BABEŞ–BOLYAI UNIVERSITY
FACULTY OF BIOLOGY AND GEOLOGY
DOCTORAL SCHOOL OF INTEGRATIVE BIOLOGY

DOCTORAL THESIS

Scientific Supervisor

PROF. DR. ELENA RAKOSY-TICAN

PhD Student

LAURA-IOANA PĂTRAȘ

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EXPLORING THE TUMOR-ASSOCIATED MACROPHAGES-MEDIATED RESISTANCE TO ANTICANCER THERAPIES IN COLORECTAL CANCER

Doctoral thesis summary

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List of abbreviations

CRC – colorectal cancer

EPR - enhanced permeability and retention

TME - tumor microenvironment

EV- extracellular vesicles

TAM – tumor-associated macrophages

LCL – long-circulating liposomes

5-FU – 5-fluorouracil

PLP – prednisolone phosphate

CAFs – cancer-associated fibroblast(s)

MSC - mesenchymal stem cells

TEV – tumor-derived extracellular vesicles

DOX- doxorubicin

MDA - malondialdehyde

IL-4 – interleukin 4

NO – nitric oxide

ROS – reactive oxygen species

bFGF - basic fibroblast growth factor

FasL - Fas ligand

EE – encapsulation efficiency

AP-1 c-Jun – c-Jun subunit of activator protein-1

iNOS – inducible nitric oxide synthase

IL - interleukin

ANOVA – analysis of variance

DT – doubling time

AUTC(s) – area under the tumor growth curve(s)

Keywords: colorectal cancer, tumor-associated macrophages, extracellular vesicles, long-circulating liposomes, 5-fluorouracil, prednisolone phosphate, resistance to therapy

Chapter I. General introduction and current state of knowledge

I.1. Introduction

Colorectal cancer (CRC) remains a leading cause of cancer-related mortality worldwide, and it is often detected at advanced stages when the outcome of conventional chemotherapy for the treatment of CRC is limited due to low drug bioavailability, severe side effects, and last but not least, the drug-induced resistance to chemotherapy. To overcome these limitations, targeted therapies based on liposomal drug formulations could facilitate the accumulation of the therapeutic agent at the tumor site via the enhanced permeability and retention (EPR) effect and could increase the therapeutic index, especially in the case of advanced or more drug-resistant cancers.

A distinctive feature of solid tumors, such as CRCs, is the crosstalk between the malignant and stromal infiltrating or resident cells that build the tumor microenvironment (TME). This bidirectional communication among cancer cells and TME cells through bioactive soluble molecules or extracellular vesicles (EV) ensures tumor progression. Tumor-associated macrophages (TAM) are major components of the innate immune system and the most abundant stromal cells. In the tumor milieu, TAM are being educated to display a behavior that supports processes associated with tumor progression (e.g. tumor growth, inflammation, angiogenesis, invasion and metastasis, cancer cell drug resistance to therapy) and can orchestrate the therapeutic outcome. Therefore, the future targeted therapies should attempt to impair the protumor action of TAM and to enhance their antitumor role.

This thesis explored the resistance of colorectal cancer cells to therapy that is mediated by the crosstalk between TAM and cancer cells. Also, in this thesis, a novel therapeutic strategy for CRC was developed and evaluated in a murine colon carcinoma model *in vivo* by employing a combination therapy of a cytotoxic drug targeted therapy using long-circulating liposomal 5-fluorouracil (LCL-5-FU) and a potentially TAM-targeted therapy using long-circulating liposomal prednisolone phosphate (LCL-PLP).

I.2. Colorectal cancer therapy

The conventional CRC chemotherapy is based on drugs with distinct mechanisms of action such as 5-FU (antimetabolite), irinotecan (topoisomerase I inhibitor), and oxaliplatin (DNA replication inhibitor) (Longley *et al.*, 2003; Xu and Villalona-Calero, 2002; Raymond *et al.*, 1998). The drawbacks that arised from the use of these drugs (e.g. low drug bioavailability, severe side effects, induction of cancer cell drug resistance) could not be entirely overcome by the addition of oral drugs (e.g. capecitabine, trifluridine/tipiracil, and folinic acid) (Machover, 1997; Cutsem *et al.*, 2004; Peeters *et al.*, 2018). Therefore, the development of targeted therapies addressing increased anti-tumor specificity and reduced side effects, revealed a significantly improved

therapeutic outcome in advanced cancers (Marabelle and Gray, 2015; van Cutsem *et al.*, 2016; Heinemann *et al.*, 2013). The most promising Food and Drug Administration (FDA)-approved targeted therapies for advanced CRC are classified into immunotherapies which employ immunomodulatory antibody-based drugs (known as immune checkpoint inhibitors) that activate the immune tumor microenvironment to exert anti-tumor responses, and into biotherapeutic drugs represented by monoclonal antibody-based drugs that target tumor growth and angiogenesis (Buchbinder and Desai, 2016; Ng and Cunningham, 2004; van Cutsem *et al.*, 2017; Cui *et al.*, 2014).

Alternatively, continuous research aims to implement nanoparticle-based therapies for CRC treatment consisting of liposomes, micelles or polymeric nanoparticles for the targeted delivery of chemotherapeutic drugs to the tumor site (Bobo et al., 2016; Ren et al., 2016). The encapsulation of drugs in nanocarriers offers several advantages which include: tumor targeting capacity, reduced toxicity, biocompatibility, bioavailability, and can be either stable to ensure sustained drug release or biodegradable in the target tissue (Bharali and Mousa, 2010). The tumor targeting capacity of nanocarriers is ensured by the EPR phenomena due to an increased tumor vascular permeability with pores ranging in size from 100 to 780 nm among the endothelial cells and a lymphatic drainage that is defective (Maeda, 2001; Cho *et al.*, 2008; Kumar, 2012). Thus, the accumulation of nanocarriers at the tumor site and passive drug delivery is facilitated by modulating their size (50-150 nm) and coating with a hydrophilic surface to evade from the reticuloendothelial system, which confers them prolongued systemic circulation time (Gabizon and Papahadjopoulos, 1988; Marcucci and Lefoulon, 2004; Kumar, 2012).

Although there is a wide variety of nanomedicines designed for tumor targeting and drug delivery, among the liposomal therapies that are FDA-approved, there are currently no approved liposomal formulations for the commonly-used chemotherapeutic drugs 5-FU, oxaliplatin, and irinotecan for the treatment of CRC.

I.3. Overview on the nanoparticle-based therapies for CRC employing 5-FU

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The most common chemotherapeutic approach for CRC therapy relies on 5-FU, which remains a cornerstone in the systemic treatment of this cancer (Wolpin and Mayer, 2008). This cytotoxic agent is a fluorinated analogue of uracil, which classifies it as an antimetabolite drug that is subjected to intercellular conversion to its three active metabolites that incorporate into the DNA and RNA, inducing apoptosis and cell death (Anitha *et al.*, 2014; Longley *et al.*, 2003). The

clinical applicability of 5-FU is limited due to the lack of tumor specificity and low bioavailability since the majority of the drug is enzymatically degraded in the liver (Zhang *et al.*, 2008b; Wigmore *et al.*, 2010). This outcome requires the use of high dosages that exert increased toxicity towards healthy cells and tissues, leading to severe side effects (Zhang *et al.*, 2008b; Wigmore *et al.*, 2010). Another major obstacle for the use of 5-FU in advanced CRC is the development of cancer cell resistance to the treatment through multiple mechanisms (Zhang *et al.*, 2008b; Ortiz *et al.*, 2012).

Several CRC-targeted therapies based on the encapsulation of 5-FU in a large variety of nanoparticulate delivery systems attempted to overcome the aforementioned limitations and to improve the therapeutic index through specific targeting of the drug at the tumor site and controlled local release of the drug (Nair *et al.*, 2011; Subudhi *et al.*, 2015). These effects were achieved by using pH-dependent drug delivery mechanisms (e.g. hydrogels and thiolated chitosan spherical nanoparticles) and microflora- (e.g. chitosan-based biodegradable nanoparticles) or hyperthermia- (e.g. engineered multilamellar magnetic liposomes) activated mechanisms (Subudhi *et al.*, 2015; Park *et al.*, 2010b; Clares *et al.*, 2013).

Despite the multitude of the nanoparticulate formulations developed for 5-FU delivery to CRC that were superior to conventional administration strategies of 5-FU, serious adverse effects could not be overcome and future studies for the optimization of the tumor-targeted therapies based on nanoparticles incorporating 5-FU are in line since there is no approved nanoparticle-based CRC therapy for this drug.

I.4. Tumor microenvironment-based modulation of cancer progression and therapeutic outcome

I.4.1. The tumor microenvironment

The TME is a major determinant of the therapeutic outcome in solid tumors due to the interplay between malignant cells and the stromal cells (e.g. immune cells, vascular endothelial cells, lymphatic endothelial cells, pericytes, cancer-associated fibroblasts (CAFs), mesenchymal stem cells (MSC) and adipocytes) (Li *et al.*, 2007; Casazza *et al.*, 2014). The bidirectional transfer of intercellular signals (e.g. soluble factors and extracellular vesicles that convey bioactive molecules) ensures tumor-stroma co-evolution and crosstalk which modulates tumor-associated processes such as tumor growth, immune evasion, angiogenesis, invasion and metastasis, as well as the development of resistance to cancer therapies (Li *et al.*, 2007; Whiteside, 2008; Egeblad *et al.*, 2010; Pitt *et al.*, 2016).

The primary antitumor role of inflammatory cells that infiltrate into tumors (macrophages, lymphocytes, dendritic cells, and natural killer cells) is shifted towards the production of immunosuppressive molecules, cytokines, chemokines, growth factors that disrupt tumor

immunity contribute to tumor progression (Zitvogel *et al.*, 2006; Li *et al.*, 2007; Whiteside, 2008; Mancino and Lawrence, 2010; Balkwill and Mantovani, 2001). The monocyte-macrophage hematopoietic cell lineage is a critical modulator of this shift due to the intratumor abundancy and high plasticity of macrophages that can be skewed towards two different activation states depending on the environmental cues (Mantovani and Locati, 2013). Classical activation of macrophages towards the M1 antitumor phenotype ensures a pro-inflammatory and immunostimulatory environment that contributes to cancer cell death (Zhong *et al.*, 2018; Mantovani *et al.*, 2004b). The alternative activation of macrophages into M2 protumor macrophages leads to an anti-inflammatory and immunosuppressive environment with tissue remodeling capacity (Mantovani *et al.*, 2004a; Mantovani, 2006; Mantovani *et al.*, 2013).

