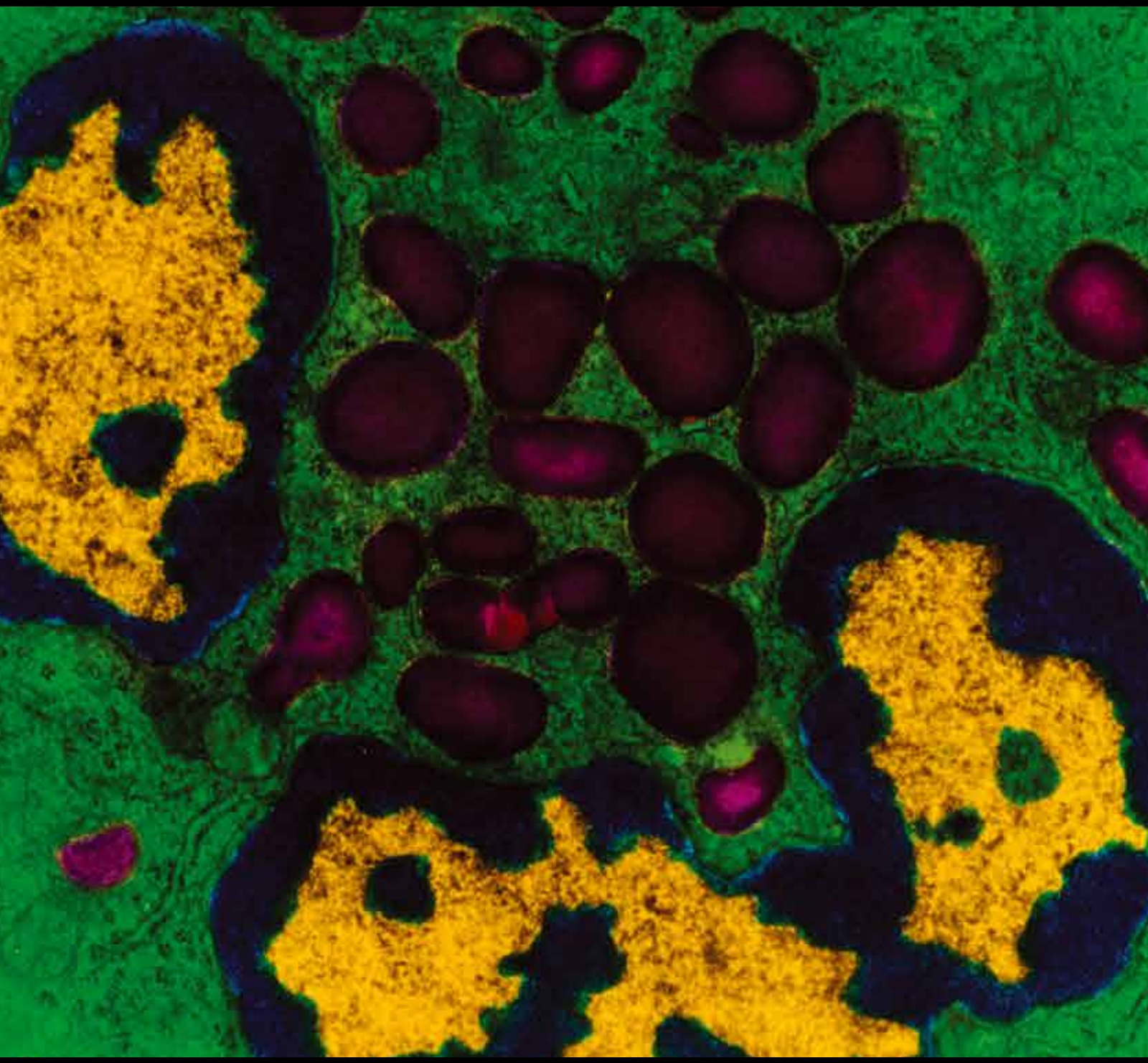
Mediators of Inflammation

Cytokines and Disease

Guest Editors: Arkadiusz Orzechowski, Agueda A. Rostagno, Sabina Pucci, and Gilles Chiocchia



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Editorial Board

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Editorial
Cytokines and Disease

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Recent evidence in biology and medicine has expanded the knowledge of cytokines function in many disease states and conditions as well as the severe adverse effects implicated in the escalated production associated with specific disease conditions. This special issue was intended to provide an updated view on the role of cytokines in the disease pathogenesis of degenerative and neoplastic disorders. Special emphasis has been dedicated to the contribution of cytokines to systemic conditions including but not limited to neurodegeneration, cancer, and graft/prosthesis rejection. To address these issues we invited authors who could shed light on the contribution of cytokines on such diseases, identify mechanisms employed by tumor cells to subvert the host cytokine response, provide new cellular and animal models to test and understand the activity of cytokines in degenerative and neoplastic diseases, highlight cytokines involved in neurodegeneration, and illustrate how cytokines affect graft and prosthesis rejection.

One of the papers of this special issue addresses the dynamics of acute local inflammatory response to the intramuscular cell transfer in autologous cell models. Early immune reaction (infiltration with neutrophils and macrophages) at the site of injection was accompanied by considerable rise of *Il-1α, Il-6, Tgf-β*, and *Tnf-α* gene expressions. Autotransplantation of muscle-derived cells into skeletal muscle resulted in marked decrease of viable transplanted cells which may contribute to the low efficacy of cellular grafts. Another paper presents the observations collected from children with allergic rhinitis (AR). The authors showed that the anti-inflammatory cytokine IL-37b, a member of the interleukin 1 cytokine family whose expression is related to the efficacy of intranasal synthetic steroid (mometasone furoate) therapy, downregulated the expression of Th2 cytokines in PBMCs through MAPK and PI3-K signaling pathways in AR. The paper entitled "*Tumour necrosis factor superfamily members in the pathogenesis of inflammatory bowel disease*" reviews the contribution of the tumor necrosis superfamily (TNFSF) to the pathogenesis of inflammatory bowel disease (IBD). Special attention was drawn on TL1A, FasL, LIGHT, TRAIL, and TWEAK as possible targets in IBD treatment through their role on apoptosis and T-cell differentiation. One of the papers of this special issue is a review featuring TNF-α as a proinflammatory cytokine exerting both homeostatic and pathophysiological roles in the central nervous system. It summarizes the current knowledge of the cellular and molecular mechanisms by which TNF-α links the neuroinflammatory and excitotoxic processes that occur in several neurodegenerative diseases, but with a special emphasis on amyotrophic lateral sclerosis (ALS). It stresses the *de novo* production of TNF-α by microglia as an important component of the so-called neuroinflammatory response which may represent a valuable target for intervention. The paper entitled "*Protective effects of lipoxin A4 in testis injury following testicular torsion and detorsion in rats*" describes the study on the protective effects of lipoxin A4 (LXA4), a lipid mediator with potent anti-inflammatory properties, in rat testis injury following testicular torsion/detorsion. Lipoxin A4 protective effect takes place via modulation of proinflammatory cytokines, oxidative stress, and NF-κB activity. The paper by J. M. Zarzynska addresses the current view on the dual role played by TGF-β1 in breast cancer insurgence and

progression. In early stages of breast cancer, TGF-β1 inhibits epithelial cell cycle progression and promotes apoptosis, showing tumor suppressive effects. However, in late stages of the disease, TGF-β1 cooperates to promote tumor progression by orchestrating the tumor microenvironment cytokines production, influencing tumor cell motility, invasion genes expression, cancer invasiveness, and metastasis. TGF-β1 is also involved in cancer microenvironment modification and promotion of epithelial to mesenchymal transition. According to current knowledge, several drugs counteracting TGF-β1 have been developed and are either in preclinical or in early stages of clinical investigation. One of the papers evaluates the importance of intestinal homeostasis for the healthy status of the large bowel. The authors present numerous data that substantiate dietary essential n-3 polyunsaturated fatty acids (PUFAs) and short-chain fatty acid butyrate as anti-inflammatory and anticancer agents. PUFAs modulate the production and activities of TNF family cytokines (TNF-α, TRAIL, and FasL) that have potent inflammatory activities. The possible application for the prevention and therapy of colon inflammation and cancer is also outlined. The final paper of this special issue outlines the effects of the antibiotic florfenicol on the expected changes in sTNF-α, damage markers of the liver and kidney, and the lipid metabolism parameters in endotoxemic brown trout. The paper concludes that florfenicol does not affect the LPS-mediated increases of sTNF-α and does not prevent liver or kidney damage indicating that the antibiotic does not have any evident positive effects on acute endotoxemia in fish.

Overall, the current issue highlights the multidisciplinary contribution of cytokines, through their broad spectrum signaling capability and regulation of cellular communication, as crucial mediators in diverse pathologic entities with broad etiology.

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Research Article

Dynamics of Acute Local Inflammatory Response after Autologous Transplantation of Muscle-Derived Cells into the Skeletal Muscle

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The vast majority of myoblasts transplanted into the skeletal muscle die within the first week after injection. Inflammatory response to the intramuscular cell transfer was studied in allogeneic but not in autologous model. The aim of this study was to evaluate immune reaction to autotransplantation of myogenic cells and to assess its dynamics within the first week after injection. Muscle-derived cells or medium alone was injected into the intact skeletal muscles in autologous model. Tissue samples were collected 1, 3, and 7 days after the procedure. Our analysis revealed the peak increase of the gene expression of all evaluated cytokines (*Il-1α, Il-1β, Il-6, Tgf-β,* and *Tnf-α*) at day 1. The mRNA level of analyzed cytokines normalized in subsequent time points. The increase of *Il-β* gene expression was further confirmed at the protein level. Analysis of the tissue sections revealed rapid infiltration of injected cell clusters with neutrophils and macrophages. The inflammatory infiltration was almost completely resolved at day 7. The survived cells were able to participate in the muscle regeneration process. Presented results demonstrate that autotransplanted muscle-derived cells induce classical early immune reaction in the site of injection which may contribute to cellular graft elimination.

1. Introduction

Adult muscle tissue is highly exposed to the damage by both internal and external factors throughout the life span and therefore requires the ability to effectively regenerate. The cells which are primarily responsible for skeletal muscle tissue restoration capacity are called satellite cells. The idea to deliver myoblasts which are satellite cells progeny into the dysfunctional muscles as a method of treatment was presented for the first time in 1978 by Partridge and colleagues [1]. Since then myoblasts transfer therapy (MTT) has been a subject of extensive studies. At the beginning, myoblasts have been considered as a very promising alternative for treatment of muscular dystrophies (MDs); however it became clear that this population is not able to engraft skeletal muscle after systemic delivery [2]. The poor migratory capabilities hinder the potential use of myoblast-based therapy in Duchenne MD and narrow the possible applications to the disorders with more focal character. Nevertheless, myoblasts transfer is still perceived as possible future alternative treatment in numerous conditions. The list includes sphincters dysfunction (urethral, anal, esophageal, and pyloric), atrophy of reinnervated muscle, rectovaginal fistulas, local muscle loss due to injury, and some types of muscular dystrophies

(like oculopharyngeal or facioscapulohumeral MDs). Unfortunately, myoblasts transfer procedure is associated with another crucial unsolved problem, the poor survival rate of donor cells. It was demonstrated that the vast majority of graft is lost within first 3 days after transplantation. The massive elimination of injected myoblasts was identified in 1996 by Fan and colleagues [3] and subsequently confirmed by many other studies. This phenomenon was observed regardless of type of animal model (murine or porcine), type of target area (skeletal muscle, myocardium, or urethral sphincter), status of host muscle (intact or injured), or status of the host organism (immunodeficient or immunocompetent) [4–9]. Moreover, Holzer and colleagues demonstrated that myoblasts are eliminated even after autologous transplantation [7]. Several potential causes of rapid myogenic cell death after transfer have been proposed. They include inflammatory response in the injection site [5], ischemia [10, 11], hypoxia, [12], and anoikis [13]. The immune reaction after myoblasts injection has been studied intensively in allogeneic models [5, 14–17] but not after autologous transplantation as this type of transfer is believed to be nonimmunogenic. However, our previous study demonstrated that oxidative stress might play a role in elimination of myogenic cells after autologous transplantation [18]. Therefore, we hypothesized that autologous transfer of myogenic cells also triggers early immune response associated with oxidative burst. As autologous transplantation is considered to be the safest option in all clinical applications listed above, it seems to be of prime importance to evaluate the local tissue response for this kind of grafting. Therefore, the aim of this study was to examine the presence and dynamics of cytokines expression and cells associated with innate immune reaction within first week after autologous MTT.

2. Materials and Methods

2.1. Animals. All experiments were performed on 3-month-old Lewis rats (in-bred strain). Animals were housed with free access to food and water and were maintained at a constant temperature. Animal housing and experimental procedures were approved by Local Ethics Animal Welfare Commission of the Medical University of Warsaw.

2.2. Isolation and Cultivation of Muscle-Derived Cells (MDCs). Skeletal muscle samples (about 0.05 g) for cell isolation were obtained from *musculus gracilis* during general anesthesia induced by administration of xylazine (10 mg/kg; Leciva, Prague, Czech Republic), ketamine (40 mg/kg; Spofa, Prague, Czech Republic), and butorphanol (1 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA, USA). Tissue sampling did not impair rats mobility after surgery. Isolation of muscle-derived cells was performed as described by Burdzińska et al. [19]. The cells were suspended in standard growth medium (GM), DMEM supplemented with 10% (v/v) fetal bovine serum and antibiotic, antimycotic mixture (all components purchased from Invitrogen, Carlsbad, CA, USA). In order to reduce number of fibroblasts in culture, the medium containing nonadherent cells was removed to another dish 24 h after cell seeding (preplating). The first change of culture medium was performed 72 h after isolation. When the culture reached 70% of confluence, cells were harvested by trypsinization (0.25% trypsin and 0.02% EDTA; Invitrogen-Gibco Carlsbad, USA) and reseeded in new dishes in a density of $5 \times 10^3/\text{cm}^2$. Majority of cells were cultured for transplantation whereas part of population were seeded separately to perform *in vitro* characterization, desmin expression and differentiation potential analysis.

2.3. Immunocytofluorescence and Differentiation Potential. To identify isolated cells, MDCs were analyzed for the presence of desmin, myogenic cells marker. Cells after the first passage were cultured in a Lab-Tek 4-chamber slide w/Cover (Permanox Slide Sterile, Nalge Nunc International, Naperville, IL, USA) until they reached 80% confluence; then they were fixed in 4% (w/v) paraformaldehyde for 15 min at room temperature and permeabilized with 70% cold methanol for 20 min in −20°C. Samples were treated with blocking solution (1% bovine serum albumin/5% normal donkey serum in phosphate-buffered saline) for 30 min in RT and then probed with mouse anti-desmin (Sigma-Aldrich, St. Louis, MO, USA, 1 : 50 v/v, 90 min, RT). Afterwards, cells were washed and probed with a secondary antibody [Alexa-Fluor 594 donkey anti-mouse (Jackson ImmunoResearch Europe, Suffolk, UK), 1 : 100 v/v, 60 min, RT]. Cells were visualized using fluorescent microscopy via Olympus IX51.

To verify myogenic potential, the other subsets of isolated cells were induced to differentiate by cultivation in DMEM supplemented with 2% of horse serum (HS) for 3 days. The differentiated cells were immunostained for desmin as described above. The fusion index was determined as the ratio of nuclei in myotubes to the total number of nuclei in the same field calculated from at least 10 fields of view per animal and was expressed as a percentage (0% to 100%). The presence of intracellular lipid droplets in MDC population was confirmed with Oil Red O staining (Sigma-Aldrich, St. Louis, MO, USA).

2.4. Cell Suspension or Vehicle Injection. For injection procedure rats were sedated with xylazine/ketamine mixture. The skin in the area of injection was shaved and disinfected. In the transplanted animals, MDCs suspended in 200 μL DMEM were administered into the gastrocnemius muscle in autologous manner. Cell suspension was given through 22 G needle in a single bolus directly to the intact muscle without any skin incision. Sham animals were treated in the same way but received 200 μL DMEM vehicle only. At the same time, additional 100 μL of cell suspensions was directed for microbiological tests (bacteria and yeast). The injected cells were either unlabeled (animals designated for gene and protein expression analyses) or labeled with fluorescent membrane linkers, PKH26 (red dye, animals designated for immunohistochemical staining) or DiD (DiIC18(5)-DS [1,1-Dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine-5,5-disulfonic acid] in animals for *in vivo* imaging). Before preparing the final suspension, the cells were washed twice in DMEM to remove serum completely.

2.5. Tissue Collection. The tissue surrounding the area of either cells or DMEM administration was harvested at day 1 (24 hours), day 3, or day 7 after the transplantation. In the untreated group, the analogous muscle fragments were collected. The tissue samples were immediately snap-frozen in liquid nitrogen and stored in −80°C until analysis.

2.6. RNA Isolation, Reverse Transcription, and Real-Time PCR Analysis. The animals designated for gene and protein expression analysis were transplanted with equal amount (1×10^6) of cells ($n = 18$, 6 in each time point). MDCs for these experiments were unlabeled to avoid additional manipulations which are always associated with increased risk of acquired immunogenicity. Untreated ($n = 7$) and sham operated groups ($n = 18$, 6 in each time point) served as controls. Tissue samples collected at days 1, 3, and 7 were homogenized in TissueLyser homogenizer (Qiagen, GmbH, Hilden, Germany) at a frequency of 25 Hz for 5 minutes. Total RNA was isolated using RNeasy Fibrous Tissue Mini Kit (Qiagen, GmbH, Hilden, Germany). RNA concentration was quantified by spectrophotometer at 260 nm using NanoDrop (ND-1000 Spectrophotometer, NanoDrop Technologies, Inc.). Reverse transcription of total mRNA into cDNA was performed using the SuperScript III (Invitrogen, Gibco, Carlsbad, USA) according to the manufacturer's instruction. Real-time PCR was performed on ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, USA). Specific primers and probes set were purchased from Applied Biosystems: *Il-1α* (Rn0055700_m1), *Il-1β* (Rn00580432_m1), *Il-6* (Rn00561420_m1), *Tgf-β1* (Rn00572010_m1), and *Tnf-α* (Rn01525859_m1). *Gapdh* gene (4352338E) was used for normalization. The values are expressed relatively to a reference sample (calibrator): not treated muscle. The Ct (threshold cycle) for the target gene and the Ct for the internal control were determined for each sample. The relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ method.

2.7. ELISA. The evaluation of *Il-1α* and *Il-1β* concentration in tissue homogenates was performed by ELISA. The muscle samples were homogenized in a buffer with phosphates and proteases inhibitors (Sigma-Aldrich, St. Louis, USA). Then probes were clarified by centrifugation at 10 000 rpm for 5 minutes and addition of PMSF. Total protein concentration was measured using NanoDrop (ND-1000 Spectrophotometer). Cytokines concentrations in tissue lysates were determined using commercial available ELISA kits (R&D System, Minneapolis, MN, USA). The results were presented as an absolute ratio: interleukin concentration/protein concentration ($\times 10^{-9}$).

2.8. Histology and Immunohistochemistry. To visualize injected cells in the host tissue cells were labeled with red fluorescent membrane linker, 5 μM PKH26 ($n = 6$, 2 in each time point). The contralateral gastrocnemius muscles in this group were injected with vehicle only. Histological and immunohistochemical staining were performed on frozen sections (10 μm thick) prepared with the use of cryostat Microm HM 525 (Microm, Walldorf, Germany). Some sections were stained with hematoxylin and eosin. Immunohistochemical staining was performed using primary antibodies against antigens: CD43 (1 : 20 v/v) and CD68 (1 : 20 v/v) (AbD Serotec, Kidlington, UK). Samples were fixed with cold acetone. Nonspecific binding sites were blocked with 5% normal donkey serum in PBS. Tissue sections were incubated with primary antibodies for 1 h at RT. Afterwards, cells were washed and probed with a secondary antibody [Alexa-Fluor 488 donkey anti-mouse] (Jackson ImmunoResearch Europe, Suffolk, UK, 1 : 100 v/v) for 1 h at RT. Finally, the slides were washed and covered with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories LTD., Peterborough, UK). The samples were evaluated with Eclipse Ni-U microscope (Nikon, Tokyo, Japan).

2.9. In Vivo Imaging. For optical imaging an average of 3.3×10^5 of MDCs were injected as described above ($n = 3$). The cells were labeled prior to the transplantation with 7.5 μM DiD, a membrane linker with Ex-max 650 nm/Em-max 670 nm (AAT Bioquest, Sunnyvale, CA). The area of imaging was carefully shaved and the adjacent part of the body was covered with dark fabric to avoid hair-derived autofluorescence. *In vivo* imaging was carried out using the IVIS Spectrum system (Caliper Life Sciences, Hopkinton, MA). Automatic algorithm for spectral unmixing of DiD dye against the food and autofluorescence backgrounds was used for visualizing the transplant-specific fluorescent signal, with excitation wavelength of 640 nm and six emission wavelengths (680, 700, 720, 740, 760, and 780 nm). Imaging data were analyzed using Living Image 4.4 software (Caliper).

2.10. Statistical Analysis. Results from RT-PCR were presented as a fold change of gene expression in relation to the calibrator, whereas data from ELISA were expressed as means (SD). Results were analyzed in pairs (untreated control versus VEH group and VEH versus MDC groups in certain time points) using nonparametric *U* Mann-Whitney test. A value of $P < 0.05$ was considered as statistically significant. For RT-PCR assay the significance of differences between groups was measured in ΔCt values. All RT-PCR and ELISA analysis were done in duplicate.

3. Results

3.1. The Isolated Muscle-Derived Population Is Heterogeneous but Consists Primarily of Myoblasts. Within first days after isolation procedure, the mononuclear, colony-forming spindle shaped cells could be observed (Figure 1(a)). The mean percentage of desmin expressing cells in obtained MDCs population after the first passage amounted to 77% (Figure 1(b)). Isolated MDCs cultured in DMEM/2% HS differentiated into myotubes (Figure 1(c)), which confirms their myogenic potential. The mean fusion index was 35%. However, some cells in culture spontaneously accumulated lipids, which was visualized by Oil Red O staining (Figure 1(d)). These cells were probably the progeny of fibro-/adipogenic progenitor cells residing in muscle tissue.

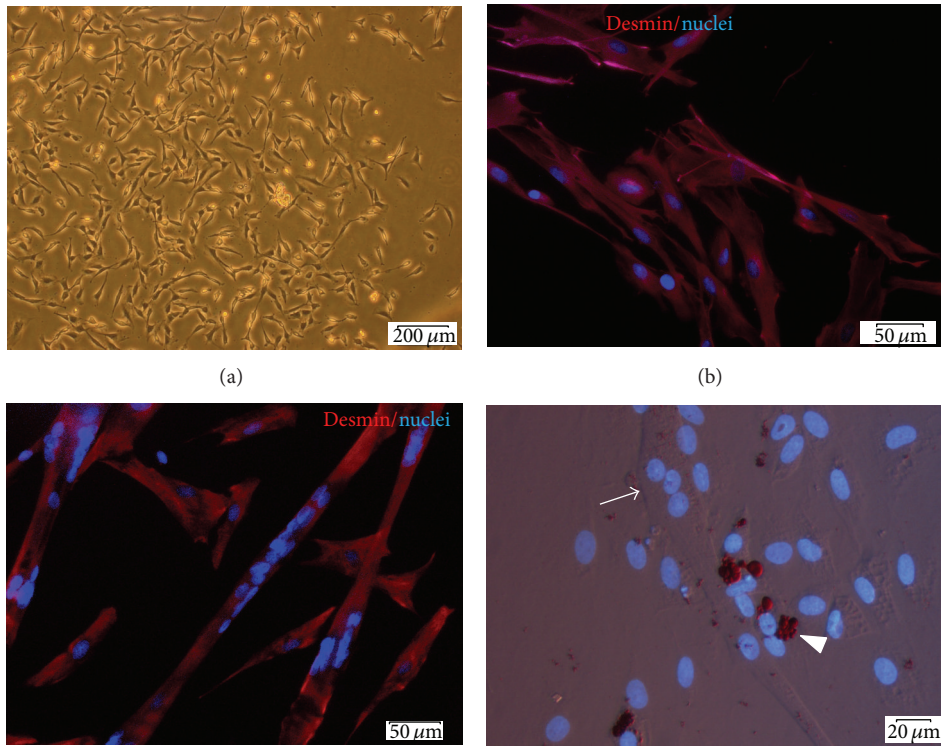

Figure 1: Identification of isolated muscle-derived cells. (a) The appearance of muscle-derived colony 6 days after isolation; (b and c) fluorescence microscopy. Desmin protein stained with Alexa 594 conjugated Ab (red) in undifferentiated MDCs (b) and in MDCs induced to myogenic differentiation for 3 days (c); (d) the presence of lipid accumulating cells within MDC population, red lipid droplets indicated with white arrowhead (Oil Red O staining), and cell nuclei stained with DAPI (blue). A multinucleated myotube can be observed in the same field of view (arrow). Scale bars in (a) 200 μm, (b and c) 50 μm, and (d) 20 μm.

3.2. Administration of Vehicle Itself Induces Significant Upregulation of Il-1α, Il-6, and Tgf-β Genes Expression. The analysis of gene expression revealed that needle insertion and administration of 200 μL vehicle into the muscle caused significant upregulation of *Il-1α, Il-6,* and *Tgf-β* (7-fold, 2.3-fold, and 2.7-fold, resp.) at day 1 in the injection site comparing to the untreated control. The significant elevation of *Il-6* gene expression in VEH group comparing to CTRL group was maintained at day 3. The expression of other evaluated cytokines normalized at day 3. One week after the injection there was no statistical differences between VEH and CTRL groups (Figure 2).

3.3. Transplantation of Muscle-Derived Cells Causes Significant Upregulation of mRNA Level for Il-α and *Il-1β Genes and Increases Il-1β Concentration at Day 1 after Injection in Comparison to the VEH Group.* Cell suspensions directed to transplantation were free from microbiological contamination. As the injection of vehicle itself caused significant differences in cytokines gene expression, the results obtained from transplanted samples were statistically analyzed in comparison to the sham control (VEH group). The administration of autologous cells caused significant increase in gene expression of *Il-1α* and *Il-1β* (Figure 2). The elevation was 5.5-fold and 5.2-fold, respectively, comparing to the VEH group and 36-fold and 17-fold, respectively, comparing to the untreated group. The expression of *Il-6, Tnf-α,* and *Tgf-β* displayed similar pattern (the elevation peak at day 1); however the differences in those cytokines were not statistically relevant. Significant changes in gene expression (both *Il-1α* and *Il-1β*) were verified by evaluation of protein level in analogous samples. In the case of *Il-1α*, the protein concentration did not confirm upregulation of gene expression; the differences between groups were not statistically significant (Figure 3(a)). In contrast, *Il-1β* protein level strictly reflects transcriptional changes. MDCs administration induced significant 4-fold increase of proinflammatory *Il-1β* mean concentration in the site of injection 24 h after transplantation in comparison to the sham control (Figure 3(b)). At the same time, vehicle injection itself also caused significant local elevation of *Il-1β* level in comparison to the untreated control (Figure 3(b)).

3.4. There Are No Significant Differences in Cytokines Expression at Day 7 after MDC Autologous Transplantation. The dynamics of evaluated genes expression clearly demonstrate that upregulation of proinflammatory cytokines after autologous MDCs transfer is acute and transient. At day 3 only *Il-1α* expression was still significantly elevated in samples from MDC group in comparison to the sham group. At day

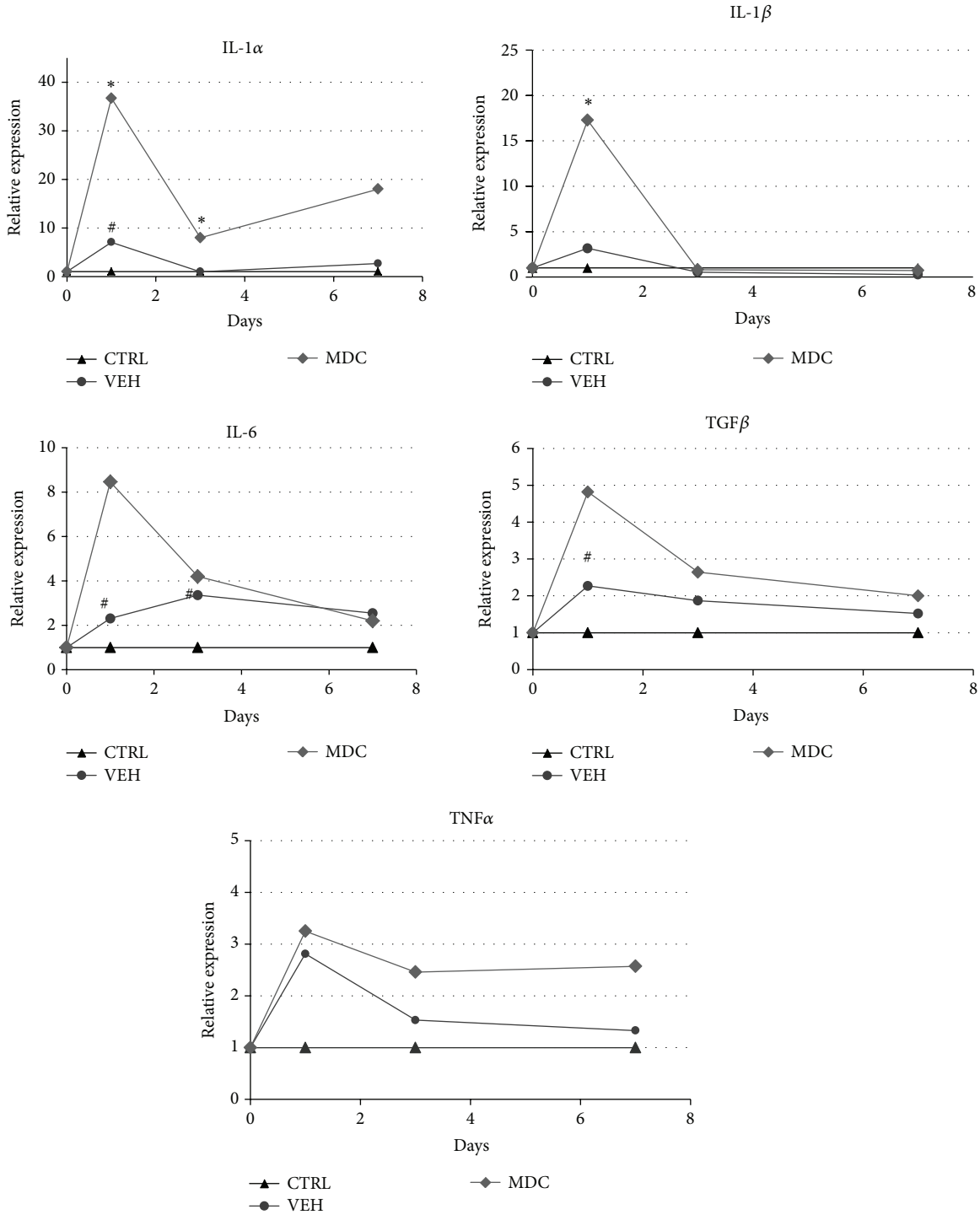

Figure 2: The analysis of cytokines gene expression in the muscle tissue determined by real-time PCR. The graphs represent relative gene expression of *Il-1α, Il-1β, Il-6, Tgf-β,* and *Tnf-α* in animals' muscles from different groups: untreated control (CTRL), after vehicle injection (VEH), and after MDCs transplantation (MDC) 1, 3, and 7 days after surgery. Results were analyzed in pairs (untreated control versus VEH and VEH versus MDC) using nonparametric *U* Mann-Whitney test. *indicates statistically significant difference between MDC and VEH groups and #indicates statistically significant difference between VEH and CTRL groups in certain time points.

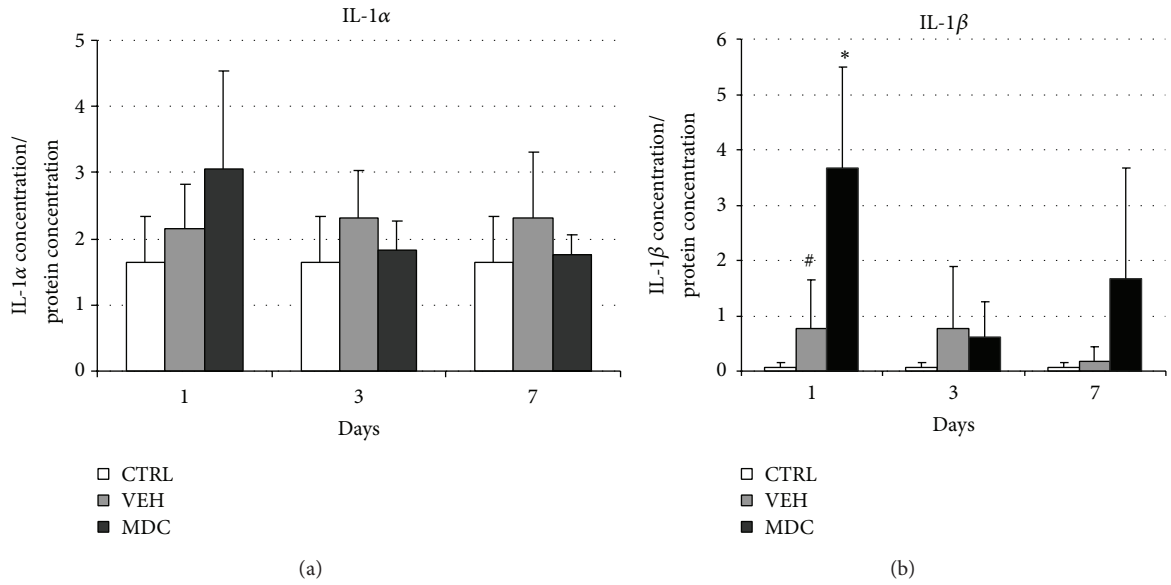

Figure 3: The concentration of cytokines: IL-1α (a), IL-1β (b) in the muscle tissue: untreated control (CTRL), after vehicle injection (VEH), and after MDCs transplantation (MDC) 1, 3, and 7 days after surgery. Protein concentrations of cytokines in tissue were determined using ELISA tests. Results were analyzed in pairs (untreated control versus VEH and VEH versus MDC) using nonparametric U Mann-Whitney test. *indicate statistically significant difference between MDC and VEH groups and $^{\#}$indicate statistically significant difference between VEH and CTRL groups in certain time points.

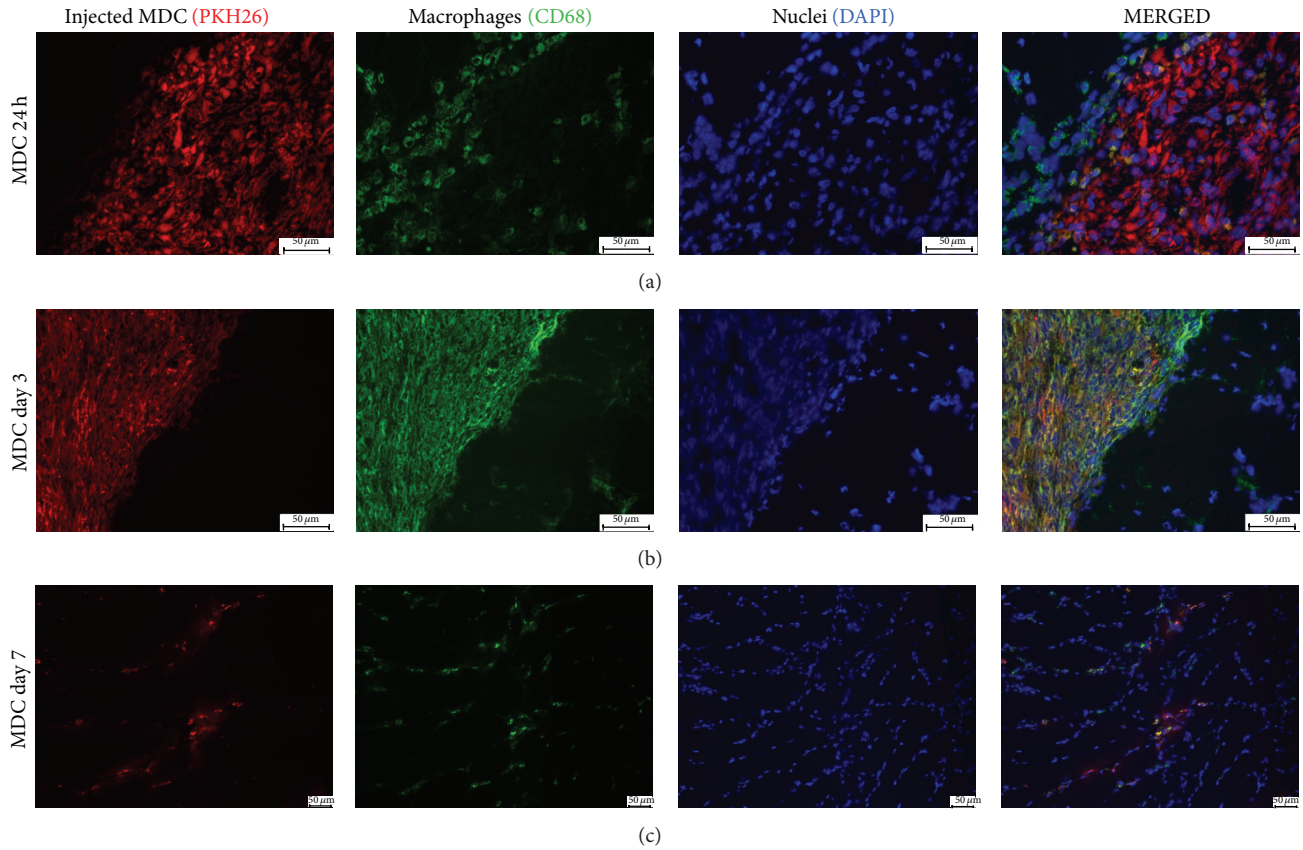

Figure 4: Immunohistochemical staining in different time points. Macrophages (CD68 antigen stained with Alexa 488 (green)) are infiltrating the cluster of transplanted MDC (PKH26 (red)). Cell nuclei stained with DAPI (blue). Scale bars: 50 μm.

7 no significant differences in the mRNA level of examined cytokines were observed either after the transplantation or after the vehicle injection (Figure 2).

3.5. Distribution of Injected Cells Differs between Analyzed Time Points. To identify injected cells at the injection site in some animals ($n = 6$) MDCs were labeled with red fluorochrome PKH26 prior to transplantation. The analysis of sectioned tissue samples revealed big, distinct clusters of PKH26 stained cells between muscle fibers (Figures 4 and 5). No red fluorescence was observed in sham operated limbs (data not shown). The transplanted cells formed dense clusters at day 1 and day 3 but not after one week after injection. PKH26 positive cells in tissue samples collected at day 7 were rather rare and diffused throughout the injection site.

3.6. Host Derived Cultured MDCs Induce Rapid Infiltration of Inflammatory Cells. To evaluate the presence of inflammatory cells in the injection site the tissue sections were stained with H&E (Figure 6). This preparation allowed for recognition of transplanted cell clusters in samples collected 1 and 3 days after injection. Numerous inflammatory cells, especially polymorphonuclear granulocytes, could be observed around and within the clusters at day 1. In specimens obtained 7 days after injection, inflammatory cells were not observed any more in relevant number. To characterize this infiltration better, the tissue sections were immunostained for CD68, macrophage marker, and CD43, leukosialin, present on majority of leukocytes and not on macrophages. The substantial number of both macrophages and leukocytes could be recognized in the direct vicinity of transplanted MDCs clusters 24 h after injection. At this time point the majority of CD68 expressing cells were surrounding the clusters, and only few macrophages could be observed between the injected cells (Figure 4(a)). At day 3 cells expressing CD68 were totally covering the area of PKH26 clusters (Figure 4(b)). One week after injection the number of macrophages was substantially reduced. In many cases, CD68 colocalized with PKH26 red fluorescence (Figures 4(b) and 4(c)). The presence of CD43 positive cells was the most prominent at day 1 (Figure 5(a)).

3.7. Muscle-Derived Cells Are Eliminated from the Injection Site after Autologous Transplantation. *In vivo* fluorescence imaging revealed the distinct reduction of DiD-derived signal in subsequent time points in all analyzed animals ($n = 3$). Images of representative rat were presented at Figure 7. Vehicle injection did not induce fluorescence in analyzed wavelengths which indicate the specificity of signal detected in the transplanted limb. These results confirm the poor cell survival after intramuscular injection, but for the first time this phenomenon is demonstrated in the same animal in subsequent time points after autologous transplantation.

3.8. Survived MDCs Were Able to Fuse with Host Muscle Fibers. Labeling of cells *ex vivo* allowed for identification of their fate *in vivo*. At day 7 PKH26 derived fluorescence was observed both in mononuclear cells located between muscle fibers and in the muscle fibers (Figures 8(a) and 8(b)) Moreover, PKH26 positive cells could be recognized in the central position of muscle fibers sections (Figures 8(c) and 8(d)) indicating that transplanted cells possessed ability to participate in the regeneration process.

4. Discussion

The poor survival of myoblasts after transplantation is well-known problem which limits the introduction of MTT into the clinic. The elimination of injected myoblasts is massive and rapid. In allogeneic models, 24 h after myogenic cells transplantation only 20% or less of initial cell number can be detected in the area of injection. This amount is further decreasing to 1–5% at days 3–5 after transfer [5, 15, 16]. Autologous way of transplantation does not solve this problem. The process of graft losing seems to be slower; Holzer et al. [7] detected 60% of injected population after 24 h, but the percentage of survived cells diminished to about 10% at day 3 [7]. Herein, we confirm the limited cell survival after autologous intramuscular transplantation by *in vivo* imaging of the same individuals in subsequent time points. The current understanding of such graft failure is not clear. Our previously published results suggest the role of oxidative stress (possibly generated by phagocytic immune cells) in cellular graft elimination after autologous transfer [18]. Therefore the aim of presented herein experiments was to describe the dynamics of acute local inflammatory reaction in response to autologous MDC transplantation.

In our experiments we isolated cells exploiting preplate (pp) technique described by Qu et al. [20]. To reduce the number of fibroblasts in culture we used a subset of cells which adhered to the plastic surface between 24 h and 72 h after seeding. According to preplating schedule presented by Qu et al. [20] we used mixed population of pp3-pp4 cells which mainly consists of satellite cells progeny, myoblasts. Indeed, our results confirm that majority of isolated cells expressed desmin. The average fusion index 3 days after induction of myogenic differentiation amounted to 35%. In comparison, the average fusion index of C2C12 cell line (mice satellite cells) was shown to be about 50% [21]. Therefore, as obtained population still contained some nonmyogenic cells like fibroblasts or fibroadipogenic progenitors, we call it muscle-derived cells rather than myoblasts.

The analysis of samples collected 24 h after transplantation indicated that autologous MDCs induce significant upregulation of *Il-1α* and *Il-1β* genes expression in comparison to the group treated with vehicle only (Figure 2). The significantly increased expression of *Il-1*, especially *Il-1β* isoform, in response to the intramuscular cell transplantation, was previously reported in several different models: allogeneic transfer of myoblasts into the intact myocardium [5], allogeneic transfer of mesenchymal stem cells (MSCs) into the infarcted myocardium [22], and syngeneic transfer of MSCs into intact skeletal muscle [23]. In the present study we demonstrate for the first time that similar response in regard to *Il-1β* expression is observed also after autologous transfer of MDCs. Moreover, we confirmed this finding at the protein

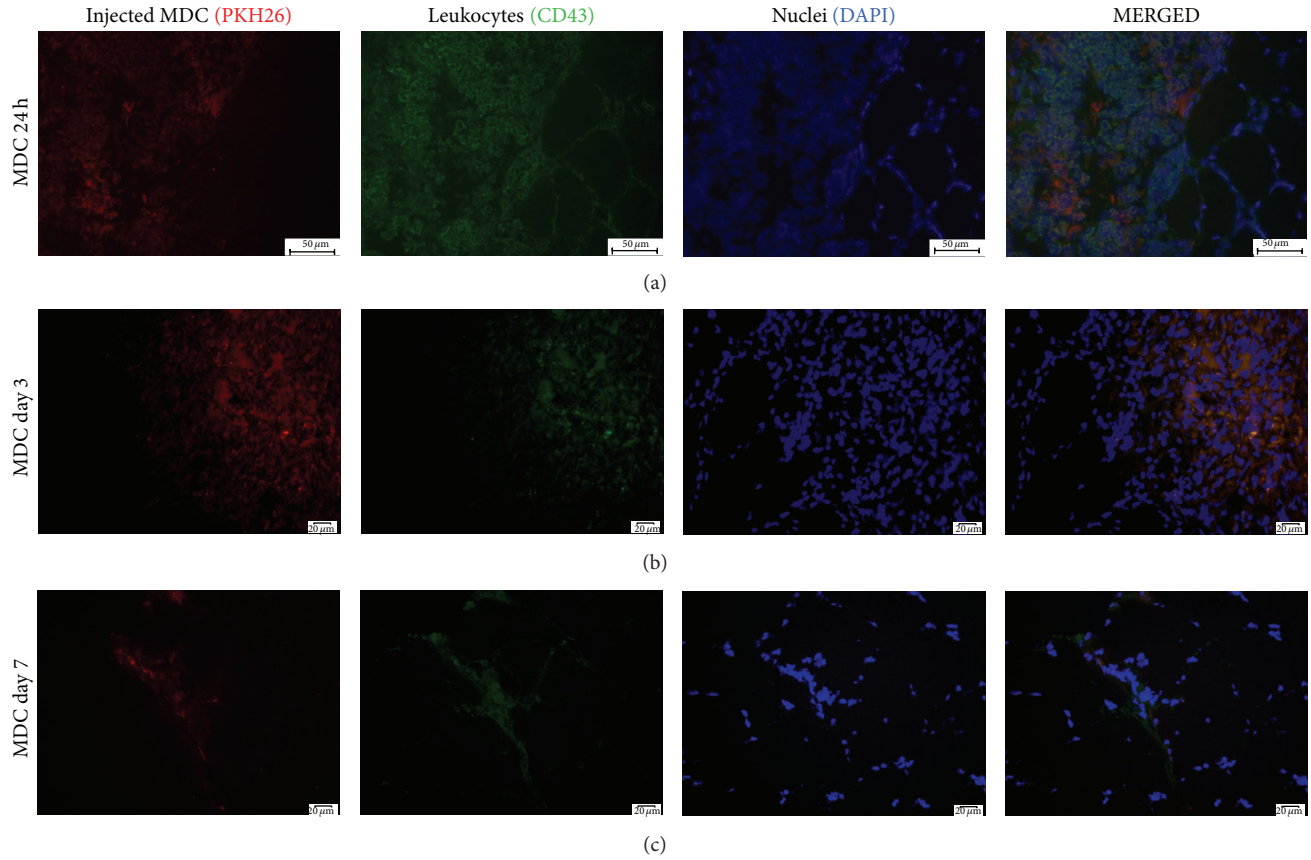

Figure 5: Immunohistochemical staining in different time points. Leukocytes (CD43 antigen stained with Alexa 488 (green)) are infiltrating the cluster of transplanted MDC (PKH26 (red)). Cell nuclei stained with DAPI (blue) in different time points. Scale bars: 50 μm.

level (Figure 3). The evaluation of *Il-1β* expression dynamics during the first week after the procedure revealed that the boost of *Il-1β* is acute and transient and the expression at day 3 is distinctly lower than at day 1 and it is not any more significantly higher comparing to the VEH group in analogous time point. Suzuki et al. [5] also evaluated the dynamics of cytokines expression in two time points (24 h and 3 days after transfer) after allogeneic myoblasts grafting into intact myocardium and reported the same pattern of changes, peak upregulation at day 1 and downregulation at day 3. As IL-1β is the key proinflammatory cytokine associated with the activity of phagocyting immune cells, those data suggest that the dynamic of early inflammatory reaction after MTT is similar in both auto- and allogeneic transplantations. It is worth noting that IL-1 was showed to be important in respect to grafted cell survival in allogeneic model. It was demonstrated that blocking of IL-1 action resulted in increased transplantation efficacy [14, 20]. Our data suggest that the same effect could be obtained in autologous transfer.

In the present study we also characterized early inflammatory infiltration in reaction to MDCs transfer. For this part of experiment cells were labeled with red membrane linker, PKH26, prior to transplantation. This method of labeling was chosen as it is described to be nonimmunogenic in both manufacturer's specification and a published report [24]. Thus it seems to be applicable for studying of immune response. The disadvantage of this cell tracker is substantial diluting during cell divisions which was analyzed in our recently published report [25]. However in short term experiment in which we did not expect intensive cell proliferation membrane linker met our needs. In the tissue samples collected from MDC group at days 1 and 3 the transplanted cells were clearly visible. In those two time points they formed dense, demarcated cell clusters (Figures 4(a) and 4(b)). Very similar appearance was observed after allogeneic transplantation of either myoblasts or muscle-derived stem cells at day 2 after injection [20]. In our experiment, the distribution of grafted cells changed in specimens collected at day 7; the cells were much more diffused throughout the site of injection (Figure 4(c)). The amount of PKH26 positive cells at this time point was distinctly lower comparing to either day 1 or day 3. At day 7 PKH26 derived fluorescence was observed both in mononuclear cells located between muscle fibers and in the muscle fibers (Figure 8(a)). Moreover, PKH26 positive cells could be recognized in the central position of muscle fiber section (Figure 8(b)) which indicated that transplanted cells which survived in the injection site were able to participate in the regeneration process. This finding is consistent with previous reports [7, 20, 26].

The results indicating limited persistence of injected cells at day 7 were confirmed by semiqualitative graft survival

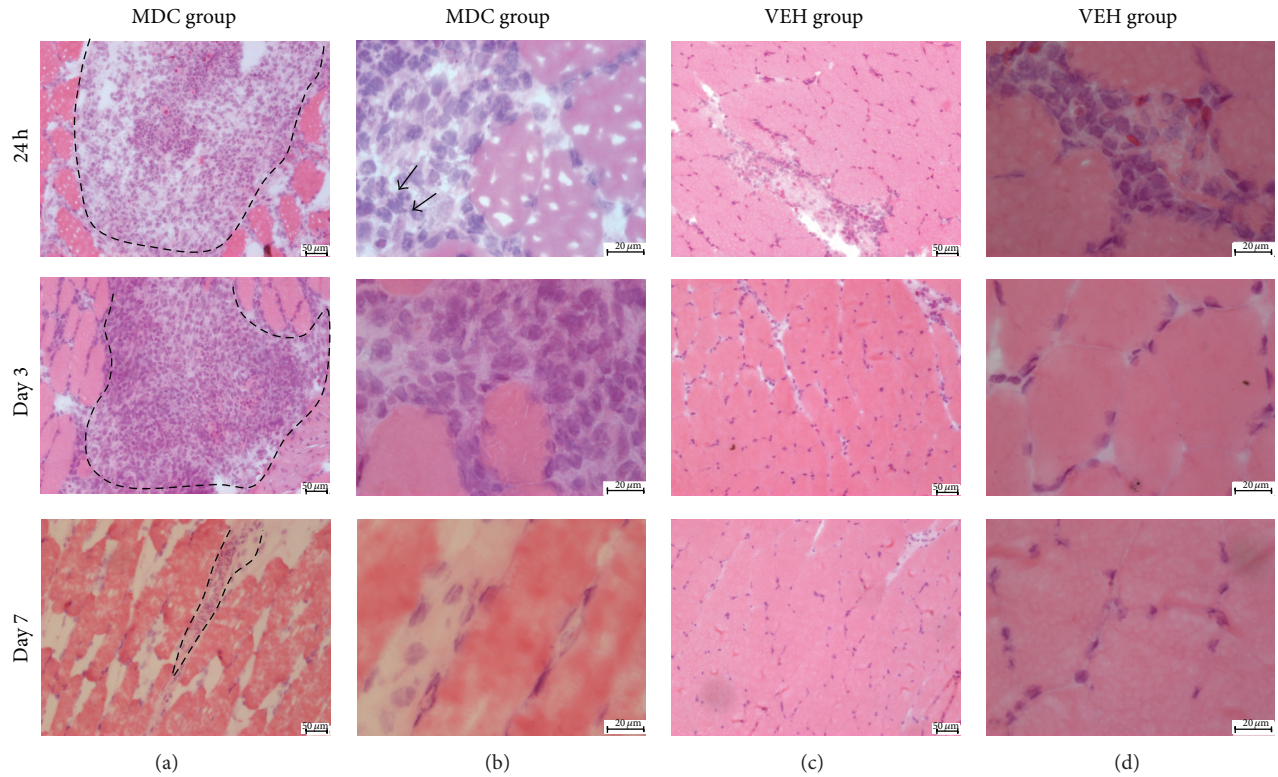

Figure 6: The skeletal muscle stained with hematoxylin and eosin. Images present the area of injection at days 1, 3, and 7 after procedure in subsequent rows. (a and b) Cross sections from transplanted group in two different magnifications. Distinct clusters of injected cells are visible. Dashed lines drawn on images in column A indicate approximate area of clusters located between muscle fibers. At day 1, neutrophils can be recognized within the cluster (arrows). At day 3, identification of neutrophils is not obvious; large mononuclear cells dominate in the cluster. At day 7, inflammatory infiltration is not clearly visible anymore; (c and d) cross sections from group injected with vehicle only in two different magnifications. Some inflammatory cells could be recognized in samples collected at day 1 time point.

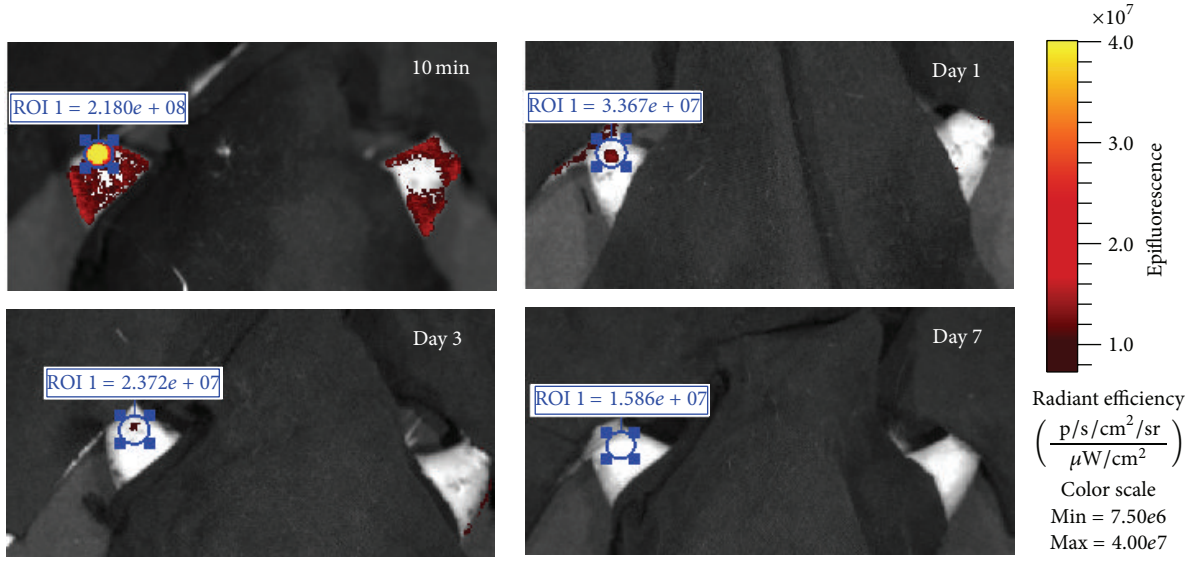

Figure 7: *In vivo* imaging of a representative animal in subsequent time points. 2.5×10^5 DiD-labeled MDCs were injected into the gastrocnemius muscle (limb on the left). Contralateral limb was injected with the equal volume of vehicle and constituted internal control. Optical imaging was carried out at 10 min, days 1, 3, and 7 after the injection. Read-out scale was unified between the subsequent images, as presented on the right panel. Values in blue rectangles indicate total radiant efficiency of DiD-derived signal measured in subsequent time points in equal regions of interest (ROI).

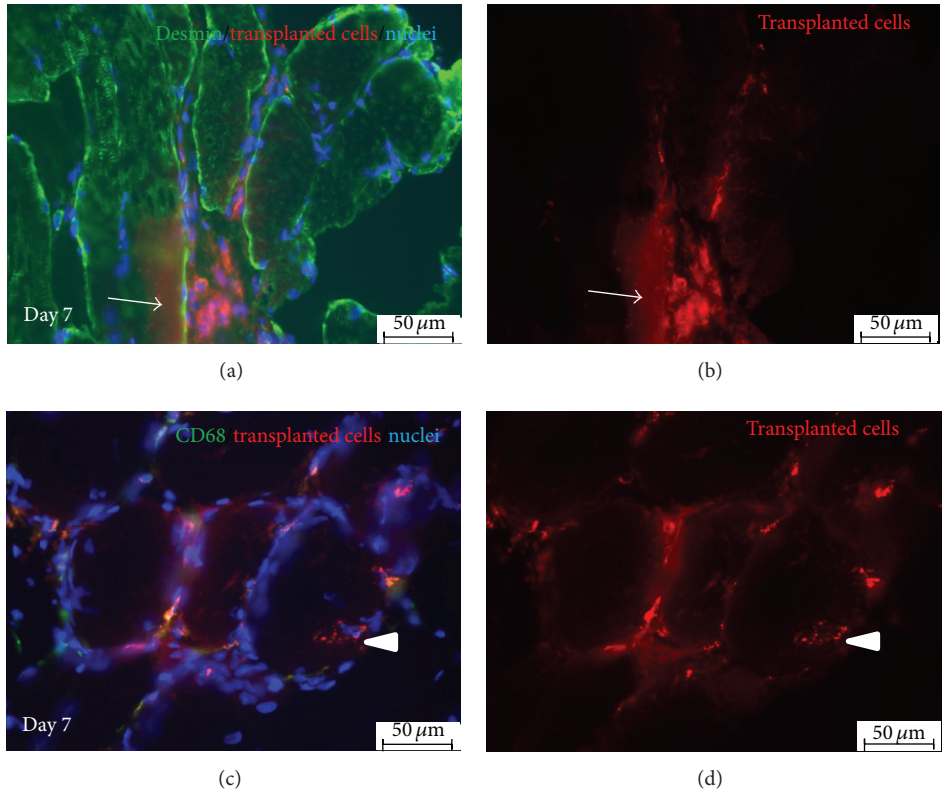

Figure 8: Immunohistochemical staining of samples collected 7 days after autologous MDCs injection. (a and b) PKH26 positive cells (red) can be recognized between and within muscle fibers (arrow), desmin stained with Alexa 488 (green); (c and d) PKH26 positive cells (red) can be recognized in the central position of muscle fiber (arrowhead) indicating the ability to contribute muscle regeneration. Few macrophages (CD68 stained with Alexa 488 (green)) are still present in the site of transplantation. Scale bars: 50 μm.

analysis using *in vivo* fluorescence imaging (FLI). Although this method has serious limitations because of considerable autofluorescence of the animal tissues, it was previously demonstrated that FLI analysis of myoblasts survival after intramuscular injection can be valid [8]. In our study, we have utilized a spectral unmixing algorithm offered by the Living Image software to alleviate this issue. Using membrane linkers for *in vivo* imaging creates an additional problem; these dyes can be absorbed by phagocytes and therefore impede interpretation of results. However, in our experiment the radiant efficiency of DiD-derived signal decreased markedly in subsequent time points; therefore we can conclude that cells were indeed eliminated from the injection site.

To confirm the presence of immune response to autologous MDCs injection the tissue sections were analyzed in order to identify inflammatory cells. Basic H&E staining revealed that clusters of transplanted cells were infiltrated with polymorphonuclear granulocytes at day 1 (Figure 6(a)). At day 3, the presence of neutrophil in H&E stained sections was not so obvious; most numerous cells within transplanted clusters at this time point were large and mononuclear which could indicate them to be macrophages. To identify the infiltration more precisely the sections were immunostained for CD43—an antigen which is present on majority of leukocytes and not on macrophages. The analysis revealed the typical sequence of events in early innate immune reaction, the rapid infiltration of target area with neutrophils (within first 24 h) and delayed appearance of macrophages with the maximal intensity at day 3. At day 7 the inflammatory reaction was almost completely resolved which is consistent with results of cytokines gene expression analysis. Thus, our results demonstrate for the first time that autologous transplantation of muscle-derived cells induces classical early immune reaction in the site of injection. It can be claimed that the presence of macrophages in the injection site is beneficial for the grafting efficacy. It was previously demonstrated that macrophages enhance or even are required for muscle regeneration [27, 28]. Furthermore, it was shown that coinjection of myoblasts and macrophages improves survival, proliferation, and migration of transplanted muscle precursor cells [9, 29]. However, results regarding coinjection were obtained after either allogeneic or xenogeneic injection into immunodeficient host. Thus, it is difficult to predict if host derived macrophages could also improve transplanted cell survival.

There are two most probable interpretations of innate response appearance. First, the inflammatory cells are activated because the injected cells become immunogenic during extracorporeal manipulations. Second, injected cells die due to other reasons like ischemia, hypoxia, or anoikis and phagocytes infiltrate the graft to clean up dead cells but not to eliminate viable ones. Verification of the mechanism which

has predominant importance is crucial for assessment of future strategies for autologous MTT efficacy improvement. Our previously published data demonstrated that cells with increased resistance to oxidative stress survive better after autologous intramuscular transplantation. Similar findings were presented before by Urish et al. [26] on allogeneic model of transplantation. As the production of reactive oxygen species is associated with phagocyting activity, thus the importance of resistance to oxidative stress supports the hypothesis that inflammation is the cause and not the consequence of grafted cell death. The role of inflammation in myoblasts elimination after autologous administration was also shown by Ito et al. [30]. The authors demonstrated that strong systemic immunosuppression improved the efficacy of transplantation in nondystrophic dogs. On the other hand some reports suggest that neutrophils and macrophages are not responsible for early death of donor myoblasts even in allogeneic model [15, 16] and thus support the theory that cells die due to other reasons and inflammation is only the secondary consequence of this death. As the two potential mechanisms are not exceptive, it is possible that only complex protective approach will be able to effectively prevent myoblasts' death after transplantation.

To conclude, presented data demonstrate that autologous intracellular MDCs transplantation induces classical early immune reaction in the site of injection. These results, when considered with previously published reports, suggest that innate inflammatory response can contribute to limited survival of theoretically nonimmunogenic autologous cellular graft.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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References

[1] T. A. Partridge, M. Grounds, and J. C. Sloper, "Evidence of fusion between host and donor myoblasts in skeletal muscle grafts," *Nature*, vol. 273, no. 5660, pp. 306–308, 1978.

[2] A. Dellavalle, M. Sampaolesi, R. Tonlorenzi et al., "Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells," *Nature Cell Biology*, vol. 9, no. 3, pp. 255–267, 2007.

[3] Y. Fan, M. Maley, M. Beilharz, and M. Grounds, "Rapid death of injected myoblasts in myoblast transfer therapy," *Muscle and Nerve*, vol. 19, no. 7, pp. 853–860, 1996.

[4] J. Huard, T. Yokoyama, R. Pruchnic et al., "Muscle-derived cell-mediated ex vivo gene therapy for urological dysfunction," *Gene Therapy*, vol. 9, no. 23, pp. 1617–1626, 2002.

[5] K. Suzuki, B. Murtuza, J. R. Beauchamp et al., "Dynamics and mediators of acute graft attrition after myoblast transplantation to the heart," *The FASEB Journal*, vol. 18, no. 10, pp. 1153–1155, 2004.

[6] C. Guo, H. K. Haider, W. S. N. Shim et al., "Myoblast-based cardiac repair: xenomyoblast versus allomyoblast transplantation," *Journal of Thoracic and Cardiovascular Surgery*, vol. 134, no. 5, pp. 1332.e2–1339.e2, 2007.

[7] N. Holzer, S. Hogendoorn, L. Zürcher et al., "Autologous transplantation of porcine myogenic precursor cells in skeletal muscle," *Neuromuscular Disorders*, vol. 15, no. 3, pp. 237–244, 2005.

[8] X. Xu, Z. Yang, Q. Liu, and Y. Wang, "In vivo fluorescence imaging of muscle cell regeneration by transplanted EGFP-labeled myoblasts," *Molecular Therapy*, vol. 18, no. 4, pp. 835–842, 2010.

[9] M. Bencze, E. Negroni, D. Vallese et al., "Proinflammatory macrophages enhance the regenerative capacity of human myoblasts by modifying their kinetics of proliferation and differentiation," *Molecular Therapy*, vol. 20, no. 11, pp. 2168–2179, 2012.

[10] K. L. Pellegrini and M. W. Beilharz, "The survival of myoblasts after intramuscular transplantation is improved when fewer cells are injected," *Transplantation*, vol. 91, no. 5, pp. 522–526, 2011.

[11] D. Skuk, M. Paradis, M. Goulet, and J. P. Tremblay, "Ischemic central necrosis in pockets of transplanted myoblasts in nonhuman primates: implications for cell-transplantation strategies," *Transplantation*, vol. 84, no. 10, pp. 1307–1315, 2007.

[12] M. Bouchentouf, B. F. Benabdallah, P. Bigey, T. M. Yau, D. Scherman, and J. P. Tremblay, "Vascular endothelial growth factor reduced hypoxia-induced death of human myoblasts and improved their engraftment in mouse muscles," *Gene Therapy*, vol. 15, no. 6, pp. 404–414, 2008.

[13] M. Bouchentouf, B. F. Benabdallah, J. Rousseau, L. M. Schwartz, and J. P. Tremblay, "Induction of anoikis following myoblast transplantation into SCID mouse muscles requires the Bit1 and FADD pathways," *American Journal of Transplantation*, vol. 7, no. 6, pp. 1491–1505, 2007.

[14] K. Suzuki, B. Murtuza, J. R. Beauchamp et al., "Role of interleukin-1β in acute inflammation and graft death after cell transplantation to the heart," *Circulation*, vol. 110, no. 11, pp. II219–II224, 2004.

[15] L. M. Sammels, E. Bosio, C. T. Fragall, M. D. Grounds, N. Van Rooijen, and M. W. Beilharz, "Innate inflammatory cells are not responsible for early death of donor myoblasts after myoblast transfer therapy," *Transplantation*, vol. 77, no. 12, pp. 1790–1797, 2004.

[16] S. I. Hodgetts, M. W. Beilharz, A. A. Scalzo, and M. D. Grounds, "Why do cultured transplanted myoblasts die in vivo? DNA quantification shows enhanced survival of donor male myoblasts in host mice depleted of CD4+ and CD8+ cells or NK1.1+ cells," *Cell Transplantation*, vol. 9, no. 4, pp. 489–502, 2000.

[17] D. Skuk, N. Caron, M. Goulet, B. Roy, F. Espinosa, and J. P. Tremblay, "Dynamics of the early immune cellular reactions after myogenic cell transplantation," *Cell Transplantation*, vol. 11, no. 7, pp. 671–681, 2002.

[18] U. Bartoszuk-Bruzzone, A. Burdziń, A. Orzechowski, and Z. Kłos, "Protective effect of sodium ascorbate on efficacy of intramuscular transplantation of autologous muscle derived cells," *Muscle and Nerve*, vol. 45, no. 1, pp. 32–38, 2012.

[19] A. Burdzińska, U. Bartoszuk-Bruzzone, M. M. Godlewski, and A. Orzechowski, "Sodium ascorbate and basic fibroblast

growth factor protect muscle-derived cells from H2O2-induced oxidative stress," *Comparative Medicine*, vol. 56, no. 6, pp. 493–501, 2006.

[20] Z. Qu, L. Balkir, J. C. T. van Deutekom, P. D. Robbins, R. Pruchnic, and J. Huard, "Development of approaches to improve cell survival in myoblast transfer therapy," *Journal of Cell Biology*, vol. 142, no. 5, pp. 1257–1267, 1998.

[21] P. Pawlikowska, B. Gajkowska, J. Hocquette, and A. Orzechowski, "Not only insulin stimulates mitochondriogenesis in muscle cells, but mitochondria are also essential for insulin-mediated myogenesis," *Cell Proliferation*, vol. 39, no. 2, pp. 127–145, 2006.

[22] Y. Imanishi, A. Saito, H. Komoda et al., "Allogenic mesenchymal stem cell transplantation has a therapeutic effect in acute myocardial infarction in rats," *The Journal of Molecular and Cellular Cardiology*, vol. 44, no. 4, pp. 662–671, 2008.

[23] K. Gala, A. Burdzinska, M. Idziak, E. Wilczek, and L. Paczek, "Transplantation of mesenchymal stem cells into the skeletal muscle induces cytokine generation," *Cytokine*, vol. 64, pp. 243–250, 2013.

[24] K. Fischer, R. Andreesen, and A. Mackensen, "An improved flow cytometric assay for the determination of cytotoxic T lymphocyte activity," *The Journal of Immunological Methods*, vol. 259, no. 1-2, pp. 159–169, 2002.

[25] A. Burdzińska, R. Crayton, B. Dybowski et al., "The effect of endoscopic administration of autologous porcine muscle-derived cells into the urethral sphincter," *Urology*, vol. 82, no. 3, pp. 743.e1–743.e8, 2013.

[26] K. L. Urish, J. B. Vella, M. Okada et al., "Antioxidant levels represent a major determinant in the regenerative capacity of muscle stem cells," *Molecular Biology of the Cell*, vol. 20, no. 1, pp. 509–520, 2009.

[27] L. Lescaudron, E. Peltékian, J. Fontaine-Pérus et al., "Blood borne macrophages are essential for the triggering of muscle regeneration following muscle transplant," *Neuromuscular Disorders*, vol. 9, no. 2, pp. 72–80, 1999.

[28] J. G. Tidball and M. Wehling-Henricks, "Macrophages promote muscle membrane repair and muscle fibre growth and regeneration during modified muscle loading in mice in vivo," *The Journal of Physiology*, vol. 578, no. 1, pp. 327–336, 2007.

[29] P. F. Lesault, M. Theret, M. Magnan et al., "Macrophages Improve Survival, Proliferation and Migration of Engrafted Myogenic Precursor Cells into MDX Skeletal Muscle," *PLoS ONE*, vol. 7, no. 10, Article ID e46698, 2012.

[30] H. Ito, J. T. Vilquin, D. Skuk et al., "Myoblast transplantation in non-dystrophic dog," *Neuromuscular Disorders*, vol. 8, no. 2, pp. 95–110, 1998.

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Research Article

Anti-Inflammatory Effect of IL-37b in Children with Allergic Rhinitis

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Background. Interleukin-37 (IL-37), a newly described member of IL-1family, functioned as a fundamental inhibitor of innate inflammatory and immune responses, especially its isoform IL-37b. *Objective*. This study was undertaken to evaluate the expression and regulation of IL-37b in children with allergic rhinitis (AR). *Methods*. Forty children with AR and twenty-five normal controls were included. The relationship between IL-37b and Th1/2 cytokines production in serum and nasal lavage was examined by enzyme-linked immunosorbent assay (ELISA). Peripheral blood mononuclear cells (PBMCs) were purified for in vitro regulation experiment of IL-37b. Intranasal mometasone furoate was given in AR children and IL-37b change after one-month treatment was detected using ELISA. *Results*. We observed significantly decreased IL-37b expression levels in both serum and nasal lavage compared to controls. IL-37b was negatively correlated with Th2 cytokines. Our results also showed that IL-37b downregulated Th2 cytokine expressed by PBMCs and this modulation was through mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathway. We also found that intranasal mometasone furoate therapy can promote nasal IL-37b expression. *Conclusion*. IL-37b may be involved in Th2 cytokine regulation in AR and its expression was related to the efficacy of intranasal steroid therapy.

