Lipid Signaling in Anti-Cancer Drug Resistance

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Printing of this thesis was financially supported by Cayman Chemical, Fresenius-Kabi, Boeringer-Ingelheim and Nutricia.

The work described in this thesis was performed at the laboratory of Medical Oncology at the University Medical Center in Utrecht (UMCU) and at the laboratory of Molecular Oncology at the Netherlands Cancer Institute in Amsterdam (NKI-AvL).

Lipid signaling in anti-cancer drug resistance

De rol van lipiden in resistentie tegen anti-kanker medicatie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 12 april 2016 des middags te 2.30 uur

door
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te Heemskerk

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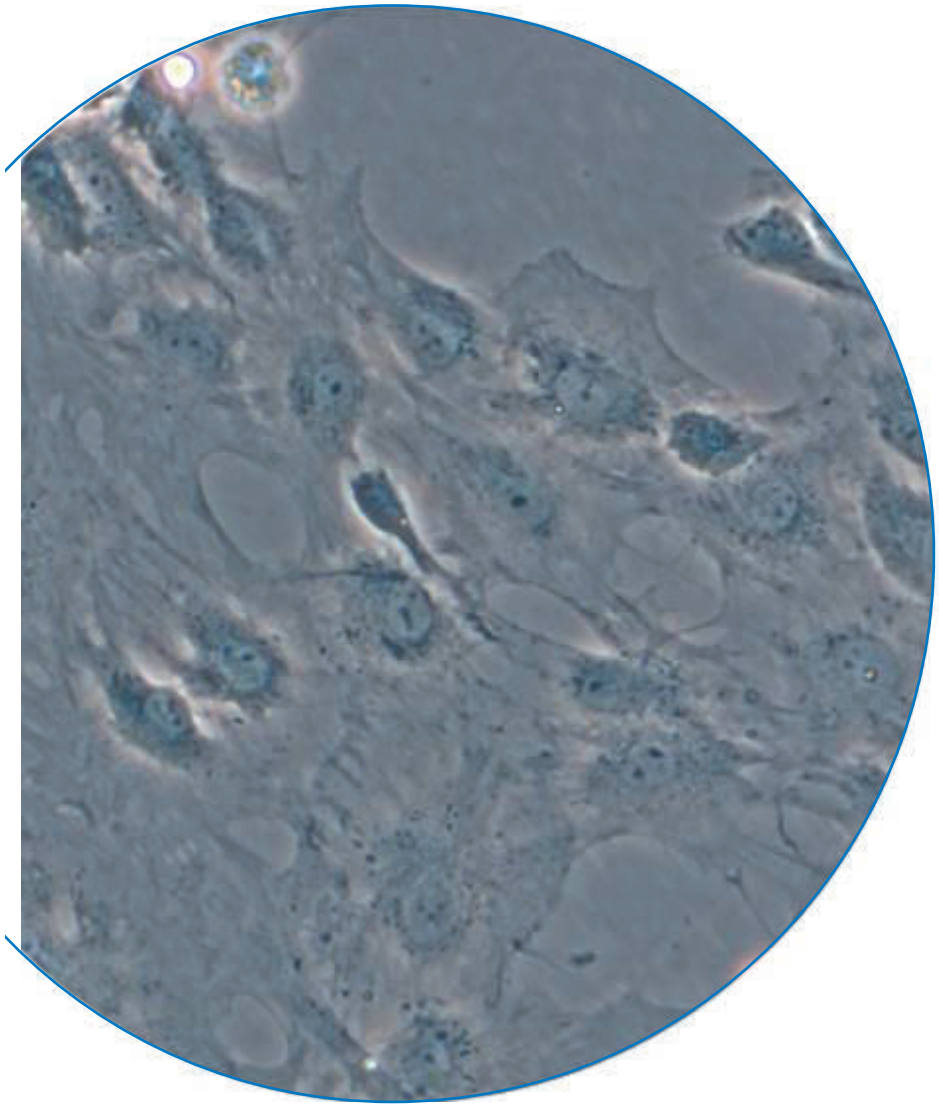
Het onderzoek beschreven in dit proefschrift is gefinancierd door KWF Kankerbestrijding (UU2009-4534), Stichting de Drie Lichten en de René Vogels beurs

Contents

CHAPTER 1



The role of mesenchymal stem cells and macrophages in treatment-induced anti-cancer drug resistance

Adapted from:

The role of mesenchymal stem cells in anti-cancer drug resistance and tumor progression

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British Journal of Cancer **106**: 1901-1906 (2012)

Treatment-induced host-mediated mechanisms reducing the efficacy of antitumor therapies

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Oncogene **33**: 1341-1347 (2014)

Abstract

It is becoming increasingly clear that the tumor microenvironment plays a very important role in tumor progression and drug resistance. In addition to its direct effects on tumor cells, chemotherapy can rapidly activate various host processes that contribute to therapy resistance and tumor regrowth. Examples include the acute mobilization and tumor homing of pro-angiogenic bone marrow-derived cells, activation of cells in the tumor microenvironment to produce systemic or paracrine factors, and tissue-specific responses that provide a protective niche for tumor cells. Recent studies have shown that these processes can reduce chemotherapy efficacy, and blocking the host response at various levels may therefore significantly improve treatment outcome. However, before the combination of conventional chemotherapy with agents blocking specific aspects of tumor microenvironment can be implemented into clinical practice, a better understanding of the molecular mechanisms is required. This review gives an overview of the effects of chemotherapeutics on normal tissues and how these host-responses can affect chemotherapy efficacy with a focus on mesenchymal stem cells and macrophages within this process.

Introduction

Anti-cancer therapies frequently induce shrinkage of tumor mass, but a subpopulation of cells will almost invariably survive therapy. The selective pressure of therapy can lead to the development of resistance in this population, which may subsequently grow out into a clinically important tumor burden. Given the continued importance of chemotherapy in cancer management, chemotherapy resistance poses a major obstacle in effective anti-cancer treatment. Resistance to chemotherapy can arise within tumor cells due to genetic changes causing for instance increased drug efflux (intrinsic resistance), or it can be the result of the tumor microenvironment protecting tumor cells against treatment (extrinsic resistance). The tumor microenvironment can promote drug resistance in a passive way, by preventing penetration of drugs into the tumor or in an active way by secreting protective cytokines or changing gene transcription within the tumor cells to override the cytotoxic effects of anti-cancer agents[1]. Here, we summarize the available literature on how anti-cancer drugs activate non-tumor host cells to the unfortunate benefit of tumors with a focus on mesenchymal stem cells and macrophages.

Host responses to chemotherapy treatment

Even though chemotherapy is designed to target rapidly dividing tumor cells, systemic administration of a cytotoxic agent will hit non-tumor cells as well. Acute side effects such as mucositis, myelosuppression and hair loss show us that rapidly regenerating healthy tissues are also damaged: the lining of the gut, the bone marrow and hair-follicle cells. A number of studies show that chemotherapy also has impact on multiple cell types in the tumor microenvironment. In some cases, the effects of chemotherapy on healthy tissues may enhance treatment benefits. For instance, conventional cytotoxic agents have been shown to target proliferating endothelial cells of the growing tumor vasculature, thereby diminishing angiogenesis. These antiangiogenic effects of chemotherapy can be optimized by prolonged administration of low-dose chemotherapy without drug-free breaks, the so-called 'metronomic' regimen[2-5]. Furthermore, some chemotherapeutic agents were shown to have immunogenic effects that enhance their anti-cancer efficacy, for example, suppression of pro-tumorigenic regulatory T cells by low-dose cyclophosphamide[6]. Other examples include selective cytotoxicity towards tumor-associated myeloid-derived suppressor cells by 5-FU[7], and the activation of tumor antigen-specific T-cell immunity due to release of HMGB1 by dying tumor cells following anthracycline-based chemotherapy[8]. On the contrary, gemcitabine and 5-FU were recently shown to induce MDSC-mediated T-cell proliferation into IL-17-producing $CD4^+$ T cells able to reduce chemotherapy efficacy[9]. Thus, in addition to the beneficial effects on the microenvironment, chemotherapy may also induce a host response with a 'malignant' character (Figure 1). Theoretically, this could lead to the paradoxical situation where treatment effects of an anti-cancer drug on the microenvironment impair its activity on the tumor.

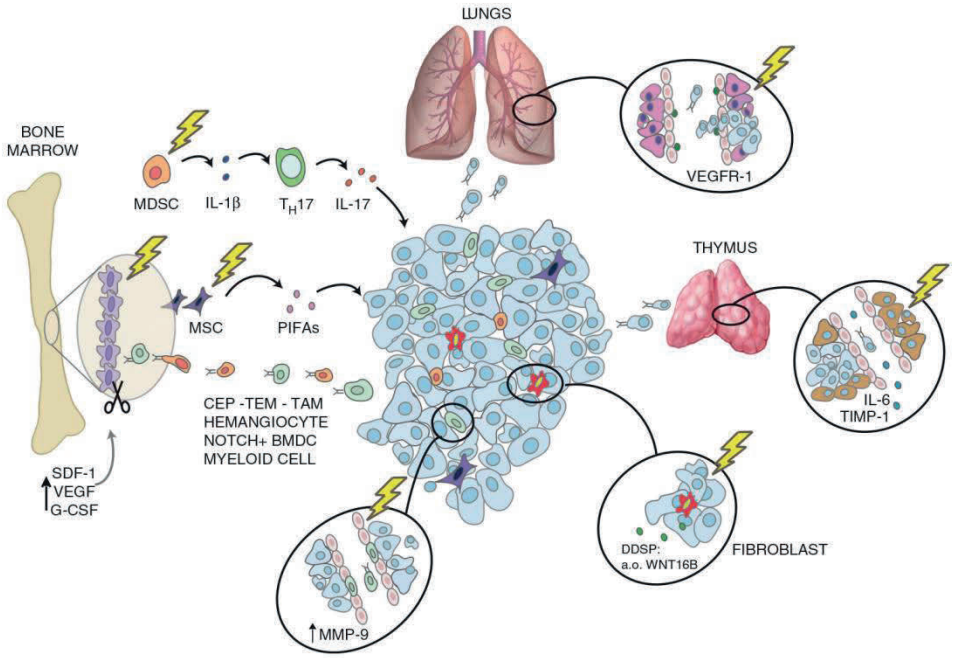

Figure 1: Various aspects of the host response to antitumor therapy may interfere with treatment outcome. The host response includes upregulation of cytokines, followed by the acute mobilization and tumor homing of pro-angiogenic BMDCs. Activation of cells in the tumor microenvironment takes place, resulting in production of systemic or paracrine factors. Furthermore, tissue-specific responses to chemotherapy in lungs and thymus can provide a protective niche to tumor cells. CEP, circulating endothelial progenitor cell; TEM, Tie2-expressing monocyte; BMDC, bone marrow-derived cell; MSC, mesenchymal stem cell; PIFA, platinum-induced fatty acid; SDF-1, stromal cell-derived factor 1; VEGF, vascular endothelial growth factor; G-CSF, granulocyte colony-stimulating factor; VEGFR-1, vascular endothelial growth factor receptor 1; IL-6, interleukin-6; TIMP-1, tissue inhibitor of metalloproteinase 1; DDSP, DNA damage secretory program; MMP-9, matrix metallopeptidase 9; MDSC, myeloid-derived suppressor cell; TH17, T helper cell type 17; IL-1β, interleukin-1β; IL-17, interleukin-17.

The role of non-neoplastic cells in chemotherapy resistance

To study the consequences of the host response on tumor growth, one has to distinguish these effects from the potent, direct antitumor effects of cytotoxic agents. Administration of chemotherapy to non-tumor-bearing mice provides an elegant solution to this challenge. Using plasma from non-tumor-bearing mice that had been treated with paclitaxel, Gingis-Velitsky *et al.* found enhanced migration and invasion of tumor cells *in vitro*[10]. Sun *et al.* analyzed DNA damage in fibroblasts in tissues from prostate, breast and ovarian cancer patients after chemotherapy. Extensive DNA damage and subsequent NF-κB activation occurred, resulting in a 'DNA damage secretory program' (DDSP). Numerous growth factors and cytokines were secreted by fibroblasts ensuing chemotherapy-induced

damage, among which was WNT16B, promoting phenotypic changes in tumor cells through paracrine mechanisms. Not only tumor cell proliferation, epithelial-to-mesenchymal transition, migration and invasion were enhanced, but response to cytotoxic therapy was impaired as well. Blocking the DDSP through either NF-κB or WNT16B inhibition restored chemotherapy sensitivity[11].

Some aspects of the host response to chemotherapy are tissue-specific. Gilbert *et al.* reported a thymus-specific response to cytotoxic drugs that impaired tumor regression specifically at that anatomical localization. In a mouse model of disseminated Burkitt's lymphoma, doxorubicin exposure resulted in tumor regression at all lymphoid tissues with the exception of the thymus. There, minimal residual disease remained from which tumors regrew. Cytotoxic agents were shown to activate stress-responsive kinases such as p38-MAPK in endothelial cells in the thymic microenvironment, which resulted in the secretion of IL-6 and TIMP-1 which subsequently protected the tumor cells against genotoxic chemotherapy[12].

Finally, another tissue-specific survival niche can be found in the pulmonary vascular bed. When chemotherapy was administered to non-tumor-bearing mice followed by intravenous tumor cell injection, the number of pulmonary tumor cell colonies was enhanced compared with mice without pretreatment[13-16]. Priming of the lungs by chemotherapy was recently shown to induce VEGFR-1 upregulation on a specific population of pulmonary endothelial cells, resulting in enhanced tumor cell retention in the lungs by a not yet uncovered mechanism. Combining chemotherapy with an antibody targeting VEGFR-1 diminished early retention of tumor cells in the lungs and prevented formation of chemotherapy-induced metastases. Thus, when the direct antitumor effects of chemotherapy were absent, the host response could be visualized, creating an environment favorable for tumor-cell colonization by directly affecting lung endothelial cells and priming the lung for tumor cell colonization[17].

Mesenchymal stem cells and anti-cancer drug resistance

Mesenchymal stem cells (MSCs) have been widely investigated for their role in tumor progression and more recently also anti-cancer drug resistance (Figure 2). The finding that enhanced numbers of MSCs are present in the circulation of cancer patients compared to healthy volunteers indicates that there is cross-talk between the tumor and MSCs[18,19]. The role of MSCs in cancer progression has often been debated since both positive and negative effects on tumor proliferation, immune suppression, metastasis and therapy resistance have been found. For a number of hematological malignancies research indicates that MSCs can confer resistance to treatment. Chronic myeloid leukemia (CML) cells can be protected from imatinib-induced cell death by co-culturing them with MSCs.

This protective mechanism was mediated by SDF-1α secretion from the MSCs following activation of the CXCR4 pathway in CML cells leading to reduced caspase 3 activity. Inhibition of CXCR4 restored the sensitivity to imatinib[20]. Furthermore, it was shown that imatinib itself also increased CXCR4 expression on CML cells, indicating that treatment itself can prime tumor cells to such a state that they are more susceptible to the protective effects of MSCs. This increased CXCR4 expression also caused migration of leukemic cells to the bone marrow resulting in stroma-mediated senescence and chemotherapy resistance[21]. MSCs are also implicated in drug resistance of chronic lymphocytic leukemia (CLL). Balakrishnan *et al*. reported that CLL cells can become resistant to the novel drug forodesine. Forodesine induces apoptosis in circulating CLL cells. However, it is unable to effectively kill CLL cells present in the bone marrow where they reside closely to MSCs and other bone marrow cells. Co-cultures of MSCs and CLL cells showed decreased forodesine-induced depletion of ATP and GTP and decreased apoptosis in the CLL cells proving that MSCs are responsible for the reduced effectiveness of the drug[22]. MSCs also play a role in drug resistance of acute lymphoblastic leukemia (ALL). ALL cells express low levels of asparagine synthase (ASNS) and can therefore not supply in their own needs of asparagine. Many patients therefore benefit from asparaginase treatment leading to the depletion of asparagine in the circulation, although resistance to treatment poses a problem. Since MSCs express high levels of asparagine synthase and ALL cells grow in close proximity to MSCs it was hypothesized that the MSCs are able to confer resistance to asparaginase treatment by supplying the ALL tumors with asparagine. Iwamoto *et al*. have showed the validity of this hypothesis by demonstrating that co-culturing MSCs with ALL cells decreased the asparaginase induced cytotoxicity[23].

Resistance against anti-cancer agents occurs not only in hematological malignancies, MSCs have also been implicated in resistance to chemotherapy of solid tumors. Head-and-neck squamous cell carcinoma (HNSCC) cell lines FaDu and HLaC78 are more resistant to paclitaxel treatment when co-cultured with bone marrow derived stem cells (BMSCs). The use of a trans-well system indicated that factors that give chemoresistance are secreted by the BMSCs and that no direct cell-cell contact is needed[24]. Evidence also exists for a role of MSCs in ovarian cancer therapy resistance. Intraperitoneal chemotherapy is an effective strategy to treat patients with ovarian cancer. Recently, studies have been conducted in which intraperitoneal chemotherapy was combined with hyperthermia (HIPEC) in ovarian cancer patients. In contrast with the high success rate of this approach in colorectal cancer, it proved unsuccessful for the treatment of ovarian cancer. Lis *et al*. demonstrated that tumor associated MSCs are able to protect ovarian cancer cells from the hyperthermia-induced cell death via SDF1α/CXCR4 signaling[25].

Another mechanism by which MSCs can confer resistance to chemotherapy is by their conversion to cancer-initiating cells or cancer stem cells. Cancer stem cells have the capacity to initiate tumor formation, metastasis and are believed to be highly resistant to anti-cancer drugs and therefore responsible for recurrence. Teng *et al.* has recently shown that targeted methylation of the promoters of two tumor suppressor genes RasF1A and HIC1 in MSCs caused their transformation towards cancer initiating cells and enabled them to grow in an anchorage independent manner, formed tumors in nude mice and showed increased resistance to cisplatin treatment[26]. This sheds a new light on the role of MSCs in tumor formation and will require further research if MSCs under physiological conditions by this mechanism can also form a pool of possible cancer initiating cells. In addition to this the hypothesis has been posed that MSCs can sustain a cancer stem cell niche *in vivo* thereby sustaining malignancy and possibly also drug resistance[27].

In contrast, there are also reports that MSCs can also inhibit tumor growth in leukemia[27] and hepatoma[28] amongst others. Mechanistically, these anti-tumor effects involve downregulation of Akt, beta-catenin, Bcl-2, c-Myc, proliferating cell nuclear antigen (PCNA) and survivin, leading to reduced proliferation, G1 arrest, suppression of oncogenes and increased apoptosis. These differences in pro- versus anti-tumor effects of MSCs can in part be explained by the tumor types investigated, isolation methods used to obtain the MSCs and numbers of MSCs used. Furthermore, there is a clear difference between the function of MSCs *in vitro* and *in vivo* as was shown for several tumor cell lines including hematopoietic and non-hematopoietic tumor cells. The MSCs exhibited anti-proliferative effects by causing a G1-arrest *in vitro* however *in vivo* injection of tumor cells combined with MSCs led to faster tumor growth[27]. These results clearly underscore the challenges ahead of us in investigating how MSCs in one situation block tumor growth and promote growth or metastasis in other situations. In spite of the studies showing a pro-tumorigenic effect, several groups have advocated the use of genetically engineered MSCs as a vehicle to target tumor cells. This approach was successful in models for glioma amongst multiple other tumor types[29]. In summary, MSCs are pluripotent cells that, once arrived in the tumor microenvironment, can secrete a variety of factors that either positively or negatively influence tumor growth.