I.4.2. TAM as critical players in the TME

TAM are associated with an M2-like phenotype and their pivotal role in orchestrating tumor progression and the therapeutic outcome is denoted from their capacity to secrete inflammatory molecules, proteolytic enzymes, pro-angiogenic growth factors (Mantovani *et al.*, 2004a; Lewis and Pollard, 2006; Mancino and Lawrence, 2010; Zhong *et al.*, 2018). These molecules promote and coordinate TME-associated processes such as immunosuppression, tumor growth, tumor inflammation and oxidative stress associated with angiogenesis, invasion and metastasis, and chemoresistance (Mantovani *et al.*, 2006; Pollard, 2004; Crowther *et al.*, 2001; De Palma and Lewis, 2013; Zhong *et al.*, 2018).

Although several studies ascribed a protumor role to TAM in solid tumors and associated their infiltration in the TME with a poor prognosis, the role of TAM in CRC progression remains controversial, since both protumor and antitumor actions have been described for these cells and could vary depending on the molecular signature of the type of cancer, the experimental conditions, and the drug used (Zhong *et al.*, 2018; Waniczek et al., 2017). Therefore, for CRC therapeutic intervention, future knowledge of the precise mechanisms by which TAM promote or reduce tumor progression could be used for the design of therapies that specifically target TAM, either by inhibition of their protumor function or by re-educating them towards an antitumor M1 phenotype (Stout *et al.*, 2009; Zhong *et al.*, 2018). Moreover, TAM re-education strategies that combine cytotoxic drug therapy with TAM-directed therapy may reach superior antitumor efficacy due to a synergistic or complementary outcome (Mantovani *et al.*, 2017).

Chapter II. Intercellular crosstalk via extracellular vesicles in tumor milieu as emerging therapies for cancer progression

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II.1. Introduction

Extracellular vesicles (EV) are heterogeneous nano-sized membranous structures with an essential role as intercellular communication tools in the TME through the bidirectional transfer of functional molecules between cancer cells and stromal cells (Yuana *et al.*, 2013; Becker *et al.*, 2016; Vader *et al.*, 2014). Increasing evidence supported the active contribution of EV to disease progression and therapeutic outcome by mediating the stromal cell-cancer cells crosstalk and creating a neoplastic milieu favorable for all protumor processes, such as immunosuppression, angiogenesis, invasion and metastasis, and cancer cell resistance to oncological drugs (Kanada *et al.*, 2016).

Besides the EV potential to enable the deciphering of crucial aspects of tumor-related intercellular communication, EV-based therapeutic strategies aim to target cancer progression by interfering with the production of protumor EV, the EV uptake by recipient cells or by altering their cargo (Peinado *et al.*, 2012; Atai *et al.*, 2013; Lunavat *et al.*, 2017). Additionally, EV are currently being employed as drug delivery systems due to their analogy to liposomes and the advantages conferred by their intrinsic properties (e.g. small size, natural protein and lipid coat) which include increased bioavailability, long circulation time, preferential uptake by tumor cells, the capacity to overcome biological barriers (El Andaloussi *et al.*, 2013; Yang *et al.*, 2015a; Kim *et al.*, 2018). Several drug loading approaches of EV have been investigated to optimize the most efficient incorporation of different therapeutic molecules in these nanovesicles (van Dommelen *et al.*, 2012; Raposo and Stoorvogel, 2013; Vader *et al.*, 2016). In general, these approaches were based either on engineering the donor cells to secrete modified EV that load the specific therapeutics or by the use of several methods for EV loading after their purification (Vader *et al.*, 2016).

II.2. EV roles in the TME

Tumor EV (TEV) have been directly involved in promoting the recruitment and polarization of macrophages towards the M2 phenotype via enhancing their anti-inflammatory and

immunosuppressive function (Chow *et al.*, 2014; Cooks *et al.*, 2018; Park *et al.*, 2019). Other studies indicated that TAM-derived EV could alter the phenotype and function of tumor microenvironmental cells in a protumor manner consisting of immunosuppression and increased invasiveness and metastatic potential (Shao *et al.*, 2018b; Shen and Ren, 2018; Zhou *et al.*, 2018). These findings suggest that the EV-mediated crosstalk in the TME between protumor M2 macrophages and cancer cells or other stromal cells is a major determinant of tumor progression and emphasize the therapeutic opportunities that may arise from TAM re-education strategies or impairment of the production of protumor EV in the TME.

In recent years, literature has reported that EVs contribute to the settlement of cancer cell resistance to the chemo-, radiation, and targeted therapies through a variety of mechanisms (Meads *et al.*, 2009; Azmi *et al.*, 2013; Sharma, 2017). For instance, TEV contribute to chemoresistance through their ability to sequestrate several cytotoxic drugs and their metabolites from tumor cells or by conveying drug resistance-inducing molecules to sensitive recipient tumor cells (Raposo and Stoorvogel, 2013; Vader *et al.*, 2013).

II.3. The highlights of using EVs as emerging therapies for cancer

EV have emerged as a promising new class of anticancer drug delivery systems due to their feasibility and breakthrough for rebalancing and re-educating the TME to overcome the main supportive processes for malignancy progression (Kooijmans *et al.*, 2012; El Andaloussi *et al.*, 2013). Since a major advantage of EV-based drug delivery is the enhanced cytotoxic effect that may avoid drug resistance in various cancers, several studies implemented the use of EV isolated from naïve cells for functionalization and loading with various chemotherapeutic agents (Mulcahy *et al.*, 2014; Kim *et al.*, 2018). Another EV-based therapeutic strategy consisted of exploiting the EV produced by engineered cancer or immune cells that could carry a specific cargo or expressed a certain ligand, for their *in vitro* and *in vivo* capacity to overcome tumor progression and to induce chemosensitivity (Lou *et al.*, 2015; Yuan *et al.*, 2017; Li *et al.*, 2018).

Nevertheless, one of the major obstacles remains the inefficient drug delivery to the sites of malignancy. Gaining more insight into fundamental EV biology, especially future knowledge about their surfaceome and cargos as well as different physiological functions of specific EV subtypes, could contribute to further improvements in the development of EV as drug delivery systems for oncological applications.

Chapter III. The aim of the thesis and general objectives

In the light of frequent therapeutic failure of CRC chemotherapy, recent evidence ascribed an increasingly important protumor role to EVs and TAM, as critical players in the TME that drive tumor progression and modulate the therapeutic outcome of cancer therapy. Although targeted therapies for CRC are under continuous development, there is currently no approved liposomal therapy for this type of cancer, and neither is any therapy targeting the TAM-mediated tumor progression. Therefore, the **aim of this thesis** was to develop a novel CRC-targeted combination therapy based on the modulation of the intercellular communication between C26 colon carcinoma cells and TME cells. For achieving this aim, two general objectives were established.

The **first objective** was to study the interaction between TAM and cancer cells to unveil the molecular mechanisms responsible for the modulation of the therapeutic response. The research in **Chapter IV** investigated the role of EVs in mediating the communication between cancer cells and TAM and their association with the settlement of CRC cells resistance to DOX treatment *in vitro*. **Chapter V** addressed the TAM-driven modulation of C26 colon carcinoma cells response to the cytotoxic drug 5-FU, in a co-culture model *in vitro*.

The **second objective** was to develop a novel liposomal combination therapy based on the simultaneous targeting of colon cancer cells using long-circulating LCL-5-FU and of TAM-driven effects using long-circulating LCL-PLP and the capacity of the proposed targeted liposomal therapy (LCL-5-FU+LCL-PLP) to enhance the cytotoxicity of the therapeutic agent by targeting TAM-associated protumor processes *in vivo* was investigated in **Chapter VI**.

Chapter IV. Doxorubicin-elicited tumor extracellular vesicles modulate the response of stromal cells to chemotherapy *in vitro*

[Chapter VI is a manuscript submitted for publication as: Patras L., Fens M.H.A.M., Vader P., Barendrecht A., Banciu M., Schiffelers R. 2019. Doxorubicin-elicited tumor extracellular vesicles modulate the response of stromal cells to chemotherapy in vitro]

The bidirectional transfer in the TME underlying the tumor-stroma crosstalk supports tumor progression via regulating the immune responses, angiogenesis, invasion, metastasis, and cancer drug resistance. The modulatory role of TEV on the therapeutic outcome stems from their ability to alter the cellular phenotype and behavior of recipient cells, thus remodeling the TME and the complex interplay between cancer and stromal cells. This study investigated the capacity of TEV isolated from C26 murine colon carcinoma cell cultures *in vitro* exposed to hypoxic (1% oxygen) and therapeutic (doxorubicin (DOX)) stress conditions to alter the response of recipient C26 and RAW 264.7 macrophage-like cells to the same drug, under normoxic and hypoxic conditions. Our results suggested the potential of normoxic TEV to render the recipient cells less

responsive to DOX and this effect was assigned to a more drug resistant phenotype in TEV donor cells that was induced by DOX treatment, and reflected through a strong anti-apoptotic response and the capacity of the cells to maintain physiological levels of oxidative stress.

Altogether, this study brings new insight into the chemotherapy-elicited TEV modulatory role in the CRC microenvironment that could better reflect the *in vivo* settings that contribute to the aquision of drug resistance by cancer cells.

Chapter V. Dual role of macrophages in the response of C26 colon carcinoma cells to 5-fluorouracil administration

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V.1. Introduction

Previous studies have shown that tumor-associated macrophages (TAM) are pivotal players in tumor progression via modulation of tumor angiogenesis, inflammation, metastasis, oxidative stress as well as of the response of cancer cells to cytotoxic drugs. Nevertheless, the TAM role in the prognosis of colorectal cancer is still controversial. Therefore, the present study aimed to investigate how TAM mediate the response of C26 colon carcinoma cells to cytotoxic drug 5-fluorouracil (5-FU), upon the TAM co-cultivation with these cancer cells *in vitro*. In this respect, we assessed 5-FU cytotoxicity in C26 cells in standard culture and co-culture of C26 cells with peritoneal macrophages and we determined the production of NF-κB by western blot analysis, as well as the production of angiogenic/inflammatory proteins in each experimental model by protein array analysis. To gain further evidence on TAM influence on oxidative stress, we measured malondialdehyde (MDA) through HPLC and the production of nitrites through a colorimetric method. Our results demonstrated that TAM displayed a dual role in the response of C26 cells to 5-FU administration in the co-culture model.