1. Introduction

Allergic rhinitis (AR) is a worldwide common disease in children with occurrence of 10–40% [1, 2]. It affects both life and performance of children and it is often accompanied by asthma [3]. T cell type-2 (Th2) inflammation accompanied by infiltration with eosinophils is characteristic pathological changes in AR [4, 5]. Infiltration of T cells, B cells, plasma cells, eosinophils, basophils, and neutrophils and activation of Th2 cells caused the occurrence of AR [6]. Most studies about pathophysiological mechanism of AR focused on proinflammatory cytokines, while anti-inflammatory cytokines received less attention.

Interleukin-37 (IL-37) is a recently discovered anti-inflammatory cytokine and can bind to IL-18-receptor (R) and IL-18-binding protein (BP) [7, 8]. IL-37 can be secreted by monocytes, macrophages, epithelial cells, and so on [9]. IL-37 is expressed in normal and tumor tissues in human and it exerts anti-inflammatory effects by suppressing innate immune responses through decreasing the production of inflammatory cytokines [10]. IL-37 has five different isoforms, of which IL-37b is the largest isoform and it is believed to suppress proinflammatory cytokine production and dendritic cell (DC) activation [8]. The role of IL-37b in autoimmune diseases was well characterized recently. It was reported that IL-37b expression was elevated in rheumatoid arthritis, in patients with *Mycobacterium avium* infections, atherosclerotic coronary and carotid artery plaques, in psoriatic plaques, and in Crohn's disease [10, 11]. However, the role of IL-37b in allergic disease is not characterized.

In our present study, we sought to determine the expression of IL-37b in serum and nasal lavage of AR children and

the effect of IL-37b on Th1/2 cytokines production and the involved signaling pathway.

2. Methods

2.1. Patients. Forty children (<18 years old) with AR were recruited in the study. The diagnosis was established on history, clinical examination, skin prick test, and specific IgE measurement, which is in accordance with Allergic Rhinitis and its Impact on Asthma (ARIA) guideline (2010) [12]. Of them, children with asthma were diagnosed according to the global initiative for asthma (GINA) criteria [13]. Twenty-five children of similar age and gender with no history of allergic disease or wheezing were selected as the control group. Those with chronic diseases (e.g., malnutrition, anatomic malformation of the respiratory system, chronic lung disease, heart disease, gastroesophageal reflux disease, and cystic fibrosis) and those with a history of chronic drug use (e.g., oral or nasal corticosteroids, antiepileptics, and immune suppressives) were excluded from the study. The tissues from the inferior turbinate from nine AR children (13–17 years old) and eight controls (13–16 years old) that underwent inferior turbinectomy due to severe nasal congestion caused by inferior turbinate hypertrophy were sampled for detection.

2.2. Blood and Nasal Lavage Samples Preparation and Analysis. Venous blood samples were collected into Vacuette tubes and centrifuged at 1000 g for 15 min at 4°C. Serum samples were stored at −80°C. These samples were used for enzyme-linked immunosorbent assay (ELISA) and qPCR measurement. The whole blood cell counts were measured by LH-785 system (Beckman Coulter, Mervue, Galway, Ireland). Total serum IgE was measured by electrochemiluminescence (ECLIA) method using an ELX-800 system.

Nasal lavage was performed using saline warmed to 37°C. The process was performed according to method described elsewhere [14]. Due to cooperation of children, 28 AR samples and 15 controls were collected. The samples were centrifuged to remove cellular debris and aliquots of the supernatants were stored at −20°C in eppendorf tubes until analysis. Total protein concentrations were determined with Bio-Rad protein assays according to Bradford [15].

2.3. ELISA for Protein Expression and ECLIA for Eosinophil Cationic Protein (ECP) Protein Expression. ELISA kits were used for measuring IL-37b, IL-4, IL-13, IL-5, IL-12, IFN-γ, and IL-10 (all from R&D systems, USA) level in serum, nasal lavage, and supernatant of peripheral blood mononuclear cells (PBMCs). ECP levels were detected by other ELISA kit (EIAab, Wuhan, China), according to the manufacturer's protocols. The detection limits of the assays were as follows: IL-37b (125 pg/mL), IL-4 (1.56 pg/mL), IL-13 (93.8 pg/mL), IL-5 (7.8 pg/mL), IL-12 (2.5 pg/mL), IFN-γ (12.5 pg/mL), and IL-10 (3.9 pg/mL).

2.4. Real-Time PCR Analysis. Real-time PCR was performed as described previously [16]. Total RNA was extracted from mucosa tissues using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription (RT) was performed, and cDNA was synthesized from 2 μg of total RNA using an oligo (dT) 18 primer and M-MLV reverse transcriptase (TAKARA, Syuzou, Shiga, Japan). The mRNA expression was determined using an ABI PRISM 7300 Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Premix Taq (TAKARA). The sequences of the primers were as follows: IL-37b, forward primer: 5′-CTCCTGGGGGTCTCTAAAGG-3′, reverse primer: 5′-TACAATTGCAGGAGGTGCAG-3′; β-actin, forward primer: 5′-CAGAGCAAGAGAGGCATCCT-3′, reverse primer: 5′-GTTGAAGGTCTCAAACATGATC-3′; PRISM samples contained 1× SYBR Green Master Mix, 1.5 μL of 5 μM primers, and 25 ng of synthesized cDNA in a 25 μL volume. Reaction mixtures were heated to 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, and annealing extension at 60°C for 60 s. All PCR reactions were performed in duplicate. Melting curve analysis was used to control for amplification specificity. The mean value of the replicates for each sample was calculated and expressed as a cycle threshold (Ct) value. The relative expression of each target gene was determined as the difference (ΔCt) between the Ct value of the target gene and the Ct value of β-actin. Fold changes in the target gene mRNA were determined as $2^{-\Delta\Delta Ct}$.

2.5. Immunohistochemical (IHC) Staining. For immunohistochemistry, the slides were placed in 0.3% H_2O_2 for 20 minutes at room temperature to reduce nonspecific background staining caused by endogenous peroxidases. After additional washing with PBS again, the slides were boiled in 10 mM citrate buffer for 15 minutes followed by cooling at room temperature. Antibodies for IL-37b (Abcam and Creative BioMart, USA), IL-12 (R&D systems, USA), IL-4 (R&D systems, USA), IL-1 (R&D systems, USA), and IgG (as control, Dako, Denmark) were incubated overnight at 4°C for immunohistochemical staining, respectively. The next day, the slides were washed with PBS and incubated with secondary antibody conjugated with streptavidin-horseradish peroxidase (Gene Tech, Shanghai, China) at room temperature for 1 hour.

After washing, DAB (Gene Tech, Shanghai, China) staining was performed under microscope. After rinsing with distilled water, the sections were counterstained with Mayer's hematoxylin (Zhongshan Goldenbridge, Beijing, China) for further 25 seconds, dehydrated with series of ethanol (v/v, 90%–100%), cleared from lipid with xylene (3 times), and mounted with neutral balsam (Zhongshan Goldenbridge, Beijing, China). Control for nonspecific staining was routinely performed with PBS but antibody and all proved negative with secondary antibody.

The sections were blindly examined and coded with no awareness of the clinical data. They were visualized with an Olympus CX40 Microscope (Olympus Europa GmbH, Germany). The number of positive cells (brown cells) was counted in 10 high-magnification visual fields (×200) and averaged.

Table 1: Demographic characteristic of AR children and normal controls.

Groups	AR without asthma group	AR with asthma group	Control
Number	30	10	25
Sex (male : female)	16 : 14	6 : 4	12 : 13
Age (months)	71.2 ± 34.0	63.2 ± 23.0	72.0 ± 31.8
History of asthma, *n* (%)	0	100	0
Family history of atopy, *n* (%)	15 (50)*	8 (80)#	3 (12)
Exposure to smoking, *n* (%)	18 (60)*	7 (70)#	1 (4)
Eosinophil[a] (count/mm^3)	145 (60–1350)*	195 (60–1350)#	73 (5–230)
Neutrophil[a] (count/mm^3)	8430 (2000–16300)	12330 (9000–25700)#	7400 (3200–12500)
Basophil[a] (count/mm^3)	50 (10–150)*	160 (70–350)#	8 (4–16)
Monocytes[a] (count/mm^3)	238 (70–2740)	268 (80–1960)	256 (60–2530)
Lymphocyte[a] (count/mm^3)	7900 (3100–15400)	7500 (4100–16600)	8300 (2500–16300)
ECP[a] (ng/mL)	38.2 (4.0–131.0)*	68.1 (35.0–188.0)#	10.9 (3.6–109.0)
IgE[a] (IU/mL)	101.1 (2.5–1200.0)*	198.2 (87.5–1600.0)#	31.0 (5.3–89.0)

[a]Data presented as median values (minimum–maximum).
*Compared with control group, $P < 0.05$.
#Compared with AR group, $P < 0.05$.

2.6. PBMCs Preparation. PBMCs were separated from 20 mL of heparinized whole blood from AR children. Specifically, cells were isolated by Lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway) density-gradient centrifugation from heparinized leucocyte-enriched buffy coats. Then, PBMCs were cultured at $2*10^6$/mL in 24-well plates in culture medium: RPMI 1640 supplemented with 5% human AB serum, 5 mmol/L glutamine, and penicillin, and streptomycin solution (all from Invitrogen, except for serum from Sigma-Aldrich). Stimulation was performed through addition of rhIL-37b (1–100 ng/mL) with or without other cytokines (Poly (I:C) (1 ng/mL), rhIL-4 (1–100 ng/mL), rhIL-5 (1–100 ng/mL), rhIL-13 (1–100 ng/mL), rhIL-12 (1–100 ng/mL), rhIFN-γ (1–100 ng/mL), rhIL-10 (1–100 ng/mL), SB203580 (10 μmol/L), and LY294002 (10 μmol/L). All the above stimulators were from R&D systems.

2.7. Human Peripheral Blood Eosinophil Purification and Assay. Eosinophils were obtained from the peripheral blood of atopic donors (blood eosinophil levels 5–10%) by MACS-negative immunomagnetic separation as described previously [17]. The purity of cells was 98–100% (Kimura staining) and the viability was larger than 98% (Trypan blue staining). The isolated eosinophils were suspended in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Eosinophils (500 μL, 10^5/mL) were treated with or without rh-IL-37 (1–100 ng/mL) and eotaxin (1 ng/mL). ECP protein changes were detected by Unicap system.

2.8. Intranasal Mometasone Furoate Therapy. All AR children underwent a 4-week course of intranasal mometasone furoate (50 μg per puff per nostril to both nostrils, once daily). At the end of treatment, the nose symptoms (runny nose, sneezing, and blocked nose), eye symptoms (streaming and swelling, redness and itching), and lung symptoms (breathlessness, cough, wheeze and chest tightness) were scored by the children as follows: 0 = no symptoms, 1 = slight symptoms, 2 = moderate symptoms, and 3 = severe symptoms. Besides, blood and nasal lavage samples were collected and changes of IL-37b protein expression were detected with ELSIA.

2.9. Statistical Analysis. All data were expressed as mean ± SD except additional note. Statistical significance between different groups was determined using nonparametric Mann-Whitney *U* test. The Spearman rank correlation test was used to analyze the correlation among the expression of biomarkers and clinical stage. $P < 0.05$ was considered as significant difference.

3. Results

3.1. Demographic and Laboratory Characteristics of the Study Population. This study was conducted with 65 children, 40 of whom suffered from AR, with ages ranging between 16 and 187 months (mean age: 73.2 ± 33.0 months, 22 males), and 25 of whom were healthy controls with ages ranging between 15 and 184 months (mean age: 72.0 ± 31.8 months, 11 males). The demographic features and laboratory parameters of the population are presented in Table 1. Asthma was found to be 25% in the AR children. When the two groups were compared, the basophil count, the eosinophil count, total serum IgE, and ECP levels were found to be higher in the AR without asthma group than those of the control group, especially in AR with asthma group.

3.2. Decreased IL-37b mRNA and Protein Levels in Relation to Th1/Th2/Treg Cytokines in AR. The serum and local IL-37b mRNA and protein expression in AR were significantly lower than those in the normal controls ($P < 0.001$, Figures 1(a)–1(d)). However, when we subdivided the AR group into asthma and nonasthma group, we found no difference of IL-37b expression levels between two groups. We also compared IL-37 expression between AR children >6 yr and <6 yr and

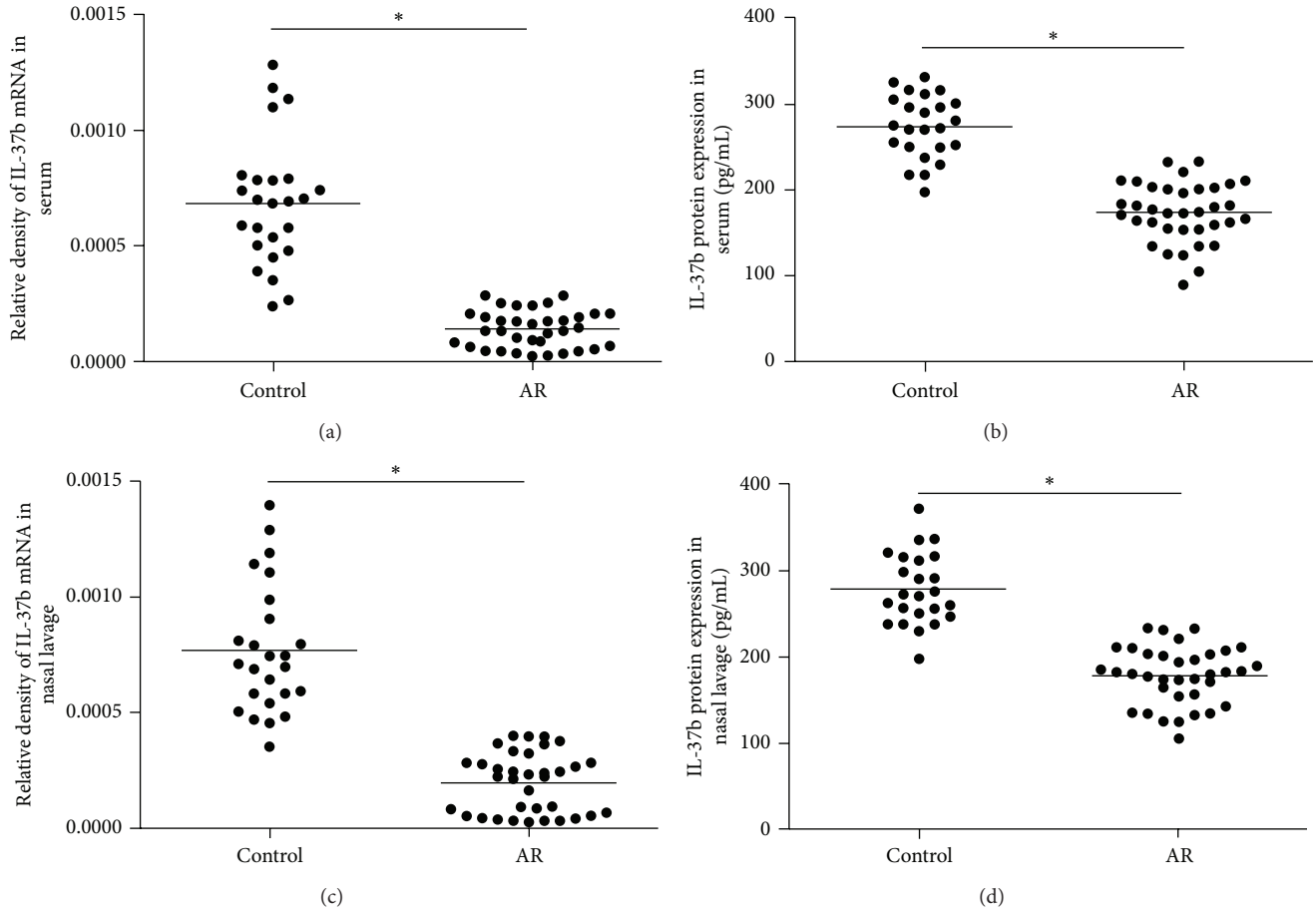


Figure 1: The serum and nasal lavage mRNA and protein expression of IL-37b in AR and normal control. (a) The serum IL-37b mRNA level in AR and normal control; (b) the serum IL-37b protein level in AR and normal control; (c) the nasal lavage IL-37b mRNA level in AR and normal control; (d) the nasal lavage IL-37b protein level in AR and normal control. $^*P < 0.05$, comparing the two groups.

no differences were found (data not shown). Our results showed enhanced IL-4, IL-13, and IL-5 protein expression, especially in children with asthma and decreased IL-12, IFN-γ, and IL-10 protein expression in serum and nasal lavage of AR children compared with control group ($P < 0.001$, Figures 2(a)–2(l)). Nasal IL-4, IL-5, and IL-13 were negatively correlated with local IL-37b ($P < 0.05$, Figures 3(a)–3(c)). The serum ECP and IgE levels as well as eosinophil counts were found to be negatively correlated with serum IL-37b expression ($P < 0.05$, Figures 3(g)–3(i)). No relationship between IL-37b and IL-12, IFN-γ, and IL-10 was found (Figures 3(d)–3(f)).

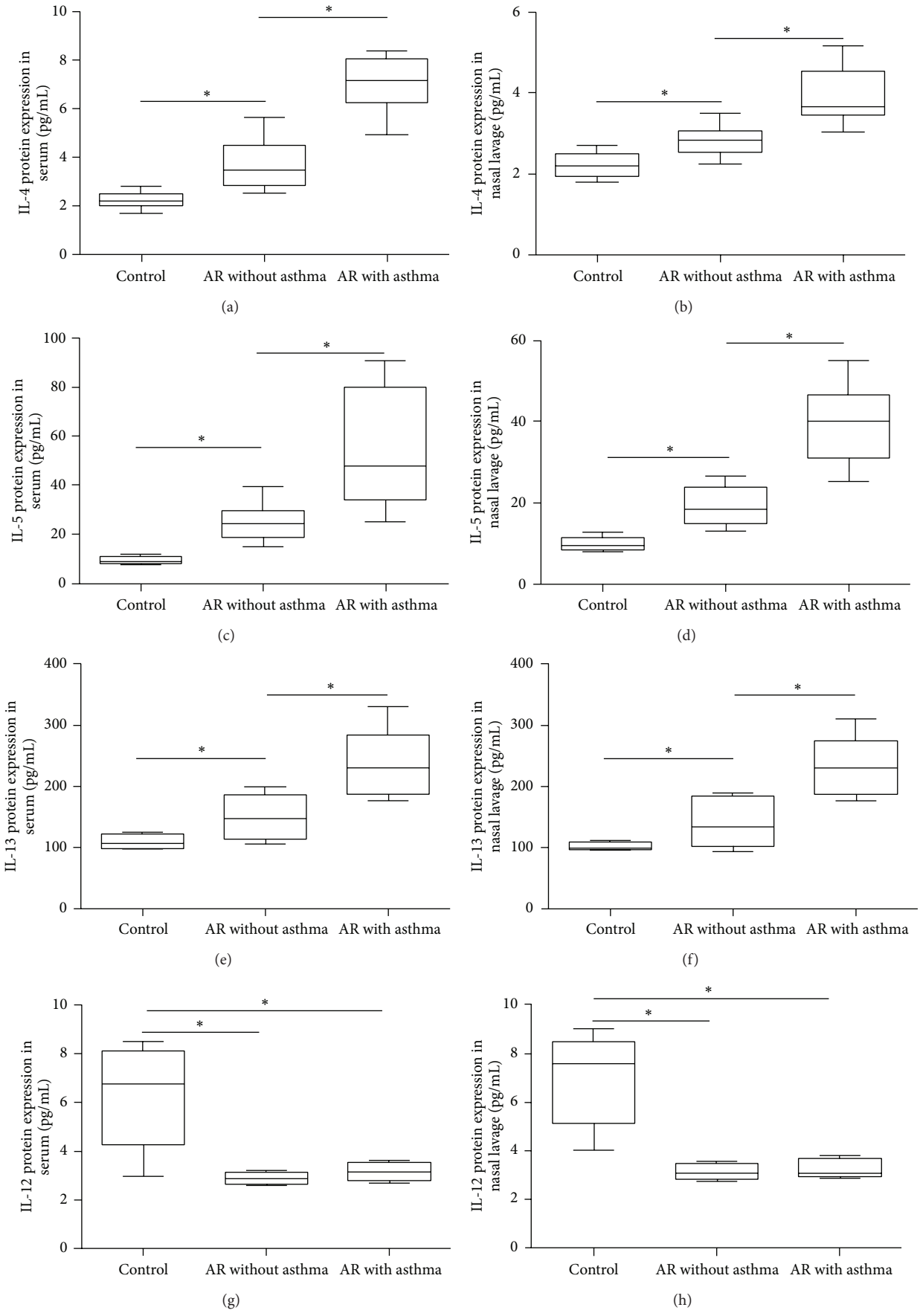
3.3. Decreased IL-37b Expression in AR by IHC. In normal tissues, some epithelial cells, interstitial cells, and glandular cells were positive for IL-37b and IL-37b immunoreactivity was enhanced significantly in controls compared with AR samples (Figures 4(a) and 4(b)). We also detected IL-12 (Figures 4(c) and 4(d)), IL-4 (Figures 4(e) and 4(f)), and IL-1 (Figures 4(g) and 4(h)) and the results showed that the number of IL-4 and IL-1 positive cells in AR was significantly higher than that of control, while the IL-12 positive cells presented as opposite trend (Table 2).

Table 2: Cell count of IL-37b and related cytokines in AR and control tissue (median ± IQR).

Groups	AR group	Control group
IL-37b	4.8 ± 2.3*	16.4 ± 4.3
IL-4	12.3 ± 4.5*	4.5 ± 1.9
IL-12	11.3 ± 5.6*	22.1 ± 7.8
IL-1	25.2 ± 8.1*	8.1 ± 2.4

*Compared with control group, $P < 0.05$.

3.4. IL-37b Regulated Th2 Cytokines Expression in PBMCs through Mitogen-Activated Protein Kinase (MAPK) and Phosphatidylinositol3-Kinase (PI3K) Pathway. After stimulation with various concentrations of rhIL-37b (1–100 μg), we found significant downregulation of Th2 cytokines (IL-4, IL-5, and IL-13) in a dose and time dependent manner and this effect was blocked after treatment with SB203580 and LY294002 (Figures 5(a)–5(f)). However, our results showed that IL-37b did not regulate Th1 cytokines and IL-10 expression. To investigate the effect of Th1/Th2/Treg cytokines on IL-37b expression, we used rhIL-4, rhIL-13, rhIL-5, rhIL-12, rhIFN-γ, and rhIL-10 to stimulate PBMCs for 24 hours



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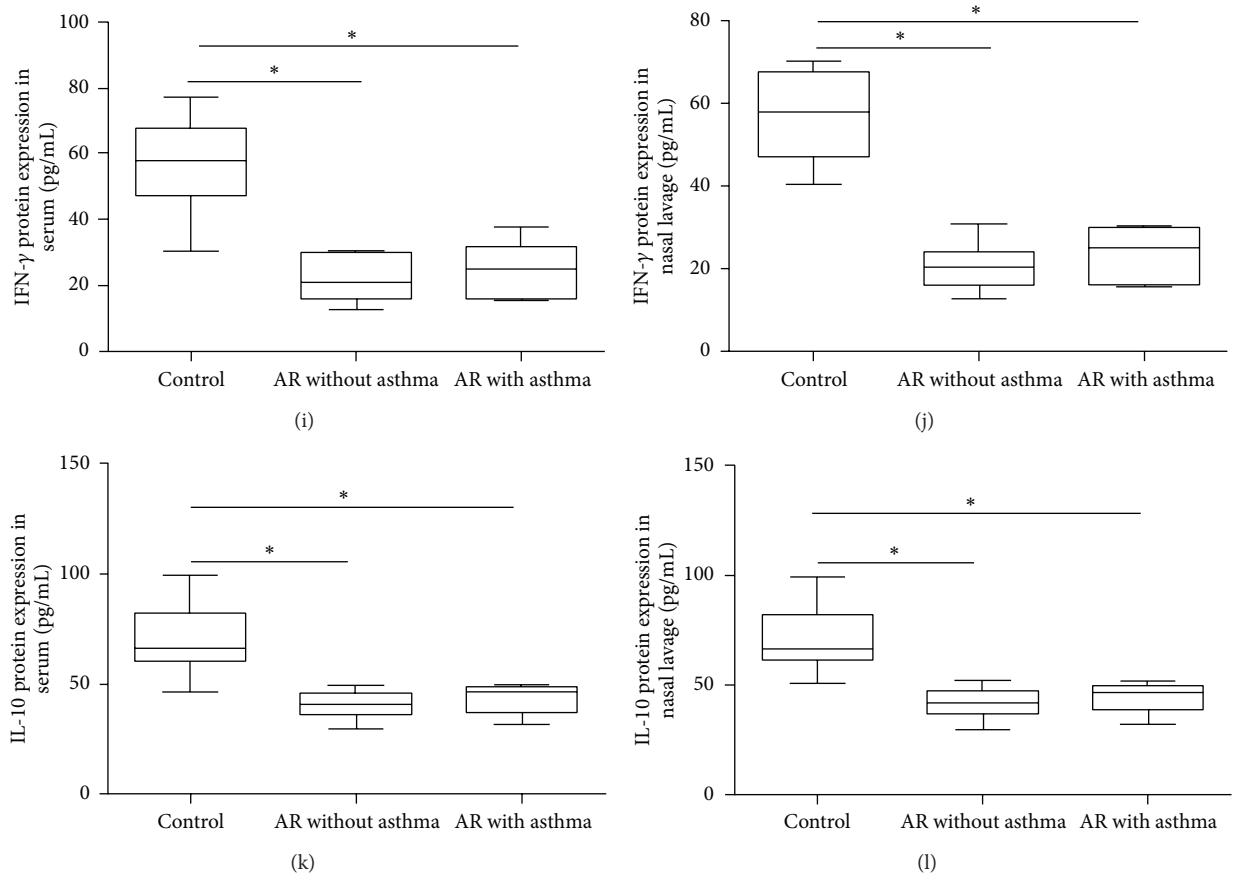


Figure 2: The serum and nasal lavage protein expression of Th2 (IL-4, IL-5, and IL-13), Th1 (IL-12, IFN-γ) and Treg (IL-10) cytokines in AR with asthma, AR without asthma, and control groups. (a)–(f) The serum and nasal lavage protein expression of Th2 (IL-4, IL-5, and IL-13) cytokines in AR with asthma, AR without asthma, and control groups; (g)–(j) the serum and nasal lavage protein expression of Th1 (IL-12, IFN-γ) cytokines in AR with asthma, AR without asthma, and control groups; (k), (l) the serum and nasal lavage protein expression of Treg (IL-10) cytokines in AR with asthma, AR without asthma, and control groups. $^*P < 0.05$, comparing the two groups.

and found that Th2 cytokines (rhIL-4, rhIL-13, and rhIL-5) decreased IL-37b expression (Figures 6(a)–6(c)), whereas Th1 cytokines (rhIL-12, rhIFN-γ) had no effect on IL-37b expression (Figures 6(e) and 6(f)). Interestingly, rhIL-10 can upregulate IL-37b expression by PBMCs (Figure 6(d)).

3.5. IL-37b Induces ECP Production by Eosinophils. After stimulation with various concentrations of rhIL-37b (1–100 ng/mL), we found significant downregulation of ECP level by eosinophils in a dose and time dependent manner (Figures 7(a) and 7(b)).

3.6. Intranasal Mometasone Furoate Therapy Downregulates Local IL-37b Expression. After 4-week course of intranasal mometasone furoate (50 μg per puff per nostril to both nostrils, once daily), we found significant relieve of symptoms of AR children (Table 3). Nasal lavage IL-37b protein expression was enhanced significantly after treatment and its expression was negatively related with total symptom scores (Figures 8(a) and 8(b)).

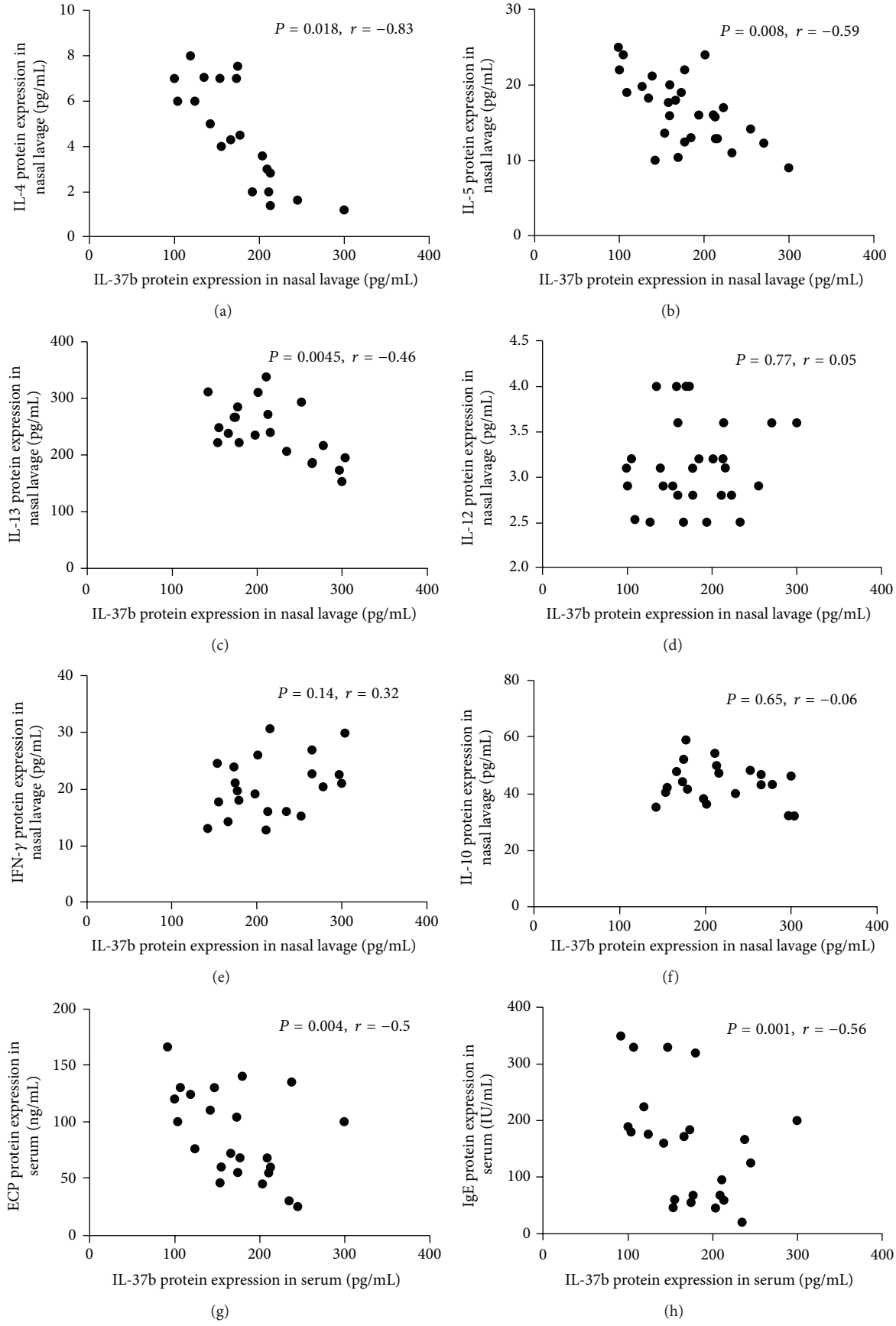
Table 3: Clinical outcome of 40 AR children after treatment (median ± IQR).

	Before treatment	After treatment
Symptom scores		
nose symptom	2.3 ± 0.5	0.7 ± 0.1*
lung symptom	1.5 ± 0.4	1.0 ± 0.2*
eye symptom	1.8 ± 0.3	0.9 ± 0.4*
Total scores	5.6 ± 1.2	2.4 ± 0.5*

*Compared with baseline level, $P < 0.05$.

4. Discussion

AR is a common disease in children which is characterized by persistent inflammation of the nasal mucosa, typically showing a Th2 skewed eosinophilic inflammation with high levels of IL-5 and IgE. Th2 cytokines play an important role in the development and deterioration of AR. At present, intranasal steroid is the first-line treatment for AR and its mechanism includes regulation of Th1/Th2 balance and inhibition of inflammatory cytokines.



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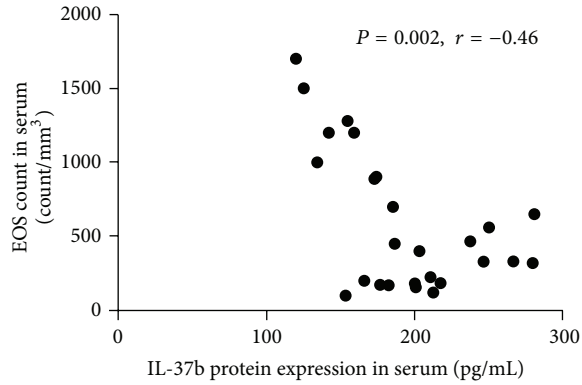
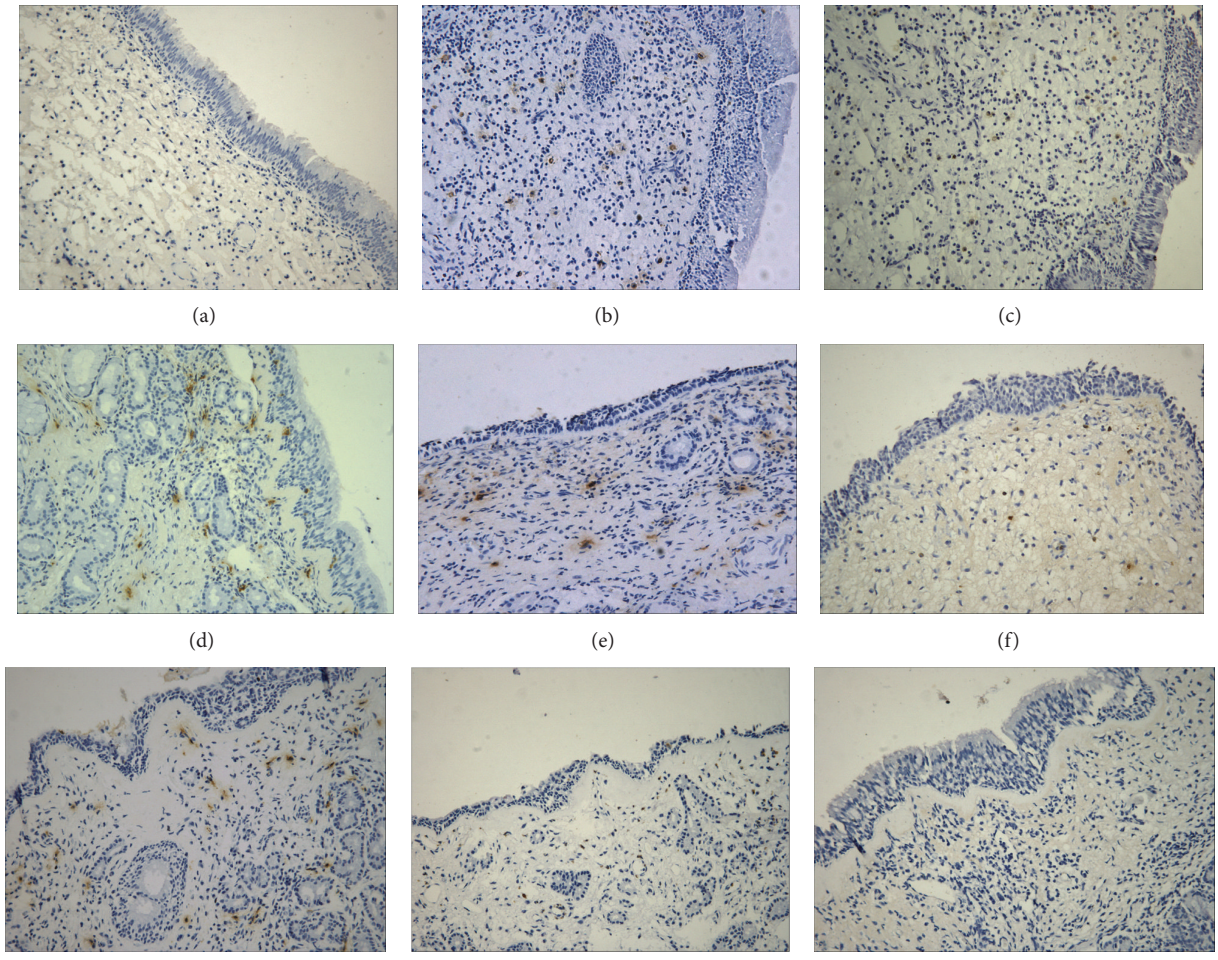


(i)

Figure 3: Correlation between IL-37b protein expression and related cytokines or protein. (a) Correlation between nasal IL-37b and IL-4 protein; (b) correlation between nasal IL-37b and IL-5 protein; (c) correlation between nasal IL-37b and IL-13 protein; (d) correlation between nasal IL-37b and IL-12 protein; (e) correlation between nasal IL-37b and IFN-γ protein; (f) correlation between nasal IL-37b and IL-10 protein; (g) correlation between serum IL-37b and ECP protein; (h) correlation between serum IL-37b and IgE protein; (i) correlation between serum IL-37b and EOS count.



(a) (b) (c)

(d) (e) (f)

(g) (h) (i)

Figure 4: The expression of IL-37b and related cytokines in inferior turbinate of AR children and normal control. (a) IL-37b staining in AR; (b) IL-37b staining in control; (c) IL-12 staining in AR; (d) IL-12 staining in control; (e) IL-4 staining in AR; (f) IL-4 staining in control; (g) IL-1 staining in AR; (h) IL-1 staining in control; (i) IgG control antibody staining. Magnification ×200.

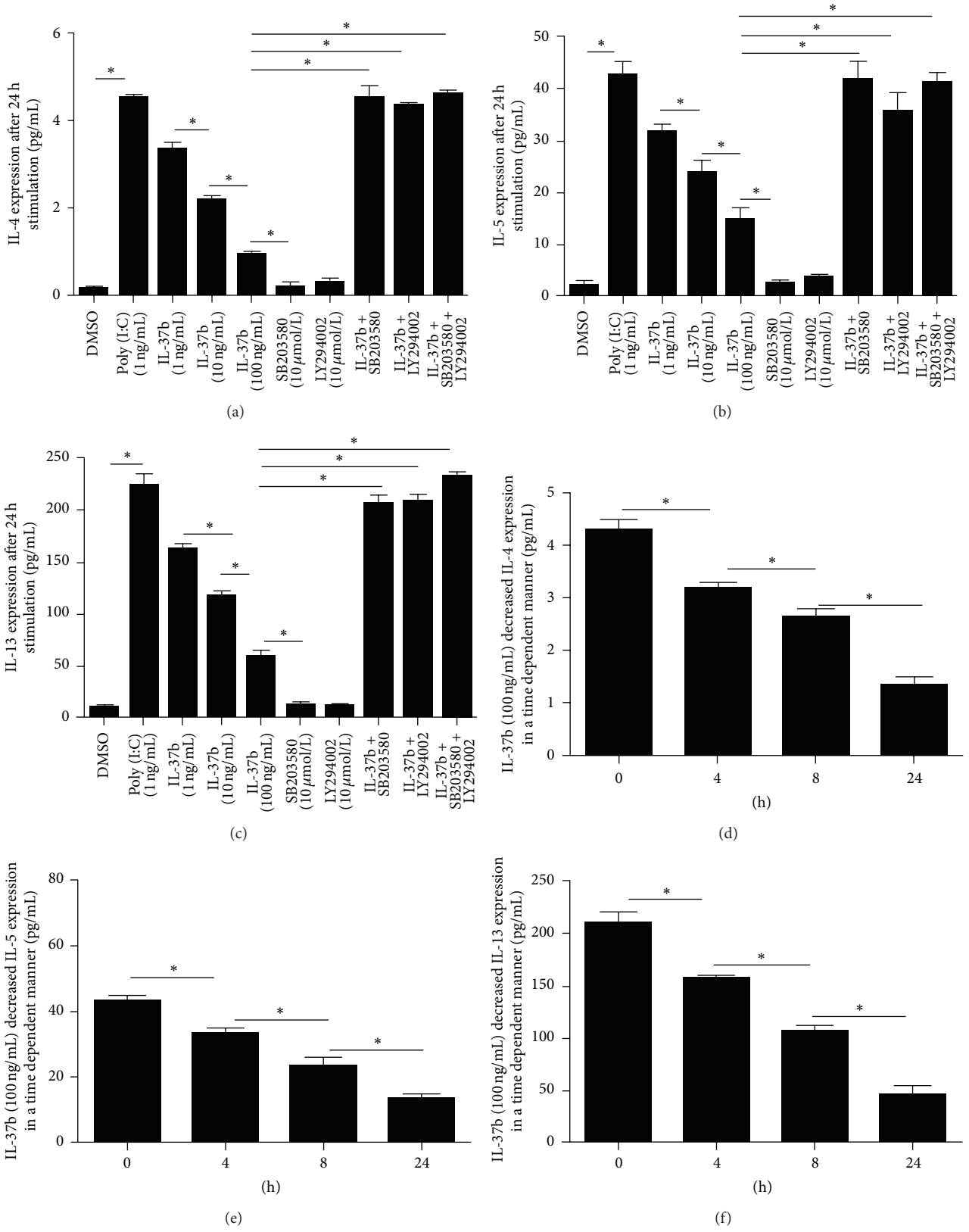


Figure 5: The regulation of Th2 cytokines (IL-4, IL-5, and IL-13) by IL-37b in PBMC in a dose and time dependent manner. (a) IL-4 expression by PBMC after stimulated with different concentration of IL-37b with or without SB203580 and LY294002; (b) IL-5 expression by PBMC after stimulated with different concentration of IL-37b with or without SB203580 and LY294002; (c) IL-13 expression by PBMC after stimulated with different concentration of IL-37b with or without SB203580 and LY294002; (d) IL-37b decreased IL-4 expression by PBMC in a time dependent manner; (e) IL-37b decreased IL-5 expression by PBMC in a time dependent manner; (f) IL-37b decreased IL-13 expression by PBMC in a time dependent manner. $^{*}P < 0.05$, comparing the two groups.

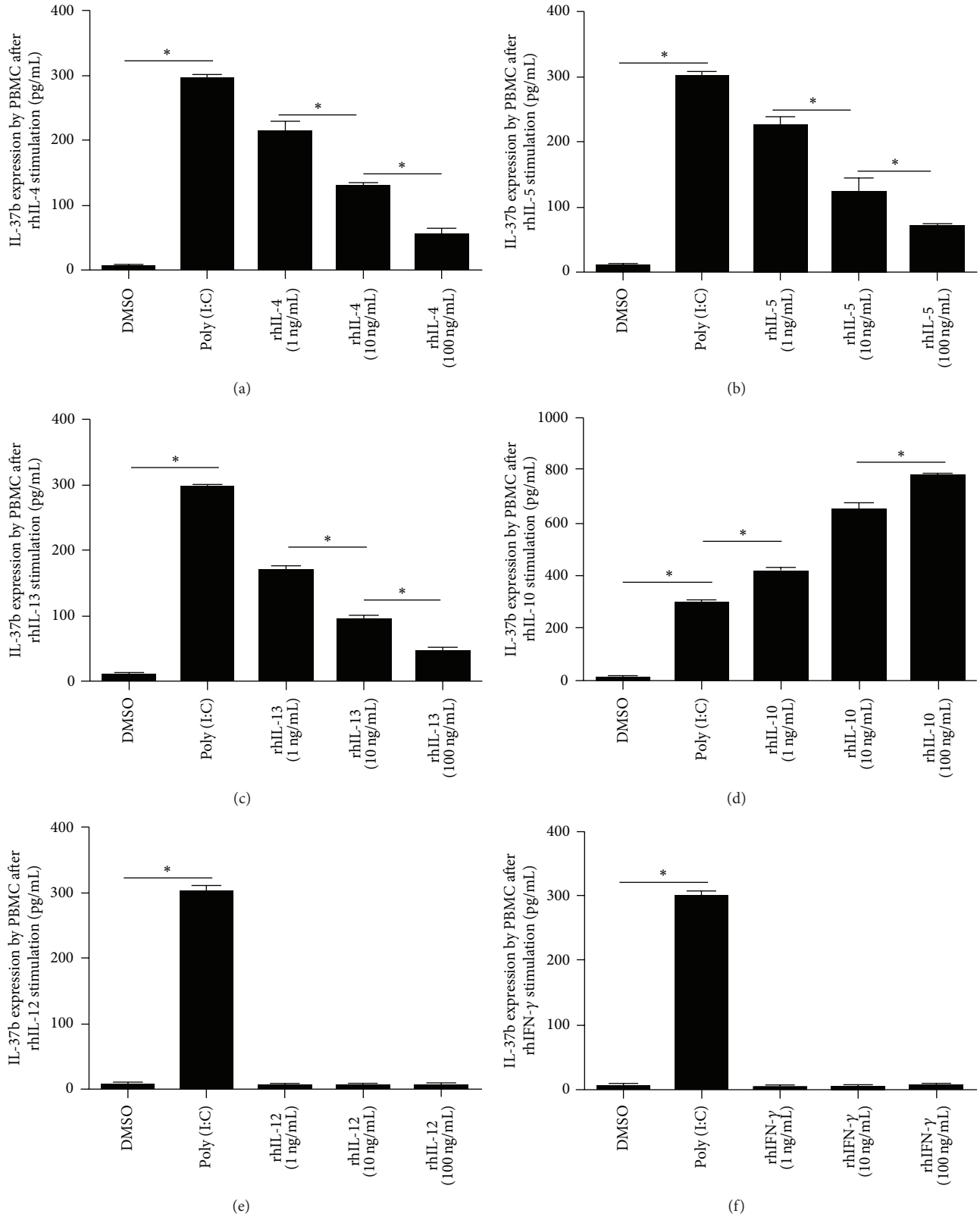


Figure 6: Regulation of IL-37b expression by Th2 cytokines (rhIL-4, rhIL-5, rhIL-13), Th1 cytokines (rhIL-12, rhIFN-γ), and Treg cytokine (rhIL-10) in PBMC. (a) IL-37b expression in a dose dependent manner by PBMC after 24 h stimulation with rhIL-4; (b) IL-37b expression in a dose dependent manner by PBMC after 24 h stimulation with rhIL-5; (c) IL-37b expression in a dose dependent manner by PBMC after 24 h stimulation with rhIL-13; (d) IL-37b expression in a dose dependent manner by PBMC after 24 h stimulation with rhIL-10; (e) IL-37b expression was not affected after 24 h stimulation with rhIL-12; (f) IL-37b expression was not affected after 24 h stimulation with rhIFN-γ. $^*P < 0.05$, comparing the two groups.

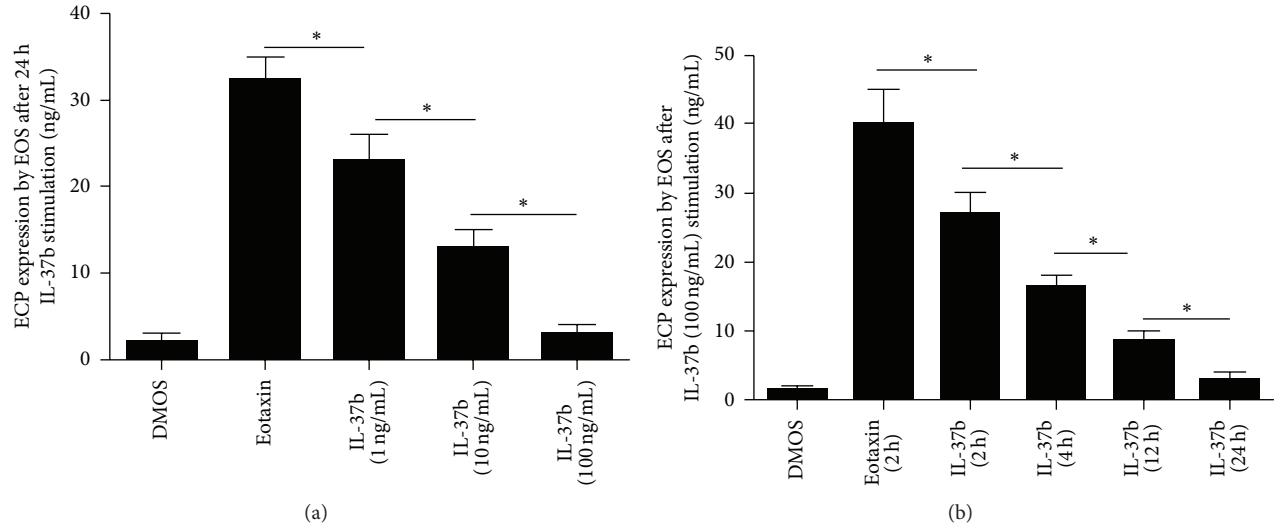


Figure 7: Regulation of ECP expression by EOS after stimulation with IL-37b. (a) ECP expression in a dose dependent manner by EOS after stimulation with rhIL-37b; (b) ECP expression in a time dependent manner by EOS after stimulation with rhIL-37b. $^{*}P < 0.05$, comparing the two groups.

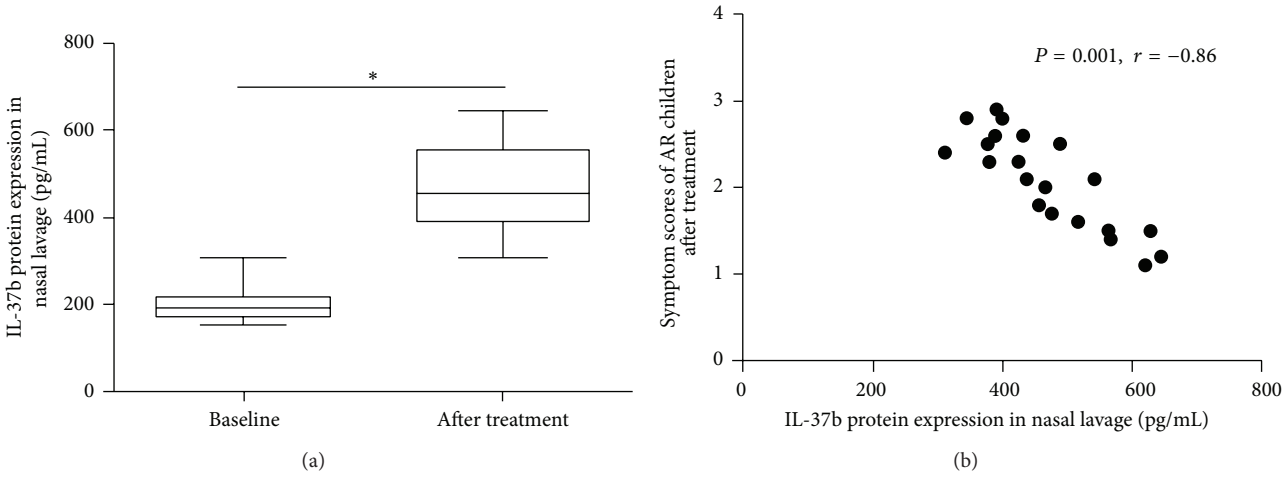


Figure 8: Nasal lavage IL-37b protein expression after treatment and its correlation with total symptom scores. (a) Nasal lavage IL-37b protein expression before and after treatment. (b) Correlation between nasal lavage IL-37b protein expression and total symptom scores after treatment. $^{*}P < 0.05$, comparing the two groups.

At present, most studies on pathogenesis of AR concentrated on the production of inflammatory cytokines instead of anti-inflammatory cytokines. IL-37 (IL-1F7) is a newly reported molecular of IL-1 family with anti-inflammatory effect [18]. IL-37 expression was found in many cancer cells such as stroma of colon carcinomas, and ductal mammary carcinoma and also in blood monocytes, fully differentiated keratinocytes in stratum granulosum of skin, PBMC, and dendritic cell [19, 20]. IL-37b has potent anti-inflammatory properties and many studies have elucidated its precise role in autoimmune disease such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), inflammatory bowel disease (IBD), guillain-Barré syndrome, and atopic dermatitis (AP) [21, 22]. However, its role in respiratory disease was not reported.

As IL-37b was believed to be the most important isoform of IL-37, thus our study focuses on it and we provide the first evidence that IL-37b expression was negatively related to enhanced Th2 inflammation in AR. In normal nasal mucosa, we found that IL-37b was expressed by epithelial, interstitial cells, and glandular cells. However, IL-37b expression in AR was relatively weak. Interestingly, we found that IL-37b expression was not affected by the state of asthma and age in children. Consistent with our results, Fujita et al. [22] detected IL-37b expression in AP and their results showed that most AP patients presented as high IL-37 level,

but several severe AD showed low level of IL-37. These results suggested that IL-37 played different roles in different disease states and IL-37 isoforms may represent even different functions. Besides, when MAPK and PI3K pathway inhibitor were added to PBMCs, the inhibitory effect on Th2 cytokines by IL-37b was significantly decreased, suggesting that MAPK and PI3K pathways were involved in IL-37b mediated Th2 inflammation regulation, which was consistent with previous report [23].

In Imaeda's study [23], IL-37b was found to inhibit CXCL10, a Th1-chemokine, suggesting its potential role in inhibiting Th1 inflammation. On the contrary, our data showed that Th1 cytokines were not correlated with IL-37b. We also found that IL-37b did not affect IL-10 expression. These results suggested that IL-37 regulation of Th2 cytokines is not IL-10 dependent, which is inconsistent with Mcnamee's study [11], who reported that IL-37 isoforms not only suppress Th2 cytokines, but also induce IL-10. However, our data showed that rhIL-10 can upregulate IL-37b expression by PBMCs and its mechanism needs further exploration.

Eosinophils mediate epithelial damage via the release of preformed effector molecules, such as major basic protein and ECP. Eosinophils play a critical role in the maintenance and progression of AR by promoting airway dysfunction and tissue remodeling. Our results show serum ECP and IgE levels and eosinophil counts were negatively correlated with serum IL-37b. Thus, we purified eosinophils and found that IL-37b can inhibit ECP expression by eosinophils directly in a dose and time dependent manner. Our results provide a new eosinophil regulator in the development of AR.

Finally, we analyzed the IL-37b expression after intranasal steroid treatment. We found the treatment decreased nasal IL-37b expression and disease symptoms, suggesting that intranasal steroid may improve symptoms through regulation of IL-37b expression. However, inconsistent with our results, Song's study [24] showed that glucocorticoid can downregulate the expression of IL-37b and other cytokines in SLE patients. These differences in expression of IL-37b in above-mentioned diseases suggested that IL-37b played various roles in different backgrounds.

5. Conclusions

In summary, we have shown the expression, distribution, and regulation of IL-37b in AR children and demonstrated the importance of decreased IL-37b level in regulation of Th1/Th2/Treg balance and activation of eosinophils. Our findings may be beneficial for designing a potential strategy for the optimal prevention and management of AR children.

Conflict of Interests

The authors declare that they have no financial disclosures or conflict of interests.

Authors' Contribution

Wenlong Liu and Li Deng contributed equally to this work.

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References

[1] D. Strachan, B. Sibbald, S. Weiland et al., "Worldwide variations in prevalence of symptoms of allergic rhinoconjunctivitis in children: the International Study of Asthma and Allergies in Childhood (ISAAC)," *Pediatric Allergy and Immunology*, vol. 8, no. 4, pp. 161–176, 1997.

[2] B. Björkstén, T. Clayton, P. Ellwoodet al., "Worldwide time trends for symptoms of rhinitis and conjunctivitis: phase III of the International Study of Asthma and Allergies in Childhood," *Pediatric Allergy and Immunology*, vol. 19, no. 2, pp. 110–124, 2008.

[3] S. Hamouda, C. Karila, T. Connault, P. Scheinmann, and J. de Blic, "Allergic rhinitis in children with asthma: a questionnaire-based study," *Clinical and Experimental Allergy*, vol. 38, no. 5, pp. 761–766, 2008.

[4] B. S. Bochner and R. P. Schleimer, "Mast cells, basophils, and eosinophils: distinct but overlapping pathways for recruitment," *Immunological Reviews*, vol. 179, pp. 5–15, 2001.

[5] I. Hansen, L. Klimek, R. Mösges, and K. Hörmann, "Mediators of inflammation in the early and late phase of allergic rhinitis," *Current Opinion in Allergy and Clinical Immunology*, vol. 4, no. 3, pp. 159–163, 2004.

[6] E. M. Ling, T. Smith, X. D. Nguyen et al., "Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease," *The Lancet*, vol. 363, no. 9409, pp. 608–615, 2004.

[7] D. Boraschi, D. Lucchesi, S. Hainzlet et al., "IL-37: a new anti-inflammatory cytokine of the IL-1 family," *European Cytokine Network*, vol. 22, no. 3, pp. 127–147, 2011.

[8] P. Bufler, T. Azam, F. Gamboni-Robertsonet et al., "A complex of the IL-1 homologue IL-1F7beta and IL-18-binding protein reduces IL-18 activity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 21, pp. 13723–13728, 2002.

[9] M. F. Nold, C. A. Nold-Petry, J. A. Zepp, B. E. Palmer, P. Bufler, and C. A. Dinarello, "IL-37 is a fundamental inhibitor of innate immunity," *Nature Immunology*, vol. 11, no. 11, pp. 1014–1022, 2010.

[10] S. Sharma, N. Kulk, M. F. Nold et al., "The IL-1 family member 7b translocates to the nucleus and down-regulates proinflammatory cytokines," *Journal of Immunology*, vol. 180, no. 8, pp. 5477–5482, 2008.

[11] E. N. McNamee, J. C. Masterson, P. Jedlicka et al., "Interleukin 37 expression protects mice from colitis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 40, pp. 16711–16716, 2011.

[12] J. L. Broek, J. Bousquet, C. E. Baena-Cagnani et al., "Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines: 2010 revision," *Journal of Allergy and Clinical Immunology*, vol. 126, no. 3, pp. 466–476, 2010.

[13] S. E. Pedersen, S. S. Hurd, R. F. Lemanske Jr. et al., "Global strategy for the diagnosis and management of asthma in children 5 years and younger," *Pediatric Pulmonology*, vol. 46, no. 1, pp. 1–17, 2011.

[14] K. Irander, J. P. Palm, M. P. Borres, and B. Ghafouri, "Clara cell protein in nasal lavage fluid and nasal nitric oxide-biomarkers

with anti-inflammatory properties in allergic rhinitis," *Clinical and Molecular Allergy*, vol. 10, article 4, 2012.

[15] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[16] W. Wen, W. Liu, L. Zhang et al., "Increased neutrophilia in nasal polyps reduces the response to oral corticosteroid therapy," *Journal of Allergy and Clinical Immunology*, vol. 129, no. 6, pp. 1522–1528, 2012.

[17] I. Puxeddu, A. Alian, A. M. Piliponsky, D. Ribatti, A. Panet, and F. Levi-Schaffer, "Human peripheral blood eosinophils induce angiogenesis," *International Journal of Biochemistry and Cell Biology*, vol. 37, no. 3, pp. 628–636, 2005.

[18] S. Kumar, P. C. McDonnell, R. Lehr et al., "Identification and initial characterization of four novel members of the interleukin-1 family," *Journal of Biological Chemistry*, vol. 275, no. 14, pp. 10308–10314, 2000.

[19] S. J. Busfield, C. A. Comrack, G. Yu et al., "Identification and gene organization of three novel members of the IL-1 family on human chromosome 2," *Genomics*, vol. 66, no. 2, pp. 213–216, 2000.

[20] G. Pan, P. Risser, W. Mao et al., "IL-1H, an interleukin 1-related protein that binds IL-18 receptor/IL-1Rrp," *Cytokine*, vol. 13, no. 1, pp. 1–7, 2001.

[21] C. Li, P. Zhao, X. Sun, Y. Che, and Y. Jiang, "Elevated levels of cerebrospinal fluid and plasma interleukin-37 in patients with Guillain-Barré syndrome," *Mediators of Inflammation*, vol. 2013, Article ID 639712, 9 pages, 2013.

[22] H. Fujita, Y. Inoue, K. Seto, N. Komitsu, and M. Aihara, "Interleukin-37 is elevated in subjects with atopic dermatitis," *Journal of Dermatological Science*, vol. 69, no. 2, pp. 173–175, 2013.

[23] H. Imaeda, K. Takahashi, T. Fujimoto et al., "Epithelial expression of interleukin-37b in inflammatory bowel disease," *Clinical and Experimental Immunology*, vol. 172, no. 3, pp. 410–416, 2013.

[24] L. Song, F. Qiu, Y. Fan et al., "Glucocorticoid regulates interleukin-37 in systemic lupus erythematosus," *Journal of Clinical Immunology*, vol. 33, no. 1, pp. 111–117, 2013.

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Review Article

Tumour Necrosis Factor Superfamily Members in the Pathogenesis of Inflammatory Bowel Disease

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Inflammatory bowel disease (IBD) is a group of inflammatory conditions of the gastrointestinal tract of unclear aetiology of which two major forms are Crohn's disease (CD) and ulcerative colitis (UC). CD and UC are immunologically distinct, although they both result from hyperactivation of proinflammatory pathways in intestines and disruption of intestinal epithelial barrier. Members of the tumour necrosis factor superfamily (TNFSF) are molecules of broad spectrum of activity, including direct disruption of intestinal epithelial barrier integrity and costimulation of proinflammatory functions of lymphocytes. Tumour necrosis factor (TNF) has a well-established pathological role in IBD which also serves as a target in IBD treatment. In this review we discuss the role of TNF and other TNFSF members, notably, TL1A, FasL, LIGHT, TRAIL, and TWEAK, in the pathogenesis of IBD.

1. Introduction

Inflammatory bowel disease (IBD) is a group of inflammatory conditions of the gastrointestinal (GI) tract. Its two major forms are ulcerative colitis (UC) and Crohn's disease (CD). Crohn's disease affects mainly small intestine and colon, although any other segment of the GI tract may also be involved. CD is characterized by discontinuous ulcerations and bowel wall inflammation. UC manifests by inflammation of the colon mucosa that in most cases extends to the rectum. Typical symptoms of IBD are abdominal pain, diarrhoea, and rectal bleeding as well as weight loss, fever, and fatigue. Furthermore, CD patients often develop strictures between segments of the bowel or between the bowel and other organs [1]. IBD is an autoimmune disorder of unknown aetiology that results from excessive immune responses to intestinal microbiota which are triggered by increased activity of effector T cells and/or decreased activity of regulatory T cells, changes in the composition of intestinal microflora, and/or damaged epithelial barrier [1, 2]. Recently, Hand et al. [3] showed in a mouse model that acute infection of the GI tract results in the loss of CD4(+) T cell tolerance of commensal antigens and priming of adaptive immune response directed against commensal bacteria which contributes to the development of IBD. Furthermore, 5–16% of IBD patients report a family history of the disease [4] which indicates that it may be associated also with a genetic background. Indeed, there are several genetic factors that contribute to the pathogenesis of the IBD which include genetic mutations leading to enhanced inflammatory response [5–7], defective elimination of intracellular bacteria [8, 9], or disruption of the intestinal epithelial barrier [10]. There are also certain environmental risk factors for IBD that include (1) treatment with nonsteroidal anti-inflammatory drugs which damage intestinal mucosa, making it more permeable to bacteria; (2) taking oral contraceptives that elevate the level of estrogens which act as enhancers of humoral immunity; (3) smoking that increases risk of acquiring CD, although it appears to play a protective role in UC through yet unknown mechanisms; and (4) limitation of exposure to enteric pathogens in childhood due to antibiotic treatment or living in hygienic environment [2]. Association of IBD with other environmental factors such as diet rich in sugars and fats and living in urban environment or stress remains currently controversial [2].

Considering the type of immune response, IBD is not a uniform disease; in CD the inflammation is mainly driven by T helper 1 (Th1) or T helper 17 (Th17) cells, while UC

is considered to be generally a T helper 2- (Th2-) mediated condition [11]. It has to be noted, however, that the strict polarization model of Th1, Th2, and Th17 is not fully applicable in IBD due to a redundancy of effector and regulatory pathways affected by factors such as the phase of the disease (remission or acute bouts), innate inflammatory mechanisms, or anti-inflammatory treatment of patients [12]. For example, during the remission phase of the disease, the level of a Th2 cytokine, interleukin 13 (IL-13), is higher in peripheral blood mononuclear cells (PBMCs) isolated from patients with CD than in PBMCs isolated from patients with UC [13]. Other reports show that the frequency of Th1 (IFN-γ(+) CD4(+)) T cells is lower in the peripheral blood of paediatric IBD patients than in healthy control subjects [14, 15] and it increases with patients' age [15]. Furthermore, the cytokine expression profile in IBD patients does not usually reflect fully differentiated Th1, Th2, or Th17 immune responses [12]. In UC, expression of a typical Th2 cytokine, IL-4, was not elevated in intestinal mucosa of UC patients [12]. Instead, it has been suggested that the central role in the pathogenesis of UC is played by IL-13 [12, 16] which not only acts as a Th2 effector cytokine [17] but also disrupts the continuity of colonic epithelium by inducing apoptosis of epithelial cells and upregulating expression of claudin-2, a pore-forming tight junction protein [18]. To add more complexity to the pathogenesis of IBD, a recent report by Mannon et al. [19] has showed that in some patients UC is characterized by elevated production of a Th17-specific cytokine, IL-17A, by intestinal lamina propria T cells.

2. Tumour Necrosis Factor Superfamily

There are 19 ligands and 29 receptors identified to date that constitute the tumour necrosis factor superfamily (TNFSF) [20]. Their expression pattern and structural attributes allow them to activate signalling pathways that lead to cell survival, proliferation, differentiation, or apoptosis. TNFSF receptors can be divided into two groups depending on the presence or absence of the intracellular death domain (DD). Signalling via the death domain requires the participation of adaptor proteins FADD (Fas-associated death domain) and TRADD (TNF receptor-associated death domain) and leads to activation of caspases which typically results in apoptotic death of a cell [21]. The second group of TNFSF receptors signals only via adaptor proteins termed TRAFs (tumour necrosis factor receptor-associated proteins), although DD-containing receptors can also utilize this pathway. TRAFs bind either to TRADD or directly to the cytoplasmic part of the receptor and initiate signal transduction pathways that lead to the activation of several transcription factors, such as AP-1 and NF-κB, responsible for the activation of prosurvival genes [21], although they are involved also in proapoptotic signalling [22–24]. Hence, functional activity of TNFSF receptors largely depends on the cellular context and the balance between pro- and antiapoptotic factors inside the cell and in the environment.

Most TNFSF members are expressed on cells of the immune system and play an important role in maintaining the equilibrium of T cell-mediated immune responses by providing direct signals required for full activation of effector and regulatory T cells, regulation of their expansion, contraction of the T cell effector pool, and survival of memory T cells [25–30]. For these reasons, members of the TNFSF are involved in the pathogenesis of many T cell-mediated autoimmune diseases, such as asthma, diabetes, or arthritis [26]. Many recent reports indicate that certain TNFSF members, notably, TNF (tumour necrosis factor, TNFSF2, also known as TNF-α) [31], TL1A (TNF-like protein 1A, TNFSF15) [32, 33], FasL (TNFSF6) [34–36], LIGHT (lymphotoxin-like inducible protein that competes with glycoprotein D for binding herpesvirus entry mediator on T cell, TNFSF14) [37], TRAIL (TNF-related apoptosis inducing ligand, TNFSF10) [38], and TWEAK (TNF-like weak inducer of apoptosis, TNFSF12) [39], contribute to the pathogenesis of IBD not only by enhancing proinflammatory function of T cells but also by direct disruption of the integrity of intestinal epithelium (Table 1).

3. TNF

Tumour necrosis factor (TNF; TNF-α; TNFSF2) is biologically active in the form of homotrimeric transmembrane or soluble protein [40]. It is expressed by macrophages, T cells, B cells, NK cells, mast cells, endothelial cells, fibroblasts, and neurons; its expression is strongly upregulated by certain proinflammatory factors such as lipopolysaccharide (LPS) or other bacterial products and IL-1β [20, 24, 41]. There are two types of TNF receptors, the death domain-containing TNFR1 (TNF receptor 1, also known as p55 or TNFRSF1A), which is constitutively expressed on most nucleated mammalian cells and is activated by both the transmembrane and soluble form of TNF [20], and TNFR2 (TNF receptor 2, also known as p75 or TNFRSF1B) which does not contain the death domain and is activated only by the transmembrane form of TNF [24]. Expression of TNFR2 is strictly regulated and found mostly on certain populations of lymphocytes, including T-regulatory cells (Tregs), endothelial cells, microglia, neuron subtypes, oligodendrocytes, cardiac myocytes, thymocytes, and human mesenchymal stem cells [20, 42].

Elevated expression of TNF was detected in IBD patients more than 20 years ago [52]. The level of TNF mRNA was upregulated in involved colonic tissue of CD patients [53] as well as in both involved and uninvolved colonic tissue of UC patients [54] compared to healthy subjects. A recent report [55] showed that elevated concentration of TNF protein that correlated with the activity of the disease was present in blood serum of CD patients while other groups [52, 56] found increased levels of TNF protein both in serum [52, 56] and in the intestinal lamina propria of both CD and UC patients as well as the intestinal submucosa of CD patients [57]. The production of TNF in the colon mucosa of UC patients was localized to lamina propria macrophages [57]. Although several groups did not detect increased levels of TNF protein or mRNA in blood serum or colon mucosa of IBD patients, respectively [58, 59], successful use of anti-TNF agents in IBD therapy [31] documents that TNF belongs to

Table 1: Overview of the expression and function of the major members of TNSF superfamily in IBD.

TNFSF member and its expression	Receptors and their expression	Role in IBD pathogenesis	References
TNF—macrophages, NK cells, T cells, and B cells	(i) TNFR1—intestinal epithelial cells (ii) TNFR2—intestinal epithelial cells	Disruption of intestinal epithelium integrity by induction of adhesion proteins rearrangement and induction of intestinal cells apoptosis	[20, 43, 44]
TL1A—antigen-presenting cells and T cells	(i) DR3—T cells, NK cells, NKT cells, and regulatory T cells (ii) DcR3 (decoy)—activated T cells	Promotion of proinflammatory activity of T cells and inhibition of suppressive activity of regulatory T cells	[45, 46]
FasL—T cells, NK cells, monocytes, and Paneth cells	(i) Fas—intestinal epithelial cells and T cells (ii) DcR3 (decoy)—activated T cells	Possible disruption of intestinal epithelium integrity by induction of epithelial cells apoptosis. Possible involvement in accumulation of proinflammatory T cells in intestinal lamina propria	[20, 47, 48]
LIGHT—T cells, monocytes, granulocytes, and dendritic cells	(i) HVEM—T cells, B cells, and monocytes (ii) LTβR—nonlymphoid hematopoietic cells and stromal cells (iii) DcR3 (decoy)—activated T cells	Possible promotion of proinflammatory activity of Th1 cells	[20, 49]
TRAIL—intestinal epithelium, T cells, NK cells, and dendritic cells	(i) TRAIL-R1—almost all cell types (ii) TRAIL-R2—almost all cell types (iii) TRAIL-R3 (decoy)—almost all cell types (iv) TRAIL-R4 (decoy)—almost all cell types (v) OPG (decoy)—osteoclasts' precursors, endothelial cells, and other cell types	Disruption of intestinal epithelium integrity by induction of epithelial cells apoptosis. Possible contribution to development of fistulas and strictures in CD patients	[20, 38, 50]
TWEAK—T cells, macrophages, and dendritic cells	Fn14—intestinal mucosa and fibroblasts	Possible upregulation of proinflammatory cytokines and infiltration of lamina propria by inflammatory cells. Induction of intestinal cells apoptosis in cooperation with IL-13	[20, 39, 51]

the major effector molecules involved in the pathogenesis of CD and UC. It is worth to note, however, that a recent study on a mouse model of T cell-mediated colitis has shown that only neutralization of the transmembrane, but not soluble, TNF form induced remission of experimental colitis [60]. Pathogenesis of IBD is associated also with altered expression of TNF receptors since both CD and UC patients showed elevated expression of TNFR2 on colonic epithelial cells [61]. Moreover, a positive correlation was observed between CD and UC activity and serum concentration of soluble forms of TNFR1 and TNFR2 [55]. Furthermore, upregulated expression of TNFR2 (but not TNFR1) was found on intestinal lamina propria $CD4^+$ cells as well as peripheral blood T cells of CD patients [62].

