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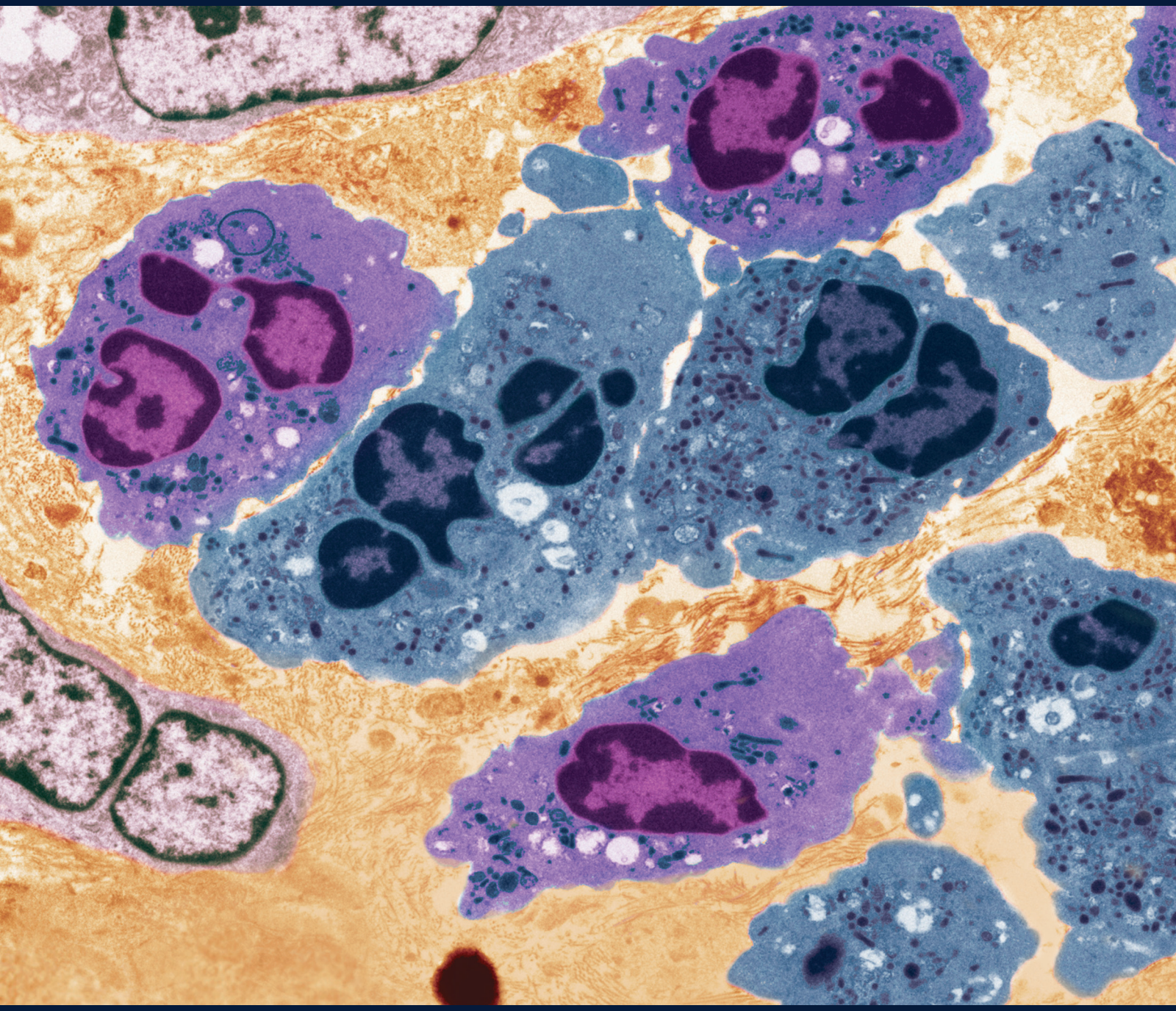
# Adaptive Immunity and Inflammation

Guest Editors: Brancaleone Vincenzo, Iqbal J. Asif, Paschalidis Nikolaos, and Maione Francesco



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# Editorial Board

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*Editorial*

# Adaptive Immunity and Inflammation

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Inflammation is part of a complex biological response to injury as a result of different stimuli such as pathogens, damaged cells, or irritants. Local signals at the sites of inflammation mediate rapid cells mobilization and recruitment and dictate differentiation programs whereby these cells drive clearance of "inflammatory inducers" and promote resolution and restoration of tissue homeostasis. However, persistent inflammatory stimuli or dysregulation of mechanisms of the resolution phase can lead to chronic inflammation. This phenomenon, from a "temporal point of view," distinguishes a first cellular subset that responds to proinflammatory stimuli, commonly referred to as innate immunity (PMN, monocytes), later followed by a second phase, classically catalogued as the adaptive immune response (T and B lymphocytes). Inflammation is more generally associated with the innate immune response, however, increasing experimental and clinical evidence has highlighted its importance in antigen driven adaptive immune responses.

Intriguingly, the hypothesis that components of adaptive immunity involve the generation of memory cells which can also fuel the chronic nature of inflammation driven by the adaptive arm of the immune response is now emerging. This "novel view" supports the view that lymphocytes cooperate with innate immune cells and collectively orchestrate the inflammatory response.

Original research and review articles successfully submitted to this special issue have stimulated the continuing efforts to understand the interaction between adaptive immunity and the inflammatory process. This special issue, also pays particular attention to the role of proinflammatory Th17 T cells and T cell derived cytokines in autoimmune, cardiovascular, and infectious diseases ("Interleukin-17A Exacerbates Ferric Chloride-Induced Arterial Thrombosis in Rat Carotid Artery," authored by F. Maione et al.; "Th17 Cells in Autoimmune and Infectious Diseases," authored by J. F. Zambrano-Zaragoza et al.; "Limited Applicability of GW9662 to Elucidate PPAR$\gamma$-Mediated Fatty Acid Effects in Primary Human T-Helper Cells," authored by A. Jaudszus et al.). Moreover, this special issue has highlighted the importance of lymphocytes involvement during acute and chronic inflammation ("Live Combined *Bacillus subtilis* and *Enterococcus faecium* Ameliorate Murine Experimental Colitis by Immunosuppression," authored by S. Chen et al.; "Contradictory Immune Response in Post Liver Transplantation Hepatitis B and C," authored by A. Takaki et al.; "Neurotensin Decreases the Proinflammatory Status of Human Skin Fibroblasts and Increases Epidermal Growth Factor Expression," authored by L. P. da Silva et al.).

We hope that this special issue will stimulate the interest of scientists working in different areas of inflammation and immunology and highlight the need for continuing research in the area.

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*Research Article*

# Live Combined *Bacillus subtilis* and *Enterococcus faecium* Ameliorate Murine Experimental Colitis by Immunosuppression

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Live combined *Bacillus subtilis* and *Enterococcus faecium* ameliorate murine experimental colitis by immunosuppression manifested by downregulation of TLRs, macrophages, Th1, and Th2 but upregulation of Tregs.

## 1. Introduction

Inflammatory bowel disease (IBD) is an idiopathic disorder of chronic inflammation of the gastrointestinal tract, which is represented mainly by ulcerative colitis and Crohn's disease. Despite scientific efforts during the last decades, etiology and pathogenesis of the two major inflammatory bowel diseases, namely, Crohn's disease and ulcerative colitis, remain rather unclear [1, 2]. The widely accepted causes are infections and immune and genetic factors. More and more recent reports have shown the importance of immune modulation in the development of IBD [3, 4]. Aminosalicylic acid, glucocorticoid, and immunosuppressant are three main therapies [5]. Recently some kinds of probiotics have been applied and shown to be significantly effective to IBD [6]. Since 2008, probiotic lactobacillus strains have been used to treat IBD as "B-grade" advice [7]. Probiotics are a group of specific nonpathogenic bacteria that are functionally and genetically defined by their ability to reduce inflammation in the intestine [8]. The mechanism is still under investigation. Recent studies relative to the mechanism of action of probiotics have shown that these organisms can have an effect on enhancing epithelial barrier function and modulating epithelial cytokine secretion into an anti-inflammatory dominant profile [9–11]. Our study aims to further investigate the effect of the probiotic lactobacillus strains Medilac-S (live combined *Bacillus subtilis* and *Enterococcus faecium* enteric-coated capsules) in murine experimental colitis and its possible mechanisms.

## 2. Materials and Methods

*2.1. Animal.* Male and female Sprague-Dawley rats (200–230 g) were obtained from the Tongji Laboratory Animal Center (Wuhan, China).

*2.2. Preparation of Murine Model of Colitis.* The murine models of colitis were randomly divided into three groups: normal group, control group, and Medilac-S group. Every group had 10 rats in the end. Experimental murine colitis models were established as follows [12]: fast 24 h, intraperitoneal anesthesia with pentobarbital (30 mg/kg), enema with 2,4,6-trinitrobenzene sulfonic acid (TNBS, 100 mg/kg, Sigma company), and equal volume anhydrous ethanol. After the establishment of experimental colitis, the saline (2 mL) and Medilac-S (20 mg/200 g, each 250 mg capsule contains $5.0e+07$ *Bacillus subtilis* and $4.5e+08$ *Enterococcus faecium*, Beijing Han Mei Pharmaceutical Co., LTD.) were administered into control group and Medilac-S groupby daily gastric irrigation.

All rats were sacrificed after 10 days. All procedures were performed in accordance with the guidelines for animal care from the Animal Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology.

*2.3. Determination of DAI, CMDI, and Histological Change.* Body weight, diarrhea, and bloody stool with naked eye were observed every day from the second day of establishment of the colitis model, which were scored as disease activity index (DAI): score 0 = weight without falling, stool normal, or occult blood (−); score 1 = weight loss 0–5%, loose stool, or occult blood (+); score 2 = weight loss 5–10%; score 3 = weight loss 10–15%, watery stools, or bloody stool with naked eye; score 4 = weight loss > 15; DAI = (weight loss score + stool score + hematochezia score)/3. After the sacrifice of the rats, the intestinal segments from the anus to ileocecal junction were taken and cut along the longitudinal mesenteric edge. The colon general state and colonic mucosa damage index (CMDI) [3] were evaluated by the anatomical microscope. The distal colon, transverse colon, and ascending colon were given a block size about 2 mm × 10 mm tissue specimens and in serious inflammation or ulcer place at least a piece of tissue sample was taken from each rat. Routine paraffin section, H&E staining, and pathology scores were performed by pathology professionals.

*2.4. Determination of SOD and MPO Activities.* SOD activity of tissue was analyzed by xanthine oxidase method and MPO activity of tissue was measured by spectrophotometry according to the specific steps for SOD, MPO detection kits (Nanjing Jiancheng Biological Engineering Research Institute).

*2.5. Reverse Transcription Quantitative Real-Time PCR.* RNA was extracted using the Trizol method (Invitrogen, UK) according to the manufacturer's instructions. A volume of 1 mg of RNA was reverse transcribed using SuperScript II reverse transcriptase and Oligo (dT) primers (Invitrogen, UK). The mRNA expressions of TLR2, TLR4, and TLR9 were quantified using SYBR green master mix (Finnzyme, New England Biolabs, UK) and $\beta$-actin for normalization among samples. The primers used are listed as below: TLR2 upstream primer 5′AAACGGTAACAATACGGAG3′, downstream primer 5′TGACAACTGTC GGGCATA3′; TLR4 upstream primer 5′CAGAGCCGTTGGTGTATC3′, downstream primer 5′CCCTGTGAGGTCGTTGA3′; and TLR9 upstream primer 5′AGTGCTTGATGTGGGTGG3′, downstream primer 5′CTGAGCGTGTTC TTGTTGA3′. PCRs were performed as 40 cycles of 95°C (15 s), 60°C (45 s), and 72°C (45 s) on a Chromo 4 cycler and recorded with MJ Opticon Monitor software V3 (Biorad Labs Inc., Hercules, CA, USA). Gene expression was calculated relative to $\beta$-actin.

*2.6. Immunohistochemistry.* 3 $\mu$m thick paraffin sections were in sequence incubated with 3% $H_2O_2$ for 10 min and 5% BSA for 30 min at room temperature and then incubated with primary antibodies which are rabbit anti-rat and polyclonal such as TLR2 (sc - 10739), TLR4 (sc - 10741), and TLR9 (sc - 25468).

The paraffin sections were then incubated with biotinylated secondary goat anti-rabbit IgG for 30 min followed by horse radish peroxidase-conjugated streptavidin (BD Pharmingen, USA) and diaminobenzidine staining; an irrelevant rabbit IgG was used for negative control. Images from the microscope were captured with a Nikon DXM 1200 digital camera using Automatic Camera Tamer (ACT-1) software and analyzed with image analysis software (Image Pro plus 5.1). The expressions of TLRs were quantified by the mean numbers of positive cells per square millimeter at 400x magnification on 5 fields per section from 10 rats per group. All scorings were carried out by observers blinded to the experimental groups.

*2.7. Immunofluorescence.* The tissue sections (6 um) were immunostained with the primary antibody, rabbit anti-rat F4-80 (1 : 100; Santa Cruz Biotechnology), PE-conjugated anti-rabbit secondary antibody (1 : 200; Invitrogen), and 40,6-diamidino-2-phenylindole (DAPI, Invitrogen). Images were analyzed using a BIOREVO immunofluorescence microscope (Keyence). Each result was obtained in at least four separate experiments. We prepared ten rats in each group for a single experiment.

*2.8. Flow Cytometry.* Spleens were harvested and single cell suspensions were prepared by processing the spleen with a 200 mm nylon mesh. The spleen cells were directly collected in a 35 mm dish which was filled with 4 mL EZ-Sep mouse 1X lymphocyte separation medium (Dakewe Biotech Company Ltd., Shenzhen, China). Then the cell suspension in lymphocyte separation medium was transferred into a 15 mL centrifuge tube. RPMI 1640 medium (1 mL) was laid on it. The tube was centrifuged at 800 g for 30 min at 4°C. Red blood cells and dead cells were deposited at the bottom. Lymphocytes at the interface were collected and washed.

The lymphocytes were labeled with anti-CD4-FITC (Biolegend, CA, USA) and anti-CD25-PE (eBioscience, CA, USA) monoclonal antibodies. After staining with CD4-FITC, the membranes of cells were fixed and ruptured and then the cells were stained with anti-intracellular cytokines monoclonal antibodies such as anti-IFN-$\gamma$-PE (eBioscience, CA, USA) or anti-IL-4-PE (eBioscience, CA, USA). Samples were acquired using a FACScan cytometer (Beckton Dickinson, Oxford, UK) and analyzed with "FCS Express V3" software (De Novo Software).

*2.9. Statistical Analysis.* Data are expressed as mean ± SD. Statistical differences were analyzed by Student's $t$-test or a one-way ANOVA using SPSS (version 13.0). A $P$ value < 0.01 was considered statistically significant.

## 3. Results

*3.1. Medilac-S Ameliorated Murine Experimental Colitis.* Congestion, edema, erosion and bleeding, and shallow small

Table 1: DAI, CDMI, and histological change in groups.

| Groups | Numbers | DAI | CDMI | Histological change |
|---|---|---|---|---|
| Normal | 10 | $0.07 \pm 0.14$ | $0.00 \pm 0.00$ | $0.20 \pm 0.42$ |
| Control | 10 | $1.72 \pm 0.58^{**}$ | $8.45 \pm 0.51^{**}$ | $6.54 \pm 0.51^{**}$ |
| Medilac-S | 10 | $0.60 \pm 0.78^{\#\#}$ | $1.20 \pm 1.40^{\#\#}$ | $1.45 \pm 1.01^{\#\#}$ |

Note: $^{**}P < 0.01$ compared with normal group; $^{\#\#}P < 0.01$ compared with control group.

Table 2: The activities of SOD and MPO in groups.

| Groups | Number | SOD (U/mg protein) | MPO (U/g wet tissue) |
|---|---|---|---|
| Normal | 10 | $674 \pm 37.78$ | $0.29 \pm 0.04$ |
| Control | 10 | $495.5 \pm 49.97^{**}$ | $1.03 \pm 0.2^{**}$ |
| Medilac-S | 10 | $585.82 \pm 95.67^{\#\#}$ | $0.48 \pm 0.16^{\#\#}$ |

Note: $^{**}P < 0.01$ compared with normal group; $^{\#\#}P < 0.01$ compared with control group.

ulceration were indicated in the intestinal tissues of experimental colitis rats, particularly in lower segments of colons. The glands were destroyed to a certain extent. An amount of neutrophils and lymphocytes was seen in intestinal tissue and a few eosinophils infiltrated into the intrinsic layer and submucosa, which could be accompanied with crypt abscesses, granuloma, and thickening of mucosa muscle layer (Figure 1). Histologic change, DAI, and CDMI scores of control group were significantly higher compared with normal group ($P < 0.01$) (Table 1). After treatment with Medilac-S, the pathologic changes in experimental colitis rats were ameliorated (Figure 1). Histologic change, DAI, and CDMI scores of Medilac-S group were significantly reduced compared with control group ($P < 0.01$) (Table 1).

The activities of SOD were lower ($P < 0.01$) and the activities of MPO were higher ($P < 0.01$) in control group compared with normal group (Table 2). After treatment with Medilac-S, the activities of SOD were significantly increased ($P < 0.01$) and the activities of MPO were decreased ($P < 0.01$) in experimental colitis rats (Table 2).

*3.2. Medilac-S Reduced the Expression of TLRs in Experimental Colitis Rats.* In the normal group TLR2 and TLR4 were lowly expressed in the intestinal tissues, while TLR9 was rarely expressed. After the establishment of experimental colitis, the expressions of TLR2, TLR4, and TLR9 were enhanced in the inflammatory cells which were located in lamina propria and submucosa (Figure 2(a)). But after the treatment of Medilac-S the expressions of TLR2, TLR4, and TLR9 were very weak (Figures 2(a) and 2(b)). In accordance with the above results, the mRNA expressions of TLRs in control group were higher compared with the normal group, but after treatment with Medilac-S the mRNA expressions of TLRs reduced (Figure 2(c)).

*3.3. Medilac-S Reduced the Infiltration of Macrophages in Experimental Colitis Rats.* The infiltration of macrophages in the local intestinal tissue is an important early event during the process of colitis [13], which could recruit other inflammatory cells into local tissue. So we compared the states of the infiltration of macrophages in the three groups. The result has shown that rare macrophages infiltrated into the intestinal tissue in the normal group. But after establishment of experimental colitis, more macrophages labeled with F4/80 infiltrated into the local intestinal. By the treatment with Medilac-S to control group, the infiltrated macrophages reduced significantly (Figure 3). These results imply that Medilac-S could prevent the infiltration of macrophages into local intestinal tissues.

*3.4. Medilac-S Decreased the Percentage of Th1 and Th2 but Increased the Percentages of Tregs in Experimental Colitis Rats.* $CD4^+$ T lymphocytes activation is a crucial immune response accompanied by inflammation and the ratios of subtypes of CD4+ T lymphocytes such as Th1, Th2, and Tregs manifest the state of immune system. We aimed to analyze the ratios of subtypes of $CD4^+$ T lymphocytes in the three groups and find the effect of Medilac-S on the state of immune response. Our results demonstrated that significant higher activation of Th1 ($P < 0.001$) and Th2 ($P < 0.001$) in control group compared with the normal group which imply excessive and continuous inflammation and immune response (Figures 4(a) and 4(b)). Medilac-S treatment decreases the ratios of Th1 ($P < 0.001$) and Th2 ($P < 0.01$) but increases the ratio of Tregs ($P < 0.005$) significantly (Figures 4(a) and 4(b)). These data indicated that Medilac-S has a role of immunosuppression manifested by downregulation of Th1 and Th2 and upregulation of Tregs.

For a more complete evaluation, all experiments consisting of rats model received heat-treated Medilac-S (121°C, 15 min in high compressed steam). Interestingly, the observed effects of live Medilac-S were all abolished (data not shown). So the heat-treatment destroys the probiotic character of Medilac-S.

## 4. Discussions

Inflammatory bowel diseases (IBD) are chronic, relapsing inflammatory disorders of the gastrointestinal tract. Although the cause of IBD remains unknown, immunological abnormalities triggered by genetic and environmental factors are thought to be important in its pathogenesis [2].

The majority of patients with IBD were used conventional therapy (viz., aminosalicylates, antibiotics, corticosteroids, and immunomodulatory agents) to both induce and maintain remission [14]. There is mounting evidence that probiotic therapy may ameliorate IBD both in animal models and in patients [8]. Probiotics, for the treatment of IBD, are a group of specific nonpathogenic bacteria that are functionally and genetically defined by their ability to reduce inflammation in the intestine. Although probiotics also seem to have broad beneficial effects in humans, there are specific identified mechanisms for the pathogenesis of IBD. Probiotics have been reported to have a direct effect on epithelial cell function and intestinal health, including enhancing epithelial barrier

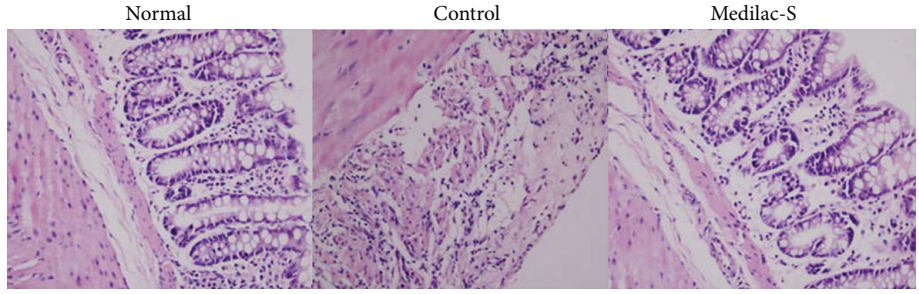


Figure 1: Morphological analysis of the lower segments of colons from normal, control, and Medilac-S rats. H&E staining with a magnified view ×200 shows significant histological changes in groups. In the control group, the glands were destroyed to a certain extent. An amount of neutrophils and lymphocytes was seen in intestinal tissue and a few eosinophils infiltrated into the intrinsic layer and submucosa. But in the Medilac-S group, few inflammation cells were seen (bars = 200 $\mu$m).