Recently, we have shown that MSCs can confer chemotherapy resistance in solid tumors in mice. Injection of MSCs intravenously or subcutaneously at a distant site from the tumor at the time of cisplatin treatment led to complete resistance to the treatment. The ability to induce resistance was unique to MSCs since differentiated progeny were not able to induce resistance, indicating that this feature is lost in the differentiation process[19]. The resistance was mediated by the release of two distinct platinum-induced polyunsaturated fatty acids (PIFAs) in response to the cisplatin. The PIFAs that were

identified are 12-S-keto-5,8,10-heptadecatrienoic acid (KHT) and 4,7,10,13-hexadecatetraenoic acid (16:4(n-3)). KHT is the oxidized metabolite of 12-S-hydroxy-5,8,10-heptadecatrienoic acid (12-S-HHT) which is a fatty acid that is formed as a by-product in the production of thromboxane A2 synthesis within the arachidonic acid (AA) pathway. Metabolism of AA leads to the production of a series of active fatty acids derivatives also known as eicosanoids including 12-S-HHT and KHT. Very little is known about 16:4(n-3), it is an omega-3 fatty acid and it has been described to be produced by some marine algae[30].

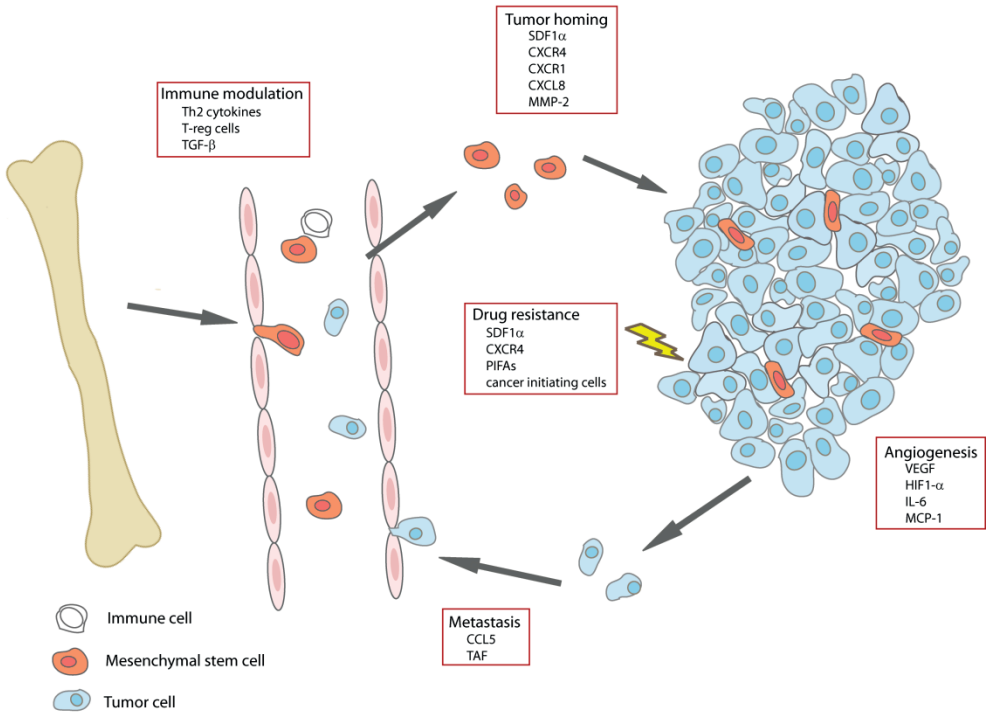

Figure 2: Schematic overview of the role of MSCs in tumor progression and drug resistance. MSCs can modulate various immune responses allowing tumor cells to evade the immune system. Pathways involved in this process include activation of regulatory T cells, secretion of Th2 cytokines and TGF-b. MSCs can also promote tumor growth by migrating to developing tumors (via SDF-1α/CXCR4 signaling, CXCL8, MMP-2) and subsequent stimulation of proliferation, angiogenesis (via VEGF, IL-6, MCP-1 and HIF-1α signaling) and inhibition of apoptosis. MSCs have also been shown to enhance metastasis directly via CCL5 signaling and indirectly via differentiation into tumor associated fibroblasts. Increasing evidence also shows that MSCs contribute to drug resistance via secretion of PIFAs, activation of SDF-1α/CXCR4 signaling or by forming a pool of cancer initiating cells themselves. TGF-β: transforming growth factor beta, MCP-1: monocyte chemotactic protein 1, MMP-2: matrix metalloproteinase 2, HIF-1α: hypoxia inducible factor 1 alpha, CCL5: Chemokine (C-C motif) ligand 5.

The PIFAs were able to induce resistance against a broad spectrum of anti-cancer agents and were functional in nanomolar concentrations. Systemic treatment of tumor-bearing mice with these PIFAs also showed resistance to chemotherapy, indicating that the presence of the MSCs itself was not needed. The production of these PIFAs was depended on cyclooxygenase 1 (COX-1), cytosolic phospholipase A2 ($cPLA_2$) and thromboxane synthase (TXAS), since inhibitors for these enzymes were able to block the formation of resistance[19]. These novel findings show that MSCs can also generate a systemic response to chemotherapy distant from the tumor, rather than initiating a response only from within the tumor microenvironment. Both ways can lead to resistance against various anti-cancer agents. Our data shows that the MSCs are activated by platinum-containing therapeutics but protect against a broad range of chemotherapeutics. This novel role of MSCs in systemic therapy resistance can possibly be placed in a bigger perspective where MSCs are functioning as guarding cells of the body protecting it from exogenous cytotoxic agents, backfiring on us in the case of anti-cancer treatment.

Macrophages and anti-cancer drug resistance

Macrophages are often found in and around the tumor. Active recruitment of macrophages to the tumor been seen in various tumor types including, but not restricted to, breast cancer, glioblastoma, lung cancer, hepatic cancer and pancreatic cancer[31-37]. DeNardo *et al*. showed that tumor cells upon chemotherapy treatment release colony stimulating factor 1 (CSF1) which attracts CSF1R expressing macrophages to the tumor. The recruitment of these tumor-associated macrophages (TAMs) led to a decrease in chemotherapy efficacy which could be restored by a CSF1R blocking antibody[38]. Other research groups have shown similar but mechanistically different mechanisms. Paclitaxel treatment can attract tumor-associated macrophages (TAMs) to mammary tumors where they interfere with the chemotherapy response by the secretion of cathepsins. Concurrent therapy with paclitaxel and cathepsin inhibitors slowed tumor growth[39]. In addition, Sahoo *et al.* showed that cross talk between tumor cells and macrophages via human chorionic gonadotrophin (hCG) can direct macrophages to produce IL-6 which in turn provided a chemoprotective niche for tumor cells[40]. Pancreatic cancer is characterized by large infiltrates of stromal cells which can severely hamper effective treatment. The TAMs present in the tumor microenvironment of pancreatic cancer have been shown to interfere with gemcitabine treatment by upregulating cytidine deaminase (CDA), an enzyme that metabolizes the drug after it has been transported into the cell. Inhibition of CDA in pancreatic cells restored sensitivity to gemcitabine[41].

TAMs can also interfere with the efficacy of targeted anti-cancer agents. Tripathi *et al*. found that hypoxic breast cancer cells can attract TAMs and polarizes them towards a M2 pro-angiogenic phenotype via eotaxin and oncostatin M. Inhibition of TAM recruitment

enhanced the efficacy of the anti-angiogenic drug bevacizumab in tumor-bearing mice[42]. In addition, resistance against the anti-IGF1R monoclonal antibody cixutumumab is mediated by macrophages and fibroblasts. Treatment with cixutumumab enhances tumor infiltration by macrophages and fibroblasts resulting in enhanced CXCL8 production and the generation of a angiogenic and metastatic microenvironment[43]. Macrophages also play an important role in the resistance of melanoma against BRAF inhibition. In the presence of a BRAF inhibitor, macrophages produce VEGF leading to activation of the VEGFR on melanoma cells and enhanced proliferation[44].

Our lab has recently shown that splenic macrophages can induce resistance against chemotherapy. The splenic F4/80^{+}/CD11b^{low} macrophages are activated by PIFAs secreted from mesenchymal stem cells and as a result produce lysophosphatidylcholines (LPCs) which reduce chemotherapy efficacy. Splenectomy or depletion of splenic macrophages by liposomal clodronate protects against PIFA-mediated chemoresistance[45]. This is an interesting finding for a number of reasons. First, the macrophages responsible for the chemoprotective effect are distant from the tumor and no active migration of these macrophages to the tumor is needed to induce resistance. Second, the signaling molecules involved are not cytokines but lipid mediators. And finally, the effect of the PIFAs is rapid, only one hour exposure of splenic macrophages to the PIFAs is enough for the production of the LPCs and subsequent chemoresistance. In addition, nanomolar concentrations of both PIFAs and LPCs are sufficient, indicating a potent protection mechanism. Taken together, it has been shown that macrophages can negative affect anti-cancer treatment and various clinical trials have found a correlation between macrophage infiltrate within the tumor and poor prognosis[46-48].

Discussion

The tumor microenvironment is an important determinant in tumor development, metastasis and response to therapy. The reaction of host cell to anti-cancer therapy can have a significant effect on the tumor. This host response can be seen as an attempt of the body to protect tissues crucial for its well-functioning and homeostasis, which is exploited by the tumor. MSCs and macrophages, for example, are recruited to wounds and other sites of injury, where they contribute to tissue repair. The massive damage exerted to a tumor by chemotherapy might elicit a similar response in these cells, thereby aiding tumor repopulation. MSCs and macrophages can promote tumor growth and drug resistance either via close contact with the tumor cells or via systemic mechanisms involving secreted factors. Targeting MSCs or macrophages as part of anti-cancer therapy can significantly inhibit tumor growth and metastasis and increase therapy response *in vitro* and in mouse models. However, both MSCs and macrophages are highly plastic cells that can have diverse ways to decrease anti-cancer therapy efficacy, inhibiting these cells in a

clinical setting will be challenging. The potent homing of MSCs to tumors is already explored to selectively target tumors. However, it should be kept in mind that these genetically engineered MSCs still contain the numerous intrinsic mechanisms that can promote tumor growth, metastasis and drug resistance indicating that using them as vehicles to target tumors may have severe side effects if not used very carefully. Despite the enormous amount of data present on the tumor promoting properties of MSCs there are also reports that MSCs can inhibit tumor growth. Perhaps depending on different stimuli the MSCs can also polarize into different states like it has been described for macrophages and more recently also neutrophils. However, what causes the MSCs to become tumor promoting or inhibiting is still unknown. The contradictions between studies could be due to the number of cells used, the source of the MSCs, the cancer types investigated or other components within the tumor microenvironment that will influence the function of the MSCs. The role of macrophages in anti-cancer therapy resistance evident from numerous studies that show that there is multi-level cross talk between tumor cells and macrophages. This data is validated by various clinical studies in which a correlation was shown between the presence of macrophages within a tumor and poor prognosis.

The challenge ahead lies in interfering with the host response to maintain its important physiological role in tissue repair of healthy tissues while preventing repair in malignant tissues. If the balance swings too much towards allowing repair, tumors may benefit. However, if too much weight is put on preventing repair, the patient might experience numerous side effects. An important question that needs to be addressed clinically is whether the host responses described above occur in all patients, or whether a subset is more at risk. We know that some patients respond better to chemotherapy than others, and some patients experience more side effects than others. Despite the use of predictive models, it is currently hard to select the patients who will benefit most from therapy. The host response to therapy will depend on the nature of the microenvironment, which is dynamic and influenced by the drug used. Also tumor type, localization, stage and treatment will affect the host response, as well as the characteristics of individual patients and tumors. Despite these challenges, we believe that further research should be focused on the combination of conventional antitumor therapies with interventions specifically aimed at preventing the host response since it has the potential to significantly improve cancer therapy.

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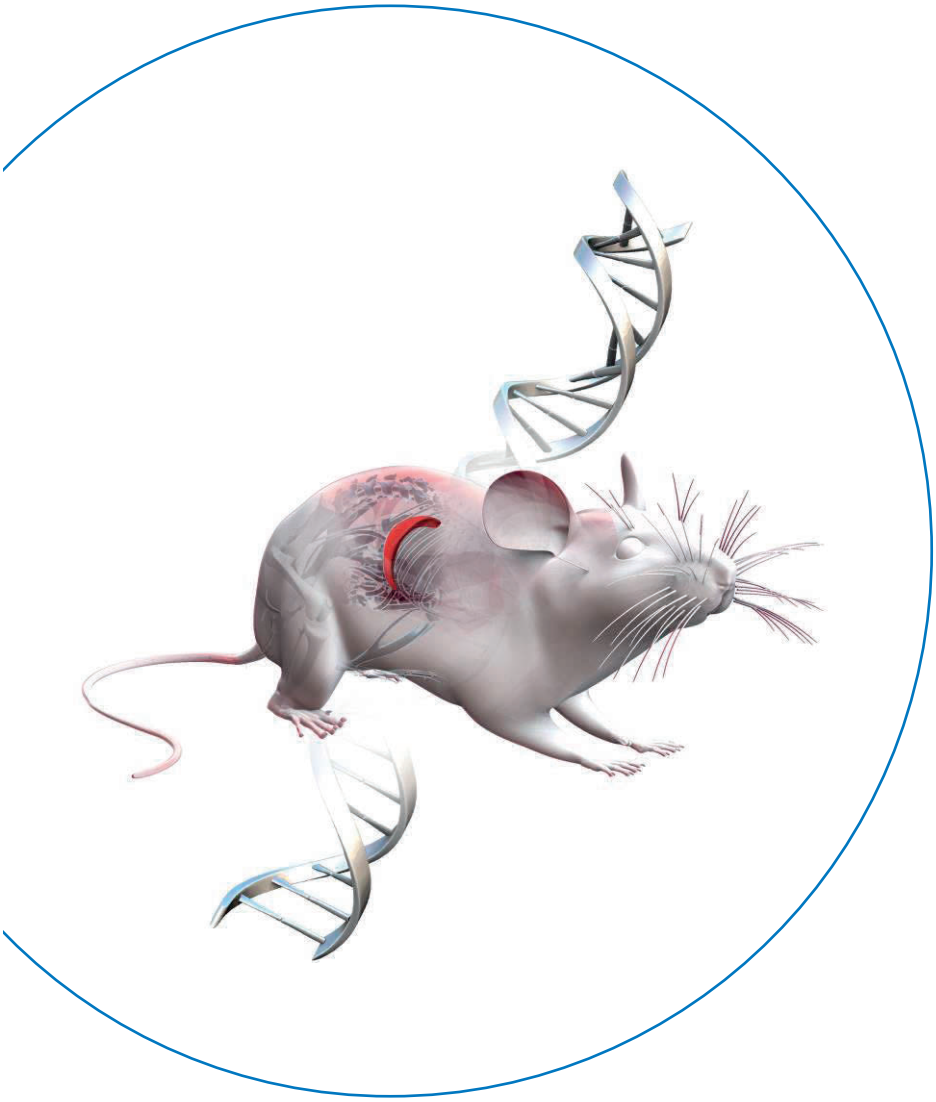
CHAPTER 2



Lysophospholipids secreted by splenic macrophages induce chemotherapy resistance via interference with the DNA damage response

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Nature Communications **5**: 5275 (2014)

Abstract

Host responses to systemic anti-cancer treatment play important roles in the development of anti-cancer drug resistance. Here we show that F4/80^{+}/CD11b^{low} splenocytes mediate the resistance to DNA-damaging chemotherapeutics induced by two platinum-induced fatty acids (PIFAs), 12-S-hydroxy-5,8,10-heptadecatrienoic acid (12-S-HHT) and 4,7,10,13-hexadecatetraenoic acid (16:4(n-3)) in xenograft mouse models. Splenectomy or depletion of splenic macrophages by liposomal clodronate protects against PIFA-induced chemoresistance. In addition, we found that 12-S-HHT, but not 16:4(n-3), functions via leukotriene B4 receptor 2 (BLT2). Genetic loss or chemical inhibition of BLT2 prevents 12-S-HHT-mediated resistance. Mass spectrometry analysis of conditioned medium derived from PIFA-stimulated splenic macrophages identified several lysophosphatidylcholines as the resistance-inducing molecules. When comparing cisplatin and PIFA-treated tumors with cisplatin alone treated tumors we found overall less γH2AX, a measure for DNA damage. Taken together, we have identified and intricate network of lysophospholipid signaling by splenic macrophages that induces systemic chemoresistance *in vivo* via an altered DNA damage response.

Editor's summary: It is known that mesenchymal stem cells contribute to chemotherapy resistance by secreting polyunsaturated fatty acids. Here, the authors show that macrophages in the spleen secrete lysophosphatidylcholines and contribute to chemotherapy resistance by altering the tumor's DNA damage response

Introduction

The development of resistance to chemotherapy is one of the most important obstacles to continued effective treatment of cancer in patients. Resistance to treatment is generally considered to be the result of tumor-intrinsic alterations in which tumor cells acquire genetic mutations leading to treatment tolerance or utilization of compensatory pathways to become drug tolerant. During recent years, it has become clear that in addition to tumor cell intrinsic resistance, signaling events from the tumor microenvironment play a major role in drug resistance[1-4]. Macrophages are highly plastic cells that adopt a tumor promoting or tumor-inhibiting phenotype, depending on the surrounding stimuli[5]. Macrophages can infiltrate developing tumors in large numbers and these tumor-associated macrophages (TAMs) can hamper chemotherapy via various mechanisms involving cathepsins, colony stimulating factor 1 (CSF1) and metalloproteases[6-9]. Recently, Cortez-Retamozo *et al.* showed that the spleen harbors TAM precursors which play an important role in tumor progression[10]. Furthermore, Ugel *et al*. have shown that $CD11b^{+}/GR1^{int}/Ly6C^{high}$ cells residing in the marginal zone of the spleen play a key role in generating tolerance to tumor antigens and that splenectomy prevents against tumor-induced tolerance[11]. In general, TAMs are considered to exert their effects locally. In this study we show that macrophages residing in the spleen can be specifically activated by circulating fatty acids to secrete secondary lysophospholipids which lead to systemic resistance to genotoxic chemotherapy.

Results

PIFAs protect only against DNA-damaging chemotherapeutics

We have found that mesenchymal stem cells (MSCs) can secrete two specific polyunsaturated fatty acids in response to platinum-containing chemotherapeutics and that these platinum-induced fatty acids (PIFAs) can independently induce resistance to various chemotherapeutics at minute quantities (supplemental figure 1 and Roodhart *et al*.[12]). To determine whether the effects of PIFAs are limited to DNA damaging agents, we tested two non-genotoxic agents, paclitaxel (spindle poison) and sorafenib (tyrosine kinase inhibitor), and found that their anti-tumor effects were not inhibited by co-administration of PIFAs (Figure 1a-b). This suggested that PIFAs specifically protect against the effects of DNA damaging agents. To further investigate this effect we analyzed γH2AX foci, which mark DNA damage. Treatment with cisplatin resulted in a strong increase in the number of γH2AX positive cells over time with a peak at 4 hours after treatment. Co-treatment with PIFAs largely reversed the cisplatin-induced DNA damage (Figure 1c). In line with these findings BrdU and phospho-histone 3 (pH3) levels were higher in tumors treated with cisplatin and PIFAs compared to tumors treated with cisplatin alone (Supplemental figure 2). Flow cytometric analyses of single cell suspensions of mouse tumors showed that eight hours after therapy, in cisplatin-treated tumors high

percentages of cells were in subG1 or in a G1/S arrest, whereas in tumors treated with cisplatin and PIFAs, cell cycle profiles were comparable to the untreated controls (Figure 1d). The difference in γH2AX was not the result of an altered exposure to cisplatin. Cytosolic, nuclear and DNA-bound platinum concentrations were similar in presence or absence of PIFAs 1 hour after treatment (Figure 1e). Previously published data showed that PIFAs exert their effects even when administered up to 3 hours after cisplatin treatment[1].