Thus, on one side TAM sensitized C26 cells to 5-FU administration through inhibition of the production of inflammatory and angiogenic proteins in these cancer cells, but on the other side protected cancer cells against 5-FU-induced oxidative stress. Collectively, our findings suggest that the combined administration of 5-FU with pharmacological agents that prevent TAM to maintain physiological range of tumor cell oxidative stress may highly improve the therapeutic potential of this drug.

V.2. Material and methods

C26 murine colon carcinoma cells (Cell Line Services GmbH, Germany) were cultured as a monolayer in complete RPMI 1640 medium (Lonza) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, GE Healthcare Life Sciences), at 37 ºC in 5% CO_2 humidified atmosphere.

Co-culture of C26 cancer cells with macrophages was obtained by seeding C26 tumor cell suspensions over a monolayer culture of thioglycolate-elicited peritoneal macrophages isolated from BALB/c mice (Cantacuzino Institute, Romania), at a cell density ratio of 1:4 that ensures macrophage polarization into TAM and is closely related to the *in vivo* colon carcinoma development conditons (Herbeuval *et al.*, 2004). Moreover, the angiogenic/inflammatory protein signature from our co-culture model was compared to the same protein signature from TAM obtained from peritoneal macrophages differentiated with IL-4 and co-cultured with cancer cells and the differences were not significant (data not shown) (Martinez et al., 2006). Experiments complied to the national regulations and were approved by the local animal experiments ethical committee (registration no.32652/01.07.2014).

Cell proliferation assay was performed using the ELISA BrdU-colorimetric immunoassay (Roche Applied Science, Germany) to determine whether TAM presence could alter the response of C26 cells to therapy. Thus, the cytotoxicity of various 5-FU concentrations (0.125-16 µM) was assessed towards $1x10^4$ C26 cells/well, cultured alone as well as in co-culture with macrophages at a density ratio of 1:4, after 72h incubation with the drug. Results were expressed as % of inhibition of C26 cells/ co-culture cell proliferation compared to control (untreated monoculture or co-culture).

Cell culture lysates were obtained for further assessment of the role of a 4 µM 5-FU treatment and TAM presence on key tumor markers associated with tumor progression. The cell lysis buffer consisted of 10 mM HEPES (pH 7), 200 mM NaCl, 1% Triton X, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and protease inhibitor cocktail tablets (Complete, Roche Diagnostics GmbH, Germany). The protein concentration was determined through the Bradford assay (Bio-Rad, Hercules, CA) (Bradford, 1976).

The expression levels of NF-κB transcription factor were determined from cell lysates via western blot analysis to test the effects of 5-FU on this key mediator of tumor inflammation and angiogenesis and whether the presence of TAM could potentiate the effects of the drug on the expression levels of NF-κB. Monoclonal mouse IgG anti-mouse NF-κB p65 primary antibody and goat IgG anti-mouse IgG secondary antibody HRP-conjugated were used (Santa Cruz Biotechnology). Proteins were detected by using Clarity™ Western ECL (Bio-Rad) and the membranes were exposed to an X-ray film (Kodak) which was developed and photographed using

BioSpectrum Imaging System (BioSpectrum AC Chemi HR 410, Cambridge, England). TotalLab Quant Software version 12 for Windows was used for the densitometric analysis and the expression levels of NF-κB in monoculture and co-culture were displayed as % of inhibition compared to untreated monoculture and co-culture.

The expression levels of inflammatory and angiogenic proteins in each experimental condition were screened by using the RayBio® Mouse Angiogenic Cytokine Antibody Array kit (RayBiotech Inc., Norcross, GA) as described previously (Banciu *et al.*, 2006). The protein expression level of 24 proteins involved in inflammatory and angiogenic processes were quantified through densitometry using TotalLab Quant Software.

Malondialdehyde (MDA) levels were measured by High Performance Liquid Chromatography (HPLC) as previously decribed (Alupei *et al.*, 2015), since MDA is the main by-product of reactive oxygen species-mediated lipid peroxidation and it is boadly used as an indicator of overall oxidative stress (Del Rio *et al.*, 2005). This determination was performed to investigate the modulatory role of TAM on the the overall oxidative stress levels after 5-FU treatment. The HPLC column type was RP18 (5 µm) (Supelco, Pennsylvania, USA) and the mobile phase consisted of 30 mM KH_2PO_4/methanol in a volume ratio of 65:35. Flow rate was set at 0,5 ml/min and MDA was measured using a UV detector (UV -2070/2075 Jasco, Tokyo, Japan) set at 254 nm. Data were expressed as µM MDA and were normalized to the protein concentration from cell lysates.

Nitric oxide (NO) metabolites levels after 5-FU treatment on both standard culture and in co-culture was assessed by measuring nitrites via colorimetric Griess assay, as previously described (Alupei *et al.*, 2015). NO is a key signaling molecule that becomes cytotoxic to cancer cells when produced in high levels, whereas low levels of NO exert tumor promoting properties (Rahat and Hemmerlein, 2013). Data were expressed as nM nitrites after normalization to the protein concentration from cell lysates.

Statistical analyses consisted of using unpaired *t* test for comparing the effects of 5-FU on either C26 cells monoculture or co-culture with macrophages, and two-way analysis of variance with Bonferroni correction for multiple comparisons for comparing the effects of 5-FU on the production of each inflammatory/angiogenic proteins in cell lysates from standard culture and co-culture. Data were expressed as mean ± standard deviation (SD) and GraphPad Software (San Diego, CA) was used. A *P* value lower than 0.05 was considered significant.

V.3. Results and discussion

V.3.1. 5-FU treatment strongly inhibits C26 cell proliferation and reduces the expression levels of NF-κB transcription factor irrespective of TAM presence

We observed that at concentrations higher than 4 μM, 5-FU inhibited strongly (by 75% compared to the proliferation of control cells) the growth of C26 cells under standard culture conditions (Fig. V.1A) as well as after C26 cell co-cultivation with TAM (Fig. V.1B).

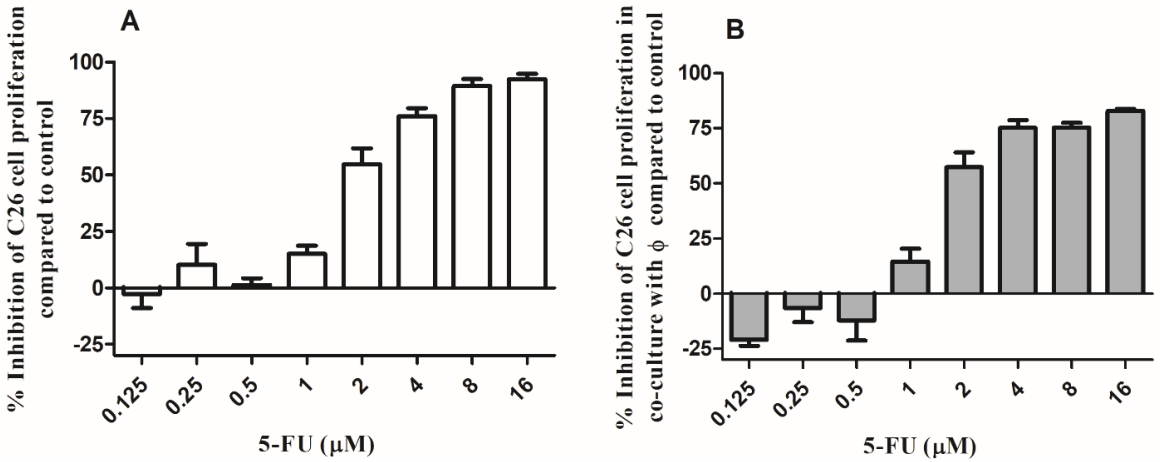

Figure V.1. The effects of 5-FU on the proliferation of C26 murine colon carcinoma cells. (A) 72h after incubation of C26 cells with increasing concentrations of 5-FU ranging between 0.125-16 μM; **(B)** 72h after incubation of co-culture of C26 cell with peritoneal macrophages (ϕ) with increasing concentrations of 5-FU ranging between 0.125-16 μM. Data are shown as mean ± SD of triplicate measurements. The results are expressed as mean % of inhibition ± SD of C26 cell proliferation after 5-FU treatment compared to control untreated cell proliferation.

The lowest concentration of 5-FU (4 μM) that exerted strong cytotoxic effects on cell proliferation (Fig. V.1A and B) was further used throughout the experiments for testing the TAM modulatory actions on the response of C26 colon carcinoma cells to 5-FU administration.

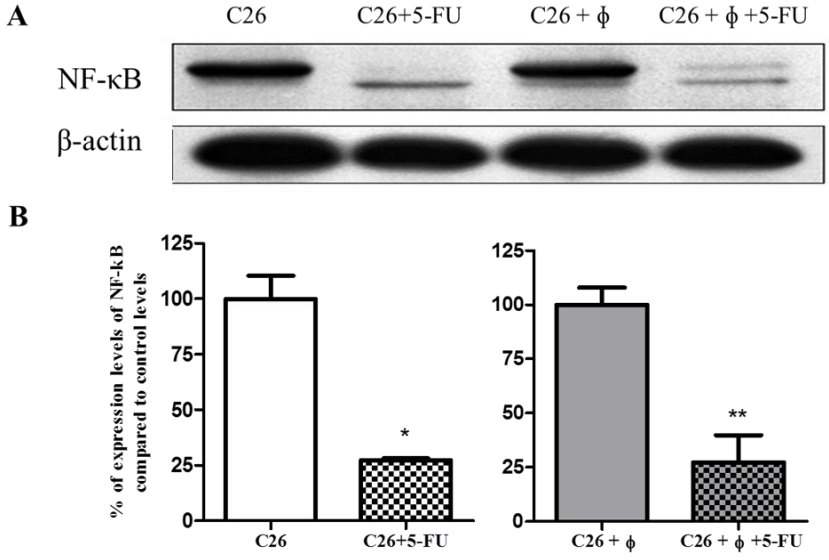

Figure V.2. Effects of 5-FU administration on cell levels of NF-κB. (A) Western blot analyses of NF-κB levels in C26 cells. C26 = untreated C26 cells; C26+5-FU = C26 cells treated with 4 μM 5-FU for 72h; C26 + ϕ = untreated co-culture of C26 cells and macrophages (ϕ); C26 + ϕ + 5-FU = co-culture of C26 cells and ϕ incubated with 4 μM 5-FU for 72h. β-actin was used as loading control; **(B)** Quantification of western blot data. The results are compared to the NF-κB levels in controls. Data are expressed as mean ± SD of two independent measurements; *ns* - not significant, P>0.05; *, P<0.05.

