

REVIEW

Milk-derived or recombinant transforming growth factor-beta has effects on immunological outcomes: a review of evidence from animal experimental studies

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Summary

Identified factors from milk have been shown to improve health outcomes. One specific factor, transforming growth factor-Beta (TGF)- β , has been identified previously as having the potential to impact on immunological outcomes in the newborn offspring. The primary objective of this review was to examine the published studies that have considered TGF- β in association with immunological outcomes of experimental models. We hypothesized that oral administration of TGF- β (through human milk, cow's milk, infant formula) or recombinant TGF- β delivered via gavage, may down-regulate immune activation in newborn offspring. Animal experimental studies were identified through MEDLINE, CAB Abstracts, Biological Abstracts and Scopus. Selection criteria included well-described animal populations, sample and study design, source of TGF- β , age and immunological outcomes measured and effect size. The findings were summarized temporally in tabular format, giving an overall measure of effect based on the literature available since 1994. Animal experimental studies ($n = 13$) were included in the review to determine an association between maternal TGF- β and immunological outcomes. Overall 92% of these studies (12/13) showed a positive association with TGF- β 1 or TGF- β 2, demonstrating protection against immunologically related outcomes in early life in an animal model. TGF- β is important in developing and maintaining appropriate immune responses in the offspring. TGF- β delivered orally to neonatal animals provides protection against adverse immunological outcomes, corroborating and supporting findings from human studies. Animal studies provide important clues to the pathogenesis and therapeutics of immune activation and allergy in early childhood. TGF- β s are important growth factors involved in maintaining homeostasis in the intestine, regulating inflammation and allergy development and promoting oral tolerance in infants. Thus, taken as a whole, these and our other findings suggest that this cytokine in milk may influence the development of immunological outcomes in offspring.

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Introduction

Growing evidence suggests that transforming growth factor-beta (TGF)- β , a multifunctional polypeptide, may be a key immunoregulatory factor for the establishment of the development and maturation of the mucosal immune system [1–8]. TGF- β has been shown to have multiple functions with regard to immunology. It can act as a trophic factor causing maturation within cells or it can specifically affect anti-inflammation in an immune-stimulated process [9]. Three highly related mammalian

isoforms of TGF- β have been identified: TGF- β 1, TGF- β 2 and TGF- β 3 [10]. Although the significance of each is unknown, TGF- β 2 is the predominant isoform in rat milk and TGF- β receptors which facilitate milk-TGF- β uptake are present in the rat pup intestine [11].

The importance of TGF- β to the development of the immune system was demonstrated in ground-breaking research [1, 12, 13]. This research showed that disruption of the TGF- β 1 gene by homologous recombination in murine embryonic stem cells enabled mice to be generated that carry the disrupted allele. Animals homozygous for

the mutated TGF- β 1 allele showed no gross developmental abnormalities; however, they succumbed to a wasting syndrome about 20 days after birth that was accompanied by a multifocal, mixed inflammatory cell response and tissue necrosis, leading to organ failure and death [12]. Subsequently, TGF- β -deficient mice (i.e. TGF- β 1 gene knockout) were observed to survive for the first few weeks of life while nursing, indicating that maternal sources of TGF- β 1, via both placental transfer and milk, provided for and supported the normal development and postnatal survival of these mice [1]. Thus, original work showed that TGF- β 1 has profound regulatory effects on many developmental and physiological processes [12, 13].

It has been almost two decades since human milk was shown to contain cytokines, small soluble glycoproteins that act in an autocrine-paracrine fashion by binding to specific cellular receptors, operating in networks, and orchestrating immune system development and function [14–19]. Reviews related to the regulation of immune responses by TGF- β and other animal studies support the importance of this milk cytokine [20–22]. Although the various mechanistic pathways by which TGF- β modulates the development and maintenance of the immune system have not been fully recognized, TGF- β appears to play an important immune-regulatory role on targets in autoimmunity, immunodeficiencies, and other immune-mediated pathologies, and we have reported previously a systematic review of the immunological effects of TGF- β in human infants [23].

The hypothesis of the current review was that administration of TGF- β (orally through maternal milk, cow's milk, formula or recombinant TGF- β via gavage) may bring balance to the immune system through its specific actions in immune function. Our objective was to provide a temporal perspective of the importance of TGF- β in relation to immunological outcomes, as shown from the results of animal experimental studies. Therefore, the review was based on results from published studies only, to address the specific question, 'does TGF- β bring balance to the immune system function and development through its specific actions in the offspring?'.