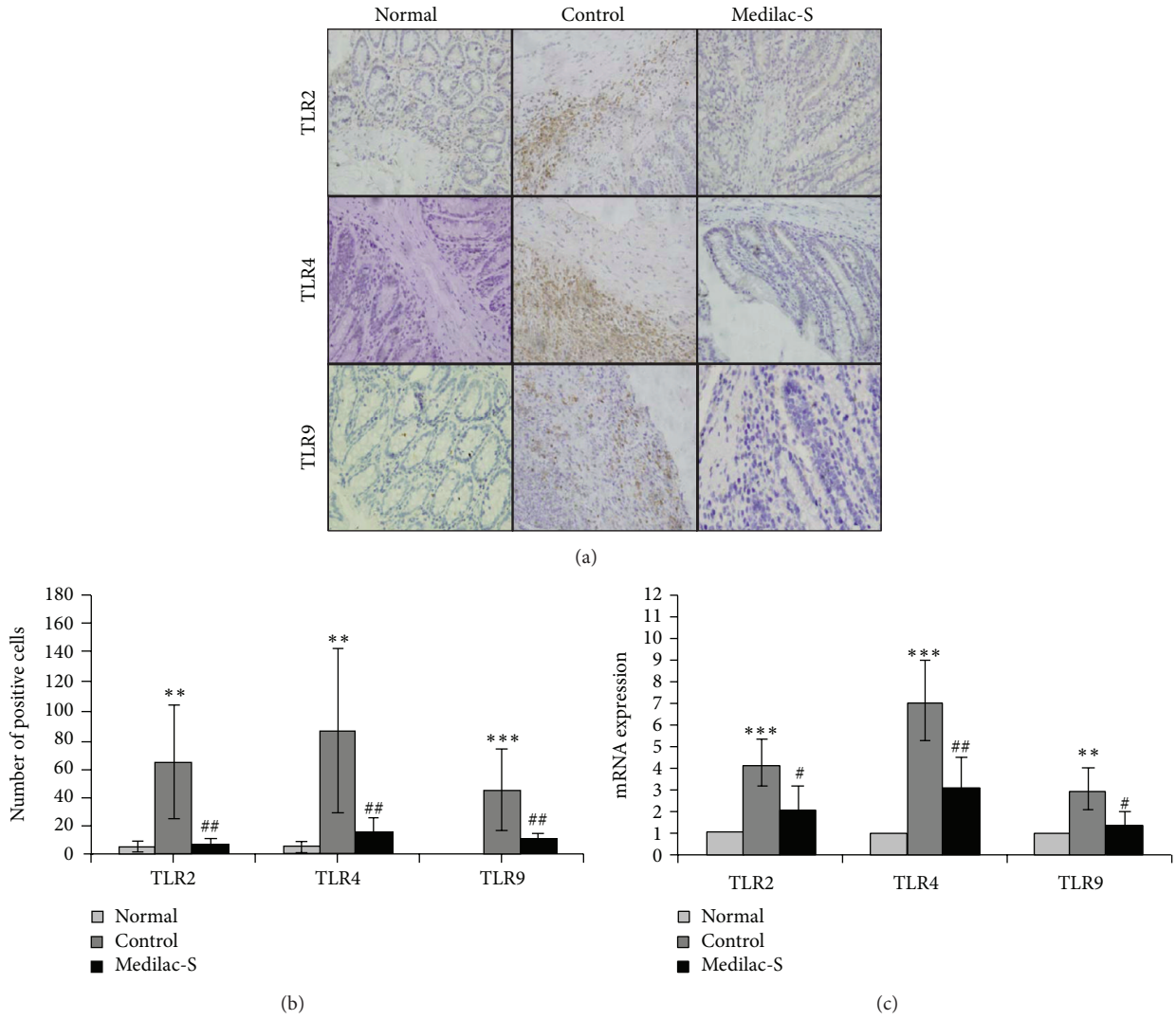


Figure 2: The expressions of TLRs in groups. (a) Immunochemistry of TLRs expressions in groups. (b) The mean numbers of positive cells per square millimeter of TLRs expressions were quantificated. (c) qRT-PCR analyzed the mRNA expressions of TLRs in groups. TLR2 and TLR4 were lowly expressed in normal intestinal tissues, while TLR9 was rarely expressed. After the establishment of experimental colitis, the expressions of TLR2, TLR4, and TLR9 were enhanced in the inflammatory cells which were located in lamina propria and submucosa. But after the treatment of Medilac-S the expressions of TLR2, TLR4, and TLR9 were all weakened. And the mRNA expressions in groups had the similar phenomenon ($n$ = 10 per group; $^{*}$compared with normal group, $^{\#}$compared with control group, $^{*}$ or $^{\#}P < 0.05$, $^{**}$ or $^{\#\#}P < 0.01$ and $^{***}$ or $^{\#\#\#}P < 0.001$).

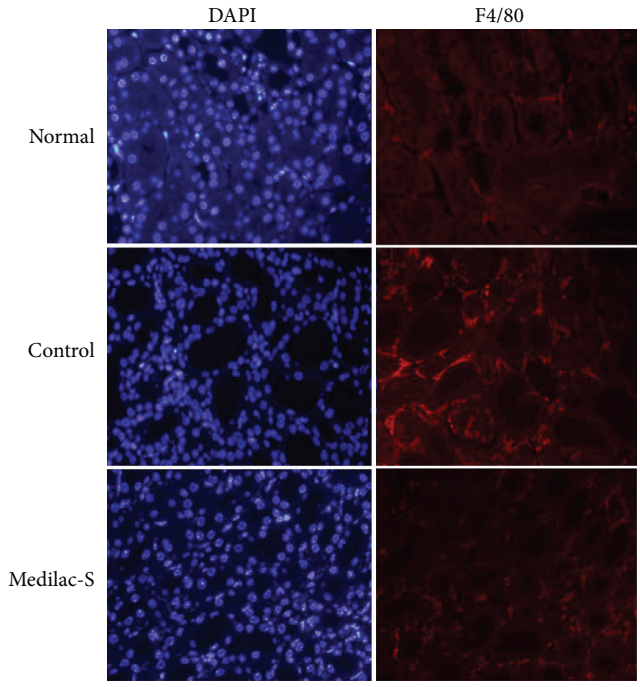


Figure 3: Infiltration of macrophages into the lower segments of colons from normal, control, and Medilac-S rats. Representative images of lower segments of colons tissue stained with DAPI (blue) and F4/80 (red). The result has shown that rare macrophages labeled with F4/80 infiltrated in the intestinal tissue in the normal group. But after establishment of murine model of colitis, a few macrophages infiltrated into the local intestinal. And the treatment with Medilac-S reduced the infiltrated macrophages significantly.

function, modulating epithelial cytokine secretion into an anti-inflammatory dominant profile, and altering mucus production [15, 16]. But the effect of probiotics on the innate or adaptive immune in IBD remains unclear since immunological abnormalities are important in its pathogenesis.

The group chose Medilac-S (live combined *Bacillus subtilis* and *Enterococcus faecium* enteric-coated capsules) to study the probiotic effect and mechanism. The *Bacillus subtilis* species has a long history of safe use. In view of the fact, some strains of *Enterococcus* can display a high level of resistance to vancomycin or can acquire such resistance and that certain strains of vancomycin resistant enterococci are commonly associated with nosocomial infections in hospitals. Meanwhile, some strains of *Enterococcus* display probiotic properties and may not at the point of inclusion in a product display vancomycin resistance. So we choose normal gut bacteria *Enterococcus faecium*, which is of course vancomycin sensitive.

TLRs represent key mediators of innate host defense in the intestine, involved in maintaining mucosal as well as commensal homeostasis [17]. The TLR family includes cellular signatures (mainly TLR2, TLR4, and TLR9) of microbial pathogens and plays a fundamental role in innate immune responses. The signal transduction is initiated from the Toll/interleukin-1 receptor (TIR) domain of TLRs after pathogen recognition. Almost all TLRs use a TIR-containing adapter MyD88 to activate a common signaling pathway that results in the activation of NF-kappaB to express cytokine genes relevant to inflammation leading to the production of inflammatory cytokines, chemokines, and interferons [18]. But inappropriate natural immune response induced by TLR4 may lead to serious tissue organ damage. Undue TLR stimulation may disrupt the fine balance between pro- and anti-inflammatory responses. Such disruptions may harm the host through the development of autoimmune and inflammatory diseases, such as rheumatoid arthritis and systemic lupus erythematosus [19]. Our previous study has shown that high expressions of TLR2, TLR4, and TLR9 in the colonic mucosa of experimental colitis rats and a positive correlation between intestinal damage degrees, which implied TLR2, TLR4, and TLR9, prompt the immune injury of murine intestinal in experiment colitis [20]. In this study, our results have shown that Medilac-S alleviated the protein and mRNA expressions of TLR2, TLR4, and TLR9 in the colon mucosa in experimental colitis. We assume that Medilac-S ameliorated murine experimental colitis by downregulation of TLRs which would suppress inflammation and innate immune response.

Our results also show that fewer macrophages were infiltrated after Medilac-S treatment in experimental colitis. Reports have shown that TLRs could induce neutrophil recruitment in lungs [21]. Medilac-S may decrease the recruitment of macrophages in intestinal by suppression of chemokines induced by TLRs, which would be further investigated.

TLRs also have crucial roles in initiating and shaping the adaptive immune response. Our results also have shown that the percentages of Th1 and Th2 cells were decreased but the percentage of Tregs was increased after Medilac-S treatment in experimental colitis. It seems that Medilac-S could play a role in the immune shift from T helper cells to Tregs. Recent researches have shown that TLRs could regulate the activation of T cells. TLRs also play an important, indirect role in the initiation of subsequent adaptive T cell responses. TLRs can function as costimulatory receptors that complement TCR-induced signals to enhance effector T cell proliferation, survival, and cytokine production [22–24]. But whether Medilac-S regulates T cell response via TLRs is still uncertain.

In conclusion, Medilac-S has protective effect against murine experimental colitis with decreased expression of TLRs, less infiltration of macrophages, and regulation of T subtypes. The accurate signaling mediated by Medilac-S needs to be further investigated.

## Conflict of Interests

The authors declare that they have no competing interests.

## Authors' Contribution

S. Chen and Y. Fu contributed equally to this work.

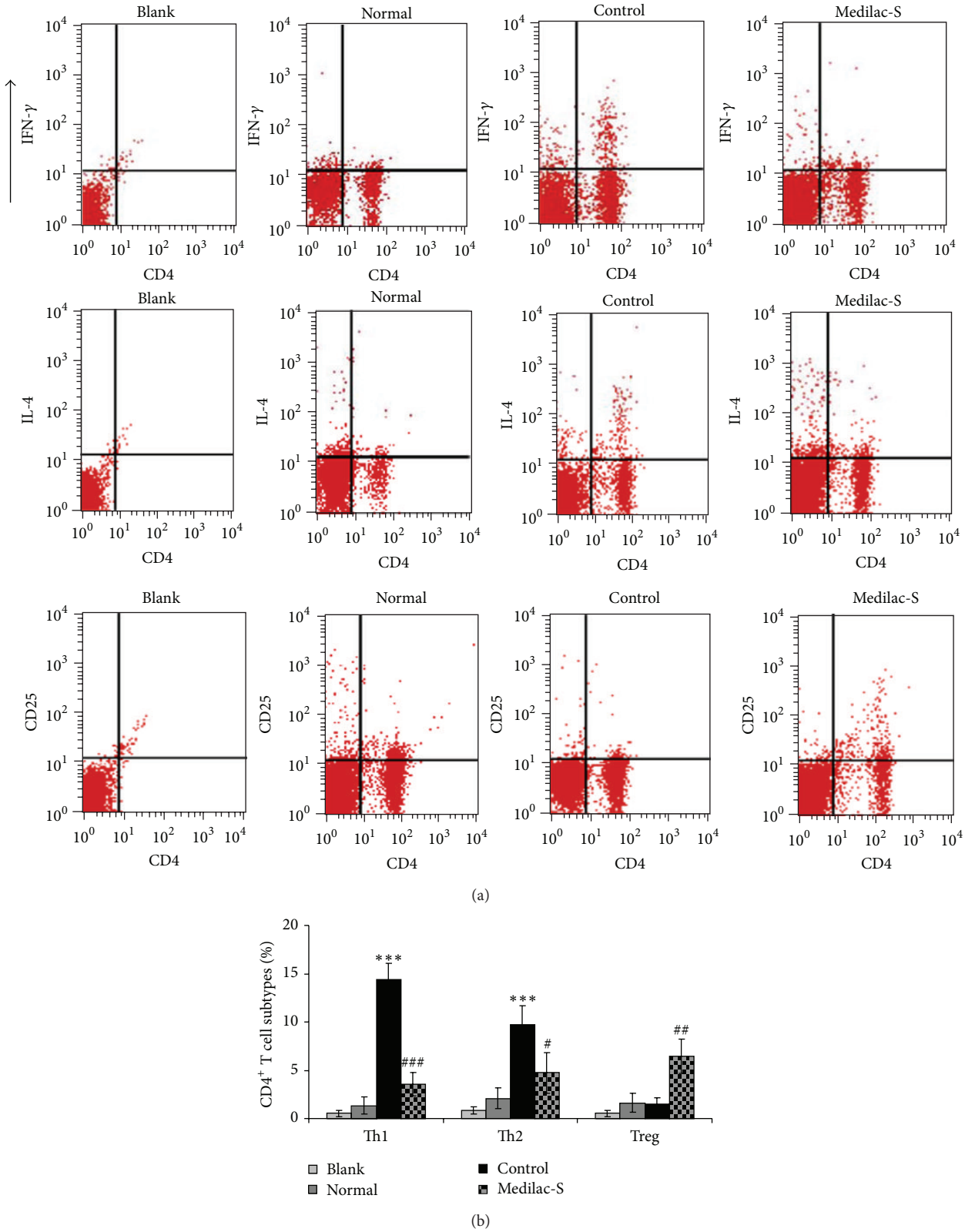


Figure 4: Analysis of subtypes of $CD4^+$ T lymphocytes from the spleens of rats in groups. (a) FACS analysis of Th1 ($CD4^+$ IFN-$\gamma^+$), Th2 ($CD4^+$ IL-$4^+$), and Treg ($CD4^+$ $CD25^+$). (b) Quantification of the percentages of Th1, Th2, and Treg in lymphocytes of the spleen. Our results demonstrated that significant higher activation of Th1 and Th2 in control group compared with the normal group. Medilac-S treatment decreased the ratios of Th1 and Th2 but increased the ratio of Tregs significantly compared with the control group. ($n = 10$ per group; $^*$compared with normal group, $^\#$compared with control group, $^*$ or $^\# P < 0.05$, $^{**}$ or $^{\#\#} P < 0.01$, and $^{***}$ or $^{\#\#\#} P < 0.001$).

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*Review Article*

# Contradictory Immune Response in Post Liver Transplantation Hepatitis B and C

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Hepatitis B and C often progress to decompensated liver cirrhosis requiring orthotopic liver transplantation (OLT). After OLT, hepatitis B recurrence is clinically controlled with a combination of hepatitis B immunoglobulin (HBIG) and nucleos(t)ide analogues. Another approach is to induce self-producing anti-hepatitis B virus (HBV) antibodies using a HBV envelope antigen vaccine. Patients who had not been HBV carriers such as acutely infected liver failure or who received liver from HBV self-limited donor are good candidate. For chronic HBV carrier patients, a successful response can only be achieved in selected patients such as those treated with experimentally reduced immunosuppression protocols or received an anti-HBV adaptive memory carrying donor liver. Hepatitis C virus (HCV) reinfects transplanted livers at a rate of >90%. HCV reinfected patients show different severities of hepatitis, from mild and slowly progressing to severe and rapidly progressing, possibly resulting from different adaptive immune responses. More than half the patients require interferon treatment, although the success rate is low and carries risks for leukocytopenia and rejection. Managing the immune response has an important role in controlling recurrent hepatitis C. This study aimed to review the adaptive immune response in post-OLT hepatitis B and C.

## 1. Introduction

Hepatitis B virus (HBV) infection and hepatitis C virus (HCV) infection are the main causes of end-stage liver disease requiring orthotopic liver transplantation (OLT). However, the post-OLT course is quite different between the two types of hepatitis. The post-OLT hepatitis B recurrence rate is >80% without any prevention, while >90% of recurrent infections can be controlled with a combination of hepatitis B immunoglobulin (HBIG) and nucleos(t)ide analogues (NAs) [1]. Non-OLT chronic hepatitis B patients are treated with antiviral proliferative NA agents, with >90% long-term control with minute side effects. The first commercially available NA, lamivudine (LAM), produced a rapid and definite short-term antiviral response, but 15–20% of the patients experienced annual recurrence of resistant virus and 70% of them did so after 5 years [2]. Fewer than 3% of patients treated with newer NAs such as entecavir (ETV) or tenofovir (TDF) experience resistant virus; these newer NAs are accepted as first-line and long-term treatment [3, 4].

After OLT, more than half of the patients become reinfected. It is difficult to eradicate the virus once these patients become reinfected [5].

The mechanism for controlling HBV viral recurrence is direct viral replication control by a combination of NAs and HBIG as passive immunoprophylaxis [6]. As HBIG combination therapy has important roles, the B-cell-related adaptive immune response appears to play a role in controlling HBV after OLT. However, as HBV-induced hepatitis is characterized by T-cell immune response, both B- and T-cell adaptive immune responses have vital roles [7]. When active immunization of patients with HBV vaccine is performed, HBV-specific and non-HBV-specific immune responses can be obtained.

HCV reinfects >90% of patients, with more than half of these patients developing chronic hepatitis requiring interferon- (IFN-) based antiviral treatment [8]. Non-OLT chronic hepatitis C patients have been treated with IFN-based immune reaction-related treatment. Recently, pegylated IFN (Peg-IFN) has been used in combination with ribavirin and, more recently, with the addition of a direct-acting antiviral agent (DAA) targeting the HCV nonstructural protein (NS) 3/4A protease [9]. Single Peg-IFN resulted in only 30% of patients experiencing sustained viral response (SVR), representing undetectable HCV-RNA longer than 24 weeks after finishing IFN [10]. This demonstrated >99% viral eradication, while the Peg-IFN and ribavirin combination resulted in 50% SVR [10, 11]. Peg-IFN plus ribavirin and telaprevir or simeprevir resulted in >80% SVR for patients with genotype 1, which is the more difficult-to-treat genotype of HCV [12]. All-oral, IFN-free regimens are expected to become commercially available in the near future [13].

HCV-related liver cirrhosis is a common indication for OLT [14]. However, HCV persists in almost all post-OLT patients. Graft reinfection is universal after OLT [15], leading to high-titer HCV viremia, with cirrhosis developing within 5 years of transplantation in approximately 20% of patients and within 10 years in 50% [16]. Thus, HCV infection after OLT differs completely from chronic hepatitis C (CHC) without transplantation. However, the mechanisms underlying accelerated HCV-induced liver damage after OLT are poorly understood. Several factors appear to be involved in the risk of hepatitis C recurrence, particularly those related to viral and immune responses. Immunosuppressive therapy is a likely cause for the severe accelerated course of HCV-related hepatitis after OLT [16, 17]. In particular, high-dose steroids, immunosuppressive drug combinations, powerful induction treatments, and acute rejection can worsen patient outcomes [18]. The pathology of HCV-related disease reflects immune reactions to virus-infected hepatocytes [7]. In post-OLT settings, immunosuppressive drugs definitely affect the clinical course. The effects of interferon-based treatment are limited to 30–50% of patients, with especially poorer results in post-OLT patients, and also carry the possibility of inducing mortal chronic rejection that should be avoided [19].

In this review, we summarize the aberrant immune system in HBV- and HCV-related hepatitis, together with the changes in these diseases after OLT.

## 2. Immune Mechanisms in Non-OLT HBV- and HCV-Related Hepatitis

*2.1. Immune Mechanisms in HBV-Related Hepatitis.* After infecting a patient once , HBV persists in the liver for the rest of a person's life, even after the patient achieves a clinically cured condition with seroclearance of HBV envelope antigen (hepatitis B surface antigen, HBsAg) and emergence of HBs antibody (HBsAb) [20]. In controlling viral replication, immune function has been revealed to be important, as immunosuppressive treatment for cancer chemotherapy or organ transplantation can induce viral replication even in HBsAg negative with HBsAb positive clinically cured patients and organ transplant recipients [21, 22]. HBV is an enveloped DNA virus containing a relaxed circular DNA genome, which is converted into a covalently closed circular (CCC) DNA that persists in the nucleus of infected cells as minichromosomes [23].

Natural killer (NK) cells work as the innate immune modulator to induce the death of microbial-infected cells with strong cytotoxic activity and the production of high levels of certain cytokines and chemokines in a nonmajor histocompatibility complex- (MHC-) restricted manner distinct from T and B cells [24]. Upon HBV infection, NK cells migrate to the liver, with a decrease in their numbers in the spleen and bone marrow, suggesting the recruitment of NK cells from these organs [24]. As hepatocytes normally express little MHC class I, NK cells may play a more important role in the early defense against HBV infection before the MHC class I expression is upregulated after viral replication in hepatocytes [25].

Antigen-presenting cells (APCs), such as Kupffer cells (liver resident macrophages) and dendritic cells, have important roles in intermediating the innate to adaptive immune responses [26]. Kupffer cells or macrophages behave in both an immunostimulatory and immunoregulatory fashion upon HBV exposure. The addition of HBV particles and HBsAg induces the production of proinflammatory cytokines interleukin- (IL-) 1$\beta$, IL-6, CXCL-8, and tumor necrosis factor (TNF)-$\alpha$ by human CD68$^+$ macrophage-enriched cells via NF-$\kappa$B (nuclear factor kappa-light-chain-enhancer of activated B cells) activation [27]. However, another study reported no such cytokine production with immunoregulatory cytokine transforming growth factor- (TGF-) $\beta$ production [28]. The immune system activates Kupffer cells to eradicate HBV, while HBV evades the Kupffer cell-related pathway to reduce the inflammatory pathway and change the environment to be favorable for survival. Pretreatment of nonparenchymal cells, including Kupffer cells, with HBsAg or HBV virion, abrogates the Toll-like receptor- (TLR-) related antiviral response such as IFN-$\beta$, interferon-stimulated gene (ISG), or NF-$\kappa$B. In the liver biopsy specimens of patients with active hepatitis B, Kupffer cells have been revealed to possess higher expression of galectin-9, which is an immunoregulatory molecule [29]. Kupffer cells accumulate around injured hepatic loci and produce several cytotoxic and fibrosis progression-related molecules [30]. However, they also have an important function in scavenging apoptotic hepatocytes, which could function as a bait for inflammation, and depletion of Kupffer cells could induce worsening of hepatitis [31, 32]. Kupffer cells function as both proinflammatory and anti-inflammatory and profibrotic and antifibrotic cells in their environment. Both the hepatitis state and Kupffer cell polarity are needed to understand the immunological pathogenesis in HBV-related hepatitis.

Strong HBV-specific CD8$^+$ T-cell responses have been shown to correlate with viral and hepatitis control during acute infection [33]. In chronically infected patients, HBV-specific CD8$^+$ T-cell responses are weak and occur with low frequency, while patients with low viral load exhibit multispecific strong responses [34]. The epitopes targeted by

the $CD8^+$ T cells have been deeply analyzed in HLA-A and -B restricted epitopes, as these have been believed to have antiviral impact [35, 36]. However, a recently characterized HLA-C restricted epitope has also been revealed to have a clinical impact and is an especially frequent allele in patients who live in Southeast Asia [37]. Several factors have been suggested to explain this phenomenon. In HBV-specific $CD8^+$ T cells, proapoptotic protein Bcl2-interacting mediator (BIM) is upregulated, naïve T-cell phenotypes such as CD45RA, CD27, CD28, and CCR7 are highly expressed, and immune regulatory molecules such as programmed death-1 (PD-1), cytotoxic lymphocyte antigen 4 (CTLA-4), and T-cell immunoglobulin mucin-3 (TIM-3) are also highly expressed [29, 38–41]. Several experimental trials that were conducted to block such immunoregulatory exhaustion molecules showed reversal of these immunoregulatory conditions [41, 42].

Similar to the $CD8^+$ T cells, $CD4^+$ T cells have also been found to exhibit a lower response in the acute phase of infection in patients who developed chronic hepatitis later [43]. The $CD4^+$ T-cell response in patients who recovered was found to be more frequent, stronger, and more multispecific than that observed later in patients with chronic hepatitis [40]. The IFN-$\gamma$ producing antiviral Th1 response against HBV core has been revealed to be stronger in patients with resolved infection even several years after infection [44].

The humoral immune response has been acknowledged as an avenue for understanding the clinical course of acute and chronic hepatitis B [45]. The antibody responds against viral structural antigens such as the core antigen (HBcAg) and the envelope antigen (HBsAg). Anti-HBcAg IgM antibody (IgM-HBcAb) is accepted as the earliest and most diagnostic marker of acute infection. Anti-HBc IgG antibody (IgG-HBcAb) develops during acute infection and remains positive for the duration of the patient's life [46]. HBsAg emerges in serum from the acute phase of infection and remains when the patient exhibits chronic hepatitis B, while in patients who experience an acute self-limiting course HBsAg could be cleared. Anti-HBs antibody (HBsAb) is a virus-neutralizing antibody recognized as having lower viral and disease activities. The lack of anti-HBs in chronic infection can be attributed to a selective exhaustion of B cells and IL-10 secreting immunoregulatory B-cell expansion [45, 46].

*2.2. Immune Mechanisms in HCV-Related Hepatitis.* Since HCV is not also a cytopathic virus, immune reactions play a central role in the development of chronic hepatitis (Figure 1) [47, 48]. Innate antiviral responses constitute the first-line defense system against infected virus. HCV disables some innate antiviral systems to escape from the immune pressure [48]. The lack of a strong Th1-type helper T-cell response and cytotoxic T-cell response against HCV leads to chronic infection with this virus [49–51]. High-magnitude, broad, polyfunctional, and sustained T-cell responses correlate with spontaneous recovery [35, 49, 52], but these responses are not correlated with interferon-induced viral clearance [53].

