Journal of Immunology Research

Inflammation in Cancer: Part of the Problem or Part of the Solution?

Lead Guest Editor: Monica Neagu
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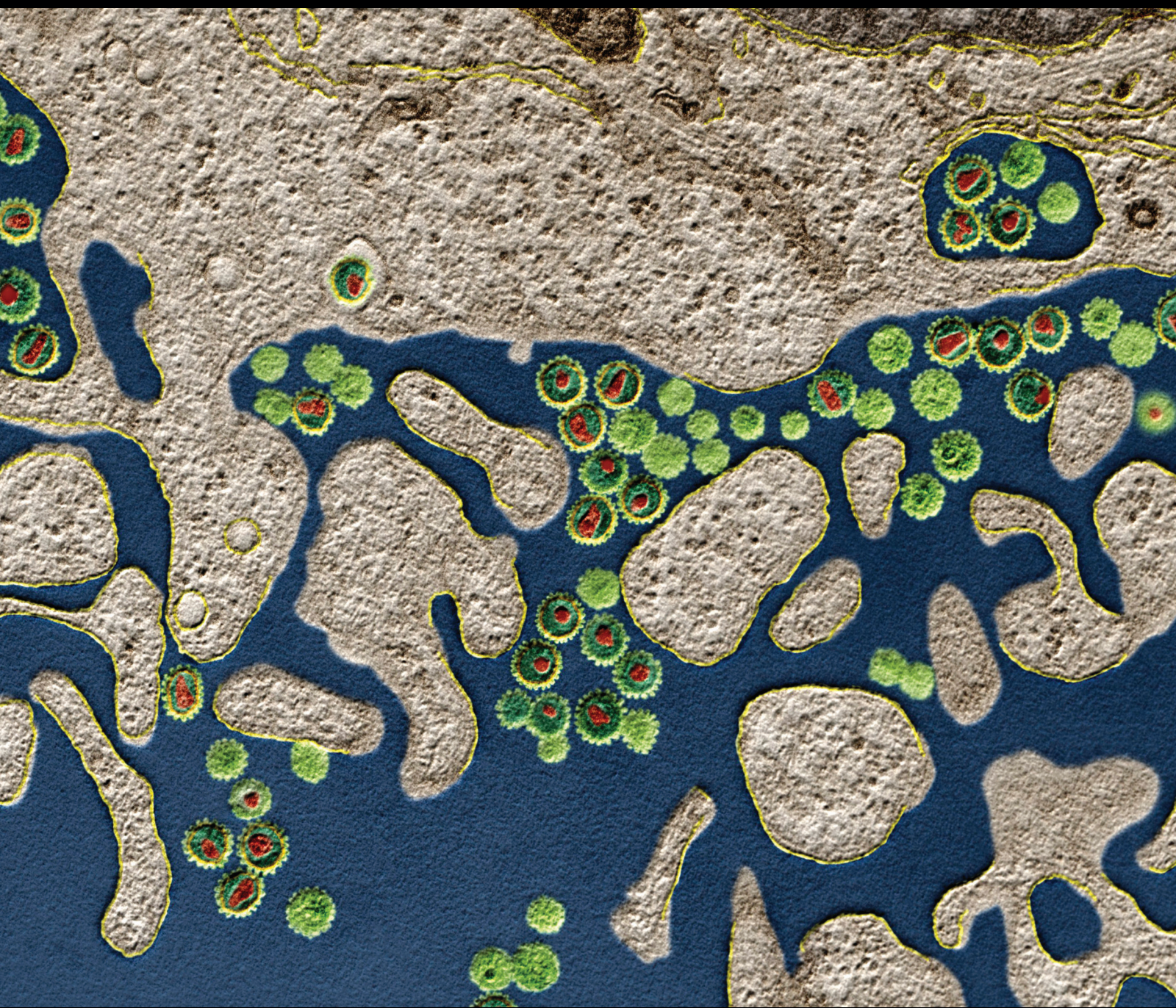

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Editorial Board

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Hindawi
Journal of Immunology Research
Volume 2019, Article ID 5403910, 2 pages
https://doi.org/10.1155/2019/5403910

Editorial

Inflammation in Cancer: Part of the Problem or Part of the Solution?

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Received 2 December 2018; Accepted 2 December 2018; Published 7 February 2019

With the advent of the new era of immune therapy, new aspects of inflammation as a process involved in therapy, in tumorigenesis, and in several other human pathologies have been highlighted [1]. This special issue attempted to cover as many of these inflammation aspects in original papers and in extended reviews.

It is possible to trait two distinct sections discussing inflammation aspects; a series of papers focusing on oncology and a series of papers focusing on other inflammation-related pathologies. From the first series of papers, M. Tampa et al. review the inflammatory markers of oral squamous cell carcinoma highlighting the main markers of inflammation that could improve early diagnosis. In esophageal squamous cell carcinoma cells, M. Wang et al. show that in *in vitro* studies, PAR4 can have a tumor suppressor role and can be used as a future therapeutic target. In endometrial cancer, S. Gu et al. show that tumor-associated macrophages are predominantly type 2 (M2) that contribute to the progression of this type of cancer and that the anti-CD27 therapy could have an antitumoral effect. Similarly, tumor-associated macrophages are reviewed by H. Degroote et al. in another type of cancer, hepatocellular carcinoma. The preclinical evolution and hitherto clinical trials for TAM-targeted therapy in HCC have been highlighted.

A syndrome associated with cancer progression is cachexia, reviewed by E. Manole et al. The paper is describing several myokines produced and released by myocytes that can become potential biomarkers and future therapeutic targets.

As already mentioned, immune therapy would be probably one of the major breakthroughs in oncology. C. Vajaitu et al. review checkpoint inhibitor new treatments and the role of inflammation in these therapies presenting biomarkers that could predict efficacy and immune therapy resistance.

A. Calinescu et al. reviewed an inflammatory-related protein, carcinoembryonic antigen-related cell adhesion molecule 1, and its involvement in malignancies. The paper shows its importance as a prognostic factor in oncology and a future target specific cancer therapy.

The second section of the special issue consists of reviews and original papers evaluating inflammation in various nononcological diseases. A. Pedrinolla et al. evaluate proinflammatory markers of age-related obesity. M. Bucur et al. highlight in their original paper that there are clear profibrotic and antifibrotic factors expression differences in oral mucosa and skin scars.

M. Dobre et al. show that differences found in various transcript levels of inflammatory molecules could aid the differential diagnosis between ulcerative colitis and Crohn's disease. Chronic kidney disease could benefit from current proteomic approaches, as S. Mihai et al. are describing the inflammasomes and gut microbiota dysbiosis involvement in this disease and moreover in the renal malignancy.

An in vivo animal model is shown by E. Codrici et al. where a caveolin-1-knockout mouse is thoroughly characterized and presented as a good inflammatory disease model. Another original paper by V.M. Anghelescu et al. shows the inflammatory pattern evaluation in animal models associated with various bone implants. Last, but not least, S.R. Georgescu et al. revise the traits of chronic inflammation in HPV infection that can lead to tumorigenesis.

We are very satisfied that our subject resulted in so many valuable papers, and we would like to thank all the authors who submitted their work for consideration to this special issue. Without their effort, this special issue would have not taken place. Editors would like to thank the reviewers who thoroughly revised the papers and provided important suggestions that significantly improved the papers.

Conflicts of Interest

The Guest Editorial team gathered for developing the mentioned special issue comprising Prof. Monica Neagu, Prof. Donato Zipeto, and Assoc. Prof. Iulia Dana Popescu provide a clear declaration that they do not have any conflict of interest or do not have any private agreements with companies.

Monica Neagu
Donato Zipeto
Iulia D. Popescu

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Hindawi
Journal of Immunology Research
Volume 2019, Article ID 4513108, 13 pages
https://doi.org/10.1155/2019/4513108

Research Article

Efficiency of Multiparticulate Delivery Systems Loaded with Flufenamic Acid Designed for Burn Wound Healing Applications

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Received 13 July 2018; Accepted 21 November 2018; Published 5 February 2019

Guest Editor: Iulia D. Popescu

Burns are soft tissue injuries that require particular care for wound healing. Current tissue engineering approaches are aimed at identifying the most efficient treatment combinations to restore the tissue properties and function by using adapted scaffolds or delivery platforms for tissue repair and regeneration by triggering molecules. To reduce the inflammation associated with skin burns, the addition of an anti-inflammatory factor in these scaffolds would greatly increase the quality of the therapy. Therefore, this study is aimed at obtaining and validating a novel multiparticulate system based on a collagen matrix with controlled delivery of flufenamic acid anti-inflammatory drug for burn wound healing applications. In this work, we have characterized the properties and biocompatibility of these multiparticulate drug delivery systems (MDDS) and we have demonstrated their efficiency against burns and soft tissue lesions, particularly when the drug was microencapsulated, and thus with a controlled release. This study contributes to the advancement in therapy of burns and burn wound healing applications.

1. Introduction

Skin burns are tissue injuries generally caused by heat due to the contact with boiling liquids (scalds), hot solids, or flames. According to the WHO report in 2018, 180,000 deaths are estimated to occur annually worldwide with a higher rate in low- and middle-income countries [1].

The skin has critical roles in maintaining the body fluid homeostasis and thermoregulation, being considered the body's largest and active immune organ involved in the first defense barrier. Thermal burns are complex processes that demand careful guided treatment to promote wound healing, reestablishing the immune barrier, and fast tissue regeneration with minimum scaring.

The healing process of the thermal burns comprises four overlapping phases including an initial phase of tissue homeostasis activated in the first couple of minutes after injury followed by posttraumatic inflammation and, in a couple of days, by the proliferation and skin remodeling phases [2].

The first two phases of activated postinjury are critical for the wound healing evolution and scaring. Among the processes activated immediately after injury are immune activation and platelet aggregation with blood clotting in order to

protect the affected area and supply the scaffold-like matrix for the migration of cells responsible for skin integrity like dermal fibroblasts, keratinocytes, leucocytes, and endothelial cells. The posttraumatic inflammatory phase is generally initiated by some cytokines such as tumor necrosis factor (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) to promote local inflammation with neutrophil extravasation and macrophage activation. An impaired inflammatory process prolonged local pain and stimulates the cellular secretion of extracellular matrix with consequences on thick scars [2, 3].

Dressings like gauzes and hydrogels are formulations that can be made from natural (alginate, collagen, and cellulose) and synthetic (polyvinyl alcohol, caprolactone, and polylactic acid) polymers: foams and hydrocolloids by carboxymethyl cellulose, transparent films by polyurethane. The research is focused to develop combinations of polymers as candidates to obtain ideal dressings to stimulate the wound healing [4–7].

Because of their high biocompatibility and similarity to the extracellular matrix (ECM), the natural polymers are widely used in wound dressing preparation [8–11]. Collagen presents low antigenicity and low inflammatory properties, good biocompatibility, water-absorbing ability, and haemostatic properties and has the ability to promote cell attachment [12–15]. Pure collagen may not satisfy all requirements that an ideal wound dressing should have; therefore, it needs to be combined with some anti-inflammatory agents, like drugs or natural plant extracts. Providing the biological scaffold like collagen and administrating a short-term anti-inflammatory agent are helpful for cellular migration to promote the skin regeneration, decrease the local pain, and maintain a balanced inflammatory level favorable in a long-term cicatrizing process [2, 3, 16].

So far, only few biopolymer-based wound dressings with nonsteroidal anti-inflammatory drugs (NSAIDs) were developed [17–20].

A limitation of some of these formulations is that the rapid release effect is too pronounced during the first hours, with the risk of an important amount of drug to be released before the inflammation is controlled [17, 21–23].

In this respect, the vehicle in the drug delivery sciences is a critical quality attribute which needs special attention for a tailor-made design to rationalize the formulation development. Thus, new drug delivery systems have to be developed in order to ensure a proper delivery of the anti-inflammatory drug from spongious matrices using different techniques that allow a gradual degradation of collagen support and a slow release of drug.

In this context, to reduce the burst release effect and to provide a controlled drug release, we propose in this study the microencapsulation of an anti-inflammatory drug in biodegradable polymeric supports which results in topical multiparticulate drug delivery systems (MDDS).

Based on our previous studies concerning some designed collagen-dextran-flufenamic acid collagen sponges, the aim of this work was (1) to obtain multiparticulate systems based on the collagen matrix with controlled delivery of an anti-inflammatory drug (flufenamic acid was selected as a model NSAID) embedded in gelatin-alginate-carboxymethyl cellulose microcapsules, (2) to investigate their biocompatibility for future wound healing applications, and (3) to evaluate the release mechanism, degradation rate, absorption capacity, and efficiency against *in vitro* inflammation modelling *in vivo* behavior. The preclinical *in vivo* studies involving animal models are very important and frequently used following the *in vitro* studies to evaluate the efficacy of a novel product designed for burn healing bringing substantial advancements in the therapy of burns [2, 3, 16, 24–26].

2. Materials and Methods

2.1. Achievement of MDDS

2.1.1. Materials. The type I fibrillar collagen gel (Col) having an initial concentration of 1.92% (*w*/*v*) and gelatin (Gel) with an initial concentration of 2.5% were extracted from calf hide using technology currently available in Collagen Department from Research-Development Textile Leather National Institute Division Leather and Footwear Research Institute [25]. The collagen gel, dextran sulfate sodium salt (Dex) with a molecular weight of 40,000 (Alfa Aesar, Germany), and flufenamic acid (FA) (MP Biomedicals, USA) were raw materials for matrix (spongious form) preparation. Gelatin, sodium carboxymethyl cellulose (CMCNa) (Fluka), and alginic acid sodium salt from brown algae (Alg) (Sigma-Aldrich, Germany) were chosen to prepare microcapsules. Glutaraldehyde (GA) from Sigma-Aldrich (Germany) has been used for matrix cross-linking, and calcium chloride ($CaCl_2$) from Merck has been used for microcapsule cross-linking. Sodium hydroxide from Merck (Germany) was of analytical grade, and the water was distilled.

2.1.2. Preparation of Microcapsules. 1 g of sodium alginate, 0.25 g CMCNa, and 2.5 g gelatin were solubilized in distilled water, and 2.4 g FA previously solubilized in 1 M NaOH solution was added and homogenized together in distilled water, at 60°C, to obtain 100 ml gel for microcapsule preparation. The gel was dropped with a syringe into a 1 M $CaCl_2$ solution for cross-linking, and spherical capsules were obtained.

2.1.3. Preparation of Matrices. The previously obtained microcapsules (MC) were embedded in the composite gel consisting in 0.8% collagen (Col), 1.2% dextran (Dex) reported to collagen (which means 0.96 g in 100 ml gel), and 0.5% flufenamic acid (FA) reported to collagen (which means 0.4 g in 100 ml gel) and then cross-linked with 0.006% glutaraldehyde (GA) reported to collagen (which means 0.0048 g in 100 ml gel). All the samples consist in the same concentration of collagen, dextran, and crosslinking agent, the difference between them being given by FA concentration and microcapsule amount: M1 consisting in FA and no microcapsules, M2 consisting in 30 g microcapsules and 70 g composite gel without FA, M3 consisting in 15 g microcapsules and 85 g composite gel without FA, M4 consisting in 30 g microcapsules and 85 g composite gel with FA, and M5 consisting in 15 g microcapsules and 85 g composite gel with FA.

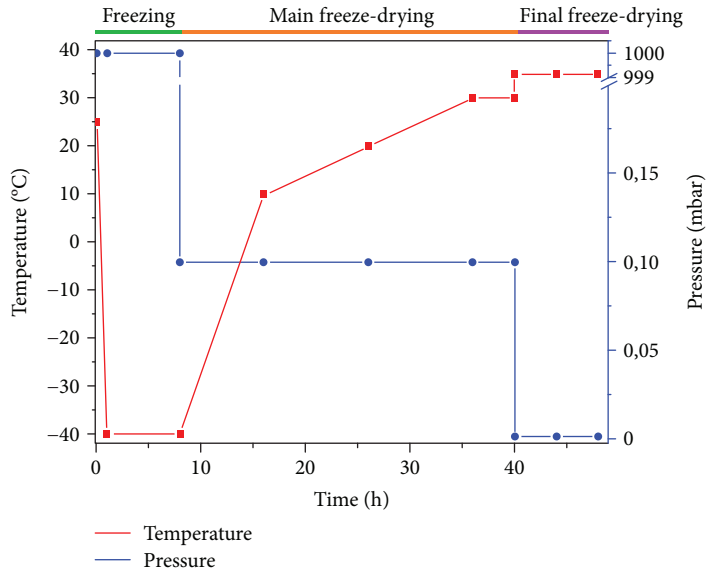

Figure 1: Graph chart of the freeze-drying process.

The gel compositions were cast in glass dishes (5 cm diameter and 0.5 cm height) and kept at 4°C for 24 hours for cross-linking. Then they were freeze-dried (48 hours) as we present in Figure 1, in order to obtain porous scaffolds, as follows: cooling to −40°C (4 h), keeping up for 8 h, then freeze-dried at −40°C and 0.12 mbar for 8 hours; then heating to +20°C at a rate of 3°C/hour (10 h) at 0.12 mbar, then heating (10 h) to 30°C, other 4 h at 30°C and the same pressure; and finally freeze-dried at +35°C at 0.01 mbar for 8 hours, using the Christ Model Delta 2–24 LSC freeze-dryer (Germany).

The matrices M1 ÷ M5 in a spongious form were obtained by lyophilization as multiparticulate drug delivery systems (MDDS) and tested by water uptake and enzymatic degradation.

2.1.4. Water Uptake of MDDS. The water uptake capacity was carried out using phosphate buffer pH 7.4 as the immersion medium ($n = 3$) and calculated using the previously described methods [17]. Briefly, the samples were first immersed in PBS at 370°C. At scheduled time intervals, the samples were withdrawn, wiped (to remove the surface water), and weighed. The water uptake ability was monitored using the following equation:

$$\text{Water uptake} = \frac{(W_t - W_0)}{W_0}, \quad (1)$$

where W_0 is the dried sponge weight at the initial time and W_t is the sponge weight after immersion at time t [17, 26].

2.1.5. Enzymatic Degradation of MDDS. In vitro enzymatic degradation of MDDS sponges by collagenase was also investigated by monitoring the mass loss of samples as a function of exposure time to a collagenase solution according to a procedure described in the literature [17, 26]. Pieces of collagen scaffolds (1 cm in diameter) were accurately weighed (wet weight without excess of water), placed in a solution of PBS and collagenase (1 μg/ml) at pH 7.4, and incubated at 37°C. At regular intervals, the swollen sponges were removed from the collagenase solution, wiped, and weighed. The resistance to enzymatic degradation was determined through a weight loss parameter computed using the following equation:

$$\%\text{weight loss} = \frac{W_i - W_t}{W_i} \times 100, \quad (2)$$

where W_i is the sponge initial weight and W_t is the weight of the samples after time t [17, 26]. Each biodegradation experiment was repeated 3 times. The final percentage of biodegradation was calculated as the average values.

2.2. In Vitro Drug Release Study and Data Modelling. The studies of FA release from the collagen sponges incorporating the drug in various forms (free form, free and encapsulated form, and encapsulated form in spongious matrices) were carried out using a dissolution equipment in conjunction with paddle stirrers (Esadissolver), as previously reported [16]. Briefly, the sponge samples were fixed in a transdermal sandwich device and then immersed in apparatus dissolution vessels. The kinetic studies were performed at 37°C ± 0.5°C with a rotational speed of 50 rpm. The release medium was a phosphate buffer solution of pH 7.4. At predetermined time intervals, samples of 5 ml were collected from the receiving medium and replaced with an equal volume of fresh phosphate buffer solution, kept at 37°C ± 0.5°C, to maintain a constant volume in the release vessel. The concentration of FA was spectrophotometrically assessed (Perkin-Elmer UV-vis spectrophotometer) using the standard curve ($A^{1\,\text{cm}}_{1\%} = 534$) determined at $\lambda_{\text{masx}} = 288$ nm, and the cumulative released drug percentage was determined.

2.3. In Vitro Assessment of Material Biocompatibility. A culture of human adipose-derived stem cells (hASCs) was

obtained (Gibco, Thermo Fisher, USA) and maintained in standard conditions (370°C, 5% CO_2). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotic antimycotic solution (Sigma Aldrich, Germany). The cells were seeded and allowed to distribute inside the 3D materials, and the biocompatibility of the materials was assessed 3 and 6 days after cell seeding. All the tests were realized in triplicate.

The viability and the proliferation rate of the cells in contact with the materials were evaluated by the MTT test (Sigma-Aldrich, Germany). The materials seeded with cells were washed with PBS before incubation with MTT solution 1 mg/ml at 370°C for 4 hours. The formazan produced by the metabolic active cells was solubilized in isopropanol, and its absorbance was measured by spectrophotometry at 550 nm. The values obtained were proportional with the number of viable cells in the materials.

The levels of cytotoxicity induced by the materials were obtained by LDH assay (TOX7-1KT, Sigma-Aldrich, Germany). The LDH released by the cells that lost membrane integrity was quantified by spectrophotometry, measuring the absorbance at 490 nm. The values obtained were proportional with the amount of cells that died in contact with the materials.

For the qualitative evaluation of the behavior of the cells in contact with the materials, the dead and live cells were visualized by fluorescence microscopy. The solution with calcein and ethidium homodimer obtained from the Live Dead kit (ThermoFisher, USA) allowed staining and visualization of the live cells in green fluorescence and dead cells in red fluorescence.

2.4. In Vitro Evaluation of the Anti-inflammatory Potential of the MDDS. In order to evaluate the anti-inflammatory properties of the M1—M5 systems, a culture of murine macrophages from RAW 264.7 cell line was obtained in standard conditions. Twenty-four hours before putting the cells in contact with the materials, the macrophages were activated with 1 μg/ml lipopolysaccharide (LPS) (Sigma-Aldrich, Germany). The levels of proinflammatory mediators in response to the FA anti-inflammatory properties were evaluated 48 hours after cell seeding both at gene and protein levels of expression. A control sample of activated macrophages exposed to no other treatment was considered positive reference in analysis.

2.5. Screening of Proinflammatory Mediator Levels of Expression Multiplex Assay. The levels of IL6, TNFα, MCP1, and RANTES cytokines and chemokines were measured from cell culture media 48 hours after induced macrophages were put in contact with the M1—M5 systems, using a customized magnetic bead-based multiplex assay (Merck Millipore, Germany) and detection with a Luminex 200 system. Data was analyzed using xPONENT software.

2.6. Gene Expression Evaluation by qPCR. IL6 gene expression was analyzed by qPCR, considering a comparison between the levels of IL6 in murine macrophages exposed to M1—M5 materials and the level of IL6 in LPS-induced macrophages. Briefly, the total RNA from RAW cells was isolated using Trizol reagent (ThermoFisher, USA), according to the manufacturer's protocols and tested for integrity on a BioAnalyzer 2100 (Agilent Technologies, Germany). One microgram of total cellular RNA was reverse transcribed to the corresponding cDNA using an iScript cDNA Synthesis kit (BioRad, USA). Sequences of IL6 gene-specific primers used in q-PCR assay are 5′-AGTTGCCTTCTTGGGA CTGA-3′ and 5′-TCCACGATTTCCCAGAGAAC-3′. The relative gene expressions of IL6 was evaluated by qPCR on a LightCycler 2.0 system (Roche, Germany) and was normalized to GAPDH reference gene.

2.7. Cytokine Expression Evaluation by Fluorescence Microscopy. IL6 protein expression was investigated by immunostaining and fluorescence microscopy 48 hours after induced macrophages were put in contact with M1—M5 materials. Briefly, cells in matrices were fixed with 4% paraformaldehyde for 2 hours and then permeabilized for 1 hour with a 0.1% Triton X100 solution in 2% BSA. The systems were further immunostained overnight using a primary anti-IL6 antibody (sc-1266, 1 : 100 dilution, Santa Cruz, USA) and then secondary antibody conjugated with FITC (sc-2777, 1 : 100 dilution, Santa Cruz, USA) for 1 hour at 4°C. Cell nuclei were stained with DAPI. Staining was visualized in an Olympus IX73 fluorescence microscope.

2.8. Animal Model of Experimentally Induced Burns. The experiment was performed on 30 Wistar rats weighing 160 ± 10 g purchased from the Animal Biobase of the "Carol Davila" University of Medicine and Pharmacy, Bucharest.

All animals used in the study were kept in standard laboratory conditions, were fed twice a day, and received water *ad libitum.* The experiment was performed in compliance with the European Communities Council Directive 2010/63/UE and Law No. 43 of the Romanian Parliament from 11.04.2014.

The animals were distributed in 7 groups of 5 individuals each as follows: group 1—M1, group 2—M2, group 3—M3, group 4—M4, group 5—M5, group 6—control (burns), and group 7—negative control (healthy animals used for hematological analyses).

The animals belonging to groups 1–6 were anesthetized with ether ethylic, and the hair was removed from the dorsal area. A special metallic device containing a 10 mm diameter disc was used for inducing the experimental wound. The device was heated in boiling physiological serum and applied on the shaved dorsal area for 15 seconds. The lesions measuring 10 mm diameter were sterilized, and the collagen scaffolds were applied and fixed with a silk plaster for groups 1–5. The control group (group 6) used in the study received the classical burn treatment by covering the wounds with sterile cotton dressing.

The surface morphology evolution of the wounds was recorded using a digital camera (Olympus SP-590UZ), and the wound diameter was measured for 17 days.

The healing process was evaluated according to the size profile of the wound as described by the following equation:

$$\text{Healing process\%} = \frac{(\text{wound diameter at } t = 0) - (\text{wound diameter at } t)}{\text{wound diameter at } t = 0} \times 100, \quad (3)$$

where the wound size was an average measurement of the longest and shortest dimensions of the affected area [2, 3, 12, 16]. The wound was considered healed after the crust of the lesion fell off.

Any secondary effects such as inflammation or infection of the wounds, as well as any variations on the animal health status, were also monitored. In the last day of the monitoring, the animals were ethyl-ether anesthetized and slaughtered. The blood was collected in K_3EDTA anticoagulant vacutainers for hematological tests to evaluate any possible secondary effects like inflammation or anemia induced by the treatment. A supplementary negative control group (group 7) ($n = 5$) monitored for 17 days with no experimentally induced burn or treatment was used for the comparison of the hematological data.

The blood analyses were performed using an Abacus Junior hematological equipment. The specific reagents were purchased from the Diatron company.

2.9. Statistical Analyses. Statistical analyses were performed using the GraphPad Prism 6 software. All data were expressed in the mean and standard deviations (SD). Normal distribution was calculated using the Kolmogorov–Smirnov test. The experimental data were evaluated using the student t-test and analyses of variance followed by Dunnett's multiple comparison test.

For in vitro biocompatibility tests and anti-inflammatory potential of the MDDS, a one-way ANOVA algorithm of analysis was used, followed by Bonferroni's posttest. The results were considered significant at $p < 0.05$, highly significant at $p < 0.01$, and not significant at $p > 0.05$.

3. Results and Discussion

3.1. Evaluation of Water Uptake and Resistance to Enzymatic Degradation. Water uptake of obtained matrices was investigated by gravimetric analysis in order to evaluate how the microcapsules influenced the MDDS.

According to our results, the highest amount of water was absorbed by M1 sample, having no microcapsules in the composition (Figure 2). The samples with a low amount of microcapsules (M3 and M5) absorbed higher amount of water than samples with a higher amount of microcapsules (M2 and M4). This is because the microcapsules swell themselves in contact with water and perform a denser and compact structure of sponges with smaller porosity.

Multiparticulate systems absorbed water quickly in the first 30 min and then continued gradually, until it reached equilibrium after 24 h.

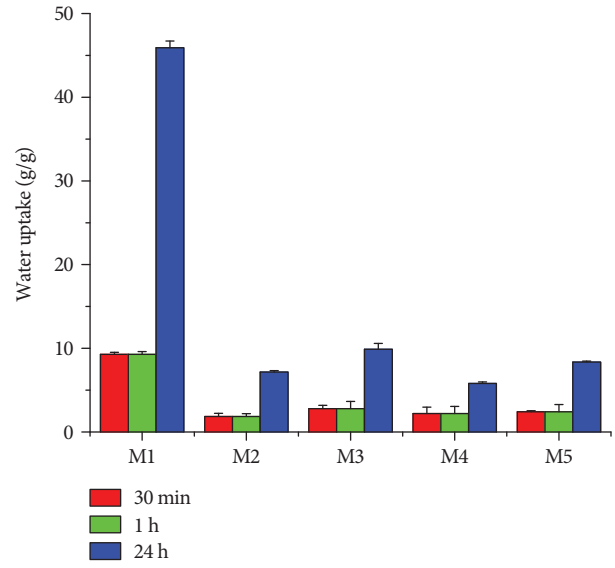

Figure 2: Water uptake of multiparticulate systems.

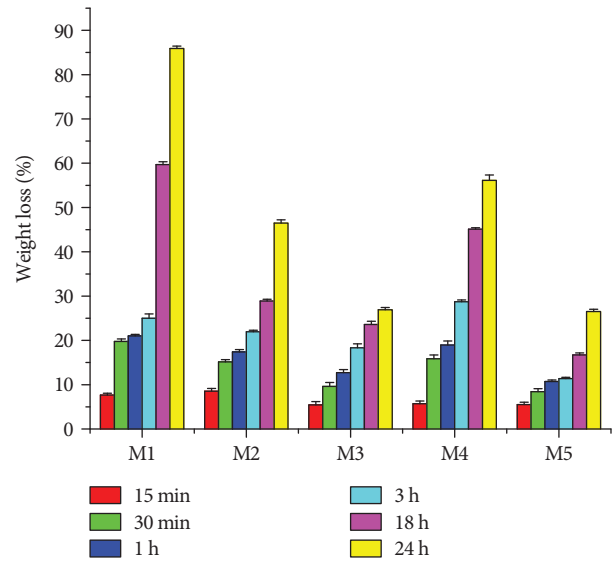

Figure 3: Enzymatic degradation of multiparticulate systems.

After swelling for 24 hours, the MDDS were evaluated by degradation in collagenase solution in order to simulate their *in vivo* behavior and to investigate their stability (Figure 3).

The sample without microcapsules (M1) was degraded faster than the multiparticulate systems (Figure 3), about 85% in 24 hours. The microcapsules swollen and make the sponge more resistant to degradation.

The release of FA from the designed supports is illustrated in the cumulative kinetic profiles given in Figure 4.

Correlating data from Figures 2 and 3, the water uptake ability and the resistance to enzymatic degradation of the designed systems are strongly influenced by the presence of

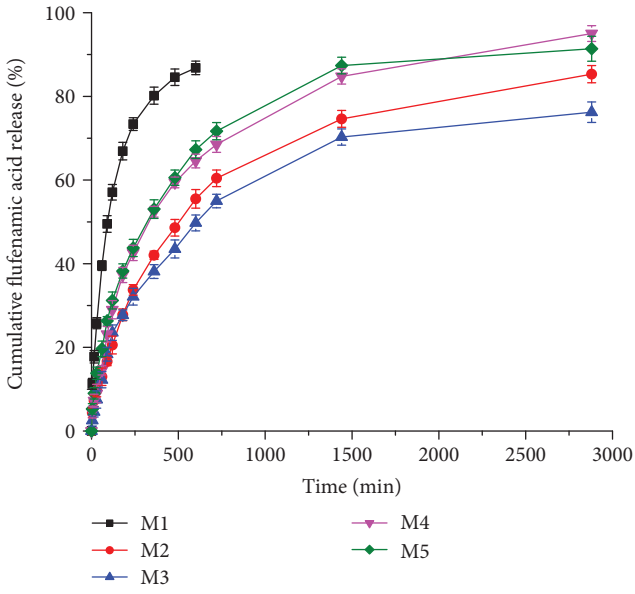

FIGURE 4: Cumulative release profiles of FA from topical spongious matrices as a function of time.

microcapsules in the formulation, the amount of FA, and the ratio between collagen gel and microcapsules.

3.2. Release of FA from MDDS. Regarding the release of FA from the multiparticulate systems, a similar biphasic shape was noticed for the kinetic profiles recorded at the release in phosphate buffer pH 7.4 (Figure 4). Thus, FA was released faster in the first 60 min, followed by a slow release during several hours of experiment (up to 10 hours for the supports incorporating FA in a free form and up to 48 hours for the multiparticulate systems; FA incorporated in the spongious matrix in an encapsulated form and in a free and encapsulated form, respectively). This initial step of drug release, known as the "burst release" effect, is more evident for the support incorporating FA in a free form (M1), the drug being released in a percentage of 39.54% in comparison with the multiparticulate systems, whose released FA percentage varies between 12.25% (M3) and 19.65% (M5). After 10 hours, the released FA percentage reach 86.81%. After 48 hours, the released FA percentage varies between 76.21% (M3) and 95.01 (M4) (Table 1). The decrease of drug amount released fast in the first hour is due to a lower absorption capacity of the multiparticulate systems as a consequence of the encapsulation technique. The burst release effect reduces the pain sensation and the amount of proinflammatory mediators released at the burn wound site, while the slow and gradual release during the following hours ensured an anti-inflammatory and analgesic effect for a longer period of time needed for burn healing. Such kinetic profiles with drug biphasic release are targeted for the control of local inflammation and pain associated to a cutaneous wound because the first 12–48 hours are critical in a wound healing process.

To evaluate the mechanism of the FA release from the supports tested, various kinetic models were applied to the experimental data: Power law model (4), as well as its particular cases, Higuchi ($n = 0.5$) and Zero-order ($n = 1$).

$$\frac{m_t}{m_\infty} = k \cdot t^n, \tag{4}$$

where m_t/m_∞ is the fractional drug released in time t, k is the kinetic constant, and n is the release exponent characteristic to the drug transport mechanism.

The values recorded for the correlation coefficient were higher for the Power law model ($R > 0.97$) compared to the Higuchi and Zero-order models, indicating a non-Fickian *release kinetic mechanism* of FA from the spongious matrices loaded with FA in a free form, i.e., from the multiparticulate systems, the release exponent values ranging from 0.36 to 0.41. The kinetic constant and the release exponent value characteristics to this model are given in Table 1.

The kinetic mechanism associated to the release profiles is presented in Figure 5. Thus, an important amount of exudate can be initially found at the burn wound level, wetting the spongious matrix surface, being then absorbed in and penetrating the matrix porous structure. Upon contact between the spongious matrix and wound, a gel layer is formed favoring the diffusion of the free-form drug entrapped at and closed to the surface and released through desorption (for the matrices where FA is incorporated in free form and free and encapsulated form, respectively), explaining the FA fast release effect ("burst release" effect) in the first 60 minutes. For the multiparticulate supports, this initial drug release step is slower. The wound exudate absorption process is followed by polymeric network hydration and relaxed polymer swelling implying a slower, gradual diffusion of the free-form drug incorporated (partially immobilized in collagen fibrillary structure) and of the drug from the microcapsules entrapped in the polymer matrix during the lyophilization process, respectively, concomitantly with the much slower process of release support degradation compared to the matrices incorporating FA only in a free form.

All these physical-chemical processes are explaining the deviation from the Higuchi model, characterized by a slower drug diffusion rate in comparison with polymer relaxation rate and also from the Zero-order model for which the drug diffusion rate is higher than the polymer relaxation rate.

3.3. In Vitro Biocompatibility of the Multiparticulate Systems. Multiparticulate drug delivery systems (MDDS) were evaluated for biocompatibility in terms of cell viability and MDDS cytotoxicity.

Quantitative results obtained by MTT assay revealed an overall good biocompatibility of the materials. Three days after cell seeding in contact with the materials, a similar cell viability was found for the M2 and M3 systems, as compared to M1 material, considered as a control. However, in case of M4 and M5 MDDS having FA anti-inflammatory agent encapsulated, an increased cell viability was registered ($p < 0.05$). After 6 days of culture, a similar cell viability profile was found (Figure 6(a)). Cells cultured in contact with the M2 system revealed similar viability to those cultured in contact with M1 control, thus proving that the addition of

Table 1: Correlation coefficients for FA release from collagen spongious supports determined by application of Higuchi, Zero-order, and Power law models; kinetic parameters for Power law model; drug-released percentage.

Spongious matrices	Higuchi model	Zero-order model	Power law model	Release exponent	Kinetic constant ($1/min^n$)	Drug-released percentage (%)
M1	0.9722	0.8741	0.9864	0.37	0.089	86.81*
M2	0.9744	0.8566	0.9828	0.41	0.034	85.30**
M3	0.9697	0.8430	0.9815	0.40	0.033	76.20**
M4	0.9621	0.8264	0.9784	0.39	0.048	95.01**
M5	0.9525	0.8041	0.9772	0.36	0.059	91.39**

*Released drug percentage after 10 hours—FA incorporated in free form; **released drug percentage after 48 hours—FA incorporated in multiparticulate systems.

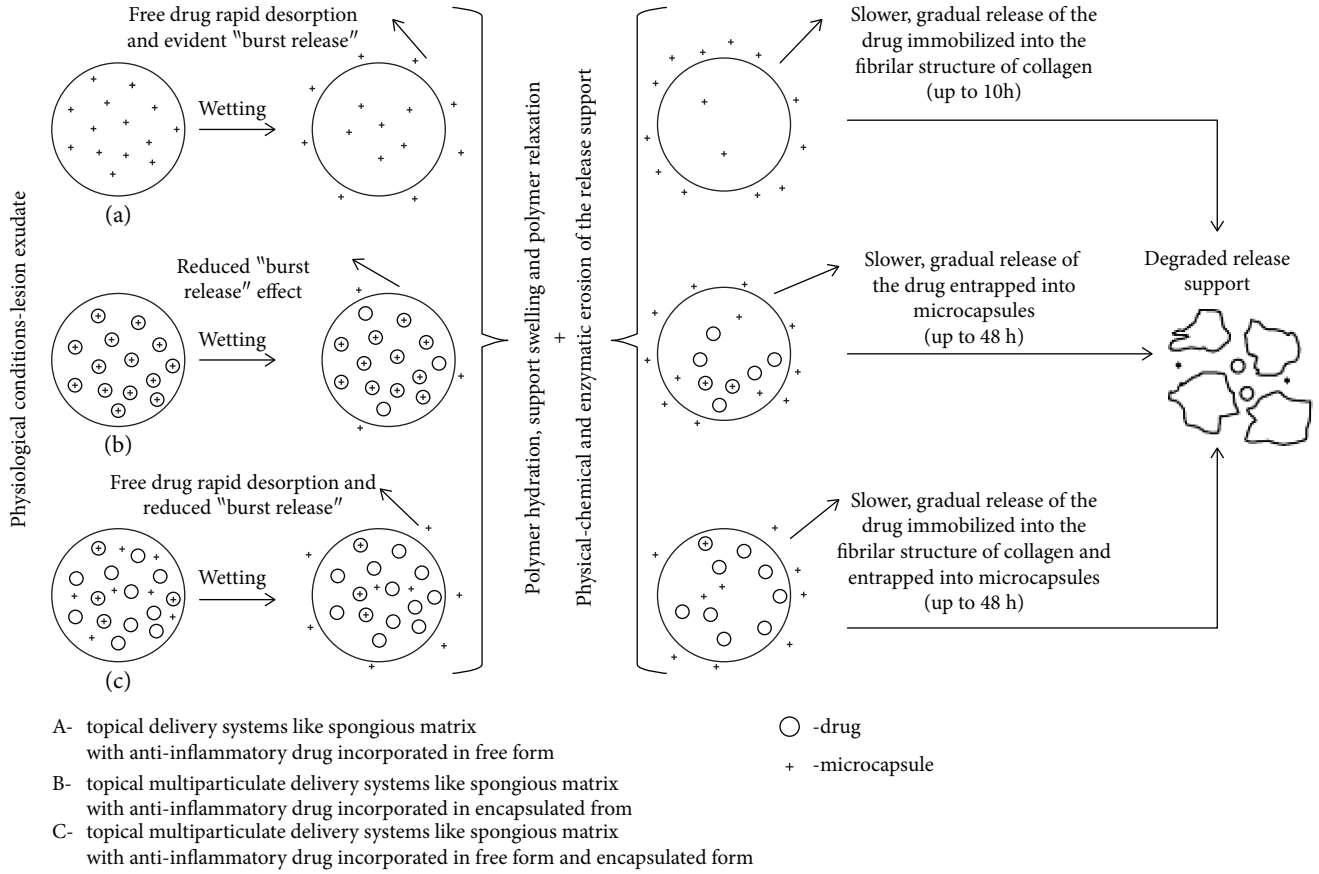

Figure 5: The drug kinetic release mechanism form of different topical delivery systems: (a) spongious matrix with an anti-inflammatory drug incorporated in a free form—M1; (b) multiparticulate delivery systems like the spongious matrix with an anti-inflammatory drug incorporated in an encapsulated form—M2 and M3; (c) multiparticulate delivery systems like the spongious matrix with an anti-inflammatory drug incorporated in a free form and encapsulated form—M4 and M5.

microcapsules (MC) in the composition of the matrices did not influence the biocompatibility of the system. Interestingly, a statistically significant decrease in cell viability was observed in case of the M3 system, as compared to the control. An increase in cell viability was registered for M4 and M5 MDDS ($p < 0.01$ and $p < 0.05$, respectively), suggesting that the incorporation of FA in the microcapsules and resulted MDDS structure and properties displayed a positive effect on cell viability.

By contrast, LDH assay offered quantitative information on the cytotoxic potential of the MDDS (Figure 6(b)). After 3 days of culture, the LDH levels released in the culture media were similar for all M1-M5 matrices, suggesting a low and comparable degree of cytotoxicity for the systems. After 6 days of culture, materials exerted almost no increase in the cytotoxicity profile for M2, M4, and M5, as compared to M1 control, showing that even if the cells registered proliferation from 3 to 6 days of culture (Figure 6(a)), the cytotoxic

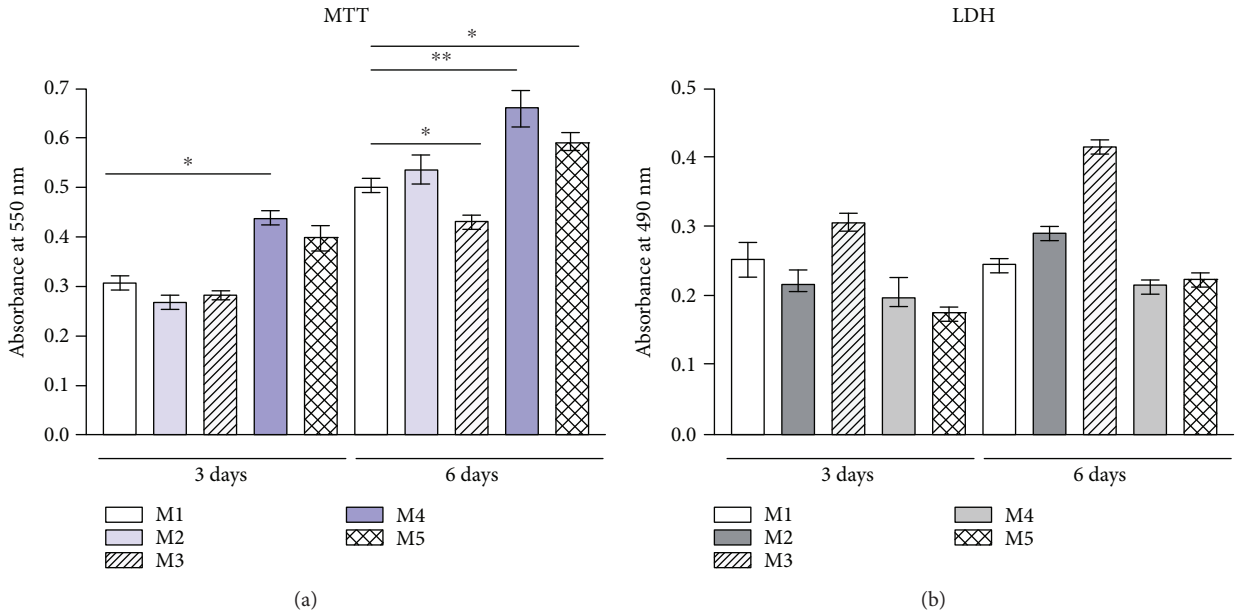

Figure 6: (a) Cell viability obtained after 3 and 6 days of hASCs contact with M1—M5 matrices, as shown by MTT quantitative assay. (b) Cytotoxic potential of M1-M5 matrices, as revealed by LDH assay after 3 and 6 days of culture in standard conditions.

levels remained to the same levels. A slight increase in material cytotoxicity was found for M3 after 6 days of culture, resulting in the lowest biocompatibility of this material among all tested compositions.

The visualization of both live and dead cells in the three-dimensional cell cultures was possible by Live Dead assay and visualization in confocal microscopy (Figure 7). Live Dead assay results are in accordance with the quantitative data obtained from MTT and LDH assays, revealing the most biocompatible system to be M4. All tested MDDS revealed an overall good biocompatibility, particularly the M4 and M5 systems, where a strong positive ratio between live green cells and dead red cells was observed. Additionally, the highest amount of dead cells marked by red fluorescence was found in contact with M3 composition after 6 days of culture, confirming the LDH results. Generally, a higher amount of live cells and a tendency to group was found in M4 and M5 materials, in comparison with M1 control, thus suggesting that the incorporation of FA in microcapsules favored material biocompatibility.

3.4. Evaluation of the Anti-inflammatory Potential of MDDS. Screening among several cytokine and chemokine levels was performed in order to investigate the effect of FA on inflammation and the anti-inflammatory potential of the M1–M5 systems (Figure 8(a)).

Screening results indicated a clear decrease in proinflammatory cytokine and chemokine levels secreted by cells in contact with M4 and M5, as compared to M1 control and M2 and M3. This suggests that the encapsulation and gradual release of FA up to 48 hours result in a more efficient anti-inflammatory response. In particular, the lowest levels of IL6 and TNFα were registered for M4 composition, which contains the highest amount of microcapsules filled with FA. The levels of MCP1 and RANTES

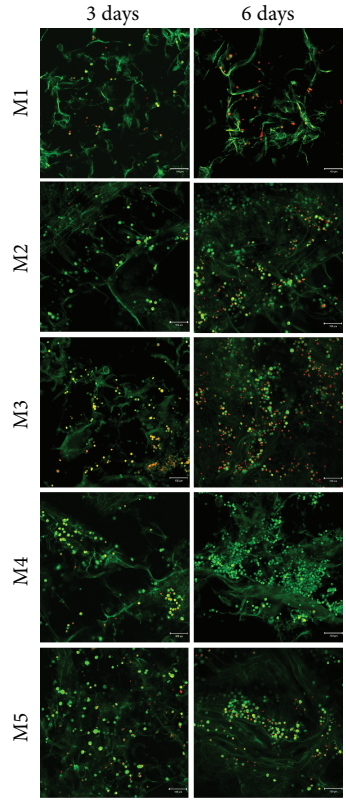

Figure 7: Live (green) cells and dead (red) cells visualized after 3 and 6 days of culture in the M1–M5 systems by confocal microscopy.

remained approximatively constant among all studied compositions, but showing a tendency to decrease also in the systems with encapsulated FA. The most significant changes were found in IL6 levels; therefore, IL6 expression was further assessed at gene level and by immunostaining.

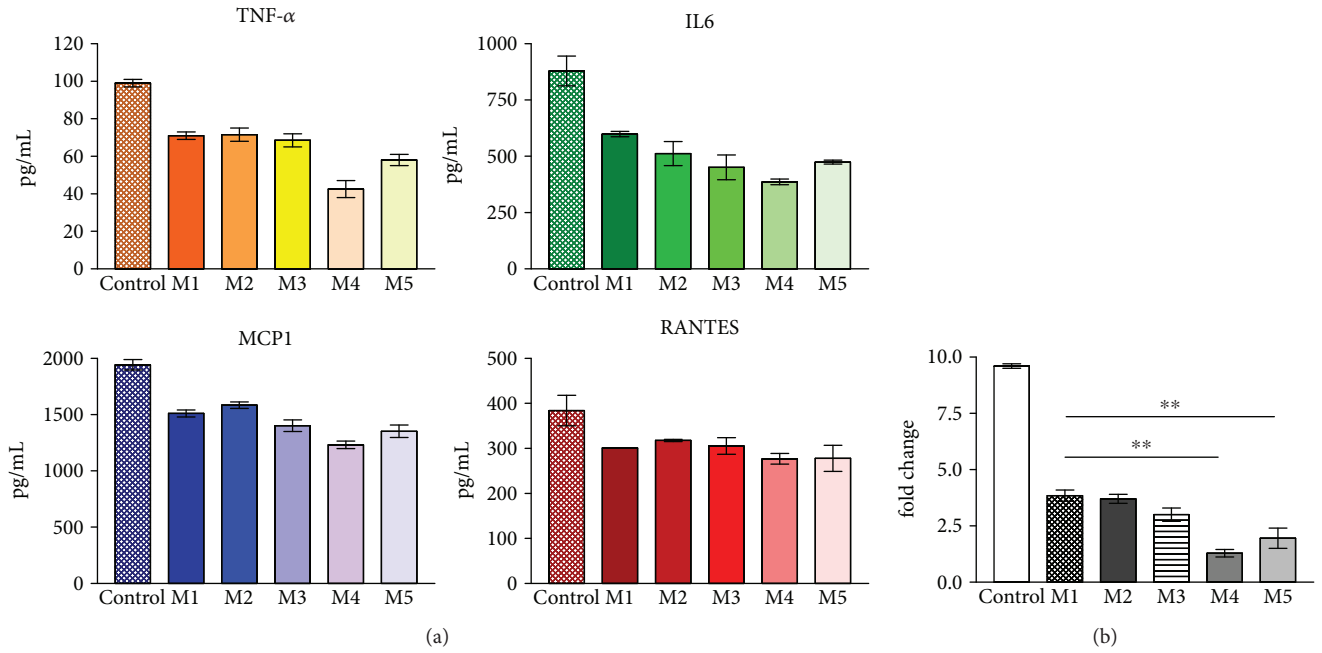

Figure 8: (a) Protein levels of TNFα, IL6, MCP1, and RANTES proinflammatory mediators found in cell culture media of activated macrophages after contact with the M1–M5 systems, as compared to the positive control. (b) IL6 gene expression in activated macrophages exposed to M1-M5, as compared to the activated control.

IL6 gene level of expression was evaluated by qPCR and revealed a statistically significant lower mRNA level after activated macrophage exposure to M4 material (Figure 8(b)). The relative gene expression registered a fold change of 10 when comparing control-activated macrophages with the cells exposed to M4 material. This decrease in IL6 expression could be correlated to the higher content of microcapsules with FA in the M4 system and could be probably attributed to the gradual controlled released of the anti-inflammatory agent from the matrices. IL6 transcript level detected by qPCR is in accordance with IL6 profile of protein expression and revealed a higher anti-inflammatory efficiency of the multiparticulate drug delivery systems containing microcapsules loaded with FA than the matrices incorporating free FA in the composition.

Immunostaining of IL6 and visualization in fluorescence microscopy (Figure 9) showed a significant decrease in IL6 level of expression in cells exposed to the M1–M5 systems, whereas all cells in positive control activated with LPS expressed IL6. Thus, it can be concluded that all analyzed materials have an anti-inflammatory effect to a lower or higher degree. The lowest IL6 expression was detected for M4 material, confirming previous gene expression results. Results obtained for M1, M2, M3, and M5 are comparable in terms of IL6 expression.

3.5. In Vivo Evaluation of MDDS Efficiency for Burn Treatment. The evolution of the wound diameter after the treatment with collagen matrices loaded with FA incorporated in various forms is presented in Table 2 and Figure 10, and the healing process is presented in Figure 11. The macroscopic morphology of the experimentally induced burns is presented in Figure 12.

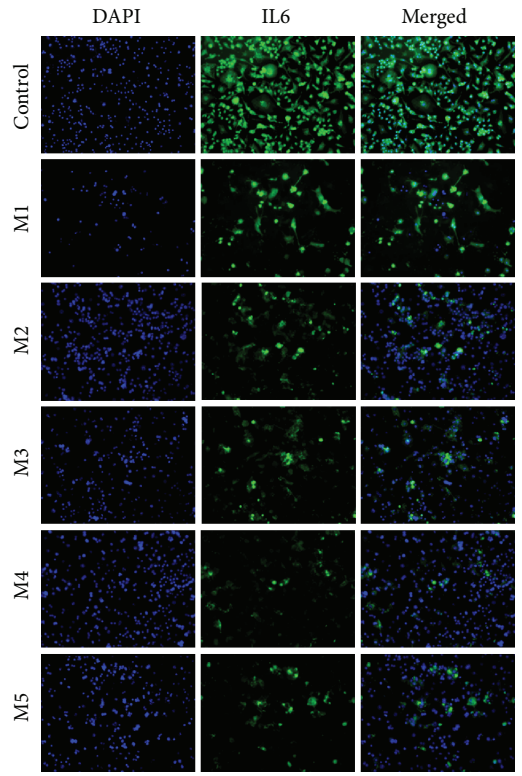

Figure 9: Expression of IL6 in macrophages exposed for 48 h to the M1–M5 systems, as compared to the positive control.

The evolution of the burn is a complex process comprising generally the following overlapping phases: an initial local homeostasis followed by inflammation, proliferation, and tissue remodeling.

Table 2: Wound diameter (mm) evolution after treatment with spongious matrices loaded with FA in experimentally induced burn to Wistar rats.

	Day 1 Mean ± SD	Day 3 Mean ± SD	Day 5 Mean ± SD	Day 7 Mean ± SD	Day 10 Mean ± SD	Day 12 Mean ± SD	Day 14 Mean ± SD	Day 17 Mean ± SD
M1	10 ± 0	9 ± 0.7***	7 ± 1.9***	5.2 ± 2.8**	3.6 ± 2.9***	1.4 ± 1.9**	1 ± 1.4*	0.2 ± 0.4
M2	10 ± 0	9.5 ± 0.6***	8 ± 0.8	5.75 ± 2.2	4.25 ± 1.0**	2.25 ± 1.0	0 ± 0	0 ± 0
M3	10 ± 0	10 ± 0.8**	6.75 ± 1***	6.25 ± 0.5	4.75 ± 1.0*	2.25 ± 2.6	1.75 ± 2.1	0 ± 0
M4	10 ± 0	9 ± 0.1***	6 ± 0.6***	5.25 ± 1.2*	3.75 ± 1.1***	1.75 ± 0.8	1 ± 1.1	0 ± 0
M5	10 ± 0	9.25 ± 0.1***	7 ± 0.6**	6 ± 1.2	5.25 ± 1.1	0.75 ± 0.8**	0.5 ± 1.1*	0 ± 0
Control	10 ± 0	11.75 ± 0.4	10.75 ± 0.4	8.67 ± 1.2	7.83 ± 1.1	5.5 ± 0.5	2 ± 2.0	1 ± 1.4
ANOVA (P)	NS	$p < 0.0001$	$p < 0.0001$	$p = 0.001$	$p < 0.0001$	$p < 0.005$	NS	NS

SD = standard deviation, Dunnett's multiple comparison test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and ANOVA (NS = not significant at $p > 0.05$). Control = animals with burn induced but without spongious matrix treatment.

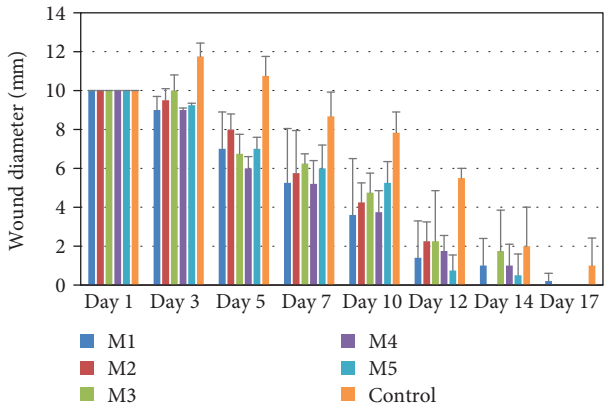

Figure 10: Wound diameter (mm) evolution after treatment with spongious matrices loaded with FA in experimentally induced burn to Wistar rats (bars in the graph represent standard deviation).

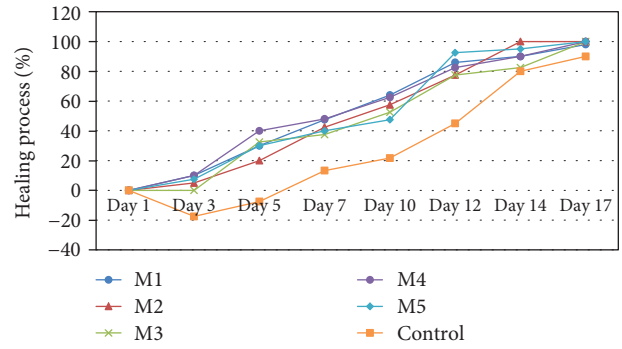

Figure 11: The evolution of the healing process (%) in experimentally induced burns to Wistar rats after the treatment with a biopolymeric collagen matrix loaded with an anti-inflammatory drug.

The first two phases (homeostasis and inflammation) occurring in the initial 1–5 days are critical for the cicatrizing process of the wounds.

After inducing the burn on experimental animals, the local area was characterized by a white eschar, the surface skin layers (epidermis and dermis) were damaged, and during the next hours, the lesion became fully hyperemic due to erythrocyte extravasation. Several homeostatic processes are activated, like platelet aggregation, immune defense, and blood clotting.

In the first three days, the posttraumatic inflammation is generally characterized by an increased level of proinflammatory cytokines stimulating the neutrophil migration and the monocytes switching into activated macrophages [27]. Releasing of strong inflammatory signals could interfere the skin remodeling and regeneration processes, promoting the formation of thick scars by increasing the secretion of the extracellular matrix. The use of NSAIDs for a short term applied in different topic formulas decreases the local pain and favors the generation of a normal skin with minimal scarring comparing to a long-term use of with impaired wound healing [2, 16, 23].

The treatment with collagen sponges applied in case of groups 1–5 decreased the local damage in the initial homeostatic phase by supplying a scaffold-like matrix for the migration of the first-line defense cells like dermal fibroblasts, keratinocytes, leukocytes, and endothelial cells [2, 16].

Following the next days, the reepithelization process was accelerated in treated groups and was associated with decreasing wound diameters in these groups comparing to the control group (animals with experimentally induced burns without spongious matrix treatment).

The treatment with a collagen spongious matrix was proved to be efficient in the posttraumatic inflammatory phase by decreasing the wound diameter favoring the healing process in days 3, 5, and 7, a critical important period in the cicatrizing process. For 5 days, a slight inflammatory posttraumatic effect was noticed in case of the control group reflected by an increased wound diameter in comparison with all treated groups.

After 5 days (Table 2, Figure 12), the most significant decrease of the wound diameter by 40% was noticed in case of the treatment with an M4 spongious matrix followed by M3 (32.5%), M1 (30%), and M5 (30%).

All treatments significantly favored the wound healing process ($p < 0.05$) after the first week. The most significant

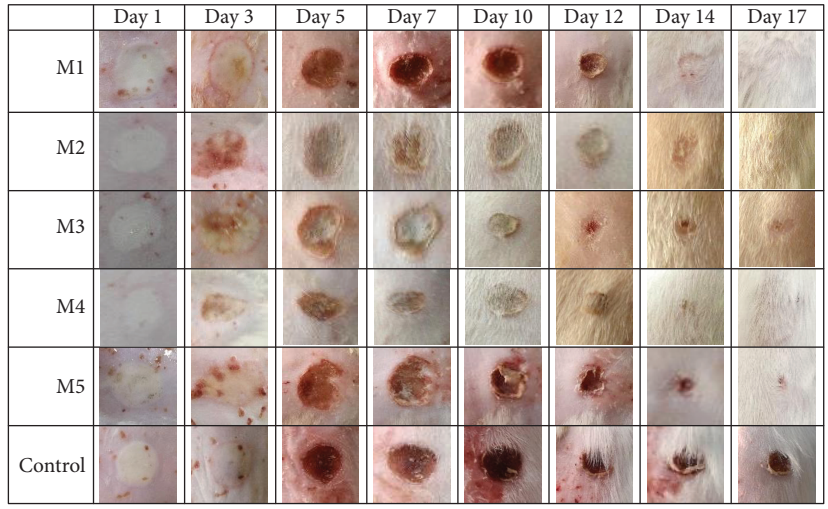

Figure 12: The macroscopic morphology of the experimentally induced burns to Wistar rats after the treatment with spongious collagen matrices loaded with an anti-inflammatory drug and a classical treated control group.

Table 3: The variation of the red blood cell level, hemoglobin, and erythrocyte indices after experimentally induced burns to Wistar rats and treated with biopolymeric spongious matrices loaded with FA.

	RBC ($\times 10^6/\mu$l) Media ± SD		HGB (g/dl) Media ± SD		HCT (%) Media ± SD		MCV (fl) Media ± SD		MCH (pg/cel) Media ± SD		MCHC (g/dl) Media ± SD		RDW Media ± SD	
M1	8.44	±0.4	15.40	±0.7	41.60	±1.0	49.33	±3.1	18.20	±0.2	36.97	±2.4	18.07	±0.4
M2	8.06	±0.5	15.03	±0.1	40.01	±1.9	49.67	±1.7	18.73	±1.2	37.67	±1.7	18.23	±0.3
M3	8.26	±0.1	15.07	±1.1	41.01	±2.4	49.33	±3.4	18.10	±0.9	36.67	±3.2	17.63	±0.5
M4	8.26	±1.1	15.87	±0.8	39.59	±4.9	48.33	±3.5	18.77	±1.6	39.23	±3.3	18.50	±0.8
M5	8.58	±0.4	15.17	±0.7	41.03	±1.8	47.67	±2.1	17.67	±1.0	37.03	±3.1	18.63	±0.2
Control	8.04	±0.4	15.47	±1.1	38.32	±2.5	47.67	±0.6	19.20	±0.5	37.32	±5.3	18.42	±0.7
Negative control	8.10	±0.8	15.43	±1.2	38.32	±2.4	47.33	±2.1	19.03	±0.6	40.17	±0.7	18.20	±0.7

RBC = red blood cell, HGB = hemoglobin, HCT = hematocrit, MCV = mean cell volume, MCH = mean cell hemoglobin, MCHC = mean cell hemoglobin concentration, RDW = red blood cell distribution width, SD = standard deviation. Control = group of animals with induced burns without spongious matrix treatment. Negative control = healthy animals.

results were noticed in case of M1 and M4 treatments with a healing process of almost 48% ($p < 0.05$).

After 14 days, most of the animals from the treated groups were completely healed with reepithelized lesions (Table 2, Figures 10–12) compared to the control group characterized by wounds with thick scars and a healing process extending the 17 days of monitoring.

The most commonly developed secondary effects after burn injuries are inflammation, infections, or anemia. The hematological analysis of some blood parameters is usually performed to identify any possible systemic effects after inducing the burns and treating new developed biopolymeric collagen sponges loaded with FA (Tables 3 and 4).

The following classical hematological parameters were used to detect the presence of anemia: red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), and red blood cell distribution width (RDW).

The hematological parameters correlated with anemia or other erythrocyte pathologies presented no significant variation in case of treated groups (M1–M5) and control group of animals with experimentally induced burns compared to the negative control group of healthy animals (Table 3).

The following leucocyte formula parameters are frequently clinically used for detecting systemic infection or inflammation and were analyzed to identify any potential secondary effects induced by the treatment: white blood cells count (WBC), lymphocytes % (Ly%), monocytes % (Mi%), and granulocytes % (Gr%) are presented in Table 4. No significant changes of the leucocyte formula were noticed after the treatment with biopolymeric wound dressings containing or not the mefenamic acid.

4. Conclusions

Multiparticulate drug delivery systems (MDDS) consisting in the polymeric matrix based on collagen-dextran and/or

Table 4: The variation of the white blood cell count after experimentally induced burns to Wistar rats and treated with biopolymeric spongious matrices.

	WBC ($\times 10^3/\mu$l) Media ± SD		Ly% Media ± SD		Mo% Media ± SD		Gr% Media ± SD	
M1	3.90	±0.8	55.03	±6.8	13.63	±2.6	31.30	±7.4
M2	4.41	±1.0	54.83	±7.1	15.03	±3.0	30.13	±5.8
M3	4.10	±0.4	55.30	±2.8	13.28	±1.0	31.38	±1.9
M4	4.18	±1.6	33.63	±1.2	17.23	±4.5	49.10	±1.6
M5	4.56	±1.1	45.88	±1.6	13.84	±3.1	40.27	±7.9
Control	4.04	±0.7	43.17	±1.0	14.80	±2.1	42.07	±2.9
Negative control	3.89	±0.5	40.71	±5.6	15.42	±1.7	40.28	±1.3

WBC = white blood cell, Ly% = lymphocyte %, Mo% = monocyte %, Gr% = granulocyte %, SD = standard deviation. Control = group of animals with induced burns without spongious matrix treatment. Negative control = healthy animals.

microcapsules based on gelatin-CMCNa-alginate with embedded anti-inflammatory drug (FA) were developed as the local treatment for burn. These MDDS were obtained in a sponge form by freeze-drying, showed good absorbent properties, and degraded in time giving possibility to control the release of drugs. The kinetic profiles recorded a reduced burst release effect and a prolonged drug release for 48 hours for the MDDS in comparison with sponge with drug incorporated in free form. All studied MDDS displayed good biocompatibility, but in particular the matrix with 30% microcapsules and FA (M4). This also revealed the highest anti-inflammatory potential, probably due to the encapsulated FA and controlled release over 48 hours. The collagen biopolymers associated with anti-inflammatory agent accelerated the healing process with beneficial effects on the critical phases of the cicatrizing process with a faster epithelial regeneration and a minimal scarring compared to the control group. The best results were obtained with that system supported by the *in vitro* studies regarding the physical-chemical properties and resistance to enzymatic biodegradation. Besides the beneficial cicatrizing effect on experimental animals, the treatment with collagen sponges was associated with no secondary systemic or topic effects comparing to the control group which developed impaired topic inflammation process in the first days and a longer period of healing. In conclusion, this study validated novel MDDS for drug delivery in skin burn wound care, revealing a polymeric matrix base on collagen/dextran with the release of flufenamic acid in a free and encapsulated form as the most promising composition for future burn wound healing application.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

There are no conflicts of interest to report.

Authors' Contributions

Sorina Dinescu and Simona Ignat contributed equally to this work.

Acknowledgments

This work was financially supported by a grant of the Romanian Ministry of Research and Innovation, CCCDI-UEFISCDI, project number PN-III-P1-1.2-PCCDI-2017-0782/REGMED Project 3, within PNCDI III (*in vitro* biological studies). The study was also financially supported by the Ministry of Research and Innovation, Program 1: Development of National System of Research-Development, Subprogram 1.2: institutional performance projects for funding the excellence in RDI, contract no 6PFE/16.10.2018 (material synthesis and characterization). The authors acknowledge the collaboration with the Faculty of Pharmacy, "Carol Davila" University of Medicine and Pharmacy for this study, which was in the frame of PN-III-Experimental Demonstration Project, project number PED 160/03.01.2017, code PN-III-P2-2.1-PED-2016-0813.

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 5196023, 14 pages
https://doi.org/10.1155/2018/5196023

Research Article

Variation in Expression of Inflammation-Related Signaling Molecules with Profibrotic and Antifibrotic Effects in Cutaneous and Oral Mucosa Scars

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Received 2 May 2018; Revised 6 October 2018; Accepted 11 October 2018; Published 28 November 2018

Guest Editor: Iulia D. Popescu

Wound healing is a complex biologic process evolving in three phases: inflammation, proliferation, and tissue remodeling controlled by numerous growth factors and cytokines. Oral mucosa wounds heal with significantly less important scars with less numerous macrophages and mast cells and more numerous myofibroblasts than cutaneous counterparts. We analyzed 32 cutaneous and 32 oral mucosa scars for TGFbeta1, TGFbeta2, TGFbeta3, TNFalpha, PDGF BB and FGF1 expression in mesenchymal cells, endothelial cells, macrophages, and multinucleated giant cells. We identified differences in the expression of profibrotic and antifibrotic factors in oral mucosa and skin scars; TGFbeta2 was positive in cutaneous multinucleated giant cells, TNFalpha was positive in cutaneous macrophages, and both were negative in oral mucosa while TGFbeta3 was positive in oral macrophages and mostly negative in cutaneous ones. PDGF BB and FGF1 were positive in oral endothelial cells and oral macrophages and negative in macrophages with opposite positivity pattern in cutaneous scars. Based on these findings, macrophage seems to be the key player in modulating pro- and antifibrotic processes in wound regeneration.

1. Introduction

Wound healing is a complex biologic process evolving in three phases: inflammation, proliferation, and tissue remodeling [1]. These phases are controlled by myriad of growth factors and cytokines and, to some extent, overlap in time. *Inflammation* occurs almost immediately after wound formation—after the blood coagulation and lasts 2–4 days; several cells are chemotactically attracted to the wound site—neutrophils (they engulf bacteria and foreign bodies) and monocytes (they differentiate into macrophages that phagocyte necrotic debris, neutrophils, and foreign bodies); later on, macrophages secrete growth factors that stimulate the formation of granulation tissue [2–4]. *Proliferation* includes several processes such as angiogenesis and fibroblasts proliferation and differentiation towards myofibroblasts (formation of granulation tissue) and epithelial cells proliferation (reepithelization) [5–9]. *Tissue remodeling* (contraction phase, "maturation") is the last and the longest phase of tissue healing; it consists of collagen synthesis and

degradation in order to align the newly formed collagen bundles along tension lines [10–12].