Methods

Literature sources and search terms

The studies considered for inclusion in this review were identified by multiple pass searches of computerized bibliographic databases conducted in March 2007 through March 2010. These bibliographic databases included searches of MEDLINE (National Library of Medicine, Bethesda, MD, USA), CAB Abstracts (applied life sciences search site), Biological Abstracts and Scopus. For each of the multiple searches, all articles that included the words TGF- β , cytokines, growth factors, human milk, milk,

immunological outcomes, infants, asthma, allergy, atopy, or infections in the title or in the key words were selected. The lists of papers from the various searches were merged, and the resulting articles were examined for inclusion or exclusion. The bibliographic citations of each of the articles ultimately selected for inclusion in the analyses were further examined to identify other acceptable studies not captured by the bibliographic data searches.

Study selection

Our systematic review of human studies is reported elsewhere [23]. Here, we report on studies that were conducted in appropriate animal experimental populations only. When studies identified during the bibliographic search were excluded from the review, a record was maintained and the reason for exclusion was categorized. Usually a paper was excluded because the objectives did not pertain to the purpose of this review. Although many studies were identified and excluded, they were not all listed herein because the list was extensive (> 12 000 hits).

Data abstraction

The suitability of each study for inclusion in the review was determined on a minimum of three phases, and followed an intensive examination of the reported data. Inclusion criteria for animal experimental studies were well-described populations of animals, well-described study design, outcome measures and analytical method of determination of TGF- β (bioassay, ELISA) in milk, in oral administration or TGF- β in a recombinant form. This limited the number of studies available but ensured the quality of those that were included. The data from each study were extracted and tabulated and included author and year of publication, aim of the study, descriptive information concerning the country and setting, study design and sample, exposure measure such as source and how TGF- β was determined (bioassay, ELISA), isoform and type of TGF- β (free, latent, active), how the TGF- β was administered (oral or gavage), age and the outcome measured, statistical effect measured and the effect size, additional comment, and an indication of overall effect (positive+, negative– or not significant NS). The primary immunological outcome variables measured in the studies included immunoglobulin titres such as IgA, IgG or IgE, leucocyte counts, oral tolerance IgE, natural killer cells, eosinophils, mast cells, bronchoalveolar lavage (BAL) fluid (typically performed to diagnose lung disease) and allergy-related immune reactions. Also accepted for inclusion were induced colitis and induced endotoxemia. The relevant studies were all published in the peer-reviewed scientific literature.

Results

An OVID search between 1994 and 2007 for TGF- β showed 25 959 results. A search for TGF- β 1 or TGF- β 2 showed 6858 results. A combination search of 'milk' and 'TGF- β 1 or TGF- β 2' and 'animal experimental studies' showed 11 results, all of which were included within the review and identified with cross-referencing. In March 2010, a final search was conducted to identify any published literature that had not been included in the initial review and two additional studies were identified and included. The conclusion from this search process was that all relevant papers ($n = 13$) were included within the review. Therefore, the results from each original study were tabulated (Table 1). Owing to the heterogeneous nature of the studies, they are discussed temporally in order of publication year.

The first study to examine maternal sources of TGF- β during development in the foetus [1] showed that TGF- β 1 null embryos and newborn mouse pups born to TGF- β 1 heterozygotes immunostained positive for TGF- β 1 whereas those born to a null female were negative and had severe cardiac abnormalities. These results suggested an important role for maternal sources of TGF- β 1 during development and provided evidence for maternal rescue of targeted gene disruption in the foetus. It was not clear whether active or latent TGF- β 1 was the most physiologically relevant form transferred to the foetus and whether this protein was transferred in a complex with carrier proteins. IgG has been seen to associate with TGF- β 1 in a form that is capable of regulating lymphocyte function; therefore, IgG transferred from mother to foetus through the placenta and to the neonate through the milk [1] may also be a source of TGF- β 1. Additionally maternal cells delivered to the infant via suckling may provide another source of TGF- β . Gastrointestinal absorption of intact TGF- β was observed after administration of radiolabelled TGF- β 1 into the stomach of 5-day-old rat pups. These data identified maternal milk as a source of TGF- β 1 for the neonate. Although the precise contribution of maternal TGF- β 1 to normal post-partum development was not defined, the hypothesis that maternal TGF- β 1 is critical for the null foetus was substantiated.

Following Letterios discoveries, Ishizaka and team examined the immuno-regulatory effect of oral administration of TGF- β to mice on the activities of natural killer (NK) cells, which can act as antiviral effector cells against viral infections [24]. They showed that large amounts of TGF- β in human milk were involved in early antiviral protection through the augmentation of NK-cell activities ($P < 0.001$ compared with controls) when tested in the mouse model. These investigators showed that a large amount of the latent form of TGF- β 1 was converted to the active form due to acid in the stomach. They noted a relatively low conversion rate of the latent form to the

active form of TGF- β in human milk, possibly resulting from the buffering capacity of gastric acid by milk.