3.1. Role of TNF in the Dysregulation of Intestinal Barrier Permeability. Several studies showed that TNF contributes to the disruption of intestinal epithelial barrier which allows for intestinal penetration of luminal antigens and promotes intestinal inflammation (Table 2) [63–65]. Intestinal epithelium integrity is provided by the presence of tight junctions (TJ) located in the apical region of intestinal epithelial cells. Data obtained *in vitro* by Ma et al. [63] showed that stimulation of colonic epithelial Caco-2 cells with TNF downregulated the expression of TJ-associated zonula occludens-1 proteins and altered their junctional localization in an NF-κB-dependent manner.

Transmembrane expression of TNF is regulated by a pleiotropic metalloproteinase ADAM17 which is involved in the cleavage of transmembrane TNF and its shedding from the cell surface [66]. Cesaro et al. [67] reported early posttranscriptional upregulation of ADAM17 in intestinal mucosa of patients with highly active CD and, in an *in vitro* model, in intestinal epithelial cells, which was linked to transepithelial migration of polymorphonuclear neutrophils. Treatment of TIMP3-deficient colonic epithelial cell line HT29-C1.16E with TIMP3, an inhibitor of ADAM17 activity, decreased TNF shedding and sensitized the cells to TNF-mediated epithelial hyperpermeability due to the downregulation of zonula occludens-1 proteins [64]. Other reports showed that IBD patients had also elevated mucosal expression of another TNF sheddase, metalloprotease ADAM19, localized mainly in epithelial cells [68], whereas a mouse study demonstrated that shedding of TNF can be mediated also by matrix metalloproteinase 13 (MMP13) [69].

Epithelial barrier dysfunction can be mediated also by increased expression of myosin light chain kinase (MLCK) followed by subsequent phosphorylation of myosin II regulatory light chain (MLC) which results in the contraction of the perijunctional ring composed of actin and myosin

Table 2: Biological effects of TNF exerted on intestinal epithelium.

TNF function	Model	References
(1) Rearrangement of cytoskeletal elements		
(i) Downregulation of zonula occludens-1 expression and alteration of its intracellular localization	(i) Caco-2 cells (*in vitro*) (ii) HT29-C1.16E cells (*in vitro*)	[63, 64]
(ii) Upregulation of myosin light chain kinase expression	(i) Caco-2 cells (*in vitro*) (ii) Mouse model (*in vivo*)	[65, 70]
(iii) Redistribution of zonula occludens-1, occludins, claudins, E-cadherins, and myosin light chain kinase to basolateral membranes of intestinal cells	Mouse model (*in vivo*)	[71]
(2) Induction of intestinal epithelial cells' apoptosis		
(i) Induction of intestinal cells' apoptosis via activation of caspase-3	Mouse model (*in vivo*)	[71]
(ii) Induction of intestinal epithelial cells' apoptosis via upregulation of iNOS and p53	Mouse model (*in vivo*)	[44, 72]

filaments [73]. Expression of MLCK was elevated in ileal and colonic epithelium of CD and UC patients and correlated with the activity of the disease [74]. *In vitro* investigation showed that TNF upregulated expression of MLCK in Caco-2 cells pretreated with IFN-γ which increased expression of TNF receptors on the cell surface [65]. A recent study on TNFR1 or TNFR2-deficient mice showed that upregulation of MLCK and the loss of intestinal epithelial barrier in CD4(+) CD45RB (high) T cell transfer model of intestinal inflammation were dependent on TNFR2 expressed on intestinal epithelium but not TNFR1 [70].

In an elegant *in vivo* study on mouse models, Marchiando et al. [71] showed that TNF induced redistribution of several TJ and adherens junction proteins, including zonula occludens-1, occludins, claudins, and E-cadherin, as well as MCLK, to basolateral membranes of intestinal epithelial cells. Furthermore, administration of TNF resulted not only in the rearrangement of junctional proteins but also in the shedding of whole cells from intestinal epithelium. These events were preceded by caspase-3 activation due to the TNF-induced activation of NF-κB-dependent signalling pathway and of proapoptotic pathways [71]. These data suggest that TNF-triggered loss of intestinal epithelium integrity is a complex process which involves not only rearrangement of cytoskeletal elements but also direct induction of intestinal cells' apoptosis by TNF. Indeed, studies on mice showed TNF-induced apoptosis of intestinal epithelial cells in a TNFR1- and TNFR2-dependent manner [44, 72] which resulted in increased intestinal permeability *in vivo* [44]. TNF signalling upregulated expression of inducible nitric oxide synthase (iNOS) which led to enhanced expression of a proapoptotic protein p53 [72]. On the other hand, TNF participates also in transactivation of epidermal growth factor receptor (EGFR) [75] which signaling upregulates the expression of cyclooxygenase-2 (COX-2) [76]. Increased expression of COX-2 has been associated with enhanced cell resistance to apoptosis, inflammation, and promotion of tumour progression [77]; therefore this aspect of TNF activity might have relevance to development of IBD-associated cancers of the GI tract [78].

3.2. Anti-TNF Agents in IBD Therapy. Currently, IBD therapy based on blocking biological activity of TNF involves the use of the following anti-TNF agents approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA): (1) infliximab: chimeric monoclonal anti-TNF antibody (approved by FDA and EMA for treatment of CD and UC); (2) adalimumab: human monoclonal anti-TNF antibody (approved by FDA for treatment of CD in adults and by EMA for treatment of CD and UC); (3) certolizumab pegol: humanized Fab' fragment of anti-TNF antibody conjugated to polyethylene glycol (approved by FDA only for treatment of CD) [79]; (4) golimumab: human monoclonal anti-TNF antibody (approved by FDA and EMA for treatment of UC) [80]. Infliximab, adalimumab [81], and certolizumab pegol [82] are effective in the treatment of patients with moderate and severe CD who do not respond to standard anti-inflammatory drugs and also when used as a first-line therapy in CD. Moreover, randomised, controlled trials showed also that infliximab [83, 84], adalimumab [85, 86], and golimumab [87, 88] induced remission in steroid- or immunosuppressant-refractory patients with moderate or severe UC. However, 10 to 40% of CD patients (depending on selection criteria) and up to 50% of UC patients do not respond to anti-TNF therapy (primary resistance) and about one-third become resistant (secondary loss of response) at 12 months after initiation of anti-TNF treatment [89–91]. Interestingly, switching to another anti-TNF agent is effective in over 50% of nonresponsive patients [92, 93]. Failure to respond to anti-TNF therapy may result from pharmacokinetics of drugs, development of antibodies against the drugs, or activity of other, TNF-independent, proinflammatory pathways in IBD patients [91, 94, 95].

It has also to be noted that blockade of TNF biological activity in IBD therapy may result in several adverse side effects [82], including acute or delayed hypersensitivity reactions to anti-TNF agents [96, 97], elevated risk of bacterial, mycobacterial, viral, and fungal infections [98] (although meta-analysis of clinical trials did not show increased rate of infections in the course of anti-TNF treatment [89]), or neurological complications [99–101]. Combinatory therapy of CD patients with glucocorticoids, immunomodulators,

and TNF inhibitors may be associated with an increased risk of non-Hodgkin's lymphoma, lung, skin, and other types of cancers, although no causative relationship of anti-TNF antibodies and carcinogenesis has been proven [90]. Anti-TNF therapy may lead also to paradoxical inflammatory skin (eczema and psoriasis) and joint (polyarthralgia) or ocular (uveitis and scleritis) manifestations [102]. Other paradoxical reactions include also demyelinating central nervous system disorders, sarcoidosis, development of anti-nuclear antibodies, and, in rare cases, lupus [89]. Mechanisms leading to these paradoxical reactions are not currently well known and most probably involve multiple pathogenic pathways. For example, it has been recently reported that psoriasiform skin lesions characterised by Th17 and Th1 cell infiltrates developed in nearly 5% of anti-TNF-treated patients with IBD and that smoking was identified as a main risk factor. Interestingly, anti-IL-12/IL-23 antibody treatment was found to be a highly effective therapy for these lesions [103].

Despite high efficacy of the majority of anti-TNF antibodies in the therapy of a considerable proportion of both CD and UC patients [90], the precise mechanisms of action underlying the efficacy of anti-TNF agents in IBD therapy have not been fully explained. In the last decade multiple mechanisms of the anti-TNF antibodies such as blocking and neutralizing of TNF molecules, regulation of cell adhesion molecule expression, induction of regulatory macrophages, or direct induction of apoptosis of T lymphocytes and macrophages in the mucosal lamina propria and peripheral blood have been proposed [104, 105]. However, the results of newer studies suggest that increased apoptosis of Treg cells, an important subset of T lymphocytes, may play an important role in the pathogenesis of IBD and can be reversed by anti-TNFα treatment [106, 107]. Moreover, infliximab and adalimumab (but not etanercept and certolizumab) were shown to induce regulatory macrophages (CD206+) in an Fc region-dependent manner. *In vitro* these macrophages produced anti-inflammatory cytokines and inhibited proliferation of activated T cells [108], whereas *in vivo* a significant induction of regulatory macrophages was observed in IBD patients with mucosal healing after treatment with infliximab and this induction was absent in patients without mucosal healing response [109].

Recently, Leal et al. [110] using whole-genome transcriptional analysis have found that anti-TNF treatment reduced expression of a set of proinflammatory genes (including IL-6, IL-23p19, and MMP9) as well as genes of cell-activation markers (CD69, CD83, and VCAM-1) in patients who both did and did not respond to this kind of therapy, suggesting that it is not only the proinflammatory function of TNF that is targeted by anti-TNF therapy. Moreover, they identified IL1B and IL17A as genes that remained altered in nonresponders, which suggests that respective proteins or their signaling pathways may present a novel therapeutic target in IBD.

Since many studies have linked TNF to increased permeability of intestinal epithelium [63–65], it is highly possible that anti-TNF agents are involved in the protection of epithelial barrier. Indeed, administration of infliximab restored the proper function of intestinal epithelium in CD patients [42, 111] and prevented TNF-induced rearrangement of tight junction proteins (notably, occludin and zonula occludens-1) in dinitrobenzene sulfonic acid- (DNBS-) induced colitis in mice [112]. These findings have been supported by a recent *in vitro* study on intestinal epithelial cell lines Caco-2 and T84 which showed that adalimumab restored expression of tight junction proteins claudin-1, claudin-2, and claudin-3 downregulated by exposure to TNF and IFN-γ [113]. Other studies demonstrated that infliximab and adalimumab induced apoptosis of CD4(+) helper T cells expressing TNFR2 and macrophages isolated from colonic lamina propria of CD patients but not healthy subjects [23]. Furthermore, Eder et al. [114, 115] found that infliximab and adalimumab promoted apoptosis of intestinal lamina propria mononuclear cells present in inflamed but not noninflamed areas of CD patients' colonic mucosa via intrinsic pathway mediated by Bcl-2 family proteins. Thus, infliximab and adalimumab not only protect intestinal epithelial integrity but also may suppress inflammatory process by inducing apoptosis of immune cells present in intestinal mucosa.

The ongoing research aimed at the elucidation of the cellular and molecular mechanisms of the anti-inflammatory activity of some but not all anti-TNF antibodies in IBD should help in designing more target-effective biological drugs. Etanercept, a nonantibody soluble fusion protein composed of the extracellular domain of TNFR2 and the hinge and Fc fragments of human IgG1 antibody [116], is an anti-TNF agent approved by FDA and EMA for treatment of rheumatoid arthritis, psoriasis, psoriatic arthritis, ankylosing spondylitis, and juvenile idiopathic arthritis but not IBD. Even though experiments on mice with DNB-induced colitis showed that etanercept reduced levels of circulating TNF and prevented apoptosis of enterocytes equally well as infliximab [112], studies on humans proved that response rates to etanercept in CD treatment were comparable to placebo [117]. A study by Scallon et al. [116] showed that infliximab binds both monomeric and trimeric forms of soluble and transmembrane TNF, whereas etanercept forms only unstable complexes with soluble TNF which may contribute to prolonged half-life of circulating TNF. Furthermore, in contrast to infliximab, etanercept did not induce apoptosis of activated T cells isolated from CD patients and healthy control subjects [104]. The failure of etanercept in IBD therapy can be attributed to its inability, in contrast to infliximab and adalimumab, to inhibit T cell proliferation and to induce regulatory macrophages [108], caused probably by differences in infliximab and etanercept binding to TNF.

4. TL1A

TL1A (TNF-like molecule 1A; TNFSF15) is the most recently discovered member of the TNF superfamily, identified for the first time in 2002 [118]. In humans, there are three different isoforms of the protein generated from TNFSF15gene as a result of alternative splicing: VEGI-174 (174 amino acids), VEGI-192 (192 amino acids), and the full-length product, TL1A (VEGI-252; 252 amino acids) [118–120], although VEGI-174 is most probably a cloning artefact [118]. Primary function of VEGI-192 is the inhibition of

angiogenesis [121], whereas TL1A is a proinflammatory factor involved in the pathogenesis of several autoimmune diseases, including arthritis, allergic lung inflammation, autoimmune encephalomyelitis, and inflammatory bowel disease [26, 122–124].

Similarly to TNF, TL1A exists in a soluble or transmembrane form [125–128]. It has been shown that recombinant human TL1A forms a homotrimer resembling the trimeric structure of other TNF superfamily members [129]; however, still very little is known about the quaternary structure of the native form of TL1A molecule.

TL1A expression is primarily found on activated cells of the immune system, such as dendritic cells, macrophages [128], and CD4(+) and CD8(+) T cells [122, 123], whereas very little TL1A was found on nonactivated immune cells [118]. Known inducers of TL1A expression are TNF, IL-1 [118], Fc fragments of IgG1 antibodies [128], and certain parasite- or bacteria-related toll-like receptors (TLR) ligands, including synthetic bacterial lipoprotein Pam3CSK4 (ligand for TLR1 and TLR2), lipopeptide FSL (ligand for TLR2), polyinosinic-polycytidylic acid (ligand for TLR3), LPS (ligand for TLR4), single-stranded RNA (ligand for TLR7), unmethylated DNA sequences (ligands for TLR9), and tachyzoite antigen (ligand for TLR11) [123, 130].

The main receptor for TL1A is death receptor 3 (DR3; TNFRSF25) [118], structurally similar to TNFR1 [131]. DR3 was found to be strongly upregulated on activated monocytes [132], NK cells [133], NKT cells [122], and B cells [134], as well as CD4(+) T helper and CD8(+) T cytotoxic cells [133, 135]. TL1A, similarly to FasL and LIGHT, binds also soluble decoy receptor 3 (DcR3, TNFRSF6B) which prevents functional TL1A/DR3 signalling [127, 136].

Even though DR3 contains the death domain, TL1A has been shown to induce apoptosis only in the erythroleukaemic cell line TF-1 treated with an inhibitor of protein synthesis, cycloheximide (CHX) [118, 137]. In activated T cells, however, TL1A did not induce apoptosis even in the presence of CHX [118]. Instead, TL1A/DR3 interactions in lymphocytes triggered proliferative and costimulatory signals through activation of NF-κB-mediated pathways [123, 133]. Thus, TL1A is a proinflammatory molecule which primarily costimulates proliferation and effector functions of CD8(+) cytotoxic T cells [138] as well as Th1, Th2, and Th17 [30, 123, 126, 139, 140] cells in the presence of TCR stimulation; however, in physiological conditions, TL1A is not required for the differentiation of these lymphocytes [123]. Furthermore, TL1A promotes also maturation of dendritic cells [141, 142] and production of proinflammatory cytokines (TNF, IL-8, and monocyte chemotactic protein 1, MCP-1) by macrophages [132]. Apart from conventional CD4(+) and CD8(+) T cells, TL1A/DR3 interaction promotes also proliferation of regulatory T cells (Tregs) [143, 144], although sustained TL1A stimulation *in vitro* dampens suppressive activity of Tregs [143–145]. Interestingly, *in vitro* studies also showed that TL1A inhibited differentiation of Tregs from their precursor cells [143, 145].

Certain alleles of TNFSF15gene which encode TL1A are associated with enhanced activity of TNFSF15promoter region and are considered to increase susceptibility to Crohn's disease [146]. TL1A protein and mRNA were upregulated in IBD and their synthesis was localized in CD patients to lamina propria infiltrating cells such as macrophages, dendritic cells, and CD4(+) and CD8(+) T cells [139, 140, 147] as well as plasma cells isolated from colon mucosa of UC patients [125]. IBD patients had also a higher proportion of DR3-expressing lamina propria T cells than healthy subjects [125, 126] and the amount of TL1A protein as well as the number of TL1A-positive cells correlated positively with the severity of inflammation, most significantly in CD [125]. Furthermore, studies on transgenic mice showed that constitutive elevated expression of TL1A on T cells or dendritic cells resulted in enhanced T cell activation and upregulation of IL-13, IL-17A, and IFN-γ mRNA levels in intestinal mucosa and mesenteric lymph nodes as well as spontaneous development of bowel inflammation [143, 144].

Recently, a population of CD161(+)CD4(+) T cells has been identified as a primary target of TL1A in IBD [148], although other subpopulations of T cells may also respond to TL1A costimulation. CD161(+)CD4(+) T cells express DR3 [149] and their gut tropism is established by high expression of intestine-homing molecules such as integrin β7 and chemokine CCR6 [149, 150]. They bear characteristics of Th17 cells and have been shown to produce proinflammatory cytokines IL-17, IL-22, and IL-13. In inflammatory conditions, however, they may revert their phenotype to Th1 type and produce IFN-γ [149–151]. In synergy with other proinflammatory cytokines, such as IL-12 and IL-18 or IL-23, TL1A further enhances the inflammatory process by increasing production of IFN-γ, IL-8, and IL-6 by lymphocytes [30, 125, 139, 140]. Thus, TL1A involvement in IBD pathomechanisms may result from enhanced costimulation of effector T cells and local upregulation of proinflammatory cytokines production in parallel to defective generation of peripheral Tregs and inhibition of suppressive activity of preexisting Tregs [45, 46].

As one of the key regulators of inflammatory pathways, TL1A appears to be a promising therapeutic target for patients with T cell-mediated autoimmune diseases, including IBD, although to this day none of TL1A blocking agents has yet been tested in clinical trials. There are, however, reports showing that antibody-mediated inhibition of TL1A biological activity prevents the development of dextran sodium sulphate- (DSS-) induced and T cell transfer-induced experimental bowel inflammation in mice [139].

5. FasL

Fas ligand (FasL, CD95L, and TNFSF6) and its receptor Fas (CD95, TNFRSF6) are other members of the TNF superfamily involved in the pathogenesis of IBD. Cytotoxic T cells and natural killer (NK) cells use FasL to kill tumour cells or viruses-infected cells which express Fas. FasL is involved also in maintaining immune homeostasis and preventing autoimmunity via a mechanism known as activation-induced cell death (AICD) which relies on killing activated T cells following their expansion and differentiation in a FasL-/Fas-dependent manner, thus preventing hyperactivation of T cell-mediated immunity [47, 152].

FasL is a transmembrane molecule, although it can be enzymatically cleaved from cells [153]. An *in vivo* mouse study showed, however, that only the transmembrane, but not soluble, FasL was capable of triggering cell death [154]. In contrast to Fas which is constitutively or inducibly expressed on many different cell types, including colon epithelial cells [155], FasL expression is tightly regulated and limited to activated CD4(+) and CD8(+) T cells, NK cells, and monocytes [47]. In physiological conditions, Paneth cells are the only cells of the intestinal epithelium which express FasL [156]. Expression of FasL was found also in tissues and organs that lack resident or infiltrating lymphocytes (e.g., eye, trophoblast, or testis) and on neurons and astrocytes as well as in several tumours where it may contribute to the suppression of local immune responses *via* induction of T cell apoptosis [47].

The majority of studies concerning the role of FasL and Fas in IBD have been conducted in patients with ulcerative colitis rather than Crohn's disease. Expression of FasL was significantly elevated on CD3(+) lymphocytes infiltrating colonic lamina propria in patients with active UC but not in UC remission, active or remission CD, or healthy subjects [34, 35]. Furthermore, serum concentration of systemic soluble Fas was lower in patients with active UC compared to healthy controls [35]. Nevertheless, there are also studies which showed upregulation of FasL in colonic lamina propria and intraepithelial lymphocytes of CD patients' mucosa [36].

The exact role of Fas/FasL system in IBD has not been fully elucidated. Taking into account the primary, proapoptotic function of Fas/FasL signalling, its possible role in IBD initially appeared to be similar to TNF/TNFR1 signalling: intestinal epithelial cells expressing Fas targeted by FasL(+) lymphocytes undergo apoptosis which may lead to the increased permeability of intestinal epithelium [157]. Indeed, an *in vitro* study demonstrated that ligation of Fas resulted in apoptotic death of intestinal epithelial cells isolated from mucosa of UC patients [158]. This concept was supported also by the fact that in healthy colon expression of FasL was restricted only to few mononuclear cells of lamina propria, suggesting that proapoptotic function of Fas/FasL system was not involved in regeneration of colonic epithelium but in pathogenesis of IBD [159]. A more recent study, however, showed that colonocytes isolated from patients with active UC had attenuated response to Fas-mediated apoptosis induction compared to healthy subjects and patients in remission [160]. Furthermore, authors of two mouse studies demonstrated that Fas-deficient mice were hypersensitive to dextran sodium sulphate- (DSS-) induced colitis [161] and did not show any significant reduction in tissue damage, even though they exhibited an increased rate of intestinal epithelial cell apoptosis in gut inflammation model based on administration of T cell activating anti-CD3 antibody [162]. These findings suggest that colonocytes may activate cytoprotective programs in response to inflammation and may not be oversensitive to Fas-dependent apoptosis as had been initially proposed [160].

Several studies showed that T cells from inflamed mucosa of CD and UC patients were more resistant to Fas-mediated apoptosis than control T cells from healthy individuals [163–165]. Suzuki et al. [166] found that in UC mucosa the population of CD45RO(+)CD4(+) T cells was less prone to Fas-mediated cell death than the population of CD45RO(+)CD8(+) T cells. Thus, potentially harmful, proinflammatory T cells may accumulate in the intestinal mucosa of IBD patients and induce tissue damage.

Fas and FasL, while playing an important role in the regulation of apoptosis, have also nonapoptotic functions. Fas contains the death domain and, in contrast to TNFR1, had been thought to be involved only in proapoptotic but not prosurvival signalling [167]. However, it has been shown recently that although strong Fas stimulation blocked activation of human CD4(+) helper T cells, weak Fas stimulation together with TCR signalling augmented their proliferation via activation of MAP kinases, transcription factors, and cell cycle activators [168].

FasL contributes to costimulation of T cells also by a phenomenon termed "reverse signalling." Under this condition ligation of transmembrane FasL by functional Fas or DcR3 (a soluble decoy receptor for FasL, TL1A, and LIGHT) triggers signal transduction from FasL, resulting in the enhanced proliferation of mouse CD8(+) cytotoxic T cells [169–172]. These findings add much more complexity to possible roles of Fas/FasL system in the pathomechanisms of IBD which, theoretically, can be involved not only in direct disruption of epithelial continuity but also in costimulation of proinflammatory T cells. Since the details of Fas/FasL role in IBD still remain largely unknown, agents directly interfering with Fas signalling have yet not been tested for IBD treatment.

6. LIGHT

LIGHT (lymphotoxin-like inducible protein that competes with glycoprotein D for binding herpesvirus entry mediator on T cell, TNFSF14), ligand for the lymphotoxin beta receptor (LTβR, TNFRSF3), and the herpesvirus entry mediator (HVEM; TNFRSF14) are expressed mostly on activated T cells, although they were found also on monocytes, granulocytes, and immature dendritic cells [173, 174]. LTβR and HVEM receptors do not contain the death domain; therefore they are considered to be involved in prosurvival signalling [167]. Indeed, interaction between LIGHT and HVEM was found to enhance proliferation and effector functions of CD8(+) cytotoxic T cells [25], stimulate expansion of CD4(+) helper T cells, and promote their differentiation into Th1 cells [28].

Several studies indicate that LIGHT contributes to the development of intestinal inflammation. Transgenic mice with elevated expression of LIGHT spontaneously develop colitis [175]. Adoptive transfer of mesenteric lymph node cells expressing LIGHT into immunodeficient RAG−/− mice resulted in Th1-mediated intestinal inflammation dependent on both LIGHT receptors (LTβR and HVEM) [49]. Furthermore, induction of colitis in mice by DSS resulted in strong upregulation of LIGHT mRNA in colon mucosa, whereas LIGHT-deficient mice showed significantly reduced symptoms of DSS-induced colon inflammation [176].

The pathological role of LIGHT in human IBD has been hardly investigated, although in IBD patients upregulation of LIGHT mRNA in inflamed intestinal mucosa when compared to noninflamed areas has been demonstrated [37]. Blockade of LIGHT as a way of IBD treatment has not been tested in clinical settings, although administration of anti-LIGHT antibodies reduced symptoms of DSS-induced colon inflammation in mice [176].

7. DcR3

DcR3, soluble receptor for TL1A, FasL, and LIGHT, is a member of the TNF receptor superfamily that does not contain the transmembrane domain [127, 136]. As a soluble receptor, it inhibits the interaction between its ligands and their membrane-bound receptors, thus suppressing their biological activity. Elevated expression of DcR3 was detected in inflamed mucosa and serum of CD and UC patients [177–179]. The biological significance of this phenomenon remains unclear, although DcR3 is thought to play a protective role in IBD. For example, Funke et al. [179] showed that DcR3, acting as a soluble decoy receptor, limited the bioavailability of FasL and protected intestinal epithelial cells from FasL-mediated apoptosis. In a similar way, DcR3 may also prevent the proinflammatory effect exerted by TL1A; thus, it has been proposed that upregulation of DcR3 expression during intestinal inflammation may have a compensatory, protective effect [45].

8. TRAIL

Certain reports indicate that another member of the TNF superfamily, TRAIL (TNF-related apoptosis inducing ligand, TNFSF10), expressed in a large variety of tissues including intestines may be involved in the pathogenesis of IBD [159]. Similarly to TNF, TRAIL is able to induce apoptosis and can also activate the prosurvival transcription factor NF-κB. Five receptors of TRAIL have been identified to date. TRAIL-R1 (TNFRSF10A; DR4) and TRAIL-R2 (TNFRSF-10B; DR5) contain the death domain in their cytoplasmic fragments and are involved in functional TRAIL signalling [128]. Other molecules, TRAIL-R3 (TNFRSF10C; DcR1) without the death domain and TRAIL-R4 (TNFRSF10D; DcR2) with defective death domain and soluble osteoprotegerin (OPG, TNFRSF11B), are considered to be decoy receptors [167].

Expression of TRAIL was found to be downregulated in intestinal epithelial cells of IBD patients [180]; however, it was significantly elevated in mononuclear cells of the resected inflamed mucosa in both CD and UC patients with highly active, steroid-refractory disease [38, 180].

Even though the exact role of TRAIL in IBD pathogenesis remains undefined, the available data suggest that TRAIL-expressing mononuclear cells present in lamina propria disrupt the integrity of intestinal epithelium by inducing apoptosis of enterocytes. This notion was supported by an *ex vivo* study on ileal organ cultures which revealed that under inflammatory conditions TRAIL became a potent inducer of apoptosis in intestinal epithelial cells [38]. TRAIL is also a potent mediator of apoptotic death of intestinal fibroblasts in fibrostenosing intestinal areas in CD. Since collagen deposits and fibroblast proliferation are factors contributing to the development of strictures and fistulas, relatively common in CD patients, TRAIL can be involved also in tissue remodelling associated with CD [50].

9. TWEAK

TWEAK (TNF-like weak inducer of apoptosis, TNFSF12) acts through its receptor Fn14 (TNFRSF12; TWEAK-R) and has multiple biological activities, including stimulation of cell growth, induction of proinflammatory cytokines, and, in certain experimental settings, induction of apoptosis. TWEAK protein is expressed mostly in immune cells such as T cells, macrophages, or dendritic cells, although it was found also in nonhematopoietic cell types like astrocytes or endothelial cells [181]. Expression of TWEAK receptor, Fn14, was found on a variety of cells, including cells of intestinal mucosa. Fn14 does not contain the death domain and its stimulation with TWEAK results in activation of the transcription factor NF-κB [181, 182].

There are very few studies concerning the role of TWEAK in IBD pathogenesis; however in the intestinal mucosa of UC patients messenger RNA levels of IL-13, TWEAK, and Fn14 increased with disease activity [39]. TWEAK deficiency or reduction of its biological activity by anti-TWEAK monoclonal antibodies reduced expression of proinflammatory cytokines, neutrophil, and macrophage infiltration decreasing severity of trinitrobenzenesulfonic (TNBS) acid-induced colitis in mice [51]. Furthermore, even though TWEAK alone did not induce damage or apoptosis of intestinal epithelial cells, it was required, together with Fn14 and TNF, for IL-13-induced activation of caspase-3 in enterocytes isolated from γ-irradiated mice [39]. *In vitro* studies on rhabdomyosarcoma cell line Kym-1 showed that TWEAK upregulated expression of transmembrane TNF which in turn induced cell apoptosis via TNFR1 [183]. Furthermore, TWEAK stimulation synergistically enhanced TNFR1-mediated apoptotic cell death of Kym-1 cells [183] which can be explained by the fact that TWEAK induces translocation of antiapoptotic adaptor protein TRAF2 from the TNFR1 signalling complex, enhancing proapoptotic signalling of this receptor [184]. The question whether these mechanisms are active also *in vivo* in intestinal epithelium has not yet been studied.

10. Conclusions

Members of the TNF superfamily contribute to the pathogenesis of IBD in two ways. (i) They disrupt the integrity of intestinal epithelium by altering the arrangement of adhesion proteins in enterocytes (TNF), inducing apoptotic death of enterocytes (TNF, FasL, TRAIL, and TWEAK), and/or (ii) they promote the proinflammatory activity of mucosa-infiltrating mononuclear cells (TNF, TL1A, LIGHT, TWEAK, and possibly FasL) and affect the activity of regulatory T cells and regulatory macrophages (Figure 1).

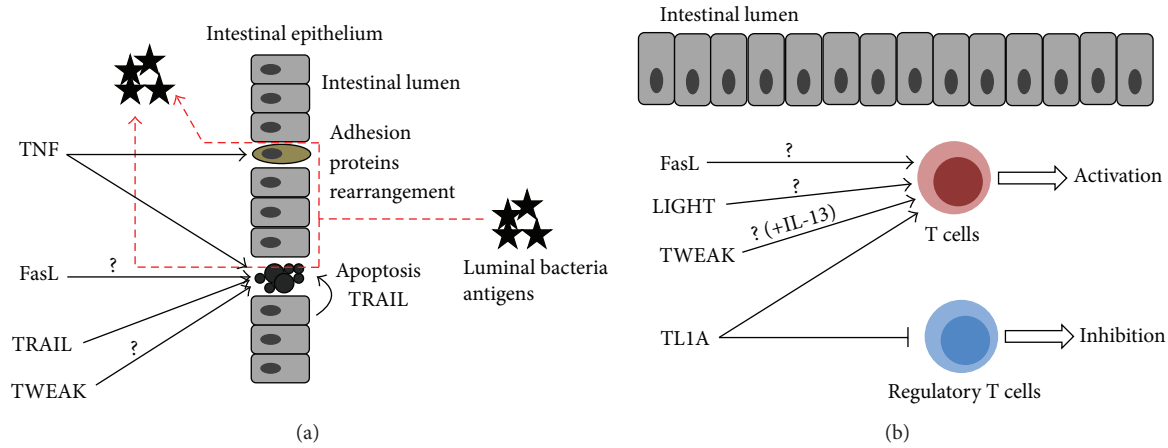

FIGURE 1: Two major mechanisms which implicate the molecules belonging to the TNFSF in the pathomechanisms of IBD. (a) Disruption of intestinal epithelium integrity allows luminal bacterial antigens to cross the epithelial barrier and migrate into the intestinal mucosa where they elicit immune responses. (b) Activation of mucosa-infiltrating T lymphocytes.

TNF superfamily members have attracted large attention as potential therapeutic targets in IBD treatment. Currently, however, the only TNFSF member targeted in clinical treatment of IBD is TNF. Another promising target, although still not tested in clinical trials, is TL1A which appears to be one of the key factors regulating the inflammatory pathways in IBD. The other members of TNF superfamily involved in IBD pathogenesis (FasL, LIGHT, TRAIL, and TWEAK) still require more in-depth studies to clearly define their function in intestinal inflammation. It has to be emphasized, however, that inflammatory injury of intestinal mucosa, a major feature of IBD, is mediated not only by the cross talk between various TNF superfamily members and their respective receptors since it results from the interactions of many cell types and inflammatory mediators which trigger multiple intracellular signalling pathways. Therefore, despite great therapeutic progress achieved in the treatment of Crohn's disease and ulcerative colitis by targeting TNF with various types of antibodies, further detailed studies are necessary to better understand the pathomechanisms of tissue injury in IBD aimed at defining more specific therapeutic targets.

Abbreviations

AICD: Activation-induced cell death
AP-1: Activator protein 1
CD: Crohn's disease
CHX: Cycloheximide
DcR3: Soluble decoy receptor 3
DD: Death domain
DNBS: Dinitrobenzenesulfonic acid
DR3: Death receptor 3
DSS: Dextran sodium sulphate
EGFR: Epidermal growth factor receptor
EMA: European Medicines Agency
FADD: Fas-associated death domain
FDA: Food and Drug Administration
GI: Gastrointestinal
IBD: Inflammatory bowel disease
IFN-γ: Interferon gamma
IL: Interleukin
iNOS: Inducible nitric oxide synthase
LIGHT: Lymphotoxin-like inducible protein that competes with glycoprotein D for binding herpesvirus entry mediator on T cell
LPS: Lipopolysaccharide
MCP-1: Monocyte chemotactic protein 1
MLC: Myosin light chain
MLCK: Myosin light chain kinase
MMP: Matrix metalloproteinase
mRNA: Messenger RNA
NF-κB: Nuclear factor kappa B
NK: Natural killer
PBMCs: Peripheral blood mononuclear cells
TJ: Tight junction
TL1A: TNF-like protein 1A
TLR: Toll-like receptor
TNBS: Trinitrobenzenesulfonic acid
TNF: Tumour necrosis factor
TNFR1: TNF receptor 1
TNFR2: TNF receptor 2
TNFSF: Tumour necrosis factor superfamily
TRADD: TNF receptor-associated death domain
TRAF: Tumour necrosis factor receptor-associated protein
TRAIL: TNF-related apoptosis inducing ligand
TWEAK: TNF-like weak inducer of apoptosis
UC: Ulcerative colitis
VEGI: Vascular endothelial growth inhibitor.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] W. Strober, I. Fuss, and P. Mannon, "The fundamental basis of inflammatory bowel disease," *Journal of Clinical Investigation*, vol. 117, no. 3, pp. 514–521, 2007.

[2] N. A. Molodecky and G. G. Kaplan, "Environmental risk factors for inflammatory bowel disease," *Gastroenterology and Hepatology*, vol. 6, no. 5, pp. 339–346, 2010.

[3] T. W. Hand, L. M. Dos Santos, N. Bouladoux et al., "Acute gastrointestinal infection induces long-lived microbiota-specific T cell responses," *Science*, vol. 337, no. 6101, pp. 1553–1556, 2012.

[4] L. Halme, P. Paavola-Sakki, U. Turunen, M. Lappalainen, M. Färkkilä, and K. Kontula, "Family and twin studies in inflammatory bowel disease," *World Journal of Gastroenterology*, vol. 12, no. 23, pp. 3668–3672, 2006.

[5] E. O. Glocker, D. Kotlarz, K. Boztug et al., "Inflammatory bowel disease and mutations affecting the interleukin-10 receptor," *New England Journal of Medicine*, vol. 361, no. 21, pp. 2033–2045, 2009.

[6] K. Yamazaki, D. McGovern, J. Ragoussis et al., "Single nucleotide polymorphisms in TNFSF15 confer susceptibility to Crohn' s disease," *Human Molecular Genetics*, vol. 14, no. 22, pp. 3499–3506, 2005.

[7] S. Maeda, L. Hsu, H. Liu et al., "Nod2 mutation in Crohn's disease potentiates NF-κB activity and IL-1β processing," *Science*, vol. 307, no. 5710, pp. 734–738, 2005.

[8] J. Hampe, A. Franke, P. Rosenstiel et al., "A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1," *Nature Genetics*, vol. 39, no. 2, pp. 207–211, 2007.

[9] M. Parkes, J. C. Barrett, N. J. Prescott et al., "Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility," *Nature Genetics*, vol. 39, no. 7, pp. 830–832, 2007.

[10] Z. Lin, L. Poritz, A. Franke et al., "Genetic association of DLG5 R30Q with familial and sporadic inflammatory bowel disease in men," *Disease Markers*, vol. 27, no. 5, pp. 193–201, 2009.

[11] S. Brand, "Crohn's disease: Th1, Th17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease," *Gut*, vol. 58, no. 8, pp. 1152–1167, 2009.

[12] G. Bamias, G. Kaltsa, and S. D. Ladas, "Cytokines in the pathogenesis of ulcerative colitis," *Discovery Medicine*, vol. 11, no. 60, pp. 459–467, 2011.

[13] J. Šventoraityte, A. Žvirbliene, G. Kiudelis et al., "Immune system alterations in patients with inflammatory bowel disease during remission," *Medicina*, vol. 44, no. 1, pp. 27–33, 2008.

[14] S. Pak, N. Holland, E. A. Garnett et al., "Cytokine profiles in peripheral blood of children and adults with Crohn disease," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 54, no. 6, pp. 769–775, 2012.

[15] N. Holland, J. Dong, E. Garnett et al., "Reduced intracellular T-helper 1 interferon-gamma in blood of newly diagnosed children with Crohn's disease and age-related changes in Th1/Th2 cytokine profiles," *Pediatric Research*, vol. 63, no. 3, pp. 257–262, 2008.

[16] I. J. Fuss and W. Strober, "The role of IL-13 and NK T cells in experimental and human ulcerative colitis," *Mucosal Immunology*, vol. 1, supplement 1, pp. S31–S33, 2008.

[17] C. J. Oliphant, J. L. Barlow, and A. N. J. Mckenzie, "Insights into the initiation of type 2 immune responses," *Immunology*, vol. 134, no. 4, pp. 378–385, 2011.

[18] F. Heller, A. Fromm, A. H. Gitter, J. Mankertz, and J.-D. Schulzke, "Epithelial apoptosis is a prominent feature of the epithelial barrier disturbance in intestinal inflammation: effect of pro-inflammatory interleukin-13 on epithelial cell function," *Mucosal Immunology*, vol. 1, supplement 1, pp. S58–S61, 2008.

[19] P. J. Mannon, R. L. Hornung, Z. Yang et al., "Suppression of inflammation in ulcerative colitis by interferon-β-1a is accompanied by inhibition of IL-13 production," *Gut*, vol. 60, no. 4, pp. 449–455, 2011.

[20] B. B. Aggarwal, S. C. Gupta, and J. H. Kim, "Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey," *Blood*, vol. 119, no. 3, pp. 651–665, 2012.

[21] B. B. Aggarwal, "Signalling pathways of the TNF superfamily: a double-edged sword," *Nature Reviews Immunology*, vol. 3, no. 9, pp. 745–756, 2003.

[22] R. H. Arch, R. W. Gedrich, and C. B. Thompson, "Tumor necrosis factor receptor-associated factors (TRAFs)—a family of adapter proteins that regulates life and death," *Genes and Development*, vol. 12, no. 18, pp. 2821–2830, 1998.

[23] R. Atreya, M. Zimmer, B. Bartsch et al., "Antibodies against tumor necrosis factor (TNF) induce T-cell apoptosis in patients with inflammatory bowel diseases via TNF receptor 2 and intestinal CD14 + macrophages," *Gastroenterology*, vol. 141, no. 6, pp. 2026–2038, 2011.

[24] H. Wajant, K. Pfizenmaier, and P. Scheurich, "Tumor necrosis factor signaling," *Cell Death and Differentiation*, vol. 10, no. 1, pp. 45–65, 2003.

[25] T. H. Watts, "TNF/TNFR family members in costimulation of T cell responses," *Annual Review of Immunology*, vol. 23, pp. 23–68, 2005.

[26] M. Croft, "The role of TNF superfamily members in T-cell function and diseases," *Nature Reviews Immunology*, vol. 9, no. 4, pp. 271–285, 2009.

[27] M. A. Nolte, R. W. Van Olffen, K. P. J. M. Van Gisbergen, and R. A. W. Van Lier, "Timing and tuning of CD27-CD70 interactions: the impact of signal strength in setting the balance between adaptive responses and immunopathology," *Immunological Reviews*, vol. 229, no. 1, pp. 216–231, 2009.

[28] C. Wang, G. H. Lin, A. J. McPherson, and T. H. Watts, "Immune regulation by 4-1BB and 4-1BBL: complexities and challenges," *Immunological Reviews*, vol. 229, no. 1, pp. 192–215, 2009.

[29] M. Croft, T. So, W. Duan, and P. Soroosh, "The significance of OX40 and OX40L to T-cell biology and immune disease," *Immunological Reviews*, vol. 229, no. 1, pp. 173–191, 2009.

[30] B. P. Pappu, A. Borodovsky, T. S. Zheng et al., "TL1A-DR3 interaction regulates Th17 cell function and Th17-mediated autoimmune disease," *Journal of Experimental Medicine*, vol. 205, no. 5, pp. 1049–1062, 2008.

[31] Z. Kmiec, "Cytokines in inflammatory bowel disease," *Archivum Immunologiae et Therapia Experimentalis*, vol. 46, pp. 143–155, 1998.

[32] G. Bamias, D. Corridoni, T. T. Pizarro, and F. Cominelli, "New insights into the dichotomous role of innate cytokines in gut homeostasis and inflammation," *Cytokine*, vol. 59, no. 3, pp. 451–459, 2012.

[33] F. Meylan, A. C. Richard, and R. M. Siegel, "TL1A and DR3, a TNF family ligand-receptor pair that promotes lymphocyte costimulation, mucosal hyperplasia, and autoimmune inflammation," *Immunological Reviews*, vol. 244, no. 1, pp. 188–196, 2011.

[34] H. Ueyama, T. Kiyohara, N. Sawada et al., "High Fas ligand expression on lymphocytes in lesions of ulcerative colitis," *Gut*, vol. 43, no. 1, pp. 48–55, 1998.

[35] M. Yukawa, M. Iizuka, Y. Horie et al., "Systemic and local evidence of increased Fas-mediated apoptosis in ulcerative colitis," *International Journal of Colorectal Disease*, vol. 17, no. 2, pp. 70–76, 2002.

[36] H. S. P. Souza, C. J. A. Tortori, M. T. L. Castelo-Branco et al., "Apoptosis in the intestinal mucosa of patients with inflammatory bowel disease: evidence of altered expression of FasL and perforin cytotoxic pathways," *International Journal of Colorectal Disease*, vol. 20, no. 3, pp. 277–286, 2005.

[37] O. Cohavy, J. Zhou, C. F. Ware, and S. R. Targan, "LIGHT is constitutively expressed on T and NK cells in the human gut and can be induced by CD2-mediated signaling," *Journal of Immunology*, vol. 174, no. 2, pp. 646–653, 2005.

[38] B. Begue, H. Wajant, J. Bambou et al., "Implication of TNF-related apoptosis-inducing ligand in inflammatory intestinal epithelial lesions," *Gastroenterology*, vol. 130, no. 7, pp. 1962–1974, 2006.

[39] R. Kawashima, Y. I. Kawamura, T. Oshio et al., "Interleukin-13 damages intestinal mucosa via TWEAK and Fn14 in MiceA pathway associated with ulcerative colitis," *Gastroenterology*, vol. 141, no. 6, pp. e2118–e2119, 2011.

[40] L. Cabal-Hierro and P. S. Lazo, "Signal transduction by tumor necrosis factor receptors," *Cellular Signalling*, vol. 24, no. 6, pp. 1297–1305, 2012.

[41] R. M. Locksley, N. Killeen, and M. J. Lenardo, "The TNF and TNF receptor superfamilies: integrating mammalian biology," *Cell*, vol. 104, no. 4, pp. 487–501, 2001.

[42] D. L. Faustman and M. Davis, "TNF receptor 2 and disease: autoimmunity and regenerative medicine," *Frontiers in Immunology*, vol. 4, p. 478, 2013.

[43] M. Leppkes, M. Roulis, M. F. Neurath, G. Kollias, and C. Becker, "The pleiotropic functions of TNF-alpha in the regulation of the intestinal epithelial response to inflammation," *International Immunology*, 2014.

[44] F. Van Hauwermeiren, M. Armaka, N. Karagianni et al., "Safe TNF-based antitumor therapy following p55TNFR reduction in intestinal epithelium," *Journal of Clinical Investigation*, vol. 123, no. 6, pp. 2590–2603, 2013.

[45] G. Bamias, L. G. Jia, and F. Cominelli, "The tumor necrosis factor-like cytokine 1A/death receptor 3 cytokine system in intestinal inflammation," *Current Opinion in Gastroenterology*, vol. 29, no. 6, pp. 597–602, 2013.

[46] T. H. Schreiber and E. R. Podack, "Immunobiology of TNFSF15 and TNFRSF25," *Immunologic Research*, vol. 57, no. 1–3, pp. 3–11, 2013.

[47] M. Lettau, M. Paulsen, D. Kabelitz, and O. Janssen, "FasL expression and reverse signalling," *Results and Problems in Cell Differentiation*, vol. 49, pp. 49–61, 2009.

[48] L. Chen, S. Park, J. R. Turner, and M. E. Peter, "Cell death in the colonic epithelium during inflammatory bowel diseases: CD95/Fas and beyond," *Inflammatory Bowel Diseases*, vol. 16, no. 6, pp. 1071–1076, 2010.

[49] J. Wang, R. A. Anders, Y. Wang et al., "The critical role of LIGHT in promoting intestinal inflammation and Crohn's disease," *Journal of Immunology*, vol. 174, no. 12, pp. 8173–8182, 2005.

[50] C. Reenaers, N. Franchimont, C. Oury et al., "Sensitivity of intestinal fibroblasts to TNF-related apoptosis-inducing ligand-mediated apoptosis in Crohn's disease," *Scandinavian Journal of Gastroenterology*, vol. 43, no. 11, pp. 1334–1345, 2008.

[51] T. Dohi, A. Borodovsky, P. Wu et al., "TWEAK/Fn14 pathway: a nonredundant role in intestinal damage in mice through a TWEAK/intestinal epithelial cell axis," *Gastroenterology*, vol. 136, no. 3, pp. 912–923, 2009.

[52] S. H. Murch, V. A. Lamkin, M. O. Savage, J. A. Walker-Smith, and T. T. MacDonald, "Serum concentrations of tumour necrosis factor α in childhood chronic inflammatory bowel disease," *Gut*, vol. 32, no. 8, pp. 913–917, 1991.

[53] S. Dionne, J. Hiscott, I. D'Agata, A. Duhaime, and E. G. Seidman, "Quantitative PCR analysis of TNF-α and IL-1β mRNA levels in pediatric IBD mucosal biopsies," *Digestive Diseases and Sciences*, vol. 42, no. 7, pp. 1557–1566, 1997.

[54] R. Matsuda, T. Koide, C. Tokoro et al., "Quantitive cytokine mRNA expression profiles in the colonic mucosa of patients with steroid Naïve ulcerative colitis during active and quiescent disease," *Inflammatory Bowel Diseases*, vol. 15, no. 3, pp. 328–334, 2009.

[55] D. Owczarek, D. Cibor, M. K. Głowacki, A. Cieśla, and P. Mach, "TNF-α and soluble forms of TNF receptors 1 and 2 in the serum of patients with Crohn's disease and ulcerative colitis," *Polskie Archiwum Medycyny Wewnetrznej*, vol. 122, no. 12, pp. 616–623, 2012.

[56] M. Maeda, N. Watanabe, H. Neda et al., "Serum tumor necrosis factor activity in inflammatory bowel disease," *Immunopharmacology and Immunotoxicology*, vol. 14, no. 3, pp. 451–461, 1992.

[57] S. H. Murch, C. P. Braegger, J. A. Walker-Smith, and T. T. MacDonald, "Location of tumour necrosis factor α by immunohistochemistry in chronic inflammatory bowel disease," *Gut*, vol. 34, no. 12, pp. 1705–1709, 1993.

[58] C. Stevens, G. Walz, C. Singaram et al., "Tumor necrosis factor-α, interleukin-1β, and interleukin-6 expression in inflammatory bowel disease," *Digestive Diseases and Sciences*, vol. 37, no. 6, pp. 818–826, 1992.

[59] J. S. Hyams, W. R. Treem, E. Eddy, N. Wyzga, and R. E. Moore, "Tumor necrosis factor-α is not elevated in children with inflammatory bowel disease," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 12, no. 2, pp. 233–236, 1991.

[60] C. Perrier, G. De Hertogh, J. Cremer et al., "Neutralization of membrane TNF, but not soluble TNF, is crucial for the treatment of experimental colitis," *Inflammatory Bowel Diseases*, vol. 19, no. 2, pp. 246–253, 2013.

[61] E. Mizoguchi, A. Mizoguchi, H. Takedatsu et al., "Role of tumor necrosis factor receptor 2 (TNFR2) in colonic epithelial hyperplasia and chronic intestinal inflammation in mice," *Gastroenterology*, vol. 122, no. 1, pp. 134–144, 2002.

[62] M. H. Holtmann, E. Douni, M. Schutz et al., "Tumor necrosis factor-receptor 2 is up-regulated on lamina propria T cells in Crohn's disease and promotes experimental colitis in vivo," *European Journal of Immunology*, vol. 32, no. 11, pp. 3142–3151, 2002.

[63] T. Y. Ma, G. K. Iwamoto, N. T. Hoa et al., "TNF-α-induced increase in intestinal epithelial tight junction permeability requires NF-κB activation," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 286, no. 3, pp. G367–G376, 2004.

[64] T. Fréour, A. Jarry, K. Bach-Ngohou et al., "TACE inhibition amplifies TNF-α-mediated colonic epithelial barrier disruption," *International Journal of Molecular Medicine*, vol. 23, no. 1, pp. 41–48, 2009.

[65] F. Wang, B. T. Schwarz, W. V. Graham et al., "IFN-γ-induced TNFR2 expression is required for TNF-dependent intestinal epithelial barrier dysfunction," *Gastroenterology*, vol. 131, no. 4, pp. 1153–1163, 2006.

[66] S. Rose-John, "ADAM17, shedding, TACE as therapeutic targets," *Pharmacological Research*, vol. 71, pp. 19–22, 2013.

[67] A. Cesaro, A. Abakar-Mahamat, P. Brest et al., "Differential expression and regulation of ADAM17 and TIMP3 in acute inflamed intestinal epithelia," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 296, no. 6, pp. G1332–G1343, 2009.

[68] E. Franzè, R. Caruso, C. Stolfi et al., "High expression of the "A disintegrin and metalloprotease" 19 (ADAM19), a sheddase for TNF-a in the mucosa of patients with inflammatory bowel diseases," *Inflammatory Bowel Diseases*, vol. 19, no. 3, pp. 501–511, 2013.

[69] R. E. Vandenbroucke, E. Dejonckheere, F. Van Hauwermeiren et al., "Matrix metalloproteinase 13 modulates intestinal epithelial barrier integrity in inflammatory diseases by activating TNF," *EMBO Molecular Medicine*, vol. 5, no. 7, pp. 932–948, 2013.

[70] L. Su, S. C. Nalle, L. Shen et al., "TNFR2 activates mlck-dependent tight junction dysregulation to cause apoptosis-mediated barrier loss and experimental colitis," *Gastroenterology*, vol. 145, no. 2, pp. 407–415, 2013.

[71] A. M. Marchiando, L. Shen, W. V. Graham et al., "The epithelial barrier is maintained by in vivo tight junction expansion during pathologic intestinal epithelial shedding," *Gastroenterology*, vol. 140, no. 4, pp. 1208–1218, 2011.

[72] T. Goretsky, R. Dirisina, P. Sinh et al., "P53 mediates TNF-induced epithelial cell apoptosis in IBD," *American Journal of Pathology*, vol. 181, no. 4, pp. 1306–1315, 2012.

[73] K. E. Cunningham and J. R. Turner, "Myosin light chain kinase: pulling the strings of epithelial tight junction function," *Annals of the New York Academy of Sciences*, vol. 1258, no. 1, pp. 34–42, 2012.

[74] S. A. Blair, S. V. Kane, D. R. Clayburgh, and J. R. Turner, "Epithelial myosin light chain kinase expression and activity are upregulated in inflammatory bowel disease," *Laboratory Investigation*, vol. 86, no. 2, pp. 191–201, 2006.

[75] T. Yamaoka, F. Yan, H. Cao et al., "Transactivation of EGF receptor and ErbB2 protects intestinal epithelial cells from TNF-induced apoptosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 33, pp. 11772–11777, 2008.

[76] S. S. Hobbs, J. A. Goettel, D. Liang et al., "TNF transactivation of EGFR stimulates cytoprotective COX-2 expression in gastrointestinal epithelial cells," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 301, no. 2, pp. G220–G229, 2011.

[77] K. Xu, L. Wang, and H. K. Shu, "COX-2 overexpression increases malignant potential of human glioma cells through Id1," *Oncotarget*, vol. 5, no. 5, pp. 1241–1252, 2014.

[78] M. Lukas, "Inflammatory bowel disease as a risk factor for colorectal cancer," *Digestive Diseases*, vol. 28, no. 4-5, pp. 619–624, 2010.

[79] I. Pache, G. Rogler, and C. Felley, "TNF-α blockers in inflammatory bowel diseases: practical consensus recommendations and a user's guide," *Swiss Medical Weekly*, vol. 139, no. 19-20, pp. 278–287, 2009.

[80] E. G. Boyce, J. Halilovic, and O. Stan-Ugbene, "Golimumab: review of the efficacy and tolerability of a recently approved tumor necrosis factor-α inhibitor," *Clinical Therapeutics*, vol. 32, no. 10, pp. 1681–1703, 2010.

[81] J. Dretzke, R. Edlin, J. Round et al., "A systematic review and economic evaluation of the use of tumour necrosis factor-alpha (TNF-α) inhibitors, adalimumab and infliximab, for crohn's disease," *Health Technology Assessment*, vol. 15, no. 6, pp. 1–250, 2011.

[82] O. H. Nielsen, J. B. Seidelin, L. K. Munck, and G. Rogler, "Use of biological molecules in the treatment of inflammatory bowel disease," *Journal of Internal Medicine*, vol. 270, no. 1, pp. 15–28, 2011.

[83] M. M. Lawson, A. G. Thomas, and A. K. Akobeng, "Tumour necrosis factor alpha blocking agents for induction of remission in ulcerative colitis," *Cochrane Database of Systematic Reviews*, vol. 3, Article ID CD005112, 2006.

[84] S. M. Wilhelm, K. A. McKenney, K. N. Rivait, and P. B. Kale-Pradhan, "A review of infliximab use in ulcerative colitis," *Clinical Therapeutics*, vol. 30, no. 2, pp. 223–230, 2008.

[85] W. Reinisch, W. J. Sandborn, D. W. Hommes et al., "Adalimumab for induction of clinical remission in moderately to severely active ulcerative colitis: results of a randomised controlled trial," *Gut*, vol. 60, no. 6, pp. 780–787, 2011.

[86] W. J. Sandborn, G. Van Assche, W. Reinisch et al., "Adalimumab induces and maintains clinical remission in patients with moderate-to-severe ulcerative colitis," *Gastroenterology*, vol. 142, no. 2, pp. 257–265, 2012.

[87] W. J. Sandborn, B. G. Feagan, C. Marano et al., "Subcutaneous golimumab induces clinical response and remission in patients with moderate-to-severe ulcerative colitis," *Gastroenterology*, vol. 146, no. 1, pp. 85–95, 2014.

[88] W. J. Sandborn, B. G. Feagan, C. Marano et al., "Subcutaneous golimumab maintains clinical response in patients with moderate-to-severe ulcerative colitis," *Gastroenterology*, vol. 146, no. 1, pp. 96.e1–109.e1, 2014.

[89] L. Peyrin-Biroulet, P. Deltenre, N. de Suray, J. Branche, W. J. Sandborn, and J. Colombel, "Efficacy and safety of tumor necrosis factor antagonists in Crohn's disease: meta-analysis of placebo-controlled trials," *Clinical Gastroenterology and Hepatology*, vol. 6, no. 6, pp. 644–653, 2008.

[90] O. H. Nielsen and M. A. Ainsworth, "Tumor necrosis factor inhibitors for inflammatory bowel disease," *New England Journal of Medicine*, vol. 369, no. 8, pp. 754–762, 2013.

[91] A. J. Yarur, M. T. Abreu, A. R. Deshpande et al., "Therapeutic drug monitoring in patients with inflammatory bowel disease," *World Journal of Gastroenterology*, vol. 20, no. 13, pp. 3475–3484, 2014.

[92] S. Danese, G. Fiorino, L. Peyrin-Biroulet et al., "Biological agents for moderately to severely active ulcerative colitis: a systematic review and network meta-analysis," *Annals of Internal Medicine*, vol. 160, no. 10, pp. 704–711, 2014.

[93] S. Ben-Horin, U. Kopylov, and Y. Chowers, "Optimizing anti-TNF treatments in inflammatory bowel disease," *Autoimmunity Reviews*, vol. 126, no. 6, pp. 1593–1610, 2014.

[94] R. Altwegg and T. Vincent, "TNF blocking therapies and immunomonitoring in patients with inflammatory bowel disease," *Mediators of Inflammation*, vol. 2014, Article ID 172821, 7 pages, 2014.

[95] G. R. Lichtenstein, "Comprehensive review: antitumor necrosis factor agents in inflammatory bowel disease and factors implicated in treatment response," *Therapeutic Advances in Gastroenterology*, vol. 6, no. 4, pp. 269–293, 2013.

[96] P. Campi, M. Benucci, M. Manfredi, and P. Demoly, "Hypersensitivity reactions to biological agents with special emphasis on tumor necrosis factor-α antagonists," *Current Opinion in Allergy and Clinical Immunology*, vol. 7, no. 5, pp. 393–403, 2007.

[97] A. Barbaud, F. Granel, J. Waton, and C. Poreaux, "How to manage hypersensitivity reactions to biological agents?" *European Journal of Dermatology*, vol. 21, no. 5, pp. 667–674, 2011.

[98] P. Deepak, D. J. Stobaugh, and E. D. Ehrenpreis, "Infectious complications of TNF-alpha inhibitor monotherapy versus combination therapy with immunomodulators in inflammatory bowel disease: analysis of the Food and Drug Administration Adverse Event Reporting System," *Journal of Gastrointestinal and Liver Diseases*, vol. 22, no. 3, pp. 269–276, 2013.

[99] P. Lozeron, C. Denier, C. Lacroix, and D. Adams, "Long-term course of demyelinating neuropathies occurring during tumor necrosis factor-a-blocker therapy," *Archives of Neurology*, vol. 66, no. 4, pp. 490–497, 2009.

[100] P. Deepak, D. J. Stobaugh, M. Sherid, H. Sifuentes, and E. D. Ehrenpreis, "Neurological events with tumour necrosis factor alpha inhibitors reported to the Food and Drug Administration Adverse Event Reporting System," *Alimentary Pharmacology and Therapeutics*, vol. 38, no. 4, pp. 388–396, 2013.

[101] G. Moris, "Inflammatory bowel disease: an increased risk factor for neurologic complications," *World Journal of Gastroenterology*, vol. 20, no. 5, pp. 1228–1237, 2014.

[102] G. Fiorino, S. Danese, B. Pariente, and M. Allez, "Paradoxical immune-mediated inflammation in inflammatory bowel disease patients receiving anti-TNF-α agents," *Autoimmunity Reviews*, vol. 13, no. 1, pp. 15–19, 2014.

[103] C. Tillack, L. M. Ehmann, M. Friedrich et al., "Anti-TNF antibody-induced psoriasiform skin lesions in patients with inflammatory bowel disease are characterised by interferon-gamma-expressing Th1 cells and IL-17A/IL-22-expressing Th17 cells and respond to anti-IL-12/IL-23 antibody treatment," *Gut*, vol. 63, no. 4, pp. 567–577, 2014.

[104] J. M. H. Van den Brande, H. Braat, G. R. Van den Brink et al., "Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease," *Gastroenterology*, vol. 124, no. 7, pp. 1774–1785, 2003.

[105] J. Mudter and M. F. Neurath, "Apoptosis of T cells and the control of inflammatory bowel disease: therapeutic implications," *Gut*, vol. 56, no. 2, pp. 293–303, 2007.

[106] C. Veltkamp, M. Anstaett, K. Wahl et al., "Apoptosis of regulatory T lymphocytes is increased in chronic inflammatory bowel disease and reversed by anti-TNFα treatment," *Gut*, vol. 60, no. 10, pp. 1345–1353, 2011.

[107] G. Boschetti, S. Nancey, F. Sardi, X. Roblin, B. Flourié, and D. Kaiserlian, "Therapy with anti-TNFα antibody enhances number and function of Foxp3+ regulatory T cells in inflammatory bowel diseases," *Inflammatory Bowel Diseases*, vol. 17, no. 1, pp. 160–170, 2011.

[108] A. C. Vos, M. E. Wildenberg, M. Duijvestein, A. P. Verhaar, G. R. Van Den Brink, and D. W. Hommes, "AntiTumor necrosis factor-α antibodies induce regulatory macrophages in an Fc region-dependent manner," *Gastroenterology*, vol. 140, no. 1, pp. 221–230, 2011.

[109] A. C. Vos, M. E. Wildenberg, I. Arijs et al., "Regulatory macrophages induced by infliximab are involved in healing in vivo and in vitro," *Inflammatory Bowel Diseases*, vol. 18, no. 3, pp. 401–408, 2012.

[110] R. F. Leal, N. Planell, R. Kajekar et al., "Identification of inflammatory mediators in patients with Crohn's disease unresponsive to anti-TNFalpha therapy," *Gut*, 2014.

[111] P. Suenaert, V. Bulteel, L. Lemmens et al., "Anti-tumor necrosis factor treatment restores the gut barrier in Crohn's disease," *American Journal of Gastroenterology*, vol. 97, no. 8, pp. 2000–2004, 2002.

[112] W. Fries, C. Muja, C. Crisafulli et al., "Infliximab and etanercept are equally effective in reducing enterocyte apoptosis in experimental colitis," *International Journal of Medical Sciences*, vol. 5, no. 4, pp. 169–180, 2008.

[113] A. Fischer, M. Gluth, U. Pape, B. Wiedenmann, F. Theuring, and D. C. Baumgart, "Adalimumab prevents barrier dysfunction and antagonizes distinct effects of TNF-α on tight junction proteins and signaling pathways in intestinal epithelial cells," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 304, no. 11, pp. G970–G979, 2013.

[114] P. Eder, L. Lykowska-Szuber, I. Krela-Kazmierczak et al., "Anti-TNF antibodies do not induce the apoptosis of lamina propria mononuclear cells in uninflamed intestinal tissue in patients with Crohn's disease," *Folia Histochemica et Cytobiologica*, vol. 51, pp. 239–243, 2013.

[115] P. Eder, L. Lykowska-Szuber, I. Krela-Kazmierczak, K. Stawczyk-Eder, M. Zabel, and K. Linke, "The influence of infliximab and adalimumab on the expression of apoptosis-related proteins in lamina propria mononuclear cells and enterocytes in Crohn's disease-an immunohistochemical study," *Journal of Crohn's and Colitis*, vol. 7, no. 9, pp. 706–716, 2013.

[116] B. Scallon, A. Cai, N. Solowski et al., "Binding and functional comparisons of two types of tumor necrosis factor antagonists," *Journal of Pharmacology and Experimental Therapeutics*, vol. 301, no. 2, pp. 418–426, 2002.

[117] W. J. Sandborn, S. B. Hanauer, S. Katz et al., "Etanercept for active Crohn's disease: a randomized, double-blind, placebo-controlled trial," *Gastroenterology*, vol. 121, no. 5, pp. 1088–1094, 2001.

[118] T. S. Migone, J. Zhang, X. Luo et al., "TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator," *Immunity*, vol. 16, no. 3, pp. 479–492, 2002.

[119] L. J. Metheny-Barlow and L. Y. Li, "Vascular endothelial growth inhibitor (VEGI), an endogenous negative regulator of angiogenesis," *Seminars in Ophthalmology*, vol. 21, no. 1, pp. 49–58, 2006.

[120] L. J. Chew, H. Pan, J. Yu et al., "A novel secreted splice variant of vascular endothelial cell growth inhibitor," *The FASEB Journal*, vol. 16, no. 7, pp. 742–744, 2002.

[121] N. Zhang, A. J. Sanders, L. Ye et al., "Vascular endothelial growth inhibitor in human cancer (Review)," *International Journal of Molecular Medicine*, vol. 24, no. 1, pp. 3–8, 2009.

[122] L. Fang, B. Adkins, V. Deyev, and E. R. Podack, "Essential role of TNF receptor superfamily 25 (TNFRSF25) in the development of allergic lung inflammation," *Journal of Experimental Medicine*, vol. 205, no. 5, pp. 1037–1048, 2008.

[123] F. Meylan, T. S. Davidson, E. Kahle et al., "The TNF-family receptor DR3 is essential for diverse T cell-mediated inflammatory diseases," *Immunity*, vol. 29, no. 1, pp. 79–89, 2008.

[124] J. Zhang, X. Wang, H. Fahmi et al., "Role of TL1A in the pathogenesis of rheumatoid arthritis," *Journal of Immunology*, vol. 183, no. 8, pp. 5350–5357, 2009.

[125] G. Bamias, C. Martin III, M. Marini et al., "Expression, localization, and functional activity of TL1A, a novel Th1-polarizing cytokine in inflammatory bowel disease," *Journal of Immunology*, vol. 171, no. 9, pp. 4868–4874, 2003.

[126] J. L. Prehn, S. Mehdizadeh, C. J. Landers et al., "Potential role for TL1A, the new TNF-family member and potent costimulator of IFN-γ, in mucosal inflammation," *Clinical Immunology*, vol. 112, no. 1, pp. 66–77, 2004.

[127] S. Kim and L. Zhang, "Identification of naturally secreted soluble form of TL1A, a TNF-like cytokine," *Journal of Immunological Methods*, vol. 298, no. 1-2, pp. 1–8, 2005.

[128] J. L. Prehn, L. S. Thomas, C. J. Landers, Q. T. Yu, K. S. Michelsen, and S. R. Targan, "The T cell costimulator TL1A is induced by FcγR signaling in human monocytes and dendritic cells," *Journal of Immunology*, vol. 178, no. 7, pp. 4033–4038, 2007.

[129] T. Jin, F. Guo, S. Kim, A. Howard, and Y. Zhang, "X-ray crystal structure of TNF ligand family member TL1A at 2.1 Å," *Biochemical and Biophysical Research Communications*, vol. 364, no. 1, pp. 1–6, 2007.

[130] D. Q. Shih, L. Y. Kwan, V. Chavez et al., "Microbial induction of inflammatory bowel disease associated gene TL1A (TNFSF15) in antigen presenting cells," *European Journal of Immunology*, vol. 39, no. 11, pp. 3239–3250, 2009.

[131] A. M. Chinnaiyan, K. O'Rourke, G. Yu et al., "Signal transduction by DR3, a death domain-containing receptor related to TNFR-1 and CD95," *Science*, vol. 274, no. 5289, pp. 990–992, 1996.

[132] Y. J. Kang, W. J. Kim, H. U. Bae et al., "Involvement of TL1A and DR3 in induction of pro-inflammatory cytokines and matrix metalloproteinase-9 in atherogenesis," *Cytokine*, vol. 29, no. 5, pp. 229–235, 2005.

[133] K. A. Papadakis, J. L. Prehn, C. Landers et al., "TL1A synergizes with IL-12 and IL-18 to enhance IFN-γ production in human T cells and NK cells," *Journal of Immunology*, vol. 172, no. 11, pp. 7002–7007, 2004.

[134] C. Cavallini, O. Lovato, A. Bertolaso et al., "The TNF-Family Cytokine TL1A Inhibits Proliferation of Human Activated B Cells," *PLoS ONE*, vol. 8, no. 4, Article ID e60136, 2013.

[135] K. A. Papadakis, D. Zhu, J. L. Prehn et al., "Dominant role for TL1A/DR3 pathway in IL-12 plus IL-18-induced IFN-γ production by peripheral blood and mucosal CCR9+ T lymphocytes," *Journal of Immunology*, vol. 174, no. 8, pp. 4985–4990, 2005.

[136] C. R. Yang, S. L. Hsieh, C. M. Teng, F. Ho, W. Su, and W. Lin, "Soluble decoy receptor 3 induces angiogenesis by neutralization of TL1A, a cytokine belonging to tumor necrosis factor superfamily and exhibiting angiostatic action," *Cancer Research*, vol. 64, no. 3, pp. 1122–1129, 2004.

[137] L. Wen, L. Zhuang, X. Luo, and P. Wei, "TL1A-induced NF-κB activation and c-IAP2 production prevent DR3-mediated apoptosis in TF-1 cells," *Journal of Biological Chemistry*, vol. 278, no. 40, pp. 39251–39258, 2003.

[138] T. J. Slebioda, T. F. Rowley, J. R. Ferdinand et al., "Triggering of TNFRSF25 promotes CD8+ T-cell responses and anti-tumor immunity," *European Journal of Immunology*, vol. 41, no. 9, pp. 2606–2611, 2011.

[139] H. Takedatsu, K. S. Michelsen, B. Wei et al., "TL1A (TNFSF15) regulates the development of chronic colitis by modulating both T-helper 1 and T-helper 17 activation," *Gastroenterology*, vol. 135, no. 2, pp. 552–567, 2008.

[140] N. Kamada, T. Hisamatsu, H. Honda et al., "TL1A produced by lamina propria macrophages induces Th1 and Th17 immune responses in cooperation with IL-23 in patients with Crohn's disease," *Inflammatory Bowel Diseases*, vol. 16, no. 4, pp. 568–575, 2010.

[141] S. F. Wu, T. M. Liu, Y. C. Lin et al., "Immunomodulatory effect of decoy receptor 3 on the differentiation and function of bone marrow-derived dendritic cells in nonobese diabetic mice: from regulatory mechanism to clinical implication," *Journal of Leukocyte Biology*, vol. 75, no. 2, pp. 293–306, 2004.