To address the controversial role of TAM in CRC development we investigated whether this cell type influences the effects of 5-FU on the expression levels of the transcription factor NF-κB – a key mediator of the proliferative, anti-apoptotic, and angiogenic potential, which is constitutively activated in most CRC lines, as well as in our *in vitro* model (Fig. V.2) (Uetsuka *et al.*, 2003; Sakamoto *et al.* 2009). Notably, the treatment with 4 μM 5-FU exerted strong inhibitory effects on the expression of NF-κB (by 70% compared to control) irrespective of TAM presence (Fig. V.2A and B), which may be also linked with the observed inhibitory effect on cell proliferation (Fig. V.1A and B).

Our findings are consistent with previous reports that associated high drug cytotoxicity with the inhibition of NF-κB leading to cancer cell death by apoptosis (Azuma *et al.*, 2001; Uetsuka *et al.*, 2003; Nowis *et al.*, 2007).

V.3.2. TAM rendered C26 cancer cells more susceptible to 5-FU treatment by mediating to a strong anti-angiogenic and anti-inflammatory effect

The protein array screening for 24 proteins involved in inflammation and angiogenesis was conducted to investigate whether TAM could modulate the 5-FU actions on these processes and an overview on the levels of these proteins detected in each experimental condition is illustrated in Fig. V.3. In line with previous studies our data confirmed that TAM play a crucial role in supporting tumor angiogenesis, inflammation and metastasis, since TAM presence in the untreated co-culture stimulated the overall production of these proteins by 2-fold (Fig. V.3) (Choo *et al.*, 2005; Banciu *et al.*, 2008b; Solinas *et al.*, 2009; Alupei *et al.*, 2015).

However, in the presence of macrophages, 5-FU treatment determined an overall reduction of the levels of screened proteins (by 44%, $P<0.0001$) compared to their production in untreated co-culture (Fig. V.3). The observed anti-angiogenic and anti-inflammatory effect together with the suppression of NF-κB could account for an antiproliferative environment where TAM mediate C26 cells susceptibility to the drug (Zins *et al.*, 2007; Hagemann *et al.*, 2008; Quatromoni and Eruslanov, 2012; Gutschalk *et al.*, 2013; Ryan *et al.*, 2014).

In the absence of TAM, 5-FU-treated C26 cells displayed increased levels of the majority of the angiogenic and inflammatory proteins compared to untreated C26 cells (by 84 %, $P<0.0001$). A more resistant phenotype could be assigned to these cells due to the significant 3-fold and 5-fold increase in the levels of basic fibroblast growth factor (bFGF) and, respectively, Fas ligand (FasL), as these proteins were previously associated with increased aggressiveness and metastatic potential of cancer cells (Figure V.3) (Casanovas *et al.*, 2005; Igney and Krammer, 2005).

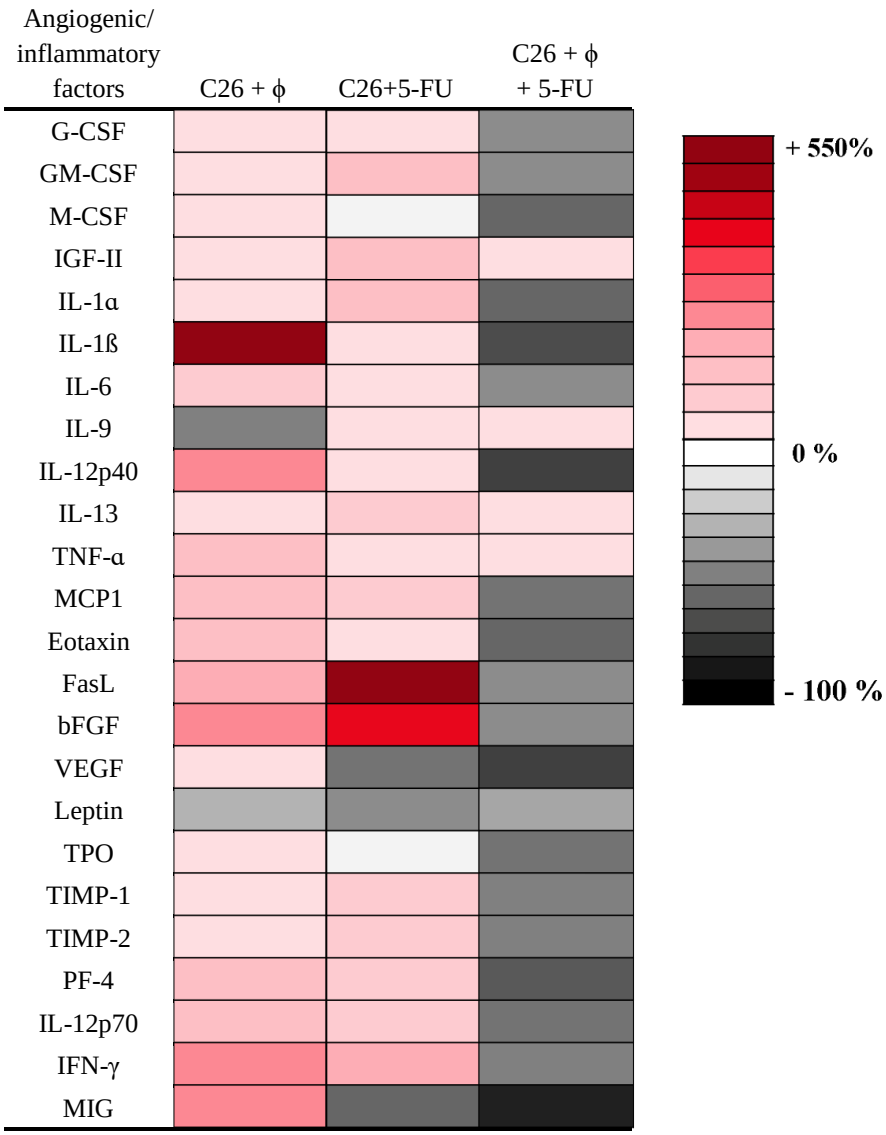

Figure V.3. Effects of various experimental conditions on the levels of angiogenic/inflammatory proteins in cell lysates. Results are presented either as percentage (%) of reduction (-) of protein levels ranging from 0% (white) to 100% (black) or as % of stimulation (+) of production of proteins ranging from 0% (white) to 550 % (red) in: C26 + ϕ = co-culture compared to levels of angiogenic/inflammatory proteins in lysates from untreated C26 cells; C26 + 5-FU = C26 cells treated with 4 µM 5-FU compared to levels of angiogenic/inflammatory proteins in lysates from untreated C26 cells; C26 + ϕ + 5-FU = co-culture treated with 4 µM 5-FU compared to levels of angiogenic/inflammatory proteins in lysates from untreated C26 cells.

V.3.3. TAM displayed a protective effect on 5-FU-exposed C26 cells by maintaining physiological oxidative stress levels

Since several studies suggested that TAM are able to maintain phyisiological levels of oxidative stress, we assessed the modulatory role of TAM on oxidative stress in 4 µM 5-FU-treated C26 cells by quantifying important oxidative stress markers from cell lysates (MDA and nitrites) (Kundu *et al.*, 1995; Siegert *et al.*, 1999; Wartenberg *et al.*, 2003; Del Rio *et al.*, 2005; Nowis *et al.*, 2007) (Fig. V4). Notably, only the treatment with 5-FU increased significantly the level of MDA in C26 cells cultivated alone (by 45%, $P<0.05$, Fig. V.4A) compared to untreated cells. The

capacity of 5-FU to exert pro-oxidant effects on CRC cells, was also reported by other studies and the oxidative stress increase over the physiological are associated with cell death via reactive oxygen species (ROS)-induced apoptosis (Hwang *et al.*, 2001; Fu *et al.*, 2014; Sun *et al.*, 2014).

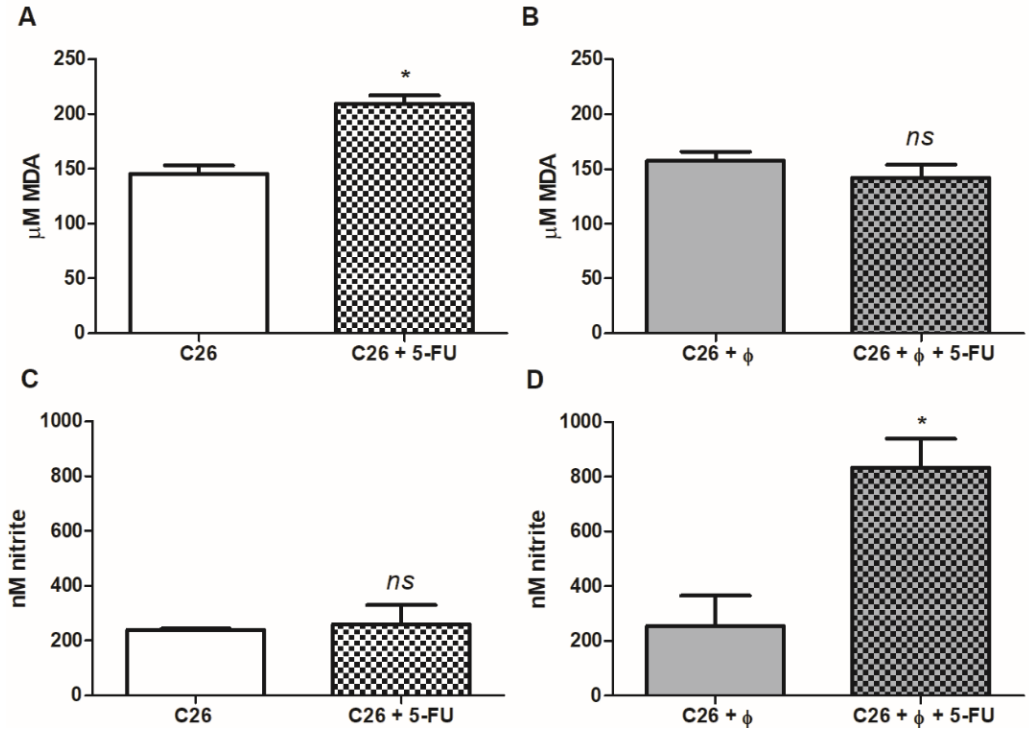

Figure V.4. The effects of 5-FU treatment on the oxidative stress markers in lysates from standard cultures and co-cultures. (A) MDA levels in C26 cell lysates after 72h of incubation with 4 µM 5-FU; **(B)** MDA levels in cell lysates obtained from co-culture after 72h of incubation with 4 µM 5-FU; **(C)** nitrite levels in C26 cell lysates after 72h of incubation with 4 µM 5-FU; **(D)** nitrite levels in cell lysates obtained from co-culture after 72h of incubation with 4 µM 5-FU. Data are shown as mean ± SD of two independent experiments; *ns* - not significant, $P>0.05$; *, $P<0.05$.