The role of NK cells in chronic hepatitis C is not completely understood. However, as NK cells are the first immunological walls against HCV, much evidence has been uncovered. An NK-cell activating and inhibitory receptor gene polymorphism has been discovered to have roles in the course of HCV infection [54, 55]. As IFN-$\alpha$ is the basic treatment for chronic hepatitis C, IFN-producing NK cells have been defined as key immune cells. NK cells can produce IFN-$\alpha$, IFN-$\gamma$, and TNF-$\alpha$ and induce dendritic cell activation and support innate to adaptive immune response bridging [56]. NK cells can also lyse HCV-infected hepatocytes, T cells, and APCs and modulate immune responses [57]. However, HCV itself has been revealed to have a role in the potential inhibition of NK cell function, resulting in chronic hepatitis [58].

Antigen presenting cells such as Kupffer cells, macrophages, or dendritic cells (DCs) behave in both an immunostimulatory and immunoregulatory manner upon HCV exposure, as in HBV [26]. In chronic hepatitis C patients, Kupffer cells are increased and activated as the higher expression markers CD163 and CD33 [59, 60]. *In vitro* analysis has revealed that HCV core and NS3-affected Kupffer cells secrete proinflammatory cytokines such as IL-1$\beta$, IL-6, and TNF-$\alpha$ and also immunosuppressive cytokine IL-10 [61]. Proinflammatory cytokine release might explain the induction and persistent inflammation in chronic hepatitis C, while immunosuppressive cytokine release explains the difficulty in the eradication of HCV-infected hepatocytes. The direct effects of HCV on the inflammatory signal in Kupffer cells have been revealed to upregulate the immunoregulatory molecule PD-L1 [62]. Probably, HCV interferes with Kupffer cell-related antiviral activities but induces strong enough inflammatory cytokines to result in chronic inflammation. The effects of Kupffer cells on liver fibrosis progression are similar to those in HBV infection. The Kupffer cells accumulate around inflammatory foci and express cytotoxic molecules such as granzyme B, perforin, and reactive oxygen species to induce inflammation and fibrosis [63].

There are a minimum of two subsets of DCs. Myeloid DCs (mDCs) produce a large amount of IL-12 upon stimulation, while plasmacytoid DCs (pDCs) produce a large amount of IFN-$\alpha$ in viral infection [64]. DC function has been reported as broadly impaired in CHC patients [65–68]. However, several reports have indicated contradictory results that DC function is not impaired in CHC patients [69–73]. Most of these reports are studies with mDCs; however, pDCs are also reportedly functionally impaired and reduced by increased apoptosis [74]. Since culture conditions and chronic hepatitis conditions in the patient may change the phenotype of immune cells, functional differences in DCs during chronic HCV infection remain contentious. *In vitro* transfection or the addition of HCV proteins such as core, NS3, or NS4 has been reported to result in reduced function of DCs [75]. Because of the scarcity of *in vitro* culture systems for HCV, these experimental results are also contentious. With the recent establishment of infectious cell culture-produced HCV, impaired pDC functions have been revealed [64].

The role of the humoral immune response in the clearance of HCV is not well understood. After viral clearance, most

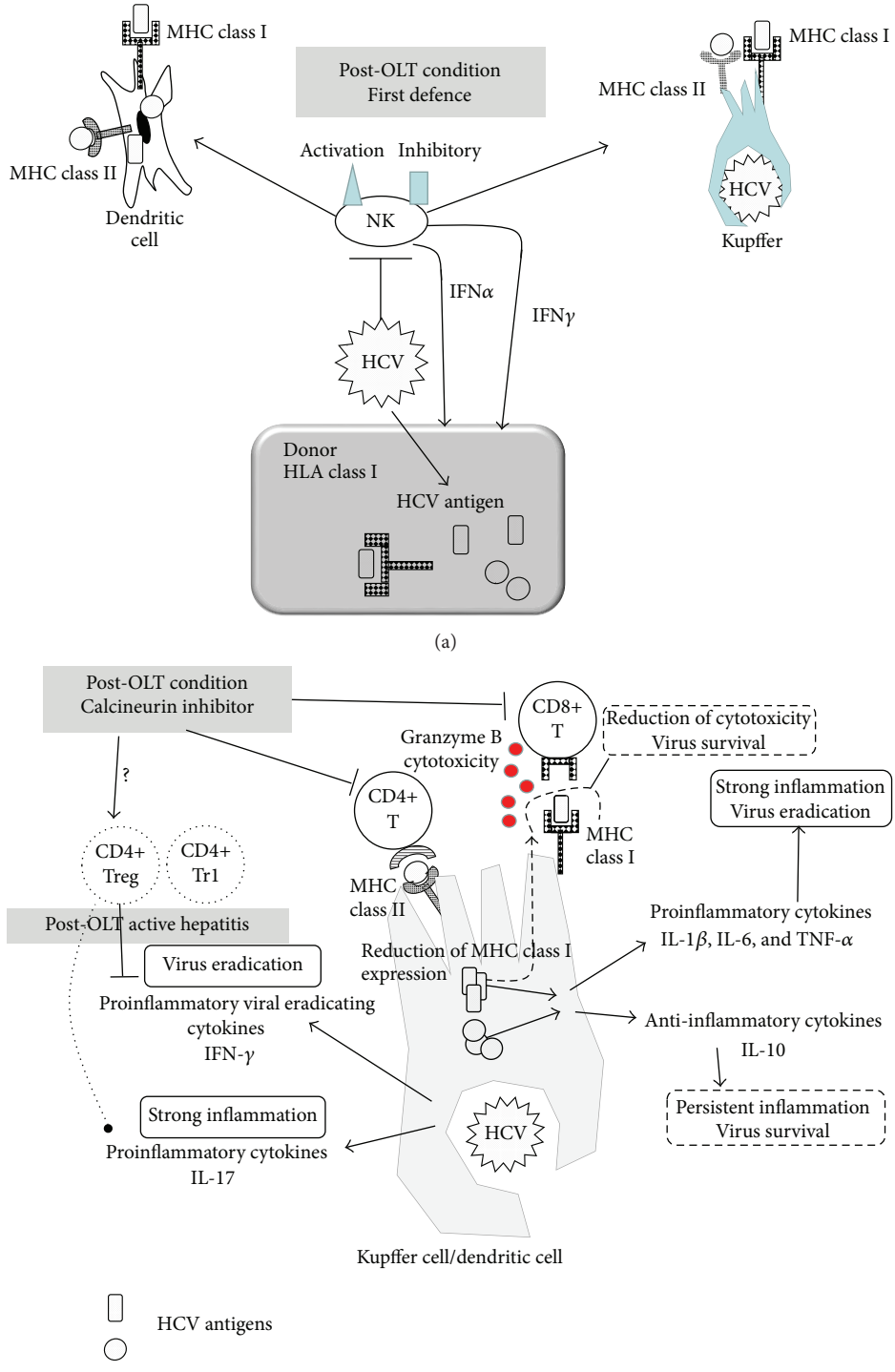


Figure 1: Immune status in chronic hepatitis C and postorthotopic liver transplantation (OLT) hepatitis C. (a) NK cells are the first immunological defense system from hepatitis C virus (HCV). The phenotype defined with the activation or inhibitory receptor gene polymorphism affects the chronic hepatitis C activity and the post-OLT hepatitis activity. The interferon producing function is decreased by HCV proteins. (b) Kupffer cells or dendritic cells (DCs) have important roles in bridging innate and adaptive immune responses. These cells show proinflammatory and anti-inflammatory functions when infected with HCV. These cytokines' balance is well controlled for viral persistence and chronic inflammatory state by viral antigens. After OLT, regulatory T cells might affect to reduce antiviral defence but not to reduce inflammatory cytokines resulting in severer chronic hepatitis. The type 1 regulatory T cells (Tr1) may induce severe hepatitis, while a lower frequency of Tr1 is correlated with hepatitis control with HCV positive status. NK: natural killer cell, OLT: orthotopic liver transplantation, HCV: hepatitis C virus, MHC: major histocompatibility complex, IFN: interferon, Treg: regulatory T cell, and Tr1: type 1 regulatory T cell.

antibody titers wane despite the persistent T-cell response [35]. A neutralizing antibody response is detectable, even in chronic hepatitis C patients [76]. The target of the response is placed in and around the envelop proteins E1 and E2 and the hypervariable region near the amino terminus of E2 [77]. Neutralization epitopes have been revealed to be masked by extensive glycosylation and by virions covered with lipid droplets and might not be effectively targeted [78, 79]. In addition, as the RNA-dependent RNA polymerase of HCV lacks proofreading activity, it is easy for HCV-RNA to mutate and escape from the host immune pressures [80]. Although 20–30% of infected patients recover from the infection with strong T-cell response memory and possible neutralization antibody B-cell response, they could be reinfected with the virus, indicating difficulties in producing disease-controlling vaccines [81].

Strong HCV-specific $CD4^+$ T-cell and $CD8^+$ T-cell responses have been shown to be evident in HCV patients with resolved infection, while diminished in patients with chronic hepatitis C [7]. To recognize viral-infected hepatocytes or APCs, viral epitopes should be expressed on the MHC. Interferon upregulates MHC class I expression; however, replicating HCV-RNA reduces that expression [82]. Interferon is released from NK cells and DCs during an early phase of viral infection and has important roles in eradicating HCV, as this is the key drug for treatment [83]. This HCV interference with MHC expression must be one reason why CHC patients show reduced $CD8^+$ T-cell responses.

Recent attention has focused on regulatory T cells (Tregs) and their contribution to CHC. Their mechanism of immunosuppression depends on both cell-cell contact and immunosuppressive cytokine secretion [84]. A subpopulation of Tregs that express CD18 and also CD49b-expressing type 1 regulatory T (Tr1) cells have also attracted attention [85], because they produce large amounts of immunosuppressive cytokines such as IL-10 and TGF-$\beta$, with which they inhibit type 1 and 2 helper responses [86]. Tregs and Tr1 cells may contribute to HCV persistence by suppressing HCV-specific T-cell responses [87–89]. Treg frequencies and activities are apparently higher in CHC patients than in those who have achieved viral clearance [90]. Recently discovered T-cell regulatory molecules such as PD-1, 2B4, and TIM-3 have been revealed to be coexpressed in intrahepatic HCV-specific CD8 T cells, indicating that HCV-induced T-cell functional exhaustion represses viral eradication [91].

Strong innate and adaptive immune responses are responsible for HCV clearance; however, the virus itself affects many sites of the immune system, ameliorating the effective antiviral immune functions. To control NK, Kupffer cells, B cells, or T cells might be difficult as they act in different ways in different CHC conditions.

## 3. Immune Responses in Post-OLT HBV Recurrence Control

*3.1. Overview of Post-OLT HBV Control with Nucleos(t)ide Analogues and Hepatitis B Immunoglobulin.* A multicenter study in Europe in 1993 identified the risk of post-OLT HBV recurrence [92]. The risk was low in patients with acute liver failure who were intolerant of HBV. However, the recurrence rate in patients with liver cirrhosis, especially with high serum HBV-DNA at OLT, was >80% [92]. As the immune system is repressed with steroids and calcineurin inhibitors, recurrent hepatitis B produces severe hepatitis with a high incidence of mortal liver failure. However, present protocols that use NA in combination with long-term HBIG have resulted in >90% control of HBV recurrence [1].

The first trial of long-term HBIG combined with the first-generation NA lamivudine (LAM) was conducted in 1998. Monthly HBIG administration with LAM resulted in all patients surviving for 1 year after OLT without serum HBV-DNA positivity [93]. Subsequent reports also described successful control of HBV recurrence with this combination [94]. The historical progression of controlling post-OLT HBV recurrence is summarized in Table 1. As patients with positive HBV-DNA before OLT were more likely to later have HBV recurrence, to maintain anti-HBs antibody titers >500 IU/L was recommended. If HBV-DNA was negative before OLT, the anti-HBs antibody titer could be reduced to 100–150 IU/L with or without LAM. From the standpoint of cost savings, the HBIG dose requirement was able to be decreased as clinical data accumulated [95–97]. Currently, HBIG is administered as required only when anti-HBs antibody titers fall below target levels. Some reports indicate that only a short duration of HBIG administration is required and that it can be withdrawn several months after OLT [98]. If HBV-DNA was negative at the time of OLT, HBIG could be withdrawn at several months after OLT. For acute liver failure patients who had been infected with the virus shortly before hepatitis development, HBIG could also be withdrawn. Of course, strict monitoring of HBV-DNA and HBV surface antigen (HBsAg) titers should be continued throughout the patient's life.

The mechanism of protection against HBV reactivation by the combination of drugs is not well defined. The cccDNA episome is the transcriptional template for HBV messenger RNA transcripts that encode viral structural and NS proteins and the pregenomic RNA template for reverse transcription and synthesis of the viral genome [5]. NAs inhibit the reverse transcription of pregenomic RNA, resulting in a rapid decrease in serum HBV-DNA, but cannot eliminate the cccDNA reservoir [99]. HBIG contains high-titer antibodies against HBsAg, which is the major component of the envelope of the HBV virion.

The possible mechanisms through which HBIG prevents HBV transmission are that it neutralizes circulating virus by immune complex formation, protects naïve hepatocytes against HBV released from extrahepatic sites through blocking the putative HBV receptor, or anti-HBs antibody internalizes into hepatocytes, interacts with HBsAg, and inhibits HBsAg secretion from cells [100]. To protect against HBV infection of naïve hepatocytes might be difficult, since recent studies have revealed that intrahepatic HBV-DNA is detectable in >50% of even well-controlled patients after OLT [5]. The HBV virion released from the infected cells could be blocked with anti-HBs antibody. In an *in vitro* assay, the internalized antibody was seen to induce the accumulation

Table 1: Recent post-OLT HBV prophylaxis with nucleos(t)ide analogue and/or HBIG combination.

| | HBV-DNA recurrence (%) | Followup (months) | Reference number | Reported year |
|---|---|---|---|---|
| Lamivudine + HBIG | | | | |
| HBIG IV 10000 IU/month | 0 | 13 | [93] | 1998 |
| HBIG IV to maintain HBsAb >200 IU/L | 9.5 | 21 (2.4–49.1) | [96] | 2001 |
| HBIG to maintain HBsAb >70 IU/L | 0 | 16 (9–22) | [97] | 2004 |
| HBIG IV to maintain HBsAb >10 IU/L | 0 | 30 (7–73) | [6] | 2007 |
| Short course (1 month) HBIG | 7 | 18 | [98] | 2003 |
| Entecavir + HBIG | | | | |
| HBIG; IM to maintain HBsAb >100 IU/L | 0 | 41.2 (33–54) | [107] | 2012 |
| One year HBIG IM; 2000 IU/month<br>Lamivudine + adefovir or tenofovir, tenofovir, entecavir | 0 | 24 (6–40) post HBIG withdrawal | [106] | 2012 |
| One year HBIG IV; dose not specified | 3.8 | 24 | [105] | 2013 |
| HBIG free with newer nucleos(t)ide analogues regimen | | | | |
| Lamivudine + adefovir<br>(no HBIG when HBV-DNA below 3 log(10)IU/mL) | 0 | 22 (10–58) | [103] | 2013 |
| Entecavir, lamivudine + adefovir, tenofovir, entecavir + tenofovir<br>(no HBIG when HBV-DNA below 3.3 log(10)IU/mL) | 8 (5/6 withdrawn NAs) | 21 (1–83) | [110] | 2013 |

OLT; orthotopic liver transplantation, HBV: hepatitis B virus, HBIG; hepatitis B immunoglobulin, HBV-DNA: hepatitis B virus DNA, IV: intravenous administration, IM: intramuscular administration, IU: international unit, HBsAb: anti-hepatitis B s antibody, and NAs: nucleos(t)ide analogues.

of intracellular viral particles even after the antibody was removed from the cell culture supernatant [101].

Several new NAs such as adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (LdT), and tenofovir (TDF) have become commercially available [102]. Because of the risk of developing resistance, LAM is no longer recommended as a first-line treatment for hepatitis B. The currently recommended first-line agents are ETV and TDF, which have resulted in a very low emergence of resistance [3, 4]. Such newer NAs are very effective when combined with HBIG even during short duration, post-OLT HBV control [103–109]. Because of low resistance and the powerful antiviral response evoked by ETV and TDF or a combination of NAs, several institutions have developed successful HBIG-free protocols if the HBV-DNA titer is low enough at the time of OLT [103, 110].

As the strong NAs are very effective in HBV control, immune cell-related treatments are not administered, although hepatitis B infection is an immune mediated disease [1].

*3.2. Adaptive Immune System to Get Anti-HBs Antibody Response with HBV Vaccine.* The practice of active immunization of post-OLT recipients with HBV vaccine is emerging. For a successful vaccine response, the immune system has important roles. Most studies report low response rates, even with doubled concentrations or prolonged injections of vaccines (Table 2) [111–115]. Patients who had not been HBV carriers (such as adult patients with acute liver failure due to sexual transmission and nonchronic HBV carriers with anti-HBc antibody-positive donor livers) are good candidates for vaccine administration [112, 116–121]. Patients with acute HBV infection who undergo OLT are often positive for anti-HBs antibody even before OLT and have powerful immune responses. Such patients should respond well to vaccination since they have not developed tolerance to HBV, unlike chronic carriers. However, some HBV carriers have responded to vaccination.

Since noncarriers respond well to HBV vaccination, even under prednisolone and calcineurin inhibitor usage, immune tolerance is expected to play a large role in this process. In non-OLT HBV patients, analysis has revealed that HBsAg-positive newborns had higher regulatory T-cell frequencies and dysfunctional CD8 T cells, which represent immune tolerant status [122]. However, another report analyzing the immunological characteristics of HBsAg-positive young carriers and aged patients with active hepatitis revealed comparable peripheral T-cell proinflammatory cytokine production capacity and HBV-specific IFN-$\gamma$ responses [123]. These findings indicate that tolerant carriers can react with HBV antigens and can show active immunity against HBV vaccination, if regulatory T-cell function diminishes. With good responses to newer NAs after OLT, HBV-DNA decreases even in the liver, and this might recover compressed HBV-specific T cells to react with HBV.

Chronic HBV carrier recipients, including patients with positive HBV-DNA at OLT, do not respond well to HBs-antigen-containing vaccine, with response rates being mostly <30% [114, 115, 119, 124, 125]. Tahara et al. reported 64.7% positive responses to experimentally minimized immunosuppressant treatment [118]. The immune status of these patients was evaluated by a mixed lymphocyte reaction (MLR) assay in response to antidonor and anti-third-party allostimulation using an intracellular carboxyfluorescein diacetate succinimidyl ester- (CFSE-) labeling technique. "Third-party" refers to healthy volunteers with the same blood type as the patients. The autologous lymphocytes, the donor lymphocytes, and the third-party lymphocytes were irradiated and used as the stimulator cells, and the recipients' lymphocytes were used as the responder cells in MLR. The investigators minimized immunosuppression until the donor lymphocytes showed no response as autologous lymphocytes, but third-party lymphocytes showed a positive response. The investigators found that vaccination was successful in patients showing a donor-specific MLR hyporesponse, with a well-maintained response to the third-party stimulus. The vaccine was not successful in patients showing hyporesponse to both the donor and the third party. These results provide encouragement that even immune tolerant liver cirrhosis patients can react with HBV vaccines under lower immunosuppressant protocols after OLT.

Another protocol of repeated vaccine administration resulted in successful immunization in 40% of patients with post-OLT liver cirrhosis [117]. The donors to good responders were the spouses of recipients and had high anti-HBs antibody titers before donation. The spouses with high-titer anti-HBs antibodies were probably infected with HBV by the recipients after marriage, resulting in the anti-HBs antibody boost. The immune systems of these donors should not have developed tolerance to the virus [126]. The adoptive immune transfer of the HBV-specific immune response could be achieved [127].

To successfully transfer immune memory to recipients, the anti-HBs antibody titer of the donors should be high. Luo et al. have shown that a high anti-HBs antibody titer (>1000 IU/L) in donors is essential for adoptive transfer [128]. These results suggest that pre-OLT HBV vaccination for candidate living donors might facilitate improved post-OLT vaccine responses in recipients with liver cirrhosis. Several experimental adjuvant vaccines have also been tried with up to 44.8% success rates [111, 119, 129].

The vaccine response depends on immune tolerance to the virus in both recipients and donors. The liver is the largest immune organ in the abdomen; therefore, it plays a critical role in immune responses. Multiple populations of nonhematopoietic liver cells, including sinusoidal endothelial cells, stellate cells located in the subendothelial space, and liver parenchymal cells, can function as APCs [130]. The viral-specific immune competence of the grafted liver might overcome general immune tolerance to the virus in chronic HBV carriers.

*3.3. Adaptive Immune System to Get Anti-HBs Antibody Response with HBV Vaccine in HBV Naïve Recipients Who Received Livers from Anti-HBc Antibody Positive Donors.* As a shortage of donor organs is a universal problem, anti-HBc

Table 2: Post-OLT HBV vaccine administration trials.

| | Methods | Definition of success | Success rate (%) | Reference number | Reported year |
|---|---|---|---|---|---|
| Liver cirrhosis | 10–20 $\mu$g monthly with experimentally minimized immunosuppressant | HBsAb >100 IU/L without HBIG | 64 | [118] | 2009 |
| | 20 $\mu$g monthly | HBsAb >100 IU/L 6 months without HBIG | 40 | [117] | 2013 |
| | 20 $\mu$g monthly | HBsAb >100 IU/L 3 months without HBIG | 0 | [112] | 2011 |
| | 40 $\mu$g 0, 1, 2, 6, 7, and 8 months | HBsAb >100 IU/L without HBIG | 0 | [114] | 2009 |
| | 40 $\mu$g 0, 1, and 6 months | HBsAb >10 IU/L without HBIG | 82 | [113] | 2000 |
| | 20–40 $\mu$g 0, 1, and 6 months | HBsAb >10 IU/L 16 months without HBIG | 0 | [115] | 2010 |
| | 20 $\mu$g with MPL adjuvant 12 monthly | HBsAb >100 IU/L 18 months without HBIG | 44.8 | [129] | 2010 |
| | Experimental adjuvant vaccine 0, 1, 2, 6, and 12 months | HBsAb >500 IU/L 18 months without HBIG | 25 | [119] | 2005 |
| Acute liver failure | 20 $\mu$g monthly | HBsAb >100 IU/L 6 months without HBIG | 100 | [117] | 2013 |
| | Experimental adjuvant vaccine 0, 1, 2, 6, and 12 months | HBsAb >500 IU/L 18 months without HBIG | 100 | [119] | 2005 |
| | 10–20 $\mu$g monthly | HBsAb >100 IU/L without HBIG | 66 | [118] | 2009 |
| Non-HBV-related patients with HBcAb positive donors | 20 $\mu$g monthly | HBsAb >100 IU/L 3 months without HBIG | 83 | [112] | 2011 |
| | Infant 20–40 $\mu$g according to body weight 2–4 times/year | HBsAb >100 IU/L without HBIG | 75 | [121] | 2007 |
| | 20 $\mu$g 0, 1, and 6 months | HBsAb >100 IU/L 6 months without HBIG | 50 | [120] | 2007 |

OLT: orthotopic liver transplantation, HBV: hepatitis B virus, HBIG: hepatitis B immunoglobulin, IU: international unit, and HBsAb: anti-hepatitis B s antibody.

positive healthy carriers could be candidate donors. With regard to the above vaccination protocols, non-HBV-related patients who received anti-HBc antibody positive donor livers have fared quite favorably. The post-OLT incidence of *de novo* hepatitis B occurring in anti-HBc antibody-positive donors without prophylaxis is high (33–100%) [22, 131, 132]. These HBV-naïve patients are good candidates for the HBV vaccine because 50–80% tend to respond well [112, 120, 121]. Pre-OLT vaccination is also possible if patients have sufficient time before undergoing OLT. In countries with universal vaccination programs, the recipients might already have anti-HBs antibody and could be boosted with additional vaccination before OLT, resulting in 78% of prospective recipients having a high titer of anti-HBs antibody (>1000 IU/L) [133]. In pediatric patients, the vaccination responses were observed to be good in recipients with higher anti-HBs titers at the time of OLT and lower tacrolimus levels at the time of vaccination [134].

