cells

IL-1 β induced a significant IL-8 response in the nontransformed adult human intestinal epithelial cell line NCM460 (A). Exposure to TGF- β 2 simultaneously with IL-1 β or conditioning with TGF- β 2 for 48 hours had no impact on this inflammatory response. In a similar manner, the human adult colon cancer cell line T84 responded to stimulation with TNF- α by a significant increase in IL-8 secretion, which was not affected by TGF- β 2 (B).

A. IL-8 secretion.

*** p<0.001 compared to IL-1 β -induced secretion of IL-8

B. IL-6 secretion

Figure 4. Attenuation of IL-1β-induced IL-8 and IL-6 secretion in H4 cells after conditioning with TGF-β2 remains unaltered up to 24 hours after exposure

IL-1β induced a significant increase in IL-8 (A) and IL-6 (B) secretion in H4 cells. Conditioning with TGF-β2 for 24 hours significantly attenuated these responses. The effect was not dependent on TGF-β2 being present at the time of stimulation and remained unaltered after up to a 24-hour washout period.

A. IL-8 mRNA

*** p<0.001 compared to IL-1β-induced IL-8 mRNA expression

B. IL-6 mRNA

*** $p < 0.001$ compared to IL-1 β -induced IL-6 mRNA expression

Figure 5. Conditioning with TGF- β 2 reduces subsequent IL-1 β -induced inflammatory cytokine gene expression in H4 cells

IL-1 β induced a marked increase in the expression of IL-8 (A) and IL-6 (B) mRNA 6 hours after stimulation. This response was significantly reduced when the cells were conditioned with TGF- β 2 48 hours prior to stimulation.

A. Nuclear translocation of NF- κ B p65.

6B. Degradation of I κ B- α in H4 cells.

Figure 6. Conditioning with TGF- β 2 reduces IL-1 β -induced NF- κ B p65 nuclear translocation by inhibiting degradation of I κ B- α in H4 cells

Stimulation with IL-1 β rapidly induced nuclear translocation of the transcription factor NF- κ B p65 subunit in H4 cells (**A**) as visualized by immunofluorescence. This phenomenon was partially inhibited in H4 cells conditioned with TGF- β 2 48 hours prior to stimulation. Rapid degradation of the NF- κ B inhibitor I κ B- α was detected by immunoblotting in H4 cells stimulated with IL-1 β (**B**), and this degradation was partially inhibited in H4 cells conditioned with TGF- β 2 48 hours prior to stimulation.

7A. ERK phosphorylation in H4 cells.

B. IL-8 secretion

** p<0.01 compared to IL-1 β -induced IL-8 secretion
 *** p<0.001 compared to IL-1 β -induced IL-8 secretion
 # p<0.05 compared to IL-1 β -induced IL-8 secretion in cells treated with PD 98.059

C. IL-6 secretion

Figure 7. Modulation of ERK activation is not the primary mechanism by which conditioning with TGF- β 2 attenuates subsequent IL-1 β -induced inflammatory cytokine secretion in H4 cells Stimulation with IL-1 β rapidly induced phosphorylation of ERK as demonstrated by immunoblotting for p-ERK, ERK and GAPDH at the indicated time points in H4 cells (A). As reported previously, TGF- β 2 significantly reduced IL-1 β -induced ERK activation when administered simultaneously with the proinflammatory insult. Conditioning with TGF- β 2 48 hours prior to stimulation had no additional impact on IL-1 β -induced ERK activation (A). When ERK signaling was inhibited in H4 cells by the specific inhibitor PD 98059, IL-1 β -induced secretion of IL-8 (B) and IL-6 (C) were significantly reduced. However, in H4 cells conditioned with TGF- β 2 48 hours prior to stimulation, secretion of IL-8 (B) and IL-6 (C)

were reduced even further. These data suggest that attenuation of IL-1 β -induced inflammatory cytokine secretion by TGF- β 2 conditioning is not primarily mediated by inhibition of ERK signaling.

Table 1

Physiological function pathways in primary hFIECs most significantly ($p < 0.01$) affected by 48-hour exposure to TGF- β 2 as assessed by MetaCore analysis of cDNA microarray data.

Pathway	Function
1. TGF, WNT and cytoskeletal remodeling	Cytoskeleton remodeling
2. Cytoskeleton remodeling	Cytoskeleton remodeling
3. Chemokines and adhesion	Cell adhesion
4. Clathrin-coated vesicle cycle	Intracellular Transport
5. Anti-apoptotic TNFs/NF- κ B/Bcl-2 pathway	Apoptosis and survival
6. Receptor-mediated axon growth repulsion	Neurophysiological process
7. WNT signalling pathway, Part 2	Development
8. IL-1 signalling pathway	Immune response
9. ECM remodelling	Cell adhesion
10. Sin3 and NuRD in transcription regulation	Transcription

Table 2

Exposure to TGF- β 2 modulates expression of genes involved in the IL-1 signalling pathway in immature human intestinal epithelial cells.

Positive regulator	Fold change	Negative regulator	Fold change
<u>IL-1RI</u> Interleukin-1 receptor type I	-2.69	<u>IL1RAP</u> Interleukin-1 receptor accessory protein	1.56
<u>MyD88</u> Adaptor protein involved in IL-1 signalling	-1.39	<u>IκB-α</u> Inhibitor of NF- κ B	1.49
<u>IRAK4</u> Required for optimal IL-1 signalling	-1.27		
<u>MEKK1</u> Phosphorylates IKK- α and IKK- β	-1.46		
<u>NIK</u> Phosphorylates IKK- α	-1.25		
<u>IKK-β</u> Phosphorylates I κ B- α leading to degradation	-1.50		
<u>STAT1</u> Activator of transcription	-1.92		
<u>c-Jun</u> Transcription factor	-1.31		
<u>AP-1</u> Transcription factor	-2.67		

Exposure to TGF- β 2 for 48 hours significantly modulated expression of a number of genes in the IL-1 signalling pathway as assessed by cDNA microarray analysis in primary fetal intestinal epithelial cells. Statistically significant ($p < 0.01$) gene expression changes induced by exposure to TGF- α 2 as detected using GeneGo Pathway Maps analysis software are presented.