However, in the presence of TAM, 5-FU treatement failed to increase the oxidative stress levels, suggesting that TAM might protect cancer cells against 5-FU-induced oxidative stress counteracting the effects of the drug (Fig. V.4B). This effect is further enforced by our findings that show that the nitrite production was significantly increased by 5-FU (by 4-fold, $P=0.0329$, Fig. V.4D) only in the presence of macrophages compared to untreated co-culture. Thus, since the nitrosative stress levels did not exceed the physiological range of NO (nM range) necessary for antitumor effects, this effect could reportedly be in tight connection with the protection against ROS-induced apoptosis (Rahat and Hemmerlein, 2013; Krishnaiah *et al.*, 2002; Wartenberg *et al.*, 2003; Riganti *et al.*, 2005; Leung *et al.*, 2008).

V.4. Conclusions

Taken together, this study proved the dual role of TAM in the modulation of the response of C26 cells to 5-FU treatment. On one side, TAM increase chemosensitivity of these cancer cells to 5-FU treatment via mediating an overall strong reduction of inflammatory and angiogenic

proteins, but on the other side TAM protect cancer cells against pro-oxidant effect of 5-FU by maintaining ROS levels in the physiological range of C26 cell oxidative stress. These findings suggest that therapeutic strategies for CRC should further exploit the intrinsic oxidative stress of cancer cells by combining the administration of 5-FU with pharmacological agents that prevent TAM to maintain physiological range of tumor oxidative stress.

Chapter VI. Liposomal prednisolone phosphate potentiates the antitumor activity of liposomal 5-fluorouracil in C26 murine colon carcinoma *in vivo*

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VI.1. Introduction

The antitumor efficacy of 5-fluorouracil (5-FU) in advanced colorectal cancer (CRC) is hindered not only by the low therapeutic index, but also by tumor cell resistance to this cytotoxic drug. Therefore, to enhance the 5-FU antitumor activity, the present research employed a novel tumor-targeted therapy based on the co-administration of 5-FU encapsulated in long-circulating liposomes (LCL-5-FU) together with liposomal prednisolone phosphate (LCL-PLP), a formulation with known anti-angiogenic actions on C26 murine colon carcinoma cells. Thus, we assessed the in vivo effects of the combined liposomal drug therapy on C26 carcinoma growth as well as on the production of molecular markers with key roles in tumor development such as angiogenic, inflammatory, and oxidative stress molecules. To get further insight into the polarization state of tumor microenvironment after the treatment, we determined the IL-10/IL-12p70 ratio in tumors. Our results showed that combined liposomal drug therapy inhibited almost totally tumor growth and was superior as antitumor activity to both single liposomal drug therapies tested. The antitumor efficacy of the combined therapy was mainly related to the anti-angiogenic and anti-inflammatory actions on C26 carcinoma milieu, being favoured by its controlling effect on intratumor oxidative stress and the skewing of polarization of tumor microenvironmental cells towards their antineoplastic phenotypes. Thus, our study unveils a promising treatment strategy for CRC that should be furthermore considered.

VI.2. Material and methods

Liposomal formulations of LCLs were prepared using the lipid film hydration method with a lipid molar ratio of 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine: N-(Carbonyl-methoxypolyethyleneglycol-2,000)-1,2-distearoylsn-glycero-3 phosphoethanolamine (Na-salt):

cholesterol (DPPC:PEG-2,000-DSPE:CHL) of 9.5:0.5:1 and contained 4.5 mol % PEG 2,000 (as PEG-2,000-DSPE), as previously described (Schiffelers *et al.*, 2005; Alupei *et al.*, 2015; Sylvester *et al.*, 2016; Achim *et al.*, 2016). LCL-PLP mean size was about 100 nm with a polydispersity value < 0.1 and the PLP encapsulation efficiency (EE) was 22% (9 mg PLP/ml) (Sylvester *et al.*, 2016). LCL-5-FU mean size was about 180 nm, with a polydispersity index < 0.10 and EE was 1.4% (150 µg/ml) (Achim *et al.*, 2016). The advantage of these nano-sized liposomes that are below tumor vasculature cutoff limits (200–800 nm) enabled their passive accumulation within the tumor tissue (Porfire *et al.*, 2014).

C26 murine colon carcinoma cells (Cell Line Services, 440156) were cultured in complete RPMI 1640 medium (Lonza, 09-774F) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HyClone, SV30160.03), at 37 ºC in 5% CO_2 humidified atmosphere.

Murine tumor model of C26 colon carcinoma was generated by subcutaneous inoculation of 1 x 10^6 C26 cells in the right flank of syngeneic BALB/c mice (6-8 weeks old) (Cantacuzino Institute, Romania). Experiments were performed according to the national regulations and were approved by the local animal experiments ethical committee (registration no. 31375/06.04.2015). Tumor size was measured regularly starting with day 7 tumors became palpable, and the tumor volume was calculated according to the formula $V=0.52xa^2xb$, where a is the smallest and b is the largest tumor superficial diameter (mm).

Antitumor activity of the proposed liposomal therapy was determined through the inhibitory effects on tumor growth. The following experimental groups were assessed for their inhibitory effect on tumor growth:

- 20 mg/kg PLP (as LCL or free form as control)
- 1.2 mg/kg 5-FU (as LCL or free form as control)
- 20 mg/kg PLP + 1.2 mg/kg 5-FU (as LCL combination therapy)
- 20 mg/kg PLP + 1.2 mg/kg 5-FU (free form as control for LCL combination therapy)

The same dosing schedule was used for the experimental groups presented above (injection of the treatment at days 8 and 11 after tumor cell inoculation and mice sacrification at day 12).

Antitumor activity of two diferent combined administratios of liposomal formulations on tumor growth was tested to compare simoultaneous administration of 20 mg/kg PLP + 1.2 mg/kg 5-FU (as LCL or free form at days 8 and 11) with sequential administration of the same treatments, which consisted of PLP pretreatment (as LCL or free form at days 7 and 10) with 24h before 5-FU treatment (as LCL or free form at days 8 and 11), as previous studies suggested that corticosteroid pretreatment enhances the efficacy of cytotoxic therapy in CRC models (Wang, 2004; Rayburn, 2009). Mice were sacrificed at day 18 when tumors reached 2,000 mm^3.

Determination of NF-κB and AP-1 c-Jun key inflammatory transcription factors production was performed by western blot analysis from tumor tissue homogenates after protein content determination by biuret method (Gornall *et al.*, 1949). Primary antibodies for NF-κB p65 (sc-56735), c-Jun (sc-45) or β-actin (sc-130656) (Santa Cruz Biotechnology) were used. The immunocomplexes were developed using Clarity™ Western ECL (Bio-Rad, 170-5061) and the blots were exposed to an X-ray film (Kodak, Z358487) for about 1-2 min. Results were expressed as mean ± SD of two independent experiments.

The expression levels of inflammatory and angiogenic proteins in tumor homogenates from each experimental condition were screened for 24 proteins by using the RayBio® Mouse Angiogenic Cytokine Antibody Array kit (RayBiotech Inc., Norcross, GA) as described previously (Banciu *et al.*, 2006). The expression levels were quantified through densitometry using TotalLab Quant Software.

Immunohistochemical examination of tumor tissue after each therapy was conducted to get insight into the intratumor TAM infiltration by examining the presence of macrophages using F4/80 murine tissue macrophage marker, and the presence of M1 macrophages using inducible NO synthase (iNOS) marker (Austyn and Gordon, 1981; Kou *et al.*, 2015). The numbers of F4/80 and iNOS positive cells were counted on several non-overlapping fields and categorized into four-score cathegories depending on the abundance of positively stained cells (Banciu *et al.*, 2008a).

The effects of LCL-PLP+LCL-5-FU treatment on the IL-10/IL-12p70 production ratio were determined as this ratio is representative for overall polarization of the TME towards an antitumor or a protumor phenotype (Liu et al., 2013). Here, interleukin 10 (IL-10) levels were measured using mouse inflammatory cytokines multi-analyte ELISArray kit (Qiagen, MEM-004A) and expressed as % compared to its levels in control, and IL-12p70 levels were determined via protein array analysis and expressed as % compared to its levels in control.

Determination of MDA levels in C26 colon carcinoma tumors were determined by HPLC to investigate the effects of different treatments on the tumor oxidative stress, as previously described (Alupei *et al.*, 2015; Patras *et al.*, 2016). Data were expressed as μM MDA ± SD and were normalized to protein concentration.

Statistical analyses consisted of using one-way ANOVA with Bonferroni post-test for multiple comparisons for comparing the overall effects of different treatments on tumor growth and on the tumor markers tested, and two-way analysis of variance with Bonferroni correction for multiple comparisons for comparing the differences between the effects of various treatments on the production of angiogenic/inflammatory proteins. The DT of tumor volumes was estimated by using an exponential tumor growth equation. Data were expressed as mean ± SD and GraphPad Software (San Diego, CA) was used. A P value < 0.05 was considered significant.

VI.3. Results and discussion

VI.1. Simoultaneous administration of the combined liposomal drug therapy inhibited more efficiently the C26 colon carcinoma growth compared to single or sequential liposomal drug therapies

The effects of different treatments on the tumor development suggested that the growth of C26 colon carcinoma was affected moderately after LCL-5-FU administration (by 53%, $P<0.01$) to strongly after LCL-PLP treatment (by 70%) when compared to control tumors (PBS-/LCL-treated groups) growth or the effects of the free form drugs, which underlines the tumor-targeting capacity of the liposomal formulations tested (Fig. VI.1).