The previous published studies highlighted the importance of TGF- β having essential immunoregulatory roles. In 1998, Pentilla determined the ontogeny of TGF- β 1-producing cells in the rat pup small intestine and assessed maternal milk concentrations of TGF- β [11]. Intestinal tissue samples of duodenum and ileum were collected, processed, and immunostained for TGF- β , and *in situ* hybridization for TGF- β 1 mRNA was performed on the duodenum. TGF- β levels in milk were assayed by ELISA. TGF- β 2 levels in milk were high at day 6 but was not detected by days 10 and 19. In contrast, the cell number and intensity of staining of TGF- β 1 peptide in the small intestine was low in 3- and 10-day-old rats and increased markedly by 19 days of life. In the duodenum, mRNA levels mirrored this trend. TGF- β expression in the lamina propria was absent before day 19, and increased progressively over time. This study showed that maternal milk TGF- β 2 levels were high in early milk and decreased during the weaning period, whereas in contrast, endogenous TGF- β production in the small intestine of the rat pup was low in the early days and increased during the weaning period. Although this study showed non-significant results it was informative for subsequent studies.

Oral whey protein extract may induce TGF- β secreting cells after oral sensitization with ovalbumin (OVA). In 2001, Pentilla tested the effect of daily oral administration of a growth factor enriched fraction from milk whey to naturally suckling rat pups on immune activation to OVA assessed by lymphocyte proliferation [25]. The role of TGF- β requires confirmation but TGF- β is known to be secreted by lymphocytes during tolerance induction and to down-regulate immune response [3]. Conclusions from this study were that oral administration of growth factor from whey can significantly down-regulate immune activation in rat pups ($P < 0.05$ compared with controls).

In 2003, Pentilla assessed the role of TGF- β 2 in mediating mucosal immune development and specifically the effect on IL-18 in suckling rat pups [26]. An important role for maternal milk was suggested in regulating immune responses after exposure to food antigens, which might otherwise induce damaging immune responses in the neonatal intestine. This regulation was potentially mediated by milk TGF- β 2 as well as endogenous IL-18, as these were significant for both tests ($P = 0.002$). The authors hypothesized that, in formula-fed infants, the potential for appropriate immune development to oral antigen exposure may be decreased as the cytokine milieu provided by breast milk is not present to help the process of immunoregulation [26, 27].

Further in 2003, Ling proposed that exogenous fatty acids in general can be designed to potentiate TGF- β and other growth factor activities in order to treat disease [28]. These investigators determined the effects of fatty acids

Table 1. Animal experimental studies – characteristics of the studies that examined milk-derived or recombinant TGF- β and immune outcomes in animals included in the systematic review