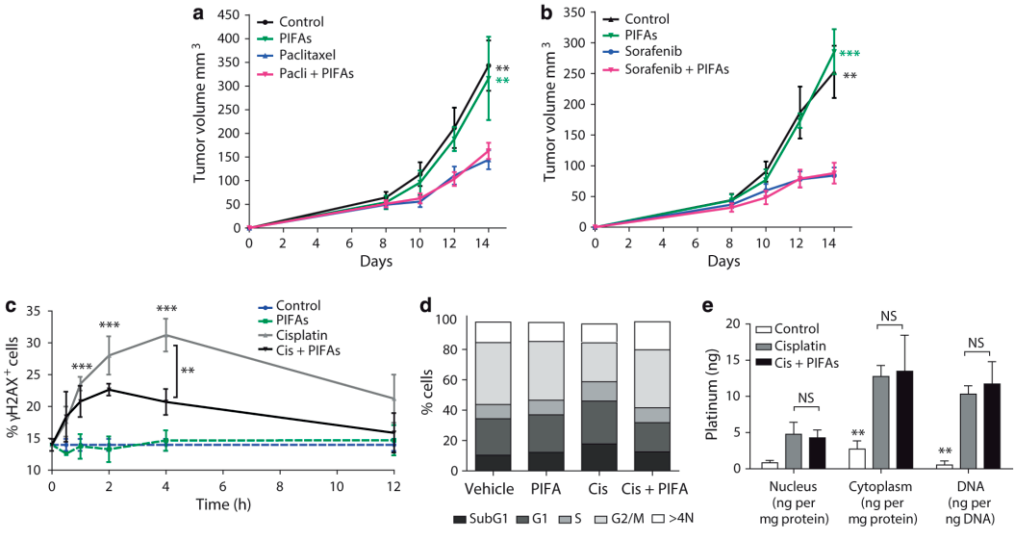

Figure 1: PIFAs induce chemoresistance by interfering with the DNA damage response. Co-administration of PIFAs with non-DNA damaging agents paclitaxel (a) or sorafenib (b) did not induce chemoresistance. Immunohistochemical analysis of tumors stained for γH2AX showed decrease in γH2AX over time after co-treatment with cisplatin and PIFAs compared to cisplatin treatment (c). Single cell suspensions were prepared from tumors and stained with propidium iodide for flow cytometric analysis of cell cycle profiles. Cisplatin treated tumors are arrested in subG1 and G1/S phase whereas the tumors receiving a combination of cisplatin and PIFAs show cell cycle profiles comparable to control animals (d). Platinum concentration in the cytoplasm, nucleus or bound to DNA was determined by ICP-MS, revealing no differences between cisplatin alone treatment and cisplatin + PIFAs (e). All graphs show results of 2 independent experiments (n=8 per group). All experiments were performed in C26 tumor-bearing balb/c mice. Data are represented as mean ± SEM. Statistical significance was determined by one-way ANOVA or two-tailed students T-test. All compared to cisplatin alone unless indicated otherwise * P<0.05, ** P<0.01, *** P<0.001.

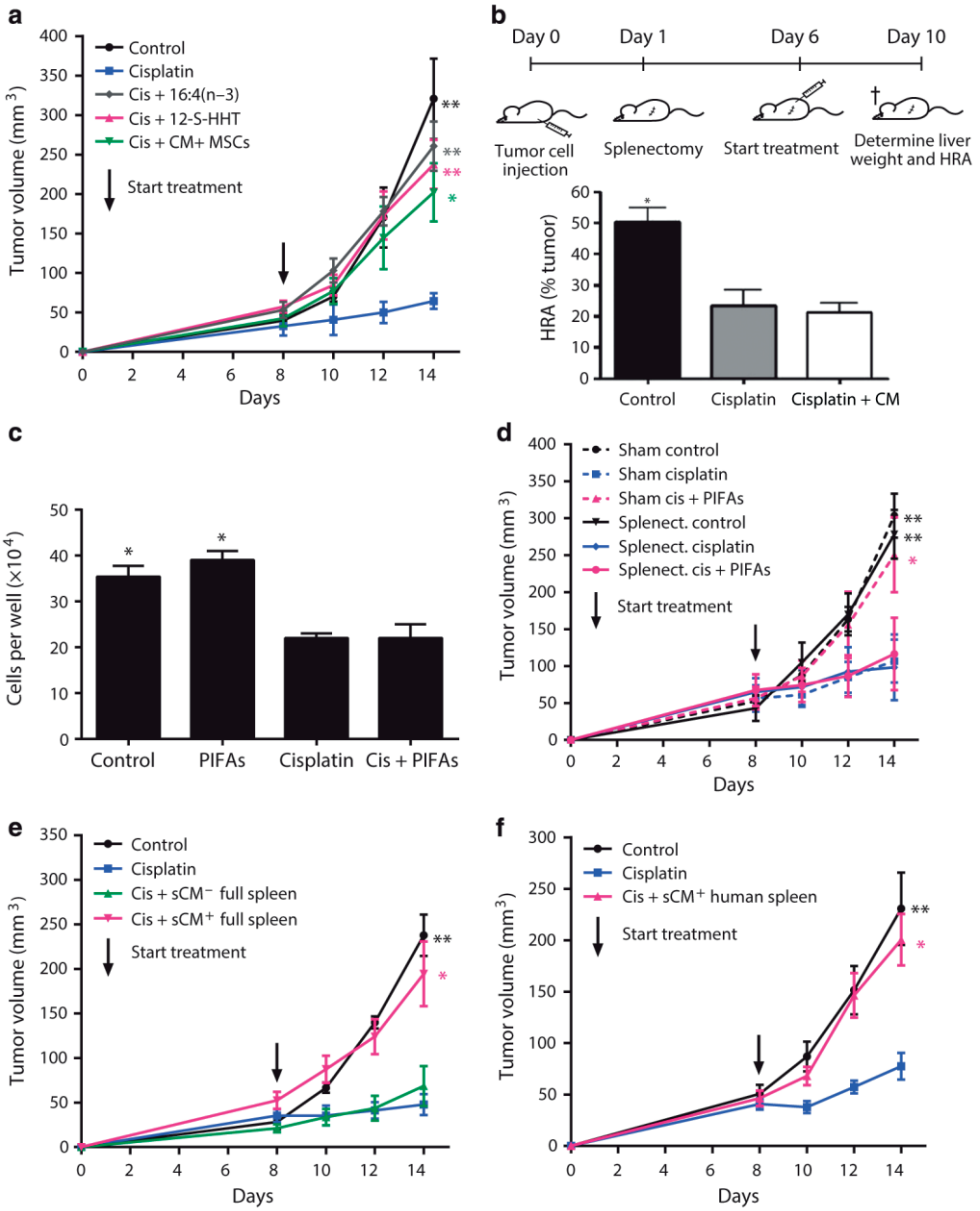

Figure 2: The spleen is required for PIFA-induced chemoresistance. 12-S-HHT and 16:4(n-3) induce chemoresistance individually *in vivo* in C26-tumor bearing balb/c mice. CM^+ derived from MSCs stimulated with cisplatin also induces chemoresistance *in vivo* (a). CM^+ from MSCs does not cause resistance in liver metastasis model in which the spleen is removed (b). PIFAs cannot induce resistance directly on C26 tumor cells *in vitro* (c). Cells were plated in 24-wells plate on day prior to cisplatin and/or PIFAs treatment. 24 hours after treatment with cisplatin (3µM) alone or in combination with PIFAs (HHT 20 nM, 16:4(n-3) 25 nM) the cells were harvested and counted. Splenectomy protects against PIFA-mediated chemoresistance in C26 tumor-bearing balb/c mice (d).

sCM$^+$ from full spleen can restore resistance in splenectomized tumor-bearing mice whereas sCM$^-$ cannot (e). sCM$^+$ from human splenocytes induces resistance in splenectomized mice (f). For all experiments C26-tumor bearing balb/c mice were used. Panel a, c, d and e show results of 3 independent experiments with similar outcome (n=12 per group). Panel b and f shows results from 2 independent experiments with similar outcome (n=8 per group for panel b, n=9 per group for panel f). Data are represented as mean ± SEM. Statistical significance was determined by one-way ANOVA. All compared to cisplatin alone, * P<0.05,**P<0.01.

The spleen is involved in PIFA-mediated chemoresistance

In search for a mechanism we noted that the PIFAs failed to induce chemoresistance in an experimental liver metastasis model using the same tumor cells and mouse strain as our subcutaneous model in which resistance is seen (Figure 2a, b). In this metastasis model tumor cells are injected in the spleen to allow venous access to the liver, after which the spleen is removed to avoid local tumor growth. This observation combined with our findings that PIFAs could not cause resistance directly to tumor cells *in vitro* (Figure 2c) suggested an indirect mechanism involving the spleen. Indeed, 16:4(n-3) and 12-S-HHT failed to induce resistance to chemotherapy in splenectomized, tumor-bearing mice (Figure 2d). In order to re-introduce chemoresistance we treated splenectomized tumor-bearing mice with chemotherapy alone or in combination with conditioned medium obtained from full spleen cell suspensions incubated in the presence (sCM$^+$) or absence (sCM$^-$) of both PIFAs. sCM$^+$ from full spleen was able to induce chemoresistance *in vivo* in splenectomized mice, whereas sCM$^-$ was not (Figure 2e). In addition, we found that sCM$^+$ from human splenocytes incubated with both PIFAs could also induce chemoresistance in splenectomized mice indicating potential clinical translation (Figure 2f).

Spleen macrophages are required for PIFA-mediated resistance in vivo

Next, we tested individual splenic cell populations for their potential to induce resistance *in vivo*. We tested natural killer cells (CD49$^+$ cells), dendritic cells (CD11c$^+$ cells), macrophages (F4/80$^+$ cells), monocytes (CD11b$^+$ cells), metallophilic marginal zone macrophages (CD169$^+$ cells) and T and B cells (CD3$^+$ and B220$^+$ cells). Only the sCM$^+$ of F4/80$^+$ splenocytes was able to significantly induce resistance *in vivo* (Figure 3a). Furthermore, none of the sCM$^-$ samples were able to induce resistance *in vivo* (Figure 3b). Importantly, treatment with either PIFAs or sCM alone did not alter tumor growth (Figure 3c). Further refinement of the F4/80$^+$ cell population by FACS sorting indicated a role for F4/80$^+$/CD11b^{low} macrophages (Figure 3d). In addition, we used liposomal clodronate to deplete F4/80$^+$/CD11b^{low} splenocytes. Depletion of F4/80$^+$/CD11b^{low} splenocytes had no direct effect on tumor growth *in vitro* and *in vivo* (Supplemental figure 3), but potently reversed the PIFA-induced chemoresistance to cisplatin in both C26-tumor bearing balb/c mice and LLC-tumor bearing C57bl/6 mice (Figure 3e-f). Chemoresistance was re-instated when liposomal clodronate-treated mice were injected with sCM$^+$ from PIFA-stimulated F4/80$^+$ splenocytes (Figure 3e-f). Further characterization of the F4/80$^+$/CD11b^{low}

splenocytes showed that they are localized in the red pulp and lack Ly6G and Ly6C expression (Supplemental figure 4). Analysis of the number of F4/80$^+$ infiltrating cells in both our subcutaneous tumors showed very little infiltrating macrophages in both LLC and C26 tumors (Supplemental figure 5), suggesting that in our xenograft models the contribution of TAMs is unlikely. We thus conclude that 12-S-HHT and 16:4(n-3) signal via macrophages residing in the spleen to confer chemoresistance.

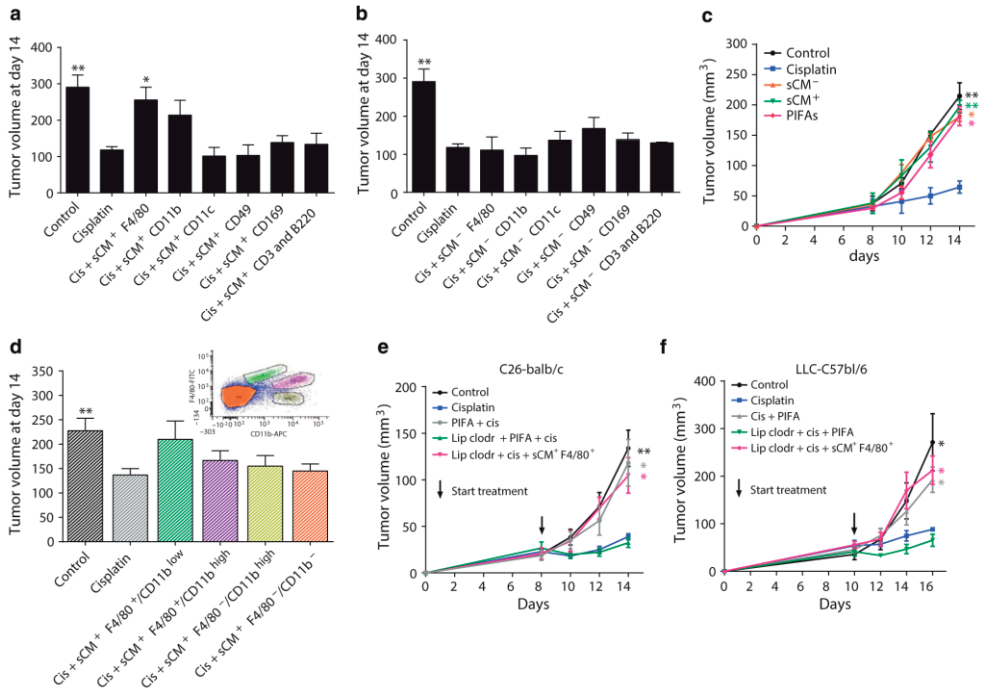

Figure 3: Splenic F/80$^+$/CD11b^{low} macrophages play a key role in chemoresistance induced by PIFAs. sCM$^+$ from F4/80$^+$ splenocytes significantly restored resistance in splenectomized tumor bearing mice, whereas sCM$^+$ from other splenic populations cannot (a). sCM$^-$ from any of the isolated populations is unable to induce resistance (b). In addition, PIFAs, sCM$^-$ or sCM$^+$ alone do not affect normal tumor growth (c). sCM$^+$ from FACS-sorted F4/80$^+$/CD11b^{low}, F4/80$^+$/CD11b^{high}, F4/80$^-$/CD11b^{high} and F4/80$^-$/CD11b$^-$ splenocytes did not significantly induce chemoresistance, however F4/80$^+$/CD11b^{low} cells were most potent (d, insert shows gated populations). Animals treated with liposomal clodronate and cisplatin + PIFAs remained chemosensitive. sCM$^+$ from F4/80$^+$ cells rescued loss of resistance in liposomal clodronate-treated mice (lip clodr + cis + sCM$^+$ F4/80$^+$). Panel e shows results from C26-balb/c model and panel f shows results from LLC-C57bl/6 model. All graphs show data from C26-balb/c model unless indicated otherwise. Graphs in panel a, d, e and f show results of 3 independent experiments with similar outcome (n=12 per group). Graphs in panel b and c show results of 2 independent experiments with similar outcome (n=10 per group). Data are represented as mean ± SEM. Statistical significance was determined by one-way ANOVA. All compared to cisplatin alone, * P<0.05,**P<0.01.

Loss of BLT2 blocks 12-S-HHT mediated chemoresistance

Leukotriene B4 receptor 2 (BLT2) is the low affinity receptor for leukotriene B4, but also specifically binds 12-S-HHT and initiates distinct events, setting it apart from BLT1[13]. Given the fact that 12-S-HHT is a natural ligand of BLT2[14] we focused on the interaction between BLT2 and 12-S-HHT in splenic macrophage-induced resistance. Analysis of expression levels showed that BLT2 is exclusively expressed on F4/80^{+}/CD11b^{low} splenocytes (Figure 4a). sCM^{+} from splenic macrophages cultured in the presence of LY255283, a BLT2 antagonist, and 12-S-HHT failed to induce resistance in splenectomized mice. In contrast, sCM^{+} from spleen macrophages cultured in the presence of BLT1 antagonist U-75302 and 12-S-HHT retained resistance-inducing potential (Figure 4b). Importantly, 16:4(n-3)-mediated resistance was not hampered by the two antagonists (Figure 4c). To further confirm the interaction between 12-S-HHT and BLT2 we tested the PIFAs in *BLT2*$^{-/-}$ mice. We found that 12-S-HHT-mediated resistance was lost in *BLT2*$^{-/-}$ LLC tumor-bearing mice whereas 16:4(n-3) fully retained its ability to induce resistance (Figure 4d). We also showed that sCM^{+} from *BLT2*$^{-/-}$ splenocytes incubated with 12-S-HHT was unable to cause resistance in wild type C57bl/6 mice, whereas the resistance inducing potential of sCM^{+} from *BLT2*$^{-/-}$ spleens incubated with 16:4(n-3) was unaffected (Figure 4e). Noteworthy, the tumor growth was delayed in *BLT2*$^{-/-}$ mice compared to wild type mice (Supplemental Figure 6). As expected, 12-S-HHT and 16:4(n-3) could reduce γH2AX levels in cisplatin treated tumors in the parental C57bl/6 strain (Figure 5a). Interestingly, tumors in *BLT2*$^{-/-}$ mice that received cisplatin and 12-S-HHT had comparable γH2AX levels to mice treated with cisplatin alone, whereas 16:4(n-3) and cisplatin-treated tumors showed decreased γH2AX levels (Figure 5b). This further emphasizes that BLT2 signaling is essential in facilitating 12-S-HHT-mediated chemoresistance via alteration of the DNA damage response.

Splenic chemoresistance factors are lysophosphatidylcholines

To identify the secondary molecules secreted by PIFA-stimulated splenic macrophages we applied a stepwise fractionation of the sCM^{+} (Figure 6a). We found that the total lipids fraction, but not the protein-containing fraction, extracted from sCM^{+} induced resistance in splenectomized mice, whereas the total lipids fraction from sCM^{-} did not. In line with these results we found that delipidation of sCM^{+} resulted in a loss of chemoresistance-inducing potential (Figure 6b), indicating that the secondary molecules are lipids. A solid phase-based fractionation of the total lipids fraction into neutral lipids (NL), free fatty acids (FFA) and two phospholipids fractions (PC/PE/SM: phosphatidylcholines, phoshatidylethanolamines and sphingomyelines and PI/PS/PA: phosphatidylinositol, phosphatidylserines and phosphatidic acids) revealed that only the PC/PE/SM fraction significantly induced resistance (Figure 6c-f). Additional mass spectrometry-guided subfractionation of PC/PE/SM revealed that subfractions F3 and F6 were able to induce

resistance *in vivo* (Figure 6g). Mass spectrometry analysis of subfraction F3 derived from sCM$^+$ showed increased levels of phosphatidylethanolamines (PEs) compared to F3 from sCM$^-$. In subfraction F6 derived from sCM$^+$ we found increased levels of lysophosphatidylcholines (LPCs) compared to F6 from sCM$^-$ (Table 1). Since not all identified PEs and LPCs are commercially available for individual testing in our animal models we decided to isolate the PEs and LPCs from sCM$^+$ and test them *in vivo*. In parallel, we obtained purified LPC(6:0), LPC(22:0), LPC(24:0) and LPC(26:0) and mixtures of LPC and PE isolated from chicken egg and liver, respectively. Importantly, the mixtures of LPC and PE did not contain any species identified in the screen and therefore served as a control. We found that only LPCs isolated from sCM$^+$ were able to induce resistance, whereas PEs extracted from sCM$^+$ did not induce resistance *in vivo* (Figure 7a).