Simoultaneous administration of 20 mg/kg LCL-PLP with 1.2 mg/kg LCL-5-FU (LCL-PLP+LCL-5-FU) decelerated almost totally (by over 80%, $P<0.001$, Fig. VI.1E and F) the growth of C26 tumors, while the combination of the free drugs inhibited only slightly the tumor growth (by 30%, $P<0.01$, Fig. VI.1E and F). Noteworthy that DT of tumors treated with LCL-PLP+LCL-5-FU was about 2.5-3.5 times longer than the DT for C26 tumors after any other treatment tested (Table VI.1).

Two different administration regimens of the liposomal formulations (20 mg/kg LCL-PLP + 1.2 mg/kg LCL-5-FU versus 20 mg/kg LCL-PLP / 1.2 mg/kg LCL-5-FU) were tested for their antitumor efficacy (Fig. VI.2). The results revealed that the simultaneous administration of the liposomal drugs exerted stronger suppression of the C26 tumor growth than that induced by their sequential administration (by 72% *vs* 50% inhibition compared to control tumors, $P=0.02$).

Taking into consideration these findings, the treatment approach based on the concurrent administration of LCL-PLP and LCL-5-FU might be exploited for future therapeutic strategies applied in CRC. Therefore, the main mechanisms of the antitumor activity of LCL-PLP+LCL-5-FU in C26 colon carcinoma-bearing mice were further investigated with regard to intratumor production of inflammatory, angiogenic and oxidative stress markers, as earlier studies suggested their antitumor activities via modulation of key protumor processes (Schiffelers *et al.*, 2005; Banciu *et al.*, 2008a; Sylvester *et al.*, 2016; Patras *et al.*, 2016).

VI.2. The combination therapy employing LCL-PLP+LCL-5-FU exerted strong anti-angiogenic and anti-inflammatory effects in the C26 colon carcinoma environment

Our data provided confirmatory evidence for the anti-inflammatory and anti-angiogenic mode of action of the antitumor activity of the combined liposomal drug therapy in colon carcinoma *in vivo*. Hence, LCL-PLP+LCL-5-FU treatment reduced the expression levels of two key transcription factors associated with inflammation, NF-κB p65 subunit (by 35%) and AP-1 c-Jun subunit (by 60%) (Fig. VI.3), which could further explain the marked suppression of the

majority of the pro-angiogenic and pro-inflammatory protein production, while antitumor protein levels were only slighly affected (Fig. VI.4) (Wang *et al.*, 2000; Lind *et al.*, 2001; Ashida *et al.*, 2005; Sakamoto *et al.*, 2009).

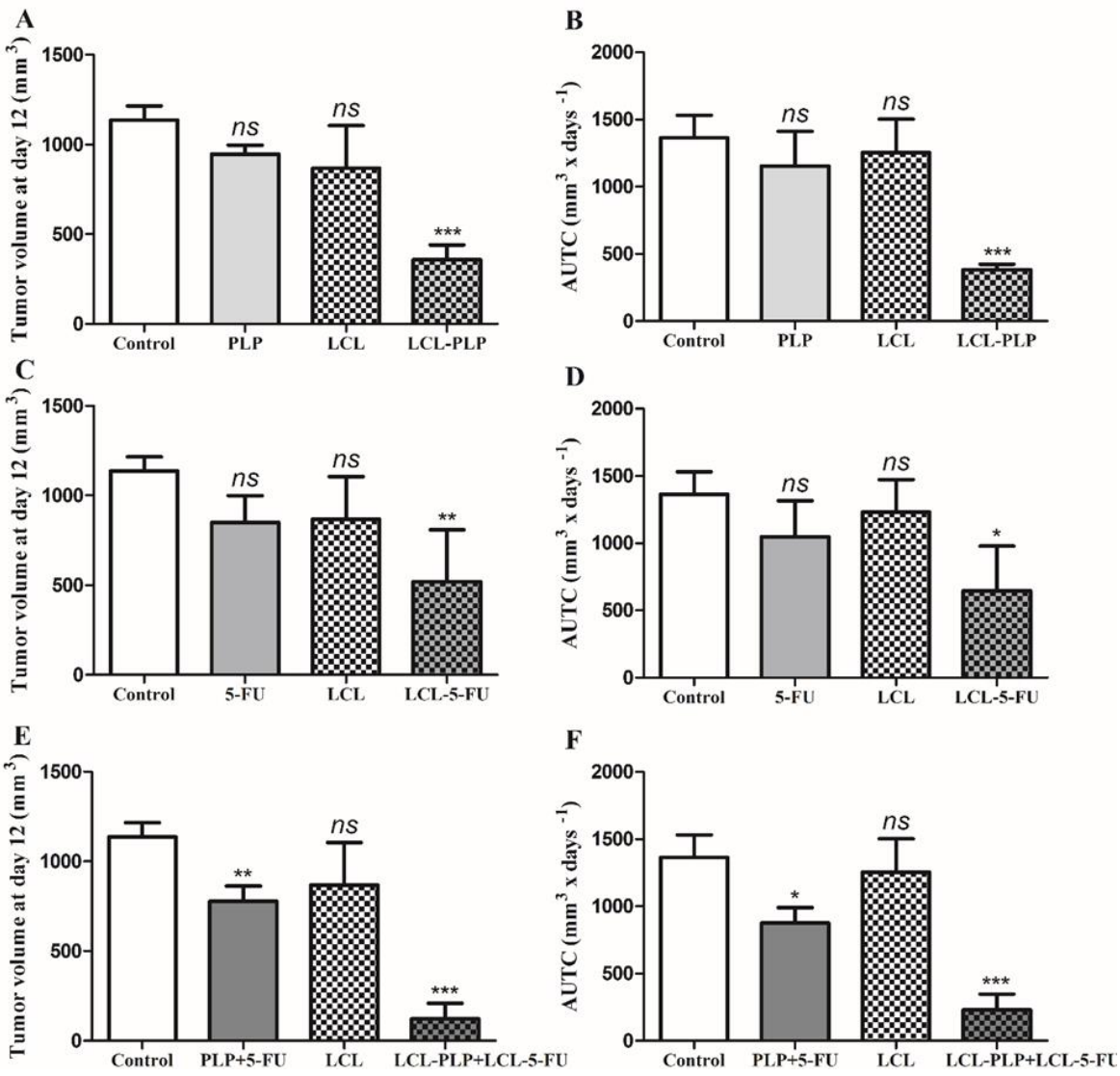

Figure VI.1. Effects of the combined administration of LCL-PLP and LCL-5-FU on the growth of *s.c.* C26 colon carcinoma. Tumor volumes at day 12 after tumor cell inoculation (when mice were sacrificed) after different treatments were presented in panels **A**, **C**, and **E**. AUTCs after various treatments were presented in panels **B**, **D** and **F**. The treatments were administered twice at days 8 and 11 after tumor cell inoculation in each experimental condition. The dose used for PLP was 20 mg/kg in either free or LCL form. The dose used for 5-FU was 1.2 mg/kg in either free or LCL form. The results were compared to PBS-treated groups (controls) and expressed as mean ± SD of tumor volumes of 5-6 mice.; *ns* - not significant, $P>0.05$; **, $P<0.01$; ***, $P<0.001$; Control = untreated group; LCL = group treated with empty liposomes.

Moreover, the reduction of both transcription factors and certain pro-angiogenic (bFGF, VEGF) and pro-inflammatory proteins (IL-1α, IL-12p40, TNF-α, MCP-1, FasL) suggest that the combined tumor-targeted therapy might also for an altered tumor cell proliferation and induction of apoptosis in these tumor cells (Takahashi *et al.*, 1996; Salven *et al.*, 1998; Casanovas *et al.*,

2005; Ashida *et al.*, 2005; Igney and Krammer, 2005; Tammali *et al.*, 2006; Nai *et al.*, 2007; Nowis *et al.*, 2007; Sakamoto *et al.*, 2009).

Table VI.1.

The doubling time of C26 colon carcinoma growth after different treatments.

Experimental groups	Tumor doubling time (days)
Control	1.864
LCL	1.531
PLP	1.771
LCL-PLP	2.040
5-FU	1.764
LCL-5-FU	1.996
PLP+5-FU	1.905
LCL-PLP+LCL-5-FU	5.364

Control = untreated group; LCL = group treated with empty liposomes; PLP or LCL-PLP were administered at a dosage of 20 mg/kg drug at days 8 and 11 after tumor cell inoculation; 5-FU or LCL-5-FU was administered at a dosage of 1.2 mg/kg 5-FU at days 8 and 11 after tumor cell inoculation.

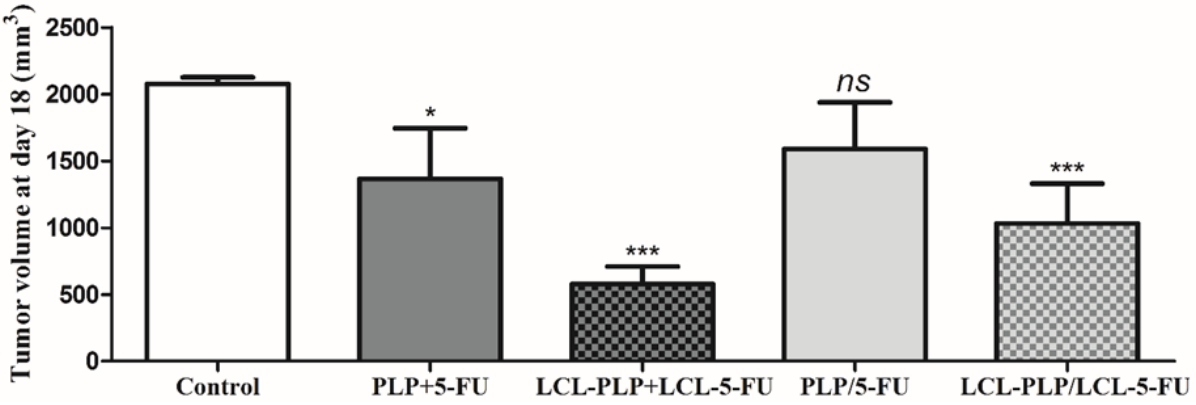

Figure VI.2. Effects of two different administration regimens of combined therapy (LCL-PLP+LCL-5-FU *versus* LCL-PLP/LCL-5-FU) on *s.c.* C26 colon carcinoma growth. Tumor volumes at day 18 after tumor cell inoculation (when tumors from control group reached 2,000 mm^3) were compared to the tumor volumes from control group. The results were expressed as mean ± SD of tumor volumes of 5-6 mice. *ns* - not significant, P>0.05; *, *P*<0.05; ***, *P*<0.001; Control = untreated group; LCL-PLP+LCL-5-FU = group treated with 20 mg/kg LCL-PLP and 1.2 mg/kg LCL-5-FU at days 8 and 11 after tumor cell inoculation; LCL-PLP/LCL-5-FU = group pretreated with 20 mg/kg LCL-PLP at days 7 and 10 after tumor cell inoculation with 24h before administration of 1.2 mg/kg LCL-5-FU.