References	Aim of study	Country	Study design, sample and exposure measure (type of TGF- β and how administered)	Age and outcome measured	Statistical effect measured	Comment Effect size	Overall effect
Letterio et al. [1]	To test whether TGF- β supplied to foetus and newborn from maternal sources is essential for normal development	USA	TGF- β null newborn mouse pups fed milk-derived TGF- β orally Active and latent TGF- β 1 and 2	Embryos and newborns TGF- β 1 protein	Identification of maternal milk as source of TGF- β for neonate	TGF- β null embryos and newborn pups born to TGF- β heterozygotes immunostained + for TGF- β , whereas those born to a null female were negative and had severe cardiac abnormalities. Results suggest an important role for maternal sources of TGF- β during development and provide evidence for maternal rescue of targeted gene disruption in foetus Large amounts of TGF- β 1 in human milk are involved in early antiviral protection through the augmentation of NK-cell activities. Types 1, 2 and 3 isoforms were detected in bioassay. There appears to be a threshold effect of TGF- β administration $P < 0.05$ compared with control	+
Ishizaka et al. [24]	To examine the immuno-regulatory effect of oral administration of TGF- β on the activities of NK cells, which can act as antiviral effector cells against viral infections	Japan	Pathogen free female mice healthy mothers Bioassay latent form to active form in which at least 3 isoforms of TGF- β 1 from human platelets, milk or pre-formed were orally administered	1 month after delivery Natural killer cells acting as antiviral effector cells	Mean $\pm$ SD Students T test	Large amounts of TGF- β 1 in human milk are involved in early antiviral protection through the augmentation of NK-cell activities. Types 1, 2 and 3 isoforms were detected in bioassay. There appears to be a threshold effect of TGF- β administration $P < 0.05$ compared with control	+
Penttila et al. [11]	To determine the ontogeny of TGF- β 1-producing cells in the rat pup small intestine and assess maternal milk concentrations of TGF- β	Australia	8 rat pups Human TGF- β 1 ELISA (R&D Systems, UK)	3, 10, 19, 22, 29 and 84 days after birth GI segments	Mean $\pm$ SD Mann-Whitney U -test T -test	Although the results of this study were non-significant they were informative for the studies to follow by this investigator	NS
Penttila [25]	To study effect of daily oral administration of a growth factor enriched fraction from milk whey to naturally suckling rat pups on immune activation to OVA assessed by lymphocyte proliferation	Australia	Rat pups Daily oral administration of GF from whey – TGF- β 1 activated cells were measured	Days 4–9 postnatal <i>In vitro</i> immune response	Mann-Whitney U -test	Oral administration of GF from whey can significantly down-regulate immune activation in rat pups. $P < 0.05$ compared with control	+
Penttila et al. [26]	To assess the role of TGF- β in mediating mucosal immune development and specifically the effect on IL-18	Australia	Random group assignment of rat pups (1) naturally suckled (2) maternal milk with canula (3) formula fed (FF) (4) FF w/recombinant TGF- β 2 (R&D Systems, Minneapolis, MN, USA) – a physiological concentration equivalent to amount of TGF- β 2 in mid-lactation rat milk	14 days Immune response profile – eosinophils, mast cells	Median test Kruskal-Wallis test and Dunn's post-test	An important role for maternal milk is suggested in regulating immune responses after exposure to food antigens, which might otherwise induce damaging immune responses in the neonatal intestine. This regulation is potentially mediated by milk TGF- β 2, as well as endogenous IL-18. Concentration rather than dose was tested due to the high TGF- β cost. $P = 0.002$ for both tests This study was important	+
Ling et al. [28]	To determine effect of fatty acids on complex formation of TGF- β	Taiwan	Mice Mink lung epithelial cells Fatty acids effects on TGF- β 1 and TGF- β 2 activity	Lab based research – age NA	Mean $\pm$ SD	Exogenous fatty acids can be designed to potentiate TGF- β and other growth factor activities in order to treat	+

Zemann et al. [29]	isoforms and plasma clearance of complexes To investigate primary oral tolerance to OVA in an IGE high-responder dog model, which is more predictive of human allergic disease than rodent models. This study did not consider milk-derived TGF- β effects	Novartis Research Institute	IgE high responder dog model – OVA fed beagles – high and low responders – tolerized and control dogs (6 each) mRNA expression of TGF- β (type not specified)	Newborn puppies BAL fluid cell Oral tolerance IgE, IgG	Wilcoxon Mann-Whitney <i>U</i> -test Student <i>T</i> -test	+	disease. No milk-derived TGF- β effects were studied The purpose of this study was to demonstrate a link between the suppression of allergic symptoms and induction of regulatory IL-10 and TGF- β . An increased TGF- β expression in tolerized BAL cells could be the mechanism of oral tolerance against OVA in the dog model. $P < 0.02$ Oral administration of high dose TGF- β simultaneously with OVA feeding significantly inhibited the OVA specific IgE elevation and anaphylactic reaction in OVA-TCR transgenic D011.10 mice. $P < 0.05$
Okamoto et al. [30]	To investigate whether TGF- β when taken orally can affect allergic immune responses	Japan	Transgenic D011.10 mice OVA TCR fed OVA and recombinant human latent TGF- β 1 or OVA and control (R&D Systems) – orally and through gavage	6–10 week old Food allergy related immune reactions	Mean $\pm$ SD	+	Beneficial effect of casein containing TGF- β 2 on the modulation of intestinal and systemic inflammation in HLA-B27 transgenic rats. $P < 0.05$
Schiffirin et al. [31]	To examine the capacity of TGF- β 2 containing diet to control the intestinal inflammation in HLA-B27 transgenic rats, and compared its effects with a similar diet devoid of TGF- β 2	Nestle Research Centre, France	3 groups of rats fed milk casein based diet orally with or without TGF- β 2. HLA-B27 transgenic rat model	Weaning Leucocyte counts, acute phase reactants, myeloperoxidase, cytokine levels – histology score, colon width and weight Weaning – post-weaning Immune response profile	ANOVA Student's <i>T</i> -Test Mann-Whitney <i>U</i> -test	+	A down-regulation of the immune response was observed between TGF- β 2 fed vs. formula fed rat pups at day 18 and 28 $P < 0.005$ TGF- β retains biologic activity and enhances induction of oral tolerance. First study to show that orally administered TGF- β acts on intestinal mucosa while also affecting the immune responses to dietary antigens $P < 0.05$
Penttila [27]	To assess the role of TGF- β in mediating mucosal immune development and specifically the effect of endogenous IL-18	Australia	Allergy prone Brown Norway rat pups suckled naturally or formula fed with and without TGF- β 2. (R&D Systems)	24 days Oral tolerance IgE	ANOVA Newman-Keuls multiple comparison test Mean $\pm$ SD Unpaired Student <i>T</i> -test	+	
Ando et al. [32]	To determine whether orally administered TGF- β is biologically active in intestinal mucosa and affects oral tolerance induction to dietary antigens	Japan and USA	Recombinant TGF- β 1 only OVA fed Transgenic mice – wild type – administered by gastric intubation or orally			+	
Verhasselt et al. [33]	To assess the role of milk borne TGF- β 1 and 2 in protection against allergic airway inflammation	France	Lactating mice TGF-B1 and 2 assessed Oral TGF β antibody assessed	Neonatal period Breast milk induced tolerance	Mann-Whitney <i>U</i>	+	New insights into mechanisms underlying tolerance induction in neonates and pinpoints maternal influence through breast milk mediated antigen transfer as a crucial factor in this process $P = 0.001$
Ozawa et al. [35]	To investigate whether commercially available pasteurized cow milk retains TGF β activity both <i>in vitro</i> and <i>in vivo</i>	Japan	Female 4–6 week old mice fed varying dosages of cow milk samples TGF B1 and 2 orally administered and tested in mice	DSS-induced colitis and LPS-induced endotoxemia	Survival rates in mice	+	Endogenous TGF-B activity was shown to protect against DSS-induced colitis and LPS-induced endotoxemia shock. $P < 0.05$ 12/13 = + 1/13 = NS
Overall effect						+	92.31% (92%)