Immune system intervenes in the regeneration of different processes such as inflammation and debris clearance in the first stages and proliferation and differentiation of the stem cells in further steps [13, 14]. Macrophages present two functional variants: M1 ("proinflammatory" macrophages with high interleukin- (IL-) 12, low IL-10, no IL-13α1, and MS4A4A production) and M2 ("anti-inflammatory" macrophages with IL-13α1, MS4A4A, high IL-10, and low IL-12 production) [15, 16]. Both types of macrophages intervene in regeneration in different moments and have to be "switched on/off" accordingly—M1 macrophages have to be active in the beginning of the process (mainly local debris clearance and subsequent cytokines secretion—IL6, TNFalpha, IL1beta, and G-CSF stimulate skeletal muscle proliferation), while M2 macrophages are involved in tissue remodeling (in skeletal muscle lesion they promote myogenic differentiation) [17]; prolonged activity of M1 macrophages or too early activation of M2 macrophages will have negative effects on wound healing [13].

Lymphoid cells are involved in wound healing mainly by cytokine secretion. NK T cells and gamma-delta T cells intervene in liver regeneration, and T-reg cells are involved in muscle regeneration and in oligodendrocyte differentiation and myelin regeneration [18–20]. Mast cells intervene in wound healing by releasing several factors with diverse functions including cytokines (IL-1 and TNFalpha) and growth factors (TGFbeta1 or PDGF) and favor collagen deposition [21, 22]. They are denser in more mature mice embryos than in younger ones, in dermis than oral lamina propria in adult pigs, and in hypertrophic than normotrophic human scars [23–25]; also, drugs with effects of inhibiting mast cell degranulation determine reduced contraction in pigs' wounds [26]. All these arguments favor the hypothesis that mast cells are directly related to fibrotic processes.

From all these data, it is obvious that inflammation can have both positive and adverse effects on wound healing, possibly some data obtained in animals being irreproducible in humans. The study of wound healing in human is extremely difficult due to ethical issues that hamper systematic approach; animal models are also difficult to use due to significant differences in healing process between species; data gathered in small mammals (mice, rats, and/or rabbits) are not reproducible in humans; pigs, however, have similar cutaneous structure and relatively similar healing mechanisms [27]. Systematic studies in pigs and studies in humans reveal differences in wound healing between cutaneous lesions and lesions in the oral mucosa. Oral mucosa wounds heal with significantly less important scars, thus deciphering specific healing mechanisms will offer valuable lessons to be applied to prevent severe scarring [28].

Healing in the oral mucosa involves less numerous macrophages and mast cells and more numerous myofibroblasts; also, TGFbeta expression is reduced in oral mucosa lesions; diminished inflammation and reduced tissue remodeling (wound contraction) favor a scarless healing. Local microenvironment is especially important in regulating myofibroblastic function since oral mucosa lesions heal with less collagen deposition than cutaneous ones despite the increased number of myofibroblasts [29]. Local inflammatory response vary according to the moment of loading of dental implants [30]. Cytokines influence on oral mucosa healing may represent the rationales for using autolog platelet-rich fibrin concentrates to favor local repair [31].

Considering all these pro's and con's, we decide to study the expression of several growth factors and cytokines in cutaneous and oral mucosa scars in humans.

2. Material and Methods

We analyzed 32 cutaneous scars and 32 mucosal scars. The cases were selected from the archives of the Department of Pathology of Colentina University Hospital; cutaneous scars specimens were reexcisions for previously resected cutaneous tumors; some specimens presented residual tumors but fragments without tumors were selected for this study (at least 4 mm distance between the residual tumor and the area of the scar selected for analysis).

All the tissue fragments were routinely processed (fixation for 24–72 hours in 10% buffered formalin; washing for 1–2 hours in running tap water); automatic histopathologic processing on a Leica ASP200S tissue processor (90 min ethanol 70° at 40°C, 105 min ethanol 80° at 40°C, 105 min ethanol 96° at 40°C, 60 min ethanol 100° at 40°C, 90 min ethanol 100° at 40°C, 90 min ethanol 100° at 40°C, 2 hours xylene at 52°C 3 bath, 1 hour paraffin 58°C, 2 hours paraffin 58°C, and 3 hours paraffin 58°C). For paraffin embedding, we used a Thermo Fisher Microm EC 1150 H embedding station; 30 slides of 3 microns thick sections were cut with a Leica RM 2265 rotary microtome; routine stains (hematoxylin and eosin (HE)), special stains (periodic acid-Schiff (PAS)), and immunohistochemical (IHC) tests were performed.

IHC tests used several primary antibodies: TGFbeta1, TGFbeta2, TGFbeta3, TNFalpha, PDGF BB, and FGF1; source, clones, specific pretreatments, and dilutions are specified in Table 1. The IHC stains were performed on an automated immunostainer Leica Bond III using Bond TM polymer refine detection (with DAB chromogen) and Bond TM polymer refine red detection. All the stains with one antibody were performed in the same day (to minimize technique-induced variations) with one negative and positive external control for each antibody; negative control consisted of IHC stains without primary antibody; positive external controls consist of normal human spleen for TGFbeta1, TGFbeta2, and TGFbeta3, human breast cancer for TNFalpha, normal human pancreas for PDGF BB, and normal human kidney for FGF1.

Two independent pathologists examined the slides; a three-grade semiquantitative scale for positivity was used: 0, negative; 1, faint positive (positivity evident when slides were examined with 40x); 2, intense positive (positivity evident when slides were examined with 10x) no matter the number of positive cells; the positivity was recorded for mesenchymal cells (fibroblasts and fibrocytes), endothelial cells, macrophages, and, when present, multinucleated giant cells. The level of positivity was interpreted in correlation with gender, localization, presence of residual tumor, and age of the scar.

Table 1: Primary antibodies: technical specifications.

Primary antibody	Source	Clone	Epitope retrieval*	Dilution	Incubation period (min)
TGFbeta1	ABCAM	Policlonal	HIER pH 6	8/200	60
TGFbeta2	ABCAM	ab36495	HIER pH 6	0.5/250	60
TGFbeta3	ABCAM	Policlonal	HIER pH 6	8/200	60
TNFalpha	ABCAM	Policlonal	HIER pH 6	1/250	60
PDGF BB	ABCAM	Policlonal	HIER pH 6	1/250	30
FGF1	ABCAM	Policlonal	HIER pH 6	1/200	60

*HIER = heat-induced epitope retrieval.

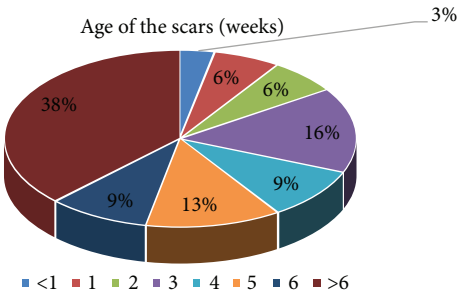


Figure 1: Age of the scars (weeks).

The statistical analysis of data was performed using EXCEL and EPIINFO programs; the results were considered statistically significant for P ($\chi 2$) was lower than 0.05.

This study was approved by Colentina University Hospital Ethic Committee, and all patients included agreed to participate in research studies.

3. Results

The cases were included in two groups: group A for cutaneous scars and group B for oral mucosa scars.

3.1. General Data. Group A included 32 patients, 13 males (40.62%) and 19 females (59.38%), between 8 and 79 years old (median 53 yrs, medium age 50.84 years). The scars were located on the head (5 cases, 15.62%), trunk (13 cases, 40.62%), limbs (10 cases, 31.25%; one case right arm and 9 cases inferior limbs), and special areas (4 cases, 12.50%; 3 cases from the skin of the breasts and one case from the axilla). The scars were as old as 3 to 504 days (Figure 1).

The scars were reexcised after previous resection of benign lesions (14 cases, 43.74%) or malignant tumor (18 cases, 56.25%; 13 melanomas, one basal cell carcinoma, 2 squamous cell carcinomas, one dermatofibrosarcoma protuberans, and one myxofibrosarcoma). Six cases (18.75%) had residual tumors (melanoma, squamous cell carcinoma, dermatofibrosarcoma protuberans, myxofibrosarcoma, melanocytic nevus, and capillary hemangioma), but, as we stated before, the area selected for analysis in our study was located at some distance from the residual tumor.

Group B (oral mucosa biopsies) included 32 patients; 11 patients were males (34.37%) and 21 females (65.62%), between 14 and 64 years old (median 22 yrs, medium age 25.19 years). All the biopsies originated from gingival areas.

All the cases presented granulation tissue or cicatricial collagen within the dermis/corion. In cutaneous scars, the inflammatory infiltrate consisted of lymphocytes and macrophages; 17 cases (53.12%) presented multinucleated giant cells, in some cases, in relation to translucid not structured material (suture material). In oral mucosa scars, the inflammatory infiltrate consisted of lymphocytes, macrophages, and, in frequent cases (18 cases, 56.25%), numerous plasma cells were present, occasionally with intracytoplasmic hyaline inclusions (Russell's bodies) as evidence of immunoglobulin production; no multinucleated giant cells were present in oral scars. Very scanty plasma cells were present in cutaneous scars.

3.2. Immunohistochemical Expression of Signaling Molecules in Cutaneous versus Oral Mucosa Scars (Data Are Summarized in Table 2)

3.2.1. TGFbeta1, TGFbeta2, and TGFbeta3. TGFbeta1 was expressed mainly in endothelial cells (27 positive cases, 84.37%; 17 cases 1+ and 10 cases 2+) and mesenchymal cells (25 cases, 78.12%; 24 cases 1+ and one case 2+) and less frequently and fainter in macrophages (12 cases, 37.5%; all of them 1+ positive) and multinucleated giant cells (9 cases, 1+ of 17 cases with multinucleated giant cells) (Figure 2(a)).

TGFbeta2 was negative in mesenchymal cells, endothelial cells, and macrophages. Multinucleated giant cells were usually faint positive (13 cases, 1+ of 17 cases with multinucleated giant cells, 76.47%) (Figure 2(b)).

TGFbeta3 was expressed mainly in endothelial cells (26 positive cases, 81.25%; 12 cases 1+ and 14 cases 2+) and mesenchymal cells (23 cases, 71.87%, all faint positive 1+) and less frequently in macrophages (14 positive cases, 43.74%; 7 cases 1+ and 7 cases 2+) and multinucleated giant cells (10 positive cases, 58.82%; 5 cases 1+ and 5 cases 2+ of 17 cases with multinucleated giant cells) (Figure 2(c)).

In the case of TGFbeta1, the only statistical association was recorded for TGFbeta1 expression in mesenchymal cells in correlation with the age of the scar—we noticed a tendency towards lack and/or diminishing of TGFbeta1 expression in mesenchymal cells in the scars of 2–5 weeks compared with younger (less than 2 weeks) or older (more than 5 weeks) cutaneous scars—$P = 0.05$ (Figure 2(d)). There was a statistically significant correlation between TGFbeta3 expression in

Table 2: Comparative summary of the differences between the findings of the expression of signaling molecules in cutaneous versus oral mucosa scars.

Type of cells	Cutaneous scars	Oral mucosa scars
TGFbeta1		
Mesenchymal cells	74.99% 1+ 3.12% 2+	100% 2+
Endothelial cells	53.12% 1+ 31.25 2+	100% 2+
Macrophages	37.5% 1+	100% 2+
Multinucleated giant cells	52.94% 1+	Not present
Plasma cells	Not present	56.25% 2+
TGFbeta2		
Mesenchymal cells	Negative	Negative
Endothelial cells	Negative	Negative
Macrophages	Negative	Negative
Multinucleated giant cells	76.47% 1+	Not present
Plasma cells	Not present	Negative
TGFbeta3		
Mesenchymal cells	71.87% 1+	Negative
Endothelial cells	37.5% 1+ 43.75% 2+	100% 2+
Macrophages	21.87% 1+ 21.87% 2+	50.00% 1+ 50.00% 2+
Multinucleated giant cells	29.41% 1+ 29.41% 2+	Not present
Plasma cells	Not present	56.25% 2+
TNFalpha		
Mesenchymal cells	46.87% 1+	Negative
Endothelial cells	25.00% 1+	Negative
Macrophages	43.74% 1+	Negative
Multinucleated giant cells	70.58% 1+	Not present
Plasma cells	Not present	28.12% 1+ 28.12% 2+
PDGF BB		
Mesenchymal cells	43.75% 1+ 6.25% 2+	Negative
Endothelial cells	59.36% 1+ 21.87 2+	100% 2+
Macrophages	65.63% 1+ 21.87% 2+	100% 2+
Multinucleated giant cells	70.58% 1+ 23.53% 2+	Not present
Plasma cells	Not present	56.25% 2+
FGF1		
Mesenchymal cells	34.37% 1+ 15.62% 2+	Negative
Endothelial cells	52.38% 1+ 16.37% 2+	100% 2+
Macrophages	71.87% 1+ 6.25% 2+	100% 1+
Multinucleated giant cells	82.34% 1+ 11.76% 2+	Not present
Plasma cells	Not present	56.25% 1+

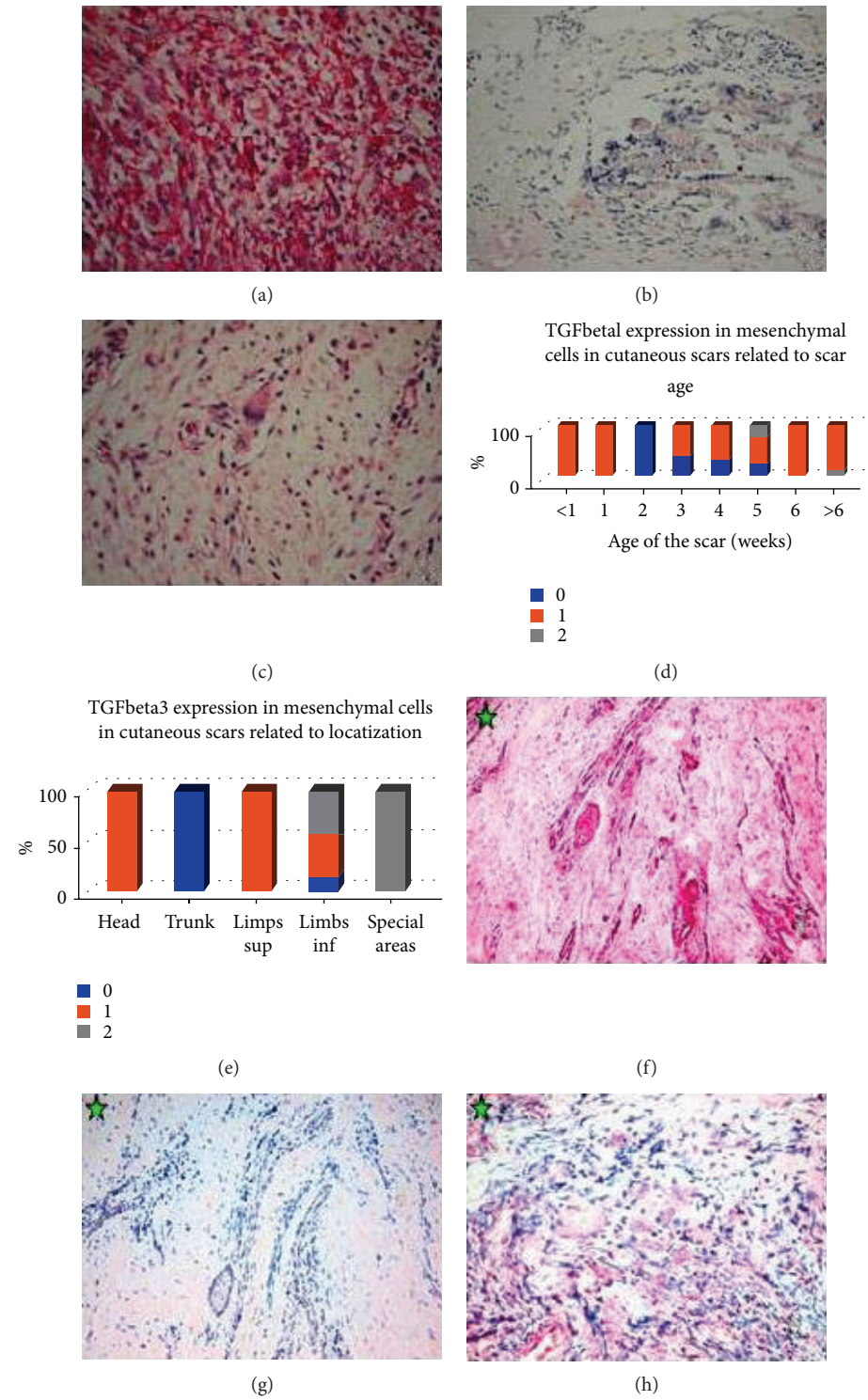
mesenchymal cells and localization of the scar—no scars on the trunk had TGFbeta3 expression in mesenchymal cells while all the scars from special areas had intense diffuse positivity—$P < 0.001$ (Figure 2(e)).

In oral mucosa biopsies, TGFbeta1 was expressed in all the cases in mesenchymal cells, endothelial cells, macrophages, and plasma cells in all cases with obvious positivity in low power (2+) in almost all the cells (Figure 2(h)). TGFbeta2 was negative in all the cases (Figure 2(i)). TGFbeta3 was negative in mesenchymal cells and intensely diffuse positivity (2+) in endothelial cells and plasma cells; all the cases had TGFbeta3 positivity in macrophages, half of them (50.00%) being 1+, while the other half were intense diffuse positive (2+) (Figures 2(j)–2(k)).

3.2.2. TNFalpha. TNFalpha expression was faint in each type of cells we investigated (1+). Most frequent macrophages and macrophage-derived cells showed TNFalpha positivity (14 cases, 43.74% showed TNFalpha faint positivity in macrophages and 12 cases of 17, 70.58% showed similar positivity in multinucleated giant cells); less numerous cases had mesenchymal cells positivity (15 cases, 46.875%) and endothelial cells positivity (8 cases, 25.00%) (Figure 2(f)). We identified a statistically significant association between TNFalpha expression in endothelial cells and the age of the scar—scars 2 to 4 weeks old tend to express TNFalpha compared with very recent scars or older ones—$P = 0.036$ (Figure 2(g)). Interestingly, in week 4, all the cases showed TNFalpha positivity within endothelial cells (all male patients, scar located on the head, trunk, and axilla, resections after melanoma or basal cell carcinoma, no residual tumor present in either case).

In oral mucosa biopsies, TNFalpha was negative in all the cases in mesenchymal cells, endothelial cells, and macrophages; cases with plasma cells within the inflammatory infiltrate showed 1+ or 2+ positivity in plasma cells (Figure 2(l)).

3.2.3. PDGF BB. PDGF BB was expressed mostly in macrophages and multinucleated giant cells (macrophages; 28 cases, 87.50% (21 cases 1+ and 7 cases 2+) and multinucleated giant cells; 16 of 17 cases, 94.11% (12 cases 1+ and 4 cases 2+)) and also in endothelial cells in 26 cases, 81.25% (19 cases 1+ and 7 cases 2+) and mesenchymal cells in 16 cases, 50.00% (14 cases 1+ and 2 cases 2+) (Figures 3(a)–3(d)). There was a tendency towards PDGF BB overexpression in mesenchymal cells in scars located in the head area ($P < 0{,}001$) (Figure 3(e)) and PDGF BB overexpression in endothelial cells in scars located in the limbs and special areas ($P = 0, 05$) (Figure 3(f)); more powerful statistically significant figures were obtained when PDGF BB overexpression in endothelial cells in scars located in the limbs or special



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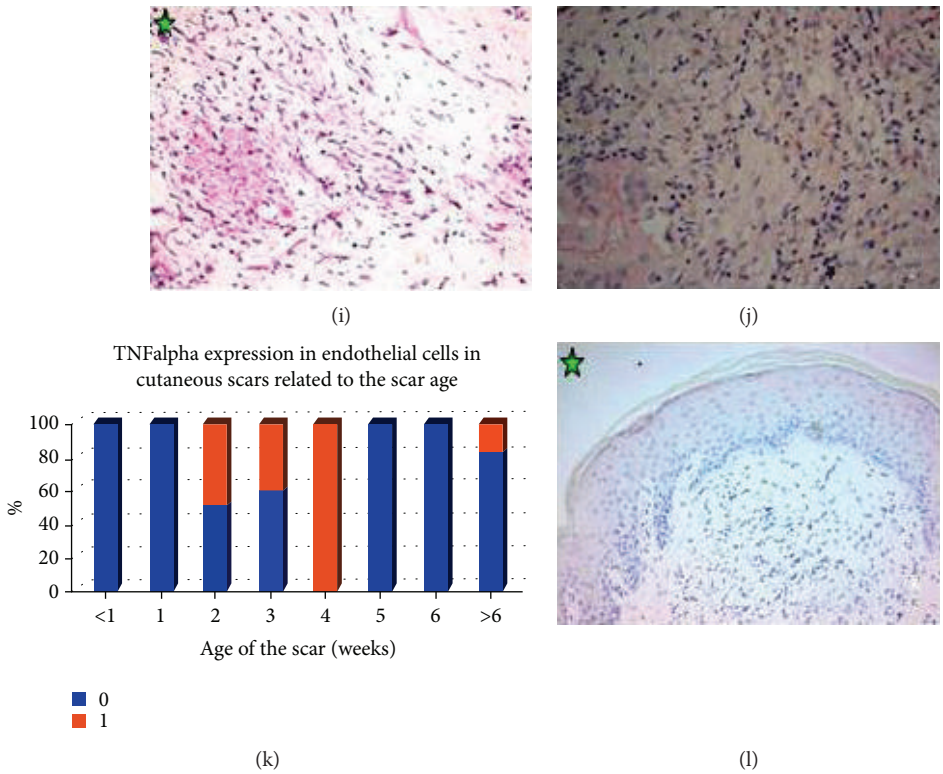
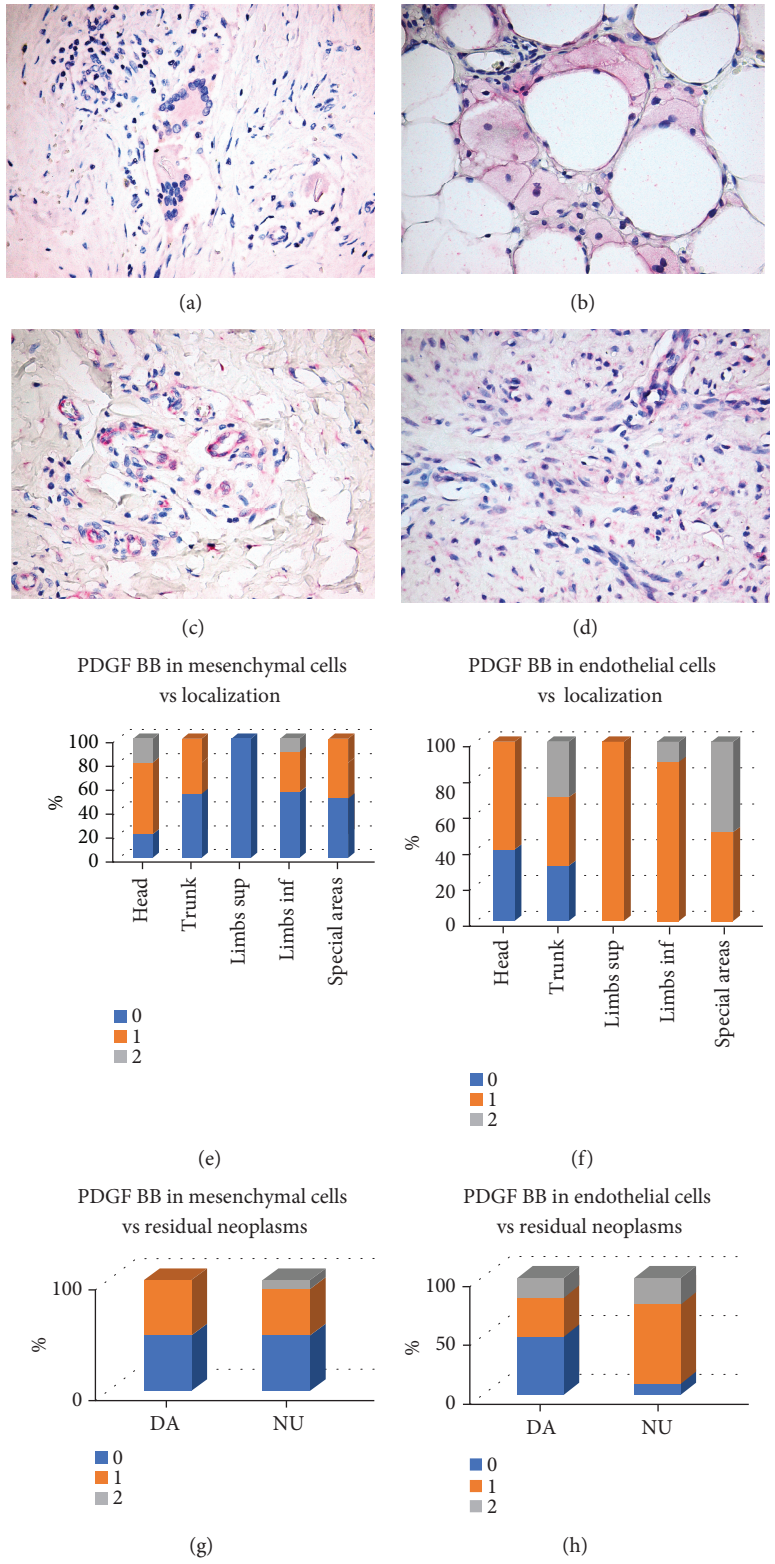


Figure 2: TGFbeta1, TGFbeta2, TGFbeta3, and TNFalpha expression. (a) Cutaneous scar. Intense positivity of TGFbeta1 (2+) in mesenchymal and endothelial cells. TGFbeta1 x400. (b) Cutaneous scar. Very faint positivity of TGFbeta2 in multinucleated giant cells engulfing foreign material (suture); macrophages, mesenchymal, and endothelial cells are negative. TGFbeta2 x200. (c) Cutaneous scar. TGFbeta3 intense positivity in macrophages, multinucleated giant cells, mesenchymal, and endothelial cells. TGFbeta3 x400. (d). TGFbeta1 expression in mesenchymal cells in cutaneous scars related to scar age. (e). TGFbeta3 expression in mesenchymal cells in cutaneous scars related to localization. (f). Oral mucosa scar. Intense positivity of TGFbeta1 (2+) in mesenchymal, endothelial cells, and macrophages. Small island of odontogenic epithelium also positive. TGFbeta1 x200. (g) Oral mucosa scar. Negativity for TGFbeta2. The island of odontogenic epithelium also negative TGFbeta2 x200. (h). Oral mucosa scar. Intense positivity of TGFbeta3 (2+) in endothelial cells and macrophages. TGFbeta3 x400. (i) Oral mucosa scar. Intense positivity of TGFbeta3 (2+) in endothelial cells and faint positivity in macrophages and multinucleated giant cells. TGFbeta3 x400. (j). Cutaneous scar. Faint positivity of TNFalpha in macrophages and multinucleated giant cells; mesenchymal and endothelial cells are negative. TNFalpha x400. (k) TNFalpha expression in endothelial cells in cutaneous scars related to the scar age. (l) Oral mucosa scar. Negativity for TNFalpha. TNFalpha x200.

areas were compared with PDGF BB expression in same cells in scars originating from the head or trunk (P trunk vs. limbs 0.012; P trunk vs. special areas <0.001; P head vs. limbs 0.002; P head vs. special areas 0.0001) (Figure 3(f)).

Also, PDGF BB was overexpressed in all types of cells (mesenchymal cells $P = 0.009$, endothelial cells $P = 0.001$, and macrophages $P = 0.0001$) in scars with no residual tumor (Figure 3(g)–3(i)) and in scars in female patients (mesenchymal cells $P = 0014$, endothelial cells $P < 0.001$, and macrophages $P < 0.0001$) (Figures 3(j)–3(l)). Interestingly, PDGF BB overexpression was noted in multinucleated giant cells in a scar with residual tumors and also in scars in male patients (in both circumstances $P < 0.001$); we looked in the group of scars with multinucleated giant cells that showed a female predominance compared with the general data of group A (70.58% females in scars with multinucleated giant cells compared with 59.38% in group A) and more numerous cases with residual tumor (23.53% in scars with multinucleated giant cells compared with 18.75% in group A), thus explaining the differences with PDGF BB expression in macrophages. In oral biopsies, PDGF BB was positive 2+ in all cases in endothelial cells, macrophages, and also in plasma cells when present; oral mesenchymal cells were negative for PDGF BB (Figures 3(m)–3(n)).

3.2.4. FGF1. FGF1 expression was noted in half of the cases (50.00%) in mesenchymal cells (11 cases 1+ and 5 cases 2 +), two-thirds (68.75%) in endothelial cells (16 cases 1+ and 5 cases 2+), and almost three quarters of cases (78.12%) in macrophages (23 cases 1+ and 2 cases 2+). All but one case (94.11%) showed FGF1 positivity in multinucleated giant cells (14 cases 1+ and 2 cases 2+) (Figures 4(a)–4(b)). We looked for FGF1 expression related to location. There was a statistically significant overexpression in the mesenchymal cells of scars from the limbs compared with the mesenchymal cells of scars from special areas ($P < 0.001$) and in the endothelial cells of scars from the head and trunk compared with the limbs and special areas ($P = 0.05$). Macrophages expressed more intense FGF1 in scars from the trunk or limbs compared with the head ($P = 0.007$ and $P = 0.0005$,



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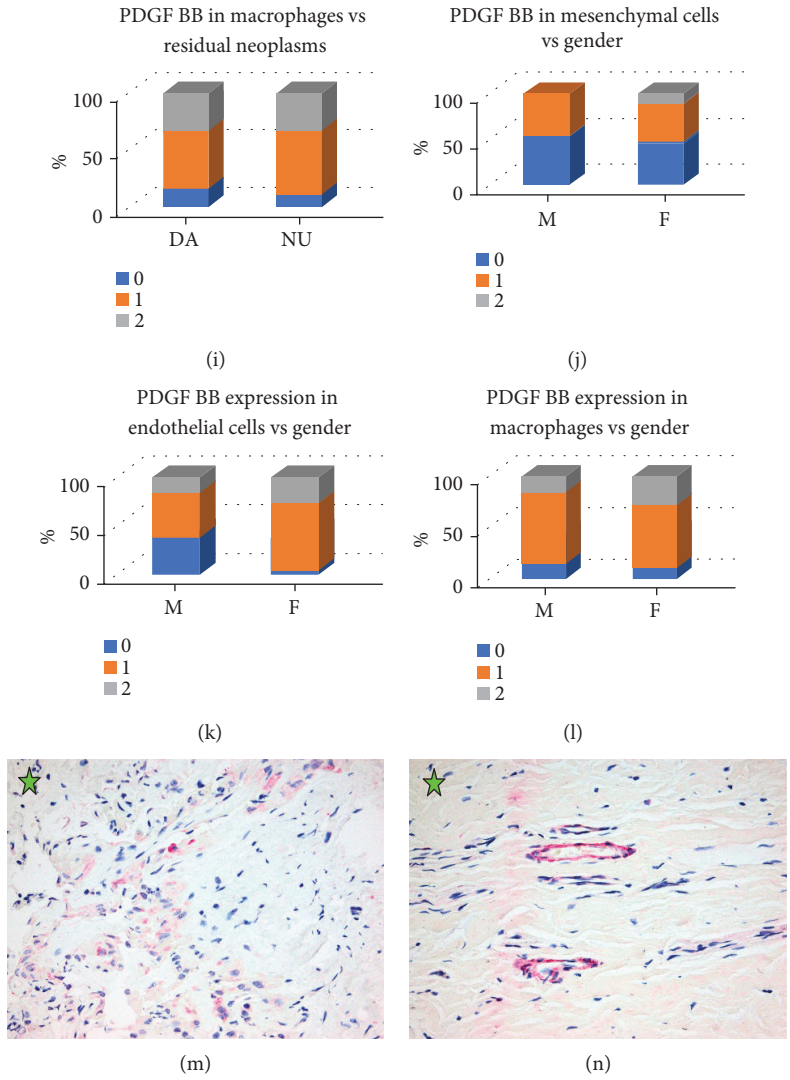

Figure 3: PDGF BB expression. (a) Faint positivity of PDGF BB (1+) in multinucleated giant cells. Cutaneous scar. PDGF BB x400. (b) Faint positivity of PDGF BB (1+) in macrophages. Cutaneous scar. PDGF BB x400. (c) Intense positivity of PDGF BB (2+) in mesenchymal and endothelial cells. Cutaneous scar. PDGF BB x400. (d). Faint positivity of PDGF BB (1+) in mesenchymal and endothelial cells. Cutaneous scar. PDGF BB x400. (e) PDGF BB expression in mesenchymal cells in cutaneous scars related to localization. (f) PDGF BB expression in endothelial cells in cutaneous scars related to localization. (g) PDGF BB expression in mesenchymal cells in cutaneous scars related to presence of residual neoplasms. (h) PDGF BB expression in endothelial cells in cutaneous scars related to presence of residual neoplasms. (i) PDGF BB expression in macrophages in cutaneous scars related to the presence of residual neoplasms. (j) PDGF BB expression in mesenchymal cells in cutaneous scars related to patient's gender. (k) PDGF BB expression in endothelial cells in cutaneous scars related to patient's gender. (l) PDGF BB expression in macrophages in cutaneous scars related to patient's gender. (m) Oral mucosa scar. Intense positivity of PDGF BB (2+) in macrophages. PDGF BB x400. (n) Oral mucosa scar. Intense positivity of PDGF BB (2+) in endothelial cells. PDGF BB x400.

respectively); also, they expressed FGF1 more often in scars from the limbs or special areas compared with the trunk or in scars from the limbs compared with special areas ($P < 0.001$ in all circumstances) (Figures 4(c)–4(e)). Moreover, FGF1 was overexpressed in all types of cells (mesenchymal cells, endothelial cells, macrophages, and multinucleated giant cells) in cases with residual neoplasm present in the vicinity of the scar tissue we examined ($P = 0.008$, $P = 0.012$, $P = 0.002$, $P < 0.001$, respectively) (Figures 3(f)–3(i)) and in female patients ($P = 0.009$, $P = 0.009$, $P = 0.001$, $P < 0.001$, respectively) (Figures 4(j)–4(m)). FGF1 expression in oral mucosa scars had similar positivity as PDGF BB: positive 2+ in all cases in endothelial cells, positive 1+ in macrophages, and negative in mesenchymal cells (Figures 4(n)–4(o)).

4. Discussions

We studied several growth factors expression in cutaneous and oral mucosa humans scars: TGFbeta1, TGFbeta2, TGFbeta 3, TNFalpha, PDGF BB, and FGF1.

4.1. TGFbeta1, TGFbeta2, and TGFbeta3. Our data showed the expression of TGFbeta1 (profibrotic growth factor) in all cases in oral specimens and numerous cases in skin fragments; however, there is an obvious relation to the age of the cutaneous scars, the young and old ones having less if any TGFbeta1 expression. TGFbeta2 (the other profibrotic factor) was positive in more than ¾ of the cases of skin specimens in multinucleated giant cells and absent in oral specimens. TGFbeta3 (with probable antifibrotic effects) was positive in all oral specimens in macrophages either intense or mild positivity, while less than half of the cutaneous fragments showed macrophagic positivity.

TGFbeta1, TGFbeta2, and TGFbeta3 belong to the TGFbeta/activin/Nodal subfamily of TGFbeta superfamily [32]. They are involved in numerous biological processes such as cellular differentiation, cell migration, apoptosis, cell-cycle arrest, production of extracellular matrix, epithelial to mesenchymal transition, or wound healing [33–35]. The overexpression of TGFbeta determines pathologic tissue fibrosis. TGFbeta1 is upregulated in early granulation tissue thus determining an increase in number and activity of SMA positive myofibroblasts thus stimulating neovascularization, collagen deposition, and wound contraction; all these processes are responsible for scar formation in adulthood. Wound in embryos predominantly express TGFbeta3 and to a significantly lesser extent, TGFbeta1; moreover, adding TGFbeta3 to a wound in adult tissues (or neutralizing TGFbeta1 and TGFbeta2) determines less or none scarring [36–42].

It is known that the major source of TGFbeta in a wound is represented by platelets; platelets release TGFbeta from their secretory granules in inactive form; part of it will be immediately activated (in the first moments of wound appearance) by trombospondin-1 and released together with TGFbeta from platelets secretory granules and also some time later by plasmin which disintegrates blood clot [43, 44]. The second wave of TGFbeta activation occurs after macrophages also occupy the wound territory via plasmin (macrophages secrete plasminogen activators) [45, 46].

TGFbeta is involved in all phases of wound healing. In the first phase (inflammation), TGFbeta are present from the very beginning. They are supplied in their inactive forms by platelets and are activated by thrombospondin1 and plasmin; in this stage, they act as chemotactic cytokines for neutrophils, monocytes, and macrophages [43, 47]. Later on, activated macrophages (local cells or differentiated from recruited circulating monocytes) release supplementary quantities of TGFbeta that stimulates angiogenesis, fibroblasts chemotaxis, and proliferation in second phase (proliferation, "granulation tissue formation") [48]. TGFbeta stimulates the differentiation of fibroblasts into myofibroblasts [40, 49] and stimulates the production of components of extracellular matrix (ECM): collagen, fibronectin, and fibronectin receptor [50]; also, by inhibiting both production and activity of matrix metalloproteinases (MMPs) and stimulating the expression of tissue inhibitors of matrix metalloproteinases (TIMPs), they determine a reduction of ECM components degradation [5]. TGFbeta activity in angiogenesis involves all aspects of the process; they promote endothelial cell migration and differentiation and also tubule formation [7].

In maturation phase, TGFbeta intervenes in a delicate balance between MMPs and TIMPs activation and also in increasing lysyl oxidases responsible of cross-linking of collagen with subsequent increase of tensile strength of the wound [51]. Studies performed on animals showed that TGFbeta1 occurs in the wound almost immediately after wound occurrence (5 minutes), then TGFbeta2 and TGFbeta3 occur, surpassing by far TGFbeta1 levels at 24 hours after wound occurrence; there is another peak of TGFbeta1 at 5 days after wound occurrence [52–54]. Also, in embryos, wounds express mainly TGFbeta3 (both in epithelial cells and fibroblasts), while TGFbeta1 and TGFbeta2 have very low levels of expression; in adults, wounds express mainly TGFbeta1 and TGFbeta2. Since wound in embryo heal without scarring while those in adults do not, it is a sound presumption that TGFbeta3 favor scar-free healing opening the gates for new therapies [55, 56].

4.2. TNFalpha. In our study, TNFalpha was negative in all cells but positive in plasma cells in oral specimens, while almost half of the cutaneous specimens showed TNFalpha positivity in macrophages; also, some skin cells (both mesenchymal and endothelial cells) had some positivity for TNFalpha also with a tendency towards expression in not so young or old scars. In week 4, all the patients (males, head, trunk, or axilla scars, resection after tumor without residual tumor present) showed TNFalpha expression in endothelial cells.

TNFalpha enhances the inflammation in wound repair, hence contributing to an impaired healing both in chronic and acute wounds. Studies revealed and increased TNFalpha levels (locally, in the wound territory, and systemically) in otherwise healthy elderly patients with active chronic venous ulcers. Also, in animals, experiments with secretory leukocyte protease inhibitor (SLPI) null mice showed that wound healing in these animals is deficient, and TNFalpha is increased in wound area (demonstrated both by RT-PCR and immunohistochemically in local inflammatory cells and epithelia); moreover, local TNFalpha inhibition with anti-TNFalpha antibody accelerates the rate of healing in a dose-dependent manner in SLPI null mice and also improve healing in wild-type mice used as controls [57].

TNFalpha induces the expression of MMP2 and MMP9 and inhibits the local accumulation of fibroblasts either by direct inhibition of chemoattraction or by attracting an impressive number of inflammatory cells in the wound area; both these actions diminish the collagen deposition and impair the wound healing [58, 59].

4.3. PDGF BB. Our study revealed PDGF BB positivity in all oral specimens in endothelial cells and macrophages but not in mesenchymal cells. In skin scars fragments, there were some expression in mesenchymal cells (half of the scars) with statistic association with feminine gender, head localization, and absence of residual tumor. Few cases of cutaneous scars had no PDGF BB expression in endothelial cells (18.75%) or macrophages (12.5%).

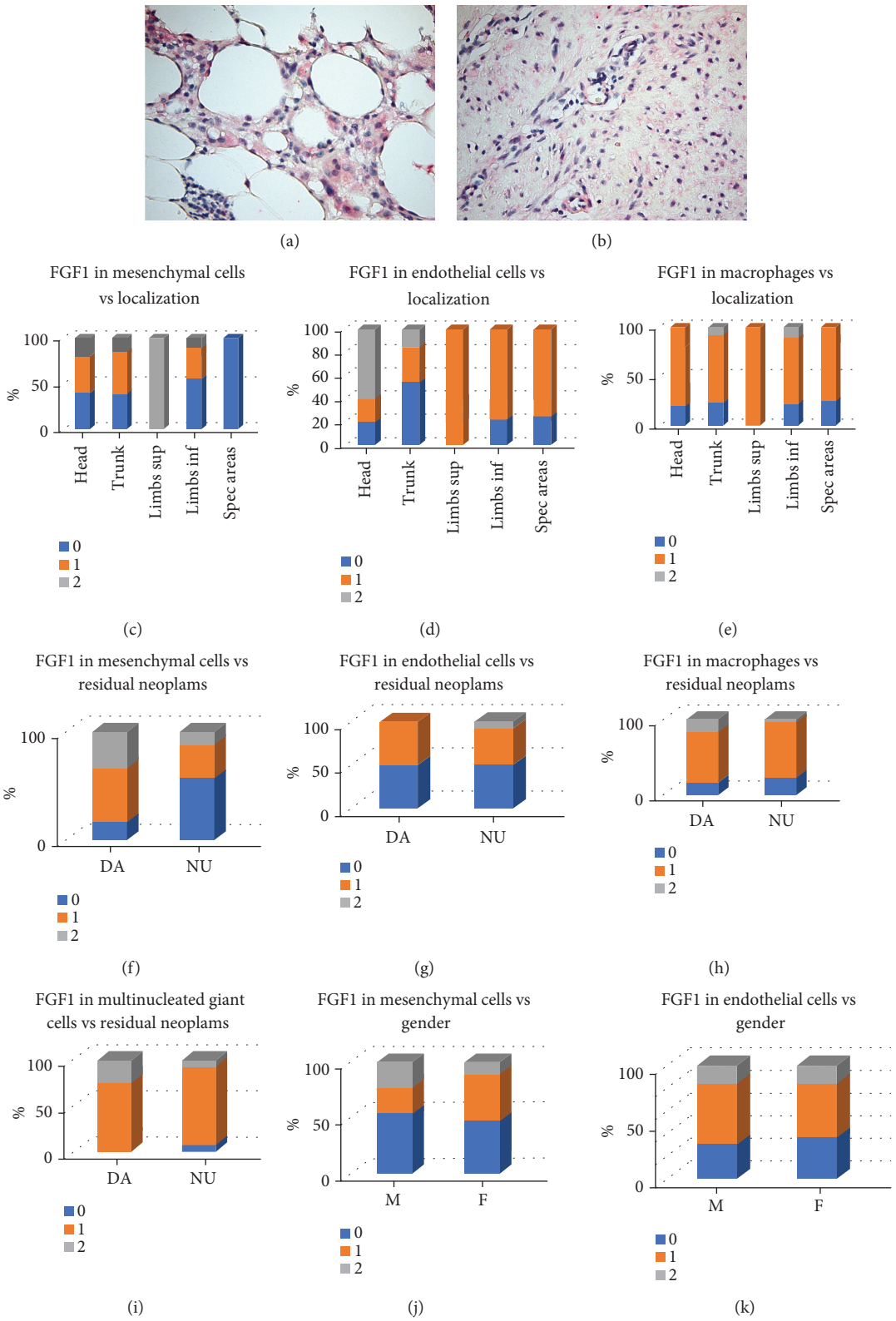

Figure 4: Continued.

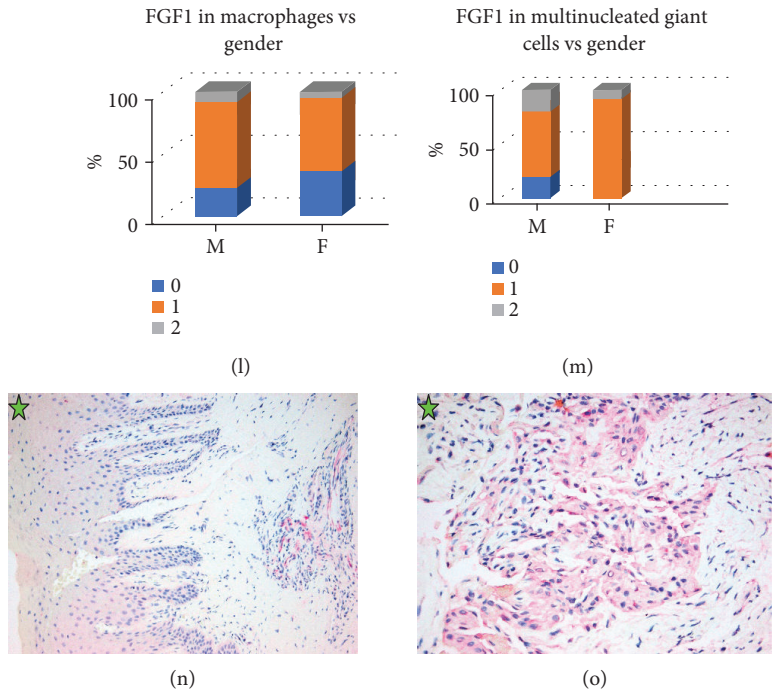

Figure 4: FGF1 expression. (a) Faint positivity of FGF1 (1+) in macrophages and multinucleated giant cells. Cutaneous scar. FGF1 x400. (b) Faint positivity of FGF1 (1+) in mesenchymal and endothelial cells. Cutaneous scar. FGF1 x400. (c) FGF1 expression in mesenchymal cells in cutaneous scars related to localization. (d) FGF1 expression in endothelial cells in cutaneous scars related to localization. (e) FGF1 expression in macrophages in cutaneous scars related to localization. (f) FGF1 expression in mesenchymal cells in cutaneous scars related to the presence of residual neoplasms. (g) FGF1 expression in endothelial cells in cutaneous scars related to the presence of residual neoplasms. (h) FGF1 expression in macrophages in cutaneous scars related to the presence of residual neoplasms. (i) FGF1 expression in multinucleated giant cells in cutaneous scars related to the presence of residual neoplasms. (j) FGF1 expression in mesenchymal cells in cutaneous scars related to patient's gender. (k) FGF1 expression in endothelial cells in cutaneous scars related to patient's gender. (l) FGF1 expression in macrophages in cutaneous scars related to patient's gender. (m) FGF1 expression in multinucleated giant cells in cutaneous scars related to patient's gender. (n) Oral mucosa scar. Intense positivity of FGF1 (2+) in endothelial cells. Hyperplasia of rete ridges specific to alveolar mucosa. FGF1 x200. (o) Oral mucosa scar. Faint positivity of FGF1 (1+) in macrophages. FGF1 x400.

PDGF is a dimeric glycoprotein with mitogenic effects on mesenchymal cells such as fibroblasts, osteoblasts, and smooth muscle cells; also, it is implicated in angiogenesis and fibrosis. Two types of subunits are identified A and B, three variants of PDGF being thus possible: PDGF-AA, PDGF-BB, and PDGF-AB [60]. PDGF stimulates reepithelialization, revascularization, and complete wound closure in ischemic skin and hyperglicemic mice, mainly due to its mitogenic effects on both keratinocytes and endothelial cells [61]. The association of PDGF-BB with TGF-alpha in topic application on wounds in genetically diabetic (C57BL/KsJ-db/db) mice induces the acceleration of healing to a level almost similar to nondiabetic mice [62]. The treatment of leg ulcers in diabetic patients with becaplermin gel (a recombinant human PDGF approved for topical applications) stimulates complete healing and shortens the time to healing [63]. However, caution should be exerted in becaplermin treatment due to the increased risk of both local infections (infected skin ulcer, cellulitis, and osteomyelitis) and death due to malignancies, currently the drug being no longer authorized [64].

4.4. FGF1. FGF1 expression was noted in our study in similar manner as PDGF BB—oral scars lack FGF1 in mesenchymal cells, while 50% of the skin scars had FGF1 mesenchymal cells positivity, statistically associated with the limbs location and residual tumor present in the vicinity of the scar. More numerous cases of skin scars lack FGF1 expression in endothelial cells and/or macrophages than PDGF BB, but all oral scars show FGF1 expression in these types of cells.

Fibroblast growth factor (FGF) family includes 22 members designated by numbers from FGF1 to FGF23 (FGF15 is lacking in humans). FGF1 (acidic FGF) has a large variety of functions; the most important ones being related to angiogenesis. It induces proliferation of endothelial cells with subsequent organization in tubes and formation of new vessels in myocardium [65]; its function as angiogenic factor overcomes that of consecrated angiogenic factors as vascular endothelial growth factor (VEGF) or platelet-derived growth factor (PDGF) [66]. Based on its angiogenic function, FGF1 is a major player in wound healing [67, 68]; it has decreased gene expression in early phases of wound healing in diabetic patients [69], and it

has a proven effect on reepitelization in NONcNZO10/LtJ mouse (model for impaired wound healing in type 2 diabetes) [70]. Also, FGF1 is involved in bone regeneration, and it is a promising biomolecule to be used in humans [71].

5. Conclusions

We identified the differences in the expression of profibrotic and antifibrotic factors in oral mucosa and skin scars; TGFbeta2 was positive in cutaneous multinucleated giant cells, TNFalpha was positive in cutaneous macrophages, and both were negative in oral mucosa while TGFbeta3 was positive in oral macrophages and mostly negative in cutaneous ones. PDGF BB and FGF1 were positive in oral endothelial cells and oral macrophages and negative in macrophages with opposite positivity pattern in cutaneous scars. Based on these findings, macrophage seems to be the key player in modulating pro- and antifibrotic processes in wound regeneration. Further studies are needed in order to establish the mechanisms favoring scarless healing and subsequent application in daily practice.

Data Availability

The datasets used and/or analyzed during the present study are included within the article; if supplemental information is needed, it is available from the corresponding author upon request except confidential data to whom access is restricted in order to protect patients' privacy.

Disclosure

The presented study will be integrated in the original part of the PhD thesis of author Mihai Bucur.

Conflicts of Interest

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

Authors' Contributions

Mihai Bucur, Octavian Dinca, and Cristian Vladan equally contributed to this work.

Acknowledgments

This work was partially supported by a grant of the Romanian Ministry of Research and Innovation, CCCDI-UEFISCDI (project number 61PCCDI/2018 PN-III-P1-1.2-PCCDI-2017-0341) within PNCDI-III.

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 1061780, 12 pages
https://doi.org/10.1155/2018/1061780

Review Article

Mediators of Inflammation – A Potential Source of Biomarkers in Oral Squamous Cell Carcinoma

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Received 11 July 2018; Accepted 25 October 2018; Published 12 November 2018

Academic Editor: Nejat K. Egilmez

Oral squamous cell carcinoma (OSCC) is the most common tumour of the oral cavity, associated with significant morbidity and mortality. It is a multifactorial condition, both genetic and environmental factors being involved in its development and progression. Its pathogenesis is not fully elucidated, but a pivotal role has been attributed to inflammation, strong evidence supporting the association between chronic inflammation and carcinogenesis. Moreover, an increasing number of studies have investigated the role of different mediators of inflammation in the early detection of OSCC. In this review, we have summarized the main markers of inflammation that could be useful in diagnosis and shed some light in OSCC pathogenesis.

1. Introduction

Oral cancer accounts for about 4% of all cancers. Histologically, over 90% of cases are diagnosed as oral squamous cell carcinoma (OSCC) [1]. OSCC is a destructive malignant tumour with an invasive behaviour and significant risk of metastases [2]. The most common localization is the tongue, but other frequent sites for tumour formation are the lips and floor of the mouth [3, 4]. OSCC is particularly diagnosed in the elderly, and although its pathogenesis is not fully understood, the main risk factors postulated include smoking, alcohol use, exposure to radiation and chemical carcinogens, infections, and immunosuppression [5–7]. With respect to infectious agents, a recent meta-analysis has shown that the human papillomavirus (HPV) genome is present in approximately one third of OSCC samples, HPV types 16 and 18 being the most commonly detected [8]. The most frequent premalignant lesions which can progress to OSCC are oral leukoplakia (OLK), oral lichen planus (OLP), and erythroplasia [5, 9]. A surgical approach of the tumour is the mainstay of treatment, but new therapies such as photodynamic therapy are promising, particularly in early-stage OSCC [10, 11].

The process of malignant transformation is complex and incompletely elucidated [12, 13]. The role of inflammation in carcinogenesis was first suggested by Rudolf Virchow in 1963 [14]. Since then, various studies have shown that chronic inflammation is a pathological response that can act to the detriment of the host and influences cell homeostasis and

various metabolic processes, inducing changes even at the genomic level, which can promote carcinogenesis [15]. Moreover, several studies have suggested a pivotal role of chronic inflammation in carcinogenesis through the modulation of proinflammatory cells and cytokine production [12, 16]. In this review, we summarize the main mediators of inflammation that might be involved in the pathogenesis of OSCC and could represent biomarkers for the early diagnosis of the tumour.

2. Tumour Inflammatory Cell Infiltrate

The cells of the inflammatory infiltrate along with the mediators they release play an essential role in the formation of a suitable microenvironment that allows an uncontrolled cell proliferation [17, 18]. Two pathways of inflammation involved in the promotion of carcinogenesis, namely, the intrinsic pathway mediated by tumour cells and the extrinsic pathway mediated by tumour-infiltrating immune cells, have been described [19]. The tumour environment is an important entity, including both immature and adaptive immune cells; among them, the main role is played by tumour-associated macrophages (TAMs) and T lymphocytes. TAMs correlate with tumour progression, their presence in the inflammatory infiltrate being an unfavourable prognostic factor and represent an important source of cytokines [20]. Macrophages have the ability to release numerous enzymes and mediators which interfere with angiogenesis, cell proliferation, and metastasis. For example matrix metalloproteinases lyse the extracellular matrix and subsequently promote metastasis, and growth factors (epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), etc.) favour cell proliferation [19, 21].

T lymphocytes can exert both inhibitory and stimulating effects on carcinogenesis through the cytokines they release; interleukin- (IL-) 6, IL-17, and IL-23 have a proinflammatory effect, favouring tumour progression, IL-12 and interferon-gamma (IFN-γ) exhibit an antitumour effect and tumour necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β), and IL-6 exert direct action on cell survival [20]. The development of a proinflammatory environment will enhance the interaction between tumour cells and their stromal cells that will underlie the initiation of carcinogenesis [22, 23]. Kullage et al. analysed the inflammatory infiltrate in OSCC and observed that lymphocytes represent the most abundant cell population. However, in poorly differentiated OSCC, the infiltrate was scarce. Therefore, they noticed a correlation between the infiltrate size and cell differentiation. This can be the result of the immunosuppression generated by malignant cells [24]. Malignant cells promote the migration of immunosuppressive cells into the tumour microenvironment resulting in the inhibition of immune response. The main recruited cells are regulatory T cells [25]. Thus, an increased number of lymphocytes in the inflammatory infiltrate is associated with a better prognosis while a decreased number of lymphocytes is associated with a lower survival rate. Other unfavourable prognostic factors include smoking, poorly differentiated tumours, and tumour localization (tongue) [26].

In order to establish the role of the inflammatory infiltrate, Mashhadiabbas analysed 125 samples from patients diagnosed with dysplasia (mild, moderate or severe) or OSCC, and found a positive correlation between the intensity of inflammatory infiltrate and lesion severity. The most abundant inflammatory infiltrate was observed among OSCC patients [27]. Lo Muzio et al. also studied the inflammatory infiltrate that accompanies OSCC and noticed a dense inflammatory infiltrate in well- and moderately differentiated tumours. In contrast, in the case of poorly differentiated tumours, a small amount of inflammatory infiltrate was revealed [28].

The study by Pellicioli et al. found an increased level of CD8 lymphocytes in OSCC compared to dysplastic lesions. In addition, they found a lower number of mature dendritic cells and an increased number of immature dendritic cells in OSCC samples compared to oral epithelial dysplasia (OED), a fact that might be involved in carcinogenesis [29]. Furthermore, Fang et al. have shown that the increased expression of CD8 lymphocytes in inflammatory infiltrate in OSCC specimens may be seen as a favourable predictor of survival. However, they have noticed that CD57 expression is a better survival marker than CD8 and CD4 [30].

Numerous studies have established the role of mast cells in allergies and inflammation, but recent research has shown that mast cells are also involved in angiogenesis and carcinogenesis [31]. Their role in tumour development and progression is debatable given that in some cancers, mast cell infiltration was correlated with a good prognosis but in others with an unfavourable outcome [32]. The role of mast cells in carcinogenesis is supported by the release of numerous cytokines, chemokines, and angiogenic factors [33–35]. Histamine, heparin, and FGF are among the most important mediators involved in angiogenesis [36].

The study by Kabiraj et al. revealed a positive correlation between mast cell infiltration and microvessel density in OSCC samples, suggesting the possible role of mast cells in tumour angiogenesis [37]. In contrast, the study by Kalra et al. showed a decrease in mast cell infiltration and an increase in the number of blood vessels from well-differentiated to poorly differentiated OSCC and concluded that mast cells are not the main regulators of tumour angiogenesis [36]. However, the study by Jain et al. highlighted that the presence of a high amount of mast cells and eosinophils in the tumour microenvironment is associated with a good prognosis in OSCC [38].

3. Markers of Inflammation in OSCC

To date, there are no reliable biomarkers for the detection of malignant transformation in early stages. In this sense, a series of markers have been studied; among them, markers of inflammation have attracted attention given the role of inflammation in carcinogenesis [39].

3.1. Systemic Inflammatory Response. An increasing number of studies have focused on the role of the systemic inflammatory response (SIS) as a prognostic factor in cancer [40]. The pathogenesis of SIS is unknown. However, some hypotheses have been postulated. It has been suggested that SIS may be

the effect of the tumour hypoxia or necrosis or the consequence of the local tissue injury [41]. The most used biomarkers that reflect a SIS are white blood cell subtypes and C-reactive protein (CRP).

3.1.1. NLR, LMR, and PLR. One of the most studied markers is the neutrophil-to-lymphocyte ratio (NLR). In many studies, NLR has been shown to be an unfavourable prognostic indicator for various diseases including neoplasia. Moreover, an increased NLR correlates with chronic inflammation [42]. A systematic review by Guthrie et al. showed that NLR is increased in patients with advanced and aggressive cancers [40].

Charles et al. performed a study on 145 patients with head and neck squamous cell carcinoma (HNSCC) and concluded that NLR can be used as a prognostic factor and a value higher than 5 is associated with a shorter survival; therefore, these patients should receive additional therapies that might prevent this outcome. In addition, it seems that NLR could be used to identify those patients at risk of relapse [43]. In line with this, in patients with oral cancer, Tsai et al. identified leukocytosis, monocytosis, neutrophilia, and elevated values of NLR associated with advanced cancer and undifferentiated tumour [44]. Moreover, Perisanidis et al. analysed 97 patients with OSCC with local invasion who were preoperatively treated with chemotherapy and observed that NLR is an independent marker for an unfavourable prognosis [45].

Eltohami et al. proposed SIS as a predictive factor in OSCC. To assess SIS, they determined the albumin level and lymphocyte-to-monocyte ratio (LMR). Low values of albumin level and LMR have been associated with advanced stages of the tumour. The low level of albumin was explained by the protein loss that accompanied advanced tumours [46]. Park et al. suggested a prognostic score system, which included the determination of NLR, LMR, and platelet-to-lymphocyte ratio (PLR). To assess the effectiveness of the score, they performed a study on 69 patients with OSCC and determined the aforementioned parameters. Low LMR and increased NLR and PLR were associated with high-grade lesions. Decreased LMR and increased NLR correlated with tumour size and increased PLR with significant lymph node involvement. These biomarkers could be useful in the follow-up of patients with OSCC [47].

3.1.2. CRP. CRP is an acute-phase protein belonging to the pentraxin family. CRP is synthesized in the hepatocytes, primarily under the stimulation of IL-1 and IL-6. Elevated serum levels occur in case of inflammation, infection, or injury [48]. It is worth pointing out that a tumour can trigger an inflammatory response, resulting in the release of proinflammatory cytokines such as IL-6 and IL-1β. Thus, an increasing number of studies have revealed elevated levels of CRP in cancer [49, 50]. However, the direct link between CRP and cancer is still a debatable topic.

Allin et al. conducted a study that included 10,408 individuals, selected from the general population, and measured the serum levels of CRP. The subjects were followed for up to 16 years, and 1624 of them developed a type of cancer. The study demonstrated that elevated levels of CRP in individuals without cancer represent a risk factor for the development of a type of cancer [51]. In line with this, the meta-analysis by Guo et al. has shown that elevated levels of CRP are associated with an increased risk of cancer. In fact, the relationship between cancer and inflammation is bidirectional. The tumour leads to an inflammatory response including increased serum levels of CRP on the one hand, and chronic inflammation can be involved in the development of a malignant process, on the other hand [52].

The study by Metgud and Bajaj assessed serum and salivary levels of CRP in 20 patients with premalignant lesions, 20 OSCC patients, and 20 healthy subjects. Serum and salivary levels of CRP were highest in OSCC patients, and the lowest values were recorded in healthy subjects. CRP levels showed a graded increase according to the severity of the tumour. They suggested that CRP could be regarded as a prognosis marker in OSCC [53]. Khandavilli et al. highlighted that the elevated level of preoperative CRP is an unfavourable prognostic indicator [54].

Moreover, Chen et al. proposed CRP as a potential marker of OSCC aggressiveness. They found elevated levels of CRP in patients with advanced disease and lymph nodes or bone involvement. In addition, there was an association with the prognosis of the disease, patients with elevated CRP levels having a lower survival rate [55]. The same idea is supported by the study of Tai et al. on 343 patients with OSCC, which has shown a positive correlation between high CRP levels ($\geq$5.0 mg/L) and oral cancer and revealed that the CRP level correlated with local and lymph nodes invasion. Regarding OSCC localisation, the best correlations were obtained in the case of buccal mucosa involvement [56].

Some investigators studied CRP in conjunction with other parameters. The study by Blatt revealed that CRP, hemoglobin, and ferritin could be used as biomarkers in prognosis and disease progression [57]. In the study by Park, the increased CRP/albumin ratio was associated with disease extension and a low survival rate, proving to be an accessible and useful marker for prognosis [58]. In addition, another study identified that elevated levels of CRP; a high number of total leukocytes, monocytes, and neutrophils; and a low number of lymphocytes correlate with a low survival rate in OSCC patients [59].

3.2. NF-κB. The nuclear factor kappa-beta (NF-κB) belongs to the Rel/NF-κB family of transcription factors [60]. The activation of NF-κB, a key event in the inflammatory process, is identified in various tumours and linked to carcinogenesis. NF-κB has been shown to be involved in angiogenesis, inhibition of apoptosis, and cell proliferation [61, 62]. The activation of NF-κB is initiated by various stimuli such as carcinogens, viral proteins, oncogenes, or infectious stimuli. The overexpression of NF-κB has been identified in many tumours, and its suppression has been associated with the inhibition of cell proliferation and promotion of apoptosis [63].

However, the role of NF-κB in carcinogenesis is controversial. The study by Piva et al. revealed a positive correlation between the overexpression of NF-κB and the amount of inflammatory infiltrate in oral dysplastic lesions [64]. The study conducted by Bancroft emphasized that the increased

expression of IL-1α in HNSCC induces the activation of transcriptional factors, NF-κB and AP-1, and IL-8 expression. Furthermore, they observed that the epidermal growth factor receptor (EGFR) promotes NF-κB activation in a murine model of SCC. Studies have shown that the inhibition of NF-κB and EGFR is associated with beneficial effects in HNSCC resulting in the prevention of cell proliferation and stimulation of a cytotoxic response [65]. In contrast, van Hogerlinden et al. found that the inhibition of Rel/NF-κB signalling in epithelial cells leads to an imbalance in cell development and differentiation with increased apoptosis of keratinocytes and the de novo appearance of squamous cell carcinomas. Thus, in a particular context, NF-κB inhibition may play a role in the development of a malignant tumour [66].

The study by Alam et al. analysed whether there is a correlation between B-cell lymphoma protein 2 (BCL-2) gene expression and AP-1 and NF-κB transcription factors. They evaluated samples of normal mucosa, primary oral tumour (PT), and recurrent chemo- and radioresistant oral tumour (RCRT). Regarding NF-κB, the best correlation was observed with BCL-2 protein in the PT group and in the case of AP-1 in the RCRT group, suggesting the role of the two transcription factors in tumour progression or treatment resistance [67]. Wei et al. demonstrated that activation of NF-κB and hedgehog signalling pathways is associated with lower survival in those patients with esophageal SCC [68].

3.3. Cytokines. Cytokines are small proteins that in the past were called lymphokines or monokines depending on the cells that produced them. It is now known that any nucleated cell has the ability to secrete cytokines, but their main cell source is represented by helper T cells and macrophages [69, 70].

Cytokines have a key role in modulation of the immune response and are classified into two broad groups, proinflammatory (IL-1, IL-6, IL-8, TNF-α, and TGF-β) and anti-inflammatory (IL-2, IL-12, IL-4, IL-10, and IFN-γ) cytokines [71]. Among the cytokines that may be involved in oral cancer, interleukins seem to have a crucial function, the most studied being IL-4, IL-6, IL-8, and IL-10 [72].

3.3.1. Proinflammatory Cytokines – IL-6 and IL-8. IL-6 is synthesized in acute inflammatory response contributing to host defence. It has been shown to be involved in processes such as inflammation, immune response control, hematopoiesis, and oncogenesis. However, under certain conditions, elevated levels of IL-6 may lead to disturbances of the immune response [71, 73–75]. IL-6 can induce the transition from acute to chronic inflammation by recruiting monocytes to the site of inflammation through monocyte chemoattractant protein-1 (MCP-1) secretion [76].

Another proinflammatory cytokine which attracted the attention of investigators is IL-8, considered the prototype molecule in the chemokine class. IL-8 plays also an important role in the acute inflammatory response and persists for a relatively long time at the site of inflammation [77]. Its release from macrophages and neutrophils is activated by NF-κB. Moreover, its expression is modulated by other various stimuli, such as inflammation, hypoxia, or steroid hormones. IL-8 binds to CRCX-1 and CRCX-2 receptors which have been identified both on inflammatory cells from the tumour-associated infiltrate and tumour cells [78, 79].

Wang et al. analysed 86 samples of OSCC and noticed higher expression of IL-6 receptor (IL-6R) and IL-6 mRNA compared to samples from tumour-free mucosa. The study revealed a positive association between IL-6R expression, tumour size, and histopathological stage. Moreover, expression of IL-6 mRNA was correlated with advanced disease (lymph node involvement and distant metastases) [80]. Sato et al. suggested that the posttreatment level of IL-6 might be a marker for the early detection of a locoregional recurrence [81]. In addition, it seems that IL-6 expression is associated with resistance to chemotherapy [82].

Schiegnitz et al. showed higher serum levels of IL-6, IL-8, and soluble IL-2 receptor (sIL-2R) in patients with OSCC compared to those with oral premalignant lesions and healthy subjects. Regarding the differentiation of oral premalignant lesions by OSCC, the best results were based on the IL-6 level. Higher sensitivity and specificity were obtained by measuring the levels of both IL-6 and IL-8. [83]. Similarly, Punyani and Sathawane analysed the salivary level of IL-8 in patients with premalignant oral lesions and OSCC and in a control group. They found a statistically significantly higher salivary level of IL-8 in patients with OSCC than in those with premalignant lesions. However, no statistical significance was obtained when comparing the premalignant group with the control group. They suggested that the salivary level of IL-8 could be used as a biomarker for OSCC, but not for premalignant lesions [84]. The study by Rao et al. showed that IL-8 could be involved in tumourigenesis by activating NF-κB and STAT signalling pathways and concluded that chronic inflammation plays a key role in malignant transformation, IL-8 being a modulator of inflammation that should not be neglected [85].

Other research has also investigated the possible role of salivary cytokines in OSCC detection. The study included 9 patients with OSCC and 9 healthy subjects; TNF-α, IL-1α, IL-6, and IL-8 were measured. However, only the salivary levels of IL-6 were statistically significantly higher in patients with OSCC compared to the control group [39].

The study by Lee et al. on 41 patients with OSCC and 24 patients without oral malignant lesions evaluated an extended group of biomarkers in both plasma and saliva. The salivary determinations of eotaxin, IFN-γ, macrophage inflammatory proteins- (MIP-) 1β, IL-1β, IL-6, IL-8, and TNF-α were significantly higher in OSCC compared to controls. Regarding plasma determinations, significant differences between groups were observed only for IFN-γ-inducible protein 10 (IP10). Elevated plasma levels of eotaxin, granulocyte colony-stimulating factor (GCSF), and IL-6 were detected in advanced stages, and they have been proposed as markers of advanced OSCC [86].

In contrast, Czerninski et al. investigated the serum levels of IL-1, IL-6, IL-8, IL-10, and soluble IL-2 receptor in patients with precancerous oral lesions, OSCC, and post-OSCC status and they did not find statistically significant differences between the studied groups. They concluded that the investigation of serum levels of these parameters has little utility in the early detection of OSCC [87].