[142] T. L. Hsu, Y. C. Chang, S. J. Chen et al., "Modulation of dendritic cell differentiation and maturation by decoy receptor 3," *Journal of Immunology*, vol. 168, no. 10, pp. 4846–4853, 2002.

[143] F. Meylan, Y.-J. Song, I. Fuss et al., "The TNF-family cytokine TL1A drives IL-13-dependent small intestinal inflammation," *Mucosal Immunology*, vol. 4, no. 2, pp. 172–185, 2011.

[144] V. Y. Taraban, T. J. Slebioda, J. E. Willoughby et al., "Sustained TL1A expression modulates effector and regulatory T-cell responses and drives intestinal goblet cell hyperplasia," *Mucosal Immunology*, vol. 4, no. 2, pp. 186–196, 2011.

[145] S. Q. Khan, M. S. Tsai, T. H. Schreiber, D. Wolf, V. V. Deyev, and E. R. Podack, "Cloning, expression, and functional characterization of TL1A-Ig," *Journal of Immunology*, vol. 190, no. 4, pp. 1540–1550, 2013.

[146] Y. Kakuta, N. Ueki, Y. Kinouchi et al., "TNFSF15 transcripts from risk haplotype for Crohn's disease are overexpressed in stimulated T cells," *Human Molecular Genetics*, vol. 18, no. 6, pp. 1089–1098, 2009.

[147] K. S. Michelsen, L. S. Thomas, K. D. Taylor et al., "IBD-associated TL1A gene (TNFSFI5) haplotypes determine increased expression of TL1A protein," *PLoS ONE*, vol. 4, no. 3, Article ID e4719, 2009.

[148] S. Jin, J. Chin, S. Seeber et al., "TL1A/TNFSF15 directly induces proinflammatory cytokines, including TNFα, from CD3+CD161+ T cells to exacerbate gut inflammation," *Mucosal Immunology*, vol. 6, no. 5, pp. 886–899, 2013.

[149] O. Cohavy, D. Q. Shih, T. M. Doherty et al., "Cd161 defines effector T cells that express light and respond to Tl1a-Dr3 signaling," *European Journal of Microbiology and Immunology*, vol. 1, no. 1, pp. 70–79, 2011.

[150] M. A. Kleinschek, K. Boniface, S. Sadekova et al., "Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation," *Journal of Experimental Medicine*, vol. 206, no. 3, pp. 525–534, 2009.

[151] W. Strober and I. J. Fuss, "Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases," *Gastroenterology*, vol. 140, no. 6, pp. 1756–1767, 2011.

[152] S. Tauzin, L. Debure, J. Moreau, and P. Legembre, "CD95-mediated cell signaling in cancer: mutations and post-translational modulations," *Cellular and Molecular Life Sciences*, vol. 69, no. 8, pp. 1261–1277, 2012.

[153] M. Lettau, M. Paulsen, H. Schmidt, and O. Janssen, "Insights into the molecular regulation of FasL (CD178) biology," *European Journal of Cell Biology*, vol. 90, no. 6-7, pp. 456–466, 2011.

[154] L. A. O'Reilly, L. Tai, L. Lee et al., "Membrane-bound Fas ligand only is essential for Fas-induced apoptosis," *Nature*, vol. 461, no. 7264, pp. 659–663, 2009.

[155] P. Moller, K. Koretz, F. Leithauser et al., "Expression of APO-1 (CD95), a member of the NGF/TNF receptor superfamily, in normal and neoplastic colon epithelium," *International Journal of Cancer*, vol. 57, no. 3, pp. 371–377, 1994.

[156] P. Möller, H. Walczak, S. Riedl, J. Sträter, and P. H. Krammer, "Paneth cells express high levels of CD95 ligand transcripts:

a unique property among gastrointestinal epithelia," *American Journal of Pathology*, vol. 149, no. 1, pp. 9–13, 1996.

[157] M. J. Pinkoski, T. Brunner, D. R. Green, and T. Lin, "Fas and Fas ligand in gut and liver," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 278, no. 3, pp. G354–G366, 2000.

[158] J. Strater, I. Wellisch, S. Riedl et al., "CD95 (APO-1/Fas)-mediated apoptosis in colon epithelial cells: a possible role in ulcerative colitis," *Gastroenterology*, vol. 113, no. 1, pp. 160–167, 1997.

[159] J. Strater and P. Moller, "Expression and function of death receptors and their natural ligands in the intestine," *Annals of the New York Academy of Sciences*, vol. 915, pp. 162–170, 2000.

[160] J. B. Seidelin and O. H. Nielsen, "Attenuated apoptosis response to Fas-ligand in active ulcerative colitis," *Inflammatory Bowel Diseases*, vol. 14, no. 12, pp. 1623–1629, 2008.

[161] S. M. Park, L. Chen, M. Zhang, P. Ashton-Rickardt, J. R. Turner, and M. E. Peter, "CD95 is cytoprotective for intestinal epithelial cells in colitis," *Inflammatory Bowel Diseases*, vol. 16, no. 6, pp. 1063–1070, 2010.

[162] M. Merger, J. L. Viney, R. Borojevic et al., "Defining the roles of perforin, Fas/FasL, and tumour necrosis factor α in T cell induced mucosal damage in the mouse intestine," *Gut*, vol. 51, no. 2, pp. 155–163, 2002.

[163] I. Monteleone, G. Monteleone, D. Fina et al., "A functional role of flip in conferring resistance of Crohn's disease lamina propria lymphocytes to FAS-mediated apoptosis," *Gastroenterology*, vol. 130, no. 2, pp. 389–397, 2006.

[164] K. Ina, J. Itoh, K. Fukushima et al., "Resistance of Crohn's disease T cells to multiple apoptotic signals is associated with a Bcl-2/Bax mucosal imbalance," *Journal of Immunology*, vol. 163, no. 2, pp. 1081–1090, 1999.

[165] M. Boirivant, M. Marini, G. Di Felice et al., "Lamina propria T cells in Crohn's disease and other gastrointestinal inflammation show defective CD2 pathway-induced apoptosis," *Gastroenterology*, vol. 116, no. 3, pp. 557–565, 1999.

[166] A. Suzuki, K. Sugimura, K. Ohtsuka et al., "Fas/Fas ligand expression and characteristics of primed CD45RO+ T cells in the inflamed mucosa of ulcerative colitis," *Scandinavian Journal of Gastroenterology*, vol. 35, no. 12, pp. 1278–1283, 2000.

[167] T. Hehlgans and K. Pfeffer, "The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games," *Immunology*, vol. 115, no. 1, pp. 1–20, 2005.

[168] M. Paulsen, S. Valentin, B. Mathew et al., "Modulation of CD4+ T-cell activation by CD95 co-stimulation," *Cell Death and Differentiation*, vol. 18, no. 4, pp. 619–631, 2011.

[169] I. Suzuki and P. J. Fink, "Maximal proliferation of cytotoxic T lymphocytes requires reverse signaling through Fas ligand," *Journal of Experimental Medicine*, vol. 187, no. 1, pp. 123–128, 1998.

[170] I. Suzuki and P. J. Fink, "The dual functions of Fas ligand in the regulation of peripheral CD8+ and CD4+ T cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 4, pp. 1707–1712, 2000.

[171] I. Suzuki, S. Martin, T. E. Boursalian, C. Beers, and P. J. Fink, "Fas ligand costimulates the in vivo proliferation of CD8+ T cells," *Journal of Immunology*, vol. 165, no. 10, pp. 5537–5543, 2000.

[172] G. Shi, H. Luo, X. Wan, T. W. Salcedo, J. Zhang, and J. Wu, "Mouse T cells receive costimulatory signals from LIGHT, a TNF family member," *Blood*, vol. 100, no. 9, pp. 3279–3286, 2002.

[173] D. N. Mauri, R. Ebner, R. I. Montgomery et al., "LIGHT, a new member of the TNF superfamily, and lymphotoxin α are ligands for herpesvirus entry mediator," *Immunity*, vol. 8, no. 1, pp. 21–30, 1998.

[174] Y. Zhai, R. Guo, T. Hsu et al., "LIGHT, a novel ligand for lymphotoxin β receptor and TR2/HVEM induces apoptosis and suppresses in vivo tumor formation via gene transfer," *Journal of Clinical Investigation*, vol. 102, no. 6, pp. 1142–1151, 1998.

[175] R. B. Shaikh, S. Santee, S. W. Granger et al., "Constitutive expression of LIGHT on T cells leads to lymphocyte activation, inflammation, and tissue destruction," *Journal of Immunology*, vol. 167, no. 11, pp. 6330–6337, 2001.

[176] M. Jungbeck, B. Daller, J. Federhofer et al., "Neutralization of LIGHT ameliorates acute dextran sodium sulphate-induced intestinal inflammation," *Immunology*, vol. 128, no. 3, pp. 451–458, 2009.

[177] G. Bamias, G. Kaltsa, S. I. Siakavellas et al., "Differential expression of the TL1A/DcR3 system of TNF/TNFR-like proteins in large vs. small intestinal Crohn's disease," *Digestive and Liver Disease*, vol. 44, no. 1, pp. 30–36, 2012.

[178] G. Bamias, G. Kaltsa, S. I. Siakavellas et al., "High intestinal and systemic levels of decoy receptor 3 (DcR3) and its ligand TL1A in active ulcerative colitis," *Clinical Immunology*, vol. 137, no. 2, pp. 242–249, 2010.

[179] B. Funke, F. Autschbach, S. Kim et al., "Functional characterisation of decoy receptor 3 in Crohn's disease," *Gut*, vol. 58, no. 4, pp. 483–491, 2009.

[180] S. Brost, R. Koschny, J. Sykora et al., "Differential expression of the TRAIL/TRAIL-receptor system in patients with inflammatory bowel disease," *Pathology Research and Practice*, vol. 206, no. 1, pp. 43–50, 2010.

[181] S. R. Wiley and J. A. Winkles, "TWEAK, a member of the TNF superfamily, is a multifunctional cytokine that binds the TweakR/Fn14 receptor," *Cytokine and Growth Factor Reviews*, vol. 14, no. 3-4, pp. 241–249, 2003.

[182] J. S. Michaelson, N. Wisniacki, L. C. Burkly, and C. Putterman, "Role of TWEAK in lupus nephritis: a bench-to-bedside review," *Journal of Autoimmunity*, vol. 39, no. 3, pp. 130–142, 2012.

[183] P. Schneider, R. Schwenzer, E. Haas et al., "TWEAK can induce cell death via endogenous TNF and TNF receptor 1," *European Journal of Immunology*, vol. 29, no. 6, pp. 1785–1792, 1999.

[184] A. Wicovsky, S. Salzmann, C. Roos et al., "TNF-like weak inducer of apoptosis inhibits proinflammatory TNF receptor-1 signaling," *Cell Death and Differentiation*, vol. 16, no. 11, pp. 1445–1459, 2009.

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Review Article

Tumor Necrosis Factor Alpha: A Link between Neuroinflammation and Excitotoxicity

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Tumor necrosis factor alpha (TNF-α) is a proinflammatory cytokine that exerts both homeostatic and pathophysiological roles in the central nervous system. In pathological conditions, microglia release large amounts of TNF-α; this *de novo* production of TNF-α is an important component of the so-called neuroinflammatory response that is associated with several neurological disorders. In addition, TNF-α can potentiate glutamate-mediated cytotoxicity by two complementary mechanisms: indirectly, by inhibiting glutamate transport on astrocytes, and directly, by rapidly triggering the surface expression of Ca^{+2} permeable-AMPA receptors and NMDA receptors, while decreasing inhibitory $GABA_A$ receptors on neurons. Thus, the net effect of TNF-α is to alter the balance of excitation and inhibition resulting in a higher synaptic excitatory/inhibitory ratio. This review summarizes the current knowledge of the cellular and molecular mechanisms by which TNF-α links the neuroinflammatory and excitotoxic processes that occur in several neurodegenerative diseases, but with a special emphasis on amyotrophic lateral sclerosis (ALS). As microglial activation and upregulation of TNF-α expression is a common feature of several CNS diseases, as well as chronic opioid exposure and neuropathic pain, modulating TNF-α signaling may represent a valuable target for intervention.

1. Introduction

Tumor necrosis factor alpha (TNF-α) was originally identified as a factor that leads to rapid necrosis of transplantable tumors in mice [1] and now it is considered a proinflammatory cytokine involved in the innate immune response [2]. In the central nervous system (CNS) TNF-α exerts both homeostatic and pathophysiological roles [3, 4]. In the healthy CNS TNF-α has regulatory functions on crucial physiological processes such as synaptic plasticity [5, 6], learning and memory [7, 8], sleep [9], food and water intake [10], and astrocyte-induced synaptic strengthening [11]. In pathological conditions, astrocytes and mainly microglia release large amounts of TNF-α; this *de novo* production of this cytokine is an important component of the so-called neuroinflammatory response that is associated with several neurological disorders [3, 12–14]. In addition, TNF-α can potentiate glutamate-mediated cytotoxicity by two complementary mechanisms: indirectly, by inhibiting glutamate transport on astrocytes, and directly, by increasing the localization of ionotropic glutamate receptors to synapses [15]. Neuroinflammation and excitotoxicity have key roles as triggers and sustainers of the neurodegenerative process and thus, elevated levels of TNF-α have been found in traumatic brain injury [16], ischemia [17, 18], Alzheimer's disease (AD) [19, 20], Parkinson's disease (PD) [21, 22], multiple sclerosis (MS) [23, 24], and amyotrophic lateral sclerosis (ALS) [25, 26]. This review summarizes the current knowledge of the cellular and molecular mechanisms by which TNF-α potentiates excitotoxicity and describes its key role in linking the neuroinflammatory and excitotoxic processes that take place not only in ALS but also in other common neurodegenerative diseases.

2. TNF-α Signaling

TNF-α is first synthesized as a transmembrane protein (tmTNF-α). The cleavage of the extracellular domain of

tmTNF-α by the matrix metalloprotease TNF-α-converting enzyme (TACE) releases a soluble TNF-α (sTNF-α) homotrimer. Remarkably, both tmTNF-α and sTNF-α are biologically active and their signal transduction involves binding to two distinct surface receptors, TNF-α receptor 1 (TNFR1 or p55TNFR) and TNF-α receptor 2 (TNFR2 or p75TNFR), which are different in their expression pattern, downstream signal-transduction cascades, and binding affinity for TNF-α [27–29]. The cytoplasmic tail of TNFR1 contains a death domain; however, this motif is missing in TNFR2. Although initially it was considered that TNFR1 activation was involved in the cytotoxic and apoptotic effects of TNF-α, and those related to cell survival and proliferation involved TNFR2 activation, now it is becoming clear that TNFR2 can also induce cell death [30]. Binding of the TNF-α trimer to the extracellular domain of TNFR1 induces receptor trimerization and recruitment of the adaptor protein TNF receptor-associated death domain (TRADD), which in turn recruits additional adaptor proteins: receptor-interacting protein (RIP), TNF receptor-associated factor 2 (TRAF2), and Fas-associated death domain (FADD). This latter protein mediates recruitment and activation of caspases 8 and 10 that initiate a protease cascade that leads to apoptosis [31]. TNFR1 signaling also results in the activation of the following signal transduction pathways: the nuclear factor-kappa B (NF-κB), the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK), the p38 mitogen-activated protein kinase (p38 MAPK), the acidic sphingomyelinase (A-SMase), and the neutral sphingomyelinase (N-SMase) pathways. These pathways regulate the expression of several genes, and some of them, particularly those regulated by the NF-κB pathway, have antiapoptotic effects. With the exception of the SMase pathways, the above signal transduction pathways can also be induced through TNFR2 signaling because TRAF2 (along with TRAF1) can directly associate to the intracellular domain of the TNFR2 receptor (reviewed by [32, 33]).

3. TNF-α and Neuroinflammation

Neuroinflammation in the CNS refers to the collective response of microglia, and to a lesser extent of astrocytes and oligodendrocytes, against diverse insults (invading pathogens, trauma, aggregated or modified proteins, stroke, etc.) designed to remove or inactivate the noxious agents and to inhibit and reverse their detrimental effects. The glial response can be considered as an innate immune mechanism, whereas the participation in the neuroinflammatory process of lymphocytes (mainly T cells) carrying binding sites for specific antigens is an acquired immune mechanism [14]. In neurodegenerative diseases, both innate and acquired immune mechanisms are unable to resolve the triggers, creating a self-sustaining environment where the neuroinflammation persists, thus leading to a chronic neuroinflammation.

Although astrocytes and neurons are able to produce TNF-α [34–36], it is assumed that microglia are the major source of this cytokine during neuroinflammation [37, 38]. The cytokine interferon gamma (IFN-γ) is a potent inducer of TNF-α gene expression in microglia [38–40], and also it upregulates the expression of adhesion/costimulatory molecules, like the major histocompatibility complex (MHC) class II molecules to sustain antigen-dependent T-cell activation [41, 42]. The different inflammatory stimuli that activate microglia during neuroinflammation trigger different signaling pathways including p38 MAPK, JNK, NF-κB, and ERK1/2 [43–46], making it difficult to determine which of them is in fact implicated in the induction of TNF-α expression. In our laboratory, we demonstrated that the sole inhibition of the mitogen-activated protein kinase and ERK kinase (MEK)/ERK signaling pathway with U0126 or apigenin was enough to inhibit the LPS or the IFN-γ-stimulated TNF-α expression in the BV-2 microglial cell line [47]. Similar results had been previously published in human monocytes [48]. As IFN-γ is produced by T cells but not in significant amounts by any CNS resident cells, including microglia [49, 50], it has been proposed that, in neurodegenerative diseases, activated T cells would infiltrate into the parenchyma of the CNS [51–54] releasing their own inflammatory mediators, including IFN-γ [55, 56]. In this sense, in ALS, lymphocytic infiltrates and antibody deposits were detected in postmortem CNS tissues [57] and, more recently, increased $CD4^+$ and $CD8^+$ T cells were found to invade the brain in postmortem human specimens and in a mouse model of PD [58]. Although the role of the infiltrated T cells in the CNS is controversial, since both $CD4^+$ and $CD8^+$ T cells can have detrimental [59] or protective effects [60–62], it may be proposed that, during the neuroinflammatory process, these infiltrated cells release the cytokine IFN-γ which, via the MEK/ERK signaling pathway, induces in microglia an increased *de novo* TNF-α production and release (Figure 1). However, T cells may not be present in the CNS at early disease stages; for example, in ALS, T cells infiltrate the spinal cord as disease progresses [63]. Thus, microglia can be activated to release TNF-α at early asymptomatic disease stages by sensing the earliest neuronal stress and later, infiltrated T cells releasing IFN-γ would keep microglia in an active state [64, 65].

These findings indicate that IFN-γ and TNF-α have key roles in the glial-T-cell dialogue that occurs during neuroinflammation, as they are involved in T-cell adhesion to endothelial cells, extravasation, and T-cell and glial activation [54]. In this regard, we have demonstrated that IFN-γ and TNF-α have complementary roles in inducible microglial nitric oxide generation [47] and that both cytokines, through the induction of the expression of several prooxidative enzymes, cooperatively induce oxidative stress and motoneuron death [66].

4. TNF-α and Excitotoxicity

Glutamate is the main excitatory neurotransmitter in the mammalian CNS and is involved in many aspects of normal brain function [67]. Excitotoxicity refers to a process of neuronal death caused by excessive or prolonged activation of receptors for this excitatory amino acid [68]. A role for excitotoxicity in the aetiology or progression of many human acute or chronic neurodegenerative diseases, including ischemia, AD, PD, MS, and ALS has been proposed (reviewed by [69]).

The first reports demonstrating that TNF-α was able to potentiate excitotoxicity were performed in human neuronal cultures. Gelbard et al. demonstrated that subtoxic doses of both TNF-α and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) were neurotoxic when combined [70]. Similar results were published one year later showing that TNF-α potentiated glutamate neurotoxicity and that this effect could be blocked by competitive (2-APV) and noncompetitive (MK-801) NMDA receptor antagonists [71]. Later, and by working on rat spinal cord, it was demonstrated that nanoinjections of nontoxic doses of either TNF-α or kainate (KA) alone into the thoracic gray matter resulted in almost no tissue damage; however, the combination of these substances at the same doses produced a large area of tissue necrosis and neuronal cell death, an effect that could be reverted by the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline 2,3-(1*H*,4*H*)-dione (CNQX) [72].

5. Potentiation of Excitotoxicity by TNF-α: Role of Glial Cells

After the above previous works, the role of glial cells in TNF-α induced neuronal death was investigated. In mouse primary microglia it was demonstrated that TNF-α, through the TNFR1 pathway, induces excitotoxicity by promoting microglial glutamate release from hemichannels of gap junctions in an autocrine manner [73]. Moreover, in rat primary microglia it was found that agonist stimulation of group 2 metabotropic glutamate receptors (mGluR2) induced TNF-α release, and when this microglial-conditioned medium was added to cerebellar granule neurons in culture resulted in caspase-3 activation and apoptotic cell death. The authors also identified microglial-released Fas ligand as an essential cofactor for the TNF-α-induced neurotoxicity [74]. Similar results were obtained on hippocampal neurons where TNF-α derived from KA-activated microglia also resulted in apoptotic neuronal cell death [75]. Thus, two potential microglial autocrine loops participating in excitotoxicity can be identified: first, TNF-α promotes further microglial TNF-α production and release through TNFR1 signaling [76] and second, TNF-α induces glutamate release that acts on microglial mGluR2 to induce more TNF-α production (Figure 1).

In astroglia, the interaction of TNF-α with TNFR1 initiates a sequence of intracellular signaling events that leads to generation of prostaglandin E_2 that, in turn, activates the elevation of intracellular calcium followed by glutamate exocytosis [77, 78]. The excessive glutamate concentrations resulting from TNF-α stimulation of astroglial and microglial TNFR1 could be diminished by the glutamate uptake system [79, 80]; however, TNF-α has a detrimental effect on astroglial glutamate uptake (reviewed by [81]) (Figure 1). At least five sodium-dependent glutamate transporters have been cloned. The transporters (human/rat) EAAT1/GLAST and EAAT2/GLT-1 are predominantly located on astrocytes and GLT-1 is the most abundant glutamate transporter in the adult brain [82, 83]. In human H4 astroglioma cells and rat astrocytes, it has been shown that exposure for four to six hours to TNF-α (10 or 20 ng/mL) inhibits glutamate uptake by inducing a downregulation of EAAT2/GLT-1 mRNA [84, 85]. In H4 astroglioma cells, downregulation of EAAT2 was dependent on the TNF-α induced binding of NF-κB to the EAAT2 promoter [84]. The role of NF-κB in regulating GLT-1 expression was further confirmed in our laboratory. We used spinal cord organotypic cultures to create a model of chronic glutamate excitotoxicity in which glutamate transporters were inhibited by threohydroxyaspartate (THA) to induce motoneuron death. Exposure to THA induced microglial activation and TNF-α release. In the presence of exogenous TNF-α (20 ng/mL), THA-induced excitotoxic motoneuron death was potentiated. Coexposure to TNF-α and THA also resulted in downregulation of GLT-1 and in increased extracellular glutamate levels. The downregulation of GLT-1, as well as the excitotoxic motoneuron death, could be prevented by NF-κB inhibition [86].

When TNF-α (20 ng/mL) was applied for a short time period (30 minutes) in hippocampal-entorhinal complex slice cultures, it reduced glutamate transport without affecting GLT-1 or GLAST expression [87]. The mechanisms of this rapid, and apparently, nongenomic effect of TNF-α are not clear. On the one hand, TNF-α is a clear inducer of oxidative stress in the CNS [66, 88, 89], and evidences indicate that glutamate transporters are vulnerable to the action of reactive oxygen and nitrogen species that inhibit glutamate uptake within minutes [90, 91], thus providing a link between oxidative stress and excitotoxicity. In addition, reactive oxygen species generated within neurons in response to an excitotoxic insult can pass across the plasma membrane and disrupt glutamate transport in neighboring astrocytes [92]. On the other hand, TNF-α, as explained before, can activate caspases, including caspase-3, which can also be activated by oxidative stress [93]. In this sense, caspase-3 mediated cleavage of GLT-1 results in the inhibition of its activity [94].

It is interesting to note that, in those neurological disorders in which neuroinflammation and increased levels of TNF-α have been described (see Section 1), it has also been reported a reduced expression of GLT-1, GLAST, or both (reviewed by [81]). As an example, in ALS, where neuroinflammation and excitotoxicity are fundamental mechanisms involved in motoneuron degeneration [65, 95], decreased GLT-1 expression has been reported both in patients [96] and rat models [97, 98]. Moreover, the intrathecal injection of cerebrospinal fluid from ALS patients in the rat spinal cord or the *in vitro* exposure to this fluid of mixed spinal cord cultures also resulted in a decrease of GLT-1 expression [99].

6. Potentiation of Excitotoxicity by TNF-α: Modulation of Glutamate and $GABA_A$ Receptors

AMPA-type glutamate receptors (AMPARs) are ligand-gated channels that mediate fast excitatory synaptic transmission in the vertebrate CNS. These receptors are tetramers assembled from glutamate receptor (GluR) 1, 2, 3, and 4 (or GluR-A, -B, -C, and -D) subunits around an aqueous pore

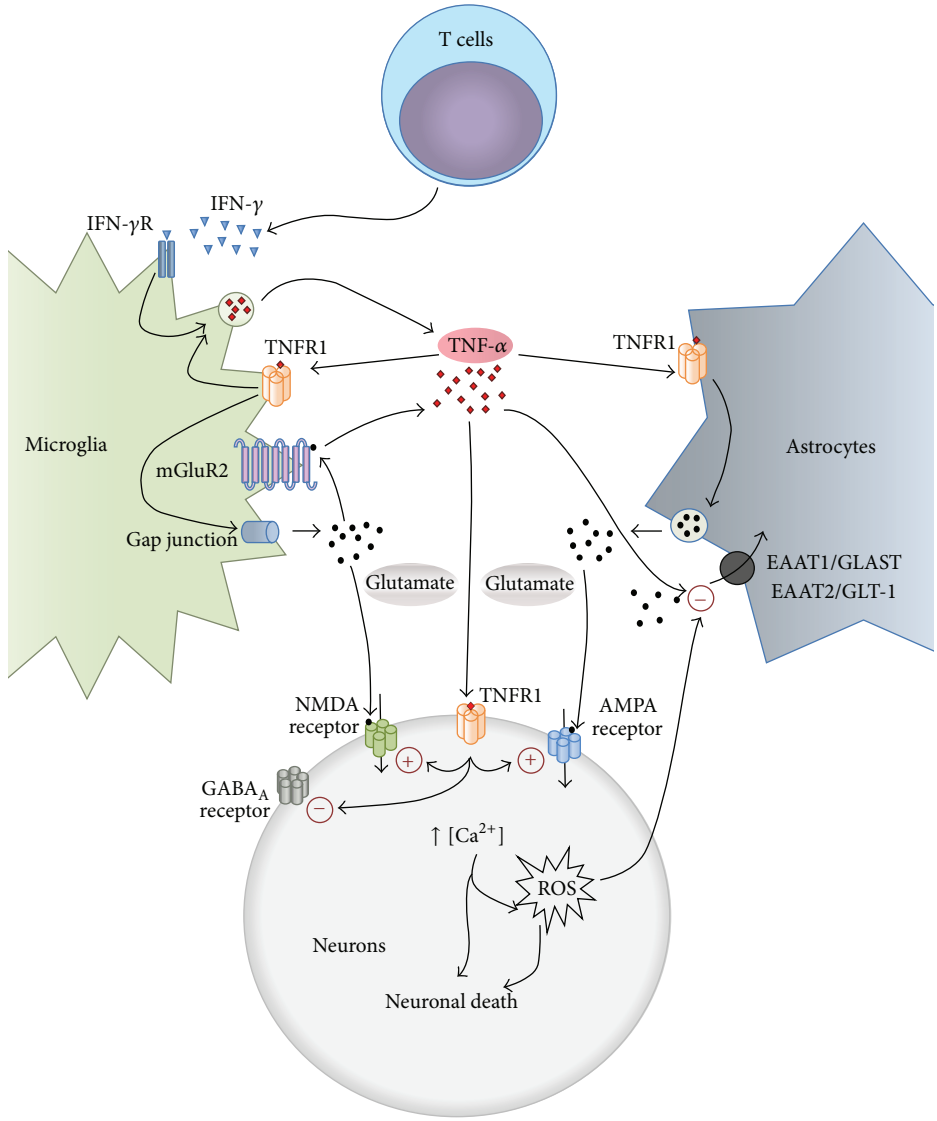

Figure 1: Proposed mechanisms by which TNF-α links the neuroinflammatory and the excitotoxic processes. The cytokine IFN-γ, released by infiltrated T cells, activates TNF-α production and release in microglia. TNF-α, through TNFR1 signaling, promotes further microglial TNF-α release and also induces glutamate release from hemichannels of gap junctions. In astrocytes, TNF-α stimulates TNFR1 to induce glutamate exocytosis and also inhibits glutamate uptake, thus increasing extracellular glutamate levels. In neurons TNF-α, via TNFR1, rapidly increases the excitatory synaptic strength by inducing increased Ca^{2+} permeable-AMPA receptors and/or NMDA receptors and also decreases the surface expression of inhibitory $GABA_A$ receptors. The excessive Ca^{2+} input to neurons induces neuronal death and generates excessive ROS that disrupt glutamate transport in neighboring astrocytes. The dying neurons maintain microglia in an active state, releasing TNF-α.

in the membrane [100, 101]. The trafficking of AMPARs with different subunit composition was initially described in hippocampal CA1 pyramidal cells [102], and now it is generally accepted that such trafficking is critical for the modulation of synaptic strength during learning and memory. Thus, AMPARs trafficking has been implicated in homeostatic synaptic scaling and other forms of long-term synaptic strength adjustments [4, 103, 104]. The GluR2 subunit has a key role in determining the permeability to Ca^{2+} of AMPARs. GluR2 in combination with other GluR subunits forms channels that are Ca^{2+}-impermeable [105, 106]. In this regard, in 1997 it was proposed the "GluR2 hypothesis" suggesting that the selective vulnerability of specific neuron populations, described in some neurological disorders, was due to a reduction in the AMPARs expressing the GluR2 subunits, resulting in increased density of Ca^{2+}-permeable AMPARs. The consequent increase in Ca^{2+} influx through these channels would result in a loss of Ca^{2+} homeostasis, thus contributing to the delayed neurodegeneration seen in those conditions [107]; see also [108].

TNF-α has an important role in the regulation of AMPARs trafficking being a critical component of the homeostatic regulatory system controlling synaptic plasticity [4]. In 2002, Yu et al. demonstrated that human NT2-N

neurons exposed to TNF-α increased their expression of the GluR1 subunit, resulting in an increased susceptibility to KA-induced necrosis. The effect of TNF-α implicated both the A-SMase and the NF-κB signaling pathways [109]. Similar results were obtained in hippocampal neurons where TNF-α, within 15 min, increased the surface expression of GluR1-containing AMPARs, and these changes were accompanied by dramatic changes in AMPAR-mediated excitatory postsynaptic currents [5]. Later, it was demonstrated a dual role for TNF-α on AMPA-induced excitotoxicity. In mouse hippocampal slice cultures it was reported that pretreatment (24 h) of cultures with 10 ng/mL TNF-α potentiated AMPA-induced neuronal death; however, decreasing the concentration of TNF-α to 1 ng/mL resulted in neuroprotection. The authors demonstrated that the "high-dose" toxic effect was mediated by TNFR1 whereas the "low-dose" protective effect implied the TNFR2 [110]. The role of TNFR1 in the potentiation by TNF-α of AMPA toxicity was further confirmed in a work performed also in hippocampal cells and demonstrating that, by activating neuronal TNFR1, TNF-α increased the surface AMPARs, but remarkably, TNF-α preferentially increased the synaptic expression of GluR2-lacking (Ca^{2+} permeable) AMPARs. This effect was mediated through a phosphatidylinositol 3-kinase- (PI3-K-) dependent process [111]. The role of PI3-K in the potentiation by TNF-α of KA-induced neuronal death was confirmed later by the same group; they demonstrated that the specific PI3-K inhibitor LY294002 reverted the TNF-α effect on hippocampal neurons. Moreover, and in agreement with the "GluR2 hypothesis," the potentiating effect was also reverted by the synthetic joro spider toxin analog NASPM, which selectively blocks Ca^{2+} permeable-AMPARs [112]. These results were also confirmed by others showing that TNF-α triggers a rapid induction of Ca^{2+} permeable-AMPARs in hippocampal pyramidal neurons; the effect was rapid (15 min) and since TNF-α exposure did not alter mRNA levels for either GluR1 or GluR2 subunit, it was proposed that TNF-α acts at posttranscriptional level to induce rapid increases in surface subunit expression [113].

The pathological relevance of the above findings was first described in a model of cervical spinal cord contusion injury. In this model, increased synaptic AMPAR numbers were found at synapses ipsilateral to the lesion at 90 min and 3 h after injury. Interestingly, *in vivo* nanoinjections of TNF-α into the ventral horns resulted in increased GluR1 and decreased GluR2 at both extrasynaptic and synaptic plasma membrane sites. The effect was seen in the neuropil 60 min after TNF-α nanoinjection and could also be detected in the somata of large spinal motoneurons [114]. In a subsequent study, using whole cell recording from lumbar motoneurons, it was demonstrated that both AMPA and NMDA receptor-mediated excitatory postsynaptic currents were rapidly increased following bath application of TNF-α [115]. Together, these results suggested that TNF-α induced GluR2-lacking AMPARs trafficking to the membrane is likely to contribute to postinjury excitotoxicity in spinal cord motoneurons. However, another study has reported TNF-α to reduce AMPAR-mediated calcium entry in cultured motoneurons by increasing cell surface expression of the GluR2 subunit [116].

Adult spinal cord motoneurons possess significant numbers of Ca^{2+} permeable-AMPARs under basal conditions, and it has been proposed that this circumstance would render them more susceptible to neurodegeneration in ALS [117–120]. Activation of both microglia and astrocytes occurs prominently in both human disease and animal models of ALS [121, 122]; these activated cells may contribute to motoneuron injury by releasing TNF-α [89, 123]. In this sense, TNF-α has been shown to potentiate AMPAR-mediated excitotoxicity on lumbar spinal cord motoneurons both by decreasing GLT-1 expression [86], and also by inducing a rapid membrane insertion of Ca^{2+} permeable-AMPARs via a PI3-K and protein kinase A- (PKA-) dependent mechanism [124]. Interestingly, vascular endothelial growth factor (VEGF) has neuroprotective effects on ALS (reviewed by [125]), and it has been shown, both *in vitro* and *in vivo*, that VEGF increases the expression of GluR2 subunit of AMPARs of spinal cord motoneurons, thus minimizing their vulnerability to AMPA-mediated excitotoxicity [126].

The effects of TNF-α on N-methyl-D-aspartate receptors (NMDARs) trafficking are less studied; however, the results obtained are similar to those on AMPARs. Thus, in hippocampal neurons TNF-α induced a rapid increase in the surface expression of the NR1 subunit of NMDARs and also, via N-SMase2, promoted a specific clustering of phosphorylated NR1 subunits into lipid rafts [127]. Similarly to that described above for motoneurons, TNF-α has also been shown to potentiate NMDAR-mediated excitotoxicity in cortical neurons [128].

TNF-α also regulates inhibitory synapse function. An *in vivo* study in the rat spinal cord indicated that TNF-α increased within 60 min synaptic and total gamma-aminobutyric acid A receptors ($GABA_A$Rs) in the neuropil and in the plasma membrane of motoneurons. However, the effect of TNF-α on $GABA_A$R trafficking was complex, displaying a nonlinear dose-dependent relationship [129]. The authors suggests that under certain physiological conditions GABAergic synapses can be excitatory and that excitatory effects of $GABA_A$Rs have been implicated in maladaptive spinal plasticity in a model of instrumental learning [130]. Interestingly, the same group has also reported that TNF-α is necessary and sufficient for generating lasting inhibition of spinal learning and that the effect of this cytokine also involves Ca^{2+} permeable-AMPARs, since it was reverted by a GluR2-lacking AMPA receptor antagonist [131]. More recently, an *in vitro* study in mature rat and mouse hippocampal neurons in culture demonstrated that acute (45 min) application of TNF-α induced a rapid and persistent decrease of inhibitory synaptic strength as well as a downregulation of cell-surface levels of $GABA_A$Rs. The trafficking of these receptors in response to TNF-α was mediated through the activation of neuronally expressed TNFR1 and required the activation of PI3-K, p38 MAPK, protein phosphatase 1, and dynamin GTPase [132].

Together, the findings presented here indicate that TNF-α potentiates excitotoxicity by rapidly increasing excitatory synaptic strength through increased AMPA and NMDA receptors surface expression and also that neurons respond to elevated levels of the cytokine weakening their inhibitory synaptic strength through a decreased presence of $GABA_A$Rs in the plasma membrane. Thus, the net effect of TNF-α is to alter the balance of excitation and inhibition resulting in a higher synaptic excitatory/inhibitory ratio [111] (Figure 1). Interestingly, it has been proposed that an elevation of this ratio is a major cause of autism spectrum disorder [133, 134]; a pathology in that elevated levels of TNF-α in cerebrospinal fluid has been described [135].

7. TNF-α Links Neuroinflammation and Excitotoxicity

It is now widely accepted that most developmental, lesional, and degenerative nervous system disorders involve common interconnected neurotoxic mechanisms. Figure 1 summarizes the proposed mechanisms by which the cytokine TNF-α links the neuroinflammatory response to glutamate-mediated toxicity. The scheme can also be regarded as three interrelated vicious circles. The first is a microglial vicious circle in which TNF-α stimulates its own release. Then, it also stimulates glutamate release that acts on microglial metabotropic glutamate receptors to stimulate more TNF-α release. The second is an astroglial vicious circle in which TNF-α stimulates astrocytes to release glutamate that cannot be efficiently taken up by their glutamate transporters, thus increasing more and more the extracellular glutamate concentrations. The third is a neuronal vicious circle in which TNF-α, by increasing the synaptic excitatory/inhibitory ratio, induces an excessive calcium entry that results in excitotoxic neuronal death; the dying neurons keep microglia in an active state that maintains their increased TNF-α production and release. As TNF-α is released by activated microglia these mechanistic links between neuroinflammation and excitotoxicity can be considered as a crosstalk between microglia and astrocytes (modulating astrocytic glutamate uptake) and microglia and neurons (modulating neuronal glutamate and GABA receptors).

It is noteworthy that the scheme shown in Figure 1 not only accounts for the most common acute or chronic neurodegenerative diseases in which increased levels of TNF-α, associated with neuroinflammation and excitotoxicity, have been reported, but also describes a broader situation in which activated microglia releases significant amounts of TNF-α. This is the case of opioid tolerance and neuropathic pain, two situations that are modulated by TNF-α [136, 137]. Chronic morphine exposure induces microglial activation and a significant increase in TNF-α mRNA expression in the rat spinal cord [138]; this effect is associated with a downregulation of GLT-1 and GLAST glutamate transporters and with an increase in the surface expression of Ca^{2+} permeable-AMPA and NMDA receptors [139]. All the above effects of chronic morphine, and remarkably, the loss of its antinociceptive effect, can be reverted by a TNF-α antagonist [138, 139]. Similarly, in mechanical allodynia, TNF-α mediated increased insertion of Ca^{2+} permeable-AMPARs in spinal cord neurons plays a major role in inflammatory pain and may represent a path by which glia contribute to neuronal sensitization and pathological pain [140].

8. Therapy Targeting TNF-α

As TNF-α is a key mediator in the pathological mechanisms of a large number of neurological disorders including ischemia, AD, PD, MS, and ALS [3] and also in peripheral autoimmune disorders including rheumatoid and juvenile arthritis, ankylosing spondylitis, and Crohn's disease, targeting TNF-α action seems to be an attractive disease-modifying strategy. The different strategies employed for TNF-α inhibition have been reviewed elsewhere [12] and include the use of humanized IgG antibodies (infliximab, adalimumab, and etanercept) that sequestrate sTNF-α and tmTNF-α, the antibiotic minocycline that decreases TNF-α synthesis, the immunomodulatory drug thalidomide and its derivatives that enhance the degradation of TNF-α mRNA [13], and TACE inhibitors that inhibit sTNF-α production. Clinical trials examining the effects of TNF-α inhibition have been conducted on patients with MS, AD, and ALS. Although promising effects were obtained in AD patients with substantial cognitive and behavioral improvements [141, 142], the treatment failed in MS and ALS patients [143, 144]. Moreover, TNF-α gene knockout did not affect life span or the extent of motoneuron loss in the superoxide dismutase 1 (SOD1) transgenic mice model of ALS, thus suggesting that TNF-α alone is not a key factor in motoneuron degeneration [145].

The above findings can be explained first because TNF-α has both neuroprotective and neurotoxic effects related to the different signaling pathways activated by their receptors [146]. In this sense, mice lacking TNF-α receptors were more susceptible to ischemia and excitotoxic injury [147, 148]. Second, because some proinflammatory cytokines (i.e., IL-1β and TNF-α) have redundant functions *in vivo*; thus, in the TNF-α knockout mice an increase in the transcripts encoding for IL-1β was detected [145]; and, third, because TNF-α often works in concert with other cytokines (i.e., IFN-γ and IL-1β) to promote neuronal death [66, 149, 150]. Nevertheless, the identification of novel agents that can restore the normal function of activated glial cells by means of reducing the production of TNF-α and/or its potentiation of excitotoxicity will be essential in the management of chronic and acute neurodegenerative diseases.

9. Conclusion

TNF-α plays a physiological role in controlling synaptic transmission and plasticity in the healthy CNS by modulating ionotropic glutamate receptors trafficking. However, excessive TNF-α levels, as a result of different types of injury, have an inhibitory effect on glutamate transporters, resulting in increased glutamate concentration in the CNS

parenchyma. In this context, even slight increases in TNF-α induced Ca^{2+} permeable-AMPA and/or NMDA receptors trafficking become toxic for neurons. As microglial activation and upregulation of TNF-α expression is a common feature of several CNS diseases, as well as chronic opioid exposure and neuropathic pain, modulating TNF-α signaling may represent a valuable target for intervention.

Abbreviations

A-SMase:	Acidic sphingomyelinase
AD:	Alzheimer's disease
ALS:	Amyotrophic lateral sclerosis
AMPA:	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic
AMPAR:	AMPA-type glutamate receptor
CNS:	Central nervous system
EAAT:	Excitatory amino acid transporter
ERK:	Extracellular signal-regulated kinase
$GABA_AR$:	Gamma-aminobutyric acid A receptor
GLAST:	Glutamate/aspartate transporter
GLT-1:	Glutamate transporter 1
GluR:	Glutamate receptor subunit
IFN-γ:	Interferon gamma
IFN-γR:	Interferon gamma receptor
JNK:	c-Jun N-terminal kinase
KA:	Kainate
MEK:	Mitogen-activated protein kinase and ERK kinase
mGluR2:	Group 2 metabotropic glutamate receptor
MHC:	Major histocompatibility complex
MS:	Multiple sclerosis
N-SMase:	Neutral sphingomyelinase
NF-κB:	Nuclear factor-kappa B
NMDAR:	N-methyl-D-aspartate receptor
p38 MAPK:	p38 mitogen-activated protein kinase
PD:	Parkinson's disease
PI3-K:	Phosphatidylinositol 3-kinase
ROS:	Reactive oxygen species
sTNF-α:	Soluble TNF-α
THA:	Threohydroxyaspartate
tmTNF-α:	Transmembrane TNF-α
TNF-α:	Tumor necrosis factor alpha
TNFR1:	TNF-α receptor 1
TNRF2:	TNF-α receptor 2.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] E. A. Carswell, L. J. Old, R. L. Kassel, S. Green, N. Fiore, and B. Williamson, "An endotoxin induced serum factor that cuases necrosis of tumors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 72, no. 9, pp. 3666–3670, 1975.

[2] I. A. Clark, "How TNF was recognized as a key mechanism of disease," *Cytokine and Growth Factor Reviews*, vol. 18, no. 3-4, pp. 335–343, 2007.

[3] S. L. Montgomery and W. J. Bowers, "Tumor necrosis factor-alpha and the roles it plays in homeostatic and degenerative processes within the central nervous system," *Jounal of Neuroimmune Pharmacology*, vol. 7, no. 1, pp. 42–59, 2012.

[4] M. Santello and A. Volterra, "TNFalpha in synaptic function: switching gears," *Trends in Neurosciences*, vol. 35, no. 10, pp. 638–647, 2012.

[5] E. C. Beattie, D. Stellwagen, W. Morishita et al., "Control of synaptic strength by glial TNFα," *Science*, vol. 295, no. 5563, pp. 2282–2285, 2002.

[6] M. Kaneko, D. Stellwagen, R. C. Malenka, and M. P. Stryker, "Tumor necrosis factor-α mediates one component of competitive, experience-dependent plasticity in developing visual cortex," *Neuron*, vol. 58, no. 5, pp. 673–680, 2008.

[7] B. T. Baune, F. Wiede, A. Braun, J. Golledge, V. Arolt, and H. Koerner, "Cognitive dysfunction in mice deficient for TNF- and its receptors," *American Journal of Medical Genetics B: Neuropsychiatric Genetics*, vol. 147, no. 7, pp. 1056–1064, 2008.

[8] C. Beste, B. T. Baune, M. Falkenstein, and C. Konrad, "Variations in the TNF-α gene (TNF-α -308G $\rightarrow$ A) affect attention and action selection mechanisms in a dissociated fashion," *Journal of Neurophysiology*, vol. 104, no. 5, pp. 2523–2531, 2010.

[9] J. M. Krueger, "The role of cytokines in sleep regulation," *Current Pharmaceutical Design*, vol. 14, no. 32, pp. 3408–3416, 2008.

[10] C. R. Plata-Salamán, "Cytokines and feeding," *International Journal of Obesity*, vol. 25, supplement 5, pp. S48–S52, 2001.

[11] M. Santello, P. Bezzi, and A. Volterra, "TNFα controls glutamatergic gliotransmission in the hippocampal dentate gyrus," *Neuron*, vol. 69, no. 5, pp. 988–1001, 2011.

[12] M. K. McCoy and M. G. Tansey, "TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease," *Journal of Neuroinflammation*, vol. 5, article 45, 2008.

[13] K. A. Frankola, N. H. Greig, W. Luo, and D. Tweedie, "Targeting TNF-alpha to elucidate and ameliorate neuroinflammation in neurodegenerative diseases," *CNS and Neurological Disorders—Drug Targets*, vol. 10, no. 3, pp. 391–403, 2011.

[14] T. Wyss-Coray and L. Mucke, "Inflammation in neurodegenerative disease—a double-edged sword," *Neuron*, vol. 35, no. 3, pp. 419–432, 2002.

[15] M. Pickering, D. Cumiskey, and J. J. O'Connor, "Actions of TNF-α on glutamatergic synaptic transmission in the central nervous system," *Experimental Physiology*, vol. 90, no. 5, pp. 663–670, 2005.

[16] J. C. Goodman, C. S. Robertson, R. G. Grossman, and R. K. Narayan, "Elevation of tumor necrosis factor in head injury," *Journal of Neuroimmunology*, vol. 30, no. 2-3, pp. 213–217, 1990.

[17] T. Liu, R. K. Clark, P. C. McDonnell et al., "Tumor necrosis factor-α expression in ischemic neurons," *Stroke*, vol. 25, no. 7, pp. 1481–1488, 1994.

[18] G. Z. Feuerstein, T. Liu, and F. C. Barone, "Cytokines, inflammation, and brain injury: role of tumor necrosis factor-alpha," *Cerebrovascular and Brain Metabolism Reviews*, vol. 6, no. 4, pp. 341–360, 1994.

[19] H. Fillit, W. Ding, L. Buee et al., "Elevated circulating tumor necrosis factor levels in Alzheimer's disease," *Neuroscience Letters*, vol. 129, no. 2, pp. 318–320, 1991.

[20] A. Álvarez, R. Cacabelos, C. Sanpedro, M. García-Fantini, and M. Aleixandre, "Serum TNF-alpha levels are increased and correlate negatively with free IGF-I in Alzheimer disease," *Neurobiology of Aging*, vol. 28, no. 4, pp. 533–536, 2007.

[21] G. Boka, P. Anglade, D. Wallach, F. Javoy-Agid, Y. Agid, and E. C. Hirsch, "Immunocytochemical analysis of tumor necrosis factor and its receptors in Parkinson's disease," *Neuroscience Letters*, vol. 172, no. 1-2, pp. 151–154, 1994.

[22] M. Mogi, M. Harada, P. Riederer, H. Narabayashi, K. Fujita, and T. Nagatsu, "Tumor necrosis factor-α (TNF-α) increases both in the brain and in the cerebrospinal fluid from parkinsonian patients," *Neuroscience Letters*, vol. 165, no. 1-2, pp. 208–210, 1994.

[23] M. K. Sharief and R. Hentges, "Association between tumor necrosis factor-α and disease progression in patients with multiple sclerosis," *The New England Journal of Medicine*, vol. 325, no. 7, pp. 467–472, 1991.

[24] P. Rieckmann, M. Albrecht, B. Kitze et al., "Tumor necrosis factor-α messenger RNA expression in patients with relapsing-remitting multiple sclerosis is associated with disease activity," *Annals of Neurology*, vol. 37, no. 1, pp. 82–88, 1995.

[25] M. Poloni, D. Facchetti, R. Mai et al., "Circulating levels of tumour necrosis factor-α and its soluble receptors are increased in the blood of patients with amyotrophic lateral sclerosis," *Neuroscience Letters*, vol. 287, no. 3, pp. 211–214, 2000.

[26] G. N. Babu, A. Kumar, R. Chandra, S. K. Puri, J. Kalita, and U. K. Misra, "Elevated inflammatory markers in a group of amyotrophic lateral sclerosis patients from northern India," *Neurochemical Research*, vol. 33, no. 6, pp. 1145–1149, 2008.

[27] H. T. Idriss and J. H. Naismith, "TNF alpha and the TNF receptor superfamily: structure-function relationship(s)," *Microscopy Research and Technique*, vol. 50, no. 3, pp. 184–195, 2000.

[28] D. J. MacEwan, "TNF receptor subtype signalling: differences and cellular consequences," *Cellular Signalling*, vol. 14, no. 6, pp. 477–492, 2002.

[29] L. A. Tartaglia and D. V. Goeddel, "Two TNF receptors," *Immunology Today*, vol. 13, no. 5, pp. 151–153, 1992.

[30] B. Depuydt, G. van Loo, P. Vandenabeele, and W. Declercq, "Induction of apoptosis by TNF receptor 2 in a T-cell hybridoma is FADD dependent and blocked by caspase-8 inhibitors," *Journal of Cell Science*, vol. 118, part 3, pp. 497–504, 2005.

[31] G. Chen and D. V. Goeddel, "TNF-R1 signaling: a beautiful pathway," *Science*, vol. 296, no. 5573, pp. 1634–1635, 2002.

[32] Z.-G. Liu and J. Han, "Cellular responses to tumor necrosis factor," *Current Issues in Molecular Biology*, vol. 3, no. 4, pp. 79–90, 2001.

[33] H. Wajant, K. Pfizenmaier, and P. Scheurich, "Tumor necrosis factor signaling," *Cell Death and Differentiation*, vol. 10, no. 1, pp. 45–65, 2003.

[34] I. Y. Chung and E. N. Benveniste, "Tumor necrosis factor-α production by astrocytes. Induction by lipopolysaccharide, IFN-γ, and IL-1β," *Journal of Immunology*, vol. 144, no. 8, pp. 2999–3007, 1990.

[35] L. C. Gahring, N. G. Carlson, R. A. Kulmar, and S. W. Rogers, "Neuronal expression of tumor necrosis factor alpha in the murine brain," *Neuroimmunomodulation*, vol. 3, no. 5, pp. 289–303, 1996.

[36] C. D. Breder, M. Tsujimoto, Y. Terano, D. W. Scott, and C. B. Saper, "Distribution and characterization of tumor necrosis factor-α-like immunoreactivity in the murine central nervous system," *Journal of Comparative Neurology*, vol. 337, no. 4, pp. 543–567, 1993.

[37] J. V. Welser-Alves and R. Milner, "Microglia are the major source of TNF-alpha and TGF-beta1 in postnatal glial cultures, regulation by cytokines, lipopolysaccharide, and vitronectin," *Neurochemistry International*, vol. 63, no. 1, pp. 47–53, 2013.

[38] U.-K. Hanisch, "Microglia as a source and target of cytokines," *Glia*, vol. 40, no. 2, pp. 140–155, 2002.

[39] V. T. Nguyen and E. N. Benveniste, "Critical role of tumor necrosis factor-α and NF-κB in interferon-γ-induced CD40 expression in microglia/macrophages," *The Journal of Biological Chemistry*, vol. 277, no. 16, pp. 13796–13803, 2002.

[40] E. N. Mangano, D. Litteljohn, R. So et al., "Interferon-gamma plays a role in paraquat-induced neurodegeneration involving oxidative and proinflammatory pathways," *Neurobiology of Aging*, vol. 33, no. 7, pp. 1411–1426, 2012.

[41] K. Frei, C. Siepl, P. Groscurth, S. Bodmer, C. Schwerdel, and A. Fontana, "Antigen presentation and tumor cytotoxicity by interferon-γ-treated microglial cells," *European Journal of Immunology*, vol. 17, no. 9, pp. 1271–1278, 1987.

[42] A. S. Harms, S. Cao, A. L. Rowse et al., "MHCII is required for alpha-synuclein-induced activation of microglia, CD4 T cell proliferation, and dopaminergic neurodegeneration," *The Journal of Neuroscience*, vol. 33, no. 23, pp. 9592–9600, 2013.

[43] K. Byun, J. Young Kim, E. Bayarsaikhan et al., "Quantitative proteomic analysis reveals that lipopolysaccharide induces mitogen-activated protein kinase-dependent activation in human microglial cells," *Electrophoresis*, vol. 33, no. 24, pp. 3756–3763, 2012.

[44] B. W. Kim, S. Koppula, S. S. Hong et al., "Regulation of microglia activity by glaucocalyxin-A: attenuation of lipopolysaccharide-stimulated neuroinflammation through NF-kappaB and p38 MAPK signaling pathways," *PLoS ONE*, vol. 8, no. 2, Article ID e55792, 2013.

[45] R. Pangestuti, S.-S. Bak, and S.-K. Kim, "Attenuation of pro-inflammatory mediators in LPS-stimulated BV2 microglia by chitooligosaccharides via the MAPK signaling pathway," *International Journal of Biological Macromolecules*, vol. 49, no. 4, pp. 599–606, 2011.

[46] S. Zhao, L. Zhang, G. Lian et al., "Sildenafil attenuates LPS-induced pro-inflammatory responses through down-regulation of intracellular ROS-related MAPK/NF-κB signaling pathways in N9 microglia," *International Immunopharmacology*, vol. 11, no. 4, pp. 468–474, 2011.

[47] M. Mir, L. Tolosa, V. J. Asensio, J. Lladó, and G. Olmos, "Complementary roles of tumor necrosis factor alpha and interferon gamma in inducible microglial nitric oxide generation," *Journal of Neuroimmunology*, vol. 204, no. 1-2, pp. 101–109, 2008.

[48] M. Guha, M. A. O'Connell, R. Pawlinski et al., "Lipopolysaccharide activation of the MEK-ERK1/2 pathway in human monocytic cells mediates tissue factor and tumor necrosis factor α expression by inducing Elk-1 phosphorylation and Egr-1 expression," *Blood*, vol. 98, no. 5, pp. 1429–1439, 2001.

[49] H. A. Young and K. J. Hardy, "Role of interferon-γ in immune cell regulation," *Journal of Leukocyte Biology*, vol. 58, no. 4, pp. 373–381, 1995.

[50] F. Aloisi, B. Serafini, and L. Adorini, "Glia-T cell dialogue," *Journal of Neuroimmunology*, vol. 107, no. 2, pp. 111–117, 2000.

[51] W. F. Hickey, B. L. Hsu, and H. Kimura, "T-lymphocyte entry into the central nervous system," *Journal of Neuroscience Research*, vol. 28, no. 2, pp. 254–260, 1991.

[52] A. S. Archambault, J. Sim, M. A. T. Gimenez, and J. H. Russell, "Defining antigen-dependent stages of T cell migration from the blood to the central nervous system parenchyma," *European Journal of Immunology*, vol. 35, no. 4, pp. 1076–1085, 2005.

[53] R. M. Ransohoff and B. Engelhardt, "The anatomical and cellular basis of immune surveillance in the central nervous system," *Nature Reviews Immunolology*, vol. 12, no. 9, pp. 623–635, 2012.

[54] J. E. Merrill and E. N. Benveniste, "Cytokines in inflammatory brain lesions: helpful and harmful," *Trends in Neurosciences*, vol. 19, no. 8, pp. 331–338, 1996.

[55] H. Wekerle, D. Sun, R. L. Oropeza-Wekerle, and R. Meyermann, "Immune reactivity in the nervous system: modulation of T-lymphocyte activation by glial cells," *Journal of Experimental Biology*, vol. 132, pp. 43–57, 1987.

[56] S. H. Appel, "$CD4^+$ T cells mediate cytotoxicity in neurodegenerative diseases," *The Journal of Clinical Investigation*, vol. 119, no. 1, pp. 13–15, 2009.

[57] D. Troost, J. J. van den Oord, J. M. B. V. de Jong, and D. F. Swaab, "Lymphocytic infiltration in the spinal cord of patients with amyotrophic lateral sclerosis," *Clinical Neuropathology*, vol. 8, no. 6, pp. 289–294, 1989.

[58] V. Brochard, B. Combadière, A. Prigent et al., "Infiltration of $CD4^+$ lymphocytes into the brain contributes to neurodegeneration in a mouse model of Parkinson disease," *The Journal of Clinical Investigation*, vol. 119, no. 1, pp. 182–192, 2009.

[59] I. Medana, M. A. Martinic, H. Wekerle, and H. Neumann, "Transection of major histocompatibility complex class I-induced neurites by cytotoxic T lymphocytes," *The American Journal of Pathology*, vol. 159, no. 3, pp. 809–815, 2001.

[60] R. Hohlfeld, M. Kerschensteiner, C. Stadelmann, H. Lassmann, and H. Wekerle, "The neuroprotective effect of inflammation: implications for the therapy of multiple sclerosis," *Journal of Neuroimmunology*, vol. 107, no. 2, pp. 161–166, 2000.

[61] M. Schwartz, I. Cohen, O. Lazarov-Spiegler, G. Moalem, and E. Yoles, "The remedy may lie in ourselves: prospects for immune cell therapy in central nervous system protection and repair," *Journal of Molecular Medicine*, vol. 77, no. 10, pp. 713–717, 1999.

[62] D. R. Beers, J. S. Henkel, W. Zhao, J. Wang, and S. H. Appel, "$CD4^+$ T cells support glial neuroprotection, slow disease progression, and modify glial morphology in an animal model of inherited ALS," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 40, pp. 15558–15563, 2008.

[63] I. M. Chiu, A. Chen, Y. Zheng et al., "T lymphocytes potentiate endogenous neuroprotective inflammation in a mouse model of ALS," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 46, pp. 17913–17918, 2008.

[64] B. Liu and J.-S. Hong, "Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention," *Journal of Pharmacology and Experimental Therapeutics*, vol. 304, no. 1, pp. 1–7, 2003.

[65] T. Philips and W. Robberecht, "Neuroinflammation in amyotrophic lateral sclerosis: role of glial activation in motor neuron disease," *The Lancet Neurology*, vol. 10, no. 3, pp. 253–263, 2011.

[66] M. Mir, V. J. Asensio, L. Tolosa et al., "Tumor necrosis factor alpha and interferon gamma cooperatively induce oxidative stress and motoneuron death in rat spinal cord embryonic explants," *Neuroscience*, vol. 162, no. 4, pp. 959–971, 2009.

[67] J. C. Watkins and R. H. Evans, "Excitatory amino acid transmitters," *Annual Review of Pharmacology and Toxicology*, vol. 21, pp. 165–204, 1981.

[68] J. W. Olney, "Neurotoxicity of excitatory amino acids," in *Kainic Acid as a Tool in Neurobiology*, E. G. McGeer, J. W. Olney, and P. L. McGeer, Eds., pp. 95–112, Raven Press, New York, NY, USA, 1978.

[69] A. Doble, "The role of excitotoxicity in neurodegenerative disease: implications for therapy," *Pharmacology and Therapeutics*, vol. 81, no. 3, pp. 163–221, 1999.

[70] H. A. Gelbard, K. A. Dzenko, D. DiLoreto, C. del Cerro, M. del Cerro, and L. G. Epstein, "Neurotoxic effects of tumor necrosis factor alpha in primary human neuronal cultures are mediated by activation of the glutamate AMPA receptor subtype: implications for AIDS neuropathogenesis," *Developmental Neuroscience*, vol. 15, no. 6, pp. 417–422, 1993.

[71] C. C. Chao and S. Hu, "Tumor necrosis factor-alpha potentiates glutamate neurotoxicity in human fetal brain cell cultures," *Developmental Neuroscience*, vol. 16, no. 3-4, pp. 172–179, 1994.

[72] G. E. Hermann, R. C. Rogers, J. C. Bresnahan, and M. S. Beattie, "Tumor necrosis factor-α induces cFOS and strongly potentiates glutamate-mediated cell death in the rat spinal cord," *Neurobiology of Disease*, vol. 8, no. 4, pp. 590–599, 2001.

[73] H. Takeuchi, S. Jin, J. Wang et al., "Tumor necrosis factor-α induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner," *The Journal of Biological Chemistry*, vol. 281, no. 30, pp. 21362–21368, 2006.

[74] D. L. Taylor, F. Jones, E. S. F. Chen Seho Kubota, and J. M. Pocock, "Stimulation of microglial metabotropic glutamate receptor mGlu2 triggers tumor necrosis factor α-induced neurotoxicity in concert with microglial-derived Fas ligand," *The Journal of Neuroscience*, vol. 25, no. 11, pp. 2952–2964, 2005.

[75] W. Zhu, H. Zheng, X. Shao, W. Wang, Q. Yao, and Z. Li, "Excitotoxicity of TNFα derived from KA activated microglia on hippocampal neurons in vitro and in vivo," *Journal of Neurochemistry*, vol. 114, no. 2, pp. 386–396, 2010.

[76] R. Kuno, J. Wang, J. Kawanokuchi, H. Takeuchi, T. Mizuno, and A. Suzumura, "Autocrine activation of microglia by tumor necrosis factor-α," *Journal of Neuroimmunology*, vol. 162, no. 1-2, pp. 89–96, 2005.

[77] P. Bezzi, M. Domercq, L. Brambilla et al., "CXCR4-activated astrocyte glutamate release via TNFa: amplification by microglia triggers neurotoxicity," *Nature Neuroscience*, vol. 4, no. 7, pp. 702–710, 2001.

[78] P. Bezzi, G. Carmignoto, L. Pasti et al., "Prostaglandins stimulate calcium-dependent glutamate release in astrocytes," *Nature*, vol. 391, no. 6664, pp. 281–285, 1998.

[79] G. Gegelashvili and A. Schousboe, "High affinity glutamate transporters: regulation of expression and activity," *Molecular Pharmacology*, vol. 52, no. 1, pp. 6–15, 1997.

[80] Y. Zhou and N. C. Danbolt, "GABA and glutamate transporters in brain," *Frontiers in Endocrinology*, vol. 4, Article ID 165, 2013.

[81] S. Tilleux and E. Hermans, "Neuroinflammation and regulation of glial glutamate uptake in neurological disorders," *Journal of Neuroscience Research*, vol. 85, no. 10, pp. 2059–2070, 2007.

[82] N. C. Danbolt, "Purification and reconstitution of the sodium- and potassium-coupled glutamate transport glycoprotein from rat brain," *Biochemistry*, vol. 29, no. 28, pp. 6734–6740, 1990.

[83] N. C. Danbolt, "Glutamate uptake," *Progress in Neurobiology*, vol. 65, no. 1, pp. 1–105, 2001.

[84] R. Sitcheran, P. Gupta, P. B. Fisher, and A. S. Baldwin, "Positive and negative regulation of EAAT2 by NF-κB: a role for N-myc in TNFα-controlled repression," *The EMBO Journal*, vol. 24, no. 3, pp. 510–520, 2005.

[85] Z. Wang, O. Pekarskaya, M. Bencheikh et al., "Reduced expression of glutamate transporter EAAT2 and impaired glutamate transport in human primary astrocytes exposed to HIV-1 or gp120," *Virology*, vol. 312, no. 1, pp. 60–73, 2003.

[86] L. Tolosa, V. Caraballo-Miralles, G. Olmos, and J. Lladó, "TNF-α potentiates glutamate-induced spinal cord motoneuron death via NF-κB," *Molecular and Cellular Neuroscience*, vol. 46, no. 1, pp. 176–186, 2011.

[87] J. Y. Zou and F. T. Crews, "TNFα potentiates glutamate neurotoxicity by inhibiting glutamate uptake in organotypic brain slice cultures: neuroprotection by NFκB inhibition," *Brain Research*, vol. 1034, no. 1-2, pp. 11–24, 2005.

[88] K. Hensley, M. Mhatre, S. Mou et al., "On the relation of oxidative stress to neuroinflammation: lessons learned from the G93A-SOD1 mouse model of amyotrophic lateral sclerosis," *Antioxidants & Redox Signaling*, vol. 8, no. 11-12, pp. 2075–2087, 2006.

[89] M. Mhatrea, R. A. Floyd, and K. Hensley, "Oxidative stress and neuroinflammation in Alzheimer's disease and amyotrophic lateral sclerosis: common links and potential therapeutic targets," *Journal of Alzheimer's Disease*, vol. 6, no. 2, pp. 147–157, 2004.

[90] D. Trotti, N. C. Danbolt, and A. Volterra, "Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration?" *Trends in Pharmacological Sciences*, vol. 19, no. 8, pp. 328–334, 1998.

[91] V. J. Miralles, I. Martínez-López, R. Zaragozá et al., "Na^+ dependent glutamate transporters (EAAT1, EAAT2, and EAAT3) in primary astrocyte cultures: effect of oxidative stress," *Brain Research*, vol. 922, no. 1, pp. 21–29, 2001.

[92] S. D. Rao, H. Z. Yin, and J. H. Weiss, "Disruption of glial glutamate transport by reactive oxygen species produced in motor neurons," *Journal of Neuroscience*, vol. 23, no. 7, pp. 2627–2633, 2003.

[93] P. Pasinelli, M. K. Houseweart, R. H. Brown Jr., and D. W. Cleveland, "Caspase-1 and -3 are sequentially activated in motor neuron death in Cu,Zn superoxide dismutase-mediated familial amyotrophic lateral sclerosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 25, pp. 13901–13906, 2000.

[94] W. Boston-Howes, S. L. Gibb, E. O. Williams, P. Pasinelli, R. H. Brown Jr., and D. Trotti, "Caspase-3 cleaves and inactivates the glutamate transporter EAAT2," *The Journal of Biological Chemistry*, vol. 281, no. 20, pp. 14076–14084, 2006.

[95] L. van den Bosch, P. van Damme, E. Bogaert, and W. Robberecht, "The role of excitotoxicity in the pathogenesis of amyotrophic lateral sclerosis," *Biochimica et Biophysica Acta*, vol. 1762, no. 11-12, pp. 1068–1082, 2006.

[96] J. D. Rothstein, M. van Kammen, A. I. Levey, L. J. Martin, and R. W. Kuncl, "Selective loss of glial glutamate transporter GLT-1 amyotrophic lateral sclerosis," *Annals of Neurology*, vol. 38, no. 1, pp. 73–84, 1995.

[97] L. I. Bruijn, M. W. Becher, M. K. Lee et al., "ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions," *Neuron*, vol. 18, no. 2, pp. 327–338, 1997.

[98] D. S. Howland, J. Liu, Y. She et al., "Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS)," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 3, pp. 1604–1609, 2002.

[99] K. Shobha, K. Vijayalakshmi, P. A. Alladi, A. Nalini, T. N. Sathyaprabha, and T. R. Raju, "Altered in-vitro and in-vivo expression of glial glutamate transporter-1 following exposure to cerebrospinal fluid of amyotrophic lateral sclerosis patients," *Journal of the Neurological Sciences*, vol. 254, no. 1-2, pp. 9–16, 2007.

[100] S. Nakanishi, "Molecular diversity of glutamate receptors and implications for brain function," *Science*, vol. 258, no. 5082, pp. 597–603, 1992.

[101] P. H. Seeburg, "The TINS/TiPS lecture: the molecular biology of mammalian glutamate receptor channels," *Trends in Neurosciences*, vol. 16, no. 9, pp. 359–365, 1993.

[102] S.-H. Shi, Y. Hayashi, J. A. Esteban, and R. Malinow, "Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons," *Cell*, vol. 105, no. 3, pp. 331–343, 2001.

[103] M. S. Beattie, A. R. Ferguson, and J. C. Bresnahan, "AMPA-receptor trafficking and injury-induced cell death," *The European Journal of Neuroscience*, vol. 32, no. 2, pp. 290–297, 2010.

[104] G. G. Turrigiano, "The self-tuning neuron: synaptic scaling of excitatory synapses," *Cell*, vol. 135, no. 3, pp. 422–435, 2008.

[105] M. Hollmann, M. Hartley, and S. Heinemann, "Ca^{2+} permeability of KA-AMPA—gated glutamate receptor channels depends on subunit composition," *Science*, vol. 252, no. 5007, pp. 851–853, 1991.

[106] T. A. Verdoorn, N. Burnashev, H. Monyer, P. H. Seeburg, and B. Sakmann, "Structural determinants of ion flow through recombinant glutamate receptor channels," *Science*, vol. 252, no. 5013, pp. 1715–1718, 1991.

[107] D. E. Pellegrini-Giampietro, J. A. Gorter, M. V. Bennett, and R. S. Zukin, "The GluR2 (GluR-B) hypothesis: Ca^{2+}-permeable AMPA receptors in neurological disorders," *Trends in Neurosciences*, vol. 20, no. 10, pp. 464–470, 1997.

[108] J. H. Weiss and S. L. Sensi, "Ca^{2+}-Zn^{2+} permeable AMPA or kainate receptors: possible key factors in selective neurodegeneration," *Trends in Neurosciences*, vol. 23, no. 8, pp. 365–371, 2000.

[109] Z. Yu, G. Cheng, X. Wen, G. D. Wu, W.-T. Lee, and D. Pleasure, "Tumor necrosis factor α increases neuronal vulnerability to excitotoxic necrosis by inducing expression of the AMPA-glutamate receptor subunit GluR1 via an acid sphingomyelinase-and NF-κB-dependent mechanism," *Neurobiology of Disease*, vol. 11, no. 1, pp. 199–213, 2002.

[110] L. Bernardino, S. Xapelli, A. P. Silva et al., "Modulator effects of interleukin-1β and tumor necrosis factor-α on AMPA-induced excitotoxicity in mouse organotypic hippocampal slice cultures," *The Journal of Neuroscience*, vol. 25, no. 29, pp. 6734–6744, 2005.

[111] D. Stellwagen, E. C. Beattie, J. Y. Seo, and R. C. Malenka, "Differential regulation of AMPA receptor and GABA receptor trafficking by tumor necrosis factor-α," *The Journal of Neuroscience*, vol. 25, no. 12, pp. 3219–3228, 2005.