on the complex formation of TGF- β 1 and TGF- β 2 isoforms and plasma clearance of complexes and showed that fatty acids did potentiate TGF- β and other growth factor activities. They also showed that the ability of fatty acids to inhibit complex formation of TGF- β isoforms depends on carbon chain length, degree of unsaturation (polyunsaturated and saturated fatty acids) and TGF- β isoforms.

In ongoing research in this area Zemmann and team proposed that the IgE high-responder dog model was more predictive of human allergic disease than rodent models [29] and showed that an increased TGF- β expression in tolerized bronchoalveolar lavage cells could be the mechanism of oral tolerance against OVA ($P < 0.02$). Controversy exists over the mechanism of induction of oral tolerance that could be related to different experimental designs. In this study, dogs were used in spite of the complexity associated with their larger size, because of several advantages over the rodent model. However, elucidation of the exact mechanism in the dog is limited by the lack of reagents specific for cytokines and cellular receptors of this species, a problem overcome by the use of RT-PCR techniques. Although this study did not consider milk TGF- β in association with immunological outcomes, these investigators showed that IL-10 and TGF- β are important mediators of immunologic suppression.

In 2005 Okamoto [30] investigated whether TGF- β when taken orally could affect allergic immune responses using a murine model. The results of this study showed that oral administration of high dose TGF- β simultaneously with OVA feeding significantly inhibited the OVA-specific IgE elevation and anaphylactic reaction in OVA-TCR transgenic DO11.10 mice ($P < 0.05$). These innovative results clearly demonstrated that TGF- β , when taken orally as well as when applied systemically (by subcutaneous injection), had the capacity to modulate a food allergy-related immune response. Orally administered TGF- β suppressed T helper type (Th)2-type immune responses with significant Th1 skewing, whereas systemically administered TGF- β suppressed both Th1- and Th2-type immune responses induced by high-dose OVA feeding in the mice. The outcome of these TGF- β effects on T cells was strongly dependent on the cellular context, such as stage of differentiation and cytokine milieu.

In that same year, Schiffrin et al. [31] reported on the capacity of a TGF- β 2-containing diet to control intestinal inflammation in HLA-B27 transgenic rats, and compared its effects with a similar diet devoid of TGF- β 2. A beneficial effect of casein containing TGF- β 2 was observed on the modulation of intestinal and systemic inflammation in the HLA-B27 transgenic rats ($P < 0.05$). These findings led to the conclusion that TGF- β was required to control intestinal inflammation.