3.3.2. Anti-inflammatory Cytokines - IL-10 and IL- 4. IL-10 is one of the most important interleukins with an anti-inflammatory role, being produced by numerous activated cells (B and T lymphocytes, macrophages, mast cells, etc.). IL-10 inhibits the release of proinflammatory mediators from macrophages and the antigen presentation [88]. IL-4 also exhibits an inhibitory effect on inflammation and angiogenesis and is particularly secreted by activated memory T cells [89]. IL-10 and IL-4 suppress the local immune response and mediate the recruitment of regulatory T cells and TAM in the tumour microenvironment [12]. Moreover, it seems that IL-4 may inhibit the invasion in oral cancers by decreasing matrix-metalloproteinase-(MMP-) 9 expression [90].

The study by Arantes et al., which focused on the analysis of anti-inflammatory cytokines in OSCC patients, showed a higher expression of IL-10 and TGF-β2 in OSCC samples compared to normal mucosa [91]. The same result regarding IL-10 was obtained by Alhamarneh et al., who suggested that IL-10 could be an unfavourable prognostic factor. The serum level of IL-10 was statistically significantly higher in OSCC patients compared to the control group [92]. Another study evaluated the role of immunosuppressive cytokines (IL-4, IL-10, IL-13, and IL-1 receptor antagonist-IL-1RA) in the early detection of OSCC on 30 patients with OSCC and 33 healthy subjects. Salivary levels of IL-10 and IL-13 were significantly higher in OSCC patients compared to normal subjects. Therefore, the study concluded that IL-10 and IL-13 could be used as biomarkers in OSCC diagnosis. In addition, it was highlighted that IL-1RA levels were higher in those with undifferentiated tumour compared to those with differentiated tumour [93]. Another study associated the increased expression of IL-10 in tumour cells with a more aggressive tumour phenotype [94]. In contrast, the study by Hamzavi et al. on 30 patients with HNSCC and 24 normal controls found no statistically significant differences between the two groups with respect to the salivary and serum IL-10 level. However, the tissue analysis revealed that IL-10 expression was positive in 86% of patients with OSCC and was not identified in controls [95]. A recent study has suggested that IL-10 could be involved in the immune evasion of tumour cells. A percentage of 91% of OSCC samples exhibited an increased expression of IL-10 [96].

Tsai et al. observed that there was an association between IL-4 genes -590 C/T polymorphism and oral cancer, suggesting that it might represent a marker in oral cancer detection [97]. The study by Sun et al. which assessed the expression of several cytokines in OLP, OLK, and OSCC revealed increased expression of IL-4 in OSCC compared to OLK and OLP and raised the hypothesis that IL-4 has a low immunosuppressive role in the tumour microenvironment [12]. The study by Beppu et al. highlighted that IL-4 can suppress NF-κB activation and increase the production of MMP-9 in TNF-α-stimulated cells. Regarding IL-10, they did not observe these effects [90].

3.4. Cyclooxygenases. It has been observed that the association between increased expression of cyclooxygenase-(COX-) 2 and chronic inflammation is involved in the initiation of a carcinogenic process. Cyclooxygenases are enzymes that convert arachidonic acid to prostaglandins. Two main isoforms, COX-1 and COX-2, have been described [98]. COX-1 is expressed by the majority of cells and participates in various physiological events [99]. In contrast, under normal conditions, COX-2 is not expressed in the cells of the body, but under the influence of certain stimuli such as inflammation, it will be expressed [100]. Thus, COX-2 plays an important role in inflammation, but in recent years its role in carcinogenesis has also been studied. It seems that COX-2 is directly or indirectly involved in cell proliferation, inhibition of apoptosis, and angiogenesis [99, 101]. The role of COX-2 in carcinogenesis is also suggested by studies which have revealed a lower risk of developing a malignant tumour in those patients receiving COX-2 inhibitors [102]. Patel et al. pointed out that COX-2 overexpression could be correlated with the risk of recurrence of OSCC [103].

Sinanoglu et al. proposed COX-2 and Ki67 as biomarkers for the malignant transformation of oral leukoplakia. They revealed a higher COX-2 expression in OSCC samples compared to oral intraepithelial leukoplakia and oral hyperkeratosis, with a positive correlation between COX-2 levels and the severity of the lesions being observed. Similar results were found for Ki67 [104]. The study by Seyedmajidi et al. revealed higher COX-2 levels in OSCC compared to dysplastic and normal mucosal lesions. COX-2 levels showed a graded increase according to the severity of dysplastic lesions, but no correlation has been obtained with the severity of OSCC lesions [105]. In contrast, the study by Shibata et al. analysed normal, dysplastic, and OSCC samples and observed higher COX-1 and COX-2 levels in dysplastic lesions compared to OSCC; the levels increased according to the severity of the lesions, a fact that suggests the role of COX-1 and COX-2 as markers of early carcinogenesis. In OSCC lesions, a negative correlation between COX-1 and COX-2 levels and tumour histological grade was observed [106].

3.5. Matrix Metalloproteinases. Matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes, which encompass over 20 members with proteolytic activity that can disintegrate almost any extracellular matrix component. MMPs take part to many physiological processes such as tissue remodelling or regeneration but also in pathological conditions such as inflammation and tumourigenesis, by abnormal activation [107–109]. MMPs are involved in cell migration and regulate the level of cytokines at the site of inflammation, where, in turn, activated inflammatory cells release MMPs. It is worth noting that under such conditions, MMPs act on non-matrix substrates. It seems that they can activate or inactivate cytokine functions and subsequently promote or inhibit the inflammatory response [110]. Studies increasingly link MMPs to cancer [111, 112]. The main mechanisms postulated which support the hypothesis that MMPs participate in carcinogenesis include regulation of tumour cell growth and angiogenesis, modulation of apoptosis, extracellular matrix degradation, which facilitates tumour invasion, and epithelial to mesenchymal transition [107, 113]. Several researchers have analysed the role of MMPs as markers in OSCC.

The study by Chang et al., which investigated markers of inflammation in patients with oral leukoplakia and OSCC, showed that the most reliable markers are MMP-2, MMP-9, CRP, TGF-β1, and E-selectin. MMP-9 was the marker that best correlated with the evolution from normal tissue to leukoplakia and neoplasm. In addition, the study showed that the risk of relapse is greatest in patients with CRP $\geq$2 mg/L and E − selectin $\geq$ 85 ng/mL at baseline. The panel of the five studied markers was associated with a sensitivity of 67.4% for premalignant lesions and 80% for malignant tumours and a specificity of 90%, for the identification of the patients at risk of malignant transformation [114]. Another recent study has suggested that MMP-10 could also be a marker of malignant transformation of normal mucosa into OSCC [115].

Makinen et al. evaluated the expression of MMP-7 and MMP-25 in 73 patients with oral tongue SCC in an early stage. A percentage of 90% of the tumours expressed MMP-7 and MMP-25. The increased expression of MMP-7 was identified in poorly differentiated tumours and correlated with a higher risk of ocular metastases and a higher degree of local invasion. In contrast, MMP-25 expression did not correlate with any prognostic factors [116]. The study by Lawal et al. highlighted the increased expression of MMP-2 in poorly differentiated OSCC and a lack of expression of MMP-8; MMP-8 was identified in well-differentiated tumours. They suggested that MMP-2 could be a marker of tumour aggressiveness, and MMP-2 inhibitors could be used in therapy [117].

One of the phenomena that occur during the local invasion and subsequent metastasis is the degradation of the extracellular matrix and basal membrane [115]. Based on this fact, Tanis et al. evaluated the potential role of MMPs in metastatic OSCC. They included 12 patients with metastatic OSCC and 12 healthy controls and emphasized that the expression of MMP-1, MMP-3, MMP-9, and MMP-10 was greatest in those with metastatic OSCC, demonstrating the role of MMPs as early markers of metastatic tumour [118].

3.6. Galectins. Galectins are animal lectins with affinity for beta-galactosides. Galectins contain carbohydrate-recognition domains, which are involved in carbohydrate binding. Carbohydrate binding activity plays an important role in the various functions of galectins [119]. Numerous studies have investigated the expression of galectins in cancer patients. To date, according to the meta-analysis of Thijssen, there are over 200 studies, and most of them (>70%) have analysed the expression of galectin- (gal-) 1 and gal-3. With regard to the type of cancer, over half of the studies included patients with cancers of digestive or reproductive system [120]. In neoplasms, galectins have a dual role, pro- or anti-tumoural, depending on the type of cancer [121]. It seems that galectins modulate various biological processes, being regulators of adaptive immunity, homeostasis, tissue regeneration, and angiogenesis [122]. Galectins participate in immune response through diverse mechanisms, including the promotion of inflammation, stimulation of T cells, and modulation of regulatory T cell activity [9].

Noda et al. showed that the evaluation of the immunohistochemical expression of gal-1 in samples taken from the oral cavity could discriminate between neoplastic processes and reactive changes, decreasing the rate of false-positive and -negative results [123]. In addition, it could be used as a prognostic factor [124]. Aggarwal et al. proposed serum levels of gal-1 and gal-3 as screening markers for those patients at high risk of developing OSCC [125]. Another study has suggested the role of gal-9 in differentiating OSCC from premalignant lesions. Muniz et al. assessed the expression of gal-1, gal-3, and gal-9 in 40 OSCC samples and 40 premalignant lesions (20 OLP and 20 OLK) and 13 normal tissue samples. The expression of gal-9 was significantly higher in OSCC samples compared to premalignant lesions and healthy tissue. With respect to gal-1 and gal-3, the results were variable [126].

Mesquita et al. assessed the immunohistochemical expression of gal-3 and gal-7 in correlation with tumour clinical stage and histopathological grade in 32 patients younger than 45 years with OSCC. Gal-3 expression was identified in 65.6% of cases and did not correlate with the two studied parameters. In contrast, gal-7 expression was observed in a higher percentage, 96.9%, and a statistically significant correlation with the histological grade was obtained [127]. Another study suggested that gal-7 could be used as a marker of resistance to chemo and/or radiotherapy [128].

3.7. Markers of Oxidative Stress. Under physiological conditions, reactive oxygen species (ROS) exert beneficial effects being involved in the fight against infectious agents and preservation of cell homeostasis. However, under pathological conditions, the most common being conditions associated with chronic inflammation, ROS levels are increased and exhibit deleterious effects on cell components (lipids, proteins, and nucleic acids) [129–131]. The main consequence of chronic inflammation is an imbalance between oxidants and antioxidants, with increased production of ROS and decreased antioxidant protection. It was demonstrated that ROS are involved in carcinogenesis exerting effects on cell proliferation and apoptosis. It seems that ROS are the main chemical effectors acting at tissue level [132–135].

Huo et al. found elevated levels of ROS associated with low levels of antioxidant enzymes, in both blood and tumour tissue of the patients with OSCC. They revealed high levels of malondialdehyde (MDA) and nitric oxide (NO) and low levels of superoxide dismutase (SOD) and catalase (CAT) compared to the control group, suggesting the role of the imbalance between oxidants and antioxidants in carcinogenesis [136]. The same idea is supported by the study of Kumar et al. on 100 patients with HNSCC and 90 healthy subjects. The increased levels of ROS were associated with reduction of salivary total antioxidant capacity (TAC) and glutathione (GSH) level [137]. The study by Subapriya et al. revealed differences between the oxidative stress markers in tumour tissue and blood. Tissue determinations showed low lipid peroxidation in association with increased GSH-dependent antioxidant capacity. In contrast, increased lipid peroxidation and low antioxidant levels were detected in blood. It was therefore suggested that increased GSH-dependent antioxidant capacity represents the basis of a selective

development of cancer cells which is detrimental to the normal cells. The results obtained from blood are explained by the sequestration phenomenon of antioxidants in the tumour cells and the susceptibility of erythrocytes to lipid peroxidation induced by ROS [138].

Manoharan et al. have identified that the levels of thiobarbituric acid reactive substances (TBARS) and antioxidants correlate with tumour status; there was a progressive increase in TBARS levels and a decrease in antioxidants levels from stage II to stage IV [139].

4. Conclusion

OSCC, a tumour with a local invasive behaviour and an important metastatic capacity, would benefit from an early diagnosis, which in turn may contribute to a more favourable outcome. Starting from the hypothesis that inflammation plays an important role in carcinogenesis, various markers have been investigated in order to elucidate the involvement of different inflammation pathways in OSCC pathogenesis. Thus, either common markers such as serum CRP or white blood cell subtypes, as well as more sophisticated markers such as MMPs or COX-2, may provide new insights into the molecular mechanisms of tumour development and progression and could open new pathways in diagnosis, prognosis, and therapeutic approach for OSCC. The quest for the ideal biomarkers in oral cancer is currently ongoing, and further studies are needed in order to establish the most reliable candidate markers with the greatest impact on both scientific research and clinical practice.

Conflicts of Interest

The authors declare no conflict of interests.

Authors' Contributions

All authors have equally contributed to writing and editing the manuscript.

Acknowledgments

This work was partially supported by a grant of the Romanian Ministry of Research and Innovation, CCCDI-UEFISCDI (project number 61PCCDI/2018 PN-III-P1-1.2-PCCDI-2017-0341), within PNCDI-III.

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 6156757, 12 pages
https://doi.org/10.1155/2018/6156757

Research Article

CD47 Blockade Inhibits Tumor Progression through Promoting Phagocytosis of Tumor Cells by M2 Polarized Macrophages in Endometrial Cancer

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Received 20 May 2018; Accepted 16 August 2018; Published 7 November 2018

Academic Editor: Monica Neagu

There are rapidly emerging efforts to explore tumor-associated macrophages (TAMs) as a tumor therapy target. Tumor cells express CD47, which can interact with the macrophages' SIRPα transmitting a "don't eat me" signal to macrophages. The expression of CD47 increases in various tumors to evade immune attack. However, the expression of CD47 in endometrial cancer (EC) and the role of CD47-SIRPα in the TAMs which mediate the progression of EC remain unclear. Our study shows that there are increased TAMs in EC which dominantly consist of M2 macrophages and contribute to the progression of EC. We confirm that CD47 is highly expressed in EC tissue using the TCGA database, qPCR, and flow cytometry. Instead of directly promoting the apoptosis of EC cells, anti-CD47 blocking antibody promoted phagocytosis of EC cells by macrophages and the increased phagocytosis ability was mediated by M2 macrophages in a coculture assay. Besides, CD47 blockade inhibited the growth of the EC tumors *in vivo* and increased the infiltration of macrophages with antitumor ability in the tumor microenvironment (TME). These findings might assist in developing promising strategies that blocked the CD47-SIRPa interaction for EC therapy.

1. Introduction

Endometrial cancer is one of the most common gynecological malignancies, with 61,380 estimated new cases and 10,920 estimated deaths in 2017 in America [1]. Patients in less developed regions have poorer prognosis [2]. Novel therapeutic options are desperately needed. Tumor immunotherapies which target the tumor microenvironment to increase the antitumor activity of the immune system elicit durable responses in many kind of tumors [3, 4]. The tumor microenvironment (TME), which is composed of tumor cells, immune cells, tumor-associated fibroblasts, the vascular network, cytokines, and so on [5], tends to be polarized to an immunosuppressive state to facilitate the tumor immune evasion [6]. In endometrial cancer, neoplastic cells can exploit a large variety of immune evasion mechanisms, including alterations in the expression of some molecules that inhibit antitumor immune response, such as programmed cell death 1 ligand 1 (PD-L1) and indoleamine-2,3-dioxygenase (IDO) [7, 8]. Accumulating evidence indicates that anti-PD-1/PD-L immune checkpoint therapy may be effective in DNA polymerase epsilon- (POLE-) mutated and microsatellite instability (MSI) EC patients [9–11]. Considering that POLE-mutated and MSI EC patients account for a small

fraction of the total EC population (7%–12% and 20%–30%, respectively) and have better prognosis [12, 13], more universal drugs should be found.

Recently, the role of immune cells in the TME is well demonstrated in tumor progression and immunotherapy [5, 14]. Macrophages infiltrating into the TME are termed the tumor-associated macrophages (TAMs), which are the major component of infiltrating leukocytes in most tumors [15]. Macrophages are characterized by considerable heterogeneity and have been divided into two general subtypes: the classically activated M1 macrophages which have the potential to exhibit antitumor activity, and the alternatively activated M2 macrophages which are considered to be involved in tumor growth and progression [16]. TAMs tend to acquire a polarized M2 phenotype in many kinds of tumors with low antitumor activity through various mechanisms [17]. It is important to investigate the phenotype, phagocytosis ability, and antigen presenting ability of TAMs in EC.

Considering that TAMs contribute to the formation of an immunosuppressed state within the TME, one of the therapeutic strategies targeting TAMs is reeducating TAMs to an antitumor phenotype, such as promoting macrophages' phagocytosis ability [18, 19]. Accumulating evidences show that the CD47-SIRPα signal participates in tumor immune evasion mediated by TAMs [20, 21]. CD47 is a broadly expressed membrane protein on various tumor cells and plays an important role in self-recognition by which normal cells protect themselves from phagocytosis [21]. Signal regulatory protein alpha (SIRPα, also known as CD172a), which mainly expresses on the surface of macrophages, is the receptor for CD47. When CD47 binds to SIRPα, the intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs) of SIRPα is phosphorylated, followed by recruitment and activation of the tyrosine phosphatases such as SHP-1 and SHP-2. Then, the phosphoprotein substrates are dephosphorylated which affect downstream signaling pathways, transmitting a "don't eat me" signal to inhibit the macrophages' phagocytosis ability [22]. Accumulating evidences showed that CD47 was upregulated in many malignancies to evade the immune attack, and its overexpression was correlated with poor prognosis [23–26]. Besides, interruption of the ligation of CD47 and SIRPα promotes the tumor cells to be phagocytosed by macrophages in various malignancies [24, 27, 28]. A number of different drugs targeting the CD47-SIRPα signal are evaluated in patients with solid tumors in clinical trials (http://clinicaltrials.gov identifiers: NCT02216409, NCT02890368, NCT02953782, and NCT03013218).

Intriguingly, researchers found that CD47 was expressed on all cancer cells from patients [25], pointing out that it is necessary to investigate the expression of CD47 in EC. To our knowledge, the role of the CD47-SIRPα signal in EC has not been studied yet. To clarify whether the CD47-SIRPα signal contributes to the immune evasion mediated by TAMs, we perform a phagocytosis assay *in vitro* and establish the xenograft EC model to test the antitumor activity of CD47 blockade therapy. Our studies highlight the potential therapeutic strategy in which reeducating TAMs may have beneficial antitumor effects in EC.

2. Materials and Methods

2.1. Preparation of Tissue Samples. All human samples were obtained from the International Peace Maternity and Child Health Hospital after receiving patients' informed consent.

2.2. Immunohistochemistry. The paraffin-embedded tissues were sectioned into 4 μm, then deparaffinized and rehydrated with xylene and graded alcohol. Antigen retrieval was used with EDTA. Sections were incubated with mouse anti-human CD68 antibody (1 : 200; Abcam), mouse anti-human CD163 antibody (1 : 1000; Bio-Rad), and anti-human CD47 antibody (1 : 1000; GeneTex) at 4°C overnight. EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC Kit (Abcam) was used for the following steps according to the manufacturer's protocol. All the samples were assessed by two pathologists in 10 different high-power fields (HPFs). The number of $CD68^+$ cells and $CD163^+$ cells were counted and the average taken. The staining intensity of CD47 was scored as 0 (no staining), 1 (weak staining), 2 (intermediate staining), or 3 (dark staining). The percentage of staining cells was scored as 0 (0–5%), 1 (1–25%), 2 (26–50%), 3 (51–75%), or 4 (76–100%). The product of the two scores were considered as the CD47 IHC score. Samples were classified as low CD47 expression (IHC score ≤ 4) or high CD47 expression (IHC score > 4).

2.3. Immunofluorescence. Sections or coculture cell (mouse macrophages + EC cells) dishes were blocked with 10% calf serum and incubated with rabbit anti-human CD68 antibody (1 : 100), mouse anti-human CD163 antibody (1 : 1000), and anti-mouse F4/80 antibody (1 : 200; Abcam) at 4°C overnight. The sections were then incubated with Alexa Fluor 488 donkey anti-mouse IgG, Alexa Fluor 594 donkey anti-mouse IgG, and Alexa Fluor 594 donkey anti-rabbit IgG (Life Technologies) at room temperature for 2 hours followed by nuclear counterstaining with DAPI (Abcam). The samples were detected by confocal microscopy.

2.4. RNA Extraction and qPCR. The tissues used for RNA extraction were ground by a TissueLyser. Total RNA was extracted using the TRIzol (Invitrogen) method. cDNA was synthesized from 1 μg total RNA using a reverse transcription kit (Tiangen Biotech (Beijing) Co. Ltd., China). Real-time PCR was performed using the SYBR Green Master Mix (Takara Bio Inc.) on a 7500 Real-Time PCR System (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method was used to calculate fold changes in the gene expression normalized to GAPDH. The primers that were used are shown Table 1.

2.5. The Preparation of Tissue Single-Cell Suspension. From May 2017 to October 2017, 27 patients who underwent hysterectomy for EC or other benign diseases were recruited into this study. Clinical endometrial tissues were obtained from the patients after getting their informed consent. Fresh endometrial tissue specimens were transported on ice to the laboratory, cut into small pieces of 2–4 mm, and enzymatically dissociated with the Tumor Dissociation Kit (Miltenyi Biotec). ACK Lysing Buffer (Thermo Fisher Scientific) was

Table 1: The primers used in this study.

Primers		
CD47 (human)	Sense primer	5′-AGAAGGTGAAACGATCATCGAGC-3′
	Antisense primer	5′-CTCATCCATACCACCGGATCT-3′
GAPDH (human)	Sense primer	5′-ACCACAGTCCATGCCATCAC-3′
	Antisense primer	5′-TCCACCACCCTGTTGCTGTA-3′
TNF-α (mouse)	Sense primer	5′-GATCTCAAAGACAACCAACTAGTG-3′
	Antisense primer	5′-AGGTCCAGACGCAGGATGGCATG-3′
iNOS (mouse)	Sense primer	5′-GGCAGCCTGTGAGACCTTTG-3′
	Antisense primer	5′-TGAAGCGTTTCGGGATCTG-3′
IL-12 (mouse)	Sense primer	5′-AAATGAAGCTCTGCATCCTGC-3′
	Antisense primer	5′-TCACCCTGTTGATGGTCACG-3′
Ym1 (mouse)	Sense primer	5′-TCTGGTGAAGGAAATGCGTAAA-3′
	Antisense primer	5′-GCAGCCTTGGAATGTCTTTCTC-3′
Fizz1 (mouse)	Sense primer	5′-CAGCTGATGGTCCCAGTGAA-3′
	Antisense primer	5′-TTCCTTGACCTTATTCTCCACGAT-3′
GAPDH (mouse)	Sense primer	5′-AGGTCGGTGTGAACGGATTTG-3′
	Antisense primer	5′-TGTAGACCATGTAGTTGAGGTCA-3′

used to remove erythrocytes. Cells were then washed twice with PBS and filtered through a 70 μm filter.

2.6. Flow Cytometry Analysis. Single-cell suspensions were stained with FITC-conjugated anti-CD47 (eBioscience). 7-AAD (eBioscience) and antibodies targeted to CD45 (BD Biosciences) and CD31 (eBioscience) were used to exclude dead, nontumor cells. Flow cytometry analyses were performed on BD FACS Canto II.

2.7. Murine BMDM Culture and Differentiation. Murine bone marrow cells were collected from 8-week old NOD/SCID/IL2γ^{null} mice (NSG, Beijing Biocytogen Co. Ltd.). 1×10^6 murine bone marrow cells were planted per well in a 24-well plate and cultured with Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplied with recombinant mouse macrophage colony-stimulating factor (M-CSF; 10 ng/mL, R&D Systems) for 7 days. The macrophages at this state were considered as M0 macrophages. Purity was verified by flow cytometry using F4/80 and CD11b. M1 MΦ were obtained by further treatment on day 7 with recombinant mouse interferon-gamma (IFN-γ, 20 ng/mL) and lipopolysaccharide (LPS; 100 ng/mL, Sigma-Aldrich) for 24 hours. M2 MΦ were obtained by further treatment on day 7 with IL-4 (20 ng/mL) for 24 hours. All cytokines were purchased from PeproTech Inc. unless otherwise stated.

2.8. Establishment of CD47 Knockdown EC Cells. We designed a shRNA to target the human CD47 gene (NM_198793.2). The CD47-shRNA and control sequences are as follows: CD47-shRNA (5′-CCGGGCACAATTACTTGGACTAGT TCTCGAGAACTAGTCCAAGTAATTGTGCTTTTT-3′), scramble-shRNA (5′-CCGGTTCTCCGAACGTGTCACGTT TCAAGAGAACGTGACACGTTCGGAGAA TTTTTG-3′). The shRNA was cloned into a lentiviral vector (pL-TO-IRES-LUC) to knockdown the expression of CD47 in EC cells (GeneChem Biotech, Shanghai, China). 1×10^5 Ishikawa cells or KLE cells were transfected with 2×10^6 TU shRNA-encoding lentivirus in the presence of polybrene (5 μg/mL) for 12 h. Then, the EC cells were cultured in DMEM with 10% FBS for 1 week. Puromycin (1 μg/mL) was used to select the cells that were successfully transfected. After 2 weeks, the CD47 protein expression on EC cells was detected by flow cytometry.

2.9. In Vitro Phagocytosis Assay. For the *in vitro* phagocytosis assay, Ishikawa cells were labeled with 1 μM CFSE using the CellTrace CFSE Cell Proliferation Kit (Invitrogen). Macrophages were incubated with 1×10^6 CFSE-labeled Ishikawa cells in serum-free medium in the presence of IgG control (10 μg/mL, eBioscience) or anti-CD47 antibodies (10 μg/mL, eBioscience) for 2 h. Then, the plate was washed for 3 times with warm PBS to remove unphagocytosed Ishikawa cells.

For the immunofluorescence assay, the cocultured cells were observed through a fluorescence microscope to investigate the phagocytosis of EC cells by macrophages. For the flow cytometry assay, the cocultured cells were digested with 0.25% Trypsin-EDTA (Gibco). A single-cell suspension was incubated with a mAb specific for mouse CD16/CD32 to prevent nonspecific binding against FcγR, and it was then incubated with F4/80 antibodies (BioLegend) for 30 min at 4°C and washed twice with 2% FBS in PBS. Stained cells were

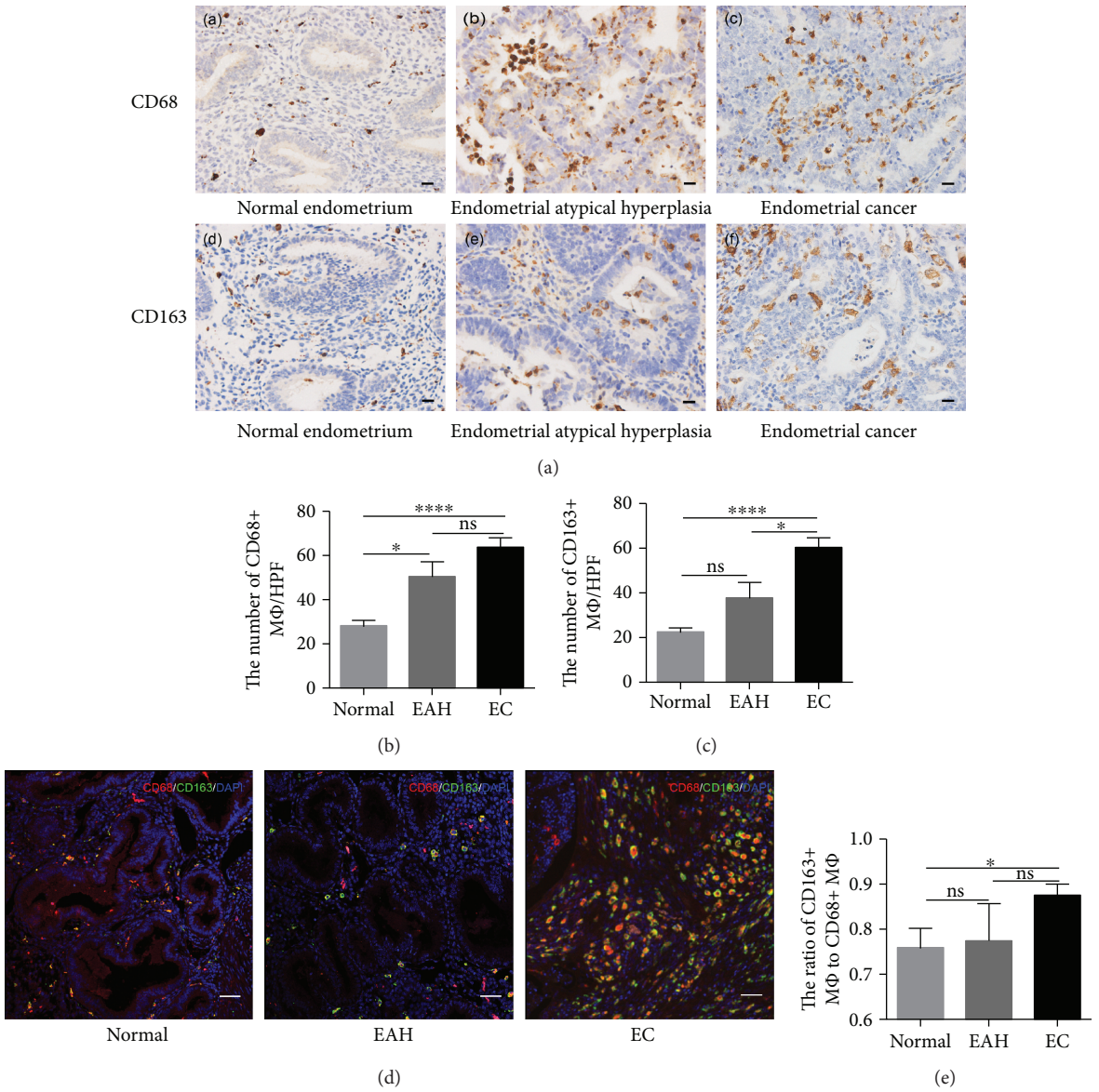

Figure 1: The majority of infiltrated macrophages in EC tissues are M2 TAMs. (a) Representative immunohistochemical staining of CD68 and CD163 (400x) in normal, EAH, and EC tissues. CD68 (a–c) and CD163 (d–f). Scale bars, 20 μm. (b–c) Cell counts of $CD68^+$ and $CD163^+$ macrophages. (d) Representative images of $CD68^+CD163^-$ macrophages (M1 macrophages) and $CD163^+CD68^+$ macrophages (M2 macrophages). Scale bars, 20 μm. (e) The ratio of $CD163^+$ to $CD68^+$ cells (M2/total macrophage ratio). There were 26 normal endometrium samples, 11 EAH samples, and 47 EC samples. Data were shown as the mean ± SEM (ns, not significant; $^*P < 0.05$ and $^{****}P < 0.0001$).

subjected to flow cytometry, and data was analyzed with FlowJo software. The phagocytic index was calculated as the percentage of $CFSE^+$ macrophages.

2.10. Apoptosis Assay. The EC cells were treated with anti-CD47 antibody (B6H12; 10 μg/mL) or control antibody (10 μg/mL) for 2 h or 12 h. Then, the EC cells were digested and the apoptosis was measured by flow cytometry using Annexin V-FITC and PI (BD Biosciences).

2.11. Tumor Xenograft Assay in NSG Mice. Twenty 7-week old female NSG mice were obtained from the Beijing Biocytogen Co. Ltd. Animal research was carried out in strict accordance with the Guideline for the Care and Use of Laboratory Animals of China. 10^7 CD47-knockdown Ishikawa cells or control Ishikawa cells were injected subcutaneously to the left flank of NSG mice, and tumor growth was monitored. The tumor volume and body weight were measured per week. After four weeks, the mice were sacrificed by cervical dislocation and tumor bulk was removed from the animals.

2.12. Statistical Analysis. Statistical analyses were performed using GraphPad Prism 6.0. Data were analyzed by unpaired

Table 2: Correlation between the number of $CD68^+$ or $CD163^+$ macrophages and relevant clinical characteristics of the EC cases.

Parameters	Patients (n)	Patients (%)	$CD68^+$ macrophages		$CD163^+$ macrophages	
			Mean ± SEM	P value	Mean ± SEM	P value
Total age (year)	47	100				
<55	16	34.0	63.37 ± 6.261	0.9676	62.44 ± 6.602	0.7301
≥55	31	66.0	63.73 ± 5.684		58.95 ± 6.336	
Grade (endometrioid = 37)	37					
G1 or G2	33	88.6	63.45 ± 5.344	0.1247	56.40 ± 4.675	0.0118[a]
G3	4	11.4	88.98 ± 15.07		99.65 ± 29.49	
FIGO stage						
I or II	26	55.3	51.19 ± 5.009	0.0007[a]	44.71 ± 3.859	< 0.0001[a]
III or IV	21	44.7	78.83 ± 5.731		79.24 ± 7.620	
Histologic type						
Endometrioid	37	78.7	65.04 ± 4.977	0.507	60.65 ± 5.361	0.8364
Nonendometrioid	10	21.3	57.98 ± 7.415		58.24 ± 10.32	
Myometrial invasion						
<1/2	31	66	62.04 ± 5.638	0.6016	58.05 ± 5.843	0.5433
≥1/2	16	34	66.81 ± 5.857		64.18 ± 8.107	
Positive lymph nodes						
No	33	64.1	54.75 ± 5.356	0.0042[a]	47.87 ± 4.430	0.0008[a]
Yes	14	35.9	81.63 ± 6.996		84.15 ± 10.81	
Lymphovascular space involvement						
No	25	53.2	52.27 ± 5.558	0.0033[a]	47.48 ± 4.536	0.0031[a]
Yes	22	46.8	76.40 ± 5.419		74.53 ± 7.646	

[a]$P < 0.05$, the difference between CD68/CD163 expression in patients and different grades, FIGO stages, with or without lymph node metastasis, or lymphovascular space involvement.

Student's t-test or one-way ANOVA and were presented as the mean ± SEM. P values < 0.05 were considered statistically significant. All experiments were repeated three times.

3. Results

3.1. M2 TAMs Are Closely Associated with the Tumor Progression in EC. To study the distribution of macrophages, immunohistochemistry was used to evaluate the infiltration of TAMs in normal endometrium, endometrial atypical hyperplasia (EAH), and EC (Figure 1(a)). CD68 and CD163 are relatively commonly accepted markers for total macrophages and M2 macrophages, respectively [19]. There were more macrophages infiltrating in EC than in the normal endometrium (Figures 1(b)-1(c)), which were mainly M2 macrophages (Figures 1(d)-1(e)). Besides, there was a progressive upregulation of M2 macrophages from the normal endometrium and EAH to EC (Figure 1(c)).

On the basis of the findings that macrophage infiltration was correlated with patient survival, we hypothesized that macrophage infiltration might play roles in the progression of EC. The relationship between total or M2 macrophages and clinicopathological features was analyzed (Table 2). The high number of macrophages, particularly M2 macrophages in EC, was strongly correlated with unfavorable prognostic factors, such as high pathological grade ($P = 0.0118$), high FIGO stage ($P < 0.0001$), lymph node metastasis ($P = 0.0008$), and lymphovascular space involvement ($P = 0.0031$). Our results showed that M2 TAM infiltration was closely associated with the progression of EC.

3.2. CD47 Is Highly Expressed in EC Compared with Normal Endometrium. Previous researches have reported that CD47 was overexpressed in various tumors [23–28]. We found that CD47 mRNA was highly expressed in EC samples using The Cancer Genome Atlas Research Network (TCGA) database (Figure 2(a)). CD47 was highly expressed in EC tissue when analyzed by qPCR (Figure 2(b)). CD47 protein expression level was increased in EC tissues by immunochemistry (Figures 2(c)-2(d)). Considering CD47 that expressed on the cell surface interacted with SIRPα, we evaluated the CD47 expression on freshly isolated cells from EC tissue and normal endometrium by flow cytometry. Although CD47 protein was detectable on all specimens, it was significantly overexpressed in tumor tissue compared with normal tissue (Figure 2(e)). Besides, CD47 was detectable in all EC cell lines that we tested (Supplementary Figure 1).

3.3. CD47 Blockade Increases Phagocytosis of EC Cells by Macrophages In Vitro. To directly study the inhibitory effect of the interaction between CD47 and SIRPα, we performed phagocytosis assays *in vitro*. The majority of the NSG or

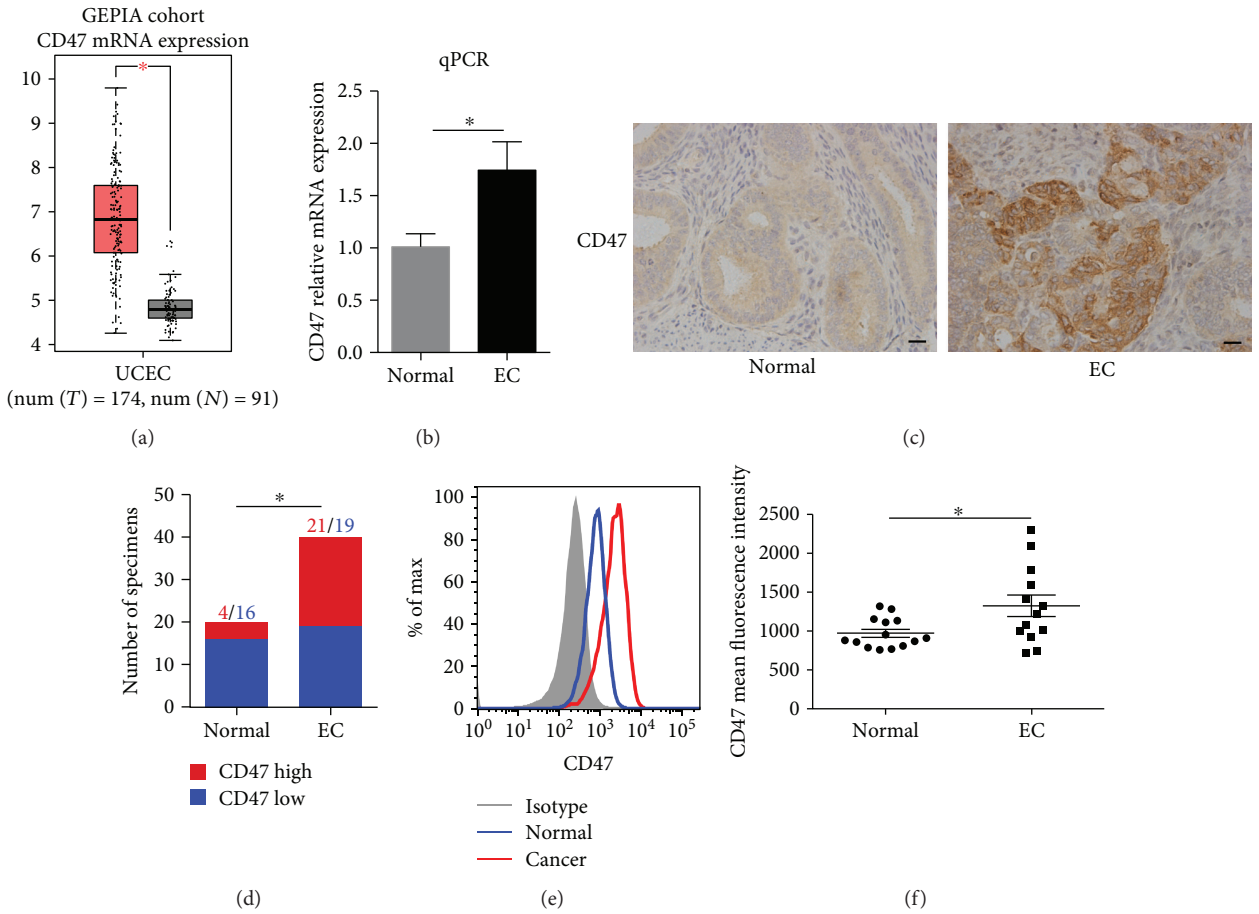

Figure 2: CD47 is highly expressed in EC tissues. (a) Analysis of CD47 mRNA expression in EC samples using TCGA RNAseq. (b) CD47 mRNA expression measured by qPCR. There were 18 normal endometrium samples and 22 EC samples. (c) The representative immunohistochemical staining of CD47 (400x) measured by immunochemistry. Scale bars, 20 μm. (d) The quantification of CD47 expression measured by immunochemistry. There were 20 normal endometrium samples and 40 EC samples. (e) The representative image of CD47 expression measured by flow cytometry. (f) The quantification of CD47 expression measured by flow cytometry. There were 14 normal endometrium samples and 13 EC samples. Data were shown as the mean ± SEM ($^*P < 0.05$).

C57BL/6 mouse bone marrow-derived macrophages (BMDMs) were $CD11b^+F4/80^+$ macrophages which suggested successful cultivation (Supplementary Figure 2A). NSG mouse BMDMs were cocultured with EC cells (Ishikawa cells or KLE cells) with or without the anti-CD47 antibody. Phagocytosis was evaluated by the percentage of macrophages engulfing EC cells. CD47 blockade with the anti-CD47 blocking antibody (B6H12) resulted in a significant increase in phagocytosis of Ishikawa cells by NSG and C57BL/6 mouse macrophages, while this effect was not observed with the anti-CD47 nonblocking antibody (2D3), which was specific to CD47 but did not interrupt the interaction between CD47 and SIRPα (Figures 3(a)-3(b), Supplementary Figures 2B-2C). CD47 blockade could increase phagocytosis of KLE cells by NSG BMDMs (Figure 3(c)). Some studies showed that anti-CD47 antibodies might directly induce the apoptosis of tumor cells [29, 30]. However, our results showed that the soluble anti-CD47 antibody (B6H12) could not promote the apoptosis of EC cells (Supplementary Figure 3).

3.4. CD47 Knockdown Increases Phagocytosis of EC Cells by Macrophages In Vitro. We performed a CD47-knockdown experiment in Ishikawa cells and KLE cells using a lentiviral-based approach and confirmed that the successful establishment of CD47-knockdown EC cells (Figures 4(a), 4(c)). There was an increase in phagocytosis to both CD47-knockdown Ishikawa cells and CD47-knockdown KLE cells by NSG mouse BMDMs in phagocytosis assays (Figures 4(b), 4(d)).

3.5. The Increased Phagocytosis Ability with CD47 Blockade Treatment Is Mediated by M2 Macrophages In Vitro. To study whether the CD47 blockade can influence the phagocytosis of the macrophages with different states, we induced NSG mouse BMDMs into different phenotypes (Supplementary Figure 4). Then, the polarized macrophages were cocultured with Ishikawa cells in the presence of anti-CD47 blocking, nonblocking antibodies, and control IgG antibody. In the control group, we found that M1 macrophages had a greater ability of phagocytosis, compared to M2 macrophages

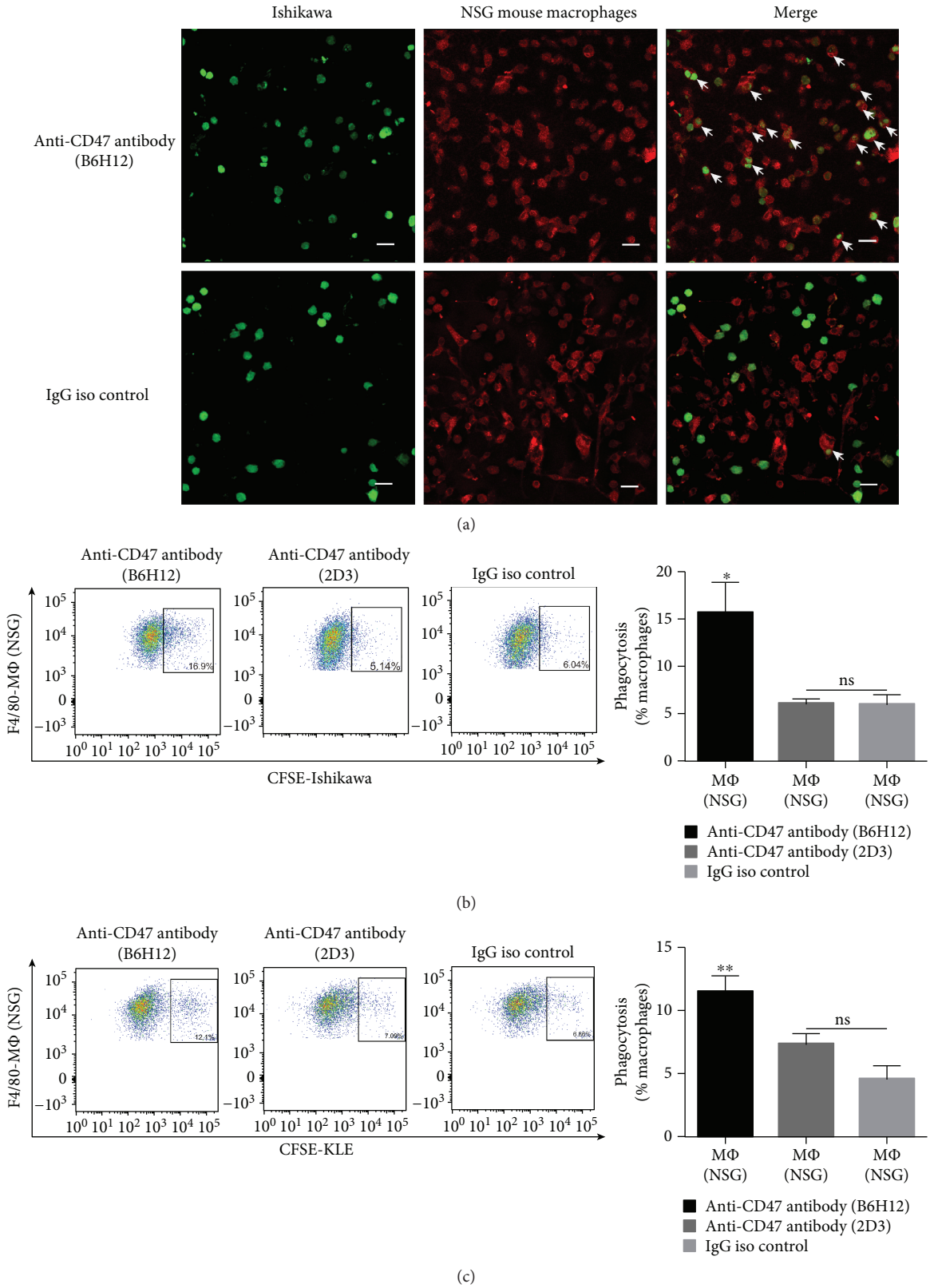

Figure 3: CD47 blockade increases phagocytosis of EC cells by macrophages. (a) Representative images of the phagocytosis assay in which Ishikawa cells were cocultured with NSG mouse BMDMs in the presence of anti-CD47 antibody or control IgG antibody. The white arrows point to the macrophages that phagocytosed Ishikawa cells. Scale bars, 20 μm. (b-c) Flow cytometry results of phagocytosis assays in which Ishikawa or KLE cells were cocultured with NSG mouse BMDMs. Percentages of $CFSE^+$ $F4/80^+$ macrophages in total macrophages were indicated beside the gated population. Data were shown as the mean ± SEM (ns, not significant; $^*P < 0.05$ and $^{**}P < 0.01$).

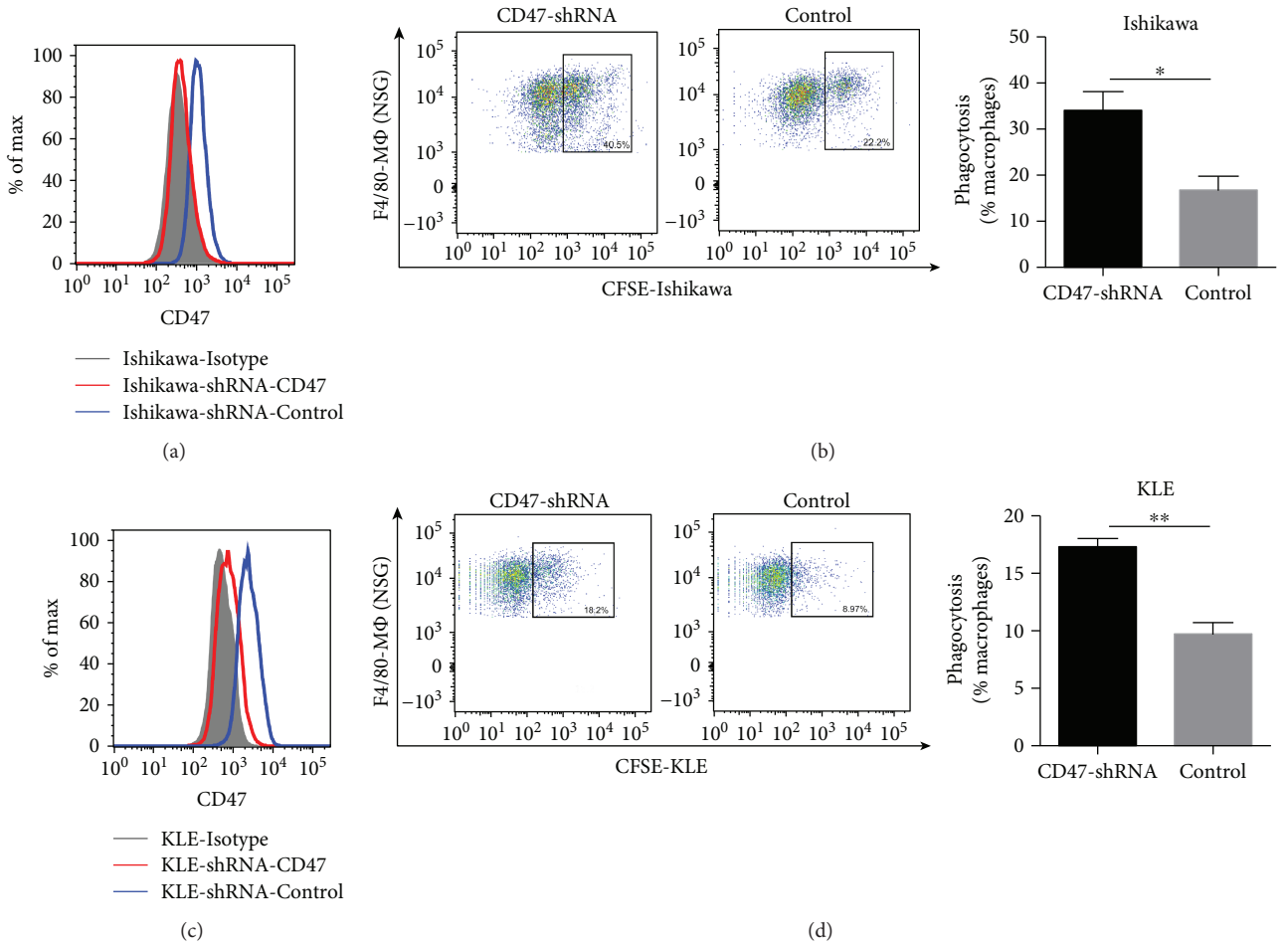

Figure 4: CD47 knockdown increases phagocytosis of EC cells by macrophages. (a, c) Successful knockdown of CD47 in EC cell lines (Ishikawa cell line and KLE cell line) measured by flow cytometry. (b, d) Representative images of the phagocytosis assay in which CD47-knockdown EC cells (Ishikawa or KLE cells) or control EC cells were cocultured with NSG mouse BMDMs. Percentages of $CFSE^+$ $F4/80^+$ macrophages in total macrophages were indicated beside the gated population. Data are shown as the mean ± SEM (ns, not significant; $^*P < 0.05$ and $^{**}P < 0.01$).

(Figures 5(a)-5(c)). However, we found that M2 macrophages display a larger phagocytic response towards EC cells than M1 macrophages when treated with anti-CD47 blocking antibody (Figures 5(a)-5(c)). In other words, $CD47^+$ Ishikawa cells mainly inhibited themselves from being engulfed by M2 macrophages rather than by M1 macrophages.

3.6. CD47 Knockdown Inhibits Tumor Growth and Promotes the Infiltration of M1 Macrophages in the TME In Vivo. To examine whether CD47 knockdown contributed to the growth of EC, we found that the size of the tumors formed by shCD47 clones in NSG mice were smaller, when compared with those in the control group (Figures 6(a), 6(c)), although there was no difference in mouse body weight between the two groups (Figure 6(b)). More importantly, we observed that there were more macrophages which were mainly M1 macrophages in xenografted tumors formed by shCD47 clones than in the control group (Figures 6(d)-6(f)). These results suggested that CD47 knockdown inhibited the growth of the EC tumors *in vivo* and promoted the infiltration of macrophages which might play an important role in antitumor activity.

4. Discussion

The underlying mechanism of tumor progression and immune evasion mediated by TAMs in EC has been poorly characterized to date. Our results showed that TAMs in EC tended to acquire a polarized M2 phenotype which might contribute to skewing the TME to a tumor-progressive condition. Besides, our results indicated that the increased number of TAMs was positively correlated with the progression of EC; therefore, they are consistent with other studies [31–33].

CD47 expressed by tumor cells interact with SIRPα transmitting a "don't eat me" signal to macrophages to avoid being eliminated. The CD47 overexpression was responsible for immune suppression and tumor progression in EC. The CD47 blockade treatment could increase the phagocytosis

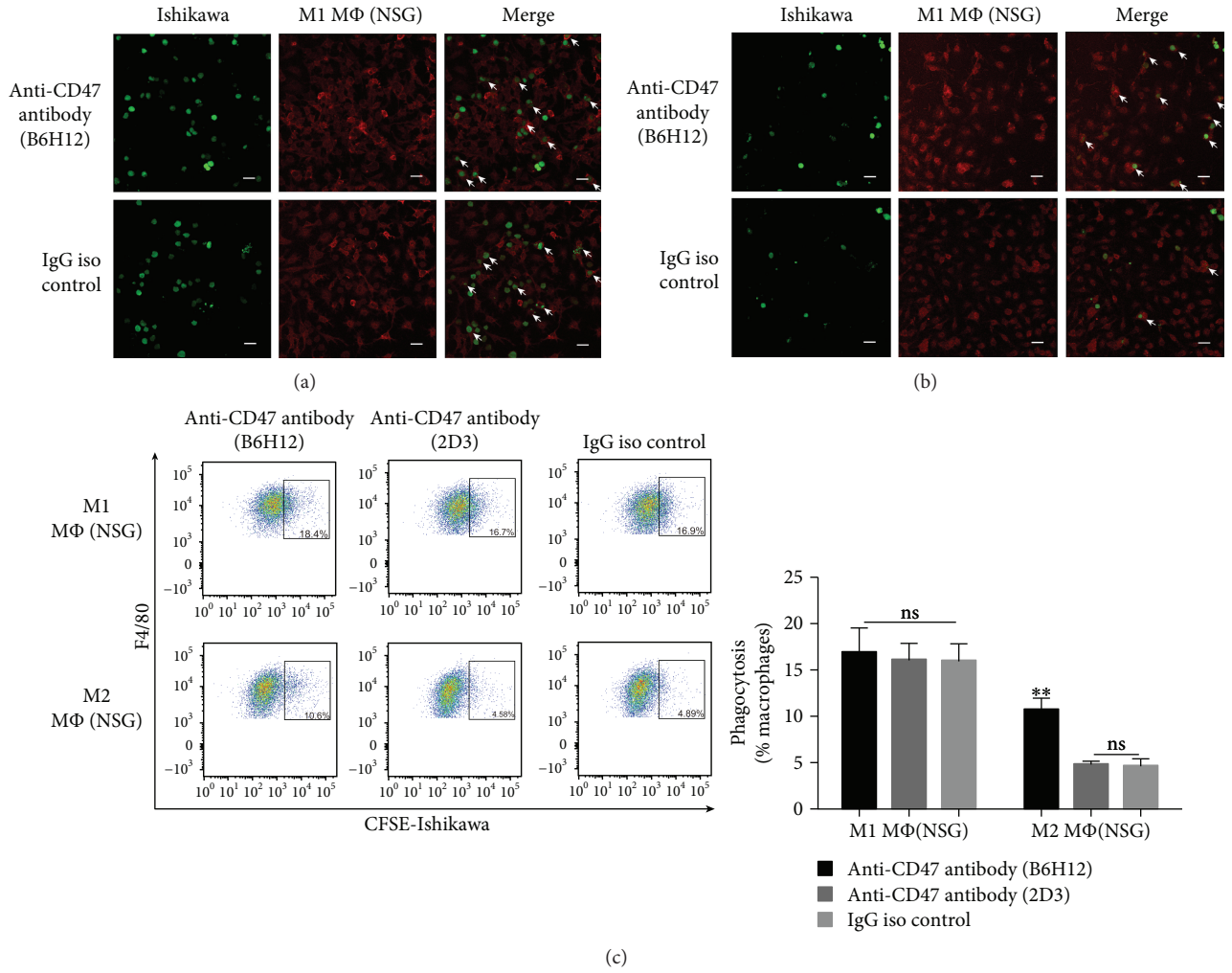

Figure 5: CD47 blockade increases phagocytosis of EC cells by M2 macrophages. (a-b) Representative images of the phagocytosis assay in which Ishikawa cells were cocultured with M1 or M2 NSG mouse BMDMs in the presence of anti-CD47 antibody or control IgG antibody. The white arrows point to the macrophages that phagocytosed Ishikawa cells. Scale bars, 20 μm. (c) Flow cytometry results of phagocytosis assays. Percentages of $CFSE^+$ $F4/80^+$ macrophages in total macrophages were indicated beside the gated population. Data were shown as the mean ± SEM (ns, not significant; $^{**}P < 0.01$).

ability of M2 macrophages instead of M1 macrophages *in vitro*. These results suggested that CD47 blockade therapy could take advantage of M2 macrophages in the TME without affecting the normal function of M1 macrophages in our bodies. Besides, the *in vivo* experiment suggested that there were smaller tumor sizes and increased TAMs which dominantly consisted of M1 macrophages in the CD47-knockdown group, indicating that these macrophages played an important role in eliminating EC cells.

Our study first revealed that CD47 was overexpressed in several EC cell lines and in all clinical specimens that we tested, regardless of pathological or molecular features. Besides, the anti-CD47 antibody could increase phagocytosis of both Ishikawa cells and KLE cells. Compared to PD-1 blockade immunotherapy which might be effective in a minority of EC patients with the polymerase epsilon (POLE) or microsatellite instability (MSI) mutations [34–36], CD47 blockade immunotherapy might be an extensive and effective choice for EC patients.

In addition to inhibiting the "don't eat me" signal, other potential mechanisms also contributed to the antitumor effects of the anti-CD47 therapy. For instance, some anti-CD47 antibodies could induce the apoptosis of tumor cells directly in several malignancies. However, our results suggested that the antitumor activity of the anti-CD47 blocking antibody was mediated by the interruption of CD47-SIRPα interaction instead of promoting the apoptosis of EC cells.

With the use of immunodeficient NSG mice completely lacking T cells, B cells, and NK cells [37], the involvement of macrophages might be the predominant mechanism to regulate the growth of EC *in vivo*. Besides, the SIRPα protein produced by NSG mice has greater reactivity with human CD47 than other strains [38] which could better reflect the effect of the CD47-SIRPα interaction. However, CD47

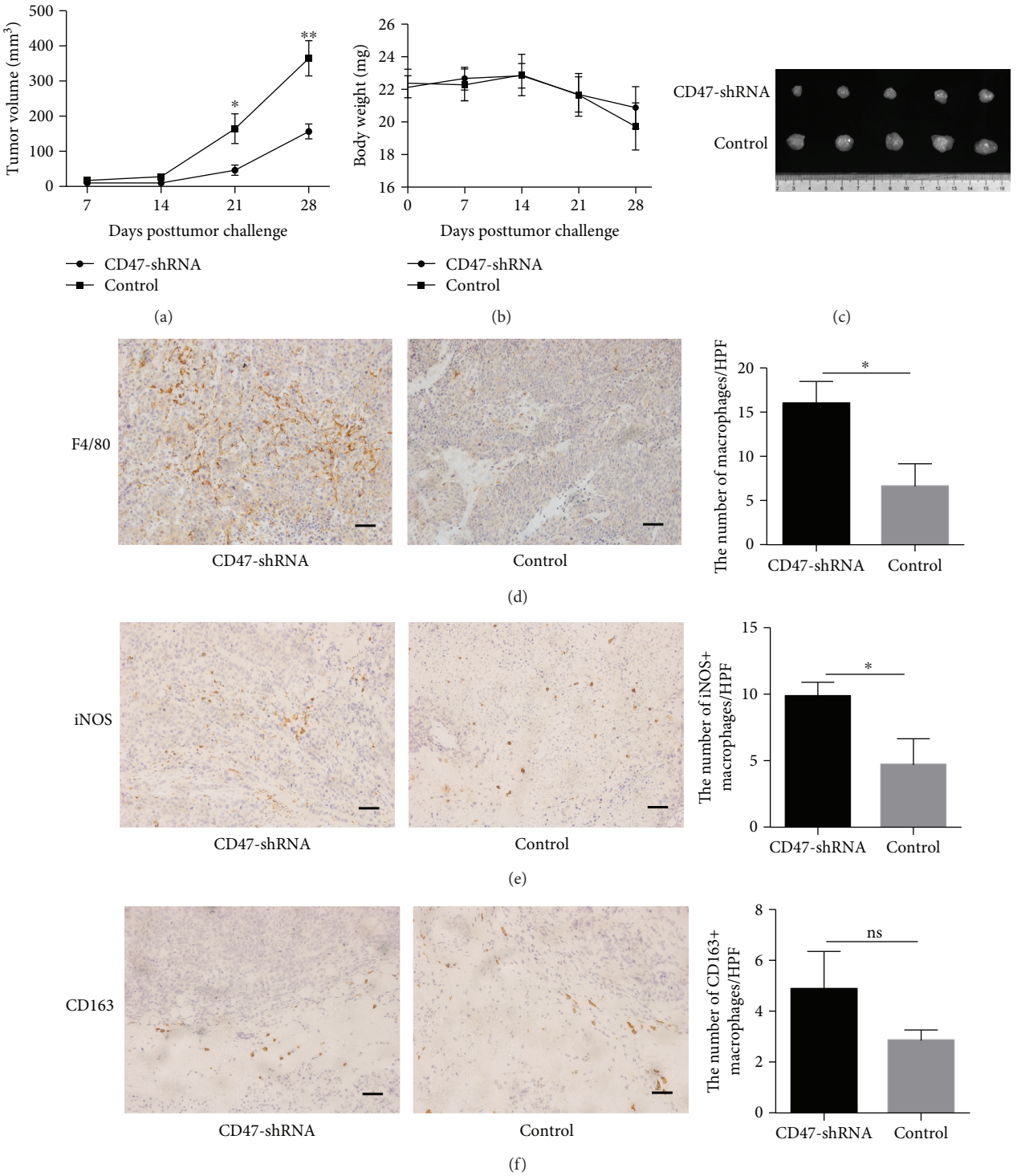

Figure 6: CD47 knockdown inhibits EC growth *in vivo* and promotes the infiltration of M1 macrophages in tumors. (a) Tumor volumes of heterotopic xenografted tumors in NSG mice which were engrafted with CD47-knockdown Ishikawa cells or control Ishikawa cells measured every 7 days. (b) The body weight of the mice measured every 7 days. (c) Tumor volumes of different groups. (d) Representative images and cell counts of F4/80$^+$ macrophages in tumor mass (200x). Scale bars, 50 μm. (e) Representative images and cell counts of iNOS$^+$ macrophages in tumor mass (200x). Scale bars, 50 μm. (f) Representative images and cell counts of CD163$^+$macrophages in tumor mass (200x). Scale bars, 50 μm. Data are shown as the mean ± SEM (ns, not significant; $^*P < 0.05$ and $^{**}P < 0.01$).

negatively regulates the function of the human T cell, dendritic cell [39, 40], NK cell [41], and B cell [42] and plays an inhibitory role in the immune response against tumor cells. The animal model we used could not reflect the role of CD47 in these immune cells. Thus, further studies which focus on CD47 in other immune cells in EC are needed.

5. Conclusions

Taken together, we have found that the overexpression of CD47 in EC protected tumor cells against phagocytosis by macrophages *in vitro* and promoted the progression of EC *in vivo*. In conclusion, the CD47 blockade therapy, which can reeducate M2 macrophages by increasing their phagocytosis ability, might be an attractive target for tumor immunotherapy for EC.

Data Availability

The TCGA database analyzed during the current study is available in the Gepia website (http://gepia.cancer-pku.cn). Other data used to support the findings of this study can be accessed by requesting the corresponding author through email (sunxiao000304@163.com).

Conflicts of Interest

The authors declare that they have no potential conflicts of interest.

Funding

This study was supported by the National Natural Science Foundation of China (nos. 81172477 and 81402135), the Project of the Science and Technology Commission of Shanghai Municipality (no. 11ZR1440800), and the Project of Outstanding Subject Leaders of the Shanghai Health System (no. XBR2013097).

Acknowledgments

The authors would like to thank Dr. Feifei Luo, Dr. Jiawen Qian, Dr. Yuedi Wang, and Dr. Weibin Wu for technical support. The authors would also like to thank Dr. Xueli Zhang for English revision.

Supplementary Materials

Supplementary Figure 1: the EC cell lines stably expressed CD47. Supplementary Figure 2: the maturation of mouse BMDMs and CD47 blockade increases phagocytosis of EC cells by C57BL/6 mouse BMDMs. Supplementary Figure 3: anti-CD47 antibody cannot promote apoptosis of EC cells. Supplementary Figure 4: NSG mouse BMDMs are polarized to different phenotypes. *(Supplementary Materials)*

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 7134235, 9 pages
https://doi.org/10.1155/2018/7134235

Research Article

Role of Exercise in Vascular Function and Inflammatory Profile in Age-Related Obesity

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Received 13 July 2018; Revised 11 September 2018; Accepted 3 October 2018; Published 28 October 2018

Guest Editor: Monica Neagu

In western countries, aging is often accompanied by obesity and age-related obesity is characterized by vascular dysfunction and a low-grade inflammatory profile. Exercise is a nonpharmacological strategy able to decrease the development and incidence of risk factors for several health-threatening diseases. Nonetheless, its long-term effect on vascular function and inflammation in age-related obesity is still unclear. The aim of this study was to investigate the effect of regular, supervised exercise on inflammatory profile and vascular function in age-related obesity. We also hypothesized that vascular function and inflammatory profile would have been correlated in overweight and obese individuals. Thirty normal weight (NW; 70 ± 5 years, 23.9 ± 2.6 BMI) and forty overweight and obese elderly (OW&OB; 69 ± 5 years, 30.1 ± 2.3 BMI) regularly taking part in a structured, supervised exercise program were enrolled in the study and evaluated for vascular function (flow-mediated dilation; FMD) and inflammatory profile (plasma CRP, IL-1β, IL-1ra, IL-6, IL-8, IL-10, TNF-α, and MCP-1). Although no differences between groups were found concerning performance and the weekly amount of physical activity, the OW&OB group compared with the NW group demonstrated higher systolic and diastolic blood pressure (+10%, $p = 0.001$; +9%, $p = 0.005$, respectively); lower FMD% (−36%, $p < 0.001$) and FMD/shear rate (−40%, $p = 0.001$); and higher levels of CRP (+33%, $p = 0.005$), IL-6 (+36%, $p = 0.048$), MCP-1 (+17%, $p = 0.004$), and TNF-α (+16%, $p = 0.031$). No correlations between vascular function and inflammation were found in OW&OB or NW. Although exercising regularly, overweight and obese elderly exhibited poorer vascular function and higher proinflammatory markers compared with the leaner group. These results support the idea that exercise alone cannot counteract the negative effect of adiposity on vascular function and inflammatory profile in elderly individuals and these two processes are not necessarily related.

1. Introduction

Aging, an inevitable and unstoppable process, is a well-documented risk factor for cardiovascular and atherosclerotic disease and it is associated with a progressive decline in endothelium-dependent vasodilation in both resistance and conduit arteries [1, 2]. These alterations are primarily related to a worsening of mitochondrial function, increased inflammatory profile, augmented free radical production, and a gradual loss of antioxidant capacity [2, 3]. Altogether, these mechanisms lead to an impairment of nitric oxide (NO) bioavailability [1–3], with reduced endothelium-dependent vasodilation, serving as a risk factor for diabetes, hypertension, dyslipidemia, and several forms of cancer [4].

Obesity is another condition known to be characterized by vascular dysfunction and, differently from aging, is a reversible state. Indeed, increased body fat is associated with increased inflammatory profile, as well as with increased reactive oxygen species (ROS) production and impaired NO-mediated endothelium-dependent vasodilation [5, 6]. Older adults represent the faster growing population in Europe and all over the world, and the prevalence of obesity among this population is about 20–30% [7]. Indeed, aging is also characterized by increased fat mass and loss of fat-free mass, with a concomitant increase of overall adiposity stored primarily as triglycerides in subcutaneous and visceral adipose tissue, as well as in nonadipose tissues such as skeletal muscles and liver [8]. Consequently, the aging process itself and the age-dependent accumulation of adipose tissue contribute both to the onset and development of NO-mediated vascular dysfunction with a detrimental effect on the incidence of cardiometabolic diseases such as hypertension, dyslipidemia, and diabetes, as well as the development of cancer [5, 9–11]. However, evidence showing the relation between higher inflammatory profile and lower vascular function in obese elderly individuals is lacking and specific studies are needed.