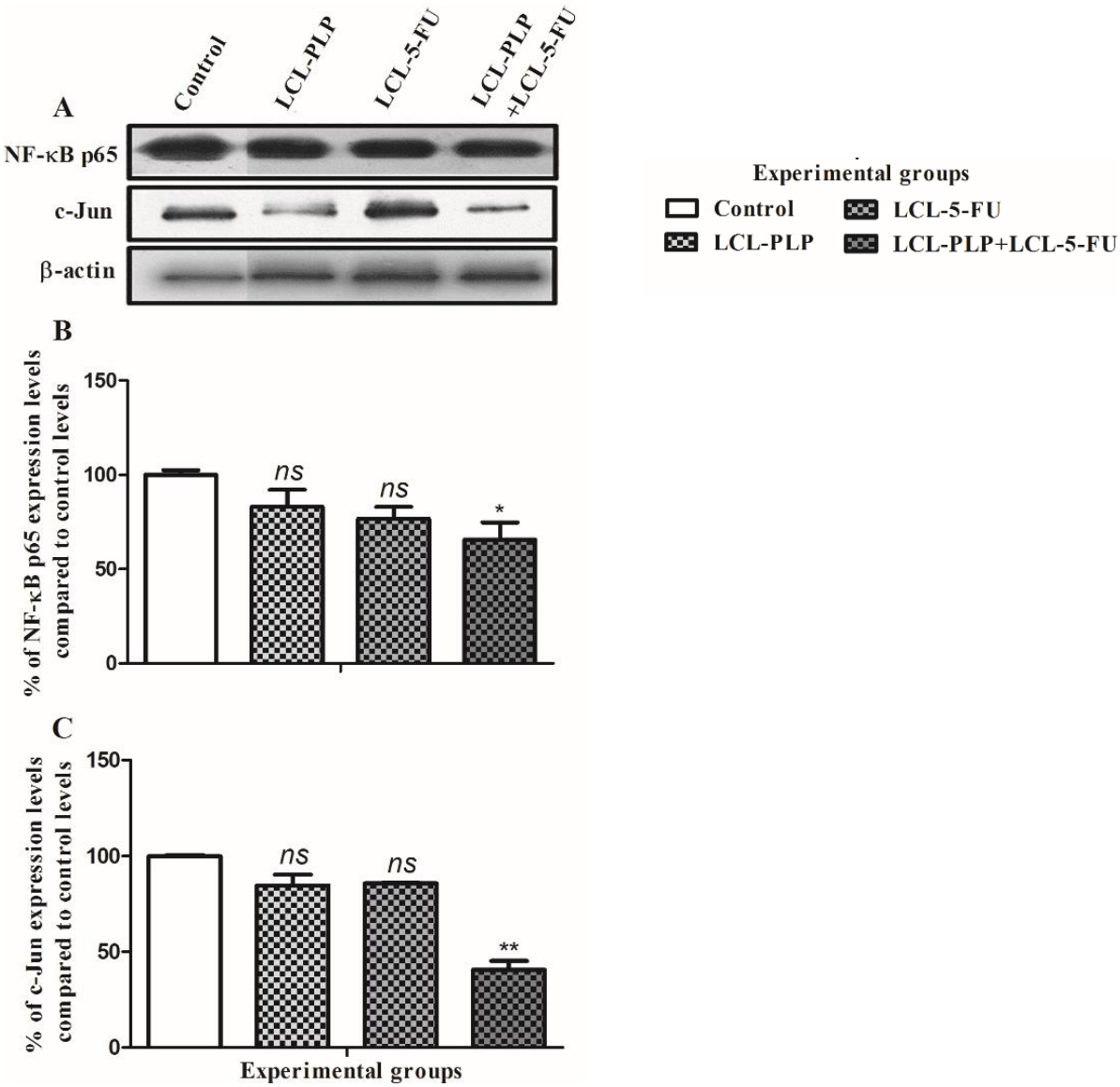

Figure VI.3. Effects of different treatments on the intratumor levels of p65 subunit of NF-κB and c-Jun subunit of AP-1. (A) Western blot analyses of NF-κB p65 and c-Jun levels in C26 tumor homogenates from each experimental group: Control = untreated group (lane 1); LCL-PLP = group treated with 20 mg/kg PLP as liposomal form at days 8 and 11 after tumor cell inoculation (lane 2); LCL-5-FU = group treated with 1.2 mg/kg 5-FU as liposomal form at days 8 and 11 after tumor cell inoculation (lane 3); LCL-PLP+LCL-5-FU = group treated with 20 mg/kg LCL-PLP and 1.2 mg/kg LCL-5-FU at days 8 and 11 after tumor cell inoculation (lane 4). β-actin was used as loading control. Quantification of western blot data for NF-κB p65 expression levels **(B)** and for c-Jun expression levels **(C)**. The levels of proteins from each experimental group are compared to the control levels of the same proteins and are expressed as mean ± SD of two independent measurements; *ns* - not significant, $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Nevertheless, for specific angiogenic and inflammatory proteins, PLP, 5-FU, PLP+5-FU, and LCL-5-FU treatments exerted moderate (by 40-100% stimulation compared to their control production) to strong (by 100-200% enhancement compared to their control production) stimulatory effects on their intratumor production (Fig. VI.4).

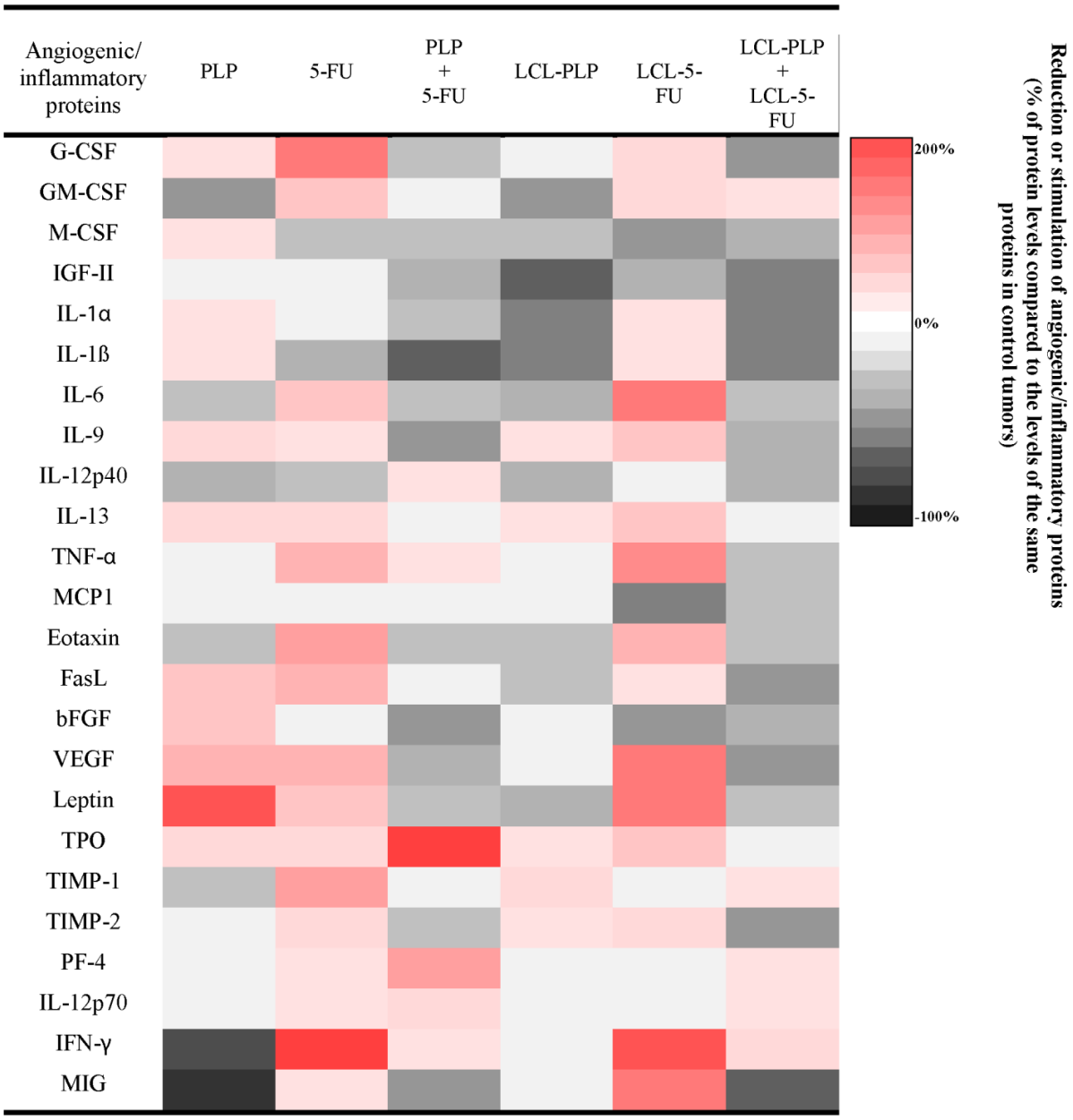

Figure VI.4. The effects of different treatments on angiogenic and inflammatory proteins production in *s.c.* C26 colon carcinoma tissue. Data are expressed as average % of reduction of tumor protein levels ranging from 0% (white) to -100% (black) or stimulation (+) of production of proteins ranging from 0% (white) to +200% (red) compared to the levels of the same proteins in control tumors. All treatments were administered at days 8 and 11 after tumor cell inoculation; PLP or LCL-PLP was administered at a dose of 20 mg/kg drug. 5-FU or LCL-5-FU was administered at a dose of 1.2 mg/kg drug.