## 4. Adaptive Immune Responses in Post-OLT Hepatitis C Recurrence Control

*4.1. Overview of Post-OLT Hepatitis C Recurrence and Treatment.* As HCV recurrence is observed in almost all the patients who receive OLT, HCV eradication before OLT has been tried, although with unsuccessful outcomes [135, 136].

Post-OLT IFN administration is the only way to achieve better outcomes. HCV genotypes 1b and 4 seem to be negative predictive factors for recurrence because of a lower response to pegylated interferon (Peg-IFN) and ribavirin combination therapy [136]. The host and donor factors associated with poorer outcomes are female gender, older donor age, steatosis of the graft, and the IL-28B single nucleotide polymorphism (SNP) [137–140]. A human genomewide association study recently uncovered many disease-susceptible genes or drug sensitivity-related genes. In CHC patients, the IL-28B gene SNP was found to be related to spontaneous clearance and susceptibility to treatment with Peg-IFN plus ribavirin [141–143]. The combination of recipient and donor IL-28B genetic polymorphism has been revealed to be important in post-OLT HCV treatment outcomes [137].

Recently, direct-acting antivirals such as NS3 protease inhibitors or NS5 polymerase inhibitors or a combination of them have come to represent a new highly effective treatment strategy [144]. The triple combination therapy of Peg-IFN, ribavirin, and a protease inhibitor (telaprevir) has been accepted as a highly effective treatment for non-OLT CHC, producing >75% sustained virological response (SVR) [145]. However, as telaprevir inhibits cytochrome P450 3A4 and reduces the metabolism of calcineurin inhibitors, the trough levels of cyclosporine A (CyA) increase to 4.6-fold and FK 506 (FK) to 70-fold [146]. This phenomenon requires that triple therapy be used with strict care. The second generation protease inhibitor simeprevir very weakly inhibits cytochrome P450 3A4 and is safer than telaprevir. Triple therapy including simeprevir is safer than triple therapy with telaprevir and is currently recommended [147].

*4.2. Adaptive Immune Responses in Hepatitis C Recurrence.* In post-OLT settings, T-cell activities are affected by immunosuppressive therapy [148]. Although the T-cell response is repressed with calcineurin inhibitors, post-OLT CHC patients often show severe hepatitis recurrence with high viral load [14]. In post-OLT CHC patients, the importance of immune reaction has been accepted. Several reports have mentioned that HCV-specific immune responses correlate with post-OLT hepatitis C progression [149, 150]. The frequency of HCV-specific IL-17-secreting $CD4^+$ T cells was shown to be increased in severe inflammation in liver fibrosis patients [150]. The serum cytokine profile of these patients with severe recurrence exhibited higher inflammatory cytokines (IL-17, IL-1$\beta$, IL-6, IL-8, and monocyte chemoattractant protein [MCP]-1), decreased antiviral cytokine IFN-$\gamma$, and increased IFN-$\gamma$ reducing cytokine IL-10, suggesting the presence of the inflammatory phenotype with repressed antiviral immune response.

Several studies have demonstrated that Tregs induce allograft tolerance [151, 152]. Moreover, Tregs and Tr1 cells are overexpressed in patients with severe hepatitis C recurrence compared with patients with no or minor recurrence [86, 153]. These results suggest that Tregs and Tr1 cells are involved in HCV recurrence after OLT. Because the strength of immunosuppressive therapy and the viral load would be changed after OLT, the time course of the immune response has important roles. Recently, we have shown that Tr1 frequency was repressed in 40 days after OLT under the condition of persistently normal alanine aminotransferase (ALT), even at 3 years after OLT [8]. Tr1, which has a strong IL-10 production capacity, may reduce HCV-specific T-cell responses and induce active hepatitis with ALT elevation. Monitoring Tr1 frequency might be a way to determine which patients would develop active hepatitis. However, HCV-specific $CD4^+$ T-cell IFN-$\gamma$ production, which was higher in patients with persistently normal ALT until 3 years after OLT, was found to diminish after 3 years (Tsuzaki R. et al. accepted manuscript for Acta Med Okayama, 2014). This result indicates that, although the adaptive immune response could control hepatitis, the strength of the response might diminish over time. These results from our experience indicate that IFN-based anti-HCV therapy could be applied for patients with higher Tr1 after OLT, who might show active hepatitis until 3 years after OLT. Whether the Tr1 reduction treatment will become the next treatment strategy is not clear, as selective reduction of Tr1 might be difficult. However, as calcineurin inhibitors reduce regulatory T cells, minimum usage of calcineurin inhibitors might be the way this can be accomplished now [154].

*4.3. Innate Immune Responses in Hepatitis C Recurrence.* Innate immune responses have also been identified as HCV targets and could be depressed with respect to their functions. Dendritic cells (DCs) and NK cells are thought to play a central role in the interplay between the innate and adaptive immune responses. Kupffer cells are also involved in post-OLT hepatitis C recurrence, as NF-$\kappa$B was highly expressed in patients with post-OLT HCV recurrence. However, the specificity for the disease state is not well characterized [155].

In post-OLT settings, blood pDCs decreased after OLT and the pDC product IFN-$\alpha$ also decreased. These decreases might affect the recurrence of post-OLT hepatitis C [156].

NK cells have also been deeply investigated with respect to their activities in HCV infection and hepatitis. NK cells are implicated in various viral infections, including HCV and front-line anticancer immune responses. The HCV-E2 protein has been revealed to bind the NK CD81 receptor and decrease the release of IFN-$\gamma$, resulting in noneffective antiviral responses [157]. Another NK cell receptor, the killer immunoglobulin receptor (KIR), which displays an inhibitory function, has been revealed to be correlated with post-OLT hepatitis C. The KIR-ligand mismatch and recipient KIR2L3 haplotype have been shown to correlate with recurrent hepatitis C [55]. IFN treatment susceptibility of post-OLT HCV recurrence has also been shown to be correlated with the NK receptor haplotype KIR2DS2 [158]. Intravenous administration of living donor perfusate of NK cells could reduce the HCV-RNA increase after OLT [159]. As acutely infected hepatitis C patients show self-recovery at a rate of 20–30%, a strong NK cell response might control hepatitis C even under immunosuppressive treatment.

Adaptive immune response in post-OLT HBV remains a problem that should be investigated, as this virus continues to be difficult to be eradicated from the infected liver. However, the anti-HCV treatment protocol is drastically changing because several clinical trials of new DAA with >80% viral eradication might result in these drugs being introduced to the market; therefore, the importance of investigating the immune system in post-OLT HCV will probably consolidate to selected refractory patients in the next 10 years.

## 5. Conclusion

The adaptive immune response in post-OLT hepatitis B recurrence is hidden under strong antiviral HBIG and NA combination treatment. However, the effectiveness of active immunization is dependent upon adaptive immune responses being effective for patients with non-HBV-related disease who have received anti-HBc antibody-positive donor livers and patients with acute liver failure who are not immune tolerant to HBV. Vaccination is not sufficiently effective for patients with liver cirrhosis; nevertheless, the donor immune memory for HBV and the strength of the immunosuppressant drugs have important roles. Adaptive immune responses, especially of the $CD4^+$ and $CD8^+$ T cells and the Treg, have strong effects in post-OLT hepatitis C viral recurrence and in recurrent hepatitis activities. The regulatory T cells and Tr1 cells affect the clinical course and could be used as prediction markers. As IFN-based treatments have risks after OLT, forecasting the patient's course with such markers could be beneficial.

## Conflict of Interests

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

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*Research Article*

# Neurotensin Decreases the Proinflammatory Status of Human Skin Fibroblasts and Increases Epidermal Growth Factor Expression

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Fibroblasts colonization into injured areas during wound healing (WH) is responsible for skin remodelling and is also involved in the modulation of inflammation, as fibroblasts are immunologically active. Herein, we aimed to determine neurotensin effect on the immunomodulatory profile of fibroblasts, both in homeostatic and inflammatory conditions. Neurotensin mediated responses occurred through NTR1 or NTR3 receptors, while under inflammatory conditions NTR1 expression increase seemed to modulate neurotensin responses. Among different immunomodulatory genes, CCL11, IL-8, and IL-6 were the most expressed genes, while CCL4 and EGF were the less expressed genes. After neurotensin exposure, IL-8 mRNA expression was increased while CCL11 was decreased, suggesting a proinflammatory upregulation and chemoattractant ability downregulation of fibroblasts. Under inflammatory conditions, gene expression was significantly increased. After neurotensin exposure, CCL4 and IL-6 mRNA expression were decreased while CCL11 was increased, suggesting again a decrease in the chemoattractant capacity of fibroblasts and in their proinflammatory status. Furthermore, the expression of EGF, a crucial growth factor for skin cells proliferation and WH, was increased in all conditions. Overall, neurotensin, released by nerve fibers or skin cells, may be involved in the decrease of the chemotaxis and the proinflammatory status in the proliferation and remodelling phases of WH.

## 1. Introduction

Neuropeptides can be produced by skin cells or be released by peripheral nerves into the skin, where they bind to respective receptors stimulating different signalling pathways and cellular responses [1, 2]. Within the cellular responses and events that can be modulated in the skin by neuropeptides, inflammatory processes are some of the most important. However, the role of neurotensin (NT) in the modulation of skin inflammation is still unclear.

The first report demonstrating NT-positive fibbers in the skin was in 1983 [3] and it was later confirmed by Donelan et al. in 2006 [4]. The interest in NT first appeared when its influence on the pathogenesis of skin disorders exacerbated by stress was discovered [5]. In fact, it has been observed that in many skin disorders worsened by stress, the number and activation status of mast cells increase. Accordingly, NT has been shown to increase the number and activation of mast cells in a skin pathogenesis, such as psoriasis [6], which is in agreement with several other reports showing that mast cell degranulation is triggered by stress and via neurotensin [7–9]. Moreover, skin vascular permeability induced by corticotropin-releasing hormone (CRH) on mast cells has been shown to occur through a neurotensin-dependent mechanism [4]. In fact, human mast cells are able to synthesize a neurotensin precursor, secrete

bioactive NT-like peptide(s), and express NT receptor NTS1 [10]. Besides mast cells, neurotensin has also been shown to enhance the chemotaxis capacity of lymphocytes and to limit the growth of cutaneous T-cell lymphoma tumor cells [11]. Most recently, we have demonstrated that NT can modulate inflammatory events on a skin dendritic cell line [12]. Fetal-skin dendritic cells expressed both NTR1 and NTR3 and neurotensin was able to downregulate the activation of inflammatory signalling pathways and the expression of cytokines IL-6, TNF-$\alpha$, and IL-10, as well as vascular endothelial growth factor (VEGF), while upregulating the survival pathway ERK and epidermal growth factor (EGF) expression [12].

In spite of these results not much has been studied regarding NT effects in fibroblasts. Therefore, in the present study we used a cell line established from the skin of normal newborn human foreskin fibroblasts. Fibroblasts are a heterogeneous population of cells of mesenchymal origin which support the development, repair, and homeostasis of their resident tissue. Dermal fibroblasts play a key role in extracellular matrix (ECM) deposition, epithelial-mesenchymal interactions, and wound healing [13]. They also contribute to the immune regulation of the skin, producing and releasing chemokines IL-8 and CCL5, cyclooxygenases, and prostanoids, after activation by inflammatory agents, such as bacterial agents [14]. By using this cell line, we intended to decrease the variability of the donors, although lacking the variability of skin from different body locations. Using fibroblasts from the foreskin ensures the use of cells from a skin area replenished of a rich and complex network of nerves with neurophysiologic parameters [15, 16].

The main aim of this work was to unravel the effect of neurotensin and its chemoattractant capacity in newborn foreskin fibroblasts, in both homeostatic and inflammatory conditions, in order to understand the possible physiological role of this neuropeptide in skin wound healing as well as speculate about the role of this neuropeptide in other skin pathologies.

## 2. Materials and Methods

*2.1. Materials.* Lipopolysaccharide (LPS) from *Escherichia coli* (serotype 026:B6) was obtained from Sigma Chemical Co. (St. Louis, MO, USA), NT was obtained from Bachem (Weil am Rhein, Germany), 30% Acrylamide/BisSolution 29 : 1, TEMED, and SYBR Green were obtained from Bio-Rad, and High Capacity cDNA Reverse Transcription kit was obtained from Applied Biosystems.

The protease inhibitor cocktail (Complete Mini) and the phosphatase inhibitor cocktail (PhosSTOP) were obtained from Roche (Carnaxide, Portugal). Bicinchoninic acid (BCA) kit assay was obtained from Novagen. The polyvinylidene difluoride (PVDF) membranes and the antibody against $\beta$-actin were purchased from Millipore Corporation (Bedford, MA, USA). The polyclonal antibodies against NTR1(H-130), NTR2(H-19), and NTR3(H-300) were purchased from Santa Cruz (Frilabo), references sc-15311, sc-31696, and sc-30217, respectively. The alkaline phosphatase-linked secondary antibody and the enhanced chemifluorescence (ECF) reagent were obtained from GE Healthcare (Carnaxide, Portugal). The Vectashield mounting medium was purchased from Vector Inc. (Burlingame, CA, USA), the Alexa Fluor 488 antibody was purchased from Molecular Probes (Eugene, OR, USA), and the Alexa Fluor 555 phalloidin antibody was purchased from Invitrogen (Barcelona, Spain). TRIzol was obtained from Invitrogen; diethyl pyrocarbonate (DEPC) was acquired from AppliChem. Methanol, ethanol, and isopropanol were obtained from Merck. All primers were obtained from MWG Biotech (Ebersberg, Germany). All other reagents were purchased from Sigma Chemical Co.

*2.2. Culture of the BJ Cell Line.* The BJ cell line (ATCC number CRL-2522) was kindly supplied by Paula Marques and João Malva (Life Sciences Department and Center for Neurosciences and Cell Biology, Coimbra University, Portugal). This cell line was established from skin taken from normal newborn human foreskin fibroblasts. The BJ cell line has the capacity to proliferate to a maximum of 72 population doublings before the onset of senescence.

BJ cells were cultured in endotoxin-free Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) of inactivated fetal calf serum, 3.02 g/l sodium bicarbonate, 100 U/mL penicillin, 100 $\mu$g/mL streptomycin, and 30 mM of glucose, in a humidified incubator with 5% $CO_2$/95% air, at 37°C. BJ fibroblasts have a doubling time of about 72 h and were used after reaching 80–90% confluence, which occurred approximately every 7 days. Along the experiments, cells were monitored by microscopic observation in order to detect any morphological change.

*2.3. Western Blot.* The cells (a confluent 75 $cm^2$ flask) were washed with ice-cold PBS and harvested in a sonication buffer. Cell lysates and protein quantification were performed as previously described [12]. NTR1, NTR2, and NTR3 levels were evaluated by Western blots. Proteins were separated by electrophoresis and transferred to PVDF membrane, as previously described [12]. The immune complexes were detected by membrane exposure to the ECF reagent, followed by scanning for blue excited fluorescence on the VersaDoc (Bio-Rad Laboratories, Amadora, Portugal). Membranes were stripped and reprobed with the antibody for $\beta$-actin. The generated signals were analysed using the Image-Quant TL software.

*2.4. RNA Extraction.* Cells ($8 \times 10^5$) were plated in 60 mm dishes in a final volume of 6 mL and were treated with 10 nM of NT during 30 h, or pretreated with 10 nM of NT during 24 h, and then stimulated with 1 $\mu$g/mL of LPS during 6 h, treated for 6 h with LPS, or left untreated (control). Total RNA was isolated from these cells with TRIzol according to the manufacturer's instructions. The RNA concentration was determined by OD260 measurement using a Nanodrop spectrophotometer (Wilmington, DE, USA). RNA was stored in RNA Storage Solution (Ambion, Foster City, CA, USA) at −80°C.

*2.5. Real-Time RT-PCR.* One microgram of total RNA was reverse-transcribed using High Capacity cDNA Reverse

Table 1: Primer sequences for targeted cDNAs.

| Primer | 5′-3′ sequence (F: forward; R: reverse) | RefSeqID |
|---|---|---|
| HPRT1 | F: TGACACTGGCAAAACAATG<br>R: GGCTTATATCCAACACTTCG | NM_000194 |
| NT | F: GCATACATCAAAGATTAGT<br>R: TAAAGCAGTAGGAAGTTT | NM_006183 |
| NTR1 | F: GTCGTCATACAGGTCAAC<br>R: GATGATGGTGTTCAGGAC | NM_002531 |
| NTR2 | F: GCAAGAATGAACAGAACA<br>R: GAATGATTAGTGATGAGGTT | NM_012344 |
| NTR3 | F: TGGGTTGGAGATAGCACTGG<br>R: ACGACTTCCTCCAGACACCT | NM_002959 |
| IL-1$\beta$ | F: GCTTGGTGATGTCTGGTC<br>R: GCTGTAGAGTGGGCTTATC | NM_000576 |
| IL-6 | F: TCTGGATTCAATGAGGAGACTTG<br>R: TCACTACTCTCAAATCTGTTCTGG | NM_000600 |
| IL-8 | F: CTTTCAGAGACAGCAGAG<br>R: CTAAGTTCTTTAGCACTCC | NM_000584 |
| CCL11 | F: ACCAGAGCCTGAGTGTTG<br>R: TGCCCTTTGGACTGATAATGA | NM_002986 |
| CCL4 | F: CGCCTGCTGCTTTTCTTACAC<br>R: CAGACTTGCTTGCTTCTTTTGG | NM_002984 |
| CCL5 | F: CAGTGAGCTGAGATTGTG<br>R: TTTGTTGTTGTTGTTGTGA | NM_002985 |
| EGF | F: TCAGAAGATAACATTACAGAAT<br>R: AATACACCGAGCATACAT | NM_001178130 |

Transcription (RT), from Applied Biosystems. Real-time RT-PCR was performed in a 20 $\mu$L volume containing 2.5 $\mu$L cDNA (25 ng), 10 $\mu$L 2X SYBR Green Supermix, 2 $\mu$L of each primer (250 nM), and 1 $\mu$L of $H_2O$ PCR grade. Samples were denatured at 95°C for 3 min. Subsequently, 40 cycles were run for 10 sec at 95°C for denaturation, 30 sec at the appropriate annealing temperature, and 30 sec at 72°C for elongation. Real-time RT-PCR reactions were run in duplicate for each sample on a Bio-Rad My Cycler iQ5. After amplification, a threshold was set for each gene and Ct-values were calculated as previously described [12].

Primers were designed using Beacon Designer Software v7.2, from Premier Biosoft International, and thoroughly tested. Primer sequences are given in Table 1. The results were normalized using a reference gene, hypoxanthine phosphoribosyltransferase 1 (HPRT-1). This reference gene was previously validated in our lab [17] and its expression did not suffer variations upon BJ cell line stimulation with LPS and NT.

*2.6. Immunocytochemistry Assay.* Cells ($2 \times 10^5$) were cultured in 24-well plates containing circular glass lamella on the bottom. Cells were washed with ice-cold PBS, fixed with 4% paraformaldehyde in PBS for 10 min, and then permeabilized with 0.1% Triton X-100 in PBS containing 200 mM glycine for 5 min. After a blocking step with PBS/1% BSA for 30 min, cells were incubated overnight with antibodies against NTR1, NTR2, and NTR3 (1 : 100 in PBS containing 0.1% BSA). After washing with PBS, cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit antibody (1 : 500) and Alexa Fluor 555 phalloidin antibody (1 : 500) for 30 min at room temperature. After a washing step, cells were then incubated for 1 min with DAPI (0.1 $\mu$g/mL in PBS) and mounted with Vectashield medium to reduce photobleaching. For image acquisition, fluorescence labelling was visualized using a fluorescence microscope—Zeiss Axiovert 200—and images captured with a coupled AxioCam HR camera. In each experiment, the optimal acquisition parameters were defined for the control cells and maintained for all the other conditions within the same experiment.

*2.7. Statistical Analysis.* The results were statistically analysed using the nonparametric Kruskall-Wallis test followed by Dunn's posttest, using Graphpad software. Results are presented as mean ± SD and the significance level was $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

## 3. Results

*3.1. Expression of Neurotensin and Its Receptors on Skin Fibroblasts.* BJ cells do not constitutively express neurotensin, under either basal conditions or LPS stimulation (data not shown).

The expression of neurotensin receptors, namely, NTR1, NTR2, and NTR3, was determined by real-time RT-PCR, Western blot, and immunocytochemistry analyses. BJ cells constitutively express the genes for NTR1, NTR2, and NTR3, with NTR2 being the most abundant, as shown in Figure 1(A).

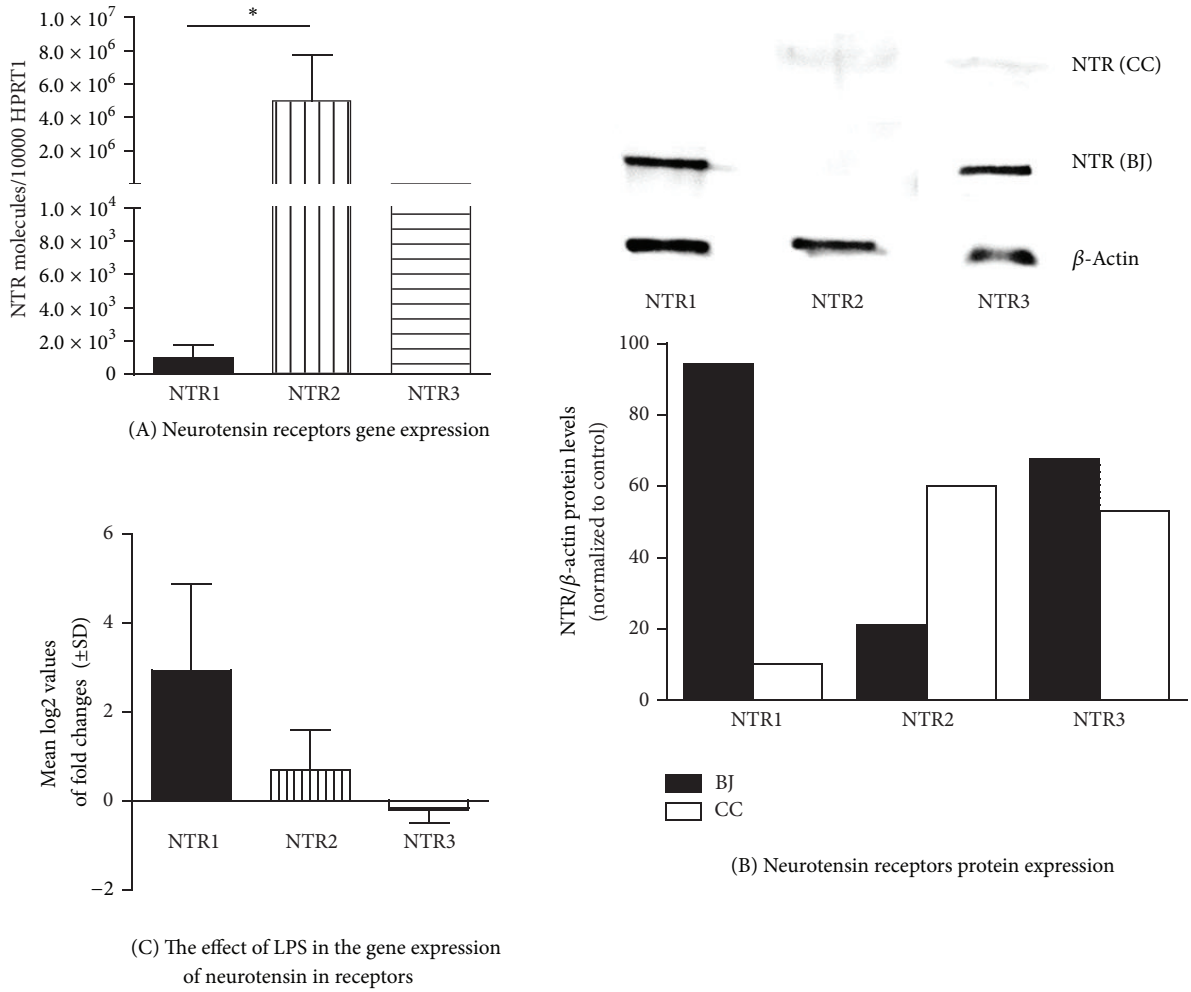


Figure 1: Neurotensin receptor mRNA and protein expression. Cells were maintained in medium (A, B) or treated with 1 $\mu$g/mL of LPS for 6 h (C), at 37°C, with 5% $CO_2$. Total RNA was isolated and retrotranscribed as indicated in Section 2. The mRNA levels were assessed by quantitative real-time RT-PCR. Gene expression is indicated as genes studied/10,000 molecules of the reference gene HPRT1 (A) or mean log2 values of fold changes relative to the control (C). Each value represents the mean ± SD from three independent experiments ($^*P < 0.05$; Kruskall-Wallis test followed by Dunn's multiple comparison posttest). Cells extracts from untreated BJ cells (BJ) and from the murine cortical cortex (CC) were subjected to Western blot analysis (B) using NTR1, NTR2, and NTR3 antibodies, with normalization to $\beta$-actin. The blot shown is representative of 3 independent experiments yielding similar results.