Recent evidence has shown that physical activity can improve the inflammatory profile and endothelium-dependent vasodilation in healthy individuals as well as in individuals with endothelial dysfunction associated with chronic conditions [1]. Physical training has beneficial effects on several cardiovascular risk factors such as dyslipidemia, hypertension, and diabetes, and can serve as an intervention to lower the risk of developing several health-threatening conditions such as cardiovascular chronic disease and cancer [2]. Studies on healthy and active aging have shown that in contrast to their sedentary counterparts, older men who performed regular aerobic exercise have largely preserved vascular function, lower low-grade inflammation, and reduced risk factors for several invalidating diseases [3, 4, 6, 12]. However, a recent study aimed at evaluating the effect of long-term physical activity intervention on vascular health and inflammatory biomarkers in the elderly did not provide further evidence for the beneficial effect of regular exercise in older adults [13]. Recently, some publications have emerged claiming that physical activity counteracts obesity as a risk factor [5, 10, 14]. Nevertheless, other studies have shown that regular physical activity protects against incident cardiovascular disease, but it does not eliminate the harmful effect of overweight and obesity [5, 11, 15–17]. Moreover, it is important to highlight that no studies have aimed at investigating the exercise-induced effects on vascular function and inflammatory profile in age-related obesity.

Consequently, due to contrasting results about the effect of regular exercise on inflammatory profile and vascular function in aging and obesity, and the lack of evidence about the effect of exercise on aging-induced obesity, the aim of this study was to investigate the effect of regular, supervised exercise on inflammatory profile and vascular function in nonobese and obese elderly who habitually take part in a supervised exercise program. The aim of this study was to investigate the effects of regular, supervised exercise on vascular function and inflammatory profile in obese and nonobese older individuals.

Table 1: Subjects characteristics and between groups comparison.

	NW ($n = 30$)	OW&OB ($n = 40$)	p
Age (years)	70.3 ± 4.7	69.4 ± 5.4	ns
Female/male (n)	16/14	23/17	ns
BMI (kg • $(m^2)^{-1}$)	23.9 ± 2.6	30.1 ± 2.3	$p < 0.001$
Sys (mmHg)	133.1 ± 14.7	147.5 ± 16.2	$p = 0.001$
Dia (mmHg)	76.7 ± 7.6	83.9 ± 10.4	$p = 0.005$
Medications			
Statin—n (%)	7 (23)	10 (25)	ns
Diuretics—n (%)	5 (17)	7 (18)	ns
Antiacid—n (%)	5 (17)	6 (15)	ns
Physical activity			
Years	5 ± 1	5 ± 2	ns
Min/week	209.4 ± 82.5	221.7 ± 71.3	ns
Days/week	3 ± 1	3 ± 1	ns
6MWT (m)	610.1 ± 68.5	610.0 ± 83.7	ns
Inflammatory profile			
IL-10 (ng/mL)	18.9 ± 22.6	16.6 ± 17.2	ns
IL-1ra (ng/mL)	61.9 ± 33.6	52.7 ± 24.1	ns
IL-1β (ng/mL)	9.1 ± 7.2	7.8 ± 5.1	ns
IL-8 (ng/mL)	5.9 ± 4.4	7.1 ± 4.1	ns

*Plus-minus values are means ± standard deviation (SD). One-way ANOVA was used to identify between-group differences for parametric variables, followed by the Holm-Sidak test. The Kruskal-Wallis one-way analysis of variance on ranks was used for nonparametric variables, followed by the Tukey test. Note: NW: normal weight; OW&OB: overweight and obese; BMI: body mass index, activity (min/week); Sys: systolic blood pressure; Dia: diastolic blood pressure; FMD: flow-mediated dilation; 6MWT: 6-minute walking test; CRP: C-reactive protein; IL: interleukin; MCP-1: monocyte chemoattractant protein-1; TNF-α: tumor necrosis factor-α; $p = p$ value.

2. Methods

2.1. Subjects. The study population included 70 physically active older adults (30 females, 40 males, mean age 70 ± 5 years, 30 nonobese, 15 overweight, and 25 obese) (Table 1) who habitually took part in a structured, supervised exercise program. Subjects were included in the study in the absence of smoking history, atherosclerotic vascular disease, heart failure, and liver, renal, or inflammatory disease, and if they did not experience great body mass index (BMI) variation in the last 5 years (≤0.7 kg • $(m^2)^{-1}$). None of the subjects was subjected to a caloric restriction diet at the time of the study. Subjects were informed about testing procedures, possible risks, and discomfort that might ensue and gave their written informed consent to participate in accordance with the Declaration of Helsinki, as part of a protocol approved by the Institutional Review Board of the Azienda Ospedaliera Universitaria Integrata of Verona, Italy (protocol # 788CESC).

One day preceding the tests, the subjects were prohibited from training and their normal diet was maintained. A

vascular test and blood sampling were carried out on the same day. Room temperature was 22–24°C, and relative humidity was 50%. All the ultrasound studies were performed by a single experienced vascular sonographer who was unaware of the clinical and laboratory characteristics of the subjects. Height and weight were recorded by means of a scale and stadiometer (Seca, Hamburg, Germany), and body mass index (BMI) was calculated as weight (kg)/height $(m)^2$.

The study was conducted in September 2017, right before the beginning of the annual exercise program (which starts in September and ends in July, having about a 4-week break in summer). The data were collected at the beginning of a new year of the exercise program from subjects who have been taking part in this program for about 5 years, 3 days a week, allowing us to consider these people as active and trained even after a break of a few weeks. Also, a battery of performance and functional tests including a 6-minute walking test (6MWT) was performed at the beginning of the exercise program in order to set the exercise goals. Briefly, 6MWT measures the maximum distance that a person can walk over 6 min and it is commonly used as an assessment of exercise capacity. The distance (m) covered in 6 min was recorded [18].

2.2. Blood Samples and Analysis. Venous blood samples were drawn from an antecubital vein and collected into EDTA tubes for analysis of inflammatory profiles. The samples were centrifuged for 10 minutes at +4°C with 2500 ×g. Plasma was kept at −80°C until analyzed for interelukine-1ra (IL1-ra; Chi, 4.67%; CV, 0.034%; DC = (0.10, 18011.00)), interleukin-1β (IL-1β; Chi, 6.86%; CV, 0.65%; DC = (0.057, 23660.71)), interleukin-6 (IL-6; Chi, 7.70%; CV, 0.36%; DC = (0.39, 47602.24)), interleukin-8 (IL-8; Chi, 2.15%; CV,0.21%; DC = (0.18, 41913.46)), interleukin-10 (IL-10; Chi, 7.29%; CV, 0.66%; DC = (0.36, 10173.63)), tumor necrosis factor-α (TNF-α; Chi, 7.02%; CV, 0.77%; DC = (0.28, 15643.48)), and monocyte chemoattractant protein-1 (MCP-1; Chi, 4.73%; CV, 0.68%; DC = (0.43, 21239.64)), which were determined by means of the commercially available MILLIPLEX multianalyte panel HCYTOMAG-60K-07 (Merck Millipore, Darmstadt, Germany) following the manufacturer's recommendation. C-reactive protein (CRP) was measured using an ELISA commercial kit, following the manufacturer's recommendation (Diagnostics Biochem Canada Inc. (DBC), London, Canada). We included both pro- and anti-inflammatory markers related with exercise as well as related with the development of risk factors for several health-threatening diseases.

2.3. Flow-Mediated Dilation (FMD) Test. The FMD test has been proposed to represent a functional bioassay for endothelium-derived NO bioavailability and vascular function in humans [19]. The FMD test was performed in a quiet room with participants in abstinence from alcohol, antioxidants (i.e., orange juice and beetroot juice), and caffeine for at least 12 h [20]. High-resolution ultrasound was used to image the brachial artery at rest and after 5 min of ischemia. All the FMD tests were performed with the participant in the supine position, with the right arm extended at an angle of ~90° from the torso. The brachial artery was imaged using the high-resolution ultrasound system LOGIQ-7 ultrasound Doppler system (General Electric Medical Systems, Milwaukee, WI, USA). The ultrasound Doppler system was equipped with a 12–14 MHz linear array transducer. The brachial artery was imaged 5–10 cm above the antecubital fossa in the longitudinal plan, and the diameter was determined at 90° angle along the central axis of the scanned area. When an optimal image was acquired, the position was maintained for the whole test and all scans were stored for later analysis. After baseline brachial artery imaging, a blood pressure cuff was placed around the forearm and inflated to 250 mmHg for 5 min. Brachial artery images and blood velocity were obtained continuously 30 s before and 2 min after cuff release [19]. The brachial artery images were analyzed by a blinded investigator by means of FloWave.US [21]. Arterial diameter was measured as the distance (mm) between the intima-lumen interfaces for the anterior and posteriors walls. Utilizing arterial diameter and the blood velocity, blood flow (BF) was calculated as follows:

$$\mathrm{BF}\ \ (\mathrm{ml/min}) = \mathrm{blood}\ \ \mathrm{velocity} \cdot \Pi \cdot \left(\frac{\mathrm{vessel}\ \ \mathrm{diameter}}{2}\right)^2 \cdot 60. \quad (1)$$

The calculation of FMD as a percentage change uses the peak diameter in response to reactive hyperemia in relation to the baseline diameter and was calculated as follows:

$$\mathrm{FMD}(\%) = \frac{(\mathrm{peak}\ \ \mathrm{diameter} - \mathrm{baseline}\ \ \mathrm{diameter})}{\mathrm{baseline}\ \ \mathrm{diameter}}, \quad (2)$$

and when multiplied by 100, FMD is expressed as a percentage of change in the vessel caliber [19].

Postcuff release shear rate was calculated using the following equation: shear rate $(s^{-1}) = 8V_{\mathrm{mean}}/\mathrm{vessel\ diameter}$. Cumulative shear rate $(s^{-1} \bullet s)$ and the reactive postcuff release (total blood flow over 2 min) were integrated using the trapezoidal rule and calculated as

$$\sum \left[y_i \left(x_{(i+1)} - x_i \right) + \left(\frac{1}{2} \right) \left(y_{(i+1)} - y_i \right) \left(x_{(i+1)} - x_i \right) \right]. \quad (3)$$

Consequently FMD was normalized for shear rate (FMD/shear rate) [19].

2.4. Supervised Exercise Training. Individuals included in the study habitually exercised at the "Silver Fitness" program going on at the Department of Neurosciences, Biomedicine and Movement Sciences, Section of Movement Sciences, at the University of Verona, Italy. All individuals exercised at least twice a week for 90 minutes combining moderate intensity endurance and resistance training. Sessions usually started with 15 minutes of warm up, which included active joint mobilization and walking on a treadmill or cycling at a preferred speed. Then, individuals performed two 15-minute endurance exercises (either on a cycle ergometer,

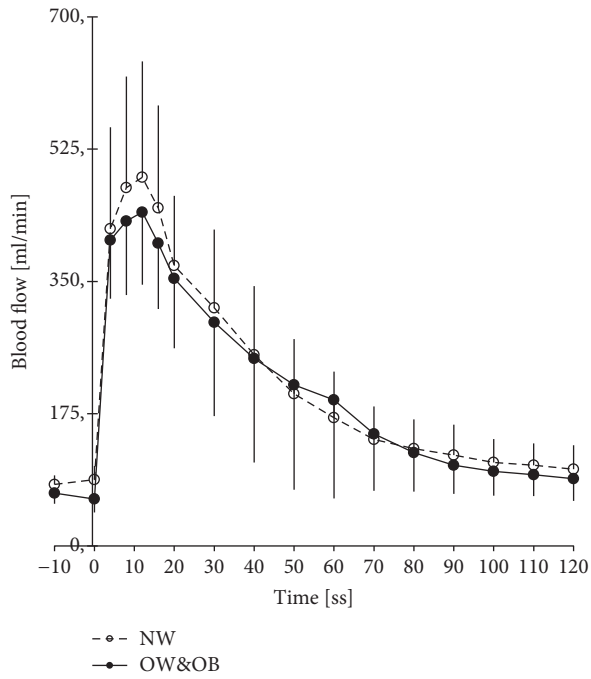

Figure 1: Reactive hyperemic response during the FMD test. Increase in brachial artery blood flow in the normal weight (NW) group and overweight and obese (OW&OB) group during the 2 minutes following a 5-minute supersystolic occlusion during the FMD test.

treadmill, or arm-cranking ergometer) at 70% of the maximal heart rate (calculated using the Karvonen formula: 220 − age in years). Subsequently, individuals performed 3 sets of 8 to 12 reps of resistance exercises at 60–75% of 1 repetition maximum (1RM). 1RM was determined by means of the Brzycki method [22] for all the isotonic ergometers included in the training (i.e., chest press, lat machine, leg press, and others, Technogym, Gambettola, Italy) every year at the beginning of the training program and every 4 months over the year to adjust the work load for the improvements achieved. Exercise sessions ended with stretching exercises for all the muscles involved in the training. All training sessions were supervised by kinesiologists, with a ratio of 1 : 6 (1 kinesiologist supervising 6 participants).

2.5. Statistical Analysis. Data are expressed as mean ± SD and minimum-maximum range. One-way ANOVA was used to identify between-group differences for parametric variables (age, systolic blood pressure, FMD%, and shear rate), followed by the Holm-Sidak test. The Kruskal-Wallis one-way analysis of variance on ranks was used for nonparametric variables (quantity of physical activity, BMI, diastolic blood pressure, FMD/shear rate, 6-MWT, CRP, IL-1ra, IL-1β, IL-6, IL-8, IL-10, MCP-1, and TNF-α), followed by the Tukey post hoc test. The Pearson product moment correlation was used to identify correlations between vascular function variables and inflammation markers. All statistical analyses were performed with SigmaPlot Windows Version 12.0 (Systat Software, Chicago, IL).

3. Results

3.1. Characteristics of the Participants. Individuals included in this study were 70 ± 5 years old and included 39 females and 31 males. The most common medications taken by the participants were statins, taken by 24% of the participants; diuretics, taken by 17% of the participants; and antiacids, taken by 16% of the participants. Interestingly, although a history of high blood pressure was not reported by the participants, on average both of the groups exhibited high values of systolic blood pressure (see Table 1). We also investigated the sex-specific differences in the variables we assessed; however, differences between males and females were not found. Referring to the quantity of physical activity, on average all individuals included in the study have been taking part in the exercise program for 5 ± 1 years and were involved 3 ± 1 days/week, for a total amount of 270 ± 60 minutes per week (see Table 1).

3.2. Lean vs. Overweight and Obese Subjects. NW and OW&OB did not differ for age, number of males and females in the groups, medicine intake, years practicing physical activity, weekly amount of physical activity, and performance measured as 6-MWT (see Table 1). As expected, the OW&OB group had a 20.6% higher BMI compared with the NW group ($p < 0.001$). Regarding the vascular function, no difference between groups was found in the hyperemic response during the FMD test (see Figure 1) or in the shear rate (see Figure 2(b)). Nevertheless, the OW&OB group exhibited a 9.8% higher systolic blood pressure ($p = 0.001$) and an 8.6% higher diastolic blood pressure ($p = 0.005$), as well as a 35.5% lower FMD% (see Figure 2(a), $p < 0.001$) and a 39.7% lower FMD/shear rate (see Figure 2(c), $p = 0.001$) than the NW group. Concerning the inflammatory profile, no significant differences between groups were found for many cytokines, such as IL-10, IL-1ra, IL-1β, and IL-8. However, the OW&OB group exhibited higher values of proinflammatory cytokines, such as a 32.9% higher CRP level (see Figure 3(a), $p = 0.005$), a 35.5% higher IL-6 level (see Figure 3(b), $p = 0.048$), a 15.8% higher TNF-α level (see Figure 3(c), $p = 0.0031$), and a 16.7% higher MCP-1 level (see Figure 3(d), $p = 0.004$) compared with the NW group. When investigating the correlation between vascular function and inflammatory profile, no significant results were found in NW or OW&OB (see Table 2).

4. Discussion

Although several studies have investigated the effect of exercise on age-related and obesity-related morbidities, no studies have aimed at investigating the exercise-induced effects on vascular function and inflammatory profile in age-related obesity without inclusion of caloric restriction. Numerous studies highlighted the fact that during the aging process there is an increase in overall adiposity and a decrement in fat-free mass contributing to lower vascular function and an increase in the low-grade inflammatory profile. Numerous reviews about vascular function and inflammatory

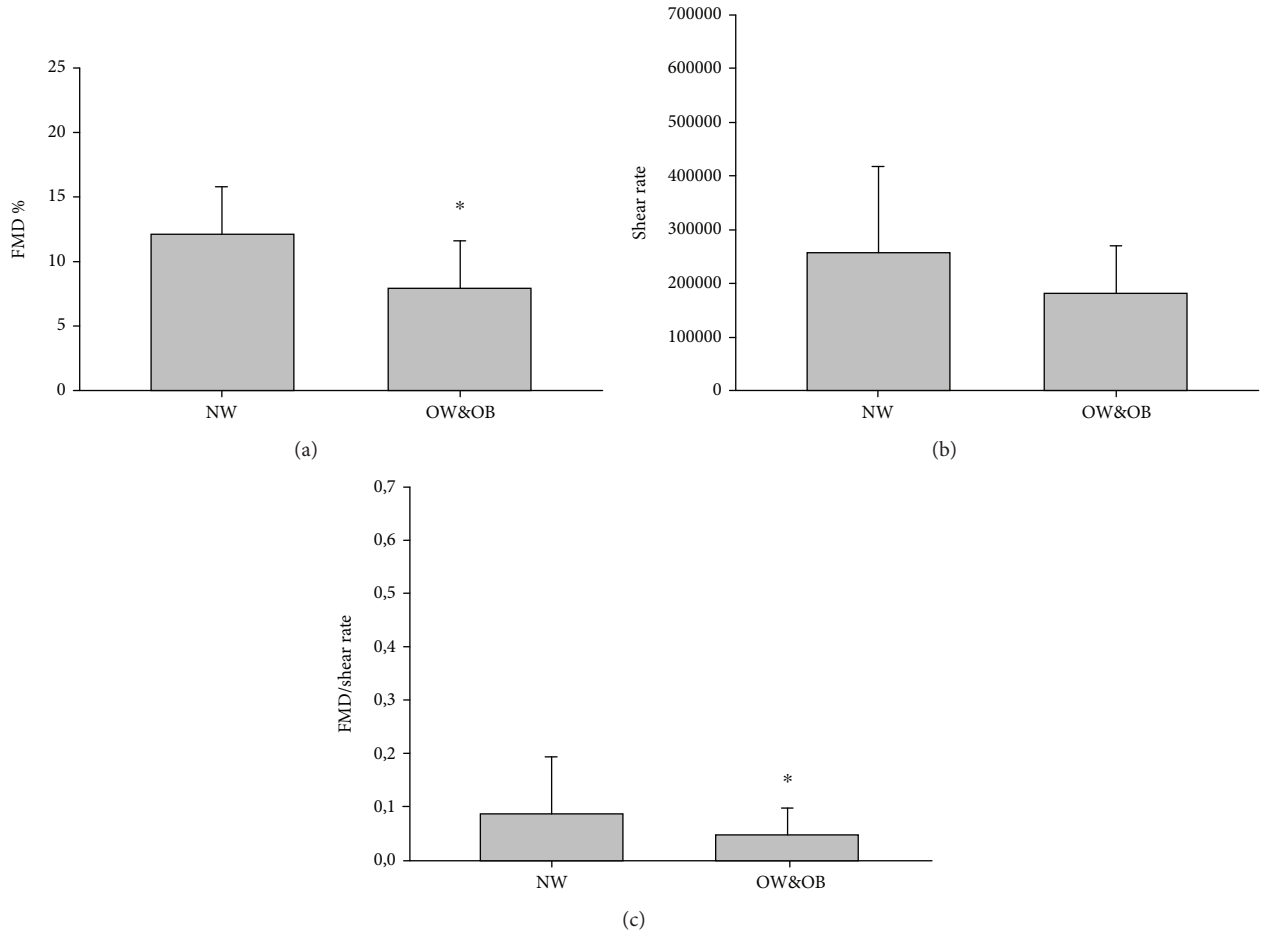

Figure 2: Flow-mediated dilation in normal weight and overweight and obese elderly subjects. *Statistical difference between groups, $p < 0.005$.

profile in obese older adults also recommend exercise as an effective intervention [7, 8, 10]. However, no studies have investigated the effect of exercise without caloric restriction on vascular function and inflammatory profile in older obese adults. In the present study, we measured endothelial function and inflammatory profile in habitually exercising obese and nonobese older adults who have been taking part in a structured, supervised exercise program for several years without caloric restriction. In accordance with our hypothesis, the effects of elevated adiposity on endothelial function and inflammatory profile were not blunted in exercising overweight and obese individuals, demonstrating that exercise alone cannot serve as a fully successful strategy. Moreover, even though obese individuals exhibited poorer vascular function and higher proinflammatory values, these two phenomena do not seem to be correlated.

4.1. Evidence about the Efficacy of Exercise on Vascular Functions in Age-Related Obesity. Although several studies have already investigated the effects of exercise intervention on vascular functions in aging and obesity supporting a positive exercise-induced adaptation, the majority of the studies investigated short-term exercise programs administered to previous sedentary obese subjects [4, 6]. Vinet et al. [6] investigated the effect of short-term low-intensity exercise training in middle-age obese men. Ten individuals were recruited and tested for brachial FMD before and after 8 weeks of an individualized low-intensity program, without any dietary intervention. Compared with normal weight controls, obese individuals exhibited poorer FMD; however, exercise training ameliorated FMD values in the obese group. The authors concluded that a short-term low-intensity exercise training improves endothelium-dependent vasodilation in sedentary middle-age obese men [6]. Another study by Dow et al. [4] showed that regular aerobic exercise reduces ET-1-mediated vasoconstrictor tone in overweight and obese middle-aged adults, and they concluded that these mechanisms may be very important in the exercise-induced improvement in endothelium-dependent vasodilator function in this population.

Our results are not in agreement with the mentioned studies. Indeed, the obese elderly individuals included in our study, who were not undertaking a caloric restriction diet, showed a significantly decreased vascular function compared with age-matched counterparts even though the subjects have been exercising regularly for several years. There was also no statistical difference in the amount of physical activity practiced in the two groups.

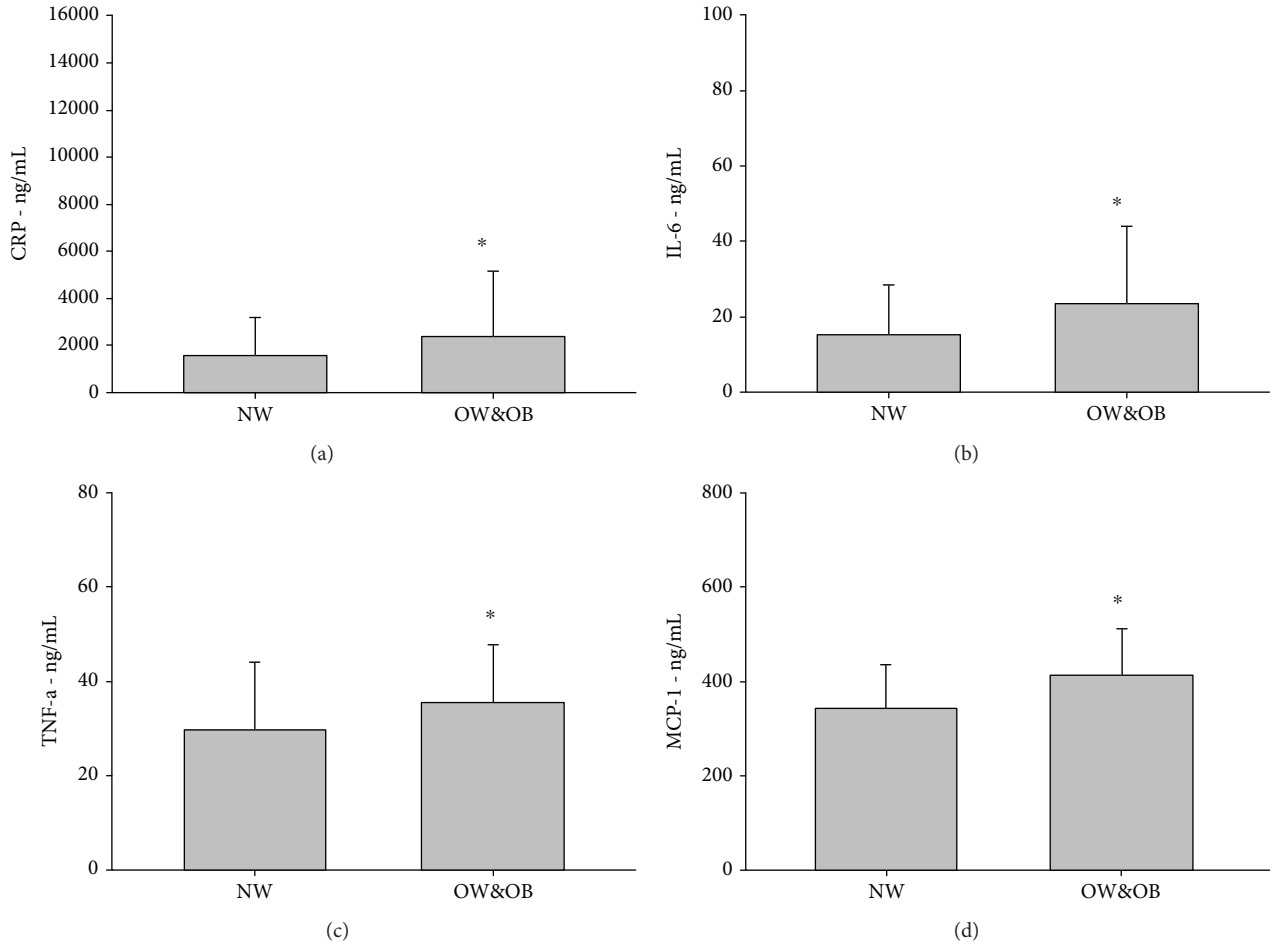

Figure 3: Proinflammatory markers in normal weight and overweight and obese elderly subjects. C-reactive protein (CRP, a); interleukin-6 (IL-6, b); tumor necrosis factor-α (TNF-α, c); and monocyte chemoattractant protein-1 (MCP-1, d). *Statistical difference between groups, $p < 0.005$.

Table 2: Pearson's correlation between vascular function and inflammatory profile in normal weight and overweight and obese groups.

	CRP	IL-10	IL-1ra	IL-1B	IL-6	IL-8	MCP-1	TNF-α
Normal weight								
FMD%	0.216	0.372	0.608	0.225	0.974	0.397	0.980	0.402
Shear rate	0.252	0.820	0.867	0.178	0.295	0.458	0.962	0.534
FMD/shear rate	0.679	0.748	0.492	0.651	0.325	0.748	0.385	0.620
Overweight & obese								
FMD%	0.237	0.800	0.430	0.383	0.248	0.177	0.121	0.709
Shear rate	0.469	0.718	0.640	0.300	0.666	0.799	0.438	0.297
FMD/shear rate	0.670	0.729	0.755	0.616	0.540	0.464	0.582	0.722

Note: CRP, C-reactive protein (ng/mL); IL, interleukin (ng/mL); MCP-1, monocyte chemoattractant protein-1 (ng/mL); TNF-α, tumor necrosis factor-α (ng/mL). No statistical correlation between variables in both groups were found.

In support of our results, Lind et al. [5] demonstrated that the increased level of self-reported physical activity does not fully eliminate the deleterious cardiovascular consequences associated with overweight and obesity. One reason supporting our findings may reside directly in the physiology of adipose tissue.

Indeed, adipose tissue acts as an endocrine organ that releases bioactive molecules known as adipokines, overexpressed in obese individuals, leading to a proinflammatory status. Among adipose tissue depots, the perivascular adipose tissue (PVAT) displays a unique physiological role that is a paracrine regulation of vascular function. At the

physiological level, PVAT exerts regulatory effects on metabolism and inflammatory response via the local release of hormones, cytokines, and reactive oxygen and nitrogen species. When PVAT exceeds the physiological level, it carries an anticontractile property that influences arteriolar responses to agonists contributing to the regulation of blood flow, nutrient uptake, and tissue homeostasis. Moreover, PVAT is susceptible to inflammation and in obesity infiltration of immune cells into the PVAT is aggravated and the amount of PVAT alongside large and small vessels is augmented, exerting anticontractile effects on conduit and resistance arteries [23]. Consequently, an excess of PVAT might contribute to explain how impaired adipose tissue homeostasis affects vascular functions in obese elderly individuals.

4.2. Evidence about the Efficacy of Exercise on Inflammatory Profile in Age-Related Obesity. Recent literature started to focus on long-term physical activity and inflammatory biomarkers in obese subjects, but results are still contrasting. Christiansen et al. [16] aimed to investigate the effect of exercise training and diet-induced weight loss alone or in combination with inflammatory biomarkers. By combining the weight loss in all three groups, the authors found a correlation between the degree of weight loss and improvement in several of the inflammatory markers. The authors concluded that the rather large weight losses (<5–7%) were found to have beneficial effects on circulating inflammatory markers in obese subjects. Furthermore, aerobic exercise for 12 weeks was found to have no effects on circulating inflammatory markers in these subjects, suggesting that a more intensive exercise may be necessary to affect systemic inflammation in obese subjects [16]. Brunn et al. [17] aimed to investigate the effect of a 15-week lifestyle intervention (hypocaloric diet and daily exercise) on inflammatory biomarkers in severely obese subjects. The intervention reduced body weight and increased insulin sensitivity. It also increased plasma adiponectin, while it decreased CRP, IL-6, IL-8, and MCP-1 levels. In agreement with the previous study, the authors concluded that the combination of hypocaloric diet and moderate physical activity resulted in a significant general decrease in the level of inflammation [17]. Pischon et al. [24] investigated the relationship between physical activity and the obesity-related inflammatory markers CRP, IL-6, and soluble TNF-receptors (sTNF-Rs) 1 and 2. The authors even examined the relationship between physical activity and insulin sensitivity and whether inflammatory markers mediate this association. In the study, 405 healthy men and 454 healthy women were included and information about physical activity and other variables were assessed by questionnaires. Results showed that physical activity was inversely associated with the plasma levels of sTNF-R1 and 2, IL-6, and CRP, but after having adjusted for BMI and leptin (as a surrogate of fat mass), most of these association were no longer significant [24]. Our results are partially in accordance with the mentioned studies. First, even though obese individuals without a caloric restriction diet included in the study are considered very active, no exercise-induced adaptation on the inflammatory profile is seen. As previously mentioned, although our subjects have been active already for several years and there is no difference in the amount of physical activity between groups, they did not experience great weight changes in the last 5 years, limiting the effects of the regular physical activity they practice. In obesity, an increased fat mass is accompanied by alterations in the cellular composition and physiology of adipose tissue. Hypertrophy and increased distribution of adipocytes, together with inflammation and dysregulated adipokine secretion, characterize a dysfunctional adipose organ which contributed to the development of several obesity-associated morbidities [23]. Furthermore, in obesity the interplay between adipocytes and immune system components changes. Immune cells secrete cytokines that enhance adipose tissue inflammation, and at the same time, adipocytes express classical macrophage features. As the adipose tissue expands, the populations of macrophages, mast cells, B cells, and T cells increase considerably. As a consequence, there is an increased secretion of the inflammatory adipokines TNF-α and CRP and a reduction of anti-inflammatory adipokines [23]. In turn, proinflammatory adipokines and CRP might express their action on endothelial cells compromising their proliferative rate and angiogenic potential, which may contribute to the decline of vascular health and development of symptoms such as hypertension [25]. Although exercise can be considered an anti-inflammatory treatment as seen in many studies, it appears that if exercise is not accompanied by an important caloric restriction with consequent weight loss, the effect of exercise on the inflammatory profile is poor. Indeed, only when exercise decreases body fat and adipocyte hypertrophy will the number of inflammatory cells contained within the adipose tissue decline [23]. This could explain the results found in the individuals included in our study. While they are very active, accumulating about 250 min of physical activity a week, this does not seem to be enough to counteract the harmful effect of age-related adiposity on the inflammatory profile of overweight and obese elderly people [24, 26].

4.3. Limitations. Although the results of our study are consistent, some limitations have to be highlighted. First, we did not have any additional control group such as a sedentary NW group and a sedentary OW&OB group. These two control groups would have allowed a better understanding of the exercise-induced adaptation in elderly obese individuals who habitually exercise. Second, we did not investigate any difference between the subgroups of overweight and obese who could exhibit different responses. Third, we did not measure any other parameter which could have helped in the description of the subjects and in the interpretation of the results such as lipid profile, hematocrit, and white blood cell analysis.

5. Conclusion

Our hypothesis was that regular exercise would not have helped in maintaining a good inflammatory profile and vascular function in overweight and obese elderly individuals.

We also hypothesized that poorer vascular function would have been related to higher proinflammatory markers in this population. Although significant differences were found for the measured variables between overweight and obese subjects compared with nonobese elderly individuals, the results of our study demonstrated that regular, supervised exercise without a caloric restriction diet is not a fully successful stimulus able to counteract the deleterious effect of age-related adiposity on vascular function and inflammatory profile. Moreover, contrary to our second hypothesis inflammation and vascular function do not seem to be correlated and further studies are needed in order to understand the mechanisms underlying the exercise-induced physiological processes that may ensure protection against aging and obesity effects, and studies should be focused on an efficient exercise program able to trigger positive physiological mechanisms against age-induced obesity-related morbidities.

Abbreviations

NW: Normal weight individuals
OW&OB: Overweight and obese individuals
NO: Nitric oxide
ROS: Reactive oxygen species
BMI: Body mass index
6MWT: 6-Minute walking test
IL-1ra: Interleukin-1ra
IL-1β: Interleukin-1β
IL-6: Interleukin-6
IL-8: Interleukin-8
IL-10: Interleukin-10
TNF-α: Tumor necrosis factor-α
MCP-1: Monocyte chemoattractant protein-1
CRP: C-reactive protein
FMD: Flow-mediated dilation
BF: Blood flow
1RM: 1 repetition maximum
ET-1: Endothelin-1
PVAT: Perivascular adipose tissue.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

The authors are indebted to Camilla Varalta, Daniela Zampieri, Marina, Lia, Francesca Dal Molin, and Sara Flamigni for their help. The authors also thank the volunteers for providing the samples used in this work. This work was supported by the Italian Diabetology Society (SID).

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 8260742, 9 pages
https://doi.org/10.1155/2018/8260742

Review Article

Myokines as Possible Therapeutic Targets in Cancer Cachexia

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Received 13 July 2018; Accepted 23 September 2018; Published 22 October 2018

Guest Editor: Donato Zipeto

Cachexia is an extremely serious syndrome which occurs in most patients with different cancers, and it is characterized by systemic inflammation, a negative protein and energy balance, and involuntary loss of body mass. This syndrome has a dramatic impact on the patient's quality of life, and it is also associated with a low response to chemotherapy leading to a decrease in survival. Despite this, cachexia is still underestimated and often untreated. New research is needed in this area to understand this complex phenomenon and ultimately find treatment methods and therapeutic targets. The skeletal muscle can act as an endocrine organ. Signaling between muscles and other systems is done through myokines, cytokines, and proteins produced and released by myocytes. In this review, we would like to draw attention to some of the most important myokines that could have potential as biomarkers and therapeutic targets: myostatin, irisin, myonectin, decorin, fibroblast growth factor 21, interleukin-6, interleukin-8, and interleukin-15.

1. Introduction

Cachexia is an extremely serious syndrome manifested by anorexia, weight loss through loss of muscle mass and fatty tissue, inflammation, and increased energy consumption that occurs in many chronic diseases, of which cancer occupies a special place (80% of patients with cancers develop cachexia) [1]. Cachexia occurs in most patients with terminal cancer and is responsible for death of approximately 22% of patients [2]. It is characterized by systemic inflammation, a negative protein and energy balance, and involuntary loss of body mass. This syndrome has a dramatic impact on the patient's quality of life, and it is also associated with a low response to chemotherapy and leads to a decrease in survival [3–5].

Cachexia is still underestimated and often untreated [6, 7] despite its association with many mechanisms, especially inflammatory, which contribute to the installation of a persistent catabolic status.

The current strategy focuses on treating cancer, with the hope that it will completely reverse cachexia syndrome. But this is not valid in advanced cancers. Another option is to increase nutritional intake, but the anorexia of cachectic patients is only part of the problem, nutrition as unimodal therapy not yielding the expected results. In addition, radiochemistry may exacerbate the progression of cachexia in a number of patients [8, 9].

Until ten years ago, cachexia was seen as an untreatable syndrome. In recent years, however, the management of cancer cachexia has greatly improved, as studies on the involved mechanisms have developed. Current treatment of cachexia in malignant neoplasm is a palliative one. Many anticancer products may have beneficial effects in treating cancer but

worsen cachexia [10]. New research is needed in this area to understand this complex phenomenon and ultimately find treatment methods, therapeutic targets that prevent cancer progression but also improve the quality of patient's life. A multidisciplinary approach to treating cachexia would be necessary: new pharmacological agents combined with diet modification and exercise.

There are papers showing that the skeletal muscle can act as an endocrine organ [11, 12], exerting its influence on other organs/systems, maintaining physical activity and ultimately life. It is a tissue energy producer and consumer that influences the energy metabolism of the whole organism. Signaling between muscles and other systems is done through myokines, bioactive substances released by the skeletal muscles [13]. These muscle cytokines exert an autocrine, paracrine, and endocrine effect and play a role as metabolic mediators between muscle tissue and other tissues such as the adipose tissue [14, 15], cardiac muscle [16], liver [17, 18], and pancreas [18].

In this review, we will refer to myokines, one of the components of this complex mechanism that leads to the appearance of muscle weakness and muscle mass loss in cancer, that have an important potential to become therapeutic targets.

2. What Are Myokines

Myokines have been defined as cytokines and proteins produced and released by myocytes [19] under the action of contractile activity [12]. They exert an autocrine, paracrine, or endocrine effect. Their receptors were found in the muscle, fat, liver, pancreas, bone tissue, heart, brain, and immune cells [20]. For the role of muscle tissue as "endocrine organ," there are several studies that address this subject from different angles, not necessarily in cachexia. Thus, the existence of myokines as metabolic mediators between skeletal muscle and other organs during exercise to maintain a healthy status is shown by Schnyder and Handschin [12]. Other articles, describing the involvement of skeletal muscle in the development of aging-related pathologies, highlight the role of myokines in inducing or protecting these pathologies, depending on the secretion amount [21]. There are studies that show the role of myokines in the general metabolism of the body and how they interact with other organs [18]. Only few papers describe the role of myokines in cancer, precisely in cancer cachexia, which is an area recently approached. Dalamaga's editorial draws attention to the interaction between adipokines and myokines in the pathophysiology of cancer, making a review of literature data related to this subject [22, 23].

For the reasons above, myokines are essential therapeutic targets in cachexia and the modulation of their expression could improve the maintenance of skeletal muscles at parameters as close as normal in cancer patients (Figure 1).

Without going into the details about the signaling pathways in myocytes, already described in other publications, we would like to draw attention to some of the most important myokines that would have potential as biomarkers and therapeutic targets.

2.1. Myokines as Potential Therapeutic Targets. The main myokines studied to date are myostatin, decorin, irisin, myonectin, interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-15 (IL-15), follistatin, fibroblast growth factor 21 (FGF21), bone morphogenetic protein (BMP), and brain-derived neurotrophic factor (BDNF) [12, 13, 22, 23]. Other possible factors have been detected in skeletal muscle, but their function, as well as their presence in the circulation, are largely unknown: musclin, nonneuronal acetylcholine [11].

Between these myokines, we would like to draw attention on some of the most studied so far (Table 1).

2.1.1. Myostatin. Also called growth differentiation factor 8 (GDF-8), it is a member of the transforming growth factor-β (TGF-β) family, expressed in developing and adult muscular tissue. It is one of the first described myokines. Contrary to other myokines, which have a high level after exercise, myostatin has a low level after sustained muscular effort [24–29].

Its main function is the negative regulation of the muscle mass [30], which means high level of myostatin, less muscle mass. It plays a role in stopping myoblast proliferation and suppressing satellite cell activation, inducing muscle atrophy [31]. In addition, it influences the differentiation of muscle fibers by types (fast and slow) [32] and the arrangement of muscle glucose [33] as well as the muscle-adipose tissue cross-talking [34].

Myostatin influences the physiology of adipocytes, but it seems in an indirect manner. Pharmacological administration of myostatin *in vivo* and *in vitro* models does not lead to the reduction of adipose tissue by lipolysis [35].

It seems that in myostatin null mice, reduced body fat is caused especially by muscle mass growth. Myostatin null mice develop a massive muscular hypertrophy resulting from an accelerated myogenesis [21, 36], accompanied by a massive reduction in fatty tissue [30]. A similar phenotype has been described in a child with a mutation in the myostatin gene [37].

Interestingly for our subject, cachexia, is that the circulating leptin level, the "satiety hormone," secreted by adipocytes, is reduced in mice with myostatin deficiency, although food intakes compared to control mice (WT) were not different [36, 38].

Although there are relatively few studies on the expression of myostatin in muscle cachexia, especially as a biomarker and therapeutic target, we consider it to be a good research approach in cachexia treatment, especially in conjunction with decorin and leptin.

2.1.2. Irisin. Discovered in 2012 as a transmembrane protein [39], FNDC5 has a cleaved soluble form, irisin, that it is released into circulation during the proteolytic process after acutely exercising of skeletal muscles. It increases the energetic and oxidative metabolism of the muscle by activating genes related to these processes. It has a high level during myogenesis and induces glucose uptake [40], improving glucose homeostasis, inhibiting lipid accumulation, and reducing body weight [41].

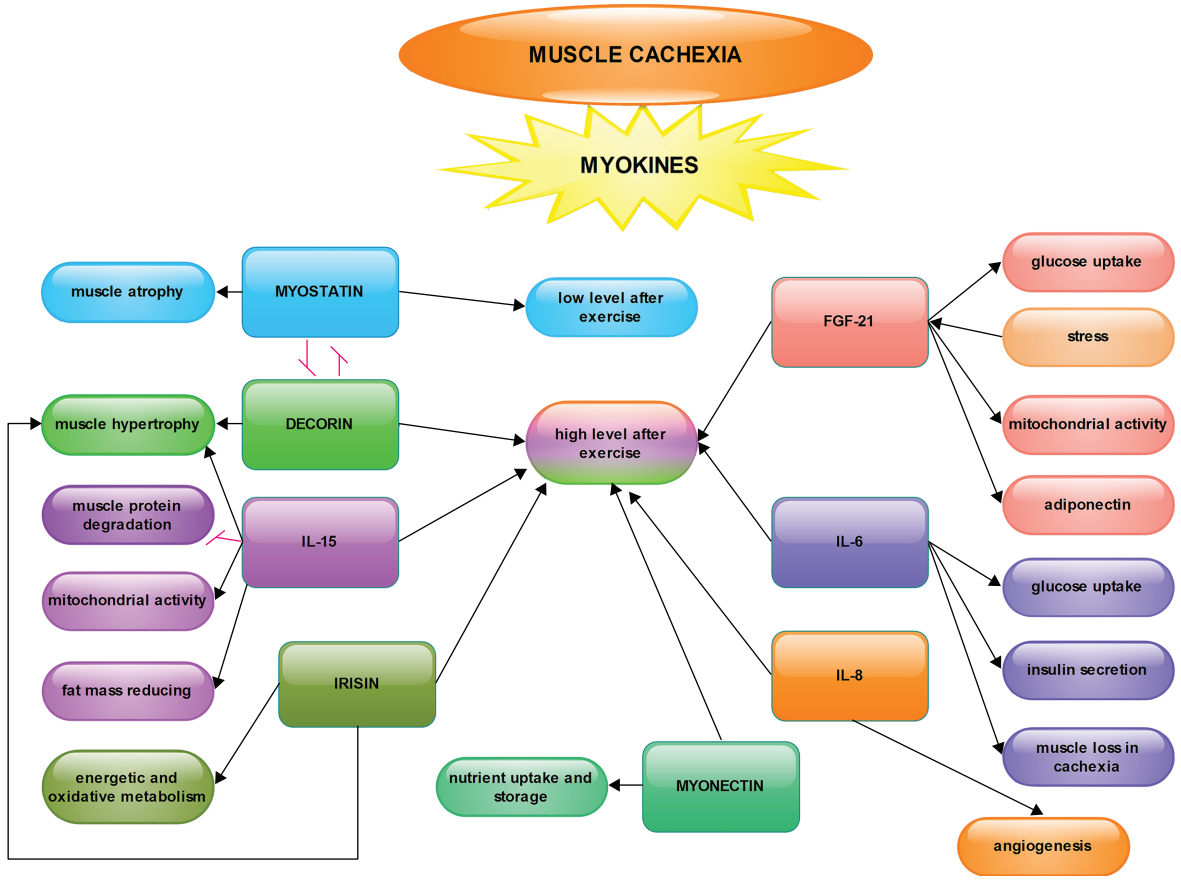

Figure 1: Effects of myokines in muscle cachexia. The schematic representation of myokine activity in the skeletal muscle shows the following: except for myostatin, which decreases after exercise, all others have a higher level after effort; between myostatin and decorin, there is an antagonistic relationship of mutual inhibition; the arrows show an activation or stimulation relationship between myokines and various metabolic processes that occur in the skeletal muscle.

It has been studied especially in relation to obesity but also with myopathies such as muscular dystrophy. In these latter studies, injection of irisin induced muscle hypertrophy, improving muscle strength and reducing necrosis and development of connective tissue in a murine model [42]. This study may be a starting point for attempts at therapeutic irisin targeting cancer cachexia as well.

2.1.3. Myonectin (CTRP15). Myonectin is a protein belonging to the C1q/TNF-related protein (CTRP) family, and it is found mainly in muscle, less in circulation, being especially related to nutritional metabolism. Thus, the expression of myonectin is stimulated by exercise and nutrients and is supposed to induce nutrient uptake and storage in other tissues, such as adipose tissue, causing a flux of glucose or fatty acids [43, 44].

It is less studied in connection with cachexia. We suppose that it could be a therapeutic target, just like other myokines, being linked to nutrient uptake.

2.1.4. Decorin. Decorin is a small leucine-rich proteoglycan released by myotubes, and as other myokines, its circulating level is increased after acute exercise. Decorin is overexpressed in the skeletal muscle in humans and mice after chronic training [45]. It directly binds myostatin which is a strong inhibitor of muscle growth [36]. Decorin acts antagonistically to myostatin and is involved in restructuring muscle during hypertrophy [45].

Considering all of this, we can say that this myokine could be taking into account as the therapeutic target along with myostatin, being able to modulate the maintenance of muscle mass in cachexia.

2.1.5. Fibroblast Growth Factor 21 (FGF 21). Fibroblast growth factors are present in many tissues as signaling proteins and are implied in development and metabolism [46]. In the skeletal muscle, it has been shown that FGF21 has a role in glucose uptake in myotubes [47].

FGF21, as a myokine, is induced by stress [48]. Mitochondrial dysfunction after an autophagy deficiency increases the FGF21 level to protect against obesity induced by diet and insulin resistance [49]. In the mitochondrial respiratory chain deficiency, there is a compensatory increase in FGF21 level resulting in an increase in mitochondrial activity [50].

There is a close link between FGF21 and adiponectin that acts as downstream effector of FGF21, controlling in an endocrine mode the lipid homeostasis and glucose in the

Table 1: The most studied myokines and their action mode in skeletal muscular tissue.

Myokine	Action	Level after muscle exercise
Myostatin	Stops myoblast proliferation Suppresses satellite cell activation Induces muscle atrophy	Lower level
Irisin	Activates genes related to oxidative metabolism Induces muscle hypertrophy Improves muscle strength Reduces necrosis	High level
Myonectin	Induces nutrient uptake Induces nutrient storage in adipose tissue	High level especially in muscle, less in circulation
Decorin	Acts antagonistically with myostatin Involved in restructuring muscle	High level
FGF21	Induces glucose uptake Increases mitochondrial activity Connected with adiponectin Implied in the control of lipid homeostasis, energetic metabolism, and insulin sensitivity	High level
IL-6	Increases glucose uptake, oxidation of fatty acids Increases insulin secretion Elevated in cancer cachexia—low level Alleviate cachexia progress	High level
IL-8	Elevated in cancer cachexia, especially like cytokine Induces angiogenesis	High level in muscle, not in plasma
IL-15	Anabolic effect Decreases muscle protein degradation Reduces fat mass Induces muscle hypertrophy Increases mitochondrial activity	High level

skeletal muscle and other organs, such as the liver. In turn, adiponectin regulates the influence of FGF21 on energetic metabolism and insulin sensitivity [51, 52].

FGF21 is a very poorly addressed myokine in the study of cachexia, although its involvement in the energy metabolism of the myocyte is demonstrated. Future research would be wanted to highlight its potential in therapeutic strategies as long as the energy metabolism of the muscle is very important in maintaining a normal state of this tissue.

2.1.6. Interleukin-6 (IL-6). IL-6 is the first myokine that has been discovered in the bloodstream, secreted by muscle cells after contraction [19], and one of the most studied.

It was originally described as a prototypic proinflammatory cytokine, then having anti-inflammatory properties also [53]. IL-6 is released by the immune system cells (monocytes/macrophages), fibroblasts, and endothelial cells [54] and also by the skeletal muscle correlated with the exercise [54–57]. Following the release of IL-6 by the muscle, it increased glucose uptake, oxidation of fatty acid, and insulin secretion. Although its release was originally linked to muscle damage [58], subsequently, a plasma increase in IL-6, less dramatic and nondamaging, was demonstrated in concentric muscular contraction and even immediately after exercise [19].

But how does IL-6 bind to cachexia and what therapeutic role can it have? A review on this subject was made by Narsale and Carson [59]. The authors show that IL-6 remains a promising therapeutic strategy for diminishing cachexia in many types of cancers. However, it is necessary to better understand the direct and indirect effects of IL-6, as well as its specific tissue actions to improve this treatment.

It is clear that diminishing this myokine can alleviate the progression of cachexia in cancer patients [60].

Numerous *in vivo* studies on rodents have been conducted to establish the mechanisms for muscle wasting producing. It has shown that there is a suppression of protein synthesis on the one hand and the activation of pathways of protein degradation on the other hand [61–64]. The muscle loss in cancer cachexia is directly or indirectly linked to overexpression of IL-6 [65–67]. But between the results obtained on murine cachexia models in different types of cancers, there are differences: in IL-6 mechanisms of action and in inhibition of various IL-6-dependent signaling pathways [68, 69] by attenuating or eradicating the progression of cachexia [67].

Unlike *in vivo* and *in vitro* investigations, studies on muscle mass recovery pathways in cancer patients are difficult to do, and the results differ from one type of cancer to another. It is certain, however, that advanced or terminal cancer patients have high levels of IL-6 in plasma, correlated with weight loss, anemia, and depression [70]. Clinical studies of an IL-6R inhibitor that inhibits the binding of IL-6 to its receptor, tocilizumab, have shown in patients with cancer cachexia the reduction of plasma IL-6 levels, the alleviation of muscle mass loss without affecting tumor proliferation [8, 71, 72]. Possible side-effects of suppression of interleukins, such as IL-6, which may be compromising patients' immune response to infections, should be monitored. Also, the effects of IL-6 signaling in organs other than muscles, such as liver and gut, should be considered [73].

2.1.7. Interleukin-8 (IL-8). IL-8 is a chemokine produced by muscle cells and also by other cells like macrophages, epithelial cells, and endothelial cells. It is a member of the CXC cytokine family and was originally described as a chemoattractant for lymphocytes and neutrophils [74, 75], and later, it was shown to be involved in angiogenesis and tumor growth [76].

In recent years, some researchers have shown that IL-8 is involved in cachexia, finding an elevated level in the serum of patients with this syndrome [77, 78], but rather like cytokine rather than myokine.

An additional argument that IL-8 plays a role in cachexia is brought by a publication that has shown that the genetic polymorphism of this myokine can contribute to the pathogenesis of cachexia in gastric cancer [79].

A team of researchers found IL-8 in the muscle, not the plasma, following exercise, indicating its local role in angiogenesis for example [80]. Although its physiological function is largely unknown, association with CXCR2 suggests its involvement in exercise-induced neovascularization in the muscle tissue [81].

It has been shown in healthy subjects that after muscle exercise, the level of myokines in the blood has increased. These include IL-8 and IL-15. Interestingly, a continuous muscle contraction with a moderate intensity induces a higher concentration of myokines than a shorter muscular contraction but with a high intensity [82]. This fact, correlated with the promotion of angiogenesis, could be a starting point for studies on IL-8 produced in muscular tissue as a therapeutic target in cancer cachexia and may be a key point in reducing muscle mass loss or in rebuilding skeletal muscle along with other factors.

Attention should also be paid to the fact that IL-8 is also produced in adipose tissue, especially the visceral one, and has a high level in obese patients [83]; the modulation of this myokine could be made from different directions/tissues.

2.1.8. Interleukin-15 (IL-15). IL-15 is present in the skeletal muscle, having an anabolic effect on the metabolism of muscle proteins, and is also modulated by exercise [20]. It decreases muscle protein degradation and reduces fat mass, playing an important role in skeletal muscle-adipose tissue interaction [84–88]. IL-15 overexpression induces muscle hypertrophy and is involved in the synthesis and inhibition of protein degradation as it is shown in an *in vitro* study [89].

This myokine is connected with the alteration of mitochondrial function, overexpression of muscle IL-15 increasing mitochondrial activity and adipose tissue mass [90].

The role of IL-15 in cachexia is not fully understood. An earlier study on a rat model with cancer cachexia showed that IL-15 decreases the rate of protein degradation without affecting protein synthesis [91]. A research conducted on adult patients with a diagnosis of recent cancer and weight loss showed that there was no difference between their serum IL-15 levels and those of healthy subjects [92].

Despite these controversial results, the potential of this interleukin is not excluded, and other studies are needed to show this.

An important idea that should be considered is that there are cytokines that can be released by both the immune system and the muscles. Inflammation occurs in cancer and may even induce cancer [93]. So when we act on the cytokines released by the muscles (myokines), we must also keep in mind that the same cytokines are released by other cells/tissues also, and they can be influenced by our action [94–96].

3. Conclusions

We cannot draw conclusions about the place and role of myokines in *cancer cachexia therapy* without reminding the complex pathophysiology they are involved in and the fact that there are many signaling pathways in this syndrome that interfere and interrelate.

One of these important *interactions is between skeletal muscle and adipose tissue*, more specifically between myokines, adipokines, and free fatty acids, as we have shown. It has been proven that 25% of cancers are caused by obesity and a sedentary life [97]. Myokines and adipokines play an essential role in maintaining the body muscle and body fat at normal levels and thus modulate the body composition [23, 98–100]. The lack of movement and the existence of a large adipose tissue contribute to the destruction of the skeletal muscle tissue that occurs in cancer cachexia.

Cachexia treatment may be a challenge because it is necessary to address multiple and complex determinant causes. It requires a therapeutic combination based on sustained research in the fields of pharmacology, nutritional intake stimulation, reduction of inflammation, etc. We would like to draw attention to the possibility of considering myokines as possible therapeutic targets. Some of them have already been considered, especially IL-6, but not all of them. They may be taken into account for targeted therapeutic interventions, especially in personalized medicine when specific tests could be performed for each patient regarding their specific released myokines, depending on the health status or muscle damage.

Consideration should be given to the possibility of cancer *cachexia prevention* also, so that patients could better respond to antitumor treatment. As we have seen, *exercise* can be a powerful tool in preventing and treating muscle cachexia, along with other therapies. Of course, the exercise should be moderate, not acute, in order to not interfere with unwanted metabolic changes in both skeletal muscle and other organs such as the liver, adipose tissue, and pancreas. Myokine expressions change after exercise and transmit signals from the muscles to the rest of the body. There are not many studies regarding this phenomenon in muscle cachexia, and research is needed in this direction to know how much exercise a cachectic patient needs to get beneficial effects in muscle recovery or even to prevent cancer cachexia.

Conflicts of Interest

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

Acknowledgments

This study was partially supported from Project IDs PN-III-P1-1.2-PCCDI-2017-0341 (PATHDERM) and PN-III-P1-1.2-PCCDI-2017-0782 (REGMED), PN 18.21.02.02/2018.

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 4625472, 10 pages
https://doi.org/10.1155/2018/4625472

Review Article

The Central Role of Inflammation Associated with Checkpoint Inhibitor Treatments

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Received 13 July 2018; Accepted 3 September 2018; Published 17 October 2018

Guest Editor: Iulia D. Popescu

An important function of the immune system is its ability to differentiate between healthy cells in the organism and "foreign" cells, allowing the latest to be attacked and the first ones to be conserved. The most important molecules in this process are considered to be checkpoint inhibitors. This review is focused on the association between cancer and inflammation, underlying the mechanisms of action of monoclonal antibodies that are targeting checkpoint inhibitors: ipilimumab against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and pembrolizumab and nivolumab against programmed cell death protein 1 (PD-1), their indications for treatment, and side effects. Presence of antibodies against checkpoint inhibitors shows promising results in the clinical trials in patients with types of cancer difficult to treat until now such as melanoma, non-small-cell lung cancer (NSCLC), and renal cell carcinoma, offering an increase in the overall survival rate, response rate, and progression-free rate. Resistance is now observed to emerge in patients treated with this therapy, showing the need for more studies in order to design a biomarker that will predict the type of response to immunotherapy.

1. Introduction

The immune system plays an important role in controlling malignant flare-ups, in this way abolishing cancer. In patients who developed cancer, there are multiple immune suppression mechanisms that prevent the development of a competent antitumor response. Current advances revealed that antibodies against negative immunologic regulators such as checkpoint inhibitors can have success in treating a wide variety of malignancies, both solid and hematologic. Even though cancer has a major impact around the globe, an increase in the survival rate of the people suffering from cancer was registered, due to the development of these new therapeutic approaches [1]. Checkpoint inhibitors have shown clinical efficacy in several solid malignancies including melanoma, renal cell carcinoma, NSCLC, bladder cancer, head and neck squamous cell carcinoma, Merkel cell carcinoma, and hematologic malignancies such as Hodgkin lymphoma. A particularly successful effect was observed in melanoma, nowadays being approved both checkpoint inhibitors anti-PD-1 or PD-L1 (nivolumab, pembrolizumab) and anti-CTLA-4 (ipilimumab), as monotherapy or in association between them [1–3]. Even though melanoma accounts only for 4% of all skin cancers, it is responsible for 80% of the deaths due to malignancies of the skin [4]. It is one of the cancer types that benefit the most from immunotherapy, especially in advanced stages. Melanoma appearance is considered to be closely bound to UV exposure [5]

and stress. In vitro, the association with stress was explained by the observation that high level of cortisone, epinephrine, and norepinephrine in cellular environment leads to an increase of murine B16F10 melanoma cells [6].

2. Inflammatory Response in Cancer

Inflammation in cancer is sustained by a series of immune and nonimmune cells and the molecules that are secreted by these.

Malignant cells secrete a wide variety of molecules such as cytokines and chemokines, which attract a diverse population of leukocytes (neutrophils, dendritic cells, macrophages, mast cells, and lymphocytes). These will in turn produce an array of molecules that contribute to the fight against malign cells, these molecules being TNF-alpha, interleukins, interferons, and several other agents that are able to damage the plasmatic membranes of the cancerous cells.

There are several immune cells that are in the tumorigenesis process and are endowed with protumor functions. Macrophages associated with the tumor have opposite roles in the malign environment: they can kill the cancerous cells after being activated by IL-2, interferon, and IL-12, but also they can secrete angiogenic and lymphangiogenic growth factors that enable the tumor cells to proliferate [7].

The mechanism of attracting immune cells to the cancerous environment is explained trough the fact that around 15% of all cancers arise from areas that were or still are subjected to infection [8]. Persistent infections will lead to a chronic inflammation. The immune cells will produce reactive oxygen and nitrogen species. These will induce permanent genetic alterations of the proliferating cells by producing a mutagenic agent called peroxynitrite.

It can be concluded that inflammatory cells play an important role in cancer development. In the first stages of cancer, these cells promote tumor growth by producing growth and survival factors that will enable angiogenesis and lymphangiogenesis. In the later stages, inflammatory cells can produce chemokines that are able to help the malignant spread, thus leading to metastasis. On the other site, the inflammatory cells will trigger the immune response which will be counterproductive for the tumor development [9].

Inflammation and tumor generation have a close causal relationship between them. On one side, an inflammatory state can precede a cancerous growth, but on the other side, a malignant transformation can lead to inflammation in the cellular environment which will maintain the cancerous state. Accumulation and production of inflammatory molecules such as cytokines over a long period of time will lead to an immunosuppressant state which is linked to tumor progression [10].

3. Types of Immunotherapies

The era of immunotherapy in cancer dates back to the late 19th century. It all started with the idea of William Coley, an American surgeon, who inoculated bacteria in a sarcoma in order to shrink the tumor. His idea was based on the fact that bacteria will be able to trigger an immune response, which will eventually lead to a sustained antitumor immune response.

For most of the 20th century, scientists struggled to convert Coley's observations into an effective cancer treatment. This struggle lead to success; thus, nowadays there is a variety of immunotherapies that are making their way to the clinic. Immunotherapies can be divided into four categories: nonspecific immune stimulation, adoptive cell transfer, checkpoint inhibitors, and vaccination.

3.1. Nonspecific Immune Stimulation. In principle, in vivo nonspecific immune stimulation leads to a general increase of the immune response. Inoculated molecules activate the antigen-presenting cells (APCs) by binding to specific membrane receptors. Activated APCs stimulate other immune cells such as T cells, the principal antitumor cells. In order to be fully activated, T cells need the presence of some cytokines such as interferon alpha (IFN-alpha) and interleukin 2 (IL-2). These cytokines have already gained their role in cancer treatments [11].

Nonspecific immune stimulation can be achieved by BCG (bacillus Calmette-Guerin) vaccination. This can give a boost of the immune system that can lead to an increase in cancerous fight. The mechanism is based on the fact that attenuated bacteria can lead to inflammation, attracting more immune cells in the tumor microenvironment. The increased number will raise the chances of attacking the cancerous cells [12].

3.2. Adoptive Cell Transfer. Adoptive cell transfer is a type of in vitro manipulated therapy, which uses cytotoxic T cells isolated from the patient. These extracted cells are activated in vitro in order to be able to specifically target the cancerous cells.

T cells can be taken either directly from the tumor or from the blood. The advantage of the first procedure is the fact that immune cells are already exposed to specific antigens from the tumor microenvironment. Then, the harvested cells are activated using cytokines and multiplied before being transferred into the patient [13].

3.3. Checkpoint Inhibitors. Nonspecific immunity can also be achieved by removing immune checkpoint inhibitors. These inhibitors normally dampen down the immune response to prevent collateral damage to healthy tissue. In order to restore the active antitumoral immune response, scientists need to remove some of these inhibitors to make the immune response stronger. This is the case of ipilimumab, an antibody which can target a blockade molecule called CTLA-4 [1].

3.4. Vaccination. In comparison to the BCG vaccine that induces a general immune response, viral vaccines can be used to direct immune cells to target the malign environment. An example of this type of vaccine is an attenuated version of herpes simplex virus adapted to induce an immune-stimulating factor at the tumor site. This therapy is suitable for melanoma or head and neck carcinoma. T-VEC (talimogene laherparepvec) is a genetically engineered form of herpes simplex virus type-1, having removed

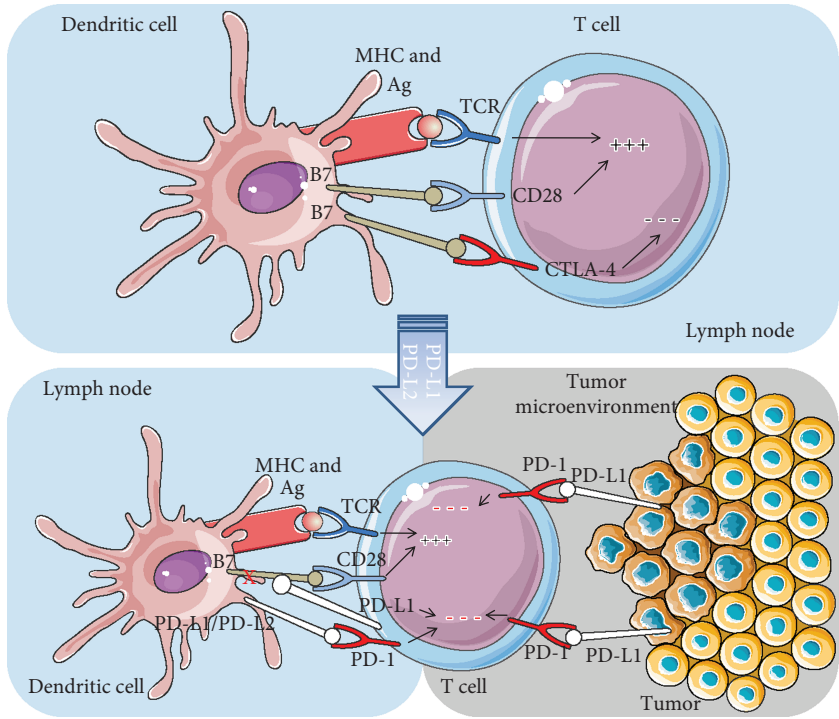

Figure 1: CTLA-4 and PD-1/PD-L1 pathways.

two genes in order to be unable to replicate intracellular in normal cells, while maintaining the cytolytic activity against cancerous cells [14, 15]. The multiplication inside cancerous cells leads to their burst (oncolytic effect) and triggers a systemic anticancerous immune response.

In nontumoral, but viral infected cells, viral replication takes place when ICP34.5, a gene that gives the herpes virus the propriety of neurovirulence, forms a complex with low levels of proliferating cell nuclear antigen (PCNA), a protein which is involved in DNA (deoxyribonucleic acid) replication and repair. In cancerous cells, the level of PCNA is high, so the herpes virus is able to replicate without the need of ICP34.5 gene [16]. The most recent clinical trials used HSV vaccine with an additional modification—the insertion of a gene which produces GM-CSF (granulocyte-macrophage colony-stimulating factor), which will improve the anticancerous effects by increasing the stimulation and recruitment of dendritic cells [17–19].

Another type of vaccination is with irradiated autologous cancerous cells engineered to secrete growth factors such as GM-CSF. Malignant cells can be extracted from the tumor, irradiated in order to stop the proliferation, and engineered in order to produce growth factors. The modification involves a retroviral-mediated gene transfer. The growth factors produced by the modified cells can alert the immune system regarding the cancer and further attacking it [20].

4. Mechanism of Action of Checkpoint Inhibitors

The immune system is regulated by a complex balance between activation and inhibition of lymphocytes. Immune system has multiple cells such as T cells, B cells, and natural killer cells that function as cancer fighters with the T cells being the main effector and regulator cell. T cells present some specific receptors that function as activators while others as inhibitors of the T cell's activity.

Checkpoint inhibitors are molecules that permit activation of T lymphocytes through inhibiting the connection between a receptor that works as an inhibitor and its ligand. In this way, the checkpoint inhibitors allow the immune system to improve the efficacy of the fight against malignant cells. Thus, it can be concluded that immune checkpoints play a central role in maintaining an immune tolerance by inhibiting the immune system and so preventing the appearance of autoimmune phenomenon. In case of their blockage, a boost of the immune system will be observed, which can lead to a tumor control, explaining thus the efficacy of antibodies against checkpoint inhibitors in treating cancer [3].

TIM-3 is a protein that is part of the TIM family and has a role in regulating the function of Th1 lymphocytes. Overexpression of TIM-3 was observed to be associated with a poor prognosis in some forms of cancer [21].

Two of the most important checkpoint inhibitors are considered to be CTLA-4 and PD-1, which will be described in the following paragraphs and illustrated in Figure 1.

4.1. CTLA-4 Pathway. The normal process following the activation of T cell leads to an upregulation of CTLA-4 (cytotoxic T lymphocyte antigen-4) on the surface of the T cell. CTLA-4 function is to be a downregulator of the T cell by outcompeting CD28 for B7 ligand, preventing in this way costimulation of the T cell and also by inducing T cell cycle arrest [22–25].

CTLA-4's affinity for B7 is higher than that of CD28. When CD28 binds to B7, it leads to a stimulatory signal, while CTLA-4 link to B7 will determine inhibitory signals [26]. Thus, the balance between the CLTA-4/B7 link and CD28/B7 link has to be carefully maintained. An imbalance in those connections can determine whether the T cell will activate or will undergo into an anergy state [27].

CTLA-4 is an important negative regulator in maintaining a normal immunologic environment. This was demonstrated by the fact that mice deficient in CTLA-4 suffered a CD28-dependent expansion of T cells in lymphatic organs. This proliferation led to death in less than 4 weeks postbirth due to lymphoproliferation [28].

Rudolph et al. noticed that blocking CTLA-4 will lead to a qualitative modification of the T memory cells. At the same time, a decrease in the number of CD4+ T cells and in this way a reduction in producing IFN-γ, IL-2, and tumor necrosis factor-alpha (TNF-α) as a response to the antigenic exposure was observed [29].

Pedicord et al. noticed that administration of anti-CTLA-4 antibodies will lead to a rise in the number of the CD8+ T cells with memory, leading to an increase in the secretion of TNF-α and IFN-γ [30].

On the basis of the preclinical studies, ipilimumab and tremelimumab were developed as anti-CTLA-4 antibodies. They function by blocking the interaction between CTLA-4 and B7, thus facilitating the linking between CD28 and B7, and leading to proliferation and activation increase of the T lymphocytes. Their action will lead to an increase in the antitumor immune response [31, 32].

Ribas et al. showed in a phase III study that the efficiency of tremelimumab was lower than that of chemotherapy with dacarbazine/temozolomide in patients with melanoma in advanced stages. Thus, not showing a statistically significant increase in overall survival rate, it was considered to be unfit for further studies [33].

On the other side, ipilimumab was discovered to significantly increase the overall survival rate in patients with advanced melanoma. Ipilimumab is a fully human IgG1 monoclonal antibody that inhibits the CTLA-4 binding to B7, and it was approved by FDA and EMA and is currently introduced in stage IV melanoma therapy.