VI.3.3. The treatment with LCL-PLP+LCL-5-FU favoured the skewing of TME cells towards an antitumor phenotype in C26 colon carcinoma milieu

The immunohistochemical analysis was used to determine the levels of macrophage infiltration into tumors, as their polarization into protumor or antitumor macrophages is a critical determinant of the therapeutic outcome (De Palma and Lewis, 2013; Zhong et al., 2018; Mantovani et al., 2006). The three-score qualitative analysis obtained from the immunostaining results that is

displayed in Table VI.3 revealed a marked F4/80 and iNOS positive macrophage infiltration into the tumors treated with LCL-5-FU (Table VI.3), most likely on the account of the overexpression of pro-angiogenic proteins VEGF and leptin (Fig. VI.4) and the low intratumor amounts of 5-FU (due to the low 5-FU dose administered) which render tumors more aggressive (Mantovani and Allavena, 2015). In contrast, tumors treated with LCL-PLP and LCL-PLP+LCL-5-FU displayed a strong tumor growth inhibition, a strong anti-angiogenic and anti-inflammatory effect (Fig. VI.1 and VI.4), which potentially led to occasional infiltration of macrophages into tumor tissue as only little or no stainings for both F4/80 and iNOS markers were observed (Table VI.3).

Table VI.3.

Immunohistochemical examination of macrophages infiltration in *s.c.* C26 colon carcinoma tumor tissues after different treatments.

	Experimental groups			
Marker	Control	LCL-PLP	LCL-5-FU	LCL-PLP+LCL-5-FU
F4/80	+	+	+++	+
iNOS	++	+	+++	+

Control = untreated group; Treatment was administered at days 8 and 11. LCL-PLP = group treated with 20 mg/kg PLP as liposomal form; LCL-5-FU = group treated with 1.2 mg/kg 5-FU as liposomal form; LCL-PLP+LCL-5-FU = group treated with 20 mg/kg LCL-PLP and 1.2 mg/kg LCL-5-FU. The numbers of F4/80 and iNOS positively stained cells in each experimental group were counted and categorized into three groups based on their density: +, few; ++, moderate; +++, many.

For a better overview on the efects of the combined therapeutic approach proposed in this study on the polarization of TME cells we also determined the levels of the antagonist cytokines IL-10 (M2 macrophage marker) and IL-12p70 (M1 macrophage marker), which were expressed as % of expression levels compared to the levels of the same proteins in control tumors, and the ratios for each experimental group were plotted as depicted in Fig. VI.5 (Michielsen *et al.*, 2011; Liu *et al.*, 2013). When liposomal combination therapy was applied, there was a 2-fold reduction of IL-10/IL-12p70 ratio (P=0.0037) compared to control ratio for the same cytokine, whereas the single liposomal therapies did not affect this value (Fig. VI.5). This effect, together with the reduction of M-CSF, IL-6 (Fig. VI.4), and NF-κB levels (Fig. VI.3A and B) induced by combined liposomal drug therapy, might suggest the conversion of the immunosuppressed phenotypes of the infiltrated immune cells to their antitumor phenotypes (Rolny *et al.*, 2011; Cook and Hagemann, 2013).

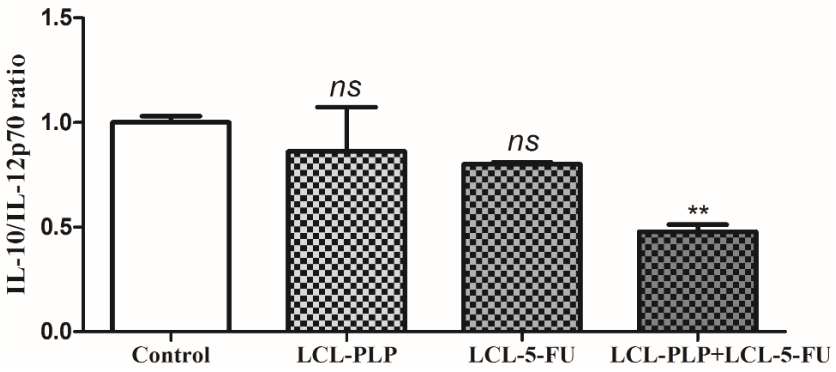

Figure VI.5. The effects of different treatments on the production ratio of IL-10/IL-12p70 in *s.c.* C26 colon carcinoma tissue. Levels of IL-10 and IL-12p70 cytokines are expressed as mean percentage ± SD compared to the expression levels of the same proteins in control tumors. Treatments were administered at days 8 and 11 for all experimental grups. Control = untreated group; LCL-PLP = group treated with 20 mg/kg PLP as liposomal form; LCL-5-FU = group treated with 1.2 mg/kg 5-FU as liposomal form; LCL-PLP+LCL-5-FU = group treated with 20 mg/kg LCL-PLP and 1.2 mg/kg LCL-5-FU; *ns* - not significant, $P>0.05$; **, $P<0.01$.

VI.3.4. The combination liposomal therapy controls tumor oxidative stress-mediated progression in the C26 colon carcinoma environment

Both single liposomal drug therapies induced a pro-oxidant effect in the proliferative range of tumor oxidative stress (μM) that might be associated with ROS-induced resistance to treatment and explain their lower antitumor activity (Fig. VI.1A-E, Fig. VI.6 and Table VI.1) (Licarete *et al.*, 2015; Boonyong *et al.*, 2017).

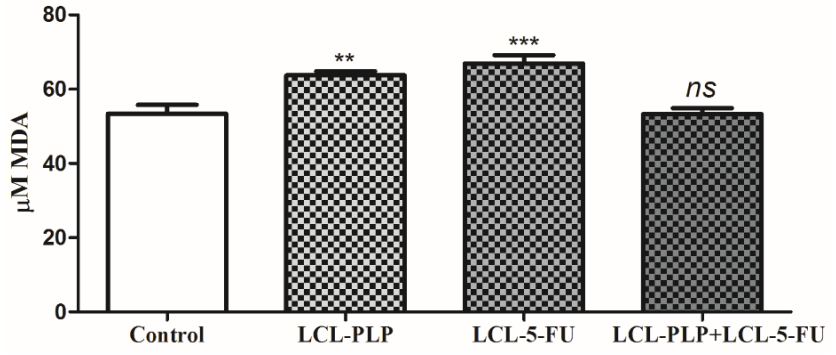

Figure VI.6. The effects of different treatments on MDA levels from C26 tumor homogenates. Data were expressed as mean ± SD of triplicate measurements. Control = untreated group; LCL-PLP = group treated with 20 mg/kg PLP as liposomal form at days 8 and 11 after tumor cell inoculation; LCL-5-FU = group treated with 1.2 mg/kg 5-FU as liposomal form at days 8 and 11 after tumor cell inoculation; LCL-PLP+LCL-5-FU = group treated with 20 mg/kg LCL-PLP and 1.2 mg/kg LCL-5-FU at days 8 and 11 after tumor cell inoculation; *ns* - not significant, $P>0.05$; **$P<0.01$; ***$P<0.001$.

Since our recent data have already proved that tumor oxidative stress potentiated angiogenic capacity of C26 colon carcinoma microenvironment (Luput *et al.*, 2017), the pro-oxidant effect of the single administration of LCL-5-FU probably determined a more agressive phenotype of C26 colon carcinoma, as also reflected by the increased levels of VEGF, leptin, IL-

6, and IL-13 in the tumor microenvironment (Fig. VI.4 and VI.6) (Becker et al., 2004; Pucci et al., 2009; Bendardaf et al., 2009; Gordon and Martinez, 2010; Gonzalez-Perez et al., 2013; Guerriero et al., 2013). However, the observed protumor effect was counteracted after simultaneous administration of both liposomal formulations, since the levels of MDA after LCL-PLP+LCL-5-FU treatment remained similar to those measured in control tumors (Fig. VI.6). This suggests the capacity of the liposomal combination therapy to control tumor oxidative stress.

VI.4. Conclusions

Altogether, the present study demonstrates the antitumor efficacy of the combined therapy based on the concurrent administration of LCL-PLP and LCL-5-FU compared to single administration of each liposomal formulation in C26 murine colon carcinoma-bearing mice. The antitumor activity of LCL-PLP+LCL-5-FU was based on the inhibition of tumor angiogenesis and inflammation in a C26 colon carcinoma microenvironment that was polarized towards an antineoplastic phenotype.

VII. General conclusions

The first study (**Chapter IV)** suggested that cytotoxic drug therapy of colon cancer cells induced the production of normoxic TEV that possessed an anti-apoptotic phenotype that could alter the response of recipient cells (cancer cells and macrophages) to the same drug, rendering them less responsive. Thus, the effects cancer cell-macrophage crosstalk on the therapeutic response were further investigated and the results reported in **Chapter V** revealed the dual role displayed by TAM in modulating the response of C26 colon carcinoma cells to cytotoxic drug 5-FU in a co-culture model *in vitro*. Besides demonstrating the pivotal role of macrophages in promoting cancer progression and protecting cancer cells against 5-FU-induced oxidative stress, we also observed a TAM-driven impairment of angiogenic and inflammatory processes. Consequently, we proposed that future therapeutic strategies should focus on preventing TAM to maintain a physiological range of tumor oxidative stress, which is known to facilitate tumor progression. The last study of the thesis (**Chapter VI**) provided evidence that the simultaneous administration of LCL-PLP and LCL-5-FU was by far superior to single liposomal therapy or sequential liposomal treatment, as it synergistically inhibited tumor growth almost entirely. We demonstrated that the molecular mechanisms underlying this effect were based on a strong anti-angiogenic and anti-inflammatory action on C26 carcinoma milieu, characterized by the polarization of TME cells towards their antineoplastic phenotypes. These results confirmed the potential of the proposed therapy to target TAM-mediated protumor processes and of the liposomal PLP therapy to enhance 5-FU antitumor efficacy *in vivo*, which together should be considered for subsequent pre-clinical studies addressing TME targeting.

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Chapter I.

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Chapter II.

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Chapter IV.

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Chapter V.

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Chapter VI.

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