[112] D. Leonoudakis, P. Zhao, and E. C. Beattie, "Rapid tumor necrosis factor α-induced exocytosis of glutamate receptor 2-lacking AMPA receptors to extrasynaptic plasma membrane

potentiates excitotoxicity," *The Journal of Neuroscience*, vol. 28, no. 9, pp. 2119–2130, 2008.

[113] F. Ogoshi, H. Z. Yin, Y. Kuppumbatti, B. Song, S. Amindari, and J. H. Weiss, "Tumor necrosis-factor-alpha (TNF-α) induces rapid insertion of Ca^{2+}-permeable α-amino-3-hydroxyl-5-methyl-4-isoxazole- propionate (AMPA)/kainate (Ca-A/K) channels in a subset of hippocampal pyramidal neurons," *Experimental Neurology*, vol. 193, no. 2, pp. 384–393, 2005.

[114] A. R. Ferguson, R. N. Christensen, J. C. Gensel et al., "Cell death after spinal cord injury is exacerbated by rapid TNFα-induced trafficking of GluR2-lacking AMPARs to the plasma membrane," *The Journal of Neuroscience*, vol. 28, no. 44, pp. 11391–11400, 2008.

[115] P. Han and P. J. Whelan, "Tumor necrosis factor alpha enhances glutamatergic transmission onto spinal motoneurons," *Journal of Neurotrauma*, vol. 27, no. 1, pp. 287–292, 2010.

[116] S. R. Rainey-Smith, D. A. Andersson, R. J. Williams, and M. Rattray, "Tumour necrosis factor alpha induces rapid reduction in AMPA receptor-mediated calcium entry in motor neurones by increasing cell surface expression of the GluR2 subunit: relevance to neurodegeneration," *Journal of Neurochemistry*, vol. 113, no. 3, pp. 692–703, 2010.

[117] T. L. Williams, N. C. Day, P. G. Ince, R. K. Kamboj, and P. J. Shaw, "Calcium-permeable alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors: a molecular determinant of selective vulnerability in amyotrophic lateral sclerosis," *Annals of Neurology*, vol. 42, no. 2, pp. 200–207, 1997.

[118] H. Z. Yin, D. T. Tang, and J. H. Weiss, "Intrathecal infusion of a Ca^{2+}-permeable AMPA channel blocker slows loss of both motor neurons and of the astrocyte glutamate transporter, GLT-1 in a mutant SOD1 rat model of ALS," *Experimental Neurology*, vol. 207, no. 2, pp. 177–185, 2007.

[119] M. Tateno, H. Sadakata, M. Tanaka et al., "Calcium-permeable AMPA receptors promote misfolding of mutant SOD1 protein and development of amyotrophic lateral sclerosis in a transgenic mouse model," *Human Molecular Genetics*, vol. 13, no. 19, pp. 2183–2196, 2004.

[120] J. C. Corona and R. Tapia, "Ca^{2+}-permeable AMPA receptors and intracellular Ca^{2+} determine motoneuron vulnerability in rat spinal cord in vivo," *Neuropharmacology*, vol. 52, no. 5, pp. 1219–1228, 2007.

[121] S. A. Sargsyan, P. N. Monk, and P. J. Shaw, "Microglia as potential contributors to motor neuron injury in amyotrophic lateral sclerosis," *Glia*, vol. 51, no. 4, pp. 241–253, 2005.

[122] L. H. Barbeito, M. Pehar, P. Cassina et al., "A role for astrocytes in motor neuron loss in amyotrophic lateral sclerosis," *Brain Research Reviews*, vol. 47, no. 1–3, pp. 263–274, 2004.

[123] D. Tweedie, K. Sambamurti, and N. H. Greig, "TNF-α inhibition as a treatment strategy for neurodegenerative disorders: new drug candidates and targets," *Current Alzheimer Research*, vol. 4, no. 4, pp. 378–385, 2007.

[124] H. Z. Yin, C. I. Hsu, S. Yu, S. D. Rao, L. S. Sorkin, and J. H. Weiss, "TNF-alpha triggers rapid membrane insertion of Ca^{2+} permeable AMPA receptors into adult motor neurons and enhances their susceptibility to slow excitotoxic injury," *Experimental Neurology*, vol. 238, no. 2, pp. 93–102, 2012.

[125] J. Llado, L. Tolosa, and G. Olmos, "Cellular and molecular mechanisms involved in the neuroprotective effects of VEGF on motoneurons," *Frontiers in Cellular Neuroscience*, vol. 7, Article ID 181, 2013.

[126] E. Bogaert, P. van Damme, K. Poesen et al., "VEGF protects motor neurons against excitotoxicity by upregulation of GluR2," *Neurobiology of Aging*, vol. 31, no. 12, pp. 2185–2191, 2010.

[127] D. Wheeler, E. Knapp, V. V. R. Bandaru et al., "Tumor necrosis factor-α-induced neutral sphingomyelinase-2 modulates synaptic plasticity by controlling the membrane insertion of NMDA receptors," *Journal of Neurochemistry*, vol. 109, no. 5, pp. 1237–1249, 2009.

[128] A. M. Floden, S. Li, and C. K. Combs, "β-Amyloid-stimulated microglia induce neuron death via synergistic stimulation of tumor necrosis factor α and NMDA receptors," *The Journal of Neuroscience*, vol. 25, no. 10, pp. 2566–2575, 2005.

[129] E. D. Stück, R. N. Christensen, J. R. Huie et al., "Tumor necrosis factor alpha mediates GABAA receptor trafficking to the plasma membrane of spinal cord neurons in vivo," *Neural Plasticity*, vol. 2012, Article ID 261345, 2012.

[130] A. R. Ferguson, S. N. Washburn, E. D. Crown, and J. W. Grau, "GABAA receptor activation is involved in noncontingent shock inhibition of instrumental conditioning in spinal rats," *Behavioral Neuroscience*, vol. 117, no. 4, pp. 799–812, 2003.

[131] J. R. Huie, K. M. Baumbauer, K. H. Lee et al., "Glial tumor necrosis factor alpha (TNFalpha) generates metaplastic inhibition of spinal learning," *PLoS ONE*, vol. 7, no. 6, Article ID e39751, 2012.

[132] H. Pribiag and D. Stellwagen, "TNF-alpha downregulates inhibitory neurotransmission through protein phosphatase 1-dependent trafficking of $GABA_A$ receptors," *The Journal of Neuroscience*, vol. 33, no. 40, pp. 15879–15893, 2013.

[133] J. L. R. Rubenstein and M. M. Merzenich, "Model of autism: increased ratio of excitation/inhibition in key neural systems," *Genes, Brain and Behavior*, vol. 2, no. 5, pp. 255–267, 2003.

[134] O. Yizhar, L. E. Fenno, M. Prigge et al., "Neocortical excitation/inhibition balance in information processing and social dysfunction," *Nature*, vol. 477, no. 7363, pp. 171–178, 2011.

[135] M. G. Chez, T. Dowling, P. B. Patel, P. Khanna, and M. Kominsky, "Elevation of tumor necrosis factor-alpha in cerebrospinal fluid of autistic children," *Pediatric Neurology*, vol. 36, no. 6, pp. 361–365, 2007.

[136] Y.-R. Wen, P.-H. Tan, J.-K. Cheng, Y.-C. Liu, and R.-R. Ji, "Microglia: a promising target for treating neuropathic and postoperative pain, and morphine tolerance," *Journal of the Formosan Medical Association*, vol. 110, no. 8, pp. 487–494, 2011.

[137] C.-H. Shen, R.-Y. Tsai, and C.-S. Wong, "Role of neuroinflammation in morphine tolerance: effect of tumor necrosis factor-alpha," *Acta Anaesthesiologica Taiwanica*, vol. 50, no. 4, pp. 178–182, 2012.

[138] C.-H. Shen, R.-Y. Tsai, M.-S. Shih et al., "Etanercept restores the antinociceptive effect of morphine and suppresses spinal neuroinflammation in morphine-tolerant rats," *Anesthesia and Analgesia*, vol. 112, no. 2, pp. 454–459, 2011.

[139] C.-H. Shen, R.-Y. Tsai, Y.-H. Tai, S.-L. Lin, C.-C. Chien, and C.-S. Wong, "Intrathecal etanercept partially restores morphines antinociception in morphine-tolerant rats via attenuation of the glutamatergic transmission," *Anesthesia and Analgesia*, vol. 113, no. 1, pp. 184–190, 2011.

[140] J. I. Choi, C. I. Svensson, F. J. Koehrn, A. Bhuskute, and L. S. Sorkin, "Peripheral inflammation induces tumor necrosis factor dependent AMPA receptor trafficking and Akt phosphorylation in spinal cord in addition to pain behavior," *Pain*, vol. 149, no. 2, pp. 243–253, 2010.

[141] E. L. Tobinick and H. Gross, "Rapid improvement in verbal fluency and aphasia following perispinal etanercept in Alzheimer's disease," *BMC Neurology*, vol. 8, article 27, 2008.

[142] E. Tobinick, H. Gross, A. Weinberger, and H. Cohen, "TNF-alpha modulation for treatment of Alzheimer's disease: a 6-month pilot study," *Medscape General Medicine*, vol. 8, no. 2, article 25, 2006.

[143] B. W. van Oosten, F. Barkhof, L. Truyen et al., "Increased MRI activity and immune activation in two multiple sclerosis patients treated with the monoclonal anti-tumor necrosis factor antibody cA2," *Neurology*, vol. 47, no. 6, pp. 1531–1534, 1996.

[144] E. W. Stommel, J. A. Cohen, C. E. Fadul et al., "Efficacy of thalidomide for the treatment of amyotrophic lateral sclerosis: a phase II open label clinical trial," *Amyotrophic Lateral Sclerosis*, vol. 10, no. 5-6, pp. 393–404, 2009.

[145] G. Gowing, F. Dequen, G. Soucy, and J.-P. Julien, "Absence of tumor necrosis factor-α does not affect motor neuron disease caused by superoxide dismutase 1 mutations," *The Journal of Neuroscience*, vol. 26, no. 44, pp. 11397–11402, 2006.

[146] P. Ghezzi and T. Mennini, "Tumor necrosis factor and motoneuronal degeneration: an open problem," *NeuroImmunomodulation*, vol. 9, no. 4, pp. 178–182, 2001.

[147] A. J. Bruce, W. Boling, M. S. Kindy et al., "Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors," *Nature Medicine*, vol. 2, no. 7, pp. 788–794, 1996.

[148] P. G. Sullivan, A. J. Bruce-Keller, A. G. Rabchevsky et al., "Exacerbation of damage and altered NF-κB activation in mice lacking tumor necrosis factor receptors after traumatic brain injury," *The Journal of Neuroscience*, vol. 19, no. 15, pp. 6248–6256, 1999.

[149] G.-H. Jeohn, L.-Y. Kong, B. Wilson, P. Hudson, and J.-S. Hong, "Synergistic neurotoxic effects of combined treatments with cytokines in murine primary mixed neuron/glia cultures," *Journal of Neuroimmunology*, vol. 85, no. 1, pp. 1–10, 1998.

[150] C. C. Chao, S. Hu, L. Ehrlich, and P. K. Peterson, "Interleukin-1 and tumor necrosis factor-α synergistically mediate neurotoxicity: Involvement of nitric oxide and of N-methyl-D-aspartate receptors," *Brain, Behavior, and Immunity*, vol. 9, no. 4, pp. 355–365, 1995.

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Research Article

Protective Effects of Lipoxin A4 in Testis Injury following Testicular Torsion and Detorsion in Rats

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Purpose. To investigate the protective effects of lipoxin A4 (LXA4) in rat testis injury following testicular torsion/detorsion. *Methods*. A rat testicular torsion model has been established as described. Rats were randomly divided into 6 groups: sham group, torsion group, torsion/detorsion (T/D) group, and T/D plus LXA4-pretreated groups (3 subgroups). Rats in LXA4-pretreated groups received LXA4 injection (0.1, 1.0, and 10 μg/kg body weight in LXA4-pretreated subgroups 1–3, resp.) at a single dose 1 h before detorsion. Biochemical analysis, apoptosis assessment, and morphologic evaluation were carried out after orchiectomies. *Results*. GPx and SOD levels significantly increased and MDA levels significantly reduced in LXA4-pretreated groups compared to T/D group. LXA4 also reverted IL-2 and TNF-α to basal levels and improved the expression of IL-4 and IL-10 in LXA4-pretreated groups. Moreover, the expression of NF-κB was downregulated in LXA4-pretreated groups. LXA4 treatment also showed an improved testicular morphology and decreased apoptosis in testes. *Conclusion*. Lipoxin A4 protects rats against testes injury after torsion/detorsion via modulation of cytokines, oxidative stress, and NF-κB activity.

1. Introduction

Testicular torsion occurs at a frequency of 1 in 4000 in men who are younger than 25 years old [1]. It is a common urologic emergency that leads to testicular necrosis and results in decreased fertility [2]. The primary pathophysiologic event in testicular torsion is ischemia followed by reperfusion; thus, testicular torsion/detorsion is considered as an ischemia/reperfusion injury (IRI) to the testis [3]. Numerous drugs have been applied to prevent testis against IRI following testicular torsion in animal models [4–7]. Those previous studies suggested that treatment against IRI following testicular torsion/detorsion may result in decreased oxidative stress, reduced inflammation, and improved testicular morphology to testis.

Lipoxins are lipoxygenase derived lipid mediators with both anti-inflammatory and proresolution properties that have been demonstrated in vivo and in vitro [8]. Lipoxin A4 (LXA4) is the major physiological lipoxin during inflammation in mammalian systems [9]. The protective effects of lipoxins against IRI have been confirmed in many organs including brain, heart, kidney, and stomach [10–13]. However, to our knowledge, the effect of LXA4 on IRI following testicular torsion/detorsion has not yet been reported. In the present study, a rat model was used to investigate the roles of LXA4 in testicular torsion/detorsion.

2. Materials and Methods

2.1. Animals and Reagents. All experiments were conducted in accordance with the guidelines of Animal Use and Care Committee of Wuhan University. 60~90-day-old specific pathogen-free (SPF) Sprague Dawley (SD) rats weighing 180 ~ 200 g were obtained from the Center for Animal Experiment/Animal Biosafety Level III laboratory (ABSL-III lab) of Wuhan University in China. Rats were housed individually in cages on a 12 h dark: 12 h light cycle at 23 ± 2°C under standard environmental conditions and had free access to pellet diet and tap water.

2.2. Animal Model and Study Design. Rats were randomly divided into 6 groups: (1) sham group ($n = 10$): rats received sham operations with no additional interventions; (2) torsion group ($n = 10$); (3) torsion/detorsion (T/D) group ($n = 10$) and (4) T/D + LXA4-pretreated groups ($n = 10$ in each subgroup, 3 subgroups in total). The torsion and detorsion protocols lasted for 6 hours. Bilateral orchiectomies were performed at the end of protocol. In detail, bilateral orchiectomies were performed 6 h after sham operation in the sham group; rats in the torsion group received bilateral orchiectomies after 6 h torsion without detorsion; rats in the T/D group and T/D + LXA4 groups received a 2 h torsion followed by a 4 h detorsion. Torsion, detorsion, and sham operations were performed on the left testis through a midscrotal vertical incision as previously described [14]. LXA4 ([5S-, 6R-, 15S-trihydroxy-7E, 9E, 11Z, 13E-eicosatetraenoic acid]; Cayman Chemical, Ann Arbor, USA) was intraperitoneally injected 1 h before detorsion in the T/D + LXA4 groups as a single dose (0.1, 1.0, and 10 μg/kg body weight in subgroup 1–3, resp.). Rats were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight; Amresco, Cleveland, USA). Tissue samples were collected for biochemical analysis and morphological evaluation at the end of study protocol. Half of each fresh testis was washed with ice-cold phosphate-buffered saline (PBS) (pH = 7.4) and then kept at −70°C for measurement of cytokines levels, MDA level, tissue SOD, and GPx activity. The remaning half of testis was divided into two parts and fixed in 10% formalin or 2.5% glutaraldehyde for light microscopy and electron microscopy, respectively.

2.3. Expression of Cytokines in Testes. Cytokines IL4, IL-10, IL-2, and TNF-α were detected using commercial ELISA kits according to the manufacturers' protocols (Beyotime Institute of Biotechnology, Jiangsu, China). Each well of the plate was coated with 100 μL of capture antibody and incubated overnight at 4°C. Plates were blocked with assay diluent for 1 h after washes. Testicular tissue homogenate (100 μL) in PBS supplemented with protease inhibitors was added to each well of the plate before incubation. Working detector (100 μL) was loaded into each well, and the plate was incubated for 1 h before the addition of substrate solution. The reaction was stopped by adding 50 μL stop solution. Calculation of the concentrations was performed in a log-log linear regression.

2.4. Expressions of Malondialdehyde and Activities of Superoxide Dismutase and Glutathione Peroxidase in Testes. Fresh testicular tissue was placed into a 1.5 mL centrifuge tube. Add 250 μL of RIPA buffer with protease inhibitors. Homogenate was then centrifuged at 11000 ×g for 10 min at 4°C. The supernatant was used for the determination of malondialdehyde (MDA) using a commercial kit (Cayman, Ann Arbor, MI) and the detection of testicular superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities. Testicular SOD activity was measured with an SOD-525TM reagent kit (OXIS International, Foster, CA); the final result was expressed as U/mg protein; tissue GPx activity was measured as described [15]. GPx catalyzes the oxidation of glutathione (GSH) by H_2O_2 in a reaction coupled with the conversion of nicotinamide adenine dinucleotide phosphate (NADPH) (reduced form) to NADPH+ (oxidized form), and the change in absorbance at 340 nm is used for detecting GPx activity.

2.5. Western-Blot Assay for NF-κB p65. The fresh testicular tissue has been collected and then was homogenized in ice-cold tissue protein extraction reagent, containing 1% protease and 1% phosphatase inhibitors. After centrifugation at 10,000 g for 5 min at 4°C, the supernatants were collected. Protein concentrations in supernatants were determined by a bicinchoninic acid protein assay kit (Boster, Wuhan, China). Western blot assay was carried out in duplicates as described [16]. Anti-NF-kappa B p65 antibody was purchased from Abcam (MA, USA). Protein bands were normalized with GAPDH, and all blots were quantified with Software Quantity One (Bio Rad).

2.6. Assessment of Apoptosis in Testes. Apoptotic activity on paraffin sections of the testis was analyzed by a TUNEL method with a commercial kit (Boster, Wuhan, China). The number of TUNEL-positive nuclei per tubule was counted. Apoptotic cells were identified by a brown stain over the nuclei. Approximately, 200 cells were counted per field; five fields were examined per slide. Apoptotic index (AI) was calculated as follows: AI = (number of apoptotic cells/the total number of counted cells) × 100%. Caspase-3 is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways. We also measured the caspase-3 protease activity using a caspase-3 colorimetric assay kit according to the manufacturer's instructions (Boster, Wuhan, China). Briefly, the homogenates of testicular tissues were centrifuged for 1 min at 10,000 g, and 100 μg of protein was incubated with reaction buffer and Asp-Glu-Val-Asp-p-nitroanilide for 90 min at 37°C. Absorbance was measured at 405 nm as caspase-3 activity.

2.7. Morphological Evaluation. Testis tissues were collected for morphological evaluation. The specimens were fixed in 10% formalin, embedded in paraffin, cut into sections 4 microns in thickness, and stained with hematoxylin and eosin (H&E). A pathologist blindly evaluated the testicular tissues in a random order under light microscope. Testicular injury and spermatogenesis were graded with Johnsen score [17]. All tubular sections in each observed area of testicular tissue are evaluated systematically and each is given a score from 1 to 10. Complete spermatogenesis with many spermatozoa present is evaluated as score 10. Additionally, electron microscopy was also carried out. Briefly, testicular tissue was fixed in 2.5% glutaraldehyde at 4°C for 24 h then washed in phosphate-buffered saline, embedded in epoxy resin, and immersed in Epon812. Sections collected with a LKB-V microtome (BROMMA, Sweden) were stained with uranium acetate and folic acid lead and captured with a transmission electron microscope (H-600, Hitachi, Japan).

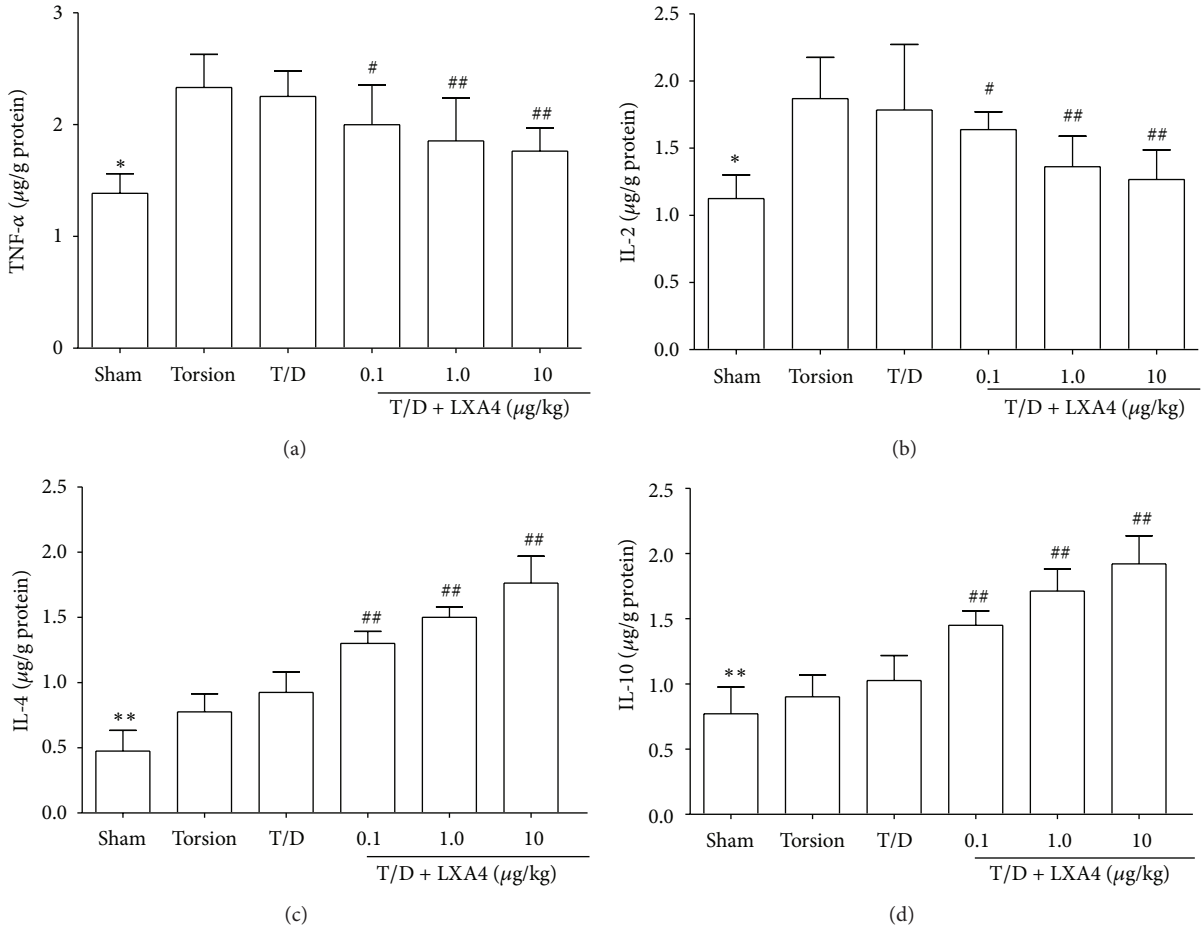

Figure 1: Changes of cytokines in testicular tissue. LXA4 treatment inhibits the increase of proinflammatory cytokines (IL-2 and TNF-α) and promotes the expression of anti-inflammatory cytokines (IL-4 and IL-10) in testes after testicular torsion. Compared with other groups, $^{*}P < 0.05$ and $^{**}P < 0.01$. Compared with torsion or torsion/detorsion (T/D) group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$.

2.8. Statistical Analysis. Data are expressed as mean ± SD. The data was processed by the statistical analysis software SPSS version 16.0 (SPSS Inc., Chicago, IL). Comparison of several means was performed using one-way and repeated measure two-way analysis of variance followed by the Tukey-Kramer test to identify significant difference between groups. All P values were two-tailed and a P value of less than 0.05 was considered significant.

3. Results

3.1. Changes of Cytokines, MDA, SOD, and GPx in Testis. Changes of cytokines, MDA, SOD, and GPx were summarized in Figures 1 and 2. Compared to the sham group, the levels of proinflammatory cytokines (IL-2 and TNF-α) and MDA were significantly increased in testes after testicular torsion, while the levels of SOD and GPx were decreased (resp., $P < 0.05$). The treatment with LXA4 reverted those parameters to basal levels. Compared to the torsion and torsion/detorsion group, the MDA and IL-2 and TNF-α levels were significantly lower in the LXA4-pretreated groups (resp., $P < 0.05$). In addition, the anti-inflammatory cytokines (IL-4 and IL-10), SOD, and GPx levels were significantly increased in the LXA4-pretreated groups than those in the torsion and torsion/detorsion groups (resp., $P < 0.05$).

3.2. NF-κB p65 Expression in Testis. Western-blot assay showed that the NF-κB p65 expression level was increased after testicular torsion. However, the NF-κB p65 expression has been inhibited by LXA4 treatment. As seen in Figure 3, the NF-κB p65 expressions in LXA4-treated groups were significantly lower than those in the torsion and torsion/detorsion groups (resp., $P < 0.05$).

3.3. Analysis of Apoptosis in Testis. The analysis of apoptosis was performed by TUNEL method and caspase-3 activity. TUNEL staining sections were shown in Figure 4. The percentage of TUNEL positive apoptotic cells was denoted as AI. The AI values of LXA4-pretreated groups were significantly lower than those in the torsion and torsion/detorsion groups (resp., $P < 0.05$, Table 1). In addition, compared to torsion and torsion/detorsion groups, the caspase-3 activity in testes was significantly decreased in the LXA4-pretreated groups (resp., $P < 0.05$, Table 1).

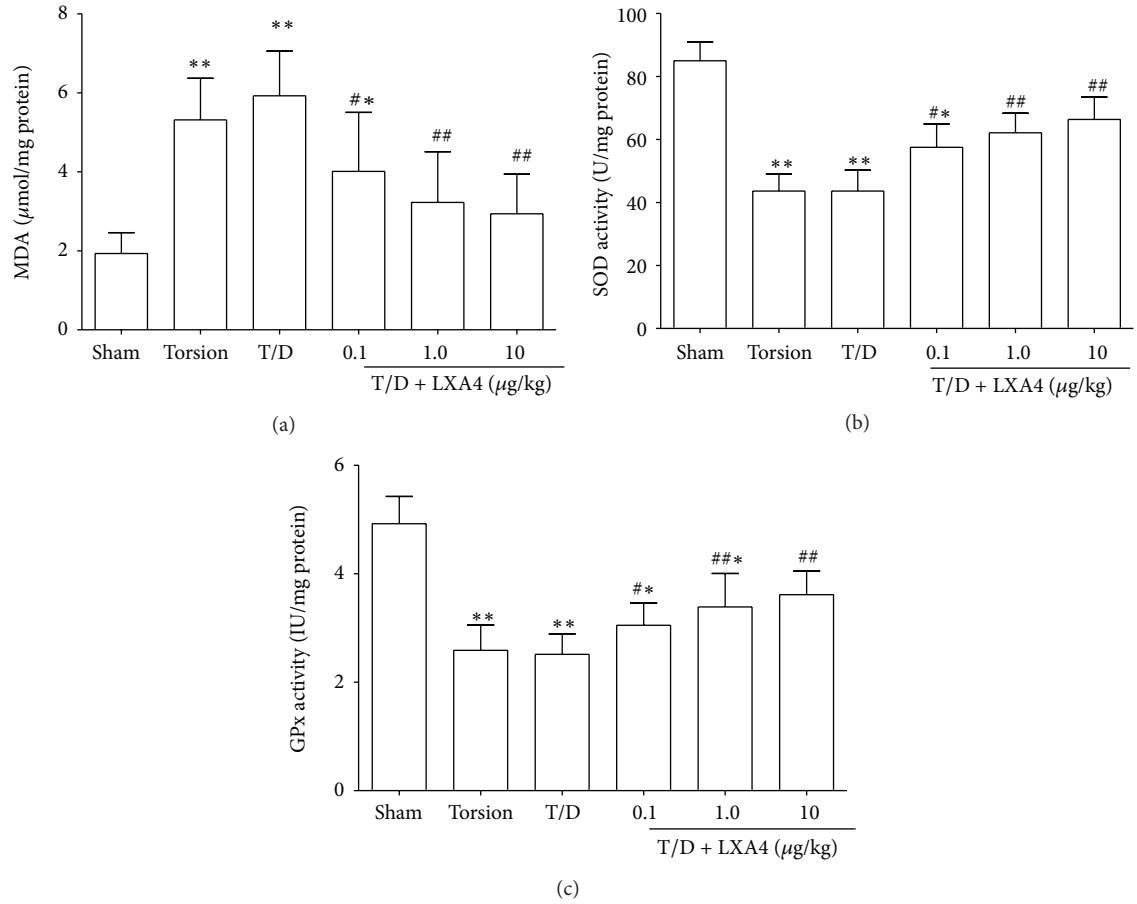

Figure 2: Changes of MDA, SOD, and GPx in testicular tissue. The levels of MDA were increased in testes after testicular torsion, while the levels of SOD and GPx were decreased. The treatment with LXA4 reverted those parameters to basal levels. Compared with Sham groups, $^{*}P < 0.05$ and $^{**}P < 0.01$. Compared with torsion or torsion/detorsion (T/D) group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$.

IOD ratio	$0.30 \pm 0.05^{*}$	0.82 ± 0.20	0.88 ± 0.15	$0.70 \pm 0.20^{\#}$	$0.65 \pm 0.10^{\#\#}$	$0.44 \pm 0.05^{\#\#}$

Figure 3: Western-blot for NF-κB p65 expression in testicular tissue. NF-κB p65 expression was increased after testicular torsion in both torsion and torsion/detorsion (T/D) groups. However, the NF-κB p65 expression has been inhibited by LXA4 treatments. Compared with other groups, $^{*}P < 0.05$. Compared with T/D group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$.

3.4. Morphological Evaluation of Testis. The findings of the light microscopy for each group are shown in Figure 5. The presence of normal testicular structure and uniform seminiferous tubular morphology was seen in the sham group. In the torsion and T/D groups, there were significant reductions in the seminiferous tubular diameter and severe distortion of tubules. Administration of LXA4 preserved the intact seminiferous tubular morphology in testes after torsion/detorsion. Furthermore, the histologic scores were significantly higher in the LXA4-treated groups compared with the torsion and torsion/detorsion groups (resp., $P < 0.05$, Table 1). Swollen mitochondria with degenerated cristae and enlarged intercellular spaces were observed under electron microscopy in both torsion and T/D groups. However, LXA4 pretreatment was effective in preventing mitochondria degeneration and dilation of intracellular spaces (Figure 6).

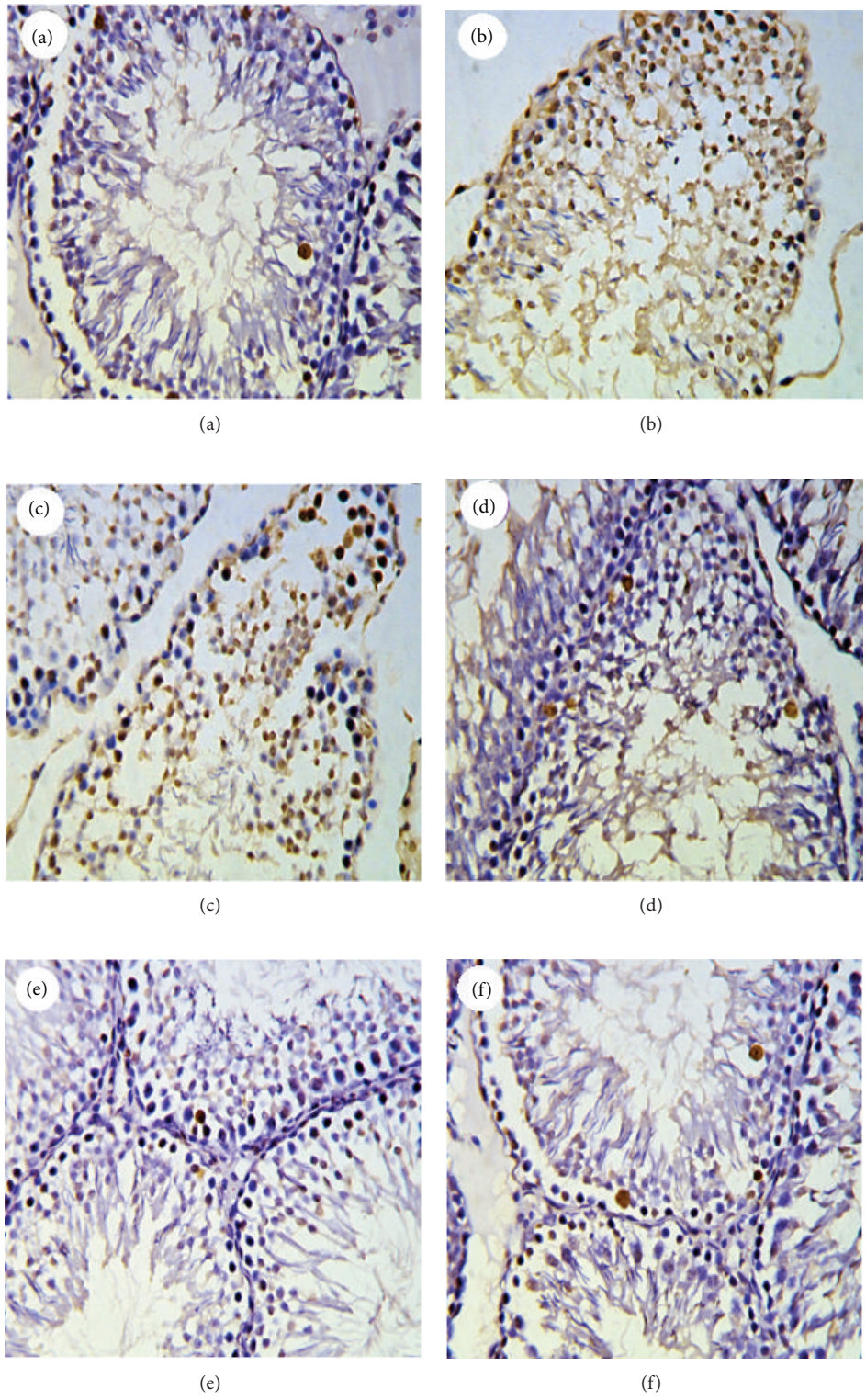

Figure 4: TUNEL staining of testicular tissue (×200). (a) Sham group: only a few TUNEL-positive cells were observed. (b) Torsion group and (c) torsion/detorsion (T/D) group: TUNEL-positive cells were mainly observed in germ cells of testis. (d), (e), and (f): T/D + LXA4 subgroup 1–3, respectively. Apoptotic cells were rarely observed in the seminiferous epithelium.

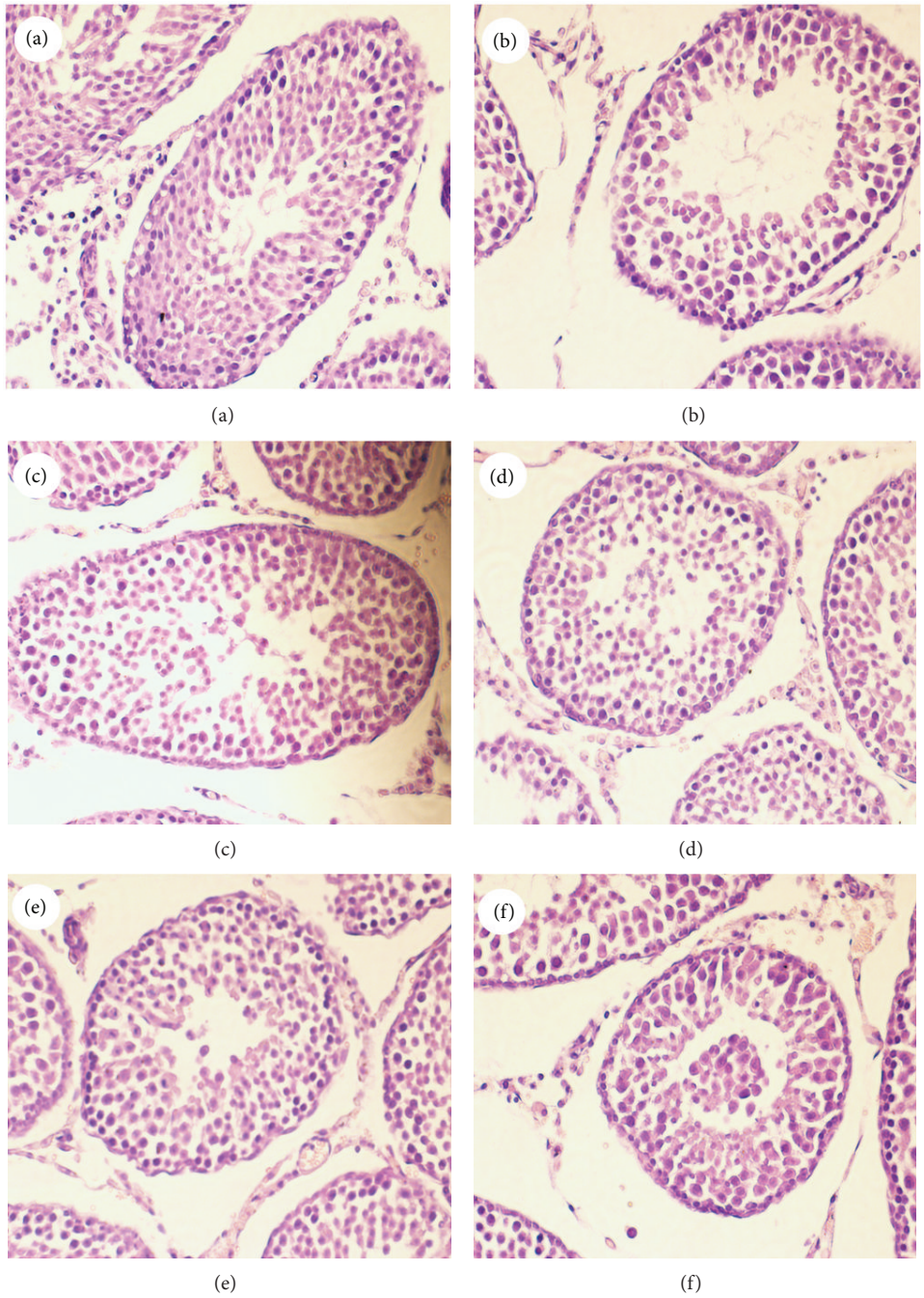

Figure 5: Light microscope observations of H&E stained sections (×200). (a) Sham group: normal testicular architecture was seen. (b) Torsion group and (c) torsion/detorsion (T/D) group: severe damage to testis was noted. (d), (e), and (f): T/D + LXA4 subgroup 1–3, respectively.

4. Discussion

In this study, we used a rat testicular torsion model to investigate the protective effects of lipoxin A4 on testicular ischemia/reperfusion injury. Various parameters such as MDA, SOD, GPx, proinflammatory cytokines, anti-inflammatory cytokines, and NF-κB have been detected. Morphologic evaluation has been also carried out after orchiectomies. Our results demonstrated that lipoxin A4 significantly reduced the inflammatory reactions, oxidative stress, and histologic damage in testes after testicular torsion.

The ischemia of the testes followed by reperfusion is the primary pathophysiological event in testicular torsion (TT) [18]. Thus, TT can be generally considered as an ischemia/reperfusion injury (IRI) to testis. Numerous studies focused on the treatment against IRI following testicular torsion and detorsion have brought beneficial effects in animal model [14, 19, 20]. The protective effects of lipoxins

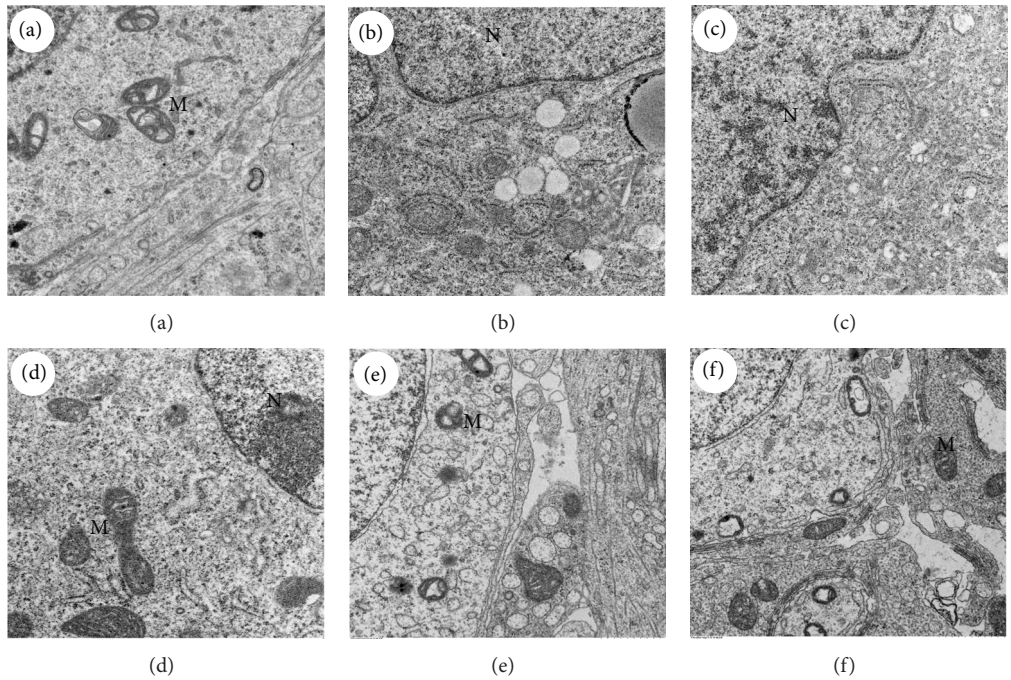

Figure 6: Electron microscopy of testicular tissue in different groups (×2000), N: nuclei, and M: mitochondria. (a) Sham group: normal testicular structure was seen in sham group. (b) Torsion group and (c) torsion/detorsion (T/D) group: swollen mitochondria with degenerated cristae and enlarged intercellular spaces were observed under electron microscopy. (d), (e), and (f): T/D + LXA4 subgroup 1–3, respectively: LXA4 pretreatment was effective in preventing mitochondria degeneration and dilation of intracellular spaces.

Table 1: Apoptotic index, Johnsen scores, and caspase-3 activities in different groups (mean ± SD).

	Apoptotic index (%)	Johnsen scores	Caspase-3 activity (absorbance 405/100 μg protein)
Sham	$8.50 \pm 4.10^{**}$	$7.50 \pm 0.80^{*}$	$0.10 \pm 0.00^{**}$
Torsion	32.40 ± 7.10	5.22 ± 1.21	0.62 ± 0.10
T/D	30.52 ± 10.20	5.50 ± 1.00	0.55 ± 0.05
T/D + LXA4 (0.1 μg/kg)	$24.60 \pm 7.70^{\#}$	$5.80 \pm 1.00^{\#}$	$0.42 \pm 0.12^{\#\#}$
T/D + LXA4 (1.0 μg/kg)	$18.50 \pm 8.12^{\#\#}$	$6.22 \pm 1.40^{\#\#}$	$0.35 \pm 0.08^{\#\#}$
T/D + LXA4 (10 μg/kg)	$14.30 \pm 6.75^{\#\#}$	$7.15 \pm 1.50^{\#\#}$	$0.22 \pm 0.05^{\#\#}$

T/D: torsion/detorsion; LXA4: lipoxin A4. Testicular injury and spermatogenesis were graded with Johnsen score. All tubular sections in each observed area of testicular tissue are evaluated systematically and each is given a score from 1 to 10. Complete spermatogenesis with many spermatozoa present is evaluated as score 10. Compared with other groups, $^{*}P < 0.05$ and $^{**}P < 0.01$. Compared with torsion or torsion/detorsion (T/D) group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$.

against IRI have been confirmed in many organs including brain, heart, kidney, and stomach [10–13]. In this study, LXA4 also showed its protective effects in the testicular IRI due to its ability of modulation of oxidative stress and inflammation.

IRI to testis is associated with the overgeneration of reactive oxygen species (ROS) and mammalian testes are highly susceptible to oxidative stress [19, 20]. MDA has been widely used as an indicator of oxidative stress in many physiopathological events including IRI [21]. Many studies proved that MDA level in testis tissue increase after testicular torsion [22]. Besides, the ability of antioxidants including SOD and GPx to prevent testis against IRI following testicular torsion has been investigated [23, 24]. In this study, treatment with LXA4 attenuated the oxidative stress in damaged testes via reducing the expression of MDA and elevating the expression of SOD and GPx. Those results showed that LXA4 might exert an antioxidative effect in testicular detorsion.

Inflammation has been established to contribute substantially to the pathogenesis of IRI. The proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) increase in IRI following testicular torsion [16]. LXA4 has been considered as a "break signal" of inflammation, and the role for LXA4 as anti-inflammatory molecules is well defined [25]. In this study, LXA4-treated rats showed lower proinflammatory cytokine levels compared to the torsion/detorsion group. Our results also suggested that the expression of NF-κB in damaged testes has been downregulated by the LXA4 pretreatment. Nuclear factor κB (NF-κB) is a primary regulator of gene expression for a large number of cytokines and is activated during IRI of testis

[26], and LXA4 have been proposed to directly stimulate gene expression of endogenous anti-inflammatory factors by regulating NF-κB activation [11]. It is suggested that the protective effect of LXA4 in testicular torsion followed IRI was partially due to its anti-inflammatory properties.

Experimental studies have shown that bilateral testicular damage and reduced fertility may result from unilateral torsion [27]. The proposed mechanism of this injury is probably due to the immune response after testicular torsion. In addition, animal experiments suggested that humoral and cellular immune mediated testicular cell damage is an important pathologic alternation in testicular torsion [28]. A few recent studies have demonstrated that lipoxins suppress antigen-presenting cell functions and regulate cytokine-driven immune reactions toward Th2 responses [29–31]. The Th1 subset mainly secretes IL-2, IFN-γ, and TNF-α. In contrast, Th2 cells mainly produce IL-4 and IL-10. In this study, we observed that the anti-inflammatory cytokines IL-4 and IL-10 were significantly increased in the LXA4-pretreated groups, while IL-2 and TNF-α were decreased. These findings suggested that LXA4 attenuates the IRI following testicular torsion and is also associated with its ability of regulating Th1/Th2 balance.

5. Conclusion

Our results suggest that LXA4 may exert an anti-inflammatory effect and reduce the histological damage in rat testicular torsion/detorsion due to its ability to regulate the production of cytokines and the NF-κB activity and to cause MDA, SOD, and GPx to revert to control levels. Thus, LXA4 may have a protective effect against IRI injury following testicular torsion/detorsion via modulation of cytokines, oxidative stress, and NF-κB activity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] R. C. Williamson, "Torsion of the testis and allied conditions," *British Journal of Surgery*, vol. 63, no. 6, pp. 465–476, 1976.

[2] M. Bozlu, D. Acar, S. Cayan, S. Aktas, and A. Tunckiran, "Protective effect of trapidil on long-term histologic damage in a rat model of testicular ischemia-reperfusion injury," *World Journal of Urology*, vol. 27, no. 1, pp. 117–122, 2009.

[3] D. Dokmeci, "Testicular torsion, oxidative stress and the role of antioxidant therapy," *Folia medica*, vol. 48, no. 3-4, pp. 16–21, 2006.

[4] D. Dokmeci, M. Kanter, M. Inan et al., "Protective effects of ibuprofen on testicular torsion/detorsion-induced ischemia/reperfusion injury in rats," *Archives of Toxicology*, vol. 81, no. 9, pp. 655–663, 2007.

[5] C. Romeo, P. Antonuccio, M. Esposito et al., "Raxofelast, a hydrophilic vitamin e-like antioxidant, reduces testicular ischemia-reperfusion injury," *Urological Research*, vol. 32, no. 5, pp. 367–371, 2004.

[6] T. Akgül, A. Ayyildiz, B. Nuhoğlu et al., "Ginkgo biloba (EGb 761) usage attenuates testicular injury induced by testicular ischemia/reperfusion in rats," *International Urology and Nephrology*, vol. 40, no. 3, pp. 685–690, 2008.

[7] P. Tsounapi, M. Saito, F. Dimitriadis et al., "Protective effect of sivelestat, a neutrophil elastase inhibitor, on ipsilateral and contralateral testes after unilateral testicular ischaemia-reperfusion injury in rats," *BJU International*, vol. 107, no. 2, pp. 329–336, 2011.

[8] C. N. Serhan, M. Hamberg, and B. Samuelsson, "Lipoxins: novel series of biologically active compounds formed from arachidonic acid in human leukocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 81, no. 17, pp. 5335–5339, 1984.

[9] N. Baker, S. J. O'Meara, M. Scannell, P. Maderna, and C. Godson, "Lipoxin A4: anti-inflammatory and anti-angiogenic impact on endothelial cells," *The Journal of Immunology*, vol. 182, no. 6, pp. 3819–3826, 2009.

[10] Y. Wu, Y.-P. Wang, P. Guo et al., "A lipoxin A4 analog ameliorates blood-brain barrier dysfunction and reduces MMP-9 expression in a rat model of focal cerebral ischemia-reperfusion injury," *Journal of Molecular Neuroscience*, vol. 46, no. 3, pp. 483–491, 2012.

[11] Z. Chen, Z. Wu, C. Huang et al., "Effect of lipoxin A4 on myocardial ischemia reperfusion injury following cardiac arrest in a rabbit model," *Inflammation*, vol. 36, no. 2, pp. 468–475, 2013.

[12] N. E. Kieran, P. P. Doran, S. B. Connolly et al., "Modification of the transcriptomic response to renal ischemia/reperfusion injury by lipoxin analog," *Kidney International*, vol. 64, no. 2, pp. 480–492, 2003.

[13] B. M. Peskar, K. Ehrlich, R. Schuligoi, and B. A. Peskar, "Role of lipoxygenases and the lipoxin A4/annexin 1 receptor in ischemia-reperfusion-induced gastric mucosal damage in rats," *Pharmacology*, vol. 84, no. 5, pp. 294–299, 2009.

[14] S. Somuncu, M. Cakmak, S. Erdogan, O. Caglayan, H. Akman, and M. Kaya, "Protective effects of trapidil in ischemia-reperfusion injury due to testicular torsion and detorsion: an experimental study," *International Journal of Urology*, vol. 13, no. 5, pp. 601–605, 2006.

[15] D. E. Paglia and W. N. Valentine, "Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase," *The Journal of Laboratory and Clinical Medicine*, vol. 70, no. 1, pp. 158–169, 1967.

[16] Y. Zhang, Y. Lv, Y. J. Liu et al., "Hyperbaric oxygen therapy in rats attenuates ischemia-reperfusion testicular injury through blockade of oxidative stress, suppression of inflammation, and reduction of nitric oxide formation," *Urology*, vol. 82, no. 2, pp. 489.e9–489.e15, 2013.

[17] S. G. Johnsen, "Testicular biopsy score count—a method for registration of spermatogenesis in human testes: normal values

and results in 335 hypogonadal males," *Hormones*, vol. 1, no. 1, pp. 2–25, 1970.

[18] T. T. Turner and K. J. Brown, "Spermatic cord torsion: loss of spermatogenesis despite return of blood flow," *Biology of Reproduction*, vol. 49, no. 2, pp. 401–407, 1993.

[19] S.-M. Wei, Z.-Z. Yan, and J. Zhou, "Beneficial effect of taurine on testicular ischemia-reperfusion injury in rats," *Urology*, vol. 70, no. 6, pp. 1237–1242, 2007.

[20] D. Dokmeci, M. Inan, U. N. Basaran et al., "Protective effect of L-carnitine on testicular ischaemia-reperfusion injury in rats," *Cell Biochemistry and Function*, vol. 25, no. 6, pp. 611–618, 2007.

[21] K. Fukuda, S. Asoh, M. Ishikawa, Y. Yamamoto, I. Ohsawa, and S. Ohta, "Inhalation of hydrogen gas suppresses hepatic injury caused by ischemia/reperfusion through reducing oxidative stress," *Biochemical and Biophysical Research Communications*, vol. 361, no. 3, pp. 670–674, 2007.

[22] A. Unsal, M. Eroglu, A. Avci et al., "Protective role of natural antioxidant supplementation on testicular tissue after testicular torsion and detorsion," *Scandinavian Journal of Urology and Nephrology*, vol. 40, no. 1, pp. 17–22, 2006.

[23] D. Avlan, K. Erdouğan, B. Cimen, D. Düşmez Apa, I. Cinel, and S. Aksöyek, "The protective effect of selenium on ipsilateral and contralateral testes in testicular reperfusion injury," *Pediatric Surgery International*, vol. 21, no. 4, pp. 274–278, 2005.

[24] D. W. Filho, M. A. Torres, A. L. Bordin, T. B. Crezcynski-Pasa, and A. Boveris, "Spermatic cord torsion, reactive oxygen and nitrogen species and ischemia-reperfusion injury," *Molecular Aspects of Medicine*, vol. 25, no. 1-2, pp. 199–210, 2004.

[25] C. N. Serhan and E. Oliw, "Unorthodox routes to prostanoid formation: new twists in cyclooxygenase-initiated pathways," *The Journal of Clinical Investigation*, vol. 107, no. 12, pp. 1481–1489, 2001.

[26] J. J. Lysiak, H. J. Bang, Q. A. Nguyen, and T. T. Turner, "Activation of the nuclear factor kappa B pathway following ischemia-reperfusion of the murine testis," *Journal of Andrology*, vol. 26, no. 1, pp. 129–135, 2005.

[27] R. Rabinowitz, H. Nagler, S. Kogan, and M. Consentino, "Experimental aspects of testicular torsion," *Dialogues in Pediatric Urology*, vol. 8, article 18, 1985.

[28] M. G. Rodriguez, C. Rival, M. S. Theas, and L. Lustig, "Immunohistopathology of the contralateral testis of rats undergoing experimental torsion of the spermatic cord," *Asian Journal of Andrology*, vol. 8, no. 5, pp. 576–583, 2006.

[29] F. S. Machado and J. Aliberti, "Lipoxins as an immune-escape mechanism," *Advances in Experimental Medicine and Biology*, vol. 666, pp. 78–87, 2009.

[30] J. F. Parkinson, "Lipoxin and synthetic lipoxin analogs: an overview of anti-inflammatory functions and new concepts in immunomodulation," *Inflammation & Allergy: Drug Targets*, vol. 5, no. 2, pp. 91–106, 2006.

[31] W. Liao, F. Zeng, K. Kang et al., "Lipoxin A4 attenuates acute rejection via shifting TH1/TH2 cytokine balance in rat liver transplantation," *Transplantation Proceedings*, vol. 45, no. 6, pp. 2451–2454, 2013.

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Review Article

Two Faces of TGF-Beta1 in Breast Cancer

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Breast cancer (BC) is potentially life-threatening malignancy that still causes high mortality among women. Scientific research in this field is focused on deeper understanding of pathogenesis and progressing of BC, in order to develop relevant diagnosis and improve therapeutic treatment. Multifunctional cytokine TGF-β1 is one of many factors that have a direct influence on BC pathophysiology. Expression of TGF-β1, induction of canonical and noncanonical signaling pathways, and mutations in genes encoding TGF-β1 and its receptors are correlated with oncogenic activity of this cytokine. In early stages of BC this cytokine inhibits epithelial cell cycle progression and promotes apoptosis, showing tumor suppressive effects. However, in late stages, TGF-β1 is linked with increased tumor progression, higher cell motility, cancer invasiveness, and metastasis. It is also involved in cancer microenvironment modification and promotion of epithelial to mesenchymal transition (EMT). This review summarizes the current knowledge on the phenomenon called "TGF-β1 paradox", showing that better understanding of TGF-β1 functions can be a step towards development of new therapeutic approaches. According to current knowledge several drugs against TGF-β1 have been developed and are either in nonclinical or in early stages of clinical investigation.

1. Introduction

Breast cancer (BC) is the most common and fatal cancer worldwide, with high morbidity and mortality in woman. It is ranked on the second place in mortality among cancer types [1], causing death of about 350,000 women in both developed and developing countries every year [2]. More than 90% of lethality in cancer patients is caused by metastasis, and the occurrence of distant metastases severely limits the prognosis of breast cancer patients [3]. The 5-year survival rate for patients with breast cancer drops precipitously from 98% for individuals with localized disease to 23% for those with metastatic disease [4]. Many factors are involved in the pathogenesis and progression of BC, including genetic, biological, and environmental factors, as well as lifestyle [2]. Cytokines belong to the biological factors, playing pivotal role in modulation of cellular growth, maturation, differentiation, and cancer progression. One of the cytokines responsible for regulation of cell behavior is Transforming Growth Factor-β (TGF-β), which has been extensively studied in regard to its various effects exerted on epithelial cells and derivative carcinoma cell populations *in vitro* and *in vivo*. TGF-β has been shown to inhibit epithelial cell cycle progression and promote apoptosis. These effects together significantly contribute to the tumor suppressive role of TGF-β during carcinoma initiation and progression. TGF-β is also able to promote epithelial to mesenchymal transition (EMT), in order to modulate immune system and tumor microenvironment, which have been associated with increased tumor cell motility, invasion, and metastasis [5–8]. In several types of human carcinomas, mutations or loss of heterozygosity (LOH) in central components of the TGF-β pathway has been observed [9, 10]. Functional insights into TGF-β pathway are vital for developing new therapeutic approaches in cancer. This publication is focused on the influence of TGF-β on human breast cancer pathophysiology.

2. TGF-β Characteristics

The superfamily of TGF-β cytokines consists of over 40 proteins, including: TGF-beta (β), activins (A, AB, B, C, E), inhibins (A, B), bone morphogenetic proteins (BMPs), and growth/differentiation factors (GDFs) [11, 12]. TGF-β is a polypeptide (112 AA), encoded by a gene located on the long arm of chromosome 19 (19q13) in humans [13]. TGF-β occurs in five isomeric forms (60–80% of homology), from β1 to

β5, produced by alternative splicing. TGF-β 1–3 are found in humans, other mammals, and birds [12]. TGF-β1 activity is pleiotropic. It is a predominant isoform in humans, synthesized by almost all cells, primarily by platelets, Treg cells, macrophages/monocytes, lymphocytes, fibroblasts, epithelial cells, and dendritic cells [12]. TGF-β1 plays pivotal roles in modulation of cellular growth, maturation and differentiation, ECM (extracellular matrix) formation, homeostasis, endothelial cell plasticity, immunoregulation, apoptosis, angiogenesis, and cancer progression [14–19]. TGF-β1, -2, and -3-specific mRNAs are detected in majority of primary breast cancers [20]. Plasma levels of TGF-β have also been reported to be elevated in patients with breast cancer. These levels correlate with disease stage and decrease following resection of primary tumor [20]. The members of TGF-β family are predictors of poor response to chemotherapy in women with BC [21].

TGF-β1 occurs as a homodimer (25 kDa molecular weight) and is released from cells as an inactive precursor, with propeptide latency-associated protein (LAP). TGF-β1 is connected by a disulphide bond through the LAP with Latent TGFβ binding protein (LTBP). LTBP1-4 is a component of the ECM and is necessary both for synthesis of TGF-β1 and its storage [12]. Thrombospondin-1 (TSP-1), an ECM protein, allows releasing TGF-β1 in an active form by changing the conformation of LTBP. Increased TGF-β1 activity has been observed in response to angiotensin II, low density lipoproteins (LDL), glucose, thromboxane A2, integrin, calpain, cathepsin D, chymase, elastase, endoglycosidase F, kallikrein, MMP-9, neuraminidase, plasmin, and TSP-1. Among the known inhibitors of this cytokine are follistatin, decorin, and α2-macroglobulin [5, 12].

Studies carried out on the mouse model indicate a key role of the members of TGF-beta family (TGF-β1, -β2, -β3) in establishing proper mammary gland architecture, regulating stem cell kinetics, maintaining the mammary epithelium in a functionally undifferentiated state, and inducing apoptosis in the involuting gland [11]. TGF-β1 inhibits mammary epithelial cells (MECs) proliferation in an auto/paracrine manner [11]. It is expressed during all stages of mammary gland development, with the lowest expression level during lactation. Studies on rodents and human mammary carcinoma cells implicate that prolactin, growth hormone (GH), EGF, and IGF-I act as inhibitors of TGF-β1 expression in MECs, while somatostatin and sex steroids are shown to stimulate the expression. Expression of TGF-β1 is also negatively regulated by ECM. Transcription of TGF-β1 is high in the absence of ECM and is considerably lower in the presence of endogenously synthesized basement membrane [22].

3. TGF-β1 Signaling

The TGF-β1 signaling pathway depends on the tissue context [23]. Specific membrane binding receptors are needed for signaling activity of TGF-β1 in the cells. The best known are dimeric proteins, TβRI (53 kDa), TβRII (75 kDa), and TβRIII (280 kDa). TβRI (also known as activin receptor-like kinase 5, ALK5) and TβRII are transmembrane receptors, which have serine-threonine kinase activity of the intracellular domains. The extracellular part of TβRII is activating the intracellular part by binding the ligand (autophosphorylation). Then the complex joins and recruits TβRI, which determines specificity of TGFβ recognition. Activated TβRII kinase phosphorylates serine fragments of sequence TTSGS-GSG in GS domain (domain rich in Gly and Ser) of TβRI, thus leading to activation of serine-threonine kinase in the receptor, and thereby the signal transduction cascade inside the cell is initiated [5, 8, 12, 23, 24]. The full heterodimeric complex is needed for correct signal transduction. Without presence of TβRI TGF-β1 can bind to the TβRII, but the transduction does not occur. In the absence of TβRII, the cells are insensitive to the action of TGF-β. TβRIII has a structure of betaglycan and has no enzymatic activity [12]. It is a coreceptor presenting TGF-β to the other receptors. TβRIII may also be an inhibitor of the signal transduction by preventing TGF-β1 binding to TβRII and TβRI in the mechanism independent of ligand binding, so in such situations it exerts regulatory function [5, 12, 23, 25].

Further signal transduction to the nucleus occurs with participation of cytoplasmic proteins, which are transcription factors and intracellular transmitters from Smad family. After activation of TβRI, the signal activates Smad2 and Smad3 proteins (R-Smad subclass; receptor regulated Smad) bound to the receptors, by phosphorylation of their C-terminal (SXS motif) residues. Phosphorylated R-Smad can be separated from the connection with the receptor and from protein SARA (Smad Anchor for Receptor Activation). SARA is a cytoplasmic protein anchored in the cell membrane that binds both the R-Smad and heteromeric complex of TGFβ1 receptors. It recognizes the nonphosphorylated R-Smad, joins it to the complex, and then dissociates. The next step is formation of a functional trimeric complex by phosphorylated R-Smad and co-Smad (common partner Smad), namely Smad4, and then this complex is translocated to the nucleus, where it regulates the transcription of TGF-β1-dependent genes, thus Smads have the activity of transcription factors [5, 24, 26, 27]. Smad4 cooperates with other transcription factors, such as FoxH1, Mixer, Runx-related proteins, and E2F, as well as transcriptional coactivators (e.g., p300 and CBP) and corepressors (e.g., SKI and SnoN, prooncoproteins) in the regulation of target genes [5, 26].

The activity of TGF-β signaling pathway is regulated by a negative regulatory feedback loop mediated by I-Smads (Smads inhibitors): Smad 6 and 7. They are able to interact with membrane receptors by forming stable complex with activated TβRI, and thus impairing their interaction with the R-Smad (inhibition of their phosphorylation). Smad7 expression is induced by TGF-β, leading to inhibition of the cellular response to this cytokine [26]. Smad7 has been shown to promote recruitment of E3 ubiquitin ligases (including Smad ubiquitin regulatory factors (SMURF1, SMURF2, PRAJA, WWP1, and Nedd4-2) into the receptor complex [8]. Binding of Smad7 and SMURF to the receptor complex also results in competitive inhibition of Smad2/3 binding to TβRI [25]. TGF-β1 signaling is also attenuated by other proteins, which interact with Smad7, like STRAP or YAP65 [5]. Therefore, there is an autoregulation of the negative feedback mechanism. Anti-TGF-β activity of Smad7 can be

negated by AMSH2 or Arkadia [28]. Under disease conditions, Smads also interact with other signaling pathways, such as the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathways [29, 30].

It is well known that TGF-β1 also signals in a Smad-independent manner (noncanonical pathways), by induction of other pathways, such as the extracellular signal-regulated kinase 1/2 (ERK1/2) and the p38 MAP kinase (p38 MAPK) [5, 18]. MAPK pathways are showing direct function in signal transduction of TGF-β1-modulated cellular migration and invasion [1]. At present, the Smad-independent pathways are known to include ShcA, RhoA-Rock1, RAC/CDC42, RAS, TRAF6-TAK1-p38/JNK, PI3 K, PAR6, MAP3 K1, DAXX, and PP2A [5, 6, 8, 18, 28]. RhoA-Rock1 signaling with engagement of TGF-β1 is required for the EMT [9, 31].

4. TGF-β1 Paradox in Cancer

The role of TGFβ-1 in cancer progression has been shown to be multifaceted, depending on the tumor stage. This cytokine acts as a potent growth inhibitor. It has been shown to inhibit epithelial cell cycle progression and promote apoptosis that together significantly contribute to the tumor suppressive role during carcinoma initiation and progression [5, 25, 32, 33]. However, the ability of TGF-β1 to induce and promote EMT associates this cytokine with increased tumor cell motility and invasion [34]. Thus, TGF-β1 is also regarded as a metastasis inducer, participating in malignant progression and angiogenesis [1, 5, 14, 25, 27, 33, 35–37]. These contrasting, dichotomous TGF-β1 behaviors in cancer development and progression are known as "TGF-β1 paradox" [17, 18].

Currently the link between tumor progression and modification of tumor microenvironment (interaction between carcinoma cells and adjacent cell populations) is under profound investigation. The role of TGF-β1 in this context has been studied by many research groups [5]. One of the predominant stromal-epithelial axes, associated with the regulation of cancer progression, involves carcinoma-immune cell interactions within the tumor microenvironment [38]. Specifically, TGF-β1 has been shown to suppress the antitumor activity of T cells, NK cells, neutrophils, monocytes, and macrophages that are known to have a significant role in the regulation of tumor progression [39].

Both tumor suppressor and tumor-promoting activities of TGF-β1 have been clearly demonstrated in a variety of genetically modified mouse lines, in which the TGF-β1 signaling pathway is ablated or modified [36]. These studies support a model, in which TGF-β1 inhibits the development of early, benign lesions, but promotes invasion and metastasis, when the tumor suppressor activity is overridden by oncogenic mutations in other pathways [5, 10, 36]. Changed expression of growth factors and their receptors has been shown to play an important role in the neoplastic formation and tumor progression.

5. TGF-β1 Role in Early Stages of Tumor Progression

In normal physiological conditions, TGF-β1 is a potent inhibitor of growth of many cell types (cytostatic effect as well as the effect on apoptosis), including neoplastic cells [26]. The TGF-β1 cytostatic responses primarily target G1 events, by regulating the expression of several genes promoting cell cycle arrest. In normal epithelial cells, TGF-β1 induces expression of p15Ink4b, which inhibits Cyclin D-Cdk 4/6 complexes, and of p21, which inhibits Cyclin E/A-Cdk2 complexes. In response to TGF-β1, activated Smad-FoxO (FoxO1, FoxO3, and FoxO4) transcriptional complexes target a region of the p21 promoter and mediate the induction of p21. On the other hand, TGF-β1 induction of p15Ink4b by activated Smad-FoxO complexes additionally requires C/EBP β for their response to TGF-β1 [26]. The TGF-β1 cytostatic effect also involves transcriptional repression of the growth-promoting transcriptional factors c-Myc and inhibitors of differentiation (ID 1,2,3) [8, 9]. TGF-β1 downregulates the expression of c-Myc in MECs and human skin keratinocytes. Repression of c-Myc is mediated by binding of a transcriptional repression complex containing Smad, E2F4/5, p107, and C/EBPβ to the TGF-β1 inhibitory element in the proximal region [26].

In the early stages of cancer development, cancer cells respond to antimitotic effect of TGF-β1. TGF-β controls cell proliferation mainly by inhibiting cell cycle progression through G1-arrest, by inducing or activating cdk inhibitors such as p16INK4A, p15INK4B, p21CIP1, and/or p27Kip1 [6, 8]. When the tumor cells are entering the phase of uncontrollable growth, the majority of them lose sensitivity to this inhibitory effect [40]. Surprisingly, this occurs despite the presence of the TGF-β1 receptors on the tumor's cell membranes. Furthermore, these cancer cells also begin to secrete TGF-β1. On the other hand, it has been noted that in early development increased TGF-β1 expression leads to inhibition of mammary epithelial outgrowth *in vivo* [41]. Also a decreased incidence of tumorigenesis induced by infection with the mouse mammary tumor virus in mammary epithelium with TGF-β1 expression was reported [41].

Indirect regulation of tumor suppression by TGF-β1 can be correlated with blockage of paracrine factor production (stromal cell-derived factor-1, SDF-1) in the tumor stroma. While the impact of the stromal fibroblast on tumor progression has been known since early studies on breast cancer, the role of TGF-β in this process emerged from mouse models in which TGF-β signaling was impaired in stromal fibroblasts [9].

The TGF-β1-dependent immunosuppressive activity stimulates angiogenesis, increasing the affinity of cancer cells to cell adhesion molecules, and creates a microenvironment favorable to tumor growth and its metastasis—increasing cancer cells invasiveness. Additionally, TGF-β1 induces death of the surrounding healthy cells and thus eliminates their effect designed to inhibit tumor growth. It appears that cancer cells need higher TGF-β1 concentrations than normal cells to receive TGF-β1-anti-mitotic stimulus [12]. The results of clinical and experimental studies indicate that the molecular background of the lack of cell response to TGF-β1 during malignant transformation is the mutations in the TβRII receptor and/or within the Smad proteins [26, 40].

6. TGF-β1, TβR and Smad Mutations and Inactivation

Many investigations among different human populations have detected different mutations in the TGF-β1 [13]. Amani et al. [13] reported, for the first time, that the TGF-β1 haplotype "GTGCCGC" might be associated with BC in Iranian woman. Several somatic mutations that disrupt the TGF β1-Smad signaling pathway have been reported in human breast tumors [26, 42, 43]. These mutations may affect the different aspects of BC, including its occurrence, prognosis, progression, and metastasis. Single nucleotide polymorphisms (SNP) of genes are widely examined. 29T → C coding cSNP (Leu10Pro, rs1800470) is mostly studied in BC field, and it is the most prevalent polymorphism of TGF-β1. The Pro-allele is considered a "high-activity" (hypermorphic) allele compared to the Leu-allele [44]. A large study of the Breast Cancer Association Consortium (BCAC) has reported an association of the Pro-allele with a moderate, but significantly increased, BC risk (e.g., Pro/Pro versus Leu/Leu: OR, 1.16; 95% confidence interval, 1.08–1.25). Other studies have either reported an increased risk, an unaltered risk, or even a decreased risk associated with the Pro-allele [44]. The findings by Taubenschuß et al. [44] indicated that the Pro-allele may also lead to higher TGF-β1 secretion *in vivo* but that the observed effects on serum levels were less pronounced and more heterogeneous than *in vitro*. In this work [44] the L10P SNP of *TGFβ*1 was genotyped in 274 breast cancer patients and 252 female controls. The frequency of the Pro-allele was 40.0% in patients and 42.3% in controls. The Pro/Pro genotype was slightly less frequent in BC patients than in controls (16.1% and 19.0%, resp.). The fraction of patients with the Pro/Pro genotype tended to be increased in several patient subgroups associated with advanced cancer progression and/or poor prognosis. The same conclusions can be found in other studies [45–47]. That could be the evidence on the dual role of TGF-β1 in different cancer stages and cancer subclasses. It has been suggested that the Pro-allele is associated with a reduced risk of *in situ* tumors, but an increased risk of invasive BC; or with a reduced risk of early-stage invasive BC, but an increased risk of BC with advanced stages [48]. The BCAC study has reported higher odds ratios associated with the Pro-allele in patients with high tumor grade and stage, and negative ER and PR status, although only the latter association was statistically significant. Similarly, the Pro-allele was associated with a reduced risk of early-stage BC, but an increased risk of BC with advanced stages [44].