Several combinations are ongoing in clinical trials to increase ipilimumab efficacy. Hodi et al. developed a clinical trial involving 676 patients with advanced melanoma whose disease progressed after treatment with at least one standard therapy. The patients were divided into 3 groups: the first group received ipilimumab in association with a vaccine (a gp100 peptide), the second group received ipilimumab only, and the third group received only the gp100 vaccine. When comparing the overall survival rate of the first group with that of the third group, a statistically significant increase was observed for the first group (10 months vs. 6.4 months). The same significant difference was noticed when confronted the data for the second group and the third group, 10.1 months vs. 6.4 months. Also, it was observed that the ORR (overall response rate) was the highest for the second group (10.9%), being almost double compared to the first one (5.7%), while the third group had an ORR of 1.5%. The longest duration of response was recorded in the second group, 11.5 months. Thus, the conclusion of the study was that ipilimumab can greatly increase the overall survival rate and response rate [34].

Wolchok et al. developed a clinical study involving 2 types of patients with metastatic melanoma: treatment-naïve and previously treated with chemotherapy. When looking at the survival rate at 4 years, it was discovered that patients therapeutically naïve had a more durable and significant survival rate in comparison to those who previously received chemotherapy [35].

A study developed by Prieto et al. has suggested that an association between ipilimumab and a high-dose IL-2 can have greater results than ipilimumab alone [36].

Looking to the survival rates in the study performed by Schadendorf et al. at both therapeutically naïve and experimented patients, a promising increase was observed compared to chemotherapy. This offers high hopes for patients with advanced melanoma [37].

Thus, ipilimumab has a long-term curative and regressive potential for advanced melanoma. Even though this molecule has serious adverse effects, if it is well monitored and treated promptly and correctly, it can be kept under control.

4.2. PD-1/PD-L1 Pathway. PD-1 (programmed death-1) receptor is a downregulator of the T cells, reducing the activity of T lymphocytes when binding to PD-L1 or PD-L2 ligands. PD-L1 is a receptor found on the plasmatic membranes of the malignant cells, while PD- L2 can be found on the surface of dendritic cells. The link between PD-1 and its ligand will inhibit kinase signaling pathways, thus leading to inactivation of the T cell. Using this mechanism of inhibiting T lymphocytes, tumor cells are able to escape the immune system and thus survive and develop further [38, 39]. Thus, it can be said that PD-1 blockade acts in the effector phase of the T cells. PD-L2 is a protein that can bind to PD-1, which has the ability to deplete T cells that present PD-1 on their surface [40].

PD-1 molecule serves as a negative regulator of the immune response, maintaining the self-tolerance of the organism. Early studies have shown that mice deficient in PD-1 developed autoimmune diseases such as lupus-like arthritis and glomerulonephritis and autoimmune dilated cardiomyopathy [41, 42].

PD-1/PD-L1 blockade leads to a successful antimalignant immune response. This involves the activation and proliferation of antigen-experienced T cells located at the malignant site [43, 44].

In order to generate CD8+ T cells that are tumor-reactive, an efficient presentation of the tumor antigens by APC is needed. A receptor found on the membrane of the T cell will recognize this tumor antigen, leading to initiation of activation of the T cell. The full activation of the T cell will happen only after the linkage between CD28 receptor and B7 ligand found on the surface of antigen-presenting cells [45].

Tumor-specific CD8+ T cells subsequently differentiate into effector T cells, undergo clonal expansion, traffic to the

tumor microenvironment, and ultimately kill tumor cells displaying tumor-associated antigen, via release of several cytolytic effector molecules among which are granzyme A/B and perforin. For long-term immunologic memory and presumably durable disease control, a subset of effector T cells must differentiate into effector memory T cells, under the guidance of CD4+ helper T cells and dendritic cells. These are maintained for life and respond to rechallenge with antigen.

Based on the preclinical studies, a number of antibodies targeting the inhibition of linkage between PD-1 and PD-L1 entered clinical development. In this review, we will focus only on two antibodies that target PD-1 molecule: nivolumab and pembrolizumab, which have shown significant increase in the response rate in patients with advanced malign tumors [46, 47].

Nivolumab is a fully human IgG4 antibody directed against PD-1 receptor. By blocking the binding of PD-1 to PD-L1, this antibody will restore the natural tumor-specific immune response. It is recommended for patients with advanced melanoma and disease progression under ipilimumab and BRAF inhibitors, in the case of the patients with BRAF mutation. BRAF is a humane protoncogene that encodes a protein named B-Raf which is involved in a signalizing cascade with roles in growth promotion and cellular proliferation and differentiation.

Prior to approval, Weber et al. elaborated a phase III clinical study to compare the efficacy of nivolumab versus chemotherapy. In this way, the patients were divided into 2 groups: one that received nivolumab in monotherapy and the other one dacarbazine with carboplatin. The conclusion of the study was that nivolumab had a greater response rate and fewer adverse effects and made the treatment of adverse effects easier than chemotherapy [48].

In numerous phase III studies, nivolumab has proven a survival advantage when compared to the conventional treatments including chemotherapy. This was valid for both therapy-naïve or experimented patients [47–50].

Nivolumab can be administered in doses of 3 mg/kg every 2 weeks, and recently a new dosing schedule of 480 mg every 4 weeks it has been established. Indications for the new schedule of nivolumab can be found in Table 1. Recent studies have shown that nivolumab 480 mg every 4 weeks has the same results in terms of overall survival rate and response rate as nivolumab 3 mg/kg every 3 weeks, and also the safety profile is considered to be similar [51].

Pembrolizumab is an engineered humanized IgG4 monoclonal antibody which acts against the PD-1 (programmed death) receptor. Thus, pembrolizumab binds to PD-1 receptor and blocks its interaction with PD-L1 or PD-L2. As a result, the antitumor immunity will be reactivated by enhancing T cells to produce several activating cytokines such as IL-2, IL-6, IL-17, IFN-gamma, and TNF-alpha.

It can be administered in doses of 2 mg/kg every 3 weeks or 10 mg/kg every 2 or 3 weeks. The recommended dose sustained by the clinical studies found in the literature is demonstrated to be 2 mg/kg every 3 weeks, an increased not being associated with additional clinical benefit [52].

Table 1: Indications for administration of nivolumab 480 mg every 4 weeks [51].

Indications—nivolumab 480 mg every 4 weeks
(i) Metastatic melanoma
(ii) Previously treated NSCLC
(iii) Advanced renal cell carcinoma treated with prior antiangiogenic therapy
(iv) Locally advanced or metastatic urothelial carcinoma which was previously treated, with progression during or after platinum-based chemotherapy
(v) Classical Hodgkin lymphoma following relapse/progression after autologous hematopoietic stem cell transplantation (HSCT) and brentuximab vedotin or three or more lines of systemic therapy that includes autologous HSCT
(vi) Recurrent/metastatic squamous cell carcinoma of the head and neck following platinum-based therapy
(vii) Hepatocellular carcinoma after prior sorafenib therapy
(viii) Adjuvant therapy for patients with completely resected melanoma with lymph node involvement or metastatic disease

Pembrolizumab is recommended to be administered until the progression of the disease is confirmed or unacceptable toxicity is observed. Atypical responses at the treatment have been noted such as an initial increase in tumor size or the appearance of new lesions of small dimensions in the first months after the initiation of treatment, followed by a decrease in tumor size.

KEYNOTE 001 is a phase 1 trial with the purpose of assessing the appropriate dose of pembrolizumab in patients with progressive, locally advanced, or metastatic melanoma unable to respond at local therapy and with ECOG (Eastern Cooperative Oncology Group performance status) < 2 who could be ipilimumab naïve, treated, or refractory. The conclusions were that the longest median progression-free survival was obtained in the group treated with 2 mg/kg every 3 weeks when discussing about naïve patients, while for treated or refractory, there was no significant difference between the dosages [53–55].

75–83% of the patients treated with pembrolizumab experienced treatment-related side effects, but the majority of them had grade 1 or 2 in severity. Most common side effects related to the pembrolizumab treatment were fatigue, diarrhea, nausea, arthralgia, rash, and pruritus, out of which the first three were more common in patients receiving 10 mg/kg every 2 weeks in comparison with those who received 2 mg/kg every 3 weeks [54].

Pembrolizumab is frequently associated with immune adverse reactions. Most of them, including severe side effects, were remitted after the initiation of adequate medical treatment or stopping of pembrolizumab. The most frequent immunologic side effects were hypothyroidism, pneumonitis, and hyperthyroidism, followed by less common side effect such as colitis, hypophysitis, hepatitis, nephritis, and infusion-related reactions. Immunosuppressants can be used during the treatment in case of appearance of an immunologic side effect, but patients should avoid using them before starting the treatment with pembrolizumab

Table 2: Adverse effects of checkpoint inhibitors [60].

Type of toxicity		Management of adverse effects
Cutaneous	Rash/inflammatory dermatitis	Rashes that can be controlled through topical treatments and oral antihistamines do not require stopping the immune therapy, but in the case of severe or unmanageable rashes, it is necessary to hold the therapy until the resolution of skin toxicity.
	Bullous dermatoses	If the blisters cover more or less than 10% of body surface area and do not affect the quality of life, the recommended treatment is topical corticosteroids. If the surface involved is more than 10%, the mucosal membranes are involved, and the lesions affect the quality of life, the immune therapy must be halted and continued only after skin resolution.
	Severe cutaneous adverse reactions (Stevens-Johnson epidermal necrolysis, acute generalized exanthematous pustulosis)	In case of maculopapular exanthem covering 10–30% of BSA (body surface area) in association with systemic symptoms, lymphadenopathy, or facial swelling, it is recommended to hold the checkpoint inhibitor therapy and give topical emollients, oral antihistamines, and topical corticosteroids with medium to high potency.
	Drug-induced hypersensitivity syndrome/ drug reaction with eosinophilia and systemic symptoms (DHIS/DRESS)	
Pulmonary	Pneumonitis (identified on CT imaging as focal or diffuse inflammation of the lung parenchyma)	If the inflammation involves more than one lobe, but is less than 50% of the total parenchyma, the therapy is withheld until the resolution of symptoms, and prednisone 1–2 mg/kg/day is administered. If the inflammation involves more than 50% of the lung parenchyma or severe symptoms are present, the treatment will be permanently discontinued and antibiotics and systemic corticosteroids will be administered.
Renal	Nephritis	In case of G1 toxicity (creatinine 1.5–2 times over the baseline), only monitorisation is required. G2 toxicity (creatinine 2–3 times above baseline) leads to the hold of therapy, and if no improvement is observed, systemic corticosteroids will be administered (prednisone 1–2 mg/kg/day or equivalents). Grade 3 toxicity (creatinine > 3x baseline) leads to permanent discontinuation of therapy. Grade 4 toxicity has indication for dialysis and also administration of corticosteroids.
Hematologic	Autoimmune hemolytic anemia	Grade 1 toxicity allows continuation of therapy and a close clinical check-up. Grade 2 needs holding therapy and also administration of 0.5–1 mg/kg/d prednisone. Grade 3 or 4 requires permanent discontinuation, with administration of prednisone 1–2 mg/kg/d and supplementation with folic acid 1 mg daily. In case of grade 4 toxicity, if no improvement is observed, initiation of immunosuppressive drugs is required (rituximab, IVIG, cyclosporin A, mycophenolate mofetil).
	Acquired thrombotic thrombocytopenic purpura	All grades need therapy holding and hematology consult. G1 and G2 require the administration of 0.5–1 mg/kg/d prednisone, while grade 3 or 4 needs administration of methylprednisolone 1 g iv daily for 3 days, taking into consideration rituximab.
	Hemolytic uremic syndrome	Grades 1 and 2 does not require stopping the therapy, while grades 3 and 4 require the stop and initiation of exulizumab therapy 900 mg weekly for 4 doses, 1200 mg week5, then 1200 mg every 2 weeks.
	Aplastic anemia	Grade 1 requires therapy hold and administration of growth factor with close clinical observation. In case of grade 2 toxicity, it is added ATG (antithymocyte globulin) and cyclosporine administration to the protocol for grade 1. Patients with grade 3 or 4 have the same management as those with grade 2. If no response is observed, it is needed to repeat immunosuppression with ATG, cyclosporine, and cyclophosphamide. In the case of refractory patients, eltrombag needs to be taken into consideration.

Table 2: Continued.

Type of toxicity	Management of adverse effects
Lymphopenia	The only situation that requires holding therapy is a grade 4 toxicity (<250 PB lymphocyte count). In this case, it has to be initiated mycobacterium avium complex prophylaxis and Pneumocystis jirovecii prophylaxis and also cytomegalovirus (CMV)/human immunodeficiency virus (HIV)/hepatitis screening.
Immune thrombocytopenia	Patients with a platelet count < 100/mcL (grade 1) need to continue the therapy with close clinical and laboratory evaluation. A count less than 75/mcL requires therapy holding with administration of oral prednisone 1 mg/kg/day 2–4 weeks, with taper over 4–6 weeks and also IVIG in case a faster increase in the platelet count is needed. Grade 4, meaning a platelet count < 25/mcL, is treated with prednisone 1–2 mg/kg/day and association with IGIV. In case of no response, rituximab or thrombopoietin receptor agonist can be used.
Acquired hemophilia	G1 toxicity (5–40% of normal factor activity in the blood) needs holding of therapy and administration of 0.5–1 mg/kg/day prednisone. G2 toxicity (1–5% of normal factor activity in the blood) requires holding the therapy, administration of factor replacement, and administration of 1 mg/kg/d prednisone and 375 mg/m2 rituximab weekly for 4 weeks and/or cyclophosphamide 1–2 mg/kg/day. In case of severe symptoms (G3 or 4, <1% of normal factor activity in the blood), permanent discontinuation of therapy is required, in association with administration of bypassing agents (factor VII, factor VIII inhibitor bypass activity) and also administration of 1 mg/kg/d prednisone and 375 mg/m2 rituximab weekly for 4 weeks and/or cyclophosphamide 1–2 mg/kg/day. In case of bleeding, transfusions are needed.

because corticosteroids can alter the pharmacodynamics of pembrolizumab [54].

Ribas et al. showed in a clinical trial a response rate at 3-year of 33% for patients treated with pembrolizumab. 70–80% out of patients who initially responded maintained a clinical response over the 3-year period [56].

Literature studies showed that association immunotherapy can increase even further the response rate in comparison to one type of monoclonal antibody alone. The downside of this association is the increase in the number of the side effects and their severity. Thus, the increase in response rate and the severity of the side effects further have to be put in balance in order to pursue this therapy in further clinical trials [57, 58].

In Table 2, we resume the main side effects of checkpoint inhibitor therapy with the corresponding management for every grade of toxicity.

5. Resistance at Checkpoint Inhibitors

Recent clinical studies have shown that immune therapy can lead to resistance development. Thus, the population receiving this therapy can be divided into 3 groups: responders, innate resistance, and acquired resistance. Responders are patients that answer initially and maintain this answer. Innate resistance characterizes the patients that fail to respond from the first dose; thus, in this case the therapy should be halted and changed. Acquired resistance is shown in patients that first answer to the therapy, but after some cycles, they start to stop responding and eventually display disease progression [43, 44, 59]. Thus, it is a great and important therapeutic challenge to define and differentiate the responders and nonresponders, especially given the heterogeneity in patterns of response that can be seen with immune checkpoint inhibitors.

Failure of response to immune checkpoint inhibitors can arise from the following alterations: insufficient generation of antitumor T cells, inadequate function of tumor-specific T cells, and impaired formation of memory T cells. All of these alterations will lead to an imbalance between protumor state and antitumor state, with an increase in the protumor state.

Also, lack of sufficient or suitable tumor antigens, inadequate tumor antigen processing, or impaired presentation of tumor antigens can all lead to impaired formation of tumor-reactive T cells.

6. Side Effects of Checkpoint Inhibitors

We have put together the main side effects developed upon checkpoint inhibitors and the management of these side effects (Table 2).

7. Conclusions

In spite of an ascending trend in the incidence of melanoma, most cases are diagnosed in initial stages during which surgery is curative for a great majority of them. In the case of patients with high risk of developing metastasis, immunotherapy can be used after surgical excision. Thus, the greatest

challenge seems to remain the disseminated type of melanoma, which seems to be the target for checkpoint inhibitors.

Immunotherapy with checkpoint inhibitors has a great potential in changing the face of oncologic treatments and so the outcomes of cancers are hard to treat until now. Numerous clinical studies proved great efficiency of checkpoint inhibitors regarding progression-free rate and overall survival rate in treating a variety of solid tumors (such as melanoma, non-small-cell lung carcinoma, and renal cell carcinoma) and hematologic cancers. Unfortunately, not all the studies confirm these results. Thus, it can be concluded that immunotherapy has limitations in cancer treatment. A further step that should be followed is the identification of biomarkers which can indicate when is best to use the checkpoint inhibitors.

Conflicts of Interest

The authors declare no conflict of interests.

Acknowledgments

This work was partially supported by a grant from the Romanian Ministry of Research and Innovation, CCCDI-UEFISCDI, (project number 61PCCDI/2018 PN-III-P1-1.2-PCCDI-2017-0341), within PNCDI-III.

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 9208274, 10 pages
https://doi.org/10.1155/2018/9208274

Research Article

Differential Intestinal Mucosa Transcriptomic Biomarkers for Crohn's Disease and Ulcerative Colitis

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Received 9 July 2018; Accepted 4 September 2018; Published 17 October 2018

Guest Editor: Donato Zipeto

Genetic research has shaped the inflammatory bowel disease (IBD) landscape identifying nearly two hundred risk loci. Nonetheless, the identified variants rendered only a partial success in providing criteria for the differential diagnosis between ulcerative colitis (UC) and Crohn's disease (CD). Transcript levels from affected intestinal mucosa may serve as tentative biomarkers for improving classification and diagnosis of IBD. The aim of our study was to identify gene expression profiles specific for UC and CD, in endoscopically affected and normal intestinal colonic mucosa from IBD patients. We evaluated a panel of 84 genes related to the IBD-inflammatory pathway on 21 UC and 22 CD paired inflamed and not inflamed mucosa and on age-matched normal mucosa from 21 non-IBD controls. Two genes in UC (CCL11 and MMP10) and two in CD (C4BPB and IL1RN) showed an upregulation trend in both noninflamed and inflamed mucosa compared to controls. Our results suggest that the transcript levels of CCL11, MMP10, C4BPB, and IL1RN are candidate biomarkers that could help in clinical practice for the differential diagnosis between UC and CD and could guide new research on future therapeutic targets.

1. Introduction

Inflammatory bowel diseases (IBD) are a distinct class of gastrointestinal diseases mainly represented by Crohn's disease (CD) and ulcerative colitis (UC). These are chronic diseases characterized by a relapsing remitting course with an increasingly high incidence and prevalence worldwide [1]. The current accepted model for IBD etiology implies the existence of a genetic predisposition, perturbations in the intestinal barrier components, and altered microbiota, which combined will lead to an aberrant immune response [2]. Distinguishing between the two diseases represents a problem in clinical practice due to some similarities in endoscopic and morphological aspects which in turn will lead to a change in diagnosis throughout the course of disease [3]. However, some fundamental differences between CD and UC have been reported: UC is characterized by diffuse inflammation confined to the colorectal mucosa, whereas in CD, the inflammation is discontinuous, transmural, and can affect the entire gastrointestinal tract. Moreover, CD patients often present complications like intestinal strictures, fistulas, and abscesses [4]. Despite these differences, physiopathological mechanisms, clinical criteria, and therapeutical strategies considerably overlap, but CD and UC seem to be triggered and maintained by differential molecular mechanisms, which are not completely known.

Genetic studies in IBD have gained importance during the past decade since endoscopic assessment and biopsies provide limited data regarding early disease activity and factors for relapse. The candidate gene approach, genome-

Table 1: Clinical and demographical parameters of individuals involved in the study.

	CTRL ($n = 21$)	UC ($n = 21$)	CD ($n = 22$)
Sex, n (%)			
Male	9 (42.9)	16 (76.2)	13 (59.1)
Female	12 (57.1)	5 (23.8)	9 (40.9)
Age, yrs, mean ± SD	46.5 ± 16.7	44.4 ± 12.8	45.1 ± 15.1
Medications at tissue acquisition n (%)			
None	21 (100)	3 (14.3)	4 (18.2)
Biological	—	2 (9.5)	4 (18.2)
5-ASA	—	14 (66.7)	7 (31.8)
Cortisone	—	—	2 (9.1)
Polytherapy	—	2 (9.5)	5 (22.7)

wide association studies, and meta-analyses have contoured the genetic background of these disorders, revealing more than 200 risk loci in both European and non-European individuals [5]. However, previous studies showed that many of these loci are shared between CD and UC [6], and no specific genetic markers entered clinical practice yet.

A number of candidate gene expression studies, RNA sequencing, and microarray studies on mucosa from IBD patients have been published in the last years with the attempt to find a specific profile able to discriminate UC and CD. Gene expression analysis of tissue samples from affected and nonaffected individuals can help in discovering important events involved in disease pathogenesis. For example, individual mRNA levels can be sensitive markers for improving classification and diagnosis, identifying new therapeutic targets, and providing prognostic information [7].

Studies conducted so far analyzing the expression levels of cytokines and transcription factors in mucosa revealed that CD has been associated with an impairment of Th1/Th17 response [8], whereas UC has been associated with a Th2/NKT cell response [9]. Other genes have been indicated as putative differential biomarkers, including α-defensin-5 [10], circadian genes [11], TNFAIP3, PIGR, TNF, and PIGR [12]. Other studies based on RNA-seq approaches revealed important transcriptomic differences between normal mucosa, noninflamed CD mucosa, and inflamed CD mucosa [13] as well as differences among colon biopsies from CD patients, UC patients, and non-IBD controls [4].

In this study, we aimed to identify the inflammatory signature specific for UC and CD both in endoscopically inflamed and not inflamed mucosa and how the type of therapy can influence the gene expression profile in Romanian patients. To address these questions, we evaluated the gene expression profile of a panel of 84 selected genes (previously associated to IBD) in paired mucosa samples of 21 UC and 22 CD patients, and we compared them with the profiles obtained in a group of 21 non-IBD healthy controls.

2. Materials and Methods

2.1. Patients. Forty-three IBD patients (21 UC and 22 CD) and 21 non-IBD controls have been enrolled in the study at the Department of Gastroenterology and Hepatology, "Elias" Emergency University Hospital and at the "Fundeni" Clinical Institute of Bucharest, Romania. In terms of disease location, patients with CD had colonic and ileocolic forms of the disease. All the patients and controls were of Romanian origin. Written informed consent was obtained from all participants prior to biopsy collection, and the study was approved by the local ethics committees. The diagnosis had been made based on clinical, endoscopic, and histological criteria according to European Crohn's and Colitis Organization Guidelines [3]. From each UC and CD patients, paired colonic inflamed mucosa (IM) and macroscopically colonic noninflamed mucosa (NM) were obtained during a colonoscopy. We defined the inflammation status based on the presence of erythema, ulcerations, and bleeding of the mucosa. A biopsy of a normal-looking colonic mucosa was obtained also from a group of non-IBD controls during a colonoscopy screening. Exclusion criteria for non-IBD controls were as follow: (1) presence of digestive symptoms, (2) current or previous nonsteroidal anti-inflammatory treatments (within the past 3 months), and (3) current or previous anticoagulant/antiplatelet treatments (within the past 3 months). The characteristics of the three groups are reported in Table 1.

2.2. Total RNA Isolation and qPCR. Total RNA isolation from fresh-frozen tissues preserved in RNA later was performed using RNeasy mini Kit (Qiagen), according to the manufacturer's protocols. The quantity and quality of RNA were determined using the NanoDrop 2000 (Thermo Scientific). An amount of 600 ng of RNA was reverse transcribed to cDNA using the RT2 First Strand Kit (Qiagen). The Human Crohn's Disease RT2 Profiler PCR Array (PAHS-169Z, Qiagen), using SYBR Green chemistry, evaluated the expression of 84 key genes, according to the manufacturer's protocol, on the ABI-7500 fast instrument (Applied Biosystems). The expression levels of each gene were normalized on the geometric mean values of two housekeeping genes (GAPDH and HPRT1) based on RefFinder algorithm (http://leonxie.esy.es/RefFinder/) [14] analysis of five candidate reference genes (ACTB, B2M, GAPDH, HPRT1, and RPLP0).

2.3. Statistical Analysis. qRT-PCR data analysis was conducted using the Statistical Package for Social Science (SPSS version 17.0). Categorical variables were tested by means of the chi-square test, and continuous variables with the t-test. Paired t-test was used to assess difference in gene expression levels of IM and NM.

3. Results

The group of patients and controls was homogeneous for age ($p > 0.05$) and sex ($\chi^2 = 4.880$, $p = 0.087$) distribution, and the UC and CD groups did not statistically differ for the class of treatment ($\chi^2 = 6.409$, $p = 0.171$).

3.1. Gene Expression Alterations in Paired Inflamed and Noninflamed Mucosa of UC and CD Patients. Gene expression analysis was performed on 21 pairs of tissues representing IMUC and NMUC and 22 pairs of tissues representing IMCD and NMCD. In IM, 11 genes out of 84 were found differentially overexpressed both in UC and CD compared with the paired NM. Thirty-three transcripts were found specifically altered only in UC patients (two downregulated and 31 upregulated). Results are shown in Table 2.

3.2. Gene Expression Alterations in CD and UC Patients Compared with Non-IBD Controls. Gene expression analysis was performed on 21 noninflamed and inflamed mucosa from UC patients, 22 from CD, and 21 from healthy controls. Considering a fold change (FC) > |2.0| and a p value below 0.05, 32 genes out of 84 were found differentially expressed both in UC and CD compared with C (two downregulated and 30 upregulated), and 17 were specifically altered only in UC patients (four downregulated and 13 upregulated). No gene was found modified only in CD. When comparing the NM tissues vs. C, we found two transcripts upregulated in UC and five upregulated in CD (Table 3). A graphic representation of the results is shown in Figure 1. Genes whose expression differed between NM and controls and are also different comparing paired IM-NM are shown in Figures 2(a) and 2(b).

3.3. Differences in Gene Expression in IBD Patients on Different Treatments. Due to the limited sample size of the UC and CD groups, we analyzed the treatment effect on gene expression levels considering the entire IBD cohort. Comparing the patients treated with 5-ASA ($n = 21$) vs. drug-free patients ($n = 7$), we found that ISG15 ubiquitin-like modifier (ISG15) was downregulated both in inflamed and not inflamed tissues with FC and p value of -2.04, $p = 0.003$ in IM and -1.84, $p = 0.033$ in NM. Moreover, we found that the six patients with biologic treatment showed lower levels of serum amyloid A1 (SAA1) with FC of -6.66 and $p = 0.025$ in IM.

Comparing patients with biological treatment vs. 5-ASA, we found that CCR1 was upregulated in IM with FC = 2.1 and $p = 0.005$ and TFF1 was downregulated both in IM and NM with FC = -2.5, $p = 0.001$ and FC = -2.4, $p = 0.004$, respectively.

Despite the limited size of the two groups, an additional analysis to find a putative effect of the treatment on the candidate genes (IL1RN and C4BP4 for UC and CCL11 and MMP10 for CD) has been performed separately both on UC and CD groups. No changes in IL1RN and C4BP4 levels were found between the three UC patients without treatment and the UC patients in treatment with 5-ASA ($p = 0.704$, $p = 0.718$), biological treatment ($p = 0.384$, $p = 0.567$), or polytherapy ($p = 0.891$, $p = 0.680$). In the CD group, no difference in CCL11 and MMP10 was found comparing the four patients without treatment and the other groups ($p > 0.05$ in all the comparisons). However, a trend toward significance was observed in MMP10 levels comparing the 4 CD patients without treatment and the group of the seven patients using 5-ASA ($p = 0.056$).

4. Discussion

Overlapping features have been reported in up to 30% of IBD [15] leading to a not accurate diagnosis and increasing the risk of inappropriate treatment. In this study, we sought to determine whether mucosal gene profile could be used to develop diagnostic biomarker(s) to discriminate between the two main inflammatory bowel diseases (UC and CD) more accurately.

To the best of our knowledge, this is the first study that evaluated 84 transcripts by qRT-PCR considering a larger cohort of participants than previous studies, including paired inflamed and not inflamed tissues from CD and UC as well as a cohort of non-IBD controls.

Using this approach, we identified 17 genes differentially expressed only in the inflamed mucosa from UC that did not differ for the CD patients. A common signature of 32 genes was identified, and no gene specific for CD inflamed mucosa was found.

Among the genes belonging to the common signature, five and two were found differentially expressed comparing the not inflamed mucosa with mucosa from non-IBD controls of CD and UC, respectively.

Interestingly, in UC, CCL11 and MMP10 were increased substantially in non-IBD controls, NM and IM, whereas in CD, this increase was observed for C4BPB and IL1RN. Hence, these four genes seem to be specific markers of UC and CD inflammation levels.

Eotaxin-1 (CCL11), a potent eosinophil chemoattractant that is considered a major contributor to tissue eosinophilia, is a key regulator of intestinal inflammation [16] and seems to be involved both in UC and CD. Indeed, unlike other chemokines, the human mRNA for eotaxin-1 is constitutively expressed in the small intestine and colon [17] where the intestinal myeloid cells seem to be a source [18].

Levels of eotaxin-1 have been found increased in sera from UC patients [19–21] as well as in colon biopsies [22]. In line with our findings that suggested an increase according to the inflammation status, a significant increase of its levels was found in patients with active UC but not in the quiescent state [23]. These data suggest that also the peripheral levels may increase accordingly to the inflammation grade as we observed in mucosa.

Increased levels of eotaxin-1 have been found also in the sera from CD patients [19, 20], and our group found that its

Table 2: The table shows the transcripts that differed by >2.0 fold with $p < 0.05$ in inflamed mucosa (IM) vs. noninflamed mucosa (NM) in UC and CD patients. Genes are arranged by alphabetic order. Italic fonts indicate genes differentially expressed only in IM from UC.

Gene	Description	UC Paired IM vs. NM		CD Paired IM vs. NM	
		FC	p value	FC	p value
C3	Complement C3	5.48	0.0106		
C4BPB	Complement component 4 binding protein beta	5.86	<0.0001	2.98	0.0090
CCL11	C-C motif chemokine ligand 11	2.01	0.0121	2.27	0.0045
CCL20	C-C motif chemokine ligand 20	4.05	0.0003		
CD55	CD55 molecule (Cromer blood group)	3.63	<0.0001		
CHI3L1	Chitinase 3 like 1	16.96	0.0045	4.19	0.0328
CR2	Complement C3d receptor 2	7.00	0.0150		
CXCL1	C-X-C motif chemokine ligand 1	17.99	<0.0001	6.82	0.0413
CXCL10	C-X-C motif chemokine ligand 10	3.23	0.0008		
CXCL11	C-X-C motif chemokine ligand 11	8.93	0.0001	4.32	0.0380
CXCL2	C-X-C motif chemokine ligand 2	14.98	<0.0001		
CXCL3	C-X-C motif chemokine ligand 3	9.79	<0.0001		
CXCL9	C-X-C motif chemokine ligand 9	5.16	0.0002	5.22	0.0059
CXCR1	C-X-C motif chemokine receptor 1	19.22	0.0131		
EDN3	Endothelin 3	−2.92	0.0048		
FPR1	Formyl peptide receptor 1	10.57	0.0035		
IFNG	Interferon gamma	2.82	<0.0001		
IL1RN	Interleukin 1 receptor antagonist	8.15	0.0018	5.68	0.0498
IL23A	Interleukin 23 subunit alpha	3.11	<0.0001		
IL2RA	Interleukin 2 receptor subunit alpha	3.45	0.0006		
CXCL8	C-X-C motif chemokine ligand 8	19.40	0.0185		
ITGB2	Integrin subunit beta 2	2.31	0.0003		
LCN2	Lipocalin 2	13.05	0.0003		
LTB	Lymphotoxin beta	4.01	0.0007		
LYZ	Lysozyme	2.06	0.0009	2.16	0.0344
MMP1	Matrix metallopeptidase 1	9.98	0.0108		
MMP10	Matrix metallopeptidase 10	14.92	0.0004		
MMP3	Matrix metallopeptidase 3	30.01	0.0016		
MMP7	Matrix metallopeptidase 7	37.37	0.0036	6.00	0.0098
NOS2	Nitric oxide synthase 2	10.99	0.0005		
PCK1	Phosphoenolpyruvate carboxykinase 1	−6.29	0.0002		
PECAM1	Platelet endothelial cell adhesion molecule 1	2.42	0.0046		
REG1A	Regenerating family member 1 alpha	10.11	0.0123		
S100A8	S100 calcium binding protein A8	17.91	0.0018		
S100A9	S100 calcium binding protein A9	9.31	0.0005		
SAA1	Serum amyloid A1	62.83	0.0016		
SELL	Selectin L	5.31	<0.0001		
SOD2	Superoxide dismutase 2	2.14	0.0003	2.03	0.0429
STAT1	Signal transducer and activator of transcription 1	2.26	<0.0001		
TDO2	Tryptophan 2,3-dioxygenase	4.00	<0.0001		
TFF1	Trefoil factor 1	2.74	0.0001		
TIMP1	TIMP metallopeptidase inhibitor 1	4.48	<0.0001		
TNF	Tumor necrosis factor	2.58	0.0022		
UBD	Ubiquitin D	7.95	0.0002	2.92	0.0116

Table 3: The table shows the transcripts that differed by >2.0 fold with $p < 0.05$ in inflamed mucosa (IM) and noninflamed mucosa (NM) of UC and CD patients compared with healthy controls. Genes are arranged by alphabetic order. Italic fonts indicate genes differentially expressed both in NM and IM compared to controls.

Gene	Description	UC				CD			
		IM ($n = 21$) vs. C ($n = 21$)		NM ($n = 21$) vs. C ($n = 21$)		IM ($n = 22$) vs. C ($n = 21$)		NM ($n = 22$) vs. C ($n = 21$)	
		FC	p value	FC	p value	FC	p value	FC	p value
ABCB1	ATP binding cassette subfamily B member 1	−7.04	0.0158			−3.76	0.0417		
ALDOB	Aldolase, fructose-bisphosphate B	−18.72	0.0269						
C3	Complement C3	3.77	0.0264						
C4BPB	Complement component 4 binding protein beta	10.05	<0.0001			8.21	<0.0001	2.76	0.0310
CCL11	C-C motif chemokine ligand 11	3.99	0.0003	2.056	0.003	3.90	<0.0001		
CCL2	C-C motif chemokine ligand 2	2.58	0.0413						
CCL20	C-C motif chemokine ligand 20	3.36	0.0009						
CCL25	C-C motif chemokine ligand 25	−13.47	0.0403						
CCR9	C-C motif chemokine receptor 9	−2.89	0.0179						
CD55	CD55 molecule (Cromer blood group)	4.63	<0.0001			2.80	0.0006		
CHI3L1	Chitinase 3 like 1	42.55	0.0016			39.26	0.0049		
CSTA	Cystatin A	2.64	0.0045						
CXCL1	C-X-C motif chemokine ligand 1	12.34	<0.0001			12.60	0.0240		
CXCL10	C-X-C motif chemokine ligand 10	2.85	0.0013			9.36	0.0416		
CXCL11	C-X-C motif chemokine ligand 11	7.28	0.0003			12.59	0.0077		
CXCL2	C-X-C motif chemokine ligand 2	9.16	<0.0001			10.09	0.0368		
CXCL3	C-X-C motif chemokine ligand 3	7.17	<0.0001			5.74	0.0123		
CXCL9	C-X-C motif chemokine ligand 9	5.36	<0.0001			11.04	0.0016		
CXCR1	C-X-C motif chemokine receptor 1	50.54	0.0103			53.31	0.0152		
DEFA5	Defensin alpha 5	−11.68	0.0422						
DEFA6	Defensin alpha 6	−12.36	0.0368						
FPR1	Formyl peptide receptor 1	11.09	0.0031						
IFNG	Interferon gamma	2.89	0.0001			3.11	0.0287		
IL13	Interleukin 13	4.08	0.0046			2.82	0.0083		
IL17A	Interleukin 17A	4.31	0.0032			2.39	0.0048		
IL1RN	Interleukin 1 receptor antagonist	12.45	0.0011			14.78	0.0233	2.6	0.0233
IL23A	Interleukin 23 subunit alpha	3.26	0.0013						
IL2RA	Interleukin 2 receptor subunit alpha	2.61	0.0026			2.44	0.0073		
CXCL8	C-X-C motif chemokine ligand 8	24.75	0.0169						
ITGB2	Integrin subunit beta 2	2.32	0.0005			1.75	0.0126		
LCN2	Lipocalin 2	19.25	0.0002			13.56	0.0006	5.957	0.0074
LTB	Lymphotoxin beta	2.91	0.0061						

Table 3: Continued.

Gene	Description	UC				CD			
		IM ($n = 21$) vs. C ($n = 21$)		NM ($n = 21$) vs. C ($n = 21$)		IM ($n = 22$) vs. C ($n = 21$)		NM ($n = 22$) vs. C ($n = 21$)	
		FC	p value	FC	p value	FC	p value	FC	p value
MMP1	Matrix metallopeptidase 1	16.79	0.0074						
MMP10	Matrix metallopeptidase 10	53.71	0.0002	3.6	0.0085	22.84	<0.0001		
MMP3	Matrix metallopeptidase 3	52.13	0.0013						
MMP7	Matrix metallopeptidase 7	286.33	0.0027			87.38	0.0030		
MUC1	Mucin 1, cell surface associated	2.65	<0.0001			2.79	0.0017	2.14	0.0024
NOS2	Nitric oxide synthase 2	11.93	0.0006			7.09	<0.0001		
PCK1	Phosphoenolpyruvate carboxykinase 1	−6.49	<0.0001			−2.36	0.0077		
PECAM1	Platelet and endothelial cell adhesion molecule 1	3.09	0.0021			2.57	0.0060		
S100A8	S100 calcium binding protein A8	28.57	0.0014						
S100A9	S100 calcium binding protein A9	16.56	0.0002			33.97	0.0364	3.61	0.0257
SELL	Selectin L	3.87	0.0003			3.97	0.0418		
SOD2	Superoxide dismutase 2	2.17	0.0005			2.33	0.0166		
TDO2	Tryptophan 2,3-dioxygenase	3.88	<0.0001						
TFF1	Trefoil factor 1	3.06	0.0001			2.91	0.0440		
TIMP1	TIMP metallopeptidase inhibitor 1	6.12	<0.0001			4.21	0.0011		
UBD	Ubiquitin D	5.19	0.0006			4.99	0.0007		
VWF	Von Willebrand factor	3.17	<0.0001			2.84	0.0022		

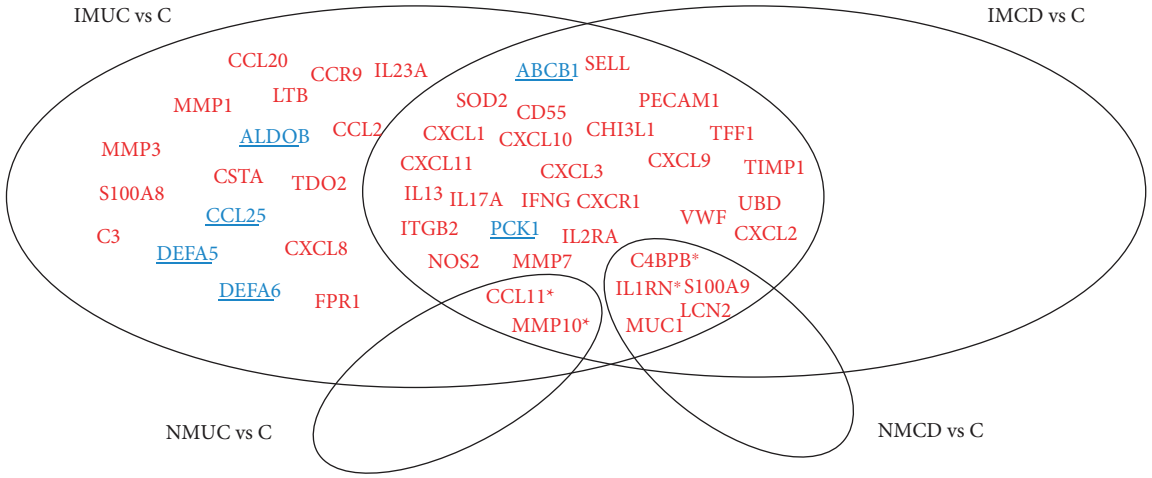

Figure 1: Venn diagram showing the genes differentially expressed across the performed different comparisons with controls. Genes marked with asterisk (*) are differentially expressed in the paired IM-NM analysis. IM = inflamed mucosa; NM = noninflamed mucosa.

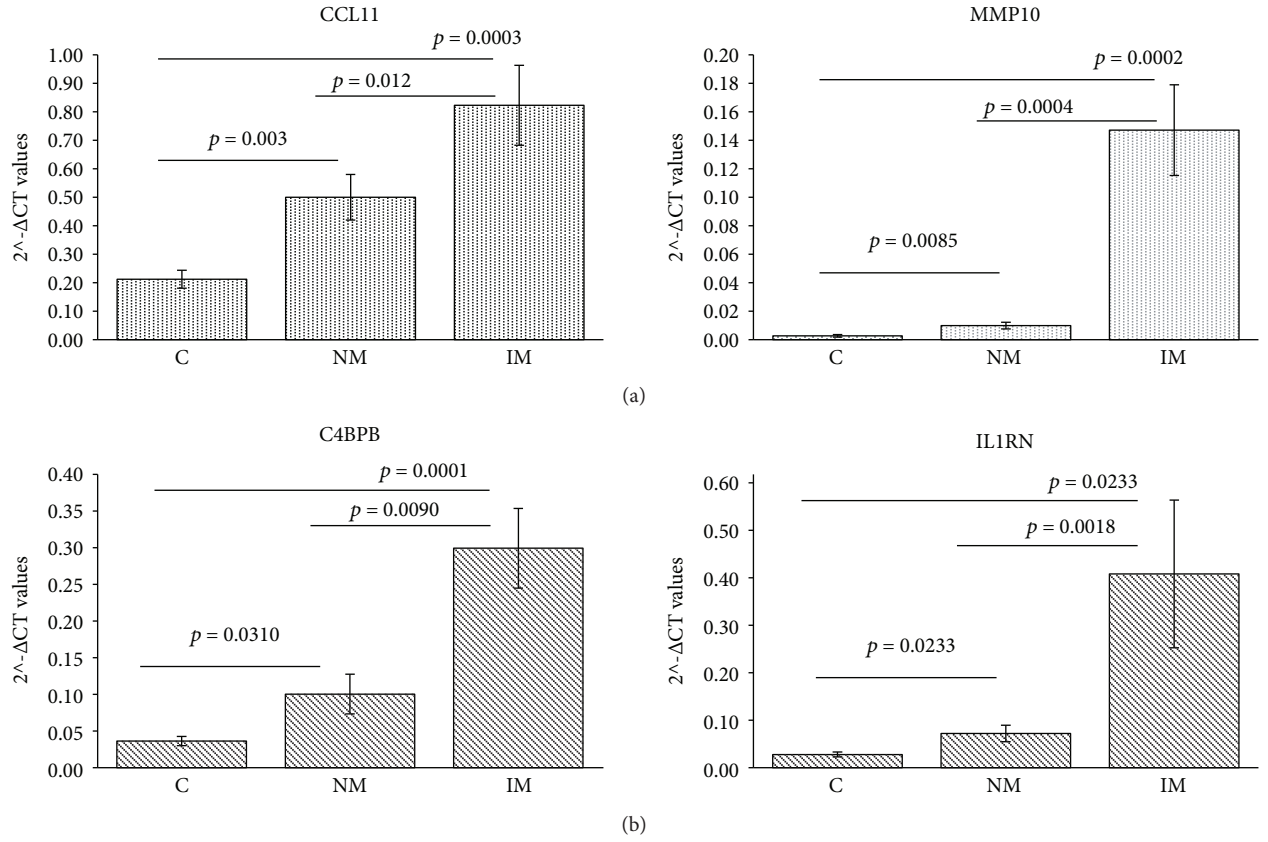

Figure 2: Bar graphs represent the mean of the 2^-ΔCT values, and error bars represent the standard error. The graphs show the genes differentially expressed comparing inflamed mucosa (IM) vs. noninflamed mucosa (NM) IM vs. NM (paired), IM vs. C, and NM vs. C in UC (a) and in CD (b). C = non-IBD controls; IM = inflamed mucosa; NM = noninflamed mucosa.

mucosal mRNA levels were higher in active CD than in controls. However, no changes were observed in the remission state [24] or in UC [25].

Another transcript having a similar trend like CCL11 in UC was MMP10. MMP10 belongs to the human matrix metalloproteinases family consisting of 24 zinc-dependent endopeptidases and is produced by infiltrating myeloid cells. Their levels are transcriptionally upregulated in response to proinflammatory cytokines, and both transcripts and protein levels of some MMPs are demonstrated to be upregulated in inflamed mucosa or serum of IBD patients [26, 27] even in the naïve to treatment subgroup [28]. In addition, increased expression of epithelial MMP10 has been found in colonic mucosa of both UC and CD pediatric patients compared to

non-IBD patients [29]. MMP10 was seen as a possible therapeutic target in IBD because its expression had been observed close to the edges of healing ulcers in human specimens of UC [30]. Its influence, however, can be debated since it could have a role in disease resolution but also in the proinflammatory process. In animal models of experimental colitis, MMP10 seems to promote mucosal healing, and in its absence due to persistent colonic inflammation, dysplastic lesions could be promoted [31]. Human genetic studies identified six SNPs across the MMP10 gene associated with UC, suggesting that these genetic variants may play a role in UC susceptibility and clinical outcome [32].

Moving forward to the specific genes associated to CD in our cohort, C4BPB and IL1RN, they will be discussed below.

The C4BPB gene encodes for C4b-binding protein, a multimeric protein that controls the complement cascade. There is one single study for this gene in CD which evaluated the serum level of C4BPB in patients treated with infliximab, revealing that upregulation of this protein is associated with primary nonresponse to this treatment [33]. Our investigation took into account current biologic treatment, but none of the patients included had had a nonresponse status declared. Thus, we can only suggest that increased expression can only be attributed to the inflammatory process.

Finally, our analysis associated the IL1RN (interleukin 1 receptor antagonist) gene with inflammation in CD. The IL1RN gene encodes for a protein member of the interleukin 1 cytokine family. This protein inhibits the interleukin 1 alpha and beta activities and modulates a variety of related immunoinflammatory responses.

Discordant results regarding the associations between IL1RN genetic variants and IBD have been published. Some studies reported significant associations with CD [34, 35] and UC predispositions [36, 37], treatment outcome [38], and age at the onset [39]; on the contrary, other studies did not find any associations [40–42]. Interestingly, IL-1RN*2 variant has been associated with reduced levels of IL-1ra protein and IL-1RN mRNA in the colonic mucosa from UC patients [43].

Summarizing, against our expectations, only four putative candidate biomarkers able to discriminate UC and CD were found. This can be due to the large gene expression intravariability observed both in the colonic mucosa from non-IBD and IBD groups. Indeed, due to a number of parameters not yet included (histologically active/in remission, duration, and response to treatment), this group intravariability might have increased. Furthermore, the raw data reported that a larger number of genes seemed to be differentially expressed (with high fold difference) without reaching statistical significance due to the high standard deviation. Accordingly, in order to find a more specific signature, the study should be validated in a larger, more homogenous cohort.

Another aim of this study was to evaluate the influence of treatment on the entire IBD cohort. Our results showed a downregulation of ISG15 in patients treated with 5-ASA and a downregulation of SAA 1 in patients with biologic treatment compared to patients without IBD treatment. The effect of different therapeutic agents on IBD gene expression should be assessed in a longitudinal cohort.

The main limitation of this study was the absence of data regarding the clinical scores (MAYO and CDAI) measuring the activity and severity of IBD.

5. Conclusions

In conclusion, we obtained differential intestinal mucosa expression signatures of 17 genes that could specifically characterize the UC inflamed mucosa. Of note, two genes in UC (CCL11 and MMP10) and two in CD (C4BPB and IL1RN) had significantly upregulated expression in the noninflamed and inflamed mucosa compared to controls. Our putative biomarkers, once validated in a larger cohort, could help in clinical practice for the differential diagnosis between UC and CD and could guide new researches on future therapeutic targets.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflict of interests.

Authors' Contributions

Maria Dobre and Elena Milanesi contributed equally to this work.

Acknowledgments

The authors thank the patients involved in the study and the endoscopy department team of the hospitals involved for the generous collaboration. This research was supported by projects PN 16.22.03.01/2016 and PN 18.21.01.05/2018 with the support of the Romanian Autoritatea Nationala pentru Cercetare Stiintifica (ANCSI through the "Nucleu" Program).

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 7819520, 9 pages
https://doi.org/10.1155/2018/7819520

Review Article

Preclinical and Clinical Therapeutic Strategies Affecting Tumor-Associated Macrophages in Hepatocellular Carcinoma

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Received 6 July 2018; Revised 7 September 2018; Accepted 27 September 2018; Published 16 October 2018

Guest Editor: Monica Neagu

Hepatocellular carcinoma (HCC) most often develops in patients with underlying liver disease characterized by chronic nonresolving inflammation. Tumor-associated macrophages (TAMs) are one of the most abundant immune cell populations within the tumoral microenvironment. As key actors of cancer-related inflammation, they promote tumor growth by suppression of effective anticancer immunity, stimulation of angiogenesis, and tissue remodeling. Therefore, they have become an attractive and promising target for immunotherapy. The heterogeneity of TAM subtypes and their origin and dynamic phenotype during the initiation and progression of HCC has been partially unraveled and forms the base for the development of therapeutic agents. Current approaches are aimed at decreasing the population of TAMs by depleting macrophages present in the tumor, blocking the recruitment of bone marrow-derived monocytes, and/or functionally reprogramming TAMs to antitumoral behavior. In this review, the preclinical evolution and hitherto clinical trials for TAM-targeted therapy in HCC will be highlighted.

1. Introduction

Hepatocellular carcinoma (HCC) lesions usually arise in patients with underlying liver cirrhosis, characterized by a chronic, dysregulated inflammatory environment that predisposes to cancer initiation. In chronic liver diseases, a predominantly proinflammatory state switches to persistent systemic inflammation and immune cell stimulation but with impairment of specific immune responses such as phagocytosis and antigen-presenting ability, a condition which is called cirrhosis-associated immune dysfunction. This is an important difference with an acute inflammatory response, regarded as protective and beneficial in the acute setting of liver damage and resolution. Chronic inflammation drives indeed a maladaptive tissue repair reaction and eventually results in the development of dysplastic nodules and cancer [1–4].

The central functions of macrophages during chronic liver diseases include the perpetuation of chronic inflammation and hepatocyte injury, activation of hepatic stellate cells with subsequent fibrogenesis, and support of tumor development by providing cytokines, chemokines, growth factors, and matrix metalloproteases, all of which are factors that favor angiogenesis and tumor cell proliferation and protection from cancer cell apoptosis and metastasis [5, 6]. Thus, hepatic macrophages provide a tumor-prone inflammatory microenvironment and at the same time respond to tumor and other stromal cell-derived signals to actively facilitate HCC progression [1, 7, 8]. Tumor-associated macrophages (TAMs) also stimulate tumor growth by acting as immune suppressor cells of the adaptive system. Not only do TAMs exhibit generally low antigen-presenting and costimulating capacity but they also actively support cancer cells to evade antitumor immunity by secreting anti-inflammatory cytokines and activating T cell checkpoint blockade. In this regard, tumor-infiltrating monocytes in HCC express high level of programmed cell death-ligand 1 (PD-L1) that binds with PD-1 on CD8+ T cells and suppresses antitumoral cytotoxic T cell responses [9–11]. This overall immune-suppressive

effect is reinforced by cross-talk with other important immune cells in the tumoral microenvironment, such as myeloid-derived suppressor cells (MDSC) and regulatory T cells (Tregs). Besides t5he suppression of cytotoxic T cells, MDSC and Tregs contribute to the dysfunctional state of dendritic cells (DCs) [1, 12–14].

Since TAMs influence various aspects of cancer progression, novel strategies to treat HCC are aimed at targeting tumor-promoting macrophages. New therapeutic development is an urgent unmet need as options are still limited for patients with advanced HCC or earlier stage progressing upon or patients unsuitable for locoregional therapies. Nowadays, cancer immunotherapy mainly focuses on immune checkpoint inhibitors. After the observed efficacy in other solid tumors, clinical trials are currently ongoing to evaluate the utility in patients with HCC. Based on promising data in the phase I/II CheckMate-040 trial, immune therapy with nivolumab (anti-PD-1 antibody) has received FDA approval in second-line treatment [15]. However, only 20% of patients are responsive. In solid tumors, recent studies suggest that the efficacy could be enhanced using coordinated strategies to counteract the TAM-dependent impairment of immune adaptive responses [16–18].

Although the clinical application of a TAM-targeted approach still has to be determined, a number of experimental preclinical studies have shown promising effects. Most studies involve other solid tumors and are described elsewhere [19–24]. In this review, the preclinical progress and limited clinical trials affecting TAMs in HCC therapy will be highlighted. Furthermore, the encountered challenges are discussed in relation to fundamental insights into the heterogeneity of TAM subtypes and their origin and dynamic phenotype and function during the initiation and progression of HCC. Lastly, we elaborate on the potential contributive effect of combinational therapies with clinically used therapies such as sorafenib and immune checkpoint inhibitors.

2. Definition and Origin of TAMs in HCC

Liver macrophages consist of ontogenically distinct populations, namely, the resident Kupffer cells (KCs) and monocyte-derived macrophages (Mo-Mfs). Kupffer cells are self-renewing and nonmigratory phagocytes. They originate from yolk sac-derived specific progenitor cells that seed the liver during embryogenesis. In the tumoral microenvironment, chemokines secreted by malignant and stromal cells recruit bone marrow-derived Ly-6c^{hi} monocytes. These infiltrating monocytes subsequently give rise to large numbers of Mo-Mfs. Monocyte-derived macrophages further differentiate and can replace and acquire a phenotype that is almost indistinguishable from resident KCs under specific circumstances [25–30]. After infiltration, Mo-Mfs even seem to acquire the ability to proliferate [27]. It is however unclear if they are able to sustain the number of TAMs in tumor lesions independently from recruitment. As a result of this continuous transition, the compartment of hepatic myeloid cells consists of subtypes of macrophages in a different state of differentiation. Each state is associated with stereotypic alterations in cell surface marker expression, which can be used for identification. In many studies, CD68 is used as an indicator for tissue macrophages, but this marker is not sufficiently specific. More recently, two markers were proposed to distinguish between Mo-Mfs and KCs. Clec4F and Tim4 are expressed by KCs but absent from infiltrating Mo-Mfs. Additionally, these markers can be used to discriminate between KCs and recently differentiated Mo-KCs as the latter do not express Tim4 in the first week postdifferentiation. However, with time, Mo-KCs will also gain expression of Tim4 [27, 28].

It is not clear to what extent TAMs are derived from tissue-resident liver cells or only represent infiltrating bone-marrow derived Mo-Mfs. In most reports, macrophages present in the tumoral microenvironment are considered and classified as "tumor-associated macrophages." Although KCs were initially thought to be only involved in antitumor immunity, there is substantial evidence that suggests that KCs are part of the TAM population and enhance tumor progression [3, 31–33]. KCs are triggered by damage-associated molecular patterns (DAMPs) released from damaged liver cells and pathogen-associated molecular patterns (PAMPs), mostly derived from the gut due to alterations in gut microbiota composition and/or increased intestinal permeability. The liver is supplied with blood via the portal vein from the intestinal tract and via hepatic arteries from the blood circulation. As such, KCs in the liver sinusoids are exposed to bacteria and associated toxins from the bloodstream [34]. DAMPs and PAMPs interact with pattern recognition receptors (PRR) on KC or directly on activating inflammasomes [34]. For example, the interaction of lipopolysaccharide (LPS) with Toll-like receptor 4 (TLR-4) on KCs showed stimulation of cancer-promoting signaling pathways in mice [35].

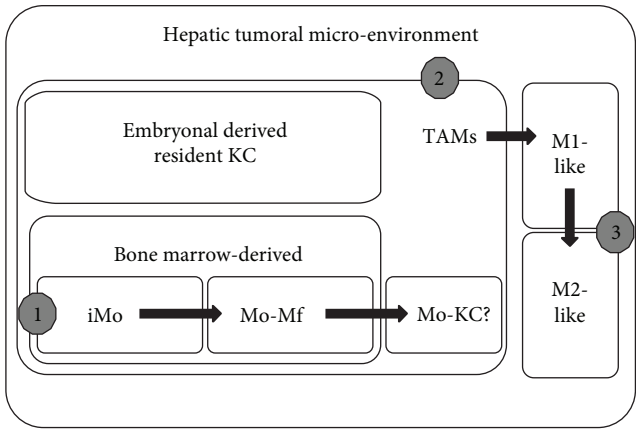
3. Phenotype and Function of TAMs in HCC

Defining TAMs as one population has limitations as shown in the contradictory results of prognostic studies, summarized in Table 1. This is mostly due to an overgeneralized definition of TAMs and indicates the need for further subdivision according to their polarization. Polarization refers to how macrophages have been activated as they can rapidly adapt to their phenotype according to signals derived from the hepatic microenvironment. Macrophages have been assigned a classically activated (proinflammatory) M1 state triggered by interferon-γ and/or lipopolysaccharide or an alternatively activated (anti-inflammatory) M2 state induced by IL-4. This traditional nomenclature however is derived from in vitro studies and does not represent chronic inflammation or the complex tumoral microenvironment. Moreover, the expression and secretory profile of macrophage subsets are not dichotomous and can differ according to the model and method of inducing polarization. There is also considerable difference between mouse and human cells in terms of molecules associated with macrophage polarization [28, 36–39].

The pro- and anti-inflammatory paradigm leads to the confusing assumption that in an inflammation-related tumor, an M2 phenotype would be beneficial. However, during tumor progression in HCC, macrophage function is

Table 1: Prognosis of HCC according to TAM identification and polarization. Immunohistochemical staining for CD68, CD86 (M1), or CD163 and CD206 (M2) is frequently used to quantify and classify TAMs [38, 41]. Expression of the used tissue markers was determined by immunohistochemical staining. Serum sCD163 levels were measured by ELISA. Defining TAMs as solely CD68+ cells gives contradictory prognostic results. When however TAMs are subdivided for location (intra- or peritumoral) and polarization (M1- or M2-like cells), a more distinct prognostic value can be attributed. Moreover, it becomes clear from the presented studies that the presence of M2-like oriented TAMs results in a poor prognostic outcome and intratumoral M1-like TAMs correlate with good prognosis.

Author	Number of patient samples	Type of sample	Marker	Definition	Prognosis
Li et al. [42]	101	Intratumoral	CD68+	TAM	Poor
Ding et al. [43]	137	Intratumoral	CD68+	TAM	Poor
Kuang et al. [11]	262	Peritumoral	CD68+	TAM	Poor
Zhang et al. [44]	149	Peritumoral	CD68+	TAM	Poor
Zhou et al. [45]	213	Intratumoral	CD68+	TAM	Poor
Wu et al. [33]	71	Intratumoral	CD68+	TAM	Poor
Minami et al. [46]	105	Intratumoral	CD68+	TAM	Poor
Liao et al. [47]	387	Intratumoral	CD68+	TAM	Not related
Dong et al. [48]	253	Intratumoral	CD68+	TAM	Not related
Yeung et al. [49]	93	Intratumoral	CD68+	TAM	Good
Yeung et al. [49]	93	Peritumoral	CD68+	TAM	Poor
Li et al. [50]	302	Intratumoral	CD68+	TAM	Good
Liao et al. [47]	387	Intratumoral	CD16+	M2-like	Poor
Waidmann et al. [51]	267	Serum	sCD163+	M2-like	Poor
Minami et al. [46]	105	Intratumoral	CD163+	M2-like	Poor
Yeung et al. [49]	93	Peritumoral	CD163+	M2-like	Poor
Dong et al. [48]	253	Intratumoral	CD206+	M2-like	Poor
Dong et al. [48]	253	Intratumoral	CD86+	M1-like	Good



(i) Inhibiting recruitment infiltrating monocytes
(ii) Depletion of TAMs present in tumor
(iii) Reprogramming polarization of TAMs

Figure 1: Origin of TAMs in the hepatic tumoral microenvironment and related TAM-targeted strategies.

skewed from M1 to M2 phenotype [10, 40]. An anti-inflammatory phenotype does not result in the resolution of inflammation but refers to the immune-deficient and immune-suppressive state of these macrophages and consequently immune evasion of cancer cells. On the other hand, a proinflammatory phenotype does not refer to inflammatory damage in an acute setting but represents a coordinated immune attack of tumor cells. Thus, the M1/M2 model is too simplistic to describe the polarization of liver macrophages in cancer. Currently, TAMs are most often defined as M1-like (leading to antitumor responses and cytotoxicity) or M2-like (tumor promotion and suppression of effective adaptive immunity) cells, taking into account the relative proportion between both characteristics as they often simultaneously express markers of both ends of the continuum [36]. The polarization of macrophages not only depends on the disease stage but also differs between tumoral nodules or within different areas of the same tumor. In human HCC, for example, most of the macrophages that are localized perivascularly are more M1-like compared to the M2-like TAM in hypoxic areas [25, 38].

4. TAM-Targeted Therapy in HCC

Current approaches for TAM-targeted therapy are aimed at decreasing the population of TAMs by eliminating TAMs present in the tumor, blocking recruitment of bone marrow-derived monocytes, and/or reprogramming TAM polarization to antitumoral behavior (Figure 1).

4.1. Preclinical Studies. In the following section, only preclinical studies in mouse models using agents with a direct effect on TAMs in HCC will be discussed and are summarized in Table 2. Gene therapy or knockout models are beyond the scope of this review.

4.1.1. Depletion of TAMs. Liposomes are artificially prepared vesicles that undergo phagocytosis by macrophages after

Table 2: Preclinical TAM-targeted therapies in HCC.

Author	Product	Mechanism of action	Animal model	Results
Wang et al. [52]	Clodronate-liposomes	Depletion of TAMs	Hepa1-6 HCC cell line xenograft and orthotopic mouse model	Inhibition of tumor growth
Zhang et al. [65]	Sorafenib and zoledronic acid or clodronate-liposomes	Depletion of TAMs	HCCLM3-R and SMMC7721 HCC cell line xenograft mouse models	Inhibition of tumor growth, lung metastasis, and angiogenesis
Li et al. [55]	CCR2 antagonist	Inhibiting recruitment of monocytes M2 polarization of TAMs	Hepa1-6 and LPC-H12 HCC cell line xenograft and Hepa1-6 orthotopic mouse model	Inhibition of tumor growth and metastasis, reduction of recurrence, enhanced survival, and activation of CD8+ T cells
Yao et al. [56]	CCR2 antagonist	Inhibiting recruitment of monocytes	Hepa1-6 or LPC-H12 HCC cell line xenograft and Hepa1-6 orthotopic mouse models	Inhibition of tumor growth, increase in CD8+ T cells, and potentiation effect of sorafenib
Teng et al. [57]	CCR2 monoclonal antibody	Inhibiting recruitment of monocytes	miR-122-knockout HCC mouse model	Inhibition of tumor growth and activation of natural killer cells
Zhou et al. [68]	Sorafenib and TACE	Inhibiting recruitment of monocytes	Walker-256 HCC cell line xenograft and orthotopic rat models	Inhibition of tumor growth and angiogenesis
Tan et al. [58]	Baicalin	Reprogramming polarization of TAMs	MHCC97L HCC cell line orthotopic mouse model	Inhibition of tumor growth
Ao et al. [59]	CSF-1 receptor antagonist	Reprogramming polarization of TAMs	Hepa1-6, HepG2, or HCCLM3 HCC cell line orthotopic mouse model	Delayed tumor growth and increase in CD8+ T cells
Sprinzl et al. [62]	Sorafenib	Reprogramming polarization of TAMs	Hepatitis B virus replicating HBV1.3.32 or albumin-promoter-controlled lymphotoxin-a/b transgenic mice	Activation of natural killer cells and cytotoxicity
Wan et al. [60]	IL-6 receptor monoclonal antibody	Blocking downstream effect of TAM products	HepG2 or human HCC cell line xenograft mouse model	Reduction of tumor growth

injection. They can be loaded with clodronate (a bisphosphonate used for osteoporosis) which induces apoptosis of macrophages after intracellular release from the liposomes. Administration of clodronate- (Cl2MDP) encapsulated liposomes partially depleted TAMs (defined as F4/80- and CD68-positive cells on immunohistochemical staining), resulting in reduced tumor growth in a murine Hepa1-6 cell-transplanted tumor model. Not only was the total amount of TAMs reduced but also the number of M2-like TAMs in tumors of liposome-treated mice was significantly lower than that in tumors of untreated mice. In contrast, the number of M1 TAMs was not significantly affected. According to the authors, these results suggest that after depleting the majority of TAMs, the remaining macrophages might undergo a phenotypical transition [52].

Selective depletion of only tumor-promoting macrophages, not just all cells with phagocyting capacity, is an encountered difficulty in TAM-targeted therapy. An excessive reduction of nontumoral macrophages might lead to safety concerns when concomitant infections occur. The use of macrophage subset-specific markers might provide a solution and has successfully been used in the field of imaging where KC-specific [53] or TAM-specific targeting by nanobodies coupled to SPECT or PET tracers allowed KC-specific and M2-like TAM-specific imaging, respectively [54]. Further research is warranted to see if this approach could be translated to pharmaceutical development. It must be emphasized that TAMs are strongly connected with other immune and stromal cells in the microenvironment and it is not clear to what extent other cells will compensate for their function after depletion.

4.1.2. Inhibiting Recruitment of Monocytes. The chemokine C-C motif ligand 2 (CCL2, also referred to as monocyte chemoattractant protein 1 or MCP-1) and the corresponding CCL2-CCR2 signaling axis are important targets to inhibit the recruitment of monocytes. Treatment with a CCR2 antagonist inhibited HCC tumor growth in different murine models. The therapy reduced the infiltration of blood $Ly6C^{high}$ inflammatory monocytes, subsequently lowered the number of TAMs (CD11b- and F4/80-positive cells) in the HCC lesions, and reduced most of the cytokines or chemokines produced by M2-like TAMs (CD206-positive cells). Moreover, the reduced number of remaining TAM shifted towards M1 phenotype. The CCR2 antagonist also supported tumor-infiltrated CD8+ T cells by blocking TAM-mediated immunosuppression [55, 56]. In addition, Teng et al. showed the tumor-inhibiting effect of a CCL2 neutralizing antibody by reducing the population of inflammatory myeloid cells in a HCC mouse model [57]. Although several chemokines are involved in attracting monocytes and targeting one pathway might not completely eliminate recruitment, blocking the CCL2-CCR2 seems to be effective in the inhibition of HCC growth.

Infiltration of monocytes is considered the most important source of TAMs in the tumoral microenvironment. It is still unclear if TAMs are able to sustain their number (or least partially) in tumors by proliferation independently from recruitment or how long TAMs survive in the tumoral microenvironment. Related to this issue, effective timing to start inhibiting recruitment of monocytes can be debated as during early stages, TAMs can also exert an antitumoral function.

4.1.3. Reprogramming Polarization of TAMs. Oral administration of baicalin, a natural flavonoid present in several medicinal plants, inhibited growth of HCC lesions in an orthotopic mouse model by initiating TAM reprogramming to an M1-like phenotype with proinflammatory cytokine production. Coculturing of HCC cells with baicalin-treated macrophages resulted in reduced proliferation and motility in vitro [58].

Colony-stimulating factor-1 (CSF-1) and its receptor, CSF-1R, regulate the differentiation and function of macrophages. CSF-1R blockade by a competitive inhibitor significantly delayed tumor growth in murine xenograft models. The compound inhibited the proliferation of macrophages in vitro, but macrophage infiltration was not decreased in vivo. Thus, the effect is not mediated by TAM depletion. Gene expression profiling showed that TAMs in the treated tumors are polarized towards an M1-like phenotype [59].

An imbalance towards M1-like macrophages might theoretically be harmful by inducing toxicity and inflammatory conditions. In the mentioned studies, no toxic effects were observed but further studies are necessary.

4.1.4. Blocking the Downstream Effect of TAM Products. TAMs represent a major paracrine IL-6 source during HCC progression, and autocrine IL-6 contributed significantly to HCC initiation from HCC progenitor cells. Blockade of IL-6 signaling using tocilizumab, an anti-IL-6 receptor antibody approved by the FDA for the treatment of rheumatoid arthritis, was able to inhibit TAM-stimulated activity of cancer stem cells in vitro and in vivo [60].

4.2. Preclinical Therapy Affecting TAMs with Currently Used Clinical Therapies. Sorafenib, an antiangiogenic oral multikinase inhibitor, is currently the standard first-line systemic treatment approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) for patients with advanced HCC. Preclinical studies show that sorafenib interferes with the polarization of TAMs and their cytokine production. In a HepG2 HCC cell line, sorafenib inhibited polarized macrophage-induced epithelial-mesenchymal transition and migration of HCC cells [61]. Administration of sorafenib reduced M2-like TAMs, inhibited their immunesuppressive effect, and stimulated antitumor natural killer (NK) cell responses in both HCC models [62]. In addition, Sprinzl et al. [63] demonstrated a decrease in CD163 serum concentration in 21 patients with HCC during treatment with sorafenib. This finding suggests that Sorafenib suppressed M2 activation in HCC patients, since soluble sCD163 is shedded into serum by activated macrophages and can serve as an indicator to follow M2 macrophage responses [51, 64]. Together, these findings indicate that macrophage modulation contributes to the anticancer activity of sorafenib.