Following on from earlier experiments, Pentilla [27] assessed the role of TGF- β 2 in mediating mucosal immune

development. A down-regulation of the immune response was observed between TGF- β supplemented formula-fed vs. non-supplemented formula-fed rat pups at days 18 and 28 ($P < 0.005$). The research group showed previously that formula feeding in Hooded Wistar (non-allergy-prone) rats resulted in an increase in Th1 cytokines and an increase in eosinophil and mast cell infiltrate in the intestine [26]. Rat pups fed formula supplemented with TGF- β 2 had an increase in splenic (systemic) Th1-like cytokines including IL-12, IL-18 and IFN- γ suggesting that supplementation of formula with TGF- β 2 has the potential to down-regulate the humoral and mast cell response to formula antigens.

Ando et al. [32] in 2007 reported the first study to show that orally administered TGF- β acts on intestinal mucosa while also down-regulating the immune responses to dietary antigens ($P < 0.05$). This group examined the effects of oral TGF- β on oral tolerance induced by high-dose OVA feeding. The team reported that TGF- β retained biologic activity and enhanced induction of oral tolerance to dietary antigens. These observations suggested that oral administration of TGF- β may be a potential strategy to prevent allergic disease through an efficient oral tolerance induction [32].

In 2008, Verhasselt and her group examined the role of milk-borne TGF- β 1 by exposing lactating mice to an airborne allergen and assessing the development of asthma in the progeny [33]. The team found that breastfeeding-induced tolerance relies on the presence of TGF- β 1 during lactation. This effect was mediated by CD4⁺ T lymphocytes and was dependent on TGF- β 1 signaling in T cells. These data showed that airborne allergens could be transferred from mother to newborn through breast milk and that this exposure could induce antigen-specific tolerance in the offspring. Breast milk-mediated transfer of an antigen and TGF- β to the neonate resulted in oral tolerance induction and antigen-specific protection from allergic airway disease. Verhasselt's study provided new insights into the mechanisms underlying tolerance induction in neonates and pinpointed a maternal influence through breast milk-mediated antigen transfer as a crucial factor in this process [34].

In 2009, Ozawa et al. [35] showed that commercially available pasteurized cows milk retains TGF- β activity *in vitro* and *in vivo*. They found that some commercial cows milk increased TGF- β /Smad-responsive reporter activity, and induced Smad2 phosphorylation and transcription of the TGF- β /Smad target genes, TGF- β itself and Smad7 *in vitro*. Mice treated orally with cows milk containing TGF- β daily for 2 weeks had increased phosphorylation of Smad2, increased TGF- β and Smad7 mRNA expression in the intestine, and greater serum TGF- β concentrations than mice treated orally with phosphate-buffered saline. Furthermore, oral administration of cows milk containing TGF- β daily for 2 weeks before the induction of dextran

sodium sulphate colitis and lipopolysaccharide-induced endotoxemia ameliorated tissue damage and mortality, respectively, in the mice. These *in vivo* effects of cows milk were relieved by the simultaneous administration of TGF- β type I receptor kinase inhibitor with the cow milk, but relief was not observed after the oral administration of milk containing little TGF- β . Thus, some commercially available pasteurized cows milk retains TGF- β activity, which may provide protection against experimental colitis and endotoxemia.

Summary of results

Overall, 92% of studies (12/13) in our review reported a positive and protective association of immunological outcomes with TGF- β 1 or TGF- β 2. The one study that did not show any effect was informative for subsequent studies. Animal experimental studies that examined the effects of TGF- β on immunological outcomes were heterogeneous, although from those reviewed it appears that the TGF- β effects were similar across species.

Discussion

We evaluated the published studies that considered milk-derived or recombinant TGF- β in association with immunological outcomes using an animal model. We found that 92% of the identified animal experimental studies published to March 2010 demonstrated an association between high TGF- β and a reduction in immunological outcomes related to allergy. Hence, this review of the associations between TGF- β and immunological outcomes in animal experimental studies provides an overall measure of effect based on the literature currently available.

Our review has several strengths. Firstly, an exhaustive search was conducted with a systematic and objective approach to avoid potential biases resulting from missed studies. Inclusion and exclusion criteria ensured a true representation of what is known. Secondly, for inclusion in the review, keywords, study questions, study design, sample size, appropriate end-points, complete follow-up, appropriate statistical analysis and applicability were considered. Hence, in spite of the dissimilar methods in diagnosing immunity, diverse animal models and studies with varied outcomes, the findings of the 13 published studies that assessed TGF- β with immunological outcomes in animal models were broadly consistent, showing that administration of TGF- β (either orally through maternal milk, cow milk or formula or as recombinant TGF- β through intubation) may significantly down-regulate immune activation and bring balance to immune system function.