However, only the NTR1 and NTR3 were effectively transduced to protein, as observed in Figure 1(B), indicating possible cellular posttranscription modification. Brain homogenates from the cortical cortex of mice were used as positive controls for NTR reactivity, since they express all neurotensin receptors, as determined by Western blot analysis (Figure 1(B)).

In addition, the expression of neurotensin receptors was studied under inflammatory conditions. The stimulation of BJ cells with LPS for 6 h caused an increase in NTR1 and NTR2 gene expression of 2.9 ± 1.9- ($^*P < 0.05$, $n = 3$) and 0.7 ± 0.9- ($n = 3$) fold above control, respectively. However, the expression of NTR3 slightly diminished by 0.2 ± 0.3- ($n = 3$) fold, as shown in Figure 1(C).

Neurotensin receptor localization was measured in these cells and it was verified that NTR1 is localized at the cell membrane, in the cytoplasm and in the nucleus, while NTR3 is mainly found in the nucleus (Figure 2).

*3.2. Modulation of Gene Expression in Fibroblasts by LPS.* To evaluate the expression of different genes under homeostatic conditions and upon 6 h of LPS exposure, real-time RT-PCR was performed for CCL4, CCL5, CCL11, IL-8, IL-1$\beta$, IL-6, and epidermal growth factor (EGF).

Nonstimulated BJ cells constitutively expressed all chemokines, cytokines, and the growth factor EGF, with IL-6, IL-8, and CCL11 being the most expressed genes, by $1.6 \times 10^4 \pm 1.1 \times 10^4$ ($^{\#\#} < 0.01$, $n = 4$), $2.8 \times 10^4 \pm 1.9 \times 10^4$ ($^{\#\#\#} < 0.001$, $n = 4$), and $2.5 \times 10^4 \pm 1.7 \times 10^4$ ($^{\#\#\#} < 0.01$, $n = 4$), respectively, relative to HPRT1 gene expression, while CCL4 was barely expressed, by 6.4 ± 5.0 ($n = 4$), relative to HPRT1 gene expression (Figure 3). When stimulated with

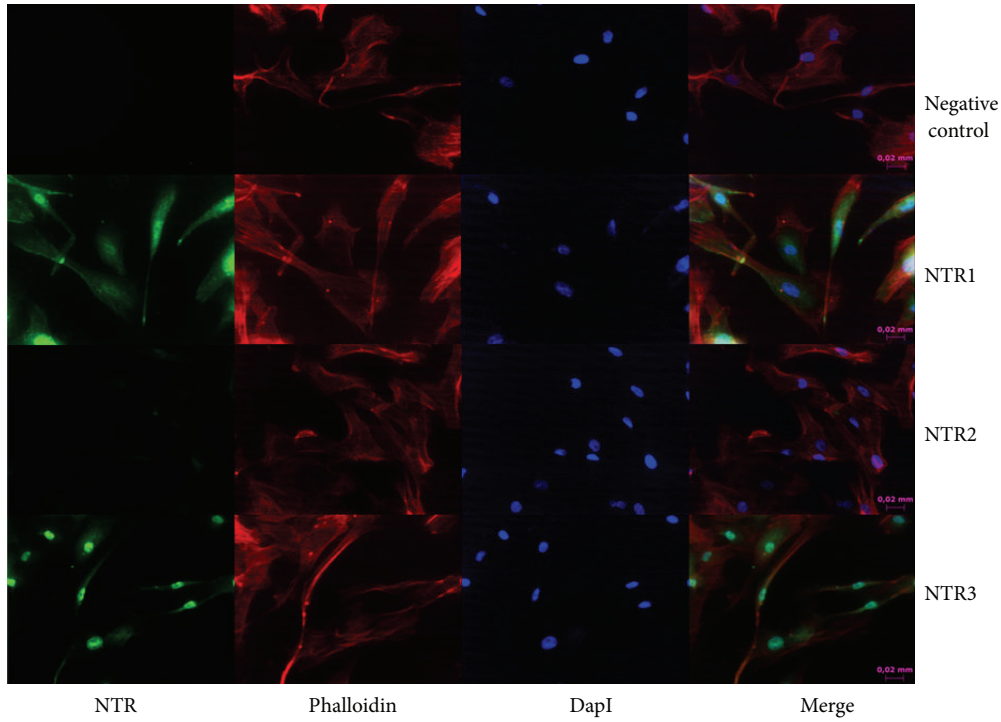


Figure 2: Neurotensin receptor localization. Cells were subjected to immunocytochemistry analysis as described in Section 2 using NTR1, NTR2, and NTR3 antibodies.

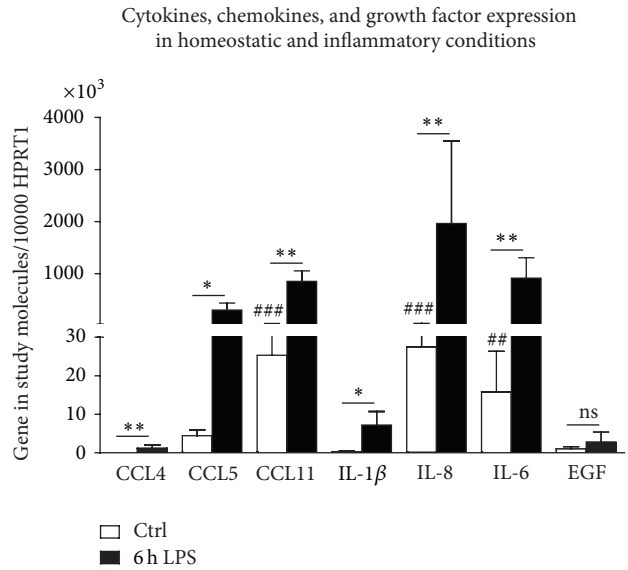


Figure 3: Expression of cytokines, chemokines, and growth factors in BJ cells, under homeostatic and inflammatory conditions. Cells were plated at $8 \times 10^5$ cells/dish in 60 mm dishes in a final volume of 6 mL of medium and treated with 1 $\mu$g of LPS during 6 h (LPS), or left untreated (Ctrl), at 37°C, with 5% $CO_2$. Total RNA was isolated and retrotranscribed as indicated in Section 2. The mRNA levels were assessed by quantitative real-time RT-PCR. Gene expression is indicated as genes studied/10 000 molecules of the reference gene HPRT1. Values represent the mean ± SD from four independent experiments. Kruskall-Wallis test followed by Dunn's posttest statistical analysis was performed between cytokines, chemokines, and growth factor expression under homeostatic and inflammatory conditions ($^{**}P < 0.01$; $^{***}P < 0.001$) and among cytokines, chemokines, and growth factor expression under homeostatic conditions ($^{\#\#} < 0.01$; $^{\#\#\#} < 0.001$). IL: interleukin; EGF: epidermal growth factor.

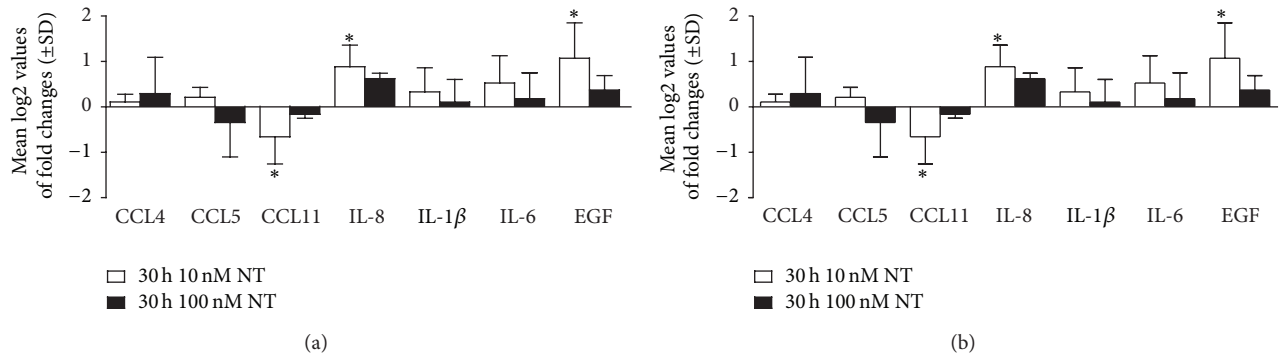


Figure 4: Modulation of gene expression by neurotensin under homeostatic and inflammatory conditions in BJ cells. Cells were maintained in culture medium (Ctrl) and treated with 10 nM of NT (white bars) or 100 nM of NT (black bars) during 30 h (a) or pretreated with 10 nM (white bars) and 100 nM (black bars) of NT during 24 h and stimulated with 1 $\mu$g of LPS during 6 h (b), at 37°C, with 5% $CO_2$. Total RNA was isolated and retrotranscribed as indicated in Section 2. The mRNA levels were assessed by quantitative real-time RT-PCR. Gene expression is indicated as mean log2 values of fold changes relative to the control. Each value represents the mean ± SD from four independent experiments ($^*P < 0.05$; Kruskall-Wallis test followed by Dunn's posttest statistical analysis between 10 and 100 nM of NT, 10, and 100 nM of NT with the corresponding control, under homeostatic and inflammatory conditions).

LPS, the expression levels of all these genes were highly upregulated, including the expression of chemokine CCL5 ($^*P < 0.05$), IL-1$\beta$ ($^*P < 0.05$, $n = 4$), CCL4 ($^{**}P < 0.01$, $n = 4$), CCL11 ($^{**}P < 0.01$, $n = 4$), IL-8 ($^{**}P < 0.01$, $n = 4$), and IL-6 ($^{**}P < 0.01$, $n = 4$), relative to nonstimulated cells (Figure 3), reinforcing the role of fibroblasts as skin immunomodulators.

*3.3. Modulation of Gene Expression in Fibroblast by Neurotensin.* To evaluate the effect of neurotensin on key inflammatory cytokines and chemokines, BJ cells were incubated with the neuropeptide alone, or with the inflammatory stimulus of LPS alone, or were incubated with the combination of both neurotensin and LPS (Figure 4). Indeed, cells were treated with (1) either 10 or 100 nM of NT for 30 h; (2) incubated with LPS alone for 6 h; (3) incubated with either 10 or 100 nM of NT for 24 h before an additional stimulus of LPS for 6 h; or (4) left untreated (control). To understand the role of neurotensin in the skin, specifically in skin fibroblasts, cells were subjected to different neurotensin concentrations (10 and 100 nM) in and out of an inflammatory environment (LPS stimulus). In fact, to better comprehend the role of neurotensin in an inflammatory environment, cells were firstly pretreated with NT and subsequently stimulated with LPS. Furthermore, this protocol may simulate an *in vivo* NT treatment immediately after an injury (before inflammation starts). After these incubation periods, total RNA was isolated, quantified, and reverse-transcribed to cDNA to finally perform real-time RT-PCR.

BJ cells treated with NT (10 nM) significantly increased the expression of EGF and IL-8 by 1.07 ± 0.45- ($^*P < 0.05$, $n = 3$) and 0.88 ± 0.48- ($^*P < 0.01$, $n = 3$) fold above control, respectively, while decreased CCL4 expression by −0.59 ± 0.27- ($^*P < 0.01$, $n = 3$) fold below the control (Figure 4(a)). However, neither the cytokine nor the chemokine profile of these cells showed significant differences in expression when cells were incubated with 100 nM of NT (Figure 4(a)). However, when cells were pretreated with NT (10 nM) during 24 h followed by a 6 h LPS stimulus, both the cytokine and chemokine profiles of these cells were significantly modulated. When cells were stimulated with both 10 nM of NT and LPS, the expression of CCL4 was significantly decreased by 0.59±0.28- ($^*P < 0.05$, $n = 3$) fold, relative to the control, as shown in Figure 4(b). In addition, when cells were incubated with 100 nM of NT plus LPS, the expression of cytokines IL-6 was significantly reduced by 1.09 ± 0.87 ($^*P < 0.05$, $n = 4$), relative to cells treated with NT (10 nM). In contrast, the chemokine CCL11 showed a significant increase in expression of 0.65 ± 0.43- ($^*P < 0.05$, $n = 3$) fold relative to cells treated with NT (10 nM). Furthermore, EGF expression was significantly increased by 0.99 ± 0.785- ($^*P < 0.05$, $n = 3$) fold relative to the control (Figure 4(b)). In conclusion, we observed that, in the presence of neurotensin (10 nM), BJ cells presented a decrease in CCL11 chemokine expression and an increase in IL-8 cytokine expression. Under inflammatory conditions and in the presence of neurotensin, BJ cells presented a decrease in CCL4 and IL-6, while an increase in CCL-11 expression. Furthermore EGF expression was increased in cells incubated with NT either alone or in combination with LPS.

## 4. Discussion

BJ cells expressed the NTR1 and NTR3; NTR1 was located at the cell membrane, cytoplasm, and nucleus while NTR3 was exclusively located in the nucleus. As NTR3 is exclusively localized in the nucleus, its mediated responses can only occur by intracellular neurotensin. Regarding that neurotensin is not expressed by BJ cells, as they do not express NT, NT-NTR3 signalling pathway can only be activated by endocytosed neurotensin previously produced by neighbouring skin cells, such as keratinocytes. Furthermore, NT-NTR3 mediated response will have a later effect in comparison to

NT-NTR1 mediated response because NTR1 is located at the cell membrane and the activation of this signalling pathway does not require NT internalization. Furthermore, upon NT binding to its receptors, NT activates different signalling pathways and cell responses, such as chemokine and cytokine expression, as previously described by us for a dendritic cell line and by others [4, 9, 18–25].

Neuropeptides receptor expression under an inflammatory environment has been shown to be regulated by neuropeptides, as observed in rat macrophages for substance P receptors [26] and in HUVECs for NPY receptors [27]. Indeed, we determined if the expression of neurotensin receptors differed in an inflammatory environment and, after cell exposure to LPS, the gene expression of NTR1 was induced while the expression of NTR3 decreased. These results suggest that the upregulation of neurotensin receptors in an inflammatory environment (LPS) will lead to a rise on NT-mediated effects and propagation of NT signalling pathways. Although endogenous NT was not expressed in BJ cells, exogenous NT produced by neighbouring cells can play an important role in NTR activation.

Regarding NT effects on the immunomodulatory function of BJ cells, NT downregulated the immunomodulatory ability of BJ cells under inflammatory conditions and significantly upregulated EGF expression, a crucial growth factor involved in cell growth and wound healing [28, 29].

Under homeostatic conditions, exogenous NT was able to significantly upregulate IL-8 and downregulate CCL11 in these cells. These cytokines are involved in the important process of immune cell recruitment to the site of inflammation [30]. IL-8 is a neutrophil-activating cytokine which induces chemotaxis and the release of granule enzymes [31] while CCL11 (or eotaxin) is an important eosinophil chemoattractant which also recruits basophils, Th2 lymphocytes, and tryptase-chymase mast cells [32–35]. Since fibroblasts orchestrate and respond to inflammatory cascades, NT upregulation of IL-8 could activate neutrophils while downregulation of CCL11 chemokine expression could decrease leukocyte recruitment in homeostatic conditions.

Under an inflammatory environment (LPS treatment), NT seemed to have the opposite effect in respect to chemokine/cytokine profile, significantly downregulating the chemoattractant CCL4 and proinflammatory cytokine IL-6. Meanwhile, NT also upregulated CCL11. Besides being a chemoattractant, CCL11 may also induce eosinophil degranulation [36] and IgE-independent degranulation of basophils [37], promoting adaptive immune responses, through the selective recruitment of Th2 lymphocytes, which is dependent on the expression of CCR4 and CCR8 on Th2 cells [38]. Indeed, the increased expression of CCL11 mediated by NT in fibroblasts could be involved in a Th2 polarized response.

Considering epidermal growth factor, a cell growth and wound healing growth factor, its expression was significantly enhanced, which is in accordance with our previous study performed in dendritic cells [12]. In fact, this same effect was already observed in granulation fibroblasts by the neuropeptide substance P [39]. EGF increase may potentially be triggering autocrine effects on BJ cells as well as paracrine effects on keratinocytes, by modulating epidermal proliferation and differentiation [40], also emphasising the importance of the EGF effect in the wound closure of wound healing [28, 29].

## 5. Conclusion

These results report the effect of exogenous NT under both homeostatic (without LPS) and inflammatory conditions (with LPS) in BJ cells. Under inflammatory conditions, NT was able to downregulate the chemoattractant function of fibroblasts, vital for the last phases of wound healing, which include its migration-proliferation and remodelling phases. Downregulation of chemokines triggered by exogenous NT could decrease the inflammatory status of the wound and could beneficially promote migration of fibroblasts to the wound site, with consequent expression of ECM proteins, important for skin repair. Furthermore, EGF-mediated effects on fibroblasts and keratinocytes will be fulcra for the remodelling phase of wound healing.

Indeed, delayed wounds like diabetic wound healing, characterized by a proinflammatory cytokine profile [1], could potentially be treated with NT, promoting the transition of the inflammatory phase to the last phases of wound healing, thus improving wound healing. Overall our results suggest that neurotensin may be of great value in therapeutic approaches for inflammatory skin diseases, through promoting wound healing. However, our *in vitro* model has limitations and treatment of fibroblasts *in vitro* with NT may not mimic its biological processes in their native environment, not taking into account the neuroendocrine, for example, sympathetic and parasympathetic, control of tissues. Thus, these findings should be confirmed with *in vivo* experiments. Meanwhile, we can hypothesize that *in vivo* NT administration, under similar conditions, may promote a decreased inflammatory response through these cells, which may be important in the later phases of healing. *In vivo* studies are needed to further confirm the potential application of NT as a therapy for diabetic foot ulcers.

## Abbreviations

NT: Neurotensin
IL: Interleukin
EGF: Epidermal growth factor
ECM: Extracellular matrix
WH: Wound healing
LPS: Lipopolysaccharide
CNS: Central nervous system
NTR: Neurotensin receptor
GPCRs: G protein coupled receptors
TNF: Tumor necrosis factor
BCA: Bicinchoninic acid
PVDF: Polyvinylidene difluoride
ECF: Enhanced chemifluorescence
DEPC: Diethyl pyrocarbonate
DMEM: Dulbecco's Modified Eagle Medium
RT-PCR: Reverse transcription polymerase chain reaction
HPRT-1: Hypoxanthine phosphoribosyltransferase 1.

## Conflict of Interests

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

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*Review Article*

# Th17 Cells in Autoimmune and Infectious Diseases

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The view of CD4 T-cell-mediated immunity as a balance between distinct lineages of Th1 and Th2 cells has changed dramatically. Identification of the IL-17 family of cytokines and of the fact that IL-23 mediates the expansion of IL-17-producing T cells uncovered a new subset of Th cells designated Th17 cells, which have emerged as a third independent T-cell subset that may play an essential role in protection against certain extracellular pathogens. Moreover, Th17 cells have been extensively analyzed because of their strong association with inflammatory disorders and autoimmune diseases. Also, they appear to be critical for controlling these disorders. Similar to Th1 and Th2 cells, Th17 cells require specific cytokines and transcription factors for their differentiation. Th17 cells have been characterized as one of the major pathogenic Th cell populations underlying the development of many autoimmune diseases, and they are enhanced and stabilized by IL-23. The characteristics of Th17 cells, cytokines, and their sources, as well as their role in infectious and autoimmune diseases, are discussed in this review.

## 1. Introduction

CD4+ T cells play an important role in the initiation of immune responses by providing help to other cells and taking on a variety of effector functions during immune reactions. Upon antigenic stimulation, naïve CD4+ T cells activate, expand, and differentiate into different effector subsets called T helpers—(Th) Th1, Th2, Th9, Th17, and Th22—that are characterized by the production of distinct cytokines and effector functions [1]. Th17 cells have been identified as one of the major pathogenic Th cell populations underlying the development of many autoimmune diseases, and it is known that IL-23 enhances and stabilizes them [2].

The main functions of the immune system are to recognize and subsequently eliminate foreign antigens, to induce immunologic memory, and to develop tolerance to self-antigens. Effective immunologic homeostasis relies on a continual balance among several factors, including Th cell activation and suppression by regulatory T cells (Treg). When homeostasis is disrupted and the immune system responds in favor of activation, the host becomes susceptible to autoimmunity [3].

The identification of the IL-17 family of cytokines and the finding that IL-23 mediates the expansion of IL-17-producing T cells led to the discovery of a new subset of Th cells designated Th17 cells. Similar to Th1 and Th2 cells, Th17 cells require specific cytokines and transcription factors for their differentiation.

Th17 cells have an important role in inducing the inflammatory process [3], the immediate protective response of the body to foreign pathogens; however, the immune response needs to be controlled to avoid injury mediated by the immune response in the form of chronic inflammation. CD4+ T cells are the first line of defense and they play a major role in the induction and regulation of immune responses, mainly by secreting cytokines. After antigenic stimulus, naïve CD4+ T cells may differentiate into effector T cells. Th1 and Th2 are the classical subsets involved in the immune response. Th1 secrete interferon-$\gamma$ (IFN-$\gamma$) and interleukin (IL)-2, while Th2 produce IL-4, IL-13, and IL-5 [4, 5]. However, the T-cell subsets have been expanded, and Th17 cells have been described as a novel subset of the specialized Th cells lineage that produces IL-17 but not IFN-$\gamma$ or IL-4 [6]. These cells are potent inducers of tissue inflammation and require TGF$\beta$ in

combination with other cytokines such as IL-6 and IL-23 for their differentiation [7].

## 2. Th17 Cells: Who Are They?

The T-cell subsets involved in inflammatory reactions are mainly Th1 and Th17. There is evidence that Th17 cells can be generated from effector memory CD4+ T cells. The involvement of such cytokines as IL-6, TGF$\beta$, IL-21, and IL-23 in the development of Th17 cells has been described clearly [8].

Th17 cells, first described in mice, are the major source of IL-17 in many types of adaptive immunity [6]. While Th1 and Th2 cells provide effector responses to intracellular bacterial infections and parasitic pathogens, respectively, Th17 cells offer protection against extracellular bacterial and fungal infections and have been implicated in autoimmunity. Th17 cells secrete different cytokines (IL-17A, IL-17F, IL-21, and IL-22) and their differentiation requires a novel set of transcription factors that includes a signal transducer and the activator of transcription 3 (Stat3), the retinoic acid receptor-related orphan receptor $\gamma$ (ROR$\gamma$), the retinoic acid receptor-related orphan receptor a, the nuclear factor kappa-light-chain-enhancer of activated B (NF-kB) cells, a zeta inhibitor (IkBf), and basic leucine zipper transcription factor (Batf) [9, 10].

Th17 differentiation in mice requires initiation by TGF$\beta$ and IL-6, expansion by IL-21, and stabilization by IL-23 [11]. In humans, the combination of TGF$\beta$ and IL-21 was sufficient to induce differentiation from naïve T cells; indeed, TGF$\beta$ plus IL-21 or TGF$\beta$ plus IL-6 and IL-23 or IL-6 and IL-21 can induce expression of ROR$\gamma$. IL-1$\beta$ plus IL-6 have been shown to be important in enhancing the amplification of Th17 cells and the production of IL-23 to maintain the Th17 cell population [4, 12].