Interestingly, the combination of sorafenib with TAM-targeting agents such as clodronate-loaded liposomes

and zoledronic acid (another bisphosphonate used for the treatment of bone metastasis) augmented the inhibitory effect of sorafenib on tumor angiogenesis, growth, and metastasis in HCC xenograft mouse models [65]. A phase II study of sorafenib combined with zoledronic acid in advanced HCC has been conducted (NCT01259193), but no results have been published yet. Besides depletion of macrophages, nitrogen-containing bisphosphonates (such as zoledronic acid) activate $\gamma\delta$ T cells, potentiating their antitumor function. This immunomodulatory effect of zoledronic acid on $\gamma\delta$ T cells is exerted through direct or indirect interaction induced by TAMs that endocytose bisphosphonate-encapsulated liposomes [66, 67]. Not only is this effect shown for sorafenib but also the combination of locoregional therapy such as transarterial chemoembolization (TACE) and zoledronic acid treatment showed enhanced therapeutic efficacy with inhibition of TAM infiltration (F4/80+) and tumor angiogenesis in a rat HCC model [68]. The enhanced efficacy of sorafenib together with a CCR2 antagonist to inhibit monocyte infiltration has been shown in a murine HCC model [56].

The effect of checkpoint blockade immunotherapy on TAMs has been shown for other solid tumors, but no data are available in HCC. Blockade of PD-1 in vivo reduced tumor growth and extended the survival in a colorectal cancer mouse model by polarization of TAMs to a phagocytic phenotype [69]. The efficacy of anticytotoxic T lymphocyte-associated antigen 4 (CTLA-4) monoclonal antibodies in a melanoma mouse model and humans is codefined by elimination of regulatory T cells by TAM targeting via antibody-dependent cellular cytotoxicity [16–18, 45]. Also, in a pancreatic cancer mouse model, the combination of depleting M2-like TAMs and repolarization towards antitumoral behavior through blockage of CSF-1/CSF-1R and immunotherapy (PD-1 and CTLA-4 antagonists) reduced tumor progression [18].

4.3. Clinical Trials. Glypican-3 is a proteoglycan that is attached to the cell surface and plays an important role in cellular growth, differentiation, and migration. Glypican-3 is highly expressed in HCC tissue and correlates with poor prognosis. It is considered a tumor-derived carcinoembryonic antigen. For example, expression of glypican-3 was associated with upregulation of CCL5, CCL3, and CSF-1 in a HCC xenograft model [70], all of which are chemokines that have been shown to enhance the recruitment of TAMs. Glypican-3 antibodies have been tested in small phase I trials for advanced HCC with promising results (in 13 and 20 patients, respectively). The antibody is well tolerated, and preliminary antitumor activity shows a threefold prolongation of the median time to progression in treated patients with advanced HCC (Child–Pugh A or B cirrhosis) [71, 72]. No phase II trials are currently registered with glypican-3 antibodies for HCC.

5. Conclusion

The tumor-promoting cascade of initial injury recognition, amplification of inflammation by monocyte recruitment, and context-dependent differentiation into functionally distinct macrophage populations in the liver offers different approaches for therapeutic interventions in HCC. Although the clinical application of TAM-targeted therapy is still in its infancy, a number of preclinical studies in HCC murine models have shown promising results. The most important obstacles to overcome are firstly the specificity of depleting only protumoral TAMs while not affecting (or even enhancing) antitumor immunity and secondly the perfect balance of their polarization towards antitumoral behavior without toxicity and side effects. The observed potential contributive effect of immune checkpoint inhibitors on solid tumors and currently used clinical therapies for HCC such as sorafenib is encouraging and must be further explored.

Abbreviations

CSF-1: Colony-stimulating factor-1
CSF-1R: Colony-stimulating factor-1 receptor
CTLA-4: Cytotoxic T lymphocyte-associated antigen 4
DAMP: Damage-associated molecular patterns
HCC: Hepatocellular carcinoma
KCs: Kupffer cells
Mo-Mfs: Monocyte-derived macrophages
Mo-KCs: Monocyte-derived Kupffer cells
PAMPs: Pathogen-associated molecular patterns
PD-1: Programmed cell death 1
PD-L1: Programmed cell death-ligand 1
TAMs: Tumor-associated macrophages.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

HD and LD are sponsored by a fund from Kom op tegen Kanker (Stand up to Cancer), a Flemish cancer society. LD received a postdoctoral fellowship from the Special Research Funds (BOF) from Ghent University. AV is sponsored by the Special Research Funds (BOF) from Ghent University. HVV and AG are sponsored by the Research Foundation Flanders (FWO).

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 7169081, 8 pages
https://doi.org/10.1155/2018/7169081

Review Article

On the Dual Role of Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 (CEACAM1) in Human Malignancies

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Received 11 July 2018; Accepted 5 September 2018; Published 14 October 2018

Guest Editor: Iulia D. Popescu

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is a glycoprotein belonging to the carcinoembryonic antigen (CEA) family that is expressed on a wide variety of cells and holds a complex role in inflammation through its alternate splicing and generation of various isoforms, mediating intricate mechanisms of modulation and dysregulation. Initially regarded as a tumor suppressor as its expression shows considerable downregulation within the epithelia in the early phases of many solid cancers, CEACAM1 has been linked lately to the progression of malignancy and metastatic spread as various papers point to its role in tumor progression, angiogenesis, and invasion. We reviewed the literature and discussed the various expression patterns of CEACAM1 in different types of tumors, describing its structure and general biologic functions and emphasizing the most significant findings that link this molecule to poor prognosis. The importance of understanding the role of CEACAM1 in cell transformation stands not only in this adhesion molecule's value as a prognostic factor but also in its promising premise as a potential new molecular target that could be exploited as a specific cancer therapy.

1. Introduction

The discovery of the carcinoembryonic antigen (CEA) as a tumor marker for colorectal carcinoma in 1965 by Gold and Freedman [1] was the milestone for identifying a much wider family of 12 carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) which mediate intricate mechanisms of modulation and dysregulation during complex biological processes regarding cancer progression, inflammation, metastasis, and angiogenesis [2].

The most vastly distributed protein within this family is CEACAM1, being expressed on various normal epithelia from the gastrointestinal tract (*intestinal and colonic superficial epithelial cells, duodenal Brunner glands, esophageal glands, bile, and pancreatic ducts*), prostatic glands, gall bladder, mammary ducts, endometrium, renal tubuli, extravillous trophoblast, etc., as well as endothelial cells, natural killer (NK) cells, T and B lymphocytes, and myeloid cells [3–9].

Subjected to alternative splicing, the CEACAM1 primary transcript generates 12 different human isoforms, 3 of which are secreted versions that play an important role in inhibition of intercellular adhesion, being a marker of melanoma, pancreatic, and urothelial bladder carcinoma (UCB) progression [10–12]. CEACAM1 alternative splicing also results in

generation of two major cytoplasmic domains, the so-called long (-L) and short (-S) tails, both of which have dysregulated expression in colorectal (CRC), breast, and non-small-cell lung carcinomas (NSCLC) [13–15].

CEACAM1 splice variants differ with respect to the number of extracellular domains and type of intracellular cytoplasmic domains [16]. The extracellular domains consist of one amino-terminal immunoglobulin variable-region-like (IgV-like) domain, which mediates hemophilic or heterophilic interactions [17, 18], and a maximum of three immunoglobulin constant-region-type-2-like (IgC2-like) domains, whose roles are still unclear. Regarding the intracellular cytoplasmic domains, splicing connects the various isoforms to either a long cytoplasmic tail (-L) or a short cytoplasmic tail (-S) [16]. (-L) tails contain two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that coordinate inhibitory signaling via Src homology 2 domain-containing tyrosine phosphatase- (SHP-) 1 or (SHP-) 2 recruitment following phosphorylation by Src family tyrosine kinases [19]; (-S) tails lack ITIMs [16].

SHP-1 and SHP-2 act as nonreceptor tyrosine phosphatases, which reverse critical tyrosine phosphorylation reactions induced by the action of tyrosine kinases [20], thus promoting signal inhibition. As a consequence, ITIM-containing family members of CEACAM1 (CEACAM1-L) generally mediate negative signaling, while ITIM-deficient CEACAM1 (CEACAM1-S) isoforms do not [5]. An increase of the CEACAM1-L/CEACAM1-S ratio is associated with decreased proliferation of tumor cells [21].

Isoform expression of CEACAM1 in tumoral tissue is particularly dynamic showing considerable downregulation within the epithelia in the early phases of many solid cancers such as prostate [22], colon [23, 24], breast [25], and liver [26] carcinomas. Restoration of CEACAM1 expression in tumor cell lines often abolishes their oncogenicity *in vivo* as indicated by studies in syngeneic or immune-deficient mice, where reinsertion of various CEACAM1 isoforms in colorectal or prostate CEACAM1-negative tumor cells proved CEACAM1-L expression to be essential for maintaining a normal phenotype with the inhibition of allograft or xenograft tumor development [27, 28]. Therefore, this adhesion molecule has been regarded as a tumor suppressor.

In contrast, studies showing CEACAM1-L overexpression in advanced stages of malignancies such as melanoma [29], NSCLC [30], bladder [31], CRC [32, 33], thyroid [34], and gastric [35] carcinomas correlate high abundance of CEACAM1-L with potential of invasiveness and metastatic spread [36], thus challenging the previously postulated concept of a tumor suppressive effect of this adhesion molecule.

This discrepancy is partially explained by the fact that ITIMs also bind Src family kinases (SFKs), which play critical signaling roles in hematopoietic cell function, such as B cells, T cells, NK cells, monocytes, granulocytes, and mast cell activation [37]. CEACAM1-SFK interactions contribute to cell adhesion properties of eosinophils as well as tumor cells [38–40]. Studies assessing CEACAM1 isoform expression in human neoplastic mast cells (mastocytosis) and medullary thyroid carcinoma cell (MTC) lines suggest that CEACAM1-L enhances cell growth in association with preferential interactions and activation of SFKs [16]. Thus, the dominantly interacting proteins SHP1 or SFK determine whether CEACAM1-L displays a positive or negative role in tumor cells [16].

2. The Role of CEACAM1 in the Regulation of Immune Surveillance, Immune Evasion, and Inflammation

The decreased expression of CEACAM1 in early malignancies and its upregulation in advanced cancers were difficult to reconcile. Further arguments supporting the molecule's role in tumor growth and progression are revealed by closer insights into the relationship of CEACAM1 with cells of the immune compartment, particularly T and NK cells, which illustrate its capacity to evade the immune system.

CEACAM1 homophilic interactions and the CEACAM1 heterophilic interactions with CEACAM5 inhibit NK-mediated killing independently of major histocompatibility complex (MHC) class I recognition and also interfere with the interferon-γ (IFN-γ) release activities of NK cells, as well as tumor-infiltrating lymphocytes (TILs) [6, 41–43]. These findings are supported by mouse cancer models, where CEACAM1 silencing results in upregulated NK cell activating ligands on the cancer cell surface [44]. In mouse melanoma cell lines, CEACAM1-4L isoform downregulates cell surface levels of NKG2D ligands MICA and ULBP2 [45] while CEACAM1-3S and -3L overexpressions in CRC cell lines cause sequestration of MICA/B intracellularly, preventing it from activating NK cells [44].

The essential interplay between CEACAM1 and cells of the immune system is well documented in melanoma. High percentages of circulating NK and CD8+ lymphocytes are CEACAM1+ in melanoma patients [10]. CEACAM1 homophilic interactions between TILs and melanoma cells appear to dampen *in vivo* TIL functions, limiting the efficacy of TIL adoptive cell transfer therapy in melanoma patients [46]. A study model investigating aspects of melanoma cell long coincubation with antigen-specific TIL demonstrated that the surviving melanoma cells increase their surface CEACAM1 expression, which in turn confers enhanced resistance against fresh TIL [46]. This appears to be an active process, driven by specific immune recognition, and is at least partially mediated by lymphocyte-derived IFN-γ [46]. These findings are consistent with results from CRC studies where CEACAM1 expression on TILs in mice and humans marks the most highly exhausted T cells [47]. Circulating CD8+ T lymphocytes and TILs from CRC patients have overexpression of CEACAM1 and TIM-3 compared with normal tissue [47]. CRC tumors among the double-positive ($CEACAM1^{+}TIM\text{-}3^{+}$) T cells exhibit a significant decrease in IFN-γ production [47]. These well-defined inhibitory roles of CEACAM1 in T and NK cells present the molecule as a valuable target for cancer immunotherapy with monoclonal antibodies in late-stage cancer.

CEACAM1's crucial role in regulating autoimmunity and antitumor immunity is also portrayed by analyzing its interaction with T cell immunoglobulin domain and mucin

domain-3 (TIM-3), which endows TIM-3 with T cell inhibition capacity [18]. TIM-3 is an activation-induced inhibitory molecule involved in tolerance shown to induce T cell exhaustion in chronic viral infection and cancers [48–52], which under some conditions may also be stimulatory. Mouse adoptive transfer colitis models show CEACAM1-deficient T cells to be hyperinflammatory with reduced cell surface expression of TIM-3 as well as regulatory cytokines, and this can be restored by T cell-specific CEACAM1 expression [53]. By forming heterodimeric interaction in both *cis* and *trans* through their (IgV)-like N-terminal domains, CEACAM1 facilitates the maturation and cell surface expression of TIM-3, resulting in its tolerance-inducing function [18]. CRC cancer mouse models demonstrate that coblockade of CEACAM1 and TIM-3 leads to enhancement of antitumor immune responses and improved elimination of tumors [18].

The involvement of CEACAM1 in angiogenesis is also important for its protumorigenic effect. CEACAM1 was shown to be a major effector of vascular endothelial growth factor (VEGF) in early tumor microvessel formation [54]. Kilic and collaborators demonstrated that VEGF increases CEACAM1 expression on both mRNA and protein levels, and the administration of a monoclonal CEACAM1 antibody blocks *in vitro* VEGF-induced endothelial tube formation [54, 55]. Furthermore, by using an experimental mouse model of cutaneous leishmaniasis, a disease known to produce severe local inflammation accompanied by accumulation of CD11b cells at the site of infection, a VEGF-independent role of CEACAM1 has been characterized [56]. Both blood and lymphatic vessel formation appear to be affected by the loss of CEACAM1/CD11b cells, which control angiogenesis during inflammation [56]. Due to its capacity to evade the immune system, as well as its potent proangiogenic effects, CEACAM1 appears to play an important role in tumor growth and progression.

The emerging picture of CEACAM1 is extremely complex as its role in tumor cells appears to be contradictory, supporting both down- and upregulation. Detailed investigation of the expression patterns of this adhesion molecule in different cancer types is crucial in determining CEACAM1 involvement in carcinogenesis and possible significance of its altered expression in terms of diagnosis, prognosis, and treatment of distinct human neoplasms.

Our review focuses on emphasizing recent insights into the role of CEACAM1 in various cancer types due to their importance in designing a more comprehensive role in cell transformation of this adhesion molecule which may be a feasible target potentially leading to promising strategies in cancer treatment.

3. Malignancies Showing Early Phase CEACAM1 Downregulation

3.1. Colorectal Carcinoma. CEACAM1-reduced expression has been reported in more than 85% of early colorectal adenomas and carcinomas [57, 58] leading to a supposed tumor growth inhibitor function of this adhesion molecule in CRC development. Hyperplastic polyp lesions and aberrant crypt foci, the earliest stages of CRC, also have reduced levels of expression of CEACAM1 [59]. Experimental murine models show similar results, with CEACAM1 knockout mice developing a significantly greater number of colonic tumors than their controls when treated with azoxymethane to induce tumorigenesis [60].

However, studies using specific anti-CEACAM1 antibodies revealed elevated expression in high-grade adenomas, adenocarcinomas, and metastatic CRC [32, 33] suggesting a bimodal role in CRC progression. In addition, Ieda et al. highlighted the CEACAM1-L isoform presence as an independent risk factor for CRC hematogenous and lymph node metastasis and for a short survival time [61].

3.2. Mammary Gland Carcinoma. While expressed prominently on the luminal side of healthy mammary epithelia, CEACAM1 becomes randomly distributed on the cell surface or is completely lost during mammary cancer progression when typical architectural features of polarized tissues decline [62]. Similar to previous observations made with colorectal cancer, a low CEACAM1 expression was found in about 65% cases among breast cancer tissues in comparison to adjacent normal breast tissue [63]. A study by Wang et al. shows cancer tissues from 60 patients with mammary carcinoma to exhibit no CEACAM1 staining (12/60 patients 20%) or weak CEACAM1 expression (13/60 patients 21.7%) while the adjacent breast tissues show moderate to intense staining in most cases, without negative expression [63].

On the other hand, a study by Gerstel and collaborators comparing the intratumoral vascular tree in spontaneous and transplanted mammary adenocarcinomas in CEACAM1 competent mice with CEACAM1 null hosts shows the former to have increased vascular densities and pericyte coverage, with increased tumor vascularization and angiogenesis [64]. CEACAM1 was only expressed in peritumoral vessels [64]. The authors conclude that endothelial expression of CEACAM1 in the vasculature of the mammary tumor periphery appears to be an important component building a proangiogenic microenvironment that supports tumor vessel stabilization [64].

3.3. Bladder and Prostate Carcinomas. Immunohistochemical studies conducted by Oliveira-Ferrer and coworkers [31] in 2004 on nonmalignant urinary bladder tissues revealed "umbrella cells"-epithelial cells lining the luminal surface of transitional epithelium to exhibit CEACAM1 staining, while blood vessels of the normal bladder appeared negative. On the contrary, in early tumor stage pTa, CEACAM1 immunostaining became negative in tumoral cells, whereas the majority of blood vessels closely associated with or growing into the epithelial layer containing tumor cells were found CEACAM1-positive. As for invasive bladder tumors, all cases showed CEACAM1-positive blood vessels in close association with the tumor cell groups, with few tumoral cells and neighboring normal urothelial area still exhibiting CEACAM1 expression. Thus, CEACAM1 expression appears to be downregulated in bladder cancer cells, while concurrently upregulated in endothelial cells of tumoral adjacent blood vessels.

Tilki and collaborators made similar observations in prostate cancer, conducting electron microscope studies on prostate intraepithelial neoplasia (PIN) specimens [65]. CEACAM1 expression was found to be downregulated in epithelial dysplastic cells and upregulated in adjacent endothelial cells, with concurrent VEGF-A, -C, and -D upregulation, indicating activation of angiogenesis.

Studies from Ergün and coworkers support the previous observations that the differential switch in CEACAM1 expression stimulates VEGF and fibroblast growth factor-dependent proangiogenic activities such as neocapillary formation, proliferation, and chemotaxis, thus, promoting angiogenesis [54].

Interestingly, CEACAM1 upregulation during endothelial cell angiogenic activation can be detected in both membrane-bound forms and supernatant of endothelial cells, suggesting that during this process, soluble CEACAM1 forms might be released into body fluids [54].

Based on this finding, another study conducted by Tilki and collaborators [12] attempted to assess whether CEACAM1 urinary levels could help differentiate patients with UCB from healthy subjects. After performing western blot analysis on urinary samples from 135 subjects (93 with UCB and 42 with no UCB), they concluded that urinary bladder carcinoma and an invasive stage are associated with higher urinary levels of CEACAM1.

The data presented displays the disappearance of CEACAM1 in the dysplastic epithelium as one of the earliest signs of tumors switching from superficial noninvasive and nonvascularized to invasive and vascularized [31]. This supports the previously postulated hypothesis that the CEACAM1 presence in normal epithelia functions as a tumor suppressor [27, 66, 67]. However, epithelial CEACAM1 downregulation increases the expression of proangiogenic factors [54, 55] and favors endothelial CEACAM1 upregulation [31], promoting invasiveness, hence the dual role of CEACAM1 expression in these malignancies.

4. Malignancies with CEACAM1 Overexpression

4.1. Non-Small-Cell Lung Carcinoma. Immunohistochemical and serum assessments of CEACAM1 in NSCLC revealed it is a valuable prognostic biomarker [68]. CEACAM1-S isoform and the CEACAM1S/CEACAM1-L ratio appear to be significantly higher in tumors than in normal tissue [68].

Sienel and collaborators found significant association between CEACAM1 expression and tumoral status, while trying to elucidate the role of CEACAM1 in the progression and survival of patients with operable NSCLC [69]. They analyzed the immunohistochemical expression of CEACAM1 in tumor samples from 145 patients with completely resected NSCLC. All sections of normal bronchiolar epithelium stained negative, 73 tumors (50.4%) displayed between 1 and 66% CEACAM1-positive tumoral cells, and the remaining 72 tumors (49.6%) showed even greater percentage of positive cells. Following a detailed statistical analysis, the authors concluded that the absence of CEACAM1 in normal lung tissue and its expression in tumor cells argue against a tumor suppressive role of CEACAM1 in NSCLC.

Furthermore, elevated CEACAM1 expression correlates with severe disease and tendency to reduced cancer-related survival of the total population, rather indicating CEACAM1 as a promoter of lung cancer progression [69]. In addition, urinary levels of CEACAM1 represent an excellent biomarker for NSCLC patients when considered alongside other signature proteins [70].

4.2. Pancreatic Adenocarcinoma. Various studies indicate CEACAM1 to be a useful biomarker for pancreatic adenocarcinomas (PAC), being present in both tumor specimens and serum of patients compared to healthy individuals [11, 71–73]. CEACAM1 is more sensitive and specific than the already consecrated CA 19-9 in differentiating cancer from normal controls, and this is improved by combining CEACAM1 and CA 19-9 [11].

Furthermore, CEACAM1 appears to be present early in the development of the disease, with most pancreatic intraductal neoplasia 3 (PanIN-3) lesions, representing pancreatic carcinoma in situ, showing elevated expression by immunohistochemical analysis [11]. As PACs have high lethality associated due to appearance of clinical manifestations late in the natural course of the disease [74], development of blood-based biomarkers capable of detecting PAC at early, preclinical stages is a necessity. A study conducted by Nolen et al. indicates CEACAM1 and prolactin as the earliest serum biomarkers to be detected at significantly altered levels, up to 35 months prior to diagnosis [75].

CEACAM1 has also been strongly correlated with distant metastasis of PAC [73].

4.3. Thyroid Carcinoma. CEACAM1 is not appreciably expressed in normal human thyroid tissue and is rarely present in benign tumors [4] but highly upregulated in thyroid carcinomas, especially in metastatic tumors [34]. To investigate the role of CEACAM1 in thyroid carcinomas, Liu and coworkers [34] compared the effects of this adhesion molecule in WRO cells (usually devoid of CEACAM1 expression) and clones with forced CEACAM1 expression. In vitro analysis revealed that forced expression of CEACAM1 caused a significant G0/G1 phase arrest, with enhanced cell–matrix adhesion and increased cell invasion, resulting in diminished tumor growth and increased tumor invasiveness when applied to a xenograft mouse model. Conversely, CEACAM1 downregulation stimulated MRO cell tumor growth with reduced invasiveness. They concluded CEACAM1 is an important cell proliferation inhibitor, retarding several parameters of tumoral growth while mediating invasion.

These findings are consistent with CEACAM1 expression correlation to invasiveness in cutaneous malignant melanoma [29] and to the molecule's implication in mediating trophoblast/endometrial interactions during trophoblastic invasion of the endometrium [76, 77].

4.4. Melanoma. Normally, melanocytes do not exhibit CEACAM1 [78, 79]. Concerning CEACAM1 expression in melanocytic nevi, few data is available. While assessing CEA family expression in melanocytic nevi using monoclonal and polyclonal panels of antibodies (none of which

directed only towards CEACAM1), Egawa et al. demonstrated an increased expression of this protein family on the surface of both acquired and congenital melanocytic nevi, in a similar distribution pattern [79]. Interestingly, blue nevi, which are melanocytes from the neural crest that failed reaching the epidermis during embryological migration, do not stain positive for CEA [79].

Immunohistochemical studies conducted by Gambichler and collaborators comparing CEACAM1 expression in benign and malignant melanocytic tumors and normal peritumoral skin reported a progressive increase of median CEACAM1 expression from 1% in benign nevi and 9.6% in dysplastic nevi to 18% in thin superficial melanomas (defined as melanomas with Breslow tumor thickness <1 mm) and 74% in thick superficial spreading melanomas (defined as melanomas with Breslow tumor thickness >1 mm) ($p < 0.0001$) [80]. Peritumoral melanocytes from normal skin stained negative [80].

In melanoma, the role of CEACAM1 is well established, with a significant number of studies unanimously demonstrating its prognostic value in diagnosis, progression, and metastasis [29, 80, 81]. There is an overwhelming expression of the CEACAM1-L isoform on melanoma cells [45, 82], and elevated serum CEACAM1 levels were found to positively correlate with decreased patient's survival, failure to respond to immunotherapy, and decreased efficacy of autologous vaccination [10, 83, 84].

In metastatic cutaneous melanoma, 89% of lesions express CEACAM1, and CEACAM1 expression increases during tumor progression [82], while soluble CEACAM1 levels significantly correlate with the level of LDH [10].

The overwhelming evidence of CEACAM1 importance in melanoma solidify the claim that this adhesion molecule could be applied as an improved prognostic and predictive biomarker for melanoma patients over the commonly used Breslow depth [85].

4.5. Squamous Cell Carcinoma (SCC). There is a lack of data concerning CEACAM1 expression in keratinocytic tumors. A study by Wang and collaborators demonstrated that CEACAM1 overexpression and abundance of neutrophils could predict a poor clinical outcome in tongue squamous cell carcinoma (TSCC) patients [86]. In this study, CEACAM1 expression on tumor cells and increased neutrophils infiltration were associated. The proposed mechanism of CEACAM1 overexpression attracting more neutrophils to tumor sites is through IL-8 and CXCL-6 upregulation. This finding is consistent with results from other studies that link cytokine-induced CEACAM1 expression on keratinocytes to a prolonged lifespan of neutrophils [87].

However, the role of CEACAM1 appears to be dependent of its distribution in oral squamous cell carcinoma. Zhou et al. showed membranous CEACAM1 expression inhibits angiogenesis and lymphangiogenesis and is associated with well-differentiated SCC [88]. Conversely, cytoplasmic CEACAM1 expression, which is associated with poorly and moderately differentiated SCCs, promotes angiogenesis and lymphangiogenesis by mediating the transformation of vascular endothelial cells into lymphatic endothelial cells.

5. Conclusions

The emerging picture of CEACAM1 in the context of cancer and the immune system is very complex. Even though decades of studies have tried to characterize its role in carcinogenesis, there is no general agreement upon this adhesion molecule's behaviour in human tumors. CEACAM1 works together with specific signaling factors, proteins and receptors, dependent on the various contexts in which it occurs; therefore, its role should be interpreted separately in each of these different cell types, tissues, and pathological conditions. CEACAM1 appears to be a valuable prognostic factor in various tumors through its different expression patterns on cancer cells. In addition, its roles in tumor progression, immune evasion, angiogenesis, and invasion make it a promising molecular target that can be exploited alongside other existing immunotherapeutics as specific cancer therapies.

Conflicts of Interest

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

Authors' Contributions

Andreea Calinescu, Gabriela Turcu, Roxana I. Nedelcu, Alice Brinzea, Anastasia Hodorogea, Mihaela Antohe, Carmen Diaconu, Coralia Bleotu, Daniel Pirici, Lucia B. Jilaveanu, Daniela A. Ion, and Ioana A. Badarau have equal contribution to this paper.

Acknowledgments

This work was partially supported by a grant of Ministry of Research and Innovation, Consiliul National al Cercetarii Stiintifice-UEFISCDI (project number PN-III-P4-ID-PCE-2016-0641) within PNCDI III, and a grant of the Romanian Ministry of Research and Innovation, CCCDI-UEFISCDI (project number 61PCCDI/2018 PN-III-P1-1.2-PCCDI-2017-0341) within PNCDI-III.

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 4735752, 10 pages
https://doi.org/10.1155/2018/4735752

Research Article

Activation of PAR4 Upregulates p16 through Inhibition of DNMT1 and HDAC2 Expression via MAPK Signals in Esophageal Squamous Cell Carcinoma Cells

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Received 4 July 2018; Revised 30 July 2018; Accepted 14 August 2018; Published 30 September 2018

Academic Editor: Monica Neagu

A previous study showed that a downexpression of protease-activated receptor 4 (PAR4) is associated with the development of esophageal squamous cell carcinoma (ESCC). In this study, we explored the relationship between PAR4 activation and the expression of p16, and elucidated the underlying mechanisms in PAR4 inducing the tumor suppressor role in ESCC. ESCC cell lines (EC109 and TE-1) were treated with PAR4-activating peptide (PAR4-AP). Immunohistochemistry for DNA methyltransferase 1 (DNMT1) and histone deacetylase 2 (HDAC2) was performed in 26 cases of ESCC tissues. We found that DNMT1 and HDAC2 immunoreactivities in ESCC were significantly higher than those in adjacent noncancerous tissues. PAR4 activation could suppress DNMT1 and HDAC2, as well as increase p16 expressions, whereas silencing PAR4 dramatically increased HDAC2 and DNMT1, as well as reduced p16 expressions. Importantly, the chromatin immunoprecipitation-PCR (ChIP-PCR) data indicated that treatment of ESCC cells with PAR4-AP remarkably suppressed DNMT1 and HDAC2 enrichments on the p16 promoter. Furthermore, we demonstrated that activation of PAR4 resulted in an increase of p38/ERK phosphorylation and activators for p38/ERK enhanced the effect of PAR4 activation on HDAC2, DNMT1, and p16 expressions, whereas p38/ERK inhibitors reversed these effects. Moreover, we found that activation of PAR4 in ESCC cells significantly inhibited cell proliferation and induced apoptosis. These findings suggest that PAR4 plays a potential tumor suppressor role in ESCC cells and represents a potential therapeutic target of this disease.

1. Introduction

Protease-activated receptors (PARs), a superfamily of G-protein-coupled receptors that are activated by thrombin, have been perceived in multiple cells affiliated with inflammatory reactions, such as macrophages, neutrophils, and mast cells [1]. The recent detection of PARs on various cancer cells suggests that PARs might be involved not only in inflammation, but also in the development of cancers [2]. Several studies suggest that PARs play roles in cancer progression including tumor growth, invasion, migration, survival, and metastasis [3, 4]. Studies investigating the role of PAR4 in cancer have had conflicting results, as they were found to be overexpressed in several malignant tumors and implicated in tumor growth and cancer metastasis [4–6]. However, other studies showed a downexpression of PAR4 in esophageal, lung, and gastric cancers [7–9]. Recently, studies demonstrated that mice with knockdown PAR4 gene could accelerate tumor growth [10] and reduce cardiomyocyte apoptosis [11]. PAR4 is highly expressed in human esophageal squamous epithelial cells [9] and frequently downregulated in esophageal squamous cell carcinoma (ESCC) tissue, which is partly the result of the hypermethylation of the PAR4 promoter [8]. However, the role of PAR4 in the progress of ESCC has not been defined.

ESCC is one of the world's most aggressive types of malignancy with a poor prognosis [12]. Tobacco smoking is one of major risk factors for ESCC [13]. Exposure to

carcinogens of tobacco smoke may result in the methylation of PAR4 gene, which is considered to be involved in carcinogenesis [14, 15]. p16, the tumor suppressor gene, is involved in the pathogenesis of esophageal cancer by influencing the cyclin kinase inhibitor cascade and DNA mismatch repair processes [16]. The promoter methylation inactivation of p16 gene can increase the risk of ESCC [17]. Previous studies have demonstrated that DNA methyltransferase 1 (DNMT1) is required for the maintenance of DNA methylation and the deactivation of p16 by DNMT1-mediated methylation that may lead to the development of ESCC [18]. At promoters, DNA methylation generally precludes transcription directly by blocking the binding of transcriptional activators or indirectly through the recruitment of methyl-binding proteins and corepressor complexes containing histone deacetylases (HDACs), which cooperatively facilitate the formation of heterochromatin [19].

In the present study, the association between the activation of PAR4 and expression of p16 protein and gene, as well as the enrichments of DNMT1 and HDAC2 on the p16 promoter, was examined by Western blotting, quantitative real-time PCR (qRT-PCR), and chromatin immunoprecipitation-PCR (ChIP-PCR) methods to identify the potentially diagnostic or therapeutic value of PAR4 in ESCC.

2. Materials and Methods

2.1. ESCC Cell Lines and Reagents. Human ESCC cell lines (EC109 and TE-1) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai) or National Infrastructure of Cell Line Resource (Beijing). The following reagents were used in this study: the selective PAR4-activating peptide (PAR4-AP) from Bachem; PAR4 control peptide from Tocric Bio-technology; PD98059 (an extracellular regulated protein kinase 1/2, ERK1/2, inhibitor), SB203580 (a p38 mitogen-activated protein kinase (MAPK), p38, inhibitor), and *t*-butylhydroquinone (tBHQ, an ERK1/2 activator) from Santa Cruz Biotechnology; and U-46619 (an ERK1/2 and p38 activator) from Millipore. This study protocol was approved by the Ethics Committee of the Taishan Medical University.

2.2. Drug Administration. Cultured ESCC cell lines were stimulated with PAR4-AP at a concentration of 100 μM for 1, 2, 6, 12, and 24 h. Cells with PAR4 control peptide (100 μM) treatment were used as control. To assess the possible effects of ERK1/2 and p38 on the regulation of p16, DNMT1, and HDAC2 expression following PAR4-AP stimulation, PD98059 (10 μM), SB203580 (10 μM), tBHQ (50 nM), or U-46619 (10 nM) was added to six-well plates 60 min prior to the addition of PAR4-AP. Cells stimulated with the PAR4 control peptide without ERK1/2 or p38 inhibitors or activators were used as controls.

2.3. Immunohistochemistry Analysis. Twenty-six ESCC tissues and their corresponding nearby nontumorous tissues were obtained from the Affiliated Hospital of Taishan Medical University, with the approval of the Local Research Ethics Committee. ESCC specimens were fixed in 10% buffered formalin. Paraffin sections were stained with anti-DNMT1 and HDAC2 (Abcam), then counterstained with hematoxylin. The immunoreaction score was then calculated by multiplying the percentage and intensity scores.

2.4. siRNA Transfection. siRNA targeting human PAR4 was synthesized by Sigma-Aldrich. A scrambled duplex siRNA was used as the negative control. ESCC cells were plated at 2×10^5/well in 6-well plates and incubated until they reached 50% confluency. Cells were transfected with PAR4-siRNA or the negative control siRNA at a final concentration of 50 nM with Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's recommendations. After 6 h of transfection, the medium was replaced with RPMI-1640 medium containing 10% fetal bovine serum. Cells were then incubated for 72 h for RNA isolation and protein extraction.

2.5. Western Blot Analysis. ESCC cells were continuously stimulated with PAR4-AP at a concentration of 100 μM for 1, 2, 6, 12, and 24 h. Then, cells were lysed, and protein was extracted. Protein lysate from each sample was separated electrophoretically in sodium dodecyl sulfate polyacrylamide gel and then transferred to polyvinylidene fluoride (PVDF) membranes. Western blot analyses were performed with anti-DNMT1, HDAC2, p16 (Abcam), PAR4 (Alomone Labs), phosphorylated-ERK1/2 (p-ERK1/2), phosphorylated-p38 (p-p38), and GAPDH (Cell Signaling Technology).

2.6. qRT-PCR Assay. Total RNA was isolated from ESCC cell lines using TRIzol reagent (Invitrogen). qRT-PCR was performed to measure the expression of p16 on the 7300 Real-Time PCR System (Applied Biosystems, CA, USA). The synthetic oligonucleotide primer sequences were as follows: DNMT1, 5′-CCT AGC CCC AGG ATT ACA AGG-3′(sense) and 5′-ACT CAT CCG ATT TGG CTC TTT C-3′ (antisense); HDAC2, 5′-TCC GCA TGA CCC ATA ACT TGC-3′ (sense) and 5′-CCG CCA GTT GAG AGC TGA C-3′ (antisense); p16, 5′-GTG TAT AGG GTC GGC CAT CAA-3′ (sense) and 5′-AGC AAA ACC AAC CTA TAC CG-3′ (antisense); β-actin, 5′-GTG TAT AGG GTC GGC CAT CAA-3′ (sense) and 5′-TTT GTT TGT GGT CTT GTC CAGT-3′ (antisense). A comparative cycle threshold fluorescence (ΔCt) method was used, and the relative transcript amount of the target gene was normalized to that of β-actin using the $2^{-\Delta\Delta CT}$ method. The final results of the real-time PCR are expressed as the ratio of the test mRNA to the control. All PCR product sizes were confirmed by electrophoresis on a 1.5% agarose gel and visualization using ethidium bromide.

2.7. ChIP-PCR. ChIPs were performed for human ESCC cell lines (EC109 and TE-1) and were analyzed essentially according to the instructions of One-Day Chromatin Immunoprecipitation Kit (Millipore). The DNA precipitated by the target antibodies (DNMT1, Abcam, ab13537; HDAC2, Abcam, ab12169) was detected with qRT-PCR. The primer sequences of the ChIP-qPCR reaction were as follows: p16-

1, 5′-CTG CTC TTA TAC CAG GCA ATG TA-3′ (sense) and 5′-CCT GTA CGA CTA GAA AGT GTC CC-3′ (antisense); p16-2, 5′-TTT CCC TAT GAC ACC AAA CAC C-3′ (sense) and 5′-CCG CGA TAC AAC CTT CCT AAC-3′ (antisense); p16-3, 5′-CCT CCT TGC GCT TGT TAT ACT CT-3′ (sense) and 5′-CCC TCC ACC ACC CTC ACT TA-3′ (antisense). Control PCRs for each antibody immunoprecipitation were performed using primers for GAPDH and IGFBP3 (DNMT1) or CD4 and von Willebrand factor (HDAC2) as negative and positive controls dependent on the antibody used. All PCR product sizes were confirmed by electrophoresis. Each ChIP experiment was done in triplicate and repeated at least three times.

2.8. Cell Viability and Apoptosis Assay. Cell growth was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT solution (0.5 mg/mL) was added to the cells and then the cells were cultured continuously for 4 h. Each sample was mixed and the optical density was measure at 570 nm. Apoptosis was assessed by an Andy Fluor 488 Annexin V and PI Apoptosis Kit (GeneCopoeia) and flow cytometry was performed using FACSCalibur (BD Biosciences) according to the manufacturers' instructions. All experiments were performed in triplicate.

2.9. Real-Time Cell Analysis (RTCA). Cells were seeded in separate electronic 16-well plates with an integrated microelectronic sensor array in 100 μl of suitable culture medium (RTCA DP, ACEA Biosciences). After 24 h, PAR4-AP was added to a total volume of 100 μl at a concentration of 100 μM. Cell proliferation and survival were monitored in real-time by measuring the cell-to-electrode responses of the seeded cells. The cell index (CI) was calculated for each E-plate well by RTCA Software. The graphs are generated in real time by the xCELLigence system.

2.10. Statistical Analysis. All experiments were repeated independently, at least three times. Values are expressed as mean ± SEM, and results were analyzed using an ANOVA followed by a Bonferroni test for comparison among groups. Significance was defined as P values < 0.05.

3. Results

3.1. Expression of DNMT1 and HADC2 in Human ESCC Tissues. Immunoreactivity for DNMT1 and HDAC2 was analyzed in 26 paired human ESCC and adjacent nontumorous tissues. DNMT1 and HDAC2 were mainly in nuclear staining in ESCC tissues and adjacent nontumorous tissues. Levels of both protein immunoreactivities in ESCC tissues were significantly higher than those in adjacent nontumorous tissues (Figure 1(a)). These suggest that interaction between DNMT1 and HDAC2 might be involved in ESCC carcinogenesis [20].

3.2. The Effect of PAR4 Activation on DNMT1, HDAC2, and p16 in ESCC Cells. The expression of DNMT1 and HDAC2 in ESCC cell lines (EC109 and TE-1) was assessed by Western blot after treatment with PAR4-AP for 1, 2, 6, 12, and 24 h, respectively. As shown in Figure 1(b), DNMT1 and HDAC2 levels were downregulated by PAR4-AP treatment. Meanwhile, PAR4-AP treatment of ESCC cells significantly increased p16 protein and mRNA levels compared with the control groups (Figures 2(a) and 2(b)). These results suggested that the upregulation of p16 protein and gene expression by PAR4 might be associated with suppression of DNMT1 and HDAC2 [21].

3.3. Effect of MAPK on DNMT1, HDAC2, and p16 Expression by PAR4-AP in ESCC Cells. To assess the possible effects of MAPK on the regulation of DNMT1, HDAC2, and p16 expression by PAR4, ESCC cells were treated with PAR4-AP for 2 h and pretreated with U-46619 (ERK1/2 and p38 activator), tBHQ (ERK1/2 activator), PD98059 (ERK1/2 inhibitor), or SB203580 (p38 inhibitor) for 60 min. Activation of PAR4 increased p-ERK1/2 and p-p38 expression in ESCC cells after PAR4-AP treatment (Figure 2(c)). Compared with PAR4-AP alone, PAR4-AP with U-46619 or tBHQ, activators for ERK1/2 and p38, could induce a decrease of DNMT1 and HDAC2 protein levels to much lower levels, which in turn markedly increased the expression of p16 protein (Figure 2(d)). Meanwhile, PD98059 or SB203580, inhibitors for ERK1/2 and p38, partially or completely blocked the increase of p16 protein expression, which in turn markedly reversed the downexpression of DNMT1 and HDAC2 proteins, compared with PAR4-AP-only groups (Figure 2(d)). These results indicated that the effect of PAR4-AP on DNMT1, HDAC2, and p16 expression is associated with MAPK signal pathways [22].

3.4. PAR4 Increased p16 Gene Expression by Attenuating DNMT1 and HDAC2 Enrichments on the p16 Promoter. To evaluate the effect of PAR4 on the physical interaction between DNMT1 and HDAC2 on promoter regions of p16, ChIP-PCR experiments for human ESCC cells were performed after treatment with PAR4-AP for 2, 6, and 12 h. We designed a series of primer coordinates to the three regions in the p16 promoter for ChIP-PCR assays (Figure 3(a)). p16-1, p16-2, and p16-3 are located upstream of the p16 promoter (−1755 bp, −551 bp and− 263 bp), representing the important regulatory regions of p16 gene. ChIP-PCRs in ESCC cell lines demonstrated that the p16 promoter regions (p16-1, p16-2, and p16-3) have less enrichments of DNMT1 and HDAC2 in ESCC cells after treatment with PAR4-AP for 2, 6, and 12 h, compared with controls ($P < 0.05$) (Figures 3(b)–3(e)). This result implicated that the promoting p16 transcription by activation of PAR4 might be associated with attenuating DNMT1 and HDAC2 enrichments on the p16 promoter.

3.5. DNMT1, HDAC2, and p16 Expression of ESCC Cell Lines following PAR4 Gene Knockdown. Western blotting and qRT-PCR assay were used to determine the effects of PAR4-siRNA-mediated PAR4 silencing on DNMT1, HDAC2, and p16 expression of ESCC cell lines. The cells were cultured for 72 h subsequent to transfection of PAR4-siRNA. The results confirmed that the expression of PAR4 protein was inhibited by transfection of PAR4-siRNA

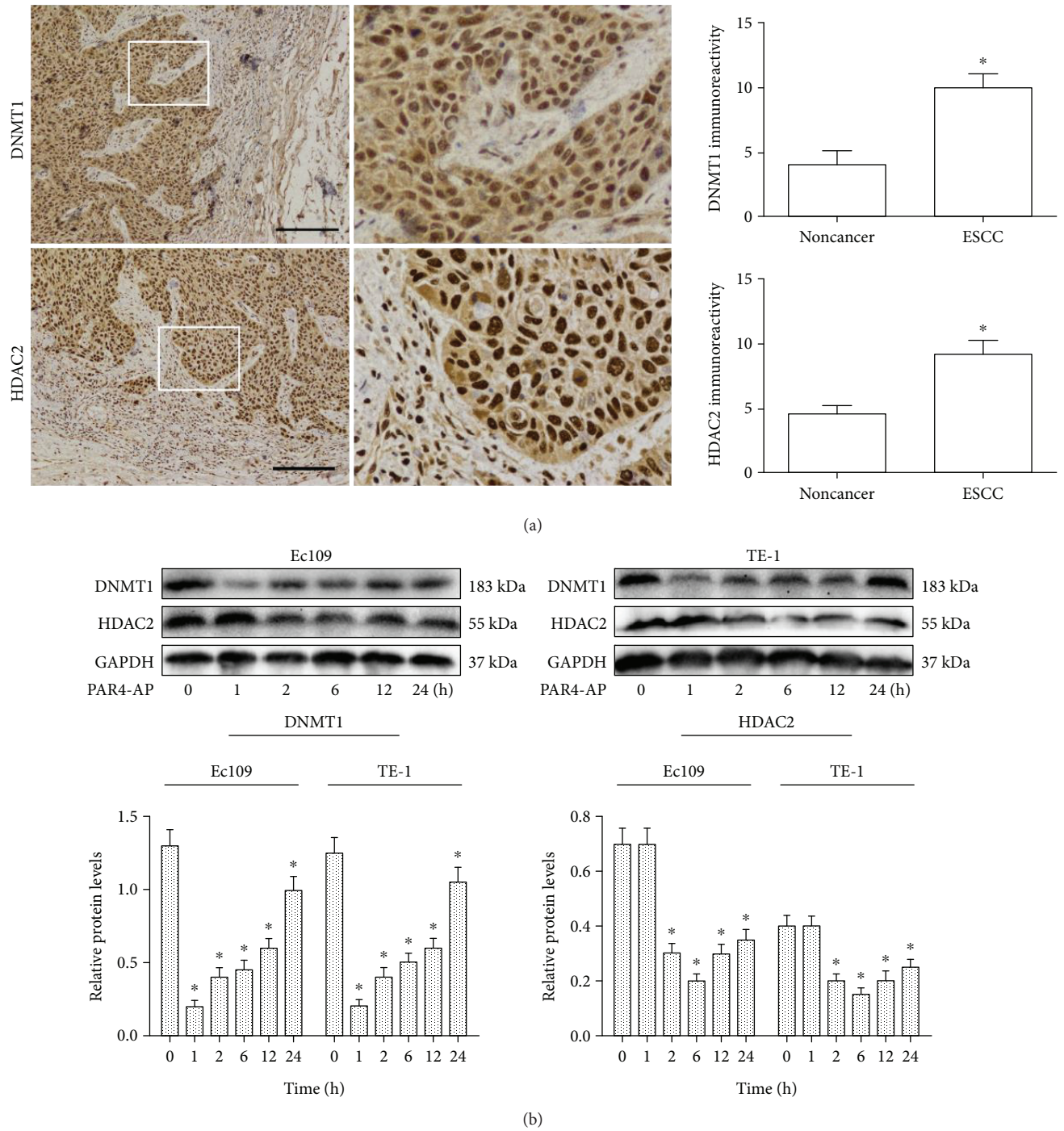

Figure 1: Immunohistochemical staining for DNMT1 and HDAC2 in ESCC tissues and effects of PAR4-AP on their expressions in ESCC cell lines after PAR4-AP treatment. (a) Representative immunostaining for DNMT1 and HDAC2 in ESCC tissues and adjacent noncancerous tissues. Sections were counterstained with hematoxylin. Scale bar: 100 μm. Graphs showing DNMT1 and HDAC2 immunostaining in ESCC tissues and adjacent noncancerous tissues. The data are expressed as the mean ± SEM. $^*P < 0.01$ (Student's t-test). Immunoreactivity was calculated by multiplying the percentage and intensity scores. (b) Examples of Western blotting showing DNMT1 and HDAC2 expressions in ESCC cell lines (EC109 and TE-1) with PAR4-AP treatment at 0, 1, 2, 6, 12, and 24 h, respectively. The mean optic densities of the proteins were calculated by normalizing to GAPDH. The data are presented as the mean ± SEM ($n = 3$), $^*P < 0.05$ versus controls.

(Figure 4(a)). The PAR4 gene knockdown was able to upregulate the levels of DNMT1 and HDAC2 proteins and genes and suppress p16 protein and gene expression following transfection of PAR4-siRNA with ESCC cells ($P > 0.05$) (Figures 4(a) and 4(b)).

3.6. Effects of PAR4 Activation on Cell Proliferation and Apoptosis in ESCC Cells. In order to determine the effect of PAR4-AP on the growth of ESCC cell lines, ESCC cells were treated with PAR4-AP and cell proliferation was assessed using RTCA and MTT assay. RTCA proliferation assay

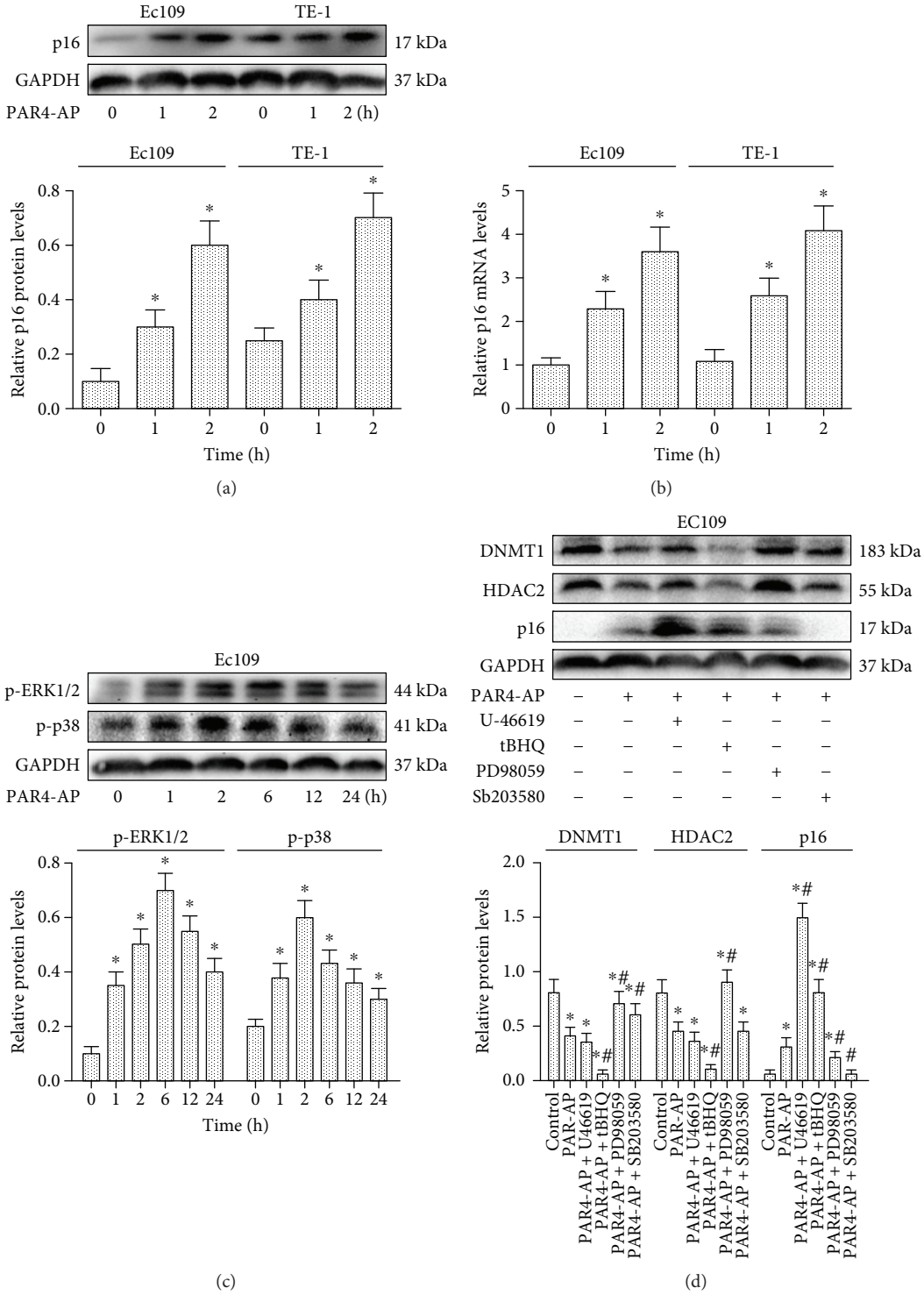

Figure 2: Effect of MAPK on DNMT1, HDAC2, and p16 expression by PAR4-AP in human ESCC cells. (a) Western blotting showing p16 protein levels in ESCC cell lines (EC109 and TE-1) with PAR4-AP treatment at 0, 1, and 2 h, respectively. (b) RT-PCR analysis showing p16 gene levels in ESCC cell lines (EC109 and TE-1) with PAR4-AP treatment at 0, 1, and 2 h, respectively. The results were calculated by normalizing to β-actin in the same sample with the ΔCt method. The changes in the relative mRNA levels are expressed as fold changes compared with the controls. (c) Western blotting showing p-ERK1/2 and p-p38 expression in ESCC cells with PAR4-AP treatment at 0, 1, 2, 6, 12, and 24 h, respectively. (d) Western blotting analyses for DNMT1, HDAC2, and p16 protein expressions in ESCC cells following treatment with PAR4-AP for 2 h and pretreatment with U-46619 (an activator of ERK1/2 and p38), tBHQ (an activator of ERK1/2), PD98059 (an inhibitor of ERK1/2), or SB203580 (an inhibitor of p38) for 60 min. The mean optic densities of the proteins were calculated by normalizing to GAPDH. All values are expressed as the means ± SEMs ($n = 3$). $^{*}P < 0.05$ versus controls; $^{\#}P < 0.05$ versus PAR4-AP-only groups.

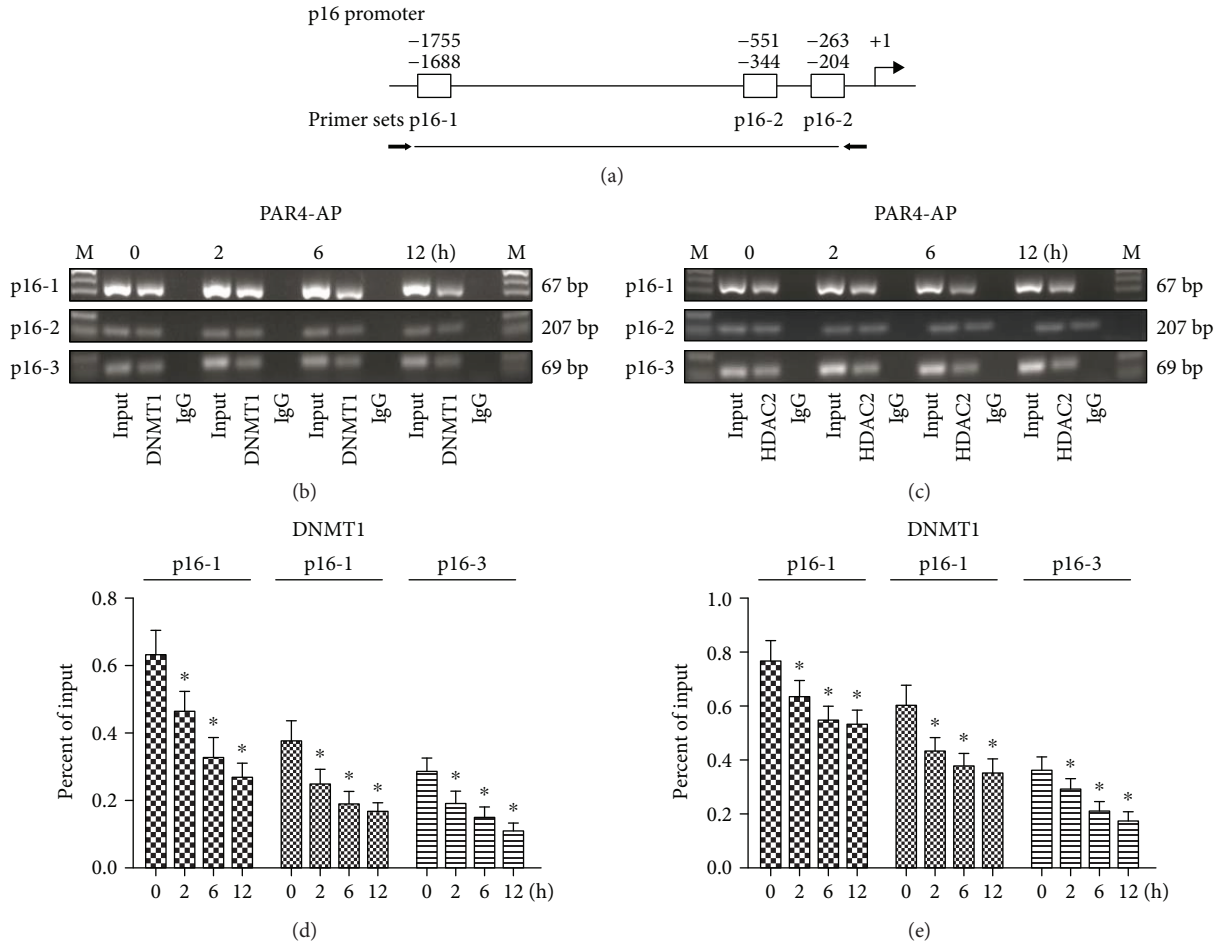

Figure 3: PAR4-AP suppressed DNMT1 and HDAC2 enrichments on the p16 promoter in ESCC cells. (a) Schematic representation of the p16 promoter region. P16-1, p16-2, and p16-3 indicate the locations of primers on the p16 promoter. (b, c) ChIP assays were performed for the p16 promoter in ESCC cells using antibodies to DNMT1 and HDAC2 after treatment of PAR4-AP at 2, 6, and 12 h. Normal rabbit IgG served as a negative control, and input chromatin (samples without immunoprecipitation (IP)) served as a positive control. (d, e) ChIP-qPCR assays to evaluate DNMT1 and HDAC2 enrichments in the p16 promoter region of ESCC cells with treatment of PAR4-AP at 2, 6, and 12 h. The data are expressed as the mean ± SEM ($n = 3$). $^*P < 0.05$ versus controls.

demonstrated that the cell index decreased in a time-dependent manner following PAR4-AP treatment and was significantly lower in PAR4-AP groups when compared with the control group following treatment for 24 h (Figure 5(a)). Similarly, MTT assay analysis demonstrated that the viability of ESCC cells decreased in a time-dependent manner following PAR4-AP treatment (Figure 5(b)). The effect of PAR4 activation on the apoptosis of ESCC cells treated with PAR4-AP was detected by flow cytometry. Graphical representation of the apoptosis assay showed that treatment with PAR4-AP led to an increase of apoptosis (Figure 5(c)). The results indicated that the apoptosis rate of ESCC cells was significantly increased in a time-dependent manner following PAR4-AP treatment.

4. Discussion

Our study demonstrated that the treatment with PAR4-AP inhibited the proliferation of ESCC cells, upregulated p16, and reduced DNMT1 and HDAC2 expression in ESCC cells. The ChIP study revealed that activation of PAR4 suppressed the enrichments of DNMT1 and HDAC2 on the p16 promoter region. These effects were associated with MAPK signals that were induced by PAR4 activation. These findings provide evidence that the inhibited proliferation of ESCC cells associated with PAR4-AP may be involved in the promotion of p16 transcription through suppressing DNMT1 and HDAC2 expression via MAPK signals in ESCC cells.

p16 is an important tumor suppressor protein that plays essential roles during cell proliferation through regulating the expression of several genes [23]. The p16 gene encodes a p16 protein that binds competitively to CDK4 and, during G1 phase, inhibits the interaction of CDK4 and cyclin D1 to stimulate passage through the cell cycle [24–26]. The p16 protein is often highly expressed in senescent cells in culture and is inactivated in a variety of human cancers, and the p16 could enhance the apoptotic functions of p53 through DNA-dependent interaction [27]. Our results showed that activation of PAR4 could inhibit proliferation and induce apoptosis, as well as upregulate p16 expression following PAR4-AP treatment of ESCC cells. Therefore, it is possible that the inhibition of proliferation and increase of apoptosis evoked

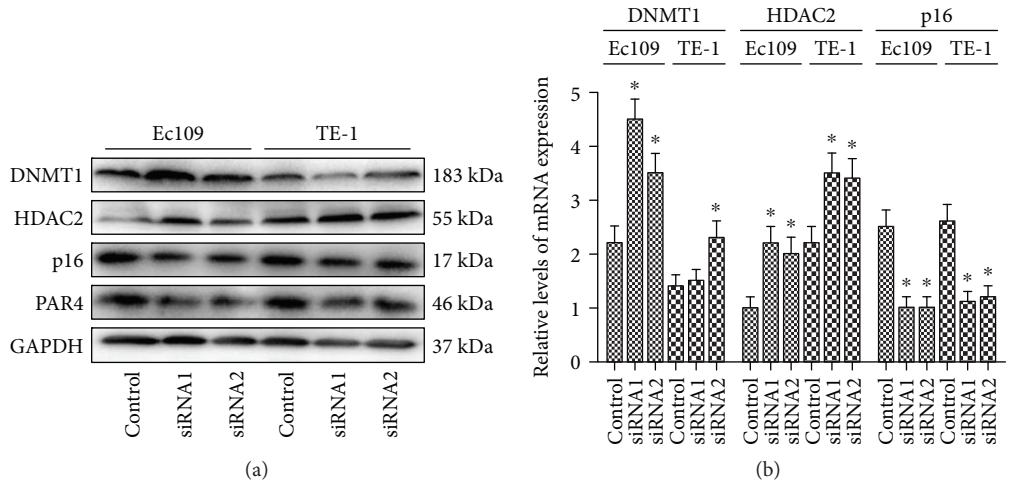

Figure 4: Silencing PAR4 induces DNMT1, HDAC2, and p16 expression in ESCC cells. (a) ESCC cells were transiently transfected with PAR4-siRNA1 or PAR4-siRNA2. Western blotting was performed to examine the expression of DNMT1, HDAC2, p16, and PAR4 at protein levels after transfection with PAR4-siRNA for 72 h. (b) qRT-PCR was performed to examine the expression of DNMT1, HDAC2, and p16 expression at mRNA levels after transfection with PAR4-siRNA for 72 h. The results were calculated by normalizing to β-actin in the same sample with the ΔCt method. The data are expressed as the mean ± SEM ($n = 3$). $^*P < 0.05$ versus controls.

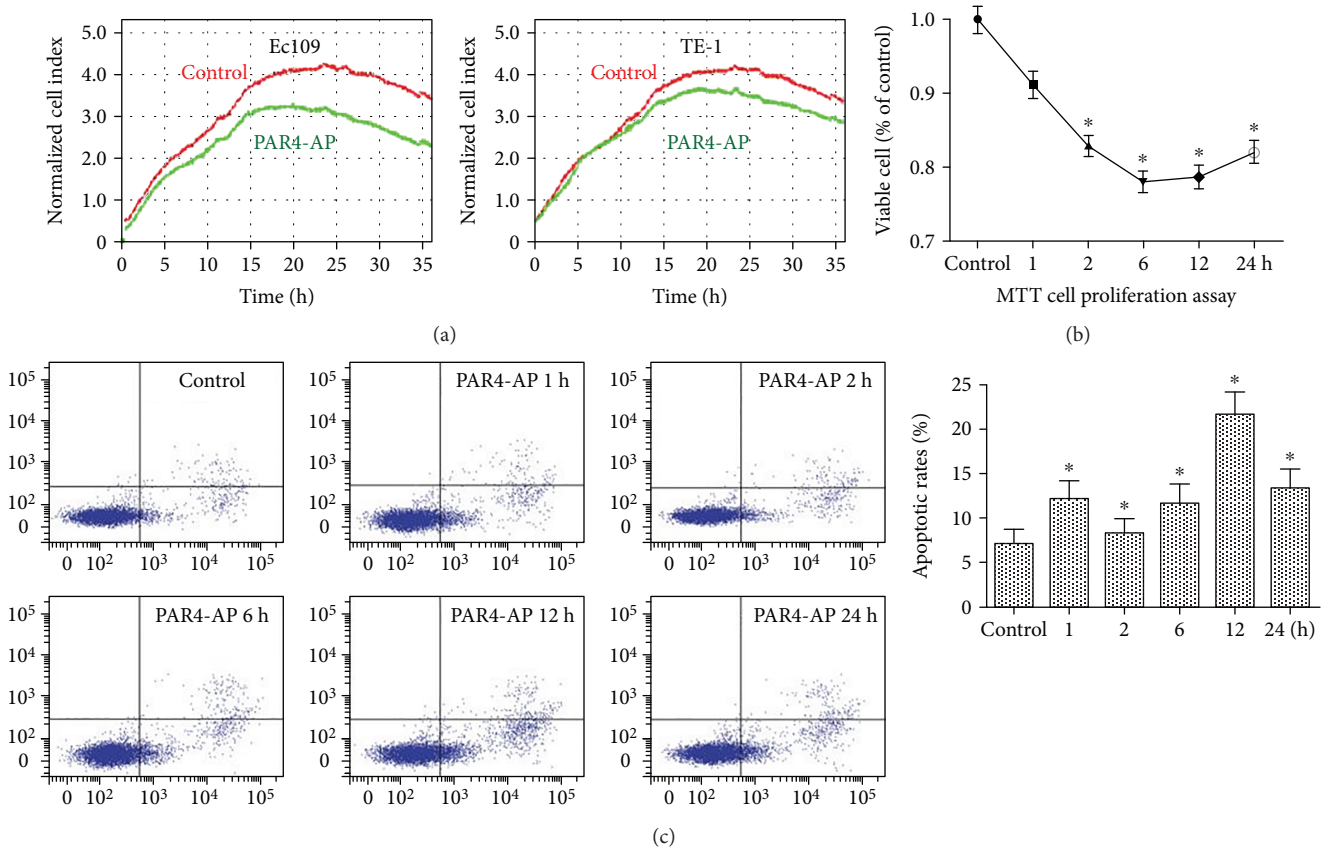

Figure 5: Effects of PAR4-AP on cell proliferation and apoptosis in ESCC cells. (a) RTCA was performed to evaluate the proliferation and viability of ESCC cells following continuous treatment with PAR4-AP. Green lines represent cell index (CI) treated with PAR4-AP, and red lines are CI treated with the PAR4 control peptide. (b) The cell viability of ESCC cells was measured by MTT assay following 1, 2, 6, 12, and 24 h of treatment with PAR4-AP, respectively. (c) Cells were stained with Andy Fluor 488 Annexin V and PI and analyzed by flow cytometry following treatment with PAR4-AP. The ratio of apoptosis in ESCC cells was detected by flow cytometry following 1, 2, 6, 12, and 24 h of treatment with PAR4-AP, respectively. The data are expressed as the mean ± SEM ($n = 3$). $^*P < 0.05$ versus controls.

by PAR4 activation is closely related to p16 gene transcription in ESCC cells.

It is believed that DNMT1 is required for the deactivation of p16 by DNMT1-mediated methylation that may lead to the development of ESCC [18]. Growing evidence shows that overexpressed HDACs are associated with tumorigenesis in ESCC [28]. Previous studies showed that HDACs form a complex with DNMT1 and that the protein stability of DNMT1 is regulated by posttranslational modifications of acetylation and ubiquitination [29]. The inhibition of DNMT1 might reduce DNA methylation in the p16 promoter and increase p16 expression [30]. HDAC2 inactivation significantly reduced G1-S cell cycle arrest, restored activity of p16, and promoted apoptosis [29]. In the present study, the activation of PAR4 was able to increase p16 expression and decrease DNMT1 and HDAC2 expression in ESCC cells. Therefore, it seems more likely that the effect of PAR4 on DNMT1 and HDAC2 expression results in promoting p16 gene transcription [18, 31]. These results suggested that PAR4 was able to upregulate p16 levels through inhibition of DNMT1 and HDAC2 expression in ESCC cells.

To understand the relationship between the activation of PAR4 and the physical interplay of DNMT1 with HDAC2 on the p16 promoter, we performed ChIP-PCR to investigate the enrichments of DNMT1 and HDAC2 on the p16 promoter in ESCC cells. The results demonstrated that activation of PAR4 could decrease the enrichments of DNMT1 and HDAC2 on the p16 promoter in ESCC cells. Suppressing DNMT1 could lead to the activation of the esophageal suppressor gene p16 [18]. DNMT1 usually forms a corepressor complex with HDACs and represses transcription [19, 31]. Therefore, it is likely that the PAR4 that led to decreased DNMT1 and HDAC2 binding to the p16 promoter of ESCC cells was likely involved in the activating transcription of this gene. The decreased DNMT1 and HDAC2 enrichments in ESCC cells after PAR4-AP treatment provided a novel molecular mechanism for promoting p16 gene transcription in ESCC cells.

In the present study, PAR4 activation increased p-ERK1/2 and p-p38 expression in ESCC cells after PAR4-AP treatment. ERK1/2 and p38 activators enhanced the effects of PAR4 activation on DNMT1, HDAC2, and p16 expression, whereas ERK1/2 and p38 inhibitors reversed these effects. Several studies have supported the idea that PAR4 activation is involved in the initiation of the MAPK signal pathways [32–34]. Previous reports implicated the involvement of the MEK/ERK pathway in the reduction of DNMT1 expression and function [35, 36]. Activation of the ERK1/2 signaling pathway evoked by PARs induces the activation of the transcriptional factor CREB, which in turn leads to gene expression [37]. CREB-binding protein (CBP), a histone acetyltransferase, could bind to CREB and enhance p16 expression [38]. Activation of the p38 pathway induces hepatocellular carcinoma cell apoptosis through suppression of DNMT1 expression [39]. It also suppresses the growth of human leukemia cells by downregulating HDAC2 expression and activity [40]. Because of the effect of MAPK on the expressions of DNMT1, HDAC2, and p16 induced by PAR4-AP, it is likely that the inhibitions of DNMT1 and HDAC2 induced by PAR4 activation are involved in the expression of p16 via MAPK signals in ESCC cells. The increase in p16 mRNA and protein levels in ESCC cells after PAR4-AP treatment provides a molecular mechanism for PAR4 in the regulation of ESCC carcinogenesis.