There is sufficient evidence from animal studies to show that milk TGF- β survives digestion and has functional

effects on neonates. When TGF- β was fed to TGF- β null mice, the cytokine was found distributed intact in lung tissue [1, 20, 36]. In fact, TGF- β appears to be rapidly endocytosed by the neonatal intestine, suggesting it has influences on immunity beyond the gut [8]. Further, oral supplementation with TGF- β induces oral tolerance in allergy-prone rats [26], and oral supplementation with TGF- β facilitates OVA-induced tolerance in mice. The pathways by which TGF- β in milk induces oral tolerance [37–39], stimulation of an IgA isotype switching in B cells [40] and maintenance of the intestinal epithelium barrier [41] are all necessary for immunological health. Recently the role of milk-borne TGF- β was assessed and it was shown that airborne antigens were efficiently transferred from the mother to the neonate through milk [33]. Tolerance induction did not require the transfer of immunoglobulins, but rather breastfeeding-induced tolerance relied solely on the presence of milk TGF- β in the presence of the antigen. However, new work from this same group showed that in milk from antigen-exposed BALB/c sensitized mice mothers IgG immune complexes were transferred to the newborn through the neonatal Fc γ RIIb resulting in the induction of antigen-specific FoxP3+CD25⁺ regulatory T cells. In contrast to tolerance induced by milk-borne free antigen, the induction of oral tolerance by milk immune complexes did not require the presence of TGF- β or IgA highlighting that IgG immune complexes found in breast milk are potent inducers of rodent oral tolerance [42].

In naturally suckling animals, the gut is exposed to maternal milk cytokines that assist in regulation of immune responses until the gut has matured enough to produce its own immunoregulatory cytokines for normal gut homeostasis [26]. It is thus possible that administration of TGF- β through an oral route stimulates many types of cells, e.g. intestinal epithelial cells, which produce various cytokines in response to TGF- β , and effects of TGF- β on T cells are affected through the cytokine milieu. TGF- β is an important family of growth factors involved in maintaining homeostasis in the intestine, regulating inflammation and allergy development, as well as promoting oral tolerance development. The mouse model has helped to delineate the functions of TGF- β in the recruitment of inflammatory cells (regulated through enhanced integrin expression, enhanced monocyte-matrix adhesion, and increase in matrix-specific collagenase secretion and chemo-taxis) and activation of mononuclear cells (result of triggering cascade of proinflammatory cytokines and differentiation of B and T cells), and has provided insight into the role of TGF- β in disorders of immunity in human infants. It is not possible to equate the total amount of orally administered TGF- β 1 (30 μ g per mouse) to the total amount of TGF- β 1 that breastfeeding human infants receive, due to the difficulty of dosage comparison between mice and humans. Thus, a more direct approach

is necessary to clarify the role of TGF- β in human milk (e.g., an experiment to test if neutralizing antibodies against TGF- β suppress the ability of breast milk to reduce allergic responses). Clarification of mechanisms underlying the specific enhancing effects of orally administered TGF- β on Th1-type immune responses is an important and interesting area of study.

The expression of TGF- β 1 in the neonatal intestine is initially very low not being detected before the age of 19 days and progressively increasing thereafter [11]. Breast-milk may therefore be an important exogenous source of TGF- β during early infancy when the mucosal and systemic immune responses are immature [43]. Indeed, rat pups fed formula devoid of TGF- β 2 exhibit more manifest activation of both innate and adaptive immune systems in comparison to those fed either TGF- β 2 supplemented formula or breast milk [26].

As elegantly described, TGF- β 2 does appear to be the most abundant TGF- β isoform in breast milk with TGF- β 1 predominantly secreted by immune cells [43]. Evolutionarily, the neonate may be adapted to immunoregulation by TGF- β 2 in breast milk. In order to initiate intracellular signaling and induce its effects, TGF- β binds to a TGF- β receptor (TGFBR) II dimer on the target cell surface that then forms a complex with a TGFBR I dimer to initiate intracellular signaling [44]. A third receptor molecule is required for optimal responses to TGF- β 2 but not to TGF- β 1 [45]. In the rat small intestine TGFBR II expression and hence intestinal TGF- β 2 responsiveness are confined to the neonatal period when the intestine is exposed to TGF- β 2 in abundance from breast milk [46]. In addition, breast milk may contain TGFBR III thus providing the infant with the receptor required for optimal responsiveness to TGF- β 2 [47].