The majority of tumor-derived mutations in Smad2 and Smad4 cluster are in the MH2 domain. Some of them have been shown to disrupt TGF-β1 signaling by blocking receptor dependent phosphorylation, or by disrupting oligomerization of the Smads. Smad4 harboring the missense mutation in the MH1 domain exhibits accelerated induction of ubiquitin dependent proteasomal degradation in comparison with wild-type Smad4. Skp2 (S-phase kinase-associated protein2) is upregulated in various human cancers and promotes the ubiquitination-dependent degradation of these Smad4 cancer mutants [26]. In the case of Smad3 no mutation has been found in human cancer. STAT pathway and NF-κB pathway also induce Smad7, thus, tumor cells with high activity of those pathways might evade the TGF-β cytostatic responses through overexpression of Smad7. The important negative regulators of TGF-β1 signaling are SKI and SnoN, which interact with Smad-2 -3 and -4. Increased expression of SKI or SnoN has been implicated in the progression of ER-positive (ER+) breast carcinoma. When SKI and SnoN are downregulated (RNAi) TGF-β1-mediated growth inhibition in BC is restored [26].

At present, a substantial number of correlative data demonstrate that TGF-β1 signaling components, including TβRI, TβRII, Smad2, and Smad4 are often lost in human cancer. Consistent with its tumor suppressor role, many cancers lose or attenuate TGF-β-mediated antimitogenic action by mutational inactivation of TGF-β receptors or Smads [49]. Studies using transgenic mice with conditional knockout of TβRII indicate that loss of TβRII in the context of polyomavirus middle T antigen (PyVmT) expression results in a shortened median tumor [49]. Mutations and loss of type I and type II TGF-β1 receptor expression have been detected in most types of common cancer, including those that occur in the biliary tract, bladder, breast, colon, esophagus, stomach, brain, liver, lung, ovary, pancreas, and prostate [5, 10, 27, 50]. In human BC, the alterations of TGF-β1 signaling molecules are relatively rare, except for TβRII downregulation [6, 51]. Pathological studies of archived breast samples, including benign lesions, ductal carcinoma *in situ* (DCIS), and invasive mammary carcinomas (IMC), indicated that TβRII downregulation is correlated with progression and aggression of both *in situ* and invasive breast carcinoma [52]. Recent studies have shown that silencing of the *TβRII* gene can occur through methylation in human breast carcinoma cells [53]. In human MECs and human mammary carcinoma cell lines the expression of TGF-β1, TβRI, and TβRII was concurrently suppressed by methylation, and these genes could be coordinately reinduced upon demethylation [54]. TβRII inactivation enhances the invasiveness of premalignant or low-grade breast tumor cells but reduces the metastasis of high-grade tumors [55]. In the opposite, TβRIII may act as a suppressor of BC, as the decrease or loss of TβRIII expression occurs in approximately 90% of BC at mRNA levels and 70% at protein levels. Additionally, TβRIII loss occurs at substantially high levels in advanced, invasive breast carcinomas. Therefore, its loss may be a negative prognostic factor for patients with invasive BC [56].

7. Role of TGF-β1 in Late Stages of Tumor Progression and Metastases

In the late stages of tumor progression TGF-β1 is changing its action into tumor promoter. Several studies have shown a broad range of potential TGF-β1 effects on cancer metastasis [21]. Immunostaining analyses revealed a correlation between TGF-β1 expression and metastasis in breast, colon, and prostate cancer. In addition, the intensity of TGF-β1 staining in invading lymph node metastases was higher in breast and colon cancers than in the primary tumors [9]. Metastasis is a multistep cascade process, including EMT, cell migration, invasion, intravasation, and extravasation from the circulation [23, 26, 52, 57]. Cheng et al. [58] demonstrated

using an *in vitro* assay that cancer cells cultured under fibroblast-conditioned medium showed increased proliferation and motility, indicating the role of stromal TGF-β1 signaling in neoplastic progression. Conversely, attenuation of carcinoma cell response to TGF-β1 by a dominant-negative type II receptor transgene (dnTβRII) significantly reduced tumor latency in the presence of TGF-α expression in mammary epithelium [52]. This indicates that specific TGF-β1 signaling in carcinoma cell is able to promote tumor cell invasion. It has been suggested that Smads are involved in the antitumor process, while the Smad-independent pathways have been implicated in induction of tumor progression [27, 59]. However, recent data also demonstrate that Smad-dependent pathways are involved in the tumor-promoting activities of TGF-β1. Smad-3 and -4 are necessary for the metastatic expansion of bone neoplasms, whereas Smad-2 in the case of lung, liver, and brain tumors, respectively [9, 60]. Approximately 40% of the human breast cancers show a positive TGF-β gene response signature, that is context dependent and appears more in ER-tumors (as opposed to ER+ tumors) and in lung metastasis (as opposed to bone metastasis) [8, 60]. The mechanism of the TGF-β induced lung metastasis in breast cancer is related to the induction of the angiopoietin-like 4 (*ANGPTL4*) gene by TGF-β Smad-dependent signaling in the primary tumor, enabling the cells which leave the breast to disrupt the lung capillary walls. The fenestrated capillaries of the bone marrow do not have any advantage from the action of *ANGPTL4*, and that might explain why the impact of TGF-β is directed to lung and not to bone metastasis [6, 8, 9, 27, 60].

At least seven genes (*IL-11*, *CTGF*, *CXCR4*, *MMP-1*, *PTHrP*, *VEGF,* and *PTGS2/COX2*) have been identified as drivers of human breast cancer bone metastases in the MDA-MB-231 model, and each of these genes is transcriptionally regulated by and dependent on TGFβ signaling *in vivo* [20]. TGF-β is a major contributor to the bone metastases, and TGF-β is released from bone matrix by the activated osteoclasts that degrade the bone matrix. Secreted TGF-β stimulates releasing of other osteolytic cytokines, such as parathyroid hormone related protein (PTH-rP), IL-11, and CTGF (Connective Tissue Growth Factor) from the metastatic cells to maintain the metastatic process.

The work of Micalizzi et al. [61] indicated that Six1 may be a critical mediator of the switch in TGF-β1 signaling from tumor suppressive to tumor promotional. However, the mechanism by which Six1 impinges on the TGF-β1 pathway remains unclear. Scientists [61] have shown in *in vivo* experiment that a target for Six1 is TβRI, and Six1 overexpression is required to switch TGF-β1 signaling to the prometastatic phenotype, showing that induction of EMT is not sufficient to induce experimental metastasis. Instead, TβRI upregulation in the absence of Six1 overexpression actually inhibited metastatic spread *in vivo* in an experimental metastasis model. Thus, Six1 is regarded as a determinant of TGF-β1 function in BC. Six1 is misexpressed in numerous cancers including breast cancer. In human BC, Six1 correlates with advanced disease and adverse patient outcomes [62].

Several studies on mouse models showed that MECs specific expression of activated TGF-β1 ligand or expression of active TβRI could enhance BC-associated lung metastases *in vivo* [57]. In the study of Darakhshan and Ghanbari [35] administration of tranilast with tamoxifen (TAM) downregulated the expression of TGF-β1, β-2, and β-3, as well as TβRI and TβRII in breast cancer cells (MCF-7 and MDA-MB-231 human breast cancer cell lines). TβRIII is a suppressor of BC progression and when its expression is restored, invasion, angiogenesis, and metastasis are inhibited *in vivo* [56]. In the study of Darakhshan and Ghanbari [35] tranilast and TAM slightly increased the expression of TβRIII.

8. TGF-β1 and EMT

BC starts as a local disease and can metastasize to distant organs. The conversion of early stage tumors into invasive malignancies has been associated with the activation of EMT, defined as changes in cell phenotype from an epithelial to a mesenchymal state, which is both a fundamental event and a hallmark in tumorigenesis [26]. Changes during EMT lead to the transition from a polarized epithelial phenotype to an elongated fibroblastoid phenotype, then cells degrade the ECM, and show invasive behavior [63–65].

TGF-β function is often accompanied by desmoplastic and fibrotic reactions, which elicit dramatic changes in the biomechanical properties of the tumor microenvironment. The elastic modulus of stroma housed within breast carcinomas is approximately 10 times more mechanically rigid than that of adjacent normal breast tissues. TGF-β potentiates these biomechanical reactions by stimulating the expression and secretion of a variety of ECM components, such as collagen I and fibronectin from stromal fibroblasts, and of ECM cross-linking enzymes, such as lysyl oxidase from mammary carcinoma cells. The formation of these rigid mammary tumor microenvironments promotes metastatic progression in breast cancers and also predicts poor clinical outcomes in patients harboring metastatic disease [66].

TGF-β1 was shown to play important regulatory role in EMT [8, 16, 67]. Identification of TGF-β as a major inducer of EMT came from *in vitro* studies on cell cultures. Treatment of normal mouse breast epithelial cells with TGF-β changes the cuboidal shape to an elongated spindle, accompanied by a decrease in epithelial markers and increased expression of mesenchymal markers [9, 28]. It induces the increase in cell size and protein content during EMT (as a result of mTOR activation) [68–70]. Its signaling downregulates claudins, occludins, and ZO1, followed by tight junctions degradation [63]. TGF-β1 also upregulates integrin-linked kinase (ILK), increasing cellular motility [23, 63, 69, 71]. Expression of integrins (e.g., α5, αv, β1, β3, β5) that bind the ECM is also enhanced by TGF-β cell signals. Ligation of these integrins ($\alpha 2\beta 1$: collagen, $\alpha 5\beta 1$: fibronectin, $\alpha v\beta 3$ or $\alpha v\beta 5$: periostin) induces the production of TGF-β, leading to a feed forward loop between tumor cells and the ECM [72].

TGF-β1-induced EMT is largely studied using NMuMG murine mammary epithelial cells, because these MECs are known to undergo EMT readily apparent 36 h after TGF-β1 treatment [68, 69, 73]. In this kind of EMT canonical Smad signaling, as well as Smad-independent signaling, (through small GTPases and the ERK1/2 and p38 MAPK pathways) is

integrated [25, 28, 73]. Increased expression of Smad3 and Smad4 in the presence of constitutively active TβRI enhances induction of EMT [9]. Smads act as transcription factors of EMT regulators, such as Snail/Slug/Twist, Cripto-1, FOXC2, and Six1 [6, 28, 61, 74]. For example, activation of Smad2/3 by TGF-β in MECs induces the expression of the nuclear high mobility group A2 (HMGA2), which promotes EMT by stimulating the expression of Snail1, Snail2/Slug, and Twist, and by inhibiting the expression of ID2 [28]. The expression of E-cadherin is repressed by TGF-β1-mediated SNAIL-Smad3/4 complex which negatively regulates E-cadherin in breast epithelial cells [24, 28, 75]. E-cadherin is also repressed by HMGA2, TBX3 (The T-box transcription factor) [76] and ZEB 1/2 (Zinc finger E-box-binding homeobox 1/2) [18, 25]. Furthermore E-cadherin is lost during EMT and cancer progression [63, 65].

Matrix rigidity converts TGF-β from a proapoptotic molecule to an inducer of EMT in NMuMG and MDCK cells, by enhanced coupling of TGF-β to the PI3 K/Akt pathway [74]. Lamouille and Derynck [68] have used the NMuMG cell model and observed that the typical loss of epithelial phenotype with concomitant acquisition of the spindle-shaped fibroblastoid phenotype, induced in the presence of TGF-β, was accompanied by an increase in cell size and protein content and correlated with rapid mTOR activation. The authors observed also that rapamycin inhibited the migration and invasion of cells after TGF-β-induced EMT, which is in agreement with recent observations that rapamycin inhibits the induced motility of some cancer cells [28, 77]. As PI3 K and mTOR activities are commonly upregulated in various cancers, PI3 K inhibitors and rapamycin analogues are investigated as inhibitors of cancer progression in preclinical and clinical trials [68, 69].

Studies suggest that Twist, Snail, and TGF-β may induce the expression of cell surface markers associated with cancer stem cells and these cells share high homology to bone marrow-derived mesenchymal stem cells [28].

9. TGF-β-Induced EMT and microRNA Regulation

There is a double negative feedback loop between microRNA: miR-200 (transcriptional targets of ZEB), miR-205, and ZEB, which allows for the plasticity existing between the cell's epithelial and mesenchymal state [28, 74, 78–80]. In addition, the same microRNAs are frequently downregulated in invasive human breast cancer cells that exhibit a mesenchymal-like morphology [28]. Recent studies demonstrated that highly metastatic 4T1 breast cancer cells are more epithelial-like as compared to their isogenic and nonmetastatic 4T07 counterparts [28]. Amongst the many unique differences between these two isogeneic cell types is the reexpression of miR-200 in metastatic 4T1 cells, leading to the synthesis and secretion of metastasis-promoting proteins necessary for metastatic outgrowth [28, 80]. In contrast to the miR-200 family, metastatic breast cancers were found to preferentially upregulate the expression of miR-10b, which promotes the invasion and metastasis of malignant MECs both *in vitro* and *in vivo* [28, 81].

In the study of Xu et al. [78] TGF-β1 secretion resulted in an increased level of ZEB1 transcription in MCF7 cells, that could reach a point, where ZEB1 transcription and protein accumulation could overcome the repression caused by miR-200, resulting in the progression of the EMT. The downregulation of paracrine TGF-β1 signaling could reduce ZEB1 and ZEB2 expression, upregulate miR-200b and miR-200c, and finally inhibit the progression of the EMT. In breast cancer progression, miR-221/222 expression is increased in aggressive basal-like subtype breast cancers. In these tumors, miR-221/222 directly represses the expression of the GATA family transcription factor TRPS1, a repressor of ZEB2. By this action miR-221/222 is promoting downregulation of E-cadherin expression, and EMT-associated increased cell migration and invasion [82].

Conversely, miR-520/373 members act as tumor-suppressive miRNAs, and increased miR-520c or miR-373 expression inhibits the invasive behavior of breast cancer cells *in vitro* and *in vivo*, in part by targeting TβRII [83]. Direct repression of Smad7 can be seen in action of miR-106b-25 cluster [84]. In the work of Kong et al. [85] administration of TGF-β to normal murine MECs (NMuMG) induced miR-155 expression through a Smad4-dependent mechanism. Once expressed, miR-155 abrogated MEC expression of RhoA and prevented their ability to undergo EMT in response to TGF-β [28, 74, 85]. miR-181a expression is also highly associated with the development of metastatic disease in BC. In the study of Taylor et al. [66], TGF-β treatment of NMuMG cells induced EMT resulting in a significant upregulation of miR-181a expression. Furthermore, it was demonstrated that inactivation of miR-181a prevented the loss of E-cadherin expression stimulated by TGF-β.

10. TGF-β1 and ECM Degradation

ECM degradation is an important part of the metastatic process. In breast cancer destabilization of p53 by Mdm2 (E3 ubiquitin-protein ligase Mdm2) is a pivotal step in EMT. Late-stage metastatic breast cancer progression can be correlated with TGF-β1-induced expression of Mdm2. Furthermore, in bones (common site of BC metastasis) cancer cells are able to activate osteoclasts influencing the extracellular matrix degradation, and releasing growth factors stored there (TGF-β, IGF, BMP). TGF-β1 was shown to stimulate cancer cells for osteolytic cytokines induction (e.g., PTHrP—stimulator of NFκB ligand RANKL production), which enhanced the osteoclast differentiation [9, 26]. In the lung metastasis by breast cancer cells, TGF-β1 signaling in the tumor microenvironment primed cancer cells for pulmonary metastasis [26, 86].

Essential regulators of ECM degradation are matrix metalloproteinases (MMPs) [63, 65], their specific inhibitors TIMPs (tissue inhibitors of MMPs), and the membrane-associated MMP inhibitor (RECK). The balance between these molecules regulates motile and invasive capacities [1, 87]. MMP-2, MMP-9, MMP-14, and TIMP-2 are linked with BC progression [88, 89]. Many scientific reports have suggested a crucial function of TGF-β1 as a modulator of MMPs [90, 91]. TGF-β enhances the tumorigenicity and

invasiveness of breast cancer cells by inducing their expression of MMPs 2 and 9 [28]. Gomes et al. [1] demonstrated for the first time that TGF-β1 is able to modulate MMP, TIMP, and RECK expression in MDA-MB-231 human breast cancer cells through ERK1/2 and p38MAPK pathways. Both of these transducer pathways were essential for the TGF-β1-enhanced migration and invasion phenotypes; however, each mediated the TGF-β1 signal for MMPs and their inhibitors in a specific manner. This study demonstrated that, similarly to MMPs, TIMPs, and RECK, the expression of TβRI and TβRII was higher in the most aggressive cell line (MDA-MB-231), as compared to the less invasive ones, except for TβRI, that was also highly expressed in ZR-75-1 cells [1]. Kim and collaborators [92] suggested that TGF-β1 also induces invasion in premalignant breast cancer cells (MCF10A), by upregulation of MMP-2 and MMP-9 [92]. Subsequent reports also indicated that MMP-2 and MMP-9 are essential in the TGF-β1-increased invasion of MCF10 cell series in a 3D *in vitro* model [24, 93].

The results suggest that TGF-β1 could suppress primary tumor growth while promoting metastasis through EMT of the responding carcinoma cells. In a mouse model of mammary carcinoma, with complete ablation of TGF-β1 response in mammary epithelium, a decrease in tumor latency was observed. Furthermore, a striking increase in pulmonary metastasis was also clearly demonstrated [94]. In this model the loss of TGF-β signaling in the mammary carcinoma cells caused also an increased abundance of smooth muscle actin positive stroma, tumor cell heterogeneity, and tumor cell survival. Additionally, TGF-β regulated chemokine expression resulting in carcinoma-immune cells, which could be related to mammary carcinoma cell metastasis [94, 95]. A reduced response of tumor cells to TGF-β signaling is often accompanied by an increase in secretion of this ligand. In breast cancer patients with poor prognosis [94], TGF-β1 levels were often elevated in plasma, tumor cells, and associated stroma [23, 96].

11. Mesenchymal-Epithelial Transition (MET)

Following EMT, metastatic cells can revert back and reacquire epithelial properties, similar to cells in the primary tumor [97]. This process is called mesenchymal-epithelial transition (MET) and contributes also to formation of tissues and organs during development [82]. It is less characterized than EMT, but MET can correlate with the establishment of secondary tumors following metastasis. Interestingly, members of the miR-106b-25 cluster can promote a MET-like process and enhance the induction of iPSCs (Induced Pluripotent Stem Cells) reprogramming through targeting TβRII [82], whereas, miR-200 family has been implicated in promoting MET through their ability to repress the expression of ZEB1 and ZEB2, leading to upregulated E-cadherin expression [74].

12. TGF-β1 and Immune Cells in Tumor Microenvironment

TGF-β1 mediates recruitment of tumor promoting myeloid cell populations. Mammary carcinoma cells specific ablation of TGF-β1 signaling led to enhanced metastasis and was associated with an increased myeloid cell infiltrate in mice [94]. Also GR1+ CD11b+ and F4/80+ myeloid cells were recruited to the leading edge of tumors exhibiting a carcinoma cell specific ablation of TGF-β1 responsiveness [98]. It was correlated with increased expression of Cxcl1 and Cxcl5 in TGF-β1 signaling deficient tissues *in vitro* and *in vivo*. *In vivo* studies showed that CxcR2 signaling significantly contributed to enhanced metastasisobserved from the TGF-β1 signaling deficient mammary carcinoma cell population, when compared with the control mammary carcinoma cells [98]. When TGF-β1 is available in the tumor microenvironment for stimulation of adjacent cell populations, including immune cell infiltrates, it can have a significant impact upon antitumor activity of T cells [5]. IL-2 dependent T-cell signaling has been reported to involve suppression of IL-2 production by Smad3. TGF-β1 was also shown to regulate T-cell growth arrest (p21Cip1 and p27Kip1 are known TGF-β1 targets) in the presence of exogenous IL-2 and IL-4, that would normally promote proliferation. TGF-β1 is also known to suppress T-cell mediated tumor rejection [3]. TGF-β1 can cause host macrophages to become suppressors of CD4+ T-cell proliferation. It has recently been shown that the CD4+ CD25+ regulatory T-cell population can provide a significant source of TGF-β1, that is responsible for attenuation of tumor antigen expanded CD8+ cytotoxic T cells (CTLs) [27, 99]. At present a number of studies have clearly demonstrated that TGF-β1 can suppress cytotoxic T-cell differentiation and cytotoxic T-cell mediated lysis of carcinoma cells [100]. Additionally, TGF-β1 was shown to prevent the expression of granzyme A, granzyme B, perforin, Fas ligand (FasL), and interferon-gamma-promoters of CTLs cytotoxicity [8, 100, 101]. Granzyme B and interferon-gamma expression was directly linked to Smad transcription factors [101]. Moreover, it has been reported that TGF-β1 stimulation inhibits NK cell and neutrophil effector functions, which contributes to tumor progression in a permissive microenvironment. In addition, TGF-β1 has been shown to suppress MHC I and MHC II expression in a number of cell populations. Importantly, the TGF-β1 dependent decrease of MHC I expression in tumor cells results in reduced tumor cell lysis by NK cells, thereby enhancing tumor growth and metastasis [102]. TGF-β1 is one of the most potent known chemoattractants for human peripheral blood neutrophils that also inhibits their ability to suppress tumorigenesis and potently regulates the interaction between neutrophils and other cell populations within the tumor microenvironment [5]. Neutrophils function in the tumor microenvironment is the recognition and destruction of carcinoma cells expressing FasL. In the presence of TGF-β1 neutrophils exhibit a decreased ability to eliminate such cells [5]. TGF-β1 is also promoting recruitment of monocytes, and it has been suggested to promote monocyte to macrophage differentiation [5]. TGF-β1 is able to block both the priming by interferon-γ and triggering by lipopolysaccharide (LPS) of macrophages, necessary for the efficient killing of tumor cells by macrophages [5]. Conversely, tumor necrosis factor alpha (TNF-α) cytotoxicity is functioning independently of TGF-β1 influence. TGF-β1 pretreatment of carcinoma cells attenuates both the cytotoxicity and cytostatic ability of macrophages *in vitro* [5]. TGF-β1 stimulation of macrophages has been shown

to attenuate macrophage-associated suppression of CD4+ T-cell proliferation [5]. TGF-β1 also suppresses MIP-1a, MIP-2, CXCL1, IL-1β, IL-8, GM-CSF, and IL-10 expression. In monocytes, TGF-β1 has been shown to promote expression of IL-1 and IL-6 and suppress oxygen free radical production [5]. It plays also a pivotal role in inducing the differentiation of Tregs (CD4+ CD25+ Foxp3+ regulatory T cells) [103], which are thought to be the main obstacle tempering antitumor immunity and immunotherapy. Their localization and the infiltrating patterns vary in BC and have different impacts on tumor progression but can be prognostic factor for BC [8].

13. Anticancer Therapeutic Strategies against TGF-β

Due to its growth-suppressive effects, in the past, TGF-β has been regarded as an attractive cytokine for the treatment of cancer. Therefore, studies were initiated to explore the potential role of TGF-β as an adjuvant for chemotherapy. TGF-β was able to protect normal cells and sensitize tumor cells towards standard chemotherapy in some preclinical models [9]. Given TGFβ's pleiotropic effects on both tumor cells and host cells, and its presumed role in tumor metastasis, detailed assessment of antitumor effects of TGFβ antagonists can only be accomplished by using models of metastatic mammary cancer: the murine metastatic mammary cancer cell lines 4T1 (Balb/C), EMT6 (Balb/C) and R3T (129S1), and the human metastatic MDAMB-231, MDA-MB-435, MCF10ACA1A, and MX-1 cell lines that are inoculated into immunodeficient mice [20].

With current knowledge about the involvement of TGF-β in progression and metastasis of cancer, there are 3 different approaches against TGF-β, which have therapeutic potential.

(1) Using antisense molecules to prevent TGF-β synthesis on ligand level; (2) blocking the ligand-receptor interactions by using ligand traps (monoclonal antibodies and soluble receptors) and antireceptor monoclonal antibodies; (3) inhibiting signaling cascade on the intracellular level (with the use of TGF-β receptor kinases inhibitors and peptide aptamers) [6–9]. For each of these approaches, several drugs have been developed and are either in nonclinical or in early stages of clinical investigation. A few examples can be found for BC therapy.

Antisense molecules are single stranded oligonucleotides (13–25 nucleotides) [8]. Since TGF-β production is usually increased during tumor progression, blocking its synthesis and TGF-β-mediated gene expression have the potential to reduce excess TGF-β levels within the tumor microenvironment. Antisense mediated inhibition of TGF-β1 gene expression has been shown to be effective in reducing malignant properties of mouse fibrosarcoma cells and murine 4T1 cells [104]. Some of the tested molecules were shown to be efficient in treatment of pancreatic cancer (AP12009 Trabedersen) or prostate carcinoma (AP11014 and AP15012) [8, 9, 104].

Ligand traps can control excess of TGF-β production in tumor microenvironment. A neutralizing monoclonal antibody (mAB), 1D11 (Genzyme Corp.,Sanofi), that binds TGF-β 1, 2, and 3, resulted in suppression of lung metastasis in metastatic breast cancer mouse model, mainly by increasing the antitumor response of CD8+ T cells [105]. It also gave decreased bone loss by reduced expression of PTHrP and its regulator Gli2 [50]. In the *in vitro* experiments of Tan et al. [20], which assessed the efficacy of the murine anti-TGFβ monoclonal antibody 1D11, the experimental metastasis models were used: bone-tropic and lung-tropic MDA-MB-231 human breast cancer cells sublines (preferentially metastasize to lungs: MDA-231-4175TR or bones: MDA-231-SCP2TR and 2860TR). Treatment with 1D11 was able to block TGFβ-induced phosphorylation of the receptor-associated Smads, Smad-2 and -3, in each of these cell lines. While 1D11 had no effect on cell growth *in vitro*, it inhibited TGFβ-stimulated tumor cell migration and invasiveness into Matrigel. Treatment with 1D11 antibody significantly reduced the burden of MDA-231-SCP2TR or 2860TR-derived metastases to bones, as well as MDA-231-4175TR-derived metastases to lungs by ~40%.

In preclinical trials another mAB, 2G7, showed efficacy in inhibiting breast cancer metastasis by increasing NK cells activity and preventing radiation induced acceleration of metastases [20, 104, 106–108]. Genzyme had developed three fully humanized mABs: GC-1008 (Fresolimumab), CAT-152 (Lerdelimimab), and CAT-192 (Metelimumab), which were tested in clinical trials [3, 104]. GC-1008 is capable of neutralizing all three TGF-β isoforms. CAT-152 is in phase III clinical trials for some metastatic tumors [3, 59]. GC-1008 was tested in phase I/II clinical trials. Two trials of GC-1008 are in recruitment phase: Fresolimumab and radiotherapy in metastatic breast cancer (NCT01401062), and safety and imaging study of GC1008 in glioma (NCT01472731). The other two mABs have not been tested yet on cancer patients.

Another way of blocking TGF-β is to use soluble receptors. For example soluble TβRII and TβRIII have been tested in preclinical studies in breast and pancreatic cancer metastasis [104, 109–111]. Muraoka et al. [109] have shown that systemic administration of Fc:TβRII increased apoptosis of primary mammary tumors expressing PyMT (polyoma middle T-antigen) and reduced tumor cell motility, intravasation, and lung metastases. Similarly, Fc:TβRII also inhibited metastases from transplanted 4T1 and EMT-6 mammary tumors in syngeneic BALB/c mice. Expression of soluble TβRII reduced BC and pancreatic cancer metastasis. No clinical trials have been undertaken with these soluble receptors until now.

In signal transduction blockade two different strategies can be proposed: the use of receptor kinase inhibitors, and targeting the intracellular TGF-β signaling pathway molecules, such as Smads, with peptide aptamers [6, 8, 50]. Peptide aptamers are small molecules containing a target binding site and a scaffolding domain that impedes the function of the target [8].

Targeting receptor kinases has been intensively investigated, because such drugs are easy to produce and can be administrated orally [50]. Ki26894, SD-208, and LY364937 are TβRI inhibitors, which appeared to be promising in terms of inhibiting metastasis to bone. Experiments were conducted using breast and gastric cell lines *in vitro* [112, 113] and xenografts mouse model *in vivo* [112, 114]. In the experiments of Ehata et al. [112] treatment with Ki26894 blocked TGFβ

signaling in MDA-MB-231-D cells, which was detected by suppression of Smad2 phosphorylation and inhibition of TGFβ-responsive target genes activity. Moreover, Ki26894 decreased the motility and the invasion of MDA-MB-231-D cells induced by TGFβ *in vitro*. Systemic Ki26894 treatment initiated 1 day before the inoculation of MDA-MB-231-D cells into the left ventricle of BALB/c nu/nu female mice resulted in decreased bone metastases and prolonged survival compared to vehicle-treated mice.

SD-208 has been shown to inhibit growth of primary tumors and pulmonary metastasis in tests with two murine mammary carcinoma lines, R3T and 4T1 [20, 104]. SB-431542, the most widely used TβR1 inhibitor, has been shown to inhibit tumor metastasis in breast cancer [3, 115], glioma, and renal cell carcinoma in the preclinical stage [3]. LY2109761 is a small molecule inhibiting the kinase activity of both TβRI and TβRII. This compound inhibits metastasis formation in mouse models of breast cancer [8, 9, 104, 116]. LY2157299 (Eli-Lilly & Co) is a TβRI kinase inhibitor that reduces growth of lung and breast cancer cell lines and has been shown to inhibit primary tumor growth induced by the Calu6 non-small lung cancer line and the MX1 breast cancer line in nude mice [8, 9, 104, 117]. This is the only TGF-β receptor kinase inhibitor that is currently tested in clinical trials.

SD-093 and LY-580276 have been shown to block EMT and tumor cell migration in pancreatic cancer and mouse mammary epithelial cells, respectively [118]. EW-7203, EW-7195, and EW-7197 inhibited Smad/TGF-β signaling, cell migration, invasion, and lung metastasis of breast cancer cells in 4T1 and MDA-MB-231 orthotropic xenograft mice and MMTV/cNeu transgenic mice. They inhibited EMT in both TGF-β treated breast cancer cells and 4T1 orthotropic xenograft mice. The dose 1.25 mg/kg of EW-7197 increased the survival time of 4T1-Luc and 4T1 breast tumor bearing mice [119]. Preclinical study with EW-7197 was completed. Fang et al. [3] have shown the efficacy of a novel small molecule YR-209, inhibitor of TβRI kinase. They have examined the effects of YR-290 on breast cancer cell migration and metastasis *in vitro* (MDA-MB-231 cell line, mouse breast tumor 4T1 cell line, human breast carcinoma cells Hs578T and BT-549, and human keratinocyte cell line HaCaT) and in tumor metastasis mouse models. YR-290 inhibited breast cancer cell migration, invasion, and EMT induced by TGF-β in a dose-dependent manner. In three different mouse tumor metastasis models YR-290 preventively and therapeutically blocked breast cancer pulmonary and skeletal metastasis by suppressing the TGF-β pathway. Treatment with YR-290 also statistically significantly prolonged the survival of tumor-bearing mice.

Trx-SARA is an example of a peptide aptamer, which reduces the levels of TGF-β-induced Smad-2/-3 in complex with Smad-4, and inhibits EMT after TGF-β stimulation in breast cancer epithelial cells [8, 120]. So far no clinical trials have been undertaken with peptide aptamers.

14. TGF-β1 and Cancer Stem-Like Cells (CSCs)

After initial response to chemotherapy many patients have recurrence of drug resistant metastatic disease, especially in triple-negative breast cancers (TNBCs). Some studies revealed that these relapses could be caused by populations of cancer stem-like cells (CSCs) with self-renewing and tumor-initiating capacities. TGF-β1 has been shown to increase stem-like properties in human breast cancer cells [121]. Bhola et al. [121] have analyzed RNA expression in matched pairs of primary breast cancer biopsies before and after chemotherapy. Biopsies after chemotherapy displayed increased RNA transcripts of genes associated with CSCs and TGF-β1 signaling. Also Shipitsin and colleagues [53] showed that subpopulations with CSC features (CD44+) within breast tumors overexpressed TGF-β1 and the TβR1. TGF-β1 ligands are often enriched in the TNBC tumor microenvironment and can be produced by tumor cells or by tumor-associated stromal and immune cells [94, 121]. These data suggest the possibility that the TGF-β pathway is involved in maintenance of CSCs in breast carcinomas. In a study of Bhola et al. [121] in TNBC cell lines (SUM 159, BT549, SUM149, MDA231) and mouse xenografts, the chemotherapeutic drug paclitaxel increased autocrine TGF-β1 signaling and IL-8 expression in CSCs, as indicated by mammosphere formation and CSC markers. The TβRI kinase inhibitor LY2157299 and SMAD4 siRNA blocked paclitaxel-induced *IL8* transcription and CSCs expansion, as well as paclitaxel-induced SUM159 and BT549 mammosphere formation. Moreover, treatment of TNBC xenografts with LY2157299 prevented reestablishment of tumors after paclitaxel treatment.

Pathways that control stem-cell proliferation are another option for cancer treatment. The canonical Wnt signaling maintains the growth of stem cells. For example in the intestine, the presence of TGF-β-signaling and the absence of Wnt signaling in the villus compartment result in rapid cell cycle arrest and differentiation. Thus, Tcf4 (affected by Wnt signaling) and Smad-4 constitute a dominant switch between the proliferative progenitor and the transitional progenitor of differentiated epithelial cell [8].

15. Combination Therapy Approaches

Investigators at Genzyme, Inc., have examined the antitumor effects of the pan-TGFβ neutralizing antibody, 1D11, in combination with various common chemotherapeutics against mammary cancer models. The combination of 1D11 with CDDP Cisplatin resulted in long-term survivors in the 4T1 murine breast cancer cells in experimental bone metastasis assay. More recently, the same investigators demonstrated at least additive dose-dependent effects of 1D11 against several human tumor xenografts (including breast and renal cell) when combined with a variety of cytotoxic agents, including paclitaxel, CDDP, doxorubicin, or CTX (cyclophosphamide). Similarly, scientists at Genentech have shown that the 2G7 potentiates the efficacy of docetaxel in 4T1 spontaneous lung metastasis assays [20].

Bhola et al. [121] determined whether the efficacy of doxorubicin in the inhibition of tumor growth and lung metastasis could be improved by simultaneous treatment with a pyrazole-based TβRI kinase inhibitor (Biogen Idec HTS466284, Eli Lilly LY364947). In these studies, murine breast cancer 4T1 cells were inoculated into both inguinal

mammary fat pads of Balb/c mice. Results of these experiments indicated that while the TβRI inhibitor alone failed to inhibit tumor growth, it significantly enhanced doxorubicin's antitumor activity.

Bandyopadhyay et al. [122] have reported that inhibiting TGF-β signaling in mammary epithelial cells using a chemical TβR-I kinase inhibitor attenuated ATM (Ataxia Telangiectasia Mutated kinase) autophosphorylation and significantly reduced its kinase activity, while adding back TGF-β1 restored functional ATM and downstream DNA damage responses. These studies have discovered a critical link between activation of TGF-β1 in the microenvironment and ATM, which directs epithelial cell genotoxic stress responses and, indirectly, tissue integrity.

In the experiments of Seth et al. [123] effects of antagonization of TGF-β were combined with the oncolytic effects of an infectious adenoviral vector. An oncolytic adenovirus expressing Fc:TβRII was constructed by homologous recombination. MDA-MB-231 and MCF-7 human breast cancer cells were infected, and the transcription of TGF-β was inhibited in targeted cells. Direct injection of virus into MDA-MB-231 human breast carcinoma xenografts caused tumor regression in more than 85% of the animals [20, 123].

16. Crosstalk between Estrogen- and TGFβ Signaling Pathways in Breast Cancer Cells

A number of studies have suggested that estrogen receptor (ER)-negative (ER−) human breast carcinoma lines were relatively more sensitive to growth inhibition by TGF-β than ER+ lines [20]. For example, estrogen dependent MCF-7 breast cancer cells were found to be quite sensitive to TGFβ-mediated growth inhibition, while estrogen-independent MCF-7 sublines were refractory to TGFβ [20]. Growth of ER-positive MCF-7 cells is stimulated by estradiol as well as by progestins in a dose-dependent manner, and this effect can be blocked by treatment with 4-hydroxy-tamoxifen (4-OH-T). Moreover, estradiol- or norethindrone-induced growth stimulation is accompanied by a dramatic decrease in TGFβ2 and -3 mRNA levels, whereas the level of TGFβ1 mRNA was not affected [20].

Manni et al. [124] have demonstrated that even though treatment with TGF-β had no effect on MCF-7 cell growth in 2D culture, it inhibited colony formation in 3D in a dose-dependent manner to a degree comparable to that observed with 4-OH-T. Furthermore, the growth inhibitory effect of 4-OH-T was completely reversed by an anti-TGFβ antibody. These observations suggested that TGFβ might act on a small TGFβ-responsive progenitor cell population, at least in 3D cultures of MCF7 cells. These findings are similar to those reported by Shipitsin et al. [53] who have examined gene expression and genetic profiles of cells isolated from cancerous and normal breast tissue using the cell surface markers, *CD44* and *CD24*. Most tumors contained cell populations that were either predominantly ER+, *CD44*−, *CD24*+ or ER−, *CD44*+, *CD24lo*. Moreover, the *TGFBR2* gene was selectively expressed in ER− *CD44*+, *CD24lo* mammary epithelial precursors, but was epigenetically silenced in differentiated, ER+ *CD44*−, *CD24*+ luminal cells. Thus, differentiation into luminal cells appeared to be associated with inactivation of TGFβ signaling.

It is worth noting that tamoxifen-responsiveness *in vivo* may depend not only on ER expression in the breast cancer cells but also on stem cell population sensitivity to TGFβ-mediated growth arrest. Thus, in some cases, ER+ tumors might become resistant to antiestrogens because the tumor stem cells no longer respond to TGFβ-mediated cell cycle arrest. In this situation, constitutive inactivation of TGF-β signaling may even contribute to antiestrogen resistance [20]. On the basis of several observations the postulate has been created that in the early stages of breast cancer development, the mammary epithelial stem cell population is still sensitive to growth inhibition by TGF-β (and, thus, sensitive to tamoxifen if there is an ER+ subpopulation). During breast cancer progression the cells escape from TGFβ-mediated growth arrest, and the higher levels of TGF-β could be associated with greater invasive and/or metastatic potential and tamoxifen-resistance [20].

17. Crosstalk between HER2 Kinase and TGFβ Signaling in Mammary Tumor Progression

HER2 gene amplification is reported in approximately 25% of metastatic breastcancers, where it is associated with poor patient outcome [125]. Studies of HER2-overexpressing breast cancer cell lines and human tumors have shown constitutive HER2 phosphorylation and activation. Overexpression of HER2 is associated with mammary epithelial cell transformation and shorter survival in breast cancer patients [20, 49, 125]. In breast cancer models (*in vitro* and *in vivo*—in mice expressing the Neu oncogene), a functional synergy between TGF-β and HER2 has been characterized. Exogenous as well as transduced TGF-β confer motility and invasiveness in MCF10A cells (HMECs), which were showing stable expression of transfected HER2 [125, 126]. Such experiments showed that TGF-β1 and TGF-β3 cDNAs cooperate with HER2 in inducing cell motility and invasion in both 2D and 3D basement membrane cultures. This cooperation between HER2 and TGF-β correlates with sustained activation of AKT, ERK, and p38 MAPK and is abolished by pharmacological inhibition of PI3 K, ERK, or p38 MAPK. Indeed, a genetic modifier screen in these cells identified TGF-β1 and TGF-β3 as molecules that cooperate with HER2 in inducing cell motility and invasion [49, 125]. Evidence suggest that blockage of HER2:TGF-β crosstalk may significantly enhance the efficiency of conventional therapies in breast cancer patients with HER2 overexpression [49].

18. TGFβ Induced Apoptosis in Tumor Suppression

In epithelial, neuronal, and haematopoietic cells, TGF-β limits cell proliferation through a coordinated program of cytostatic gene responses. So far, the unique TGF-β-induced apoptotic program characteristic for cancer cells is poorly understood [8, 9]. *In vitro* studies have shown some Smad-dependent and -independent mechanisms; for example,

TGF-β increases the expression of death associated protein kinase (DAPK) in HCC cell-lines [126]. Other apoptotic related genes affected by TGF-β pathway are DAXX (that normally activates p38MAPK), FAS, and BIM (in gastric cancer cell lines) and GADD45β (growth arrest and DNA damage inducible 45 β; in hepatocytes) [8, 9, 60]. The final targets in TGF-β-induced apoptosis are the proapoptotic caspases and several members of the BCL2 family [7]. Confirmation of the physiological relevance of these candidates awaits experimental proof using *in vivo* model systems.

19. TGFβ Induced Autophagy in Breast Cancer Cells

Autophagy is a pivotal response of normal and cancer cells to environmental stress and is induced by various stimuli [127, 128]. Otherwise, autophagy has an intrinsic function in tumor suppression [129]. Although autophagy might allow tumor cells to survive under metabolic stress [128], several genetic links have emerged between defects of autophagy and development of cancer. Metastatic cancer cells may escape from anoikis via the induction of autophagy [130, 131]. *BECLIN1* is monoallelically deleted in 40% to 75% of human breast, ovarian, and prostate cancers, and thus considered as a tumor suppressor gene [128, 132, 133]. Accordingly, heterozygous deletion of *BECLIN1* in mice (*beclin 1*+/−) resulted in increased incidence of spontaneous tumors [128, 132]. Many breast carcinoma cell lines, although polyploid for chromosome 17 (*beclin1* gene is placed on chromosome 17q21), exhibit deletions of one or more *beclin1* alleles13 and human breast tumors show decreased Beclin1 levels compared to normal adjacent tissue. Restoration of Beclin1 and autophagy in MCF-7 cells is associated with inhibition of MCF7-induced tumorigenesis in nude mice [128]. *beclin1*+/− mice do not have increased incidence of mammary tumors but rather are susceptible to lymphomas and carcinomas of the lung and liver after a long latency [134, 135]. Tumors forming in *beclin1*+/− mice express wild-type *beclin1* mRNA and protein, indicating that *beclin1* is a haploinsufficient tumor suppressor.

Autophagy activation by TGFβ1 is mediated through the Smad and JNK pathways [129]. In the work of Kiyono et al. [132] TGF-β treatment induced the formation of GFP-LC3 puncta in human MDA-MB-231 mammary carcinoma cells and in mouse mammary carcinoma cell line, JygMC. Moreover, TGF-β enhanced degradation of long-lived proteins in MDA-MB-231 cells. Autophagic cell death has been also described in anti-estrogen-treated cultured human mammary carcinoma MCF-7 cells [22].

The role of autophagy might be different in certain stages and aspects of tumor development. Various tumor suppressors (e.g., PTEN, TSC1/2, p53, and DAPK) are autophagy inducers, whereas some inhibitors of autophagy (e.g., Akt and Ras) possess oncogenic activity [136]. Because TGF-β primarily functions as a tumor suppressor in early stages of carcinogenesis, TGF-β-induced autophagy may suppress tumor initiation in cooperation with other tumor suppressors. In later stages of tumor progression, it was shown that the metabolically stressed regions of the tumor mass activate autophagy. In this scenario, autophagy activation might confer a growth advantage to these cells. Regarding the tumor-promoting aspects of TGF-β in advanced cancer, TGF-β-induced autophagy in certain tumor types, including breast cancer, might be implicated in tumor promotion in the later phase of tumorigenesis [132, 137]. In the work of Suzuki et al. [129] TGFβ-induced autophagy was suppressed by the knockdown of Smad2/3, Smad4, or DAPK, or inhibition of JNK, indicating the involvement of both Smad and non-Smad pathways. TGFβ1 activates autophagy earlier than execution of apoptosis, and silencing of autophagy genes by siRNA attenuates the cell cycle arrest and apoptosis induction by TGFβ1 in HuH7 cells (human hepatocellular carcinoma cells), indicating that autophagy activation should partially contribute to TGFβ-mediated growth inhibition.

Concepts of autophagy inhibition used in cancer therapy have led to trials testing autophagy inhibitors, such as chloroquine, as sensitizers for radio- and chemotherapy in several malignancies [138].

20. Concluding Remarks

Direct impact of TGF-β1 upon the carcinoma cells, as well as regulation of carcinoma-immune cells interactions by this cytokine, must be considered when designing relevant therapeutic approaches to manage human cancer progression and metastasis. The prognostic utility of TGF-β1 in human BC has also been described. Elucidation of the molecular mechanisms responsible for conferring oncogenic activities of TGF-β1 will undoubtedly provide new therapeutic opportunities to alleviate metastatic progression and disease recurrence of BC. Currently some anti-TGF-β1 therapies are being explored. Given the role of microRNAs in mediating EMT and TGF-β signaling, it stands to reason that identifying the micro-RNAome regulated by TGF-β during its induction of metastatic progression may also offer new inroads to enhance the overall survival of breast cancer patients.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

References

[1] L. R. Gomes, L. F. Terra, R. A. M. Wailemann, L. Labriola, and M. C. Sogayar, "TGF-β1 modulates the homeostasis between MMPs and MMP inhibitors through p38 MAPK and ERK1/2 in highly invasive breast cancer cells," *BMC Cancer*, vol. 12, article 26, 2012.

[2] P. L. Porter, "Global trends in breast cancer incidence and mortality," *Salud Pública de México*, vol. 51, pp. 141–146, 2009.

[3] Y. Fang, Y. Chen, L. Yu et al., "Inhibition of breast cancer metastases by a novel inhibitor of TGF-β receptor 1," *Journal of the National Cancer Institute*, vol. 105, no. 1, pp. 47–58, 2013.

[4] R. Siegel, D. Naishadham, and A. Jemal, "Cancer statistics," *CA Cancer Journal for Clinicians*, vol. 62, no. 1, pp. 10–29, 2012.

[5] B. Bierie and H. L. Moses, "Transforming growth factor beta (TGF-β) and inflammation in cancer," *Cytokine and Growth Factor Reviews*, vol. 21, no. 1, pp. 49–59, 2010.

[6] Y. Y. Sheen, M. J. Kim, S. A. Park, and J. S. Nam, "Targeting the transforming growth factor-β signaling in cancer therapy," *Biomolecules & Therapeutics*, vol. 21, no. 5, pp. 323–331, 2013.

[7] D. Padua and J. Massagué, "Roles of TGFβ in metastasis," *Cell Research*, vol. 19, pp. 89–102, 2009.

[8] L. H. Katz, Y. Li, J. S. Chen et al., "Targeting the TGF-β signaling in cancer," *Expert Opinion on Therapeutic Targets*, vol. 17, no. 7, pp. 743–760, 2013.

[9] E. Meulmeester and P. Ten Dijke, "The dynamic roles of TGF-β in cancer," *Journal of Pathology*, vol. 223, no. 2, pp. 205–218, 2011.

[10] L. Levy and C. S. Hill, "Alterations in components of the TGF-β superfamily signaling pathways in human cancer," *Cytokine and Growth Factor Reviews*, vol. 17, no. 1-2, pp. 41–58, 2006.

[11] J. Zarzyńska and T. Motyl, "Dissimilar effects of LY 294002 and PD 098059 in IGF-I- mediated inhibition of TGF-β1 expression and apoptosis in bovine mammary epithelial cells," *Journal of Physiology and Pharmacology*, vol. 56, no. 3, pp. 181–193, 2005.

[12] D. Kajdaniuk, B. Marek, H. Borgiel-Marek, and B. Kos-Kudła, "Transforming growth factorβ1 (TGFβ1) in physiology and pathology," *Endokrynologia Polska*, vol. 64, no. 5, pp. 384–396, 2013.

[13] D. Amani, A. Khalilnezhad, A. Ghaderi, N. Niikawa, and K. I. Yoshiura, "Transforming growth factor beta 1 (TGF-β1) polymorphisms and breast cancer risk," *Tumor Biology*, 2014.

[14] C.-H. Heldin, M. Landström, and A. Moustakas, "Mechanism of TGF-β signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition," *Current Opinion in Cell Biology*, vol. 21, no. 2, pp. 166–176, 2009.

[15] H. Ikushima and K. Miyazono, "Biology of transforming growth factor-β signaling," *Current Pharmaceutical Biotechnology*, vol. 12, no. 12, pp. 2099–2107, 2011.

[16] H. Moses and M. H. Barcellos-Hoff, "TGF-β biology in mammary development and breast cancer," *Cold Spring Harbor perspectives in biology*, vol. 3, no. 1, 2011.

[17] J. G. Parvani, M. A. Taylor, and W. P. Schiemann, "Noncanonical TGF-β signaling during mammary tumorigenesis," *Journal of Mammary Gland Biology and Neoplasia*, vol. 16, no. 2, pp. 127–146, 2011.

[18] M. A. Taylor, J. G. Parvani, and W. P. Schiemann, "The pathophysiology of epithelial-mesenchymal transition induced by transforming growth factor-β in normal and malignant mammary epithelial cells," *Journal of Mammary Gland Biology and Neoplasia*, vol. 15, no. 2, pp. 169–190, 2010.

[19] L. A. Van Meeteren and P. Ten Dijke, "Regulation of endothelial cell plasticity by TGF-β," *Cell and Tissue Research*, vol. 347, no. 1, pp. 177–186, 2012.

[20] A. R. Tan, G. Alexe, and M. Reiss, "Transforming growth factor-β signaling: emerging stem cell target in metastatic breast cancer?" *Breast Cancer Research and Treatment*, vol. 115, no. 3, pp. 453–495, 2009.

[21] C. Panis, A. C. Herrera, V. J. Victorino, A. M. Aranome, and R. Cecchini, "Screening of circulating TGF-β levels and its clinicopathological significance in human breast cancer," *Anticancer Research*, vol. 33, no. 2, pp. 737–742, 2013.

[22] T. Motyl, B. Gajkowska, J. Zarzyńska, M. Gajewska, and M. Lamparska-Przybysz, "Apoptosis and autophagy in mammary gland remodeling and breast cancer chemotherapy," *Journal of Physiology and Pharmacology*, vol. 57, no. 7, pp. 17–32, 2006.

[23] C.-F. Chang, R. Westbrook, J. Ma, and D. Cao, "Transforming growth factor-beta signaling in breast cancer," *Frontiers in Bioscience*, vol. 12, no. 12, pp. 4393–4401, 2007.

[24] X. Zu, Q. Zhang, R. Cao et al., "Transforming growth factor-β signaling in tumor initiation, progression and therapy in breast cancer: an update," *Cell and Tissue Research*, vol. 347, no. 1, pp. 73–84, 2012.

[25] J. G. Parvani, A. J. Galliher-Beckley, B. J. Schiemann, and W. P. Schiemann, "Targeted inactivation of β1 integrin induces β3 integrin switching, which drives breast cancer metastasis by TGF-β," *Molecular Biology of the Cell*, vol. 24, no. 21, pp. 3449–3459, 2013.

[26] T. Imamura, A. Hikita, and Y. Inoue, "The roles of TGF-β signaling in carcinogenesis and breast cancer metastasis," *Breast Cancer*, vol. 19, no. 2, pp. 118–124, 2012.

[27] L. Yang, Y. Pang, and H. L. Moses, "TGF-β and immune cells: an important regulatory axis in the tumor microenvironment and progression," *Trends in Immunology*, vol. 31, no. 6, pp. 220–227, 2010.

[28] M. K. Wendt, T. M. Allington, and W. P. Schiemann, "Mechanisms of the epithelial-mesenchymal transition by TGF-β," *Future Oncology*, vol. 5, no. 8, pp. 1145–1168, 2009.

[29] H. Y. Lan and A. C. Chung, "TGF-β/Smad signaling in kidney disease," *Seminars in Nephrology*, vol. 32, no. 3, pp. 236–243, 2012.

[30] T. Liu and X.-H. Feng, "Regulation of TGF-β signalling by protein phosphatases," *Biochemical Journal*, vol. 430, no. 2, pp. 191–198, 2010.

[31] N. A. Bhowmick, M. Ghiassi, A. Bakin et al., "Transforming growth factor-β1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism," *Molecular Biology of the Cell*, vol. 12, no. 1, pp. 27–36, 2001.

[32] K. E. Bachman and B. H. Park, "Duel nature of TGF-β signaling: tumor suppressor vs. tumor promoter," *Current Opinion in Oncology*, vol. 17, no. 1, pp. 49–54, 2005.

[33] E. Janda, K. Lehmann, I. Killisch et al., "Ras and TGFβ cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways," *Journal of Cell Biology*, vol. 156, no. 2, pp. 299–313, 2002.

[34] J. Massagué, "TGFβ signalling in context," *Nature Reviews Molecular Cell Biology*, vol. 13, pp. 616–630, 2012.

[35] S. Darakhshan and A. Ghanbari, "Tranilast enhance s the antitumor effects of tamoxifen on human breast cancer cells in vitro," *", J Biomed Sci*, vol. 20, no. 1, p. 76, 2013.

[36] A. M. Dunning, P. D. Ellis, S. McBride et al., "A transforming growth factorβ1 signal peptide variant increases secretion *in vitro* and is associated with increased incidence of invasive breast cancer," *Cancer Research*, vol. 63, no. 10, pp. 2610–2615, 2003.

[37] K. Miyazono, S. Ehata, and D. Koinuma, "Tumor-promoting functions of transforming growth factor-β in progression of cancer," *Upsala Journal of Medical Sciences*, vol. 117, no. 2, pp. 143–152, 2012.

[38] K. E. De Visser, A. Eichten, and L. M. Coussens, "Paradoxical roles of the immune system during cancer development," *Nature Reviews Cancer*, vol. 6, no. 1, pp. 24–37, 2006.

[39] S. H. Wrzesinski, Y. Y. Wan, and R. A. Flavell, "Transforming growth factor-β and the immune response: Implications for anticancer therapy," *Clinical Cancer Research*, vol. 13, no. 18, pp. 5262–5270, 2007.

[40] S. P. Fink, D. Mikkola, J. K. V. Willson, and S. Markowitz, "TGF-β-induced nuclear localization of Smad2 and Smad3 in Smad4 null cancer cell lines," *Oncogene*, vol. 22, no. 9, pp. 1317–1323, 2003.

[41] C. A. Boulanger, K.-U. Wagner, and G. H. Smith, "Parity-induced mouse mammary epithelial cells are pluripotent, self-renewing and sensitive to TGF-β1 expression," *Oncogene*, vol. 24, no. 4, pp. 552–560, 2005.

[42] J. Beisner, M. B. Buck, P. Fritz et al., "A novel functional polymorphism in the transforming growth factor-β2 gene promoter and tumor progression in breast cancer," *Cancer Research*, vol. 66, no. 15, pp. 7554–7561, 2006.

[43] X. Ma, A. Beeghly-Fadiel, W. Lu, J. Shi, Y. B. Xiang, Q. Cai et al., "Pathway analyses identify TGFBR2 as potential breast cancer susceptibility gene: results from a consortium study among Asians," *Cancer Epidemiology, Biomarkers & Prevention*, vol. 21, no. 7, pp. 1176–1184, 2012.

[44] E. Taubenschuß, E. Marton, M. Mogg et al., "The L10P polymorphism and serum levels of transforming growth factor β1 in human breast cancer," *International Journal of Molecular Sciences*, vol. 14, no. 8, pp. 15376–15385, 2013.

[45] H. S. Feigelson, A. V. Patel, W. Ryan Diver, V. L. Stevens, M. J. Thun, and E. E. Calle, "Transforming growth factor β receptor type I and transforming growth factor β1 polymorphisms are not associated with postmenopausal breast cancer," *Cancer Epidemiology Biomarkers and Prevention*, vol. 15, no. 6, pp. 1236–1237, 2006.

[46] P. Krippl, U. Langsenlehner, W. Renner et al., "The L10P polymorphism of the transforming growth factor-beta 1 gene is not associated with breast cancer risk," *Cancer Letters*, vol. 201, no. 2, pp. 181–184, 2003.

[47] T. R. Rebbeck, A. C. Antoniou, T. C. Llopis et al., "No association of TGFB1 L10P genotypes and breast cancer risk in BRCA1 and BRCA2 mutation carriers: a multi-center cohort study," *Breast Cancer Research and Treatment*, vol. 115, no. 1, pp. 185–192, 2009.

[48] A. Shin, X.-O. Shu, Q. Cai, Y.-T. Gao, and W. Zheng, "Genetic polymorphisms of the transforming growth factor-β1 gene and breast cancer risk: a possible dual role at different cancer stages," *Cancer Epidemiology Biomarkers and Prevention*, vol. 14, no. 6, pp. 1567–1570, 2005.

[49] S. E. Wang, "The functional crosstalk between HER2 tyrosine kinase and TGF-β signaling in breast cancer malignancy," *Journal of Signal Transduction*, vol. 2011, Article ID 804236, 8 pages, 2011.

[50] E. C. Connolly, J. Freimuth, and R. J. Akhurst, "Complexities of TGF-β targeted cancer therapy," *International Journal of Biological Sciences*, vol. 8, no. 7, pp. 964–978, 2012.

[51] S. B. Jakowlew, "Transforming growth factor-β in cancer and metastasis," *Cancer and Metastasis Reviews*, vol. 25, no. 3, pp. 435–457, 2006.

[52] A. E. Gorska, R. A. Jensen, Y. Shyr, M. E. Aakre, N. A. Bhowmick, and H. L. Moses, "Transgenic mice expressing a dominant-negative mutant type II transforming growth factor-β receptor exhibit impaired mammary development and enhanced mammary tumor formation," *American Journal of Pathology*, vol. 163, no. 4, pp. 1539–1549, 2003.

[53] M. Shipitsin, L. L. Campbell, P. Argani et al., "Molecular definition of breast tumor heterogeneity," *Cancer Cell*, vol. 11, no. 3, pp. 259–273, 2007.

[54] R. A. Hinshelwood, L. I. Huschtscha, J. Melki et al., "Concordant epigenetic silencing of transforming growth factor-β signaling pathway genes occurs early in breast carcinogenesis," *Cancer Research*, vol. 67, no. 24, pp. 11517–11527, 2007.

[55] B. Tang, M. Vu, T. Booker et al., "TGF-β switches from tumor suppressor to prometastatic factor in a model of breast cancer progression," *The Journal of Clinical Investigation*, vol. 112, no. 7, pp. 1116–1124, 2003.

[56] M. Dong, T. How, K. C. Kirkbride et al., "The type III TGF-β receptor suppresses breast cancer progression," *The Journal of Clinical Investigation*, vol. 117, no. 1, pp. 206–217, 2007.

[57] R. S. Muraoka-Cook, I. Shin, J. Y. Yi et al., "Activated type I TGFβ receptor kinase enhances the survival of mammary epithelial cells and accelerates tumor progression," *Oncogene*, vol. 25, no. 24, pp. 3408–3423, 2006.

[58] N. Cheng, N. A. Bhowmick, A. Chytil et al., "Loss of TGf-β type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-α-, MSP- and HGF-mediated signaling networks," *Oncogene*, vol. 24, no. 32, pp. 5053–5068, 2005.

[59] N. S. Nagaraj and P. K. Datta, "Targeting the transforming growth factor-β signaling pathway in human cancer," *Expert Opinion on Investigational Drugs*, vol. 19, no. 1, pp. 77–91, 2010.

[60] J. Massague, "TGFβ in cancer. A comprehensive review on TGFβsignaling and its role in cancer," *Cell*, vol. 134, no. 2, pp. 215–230, 2008.

[61] D. S. Micalizzi, C.-A. Wang, S. M. Farabaugh, W. P. Schiemann, and H. L. Ford, "Homeoprotein Six1 increases TGF-β type I receptor and converts TGF-β signaling from suppressive to supportive for tumor growth," *Cancer Research*, vol. 70, no. 24, pp. 10371–10380, 2010.

[62] K. J. Reichenberger, R. D. Coletta, A. P. Schulte, M. Varella-Garcia, and H. L. Ford, "Gene amplification is a mechanism of Six1 overexpression in breast cancer," *Cancer Research*, vol. 65, no. 7, pp. 2668–2675, 2005.

[63] S. Lamouille, E. Connolly, J. W. Smyth, R. J. Akhurst, and R. Derynck, "TGf-β-induced activation of mTOR complex 2 drives epithelial-mesenchymal transition and cell invasion," *Development*, vol. 139, no. 10, pp. 1259–1273, 2012.

[64] D. X. Nguyen and J. Massagué, "Genetic determinants of cancer metastasis," *Nature Reviews Genetics*, vol. 8, no. 5, pp. 341–352, 2007.

[65] M. Yilmaz and G. Christofori, "EMT, the cytoskeleton, and cancer cell invasion," *Cancer and Metastasis Reviews*, vol. 28, no. 1-2, pp. 15–33, 2009.

[66] M. A. Taylor, K. Sossey-Alaoui, C. L. Thompson, D. Danielpour, and W. P. Schiemann, "TGF-β upregulates miR-181a expression to promote breast cancer metastasis," *The Journal of Clinical Investigation*, vol. 123, no. 1, pp. 150–163, 2013.

[67] X. P. Shi, S. Miao, Y. Wu et al., "Resveratrol sensitizes tamoxifen in antiestrogen-resistant breast cancer cells with epithelial mesenchymal transition features," *International Journal of Molecular Sciences*, vol. 14, no. 8, pp. 15655–15668, 2013.

[68] S. Lamouille and R. Derynck, "Cell size and invasion in TGF-β-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway," *Journal of Cell Biology*, vol. 178, no. 3, pp. 437–451, 2007.

[69] Y. Katsuno, S. Lamouille, and R. Derynck, "TGF-β signaling and epithelial-mesenchymal transition in cancer progression," *Current Opinion in Oncology*, vol. 25, no. 1, pp. 76–84, 2013.

[70] K. Mandel, D. Seidl, D. Rades et al., "Characterization of spontaneous and TGF-β-induced cell motility of primary human normal and neoplastic mammary cells *in vitro* using novel real-time technology," *PLoS ONE*, vol. 8, no. 2, 2013.

[71] C. L. Arteaga, "Inhibition of TGFβ signaling in cancer therapy," *Current Opinion in Genetics & Development*, vol. 16, no. 1, pp. 30–37, 2006.

[72] S. C. Curran and P. Kelly, "Breast tumor and stromal cell responses to TGF-β and hypoxia in matrix deposition," *Matrix Biology*, vol. 32, no. 2, pp. 95–105, 2013.

[73] A. Moustakas and C.-H. Heldin, "Non-smad TGF-β signals," *Journal of Cell Science*, vol. 118, no. 16, pp. 3573–3584, 2005.

[74] C. D. Morrison, J. G. Parvani, and W. P. Schieman, "The relevance of TGF-β paradox to EMT-MET programs," *Cancer Letter*, vol. 341, no. 1, pp. 30–40, 2013.

[75] T. Vincent, E. P. A. Neve, J. R. Johnson et al., "A SNAIL1-SMAD3/4 transcriptional repressor complex promotes TGF-β mediated epithelial-mesenchymal transition," *Nature Cell Biology*, vol. 11, no. 8, pp. 943–950, 2009.

[76] J. Li, M. S. Weinberg, L. Zerbini, and S. Prince, "The oncogenic TBX3 is a downstream target and mediator of the TGF-β1 signaling pathway," *Molecular Biology of the Cell*, vol. 24, no. 22, pp. 3569–3576, 2013.

[77] L. Liu, F. Li, J. A. Cardelli, K. A. Martin, J. Blenis, and S. Huang, "Rapamycin inhibits cell motility by suppression of mTOR-mediated S6K1 and 4E-BP1 pathways," *Oncogene*, vol. 25, no. 53, pp. 7029–7040, 2006.

[78] Q. Xu, L. Wang, H. Li et al., "Mesenchymal stem cells play a potential role in regulating the establishment and maintenance of epithelial-mesenchymal transition in MCF7 human breast cancer cells by paracrine and induced autocrine TGF-β," *International Journal of Oncology*, vol. 41, no. 3, pp. 959–968, 2012.

[79] R. Derynck and R. J. Akhurst, "Differentiation plasticity regulated by TGF-β family proteins in development and disease," *Nature Cell Biology*, vol. 9, no. 9, pp. 1000–1004, 2007.

[80] P. A. Gregory, C. P. Bracken, E. Smith et al., "An autocrine TGF-β/ZEB/miR-200 signaling network regulates establishment and maintenance of epithelial-mesenchymal transition," *Molecular Biology of the Cell*, vol. 22, no. 10, pp. 1686–1698, 2011.

[81] L. Ma, J. Teruya-Feldstein, and R. A. Weinberg, "Tumour invasion and metastasis initiated by microRNA-10b in breast cancer," *Nature*, vol. 449, no. 7163, pp. 682–688, 2007.

[82] S. Lamouille, D. Subramanyam, R. Blelloch, and R. Derynck, "Regulation of epithelial-mesenchymal and mesenchymal-epithelial transitions by microRNAs," *Current Opinion in Cell Biology*, vol. 25, no. 2, pp. 200–207, 2013.

[83] I. Keklikoglou, C. Koerner, C. Schmidt et al., "MicroRNA-520/373 family functions as a tumor suppressor in estrogen receptor negative breast cancer by targeting NF-κB and TGF-β signaling pathways," *Oncogene*, vol. 31, pp. 4150–4163, 2012.

[84] A. L. Smith, R. Iwanaga, D. J. Drasin et al., "The miR-106b-25 cluster targets Smad7, activates TGF-β signaling, and induces EMT and tumor initiating cell characteristics downstream of Six1 in human breast cancer," *Oncogene*, vol. 31, no. 50, pp. 5162–5171, 2012.

[85] W. Kong, H. Yang, L. He et al., "MicroRNA-155 is regulated by the transforming growth factor β/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA," *Molecular and Cellular Biology*, vol. 28, no. 22, pp. 6773–6784, 2008.

[86] Y. Katsuno, A. Hanyu, H. Kanda et al., "Bone morphogenetic protein signaling enhances invasion and bone metastasis of breast cancer cells through Smad pathway," *Oncogene*, vol. 27, no. 49, pp. 6322–6333, 2008.

[87] E. I. Deryugina and J. P. Quigley, "Matrix metalloproteinases and tumor metastasis," *Cancer and Metastasis Reviews*, vol. 25, no. 1, pp. 9–34, 2006.

[88] A. Jezierska and T. Motyl, "Matrix metalloproteinase-2 involvement in breast cancer progression: a mini-review," *Medical Science Monitor*, vol. 15, no. 2, pp. RA32–RA40, 2009.

[89] F. D. Shah, S. N. Shukla, P. M. Shah, H. K. Shukla, and P. S. Patel, "Clinical significance of matrix metalloproteinase 2 and 9 in breast cancer," *Indian Journal of Cancer*, vol. 46, no. 3, pp. 194–202, 2009.

[90] Y. Hoshino, Y. Katsuno, S. Ehata, and K. Miyazono, "Autocrine TGF-β protects breast cancer cells from apoptosis through reduction of BH3-only protein, Bim," *Journal of Biochemistry*, vol. 149, no. 1, pp. 55–65, 2011.

[91] A. Safina, M.-Q. Ren, E. Vandette, and A. V. Bakin, "TAK1 is required for TGF-β1-mediated regulation of matrix metalloproteinase-9 and metastasis," *Oncogene*, vol. 27, no. 9, pp. 1198–1207, 2008.

[92] E.-S. Kim, M.-S. Kim, and A. Moon, "TGF-beta-induced upregulation of MMP-2 and MMP-9 depends on p38 MAPK, but not ERK signaling in MCF10A human breast epithelial cells," *International journal of oncology*, vol. 25, no. 5, pp. 1375–1382, 2004.

[93] E. Wiercinska, H. P. H. Naber, E. Pardali, G. Van Der Pluijm, H. Van Dam, and P. Ten Dijke, "The TGF-β/Smad pathway induces breast cancer cell invasion through the up-regulation of matrix metalloproteinase 2 and 9 in a spheroid invasion model system," *Breast Cancer Research and Treatment*, vol. 128, no. 3, pp. 657–666, 2011.

[94] B. Bierie, D. G. Stover, T. W. Abel et al., "Transforming growth factor-β regulates mammary carcinoma cell survival and interaction with the adjacent microenvironment," *Cancer Research*, vol. 68, no. 6, pp. 1809–1819, 2008.

[95] M. A. Taylor, L. E. E. Yong-Hun, and W. P. Schiemann, "Role of TGF-β and the tumor microenvironment during mammary tumorigenesis," *Gene Expression*, vol. 15, no. 3, pp. 117–132, 2011.

[96] P. M. McGowan and M. J. Duffy, "Matrix metalloproteinase expression and outcome in patients with breast cancer: analysis of a published database," *Annals of Oncology*, vol. 19, no. 9, pp. 1566–1572, 2008.

[97] M. Yilmaz and G. Christofori, "Mechanisms of motility in metastasizing cells," *Molecular Cancer Research*, vol. 8, no. 5, pp. 629–642, 2010.

[98] L. Yang, J. Huang, X. Ren et al., "Abrogation of TGFβ signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis," *Cancer Cell*, vol. 13, no. 1, pp. 23–35, 2008.

[99] M.-L. Chen, M. J. Pittet, L. Gorelik et al., "Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-β signals *in vivo*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 2, pp. 419–424, 2005.

[100] M. Ahmadzadeh and S. A. Rosenberg, "TGF-β1 attenuates the acquisition and expression of effector function by tumor antigen-specific human memory CD8 T cells," *Journal of Immunology*, vol. 174, no. 9, pp. 5215–5223, 2005.

[101] D. A. Thomas and J. Massagué, "TGF-β directly targets cytotoxic T cell functions during tumor evasion of immune surveillance," *Cancer Cell*, vol. 8, no. 5, pp. 369–380, 2005.

[102] J.-C. Lee, K.-M. Lee, D.-W. Kim, and D. S. Heo, "Elevated TGF-β1 secretion and down-modulation of NKG2D underlies impaired NK cytotoxicity in cancer patients," *Journal of Immunology*, vol. 172, no. 12, pp. 7335–7340, 2004.

[103] Y. Q. Li, F. F. Liu, X. M. Zhang, X. J. Guo, M. J. Ren, and L. Fu, "Tumor secretion of CCL22 activates intratumoral Treg infiltration and is independent prognostic predictor of breast cancer," *PLoS ONE*, vol. 8, no. 10, 2013.

[104] M. Korpal and Y. Kang, "Targeting the transforming growth factor-β signalling pathway in metastatic cancer," *European Journal of Cancer*, vol. 46, no. 7, pp. 1232–1240, 2010.