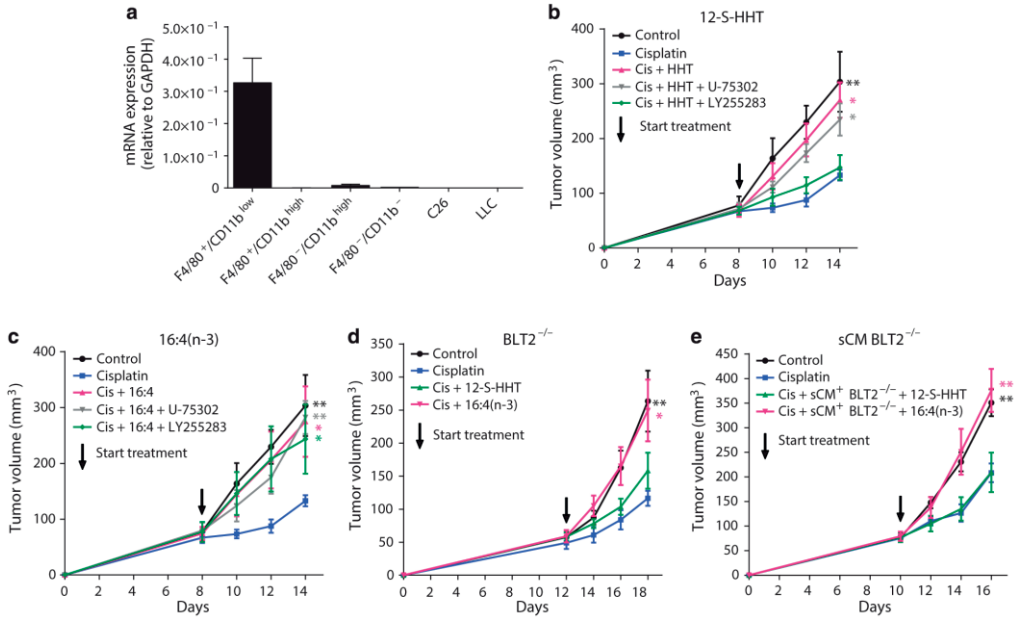

Figure 4: Inhibition or loss of BLT2 blocks formation of 12-S-HHT-induced resistance. BLT2 is exclusively expressed on F4/80$^+$/CD11b^{low} splenocytes as was shown by Q-PCR (a). Incubation of splenocytes with LY255283 and 12-S-HHT blocked 12-S-HHT-induced resistance whereas U-75302 did not (b). Neither U-75302 nor LY255283 blocked 16:4(n-3)-mediated chemoresistance (c). 12-S-HHT failed to induce resistance in BLT2$^{-/-}$ mice (d). Co-administration of cisplatin with sCM$^+$ from BLT2$^{-/-}$ mice incubated with 12-S-HHT did not induce resistance in wildtype C57bl/6 mice, whereas sCM$^+$ from BLT2$^{-/-}$ incubated with 16:4(n-3) does induce chemoresistance (e). Graphs depicted show data from 2 independent experiments (panel a n=4 per group, panels b-e n=10 per group). Statistical significance was determined by one-way ANOVA or two-tailed students T-test. All compared to cisplatin alone. *P<0.05,**P<0.01.

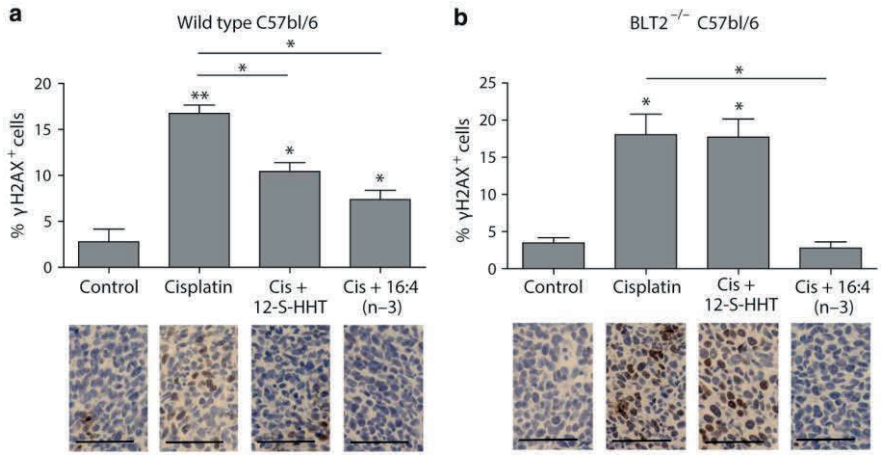

Figure 5: *BLT2*$^{-/-}$ mice are unresponsive to 12-S-HHT mediated alterations in DNA damage response. Co-treatment of wildtype mice with 12-S-HHT or 16:4(n-3) and cisplatin decreased γH2AX levels compared to cisplatin (a). In BLT2$^{-/-}$ mice γH2AX levels were comparable between 12-S-HHT and cisplatin co-treatment and cisplatin treatment (b). Mice were sacrificed 4 hours after treatment with cisplatin and/or 12-S-HHT or 16:4(n-3). Tumors were stained for γH2AX and quantified. Graphs depicted show data from 2 independent experiments (n=4 per group). Scale bars are 50 μm. Statistical significance was determined by two-tailed students T-test. All compared to control unless indicated otherwise. *P<0.05,**P<0.01.

Both mixtures of LPC and PE lacked the ability to induce chemoresistance (Figure 7b). Interestingly, the combination of saturated LPCs with cisplatin also did not induce resistance (Figure 7b), suggesting that the resistance-inducing potential is restricted to the unsaturated LPCs found in sCM^{+}. Finally, γH2AX levels in tumors treated with cisplatin in combination with LPCs isolated from sCM^{+} were decreased similar to that observed with PIFAs. PEs isolated from sCM^{+} also reduced γH2AX but to a lesser extent which is interesting since no effect of PE was seen on tumor volume. As expected, both the saturated LPCs and the commercial LPC mix were not able to significantly lower γH2AX levels (Figure 7c).

Discussion

It is becoming increasingly clear that the host plays an important role in the response to anti-cancer drugs and the development of resistance to these drugs. Here we identified a novel role for specific phospholipids released from splenic macrophages in chemotherapy resistance. Our findings show that F4/80^{+}/CD11b^{low} cells residing in the spleen become activated by circulating PIFAs, and subsequently release polyunsaturated LPCs which induced systemic chemotherapy resistance via altering the DNA damage response. We show that PIFAs interfere with the early effects of DNA damaging chemotherapeutic agents like cisplatin. In the presence of PIFAs a rapid decline in γH2AX

levels was found, alleviating cell cycle arrest and restoring tumor cell proliferation to baseline levels. This was not explained by a decreased exposure to cisplatin. Since PIFAs are endogenously produced by MSCs in response to exposure to platinum analogues, and exposure to PIFAs or sCM$^+$ results in a rapid reduction of DNA damage, this could represent a physiological stress response of the body to protect itself from genotoxic agents.

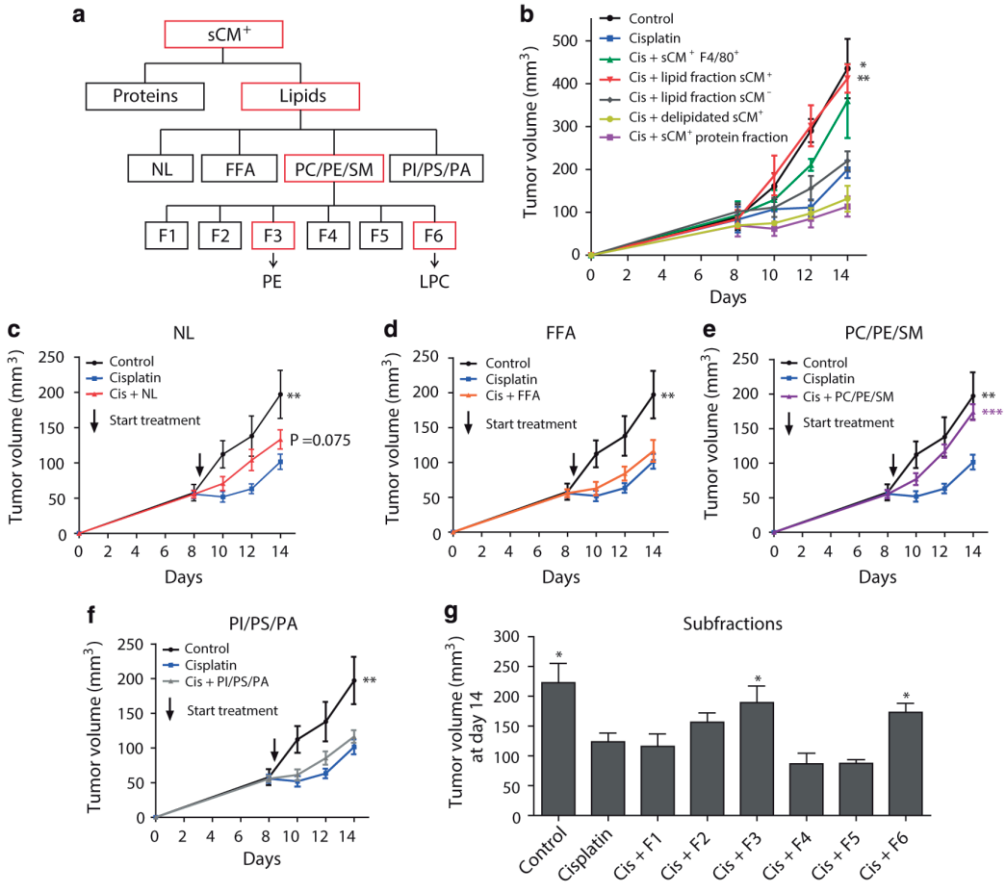

Figure 6: Fractionation showed that phospholipids present in sCM$^+$ act as resistance inducing factors. Panel a shows schematic overview of steps used to fractionate sCM$^+$. C26 tumor-bearing splenectomized balb/c mice were treated with either cisplatin alone or in combination sCM$^+$, delipidated sCM$^+$, total lipids fraction isolated from sCM$^-$ or from sCM$^+$ or with the protein fraction isolated from sCM$^+$. The total lipids fraction from sCM$^+$ and sCM$^+$ from F4/80$^+$ splenocytes was able to induce resistance, whereas the others did not (b). Fractionation of the total lipids fraction from sCM$^+$ by HPLC showed that only the fraction containing PC/PE/SM was able to induce resistance (c-f). Additional mass spec guided fractionation of the PC/PE/SM fraction revealed that only subfraction F3 and F6 were able to induce chemoresistance *in vivo* (g). All graphs depicted show data from 2 independent experiments with similar outcome (n=8 per group). Statistical significance was determined by one-way ANOVA. All compared to cisplatin alone. *P<0.05, ** P<0.01.

Table 1 | Resistance-inducing factors from sCM

Identified molecule	± Area under the curve				P-value
	sCM −		sCM +		
	Average	s.e.m.	Average	s.e.m.	
Subfraction F6					
lyso-PC(22:3)	4,20E + 01	± 0,00E + 00	5,04E + 02	± 4,20E + 01	0,008
lyso-PC(22:0)	3,43E + 03	± 5,40E + 02	3,27E + 04	± 4,20E + 03	0,020
lyso-PC(24:1)	1,09E + 03	± 8,00E + 01	1,43E + 04	± 2,00E + 03	0,022
lyso-PC(26:0)	3,89E + 03	± 2,25E + 03	5,13E + 04	± 7,30E + 03	0,025
lyso-PC(26:1)	8,07E + 03	± 4,44E + 03	7,09E + 04	± 9,95E + 03	0,029
lyso-PC(24:0)	5,19E + 03	± 2,85E + 03	6,96E + 04	± 1,13E + 04	0,031
lyso-PC(26:6)	1,04E + 03	± 2,81E + 02	2,40E + 04	± 5,55E + 03	0,054
lyso-PC(20:5)	8,40E + 01	± 4,20E + 01	3,36E + 02	± 2,10E + 02	0,360
lyso-PC(26:5)	5,22E + 02	± 6,15E + 01	9,90E + 03	± 8,30E + 03	0,376
Subfraction F3					
PE(38:9)	8,32E + 06	± 1,98E + 06	4,14E + 07	± 4,20E + 06	0,019
PE(40:9)	6,59E + 05	± 7,05E + 05	4,56E + 07	± 1,95E + 06	0,003

Mass spectrometry analysis of chemoresistance-inducing phospholipid fraction revealed undaturated LPCs as resistance-inducing factors. Mass spectrometry analysis of subfractions F3 and F6 revealed a number of significantly upregulated LPCs and PEs. Data are expressed as mean area under the curve and s.e.m.

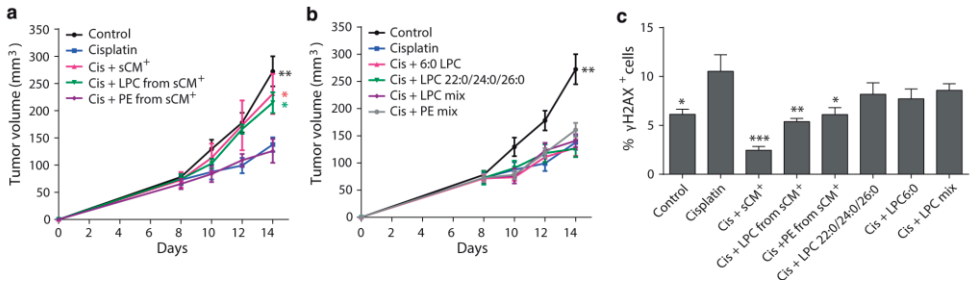

Figure 7: Unsaturated LPCs secreted by splenic macrophages induce chemoresistance *in vivo*. LPCs isolated from sCM$^+$ induces resistance in C26 tumor-bearing mice whereas PEs from sCM$^+$ cannot (a). In order to determine if the resistance-inducing potential of the identified LPCs lies in the saturated or unsaturated LPCs we tested the saturated LPCs 22:0, 24:0 and 26:0, a control short chain LPC 6:0 and commercially available LPC and PE mixtures which does not contain any of the LPCs or PEs identified in the screen, the latter three serving as negative controls. Neither of these LPCs or LPC/PE mixes induced chemoresistance when injected together with cisplatin, indicating that resistance-inducing potential of sCM$^+$ harbors in unsaturated LPCs (b). A reduction in γH2AX was seen 4 hours after co-treatment with LPCs and PEs isolated from sCM$^+$, but not with LPC(22:0), (24;0), (26:0), (6:0) or a commercially available mix of LPC (c). All graphs depicted show data from 2 independent experiments (except panel a, 4 independent experiments) with similar outcome (n=8 per group). Statistical significance was determined by one-way ANOVA or two-tailed students T-test. All compared to cisplatin alone. *P<0.05, ** P<0.01.

Our findings indicate a novel link between polyunsaturated fatty acid-signaling and the DNA damage response. Our study uncovers a number of potential drug targets to enhance chemotherapy efficacy. Here, we used chemical inhibition and a genetically engineered mouse model to show that 12-S-HHT-mediated chemoresistance is dependent on BLT2 signaling. BLT2 is the low affinity receptor for leukotriene B4, however it specifically binds 12-S-HHT and initiates distinct downstream effects, different from BLT1[13]. Overexpression of BLT2 in breast, pancreatic and bladder tumors

has been correlated to tumor progression and even to platinum resistance in ovarian cancer[15-17]. Our results combined with others identify BLT2 as a potential drug target to improve treatment outcome perhaps via a dual function by inhibiting the release of chemoresistance-inducing LPCs from the spleen and by inhibiting BLT2 pro-survival functions in the tumor cells itself. Alternatively, further insight into the production of the LPCs by the splenic macrophages could reveal potential drug targets that could enhance chemotherapy efficacy. Little is known about LPCs in cancer and chemotherapy resistance and no receptors have yet been described. It remains to be determined whether these specific LPCs act through specific receptors or directly. Taken together our results have uncovered a systemic signaling network of PIFAs and LPCs generated by mesenchymal stem cells and splenic cells that render tumor cells resistant to chemotherapy via alterations in the DNA damage response of tumor cells.

Material and Methods

Reagents

12-S-HHT, U-75302 and LY255283 were purchased from Cayman Chemicals. 16:4(n-3) was a kind gift from Dr. Ishihara[20]. The following antibodies were used for magnetic bead sorting: rat anti-mouse F4/80, rat anti-mouse CD169 (both from AbD serotec), anti-human and mouse CD11b-FITC, anti-mouse CD11c microbeads, anti-mouse CD49b (DX5) microbeads, (all from Miltenyi biotec), rat anti-mouse CD45R (B220), rat anti-mouse CD3-FITC (both from ebioscience), anti-rat IgG microbeads and anti-FITC microbeads (both from Miltenyi biotec). For FACS analysis the following antibodies were used: rat anti-mouse F4/80-FITC and rat-anti mouse CD11b-APC (both from ebioscience). For immunohistochemical staining the following antibodies were used: anti-γH2AX (Cell Signaling Inc), anti-BrdU (Abcam), anti-pH3 (Upstate/Millipore Corporation), Powervision-HRP Goat-anti-Rabbit (Novocastra/Leica Microsystems) and Goat-anti-Rabbit Alexa-568 (Life Technologies).

Animal models

C26 and LLC cells were implanted in balb/c and C57bl/6 (both from Charles River) mice respectively. For all experiments 8-10 week old male mice were used. At day 0 the mice were injected subcutaneously with $1*10^6$ (for C26) or $0.5*10^6$ (for LLC) tumor cells. Mice were splenectomized one day after tumor cell injection. At day 8 (C26) or day 10 (LLC) when the tumors were at a palpable size of 50-100 mm3 animals were randomized into groups and treatment was started. The mice received an intraperitoneal injection of cisplatin 6mg/kg and blinded tumor volume measurements were done once every two days using a caliper. Tumor volume was determined as length*width^2*0.5. Control mice received the appropriate vehicles. Concomitantly with the chemotherapy treatment, some mice received subcutaneous injection of conditioned medium from splenocytes cultured in the presence (sCM^+) or absence (sCM^-) of PIFAs. Generation of the $BLT2^{-/-}$ mice is described in Mathis *et al.* 2010[13]. Therapy was started when tumors reached 50-100 mm^3 and tumor growth was monitored as described above. Endpoints were determined as a tumor volume of >1000 mm3. All experimental animal procedures conducted in Utrecht (the Netherlands) were approved by the University Medical Center Animal Ethics Committee and were in agreement with the current Dutch Laws on animal experiments. All experimental animal procedures conducted in Louisville (KY, USA) were approved by the University of Louisville Animal Care and Use Committee. In order to show a difference of 20% in tumor volume with a standard deviation of 10% and a type I error (alfa) of 5% using a power of 90% a minimal of 8 mice per treatment group are required.