TGF- β , secreted in a latent form, is activated extracellularly by extremes of pH, by heat, and by proteases [48]. A recent report of studies in mice found that the latent form of TGF- β , such as TGF- β found in human milk, can be activated *in vivo* following oral administration [49]. The mucosal immune system constitutes a first-line defence system that protects mucosal surfaces, such as the respiratory and gastrointestinal tracts, against foreign pathogens but at the same time suppresses immune responses to dietary antigens and indigenous microflora [5, 9, 50]. At the mucosal surfaces, secretory IgA is the major effector component. Many studies have shown that TGF- β has a crucial role to play in IgA class-switch in naïve B cells [40] and IL-10 or IL-6 promote differentiation into IgA-producing cells [51]. Thus, the cytokine pattern in milk may contribute to the development of the immature gastrointestinal tract by inducing oral tolerance and promoting IgA antibody production and inhibiting IgE-, IgG- and cell-mediated reactions [52]. The results by Ando in an animal model reinforce this hypothesis as oral administration of the molecule was able to produce immune

responses in the intestinal mucosa and induce oral tolerance [32]. In humans, cytokines in milk must be partially digested before they can exert any effect upon the recipient infant, and although milk cytokines encounter a large mucosal surface in the infant's alimentary tract, they are not digested until reaching the stomach. In addition to the large number of anti-proteases found in milk, the production of pepsin, the primary gastric proteolytic enzyme, is considerably reduced in the first week of life [53]. Therefore, the gastric proteolysis that normally occurs with ingested food is considerably reduced in newborn infants. Further, during the first week after delivery the neonatal gut is 'leaky', facilitating macromolecular passage through the mucosa at a time when cytokine levels are in highest supply from milk [54], resulting in subsequent activation of extraintestinal organs [55]. However, whether the latent form of TGF- β is converted to the active form *in vivo* remains uncertain. This is of relevance for human infants whereby the passage through infant stomach may activate the latent form of TGF- β in milk, freeing active TGF- β to protect the infant intestine.

Although TGF- β production appears to be deficient in the newborn [56], maternal breast milk maintains an abundant supply during at least the first three months of lactation and apparently supplies the deficient TGF- β production of the infant [4, 5]. Of significance to humans is that the presence of TGF- β and other cytokines in milk may influence maturation and function of epithelial, inflammatory and structural components of the developing intestinal system [6]. Recent findings [57] suggest that supplementation with probiotics in pregnancy has the potential to influence foetal immune parameters as well as immunomodulatory factors in milk. Indeed, high levels of TGF- β 1 in breast milk may prevent sensitization to food allergens, acting synergistically with interleukin-10 to promote specific IgA production and inhibit T cell activation. Therefore, the maternal dyad has been formed over evolution whereby the mother maintains the abundant amount of TGF- β during lactation to the infant exogenously. The intimate environment between mother and foetus may play an additional role in genetic polymorphism for allergy and asthma in shaping and priming the offspring's immune response. Maternal factors in this context act on the prenatal and postnatal levels of the TGF- β super-family and may impact on antibodies at the materno-foetal interface [58].

Of importance is that unpasteurized cow's milk consumption has been associated with less atopy prevalence [59], and it is not only microbial load but cytokines such as TGF- β 1 that have been suggested to play a role in the effect of unpasteurized milk. Levels of TGF- β 1 in different cow's milk samples were evaluated, including raw unpasteurized milk before and after boiling, commercial pasteurized and micro-filtrated cow's milk and different

commercially available cow's milk formulas. TGF- β_1 concentration in raw unpasteurized cow's milk was highest before boiling and decreased significantly after boiling. TGF- β_1 concentrations were also significantly lower in commercial pasteurized milk and in micro-filtrated milk in comparison to unpasteurized unboiled milk ($P = 0.002$). The levels of TGF- β_1 in all formula samples tested were below the threshold of detectability for the assays. As TGF- β_1 in milk may contribute to the development of the immature gastrointestinal tract by influencing IgA production and oral tolerance induction, the authors of this study suggest consideration of not only the microbial compounds but also the cytokine patterns in milk to explain the protective effect of unpasteurized cow's milk on allergic disorders.

Conclusion and clinical relevance

Animal models are important in the context of understanding human disease, although they do not reflect specifically what is occurring in humans. However, animal studies have provided important clues and will continue to show relevance to the pathogenesis and therapeutics of a number of human diseases including

immune activation and allergy in childhood and infancy. TGF- β s are important growth factors involved in maintaining homeostasis in the intestine, regulating inflammation and allergy development and promoting oral tolerance in infants [10, 34]. Thus, taken as a whole, these and our other findings [23] suggest that this cytokine in milk may influence the development of immunological outcomes in offspring.

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