5. Conclusion

To our knowledge, we are the first to report that PAR4 activation could inhibit the viability and induce the apoptosis of ESCC cells through suppressing enrichments of DNMT1 and HDAC2 at the p16 promoter via MAPK signals. The results of this study provide new insight into the mechanisms of the promoting p16 gene transcription by PAR4 activation in ESCC cells. Therapeutically, PAR4 may be a potent target for the treatment of ESCC.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Ming Wang and Shuhong An contributed equally to this work.

Acknowledgments

The study was supported by the National Natural Science Foundation of China (81371234) and the Natural Science Foundation of Shandong Province of China (ZR2016HP26).

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 9349207, 8 pages
https://doi.org/10.1155/2018/9349207

Research Article

Inflammatory-Driven Angiogenesis in Bone Augmentation with Bovine Hydroxyapatite, B-Tricalcium Phosphate, and Bioglasses: A Comparative Study

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Received 27 April 2018; Revised 12 July 2018; Accepted 25 July 2018; Published 12 September 2018

Academic Editor: Donato Zipeto

Introduction. The clinical use of bioactive materials for bone augmentation has remained a challenge because of predictability and effectiveness concerns, as well as increased costs. The purpose of this study was to analyse the ability to integrate bone substitutes by evaluating the immunohistochemical expression of the platelet endothelial cell adhesion molecules, vascular endothelial growth factor, collagen IV, laminin, and osteonectin, in the vicinity of bone grafts, enabling tissue revascularization and appearance of bone lamellae. There is a lack of *in vivo* studies of inflammatory-driven angiogenesis in bone engineering using various grafts. *Methods*. The study was performed in animal experimental model on the standardized monocortical defects in the tibia of 20 New Zealand rabbits. The defects were augmented with three types of bone substituents. The used bone substituents were beta-tricalcium phosphate, bovine hydroxyapatite, and bioactive glasses. After a period of 6 months, bone fragments were harvested for histopathologic examination. Endothelial cell analysis was done by analysing vascularization with PECAM/CD31 and VEGF and fibrosis with collagen IV, laminin, and osteonectin stains. Statistical analysis was realized by descriptive analysis which was completed with the kurtosis and skewness as well as the Kruskal-Wallis and Mann-Whitney statistical tests. *Results*. The discoveries show that the amount of bone that is formed around beta-tricalcium phosphate and bovine hydroxyapatite is clearly superior to the bioactive glasses. Both the lumen diameter and the number of vessels were slightly increased in favor of beta-tricalcium phosphate. *Conclusion*. We can conclude that bone substitutes as bovine bone and beta-tricalcium phosphate have significant increased angiogenesis (and subsequent improved osteogenesis) compared to the bioactive glass. In our study, significant angiogenesis is linked with a greater tissue formation, indicating that in bone engineering with the allografts we used, inflammation has more benefic effects, the catabolic action being exceeded by the tissue formation.

1. Introduction

The bone augmentation, used to enhance the bone volume, needed to insert dental implants, can be done with both autologous bone and bone replacement materials. Regardless of the nature of the material, it is necessary to have angiogenesis in order to integrate it into the receptor bed and to maintain its volume. This ensures the presence and proliferation

of preosteoblasts [1]. When preosteoblasts differentiate into osteoblasts, they contribute to a much more intense lamellar bone formation around the bone substitute particles.

Inflammation and proinflammatory cytokines (IL-1, IL-4, TNF-α, IL-6, IL-5, and prostaglandins) [2] released in the area of bone augmentation are responsible for two opposite effects: bone catabolism and bone regeneration and augmentation [3]. One of the most important regenerating effects of inflammatory cytokines is promoting angiogenesis by stimulating the release of vascular endothelial growth factor (VEGF), newly formed vessels being responsible for revascularization and callus formation [4]. Understanding the inflammatory response helps rational therapeutic intervention in order to maximize benefic effects of inflammation in the healing of engineered tissue [2].

Revascularization is vital for the healing of bone defects, which in turn facilitates the development of osteoid deposition and matrix development in normal bone healing. The link between angiogenic and osteogenic cells is important because in practice, there is a greater need for vascularization where bone grafting materials are used as they are larger, more complex, and require increased control in tissue formation and development [5, 6].

There is still a lack of *in vivo* studies of inflammatory-driven angiogenesis in bone engineering using various grafts, although angiogenesis is a key factor of bone regeneration with crucial role in the success rate of bone augmentation [7].

The purpose of this study was to analyse in animal model the ability to integrate bone substitutes by evaluating the angiogenesis as revealed by immunohistochemical expression of the platelet endothelial cell adhesion molecules (PECAM-1/CD31) and vascular endothelial growth factor (VEGF) and the scaring and bone remodelling process as revealed by immunohistochemical expression of collagen IV, osteonectin, and laminin, for three materials generally used for bone augmentation: bovine hydroxyapatite, betatricalcic phosphate, and bioactive glasses. The evaluated proteins are markers of the endothelium of newly formed blood vessels in the vicinity of the bone grafts.

2. Materials and Methods

2.1. Animal Model. The study was conducted at the Cantacuzino National Research and Development Institute for Microbiology and Immunology Bucharest, on 20 mature rabbits (6–9 months old) from the New Zealand White breed (10 males and 10 females) with an average weight of about 2.5 kg. The study protocol and experimental scheme were approved by the Ethics Commission for working with laboratory animals of INCDMI Cantacuzino Bucharest in conformity with ISO 10993-2 having the number of protocol CE/37/23.02.2015. Experimental animals have been housed in special enclosures with a constant temperature between 18°C and 24°C and 55% humidity and fed a diet according to the standards. The study specimens were randomly divided according to the material with which the bone augmentation was performed in three groups (group A, group B, and group C). Surgical interventions were performed with general anaesthesia with a 3 ml solution containing a mixture of 2.3 ml of ketamine and 0.7 ml of xilazine. It was divided into 2 equal doses of 1.5 ml intramuscular injections, administered at 3-minute intervals. The control group consisted of two animals whose defects were augmented with autograft obtained by grinding the bone from the intracortical defect bone drilling.

The surgical procedure consisted in drilling of several cortical cavities in the proximal region of the tibia using rotary instruments under cooling. For the exposure of the tibial cortex, incisions of approximately 5/2 centimetres were performed and dissection of the muscular inserts from the bony plane was performed. The medial face of the tibia, in the proximal third, was exposed to a length of 6 cm. After identifying and sectioning the periosteum, it was partially cut off, respecting its integrity, for the subsequent coverage of the augmented bone defect. Using a tubular drill of 4 mm internal diameter and 2 mm outer diameter, an intracortical geode in the central area of the bone was performed to each tibia, obtaining a bone defect having the same diameter as the tubular drill (Figure 1). The bone augmentation materials used in the present study have been commercially obtained. The used bone substituents were beta-tricalcium phosphate (Cerasorb®, Curasan regenerative materials) which is a fully synthetic bioactive bone substitute, bovine hydroxyapatite (BioOSS, Geistlich Biomaterials) which consists of pure mineral content of bovine bone, and bioactive glasses (Perioglass, Novabone) having the composition 45% SiO_2 (silica dioxide), 24.5% CaO (calcium oxide), 24.5% NaO_2 (sodium oxide), and 6% P_2O_5 (phosphorus pentoxide). Six defects were augmented with bovine hydroxyapatite; another 6 defects were augmented with β-tricalcium phosphate (TCP) and another 6 with bioactive glasses. In the two remaining defects, the augmentation was done with the bone obtained by drilling the intracortical geodes, which was subjected to a grinding process. Against augmenting biomaterials, nonresorbable PTFE-based membranes were applied to obtain a barrier to connective tissue. After application of the membranes, a wound suture was made in three planes (periost, muscle tissue, and tegument). Ketoprofen 3 mg/kg was postoperatively administered to animals for three days.

Animals were euthanized 180 days after bone augmentation intervention, by intravenous administration of phenobarbital 200 mg/ml. The tissue fragments were sampled from the augmented areas, which were subsequently histopathologically examined.

Tricalcium phosphate is a bone substitute widely used due to its good biocompatibility, biological safety, virtually unlimited availability, and ease of sterilization [8]. Hydroxyapatite, although has a good biocompatibility, has a limited ability to degrade and absorb within the organism [9]. Bioactive glasses are an alternative to inert implant materials, bonding with host bone to create a stable implant [10].

2.2. Immunohistological Labeling. The specimens were decalcified in a HCL/EDTA solution for 72 hours after being immersed in 10% buffered formalin for 24 hours. Tissue fragments were selected so that the soft tissue, the graft, and its interface with the receptor bed could be studied. After

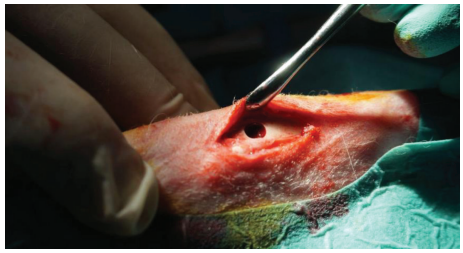

Figure 1: Bone circular defect in the proximal tibia after drilling.

decalcification, the fragments were routinely processed on a Leica ASP200S tissue processor for ethanol 70° for 90 min at 40°C, ethanol 80° for 105 min at 40°C, ethanol 96° for 105 min at 40°C, ethanol 100° for 60 min at 40°C, ethanol 100° for 90 min at 40°C, ethanol 100°C for 90 min at 40°C, xylene for 2 hours at 52°C, paraffin 58°C 1 hour, paraffin 58°C 2 hours, and paraffin 58°C 3 hours. The tissue fragments were embedded in paraffin blocks using a Thermo Fisher Microm EC 1150 H embedding station. Sections of 3-micron thickness were cut using a Leica RM 2265 rotary microtome. Slides were routinely stained with hematoxylin and eosin (HE). Immunohistochemical (IHC) tests were performed for PECAM-1/CD31 (Thermo Fisher Scientific monoclonal antibody, JC/70A clone, high-temperature antigen recovery using 0.01 M citrate recovery solution pH 6 for 15 minutes, 1 : 50 dilution for 60 minutes, and the mouse-specific HRC/DAB (ABC) Detection IHC Kit (Abcam, AB64259)), VEGF (Thermo Fisher Scientific, monoclonal antibody, MA5-12184 clone, high-temperature antigen recovery using 0.01 M citrate recovery solution pH 6 for 15 minutes, 1 : 40 dilution for 60 minutes, and the mouse-specific HRC/DAB (ABC) Detection IHC Kit (Abcam, AB64259)), laminin (Thermo Fisher Scientific, polyclonal antibody, PA1-16730 clone, high-temperature antigen recovery using 0.01 M citrate recovery solution pH 6 for 15 minutes, 1 : 50 dilution for 60 minutes, and the mouse-specific HRC/DAB (ABC) Detection IHC Kit (Abcam, AB64259)), collagen IV (Thermo Fisher Scientific, polyclonal antibody, PA1-28534 clone, high-temperature antigen recovery using 0.01 M citrate recovery solution pH 6 for 15 minutes, 1 : 400 dilution for 60 minutes, and the mouse-specific HRC/DAB (ABC) Detection IHC Kit (Abcam, AB64259)), osteonectin (Thermo Fisher Scientific, polyclonal antibody, PA1-16730 clone, high-temperature antigen recovery using 0.01 M citrate recovery solution pH 6 for 15 minutes, 1 : 50 dilution for 60 minutes, and the mouse-specific HRC/DAB (ABC) Detection IHC Kit (Abcam, AB64259)), and collagen IV (Thermo Fisher Scientific, monoclonal antibody, MA5-17180 clone, high-temperature antigen recovery using 0.01 M citrate recovery solution pH 6 for 15 minutes, 1 : 500 dilution for 60 minutes, and the mouse-specific HRC/DAB (ABC) Detection IHC Kit (Abcam, AB64259)).

Microscopic examination of the slides was performed using a Nikon Eclipse 80i microscope, and the photographs were obtained using a digital camera attached to a computer.

Endothelial cell analysis was done by analysing endothelial cell adhesion to platelets with the marker PECAM/CD31 and the proangiogenetic effect with VEGF that is strongly expressed by endothelial cells.

The amount and distribution of fibrosis were evaluated on collagen IV, osteonectin, and laminin stains.

Immunohistochemical controls were used to assess the efficacy and specificity of reactions by negative reagent controls and internal negative tissue controls.

The number of vessels and their lumen around the de novo formed bone particles was evaluated. In sections marked with anti-CD31 and VEGF antibodies, the number of vessels and the lumen diameter were comparatively analysed by visual analysis. Data were described by comparison between the three materials used, bovine bone mineral, beta-tricalcium phosphate, and bioactive glasses (Perioglass).

Histologic measuring of the new bone formation was semiquantitatively evaluated and correlated with the vessel formation around the augmented materials

2.3. Statistical Analysis. The statistical software SPSS 22 was used for data analysis. The descriptive analysis was used for primary assessment. Kurtosis and skewness tests were used to assess the asymmetrical distribution of the data. Also, the nonparametric tests for independent batches Kruskal-Wallis and Mann-Whitney were used.

For the histologic evaluation of bone formation, the nonparametric test for the independent batches Kruskal-Wallis was used. Kolmogorov-Smirnov and Shapiro-Wilk tests were performed to assess the normality.

3. Results

Following the quantitative descriptive analysis of the distribution of CD31- and VEGF-positive vessels around the augmentation material, it was noted that we maintain an increased average of the number of vessels in the bovine batch group 6.58 ± 0.59 (Figures 2 and 3) and the tricalcium phosphate 4.47 ± 0.2 (Figures 4 and 5) compared to the lot where the bioactive glass 1.58 ± 0.246 was used (Figures 6–8) (Table 1). The kurtosis and skewness tests reveal an asymmetric distribution to the right in the case of the first batch (Figure 9) and distribution to the left for the other two batches (Figures 10 and 11) with a platrictic aspect of the curve.

The amount of fibrosis, evaluated on collagen IV and laminin stains, showed no statistically significant differences between the three groups and, also, no differences comparing with the control group (Figures 12 and 13).

Bone remodelling and remineralization, as evaluated on osteonectin stain, were also similar in all groups (control group, beta-tricalcium phosphate, bovine hydroxyapatite, and bioactive glasses groups) (Figure 14).

Using the Kruskal-Wallis test, the average score in the first batch was 43.7 followed by 32.9 for tricyclic phosphate group and only 10.32 for the bioactive glass lot. From the statistical analysis, we obtained the significant difference between the three groups, chi square = 40.0 to 2 degrees of freedom with $p < 0.001$ (Figure 15) (Table 2).

Applying the Mann-Whitney test, wave $U = 79$ ($N1 = 19$, $N2 = 19$) was obtained at a significance level $p = 0.002 < 0.05$ (Tables 3 and 4).

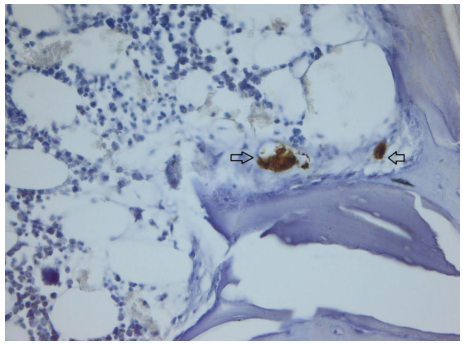

Figure 2: 40x bovine hydroxyapatite PECAM.

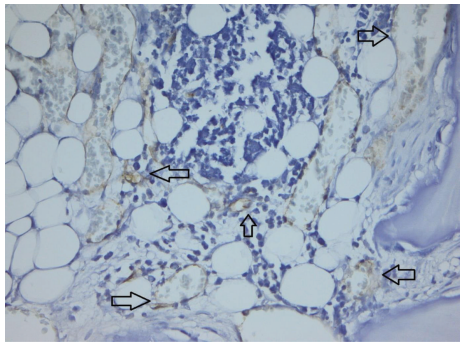

Figure 3: 40x bovine hydroxyapatite PECAM −1/CD31 reactive endothelium.

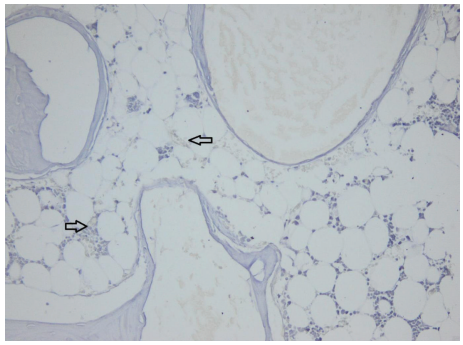

Figure 4: 40x TCP PECAM-1/CD31 reactive endothelium.

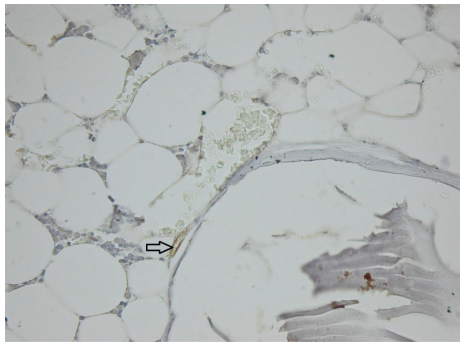

Figure 5: 40x TCP PECAM-1/CD31 reactive endothelium.

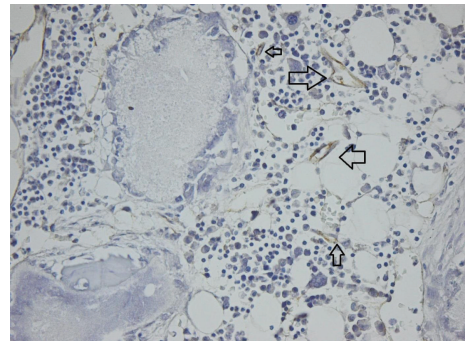

Figure 6: 40x bioglass PECAM-1/CD31 reactive endothelium.

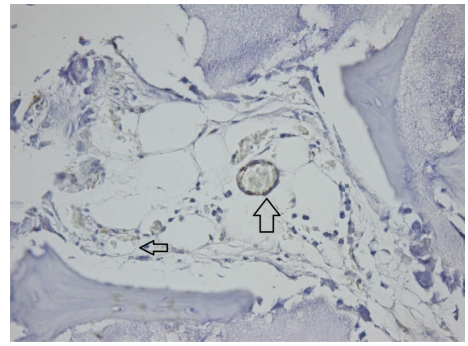

Figure 7: Bioglass PECAM-1/CD31 reactive endothelium.

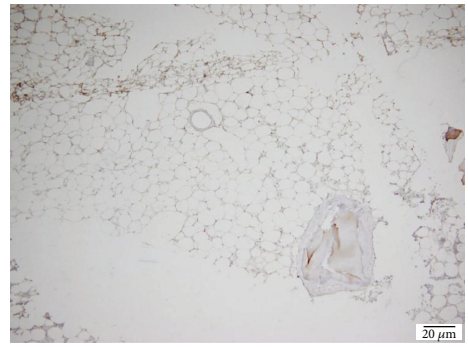

Figure 8: Bioglass 100 × VEGF reactive endothelium.

From the above analysis, statistically significant differences are observed for all three pairs. This complements the discoveries in which the amount of bone that is formed around beta-tricalcium phosphate and bovine hydroxyapatite is clearly superior to the bioactive glasses. Both the lumen diameter and the number of vessels were slightly increased on the studied slides in favour of beta-tricalcium phosphate.

Using the Kruskal-Wallis test, the average score in the first batch was 100.4, followed by 72.73 for the tricyclic phosphate group and only 34.01 for the bioactive glass batch. From the statistical analysis, we obtained the significant difference between the three groups, chi square = 59.323n at 2 degrees freedom with $p < 0.001$ (Tables 5 and 6). Following normality tests, Kolmogorov-Smirnov and Shapiro-Wilk demonstrated that the only batch that respects normality is the autologous control bone (Table 7).

Table 1: Descriptive analysis blood vessel representation.

	Bone type		Statistic	Std. error
PECAM	Bovine bone	Mean	6.58	0.599
		Median	6.00	
		Std. deviation	2.610	
		Minimum	3	
		Maximum	13	
		Skewness	1.063	0.524
		Kurtosis	0.686	1.014
	β-Tricalcium phosphate	Mean	4.47	0.208
		Median	4.00	
		Std. deviation	0.905	
		Minimum	3	
		Maximum	6	
		Skewness	0.339	0.524
		Kurtosis	−0.499	1.014
	Bioactive glass	Mean	1.58	0.246
		Median	2.00	
		Std. deviation	1.071	
		Minimum	0	
		Maximum	3	
		Skewness	−0.229	0.524
		Kurtosis	−1.102	1.014

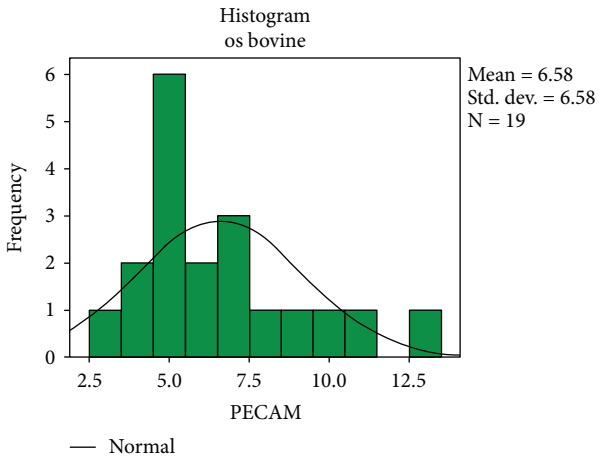

Figure 9: Histogram standard deviation bovine bone.

This complements the findings in which the amount of bone that forms around beta-tricalcium phosphate and bovine hydroxyapatite is clearly superior to bioactive glasses.

4. Discussions

The purpose of this study was to analyse the ability to integrate bone substitutes by evaluating the IHC expression of the platelet endothelial cell adhesion molecules (PECAM-1/Cd31), VEGF, collagen IV, laminin, and osteonectin for the three materials used for augmentation: bovine hydroxyapatite, beta-tricalcium phosphate, and bioactive glasses.

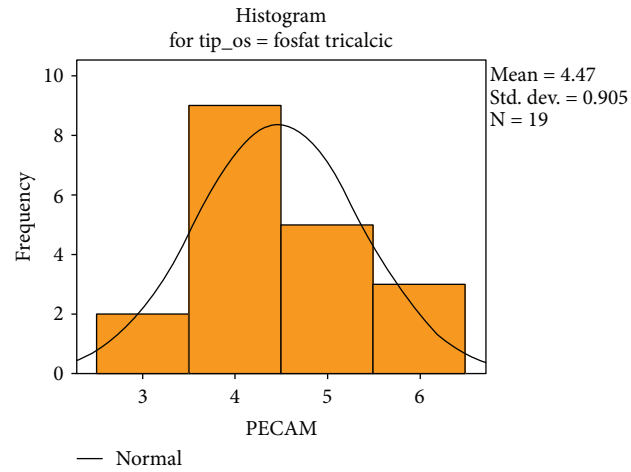

Figure 10: Histogram standard deviation B-tricalcium phosphate.

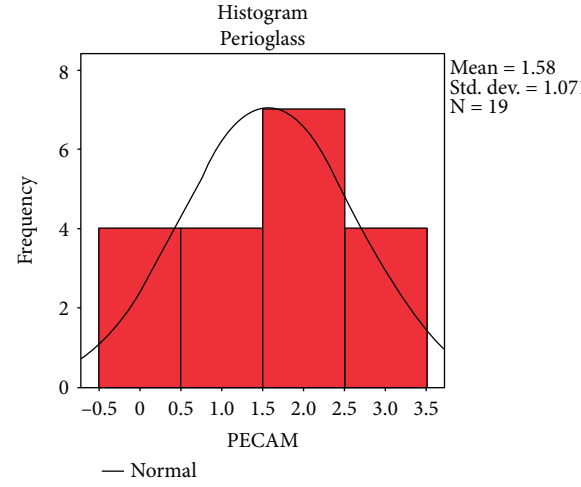

Figure 11: Histogram standard deviation bioactive glass.

Angiogenesis is the witness of inflammatory processes with both catabolic and anabolic effects, and this *in vivo* study used CD31 as an indicator of intensity of inflammatory-driven angiogenesis.

The cluster of differentiation 31 (CD31), also known as the platelet endothelial cell adhesion molecule (PECAM-1), plays a key role in removing aged neutrophils from the body. It is found on the surface of platelets, monocytes, neutrophils, and some types of T cells. The encoded protein is a member of the immunoglobulin superfamily and is probably involved in leukocyte transmigration, angiogenesis, and integrin activation. CD31 and VEGF are mainly used to demonstrate the presence of endothelial cells in histological tissue sections [11, 12].

In a bone defect, angiogenesis is a mandatory process in order to obtain osteogenesis [13–16]. Osteogenesis occurs most frequently through osteoconductivity mechanism. This is a three-dimensional process in which the biomaterial for augmentation acts as a matrix for the capillary proliferation, and the migration of proosteoblasts forms the adjacent tissue [17, 18]. Osteoconductive materials cannot be used in the

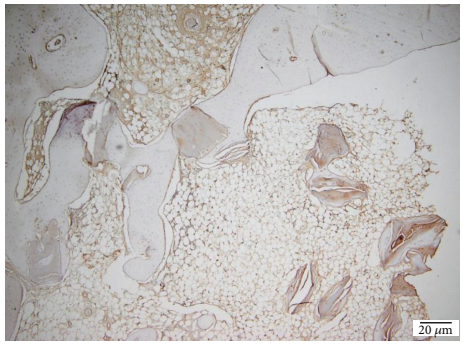

Figure 12: TCP 100x collagen IV stain showing moderate fibrosis.

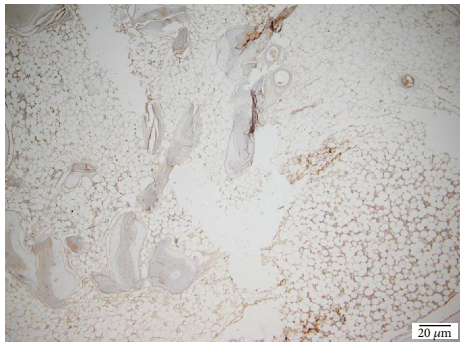

Figure 13: TCP 40x laminin stain showing moderate scaring.

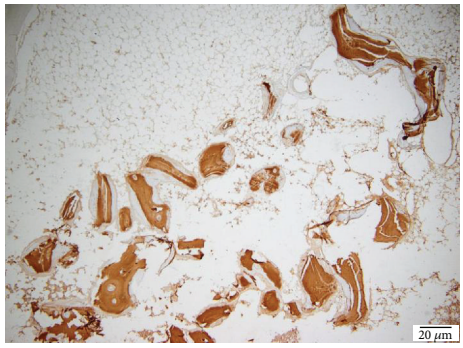

Figure 14: TCP 40x osteonectin stain showing intense bone remineralization.

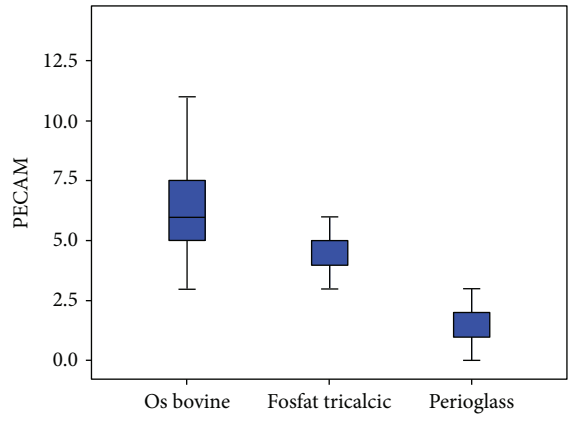

Figure 15: Representation of the mean number of blood vessels.

Table 2: Kruskal-Wallis test ranks.

	Bone type	N	Mean rank
PECAM	Bovine bone	19	43.74
	β-Tricalcium phosphate	19	32.95
	Bioactive glass	19	10.32
	Total	57	

Table 3: β-Tricalcium phosphate and bioactive glass Mann-Whitney test statistics.

	Bone type	N	Mean rank	Sum of ranks
PECAM	β-Tricalcium phosphate	19	28.79	547.00
	Bioactive glass	19	10.21	194.00
	Total	38		

Table 4: Bovine bone and bioactive glass Mann-Whitney test statistics.

	Bone type	N	Mean rank	Sum of ranks
PECAM	Bovine bone	19	28.89	549.00
	Bioactive glass	19	10.11	192.00
	Total	38		

Table 5: Kruskal-Wallis test.

Statistic test	Aria
Chi square	59.323
df	2
Asymp. sig.	0.000

Table 6: Values of newly formed bone on every type of material.

	Bone type	N	Mean rank
Aria	HA	51	100.43
	BTCP	50	72.73
	Perioglass	41	34.01
	Total	142	

Table 7: Normality test in which it can be observed that the only substitution material that respects normality is the autologous bone.

Bone type	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
TCP	0.277	51	0.000	0.634	51	0.000
Bovine	0.171	50	0.001	0.804	50	0.000
Perioglass	0.239	41	0.000	0.770	41	0.000
Autologous	0.129	10	0.200*	0.963	10	0.816

reconstruction of large bone defects, since bone formation is limited by the distance on which the replacement material is applied [19, 20]. In order to reconstruct large defects, it is preferable to use osteoinductive materials [21].

The number of vessels and lumens quantified in the studied sections stained with the CD31 and VEGF

immunomarkers was higher in bovine bone graft rabbits without a noticeable difference as compared to beta-tricalcium phosphate. Around the Perioglass material, we noticed the smallest number of vessels and the smallest amount of bone, this being consistent with other studies [22, 23]. Therefore, the superiority of the new bone formation in the case of hydroxyapatite could be justified by its osteoconductive property as well as by the larger quantity of vessels in which it was present.

Zengin et al. and Matsumoto et al. reported the existence of a cell population between the tunica media and the adventitia of adult vessels, which can behave as immature endothelial cells or osteoprogenic cells. Increased amount of CD31+ cells could induce an increase in the number of pericytes which probably contributes to osteoblast population growth [14, 24, 25]. Another study reported by Rabie has determined that anterior vascularization of the graft increases the metabolism of osteoblasts. Failure of this process initiates chondroblast and osteoclast activity and subsequent graft tissue absorption [26].

In our study, significant angiogenesis is linked with a greater tissue formation, indicating that in bone engineering with the allografts we used, inflammation has more benefic effects, the catabolic action being exceeded by the tissue formation.

5. Conclusions

Based on the results of this study, we can conclude that bone substitutes as bovine bone and beta-tricalcium phosphate have significant increased angiogenesis (and subsequent improved osteogenesis) comparing with Perioglass. There is a tendency towards better activity of bovine bone comparing with beta-tricalcium phosphate, but data are not statistically significant. Bovine bone (hydroxyapatite) has more prominent osteogenic potential because it forms a matrix that allows capillaries to develop, facilitating bone formation due to its osteoconductive property.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

The presented study will be integrated in the original part of PhD thesis of author Vlad Anghelescu.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Vlad Marian Anghelescu, Ioana Neculae, and Octavian Dincă had equal contributions.

Acknowledgments

This work was partially supported by a grant of Romanian Ministry of Research and Innovation, CCCDI-UEFISCDI, project number 61PCCDI/2018 PN-III-P1-1.2-PCCDI-2017-0341, within PNCDI-III.

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 2180373, 16 pages
https://doi.org/10.1155/2018/2180373

Review Article

Inflammation-Related Mechanisms in Chronic Kidney Disease Prediction, Progression, and Outcome

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Received 13 June 2018; Accepted 8 August 2018; Published 6 September 2018

Academic Editor: Donato Zipeto

Persistent, low-grade inflammation is now considered a hallmark feature of chronic kidney disease (CKD), being involved in the development of all-cause mortality of these patients. Although substantial improvements have been made in clinical care, CKD remains a major public health burden, affecting 10–15% of the population, and its prevalence is constantly growing. Due to its insidious nature, CKD is rarely diagnosed in early stages, and once developed, its progression is unfortunately irreversible. There are many factors that contribute to the setting of the inflammatory status in CKD, including increased production of proinflammatory cytokines, oxidative stress and acidosis, chronic and recurrent infections, altered metabolism of adipose tissue, and last but not least, gut microbiota dysbiosis, an underestimated source of microinflammation. In this scenario, a huge step forward was made by the increasing progression of omics approaches, specially designed for identification of biomarkers useful for early diagnostic and follow-up. Recent omics advances could provide novel insights in deciphering the disease pathophysiology; thus, identification of circulating biomarker panels using state-of-the-art proteomic technologies could improve CKD early diagnosis, monitoring, and prognostics. This review aims to summarize the recent knowledge regarding the relationship between inflammation and CKD, highlighting the current proteomic approaches, as well as the inflammasomes and gut microbiota dysbiosis involvement in the setting of CKD, culminating with the troubling bidirectional connection between CKD and renal malignancy, raised on the background of an inflammatory condition.

1. Introduction

Low-grade chronic systemic inflammation is a condition characterized by persistent, low to moderate levels of circulating inflammation markers. It has been long associated with coronary heart disease [1], metabolic syndrome, diabetes [2], and aging [3]. However, not only elderly pathologies are associated with the presence of low systemic inflammation. As systemic inflammation has also been reported in children and teenagers with weight problems [4], it is now clear that the persistence of the underlying condition and molecular mechanisms that trigger it should be taken into consideration in tandem with low chronic inflammation.

Whether inflammation is either a trigger or a result of a chronic underlying condition is an intensely studied topic. Studies on the impact of chronic inflammation on early stages of disease development, as well as the impact of early life nutrition on the adult inflammatory status, greatly extended the knowledge in the field (reviewed in [5]). Emergence of inflammation in childhood has been associated with obesity [6], diet [7], enteral infections [8], and even social stress [9]. Gene polymorphisms of inflammatory markers

[4, 10] and/or inflammasome components [11] are also determinants of the inflammatory response of patients in the face of chronic injuries.

The main sources of inflammatory cytokines are circulating monocytes and endothelial cells. Ubiquitous distribution of the latter could be responsible for the wide-spread impact of inflammation in almost all organs, including the bone. The kidney receives 25% of the entire blood volume, without having the benefit of antioxidant, detoxifying, and anti-inflammatory defence mechanisms developed by other intensely vascularized tissues, such as hepatic tissue. Hence, the kidney stands as a vulnerable target in front of persistent aggression.

Chronic kidney disease (CKD) is defined as "abnormalities of the kidney structure or function, present for more than 3 months, with implications for health" [12]. There is no question that inflammation plays a part in CKD progression and outcome [13], but the link between initiation of the disease and inflammation is still under debate. Similar to other chronic diseases, CKD is accompanied by a low-grade chronic inflammation, to which the kidney is vulnerable in more than one way, as discussed in the following section. Notably, distant sources of inflammation, such as a dysregulation of gut microbiota [14] or alteration of intestinal barrier [15], can negatively impact on progression of CKD and uremia-associated complications. Relationship between diet, gut microbiota, and CKD will be further detailed in one of the sections of this review. A particular issue to be addressed in the present review is the relationship between CKD, chronic inflammation, and malignancy. Similar to other chronic diseases, various types of cancer (colorectal [16], pancreatic [17], breast [18], aggressive prostate [19], lung [20], ovarian [21], or brain [22]) are associated with underlying chronic inflammation. Systemic inflammation has also been associated with renal cancers, especially in terms of prognosis [23–25], being as well a promoter of cell transformation and metastasis [26]. This review will look into more detail whether progression of CDK towards malignancy is a possibility that a clinician should consider in the context of systemic inflammation. Finally, the review will conclude with updates regarding proteomic studies of biomarkers for diagnostic, for accurate stratification, or progression from one stage to another, discussed in the framework of global search for ideal biomarkers.

2. Vulnerability of Kidneys Facing Inflammation

The role of inflammation in CKD pathogenesis and progression has been recognized since the late 1990s, when the first provocative theory was launched, in which inflammation, via monocyte release of interleukin-1 (IL-1), the master cytokine of inflammation, was the starting point concerning the major complications and the increasing rate of mortality in patients undergoing chronic dialysis [27]. It has also been described how polymorphisms in the IL-1 gene cluster influence levels of IL-1 gene products, which were later encountered in various inflammatory disease states. Since then, there has been an exponential growth of interest in deciphering the role played by the inflammatory cytokines released in the uremic milieu of CKD, as independent predictors of morbidity and mortality in CKD patients. While the release of proinflammatory cytokines could determine favourable effects, persistent inflammation is recognized to promote adverse consequences.

There are many factors that contribute to chronic inflammatory status in CKD, including increased production of proinflammatory cytokines, oxidative stress and acidosis, chronic and recurrent infections, altered metabolism of adipose tissue, and intestinal dysbiosis.

Inflammatory activation in CKD seems to be also influenced by genetic and epigenetic conditions. Therefore, several approaches have been proposed to target inflammation in CKD, including lifestyle changes, drugs, and dialysis optimization [28].

The evidence obtained so far sustains that inflammation and inflammatory reactions of any cause can modify or interfere with the intrarenal microcirculatory regulation and perfusion distribution and can induce renal damage, thus enhancing CKD progression.

It is well recognized the uniqueness of microcirculation networks in kidneys, being essential to sustain the corticomedullary osmotic gradient for fluid absorption and urine concentration. Under physiological conditions, the distribution of intrarenal vasculature is heterogeneous, and the medulla resides in a hypoxic milieu; therefore, the energy deprivation is eluded by an avalanche of regulators, such as hormones and other vasoactive molecules (prostaglandins, endothelins, kinins, medullipin, nitric oxide, and other molecules), mostly synthesized in the medulla [29]. Regardless of the highly regulated microcirculatory balance that keeps the kidneys efficient, it has to be mentioned that any slight imbalance in the interaction amongst these molecules could alter kidney function, thus rendering kidneys vulnerable to the microenvironment.

Systemic or intrarenal inflammation contributes to deregulation of the microvascular response to its regulators and sustains the production of an array of tubular toxins, including reactive oxygen species (ROS), leading to tubular injury, nephron dropout, and the onset of CKD. Circulating proinflammatory cytokines activate intrarenal microvessels, particularly endothelial cells and leukocytes, resulting in a local amplification of proinflammatory factors and ROS. These processes affect cell-surface adhesion molecules and disrupt the glycocalyx layer. Endothelial barrier function, activation of coagulation system, and receptor-mediated vasoreactivity are also compromised. These inflammation-mediated alterations can induce irreversible tubular injury and nephron failure [30].

Oxidative stress and inflammation are inseparably linked, being major characteristics of CKD and drivers of CKD progression. Systemic inflammation presence and severity contributes to CKD-associated oxidative stress, which represents a condition in which generation of ROS surpasses the capacity of the antioxidant defence system [31].

The inflammatory microenvironment, mediated by cytokines, induces overexpression of reactive oxygen/nitrogen species, bioactive lipids, and adhesion molecules. Cytokines

are also responsible for the promotion of aberrant matrix metabolism, proliferation of resident cells, and procoagulant activity of endothelium in the kidney. Cytokines control the inflammatory response and mediate some of their downstream effects through positive acute-phase proteins, such as C-reactive protein, fibrinogen, and albumin. In a recent study that analyses the association between a set of inflammatory biomarkers and progression of CKD in the Chronic Renal Insufficiency Cohort, the authors reported that elevated circulating levels of fibrinogen and TNF-α and decreased serum albumin are linked with the rapid loss of kidney function in patients with CKD, and these markers are independent predictors of CKD progression [32].

Systemic inflammation in end-stage renal disease is a well-recognized risk factor for the increased mortality in these patients and a catalyst for other complications, which are related to a premature aging phenotype, including muscle wasting, vascular calcification, and other forms of premature vascular disease, depression, osteoporosis, and frailty. Uremic inflammation is also involved in the aging process, such as telomere shortening, mitochondrial dysfunction, and altered nutrient sensing, which can have a direct effect on cellular and tissue function [33]. An in vitro study showed that circulating inflammatory monocytes from advanced CKD or hemodialysis patients transdifferentiate into osteoclasts and play a relevant role in mineral bone disorders. CKD patients, characterized by reduced renal function, frequently present an increased inflammatory state and skeletal abnormality [34].

Patients with CKD often display chronic increase in markers of inflammation, a condition that seems to be intensified by the disease progression and onset of hemodialysis. Systemic inflammation is related to malnutrition and muscle protein wasting and is involved in many morbidities including cardiovascular disease, the most common cause of mortality in this population. Investigation in the general population and other chronic disease cohorts demonstrated that an increase in habitual activity levels over a prolonged period may normalize the systemic inflammation. Furthermore, those populations with the highest baseline levels of systemic inflammation appear to have the greatest improvements from training [35]. Systemic inflammation, alongside with the loss of kidney function, can damage the resistance of the body to external and internal stressors, by reducing functional and structural tissue reserves and by impairing normal organ crosstalk, thus providing an explanation for the greatly increased risk of homeostatic breakdown in this population [35].

Overall, CKD patients show elevations in markers of chronic inflammation. Since inflammation, malnutrition, and protein-energy wasting are important contributors to mortality in CKD patients, any treatments which may positively influence these conditions should be taken into consideration [35].

Despite recent advances in the management of chronic kidney disease (CKD) and end-stage renal disease (ESRD), morbidity and mortality continue to be remarkably high in these patients. Persistent, low-grade inflammation has been recognized as an important component of the CKD scenario, leading to fibrosis and loss of renal function, and is playing a crucial role in the pathophysiology and progression of the disease, with a major impact on its complications [28].

3. Inflammasomes, Inflammation, and CKD

The inflammasomes have recently become the subject of intensive research, since they seem to play a major role in the pathogenic mechanisms in renal diseases. The inflammasomes are large, multiprotein complexes that could be induced by lipopolysaccharide (LPS). They were initially mentioned in 2002 as innate immune signaling pathways triggering activation of proinflammatory cytokines in response to various stimuli [36]. Innate immunity is an evolutionarily conserved system, the first line of host defence that supports homeostasis by regulating endogenous processes like inflammation and apoptosis. It relies on pattern recognition receptors (PRRs) that recognize damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) released in response to stress, tissue injury, or apoptosis [37]. Currently, several different classes of PRR families have been identified, which include transmembrane Toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIGs) receptors, intracellular Nod-like receptors (NLRs), and more recently included HIN-200 receptors. Extracellular PAMPs and DAMPs are recognized by TLRs and CLRs, while intracellular PAMPs are recognized by NLRs and RIGs [38, 39].

The activated innate immune system leads to activation of the prototypical proinflammatory signaling pathway, the best characterized being NF-κB (nuclear factor-kappa B) and AP-1 (activator protein-1), mainly based on the stimulation of multiple mediators, including proinflammatory cytokines such as interleukin-1 (IL-1) and tumour necrosis factor α (TNF-α). A decisive instrument in initiating the posttranscriptional processing and release of mature cytokines is represented by the development of the inflammasome complex. The human genome encodes 23 NLR proteins, from which the NLR with caspase recruitment domain (NLRC) are responsible of organizing an inflammasome complex and releasing of proinflammatory cytokines IL-1β and IL-18. There have been seven established NLRs that form an inflammasome complex: NLRP1 (NALP1), NLRP3 (NALP3 or cryopyrin), NLRP6, NLRP12, NLRC4 (with caspase recruitment domain or IPAF), AIM2 (absent in melanoma-2), and RIG-1 (retinoic acid inducible gene-1); however, the NLRP3 inflammasome is the best characterized in relation with renal diseases [40].

Activation of NLRP3 inflammasome is promoted by TLR activation, thereby triggering the NF-κB pathway and the proinflammatory cytokines being released as pro-IL-1β and pro-IL-18. In order to be converted into their active forms and be secreted, the cytokines require subsequent caspase cleavage, which determine NLRP3 to oligomerize in the presence of an adaptor molecule—ASC (apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain), and finally resulting in secretion of proinflammatory cytokines.

Despite the fact that recognition of a single unifying mechanism for the NLRP3 inflammasome activation remains elusive, several stimuli have been proposed that trigger assembly of the NLRP3 inflammasome, involving $P2X_7$ (a ligand-gated ion channel) receptor, activated via ATP, with K^+ efflux and reduction in intracellular K^+; ROS production, the release of mitochondrial DNA and cardiolipin [41]. The role of ROS as essential secondary messengers signaling NLRP3 inflammasome activation was suggested in several studies, and various pathways have been anticipated to mediate ROS production by NLRP3 activators. It was speculated that K^+ efflux could trigger ROS generation or other NLRP3 activators, such as uric acid crystals, alum, asbestos, and silica. Therefore, the so-called frustrated phagocytosis could be generated, being connected to ROS production, as well [42]. Various pathways have been proposed to mediate ROS production by NLRP3/NALP3 activators; however, the general picture of how NLRP3/NALP3 activators trigger ROS is still unclear.

Recent studies highlighted a broad role for inflammasome activation in renal diseases. Most of the studies regarding the role of NLRP3 have been performed on acute kidney injury (AKI) models, and fewer were done using models of CKD, due to the deficit of rodent models that could mimic the human CKD [43]. Among the various animal models, the unilateral ureteral obstruction (UUO) represents a suitable model of renal fibrosis, which was established as a model of CKD [44]. In a study using a UUO model, Vilaysane et al. concluded that inflammasome-dependent cytokines IL-1β and IL-18 were upregulated in association with caspase-1 activation; compared with wild-type mice, $NLRP3^{-/-}$ mice expressed less tubular injury, inflammation, and fibrosis after UUO, which highlighted the activation of NLRP3 inflammasome [45]. Using the same UUO model, Pulskens et al. concluded that the absence of NLRP3 resulted in enhanced vascular leakage and interstitial edema and revealed no effect on fibrosis and inflammation. These data showed a noncanonical effect of NLRP3 inflammasome in protecting kidney integrity following progressive renal injury [46]. It is important to note that the UUO mice model does not represent an objective readout, and the significance of inflammasome in relation to CKD remains under critical debate. Several studies in mice models and still restricted studies in humans propose an extensive role for inflammasome activation in CKD. Surprisingly, individual components of the inflammasome activation could bring their own contribution to progressive renal injury [47].

In addition to their role in mediating acute kidney disease, the IL-1β/IL-18 axis could also be involved in the development of CKD itself and its related complications—accelerated vascular calcification, fibrosis, and sepsis. It was shown that vascular inflammation is related to vascular calcification, and the proinflammatory cytokine IL-18 was the most extensively studied component of the NLRP3 inflammasome in relation to CKD. The pathophysiology behind the elevated levels of IL-18 in CKD may be related to the levels of MCP-1 (monocyte chemoattractant protein-1), since eGFR was independently associated with the serum levels of MCP-1, thereby partially explaining the increased risk of cardiovascular complications in CKD [48].

Inflammation-related vascular injury and atherosclerotic plaques in CKD were also the subject of intense research, in relation to inflammasome cytokine-mediated NLRP3, while IL-18 levels were found to be correlated with aortic pulse wave velocity. The NLRP3 inflammasome is gaining recognition for its key role in the pathogenesis of CKD and its complications; however, understanding the different pathways through which the inflammasome contributes to their genesis will supply additional insights in providing potential therapeutic targets [40].

The current understanding of CKD is based on a broad range of studies, and the inflammasomes exert a major role as guardians of the body; nevertheless, their role in regulating the intestinal microbiota and the progression of major diseases has been recently depicted.

4. An Underestimated Source of Smouldering Inflammation—Gut Microbiota

Microbiota, the microbial community which colonizes the large intestine, is nowadays considered a symbiotic "supplementary organ," consisting of trillions of microbes, which altogether contain several hundredfold more genes than the human genome. Microbiota, in terms of composition and metabolic activity, codevelops with the host even from birth and is subject to a complex interaction depending on host genome, diet, and lifestyle factors [49]. It was noticed that gut microbiota have fundamental roles in human health and disease, and the diversity of microbiota evolves over a person's life, shifting throughout childhood and adult life, continuing with elderly where it is poor in some taxonomic species, including Gram-negative Bacteroides species, and rich in Gram-positive Firmicutes species. Advances in sequencing technology (NGS) and bioinformatics have unravelled the complexity and diversity of human microbiome. Thus, the Human Microbiome Project has been launched in 2007 by the National Institutes of Health (NIH), in an effort to "characterize microbial communities found at several sites on the human body, including nasal passages, oral cavities, skin, gastrointestinal tract, and urogenital tract, and to analyse the role these microbes play in human health and disease". The NIH-funded Human Microbiome Project Consortium has been able to map the microbial signature of normal human individuals, providing a framework for current and future studies, thus leaving open future upgrades on various disease-microbiome correlations through recent research and aiming at a deeper understanding of the disease pathophysiology [50]. Recent NGS-based studies have highlighted the gut microbiome impact on different physiologies including disease, of which the gut microbiome expressed aberrant composition as compared with that of normal individuals [51].

Although the microbiota is constantly exposed to a changing environment, its composition and function in an individual remain stable, despite disturbances. Under normal conditions, the gut microbiota represents a dynamic and symbiotic ecosystem, in a continuous relationship with the host metabolism, providing trophic and protective functions.

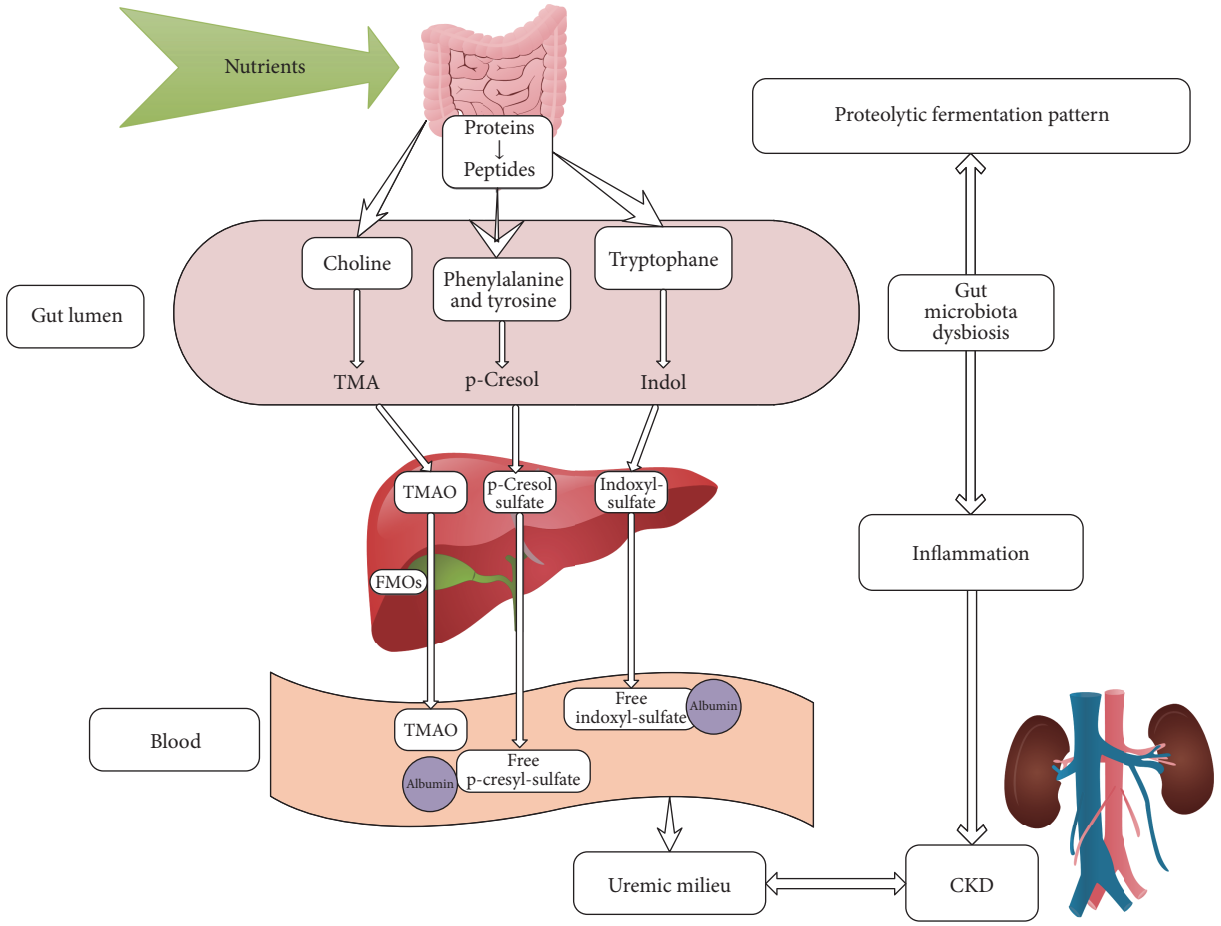

FIGURE 1: The pathway followed by the uremic metabolites (TMAO, p-cresyl sulfate, and indoxyl sulfate) in the setting of the uremic milieu, characteristic to CKD. The dysbiosis of gut microbiota contributes to the establishment of a proteolytic fermentation pattern, by enhancing the bacteria types that produce uremic toxins.

It was revealed that alterations of the commensal flora have been involved in the pathogenesis of various illnesses, including chronic inflammation and CKD.

The CKD specific uremic milieu, due to influx of urea and other retained toxins, seems to impair the intestinal barrier function and promotes inflammation throughout the gastrointestinal tract, thus being crucial in shaping the gut microbiota in terms of structure, composition, and function. Microbial diversity is significantly damaged in CKD patients, with a decreased number of beneficial bacteria that generate short-chain fatty acids (SCFAs), a fundamental nutrient for the colonic epithelium, and an increase in bacteria that produce uremic toxins such as indoxyl sulfate, p-cresyl sulfate, and trimethylamine-N-oxide (TMAO) [52]. Uremic toxicity has also been studied by the European Toxin work group (EUTox), offering novel insights into uremic milieu by developing a classification of uremic circulating components, based on their features that affect their elimination under dialysis. Thus, among small water-soluble molecules (e.g., urea and creatinine) and peptides/proteins (e.g., β2-microglobulin), a group of so-called protein bound uremic retention solutes has been identified, intriguingly generated by protein fermentation in the large intestine—namely, p-cresyl sulfate and indoxyl sulfate [53].

These uremic toxins were also evaluated in relation to kidney function (eGFR), and the results showed that their overexpression was correlated with an impaired renal function and an increased potential of all-cause mortality in CKD end-stage patients [54]. In addition, a direct connection was prominently revealed between increased levels of p-cresyl sulfate and poor prognosis on patients at CKD end stages; associations between indoxyl sulfate and unfavourable prognosis have been shown, as well, since it was demonstrated they share common ground, being both originated from bacterial protein fermentation in the large intestine. It was revealed that the circulating forms of these molecules are bound to albumin, competing for the same albumin-binding sites. Further studies have been conducted and have launched the theory by which the adsorption of indoxyl sulfate and p-cresyl sulfate at the intestinal level will lead to a delay in CKD progression. In light of these findings, it was optimistically hypothesized that these two molecules could be considered promising candidate biomarkers for evaluating the CKD progression [53] (see Figure 1).

It should be emphasized that renal phenotype is much broader than function impairment of kidneys, and most of the end-stage CKD patients are under multidrug therapy and dietary restrictions. Therefore, testing the associations

between renal function and microbiome composition could offer accurate results when assaying on experimental models. In addition, dietary restrictions in CKD end-stage patients may be associated with limited intake of potassium, sodium, phosphate, and animal proteins, as well, also restrictions in fermentable carbohydrates. As a result, the colonic transit time is prolonged, and CKD patients undergoing dialysis are suffering though of constipation. As a consequence of diet restrictions and prolonged colonic transit, the microbiota activity moves towards a proteolytic fermentation pattern. This metabolic shift represents the explanation of significant prevalence in bacterial types processing urease, uricase, and indole and p-cresol forming enzymes [55]. Microbial diversity is significantly damaged in CKD patients, with a decreased number of beneficial bacteria that generate SCFAs and an increase in bacteria that produce uremic toxins (indoxyl sulfate, p-cresyl sulfate, and TMAO) [52].

Recent evidence suggests that several circulating metabolites released by microbiota metabolism could be linked to systemic immunoinflammatory response and kidney impairment. Thus, some metabolites generated by dietary fiber fermentation in the intestinal tract (including SCFAs) could play important roles in modulating immunity, blood pressure, and lipid metabolism. Though controversial, the SCFAs could be regarded as potential therapeutic targets and seem to represent the link between the kidney malfunction and inflammatory response [56].

Inside CKD population, the interactions work bidirectionally: on one hand, the uremic milieu has a negative impact on microbiota, altering the composition and metabolism, and on the other hand, the microbiota dysbiosis releases potential uremic toxins that are normally excreted by the kidneys; thus, both conditions further lead to a toxin avalanche exposure. The generated state is also caused by the disruption of the epithelial barrier, leading to an amplified intestinal permeability, often referred to as "leaky gut," a condition that promotes inflammation and is encountered in CKD [57].

Intestinal inflammation and gut dysbiosis are nowadays considered as significant contributors in the setting of chronic inflammation and other CKD complications, thus explaining the gut-therapeutic novel approaches when designing CKD interventions [58].

4.1. Dietary Patterns in Preventing CKD Progression. Preventing the gut dysbiosis and maintaining the gut microbiota homeostasis are considered the key mechanisms for hampering the setting of chronic inflammation and CKD progression. Based on the principle that a balanced healthy microbiota is primarily saccharolytic and nutrition has significant effects on its composition, the innovative therapeutic avenues comprise special diets that successfully shape microbiota composition through a nonpharmacological approach. The Mediterranean diet, consisting mainly of carbohydrates, basically unrefined grains, fruits and vegetables, nuts, olive oil, fish, moderate red wine, dairy products, and red meat, represents one of the most promising nutritional strategies, having protective effects on CKD conditions, potentially restoring microbiota balance and slowing down CKD progression, as many studies have depicted [59–61]. Additional benefits in reducing the burden of uremic toxins, generated both by microbiota and CKD condition, were noticed under a vegetarian diet; however, increasing attention must be paid in regard to serum potassium levels [62, 63]. Other promising diets have been proposed as potential beneficent therapies, including vegan diet, DASH diet, and the modern dietary pattern, all exhibiting protective effects on both CKD progression [64, 65] or on intestinal microbiota homeostasis [62]. In contrast, the Western diet, excessively rich in proteins and low in fruits and vegetables, grains, and fibers, exerts a detrimental effect on CKD, by increasing the risk of rapid eGFR decline [66]. Along with the Western diet, other essential diets have been assessed in relation to their kidney function decline, comprising the Southern diet, DGA diet, and dal diet [67–69].

4.2. Prebiotics, Probiotics, and Synbiotics—Promising Therapies in Modulating Gut Microbiota in CKD. A promising therapeutic approach in combating CKD progression relies on targeting microbiota balance, by administrating prebiotics and probiotics and the mixture of both preparations into synbiotic compounds.

Probiotics are microorganisms that are claimed to provide beneficial effects and are defined as "live microorganisms that when administrated in adequate amounts confer a health benefit on the host" [70]. Administration of probiotics, mainly represented by Bifidobacteria, Lactobacillus, and Streptococci species, could attenuate the CKD progression. Recent studies, based on a rat model of CKD, suggest that probiotic therapy has a substantial potential in ameliorating the disease course [71]. A significant decrease in urea nitrogen circulating levels and a favourable CKD prognostic rate were reported in a multinational trial on CKD stage 3 and 4 undertaking proprietary formulation of *S. thermophilus*, L. acidophilus, and B. longum, over a period of six months. However, if these effects are due to alteration of the gut tight junction barrier remains questionable, further studies being necessary to unravel the precise mechanisms [72].

Prebiotics are typically specialized nondigestible plant fiber compounds that circulate undigested through the upper part of the gastrointestinal tract and enhance the activity of beneficial bacteria in the gut, presenting also a beneficial effect on CKD prognosis [73]. Prebiotics are commonly known as a type of fiber referred to as "oligosaccharides," and the promising therapeutic candidates are represented by inulin, fructooligosaccharides, galactooligosaccharides, soyaoligosaccharides, xyloolygosacchrides, and pyrodextrins and seem to enhance the metabolic activity of microbial species like Bifidobacteria and Lactobacillus [74]. Other relevant studies focused on the effects exerted by administration of prebiotics, probiotics, and the dual approach of combining those two preparations (synbiotics) in CKD, in both patients and animal models, have been depicted in Table 1.

Synbiotics have been the subject of different research studies, with the term pertaining to combinations in which probiotics and prebiotics strengthen each other's activity, resulting in a synergistic effect. Recent studies highlighted that administration of synbiotics has generated favourable effects, by decreasing the circulating levels of uremic

Table 1: Effects of administrating pro-, pre-, or synbiotics in CKD.

Novel therapeutic targets	Effects on CKD	Reference
Probiotics—Lactobacillus acidophilus	Nitrosodimethylamine levels decreased, and serum dimethylamine levels dropped (on humans).	[77]
Probiotics—*Bacillus pasteurii* or *Lactobacillus sporogenes*	Enhanced survival in nephrectomized rats while slowing the progress of renal injury (rat model).	[78]
Probiotics—*Sprosarcina pasteurii*	Reduced blood urea-nitrogen levels and significantly prolonged the lifespan of uremic animals (rat model).	[79]
Probiotics—oral sorbent charcoal AST-120	Delay in the progression of CKD but also in cardiovascular diseases (rat model).	[80]
Probiotics—*Bifidobacterium longum*	Reduced serum levels of indoxyl sulfate by correcting the intestinal microflora (on humans).	[81]
Probiotics—*Bifidobacterium longum*	Decreased serum levels of homocysteine, indoxyl sulfate, and triglyceride (on humans).	[82]
Prebiotics—oligofructose-enriched inulin	Significantly reduced p-cresyl sulfate generation rates (on humans).	[83]
Prebiotics—resistant starch	Reduced plasma levels of indoxyl sulfate and p-cresol sulfate (on humans).	[84]
Synbiotics	Decreased serum p-cresol sulfate and the stool microbiome modified (on humans).	[75]
Synbiotics	Normalization of bowel habits and a decrease of serum p-cresol levels (on humans).	[85]

toxins, along with a restoration in microbiota balance [75]. A meta-analysis of 12 studies on the effectiveness of pre-, pro-, and synbiotics on CKD populations has reported significantly decreased levels of the two protein-bound uremic toxins (p-cresyl sulfate and indoxyl sulfate), concluding that "there is a limited but supportive evidence for the effectiveness of pre- and probiotics on reducing p-cresyl sulfate and indoxyl sulfate in the chronic kidney disease population," but that "further studies are needed to provide more definitive findings before routine clinical use can be recommended" [76].

In conclusion, these novel promising therapeutic approaches, in which diet represents the essential factor in alleviating the disease progression, are not quite new if we go back in ancient Greece, nearly 2500 years ago, when Hippocrates postulated that "All disease begins in the gut."

5. CKD and Malignancy—Dangerous Scenarios in the Framework of Inflammation

The role of inflammation in the development of cancer has been the subject of intense research over the years, since it was noted that an inflammatory milieu arises as one of the hallmark features describing the malignancy condition. There has been over 150 years since Virchow first hypothesized the relationship between the inflammatory status and carcinogenesis, based on the assumptions that cancer regularly occurs in the setting of inflammation, and additionally, that tumour biopsy specimens reveals the presence of inflammatory cells, as well. In an attempt to establish the signature of cancer, a repertoire of six hallmarks has been initially described, in which inflammation fostered multiple hallmark functions [86]. Following these established hallmarks, Fouad and Aanei proposed a more accurate definition of cancer hallmarks as "acquired evolutionary advantageous characteristics that complementarily promote transformation of phenotypically normal cells into malignant ones, and promote progression of malignant cells while sacrificing/exploiting host tissue" [87].

Nowadays, a plethora of research studies has confirmed that mitogenesis arises within an inflammatory microenvironment [88], while chronic, low-grade inflammation accompanies the disease course. The inflammatory milieu allows tumour cells to elude host immunosurveillance, resulting in subsequent angiogenesis, tumour growth, invasion, and metastasis [23, 89].

It is widely accepted that inflammation and carcinogenesis rely on similar mechanisms in terms of development, including severe cell proliferation and angiogenesis [90]. It was hypothesized that the longer the inflammation persists, the higher the possibility of genomic instability and mutations that lead to cancer. The sustained presence of inflammatory cells in the tumour milieu can stimulate tumour growth, hindering apoptosis of atypically transformed cells [91]. Peeking behind the curtain, two compliant pathways (intrinsic and extrinsic) seem to engage inflammation in cancer development. Key players of the intrinsic pathway reside in genetic modifications such as oncogene activation and tumour suppressor gene inactivation.

The principal mechanisms involved in renal carcinoma pathogenesis seem to be mediated via PI3K-AKT-mTOR, Ras-RAF-ERK, and VEGF signaling pathways, and the level of expression of the genes that are components of these pathways was positively correlated with overall survival in these patients. Therefore, further research targeting the genes and their encoded products, within these pathways, is needed to provide more insight into the involved pathways [92, 93].

The extrinsic pathway driven by inflammatory conditions generally arises and increases the risk of cancer at certain anatomical sites. Intrinsic and extrinsic factors may cooperate towards a malignant phenotype [94].

Key orchestrators of both intrinsic and extrinsic pathways consist of transcription factors (including NF-κB) that

serve as a pivotal mediator of inflammatory responses (avalanche of cytokines, chemokines), being also an active player in cancer initiation, development, metastasis, and resistance to treatment [95, 96].

The inflammatory infiltrate is one of the examples underlying the inflammatory microenvironment generated by the avalanche of inflammatory mediators expressed along with the activation of this pathway.

Remarkably, NF-κB is constitutively active in both tumoural cells and tumour microenvironment and uncommonly activated via genetic alterations [97]. However, it was revealed that, in all malignancies, NF-κB acts in a cell type-specific fashion: stimulating survival genes within tumour cells and inflammation-promoting genes in components of the tumour milieu [98]. Hence, the active NF-κB molecule in cancer is acting like a double-edged sword: on one hand, mediating the immune responses by eliminating tumour cells and, on the other hand, being constitutively active in renal cancer, arising from a chronic low-grade inflammatory milieu or rarely being activated by oncogenic aberrations [99].

The CXCL12–CXCR4 signaling pathway is emerging as a novel potential therapeutic target for renal cancer, CXCR4 being overexpressed in renal malignant cells, contributing to tumour dissemination and metastasis. Blocking this pathway results in a decreased rate of metastasis and could also be effective when CXCR4 is administered in conjunction with other anticancer treatments [100].

It is well known that renal cell carcinoma (RCC) develops as one of the most immunogenic cancers, thus being able to induce an immune response naturally. Therefore, several immunotherapeutic strategies have been experienced by modulating the immune system with cytokines, vaccines, and T-cell modulating agents, having optimistic long-term results. It was revealed that administration of interleukin-2 (IL-2) in high doses could represent the first-line treatment approach for selected patients and was correlated with resilient complete remissions in treated patients [101].

The association between CKD in its end stage in patients demanding kidney transplantation and development of kidney malignancy has become well recognized. Unfortunately, there is mounting evidence that malignancy, overall or targeting kidneys, nests in even earlier stages of CKD [102]. Due to the insidious nature of CKD progression, it becomes even more difficult to diagnose these patients in their early stages, bringing yet additional burden. Remarkably, there is emerging evidence that consider CKD and renal carcinoma as interrelated, with 26%–44% of renal cell carcinoma cases bearing concomitant moderate or higher CKD at the time of diagnosis. In addition, patients suffering from renal cancer are more predisposed to CKD than the general population. Potentially involved mechanisms could include uremic immune inhibition or circulating toxin exposure in the background of a deficient renal function. Consequently, kidney tumour management has to consider the renal functional status in the decision of resecting the tumour or adopting a surveillance attitude. It was shown that RCC with low-grade tumours, arising in patients suffering from end-stage CKD, seems to manifest favourable outcome features compared to those diagnosed from the general population [103].

Although CKD is correlated with a high rate of progression towards end-stage renal disease and increased mortality, it was hypothesized that the etiology of renal decline could alter the CKD progression and overall survival. Therefore, data suggest that surgically induced CKD, including partial or total nephrectomy as a therapeutical option for renal tumour, present a lower rate in eGFR decline compared to CKD due to other medical causes [104].

A progressive relationship between pretreatment CKD and locally advanced RCC has been reported, possibly related to increased damage of functional renal parenchyma with tumour size or stage advancement [105]. Also, in an Australian population-based cohort analysis, Ahn et al. evaluated the predictors of new-onset CKD or moderate-severe CKD in patients surgically treated for T1 RCC and found out that the strongest associations were increasing age, decreased renal function (eGFR), and the tumour size, as well [106].

Regardless of the renal tumour size or stage migration, the survival rates are not encouraging over the last 15 years; however, a survival rate of 90% or more, depending on the tumour histology, is expected for the small tumours, when partial or total nephrectomy was performed [107].

In conclusion, a bidirectional relationship has been established for kidney disease and cancer, being intertwined in various ways. On one hand, malignancy has been recognized as a major complication in CKD end-stage patients, increasing the morbidity and mortality; on the other hand, anticancer therapies enhance the development of CKD [108]. Unfortunately, regardless of significant advances in therapy, RCC is nowadays among the 10 most prevalent malignancies, and the incidence is growing. Additionally, RCC has a poor prognosis, considering that up to 30% of patients present metastasis at the time of diagnosis and about 20% will further develop metastasis, even if they are undergoing therapy [109].

Despite the increasing body of evidence regarding the troubling connection between CKD and renal cancer, there is a lack of strong clinical trials in the efforts to decipher the underlying disease mechanisms and to offer novel insights towards early diagnostic and the best therapeutic approaches.