*2.1. The IL-17 Family.* The IL-17 family comprises cytokines that participate in inflammatory responses and in the pathogenesis of many inflammatory disorders. There are six members in this family: IL-17A (also called IL-17 or CTLA8), IL-17B, IL-17C, IL-17D, IL-17E (or IL-25), and IL-17F. Their receptors form a family that contains five members (IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE). The IL-17 cytokines show high homology to IL-17A (16% to 50% of amino acid sequence identity), while the other members of this family and the IL-17 family receptors show structural homology among their members [5, 13].

IL-17A was discovered in 1993 and was found to have a homology to an open reading frame encoded within the Herpes virus Saimiri. IL-17A can lead to neutrophil recruitment, inflammation, and host defense, but pathological production leads to excessive inflammation and overt tissue damage [5, 14].

The cellular sources and regulation of IL-17F are similar to those of IL-17A. The genes that encode IL-17A and IL-17F are located on chromosome 6. IL-17E (or IL-25) shows the lowest similarity to IL-17A in terms of the amino acid sequence and also promotes Th2 cell-mediated immune responses, thereby contributing to allergic disease and defense against helminthic parasites [10, 15]. IL-17C is produced in epithelial cells and keratinocytes in response to pathogens or inflammatory cytokines and also promotes IL-17 production. Moreover, IL-17C induced TNF$\alpha$ and IL-1$\beta$ production in the human monocytic cell line THP1 and mouse peritoneal exudate cells [16, 17]. In contrast, IL-17B and IL-17D are poorly studied and their biological functions are still unclear. However, forced expression of IL-17D in edited mouse tumor cells induced rejection by leading to the recruitment of NK cells [18].

*2.2. Biological Functions of Members of the IL-17 Family.* The IL-17 family's activities also include chronic inflammation associated with extracellular matrix destruction by activating the production of metalloproteinases and inhibiting extracellular matrix production in chondrocytes and osteoblasts. It has been reported that local mesenchymal cells promote the differentiation of naïve T cells into Th17 cells [19]. In inflammatory processes, IL-17 has shown synergistic interactions with other cytokines, such as TNF$\alpha$ and IL-1, leading to a chronic process [19].

The most thoroughly studied members of the IL-17 family are IL-17A and IL-17F; two molecules with similar biological activities that induce the production of proinflammatory cytokines, chemokines, antimicrobial peptides, and matrix metalloproteinases by activating innate and tissue resident cells, such as fibroblasts and epithelial cells. Additionally, IL-17A and IL-17F promote the recruitment and subsequent activation of neutrophils [20–22], and it has been observed that IL-17 sustains, rather than inducing, inflammation, thus amplifying the inflammatory response induced by a preexisting tissue injury [23]. On the other hand, IL-17A and IL-17F perform diverse immunoregulatory roles during infection by extracellular bacteria, fungi, and some types of viral infection [20, 21]. Interestingly, Maione et al. found evidence that IL-17A acts as a proaggregant agent by increasing platelet responses to ADP. They observed that IL-17A does not itselfcause an intra-arterial occlusive thrombus but could induce the endothelial features peculiar to a prothrombotic state, likely related to a downregulation of CD39 expression and activity in the vascular system [24, 25]. IL-17A also induces the expression of intercellular cell adhesion molecule 1 (ICAM-1) in keratinocytes and chondrocytes [21].

IL-17E (IL-25) produces a particularly important activity on acquired and innate immune responses not only because it is linked to allergic disease, but also because it plays a protective role in helminthic parasite infection. After antigen or pathogen stimulation, IL-17E induces production of Th2 cytokines such as IL-4, IL-5, and IL-13 by NKT, Th2, and Th9 cells. The role of IL-17B, IL-17C, and IL-17D in the immune system is still unclear, though they share a similar ability to induce inflammatory mediators. Both IL-17B and IL-17C induce TNF and IL-1b expression from a monocytic cell line and cause neutrophil infiltration. IL-17D induces expression of IL-6, IL-8, and GM-CSF in endothelial cells and inhibits hematopoietic progenitor colony formation [20, 21].

IL-17C is produced in epithelial cells and keratinocytes in response to pathogens or inflammatory cytokines and

promotes IL-17 production. Moreover, IL-17C induced TNF$\alpha$ and IL-1$\beta$ production in the human monocytic cell line THP1 and mouse peritoneal exudate cells [16, 17]. In contrast, IL-17B and IL-17D are poorly studied, so their biological functions remain unclear. However, forced expression of IL-17D in edited mouse tumor cells induced rejection by propitiating recruitment of NK cells [18].

The IL-17 family of cytokines mediates its biological functions via surface receptors on target cells. The IL-17R family contains 5 members that share sequence homology with IL-17RA. All members (IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE) have a fibronectin III-like domain in their extracellular part and an SEF/IL-17R (SEFIR) domain in their intracellular region. Functional receptors form heterodimers with IL-17RA as common subunit. IL-17RA is expressed constitutively in many cell types and is stimulated by IL-17 to induce production of proinflammatory molecules [13, 15].

In addition to Th17 cells, there are other immune cells that also produce IL-17, such as $\gamma\delta$ T cells [10, 19, 26], innate Th17 (iTh17) [27], natural killer (NK) cells, mast cells, and neutrophils [10, 19].

*2.3. IL-17 Signaling.* IL-17 upregulates the expression of proinflammatory chemokines and cytokines through activation of NF$\kappa$B, MAPKs, and the C/EBPs cascade. It also works with TNF$\alpha$ to induce gene expression and activates the JAK-PI3K and JAK-STAT pathways. In addition, IL-17A promotes inflammatory responses through the downregulation of microRNA-23b [5, 28]. In this way, although IL-17 does not initiate an inflammatory reaction while, if injected in preinflamed tissues, is able to further amplify biochemical and cellular events characteristic of the early stages of the inflammatory reaction [23].

Tumor-necrosis factor receptor-associated factor (TR AF6) is an E3 ubiquitin ligase essential for the activation of the NF$\kappa$B and MAPK pathways. Polyubiquitinated TRAF6 activates TGF$\beta$-activated kinase 1 (TAK1) with the subsequent NF$\kappa$B activation. However, IL-17RA does not contain a TRAF6 binding site, indicating the existence of another adaptor molecule that mediates the association of TRAF6 with IL-17RA [10, 15, 17]. At the C-terminus of the IL-17 receptor family there is a SEFIR domain. The STIR (SEFIR and TIR) domain superfamily includes TLRs, IL-1Rs, and IL-17 receptors. Interestingly, the SEFIR domain also interacts with a cytosolic protein called Act1 (NF$\kappa$B activator 1). Act1 is an NF$\kappa$B and IKK activator and an adapter for the recruitment of TRAF6. Indeed, Act1 is recruited to the IL-17 receptor complex through the homotypic interactions of the SEFIR domains upon IL-17 stimulation [13, 17]. Act1-deficient cells fail to activate NFkB and MAPKs upon IL-17A stimulation and thus cannot produce proinflammatory molecules, such as IL-6 and CXCL1. Since IL-17RA is required for IL-17F signaling, Act1 have a critical role in IL-17F signaling [10, 15].

Although the mechanism of activation of Act1 remains unclear, it is known that it mediates K63 ubiquitination and the activation of TRAF6. Moreover, IL-17A alone is a weak NF$\kappa$B activator but one that can synergize with other strong cytokines, such as TNF$\alpha$, to promote and extend proinflammatory responses [5, 10].

Another component of the IL-17 signaling pathway is HSP90, which interacts with Act1 to mediate, as a scaffold protein, IL-17 signaling [5, 29]. Ubiquitin-specific processing protease 25 (USP25) is a negative regulator of the IL-17R signal transduction pathway because it restricts the ubiquitination status of TRAF6, thereby attenuating NFkB and MAPK signal transduction [13].

### 2.4. Cytokines Involved in Th17 Differentiation

*2.4.1. TGF$\beta$.* TGF$\beta$ (transforming growth factor-beta) is a pleiotropic factor with several different roles in T-cell development, homeostasis, and tolerance [30].

The role of TGF$\beta$ in Th17 development and function has generated controversy. Recent studies support the existence of at least two functional subclasses of Th17 cells distinguished by their development in the presence or absence of TGF$\beta$, and there are reports that Th17 cells can produce their own TGF$\beta$, including TGF$\beta$1 and TGF$\beta$3, which would appear to exercise distinct programming functions [31].

The indispensability of TGF$\beta$ in Th17 differentiation resurfaced later; this time in relation to the mouse, when it was reported that there may be two pathways of Th17 differentiation: a TGF$\beta$-dependent pathway that gives rise to "nonpathogenic" Th17 cells and a TGF$\beta$-independent pathway that gives rise to "pathogenic" Th17 cells [32]. Naïve precursors polarized in the presence of IL-6, IL-1$\beta$, and IL-23, but, in the absence of TGF$\beta$ signaling, induced a population of so-called Th17 cells that induced EAE (experimental autoimmune encephalomyelitis) upon passive transfer into normal mice. In contrast, naïve cells polarized under identical conditions but with exogenous TGF$\beta$1 and no IL-23 (the so-called Th17($\beta$) cells) and failed to induce EAE following transfers, despite expressing considerably higher amounts of IL-17A [33].

*2.4.2. IL-6.* IL-6 is a pleiotropic cytokine secreted by the cells of the innate immune system such as DCs, monocytes, macrophages, mast cells, B cells, and a subset of activated T cells, though tumor cells, fibroblasts, endothelial cells, and keratinocytes also secrete IL-6 [7]. Recent studies have demonstrated that IL-6 has a very important role in regulating the balance between IL-17-producing Th17 cells and Treg. IL-6 (plus TGF$\beta$) induces the development of Th17 cells from naïve T cells; in contrast, IL-6 inhibits differentiation into Treg [34].

*2.4.3. IL-21.* IL-21 is produced by a range of differentiated CD4+ T-cell subsets and natural killer (NK) T cells [35]. IL-21 signals through a heterodimeric receptor, which is formed by a common gamma chain (shared with IL-2, IL-4, IL-7, IL-9, IL-13, and IL-15 receptors) and an IL-21 specific receptor (IL-21R) [36, 37]. Since IL-21R is expressed on CD4+, CD8+ T cells, B cells, NK cells, dendritic cells, macrophages, and keratinocytes [36], it acts on a range of lymphoid lineages and exerts pleiotropic effects. IL-21 drives differentiation of naïve

T cells into Th17 cells. IL-21 is induced by IL-6 and ROR$\gamma$t and stabilizes and maintains Th17 cells by upregulating its own expression and that of IL-23R [35, 38].

*2.4.4. IL-23.* IL-23 is produced by activated dendritic cells and macrophages in response to microbial stimulation [39]. IL-23 appears to be the critical driver behind Th17 activation and the subsequent production of IL-17. IL-23 is a heterodimer of a unique IL-23p19 and shared IL-12/23p40 chains [40].

The signaling pathway of IL-23R has been described clearly. It involves Janus-associated kinase 2 (Jak2), tyrosine kinase 2 (Tyk2), and several members of the signal transducer activator of transcription (STAT) family, including STAT1, STAT3, STAT4, and STAT5 [41].

In lymphocytes, IL-23 induces a strong phosphorylation of STAT3 and a relatively weak activation of STAT4, whereas the reverse is true for IL-12-induced phosphorylation with respect to STAT4 and STAT3. Phosphorylation of STAT3 is essential for the development of IL-17-producing T-helper (Th17) cells, whereas STAT4 is important for increasing IFN$\gamma$ production and the subsequent differentiation of Th1 cells [42].

## 3. Regulatory T Cells and Their Role in Th17 Cell Function

Regulatory T cells (Treg) are a subset of CD4+ lymphocytes involved in the maintenance of self-tolerance and the modulation of overall immune responses against infections and tumor cells by controlling CD4+ effector T cells. Treg secrete TGF$\beta$ and IL-10 and require the specific cytokine TGF$\beta$ and the transcription factor FoxP3 for their differentiation. While Th17 cells have been involved in the promotion of autoimmunity and Treg cells have been involved in the control of Th17 cells, the balance Th17/Treg has been judged important in the control of immunity mediated by Th17 cells [4]. Furthermore, both T-cell subsets require TGF$\beta$, Treg for the expression of FoxP3, and to induce the differentiation of Th17, in combination with IL-6 and IL-21. Consequently, in the proinflammatory environment (mediated by IL-6 or IL-21), ROR$\gamma$t expression is upregulated, while FoxP3 expression is reduced, and vice versa [34, 43].

On the other hand, Singh et al. have reported that aryl hydrocarbon receptor promotes epigenetic regulation thereby influencing reciprocal differentiation of Tregs and Th17 cells [44]; then it could be important in the maintenance of the Treg/Th17 ratios.

## 4. Th17 Cells in Autoimmune and Infectious Diseases

The role of Th17 cells in autoimmunity was demonstrated first in mice that were deficient for the p19 chain of the IL-23, in which the IL-17-producing T cells were significantly lower than in wild-type mice, highlighting the importance of the IL-23/Th17 axis in the pathogenicity of these autoimmune diseases [1]. Since then, the study of the pathogenic role of Th17 subset cells has focused on autoimmune inflammatory diseases, such as multiple sclerosis, rheumatoid arthritis, and psoriasis [45, 46]. The role of Th17 cells in different autoimmune, inflammatory, and infectious diseases is described below.

*4.1. Glioma.* Glioma is the most common malignant disease of the brain. Although the brain is believed to be immunologically privileged, increasing evidence shows that lymphocytes infiltrate the brain parenchyma during glioma formation and that the blood-brain barrier (BBB) is compromised under glioma stress. Few studies of the relationship between Th17 cells and this disease have been reported; however, research has shown that the numbers of Th17 cells appear to be higher than in control subjects. Moreover, Th17-related cytokines are expressed in glioma tissues, suggesting the role of these cells in glioma tumorigenesis and progression [47, 48]. Furthermore, the serum levels of IL-17 correlate with the disease, with age [49], and with the medium conditions of glioma cells that induce Th17 cell differentiation [47], thus supporting the role of Th17 cells in glioma.

*4.2. Hashimoto's Thyroiditis.* HT has long been epidemiologically associated with excessive iodine levels. However, the immunological mechanisms involved in this disease remain unclear. It has been reported that intrathyroid infiltrating Th17 cells and serum IL-17 levels increase significantly in HT patients. Moreover, the administration of moderately high levels of iodine was found to facilitate the polarization of murine splenic naïve T cells into Th17 cells, whereas extremely high levels of iodine favored Th1 polarization and inhibited Treg development, suggesting that both Th1 and Th17 cells may be involved in the pathogenesis of HT and that high levels of iodine may play a critical role in this process by modulating T-cell differentiation [50]. Additionally, IL-23 levels were found to be higher in patients with HT than controls [51, 52], while levels of IL-17A [50, 53, 54] and frequencies of Th17 cells were also higher in patients than controls [55, 56].

*4.3. Atherosclerosis.* Atherosclerosis is a chronic inflammatory disease regulated by T lymphocyte subsets. Th17 cells have been found to be elevated in patients [57, 58]. In addition, Th17-related cytokine correlates with the severity and progression of carotid artery plaques [58–61], and the Th17/Treg imbalance appears to be associated with plaque progression [62, 63]. Additionally, IL-17A has been involved in lipid metabolism and in the pathogenesis of atherosclerosis [64].

*4.4. Multiple Sclerosis.* MS is known as a neurotropic autoimmune disease in which a coordinated attack of innate and adaptive immune cells inflames the central nervous system (CNS) and interrupts signal transduction by demyelinating (destruction of the myelin sheath) the nerve fibers. This inflammatory demyelinating disease of the CNS has a certain autoimmune background [65]. T-helper cells play a critical role in disease onset and progression [66].

Several groups have studied and characterized T cells subsets and their cytokines in MS. They have reported that the frequency of Th17 [67, 68] and the levels of Th17 related cytokines [66, 69] were higher in MS patients compared to controls. Moreover, a lower Treg/Th17 ratio [65, 68] and a correlation of the severity of symptoms with the Treg/Th17 ratio [68] were also observed, suggesting their role in disease severity [65]. Additionally, it has been reported that the response of T cells to myelin antigen includes production of IL-17 [70]. Furthermore, the reduction of Th17 cells after treatment with IFN-$\beta$ [66], methylprednisolone [68], anti-TNF therapy [71], fingolimod [72], and the suppression of the production of IL-23 by IFN-$\beta$ treatment [73], together with the data described above, support the role of Th17 cells in this disease.

*4.5. Type 1 Diabetes.* DM1 is an autoimmune disease caused by T-cell-mediated destruction of insulin-producing cells. Although it has been thought that an imbalance between Th1 and Th2 is associated with the disease, the role of Th17 cells is under study [74]. As in MS, the Treg/Th17 balance has been found to be broken in DM1 patients; moreover the frequencies of TH17 cells seem to be higher in patients than controls [75].

It the case of *type 2 diabetes* (T2D), the alteration of the Th1/Th2/Th17/Treg paradigm may contribute to enhanced immune activation and inflammation and the subsequent development and progression of T2D [76]; moreover, glucoregulation may contribute to reducing IL-17 in patients [77].

*4.6. Rheumatoid Arthritis.* RA is a systemic autoimmune disease characterized by progressively destructive joint inflammation, destruction of articular cartilage, and bone and synovial hyperplasia. The chronic inflammation process is responsible for stimulating destructive mechanisms in the joint that causes structural damage and lead to functional disability and deterioration [78].

The contribution of Th17 cells to the development of chronic arthritis was first reported in mice. It was found that *in vivo* neutralization of IFN$\gamma$ exacerbates Th17 induced arthritis, and anti-IL-17A treatment delays onset of arthritis induction by Th17 cells. Thus, Th17 cells may participate in the production of autoantibodies that can induce arthritis [79].

As in other autoimmune inflammatory diseases, TH17 frequencies were found to be increased in patients compared to controls [80, 81] as were the levels of IL-17 and IL-23 [81, 82]. Also, the notion that levels of Th17 cells could be reduced by anti-TNF [71], IL-21 [83], and IL-10 [84] has been reported.

*4.7. Spondyloarthropathies.* SpAs, now better known as spondyloarthritides are a diverse group of interrelated inflammatory arthritides. This group includes not only the prototypical disease, ankylosing spondylitis (AS), but also reactive arthritis, psoriatic arthritis, Chron's disease, undifferentiated SpA, and juvenile-onset spondyloarthritis [85]. The role of the IL-23/IL-17 axis in SpAs pathology has been reviewed extensively [86]; however, it has been reported that the serum levels of IL-17 and IL-23 were elevated in SpAs [87, 88]. Moreover, the circulating Th17 cells appear to be elevated as well [84, 87].

Another finding was that serum IL-17 and IL-23 levels in AS [89, 90] and the frequency of Th17 cells [91, 92] correlate with disease activity. As reported in other autoimmune diseases, response to treatment with anti-TNF therapy significantly reduces the frequency of TH17 cells [87].

*4.8. Systemic Lupus Erythematosus.* SLE is a systemic autoimmune disease of unknown etiology. There is increasing evidence that a disturbed T-cell homeostasis plays a critical role in the development of SLE. The main T-cell subsets that are pivotal for this T-cell balance consist of T-helper cells and regulatory T cells [93]. It has been suggested that an imbalance of circulating T-helper cells and an impairment of regulatory T cells are involved in the pathogenesis of SLE as has been reported for MS and DM1 [66, 75].

The role of Th17 cells in SLE has been supported by the higher serum levels of IL-17 [94, 95] and the higher frequency of circulating Th17 cells [95–97], although no differences between patients with the active and inactive forms of the disease has been found [93]. As has been reported for other diseases, the Treg/Th17 ratio was seen to be reduced in patients [96, 98].

Also, high levels of Th17 cytokines have been found in SLE patients [82]. Additionally, cytokine levels and Th17 frequencies correlate with disease activity [99, 100], and the imbalance between Treg and Th17 cells (Treg/Th17 ratio) correlates with disease activity as well [101, 102].

*4.9. Psoriasis.* Psoriasis is a chronic, relapsing, and immune-mediated inflammatory skin disease [2]. It is characterized by hyperplasia in the epidermis, infiltration of leukocytes, including monocytes, dendritic cells and T lymphocytes into both the dermis and the epidermis, and the dilation and growth of blood vessels [103]. Psoriasis is now defined as a Th1/Th17/Th22-based inflammatory disease [104]. The role of Th17 cells has been supported by the discovery of elevated frequencies of Th17 cells in patients and the fact that the Treg/Th17 ratio correlated with the skin lesions [103]. Moreover, IL-17A, the principal effector cytokine of Th17 cells, stimulates keratinocytes to produce chemokines, cytokines, and other proinflammatory mediators, thereby enabling IL-17A to bridge the innate and adaptive immune systems to sustain chronic inflammation [105]. Finally, this has been found to be elevated in patients with psoriasis [106].

Elevated frequencies of Th17 cells have been reported in psoriatic patients [103, 107]. As in other autoimmune diseases, the Treg/Th17 cells have been found to be deregulated, and this ratio correlates with disease activity [103]. Hence, clinical trials with IL-17 pathway inhibitors may provide a new therapeutic approach for patients with psoriasis [105, 108].

*4.10. Vitiligo.* Vitiligo is a common skin disorder, characterized by progressive skin depigmentation due to the loss of cutaneous melanocytes. The exact cause of melanocyte loss

remains unclear, but a large number of observations have pointed to the important role of cellular immunity in vitiligo pathogenesis [109].

Th17 cells have been implicated in skin lesions in vitiligo [110] because of the discovery of higher levels of serum IL-17 in patients than controls [111, 112]. Th17 cell infiltration and decreased Tregs have also been reported [113]. Moreover, it has been found that levels of IL-17 decreased after treatment, while Foxp3 increased significantly [112], suggesting that the imbalance between Th17 and Treg could have an important role in vitiligo lesions.

*4.11. Inflammatory Bowel Disease.* Inflammatory bowel disease can be divided into two main forms: Crohn's disease (CD) and ulcerative colitis (UC). These are disabling diseases characterized by a chronic relapsing inflammatory response to commensal microflora in the gut [114, 115]. Although the mechanisms involved are still unclear, there is a clear genetic susceptibility [115]. In addition to the T-helper cell type (Th) 1 and Th2 immune responses, other subsets of T cells, namely, Th17 and regulatory T (Treg) cells, likely play a role in IBD, because the IL13/TH17 pathway has been postulated as an important biomarker of active IBD [17, 116], and the presence of IBD, but not the genetic load, alters mRNA expression of IBD-associated Th17/IL-13 genes [115]. Moreover, Th17 and Treg cells have been found in increased amounts in the peripheral blood of IBD patients [117], reaching levels that correlate with disease activity [118]. Also, the Treg/TH17 cell ratio was associated with disease activity in patients with Crohn's disease. Hence, together with the Treg/TH17 ratio, they could be considered as potential prognostic indicators [119].

*4.12. Cardiovascular Diseases.* The role of the IL-17 cytokine family in the pathogenesis of cardiovascular diseases has been described as one that amplifies both the inflammation induced by other cytokines in synergistic interactions [120] and the prothrombotic effects combined with the low FeCl3 concentrations that have been observed [25].

As in other pathologies, Th17 cells contribute to increasing cardiovasculopathies [121], while the Treg/Th17 imbalance has been associated with cardiovascular complications in uremic patients undergoing hemodialysis [122, 123].

*4.13. Human Immunodeficiency Virus (HIV) Infection.* The role of Th17 cells in the pathogenesis of HIV infection remains unclear. Selective depletion of this T-cell subset has been reported in gut-associated lymphoid tissue (GALT) as well as in the peripheral blood of HIV-infected individuals [124].

Th17 cells have been found to be associated with HIV patients in different ways. Studies have shown that Th17 cells are reduced in HIV patients [125, 126]. Additionally, the levels of Th17 cells appear to be higher in long-term nonprogressors compared to typical progressors [124]. Th17 cells and IL-17 levels have been shown to have a negative correlation with HIV plasma viral load [126, 127]. The Treg/Th17 ratio showed a negative correlation to viral plasma load [128, 129], although the percentage of Treg cells positively correlated with viral load before antiretroviral therapy [126]. Moreover, antiretroviral treatment normalizes the number of Th17 and the Treg/TH17 ratio in HIV patients [126, 130]. These data strongly suggest that Th17 cells and the Treg/Th17 balance could maintain HIV under control [131] and, therefore, could play a role in the pathogenesis of AIDS.