Cell lines

C26 and LLC cells (both from ATCC) were grown in DMEM (4,5 gr/L glucose) + 5% FCS at 5% CO2 and 37°C. Murine MSCs were grown in DMEM (1 gr/L glucose) + 20% FCS at 5% CO2 and 37°C.

Liver metastasis model

C26 tumor cells ($5*10^4$ cells) were injected intra-splenically in balb/c mice and allowed to migrate to the liver. One day after tumor cell injection the spleens were removed to prevent local tumor growth. Six days after tumor cell injection the mice were either left untreated, treated with cisplatin or with cisplatin and CM+ from the MSCs. After four days the livers were harvested, weighted and the hepatic replacement area was determined.

Production of sCM from splenocytes

Spleens from splenectomized mice were used to make sCM. Single cell suspensions were made from the spleens and red blood cells were lysed using red blood cell lysis buffer (RBC) (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA). Splenocytes were incubated in serum free medium (SFM) (DMEM 4,5 gr glucose/L) with 20 nM 12-S-HHT and/or 25 nM 16:4(n-3) for 1 hour at 5% CO2 and 37°C. After incubation the supernatant from the splenocytes was harvested, filtered through a 0.2μM filter and stored at -80°C until use.

MACS separation of different cell types from spleen

The spleens were processed into the same way as described for the production of sCM. After RBC lysis the splenocytes were resuspended in FACS buffer (1% BSA, 5 mM EDTA in PBS pH 7.4) and counted. Next the cells were incubated with primary antibodies rat anti-mouse F4/80, rat anti-mouse CD11b-FITC, rat anti-mouse CD169, anti-mouse CD11c microbeads, anti-mouse CD49b (DX5) microbeads, rat anti-mouse CD45R (B220), rat anti-mouse CD3-FITC at a concentration of 3 μl/$1*10^7$ cells in 100 μl for 30 minutes on ice. After incubation the cells were washed in FACS buffer and incubated with secondary antibodies (goat anti-rat microbeads or goat anti-FITC microbeads) at a concentration of 5 μl/$1*10^7$ cells in 100 μl for 15 minutes on ice. Again the cells were washed after antibody incubation and resuspended in FACS buffer and separated according to the manufacturer's instructions using LS columns (Miltenyi Biotec). Labelled cells were spun and incubated in SFM with 20 nM 12-S-HHT and/or 25 nM 16:4(n-3) for 1 hour at 5% CO2 and 37°C. The supernatant was harvested and stored as described in *Production of sCM from splenocytes*. For the BLT1 and BLT2 inhibitor experiments, the isolated macrophages were incubated with LY255283 (800 nM) or U-75302 (500 nM) for 1 hour prior to addition of HHT (20 nM) or 16:4(n-3) (25 nM).

Liposomal clodronate administration

All tumor-bearing mice received 200 µl liposomal clodronate suspension (containing 2mg clodronate) via intravenous injection 2 and 4 days prior to chemotherapy treatment to ensure maximal depletion of splenic macrophages at the time of treatment. At day 8 (for balb/c mice) or day 10 (for C57Bl/6 mice) the mice were either left untreated, treated with cisplatin (6mg/kg) alone or in combination with PIFAs (20 nM HHT and 25nM 16:4(n-3) or sCM^+ from $F4/80^+$ spleen macrophages.

FACS analysis of liposomal clodronate treated mice

To determine maximal depletion of spleen macrophages FACS profiles were made from non-tumor bearing mice 2, 4 or 8 days after a single liposomal clodronate injection. In brief, single cell suspensions were made from the spleen and red blood cells were lysed as described in *Production of sCM from splenocytes*. Next, the splenocytes were incubated with F4/80-FITC and CD11b-APC (concentration 1µl/1*10^7 cells/100µl) or corresponding isotype controls (FITC and APC both from ebiosciences) in FACS buffer for 30 minutes on ice. Samples were analyzed using a FACSCalibur from BDbioscience.

FACS sorting splenic populations

Spleens were prepared as described *in FACS analysis of liposomal clodronate treated mice* and the $F4/80^+/CD11b^{low}$, $F4/80^+/CD11b^{high}$, $F4/80^-/CD11b^{high}$ and $F4/80^-/CD11b^-$ populations were sorted using a FACSAria III from BDbioscience. Gates for sorting were set according to unstained and isotype control stained samples.

Q-PCR analysis of BLT2

RNA was extracted from FACS-sorted splenic population, C26 and LLC cells using TriZol (invitrogen). cDNA was synthesized using Superscript II (invitrogen) according to manufacturers protocol. Primers used for detection of BLT2: forward: 5'-ACCATGGAACTCCGAACTAC-3', Revers: 5'-ACTGTCTTTCTCCGTCTTGC-3'. As a control GADPH was used. Forward: 5'-ATGCAGGGATGATGTTCT-3'. Reverse: 5'-GTCATCCATGACAACTTT-3'

Immunohistochemistry and immunofluorescence

For immunohistochemistry experiments, tumors were harvested 0.5, 1, 2, 4 and 12 hours after therapy. Formaldehyde-fixed paraffin-embedded tumor sections were deparaffinated and rehydrated in a series of xylene and alcohol, incubated in a buffer containing 5% H2O2 to block endogenous peroxidase, and antigen retrieval was performed by cooking slides in citrate buffer. Tissues were blocked with goat serum (Life Technologies Europe BV) and incubated with primary antibodies: 1:200 anti-γH2AX (Cell Signaling Inc., #2577, USA) or 1:200 anti-BrdU (Abcam, #ab6326, Cambridge, UK) o/n at

4°C. Powervision-HRP Goat-anti-Rabbit (1:2000; Novocastra/Leica Microsystems, Germany) was used as secondary antibody, followed by DAB/H2O2 and counterstaining with hematoxyline. For immunofluorescent stainings, anti-pH3 (#06-570, Upstate/Millipore Corporation, USA) was used 1:250 o/n at 4°C, followed by the secondary antibody 1:500 Goat-anti-Rabbit Alexa-568 (#A11036, Life Technologies), and DAPI staining (#33342, Hoechst/Life Technologies). Analysis was performed using a grid with 36 intersection points. At least 10 fields per tumor were analyzed.

Measuring platinum concentrations

Mice were treated with cisplatin (6 mg/kg) with or without co-administration of PIFAs. Thirty minutes after therapy, tumors were harvested, weighed and snap frozen. Either DNA was isolated, or nuclear/cytosolic extractions were performed, followed by measuring platinum concentration as previously described[19-21]. In brief, for DNA isolation tumors were homogenized using Douncer homogenizers in Tris-HCl buffer, followed by addition of NH_4HCO_3, SDS and Proteinase K, overnight digestion at 42˚C and protein precipitation by adding saturated 6 M NaCl, vigorous shaking and spinning. The DNA was precipitated from the supernatant by addition of pure EtOH, washed twice in 75% EtOH, airdried and dissolved in dH2O. For nuclear and cytoplasmic extraction, snap frozen tumor tissues were homogenized in hypotonic lysis buffer with addition of NP-40 and protease/phosphatase inhibitors, which will lyse the cytoplasmic fraction. Subsequently, the nuclei were lysed with ice-cold nuclear extraction buffer (NEB) with protease/phosphatase inhibitors. Protein content was measured using a spectophotometer and control western blots were performed. DNA, nuclear and cytoplasmic fractions were hydrolyzed and analyzed by ICP-MS[22-24].

Lipid fractionation and mass spectrometry analysis of sCM$^+$

Total lipids from culture media of sCM were extracted using a modified Bligh & Dyer extraction according to Retra *et al* 2008. Total lipids fraction was separated into four fractions (neutral lipids (NL), free fatty acids (FFA), two phospholipid fractions (PC/PE/SM and PI/PS/SM) by solid phase extraction according to Kim *et al* 1990[22]. Of the PC/PE/SM fraction was further fractionated in to the separate lipid classes by hydrophilic interaction liquid chromatography (HILIC) as described by Brouwers *et al* 2013[23]. The HPLC output was split into two flows (1:10 ratio), of which one was lead to a mass spectrometer to monitor the separation process, the other major flow was used to collect to lipid classes. LPC analysis was performed using a modified method of Retra *et al* 2008[24]. Here the HPLC column was replace by a fused core HALO C18 column (Biotech, Onsala, Sweden). To increase sensitivity, LPCs were detected in multiple reaction monitoring mode, in which a collection of 36 different LPC masses (14:0 to 26:6 LPC) were monitored and confirmed by the formation of a phosphocholine fragment of 184 m/z (collision energy (CE) was 55V).

Furthermore, the fatty acid composition of the LPCs were confirmed by the presence of the corresponding fatty acid fragment in a second MS-analysis. Resulting fractions were speed-vac dried and dissolved in ethanol and diluted into PBS before injection in mice.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical significance for all animal experiments in which tumor volumes were assessed was determined by one-way ANOVA with Tukey correction for multiple testing. A value of $P<0.05$ was considered to be statistically significant. All other data was analyzed using a two-tailed students T-test. A P value <0.05 was considered statistically significant. A Levene's test was used to determine if variance between groups was comparable. Animals were excluded from analysis if two or more tumor measurements were significant outliners compared to the rest using the Grubbs outlier test (alfa: 0.05).

Funding

This research was funded by the Dutch Cancer Society (KWF), Foundation "De Drie Lichten" in the Netherlands and the Rene Vogels scholarship.

Supplementary Information

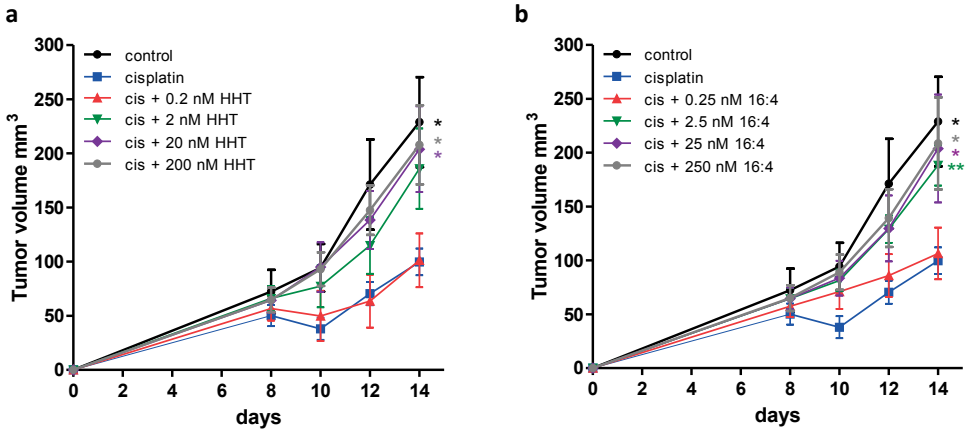

Figure 1: Dose-response curve of PIFAs shows that nanomolar quantities of 12-S-HHT or 16:4(n-3) are able to induce chemoresistance. C26-tumor bearing mice (balb/c) were treated with indicated doses of either 12-S-HHT (a) or 16:4(n-3) (b) and tumor growth was monitored over time. Both HHT and 16:4(n-3) induce resistance at 20 and 2,5 nM concentrations respectively and higher. Graph shows data from 2 independent experiments (n=8 per group). Data are represented as mean ± SEM. Statistical significance was determined by one-way ANOVA. All compared to cisplatin alone, * P<0.05,**P<0.01.

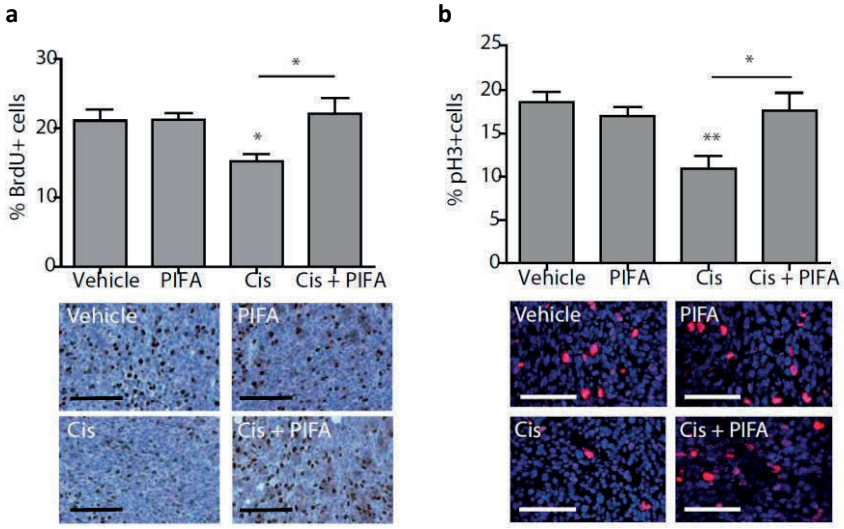

Figure 2: PIFAs induce resistance and reverse early effects of cisplatin on proliferation. C26 tumors were harvested 4 hours after treatment and stained for (a) BrdU and (b) phospho-histone H3. An increase in BrdU and phospho-histone H3 was seen after co-treatment with cisplatin and PIFAs compared to cisplatin treatment alone. Graphs show results of 2 independent experiments (n=8 per group). Data are represented as mean ± SEM. Scale bars are 100 μm. Statistical significance was determined by two-tailed students T-test. All compared to vehicle unless indicated otherwise * P<0.05, ** P<0.01.

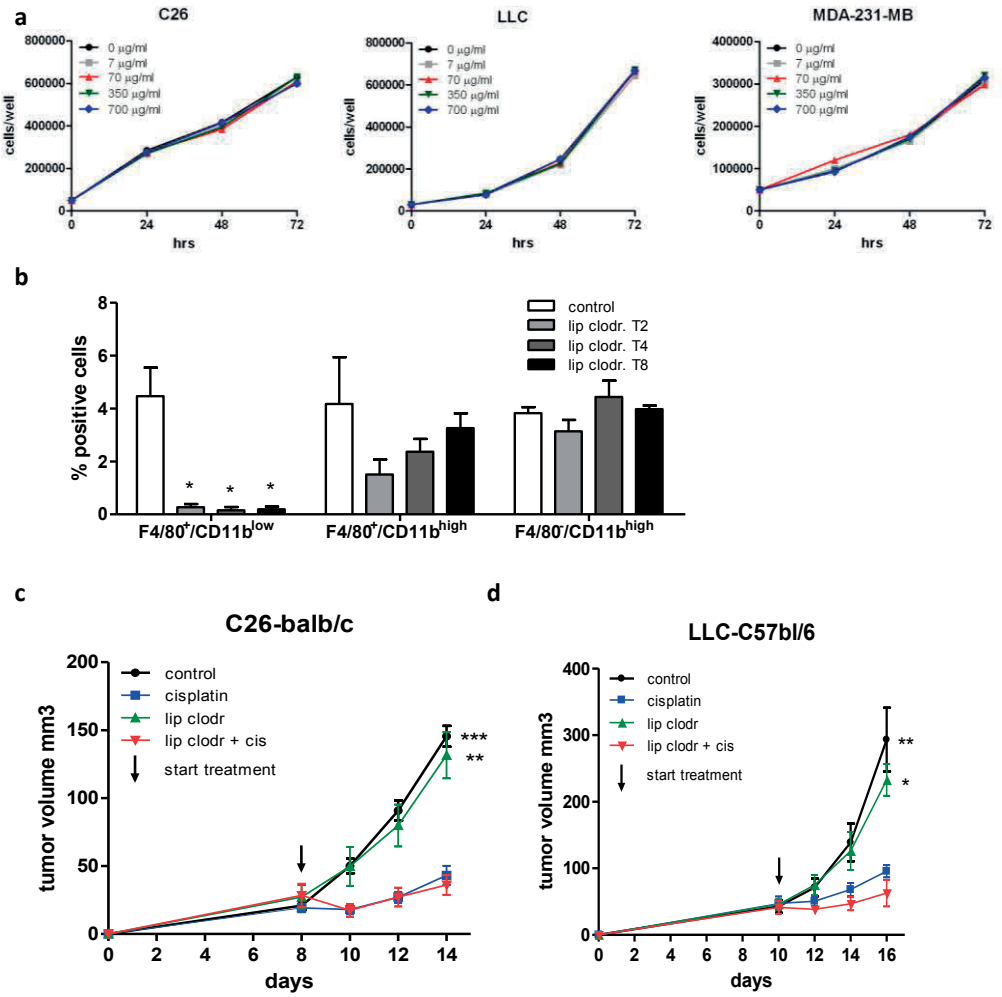

Figure 3: Liposomal clodronate has no effect on proliferation of tumor cells *in vitro* and *in vivo*. No direct anti-proliferative effect was seen on various tumor cell lines treated *in vitro* with liposomal clodronate for various concentrations and time points (a). *In vivo* depletion of splenic F4/80^{+}/CD11b^{low} macrophages was achieved in 2 days after a single dose of liposomal clodronate and depletion lasted for at least 8 days without significantly affecting the F4/80^{+}/CD11b^{high} and F4/80^{-}/CD11b^{high} populations. 0, 2, 4 and 8 days after intravenous injection of liposomal clodronate the mice were sacrificed and the depletion was analyzed by FACS (b). Liposomal clodronate administration to tumor-bearing balb/c and C57Bl/6 mice prior to treatment did not alter normal tumor growth or cisplatin response (c and d resp.) Panel a shows representative from 2 independent experiments with similar outcome. Panel b shows results from 2 individual experiments with similar outcome (n=4 per group) and panels c and c show data from 2 independent experiments (n=8 per group) with similar outcome. Data are represented as mean ± SEM. Panel c and d: statistical significance was determined by one-way ANOVA. All compared to cisplatin alone. Panel b: student's T-test all compared to control. *P<0.05,**P<0.01,***P<0.001.