[105] J.-S. Nam, M. Terabe, M. Mamura et al., "An anti-transforming growth factor β antibody suppresses metastasis via cooperative effects on multiple cell compartments," *Cancer Research*, vol. 68, no. 10, pp. 3835–3843, 2008.

[106] C. L. Arteaga, K. M. Koli, T. C. Dugger, and R. Clarke, "Reversal of tamoxifen resistance of human breast carcinomas *in Vivo* by neutralizing antibodies to transforming growth factor-β," *Journal of the National Cancer Institute*, vol. 91, no. 1, pp. 46–53, 1999.

[107] S. Biswas, M. Guix, C. Rinehart et al., "Inhibition of TGF-β with neutralizing antibodies prevents radiation-induced acceleration of metastatic cancer progression," *Journal of Clinical Investigation*, vol. 117, no. 5, pp. 1305–1313, 2007.

[108] V. Ganapathy, R. Ge, A. Grazioli et al., "Targeting the transforming growth factor-β pathway inhibits human basal-like breast cancer metastasis," *Molecular Cancer*, vol. 9, article 122, 2010.

[109] R. S. Muraoka, N. Dumont, C. A. Ritter et al., "Blockade of TGF-β inhibits mammary tumor cell viability, migration, and metastases," *The Journal of Clinical Investigation*, vol. 109, no. 12, pp. 1551–1559, 2002.

[110] M. A. Rowland-Goldsmith, H. Maruyama, K. Matsuda et al., "Soluble type II transforming growth factor-β receptor attenuates expression of metastasis-associated genes and suppresses pancreatic cancer cell metastasis," *Molecular cancer therapeutics*, vol. 1, no. 3, pp. 161–167, 2002.

[111] Y.-A. Yang, O. Dukhanina, B. Tang et al., "Lifetime exposure to a soluble TGF-β antagonist protects mice against metastasis without adverse side effects," *The Journal of Clinical Investigation*, vol. 109, no. 12, pp. 1607–1615, 2002.

[112] S. Ehata, A. Hanyu, M. Fujime et al., "Ki26894, a novel transforming growth factor-β type I receptor kinase inhibitor, inhibits *in vitro* invasion and *in vivo* bone metastasis of a human breast cancer cell line," *Cancer Science*, vol. 98, no. 1, pp. 127–133, 2007.

[113] O. Shinto, M. Yashiro, H. Kawajiri et al., "Inhibitory effect of a TGFB receptor type-I inhibitor, Ki26894, on invasiveness of scirrhous gastric cancer cells," *British Journal of Cancer*, vol. 102, no. 5, pp. 844–851, 2010.

[114] A. Bandyopadhyay, J. K. Agyin, L. Wang et al., "Inhibition of pulmonary and skeletal metastasis by a transforming growth factor-β type I receptor kinase inhibitor," *Cancer Research*, vol. 66, no. 13, pp. 6714–6721, 2006.

[115] I. M. Liu, S. H. Schilling, K. A. Knouse, L. Choy, R. Derynck, and X.-F. Wang, "TGFβ-stimulated Smad1/5 phosphorylation requires the ALK5 L45 loop and mediates the pro-migratory TGFβ switch," *EMBO Journal*, vol. 28, no. 2, pp. 88–98, 2009.

[116] D. Melisi, S. Ishiyama, G. M. Sclabas et al., "LY2109761, a novel transforming growth factor β receptor type I and type II dual inhibitor, as a therapeutic approach to suppressing pancreatic cancer metastasis," *Molecular Cancer Therapeutics*, vol. 7, no. 4, pp. 829–840, 2008.

[117] L. Bueno, D. P. de Alwis, C. Pitou et al., "Semi-mechanistic modelling of the tumour growth inhibitory effects of LY2157299, a new type I receptor TGF-β kinase antagonist, in mice," *European Journal of Cancer*, vol. 44, no. 1, pp. 142–150, 2008.

[118] G. Subramanian, R. E. Schwarz, L. Higgins et al., "Targeting endogenous transforming growth factor β receptor signaling in Smad4-deficient human pancreatic carcinoma cells inhibits their invasive phenotype," *Cancer Research*, vol. 64, no. 15, pp. 5200–5211, 2004.

[119] D. K. Kim, Y. Y. Sheen, C. Jin et al., 2011, 2-pyridyl substituted imidazoles as therapeutic ALK5 and/or ALK4 inhibitors.

[120] B. M. Zhao and F. M. Hoffmann, "Inhibition of transforming growth factor-β1-induced signaling and epithelial-to-mesenchymal transition by the Smad-binding peptide aptamer Trx-SARA," *Molecular Biology of the Cell*, vol. 17, no. 9, pp. 3819–3831, 2006.

[121] N. E. Bhola, J. M. Balko, T. C. Dugger et al., "TGFβ inhibition enhances chemotherapy action against triple- negative breast cancer," *The Journal of Clinical Investigation*, vol. 123, no. 3, pp. 1348–1358, 2013.

[122] A. M. Bandyopadhyay, L. Wang, J. K. Agyin et al., "Doxorubicin in combination with a small TGFβ inhibitor: a potential novel therapy for metastastic breast cancer in mouse model," *PLoS ONE*, vol. 5, no. 4, 2010.

[123] P. Seth, Z.-G. Wang, A. Pister et al., "Development of oncolytic adenovirus armed with a fusion of soluble transforming growth factor-β receptor II and human immunoglobulin Fc for breast cancer therapy," *Human Gene Therapy*, vol. 17, no. 11, pp. 1152–1160, 2006.

[124] A. Manni, C. Wright, and H. Buck, "Growth factor involvement in the multihormonal regulation of MCF-7 breast cancer cell growth in soft agar," *Breast Cancer Research and Treatment*, vol. 20, no. 1, pp. 43–52, 1991.

[125] A. Chow, C. L. Arteaga, and S. E. Wang, "When tumor suppressor TGFβ meets the HER2 (ERBB2) oncogene," *Journal of Mammary Gland Biology and Neoplasia*, vol. 16, no. 2, pp. 81–88, 2011.

[126] S. E. Seton-Rogers, Y. Lu, L. M. Hines et al., "Cooperation of the ErbB2 receptor and transforming growth factor β in induction of migration and invasion in mammary epithelial cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 5, pp. 1257–1262, 2004.

[127] K. Degenhardt, R. Mathew, B. Beaudoin et al., "Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis," *Cancer Cell*, vol. 10, no. 1, pp. 51–64, 2006.

[128] V. Karantza-Wadsworth and E. White, "Role of autophagy in breast cancer," *Autophagy*, vol. 3, no. 6, pp. 610–613, 2007.

[129] H. I. Suzuki, K. Kiyono, and K. Miyazono, "Regulation of autophagy by transforming growth factor-β (TGFβ) signaling," *Autophagy*, vol. 6, no. 5, pp. 645–647, 2010.

[130] C. Fung, R. Lock, S. Gao, E. Salas, and J. Debnath, "Induction of autophagy during extracellular matrix detachment promotes cell survival," *Molecular Biology of the Cell*, vol. 19, no. 3, pp. 797–806, 2008.

[131] W. Martinet, P. Agostinis, B. Vanhoecke, M. Dewaele, and G. R. Y. De Meyer, "Autophagy in disease: a double-edged sword with therapeutic potential," *Clinical Science*, vol. 116, no. 9, pp. 697–712, 2009.

[132] K. Kiyono, H. I. Suzuki, H. Matsuyama et al., "Autophagy is activated by TGF-β and potentiates TGF-β-mediated growth inhibition in human hepatocellular carcinoma cells," *Cancer Research*, vol. 69, no. 23, pp. 8844–8852, 2009.

[133] E. Morselli, L. Galluzzi, O. Kepp et al., "Oncosuppressive functions of autophagy," *Antioxidants and Redox Signaling*, vol. 14, no. 11, pp. 2251–2269, 2011.

[134] Z. Yue, S. Jin, C. Yang, A. J. Levine, and N. Heintz, "Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 25, pp. 15077–15082, 2003.

[135] X. Qu, J. Yu, G. Bhagat et al., "Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene," *The*

Journal of Clinical Investigation, vol. 112, no. 12, pp. 1809–1820, 2003.

[136] J. Botti, M. Djavaheri-Mergny, Y. Pilatte, and P. Codogno, "Autophagy signaling and the cogwheels of cancer," *Autophagy*, vol. 2, no. 2, pp. 67–73, 2006.

[137] C.-W. Jang, C.-H. Chen, C.-C. Chen, J.-Y. Chen, Y.-H. Su, and R.-H. Chen, "TGF-β induces apoptosis through Smad-mediated expression of DAP-kinase," *Nature Cell Biology*, vol. 4, no. 1, pp. 51–58, 2002.

[138] A. Fleming, T. Noda, T. Yoshimori, and D. C. Rubinsztein, "Chemical modulators of autophagy as biological probes and potential therapeutics," *Nature Chemical Biology*, vol. 7, no. 1, pp. 9–17, 2011.

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Review Article

Interaction of Dietary Fatty Acids with Tumour Necrosis Factor Family Cytokines during Colon Inflammation and Cancer

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Intestinal homeostasis is precisely regulated by a number of endogenous regulatory molecules but significantly influenced by dietary compounds. Malfunction of this system may result in chronic inflammation and cancer. Dietary essential n-3 polyunsaturated fatty acids (PUFAs) and short-chain fatty acid butyrate produced from fibre display anti-inflammatory and anticancer activities. Both compounds were shown to modulate the production and activities of TNF family cytokines. Cytokines from the TNF family (TNF-α, TRAIL, and FasL) have potent inflammatory activities and can also regulate apoptosis, which plays an important role in cancer development. The results of our own research showed enhancement of apoptosis in colon cancer cells by a combination of either docosahexaenoic acid (DHA) or butyrate with TNF family cytokines, especially by promotion of the mitochondrial apoptotic pathway and modulation of NFκB activity. This review is focused mainly on the interaction of dietary PUFAs and butyrate with these cytokines during colon inflammation and cancer development. We summarised recent knowledge about the cellular and molecular mechanisms involved in such effects and outcomes for intestinal cell behaviour and pathologies. Finally, the possible application for the prevention and therapy of colon inflammation and cancer is also outlined.

1. Introduction

Immune homeostasis in the intestine is tightly regulated by crosstalk between commensal bacteria, mucosal immune cells, and intestinal epithelial cells. These cells migrate from their place of origin at the crypt base to the villus (small intestine) or crypt (large bowel) tip, from where they are shed into the lumen. Dynamic balance between cell production at the base and cell death at the surface of the colonic crypts is precisely regulated by a number of physiological endogenous factors. In addition to hormones and cytokines, specific signalling through Wnt, Notch, and BMP pathways are essential for intestinal development and homeostasis [1]. Irregularities of such regulation might cause different pathologies including colon cancer. Recently, various types of cell death and their crosstalk were identified in the intestinal epithelium. In addition to apoptosis, autophagy and necroptosis were described as other modes of programmed colon cell death. Excessive cell death has been associated with chronic inflammation in patients with Crohn's disease and ulcerative colitis (UC) and dysregulation of cell death also plays essential role in colorectal cancer (CRC) [2].

The pathogenesis of CRC is a long and multifactorial process which involves not only mutations in specific oncogenes and tumour suppressor genes, but also alterations in gene expression which are induced by epigenetic and nongenotoxic mechanisms [3, 4]. Disturbed regulation of apoptosis and loss of sensitivity to apoptosis-inducing factors are some of the key mechanisms in CRC development [5, 6]. Further, autophagy, process of cellular self-digestion, has been demonstrated to promote cancer cell survival and drug resistance, but on the other hand it may function as a tumour

suppressor mechanism [7]. Moreover, an association between inflammation and cancer has been suggested for a long time [8]. Patients with inflammatory bowel disease (IBD) have a significantly increased risk of developing malignancy in the colon [9, 10]. An important role is especially played by increased production of various endogenous inflammatory molecules and malfunction of the immune system. However, some types of these molecules, such as tumour necrosis factor (TNF) family cytokines, may play a dual role. Their overproduction supports inflammation, but they can also induce death receptor-mediated apoptosis in certain cell types [11].

Nowadays it is clear that, in addition to endogenous regulators influencing cell and tissue homeostasis, lifestyle factors (mainly smoking, composition of the diet, and physical activity) play a role in the aetiology of colon inflammation and cancer [12, 13]. Among them, various types of fatty acids originating from dietary fat and fibre are being investigated to clarify the role and possible mechanisms by which they may influence colonic health [14–17].

The results of some studies including our own also suppose that there is mutual interaction between physiological regulators of cell behaviour and dietary lipids, which may positively or negatively influence the maintenance of intestinal tissue homeostasis. In the intestine, signals from nutritional compounds and endogenous factors, which play a role in inflammatory response and regulate cell growth, differentiation, and apoptosis, are integrated within the cell and may have a substantial impact determining the final phenotype, metabolism, and kinetics of epithelial cell population. Moreover, an altered sensitivity and response of transformed cells to these signals play the role during colon carcinogenesis.

In this review we focused on the role and mechanisms of action of dietary essential polyunsaturated fatty acids (PUFAs), short-chain fatty acid (SCFA) butyrate from fibre, and their interaction with TNF family cytokines during colon inflammation and cancer.

2. Chronic Inflammation Drives Cancer Development

Chronic inflammation is considered as one of the key mechanisms promoting and accelerating cancer development. This process mainly involves continuous activity of various cytokines, chemokines, production of reactive oxygen and nitrogen species (RONS), activity of certain enzymes, and activation of specific signalling pathways and transcription factors [18].

The inflammation present in the tumour microenvironment is characterised by leukocyte infiltration. These cells produce a variety of cytotoxic mediators such as RONS, proteases, matrix metalloproteinases, TNF-α, interleukins (IL), interferons (IFN), and enzymes such as cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), and phospholipase A2 (PLA2) responsible for eicosanoid formation. Such mediators activate specific transcription factors: nuclear factor κB (NF-κB), AP-1 or hypoxia-inducing factor 1α (HIF-1), and signal transducers like Janus kinase/Signal transducers and activators of transcription 3 (JAK/STAT3) [19]. These conditions can induce genetic and epigenetic changes including various types of mutations, chromosomal aberrations, methylation of various tumour-related genes, and modulated expression of microRNAs. These events work in concert to alter the important pathways involved in the normal cellular function and hence accelerate inflammation-associated cancer development [20].

2.1. Intestinal Inflammation and Cancer. In patients with IBD, chronic inflammation represents a major risk factor for the development of CRC [21]. This process leads to a disruption of the epithelial barrier and the formation of epithelial ulceration [22]. It permits easy access for the luminal microbiota and dietary antigens to cells resident in the lamina propria and stimulates further pathological immune cell responses [23]. However, the mechanisms underlying this neoplastic transformation are not fully understood. Studies in experimental models of CRC suggest that inflammatory cell-derived cytokines either directly or indirectly stimulate the uncontrolled growth of cancer cells [24].

Despite the differences between the molecular abnormalities found in colitis-associated dysplasia in comparison with sporadic CRC, there are many similarities (dysplasia-cancer sequence, similar frequencies of major chromosomal abnormalities, microsatellite instability, and similar glycosylation changes) that make it reasonable to suggest that also sporadic colon cancer might be largely secondary to inflammation. The fact that regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) can lower the mortality and result in regression of adenomas in familial adenomatous polyposis (FAP) patients with mutation in the adenomatous polyposis coli (APC) gene brings further evidence of the role of inflammation in CRC [25]. However, this process may function as a double-edged sword. Under specific inflammatory conditions, immune cells can boost an antitumour immune response with the downstream effect of eliminating dysplastic and cancerous cells. Thus, inflammation can play both a beneficial and a detrimental role in colon carcinogenesis [26, 27]. Since understanding of the definition and pathogenesis of CRC in IBD is crucial to optimise patient management, further investigation is necessary.

3. The Role of Cytokines in Colon Inflammation and Cancer

A variety of immune mediated bowel disorders, including celiac disease, Crohn's disease, and UC, are characterized by accelerated epithelial cell turnover and cell death leading to altered crypt morphology. These changes are mediated by the cytokines released from infiltrating inflammatory cells and enterocytes in paracrine or autocrine fashion, respectively. Similarly, various types of cytokines and chemokines, which can be produced by tumour cells themselves or by the cells in the tumour microenvironment, play an important role in colon cancer development. Using a mouse model of UC, TNF-α has been identified as a crucial mediator of the initiation and progression of colitis-associated CRC [28].

Proinflammatory molecules promote the growth of tumour cells, perturb their differentiation, and support the survival of cancer cells [23]. TNF-α, interleukins (IL-6 and IL-1β), or transcription factors (NF-κB and STATs) that are required for signalling by these cytokines are indeed emerging as potential targets for anticancer therapy. Members of the IL-12 family have been implicated in the pathogenesis of colitis, and IL-23 seems to be involved in inflammation-associated carcinogenesis [29]. The described mediators and activated signalling pathways generally delay or suppress the apoptosis of intestinal cells and modulate angiogenesis and drug-metabolising enzyme induction [30]. It was also reported that the colonic mucosa of UC patients displayed an increased T-cell infiltration. Moreover, the disbalance between CD4+ T-helper subsets (Th1 and Th2) producing specific inflammatory cytokines in the intestine and their resistance to apoptosis contributes to chronic mucosal inflammation [31]. Experiments using the Apc (Min/+) mouse model confirmed the association between intestinal cytokines and tumorigenesis. The overall polyp number and abundance of large polyps significantly correlated with inflammatory cytokine (IL-1, IL-6, and TNF-α) response. Inflammatory mediators may thus serve as important biomarkers for CRC progression [32]. Next, we focused on the role of TNF family cytokines.

3.1. Dual Role of TNF Family Cytokines. The TNF superfamily is composed of 19 ligands and 29 receptors, which play a highly diverse role in the body. This implies that at least some of the ligands have to interact with more than one receptor. All ligands and their receptors are well described in a recent work of Aggarwal et al. [33]. Without exception, the TNF superfamily exhibits a proinflammatory activity; in part through activation of the transcription factor NF-κB. However, these cytokines are involved in the regulation of not only inflammation but also cellular proliferation, apoptosis, and morphogenesis [11, 34]. Cell signalling is mediated through the interaction between transmembrane receptors and either soluble or transmembrane ligands. TNF-α, TRAIL (tumour necrosis factor-related apoptosis-inducing ligand), or Fas ligand (FasL), are expressed in both forms. Members of the TNF superfamily can be classified into those groups with or without an intracellular death domain (DD), and they have been identified as important participants in the regulation of apoptosis [35]. The death domain, a region of approximately 45 amino acids long, plays a crucial role in transmitting the death signals from the cell surface to the intracellular pathways [33, 36]. The cytokines TNF-α, FasL, and TRAIL induce apoptosis by binding to their receptors TNFR1/2, Fas, and DR4/DR5, respectively, which possess intracellular DDs recruiting certain adaptor molecules to form the death-inducing signalling complex (DISC) consisting of Fas-associated DD protein (FADD) and procaspase-8 [37]. The activation of caspase-8 at the DISC can be potently regulated by cellular FLICE-like inhibitory protein (cFLIP). In the so-called type I cells, efficient caspase-8 activation is followed by a direct cleavage of effector caspases (extrinsic apoptotic pathway), while type II cells mostly use caspase-8 to cleave the Bid protein, which is responsible for translocation of the apoptotic signal to mitochondria (intrinsic apoptotic pathway) [38]. Changes in mitochondria (mitochondrial transition pore opening, decrease of mitochondrial membrane potential, and production of ROS) are associated with the activation of pro- and antiapoptotic proteins of the Bcl-2 family, which play a crucial role in the release of proapoptotic proteins (cytochrome c, Smac/DIABLO) into the cytosol. The subsequent activation of caspase-9 is followed by activation of effector caspases, cleavage of poly ADP-ribose polymerase (PARP), and overall apoptosis. Caspase activity can be effectively modulated by the inhibitor of apoptosis proteins (IAPs) [39].

Recently, another mode of caspase-independent, non-apoptotic programmed cell death induced by stimulating death receptors named necroptosis was detected. This process shares some signalling pathways and molecules with apoptosis and its interaction with autophagy is also suggested. Necroptosis contributes to regulation of immune system and plays a role in cancer development [40]. It is associated with increased expression of receptor-interacting proteins RIP1/RIP3 and erroneous function of FADD and caspase-8 which may be critical for regulation of intestinal homeostasis [2]. Association of necroptosis and intestinal inflammation through specific activation of TNF-α synthesis by RIP1 and Akt kinase pathway has been documented [41].

In summary, TNF cytokines may play a dual role in the intestine; they have potent proinflammatory activities, but they also function as regulators of apoptosis associated with cancer development. It seems that cell proliferation, survival, and apoptosis are activated simultaneously by TNF members and the balance in their production and activation significantly determines the fate of the cells and contributes to intestinal homeostasis. Excessive programmed cell death promotes inflammation and, on the other hand, resistance to apoptosis contributes to cancer development. However, molecular mechanisms are not fully understood and may occur at different levels of intracellular signalling pathways.

3.2. TNF-α. The first member of the TNF superfamily discovered was TNF-α, a pleiotropic proinflammatory cytokine [42]. It plays a crucial role in immune and inflammatory processes, and in endotoxic shock [33, 43]. TNF-α is synthesised by macrophages and other cells in response to bacterial toxins, inflammatory products, and other invasive stimuli [44]. Its prolonged production is associated with cancer and chronic infections. It has been suggested that a gut with an active injury (e.g. in Crohn's disease or UC) contains an increased number of TNF-α secreting cells [45]. The proinflammatory cytokines, such as TNF-α, are typical activators of the canonical NF-κB signalling cascade, which is activated in response to injury, infection, inflammation, and other stress conditions [46]. In extracts of colorectal tumour tissues resected from human patients, a higher endogenous TNF-α was detected compared to adjacent normal tissue [47]. In addition to its role in inflammation, TNF-α can significantly modulate the proliferation, differentiation, and cell death of colonocytes during cancer progression [48].

3.3. TRAIL. TRAIL is an interesting candidate for anticancer therapy because of its ability to selectively induce apoptosis in cancer but not normal cells [49]. TRAIL can interact with at least five different receptors. Two of them, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), signal apoptosis, while decoy receptors DcR1 (TRAIL-R3), DcR2 (TRAIL-R4), and osteoprotegerin (TRAIL-R5) are unable to transmit the apoptotic signal [50].

An important part of TRAIL action is constituted by the regulation of intercellular interactions among the cells of the innate and adaptive immune system and the resulting apoptotic response. TRAIL is expressed on macrophages, dendritic cells, T cells, or NK cells in dependence on their stimulation status, being implicated in immunosuppressive, immunoregulatory, and immune-effector functions. Within this system, TRAIL plays an important role in autoimmune disorders, viral and bacterial infections, and immune surveillance of tumours and metastases [51]. Interestingly, a novel way to target and kill colon and prostate cancer cells in the bloodstream has been reported, using leukocytes presenting TRAIL on their surface along with E-selectin receptor [52].

In addition to acting as a potent tumour-selective inducer of apoptosis, TRAIL is also capable of efficiently triggering the nonapoptotic pathways involving extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), p38, phosphoinositide 3-kinase (PI3K)/Akt or NF-κB, which may result in increased proliferation, survival, migration, invasiveness, or inflammation in different normal and/or cancer cell types [53, 54]. Our results showed that specific inhibitors of the ERK pathway efficiently sensitised human colon cancer cells to TRAIL-induced apoptosis, which was mediated via downregulation of Mcl-1 protein. Moreover, inhibition of the PI3K/Akt pathway in colon cancer cells also resulted in sensitisation to TRAIL-induced apoptosis [55, 56]. It has recently been shown that cancer cells surviving the fractional killing by TRAIL exhibit NF-κB-dependent inflammatory phenotypes [57]. Other studies have shown an enhancement of NF-κB signalling in cancer cells resistant to TRAIL-induced apoptosis, which was associated with promotion of tumour metastasis and invasiveness [58].

The expression of TRAIL DRs, and DcRs may significantly change depending on the stage of tumorigenesis or the inflammatory disorders, suggesting their possible functional role in regulating the course of the disease. TRAIL and DR4 were downregulated in enterocytes, and TRAIL was upregulated in mononuclear cells only in IBD but not in the normal colon or appendicitis. This may point to a pathophysiological role of the TRAIL system in IBD [59]. Moreover, DR4 and DR5 were upregulated in colon adenomas and carcinomas compared with adjacent normal epithelial cells [60]. There exist contradictory data showing both a positive and a negative role of the TRAIL/TRAIL-R system in the regulation of cancer cell apoptosis, motility, invasiveness, and metastasis. DR5 has been reported to mediate anoikis (detachment-induced apoptosis) in human colon cancer cells [61]. DR deficiency in mice has been shown to enhance lymph node metastasis of epithelial cancers [62]. On the other hand, the ability of TRAIL and its DRs to promote metastasis in human and mouse CRC has also been demonstrated [63]. This dual role of DRs, and the differences observed in the cell response to TRAIL may depend on tumour type and stage and cell context, or they might be related to activation of the specific kinase pathways. It has been shown by us and others that cell sensitivity to TRAIL-induced apoptosis may be altered significantly during the colon carcinogenesis [64, 65]. Moreover, in some tumour types, the beneficial apoptosis-inducing effects of TRAIL may not be easily separated from the unfavourable nonapoptotic properties when using TRAIL in cancer therapy.

3.4. Fas/FasL. The Fas/FasL system is implicated in the aetiology of IBD because the Fas receptor (also called CD95, APO-1/Fas, or TNFRSF6) is highly expressed in the basolateral membrane of intestinal epithelial cells. During this disease, epithelial cell apoptosis is increased [7]. Fas initiates an apoptotic signal to apoptosis-sensitive cells when oligomerised by a natural ligand, FasL, or anti-Fas antibody [66]. FasL is a transmembrane protein that is expressed by lymphocytes, mainly by CD4+ and CD8+ T cells and B cells, after engagement of the Ag-specific T- or B-cell receptor and macrophages and also by NK cells [67]. It has been proposed that only membrane-bound FasL induces Fas-mediated apoptosis, whereas sFasL triggers nonapoptotic signalling pathways [68]. Activation and infiltration of tumour-specific lymphocytes expressing membrane-bound FasL, primarily tumour-specific CD8+, may be essential for Fas function. Recent results indicated that the colonic mucosa from patients with UC harbours more T cells than the normal colon tissue. This observation is consistent with human clinical data and animal-based studies, which showed a critical role of T cells in UC pathogenesis [69]. While Fas is expressed in every colonocyte of the normal colon mucosa, it is downregulated or lost in the majority of colon carcinomas. In contrast to the normal colonic epithelium, many colon carcinoma cell lines are relatively resistant to Fas cross-link [67]. Genomic data indicate that Fas is not focally amplified but significantly deleted across the entire dataset of 3131 tumours, including human CRC [70]. Thus, these data strongly suggest that Fas functions as a tumour suppressor [71, 72]. It was reported that mice with Fas deficiency in the colon tissue are hypersensitive to induced colitis and mice lacking FasL exhibited a more severe and persistent colitis than normal mice [73]. Several studies showed that cFLIP is an inhibitor of Fas signalling, which enhanced the frequency and decreased latency of subcutaneous tumour growth [67, 74]. However, recent data demonstrated that cFlip is required for intestinal tissue homeostasis in mice. This protein controls the level of activation of caspase-8 to promote the survival of intestinal epithelial cells. When cFlip was deleted from the intestinal epithelium, the animals died within a few days from severe tissue destruction, epithelial cell death, and intestinal inflammation. The death of intestinal cells was regulated extrinsically and required the presence of death receptor ligands, such as TNF-α and FasL [75]. Fas is a target for the NF-κB transcription factor but a direct interaction between NF-κB and the Fas promoter in human colon carcinoma cells was not identified [76].

Taken together, the ability of TNF family cytokines to induce apoptosis in intestinal epithelial cells seems to be very important. Their sensitivity may be influenced by many other factors and is altered during inflammation and carcinogenesis. Since many cancer cells become resistant to TNF cytokine-mediated killing, much effort has been devoted to find a possibility of overcoming it by combination with other anticancer agents. In addition to chemotherapeutics, the supportive effects of nontoxic dietary compounds seem to be a promising way. In the following parts we summarise recent knowledge about the effects of such compounds in colon inflammation and cancer and their use in combination with TNF family cytokines.

4. Dietary Factors and Colon Cancer

It is pointed out that since the Industrial Revolution, humans have fundamentally changed their dietary habits towards increased consumption of animal fat, decreased consumption of antioxidants, and an increased n-6/n-3 PUFA ratio [77]. The imbalance between energy intake and expenditure mainly due to high fat consumption and low fibre intake represents an important factor influencing colon cancer development. Nowadays, it is recognised that excessive lipid uptake in adipocytes leads to hypertrophy and consequently to metabolic dysregulation, hypoxia, inflammation, impaired adipocytokine expression and angiogenesis, insulin resistance, and macrophage recruitment. In obese patients, tumours commonly colocalise with excessive adipose tissue accumulation producing inflammatory mediators, and most of the features of hypertrophic adipose tissue are observed in cancer patients, namely, those with breast and colon cancer [78, 79]. Using the model of Apc (Min/+) mice, an association of high-fat American-type diet with obesity and an increased number of large polyps was demonstrated. On the other hand, it was also shown that calorie restriction and several bioactive food components, such as n-3 PUFA, can inhibit genetically predisposed CRC [80, 81].

4.1. The Role of Polyunsaturated Fatty Acids (PUFAs) in Colon Inflammation and Cancer. It has been shown that the quantitative and qualitative content of essential PUFAs in the diet is highly important [82, 83]. These PUFAs cannot be synthesised by mammals and their availability depends on external supply. They are divided into two main types of the n-6 and n-3 series. Precursor linoleic acid (LA, 18:2, n-6) found in many plant oils (soybean, sunflower) is desaturated and elongated to other types, mainly arachidonic acid (AA, 20:4). Alpha-linolenic acid (ALA, 18:3, n-3) is a precursor for eicosapentaenoic (EPA, 20:5) or docosahexaenoic (DHA, 22:6) acids, which are also found in fish or algal oils. Excessive amounts of n-6 PUFAs and a high n-6/n-3 ratio, found in Western diets, may promote the pathogenesis of many diseases such as cardiovascular disease, obesity, diabetes, autoimmune disease, inflammation, and cancer [84–86]. Recently, it was reported that the fatty acid profile of visceral white adipose tissue correlates with inflammatory signatures potentially associated with CRC [87].

Ingestion of PUFAs leads to their distribution to virtually every cell in the body and influences the lipid profile and fatty acid composition of plasma, nuclear, and mitochondrial cell membranes. This consequently affects the membrane structure and fluidity, the functions of membrane-bound proteins, and lipid-mediated signalling [88]. In IBD patients and human colorectal adenomas and adenocarcinomas, altered activities of enzymes metabolising endogenous AA, particularly increased expression and activity of PLA2, and COX-2 accompanied by an overproduction of prostaglandin E2 (PGE2) were detected [89] and have been shown to be responsible for immunosuppression and tumour promotion [31, 90]. Moreover, IBD colon biopsies show a marked increase in both 5-LOX and leukotriene B4 [91]. Both EPA and DHA compete for fatty acid metabolising enzymes with AA and thus are able to inhibit the formation of AA-derived proinflammatory and immunosuppressive eicosanoids [92]. Importantly, EPA and DHA form several own potent anti-inflammatory mediators (e.g., resolvins and protectins) [93]. The effects of PUFAs and their metabolites on various levels of cell organisation and their interaction with other endogenous or exogenous factors can finally significantly influence cell proliferation, differentiation, and apoptosis of various cell types [94, 95].

During the last 25 years, hundreds of papers describing the effects of PUFAs on various types of normal and cancer cells, differences between n-6 and n-3 PUFAs, and proposed mechanisms of their action have been published. This large and complex topic is beyond the scope of this review. However, in spite of much contradiction in the literature [96], it is generally thought that high calorie and fat intake are risk factors especially in colon, breast, or prostate cancer development and that n-6 PUFAs (from plant oils rich in LA) can promote inflammation and carcinogenesis [97]. Supplementation of diet with PUFAs can substantially influence cell physiology and cell kinetics, mostly by the modulation of oxidative metabolism and biosynthesis of PUFA metabolites. The particular species of free radicals affect specific phases of carcinogenesis in different ways [98]. The effects of n-6 PUFAs are primarily mediated by AA-derived eicosanoids. The anti-inflammatory and anticancer effects of NSAIDs, which function as specific inhibitors of AA metabolism, confirmed the significance of eicosanoids in cancer development [99, 100].

On the other hand, there is growing evidence that n-3 PUFAs, namely, DHA and EPA, found in fish and algal oils, exert anti-inflammatory properties, thus suppressing IBD and colon cancer [101–106]. Over the last years, these n-3 PUFA properties were confirmed by experiments with cell cultures and laboratory animals using the introduction of a newly discovered gene encoding n-3 fatty acid desaturase (fat-1), which is not normally present in mammals [107].

Nutritionally induced changes in fatty acid composition may result in an increased sensitivity to chemo- and radiotherapy and decreased undesirable side effects [108–110]. There is also evidence suggesting that n-3 PUFA may uniquely regulate stem cell signalling pathways and the increased sensitivity of colon cancer cells to chemotherapy by upregulation of colonic differentiation markers [111].

There are many papers describing the different mechanisms considered to be responsible for n-3 PUFA effects on cellular and molecular levels [101, 106, 112–114]. In general, there are three major mechanisms suggested to play the main role in n-3 PUFA effects: (1) alteration of cellular membrane phospholipid composition and lipid microdomain functionality, (2) competition for metabolisation enzymes with n-6 PUFAs and thus production of bioactive mediators, eicosanoids, and (3) modulation of intracellular signalling and nuclear receptor activation. These aspects are discussed in a further chapter dealing with the interaction of PUFAs with TNF family cytokines.

4.2. Fibre and Short-Chain Fatty Acids (SCFAs) in Colon Inflammation and Cancer. Dietary fibre increases the gastrointestinal tract biomass, changes the composition of gut flora, and may decrease the risk of metabolic disorders like dyslipidaemia, hypercholesterolaemia, and hyperglycaemia and also substantially influence immune-based actions [115]. Its fermentation by intestinal bacteria leads to the generation of SCFAs propionate, acetate, and butyrate. Butyrate, present in the colonic lumen in millimolar concentration, acts as a principal energy source and a survival factor for normal colon cells. In addition, it possesses anti-inflammatory and antineoplastic properties. Butyrate supplementation dramatically reduced proliferation and induced differentiation and/or cell death in colon cancer cells [116]. In addition to the regulation of basic cytokinetic processes, butyrate has also been shown to affect cell adhesion, morphology, invasiveness, metastasis, oxidative metabolism, angiogenesis, activity of different enzymes, gene expression and chromatin modulation, activity/expression of various transcription factors, and signal transduction molecules. These multiple effects in colon cells were reviewed by us previously [117].

The butyrate action is very complex and still not completely understood. Butyrate is a well-known histone deacetylase (HDAC) inhibitor and thereby regulates gene expression and induces sensitisation effects on cytokine action in colon cancer cells [118, 119]. Accompanying these changes butyrate influences the cell response to inflammatory stimuli mainly by the inhibition of NFκ-B and IFNγ signalling pathways and an enhancement of peroxisome proliferator-activated receptor γ (PPARγ) expression and activity, leading to the modulation of apoptosis and differentiation [120, 121]. Moreover, it was reported that HDAC inhibitors including butyrate induce autophagy which shares some common signalling pathways and is mutually regulated with apoptosis in colon cancer cells.

The putative mechanisms responsible for the different response to butyrate in normal, IBD- and tumour-derived colon cells may include changes in butyrate transport and uptake, mainly due to a different expression of sodium-coupled monocarboxylate transporters [122] and the specific G-protein coupled receptor 43 [123]. Butyrate metabolism is impaired in intestinal inflamed mucosa of patients with IBD. Disturbances in butyrate oxidation, the balance between butyrate and glucose oxidation, ROS generation in mitochondria, and differences in the overall cellular context play a role, too [124, 125].

5. Interactive Effects of Dietary Fatty Acids with TNF Family Cytokines

It is suggested by us and others that dietary fatty acids (such as essential PUFAs or butyrate) and endogenous regulators from the TNF family can mutually interact and thus modulate the behaviour of colon epithelial cells. Such interactions may result in an altered production and activity of proinflammatory TNF family cytokines or an enhancement of their antiproliferative and proapoptotic effects. Moreover, these effects may be different in normal and cancer cells. Therefore, in the following sections we summarise the knowledge about these interactions, their possible mechanisms, and outcomes for intestinal cell behaviour and pathologies. The possible application of such knowledge for the prevention and therapy of colon inflammation and cancer is also outlined. Main mechanisms supposed to play the role in the fatty acid and TNF family cytokine interaction are schematically presented in Figure 1.

5.1. Interaction of TNF Family Cytokines with PUFAs. The effects of TNF family molecules on cancer cells could be significantly modulated by PUFAs and their metabolites. Previously we evidenced the potentiating effects of various inhibitors of AA metabolism on TNF-α induced apoptosis and differentiation in human leukaemic cell lines [126–128]. This phenomenon was also verified in human colon epithelial cells [129]. Our further results showed that the pretreatment of human colon cancer HT-29 cells with low doses of AA or DHA may prepare a permissive environment for a more effective apoptotic action of TNF family molecules. Importantly, low concentrations of both AA and DHA increased the apoptosis induced by anti-Fas antibody or TNF-α, which involved enhanced ROS production and decrease of mitochondrial membrane potential and caspase activation. Compared to AA, DHA showed more pronounced effects in lower concentration [130]. Similarly, the cotreatment of HT-29 cells with DHA enhanced TRAIL-induced apoptosis supporting the mitochondrial intrinsic pathway [131]. Recently, we confirmed these effects using several other colon cell lines (including TRAIL-resistant SW620 cells), which were pretreated with DHA. We clarified the key mechanisms of DHA and TRAIL interaction including the role of mitochondria, specific lipids, and signalling pathways (submitted manuscript). These results are promising, because in spite of the fact that TRAIL may be a selective anticancer agent, many cancer cells are resistant to its effects. This resistance may occur at different levels of intracellular signalling pathways, and manipulation of their individual components such as downregulation of antiapoptotic molecules or upregulation of proapoptotic factors can change the threshold for apoptosis induction by this cytokine [55]. This can be right achieved by combined treatments of TRAIL with selected agents specifically targeting the abovementioned molecules, such as

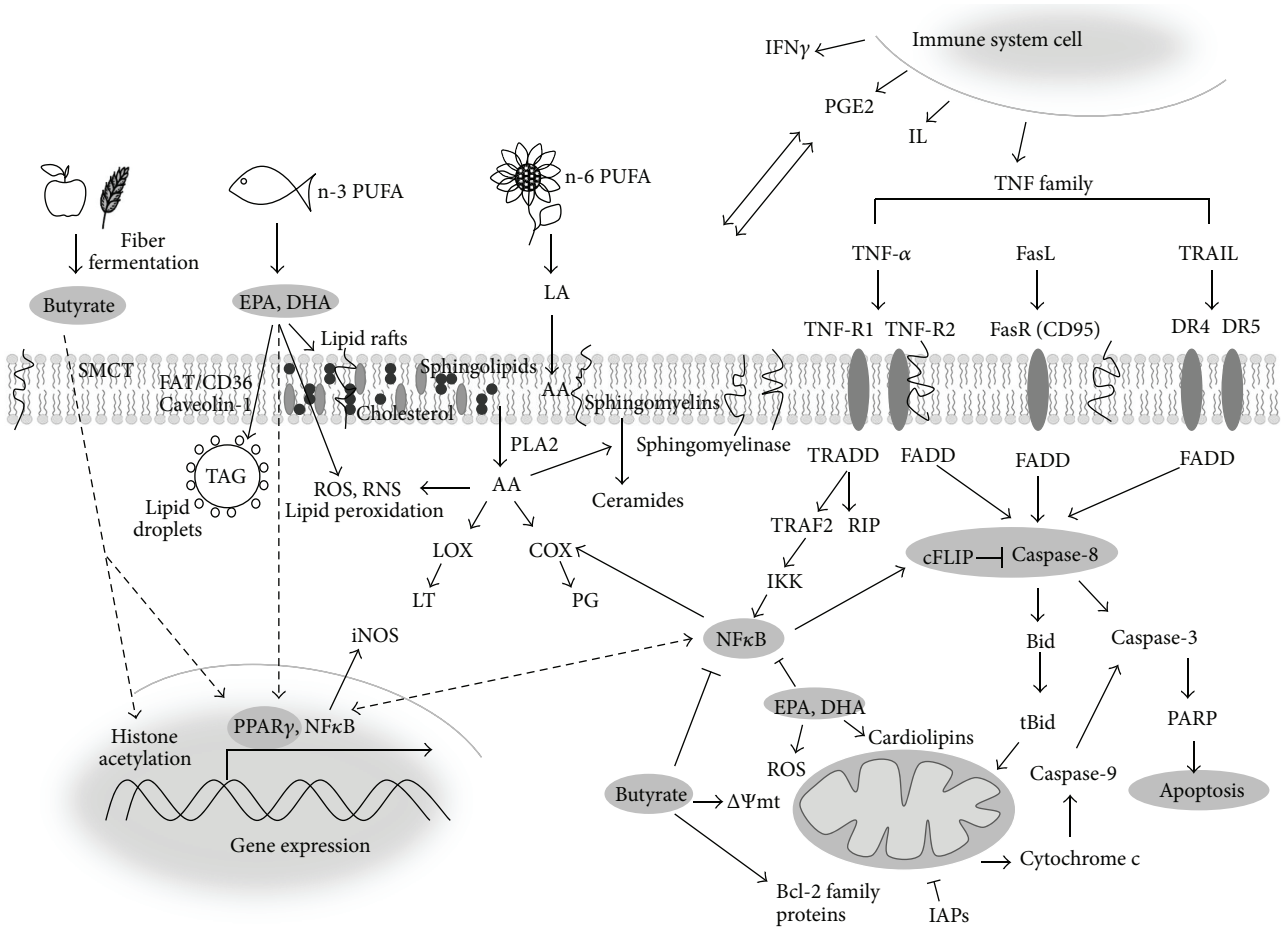

Figure 1: Schematic simplified demonstration of the main mechanisms supposed to be involved in fatty acid and TNF family interaction at different levels of colon epithelial cell organisation. Fibre fermentation product butyrate is transported by SMCT into the cells, where it may inhibit histone deacetylation, activate transcription factor PPARγ, inhibit NFκB, and affect $\Delta\Psi$mt and expression and balance of pro- and antiapoptotic members of Bcl-2 family proteins. Dietary PUFAs of the n-6 (LA, AA-plant oils) and n-3 series and (EPA and DHA, fish and algal oils) transported into the cells by FAT/CD36 are stored in lipid droplets in TAG form or incorporated into plasma and mitochondrial membrane lipids thus modulating composition and structure of lipid rafts and caveolae. PUFAs are released from membrane phospholipids by PLA2 and metabolized by COX and LOX enzymes to various types of products such as LTs and PGs. PUFAs can also modulate ceramide production and their metabolism is source of ROS and RNS and induces lipid peroxidation. Incorporation of PUFAs into mitochondrial cardiolipins influences mitochondrial metabolism and induction of apoptosis. EPA and DHA activate PPARγ and inhibit NFκB, thus suppressing iNOS expression and RNS production. TNF family cytokines TNF-α, TRAIL, and FasL bind to their receptors TNFR1 and 2, DR4 and DR5, or FasR (CD95), respectively. Programmed cell death is regulated by formation of DISC consisting of FADD and procaspase-8, whose activation may be inhibited by cFLIP. Cleavage of Bid protein to tBid mediates connection between extrinsic and intrinsic (mitochondrial) apoptotic pathway. Changes in mitochondria (involving cardiolipin modulation), mitochondrial transition pore opening, decrease of $\Delta\Psi$mt, and production of ROS influence the activation of pro- and antiapoptotic proteins of the Bcl-2 family and release of proapoptotic proteins (e.g., cytochrome c) into the cytosol. Subsequent activation of caspase-9 and caspase-3, cleavage of PARP, and execution of apoptosis can be inhibited by IAPs. TNF-α activates NFκB via pathway involving TRADD, RIP, TRAF2, and IKK proteins. The interaction between epithelial cells and cells of immune system, which are the source of inflammatory mediators (IFNγ, IL, and PGE2), is also indicated. The dashed arrows-simplified signalling pathways. AA: arachidonic acid; cFLIP: FLICE-like inhibitory protein; COX: cyclooxygenase; $\Delta\Psi$mt: mitochondrial membrane potential; DHA: docosahexaenoic acid; DISC: death-inducing signaling complex; DR: death receptor; EPA: eicosapentaenoic acid; FADD: Fas-associated DD protein; FasL: Fas ligand; FasR: Fas receptor; FAT/CD36: fatty acid translocase; IAPs: inhibitor of apoptosis proteins; IKK: inhibitor of NFκB kinase; IFNγ: interferonγ; IL: interleukin; iNOS: inducible nitric oxide synthase; LA: linoleic acid; LOX: lipoxygenase; LT: leukotrienes; NF-κB: nuclear factor κB; PARP: poly-ADP ribose polymerase; PG: prostaglandins; PGE2: prostaglandin E2; PLA2: phospholipase A2; PPARγ: peroxisome proliferator-activated receptor γ; PUFA: polyunsaturated fatty acids; RIP: receptor-interacting protein; RNS: reactive nitrogen species; ROS: reactive oxygen species; SMCT: sodium-coupled monocarboxylate transporters; TNF: tumor necrosis factor; TNFR: tumor necrosis factor receptor; TRADD: TNFR1-associated death domain protein; TRAF2: TNF receptor-associated factor 2; TRAIL: tumour necrosis factor-related apoptosis-inducing ligand.

chemotherapeutic DNA damaging drugs [53, 132] or fatty acids [131].

Summarising our results we have recognised that the cell response to molecules regulating cytokinetics as well as to lipid compounds is cell type-specific and may depend on the cell genetic background and the level of transformation. In colon cell lines derived from nontumour tissues or tissues on various stages of malignancy we detected a different sensitivity to TNF family cytokines, PUFAs as well as butyrate [65, 133, 134]. Generally, investigation of the molecular mechanisms and cellular specificity of PUFAs as well as TNF family cytokines enables us to determine also the possible mechanisms of their interaction.

PUFAs are actively transported into the cells by fatty acid translocase (FAT/CD36), and their incorporation alters the cellular lipid composition and fatty acid spectrum and shifts redox balance and the formation of various products of lipid metabolism [98]. In response to the elevated fatty acid content, complex and metabolically active organelles called lipid droplets (LDs) are formed. LDs are fundamental components of intracellular lipid homeostasis because they play a role in lipogenesis and lipolysis, serve as an important reservoir of signal molecules, and appear to be directly involved in membrane traffic and phospholipid recycling [135].

DHA, the longest (22 carbons) and the most unsaturated (6 double bonds) PUFA, is rapidly incorporated into the plasma as well as mitochondrial membrane phospholipids and also induces LD formation. It was shown to significantly alter the lipid microdomain (lipid rafts and caveolae) composition and the properties which increase or decrease specific receptors in lipid rafts accompanied by altered phosphorylation and thus activation of receptor and associated signalling kinases [136–138]. For example, after changes in membrane microdomains upon DHA treatment, epidermal growth factor receptor is excluded from caveolin-rich membrane fractions resulting in the subsequent downregulation of ERK signalling in three different cancer models [139]. The position and activity of TNFR, Fas, or DRs may be influenced in a similar way. Our results evidenced that increased DR5 surface expression, relocalisation of DRs to lipid rafts, and accelerated TRAIL internalisation are important for the sensitisation of colon and prostate cancer cells to TRAIL-induced apoptosis by platinum complexes [132]. Attention should be paid when applying TRAIL in combination with other drugs known for their ability to increase the DR surface level in cancer cells, as nonapoptotic signalling might also be increased under some circumstances.

DHA is specifically incorporated into the mitochondrial tetra-acyl phospholipid, cardiolipin (CL). Altered CL unsaturation and oxidative susceptibility of the mitochondrial membrane modulate the binding and activities of associated proteins, mainly of the pro- and antiapoptotic proteins of the Bcl-2 family. Changes of mitochondrial transition pore opening, decreased mitochondrial membrane potential, CL oxidation, and the release of proapoptotic cytochrome c and Apaf1 molecules are necessary for the execution of the intrinsic mitochondrial apoptotic pathway [140, 141]. Thus, in colonocytes and colon cancer cells, DHA alone or in combination with other agents can induce apoptosis by promotion of the mitochondrial apoptotic pathway as reported by us and others [131, 133, 142].

The beneficial effects of n-3 PUFA are of particular interest because IBD patients are very sensitive to nutraceutical approaches [143]. Using a fat-1 transgenic mice model, an inhibitory effect of endogenously synthesised n-3 PUFA on COX-2 gene expression was demonstrated during acute and chronic colitis accompanied by marked reduction in proinflammatory interleukins such as IL-1α, IL-6, and IL-18 and molecules endowed with chemotactic activity for granulocytes [144]. Moreover, endogenously synthesised n-3 PUFAs in such transgenic animals prevent colon cancer development by several mechanisms [145]. The networking of pathways of eicosanoid formation and inflammatory cytokine production represents a very important issue, but it concerns mainly the interaction of immune cells with other cell types and is not discussed in this review [146, 147].

An important consequence of PUFAs and TNF family cytokines interaction is represented by the modulation of proliferation and mainly cell death of cancer cells. Among other mechanisms, the participation of ceramides, which were already shown to play an important role in programmed cell death of cancer cells, was demonstrated [148]. Free AA has been shown to be an important mediator of TNF-α induced apoptosis via activation of sphingomyelinase and formation of ceramides [149]. TNF-α-mediated cell killing was inhibited by an increased AA metabolisation by COX-2 overexpression [150] or by blocking endogenous AA release in a mutant cell line with reduced cPLA2 activity [151]. The role of ceramide in Fas-mediated apoptosis has also been well documented [152]. Ceramide enables the Fas receptor to cluster to increase Fas-mediated apoptosis [153] and to modulate Fas receptor activation [72]. Authors identified XIAP and cIAP1 as molecular targets of ceramide.

The higher expression and secretion of various proinflammatory cytokines and their autocrine and paracrine functions play an important role in the activation of transcription factors, which in turn promote tumorigenesis. NF-κB serves as a vital biomolecule that transcribes a number of proinflammatory cytokines and antiapoptotic proteins. NF-κB is also known to play a critical role in the regulation of the inducible nitric oxide synthase (iNOS) gene. iNOS is an enzyme dominantly expressed during inflammatory reactions, and high amounts of nitric oxide (NO) have been demonstrated in pathophysiological processes, such as acute or chronic inflammation and tumorigenesis. Proinflammatory cytokines can also activate Jak3/Stat3 signalling pathways, thereby increasing inflammation and cell survival. The expression of IL-1β, IL-2, IL-4, IFNγ, TNF-α, iNOS, COX-2, Jak3, Stat3, and NF-κB was increased in the early stages of experimental CRC. N-3 PUFAs suppress the activity of NF-κB and thus reduce the production of proinflammatory enzymes and cytokines, including COX-2, TNF-α, and IL-1β [102]. The protective role of n-3 PUFAs, which suppressed the activity of NF-κB and iNOS and increased the expression of transforming growth factor β, thus preventing colitis and CRC, was confirmed using fat-1 transgene mice [154].

In vitro studies have shown that n-3 PUFAs inhibit cell proliferation and induce apoptosis in cancer cells through the activation of transcription factors PPARs [155]. They influence lipid homeostasis and may be involved in the regulation of cell differentiation and death. The differential activation of PPAR isoforms (α, β/δ, γ) and PPAR-regulated genes by specific dietary fatty acids may be central to their distinct roles in cancer [156]. The data from a human case-control study suggest that PPARγ may be associated with many aspects of CRC including insulin- and inflammation-related mechanisms [157]. In CRC patients, adipocyte dysfunctions creating a proinflammatory environment with upregulated STAT3 and the concomitant decrease of PPARγ and adiponectin in white adipose tissue were detected with respect to healthy subjects. DHA was shown to have protective effects reestablishing the equilibrium between STAT3 and PPARγ [87].

Wnt/β-catenin pathway is constitutively activated in more than 90% of human CRC. The activated β-catenin stimulates cell proliferation and survival; however, its anti-apoptotic mechanisms are not fully understood. Recently, using a mouse model of inflammation-associated CRC and human colon cancer HCT-116 cells, Han et al. evidenced that the resistance of colon cancer cells to apoptotic effects of TNF-α is mediated by the activated nuclear β-catenin which blocks caspase cleavage of retinoblastoma protein. Further, the activated β-catenin can facilitate endosomal trafficking of internalised TNF-α to suppress caspase-8 activation in colon cancer cells [158]. Since DHA was shown to inhibit the Wnt pathway and activation of β-catenin, this mechanism may also play a role in its apoptosis-supporting effects [159].

5.2. Interaction of TNF Family Cytokines with Butyrate. Butyrate has been shown to have protective effects on inflammatory diseases such as UC and inflammation-mediated CRC. The ability of butyrate to trigger cancer cell apoptosis is one of the main features of its anticancer activity [160]. It induces apoptosis via mitochondria, efficiently modulating the level of Bcl-2 family and activating of caspases. Upregulation of the proapoptotic Bak and Bax, downregulation of the antiapoptotic Bcl-x_L, XIAP, and survivin, or activation of caspase-3 and Bid after butyrate treatment were detected [161–163]. Butyrate was also shown to effectively modulate the extrinsic apoptotic pathway and to affect the initial steps of DR-mediated apoptosis at the level of DR, FADD, or cFLIP protein [49]. By affecting the level of these proteins, it could effectively contribute to the enhancement of apoptosis induced by TNF-α, TRAIL, or FasL [164].

Using various types of colon cancer cell lines, it was reported by us and others that the dual treatment with TNF-α and butyrate significantly increased apoptosis and decreased the differentiation induced by butyrate in HT-29 cells [165, 166]. Our experiments also documented that the sensitivity of colonic cells to TNF-α and various inhibitors of AA metabolism is dependent on the differentiation status. The apoptotic effect of dual treatment with butyrate and TNF-α can be potentiated by several different COX and LOX inhibitors [129].

The butyrate-mediated facilitation of the TNF-α induced death signal was also detected in COLO 205 adenocarcinoma cells, highly resistant to extrinsic apoptosis induced by death ligands [167, 168]. The immune escape of COLO 205 cells from TNF-α mediated apoptosis is probably caused by extensive shedding of TNFR1 and TNFR2 [169] and by a cFLIP protein inhibitory effect on caspase-8 activation [170]. The reduction of cFLIP protein and elevated TNFR1 expression after butyrate treatment corresponded with the higher sensitivity of COLO 205 cells to TNF-α induced apoptosis [171].

It could be supposed that butyrate and TNF-α might share similar signalling pathways including phospholipase C and protein kinase C [172, 173]. Butyrate-mediated changes of colon cancer cell sensitivity to TNF-α have been associated with the modulation of NF-κB activity, which had a significant impact on proliferation, apoptosis, and inflammation in colon cancer cells. It was demonstrated that butyrate pretreatment of HT-29, SW480, and SW620 cells inhibits the TNF-α mediated p65 and p50 translocation to the nucleus, probably by suppressing the cellular proteasome activity and subsequently IκB degradation through butyrate ability to inhibit HDAC [174–176]. Moreover, butyrate reduced inflammation in experimental colitis in rats [177] and decreased proinflammatory cytokine expression in intestinal biopsies from Crohn's disease patients via NF-κB inactivation [178].

Butyrate pretreatment can lead to a significant enhancement of TRAIL-induced apoptosis in TRAIL-resistant cells [160]. Enhancement of apoptosis after combined treatment with butyrate and TRAIL was described in an HCT-116 colon cancer cell line. Butyrate caused a downregulation of XIAP protein and an upregulation of DR5, but no changes were detected on DR4 levels. Increased levels of DR5 were initiated by the binding of transcriptional factor Sp1 to *DR5* promoter after butyrate treatment [179]. Recently, an increased toxicity against gastrointestinal tumour cells *in vitro* and *in vivo* by combined treatment with sorafenib, HDAC inhibitors, and TRAIL was reported. The enhanced TRAIL cell killing correlated with the reduced Akt, ERK 1/2, and mTOR (mammalian target of rapamycin) activity and the enhanced cleavage of caspase-3 and reduced expression of Mcl-1 and Bcl-X_L [180].

Increased apoptosis in the combined treatment of butyrate and TRAIL was studied not only in colon cancer but also in other model systems including breast, bladder, and nervous cancer. Although individual mechanisms are cell type-dependent, several molecules (DR5, DISC, caspase-8, and Bcl-2 family proteins) play a crucial role across the models. Moreover, the combined treatment was not harmful to normal cells of different tissues, compared with their cancer counterparts [181, 182]. In addition, butyrate was reported to sensitise colon cancer cells to Fas-mediated apoptosis, mainly due to increased ROS production [165, 183].

Other data showed that inflammation can be affected both on the level of the immune system and colon epithelial cell interaction. In patients with UC it was discovered that butyrate inhibited the binding of HDAC1 to Fas promoter, which was hyperacetylated and led to an upregulation of Fas in T cells. Therefore, butyrate eliminated the source of

inflammation by induction of T-cell apoptosis. Furthermore, butyrate suppressed IFN-γ-mediated inflammation in colon epithelial cells by preventing STAT-1 activation and blocking iNOS induction [69].

6. Importance of Fatty Acids for the Prevention and Therapy of Colon Inflammation and Cancer

Recently, it has been clear that dietary fatty acids not only serve as an energy source but also represent components which significantly influence physiological and pathophysiological processes from molecular and cellular to organismal levels. These compounds have also been considered as pharmaceutical agents which may have beneficial or detrimental impact on the body and may influence the effects of other drugs [108, 184]. The research concerning PUFAs as well as butyrate is closely related to the prevention and therapy of serious human diseases including cancer and interferes with other fields such as pharmacology and nutritional and health policy. Therefore, there is more need for a critical global overview and a consideration of the future perspectives [83, 185].

There are many reports showing the advantage of n-3 PUFAs as components of clinical enteral and parenteral lipid emulsions. Compared to the widely used emulsions from soybean oil containing mostly n-6 linoleic acid, other types with fish oil containing n-3 EPA and DHA were reported to have biological activities beneficial to patients [186, 187]. Many excellent reviews from experimental and clinical studies have been published on this topic, and thus we only summarise that nutritional supplementation with n-3 PUFA may influence inflammation, susceptibility to infection, and immune cell function and thus may affect immunological response, cachexia, and exert beneficial effects on the whole organism, which may then fight better against inflammation and cancer [188–191]. Besides fish oil, algal oil seems to be a better source of n-3 PUFAs due to gastrointestinal complaints of fish oil, especially in high-dose therapy [192]. To refine recommendations for the intake of individual n-3 PUFAs, differential effects of EPA, DHA, and their metabolites may be taken into account. The divergent incorporation into individual cellular lipids, activation of the signalling pathways and transcription factors, or the potency of their metabolites can contribute to diversity in the cellular response [193].

The importance of n-3 PUFAs as well as butyrate in relation to TNF family cytokines lies particularly in their ability to decrease the production of these cytokines and other factors (eicosanoids and interleukins) in the organism, to interfere with their signalling pathways, and thus to prevent the inflammatory response. Supplementation with DHA and EPA allows using lower doses of corticoids in IBD therapy [194].

Cancer anorexia and cachexia are major factors contributing to the weakening of the already compromised immune system of cancer patients. There is evidence suggesting that elevated levels of proinflammatory cytokines are associated with cancer-related cachexia [195, 196]. The treatment with fish oil or NSAIDs was shown to attenuate systemic inflammation and improve cachexia because it decreased production of active eicosanoids as well as level of proinflammatory and procachectic cytokines [197].

An important aspect is represented by the reported DHA and/or butyrate ability to support apoptosis induced by TNF cytokines, which is promising for use as a supportive anticancer agent. The therapeutic use of the TNF-α/TNFR, the FasL/Fas, or TRAIL/DRs in cancer treatment has been hampered by severe side effects [33, 198]. The systemic administration of TNF-α causes a septic shock-like response possibly mediated by NF-κB activation, and the injection of an agonist antibody to Fas can be lethal. Moreover, many cancer cells are resistant to apoptosis induced by these cytokines [43, 48]. Thus, particularly the ability of n-3 PUFAs to decrease the therapeutic doses, to overcome the resistance, or to improve the overall state of the organism could be considered. Moreover, the interaction with TRAIL overcoming cancer cell resistance and being selective for cancer cells is highly important. However, it is necessary to further investigate and verify these possibilities before future clinical use.

There are studies showing mainly preventive but also potential therapeutic applications of butyrate, which aim to exploit its ability to regulate inflammation, apoptosis, and ion uptake. Anti-inflammatory effects of butyrate were studied in patients with UC or Crohn's disease as well as on animal models, where in several studies it reduced the clinical and inflammatory index [121]. Moreover, its ability to support the effects of other anti-inflammatory and anticancer agents (such as NSAIDs and apoptotic cytokines) is promising for clinical use. The advantages of treatment with butyrate include practically no adverse side effects and its possible oral administration, although its unpleasant taste and odour make it extremely difficult [199].

7. Conclusion

From the data described above it appears that the targeted use of a specific type of fat and fibre (PUFAs and butyrate) may have a number of beneficial effects both in physiological and pathophysiological conditions particularly in the intestine. Both PUFAs and fibre (source of butyrate) are natural dietary components which represent a nontoxic way for positive modulation of intestinal cell physiology, improvement of inflammatory conditions and cancer treatment outcomes, and slowing down or preventing the recurrence of IBD or CRC. Important issue represents the ability of dietary fatty acids to modulate production and function of endogenous regulators from TNF family and thus positively or negatively influence the behaviour of intestinal cells. Understanding the molecular mechanisms of both PUFA and butyrate effects and their interaction with specific type of cytokines may help to optimise the composition of clinical nutrition and therapeutical approaches for patients with IBD and intestinal neoplasms. However, as with other agents, they have to be

applied carefully, based on solid scientific evidence of their mechanisms of action from the molecular and cellular up to the organismal levels.

Abbreviations

AA: Arachidonic acid
ALA: α Linolenic acid
APC: Adenomatous polyposis coli
cFLIP: FLICE-like inhibitory protein
CL: Cardiolipin
COX: Cyclooxygenase
CRC: Colorectal cancer
DD: Death domain
DHA: Docosahexaenoic acid
DISC: Death-inducing signaling complex
DR: Death receptor
EPA: Eicosapentaenoic acid
ERK: Extracellular signal-regulated kinases
FAP: Familial adenomatous polyposis
FADD: Fas-associated DD protein
FAT/CD36: Fatty acid translocase
HDACI: Histone deacetylase inhibitor
HIF-1α: Hypoxia-inducing factor 1α
IAPs: Inhibitor of apoptosis proteins
IBD: Inflammatory bowel disease
IL: Interleukin
iNOS: Inducible nitric oxide synthase
JAK: Janus kinase
IFN: Interferon
JNK: c-Jun N-terminal kinase
LA: Linoleic acid
LD: Lipid droplet
LOX: Lipoxygenase
mTOR: Mammalian target of rapamycin
NF-κB: Nuclear factor κB
NSAIDs: Nonsteroidal anti-inflammatory drugs
PGE2: Prostaglandin E2
PLA2: Phospholipase A2
PPARγ: Peroxisome proliferator-activated receptor γ
PUFA: Polyunsaturated fatty acids
RIP: Receptor-interacting protein
RONS: Reactive oxygen and nitrogen species
SCFA: Short-chain fatty acid
STAT: Signal transducer and activator of transcription
TNF: Tumor necrosis factor
TRAIL: Tumour necrosis factor-related apoptosis-inducing ligand
UC: Ulcerative colitis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] F. E. Bertrand, C. W. Angus, W. J. Partis, and G. Sigounas, "Developmental pathways in colon cancer: crosstalk between WNT, BMP, Hedgehog and Notch," *Cell Cycle*, vol. 11, pp. 4344–4351, 2012.

[2] C. Gunther, H. Neumann, M. F. Neurath, and C. Becker, "Apoptosis, necrosis and necroptosis: cell death regulation in the intestinal epithelium," *Gut*, vol. 62, pp. 1062–1071, 2013.

[3] F. E. Ahmed, "Colon cancer: prevalence, screening, gene expression and mutation, and risk factors and assessment," *Journal of Environmental Science and Health C: Environmental Carcinogenesis and Ecotoxicology Reviews*, vol. 21, no. 2, pp. 65–131, 2003.

[4] W. M. Grady and J. M. Carethers, "Genomic and epigenetic instability in colorectal cancer pathogenesis," *Gastroenterology*, vol. 135, no. 4, pp. 1079–1099, 2008.

[5] S. Lifshitz, S. A. Lamprecht, D. Benharroch, I. Prinsloo, S. Polak-Charcon, and B. Schwartz, "Apoptosis (programmed cell death) in colonic cells: from normal to transformed stage," *Cancer Letters*, vol. 163, no. 2, pp. 229–238, 2001.

[6] B. Zhivotovsky and S. Orrenius, "Carcinogenesis and apoptosis: paradigms and paradoxes," *Carcinogenesis*, vol. 27, no. 10, pp. 1939–1945, 2006.

[7] L. Chen, S. M. Park, A. V. Tumanov et al., "CD95 promotes tumour growth," *Nature*, vol. 465, pp. 492–496, 2010.

[8] F. Balkwill and A. Mantovani, "Inflammation and cancer: back to Virchow?" *The Lancet*, vol. 357, no. 9255, pp. 539–545, 2001.

[9] P. L. Lakatos and L. Lakatos, "Risk for colorectal cancer in ulcerative colitis: changes, causes and management strategies," *World Journal of Gastroenterology*, vol. 14, no. 25, pp. 3937–3947, 2008.

[10] S. H. Itzkowitz and X. Yio, "Inflammation and cancer—IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 287, no. 1, pp. G7–G17, 2004.

[11] U. Gaur and B. B. Aggarwal, "Regulation of proliferation, survival and apoptosis by members of the TNF superfamily," *Biochemical Pharmacology*, vol. 66, no. 8, pp. 1403–1408, 2003.

[12] P. Boyle and M. E. Leon, "Epidemiology of colorectal cancer," *British Medical Bulletin*, vol. 64, pp. 1–25, 2002.

[13] T. Yamamoto, M. Nakahigashi, and A. R. Saniabadi, "Review article: diet and inflammatory bowel disease—epidemiology and treatment," *Alimentary Pharmacology and Therapeutics*, vol. 30, no. 2, pp. 99–112, 2009.

[14] M. F. McEntee and J. Whelan, "Dietary polyunsaturated fatty acids and colorectal neoplasia," *Biomedicine and Pharmacotherapy*, vol. 56, no. 8, pp. 380–387, 2002.

[15] M. P. Weijenberg, M. Lüchtenborg, A. F. P. M. de Goeij et al., "Dietary fat and risk of colon and rectal cancer with aberrant MLH1 expression, APC or KRAS genes," *Cancer Causes and Control*, vol. 18, no. 8, pp. 865–879, 2007.

[16] D. Schrenk, "Dietary fiber, low-molecular-weight food constituents and colo-rectal inflammation in animal models—a review," *Molecular Nutrition and Food Research*, vol. 53, no. 10, pp. 1281–1288, 2009.

[17] H. Putaala, H. Mäkivuokko, K. Tiihonen, and N. Rautonen, "Simulated colon fiber metabolome regulates genes involved in cell cycle, apoptosis, and energy metabolism in human colon cancer cells," *Molecular and Cellular Biochemistry*, vol. 357, no. 1-2, pp. 235–245, 2011.

[18] G. Sethi, M. K. Shanmugam, L. Ramachandran, A. P. Kumar, and V. Tergaonkar, "Multifaceted link between cancer and inflammation," *Bioscience Reports*, vol. 32, no. 1, pp. 1–15, 2012.

[19] D. B. Vendramini-Costa and J. E. Carvalho, "Molecular link mechanisms between inflammation and cancer," *Current Pharmaceutical Design*, vol. 18, pp. 3831–3852, 2012.

[20] T. Chiba, H. Marusawa, and T. Ushijima, "Inflammation-associated cancer development in digestive organs: mechanisms and roles for genetic and epigenetic modulation," *Gastroenterology*, vol. 143, pp. 550–563, 2012.

[21] S. Danese and A. Mantovani, "Inflammatory bowel disease and intestinal cancer: a paradigm of the Yin-Yang interplay between inflammation and cancer," *Oncogene*, vol. 29, no. 23, pp. 3313–3323, 2010.

[22] K. A. Tappenden, "Inflammation and intestinal function: where does it start and what does it mean?" *Journal of Parenteral and Enteral Nutrition*, vol. 32, no. 6, pp. 648–650, 2008.

[23] A. Andoh, Y. Yagi, M. Shioya, A. Nishida, T. Tsujikawa, and Y. Fujiyama, "Mucosal cytokine network in inflammatory bowel disease," *World Journal of Gastroenterology*, vol. 14, no. 33, pp. 5154–5161, 2008.

[24] S. Kraus and N. Arber, "Inflammation and colorectal cancer," *Current Opinion in Pharmacology*, vol. 9, no. 4, pp. 405–410, 2009.

[25] L. Klampfer, "Cytokines, inflammation and colon cancer," *Current Cancer Drug Targets*, vol. 11, no. 4, pp. 451–464, 2011.

[26] G. Monteleone, F. Pallone, and C. Stolfi, "The dual role of inflammation in colon carcinogenesis," *International Journal of Molecular Sciences*, vol. 13, pp. 11071–11084, 2012.

[27] A. Rizzo, F. Pallone, G. Monteleone, and M. C. Fantini, "Intestinal inflammation and colorectal cancer: a doubleedged sword?" *World Journal of Gastroenterology*, vol. 17, no. 26, pp. 3092–3100, 2011.

[28] B. K. Popivanova, K. Kitamura, Y. Wu et al., "Blocking TNF-α in mice reduces colorectal carcinogenesis associated with chronic colitis," *The Journal of Clinical Investigation*, vol. 118, no. 2, pp. 560–570, 2008.

[29] M. J. Waldner and M. F. Neurath, "Cytokines in colitis-associated cancer: potential drug targets?" *Inflammation and Allergy—Drug Targets*, vol. 7, no. 3, pp. 187–194, 2008.

[30] S. R. Yan, R. R. Joseph, K. Rosen et al., "Activation of NF-κB following detachment delays apoptosis in intestinal epithelial cells," *Oncogene*, vol. 24, no. 43, pp. 6482–6491, 2005.

[31] R. S. Chapkin, L. A. Davidson, L. Ly, B. R. Weeks, J. R. Lupton, and D. N. McMurray, "Immunomodulatory effects of (n-3) fatty acids: putative link to inflammation and colon cancer," *Journal of Nutrition*, vol. 137, no. 1, pp. 200S–204S, 2007.

[32] J. L. McClellan, J. M. Davis, J. L. Steiner et al., "Intestinal inflammatory cytokine response in relation to tumorigenesis in the $Apc^{Min/+}$ mouse," *Cytokine*, vol. 57, no. 1, pp. 113–119, 2012.

[33] B. B. Aggarwal, S. C. Gupta, and J. H. Kim, "Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey," *Blood*, vol. 119, no. 3, pp. 651–665, 2012.

[34] A. Bhardwaj and B. B. Aggarwal, "Receptor-mediated choreography of life and death," *Journal of Clinical Immunology*, vol. 23, no. 5, pp. 317–332, 2003.

[35] H. Walczak and P. H. Krammer, "The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems," *Experimental Cell Research*, vol. 256, no. 1, pp. 58–66, 2000.