*4.14. Hepatitis C Virus (HCV) Infection.* The role of Th17 cells in HCV infection and progression remains unclear. It has been reported that Ag-specific Th17 cells are induced in patients infected by the hepatitis C virus (HCV) and that TGF$\beta$ and IL-10, which are induced by the nonstructural viral protein 4 (NS4), suppressed Th17 responses in HCV-infected patients [132]. Moreover, higher levels of IL-17 have been found in patients compared to normal controls, although no correlation with the viremic state was found [133, 134].

Considering that IL-17 serum levels show correlations with serum alanine aminotransferase levels, an association of this cytokine with control of liver injury has been proposed [134], although Th17 cell expansion appears not to be associated with patients who were cured, who became persistently infected, or who had circulating levels of IL-17 in cases of fibrosis [135].

The effect of treatment with pegylated IFN plus ribavirin appears to be controversial, because of reports indicating that it does not affect IL-17 levels, and that there are no differences between responders and nonresponders [133]. Moreover, this treatment downmodulates the secretion of key Th1 and Th17 proinflammatory mediators and profibrotic growth factors as early as 12 weeks after treatment initiation [136].

*4.15. Hepatitis B Virus (HBV) Infection.* The role of Th17 cells in HBV infection has been documented by the expression of IL-23 and IL-23R in biopsied liver tissues from HBV-infected patients. Also, IL-17 appears to be indispensable for HBsAg-stimulated differentiation of naïve CD4(+) T cells into Th17 cells [137]. Thus, Th17 cells have been shown to participate in the pathogenesis of liver damage associated with the hepatitis B virus (HBV) [138].

The frequencies of Treg and Th17 cells are reported to increase in the peripheral blood of HBV patients [139, 140]. Th17 levels [141, 142] and the Treg/TH17 ratio appear to have a crucial role in the occurrence, development, and outcome of HBV [142, 143] and could be used as indicators of inflammation that may predict progression to fibrosis [144]. Hence, Th17 cells can contribute to immune activation and disease aggravation in patients with chronic HBV infection [138, 145], because of the correlation of Th17 cells with serum alanine aminotransferase levels [139]. However, this does not appear to occur in pediatric patients [140]. Additionally, Th17 cells and the IL-23/IL-17 axis seem to be involved in the acute or chronic form of the disease [146].

On the other hand, it was also found that IL-17A decreased the levels of HBVs antigen (HBsAg) and HBVe antigen (HBeAg) in culture medium, as well as the levels of intracellular HBV DNA in infected HepG2.2.15 cells [147], although treatment with telbivudine does not affect IL-17 levels [148]. In contrast, HBVc-Ag induces the production

of IL-10, a cytokine involved in the blockage of Th17 cell activation [149]. Moreover, blockage of the IL-17 receptors (IL-17R) increased levels of HBsAg and extracellular HBV DNA in culture medium, as well as levels of intracellular HBV DNA [147].

The imbalance in the IL17/Il-13 axis has also been associated with responses to HBV vaccination in HCV-infected individuals [150].

## 5. Concluding Remarks

The role of Th17 cells in autoimmune diseases has been reported and supported with some clarity and has been shown to exhibit similar behaviors in the diseases studied. The number of diseases influenced by Th17 cells appears to be increasing. These diseases include those provoked by viral infections in which the role of Th17 cells remains unclear, though evidence suggests that they could play an important role in the control of these diseases.

## Conflict of Interests

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

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*Research Article*

# Limited Applicability of GW9662 to Elucidate PPAR$\gamma$-Mediated Fatty Acid Effects in Primary Human T-Helper Cells

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Synthetic antagonists of the nuclear receptor PPAR$\gamma$ such as GW9662 are widely used to elucidate receptor-mediated ligand effects. In addition and complementary to recent work, we examined whether GW9662 is suitable to serve for mechanistic investigation in T-helper cells. Human peripheral blood mononuclear cells (PBMC) were preincubated with increasing concentrations of GW9662 (0, 0.4, 2, and 10 $\mu$mol/L) 30 min before adding the *c*9,*t*11-isomer of conjugated linoleic acid (*c*9,*t*11-CLA) as representative of PPAR$\gamma$-activating fatty acids with immunomodulatory properties. Corresponding cultures were incubated with GW9662 in the absence of the fatty acid. After 19 h, cells were mitogen stimulated for further 5 h. Subsequently, intracellular IL-2 was measured in $CD3^+CD4^+$ lymphocytes by means of flow cytometry. 100 $\mu$mol/L *c*9,*t*11-CLA reduced the number of T-helper cells expressing IL-2 by 68%. GW9662 failed to abrogate this fatty acid effect, likely due to the fact that the compound exerted an own inhibitory effect on IL-2 production. Moreover, GW9662 dose-dependently induced cell death in human leukocytes. These results suggest that application of GW9662 is not conducive in this experimental setting.

## 1. Introduction

During the last decades, the scientific knowledge about the role of peroxisome proliferator-activated receptors (PPARs) in controlling metabolic and inflammatory processes has increased steadily. Among the three isoforms of the PPAR family, designated PPAR$\alpha$ (NR1C1), PPAR$\beta/\delta$ (NR1C2; NUC1), and PPAR$\gamma$ (NR1C3), the latter has been specifically implicated in the regulation of immune cell function, for example, in macrophages [1] and T-helper cells [2]. In T-helper cells, the predominately expressed splice variant $\gamma$1 is inducible by agonist ligation [3]. Its activation by ligand binding antagonizes the proinflammatory capability of several transcription factors such as nuclear factor-$\kappa$B (NF-$\kappa$B), signal transducer and activator of transcription (STAT) [4, 5], and nuclear factor of activated T cells (NFAT) to control the expression of immunostimulatory cytokines such as IL-2 and IL-4 [6, 7].

Due to their ability to activate PPAR$\gamma$ with micromolar affinity [8], conjugated linoleic acids (CLA), naturally occurring fatty acids in ruminant fats, aroused scientific interest as potentially anti-inflammatory agents. For instance, we have previously shown that the predominant natural isomer *c*9,*t*11-CLA reduces expression of the chemokine IL-8 in airway epithelial cells [9], inhibits IL-2 and TNF-$\alpha$ in T-helper cells [10], and prevents experimentally induced airway inflammation in mice at least in part via a PPAR$\gamma$-dependent mechanism [11].

GW9662 is widely used to elucidate PPAR$\gamma$-dependent anti-inflammatory mechanisms *in vitro* [12, 13] and *in vivo* [14–16]. This molecule covalently modifies the ligand-binding domain by arylation on the cysteine residue $Cys^{285}$ [17] and

thereby inhibits irreversibly ligand binding to and activation of PPAR$\gamma$.

In the present study, which is complementary to previously published work of our group [10], we examined whether GW9662 is suitable to explain PPAR$\gamma$-mediated effects of *c*9,*t*11-CLA in primary human T-helper cells.

## 2. Materials and Methods

*2.1. Chemicals.* Lyophilized 2-chloro-5-nitrobenzanilide (GW9662) was solubilized in sterile dimethylsulfoxide (DMSO) according to the manufacturer's instruction (Enzo, Lörrach, Germany, and Sigma, Taufkirchen, Germany) and stored in aliquots at −20°C. *c*9,*t*11-CLA (Matreya LLC, Pleasant Gap, USA) in free fatty acid form, phorbol 12-myristate 13-acetate (PMA), ionomycin, and brefeldin A (all Enzo) were likewise dissolved in DMSO, aliquoted, and stored at −20°C.

*2.2. Purification of PBMC.* Mononuclear cells were isolated from buffy coats obtained from peripheral blood of healthy volunteers who gave their written consent for blood donation. Buffy coat blood was 1 : 1 diluted with PBS (PAA, Cölbe, Germany), layered onto Lymphocyte Separation Medium (LSM) 1077 (1.077 g/mL; PAA; ratio 1 : 1), and centrifuged at 700 ×g for 20 min at 20°C. The PBMC interphase was collected, washed three times with PBS, and resuspended in RPMI 1640 medium supplemented with 10% FBS Gold (PAA).

*2.3. Cytokine Production.* PBMC ($1 \times 10^6$/mL) were preincubated for 30 min without or with different concentrations of GW9662 (0.4, 2, and 10 $\mu$mol/L) before 100 $\mu$mol/L *c*9,*t*11-CLA was added. After 19 h of incubation, cells were stimulated with PMA (2.5 ng/mL) and ionomycin (0.5 $\mu$g/mL) in the presence of brefeldin A (5 $\mu$g/mL) for another 5 h. Control cultures contained maximum 0.2% DMSO. Afterwards, aliquots were stained with anti-human CD3 mAb (PE-Dy647, clone MEM-57, Immunotools, Friesoythe, Germany) and anti-human CD4 mAb (FITC, clone MEM-241, Immunotools) before cells were fixed with 2% formaldehyde (Histofix, Roth, Karlsruhe, Germany). For intracellular cytokine quantification, cells were permeabilized by washing with PBS/0.1% BSA/0.1% saponin, stained with anti-human IL-2 mAb (PE, clone MQ1-17H12, eBioscience), and analyzed in reference to FMO-controls by means of flow cytometry. Nonspecific fluorescence was controlled by incubation with isotype-matched antibodies. Data were assessed and illustrated by WinMDI v.2.8 software (J. Trotter, Scripps Research Institute).

*2.4. Cell Viability.* To assess the impact of GW9662 on cell viability, PBMC ($1 \times 10^6$/mL) were incubated without or with 0.4, 2, and 10 $\mu$mol/L of this compound for 19 h, followed by 5 h stimulation with PMA (2.5 ng/mL) and ionomycin (0.5 $\mu$g/mL) in the presence of brefeldin A (5 $\mu$g/mL). Control cultures contained the according volume of DMSO. Cell viability was analyzed by annexin-V (Immunotech, Marseille, France) and propidium iodide (PI; Sigma-Aldrich, Munich, Germany) exclusion double staining as previously described [10].

*2.5. Statistics.* Differences in the percentages of IL-2 positive cells were evaluated using a linear mixed model with the fixed factors "fatty acid treatment" (*c*9,*t*11-CLA and DMSO) and "PPAR$\gamma$ antagonist treatment" (GW9662 and control) and the interaction of these two factors. The assumption of normality and homoscedasticity was justified by visual inspection of QQ-plots and predicted versus residual plots. A random intercept specific for each subject was included to control for interindividual differences. Tukey-Kramer was conducted as posthoc test and $P$ values were adjusted for multiple comparisons. For evaluation of data obtained in the absence of *c*9,*t*11-CLA, the concentration of GW9662 was entered into the model as fixed factor while IL-2 positive cells, MFI, and viability were defined as dependent variables, respectively. Because the distribution of viability was skewed, a log-transform was applied. For the latter outcome, differences between concentrations 0 $\mu$mol/L and 0.4 $\mu$mol/L were additionally evaluated by defining posthoc contrasts between these two concentration levels. Significance of difference was set at $P < 0.05$. All calculations were carried out using SAS 9.3 (PROC MIXED).

## 3. Results

*3.1. GW9662 Fails to Abrogate the Inhibitory Effect of c9,t11-CLA on IL-2 Expression in T-Helper Cells.* In stimulated control cultures, 15 ± 2% of the T cells ($CD3^+$) were identified as IL-2 positive T-helper cells ($CD3^+CD4^+$; Figure 1(a)). Incubation with 100 $\mu$mol/L *c*9,*t*11-CLA for 24 h significantly reduced the intracellular content of IL-2 in stimulated T-helper cells by 68% to 5 ± 1%. Preincubation with 0.4 $\mu$mol/L GW9662 did not result in reexpansion of the IL-2 positive T-helper cell population. This was unexpected as preincubation with 0.4 $\mu$mol/L of the PPAR$\gamma$ antagonist T0070907, a compound with similar molecular structure to GW9662 except for one single N atom, did so in the aforementioned similar approach [10].

We further tested in a range of fivefold increases of the concentration of GW9662 whether a reversal of the fatty acid effect, in terms of blocked PPAR$\gamma$, was achieved. Interestingly, pretreatment with increasing concentrations of GW9662 did not lead to increased IL-2 production but even to a reduction. At 10 $\mu$mol/L and in the presence of *c*9,*t*11-CLA, GW9662 caused a drop in the percentage of IL-2 positive T-helper cells even stronger than did the *c*9,*t*11-CLA treatment alone (Figure 1(b)).

*3.2. GW9662 Dose-Dependently Downregulates IL-2 Expression in T-Helper Cells.* We next examined whether the PPAR$\gamma$ antagonist exerted a fatty acid independent effect itself. Indeed, with increasing concentrations of GW9662 we found a continuous reduction in the IL-2 expressing T-helper cell population. Simultaneously, mean fluorescence intensity (MFI) reflecting the cytokine levels on a per-cell basis dose-dependently decreased (Figure 2).

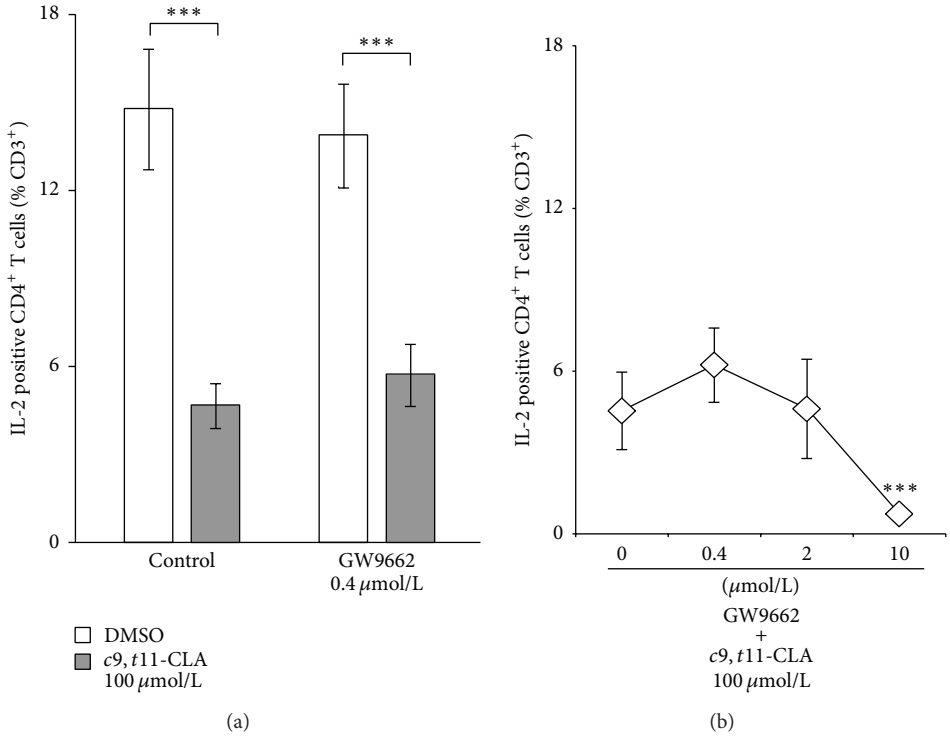


Figure 1: GW9662 exerts no effect up to 2 $\mu$mol/L and an additive effect on IL-2 inhibition in T-helper cells at 10 $\mu$mol/L. PBMC were pretreated for 30 min with GW9662 before 100 $\mu$mol/L *c*9,*t*11-CLA was added. After 19 h, cells were activated for subsequent 5 h. Intracellular IL-2 was flow cytometrically analyzed in lymphocytes gated for CD3 and CD4. $^{***}P \leq 0.001$. Data are expressed as means ± SEM of $n = 6$ (a) and $n = 5$ (b).

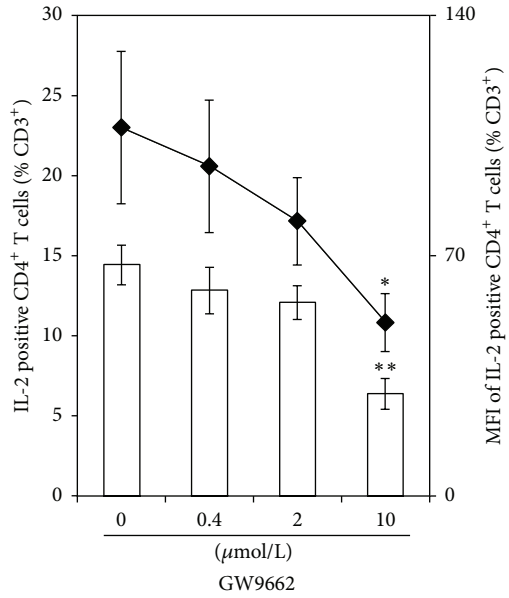


Figure 2: GW9662 dose-dependently downregulates IL-2 expression in T-helper cells. PBMC were incubated for a total of 24 h with increasing concentrations of GW9662. After 19 h, cells were activated for further 5 h. IL-2 expression of T-helper cells was flow cytometrically analyzed. Data are expressed as means ± SEM of $n = 6$. Right scales denote mean fluorescence intensity (MFI) depicted as aligned dots. The dose-dependent effect is statistically significant with $^{**}P < 0.01$ and $^{*}P < 0.05$.

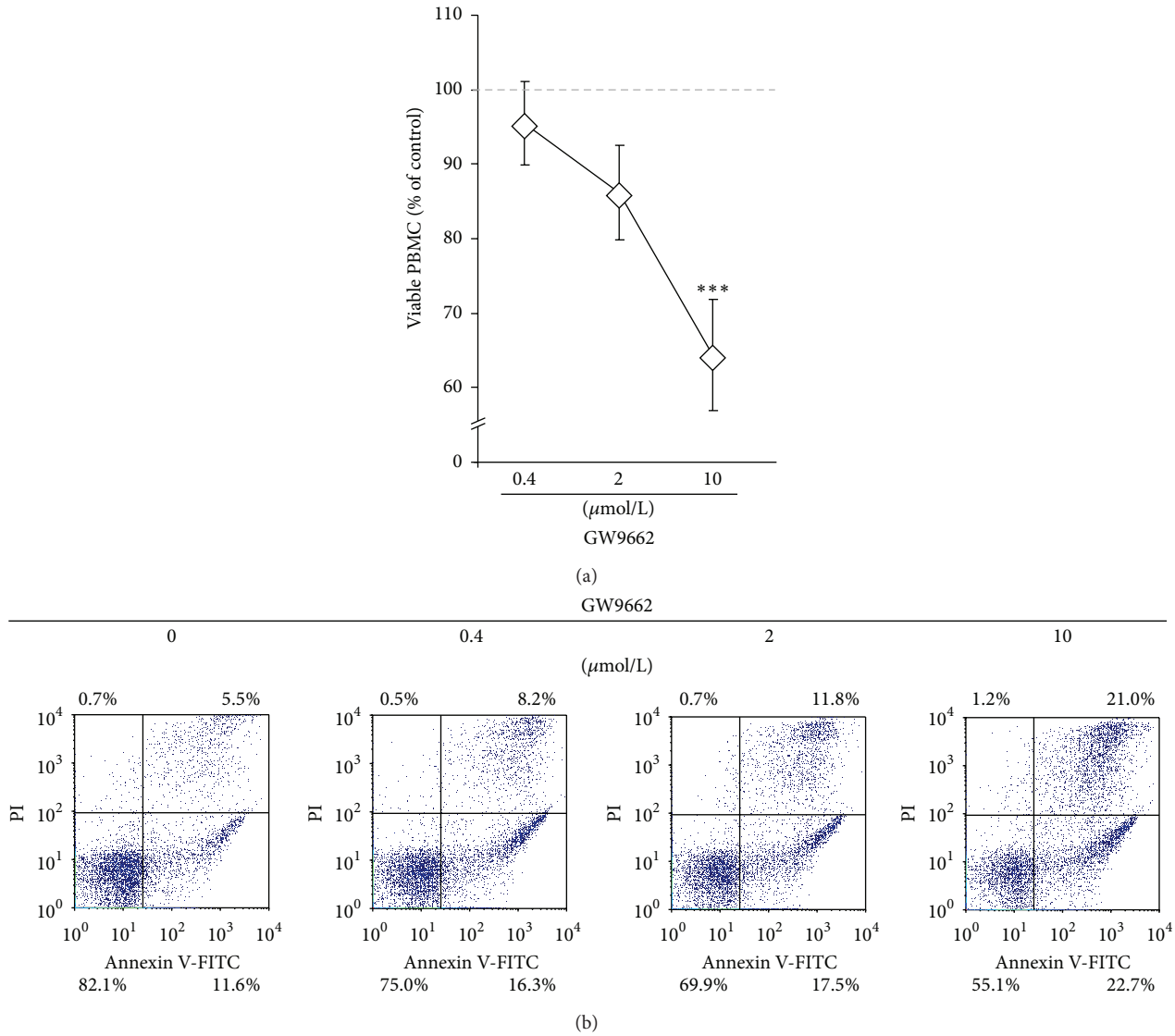


Figure 3: GW9662 dose-dependently causes cell death in leukocytes. PBMC were incubated for a total of 24 h with increasing concentrations of GW9662. After 19 h, stimulants were added for further 5 h. Cell viability was flow cytometrically assessed by annexin-V and propidium iodide exclusion double staining and is expressed as % of control without GW9662 (dotted line). Annexin-V positive and PI negative cells were defined as early apoptotic cells; annexin-V positive and PI positive cells were defined as late apoptotic and necrotic cells. (a) Data are expressed as means ± SEM of $n = 4$. The dose-dependent effect is statistically significant with $^{***}P < 0.001$. (b) Representative dot plots of GW9662 treated PBMC, gated for lymphocytes.

*3.3. GW9662 Dose-Dependently Induces Cell Death of Human Primary Leukocytes.* We further assessed whether putative cytotoxic effects underlie the failure of GW9662 to restore the cytokine production inhibited by *c*9,*t*11-CLA. As revealed by annexin-V and PI exclusion double staining, GW9662 dose-dependently caused cell death in PBMC (Figures 3(a) and 3(b)). After 24 h in the presence of GW9662, viability decreased by up to 35 ± 8% at 10 μmol/L. However, at 0.4 μmol/L GW9662 did not affect cell viability significantly (>95% of the control, $P = 0.531$).

## 4. Discussion

In line with previous work of our group [10], we demonstrated at first that *c*9,*t*11-CLA reduces the expression of the immunostimulatory cytokine IL-2 in T-helper cells. We have previously shown that *c*9,*t*11-CLA acts at least in part via a PPARγ-mediated pathway, since low-dose cotreatment with the PPARγ inhibitor T0070907 largely reverted this fatty acid effect [10]. Though intended to be likewise applicable, GW9662 failed to abrogate the fatty acid effect at all tested concentrations in the present approach. This outcome was unexpected, as a large body of evidence exists that indicates suitability of GW9662 to elucidate PPARγ-dependent mechanisms when used at concentrations within the single- to double-digit micromolar range, including own results from *in vitro* studies in human epithelial cells [9]. However, we have indications that GW9662 acts differently from T0070907 not only in primary lymphocytes but also in other cells

such as macrophages (unpublished findings). Nevertheless, in agreement with the literature, in a similar designed study like the one herein, GW9662 completely negated the modulating effects of *t*10,*c*12-CLA, a synthetic CLA isomer, on TNF-$\alpha$ expression in stimulated porcine PBMC [18]. However, corroborating our findings, Raman et al. recently reported in the Jurkat T-cell line that not only PPAR$\gamma$ agonists but also its antagonists decreased the mitogen stimulated elevation in intracellular $Ca^{2+}$, which could lead to IL-2 suppression via decreased transcriptional activity of NFAT [19].