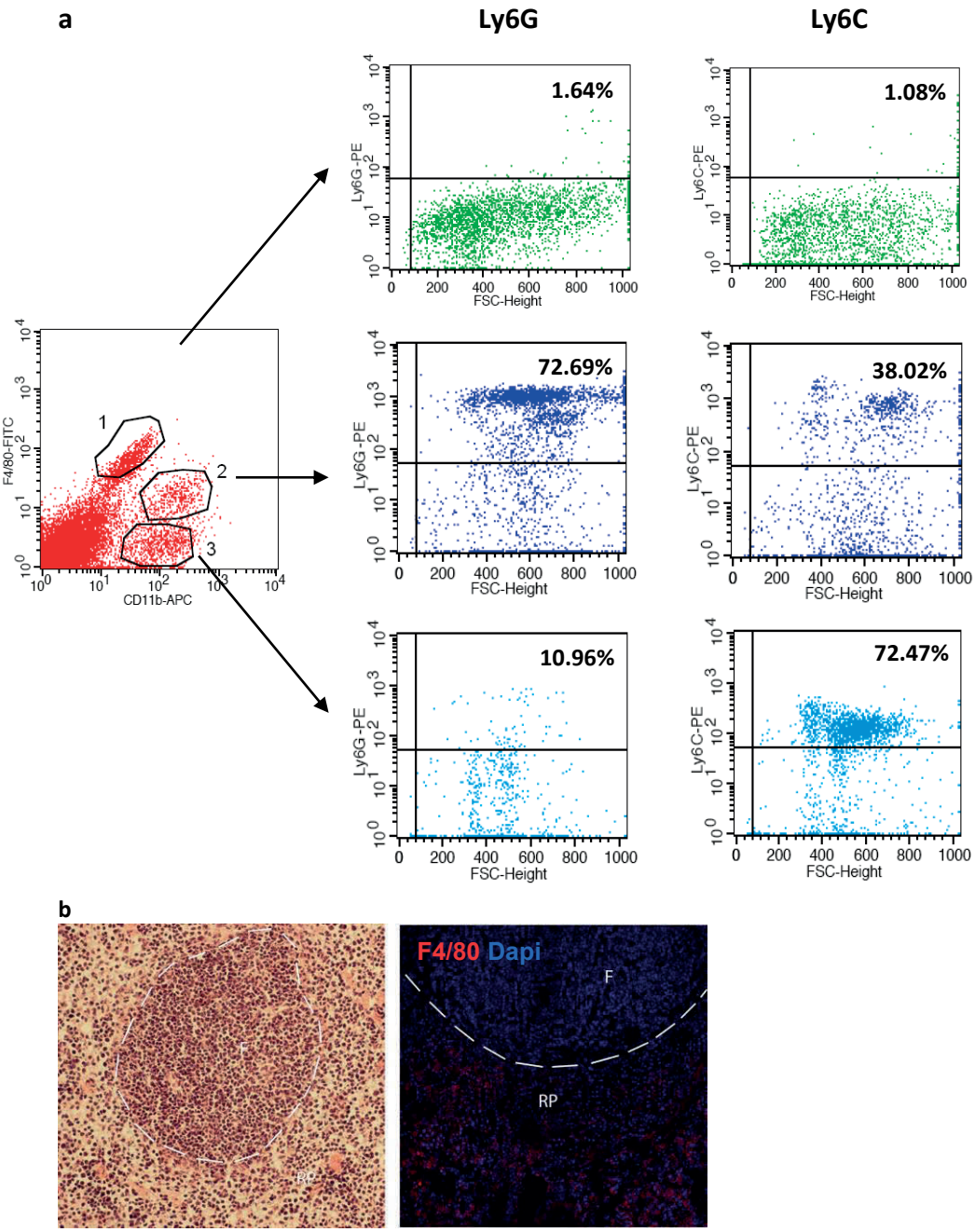

Figure 4: PIFA-responsive F4/80^{+}/CD11b^{low} splenocytes are negative for Ly6G and Ly6C. FACS analysis of F4/80^{+}/CD11b^{low} splenocytes reveals that these cells lack Ly6G and Ly6C (a). Panel b shows H&E staining (left) and F4/80 staining (right) of spleen. F=follicle, RP=red pulp. Data shows representative images of 3 independent experiments**.**

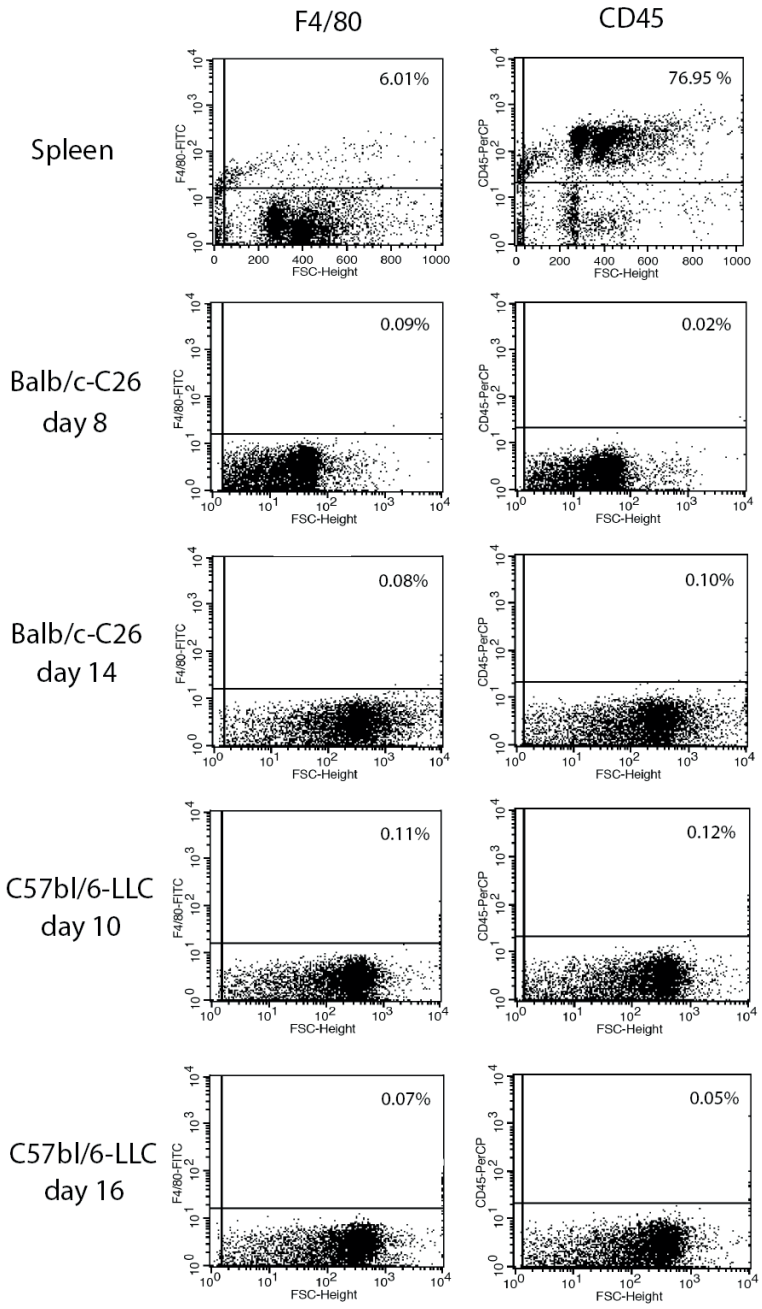

Figure 5: C26 and LLC subcutaneous tumors harbor very little infiltrating leucocytes and macrophages. FACS analysis of F4/80$^+$ and CD45$^+$ cells in C26 tumors at day 8 and 14 and in LLC tumors at day 10 and 16 show low numbers of positive cells. Spleen was used as a positive control.

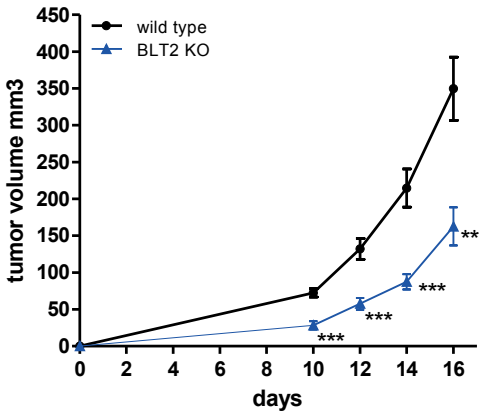

Figure 6: LLC tumors have a delayed tumor growth in *BLT2*$^{-/-}$ mice compared to wild type mice. Mice were injected at day 0 with 500.000 LLC cells/mouse and tumor growth was monitored over time by caliper measurement. *BLT2*$^{-/-}$ mice showed a delayed tumor growth compared to wild type C57bl/6 mice. Graph depicted shows combined data of 2 independent experiments (n= 12 mice per group). Data are represented as mean ± SEM. Statistical significance was determined by one-way ANOVA. **P<0.01, ***P<0.001.

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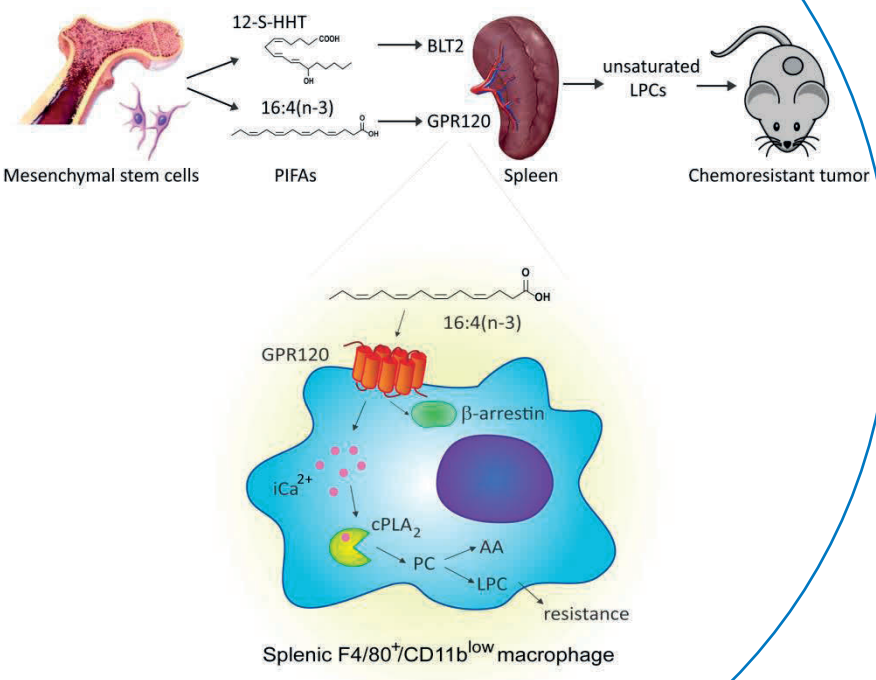
CHAPTER 3


12-S-HHT
BLT2
16:4(n-3)
GPR120
unsaturated
LPCs
Mesenchymal stem cells
PIFAs
Spleen
Chemoresistant tumor
16:4(n-3)
GPR120
β-arrestin
iCa2+
cPLA2
PC
AA
LPC
resistance
Splenic F4/80+/CD11blow macrophage

GPR120/FFAR4 activation by fatty acid 16:4(n-3) plays a key role in resistance to chemotherapy

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Under review at Nature Communications

Abstract

Although chemotherapy is designed to eradicate tumor cells it also has significant effects on normal tissues. These host-responses can impair the efficacy of treatment and overall survival. 12-S-HHT and 16:4(n-3) are two platinum-induced fatty acids (PIFAs) released by mesenchymal stem cells that induce systemic resistance to a broad range of DNA-damaging chemotherapeutics. PIFAs exert their chemoprotective effect via an indirect mechanism involving splenic F4/80^{+}/CD11b^{low} macrophages. Here, we identified GPR120 on splenic macrophages as the relevant receptor for 16:4(n-3). Both GPR40 and GPR120 can be activated by 16:4(n-3) *in vitro.* However, only inhibition or genetic loss of GPR120 was able to block 16:4(n-3)-mediated chemoresistance *in vivo*. Activation of the 16:4(n-3)-GPR120 axis led to enhanced cPLA$_2$ activity in splenic macrophages resulting in the secretion of resistance-inducing lysophosphatidylcholine 24:1 (LPC(24:1)). Taken together, we identified a novel function for GPR120. Activation by 16:4(n-3) enhances cPLA$_2$ activity and production of LPC(24:1) resulting in chemotherapy resistance.

Introduction

Chemotherapy remains one of the main therapies for the treatment of cancer. It is designed to eradicate tumor cells and preserve the host. However, as the numerous side effects of chemo already predict, the host is not left unharmed by the treatment. Many different host responses occur during chemotherapy treatment and these can have large effects on the efficacy of the treatment and overall survival[1]. For instance large infiltrates of stromal cells within pancreatic tumors can inhibit penetration of cytostatic drugs and elimination of these cells enhances chemotherapy efficacy[2]. However, there are more active ways in which host cells can influence therapy outcome. Mesenchymal stem cells (MSCs) have been shown to home to developing tumors and protect tumor cells against various chemotherapeutics by secretion of multiple cytokines and chemokines[3]. Similar chemoprotective functions have been described for macrophages[4].

Recently our lab has identified a mechanism of reversible and systemic chemotherapy resistance involving MSCs and splenic macrophages. We have showed that MSCs can secrete two distinct polyunsaturated fatty acids (12-S-HHT and 16:4(n-3)) upon stimulation with platinum-containing chemotherapeutics. These platinum-induced fatty acids (PIFAs) convey resistance to multiple types of DNA-damaging chemotherapeutics in several mouse models[5]. The PIFAs do not induce resistance directly to the tumor cells but function via F4/80^{+}/CD11b^{low} macrophages located in the red pulp of the spleen. Removal of the spleen or depletion of these macrophages using liposomal clodronate can inhibit PIFA-induced chemoresistance[6].

Here we found that 16:4(n-3)-mediated resistance is dependent on GPR120 on splenic macrophages. GPR120 is a free fatty acid receptor that binds medium- to long-chain fatty acids and omega-3 fatty acids. It is expressed in adipose tissue and on macrophages and is involved in anti-inflammatory response and metabolic control[7]. Here we show that GPR120 is also expressed on splenic red pulp macrophages and that 16:4(n-3)-GPR120 signaling is involved in chemotherapy resistance via the secretion of lysophosphatidylcholine 24:1 (LPC(24:1)).

Results

GPR40 and GPR120 are expressed on splenic macrophages

In order to identify potential receptors for 16:4(n-3) we investigated the expression levels of free fatty acid receptors GPR40, GPR41, GPR43, GPR84 and GRP120 and looked for differential expression on the splenic cell population known to induce resistance. We found that GPR40, GPR41 and GPR120 were expressed exclusively in the spleen on F4/80^{+}/CD11b^{low} splenocytes (Figure 1a-b). Since 16:4(n-3) is a long-chain omega-3 fatty acid and GPR41 a receptor for short-chain fatty acids we decided to investigate the

potential role of GPR40 and GPR120 in 16:4(n-3)-mediated chemoresistance. Although GPR40 and GPR120 display little structural homology they show substantial overlap in their ligands[8]. GPR40, also referred to as FFAR1, binds saturated and unsaturated medium- to long-chain fatty acids. GPR120, also referred to as FFAR4, binds medium- to long-chain fatty acids including omega-3 fatty acids[8].

16:4(n-3) activates both GPR40 and GPR120 in vitro

To investigate if 16:4(n-3) could activate GPR40 and GPR120 we performed intracellular calcium (iCa^{2+}) flux experiments using HEK293T cells with doxycycline-inducible expression of either GPR40 or GPR120. Interestingly, 16:4(n-3) was able to elicit an iCa^{2+} response in both GPR40 and GPR120 expressing cells (Figure 1c-d). We found that the 16:4(n-3)-induced iCa^{2+} response in GPR40 expressing cells could only be blocked with a GPR40 specific antagonist (GW1100) and not with a GPR120 specific antagonist (AH-7614). In addition, the iCa^{2+} response in the GPR120 expressing cells could be blocked with AH-7614 but not with GW1100 (Figure 1e-f). AH-7614 features a tricyclic xanthene unit also found in various other anticancer drugs[9]. To exclude the possibility of off-target effects, we synthesized the close analogue TUG-1387 which also contained a xanthene unit but lacks activity at either GPR40 or GPR120. As expected TUG-1387, did not inhibit 16:4(n-3)-mediated iCa^{2+} response in either of the cell lines (Figure 1e-f). All compounds used in this study are listed in Supplemental table 1 and Supplemental figure 1a. To confirm our findings we performed a β-arrestin2 recruitment assay and found that 16:4(n-3) recruited β-arrestin2 to both GPR40 and GPR120 (Supplemental figure 1b-c). Since both receptors are expressed by splenic macrophages and both are activated by 16:4(n-3) we set out to determine which receptor is involved in 16:4(n-3)-mediated chemoresistance *in vivo*.

Inhibition of GPR120 prevented 16:4(n-3)-mediated chemoresistance in vivo

Next, we assessed whether synthetic dual GPR40/GPR120 agonists GW9508 and TUG-891 could induce chemotherapy resistance *in vivo*. We found that *ex vivo* stimulation of splenic macrophages with either GW9508 or TUG-891 and subsequent injection of this splenic conditioned medium (sCM) with cisplatin into tumor-bearing mice induced chemoresistance. The observed resistance was similar to that obtained with sCM from splenic macrophages stimulated with 16:4(n-3) implying that GPR40 and/or GPR120 is involved in 16:4(n-3)-mediated chemoresistance *in vivo* (Figure 2a). NCG21, another GPR40/GPR120 agonist, also induced chemoresistance (Figure 2b). In order to further test the contribution of GPR40 and GPR120 to chemotherapy resistance we tested a GPR40-specific and a GPR120-specific antagonist. We found that pre-incubation of splenic macrophages with AH-7614 (GPR120 antagonist) completely prevented 16:4(n-3)-mediated chemoresistance whilst this was not achieved with the GPR40 specific antagonist GW1100 (Figure 2c). In order to exclude potential off-target effects of AH-7614

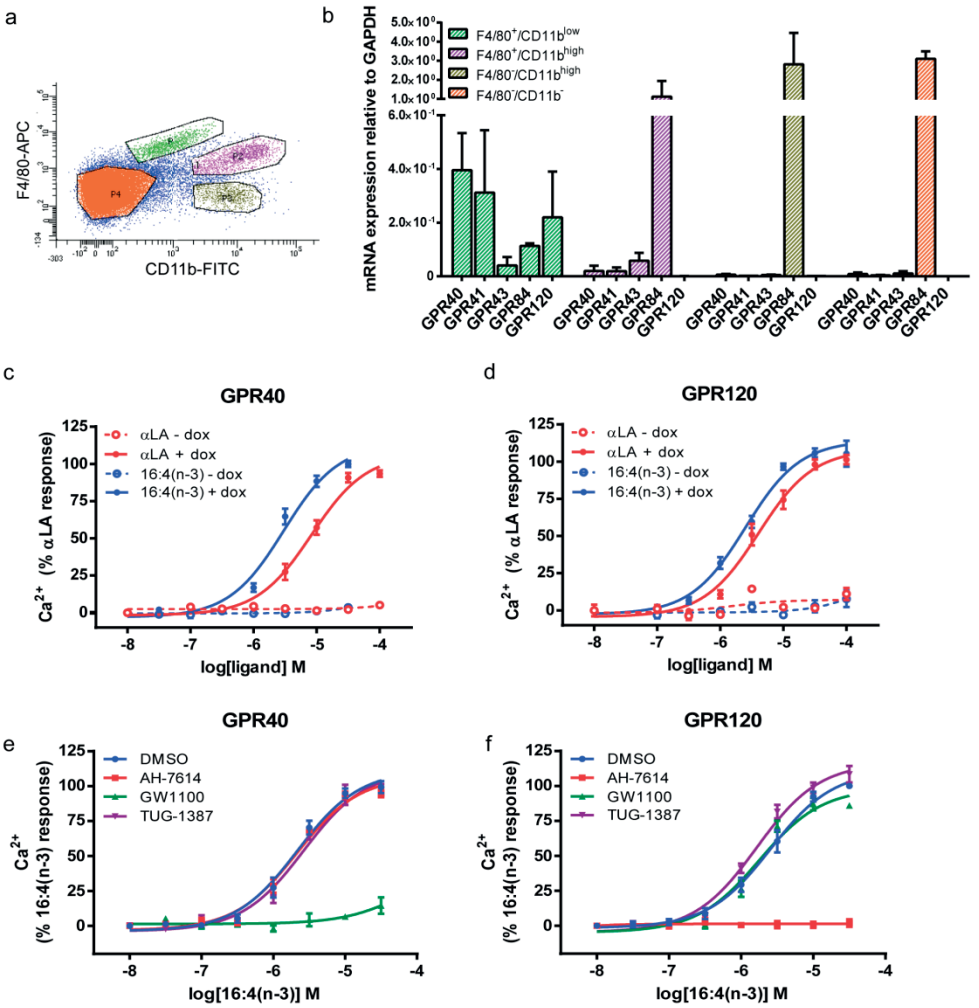


Figure 1: 16:4(n-3) activates both GPR40 and GPR120 and both receptors are expressed on splenic macrophages. Panel a shows FACS analysis of BALB/c mouse spleen stained for F4/80 and CD11b; indicated populations were sorted and relative expression of the free fatty acid receptors GPR40, GPR41, GPR43, GPR84 and GPR120 on these populations was determined by q-PCR (panel b). mRNA expression levels were normalized to GAPDH control. 16:4(n-3) is able to induce an intracellular calcium response in GPR40 or GPR120 expressing Flp-In T-Rex 293 cells. 16:4(n-3) activates both receptors with a higher potency than αLA, which was used as a positive control, Flp-In T-rex 293 cells without doxycycline (i.e. no GPR40/120 expression) were used as a negative control (panels c and d). A GPR40 specific antagonist (GW1100, 10 μM) was able to block 16:4(n-3)-mediated activation of GPR40, whereas a GPR120 specific antagonist (AH-7614, 10 μM) had no effect (panel e). AH-7614 was able to block 16:4(n-3)-mediated activation of GPR120 whereas GW1100 had no effect (panel f). Compound TUG-1387 (10 μM) which is structurally related to AH-7614 but is inactive had no effect on 16:4(n-3) induced activation of GPR40 or GPR120. Panel a and b show representative results of two independent experiments with similar outcome, panel c-f shows grouped results of three independent experiments with similar outcome. Data are represented as mean ± SEM.