[36] S. Nagata, "Apoptosis by death factor," *Cell*, vol. 88, no. 3, pp. 355–365, 1997.

[37] M. MacFarlane, "TRAIL-induced signalling and apoptosis," *Toxicology Letters*, vol. 139, no. 2-3, pp. 89–97, 2003.

[38] N. Özören and W. S. El-Deiry, "Defining characteristics of types I and II apoptotic cells in response to TRAIL," *Neoplasia*, vol. 4, no. 6, pp. 551–557, 2002.

[39] M. R. Sprick and H. Walczak, "The interplay between the Bcl-2 family and death receptor-mediated apoptosis," *Biochimica et Biophysica Acta*, vol. 1644, no. 2-3, pp. 125–132, 2004.

[40] W. Wu, P. Liu, and J. Li, "Necroptosis: an emerging form of programmed cell death," *Critical Reviews in Oncology/Hematology*, vol. 82, pp. 249–258, 2012.

[41] P. S. Welz, A. Wullaert, K. Vlantis et al., "FADD prevents RIP3-mediated epithelial cell necrosis and chronic intestinal inflammation," *Nature*, vol. 477, no. 7364, pp. 330–334, 2011.

[42] B. Beutler and A. Cerami, "Cachectin and tumour necrosis factor as two sides of the same biological coin," *Nature*, vol. 320, no. 6063, pp. 584–588, 1986.

[43] M. Jäättelä, "Biology of disease: biologic activities and mechanisms of action of tumor necrosis factor-α/cachectin," *Laboratory Investigation*, vol. 64, no. 6, pp. 724–742, 1991.

[44] K. J. Tracey and A. Cerami, "Tumor necrosis factor: a pleiotropic cytokine and therapeutic target," *Annual Review of Medicine*, vol. 45, pp. 491–503, 1994.

[45] H. C. Reinecker, M. Steffen, T. Witthoeft et al., "Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1β by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease," *Clinical and Experimental Immunology*, vol. 94, no. 1, pp. 174–181, 1993.

[46] C. Scheidereit, "IκB kinase complexes: gateways to NF-κB activation and transcription," *Oncogene*, vol. 25, no. 51, pp. 6685–6705, 2006.

[47] A. Numata, T. Minagawa, M. Asano, A. Nakane, H. Katoh, and T. Tanabe, "Functional evaluation of tumor-infiltrating mononuclear cells: detection of endogenous interferon-γ and tumor necrosis factor-α in human colorectal adenocarcinomas," *Cancer*, vol. 68, no. 9, pp. 1937–1943, 1991.

[48] C. V. Zhang, *Cell Apoptosis*, Nova Science Publishers, Inc., New York, NY, USA, 2007.

[49] G. Mellier, S. Huang, K. Shenoy, and S. Pervaiz, "TRAILing death in cancer," *Molecular Aspects of Medicine*, vol. 31, no. 1, pp. 93–112, 2010.

[50] S. R. Wiley, K. Schooley, P. J. Smolak et al., "Identification and characterization of a new member of the TNF family that induces apoptosis," *Immunity*, vol. 3, no. 6, pp. 673–682, 1995.

[51] C. Falschlehner, U. Schaefer, and H. Walczak, "Following TRAIL's path in the immune system," *Immunology*, vol. 127, no. 2, pp. 145–154, 2009.

[52] M. J. Mitchell, E. Wayne, K. Rana, C. B. Schaffer, and M. R. King, "TRAIL-coated leukocytes that kill cancer cells in the circulation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, pp. 930–935, 2014.

[53] A. Vaculova, V. Kaminskyy, E. Jalalvand, O. Surova, and B. Zhivotovsky, "Doxorubicin and etoposide sensitize small cell lung carcinoma cells expressing caspase-8 to TRAIL," *Molecular Cancer*, vol. 9, article 87, 2010.

[54] P. Secchiero, A. Gonelli, E. Carnevale et al., "TRAIL promotes the survival and proliferation of primary human vascular endothelial cells by activating the Akt and ERK pathways," *Circulation*, vol. 107, no. 17, pp. 2250–2256, 2003.

[55] A. Vaculová, J. Hofmanová, K. Souček, and A. Kozubík, "Different modulation of TRAIL-induced apoptosis by inhibition of pro-survival pathways in TRAIL-sensitive and TRAIL-resistant colon cancer cells," *FEBS Letters*, vol. 580, no. 28-29, pp. 6565–6569, 2006.

[56] A. Vaculová, J. Hofmanová, J. Zatloukalová, and A. Kozubík, "Differences in TRAIL-induced changes of Mcl-1 expression among distinct human colon epithelial cell lines," *Experimental Cell Research*, vol. 315, no. 19, pp. 3259–3266, 2009.

[57] D. A. Flusberg, J. Roux, S. L. Spencer, and P. K. Sorger, "Cells surviving fractional killing by TRAIL exhibit transient but sustainable resistance and inflammatory phenotypes," *Molecular Biology of the Cell*, vol. 24, pp. 2186–2200, 2013.

[58] C. Falschlehner, C. H. Emmerich, B. Gerlach, and H. Walczak, "TRAIL signalling: decisions between life and death," *International Journal of Biochemistry and Cell Biology*, vol. 39, no. 7-8, pp. 1462–1475, 2007.

[59] S. Brost, R. Koschny, J. Sykora et al., "Differential expression of the TRAIL/TRAIL-receptor system in patients with inflammatory bowel disease," *Pathology Research and Practice*, vol. 206, no. 1, pp. 43–50, 2010.

[60] J. J. Koornstra, J. H. Kleibeuker, C. M. M. van Geelen et al., "Expression of TRAIL (TNF-related apoptosis-inducing ligand) and its receptors in normal colonic mucosa, adenomas, and carcinomas," *Journal of Pathology*, vol. 200, no. 3, pp. 327–335, 2003.

[61] L. M. Laguinge, R. N. Samara, W. Wang et al., "DR5 receptor mediates anoikis in human colorectal carcinoma cell lines," *Cancer Research*, vol. 68, no. 3, pp. 909–917, 2008.

[62] A. Grosse-Wilde, O. Voloshanenko, S. Lawrence Bailey et al., "TRAIL-R deficiency in mice enhances lymph node metastasis without affecting primary tumor development," *The Journal of Clinical Investigation*, vol. 118, no. 1, pp. 100–110, 2008.

[63] F. J. H. Hoogwater, M. W. Nijkamp, N. Smakman et al., "Oncogenic K-Ras turns death receptors into metastasis-promoting receptors in human and mouse colorectal cancer cells," *Gastroenterology*, vol. 138, no. 7, pp. 2357–2367, 2010.

[64] A. Hague, D. J. Hicks, F. Hasan et al., "Increased sensitivity to TRAIL-induced apoptosis occurs during the adenoma to carcinoma transition of colorectal carcinogenesis," *British Journal of Cancer*, vol. 92, no. 4, pp. 736–742, 2005.

[65] J. Hofmanová, A. Vaculová, M. Hyžďalová, and A. Kozubík, "Response of normal and colon cancer epithelial cells to TNF-family apoptotic inducers," *Oncology Reports*, vol. 19, pp. 567–573, 2008.

[66] P. H. Krammer, R. Arnold, and I. N. Lavrik, "Life and death in peripheral T cells," *Nature Reviews Immunology*, vol. 7, no. 7, pp. 532–542, 2007.

[67] S. I. Abrams, "Positive and negative consequences of Fas/Fas ligand interactions in the antitumor response," *Frontiers in Bioscience*, vol. 10, pp. 809–821, 2005.

[68] I. N. Lavrik and P. H. Krammer, "Regulation of CD95/Fas signaling at the DISC," *Cell Death and Differentiation*, vol. 19, no. 1, pp. 36–41, 2012.

[69] M. A. Zimmerman, N. Singh, P. M. Martin et al., "Butyrate suppresses colonic inflammation through HDAC1-dependent Fas upregulation and Fas-mediated apoptosis of T cells," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 302, no. 12, pp. G1405–G1415, 2012.

[70] Broad Institute, 2014, http://www.broadinstitute.org/tumorscape/pages/portalHome.jsf.

[71] C. Ruiz-Ruiz, G. Robledo, E. Cano, J. M. Redondo, and A. Lopez-Rivas, "Characterization of p53-mediated up-regulation of CD95 gene expression upon genotoxic treatment in human breast tumor cells," *The Journal of Biological Chemistry*, vol. 278, no. 34, pp. 31667–31675, 2003.

[72] A. V. Paschall, M. A. Zimmerman, C. M. Torres et al., "Ceramide targets xIAP and cIAP1 to sensitize metastatic colon and breast cancer cells to apoptosis induction to suppress tumor progression," *BMC Cancer*, vol. 14, article 24, 2014.

[73] F. A. Sayani, C. M. Keenan, M. D. van Sickle et al., "The expression and role of Fas ligand in intestinal inflammation," *Neurogastroenterology and Motility*, vol. 16, no. 1, pp. 61–74, 2004.

[74] J. P. Medema, J. de Jong, T. van Hall, C. J. M. Melief, and R. Offringa, "Immune escape of tumors in vivo by expression of cellular FLICE- inhibitory protein," *Journal of Experimental Medicine*, vol. 190, no. 7, pp. 1033–1038, 1999.

[75] N. Wittkopf, C. Gunther, E. Martini et al., "Cellular FLICE-like inhibitory protein secures intestinal epithelial cell survival and immune homeostasis by regulating caspase-8," *Gastroenterology*, vol. 145, no. 6, pp. 1369–1379, 2013.

[76] F. Liu, K. Bardhan, D. Yang et al., "NF-kappaB directly regulates Fas transcription to modulate Fas-mediated apoptosis and tumor suppression," *The Journal of Biological Chemistry*, vol. 287, pp. 25530–25540, 2012.

[77] A. P. Simopoulos, "Omega-3 fatty acids in health and disease and in growth and development," *American Journal of Clinical Nutrition*, vol. 54, no. 3, pp. 438–463, 1991.

[78] P. Matafome, D. Santos-Silva, C. M. Sena, and R. Seica, "Common mechanisms of dysfunctional adipose tissue and obesity-related cancers," *Diabetes/Metabolism Research and Reviews*, vol. 29, no. 4, pp. 285–295, 2013.

[79] S. D. Day, R. T. Enos, J. L. McClellan, J. L. Steiner, K. T. Velazquez, and E. A. Murphy, "Linking inflammation to tumorigenesis in a mouse model of high-fat-diet-enhanced colon cancer," *Cytokine*, vol. 64, pp. 454–462, 2013.

[80] A. E. Tammariello and J. A. Milner, "Mouse models for unraveling the importance of diet in colon cancer prevention," *Journal of Nutritional Biochemistry*, vol. 21, no. 2, pp. 77–88, 2010.

[81] H. Zeng and D. L. Lazarova, "Obesity-related colon cancer: dietary factors and their mechanisms of anticancer action," *Clinical and Experimental Pharmacology and Physiology*, vol. 39, no. 2, pp. 161–167, 2012.

[82] P. Flachs, M. Rossmeisl, M. Bryhn, and J. Kopecky, "Cellular and molecular effects of n-3 polyunsaturated fatty acids on adipose tissue biology and metabolism," *Clinical Science*, vol. 116, no. 1, pp. 1–16, 2009.

[83] A. P. Simopoulos, "Genetic variants in the metabolism of omega-6 and omega-3 fatty acids: their role in the determination of nutritional requirements and chronic disease risk," *Experimental Biology and Medicine*, vol. 235, no. 7, pp. 785–795, 2010.

[84] A. P. Simopoulos, "The importance of the ratio of omega-6/omega-3 essential fatty acids," *Biomedicine and Pharmacotherapy*, vol. 56, no. 8, pp. 365–379, 2002.

[85] J. X. Kang, "Balance of omega-6/omega-3 essential fatty acids is important for health. The evidence from gene transfer studies," *World Review of Nutrition and Dietetics*, vol. 95, pp. 93–102, 2005.

[86] L. S. Kremmyda, E. Tvrzicka, B. Stankova, and A. Zak, "Fatty acids as biocompounds: their role in human metabolism, health and disease: a review—part 2: fatty acid physiological roles and applications in human health and disease," *Biomedical Papers of the Medical Faculty of the University Palacký, Olomouc, Czechoslovakia*, vol. 155, no. 3, pp. 195–218, 2011.

[87] M. D'Archivio, B. Scazzocchio, S. Giammarioli et al., "omega3-PUFAs exert anti-inflammatory activity in visceral adipocytes from colorectal cancer patients," *PLoS ONE*, vol. 8, Article ID e77432, 2013.

[88] D. A. Los and N. Murata, "Membrane fluidity and its roles in the perception of environmental signals," *Biochimica et Biophysica Acta*, vol. 1666, no. 1-2, pp. 142–157, 2004.

[89] F. A. Sinicrope and S. Gill, "Role of cyclooxygenase-2 in colorectal cancer," *Cancer and Metastasis Reviews*, vol. 23, no. 1-2, pp. 63–75, 2004.

[90] A. Medeiros, C. Peres-Buzalaf, F. Fortino Verdan, and C. H. Serezani, "Prostaglandin E2 and the suppression of phagocyte innate immune responses in different organs," *Mediators of Inflammation*, vol. 2012, Article ID 327568, 13 pages, 2012.

[91] A. Barajas-Espinosa, F. Ochoa-Cortes, M. P. Moos, F. D. Ramirez, S. J. Vanner, and C. D. Funk, "Characterization of the cysteinyl leukotriene 2 receptor in novel expression sites of the gastrointestinal tract," *American Journal of Pathology*, vol. 178, no. 6, pp. 2682–2689, 2011.

[92] G. Hawcroft, P. M. Loadman, A. Belluzzi, and M. A. Hull, "Effect of eicosapentaenoic acid on E-type prostaglandin synthesis and EP4 receptor signaling in human colorectal cancer cells," *Neoplasia*, vol. 12, no. 8, pp. 618–627, 2010.

[93] C. N. Serhan and N. Chiang, "Endogenous pro-resolving and anti-inflammatory lipid mediators: a new pharmacologic genus," *British Journal of Pharmacology*, vol. 153, supplement 1, pp. S200–S215, 2008.

[94] I. L. Rudolph, D. S. Kelley, K. C. Klasing, and K. L. Erickson, "Regulation of cellular differentiation and apoptosis by fatty acids and their metabolites," *Nutrition Research*, vol. 21, no. 1-2, pp. 381–393, 2001.

[95] C. P. Diggle, "In vitro studies on the relationship between polyunsaturated fatty acids and cancer: tumour or tissue specific effects?" *Progress in Lipid Research*, vol. 41, no. 3, pp. 240–253, 2002.

[96] C. H. MacLean, S. J. Newberry, W. A. Mojica et al., "Effects of omega-3 fatty acids on cancer risk: a systematic review," *Journal of the American Medical Association*, vol. 295, no. 4, pp. 403–415, 2006.

[97] M. A. Moyad, "An introduction to dietary/supplemental omega-3 fatty acids for general health and prevention—part II," *Urologic Oncology: Seminars and Original Investigations*, vol. 23, no. 1, pp. 36–48, 2005.

[98] J. Hofmanová, K. Souček, A. Vaculová, and A. Kozubík, "Fatty acids in the modulation of reactive oxygen species balance in cancer," in *Oxidants in Biology: A Question of Balance*, G. Valacchi and P. A. Davis, Eds., chapter 6, pp. 129–153, Springer, Dordrecht, The Netherlands, 2008.

[99] F. Abir, S. Alva, D. L. Kaminski, and W. E. Longo, "The role of arachidonic acid regulatory enzymes in colorectal disease," *Diseases of the Colon and Rectum*, vol. 48, no. 7, pp. 1471–1483, 2005.

[100] A. Lanas and F. Sopeña, "Nonsteroidal anti-inflammatory drugs and lower gastrointestinal complications," *Gastroenterology Clinics of North America*, vol. 38, no. 2, pp. 333–352, 2009.

[101] J. E. Teitelbaum and W. Allan Walker, "Review: the role of omega 3 fatty acids in intestinal inflammation," *Journal of Nutritional Biochemistry*, vol. 12, no. 1, pp. 21–32, 2001.

[102] D. H. Jho, S. M. Cole, E. M. Lee, and N. J. Espat, "Role of omega-3 fatty acid supplementation in inflammation and malignancy," *Integrative Cancer Therapies*, vol. 3, no. 2, pp. 98–111, 2004.

[103] S. C. Mills, A. C. Windsor, and S. C. Knight, "The potential interactions between polyunsaturated fatty acids and colonic inflammatory processes," *Clinical and Experimental Immunology*, vol. 142, no. 2, pp. 216–228, 2005.

[104] B. S. Reddy, C. Burill, and J. Rigotty, "Effect of diets high in ω-3 and ω6 fatty acids on initiation and postinitiation stages of colon carcinogenesis," *Cancer Research*, vol. 51, no. 2, pp. 487–491, 1991.

[105] L. A. Davidson, D. V. Nguyen, R. M. Hokanson et al., "Chemopreventive n-3 polyunsaturated fatty acids reprogram genetic signatures during colon cancer initiation and progression in the rat," *Cancer Research*, vol. 64, no. 18, pp. 6797–6804, 2004.

[106] G. Calviello and S. Serini, *Dietary Omega-3 Polyunsaturated Fatty Acids and Cancer*, Springer, New York, NY, USA, 2010.

[107] C. A. Hudert, K. H. Weylandt, Y. Lu et al., "Transgenic mice rich in endogenous omega-3 fatty acids are protected from colitis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 30, pp. 11276–11281, 2006.

[108] R. S. Pardini, "Nutritional intervention with omega-3 fatty acids enhances tumor response to anti-neoplastic agents," *Chemico-Biological Interactions*, vol. 162, no. 2, pp. 89–105, 2006.

[109] M. E. Surette, "The science behind dietary omega-3 fatty acids," *Canadian Medical Association Journal*, vol. 178, no. 2, pp. 177–180, 2008.

[110] G. Calviello, S. Serini, E. Piccioni, and G. Pessina, "Antineoplastic effects of N-3 polyunsaturated fatty acids in combination with drugs and radiotherapy: preventive and therapeutic strategies," *Nutrition and Cancer*, vol. 61, no. 3, pp. 287–301, 2009.

[111] F. de Carlo, T. R. Witte, W. E. Hardman, and P. P. Claudio, "Omega-3 eicosapentaenoic acid decreases CD133 colon cancer stem-like cell marker expression while increasing sensitivity to chemotherapy," *PLoS ONE*, vol. 8, Article ID e69760, 2013.

[112] S. C. Larsson, M. Kumlin, M. Ingelman-Sundberg, and A. Wolk, "Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms," *American Journal of Clinical Nutrition*, vol. 79, no. 6, pp. 935–945, 2004.

[113] R. S. Chapkin, J. Seo, D. N. McMurray, and J. R. Lupton, "Mechanisms by which docosahexaenoic acid and related fatty acids reduce colon cancer risk and inflammatory disorders of the intestine," *Chemistry and Physics of Lipids*, vol. 153, no. 1, pp. 14–23, 2008.

[114] B. Skender, A. H. Vaculová, and J. Hofmanová, "Docosahexaenoic fatty acid (DHA) in the regulation of colon cell growth and cell death: a review," *Biomedical Papers of the Medical Faculty of the University Palacký, Olomouc, Czechoslovakia*, vol. 156, no. 3, pp. 186–199, 2012.

[115] M. M. Kaczmarczyk, M. J. Miller, and G. G. Freund, "The health benefits of dietary fiber: beyond the usual suspects of type 2 diabetes mellitus, cardiovascular disease and color cancer," *Metabolism: Clinical and Experimental*, vol. 61, pp. 1058–1066, 2012.

[116] H. M. Hamer, D. Jonkers, K. Venema, S. Vanhoutvin, F. J. Troost, and R.-J. Brummer, "Review article: the role of butyrate on colonic function," *Alimentary Pharmacology and Therapeutics*, vol. 27, no. 2, pp. 104–119, 2008.

[117] J. Hofmanová, A. Vaculová, and A. Kozubík, "Regulation of the metabolism of polyunsaturated fatty acids and butyrate in colon cancer cells," *Current Pharmaceutical Biotechnology*, vol. 14, no. 3, pp. 274–288, 2013.

[118] X. Tong, L. Yin, and C. Giardina, "Butyrate suppresses Cox-2 activation in colon cancer cells through HDAC inhibition," *Biochemical and Biophysical Research Communications*, vol. 317, no. 2, pp. 463–471, 2004.

[119] N. T. Ventham, N. A. Kennedy, E. R. Nimmo, and J. Satsangi, "Beyond gene discovery in inflammatory bowel disease: the emerging role of epigenetics," *Gastroenterology*, vol. 145, pp. 293–308, 2013.

[120] M. C. Daroqui and L. H. Augenlicht, "Transcriptional attenuation in colon carcinoma cells in response to butyrate," *Cancer Prevention Research*, vol. 3, no. 10, pp. 1292–1302, 2010.

[121] R. B. Canani, M. D. Costanzo, L. Leone, M. Pedata, R. Meli, and A. Calignano, "Potential beneficial effects of butyrate in intestinal and extraintestinal diseases," *World Journal of Gastroenterology*, vol. 17, no. 12, pp. 1519–1528, 2011.

[122] V. Ganapathy, M. Thangaraju, E. Gopal et al., "Sodium-coupled monocarboxylate transporters in normal tissues and in cancer," *AAPS Journal*, vol. 10, no. 1, pp. 193–199, 2008.

[123] S. I. Karaki, H. Tazoe, H. Hayashi et al., "Expression of the short-chain fatty acid receptor, GPR43, in the human colon," *Journal of Molecular Histology*, vol. 39, no. 2, pp. 135–142, 2008.

[124] R. Thibault, F. Blachier, B. Darcy-Vrillon, P. de Coppet, A. Bourreille, and J.-P. Segain, "Butyrate utilization by the colonic mucosa in inflammatory bowel diseases: a transport deficiency," *Inflammatory Bowel Diseases*, vol. 16, no. 4, pp. 684–695, 2010.

[125] M. Andriamihaja, C. Chaumontet, D. Tome, and F. Blachier, "Butyrate metabolism in human colon carcinoma cells: implications concerning its growth-inhibitory effect," *Journal of Cellular Physiology*, vol. 218, no. 1, pp. 58–65, 2009.

[126] J. Vondráček, M. A. Sheard, P. Krejčí, K. Minksová, J. Hofmanová, and A. Kozubík, "Modulation of death receptor-mediated apoptosis in differentiating human myeloid leukemia HL-60 cells," *Journal of Leukocyte Biology*, vol. 69, pp. 794–802, 2001.

[127] J. Štika, J. Vondráček, J. Hofmanová, V. Šimek, and A. Kozubík, "MK-886 enhances tumour necrosis factor-alpha-induced differentiation and apoptosis," *Cancer Letters*, vol. 237, pp. 263–271, 2006.

[128] J. Procházková, L. Stixová, K. Souček, J. Hofmanová, and A. Kozubík, "Monocytic differentiation of leukemic HL-60 cells induced by co-treatment with TNF-α and MK886 requires activation of pro-apoptotic machinery," *European Journal of Haematology*, vol. 83, no. 1, pp. 35–47, 2009.

[129] M. Kovaříková, J. Hofmanová, K. Souček, and A. Kozubík, "The effects of TNF-α and inhibitors of arachidonic acid metabolism on human colon HT-29 cells depend on differentiation status," *Differentiation*, vol. 72, no. 1, pp. 23–31, 2004.

[130] J. Hofmanová, A. Vaculová, and A. Kozubík, "Polyunsaturated fatty acids sensitize human colon adenocarcinoma HT-29 cells to death receptor-mediated apoptosis," *Cancer Letters*, vol. 218, no. 1, pp. 33–41, 2005.

[131] A. Vaculová, J. Hofmanová, L. Anděra, and A. Kozubík, "TRAIL and docosahexaenoic acid cooperate to induce HT-29 colon cancer cell death," *Cancer Letters*, vol. 229, pp. 43–48, 2005.

[132] O. Vondálová Blanářová, I. Jelínková, A. Szöor et al., "Cisplatin and a potent platinum(IV) complex-mediated enhancement of TRAIL-induced cancer cells killing is associated with modulation of upstream events in the extrinsic apoptotic pathway," *Carcinogenesis*, vol. 32, no. 1, pp. 42–51, 2011.

[133] J. Hofmanová, A. Vaculová, Z. Koubková, M. Hýžďalová, and A. Kozubík, "Human fetal colon cells and colon cancer cells respond differently to butyrate and PUFAs," *Molecular Nutrition & Food Research*, vol. 53, supplement 1, pp. S102–S113, 2009.

[134] L. Kočí, M. Hýžďalová, A. Vaculová, J. Hofmanová, and A. Kozubík, "Detachment-mediated resistance to TRAIL-induced apoptosis is associated with stimulation of the PI3K/Akt pathway in fetal and adenocarcinoma epithelial colon cells," *Cytokine*, vol. 55, no. 1, pp. 34–39, 2011.

[135] M. T. Accioly, P. Pacheco, C. M. Maya-Monteiro et al., "Lipid bodies are reservoirs of cyclooxygenase-2 and sites of prostaglandin-E2 synthesis in colon cancer cells," *Cancer Research*, vol. 68, no. 6, pp. 1732–1740, 2008.

[136] D. W. L. Ma, J. Seo, L. A. Davidson et al., "n-3 PUFA alter caveolae lipid composition and resident protein localization in mouse colon," *The FASEB Journal*, vol. 18, no. 9, pp. 1040–1042, 2004.

[137] W. Stillwell, S. R. Shaikh, M. Zerouga, R. Siddiqui, and S. R. Wassal, "Docosahexaenoic acid affects cell signaling by altering lipid rafts," *Reproduction Nutrition Development*, vol. 45, no. 5, pp. 559–579, 2005.

[138] J. Seo, R. Barhoumi, A. E. Johnson, J. R. Lupton, and R. S. Chapkin, "Docosahexaenoic acid selectively inhibits plasma membrane targeting of lipidated proteins," *The FASEB Journal*, vol. 20, no. 6, pp. 770–772, 2006.

[139] K. R. Rogers, K. D. Kikawa, M. Mouradian et al., "Docosahexaenoic acid alters epidermal growth factor receptor-related signaling by disrupting its lipid raft association," *Carcinogenesis*, vol. 31, no. 9, pp. 1523–1530, 2010.

[140] V. E. Kagan, Y. Y. Tyurina, H. Bayir et al., "The "pro-apoptotic genies" get out of mitochondria: oxidative lipidomics and redox activity of cytochrome c/cardiolipin complexes," *Chemico-Biological Interactions*, vol. 163, no. 1-2, pp. 15–28, 2006.

[141] S. Rohrbach, "Effects of dietary polyunsaturated fatty acids on mitochondria," *Current Pharmaceutical Design*, vol. 15, no. 36, pp. 4103–4116, 2009.

[142] M. Y. Hong, R. S. Chapkin, R. Barhoumi et al., "Fish oil increases mitochondrial phospholipid unsaturation, upregulating reactive oxygen species and apoptosis in rat colonocytes," *Carcinogenesis*, vol. 23, no. 11, pp. 1919–1925, 2002.

[143] K. Mbodji, C. Charpentier, C. Guerin et al., "Adjunct therapy of n-3 fatty acids to 5-ASA ameliorates inflammatory score and decreases NF-kappaB in rats with TNBS-induced colitis," *The Journal of Nutritional Biochemistry*, vol. 24, pp. 700–705, 2013.

[144] C. Gravaghi, K. M. D. la Perle, P. Ogrodwski et al., "Cox-2 expression, PGE2 and cytokines production are inhibited by endogenously synthesized n-3 PUFAs in inflamed colon of fat-1 mice," *Journal of Nutritional Biochemistry*, vol. 22, no. 4, pp. 360–365, 2011.

[145] A. Algamas-Dimantov, D. Davidovsky, J. Ben-Ari et al., "Amelioration of diabesity-induced colorectal ontogenesis by omega-3 fatty acids in mice," *The Journal of Lipid Research*, vol. 53, pp. 1056–1070, 2012.

[146] S. N. Meydani, "Dietary modulation of cytokine production and biologic functions," *Nutrition Reviews*, vol. 48, no. 10, pp. 361–369, 1990.

[147] D. A. Ribardo, J. W. Peterson, and A. K. Chopra, "Phospholipase A2-activating protein—an important regulatory molecule in modulating cyclooxygenase-2 and tumor necrosis factor production during inflammation," *Indian Journal of Experimental Biology*, vol. 40, no. 2, pp. 129–138, 2002.

[148] T. Sassa, S. Suto, Y. Okayasu, and A. Kihara, "A shift in sphingolipid composition from C24 to C16 increases susceptibility to apoptosis in HeLa cells," *Biochimica et Biophysica Acta*, vol. 1821, no. 7, pp. 1031–1037, 2012.

[149] M. Hayakawa, N. Ishida, K. Takeuchi et al., "Arachidonic acid-selective cytosolic phospholipase A2 is crucial in the cytotoxic action of tumor necrosis factor," *The Journal of Biological Chemistry*, vol. 268, no. 15, pp. 11290–11295, 1993.

[150] Y. Cao, A. T. Pearman, G. A. Zimmerman, T. M. McIntyre, and S. M. Prescott, "Intracellular unesterified arachidonic acid signals apoptosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 21, pp. 11280–11285, 2000.

[151] G. L. Bannenberg, "Therapeutic applicability of anti-inflammatory and proresolving polyunsaturated fatty acid-derived lipid mediators," *TheScientificWorldJOURNAL*, vol. 10, pp. 676–712, 2010.

[152] J. E. Kinsella, "Lipids, membrane receptors, and enzymes: effects of dietary fatty acids," *Journal of Parenteral and Enteral Nutrition*, vol. 14, no. 5, pp. 200S–217S, 1990.

[153] V. di Marzo, "Arachidonic acid and eicosanoids as targets and effectors in second messenger interactions," *Prostaglandins Leukotrienes and Essential Fatty Acids*, vol. 53, no. 4, pp. 239–254, 1995.

[154] J. Nowak, K. H. Weylandt, P. Habbel et al., "Colitis-associated colon tumorigenesis is suppressed in transgenic mice rich in endogenous n-3 fatty acids," *Carcinogenesis*, vol. 28, no. 9, pp. 1991–1995, 2007.

[155] H. Sampath and J. M. Ntambi, "Polyunsaturated fatty acid regulation of genes of lipid metabolism," *Annual Review of Nutrition*, vol. 25, pp. 317–340, 2005.

[156] I. J. Edwards and J. T. O'Flaherty, "Omega-3 fatty acids and PPAR γ in cancer," *PPAR Research*, vol. 2008, Article ID 358052, 14 pages, 2008.

[157] S. A. Kliewer, S. S. Sundseth, S. A. Jones et al., "Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 9, pp. 4318–4323, 1997.

[158] J. Han, R. C. Soletti, A. Sadarangani et al., "Nuclear expression of beta-catenin promotes RB stability and resistance to TNF-induced apoptosis in colon cancer cells," *Molecular Cancer Research*, vol. 11, pp. 207–218, 2013.

[159] B. A. Narayanan, N. K. Narayanan, B. Simi, and B. S. Reddy, "Modulation of inducible nitric oxide synthase and related proinflammatory genes by the omega-3 fatty acid docosahexaenoic acid in human colon cancer cells," *Cancer Research*, vol. 63, no. 5, pp. 972–979, 2003.

[160] A. Hernandez, R. Thomas, F. Smith et al., "Butyrate sensitizes human colon cancer cells to TRAIL-mediated apoptosis," *Surgery*, vol. 130, no. 2, pp. 265–272, 2001.

[161] F. M. Ruemmele, S. Dionne, I. Qureshi, D. S. R. Sarma, E. Levy, and E. G. Seidman, "Butyrate mediates Caco-2 cell apoptosis via up-regulation of pro-apoptotic BAK and inducing caspase-3 mediated cleavage of poly-(ADP-ribose) polymerase (PARP)," *Cell Death and Differentiation*, vol. 6, no. 8, pp. 729–735, 1999.

[162] S. A. Jones, R. N. Butler, I. R. Sanderson, and J. W. Wilson, "The effect of specific caspase inhibitors on TNF-α and butyrate-induced apoptosis of intestinal epithelial cells," *Experimental Cell Research*, vol. 292, no. 1, pp. 29–39, 2004.

[163] S. Orzechowska, B. Pajak, B. Gajkowska, and A. Orzechowski, "Cholesterol level determines viability and mitogenicity, but it does not affect sodium butyrate-dependent sensitization of Colo 205 cells to TNF-α-induced apoptosis," *Oncology Reports*, vol. 25, no. 2, pp. 573–582, 2011.

[164] S. E. F. Tran, T. H. Holmström, M. Ahonen, V.-M. Kähäri, and J. E. Eriksson, "MAPK/ERK overrides the apoptotic signaling from Fas, TNF, and TRAIL receptors," *The Journal of Biological Chemistry*, vol. 276, no. 19, pp. 16484–16490, 2001.

[165] C. Giardina, H. Boulares, and M. S. Inan, "NSAIDs and butyrate sensitize a human colorectal cancer cell line to TNF-α and Fas ligation: the role of reactive oxygen species," *Biochimica et Biophysica Acta*, vol. 1448, no. 3, pp. 425–438, 1999.

[166] M. Kovaříková, J. Pacherník, J. Hofmanová, Z. Zadák, and A. Kozubík, "TNF-alpha modulates the differentiation induced by butyrate in the HT-29 human colon adenocarcinoma cell line," *European Journal of Cancer*, vol. 36, no. 14, pp. 1844–1852, 2000.

[167] J. Gong, D. Yang, S. Kohanim, R. Humphreys, L. Broemeling, and R. Kurzrock, "Novel in vivo imaging shows up-regulation of death receptors by paclitaxel and correlates with enhanced antitumor effects of receptor agonist antibodies," *Molecular Cancer Therapeutics*, vol. 5, no. 12, pp. 2991–3000, 2006.

[168] B. Pajak, B. Gajkowska, and A. Orzechowski, "Position of STAT-1α in cycloheximide-dependent apoptosis triggered by TNF-α in human colorectal COLO 205 cancer cell line; role of polyphenolic compounds," *Journal of Physiology and Pharmacology*, vol. 56, supplement 3, pp. 119–141, 2005.

[169] M. A. Lombard, T. L. Wallace, M. F. Kubicek et al., "Synthetic matrix metalloproteinase inhibitors and tissue inhibitor of metalloproteinase (TIMP)-2, but not TIMP-1, inhibit shedding of tumor necrosis factor-α receptors in a human colon adenocarcinoma (Colo 205) cell line," *Cancer Research*, vol. 58, no. 17, pp. 4001–4007, 1998.

[170] B. Pajak, B. Gajkowska, and A. Orzechowski, "Cycloheximide-mediated sensitization to TNF-α-induced apoptosis in human colorectal cancer cell line COLO 205; role of FLIP and metabolic inhibitors," *Journal of Physiology and Pharmacology*, vol. 56, supplement 3, pp. 101–118, 2005.

[171] B. Pajak and A. Orzechowski, "IFN-alpha competes with TNF-alpha for STAT-1alpha; molecular basis for immune escape of human colon adenocarcinoma COLO 205 cells," *Oncology Reports*, vol. 18, no. 4, pp. 1039–1045, 2007.

[172] P. Cesaro, E. Raiteri, M. Démoz et al., "Expression of protein kinase C β1 confers resistance to TNFα- and paclitaxel-induced apoptosis in HT-29 colon carcinoma cells," *International Journal of Cancer*, vol. 93, no. 2, pp. 179–184, 2001.

[173] O. C. Velázquez, R. W. Seto, and J. L. Rombeau, "The scientific rationale and clinical application of short-chain fatty acids and medium-chain triacylglycerols," *Proceedings of the Nutrition Society*, vol. 55, no. 1, pp. 49–78, 1996.

[174] M. S. Inan, R. J. Rasoulpour, L. Yin, A. K. Hubbard, D. W. Rosenberg, and C. Giardina, "The luminal short-chain fatty acid butyrate modulates NF-κB activity in a human colonic epithelial cell line," *Gastroenterology*, vol. 118, no. 4, pp. 724–734, 2000.

[175] L. Yin, G. Laevsky, and C. Giardina, "Butyrate suppression of colonocyte NF-κ B activation and cellular proteasome activity," *The Journal of Biological Chemistry*, vol. 276, no. 48, pp. 44641–44646, 2001.

[176] M. Hýžďalová, J. Hofmanová, J. Pacherník, A. Vaculová, and A. Kozubík, "The interaction of butyrate with TNF-alpha during differentiation and apoptosis of colon epithelial cells: role of NF-kappaB activation," *Cytokine*, vol. 44, pp. 33–43, 2008.

[177] J. D. Butzner, R. Parmar, C. J. Bell, and V. Dalal, "Butyrate enema therapy stimulates mucosal repair in experimental colitis in the rat," *Gut*, vol. 38, no. 4, pp. 568–573, 1996.

[178] J. P. Segain, D. Raingeard de la Blétière, A. Bourreille et al., "Butyrate inhibits inflammatory responses through NFκB inhibition: implications for Crohn's disease," *Gut*, vol. 47, no. 3, pp. 397–403, 2000.

[179] Y. H. Kim, J. W. Park, J. Y. Lee, and T. K. Kwon, "Sodium butyrate sensitizes TRAIL-mediated apoptosis by induction of transcription from the DR5 gene promoter through Sp1 sites in colon cancer cells," *Carcinogenesis*, vol. 25, no. 10, pp. 1813–1820, 2004.

[180] H. A. Hamed, Y. Yamaguchi, P. B. Fisher, S. Grant, and P. Dent, "Sorafenib and HDAC inhibitors synergize with TRAIL to kill tumor cells," *Journal of Cellular Physiology*, vol. 228, no. 10, pp. 1996–2005, 2013.

[181] J. K. Earel Jr., R. L. VanOosten, and T. S. Griffith, "Histone deacetylase inhibitors modulate the sensitivity of tumor necrosis factor-related apoptosis-inducing ligand-resistant bladder tumor cells," *Cancer Research*, vol. 66, no. 1, pp. 499–507, 2006.

[182] A. Bangert, S. Cristofanon, I. Eckhardt et al., "Histone deacetylase inhibitors sensitize glioblastoma cells to TRAIL-induced apoptosis by c-myc-mediated downregulation of cFLIP," *Oncogene*, vol. 31, pp. 4677–4688, 2012.

[183] C. Giardina and M. S. Inan, "Nonsteroidal anti-inflammatory drugs, short-chain fatty acids, and reactive oxygen metabolism in human colorectal cancer cells," *Biochimica et Biophysica Acta*, vol. 1401, no. 3, pp. 277–288, 1998.

[184] Y. M. Dupertuis, M. M. Meguid, and C. Pichard, "Advancing from immunonutrition to a pharmaconutrition: a gigantic challenge," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 12, no. 4, pp. 398–403, 2009.

[185] B. Lands, "A critique of paradoxes in current advice on dietary lipids," *Progress in Lipid Research*, vol. 47, no. 2, pp. 77–106, 2008.

[186] A. Hagi, M. Nakayama, W. Shinzaki, S. Haji, and H. Ohyanagi, "Effects of the ω-6:ω-3 fatty acid ratio of fat emulsions on the fatty acid composition in cell membranes and the anti-inflammatory action," *Journal of Parenteral and Enteral Nutrition*, vol. 34, no. 3, pp. 263–270, 2010.

[187] Y. Y. Han, S. L. Lai, W. J. Ko, C. H. Chou, and H. S. Lai, "Effects of fish oil on inflammatory modulation in surgical intensive care unit patients," *Nutrition in Clinical Practice*, vol. 27, no. 1, pp. 91–98, 2012.

[188] G. J. A. Wanten and P. C. Calder, "Immune modulation by parenteral lipid emulsions," *American Journal of Clinical Nutrition*, vol. 85, no. 5, pp. 1171–1184, 2007.

[189] I. M. Berquin, I. J. Edwards, and Y. Q. Chen, "Multi-targeted therapy of cancer by omega-3 fatty acids," *Cancer Letters*, vol. 269, no. 2, pp. 363–377, 2008.

[190] P. C. Calder, "Symposium 4: hot topics in parenteral nutrition. Rationale for using new lipid emulsions in parenteral nutrition and a review of the trials performed in adults," *Proceedings of the Nutrition Society*, vol. 68, no. 3, pp. 252–260, 2009.

[191] D. L. Waitzberg and R. S. Torrinhas, "Fish oil lipid emulsions and immune response: what clinicians need to know," *Nutrition in Clinical Practice*, vol. 24, no. 4, pp. 487–499, 2009.

[192] V. A. van Beelen, B. Spenkelink, H. Mooibroek et al., "An n-3 PUFA-rich microalgal oil diet protects to a similar extent as a fish oil-rich diet against AOM-induced colonic aberrant crypt foci in F344 rats," *Food and Chemical Toxicology*, vol. 47, no. 2, pp. 316–320, 2009.

[193] F. D. Russell and C. S. Burgin-Maunder, "Distinguishing health benefits of eicosapentaenoic and docosahexaenoic acids," *Marine Drugs*, vol. 10, pp. 2535–2559, 2012.

[194] R. Garib, P. Garla, R. S. Torrinhas, P. L. Bertevello, A. F. Logullo, and D. L. Waitzberg, "Effects of parenteral fish oil lipid emulsions on colon morphology and cytokine expression after experimental colitis," *Nutrición Hospitalaria*, vol. 28, no. 3, pp. 849–856, 2013.

[195] M. J. Tisdale, "The 'cancer cachectic factor'," *Supportive Care in Cancer*, vol. 11, no. 2, pp. 73–78, 2003.

[196] B. Pajak, S. Orzechowska, B. Pijet et al., "Crossroads of cytokine signaling—the chase to stop muscle cachexia," *Journal of Physiology and Pharmacology*, vol. 59, supplement 9, pp. 251–264, 2008.

[197] C. Deans and S. J. Wigmore, "Systemic inflammation, cachexia and prognosis in patients with cancer," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 8, no. 3, pp. 265–269, 2005.

[198] F. Balkwill, "TNF-α in promotion and progression of cancer," *Cancer and Metastasis Reviews*, vol. 25, no. 3, pp. 409–416, 2006.

[199] R. Raqib, P. Sarker, A. Mily et al., "Efficacy of sodium butyrate adjunct therapy in shigellosis: a randomized, double-blind, placebo-controlled clinical trial," *BMC Infectious Diseases*, vol. 12, article 111, 2012.

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Research Article

The Effects of Florfenicol on the Values of Serum Tumor Necrosis Factor-α and Other Biochemical Markers in Lipopolysaccharide-Induced Endotoxemia in Brown Trout

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The aim of the present study was to determine the effects of florfenicol on the expected changes in sTNF-α, damage markers of the liver and kidney, and the lipid metabolism parameters in endotoxemic brown trout. Ninety-six brown trout were included in this study. After six of the fish were reserved as the control group, the remaining 90 fish were divided equally into 3 groups as follows: LPS (2 mg/kg, IP), LPS (2 mg/kg, IP) + florfenicol (40 mg/kg, IM), and florfenicol (40 mg/kg, IM). Blood samples were obtained from the tail of the fish at 1.5, 3, 6, 10, and 24 hours. The levels of sTNF-α were determined by ELISA and biochemical markers were evaluated with an autoanalyzer. A significant increase was observed in the values of sTNF-α in the LPS and LPS + florfenicol groups ($P < 0.05$). Significant increases were found in the kidney and liver damage determinants in the LPS and LPS + florfenicol groups ($P < 0.05$). Irregular changes in the lipid metabolism parameters were observed in all the subgroups. In conclusion, florfenicol does not affect the increases of sTNF-α caused by LPS and does not prevent liver or kidney damage; at least, it can be said that florfenicol does not have any evident positive effects on the acute endotoxemia of fish.

1. Introduction

Florfenicol (d-threo chloramphenicol) is a broad spectrum antibiotic that belongs to the amphenicol group that inhibits protein synthesis through its bacteriostatic activity. Its spectrum of effect includes many gram negative and positive bacteria including *Escherichia coli (E. coli)*, *Salmonella* species (*Salmonella* sp.), *Pasteurella* sp., *Shigella* sp., *Bordetella* sp., *Chlamydia* sp., and *Mycoplasma* species. Although florfenicol is an analog of chloramphenicol, it has two main structural differences. It includes a p-methyl sulfonyl group in place of the p-nitro group seen in chloramphenicol and a fluorine atom instead of a hydroxyl group. The use of florfenicol in cows, pigs, chickens, and fish has been approved by the European Medicines Agency (EMEA) [1, 2]. It is recommended in the treatment of bacterial infections caused by *Vibrio anguillarum, Edwardsiella* sp., and *Flavobacterium* sp. in fish [3]. It has been reported that florfenicol can be safely used at differing doses in tilapia fish and dose related mild decreases in the hematopoietic/lymphopoietic tissues have been observed with its use in channel catfish [4].

Lipopolysaccharide (LPS, endotoxin), which is a component in the wall of gram negative bacteria, is quite dangerous for living organisms [5]. LPS may be used in experimental modelling to either induce a model of local inflammation [6] or generate a model of systemic endotoxemia [7]. Induction of cytokine synthesis and alterations in complete blood count (haemogram), markers of organ damage, and biochemical parameters related to lipid metabolism may be observed in organisms in response to the application of LPS [7–9]. LPS-induced endotoxemia may lead to a variety of clinical manifestations including fever, multiple organ failure, septic shock, and death [5, 10]. In living organisms, LPS is recognized by immune cells and, as a result, the immune system is activated [5]. Activated cells synthesize a number of cytokines including sTNF-α and interleukins [8, 11]. TNF-α alone has the ability to initiate the pathology of sepsis such as decreased blood pressure (hypotension), multiple organ insufficiency, and fever [10]. It has been determined in mice that florfenicol decreases the levels of interleukin-4, -5, and -13 (IL-4, -5, and -13) and can have an anti-inflammatory effect [12].

Drugs can also have a number of clinical and biochemical side-effects that may present as alterations in the values of some biochemical parameters from both the plasma and serum. These biochemical changes are accepted as warnings for the start of structural damage in some tissue and organs [13, 14]. While increases in alkaline phosphatase (ALP) are expected in damage of the biliary tract, values of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are determined to be elevated in liver damage. With insufficiencies of the livers synthesis ability, changes in the levels of serum total protein and albumin can be determined. Increased levels of blood urea nitrogen (BUN) and creatinine in the serum demonstrate a decline in kidney function. Serum high density lipoprotein (HDL), low density lipoprotein (LDL), and triglyceride (TG) values provide information regarding lipid metabolism [15–17].

In this study, it was hypothesized that the application of LPS in fish and the resulting endotoxemia would increase the levels of both sTNF-α and organ damage markers as is observed in mammals [7, 9] and would cause changes in lipid metabolism [8] and these changes would be improved with florfenicol, which has effects on the immune system [12].

The aim of the present study was to determine the effects of florfenicol on the expected changes in serum of sTNF-α, liver (ALP, AST, ALT, total protein, and albumin) and kidney (BUN and creatinine) damage markers, and lipid metabolism parameters (TG, HDL, and LDL) in the endotoxemia induced with LPS in brown trout.

2. Materials and Methods

2.1. Animals. Ninety-six brown trout (166–267 g, Dumlupinar fish facility, Bozkir, Konya, Turkey) were used in this study. The presented study was organized in accordance with the guidelines provided by Selçuk University, Experimental Medicine, Research and Application Center, Konya, Turkey. The fish were kept in tanks with fresh open water circuits under natural conditions of light and temperature. The fish were fed daily with commercial trout pellets (Blueaq, Abalioglu, Turkey).

2.2. Experimental Procedure. Following the randomized separation of six fish for the control group, the rest of the fish were divided into three equal groups: the LPS (*E. coli*, serotype O111: B4, Sigma-Aldrich Chemie, Deisenhofen, Germany, 2 mg/kg, IP) group, the LPS (2 mg/kg IP) + florfenicol (Nuflor flk, Intervet Ilac, Istanbul, 40 mg/kg, IM) group, and the florfenicol (40 mg/kg, IM) group, respectively. Following these applications, the fish were anesthetized with quinaldine (25 mg/L) for anesthesia and blood samples were taken from their tails at 1.5, 3, 6, 10, and at 24 hours.

2.3. Measurements. The serum samples were kept at −70°C until analysis. The sTNF-α levels were measured by commercial test kit, and analytical procedure were performed according to the manufacturer recommendations. The ELISA plate reader (MWGt Lambda Scan 200, USA) was used to evaluate the value of sTNF-α and an autoanalyzer (ILab-300 bioMérieux Diagnostics, Milan, Italy) was used to measure biochemical parameters including ALP, AST, ALT, total protein, albumin, BUN, creatinine TG, HDL, and LDL.

2.4. Statistical Analysis. Findings were statistically evaluated by the one-way ANOVA followed by Duncan post hoc test (SPSS 19.0). P values lower than 0.05 ($P < 0.05$) were accepted as being statistically significant.

3. Results

The effects of florfenicol on the sTNF-α and biochemical parameters of the endotoxemic fish are presented in Table 1. The sTNF-α values were found to be significantly increased in the LPS (1.5 and 3 hours) and LPS + florfenicol (1.5 hour) groups compared to the control hour (0 hour) ($P < 0.05$).

While ALP, AST, and ALT values were found to be increased in the LPS group ($P < 0.05$), ALT and AST values were increased in the LPS + florfenicol group only ($P < 0.05$). It was determined that florfenicol significantly increased the values of ALP, AST, and ALT in healthy fish ($P < 0.05$). There were a significant elevation in total protein values ($P < 0.05$) and a reduction in albumin levels ($P < 0.05$) in the LPS group and a significant decrease in the values of total protein in the florfenicol group ($P < 0.05$). LPS application was determined to significant increase serum BUN and creatinine ($P < 0.05$), whereas, with LPS + florfenicol application, increases were observed only in creatinine values ($P < 0.05$).

In the LPS group, HDL, LDL, and TG levels were increased ($P < 0.05$). In the LPS + florfenicol group, HDL levels were found to be increased ($P < 0.05$) and, in the florfenicol group, HDL and LDL levels were increased ($P < 0.05$) and TG levels were decreased ($P < 0.05$).

4. Comment

Florfenicol is a broad spectrum antibiotic used for the treatment of bacterial infections in mammals, birds, fish, and lobsters [1, 2, 4, 18, 19]. In this study, sTNF-α values were found to increase after LPS application ($P < 0.05$) (Table 1). LPS induces production of inflammation mediators such as cytokines by stimulating the transcription factor nuclear factor-kappaB (NF-κB) in cells [8, 20] and, after LPS application to fish, TNF-α levels have been reported to increase [8]. We determined that LPS-induced production of sTNF-α could not be suppressed by the administration of florfenicol in brown trout (Table 1).

Some antibiotics have a suppressive effect on the increases of sTNF-α [21, 22]. Florfenicol suppresses humoral and cellular immune responses in mice [23, 24] and reduces the levels of sTNF-α by inhibiting the efficiency of NF-κB [25, 26]. Moreover, florfenicol has an anti-inflammatory and a dose-dependent downregulatory effect on the synthesis of some interleukins [12]. Florfenicol also inhibits the synthesis of prostaglandin E2 [27]. In the present study, the reasons for not determining sTNF-α suppression with florfenicol may be due to the used dose of florfenicol or differences in the animal

Table 1: The effect of florfenicol (F) on sTNF-α and serum biochemical indices in endotoxemic fish (mean ± SE).

	Groups	0 hour	1.5 hour	3 hours	6 hours	10 hours	24 hours
TNF-α (pg/mL)	LPS	728 ± 188^{b}	2064 ± 244^{a}	2094 ± 474^{a}	1022 ± 412^{ab}	1642 ± 606^{ab}	736 ± 232^{b}
	LPS + F	728 ± 188^{b}	2313 ± 321^{a}	810 ± 425^{b}	1254 ± 440^{ab}	1101 ± 480^{ab}	1876 ± 536^{ab}
	F	728 ± 188^{a}	1681 ± 545^{a}	1462 ± 439^{a}	1196 ± 264^{a}	976 ± 431^{a}	1208 ± 228^{a}
ALP (U/L)	LPS	90.0 ± 11.4^{b}	87.0 ± 16.8^{b}	87.0 ± 11.1^{b}	160 ± 15.0^{a}	94.0 ± 7.77^{b}	123 ± 11.7^{b}
	LPS + F	90.0 ± 11.4^{a}	93.0 ± 13.0^{a}	90.0 ± 5.46^{a}	128 ± 18.7^{a}	116 ± 17.3^{a}	103 ± 13.8^{a}
	F	90.0 ± 11.4^{ab}	82.0 ± 17.0^{b}	109 ± 23.2^{ab}	132 ± 12.9^{a}	107 ± 13.5^{ab}	106 ± 3.57^{ab}
ALT (U/L)	LPS	4.50 ± 0.43^{b}	4.83 ± 0.95^{b}	4.67 ± 0.49^{b}	18.7 ± 4.70^{a}	8.83 ± 2.18^{b}	4.83 ± 0.31^{b}
	LPS + F	4.50 ± 0.43^{d}	7.17 ± 1.11^{cd}	10.3 ± 0.72^{bcd}	14.8 ± 2.24^{ab}	18.2 ± 4.37^{a}	12.7 ± 2.01^{abc}
	F	4.50 ± 0.43^{c}	9.67 ± 1.15^{ab}	7.67 ± 0.92^{bc}	12.8 ± 2.20^{a}	12.2 ± 0.95^{a}	11.5 ± 1.09^{a}
AST (U/L)	LPS	223 ± 27.3^{c}	220 ± 27.2^{c}	242 ± 16.0^{bc}	477 ± 51.6^{a}	323 ± 25.2^{b}	325 ± 18.0^{b}
	LPS + F	223 ± 27.3^{c}	281 ± 41.5^{c}	343 ± 33.9^{bc}	469 ± 43.7^{a}	405 ± 54.8^{ab}	466 ± 30.7^{a}
	F	223 ± 27.3^{d}	344 ± 44.5^{bc}	285 ± 12.3^{cd}	499 ± 48.5^{a}	440 ± 34.5^{ab}	523 ± 41.3^{a}
Total protein (g/dL)	LPS	3.80 ± 0.48^{b}	5.40 ± 0.51^{a}	3.70 ± 0.31^{b}	3.60 ± 0.30^{b}	3.40 ± 0.36^{b}	3.30 ± 0.08^{b}
	LPS + F	3.80 ± 0.48^{a}	3.40 ± 0.38^{a}	3.40 ± 0.14^{a}	3.10 ± 0.17^{a}	3.70 ± 0.26^{a}	3.30 ± 0.15^{a}
	F	3.80 ± 0.48^{a}	3.00 ± 0.21^{ab}	3.20 ± 0.13^{ab}	2.70 ± 0.18^{b}	3.30 ± 0.25^{ab}	3.30 ± 0.13^{ab}
Albumin (g/dL)	LPS	1.90 ± 0.12^{a}	1.90 ± 0.08^{a}	1.50 ± 0.03^{b}	1.90 ± 0.14^{a}	1.70 ± 0.10^{ab}	1.80 ± 0.05^{a}
	LPS + F	1.90 ± 0.12^{a}	2.00 ± 0.24^{a}	1.70 ± 0.03^{a}	1.80 ± 0.02^{a}	2.00 ± 0.09^{a}	1.80 ± 0.05^{a}
	F	1.90 ± 0.12^{a}	1.70 ± 0.14^{a}	1.70 ± 0.07^{a}	1.90 ± 0.22^{a}	1.90 ± 0.07^{a}	1.80 ± 0.09^{a}
Creatinine (mg/dL)	LPS	0.26 ± 0.03^{cd}	0.93 ± 0.12^{a}	0.38 ± 0.02^{bc}	0.51 ± 0.03^{b}	0.35 ± 0.03^{c}	0.15 ± 0.01^{d}
	LPS + F	0.26 ± 0.03^{bc}	0.22 ± 0.01^{cd}	0.20 ± 0.02^{cd}	0.38 ± 0.03^{a}	0.33 ± 0.04^{ab}	0.17 ± 0.01^{d}
	F	0.26 ± 0.03^{a}	0.21 ± 0.02^{ab}	0.20 ± 0.01^{ab}	0.16 ± 0.01^{b}	0.21 ± 0.02^{ab}	0.22 ± 0.02^{ab}
BUN (mg/dL)	LPS	4.50 ± 0.67^{b}	6.67 ± 0.99^{a}	3.83 ± 0.40^{b}	6.83 ± 0.60^{a}	4.33 ± 0.80^{b}	4.33 ± 0.33^{b}
	LPS + F	4.50 ± 0.67^{a}	5.83 ± 1.72^{a}	3.83 ± 0.48^{a}	6.33 ± 0.76^{a}	3.50 ± 0.56^{a}	3.83 ± 0.95^{a}
	F	4.50 ± 0.67^{a}	5.17 ± 0.70^{a}	4.67 ± 0.42^{a}	5.83 ± 0.91^{a}	6.00 ± 0.68^{a}	6.00 ± 0.58^{a}
HDL (mg/dL)	LPS	123 ± 3.61^{bc}	101 ± 7.34^{c}	108 ± 8.00^{c}	151 ± 9.33^{a}	132 ± 9.34^{ab}	147 ± 5.69^{a}
	LPS + F	123 ± 3.61^{bc}	109 ± 10.7^{c}	140 ± 5.49^{ab}	133 ± 5.43^{ab}	152 ± 6.41^{a}	131 ± 7.70^{ab}
	F	123 ± 3.61^{b}	131 ± 11.1^{b}	134 ± 3.46^{b}	193 ± 18.8^{a}	151 ± 2.31^{b}	150 ± 5.71^{b}
LDL (mg/dL)	LPS	35.0 ± 1.67^{bc}	39.0 ± 4.95^{bc}	32.0 ± 2.47^{c}	46.0 ± 4.62^{ab}	39.0 ± 2.18^{bc}	51.0 ± 4.30^{a}
	LPS + F	35.0 ± 1.67^{a}	34.0 ± 3.20^{a}	46.0 ± 5.16^{a}	48.0 ± 6.21^{a}	47.0 ± 3.94^{a}	39.0 ± 3.75^{a}
	F	35.0 ± 1.67^{bc}	33.0 ± 5.00^{c}	35.0 ± 3.77^{bc}	46.0 ± 1.38^{a}	42.0 ± 3.23^{abc}	44.0 ± 2.35^{ab}
TG (mg/dL)	LPS	377 ± 55.3^{b}	562 ± 95.1^{a}	243 ± 19.7^{b}	340 ± 59.9^{b}	245 ± 23.1^{b}	239 ± 23.1^{b}
	LPS + F	377 ± 55.3^{a}	369 ± 66.1^{a}	241 ± 33.6^{a}	249 ± 20.5^{a}	291 ± 33.9^{a}	257 ± 21.7^{a}
	F	377 ± 55.3^{a}	194 ± 25.6^{c}	284 ± 34.2^{abc}	340 ± 42.1^{ab}	185 ± 16.1^{c}	260 ± 26.2^{bc}

LPS: lipopolysaccharide, F: florfenicol, TNF-α: tumor necrosis factor alpha, ALP: alkaline phosphatase, ALT: alanine aminotransferase, AST: aspartate aminotransferase, BUN: blood urea nitrogen, HDL: high density lipoproteins, LDL: low density lipoproteins, TG: triglycerides, and $^{a, b, c, d}$different letters on the same line refer to statistical significance ($P < 0.05$).

species. We determined that florfenicol applied alone does not have any effect on the sTNF-α levels in healthy brown trouts (Table 1). Although it is known that antibiotics affect the synthesis of cytokines in living organisms [21, 28], Lunden et al. reported that florfenicol does not have a significant influence on antibody synthesis and leukocyte number in rainbow trout [29].

In this study, it was determined that LPS application significantly increases levels of the serum liver damage markers (ALP, ALT, and AST) ($P < 0.05$) and, with the application of LPS + florfenicol, there was no decrease in the elevated liver damage markers (ALT and AST) except for ALP (Table 1). In addition, LPS was found to affect protein synthesis, which is accepted as being a parameter of the synthesis ability of the liver (Table 1).

Swain et al. [8] determined an accumulation of LPS both in the liver and in the kidney shortly after its intravenous (IV) application in fish. Additionally, an increase in the expression of damage markers of the liver was determined in response to the administration of LPS [6, 9]. These results suggest that LPS could cause hepatotoxicity in fish as it does in mammals.

It was noticed that the administration of florfenicol in healthy fish did cause increases in the liver damage markers (AST and ALT) ($P < 0.05$) (Table 1). It has been reported that many antibiotics [30] as well as amphenicols [31] are hepatotoxic and florfenicol can cause an increase in the weight of the liver [32]. A twofold increase in the values of the serum ALP is accepted as cholestatic damage while a twofold increase in serum ALT is synonymous for hepatocellular damage. When it is taken into consideration that, in drug

related liver damage, treatment is terminated when ALT levels are increased 5 times those of normal [33], it can be said that florfenicol does not have a high potential for liver damage in brown trout.

In this study, while the markers of kidney damage (BUN and creatinine) were increased in the LPS group ($P < 0.05$), only an increase in the level of creatinine ($P < 0.05$) was observed in the LPS + florfenicol group. It has been reported that LPS accumulates quickly in the kidney of fish after intravenous administration [8] and kidney damage markers are increased in endotoxemia [9, 21]. The basic reason for kidney damage in LPS-induced endotoxemia has been shown to be due to hemodynamic changes resulting in the reduction in glomerular filtration rate, microcoagulation, and hypoxic conditions [34]. We observed a temporary decrease in creatinine levels after the administration of florfenicol in healthy fish ($P < 0.05$) (Table 1). It is a known fact that antibiotics like a number of other drugs may have effects on kidney function [35].

We determined that the administration of LPS results in an increase in the levels of HDL, LDL, and TG ($P < 0.05$). It was determined that, in endotoxemic fish, the administration of florfenicol corrected the changes in LDL and TG values but had no effect on the elevated HDL levels (Table 1). Swain et al. [8] reported that LPS can affect the parameters of lipid metabolism, but these effects can differ depending on the type of LPS and the dose given. It was observed that the value of TG was usually increased whereas the values of LDL and HDL were decreased. In rodents, it has been stated that lipoproteins can demonstrate a protective effect against the deaths due to LPS [36]. Fish are more resistant to endotoxic shock than humans and other animals [8]. This may be due to the different physiological responses that living organisms may express to LPS.

It was determined that florfenicol led to increases in the levels of both HDL and LDL ($P < 0.05$) and decreases in the values of TG ($P < 0.05$) in healthy fish (Table 1). There was no source material found on the effects of florfenicol on lipid metabolism in animals. However, it has been reported that antibiotics affect lipid metabolism [37] and florfenicol causes changes in hematological parameters [32].

In conclusion, it can be stated that, in endotoxemic brown trout, sTNF-α synthesis is stimulated, damage develops in the liver and kidney, lipid metabolism is affected, with florfenicol, these changes do not disappear, and when florfenicol is given to healthy fish, it can affect the functions of the kidney and liver and the metabolism of lipids.

Conflict of Interests

The authors declare that there is no conflict of interests.

References

[1] EMEA, 2000, http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500014280.pdf.

[2] E. Yazar, "Chemotherapeutics," in *Veterinary Drugs*, E. Yazar, Ed., pp. 21–128, Olgun-Celik Press, Konya, Turkey, 2012.

[3] K. M. Treves-Brown, "Florfenicol," in *Applied Fish Pharmacology*, pp. 151–154, Kluwer Academic, Dordrecht, The Netherlands, 2010.

[4] M. P. Gaikowski, J. C. Wolf, R. G. Endris, and W. H. Gingerich, "Safety of Aquaflor (florfenicol, 50% type A medicated article), administered in feed to channel catfish, *Ictalurus punctatus*," *Toxicologic Pathology*, vol. 31, no. 6, pp. 689–697, 2003.

[5] X. Wang and P. J. Quinn, "Lipopolysaccharide: biosynthetic pathway and structure modification," *Progress in Lipid Research*, vol. 49, no. 2, pp. 97–107, 2010.

[6] A. Er and E. Yazar, "Effects of macrolide antibiotics on blood inflammatory mediators and organ damage markers in lipopolysaccharide-induced pulmonary damage rats," *Eurasian Journal of Veterinary Sciences*, vol. 26, no. 1, pp. 7–13, 2010.

[7] F. Altan, M. Elmas, A. Er et al., "Effects of drugs on kinetic values of cytokines, adenosine deaminase and 13,14-dihydro-15-keto-prostaglandin F2α in endotoxemia: a different approach," *Eurasian Journal of Veterinary Sciences*, vol. 26, no. 1, pp. 15–19, 2010.

[8] P. Swain, S. K. Nayak, P. K. Nanda, and S. Dash, "Biological effects of bacterial lipopolysaccharide (endotoxin) in fish: a review," *Fish and Shellfish Immunology*, vol. 25, no. 3, pp. 191–201, 2008.

[9] E. Yazar, A. Er, K. Uney et al., "Effects of drugs used in endotoxic shock on oxidative stress and organ damage markers," *Free Radical Research*, vol. 44, no. 4, pp. 397–402, 2010.

[10] A. Karabacak and E. Yazar, "Current approaches in the treatment of septic shock," *Eurasian Journal of Veterinary Sciences*, vol. 22, no. 1-2, pp. 95–103, 2006.

[11] B. Henderson and M. Wilson, "Cytokine induction by bacteria: beyond lipopolysaccharide," *Cytokine*, vol. 8, no. 4, pp. 269–282, 1996.

[12] C. Xinxin, C. Chi, C. Xiao et al., "Florfenicol inhibits allergic airway inflammation in mice by p38 MAPK-mediated phosphorylation of GATA 3," *Clinical Immunology*, vol. 138, no. 2, pp. 231–238, 2011.

[13] S. Kaya and A. Unsal, "Adverse effects of drugs," in *Pharmacology in Veterinary Medicine*, S. Kaya, A. Bilgili, and I. Pirinçci, Eds., pp. 142–152, Medisan, Ankara, Turkey, 2002.

[14] F. Dogan, "Pharmacovigilance in veterinary profession," *Eurasian Journal of Veterinary Sciences*, vol. 27, no. 1, pp. 19–25, 2011.

[15] K. Turgut, "Liver disease tests," in *Veterinary Clinical Laboratory Diagnostics*, pp. 202–257, Bahçıvanlar, Konya, Turkey, 2000.

[16] K. Turgut, "Endocrine, metabolic and lipid disorders and tests," in *Veterinary Clinical Laboratory Diagnostics*, pp. 416–486, Bahçıvanlar, Konya, Turkey, 2000.

[17] M. G. Kerr, "Systematic investigation," in *Veterinary Laboratory Medicine*, pp. 199–208, Blackwell Science, London, UK, 2nd edition, 2002.

[18] D. Basti, D. Bouchard, and A. Lichtenwalner, "Safety of florfenicol in the adult lobster (*Homarus americanus*)," *Journal of Zoo and Wildlife Medicine*, vol. 42, no. 1, pp. 131–133, 2011.

[19] M. P. Gaikowski, J. C. Wolf, S. M. Schleis, D. Tuomari, and R. G. Endris, "Safety of florfenicol administered in feed to tilapia (*Oreochromis* sp.)," *Toxicologic Pathology*, vol. 41, no. 4, pp. 639–652, 2013.

[20] N. Mukaida, Y. Ishikawa, N. Ikeda et al., "Novel insight into molecular mechanism of endotoxin shock: biochemical analysis of LPS receptor signaling in a cell-free system targeting NF-κB and regulation of cytokine production/action through β2

integrin in vivo," *Journal of Leukocyte Biology*, vol. 59, no. 2, pp. 145–151, 1996.

[21] A. Er, E. Yazar, K. Uney, M. Elmas, F. Altan, and G. Cetin, "Effects of tylosin on serum cytokine levels in healthy and lipopolysaccharide-treated mice," *Acta Veterinaria Hungarica*, vol. 58, no. 1, pp. 75–81, 2010.

[22] A. Er and E. Yazar, "Effects of tylosin, tilmicosin and tulathromycin on inflammatory mediators in bronchoalveolar lavage fluid of lipopolysaccharide-induced lung injury," *Acta Veterinaria Hungarica*, vol. 60, no. 4, pp. 465–476, 2012.

[23] S. Guan, J. Lu, X. Shen, W. Qian, J. Liu, and X. Deng, "Florfenicol impairs the immune responses to vaccination against foot-and-mouth disease in mice," *Immunopharmacology and Immunotoxicology*, vol. 33, no. 4, pp. 609–613, 2011.

[24] G. Shuang, S. Yu, G. Weixiao et al., "Immunosuppressive activity of florfenicol on the immune responses in mice," *Immunological Investigations*, vol. 40, no. 4, pp. 356–366, 2011.

[25] X. Zhang, Y. Song, X. Ci et al., "Effects of florfenicol on early cytokine responses and survival in murine endotoxemia," *International Immunopharmacology*, vol. 8, no. 7, pp. 982–988, 2008.

[26] X. Zhang, K. Song, H. Xiong, H. Li, X. Chu, and X. Deng, "Protective effect of florfenicol on acute lung injury induced by lipopolysaccharide in mice," *International Immunopharmacology*, vol. 9, no. 13-14, pp. 1525–1529, 2009.

[27] X. Zhang, H. Xiong, H. Li, L. Yu, and X. Deng, "Effects of florfenicol on LPS-induced nitric oxide and prostaglandin E_2 production in RAW 264.7 macrophages," *Fundamental & Clinical Pharmacology*, vol. 25, no. 5, pp. 591–598, 2011.

[28] K. Uney, A. Er, G. E. Avci, A. Bulbul, M. Elmas, and E. Yazar, "Effect of tilmicosin on serum cytokine levels in the endotoxeniia," *Journal of Animal and Veterinary Advances*, vol. 8, no. 5, pp. 1021–1024, 2009.

[29] T. Lundén, S. Miettinen, L.-G. Lönnström, E.-M. Lilius, and G. Bylund, "Effect of florfenicol on the immune response of rainbow trout (*Oncorhynchus mykiss*)," *Veterinary Immunology and Immunopathology*, vol. 67, no. 4, pp. 317–325, 1999.

[30] C. Y. Chang and T. D. Schiano, "Review article: drug hepatotoxicity," *Alimentary Pharmacology & Therapeutics*, vol. 25, no. 10, pp. 1135–1151, 2007.

[31] F. Memik, "Opinions on drugs and liver," *The Eurasian Journal of Medicine*, vol. 4, pp. 399–402, 1975.

[32] EMEA, 1996, http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500014274.pdf.

[33] U. Oncu, "Monitoring-management of side effects of antibiotic," *Journal of ANKEM*, vol. 27, no. 2, pp. 82–84, 2013.

[34] N. Coskunfırat, M. Cengiz, and M. Yılmaz, "Animal models of acute renal failure," *Journal of the Turkish Society of Intensive Care*, vol. 8, no. 1, pp. 38–45, 2010.

[35] S. Tekin and I. Camkerten, "Use of antibiotics for kidney disease in cats and dogs," *The Journal of the Faculty of Veterinary Medicine, University of Yüzüncü Yıl*, vol. 14, no. 1, pp. 82–86, 2003.

[36] J. F. P. Berbée, L. M. Havekes, and P. C. N. Rensen, "Apolipoproteins modulate the inflammatory response to lipopolysaccharide," *Journal of Endotoxin Research*, vol. 11, no. 2, pp. 97–103, 2005.

[37] A. Krondl, "Present understanding of the interaction of drugs and food during absorption," *Canadian Medical Association journal*, vol. 103, no. 4, pp. 360–364, 1970.