In order to justify our data, we repeated the experiments with GW9662 purchased from different manufacturers (not shown). Since the results were comparable we can exclude that false-negative data have been produced. Besides PPAR$\gamma$, PPAR$\alpha$, and PPAR$\beta/\delta$ are also expressed by PBMC [20, 21] and are bound and activated by CLA [22, 23]. However, it is not plausible that the fatty acid effects have been mediated through either of these isoforms, as $Cys^{285}$, the modified residue in PPAR$\gamma$, is conserved among all three PPARs. Moreover, significantly higher concentrations of GW9662 are required for inhibition of ligand binding to PPAR$\alpha$ (factor ~10 over PPAR$\gamma$) and PPAR$\beta/\delta$ (factor ~600 over PPAR$\gamma$), respectively [17]. We clearly found that GW9662 dose-dependently exerts an own fatty acid independent diminishing effect on IL-2 production in primary T-helper cells. This finding is new and of significance since effects of the antagonist by its own might mask those which should be actually explained by its usage. Moreover, GW9662 is cell toxic in PBMC with increasing concentrations. GW9662 has previously been shown to cause apoptotic cell death in a concentration-dependent manner in oral squamous cells [24] and colon cells [25]. However, in these studies cancer cell lines were used and these cells underwent apoptosis also after treatment with T0070907 at concentrations higher than 10 $\mu$mol/L. The cell death inducing effect of high doses of PPAR$\gamma$ antagonists led to discuss them as potential therapeutic agents in the treatment of cancer [25, 26] but must be considered undesired in primary cells. However, as cell viability was not affected at 0.4 $\mu$mol/L in our experiments, other effects than cytotoxic underlie the failure of GW9662 to serve for mechanistic exploration of the fatty acid effect that remains elusive.

In summary, and with the restriction that concentrations below 0.4 $\mu$mol/L have not been tested, our data suggest that GW9662 is not valuable for determining the specific PPAR$\gamma$-mediated mode of fatty acid action in primary T-helper cells due to own regulatory and cytotoxic effects.

## Conflict of Interests

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

## Acknowledgment

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*Research Article*

# Interleukin-17A Exacerbates Ferric Chloride-Induced Arterial Thrombosis in Rat Carotid Artery

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Interleukin-17A (IL-17A), the most widely studied member of the IL-17 cytokine family, is a cytokine which emerged to be critical for host defense as well as in the pathogenesis of autoimmune disorders. Moreover, IL-17A is involved in the pathogenesis of cardiovascular diseases, such as atherosclerosis and acute coronary syndrome and in the cardiovascular risk associated with systemic immunological disorders. Consistent with this, we have recently shown that IL-17A increases human and murine platelet response to ADP. In this study we expanded our previous observation and we describe for the first time an *in vivo* prothrombotic effect of the cytokine. Our results show that IL-17A is synergic with a low $FeCl_3$ concentration in inducing carotid thrombus in rats and suggest that the effect is likely related to a downregulation of CD39 vascular expression and hydrolyzing activity. Our findings indicate that IL-17A might be an important molecule at the interface between hemostasis and inflammation.

*"This paper is dedicated to the memory of Professor Alfredo Colonna"*

## 1. Introduction

Increasing experimental and clinical evidence show that thrombosis and atherosclerosis might be closely associated with an inflammatory reaction [1]. Inflammation decreases the activity of natural anticoagulant mechanisms, initiates clotting, and impairs the fibrinolytic system. Indeed, proinflammatory molecules are involved in the activation and migration of leukocytes to sites of vascular injury and inflammation and may contribute to the release by activated cells of prothrombotic factors which, in turn, may activate platelets and other cell types [2, 3].

Recently, much attention has been driven on haemostatic disorders observed in subjects affected by immunological chronic inflammation, such as rheumatoid arthritis (RA), multiple sclerosis (MS), and inflammatory bowel disease (IBD) that may represent the cause of the increased cardiovascular risk observed in these pathologies [4–6]. Multiple factors may be implicated. Circulating cytokines and recruited inflammatory cells could cause endothelial activation and dysfunction leading toward a prothrombotic state [7, 8].

Interleukin-17A (IL-17A) is the most widely studied member of the IL-17 cytokine family. It is mainly produced by T-helper (Th)-17 lymphocytes, and also by natural killer T (NKT) cells, $\gamma\delta$ T cells ($\gamma\delta$-17), cytotoxic $CD8^+$ T cells (Tc17), and neutrophils [9–11]. IL-17A is a proinflammatory cytokine [12], highly produced in patients with chronic inflammatory

diseases, such as RA, MS, and IBD [13–15]. IL-17A is also involved in atherosclerosis [16]; furthermore, in humans a positive correlation has also been found between circulating IL-17A levels and acute coronary syndrome [17, 18]. These findings have suggested that IL-17A might play a role in the cardiovascular risk associated with systemic immunological disorders. Recently, in the attempt of finding a link between inflammatory markers and endothelial dysfunction, Marder et al. [19] demonstrated that IL-17A is an inflammatory marker that could be positively correlated with markers of impaired vascular function in subjects affected by rheumatoid arthritis. Consistent with this, it has also been shown that, on HUVEC, IL-17A reduces CD39 mRNA expression and synergistically with tumour necrosis factor $\alpha$ (TNF$\alpha$) it is able to induce tissue factor (TF) expression and to down-regulate thrombomodulin expression [20]. It is known that arterial thrombosis is primarily caused by platelet adhesion to the damaged vessel and activation [21]. Following activation, endothelium loses its antithrombotic properties and platelets may become activated [2]. Thus, IL-17A could activate endothelial cells toward a prothrombotic state; however, up to now there is no *in vivo* evidence for this. We have recently demonstrated that IL-17A increases human and murine platelet response to adenosine-5′-diphosphate (ADP). *In vitro* this effect is associated with an increased exposition of P-selectin on platelet surface [22]. Since platelets are primary involved in arterial thrombus formation [21] and an increased platelet reactivity has been found associated with several systemic immunological disorders where also IL-17A is involved [6, 7, 23], here we sought to investigate the effect of IL-17A on *in vivo* arterial thrombus formation.

## 2. Materials and Methods

*2.1. Ferric Chloride-Induced Thrombosis.* Male Wistar rats (300–350 g; Harlan Nossan, Correzzana, MI, Italy) were used for all experiments. Animals were kept under standard conditions with food *ad libitum* and maintained in a 12 h/12 h light/dark cycle at 22 ± 1°C. All the *in vivo* procedures were in accordance with the Italian legislative decree (D.L.) number 116 of January 27, 1992, and associates guidelines in the European Communities Council Directive of November 24, 1986 (8676097ECC). All efforts were made to minimize animal suffering and to reduce their number.

Rats were anaesthetized with urethane (10% wt/v; 10 mL/kg ip.) and placed on a surgical table. An arterial thrombus was induced by $FeCl_3$ application onto the surface of the right carotid artery, as described by Kurz et al. [24]. The effect of topical application of IL-17A was also evaluated. In brief, following surgery, a piece of filter paper (Whatman n°1, 3 × 5 mm) soaked in 10 $\mu$L of a solution of $FeCl_3$ (5%) or in recombinant mouse IL-17A (100 $\mu$g/mL in HCl 4 mM, R&D System, Abingdon, UK) was applied onto the external surface of the right carotid artery for 30 minutes. Afterward the paper was removed and the vessel was left *in situ* for 60 minutes, to enable thrombus formation. In another set of experiments, an IL-17A (100 $\mu$g/mL), or vehicle (HCl 4 mM), soaked filter paper was applied on the vessel for 30 minutes before applying $FeCl_3$ (5%), as described above. At the end of 60-minute period, a piece of about 2 cm in length of the right carotid artery (and its contralateral) was excised and weighed. Thrombus size was evaluated by the difference in weight between the treated vessel and its contralateral.

*2.2. Morphological Analysis.* In another group of animals, the experiment was performed as described above and a segment (2 cm) of each treated vessel was excised, rinsed in saline to remove the blood excess, and then fixed in formalin (4% v/v; Carlo Erba, Italy) for 24 hours. Samples were processed and embedded in paraffin. Sections (10 $\mu$m thick) were then stained with haematoxylin and eosin (Carlo Erba, Italy) in order to be morphologically analyzed. In all cases, a minimum of 5 sections per animal were analysed by using a standard light microscope (×5 and ×10 objective). Images were taken by a Leica DFC320 video camera (Leica, Milan, Italy) connected to a Leica DM RB microscope using the Leica Application Suite software V2.4.0.

*2.3. Western Blot Analysis of CD39.* In subsets of experiments, following local treatment with IL-17A or with its vehicle (HCl 4 mM) for 30 minutes, as described above, CD39 expression was evaluated on carotid section homogenates by Western blot analysis. For this purpose, following local treatment, carotids were immediately frozen in liquid nitrogen before being stored at −80°C. On the day of analysis, tissues were homogenized using liquid nitrogen in the following lysis buffer: Tris-HCl pH 7.5, 50 mM; NaCl, 150 mM; sodium orthovanadate, 1 mM; $\beta$ glycerophosphate, 20 mM; EDTA, 2 mM; PMSF 1 mM; leupeptin, 5 $\mu$g/mL; aprotinin, 5 $\mu$g/mL; pepstatin, 5 $\mu$g/mL. Protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad, Italy). Protein samples (35 $\mu$g) were subjected to electrophoresis on an SDS 8% polyacrylamide gel and transferred onto a nitrocellulose transfer membrane (Protran, Schleicher & Schuell, Germany). The membranes were saturated by incubation with nonfat dry milk (5% wt/v) in PBS supplemented with 0.1% (v/v) Tween 20 (PBS-T) for 1 h at room temperature and then incubated with a goat monoclonal anti-CD39 antibody (A-16, Santa Cruz, CA) (dilution 1 : 200), overnight at 4°C. Successively, membranes were washed and then incubated with the secondary antibody conjugated with horseradish peroxidase, anti-goat IgG-HRP (dilution 1 : 2000, Dako Denmark), for 2 h at room temperature. Protein bands were detected using the enhanced chemiluminescence (ECL) detection kit and Image Quant 400 GE Healthcare software (GE Healthcare, Italy). Protein bands were quantified using GS 800 imaging densitometer software (Biorad, Italy).

*2.4. Enzyme Assay.* On carotid section homogenates, ADP hydrolyzing activity was evaluated using a modification of the method described by Saucedo et al. [25]. The reaction medium used to assay ADPase activity contained 120 mM NaCl, 5.0 mM KCl, 60 mM glucose, 5 mM $CaCl_2$, and 50 mM Tris-HCl buffer, pH 7.5 in a final volume of 200 $\mu$L. A sample volume of 20 $\mu$L (10 $\mu$g proteins) was added to the reaction medium and preincubated for 10 minutes at 37°C.

The enzyme reaction was then started by the addition of ADP at a final concentration of 2 mM and incubated for 40 minutes at 37°C. Controls were performed by adding deionized $H_2O$ to each sample, instead of ADP. The reaction was stopped by the addition of 200 $\mu$L of trichloroacetic acid (TCA) 10%. Samples were then centrifuged at 3000 rpm × 10 min. As a measure of ADPase activity, inorganic phosphate (Pi) released was quantified by a colorimetric assay Sensolyte assay kit (AnaSpec) and expressed as nmol Pi released *per* $\mu$g of protein [26].

*2.5. Statistical Analysis.* Data were expressed as mean ± S.E. and analysed by one way analysis of variance (ANOVA), followed by Bonferroni's test for multiple comparisons, or by unpaired two-tailed Student's $t$-test when appropriate. In some cases, one-sample $t$-test was used to evaluate significance against the hypothetical zero value. A value of $P < 0.05$ was taken as significant.

## 3. Results and Discussion

The increased platelet reactivity, endothelial dysfunction, and atherosclerotic plaques instability are common features of subjects affected by systemic immunological inflammatory diseases and might represent the cause of the increased cardiovascular risk observed in these patients [1, 5, 23]. However, which factors are primarily responsible for predisposing to haemostasis disturbances and to cardiovascular events in chronic autoimmune diseases is still unknown. Recently, IL-17A, which is critically involved in the pathogenesis of autoimmune diseases, has been claimed as a possible candidate to participate in endothelial dysfunction and increased cardiovascular risk [19, 20]. On these bases, our work was aimed at evaluating *in vivo* the effect of IL-17A in a model of ferric chloride-induced arterial thrombosis. We found that application of $FeCl_3$ (5%) caused an intravascular thrombus of 1.10 ± 0.23 mg ($n = 9$; $P < 0.01$ one-sample $t$-test) whose mass increased significantly (1.91 ± 0.23 mg; $n = 8$. $P < 0.01$) when carotid was pretreated with IL-17A (100 $\mu$g/mL), 30 minutes before $FeCl_3$ application. Application of IL-17A alone on the external surface of rat carotid artery generated *per se* a small intravascular thrombus (0.42 ± 0.17 mg; $n = 10$; $P < 0.05$) whereas nonsignificant effects were observed after vehicle exposure (0.1 ± 0.05 mg; $n = 7$) (Figure 1).

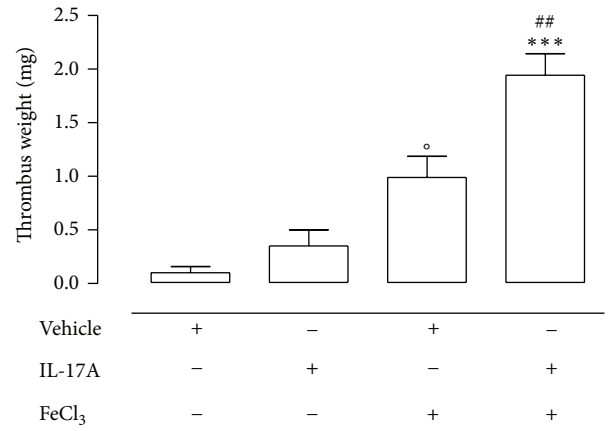


Figure 1: Effect of IL-17A on thrombus mass induced by $FeCl_3$ application on rat carotid artery. A filter paper soaked in $FeCl_3$(5%) or in recombinant mouse IL-17A (100 $\mu$g/mL) was applied onto the external surface of the right carotid artery, for 30 minutes; afterward the paper was removed and the vessel was left *in situ* for 60 minutes, to enable thrombus formation. In another set of experiments, an IL-17A (100 $\mu$g/mL), or vehicle (HCl, 4 mM), soaked filter paper was applied onto the vessel for 30 minutes before applying $FeCl_3$ (5%). At the end of 60-minute period, a piece of about 2 cm in length of the right carotid artery and of its contralateral was excised and weighed. Thrombus size was evaluated by the difference in weight between the treated vessel and its contralateral. All controls were performed by applying only vehicle ($H_2O$ or HCl 4 mM). $^{\#\#}P < 0.01$ versus $FeCl_3$, $^{***}P < 0.001$ versus IL-17A, and $^{\circ}P < 0.05$ versus vehicle (one way ANOVA followed by Bonferroni's test; $n = 8$–10).

Morphological analysis of the vessel section evidenced that the luminal surface of carotid sections from control was covered by a continuous endothelium. Moreover the vascular lumen was not characterized by aggregates (Figure 2(a)). Interestingly, sections obtained from IL-17A *plus* $FeCl_3$ showed an occluding thrombus compared with IL-17A (Figure 2(b)) or $FeCl_3$ (Figure 2(c)) treated carotids. Furthermore, the endothelium appeared damaged and vessel wall thickness extremely reduced (Figure 2(d)).

It is known that arterial thrombosis is primarily caused by platelet adhesion and activation to the damaged vessel [21]. Following activation, endothelium loses its antithrombotic properties and platelets may become activated [2]. The model of $FeCl_3$-induced thrombosis has been shown to involve platelets and several components of haemostasis [27], including TF [28].

CD39/ATP diphosphohydrolase (ATPDase) is largely expressed on vascular endothelial cells and by converting ATP and ADP to monophosphate form (AMP) it represents a key modulator of vascular haemostasis and thrombogenesis. The loss of CD39 on activated endothelial cells causes platelet sequestration and TF upregulation, key events for thrombogenesis [29]. Nonetheless, CD39 also may offer protection against myocardial ischemia-reperfusion injury since it promotes ADP degradation and thus favours, in tandem with CD73 (ecto-5′-nucleotidase), adenosine accumulation that represents a cardiovascular protective molecule [30]. It was shown that mice lacking CD39 had vascular features of a prethrombotic state, characterized by fibrin deposition in several vascular beds [31]. Recently, it has been demonstrated that IL-17A reduces CD39 mRNA expression in human endothelial cells *in vitro* and, at the same time, it increases TF expression [20]. On these bases, and considering that CD39 plays a crucial role in maintaining the endothelium antithrombotic properties, we analysed its expression and its ADP hydrolyzing activity on rat carotid artery following the *in vivo* exposure to IL-17A. Results obtained show that following the application of IL-17A on carotid surface the expression of CD39 was significantly reduced compared with the application of the only vehicle (HCl, 4 mM) (Figure 3). Concomitantly, ADP hydrolyzing activity was also reduced as demonstrated by a reduced inorganic phosphate production,

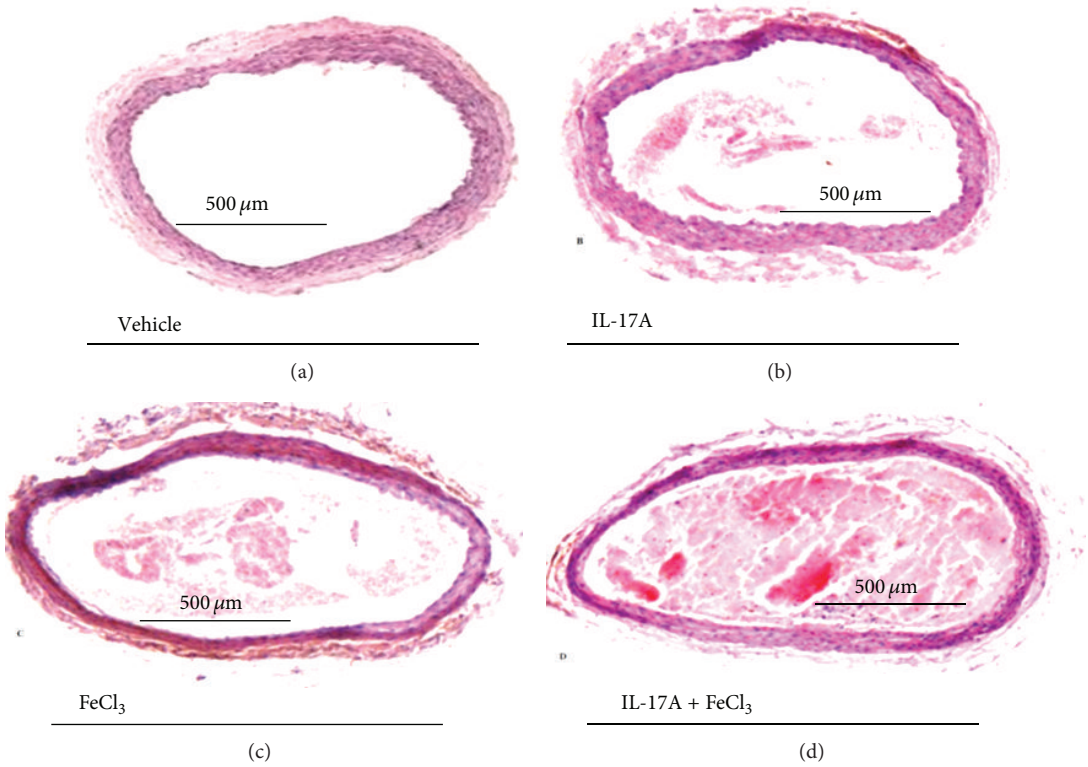


Figure 2: Photomicrographs showing hematoxylin and eosin histologic cross sections of carotid arteries following local treatment as described in the method section (magnification ×50). (a) Vehicle (HCl 4 mM); (b) IL-17A (100 $\mu$g/mL); (c) vehicle (HCl 4 mM) *plus* $FeCl_3$ (5%); and (d) IL-17A and $FeCl_3$.

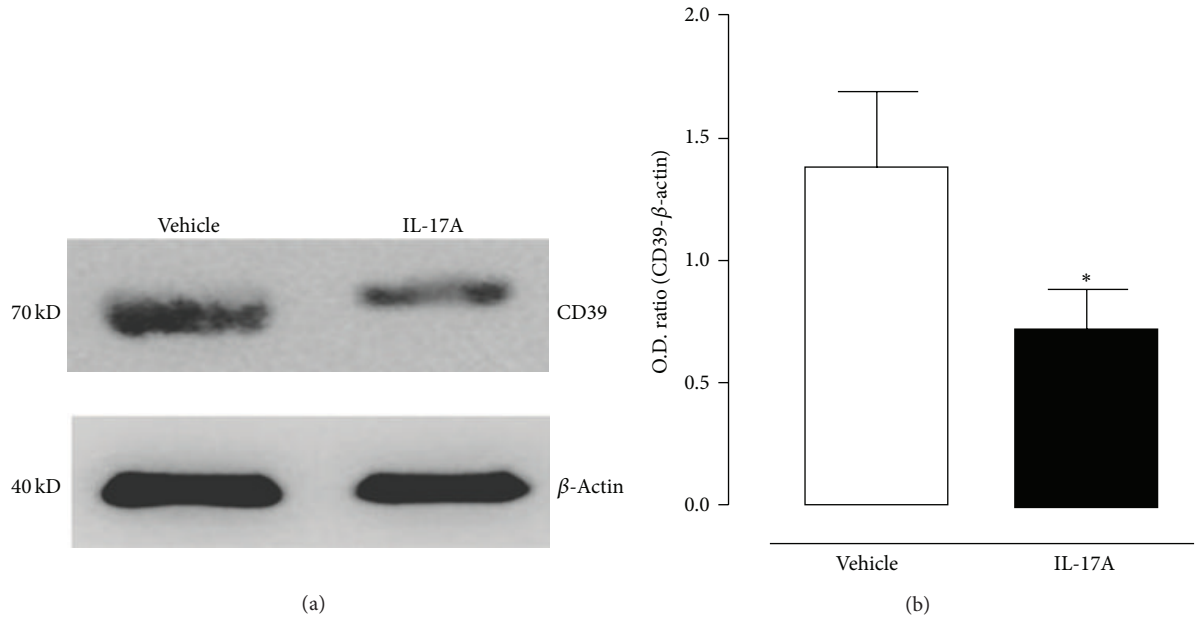


Figure 3: (a) Representative results of Western blot analyses of CD39 expression on rat carotids treated for 30 minutes with vehicle or IL-17A. (b) Densitometric analysis of Western blots; O.D. (optical density) normalized against $\beta$-actin. $^*P < 0.05$ versus vehicle (Student's $t$-test; $n = 3$).

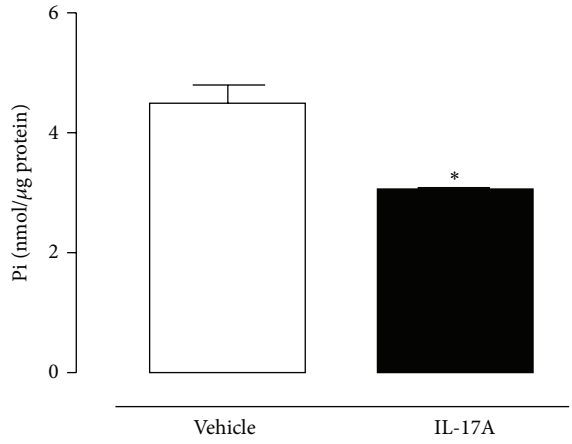


Figure 4: Quantification of inorganic phosphates (Pi) produced by rat carotid homogenates treated with vehicle or IL-17A. Pi produced was evaluated as measure of ADP hydrolyzing activity (for details, see method section). $^{*}P < 0.05$ versus vehicle (Student's $t$-test; $n = 3$).

following incubation with ADP, from IL17A treated carotids compared to vehicle (Figure 4). Thus, through the downregulation of CD39, IL-17A might contribute to priming the vessel for the effect of a minimal $FeCl_3$ concentration.

Our hypothesis would be consistent with the observation that IL-17A does not cause *per se* an intra-arterial occlusive thrombus, but it would induce those endothelial features peculiar to a prothrombotic state. Indeed, the lack of ADP hydrolysis due to CD39 downregulation on endothelial cells would not be a stimulus for thrombogenesis but, most likely, for the increase of platelet aggregates at the site of vascular injury and this would facilitate the effect of a thrombotic agent.

## 4. Conclusion

Our results give for the first time an *in vivo* evidence for a prothrombotic effect of IL-17A that is likely related to a downregulation of CD39 expression and activity in the vascular system. Accordingly with our previous results that demonstrated *in vitro* the effects of this cytokine on human and murine platelets [22], in this study we suggest that this cytokine might be an important molecule at the interface between haemostasis and inflammation. Intriguingly, the mechanistic bases of prothrombotic effect of IL-17A need further investigation.

## Conflict of Interests

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

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