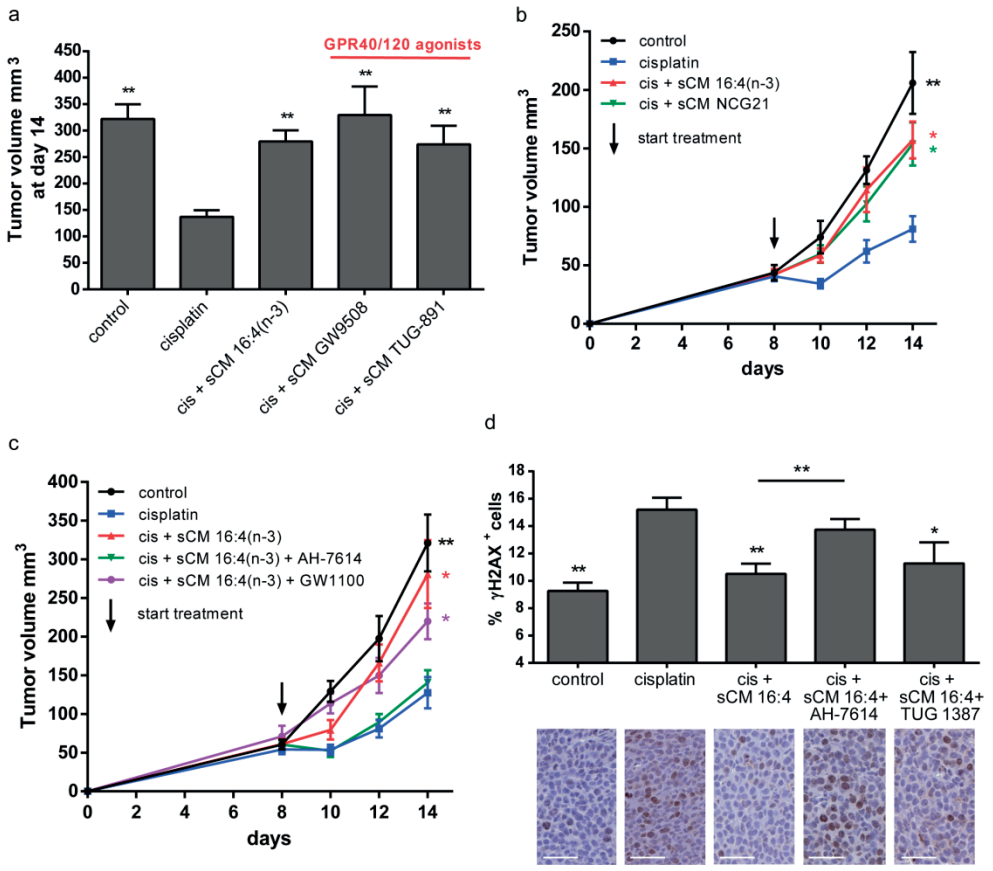


Figure 2: 16:4(n-3) induced chemotherapy resistance is mediated via GPR120 *in vivo*. sCM derived from splenocytes incubated with dual GPR40/120 agonists TUG-891 or GW9508 induce chemoresistance *in vivo* to similar extent as sCM derived from splenic macrophages incubated with 16:4(n-3) (panel a). Co-administration of cisplatin and sCM from splenocytes cultured in the presence of NCG21 (another GPR40/120 agonist) induces chemotherapy resistance in splenectomized tumor-bearing BALB/c mice (panel b). The GPR120 specific antagonist AH-7614 was able to completely block 16:4(n-3)-mediated resistance, whereas the GPR40 specific antagonist GW1100 was ineffective (panel c). Tumors of mice treated with cisplatin and sCM + 16:4(n-3) showed a decrease in γH2AX levels 4 hours after treatment compared to cisplatin alone treated animals. Mice treated with cisplatin and sCM + 16:4(n-3) + AH-7614 had comparable γH2AX levels as cisplatin alone treated mice. Again, sCM +16:4(n-3) + TUG-1387 did not alter 16:4(n-3)-mediated chemoresistance (panel d, scale bar: 80 μm). All experiment in this figure were conducted in BALB/c mice. Graphs a and c show results of three independent experiments (n=12 per group) and graphs b and d show results of two independent experiments (n=8 per group). Data are represented as mean ± SEM. Statistical significance was determined by one-way ANOVA (panel a-c) or two-tailed Students T-test (panel d). All compared to cisplatin alone unless indicated otherwise * P<0.05, ** P<0.01.

we also tested TUG-1387. As expected this structurally related but inactive molecule did not inhibit 16:4(n-3)-mediated chemoresistance (Supplemental figure 2a). Interestingly, stimulation of splenic macrophages with docosahexaenoic acid (DHA), another natural GPR120 ligand, did not elicit a chemoprotective response, indicating that 16:4(n-3) induced a specific response within these splenic macrophages (Supplemental figure 2b). PIFAs are able to alter the DNA damage response in tumor cells leading to chemoresistance. Co-administration sCM and cisplatin led to a decrease in γH2AX levels, a measure for double strand DNA breaks, compared to cisplatin monotherapy four hours after treatment[6]. In line with the tumor volumetric measurements we found that sCM from 16:4(n-3)-activated splenocytes was able to reduce γH2AX whereas mice treated with sCM from 16:4(n-3) and AH-7614 stimulated splenocytes did not. Furthermore TUG-1387 did not affect 16:4(n-3)-mediated decrease in γH2AX (Figure 2d). Inhibition of GPR120 blocks chemoresistance both in tumor volumetric experiments and at the level of γH2AX in tumors indicating that GPR120 is the functional receptor transducing 16:4(n-3)-mediated chemoresistance.

sCM derived from GPR120$^{-/-}$ mice could not induce 16:4(n-3)-mediated chemoresistance

To validate the results obtained with the agonists and antagonists we tested the resistance-inducing potential of sCM derived from splenic macrophages of wildtype and GPR120$^{-/-}$ mice stimulated with either 16:4(n-3) or 12-S-HHT. As expected we found that sCM from GPR120$^{-/-}$ mice incubated with 16:4(n-3) did not induce chemoresistance in wildtype tumor-bearing mice, whereas sCM from wildtype animals incubated with 16:4(n-3) retained the ability to induce chemotherapy resistance (Figure 3a-b). The resistance-inducing potential of 12-S-HHT was not hampered by the lack of GPR120, which is in line with our previous findings that 12-S-HHT functions via leukotriene B4 receptor 2 (BLT_2)[6] (Figure 3a-b). As expected, we found that mice treated with cisplatin and sCM from GPR120$^{-/-}$ mice incubated with 16:4(n-3) showed similar levels of DNA damage as determined by γH2AX as mice treated with cisplatin alone. Mice treated with cisplatin and sCM from wildtype mice incubated with 16:4(n-3) showed a decrease in γH2AX positive cells compared to cisplatin treated animals. The 12-S-HHT-mediated decrease in γH2AX was seen in mice treated with both GPR120$^{-/-}$ and wildtype sCM (Figure 3c).

Human splenocytes express GPR120

To investigate if our findings are relevant to the human situation we determined expression levels of GPR40, GPR41, GPR43, GPR84 and GPR120 in human spleen. Human splenocytes showed relative high expression of GPR40 and GPR120, correlating with the findings in mice. Immunohistochemistry showed GPR120 positive cells in the red pulp of the spleen (Supplemental figure 3). The splenic red pulp harbors tissue resident macrophages known for their role in iron homeostasis and has previously been identified

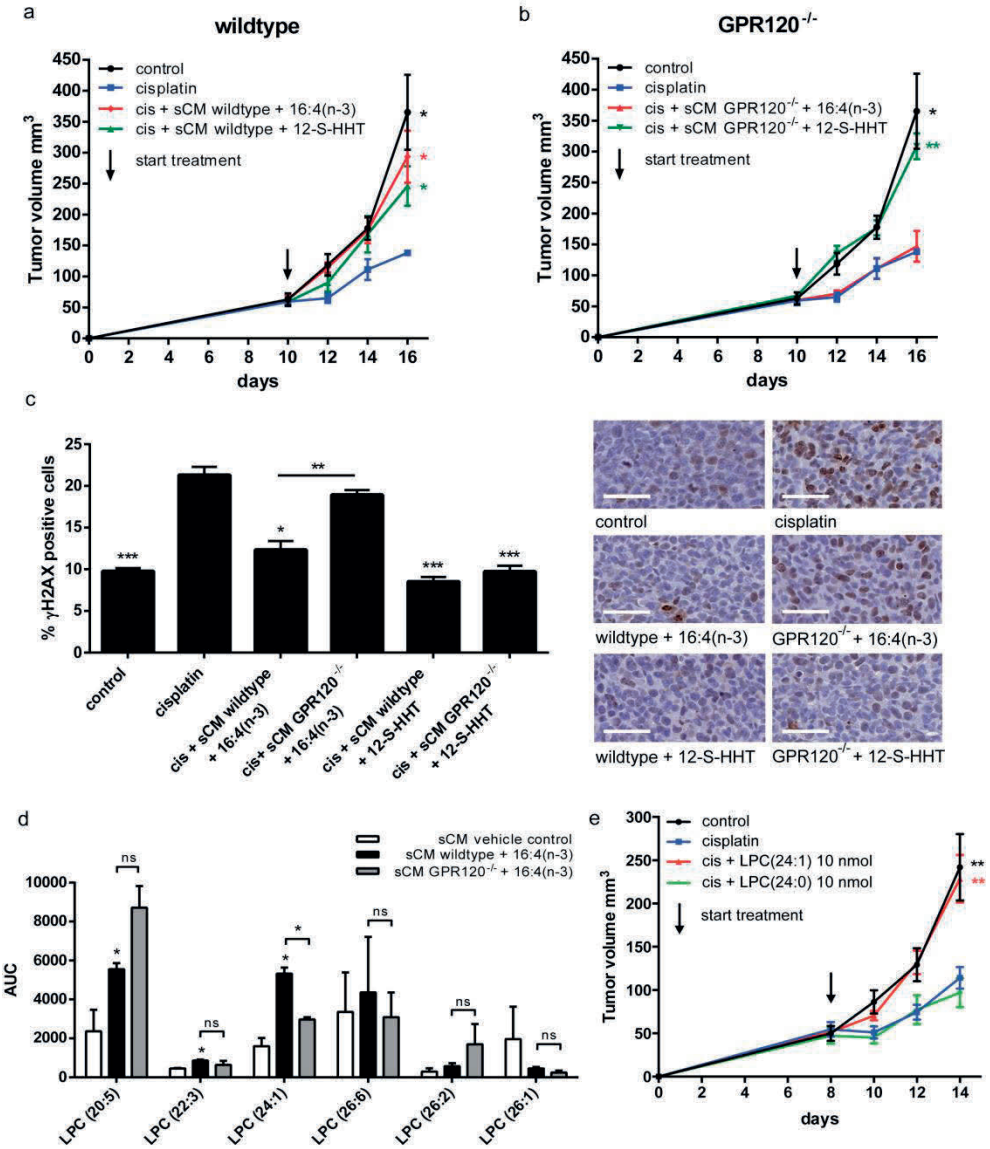


Figure 3: Genetic loss of GPR120 prevents 16:4(n-3)-mediated chemoresistance. sCM from wildtype C57BL/6 mice incubated with either 16:4(n-3) or 12-S-HHT was able to induce resistance against cisplatin in wildtype C57BL/6 splenectomized tumor-bearing mice (panel a). sCM of GPR120$^{-/-}$ mice incubated with 16:4(n-3) was unable to induce chemoresistance when co-injected with cisplatin in wildtype C57BL/6 tumor-bearing mice, whereas sCM from GPR120$^{-/-}$ mice incubated with 12-S-HHT was able to induce resistance (panel b). γH2AX levels in tumors treated with cisplatin and sCM GPR120$^{-/-}$ +16:4(n-3) showed similar levels to cisplatin monotherapy whereas tumors treated with cisplatin and sCM wildtype + 16:4(n-3) or sCM wildtype + 12-S-HHT or sCM GPR120$^{-/-}$ + 12-S-HHT showed significantly lower γH2AX levels (panel c, scale bar: 80 μm). LPC analysis of sCM from wildtype mice incubated with 16:4(n-3) revealed three significantly upregulated unsaturated LPCs

compared to vehicle control incubated splenocytes. LPC(24:1) upregulation in wildtype sCM is lost in GPR120$^{-/-}$ sCM (panel d). Co-administration of cisplatin and 10 nmol LPC(24:1) was sufficient to induce chemotherapy resistance, whereas co-administration of cisplatin and 10nmol of LPC(24:0) could not (panel e). Experiments panel a-d were conducted in C57BL/6 mice, panel e in BALB/c mice. All graphs show results of two independent experiments (n=8 per group). Data are represented as mean ± SEM. Statistical significance was determined by one-way ANOVA (panel a-b) or two-tailed Students T-test (panel c-d). All compared to cisplatin alone unless indicated otherwise * P<0.05, ** P<0.01, ***P<0.001.

as the location for the F4/80^{+}/CD11b^{low} macrophages known to induce chemoresistance[6,15]. Furthermore, we have previously established that sCM derived from human splenocytes can induce chemoresistance in mice[6].

16:4(n-3)-activated splenic macrophages produce chemoresistance-inducing LPC(24:1)

PIFA-activated splenic macrophages produce six unsaturated lysophosphatidylcholines (LPCs) as the effector molecules of resistance to tumor cells[6]. Here we identified which of these LPCs is released upon 16:4(n-3) stimulation. When measuring LPC levels in sCM incubated with 16:4(n-3) alone we found a significant upregulation of three out of these six LPCs, namely LPC(20:5), LPC(22:3) and LPC(24:1) (Figure 3d). However when comparing 16:4(n-3) stimulated sCM from wildtype animals with that from GPR120$^{-/-}$ animals we found that only LPC(24:1) was significantly downregulated in the GPR120$^{-/-}$ mice (Fig 3d). In order to test if LPC(24:1) is the resistance-inducing molecule secreted by 16:4(n-3)-activated splenic macrophages, we co-injected LPC(24:1) with cisplatin into tumor-bearing mice and found that LPC(24:1) induced chemoresistance, whereas the closely related LPC(24:0) could not (Figure 3e). LPC(20:5) and LPC(22:3) could not be tested since these could not be produced nor were commercially available.

Activation of GPR120 by 16:4(n-3) leads to enhanced cPLA$_2$ activity and LPC(24:1) production

The production of LPC from phosphatidylcholine (PC) can be mediated by various enzymes. Phospholipase A1 (PLA1) can hydrolyse PC at the sn-1 position, cytosolic phospholipase A2 (cPLA$_2$) can hydrolyse PC at the sn-2 position and phospholipase B (PLB) can cleave PC at both positions leading to structurally different LPCs (Figure 4a). Since we found that the effector molecule secreted by splenic macrophages is LPC(24:1), we examined if LPC production in these cells was mediated by PLA1 or cPLA$_2$ activity. There was a significant increase in cPLA$_2$ activity after exposure to 16:4(n-3) as measured by phospho-cPLA$_2$ and enzymatic activity. This effect could be blocked by GPR120 antagonist AH-7614 (Figure 4b-c). We found no upregulation in PLA1 activity in splenic macrophages upon 16:4(n-3) stimulation (Supplemental figure 4a).