6. Novel Promising Biomarkers Useful in CKD Management

The advent of proteomic technologies allowed novel approaches for biomarker discovery in CKD, with the end goal being early diagnosis and prognosis of CKD progression. Candidate biomarkers include molecules that were linked to different pathways, among which tubulointerstitial injury, tubulointerstitial fibrosis, and inflammation [110–115].

In a large multicentral international study of hemodialysis patients, evaluation of CRP levels, in addition to standard inflammatory biomarkers (eGFR, albumin, WBC, and ferritin), seemed to improve the mortality prediction. The

CRP level was positively and monotonically associated with mortality [116].

Another study evaluating the association between kidney function, albuminuria, and biomarkers of inflammation, in a large cohort of CKD patients, showed that plasma levels of IL-1β, IL-1RA, IL-6, TNF-α, hs-CRP, and fibrinogen were higher among participants with lower levels of estimated GFR (glomerular filtration rate). Moreover, the inflammation score was higher among the patients with lower estimated GFR and higher UACR (urine albumin to creatinine ratio). These results demonstrated that biomarkers of inflammation were inversely associated with measures of kidney function and positively with albuminuria [117]. The erythrocyte sedimentation rate, a nonspecific determinant of inflammation, has been shown to be predictive of end-stage renal disease in adolescents [118]. The level of proinflammatory cytokine IL-2 was elevated in hemodialysis patients with uremic pruritus (a common tormenting symptom among these patients) when compared to hemodialysis patient controls without pruritus [119]. The results obtained from several studies suggest that TWEAK (Tumour necrosis factor-like weak inducer of apoptosis) plays an important role in kidney injury associated with inflammation and promotes acute and chronic kidney diseases [120]. There are several studies testing different nanoconjugates that could prevent TWEAK-induced cell death and inflammatory signaling in different cell types, including renal tubular cells [121]. The results obtained from a study investigating hemodialysis patients showed that the group of patients with a specific pattern of high proinflammatory cytokines (IL-1, IL-6, and TNF-α) had increased mortality when compared to patients with a pattern of high T-cell regulatory or anti-inflammatory parameters (IL-2, IL-4, IL-5, IL-12, CH50, and T-cell number) [122]. Leptin is an adipose tissue-derived hormone shown to be associated to several inflammatory factors related to CKD. In vivo studies demonstrated that infusion of recombinant leptin into normal rats for 3 weeks results in the development of glomerulosclerosis. Moreover, higher plasma leptin levels are associated with CKD, and the authors of these studies sustain that leptin may explain part of the reported association between obesity and kidney disease [123].

Kidney injury molecule-1 (KIM-1), a type 1 transmembrane protein, has been shown to be upregulated in dedifferentiated proximal tubule epithelial cells upon ischemic or toxic injury but is undetectable in healthy kidneys or urine [124–127]. Urinary KIM-1 has been shown to predict renal injury before changes in eGFR were detectable [128, 129].

Neutrophil gelatinase-associated lipocalin (NGAL) is a protein expressed by tubular epithelial cells and neutrophils, and its expression levels were shown to predict disease severity [130, 131]. However, NGAL did not significantly improve risk prediction of progression outcomes compared to known CKD progression risk factors [132].

Epidermal growth factor (EGF) plays a role in tubular cell repair after tubulointerstitial injury. Urinary EGF expression was found to be correlated with GFR [133], and it improves CKD progression prediction when added to a conventional model including eGFR and albuminuria [134].

A candidate marker of renal fibrosis is matrix metalloproteinase-9 (MMP-9), which was found to be elevated in the urine and plasma of CKD patients compared to controls [135, 136]. Additionally, circulating MMP-9 levels improved CKD progression predictability when added to a model of conventional risk factors and eGFR [137].

Chronic low-grade inflammation is proposed to play an important role in the initiation and progression of CKD, and several candidate biomarkers have been suggested to predict GFR, as well as contribute directly to CKD progression [114, 115]. Soluble urokinase-type plasminogen activator receptor (suPAR) is involved in the pathogenesis of kidney disease. A low suPAR concentration was shown to be associated with the remission of CKD and the reduction of proteinuria (23138488). Furthermore, higher plasma suPAR was connected with CKD progression, as indicated by a stronger decline in eGFR [115]. Other inflammatory markers associated with CKD include tumour necrosis factor alpha receptor-1 and -2 (TNFR1 and TNFR2) and monocyte chemoattractant protein-1 (MCP-1). TNFR1 was found to be a strong prediction of CKD progression to ESRD [114], while circulating TNFR1 and TNFR2 were found to predict stage 3 CKD in type 1 diabetes patients. Urinary MCP-1 levels were elevated for CKD patients compared to controls [138] and were found to correlate with the rate of GFR decline [139].

Another study analysing the levels of MCP-1, MCSF, and neopterin in the serum and urine of children with CKD showed that MCP-1 levels are increased in early stages of this disease, suggesting that the inflammatory process precedes the tubular dysfunction [140].

In view of the increasing number of novel potential candidate biomarkers, advanced high-throughput research platforms are needed in order to refine the CKD diagnosis, monitoring, and follow-up.

7. Advances in Proteomic Approaches in Searching for an Ideal Biomarker

Although substantial improvements have been made in clinical care, CKD remains a major public health burden, affecting 10–15% of the population, and its prevalence is constantly growing [141]. Regardless of its etiology, CKD is defined as a "silent epidemic" disease and persistent, with low-grade inflammation reflecting a common feature in these patients. Due to its insidious nature, CKD is rarely diagnosed in early stages, as clinical symptoms occur only when kidney function has been irreversibly damaged (decreased eGFR). Unfortunately, current clinical approaches have become useful only in diagnosis of advanced CKD stages. Simply stated, once developed, CKD persists throughout the rest of the patient's life, and the single most feasible solution is likely linked to an early intervention, before irreversible nephron damage occurs [142]. In addition, nephrology lags behind other medical disciplines in terms of number, size, and quality of clinical trials undertaken, thus emerging provocative global action plans in order to improve the management of CKD and design novel therapeutic approaches to alleviate or even halt the progression of the disease [141].

In this scenario, a huge step forward was made by the increasing progression of omics approaches, designed for identification of biomarkers useful for early diagnostic and follow-up, thus exploring their potential for clinical implementation [143, 144]. In the era of omics, proteomics has risen, providing novel insights into disease mechanisms and therefore holds the promise of improving the life quality of CKD patients.

Advancements in the field of proteomics were possible by adopting a vast array of state-of-the-art technologies. Initially, two-dimensional (2D) gel electrophoresis was used, rapidly being improved by the development of two-dimensional differential gel electrophoresis (2D-DIGE), completed afterwards by employing liquid chromatography (LC) coupled with mass spectrometry (MS), enabling though untargeted protein identification. During recent years, capillary-electrophoresis (CE)-MS has been developed, combining both CE and MS advantages, providing high separation efficiency and molecular mass information within one single assay. Implementing the matrix-assisted laser desorption/ionization (MALDI) platform, by using laser energy absorbing matrix, is capable of generating ions from large molecules with minimal fragmentation, thereby moving the boundaries above. Proteomics aims to characterize the huge information flow mediated by proteins within the cell, by analysing the signaling pathways, interactions, and networks, thus enabling identification of disease specific biomarkers in order to illustrate a detailed proteomic signature for a better understanding of the molecular interactions underlying the pathogenesis of the disease. Assessing various biomarkers on multiplex proteomic platforms (Luminex xMAP array, microarrays, etc.) could unravel novel insights in deciphering the disease-specific molecular mechanisms, offering panels of biomarkers for improving the diagnosis and therapy towards a personalized approach [143, 145–147]. In the context of CKD and renal diseases, various proteomic studies have been designed, and the results were promising. Recent findings performed on MALDI suggested that molecular signatures could be generated, being capable of distinguishing between kidney disease and normal controls [148]. Siwy et al. analysed several potential urinary peptides to differentiate between distinct types of CKD, generated by capillary electrophoresis coupled to mass spectrometry [149]. Such findings are corroborated with other study results and confirm the utility of some of these urinary peptides as specific biomarkers [150]. Good et al. have developed a CKD classifier (CKD273), comprising 273 urinary peptides, specially designed for a better stratification in these patients [151]. CKD273 represents a multidimensional urinary biomarker which helps predict the renal function impairment [152]. Other studies aimed at predicting the risk of CKD progression, by determining patterns of protein expressions using mass spectrometry approaches (SELDI-TOF) [153]. CKD273 has recently received a letter of support from the US Food and Drug Administration (FDA), being now implemented in the CKD management [154]. Furthermore, CKD databases have been created; thus, KUPNetViz represents an interactive and flexible biological network tool for multiomics datasets, in the field of kidney diseases, providing biological network snapshots of the complex integrated data of the KUPKB (Kidney and Urinary Pathway Knowledge Base), thus creating the premises of generating novel in silico theories [155]. Furthermore, a CKD database (CKDdb) has been developed due to the vast amount of data generated by using high-throughput omics technologies. CKDdb represents an integrated and clustered information resource; featuring data from CKD published studies will result in deeper understanding of the molecular mechanism modulating CKD progression [156].

The translation of omics findings to clinical settings is challenging, since an ideal biomarker has not been discovered yet, thus being recommended to adopt a two-stage approach: firstly, the identification step, followed by the validation, applicable only in the framework of a well-defined clinical question and a specific phenotype [157].

8. Conclusions

Despite being a "silent epidemic" disease, CKD is now recognized as one of the major public health burden, affecting 10–15% of the population, and its prevalence is constantly growing. Mounting evidence suggests implication of inflammation in CKD pathophysiology, thereby shifting the perception of inflammation as no longer a new risk factor but rather a traditional one linked to morbidity and mortality in these patients. The pathophysiology of inflammation may not be the same in CKD patients; nevertheless, a persistent, low-grade inflammation has been established as a hallmark feature of CKD.

Among various factors that contribute to the setting of an inflammatory milieu in the context of CKD, the inflammasome has recently become the focus of extensive research, gaining recognition for its key role in the pathogenesis of CKD and its complications. As such, the inflammasome represents an attractive potential therapeutic target in renal diseases. Another underestimated source of smouldering inflammation related to CKD was assigned to gut microbiota dysbiosis, a condition intensively studied, since it was postulated that may represent the starting point of many diseases, including malignancy. Modulating the microbiota balance has become a subject of intense research; therefore, different dietary patterns have been proposed, along with administration of pre-, pro-, and synbiotics, with quite remarkable results.

In this scenario, a huge step forward was made by the increasing progression of omics approaches, specially designed for identification of biomarkers useful for early diagnostic and follow-up. Advances in proteomics, in searching for the ideal biomarker, have become increasingly popular over the last decades, offering novel insights in deciphering the CKD mechanisms, thus moving the boundaries forward. The identification of novel biomarkers using high-throughput technologies will provide the molecular signature of the disease, with impact on early diagnosis, monitoring, and prognosis.

Understanding the role of inflammation in the setting of CKD will foster the development of therapeutic strategies in order to treat and even prevent the underlying inflammation, thus improving CKD outcomes.

Conflicts of Interest

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

Authors' Contributions

Simona Mihai, Elena Codrici, Ionela Daniela Popescu, Ana-Maria Enciu, Lucian Albulescu, Laura Georgiana Necula, Cristina Mambet, Gabriela Anton, and Cristiana Tanase have contributed equally to this work.

Funding

This work was partly supported by grants from the Minister of Research and Innovation, Core Program PN 18.21.01.06.

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 2498576, 10 pages
https://doi.org/10.1155/2018/2498576

Research Article

Caveolin-1-Knockout Mouse as a Model of Inflammatory Diseases

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Received 23 May 2018; Accepted 5 August 2018; Published 29 August 2018

Academic Editor: Iulia D. Popescu

Caveolin-1 (CAV1) is the scaffold protein of caveolae, which are minute invaginations of the cell membrane that are involved in endocytosis, cell signaling, and endothelial-mediated inflammation. CAV1 has also been reported to have a dual role as either a tumor suppressor or tumor promoter, depending on the type of cancer. Inflammation is an important player in tumor progression, but the role of caveolin-1 in generating an inflammatory milieu remains poorly characterized. We used a caveolin-1-knockout ($CAV1^{-/-}$) mouse model to assess the inflammatory status via the quantification of the pro- and anti-inflammatory cytokine levels, as well as the ability of circulating lymphocytes to respond to nonspecific stimuli by producing cytokines. Here, we report that the $CAV1^{-/-}$ mice were characterized by a low-grade systemic proinflammatory status, with a moderate increase in the IL-6, TNF-α, and IL-12p70 levels. $CAV1^{-/-}$ circulating lymphocytes were more prone to cytokine production upon nonspecific stimulation than the wild-type lymphocytes. These results show that CAV1 involvement in cell homeostasis is more complex than previously revealed, as it plays a role in the inflammatory process. These findings indicate that the $CAV1^{-/-}$ mouse model could prove to be a useful tool for inflammation-related studies.

1. Introduction

A low-grade chronic inflammatory status is defined as a persistent, mild increase (2 to 4 times greater than normal) in circulating inflammation mediators [1]. Chronic inflammation is associated with a plethora of conditions, including aging (inflammaging) [2], autoimmune diseases [3], cardiovascular pathologies [4, 5], and carcinogenesis [6], as well as the formation and maintenance of a (pre)metastatic niche [7]. Systemic contributors to chronic inflammation are endothelial cells and immune cells, which are also now recognized as important players in tumorigenesis and metastasis [8, 9]. Caveolin-1 (CAV1), the scaffold protein of caveolae, could represent a link between inflammation and tumorigenesis, as it has been associated with both processes. In addition to its scaffolding role, CAV1 acts as a "guardian" by selecting the messages that are sent into cells from the outer environment. CAV1 recruits various cytoplasmic proteins involved in cell signaling via its caveolin-scaffolding domain. Loss of CAV1 has been associated with a proinflammatory status in senescent endothelial cells [10] and with premature senescence in fibroblasts [11] and was protumorigenic for selected cancers, such as prostate [12] and gastric [13] cancer and glioblastoma [14]. Loss of CAV1 in stromal cells, most notably in the cancer-associated fibroblasts, negatively affected the relapse-free survival of prostate cancer [15], breast cancer [16], and gastric cancer [17] patients. However, whether the lack of CAV1 is directly correlated with chronic inflammation has been insufficiently explored. The involvement of CAV1 in inflammation has only been sporadically addressed, with reports mainly focused on the evaluation of endothelial cells and their role in atherosclerosis [18, 19] and the lung response to sepsis [20–22]. For immune cells involved in the production of inflammatory mediators,

CAV1 has seldom been reported as related to lymphocyte migration [23, 24] and the inhibition of proinflammatory cytokine production in macrophages [18].

The aim of this study was to specifically address the hypothesis that the loss of CAV1 is involved in the pathogeny of the inflammatory response. We examined more than 30 pro- and anti-inflammatory cytokines in the plasma of $CAV1^{-/-}$ mice to assess their inflammatory status, as well as the ability of circulating leukocytes to respond to nonspecific stimuli through the production of cytokines.

2. Materials and Methods

2.1. Mice and Sample Collection. Blood samples were obtained from $CAV1^{-/-}$ mice (CAV1 KO: $CAV1^{tm1Mls}$/J) and $CAV1^{+/+}$ mice (B6129PF2/J), purchased from Jackson Laboratory (Bar Harbor, ME) ($n = 9$). For this study, we used 3-month-old male knockout mice weighing 22 ± 4 g and age-, gender-, and weight-matched control mice. All animal experiments were conducted in accordance with the respective animal welfare guidelines, the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health, and the study was approved by the Institutional Ethical Committee of "Victor Babes" National Institute of Pathology in Bucharest. The adult mice were fed with standard chow and water ad libitum.

2.2. Plasma Preparation. Collection of whole peripheral blood from knockout mice, the STOCK $CAV1^{tm1Mls}$/J and control B6129PF2/J mice, was achieved using vacuum blood tubes (Systems, Becton Dickinson) with heparin (for cell culture/plasma). For plasma extraction, the blood was allowed to clot for at least 30 min at room temperature before centrifugation at 2500 rpm for 10 min. Samples were then aliquoted and stored at −80°C until the multiplex analyses. The plasma samples were collected from mice in a consistent manner, at the same time of the day, between 10:00 a.m. and 11:00 a.m.

2.3. Cell Culture. Whole peripheral blood of both the $CAV1^{-/-}$ and control mice was obtained through retroorbital blood collection and diluted to 5% with RPMI-1640 culture medium (supplemented with 1% antibiotic), in the absence and presence of the polyclonal lymphocyte stimulator, 5 mg/L PHA (Difco, Augsburg, Germany), or 5 mg/L ConA (Difco, Augsburg, Germany) [25]. Whole-blood cell culture was performed in 96-well round bottom plates (Corning CLS3360); after the indicated exposure time to compounds, the plates were centrifuged at $250g$ for 10 minutes and 100 μL supernatants from each sample was collected and stored in 1.8 mL cryo tubes. Cultures were incubated for 24 h and 48 h at 37°C and 5% CO_2 (Shell Lab). Samples were made in triplicate. After 24 h or 48 h of treatment, the supernatant was removed, following centrifugation for 5 min at $250g$. Samples were stored at −80°C until further analysis.

2.4. Assessment of Cytokines by xMAP Analysis. The xMAP array was performed according to the manufacturer's protocols, and the plates were analyzed using a Luminex® 200™ system (Luminex, Austin, TX). Cell culture cytokine levels were determined using the Fluorokine MAP Mouse Base Kit (R&D Systems, USA), with the following analyte-specific bead sets: *GM-CSF*, *IL-1β*, *IL-2*, *IL-4*, *IL-5*, *IL-6*, *IL-10*, *IL-12p70*, *IL-13*, *IL-17*, *CCL2/JE/MCP-1*, *CXCL1/KC*, *MIP-2*, *TNF-α*, and *VEGF*. Plasma cytokine levels were determined using the MILLIPLEX MAP Cytokine Magnetic Bead Panel Kit—*GM-CSF*, *IL-1β*, *IL-2*, *IL-4*, *IL-6*, *IL-12p70*, *IL-13*, *CXCL1/KC*, *VEGF*, and *TNF-α* (Merck-Millipore, Billerica, MA, USA). Briefly, the beads were incubated with the samples, buffers, and standards in a 96-well plate at 4°C overnight. All further incubations with the detection antibodies and streptavidin phycoerythrin (SAPE) conjugate were performed at room temperature in the dark with shaking at 800 rpm. Multiplex data acquisition and analysis were performed using STarStation 2.3 (Applied Cytometry Systems, Sheffield, UK) and xPONENT 3.1 software (Millipore, Billerica, MA); the calibration curves were generated with a 5-parameter logistic fit.

2.5. Proteome Profiler™ Antibody Array: A Membrane-Based Assay. Array images were scanned with MicroChemi 4.2 (Berthold Technologies, Chennai, India), and the signal intensity of each spot was analyzed with ImageJ software; the average intensity was calculated by subtracting the average background signal. The cytokine profile assessment, including *CXCL13/BLC/BCA-1*, *IL-5*, *M-CSF*, *C5a*, *IL-6*, *CCL2/JE/MCP-1*, *G-CSF*, *IL-7*, *CCL12/MCP-5*, *GM-CSF*, *IL-10*, *CXCL9/MIG*, *CCL1/I-309*, *IL-12 p70*, *CCL3/MIP-1α*, *CCL11/eotaxin*, *IL-13*, *CCL4/MIP-1β*, *ICAM-1*, *IL-16*, *CXCL2/MIP-2*, *IFNγ*, *IL-17*, *CCL5/RANTES*, *IL-1α*, *IL-23*, *CXCL12/SDF-1*, *IL-1β*, *IL-27*, *CCL17/TARC*, *IL-1ra*, *CXCL10/IP-10*, *TIMP-1*, *IL-2*, *CXCL11/I-TAC*, *TNF-α*, *IL-3*, *CXCL1/KC*, *TREM-1*, and *IL-4*, was performed using Mouse Cytokine Array Panel A (R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer's instructions. Briefly, after the membrane blocking, the plasma samples and detection antibody cocktail were added and incubated overnight at 4°C on a rocking platform shaker. After the unbound proteins were removed by washing, the membranes were incubated with a streptavidin-HRP solution for 30 min at room temperature on a rocking platform and then washed again. Subsequently, protein spots were visualized using the chemiluminescence detection reagents.

2.6. Statistical Analysis. Data were expressed as mean ± standard error of the mean (SEM), and minimum and maximum values were provided when necessary. Duplicate/triplicate samples were used for all specimens, and the average concentrations were used for statistical analysis. Differences between groups were analyzed by two-tailed unpaired Student's t-test. Statistical significance has been indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$. Statistical analysis was performed using GraphPad Software.

3. Results

In order to evaluate the inflammatory status of the $CAV1^{-/-}$ mice, we assessed the levels of circulating pro- and anti-inflammatory cytokines and growth factors in plasma. The

pattern of the cytokine and growth factor production in the CAV1$^{-/-}$ mice, compared to the controls, was evaluated by two different multiplex analyses: xMAP technology and proteome profiler analysis. We examined adult animals, for which we confirmed the lack of CAV1 expression, before the onset of any macroscopic tumors (data not shown), in order to discriminate between existing pretumor inflammation and a tumor-driven inflammatory milieu, as these mice have been reported to be prone to tumorigenesis [26].

3.1. Increased Levels of IL-6, TNF-α, and IL-12p70 in the Plasma of CAV1$^{-/-}$ Mice. Overexpression of the plasma levels of the proinflammatory cytokines IL-6 (over a 5-fold change in the KO mice versus control, $p < 0.001$) and TNF-α and IL-12p70 (over a 3-fold change, $p < 0.05$) was detected using the xMAP Luminex 200 platform. IL-4, as well as CXCL1/KC, was also found to be upregulated in the KO mice compared to the control mice (over a 3-fold change and up to 2-fold change, resp.; $p < 0.05$) (Figure 1).

3.2. Proteome Profiler Analysis of Proinflammatory Cytokines and Chemokines in the Plasma of CAV1$^{-/-}$ Mice. In order to establish an overall perspective of the inflammatory status of the CAV1$^{-/-}$ mice, we also performed an array analysis of multiple circulating pro- and anti-inflammatory cytokines and growth factors in plasma. A dot blot assay revealed a relevant pattern for the proinflammatory status (Figures 2(a) and 2(c)). The results showed overexpression of the majority of the cytokines and growth factors in the KO mice, especially for IL-6 (8.6-fold increase in the KO mice versus the control), IL-5 (5.8-fold increase), IL-12p70 (3.8-fold increase), CXCL13/BLC (2.7-fold increase), and G-CSF, CCL2/JE/MCP-1, TARC, and TIMP-1 (~1.7-fold increase for these 4). By comparing the KO and control groups for cytokine expression, the obtained dot blot values were similar to the outline obtained by the xMAP array analysis.

Proteome profiler analysis confirmed that a set of cytokines, chemokines, and growth factors was overexpressed in the plasma of the CAV1$^{-/-}$ mice compared with that of the control mice, with significant differences for CXCL13/BLC, G-CSF, GM-CSF, CCL1/I-309, IL-3, and CXCL10/IP-10 ($p < 0.05$).

At this point in our study, we concluded that the CAV1$^{-/-}$ mice are characterized by a low-grade systemic proinflammatory status.

3.3. Nonspecific Stimulation of the Lymphocytes of KO Mice with PHA and ConA. CAV1-KO mice were previously reported to show no changes in the percentages of lymphocyte subpopulations [27]; therefore, we used whole peripheral blood to initiate cell cultures and treated them with lymphocyte-targeting stimulants, that is, concanavalin A (ConA) and phytohemagglutinin (PHA). Using whole peripheral blood was reported as a valid method to assess cytokine production [25, 28]. We assessed whether the lymphocytes' response to stimuli is modified by the chronic inflammatory milieu. We found that upon stimulation with ConA or PHA, the production of cytokines/chemokines and growth factors increased, showing that even if these cells are derived from a medium abundant with proinflammatory cytokines, their response has not reached saturation.

Overall, we noticed an activated status of the CAV1$^{-/-}$ lymphocytes, characterized by an increased response to PHA and ConA stimulation by IL-6, TNF-α, CXCL1/KC, IL-4, and IL12p70, while IL-1β did not show the same trend (Figures 3(a) and 3(f)).

Expression of IL-6 in the CAV1$^{-/-}$ lymphocytes increased at 24 h (7.4-fold compared to the control) and 48 h (17-fold versus the control), following ConA stimulation (Figure 3(a)).

Although TNF-α secretion was not inducible in the control lymphocytes, it increased in the CAV1$^{-/-}$ lymphocytes upon stimulation. The relative increase was 2.8-fold and 7-fold, at 24 h and 48 h, respectively, for ConA stimulation and 5.8-fold and 14-fold, at 24 h and 48 h, respectively, for PHA stimulation (Figure 3(b)). CXCL1/KC was also overexpressed to 1.8-fold and 3.6-fold, at 24 h and 48 h, respectively, for ConA stimulation and to 2.7-fold and 5-fold, at 24 h and 48 h, respectively, for PHA stimulation (Figure 3(c)).

Expression of IL-12p70 in the CAV1$^{-/-}$ lymphocytes increased, but only for the first 24 h after ConA stimulation (3-fold compared with the control), as well as after PHA stimulation (2.5-fold compared with the control) (Figure 3(d)).

IL-4 expression was also elevated in a time-dependent manner upon stimulation in the CAV1$^{-/-}$ mice. Thus, 24 h after ConA stimulation, we observed an increase of almost 7-fold compared to the control, and at 48 h, the increase was 10-fold (Figure 3(e)). Similar to the control lymphocytes, the CAV1$^{-/-}$ lymphocytes were nonresponsive to PHA stimulation.

For IL-1β, the response of the CAV1$^{-/-}$ lymphocytes was time-dependent following ConA stimulation (1.5-fold and 2.5-fold compared to the control at 24 h and 48 h, resp.) (Figure 3(f)).

In conclusion, the circulating levels of the proinflammatory cytokines could generate a chronic inflammatory status; additionally, the lymphocytes of these mice are readily responsive to stimuli, further contributing to the inflammatory status, which can be a useful tool for future studies of the tumor environment or other low-grade chronic inflammatory diseases.

4. Discussion

CAV1 has been repeatedly linked to cancer progression, either as a tumor suppressor, as its absence is associated with a poor prognosis [29] (e.g., aggressive prostate cancer [30, 31], breast cancer [32], and gastric cancer [33]), or as a tumor and metastasis promoter [34–38]. CAV1-KO mice have been used extensively as a model to investigate tumor-related mechanisms, such as tumor growth, pathologic angiogenesis, and tumor invasion [26, 39]. In addition, CAV1-KO cells also activate inflammation-related signaling pathways (e.g., Akt signaling, TLR4 signaling, and ERK signaling), resulting in the production of proinflammatory cytokines, chemokines, and extracellular matrix remodeling enzymes [40]. Less is known about the contribution of CAV1 to the inflammatory milieu. Thus far, studies on CAV1 and inflammation

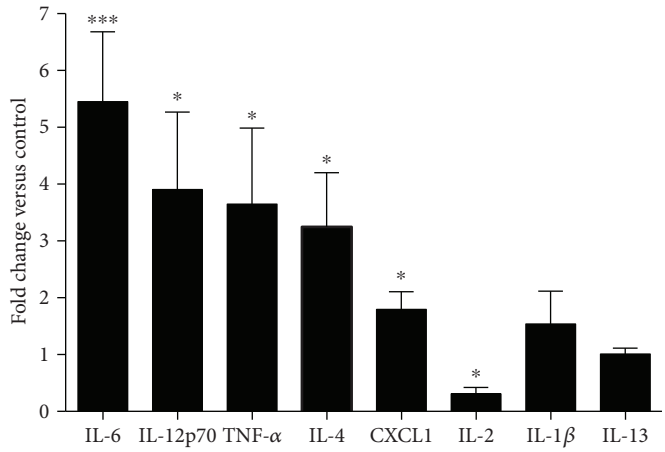

Figure 1: Cytokine levels in the plasma of the KO mice ($n = 9$) compared to the controls ($n = 9$). The data represent the average fold modification ± SEM versus the controls, as determined by the xMAP multiplex assay; molecules have been arranged in order of statistical significance. $^{*}p < 0.05$ and $^{***}p < 0.001$ indicate statistical significance compared with the controls.

have focused on organ-specific effects (lung [41, 42], colon [43, 44], and eye [45]). Additionally, different subsets of leukocytes derived from CAV1-null mice have been analyzed in terms of response to either parasitic [46] or bacterial infection [27]. Also, the ability of lymphocytes from CAV1-null mice to induce a humoral [47] or cytotoxic immune response [48] has been reported. However, studies have not focused on systemic inflammation in the absence of immune triggers.

CAV1 has also been linked with oxidative stress, in a dual manner. On the one hand, CAV1 was shown to be a "critical determinant" of oxidative stress balance. Using the same CAV1-null mouse model, Shiroto et al. found that the redox stress plasma biomarker 8-isoprostane was elevated in the blood of these mice and its involvement in oxidative stress was confirmed by knocking down CAV1 in endothelial cells in an in vitro model [49]. Furthermore, CAV1 was recently pinpointed as a target in cancer-related oxidative stress (reviewed in [50]). The relationship between CAV1, oxidative stress, and inflammation has been best studied in the tumor microenvironment, where loss of CAV1 was reported to lead to oxidative stress and to drive inflammation [51]. Moreover, loss of stromal CAV1 in a tumor model was proposed as a marker of inflammation and a predictor of poor outcome [52].

On the other hand, interesting data emerged about a direct interaction between CAV1 and nuclear factor erythroid 2-related factor 2 (NFE2L2/NRF2) [53], a transcription factor known as "master regulator of oxidative stress response" [54]. In this regard, inhibition of NRF2-mediated signaling by CAV1 activates the p53/senescence pathway [53] and inhibits antioxidant enzymes with antioxidant response element- (ARE-) dependent gene sequences [55].

In our study, we hypothesized that CAV1 has an effect on the systemic inflammation status. We addressed the involvement of CAV1 in enhancing the inflammatory response and creating a low-grade systemic inflammatory milieu. We verified the inflammation status by assessing the pro- and anti-inflammatory cytokine levels in the plasma of $CAV1^{-/-}$ mice compared with that of control mice.

4.1. $CAV1^{-/-}$ Mice Are Characterized by a Low-Grade Systemic Proinflammatory Status. To investigate whether the absence of CAV1 is related to a low-grade systemic inflammatory milieu, we performed a series of *in vitro* experiments examining the plasma protein levels of different cytokines in caveolin-1-deficient mice ($CAV1^{-/-}$). To this end, we investigated the expression of circulating proinflammatory (IL-1β, IL-2, IL-6, IL-17, TNF-α, IL-8, IFNγ, CSF, and IL-12p70) and anti-inflammatory (IL-4, IL-10, and IL-13) members of the cytokine family in CAV1-KO mice, before the onset of any clinically overt tumors.

We showed that $CAV1^{-/-}$ mice have enhanced plasma levels of a number of proinflammatory cytokines, including IL-1β, IL-2, IL-6, IL-12, and TNF-α, compared with those in the control mice (Figure 1). Inflammation is an important component of the tumor milieu and of the premetastatic niche, in which IL-6 is an important player [56, 57]. Our results are in agreement with recent findings that link CAV1 expression and IL-6 production. Lee et al. reported that the degradation of CAV1, via the ubiquitin/proteasome pathway, leads to TLR4 activation and the enhanced production of proinflammatory cytokines in bone marrow-derived macrophages [58]. *In vitro* silencing of CAV1 in mouse keratinocytes has been linked to STAT3 signaling activation, leading to increased expression of IL-6 [59]. Additionally, decreased expression of CAV1 in monocytes from diabetic peripheral neuropathy patients was negatively correlated with IL-6 and TNF-α plasma levels [60]. Taken together, these results support the fact that CAV1 expression is negatively correlated with IL-6 levels.

Weiss et al. also correlated the loss of CAV1 with increased TNF-α and other proinflammatory cytokines in a mouse colitis model [61].

Additionally, we found elevated levels of the anti-inflammatory cytokine IL-4 in the $CAV1^{-/-}$ mice. However, these levels did not increase to the same extent as IL-6, as indicated by the IL-6 : IL-4 ratio of ~1 in the control group and 1.67 in the $CAV1^{-/-}$ mice. One could speculate that IL-4 increases in the $CAV1^{-/-}$ mice as a systemic reaction to

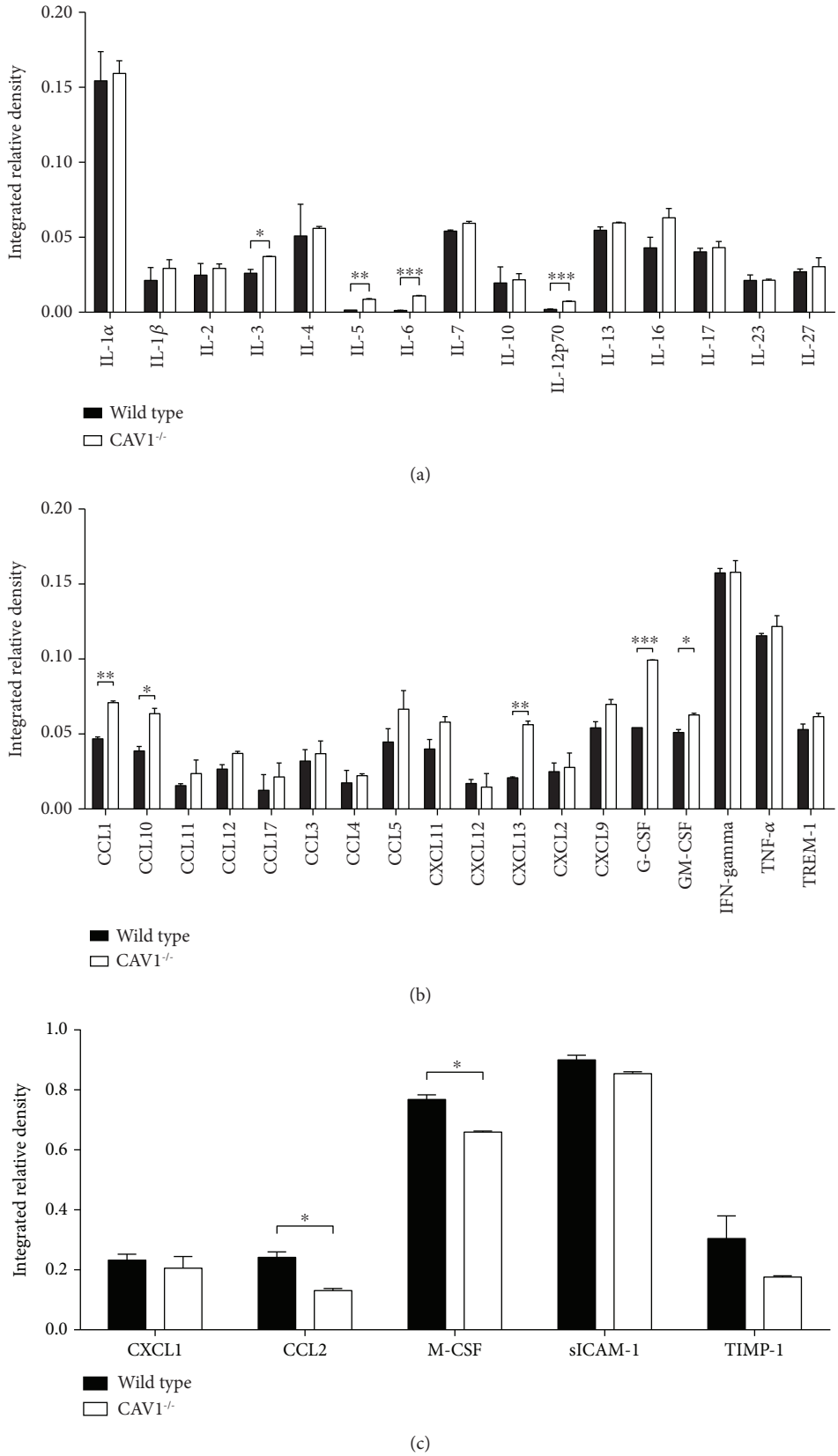

Figure 2: Relative expression levels of various mediators of inflammation. Pro- and anti-inflammatory cytokines (a), growth factors (b), and enzymes (c) involved in inflammatory processes were assessed in the plasma of the $CAV1^{-/-}$ and control mice using the Proteome Profiler. The data represent the average of the experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

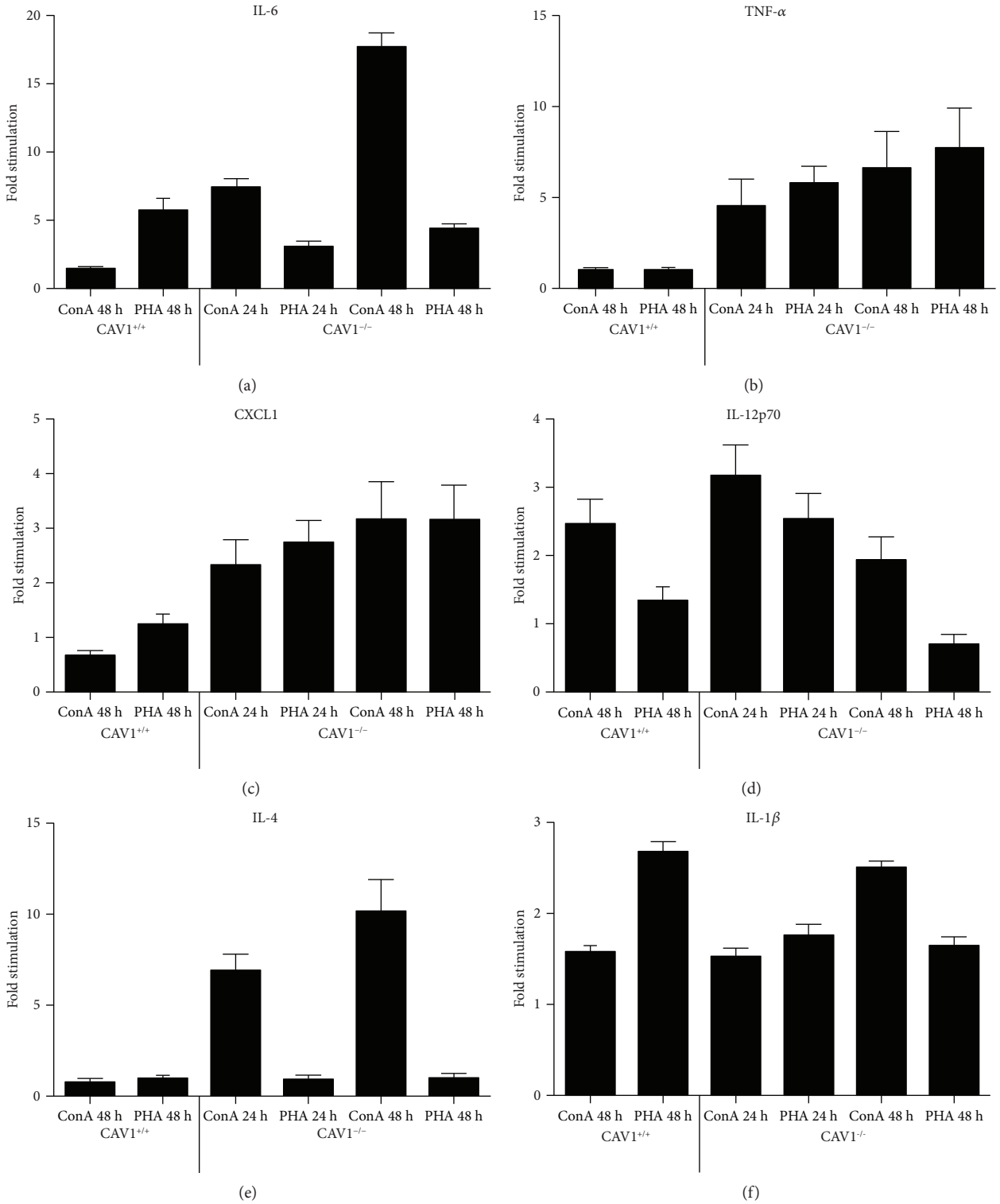

Figure 3: Cytokine production by lymphocytes upon nonspecific stimulation. Cytokine levels in the cell culture media of whole blood cells, treated with nonspecific lymphocyte stimuli at different time points after stimulation, as assessed by the xMAP multiplex assay: IL-6 (a), TNF-α (b), CXCL1 (c), IL-12p70 (d), IL-4 (e), and IL-1β (f). The data represent the fold modification of the cytokines from the CAV1$^{-/-}$-derived cells versus the controls.

compensate for the increase in the proinflammatory cytokines (IL-6 and TNF-α), but further investigation would be needed to validate such a mechanism.

The significant increase in IL-6 was confirmed by the Proteome Profiler, along with that in TNF-α and IL-12p70 (Figure 2). However, not all of the tested cytokines showed

a modification of their circulation levels, compared with those in the control. As CAV1 has been previously described as an inhibitor of cell signaling, a loss of function can be correlated with the activation of different signaling pathways that result in subsequent cytokine production [51]. The significant increase in IL-6 reported in our study correlates with the activation of the JAK2/STAT3 signaling pathway reported by Yuan et al. in the lung endothelium of CAV1-KO mice [62], which was involved in *IL-6* gene transcription.

4.2. CAV1$^{-/-}$ Lymphocytes Produce Enhanced Levels of Cytokines upon Stimulation. Endothelial cells and lymphocytes are common cellular sources of cytokines, and it has been demonstrated that endothelial inflammation is suppressed by CAV1 under physiological conditions [63]. Although uncontrolled inflammatory responses have been reported previously in relation to the loss of CAV1 [64], most of these studies have addressed the involvement of the lung endothelium [20, 21]. Owing to the abundance of caveolae in endothelial cells, these cells were the main target of CAV1 inflammation studies [10, 21, 65, 66]. To complement those studies, we addressed the contribution of other cells, namely, circulating lymphocytes, to cytokine production. Lymphocytes were initially considered negative controls for CAV1 expression, as they do not form caveolae, unless transfected with CAV1 [67]. Caveolin-1 has been detected and reported in leukemic cells [68, 69], possibly in conjunction with its involvement in cancer, and may reflect tumorigenic changes. We tested the response of CAV1-KO lymphocytes to nonspecific stimulation, which was quantified by the cytokine output in cell culture, as a possible tool to study low-grade inflammation. Our results showed that CAV1-KO lymphocytes are responsible for the production of various types of pro- and anti-inflammatory cytokines, depending on the type of stimulation and exposure time (Figures 3(a) and 3(f)). These results are supported by a number of studies reporting the role of CAV1 in primary T cells [48] and splenic B cells stimulated with LPS [27], as well as a recent report underlining the involvement of CAV1 in the regulation of B cell tolerance [47].

5. Conclusions

A CAV1-KO mouse model has been intensely used as a tool to study endothelial dysfunction, as well as tumor biology, owing to the increased susceptibility of these mice to cancer [70]. We hypothesized that CAV1 loss could also be involved in inflammation, which is a common feature of many pathologies, from cardiovascular diseases to tumor development. We demonstrated the existence of a low-grade systemic inflammatory milieu, characterized by moderately increased plasma levels of IL-6, TNF-α, and IL12-70p. Circulating lymphocytes of the CAV1$^{-/-}$ mice were overresponsive to stimuli, indicating that these cells may contribute to the maintenance of this low-grade systemic inflammatory environment. Lymphocytes could also prove to be a useful tool to assess anticancer therapies that target inflammation. Our findings showed that CAV1-KO mice can also be used as an *in vivo* model for studying inflammation and could serve in the assessment of the anti-inflammatory effect of potential novel therapies. Given the strong association between inflammation and cancer [71–73], CAV1-KO mice may be useful for studies focusing on the intricate connections between inflammation and cancer. Finally, it could be added that CAV1, besides being a tumor suppressor, can also act as an inflammation suppressor that can be considered in the studies on CAV1-null tumors.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interests regarding the publication of this paper.

Authors' Contributions

Elena Codrici, Lucian Albulescu, Ionela Daniela Popescu, and Simona Mihai contributed equally to this work.

Acknowledgments

The authors would like to thank the Animal Facility staff of the "Victor Babes" Institute for the support with the handling of the animals and sample collection. This work was partly supported by grants from the Ministry of Research and Innovation Core Program (PN 18.21.01.06 and PN 18.21.02.01).

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 5315816, 10 pages
https://doi.org/10.1155/2018/5315816

Review Article

New Insights in the Pathogenesis of HPV Infection and the Associated Carcinogenic Processes: The Role of Chronic Inflammation and Oxidative Stress

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Received 13 July 2018; Accepted 8 August 2018; Published 27 August 2018

Academic Editor: Iulia D. Popescu

Human papillomavirus (HPV) is a small double-stranded DNA virus with tropism for epithelial cells. To this date, over 150 genotypes are known and are classified into two major groups, low-risk and high-risk strains, depending on the ability of the virus to induce malignant transformation. The host's immunity plays a central role in the course of the infection; therefore, it may not be clinically manifest or may produce various benign or malignant lesions. The pathogenic mechanisms are complex and incompletely elucidated. Recent research suggests the role of chronic inflammation and oxidative stress (OS) in the pathogenesis of HPV infection and the associated carcinogenic processes. Chronic inflammation induces OS, which in turn promotes the perpetuation of the inflammatory process resulting in the release of numerous molecules which cause cell damage. Reactive oxygen species exert a harmful effect on proteins, lipids, and nucleic acids. Viral oncogenes E5, E6, and E7 are involved in the development of chronic inflammation through various mechanisms. In addition, HPV may interfere with redox homeostasis of host cells, inducing OS which may be involved in the persistence of the infection and play a certain role in viral integration and promotion of carcinogenesis. Knowledge regarding the interplay between chronic inflammation and OS in the pathogenesis of HPV infection and HPV-induced carcinogenesis has important consequences on the development of new therapeutic strategies.

1. Introduction

Numerous reports have pointed out the close link between chronic inflammation, oxidative stress (OS), and carcinogenesis [1]. A factor able to trigger and sustain an inflammatory process involving activation of various cell types, and an increase in reactive oxygen species (ROS) production, could be regarded as a potential promoter of carcinogenesis [2]. Thus, despite being a form of host defence against a pathogen, chronic inflammation can contribute to malignant progression [3].

OS is defined as the imbalance between oxidants and antioxidants in favour of the former, a condition that can lead to the alteration of various cell components and cellular signalling mechanisms [4]. It is important to emphasize that ROS should not be always regarded as harmful molecules. At

lower levels, under physiological conditions, ROS participate in various biological events (control of vascular tone, ventilation, redox homeostasis, etc.) [5]. However, elevated levels are associated with the generation of OS [6]. How cells respond and adapt to OS conditions is mainly influenced by certain cell receptors and the levels of antioxidants [7].

Several studies have suggested that in human papillomavirus (HPV) infection and the associated carcinogenic processes, chronic inflammation and OS are important cofactors, a fact which may confer new perspectives on its pathogenesis and therapeutic approach [8].

2. HPV Replication Cycle

HPV is a nonenveloped and double-stranded DNA virus with tropism for epithelial cells [9]. Its genome comprises early genes E1, E2, E4, E5, E6, and E7 that code for proteins involved in the pathogenicity of the virus and late genes, L1 and L2 that code for capsid proteins, and a noncoding regulatory long control region [10, 11].

HPV penetrates the epithelial tissues through small injuries, and its life cycle is closely related to the keratinocyte differentiation process. As keratinocytes progress to the spinous layer, amplification of gene expression accompanied by viral DNA replication occurs. The expression of viral genes was only evidenced in keratinocytes [12], but the receptor with which the virus interacts on basal cells is still unknown [13]. However, it seems that heparan sulfate proteoglycan, a structural component of the extracellular matrix, along with α6-integrin and laminin-5, has a pivotal role. The virus is internalized into the cell through clathrin- or caveolae-mediated endocytosis and penetrates the nucleus where the replication process is initiated [14, 15].

After the infection of basal cells, including stem cells, with a high rate of division, the expression of viral genes is activated, generating up to 100 extrachromosomal copies of HPV DNA per cell. E1 and E2 proteins bear an important role in this stage. These two proteins form a complex that binds to the viral replication origin participate in the recruitment of cellular polymerases and accessory proteins and modulate the DNA replication. Regarding the role of E4 and E5 proteins, further studies are required, but it seems that they modulate the late viral functions. In addition, E6 and E7 proteins exert a stimulatory effect on viral proliferation. After penetrating into the suprabasal layers, the expression of the late viral genes is initiated, resulting in a spontaneously self-assemble into an icosahedral capsid. Therefore, the virions are assembled to be released and infect new cells [13, 14, 16, 17].

3. HPV Infection and Carcinogenesis

HPV is involved in a variety of cutaneous or mucous and benign or malignant lesions [18]. To this date, over 150 genotypes are known and are classified into two major groups, low-risk and high-risk strains, depending on the ability of the virus to induce malignant transformation. In most cases, the infection is asymptomatic and spontaneously resolves through the participation of the host immune response. In some instances, the infection can be latent and reactivate under certain conditions such as immunosuppression. In a small number of cases, especially when HPV types 16 and 18 are involved, the lesions progress into invasive cancer [19, 20]. The pathogenic mechanisms of the infection are complex and incompletely elucidated.

Early onset of sexual life and multiple sexual partners facilitate the contraction of HPV; most sexually active women becoming infected. In 90% of cases, the clearance of the infection occurs in the first 2 years, but in the other 10%, the infection becomes persistent and may progress to malignant lesions [21, 22].

Cervical intraepithelial neoplasia (CIN) is a precancerous lesion, the precursor of cervical cancer. CIN is classified as CIN 1, CIN 2, and CIN 3, depending on the degree of dysplasia, namely, mild, moderate, or severe. Clinically, the lesion is asymptomatic and may regress spontaneously or progress to invasive cancer over a certain period of time [23].

Cervical cancer is an important cause of death among women worldwide. Most cases, over 80%, are noticed in developing countries where the screening programs are not correctly implemented. HPV infection is the leading cause of cervical cancer. HPV 16 and 18 are the main types involved, being identified in over 70% of cases [24, 25]. Other high-risk types involved in cervical cancer are 31, 33, 35, 39, 45, 51, 52, 56, 58, etc. [26].

The most important players involved in the malignant transformation of HPV-related lesions are E6 and E7 proteins. They have the ability to interfere with cell proliferation and differentiation. These proteins are found both in low-risk and high-risk types. E6 of the high-risk types has been found in the nucleus and cytoplasm and can be associated with 12 proteins, such as paxillin, p300/CBP, etc. [16]. E6 has the ability to induce the formation of a complex with ubiquitin ligase (UBE3A) and p53, resulting in the ubiquitination of p53 and its degradation by the 26S proteasome, which leads to a decrease in p53 turnover [27, 28].

E7 is mainly found in the nucleus and has the ability to bind to retinoblastoma (Rb) protein, inactivating its function [16]. The Rb protein is the key regulator of the cell cycle. Therefore, E7 modulates cell proliferation and promotes early cell entry into the S phase of the cell cycle. E7 also interacts with p21 and p27, important regulators of the cell cycle as well [29]. Normally, in such conditions, p53-mediated apoptosis is induced, but as previously mentioned, E6 has the ability to inhibit p53 function [30, 31]. The study by Riley et al. revealed that E7 transgenic mice develop both low-risk and potentially malignant lesions, while E6 transgenic mice only develop low-risk lesions. In combination, the two proteins lead to extensive malignant invasive lesions [32]. Moreover, by inhibiting E6 and E7 genes, some cells that had achieved malignant features returned to their previous benign phenotype [33].

4. Inflammation: A Double-Edged Sword

Inflammation is the first step of host immune defence against various harmful stimuli; it is a mechanism of innate immunity, with an important role in immunosurveillance [34].

The process starts by activating the immune cells which migrate to the site of inflammation and release various mediators, including cytokines, ROS, and hormones that will maintain the inflammatory response [35, 36]. The main cells involved are neutrophils, eosinophils, monocytes, mast cells, platelets, and fibroblasts [37]. Chemokines are the chief mediators involved in the recruitment of leukocytes, being grouped into four major classes depending on their structure and role. The largest class is represented by CC chemokines that promote the migration of mononuclear cells into inflamed tissues [38].

The persistence of a certain type of cytokines can promote chronic inflammation [39]. In those instances, in which the inflammatory process does not cease and persists, it becomes harmful to the host and leads to the alteration of numerous intracellular signalling pathways [40]. The main mechanisms involved are infectious and autoimmune, but in numerous instances, the cause of inflammatory process is unknown [41]. Chronic inflammation appears to underlie the pathogenic mechanisms of many diseases, such as cardiovascular, pulmonary, osteoarticular, or neurological diseases. It is worth noting that chronic inflammation has been shown to play a crucial role in carcinogenesis, contributing to cellular transformation, proliferation, invasion, and angiogenesis [34].

5. The Generation and Effects of Oxidative Stress

Free radicals (a term not properly used as the radicals are always free [42]) are molecules characterized by the presence of unpaired electrons in their atomic or molecular orbitals, which confer them a high reactivity [43]. The main reactive oxygen species are O_2^- (superoxide anion), H_2O_2 (hydrogen peroxide), and $OH^\bullet$ (hydroxyl radicals) [44, 45]. The mitochondrion is the primary site of ROS generation, which is the respiratory chain producing a large amount of free radicals during oxidative phosphorylation [6]. Seven major mitochondrial sites involved in the production of ROS have been described [46]. The main extramitochondrial sources are endoplasmic reticulum (ER), peroxisomes, nicotinamide adenine dinucleotide phosphate (NADPH), and xanthine oxidase [44, 47]. Regarding reactive nitrogen species (RNS), the central role is played by nitric oxide ($NO^\bullet$), which is formed by the conversion of L-arginine to citrulline, a reaction catalyzed by NO synthase. $NO^\bullet$ can react with O_2^- resulting in the formation of peroxide nitrite ($ONOO^-$), a product with toxic effect [4, 48].

Antioxidants are the most important weapons against OS damage. The main enzymes with antioxidant activity are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Other molecules involved in the antioxidant defence are glutathione, tocopherols, carotenes, and ascorbic acid [49–51].

When an inflammatory process is triggered by a certain stimulus, numerous cells are recruited and release various mediators including proinflammatory cytokines that will promote OS, which is part of the first line mechanisms of host defence [3]. Thus, a chronic inflammatory process induces the formation of ROS with harmful effects on cell structures (lipids, proteins, and nucleic acids), creating favourable conditions for malignant transformation [52, 53]. Studies have shown that ROS produce about 10,000 changes in the DNA bases at the level of a single cell per day [54]. ROS cause alterations of purine and pyrimidine bases (resulting in modified bases), loss of purines (resulting in abasic sites), and single and double strand breaks and cross-links to other molecules [55, 56].

Protein oxidation is mainly the consequence of $OH^\bullet$ attack, resulting in modified proteins, which by overcoming proteolysis mechanisms accumulate in cells and lead to alteration of cell function. The protein degradation increases up to 11 times more when the cells are exposed to ROS. Oxidation occurs directly through ROS damage or indirectly by the attack of the molecules derived from lipid peroxidation [5, 54]. The main compounds resulting from protein oxidation are carbonyl adducts [57]. Membrane structures contain significant amounts of lipids; therefore, they are highly vulnerable to OS [42]. The lipid susceptibility to ROS is closely related to the number of double bonds within the acyl chain. Thus, polyunsaturated fatty acids are more susceptible when compared to monounsaturated or saturated fatty acids [58, 59]. The two major lipid peroxidation derivatives are hydroxynonenal (HNE) and malondialdehyde (MDA), which in turn cause damage to proteins and DNA [43, 58].

6. The Link between Inflammation, Oxidative Stress, and HPV-Related Carcinogenesis

Since many cases of HPV infection regress spontaneously, it has been hypothesized that cofactors are needed for virus persistence and development of a malignant process [8]. Carcinogenesis is a complex process and the deep mechanisms involved are still investigated [60–62]. Inflammation and OS are two interconnected conditions [1, 63] and various studies have focused on their role as major cofactors in the initiation of malignant transformation [11, 57, 63].

6.1. Inflammation: A Cofactor in HPV-Associated Carcinogenesis. Exposure of a tissue to chronic inflammation will induce mutations in susceptible cell populations. Cytokines and growth factors, released during inflammation, may be involved in genetic alteration [52, 64]. Chronic inflammation impairs cell homeostasis with effects on cell DNA and subsequently on normal cell growth and, as a consequence, the malignant transformation can be initiated [65].

Histopathological analysis of severe HPV-induced lesions exhibited an increased inflammatory infiltrate [66]. Of note, regarding the pathogenesis of HPV infection, inflammation does not seem to play a central role during the initial stages because the virus infects the basal cells which are not in contact with circulating immune cells [67]. The persistent infection favours chronic inflammation, which can also induce an imbalance between prooxidants and antioxidants. The inflammatory process leads to the release of proinflammatory cytokines such as interleukin (IL)-1, IL-6, tumor necrosis factor alpha, and interferon gamma, which activate protein kinase-mediated signalling pathways, resulting in

the formation of ROS [63]. Kemp et al. have found higher levels of proinflammatory cytokines in 50 women with HPV persistent infection compared to 50 healthy subjects [68]. In addition, inflammation causes a decrease in the level of antioxidants, the main weapons of the cells against oxidative damage; studies on cervical cancer patients have revealed low levels of antioxidant systems [63, 69].

Viral oncogenes E5, E6, and E7 are involved in the development of chronic inflammation associated with cervical cancer. These oncogenes lead to the increase in cyclooxygenase- (COX-) 2 expression and, consequently, to a high amount of prostaglandins with unfavourable effects on cervical tissue [70]. The released prostaglandins may be involved in the stimulation of cell proliferation, angiogenesis, and inhibition of apoptosis, which are crucial mechanisms in carcinogenesis [71]. The attracted inflammatory cells release ROS resulting in DNA damage, which underlies the malignant transformation [70]. The study by Kulkarni et al. revealed the increased COX-2 expression in samples from patients diagnosed with CIN or neoplastic cervical lesions. HPV oncoproteins are involved in the activation of AP-1, a transcription factor which participates in the overproduction of COX-2 [72]. In addition, the action of viral oncoproteins E6 and E7 on NF-kB signalling pathway plays an important role in the ability of HPV to alter the host inflammatory response. NF-kB suppression occurs and that contributes to the HPV escape from immune surveillance [36].

The study by Castle et al., including women infected with high-risk HPV types, revealed an association between cervical inflammation and the presence of high-grade cervical lesions, drawing attention to the role of inflammation as a risk factor for the progression of HPV infection to carcinogenesis [73]. In line with this, the study by Tonon et al. has highlighted that a risky sexual behaviour (early onset of sexual life and multiple partners) and a history of other sexually transmitted infections (involving a proinflammatory status) are linked to invasive malignant lesions [74].

Taking into consideration the hypothesis that inflammation is involved in HPV-induced malignancies, Hiraku et al. evaluated whether 8-nitroguanine, a by-product of inflammation, is involved in carcinogenesis. They included patients with CIN 1, 2, and 3 and condylomata acuminata, in all cases the diagnosis being histopathologically confirmed. Immunohistochemical studies have determined the presence of 8-nitroguanine, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), and p16 in the studied samples. The expression of 8-nitroguanine was higher in CIN samples than in condylomata acuminata. Moreover, 8-oxodG and 8-nitroguanine positively correlated with the CIN grade. Regarding p16, there were no differences between the studied groups. Thus, it seems that 8-nitroguanine is a better marker to predict the risk of carcinogenesis related to inflammation than p16 in HPV-infected patients [75].

It has been found that patients with high-grade squamous intraepithelial lesions or invasive lesions have higher levels of nitrite/nitrate in plasma and elevated expression of inducible NO synthase compared to normal subjects [76]. NO can be regarded as a marker of inflammation, being produced in epithelial cells and leucocytes under inflammatory conditions [77]. Chronic inflammation is associated with increased levels of NO and inducible NO synthase. Studies on HPV-infected cells have shown that NO can induce DNA alterations and downregulation of p53 and pRb, under the action of E6 and E7 oncoproteins, suggesting the role of NO in carcinogenesis [17, 78].

6.2. Role of Nonsteroidal Anti-inflammatory Drugs in Cervical Cancer. There is growing evidence suggesting that the decrease in expression of COX-2 prevents tumour growth, in particular, by inducing apoptosis and inhibiting angiogenesis. Based on this data, several studies have been conducted in order to assess the efficacy of nonsteroidal anti-inflammatory drugs (NAISDs) in various malignancies, including cervical cancer [79].

The study by Friel et al. revealed that frequent use of aspirin (≥7 tablets/week) could reduce the risk of developing cervical cancer [80]. In contrast, the study by Wilson et al. did not reveal that association [81].

The study by Ferrandina et al. has suggested that celecoxib, a COX-2 inhibitor, can be a useful agent in both prevention and therapy of cervical cancer. There was a decrease in tumour expression of COX-2 and markers of proliferation and angiogenesis such as Ki 67 and microvessel density, respectively, in samples collected from cervical cancer patients after 10 days of celecoxib treatment (400 mg twice daily) [82]. The study by Hefler et al. evaluated the effect of another COX-2 inhibitor, rofecoxib on CIN. They conducted a prospective, randomized, placebo-controlled, and double-blind study on 16 patients with CIN 2 and 3; 8 patients were treated with rofecoxib (25 mg/day for 6 months), and 8 patients received placebo. The regression rate was 25% for rofecoxib versus 12.5% for placebo, but no statistical significance was observed. The study was discontinued following the withdrawal of rofecoxib due to its cardiovascular side effects [83]. Another recent research focused on the assessment of celecoxib efficacy in patients with CIN 3, a stage in which regression is unlikely. The results showed that celecoxib 400 mg/day for 14–18 weeks did not decrease the degree of dysplasia, except for patients with an increased baseline level of vascular endothelial growth factor (VEGF), suggesting that VEGF may be used as a marker for detecting patients who could benefit from that type of treatment [84].

Another recent study investigated the effect of both nonselective (ibuprofen) and selective (celecoxib) agents on immortalized cervical cells. Both drugs decreased growth cell and induced apoptosis. The study concluded that NSAIDs are promising drugs in cervical cancer and to avoid adverse reactions that might occur during systemic administration, topical therapies could be useful [85]. Furthermore, there are studies showing that celecoxib may be associated with a higher degree of toxicity if it is added as an adjuvant to chemoradiation in cervical cancer patients [79].

Nonetheless, the Cochrane review published in 2014 and its updated version published in 2018 investigated the ability of NAISDs to induce regression and prevent progression of CIN and have shown that there is still insufficient evidence to support that fact [86, 87].

6.3. Oxidative Stress: A Cofactor in HPV-Associated Carcinogenesis. There are numerous studies attesting an increased OS in malignant cells [88–91]. This is the result of oncogenic mutations which contribute to an aberrant cell metabolism [92]. The increased metabolic activity, cell dysfunctions due to hypoxia, increased expression of growth factors, and last but not the least excessive synthesis of ROS are the key factors contributing to elevated OS in malignant cells [93]. It seems that ROS act as the second messenger in the intracellular signalling processes promoting the persistence of cancer cells [29]. Furthermore, it has been shown that malignant cells have a higher resistance to OS compared to normal cells [2].

Interestingly, HPV has the ability to adapt to OS conditions by increasing the activity of protective mechanisms such as SOD and CAT in the infected cells [94, 95]. The main mechanisms by which cancer cells survive OS are the regulation of antioxidant activity and suppression of OS-induced apoptosis. These processes seem to be governed by HPV oncogenes, especially E7, which allows an uncontrolled proliferation of the infected cells [11]. Additionally, E7 acts on the expression of Bcl-xL, IL-18, Fas, and Bad resulting in resistant cells to OS-induced apoptosis [95].

The study by de Marco et al. has shown increased OS in samples from women with cervical dysplastic or neoplastic lesions. In the case of dysplastic samples, OS-induced alterations have been observed in the structure of some proteins such as cytoskeletal keratin 6, actin, cornulin, retinal dehydrogenase, and glyceraldehyde 3-phosphate dehydrogenase, which are involved in cell morphology and differentiation. Conversely, in cancer samples, a better control of OS was observed, demonstrating that OS participates in the formation of a favourable environment for malignant transformation [8].

The microenvironment of malignant cells is characterized by a high level of OS biomarkers. Romano et al. revealed a higher level of 8-hydroxy-2′-deoxyguanosine in dysplastic cells as compared to normal cells, attributing an important role to OS in carcinogenesis [56]. The study by Naidu et al. on cervical cancer patients has revealed a positive correlation between the MDA level and severity of the lesions, the highest levels being recorded in stage IV [96].

Siegel et al. have postulated the interesting hypothesis that markers of OS could reflect the immune host response to HPV infection. They investigated the link between the oxidant load and the clearance of the infection on a sample of 444 HPV-positive women. Anti-5-hydroxymethyl-2′-deoxyuridine autoantibody (anti-HMdU Ab) and MDA levels were measured. They observed elevated levels of the two biomarkers, which have been associated with a faster clearance of oncogenic HPV types [97]. In addition, in another study, Siegel et al. highlighted that HPV-infected women with elevated levels of ferritin were less likely to achieve viral clearance than those with low levels. Iron promotes viral replication and transcription, but at the same time, it is involved in DNA oxidation, contributing to ROS generation [98].

It seems that the deficiency of certain antioxidants might influence the course of the infection [99]. Antioxidants modulate the expression of genes associated with the transcriptional AP-1 complex [100], and in turn, ROS lead to activation of AP-1, a factor involved in the expression of E6 and E7 oncoproteins [101].

The study by Manju et al., which included patients with cervical cancer, revealed low levels of the main antioxidant enzymes, GPx, glutathione S transferase (GST), and SOD as well as vitamin C and E. That could be the consequence of both the consumption at the site of the reaction and sequestration by the tumour cells [102]. Another study on women with CIN and cervical squamous cell carcinoma showed low levels of SOD and CAT but elevated levels of GPx [103].

Gonçalves et al. conducted a study on women with untreated cervical cancer and premalignant cervical lesions and revealed low levels of vitamin C, an important antioxidant, and increased levels of erythrocyte thiobarbituric acid reactive substances (TBARS) and δ-aminolevulinate dehydratase (δ-ALA-D). Moreover, they concluded that erythrocyte TBARS and vitamin C levels might be used as OS markers in early stages of cervical cancer. Vitamin C may be valuable in the treatment of these patients, but this topic remains debatable [104]. Naidu et al. have shown an increased Cu/Zn ratio in patients with cervical cancer and emphasized the role of this parameter as a marker of tumour growth. It seems that increased levels of Cu are associated with DNA alteration, Cu being involved in the generation of superoxide anion. They have also revealed decreased levels of SOD and vitamin C [96].

6.4. Vitamin and Antioxidant Intake in Cervical Cancer. Antioxidant vitamins have the ability to neutralize free radicals capable of DNA damage, which makes cells become more vulnerable to HPV infection. The meta-analysis by Myung et al., which included 10,073 patients from 22 case-control studies, concluded that the intake of vitamin B12, vitamin E and beta-carotene was associated with a preventative effect on cervical cancer [105]. Similarly, the study by Guo et al. observed that elevated serum levels of vitamin E, vitamin C, and beta-carotene were associated with a reduced risk of cervical neoplasm [106]. In addition, the meta-analysis by Cao et al. has revealed that the association is dose-dependent; a 50 mg/day intake is correlated with a significantly lower risk [107]. Another meta-analysis has highlighted that elevated blood levels and increased intake of vitamin A reduce the risk of cervical cancer. Zhang et al. showed a strong association for carotene and a weak association for retinol [108]. Other studies have also found similar results regarding vitamin or antioxidant intake [109, 110].

The role of curcumin has been studied in several cancers with promising results. Among antioxidants, the role of polyphenols has been evaluated in several studies [29]. Curcumin, a polyphenol derived from *Curcuma longa*, has been shown to have numerous effects that interfere with HPV-related carcinogenesis, including the stimulation of apoptosis and inhibition of the expression of HPV oncoproteins [111]. In addition, in cervical cancer, it was observed that curcumin has the capacity to inhibit the proliferative effect of estradiol and induce apoptosis [112].

The study by Sedjo et al. showed that lutein and lycopene can reduce the incidence of cervical cancer due to their antioxidant properties [101]. In addition, the study by Barchitta et al. investigated the relationship between the type of diet and HPV infection and concluded that those females eating a Mediterranean diet based on fruits and vegetables had a lower risk of infection with oncogenic HPV types and a lower risk of CIN, when compared with those who adopted an unhealthy diet [113].

It is worth noting that treatments such as chemotherapy and radiotherapy induce free radical release and toxicity, thus antioxidant supplements may be adjuvant treatments associated with a better quality of life of these patients [114].

7. Conclusion

HPV infection still represents an important public health issue, affecting a considerable number of individuals, being responsible for benign and malignant lesions. The variety of the lesions and the different outcomes of the infection, from self-limiting to invasive, make difficult to understand the HPV mechanism of action. Recent research suggests the role of chronic inflammation and OS as important players involved in virus pathogenesis and promotion of carcinogenesis. The interplay between chronic inflammation and OS induces various tissue alterations facilitating HPV integration, which is a key event in the multistep process of malignant transformation. The expression of viral oncogenes produces genetic abnormalities that promote the malignant transformation. In this context, anti-inflammatory drugs and antioxidants could be a promising therapy. However, current data are scarce and further studies are needed.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

All authors have equally contributed to the writing and editing of the manuscript.

Acknowledgments

This work was partially supported by a grant of Romanian Ministry of Research and Innovation, CCCDI-UEFISCDI, (project number 61PCCDI/2018 PN-III-P1-1.2-PCCDI-2017-0341), within PNCDI-III.

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