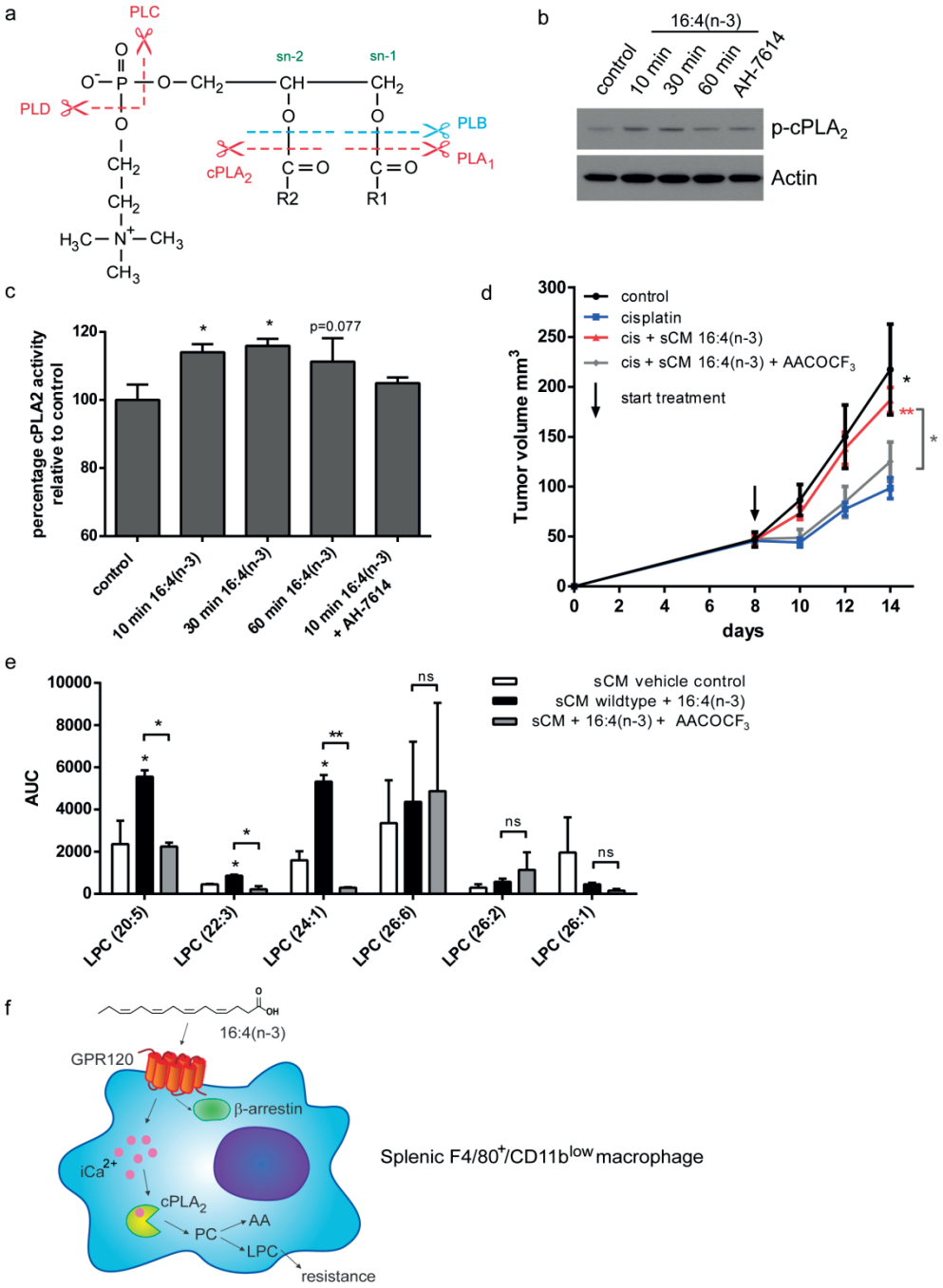


Figure 4: 16:4(n-3) stimulation increases cPLA$_2$ activity in splenic macrophages. Panel a shows a schematic overview of hydrolysis sites of phosphatidylcholine to yield metabolites like lysophosphatidylcholine. Splenic macrophages were harvested from murine BALB/c spleens by

magnetic bead sorting using F4/80 antibodies. The macrophages were incubated with either vehicle or 16:4(n-3) (25 nM) for indicated times. Cell lysates were analyzed by western blot for phospho-$cPLA_2$. Actin was used as a loading control (panel b). Fluorescent-based analysis of $cPLA_2$ enzymatic activity showed time-dependent increase in $cPLA_2$ upon stimulation with 16:4(n-3) (panel c). Co-administration of cisplatin with sCM from splenocytes incubated with 16:4(n-3) and $AACOCF_3$ showed loss of chemoresistance compared to treatment with cisplatin and sCM + 16:4(n-3) (panel d). LPC(24:1) is lost when splenocytes are incubated with 16:4(n-3) in the presence of $AACOCF_3$ (panel e). Panel f shows schematic overview of 16:4(n-3) mediated resistance in splenic macrophages. Panel b shows representative results of four experiments with similar outcomes. Graphs c-e show results of two independent experiments (n=8 per group). Data are represented as mean ± SEM. Statistical significance was determined by two-tailed Students T-test (panel c, e) or one-way ANOVA (panel d). All compared to vehicle control (panel c and e) or compared to cisplatin alone (panel d) unless indicated otherwise * $P<0.05$, ** $P<0.01$.

Next we tested if a $cPLA_2$ inhibitor ($AACOCF_3$) could block 16:4(n-3)-mediated chemoresistance. We found that sCM from splenocytes incubated with 16:4(n-3) and $AACOCF_3$ did not induce resistance (Figure 4d). When comparing LPC levels in sCM incubated with 16:4(n-3) alone or in combination with $AACOCF_3$ we found that LPC(24:1) was significantly downregulated (Figure 4e).

It has been shown that activation of $cPLA_2$ via GPR120 in the peritoneal macrophage cell line RAW264.7 induces an anti-inflammatory response[16]. To determine whether our findings can be generalized for macrophages we determined if RAW264.7 cells could upregulate phospho-$cPLA_2$ and produce LPC(24:1) after stimulation with 16:4(n-3). sCM from RAW264.7 cells stimulated with 16:4(n-3) did not induce chemoresistance in tumor-bearing splenectomized mice nor did we find an upregulation of phospho-$cPLA_2$ or LPC(24:1) (Supplemental figure 4). These findings indicate that the activation of GPR120 via 16:4(n-3), $cPLA_2$ upregulation, LPC(24:1) production and chemoresistance appears to be context dependent.

Discussion

Here we showed that the 16:4(n-3)-GPR120-LPC(24:1) signalling axis in splenic macrophages induces chemotherapy resistance. GPR120 has previously been described as a receptor for omega-3 fatty acids[7]. The relatively high expression of GPR120 in adipose tissue, macrophages and intestine has led to the discovery of its function in insulin signalling, obesity, anti-inflammatory response and dietary lipid uptake[7,17-20]. Various studies also show a role for GPR120 in tumor progression. Overexpression of GPR120 in colorectal carcinomas has been associated with angiogenic switching and cell motility[21]. Similarly, in pancreatic cancer cell lines downregulation of GPR120 prevented cell migration[22]. In contrast, in prostate cancer GPR120 signaling appears to have an anti-tumor effect[23]. Numerous ligands can activate GPR120 and the downstream effects may differ per cell type and tissue, underpinning the complexity of fatty acid signalling in

cancer biology. Here we identified 16:4(n-3) as a natural ligand of GPR120 and showed that activation of GPR120 by 16:4(n-3) leads to increased $cPLA_2$ activity and associated upregulation of LPC(24:1) and chemotherapy resistance *in vivo* (Figure 4f). Since chemotherapy resistance poses one of the main problems in effective treatment of cancer patients, insights into these mechanisms are of importance to identify new targets that could enhance chemotherapy efficacy. Our findings suggest that fatty acid signalling may provide new avenues to explore in order to increase chemotherapeutic efficacy.

Material and Methods

Reagents

16:4(n-3) was isolated from *Ulva Pertusis* as described previously[24]. GW1100 was purchased from Cayman Chemical. GW9508 and $AACOCF_3$ were purchased from Tocris. NCG21 was synthesized as described previously[13]. TUG-891 and AH-7614 were synthesized as described previously[10,14]. TUG-1387 was synthesized analogously to AH-7614. For FACS analysis the following antibodies were used: rat anti-mouse F4/80-FITC and rat-anti mouse CD11b-APC (both from eBioscience). For immunohistochemical staining the following antibodies were used: anti-γH2AX (Cell Signaling, 2577), anti-GPR120 (Novus Biologicals, NBP1-00858) and poly-HRP goat-anti-Rabbit/Rat/Mouse (Immunologic). For western blot the following antibodies were used: rabbit anti-phospho-$cPLA_2$ (Ser505, Cell Signaling, 2831), mouse anti-beta actin (Novus Biologicals, NB600-501) and goat-anti-mouse HRP (Santa Cruz).

Animal models

C26 and LLC cells were implanted in balb/c and C57bl/6 (both from Charles River) mice respectively. For all experiments 8-10 week old male mice were used. At day 0 the mice were injected subcutaneously with $1*10^6$ (for C26) or $0.5*10^6$ (for LLC) tumor cells. Mice were splenectomized one day after tumor cell injection. The spleens from the surgery were used to prepare sCM. At day 8 (C26) or day 10 (LLC) when the tumors reached a size of 50-100 mm^3 animals were randomized into groups and treatment was started. The mice received an intraperitoneal injection of cisplatin 6mg/kg alone or in combination with a subcutaneous injection of 200 µl sCM or 100 µl of LPC(24:1) or LPC(24:0) (both 10 nmol). Blinded tumor volume measurements were done once every two days using a digital caliper. Tumor volume was determined as $length*width^2*0.5$. Control mice received the appropriate vehicles. All experimental animal procedures conducted in Utrecht (the Netherlands) were approved by the University Medical Center Animal Ethics Committee and were in agreement with the current Dutch Laws on animal experiments. All experimental animal procedures conducted in Kyoto (Japan) were approved by the Kyoto University Animal Care and Use Committee. In order to show a difference of 20% in tumor volume with a standard deviation of 10% and a type I error (alfa) of 5% using a power of 90% a minimal of 8 mice per treatment group are required.

Cell lines

C26, LLC and RAW264.7 cells (all from ATCC) were grown in DMEM (4.5 gr/L glucose) + 5% FCS at 5% CO_2 and 37°C. Flp-In T-REx 293 cells with inducible overexpression of GPR120-eYFP or GPR40-eYFP were grown in DMEM (4.5 gr/L glucose) + 10% FCS at 5% CO_2 and 37°C. All cell lines were mycoplasma negative and the C26 and LLC cells were also tested for mouse pathogens according to the FELASA panel and found to be negative.

Preparation of sCM from splenocytes

Spleens from splenectomized mice were used to make sCM. Single cell suspensions were made from the spleens and red blood cells were lysed using red blood cell lysis buffer (RBC) (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA). Splenocytes were incubated in serum free medium (SFM) (DMEM 4.5 gr glucose/L) with 25 nM 16:4(n-3) or 20 nM 12-S-HHT for 1 hour at 5% CO_2 and 37°C. After incubation the supernatant from the splenocytes was harvested, filtered through a 0.2μM filter and stored at -80°C until use. For the preparation of sCM with TUG-1387 and AH-7614 the splenocytes were pre-incubated for 10 minutes with 1 μM of the indicated compounds before adding 16:4(n-3) for an additional hour. Splenocytes incubated with 16:4(n-3) and GW1100 or $AACOCF_3$ were preincubated for 30 minutes with 10 μM of the compounds before adding 16:4(n-3). GW9508 (100 μM), NCG21 (100 μM), DHA (100 μM) and TUG-891 (10μM) were incubated with the splenocytes for 1 hour before harvesting the sCM as described above. CM from RAW264.7 cells was made as follows; RAW264.7 cells were plated at a density of $3*10^5$ cells/well in a 6-wells dish. The next day culture medium was replaced by 1 ml serum-free medium with 25 nM 16:4(n-3) and incubated for 1 hour at 5% CO_2 and 37°C. After incubation the supernatant from the RAW264.7 cells was harvested and stored at -80°C until use.

FACS sorting and q-PCR analysis of GPCRs

Single cell suspensions were made from the spleen and red blood cells were lysed as described in *Preparation of sCM from splenocytes*. Next, the splenocytes were incubated with F4/80-FITC and CD11b-APC (concentration 1μl/$1*10^7$ cells/100μl) or corresponding isotype controls (FITC and APC both from eBioscience) in FACS buffer for 30 minutes on ice. $F4/80^+/CD11b^{low}$, $F4/80^+/CD11b^{high}$, $F4/80^-/CD11b^{high}$ and $F4/80^-/CD11b^-$ populations were sorted using a FACSAria III from BDbioscience. Gates for sorting were set according to unstained and isotype control stained samples. RNA was extracted from FACS-sorted splenic populations using TriZol (Invitrogen). cDNA was synthesized using Superscript II (Invitrogen) according to manufacturer's protocol using oligo dTs (Fermentas). Mouse primers used for detection of GPR40 forward: 5'-GCTATTCCTGGGGTGTGTGT-3', reverse: 5'-CCCTGTGATGAGTCCTAACT-3'. GPR41 forward: 5'-CTGCTCCTGCTCCTCTTC-3', reverse: 5'-CCAGGCGACTGTAGCAGTA-3'. GPR43 forward: 5'-GGCTTCTACAGCAGCATCTA-3', reverse: 5'-AAGCACACCAGGAAATTAAG-3'. GPR84 forward: 5'-TCCAATTCTGTCTCCATCCT-3', reverse: 5'-CTGACTGGCTCAGATGAAA-3'. GPR120 forward: 5'-CCATCCCTCTAGTGCTCGTC-3', reverse: 5'-TGCGGAAGAGTCGGTAGTCT-3'. For the q-PCR analysis of human spleen RNA was also isolated using TriZol and cDNA was synthesized using Superscript II. Primers used for detection of human GPR's are: GPR40 forward: 5'-GTGTCACCTGGGTCTGGTCT-3', reverse: 5'-GAGCAGGAGAGAGAGGCTGA -3'. GPR41 forward: 5'-TCTCAGCACCCTGAACTCCT -3', reverse: 5'-TTCTGCTCCTTCAGCTCCAT -3'.

GPR43 forward: 5'-AAGGAGAAGGGATGCCAAGT-3', reverse: 5'-GGGATACCAAGCTGGTGAAA -3'. GPR84 forward: 5'-TCAGCAGTGTTGGCATCTTC -3', reverse: 5'-TAACCTGCTGTCCAGCTCCT -3'. GPR120 forward: 5'-CTTCTTCTCCGACGTCAAGG-3', reverse: 5'-AGAGGGATAGCGCTGATGAA-3'. GAPDH forward: 5'-CGACCACTTTGTCAAGCTCA-3', reverse: 5'- AGGGGTCTACATGGCAACTG-3'.

Immunohistochemistry

For immunohistochemistry experiments, murine tumors were harvested 4 hours after therapy. Human spleen tissue was provided from the pathology biobank database. Formaldehyde-fixed paraffin-embedded tissue sections were deparaffinated and rehydrated and incubated in endogenous peroxidase blocking buffer containing 5% H_2O_2. Antigen retrieval was performed by cooking slides in citrate buffer. Tissues were blocked in 5% goat serum (Life Technologies) in TBST for the γH2AX staining or in serum-free protein block (Dako) for the GPR120 staining. Antibodies were used in following dilutions: rabbit anti-γH2AX 1:200 in 5% goat serum in TBST, overnight at 4°C, Cell Signaling, rabbit anti-GPR120 1:1000 in Bright Diluent (Immunologic). Poly-HRP goat-anti-Rabbit/Rat/Mouse (Immunologic) was used as secondary antibody, followed by DAB staining. Slides were counterstained with hematoxyline. Each tumor slide was scanned using a Leica Aperio Scanscope and a minimum of 10 images per tumor were analyzed. To determine the percentage of positive cells a grid with 54 intersection points was used. A second examiner, blinded to the treatment groups, also quantified the γH2AX stainings.

Western blot

The F4/80 positive cells were isolated from murine spleens using magnetic bead sorting. Spleens were harvested from non-tumor bearing mice and a single cell suspension was made. After RBC lysis the splenocytes were resuspended in FACS buffer (1% BSA, 5 mM EDTA in PBS pH 7.4) and counted. The splenocytes were incubated with rat anti-mouse F4/80 at a concentration of 3 μl/1*10^7 cells in 100 μl for 30 minutes on ice. After incubation the cells were washed in FACS buffer and incubated with goat anti-rat microbeads at a concentration of 5 μl/1*10^7 cells in 100 μl for 15 minutes on ice. Again the cells were washed after antibody incubation and resuspended in FACS buffer and separated according to the manufacturer's instructions using LS columns (Miltenyi Biotec). The F4/80$^+$ splenocytes were incubated for indicated times with 25 nM 16:4(n-3). After incubation the cells were lysed and subjected to SDS-PAGE and western blotting. Membranes were blocked in either 5% BSA in TBST (for phospho-cPLA2) or 5% non-fat milk in TBST (for actin) and incubated overnight with antibodies against phospho-cPLA2 (1:1000 in 5% BSA in TBST), actin (1:10.000 in 5% milk in TBST). Secondary antibodies used for detection were diluted 1:7500 in 5% BSA in TBST (goat-anti rabbit HRP) or in 5% milk in TBST (goat-anti-mouse HRP).

$cPLA_2$ and PLA1 activity measurements

$F4/80^+$ splenocytes were lysed after incubating them for the indicated times with 25 nM 16:4(n-3) (or 25 nM 16:4(n-3) and a 10 min pre-incubation of 10 μM AH-7614) and $cPLA_2$ or PLA1 activity was measured using the EnzChek phospholipase A_2 or EnzChek phospholipase A1 assay kit respectively (Life technologies) according to the manufacturer's protocol.

Ca^{2+} mobilization assay

GPR120 and GPR40 Flp-In T-REx 293 cells were plated 50,000 cells/well in black 96-well plates with clear bottoms. Cells were then treated with 100 ng/ml doxycycline to induce receptor expression and maintained overnight at 37 °C and 5% CO_2 prior to their use. Cells were labeled for 45 min with Fura2-AM followed by washing and maintenance in Hanks' balanced salt solution (HBSS). Fura-2 fluorescent emission at 510 nm resulting from 340 or 380 nm of excitation was then monitored before and for 90s after the addition of test compound using a Flexstation II plate reader (Molecular Devices). Ca^{2+} responses were taken as the peak 340/380 ratio measured after compound addition and normalized to the maximal response obtained to either αLA or 16:4(n-3) as indicated.

β-Arrestin2 recruitment assay

HEK293T cells were co-transfected using polyethyleneimine with FLAG-GPR120-eYFP and nanoluciferase(NLUC)-β-arrestin2 plasmids or FLAG-GPR40-eYFP and NLUC-β-arrestin2 plasmids in a ratio of 4:1. 24 h post-transfection, cells were plated into poly-d-lysine-coated white 96-well tissue culture plates and maintained for a further 24 hrs prior to the experiment. For the experiment cells were first washed and then incubated at 37°C for 30 min in HBSS. The NLUC NanoGlo substrate (Promega UK) was added with a final dilution of 1:800 before a further incubation for 10 min at 37°C. Test compounds were then added at the specified concentration, and cells incubated a final 5 min prior to measuring luminescent emission at 530 and 465 nm using a ClarioStar plate reader (BMG Lab Tech). The 530/465 emission ratio was calculated and corrected for the ratio obtained in cells transfected with only the NLUC-β-arrestin2 plasmid before normalizing to the maximal response obtained to 16:4(n-3).

LPC analysis of sCM samples

Total lipids from culture media of sCM were extracted using a modified Bligh & Dyer extraction according to Retra *et al.* 2008[25]. LPC analysis was performed using a modified method of Retra *et al.* 2008[25]. Here the HPLC column was replace by a fused core HALO C18 column (Biotech, Onsala, Sweden). To increase sensitivity, LPCs were detected in multiple reaction monitoring mode, in which a collection of 36 different LPC masses (14:0 to 26:6 LPC) were monitored and confirmed by the formation of a phosphocholine

fragment of 184 m/z (collision energy (CE) was 55V). Furthermore, the fatty acid composition of the LPCs was confirmed by the presence of the corresponding fatty acid fragment in a second MS-analysis.

LPC(24:1) production

1,2-dinervonoyl- phosphatidylcholine (PC(24:1/24:1)) was purchased from Avanti Polar Lipids (chloroform dissolved) and converted *in vitro* into lysophosphatidylcholine 24:1 (LPC(24:1)) by addition of $sPLA_2$. The chloroform was evaporated under a stream of nitrogen to yield 20 µmol of dried PC(24:1) after which it was sonicated in 1 ml PLA_2-buffer (10 mM TRIS-HCl, 100 mM NaCl, 2 mM $CaCl_2$ pH 7.4) containing 1% lipid-free BSA, to form liposomes. Incubation with 2 Units pancreas-$sPLA_2$ for 20 hrs at ambient temperature yielded an 80-90% conversion of PC(24:1/24:1) into LPC(24:1). The conversion was monitored by mass spectrometry. Subsequently, proteins (BSA and PLA_2) were removed by a Bligh & Dyer lipid extraction.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical significance for all animal experiments in which tumor volumes were assessed was determined by one-way ANOVA with Tukey correction for multiple testing. A value of $P<0.05$ was considered to be statistically significant. All other data was analyzed using a two-tailed Students T-test. A P value <0.05 was considered statistically significant. A Levene's test was used to determine if variance between groups was comparable. Animals were excluded from analysis if two or more tumor measurements were significant outliners compared to the rest using the Grubbs outlier test (alfa: 0.05).

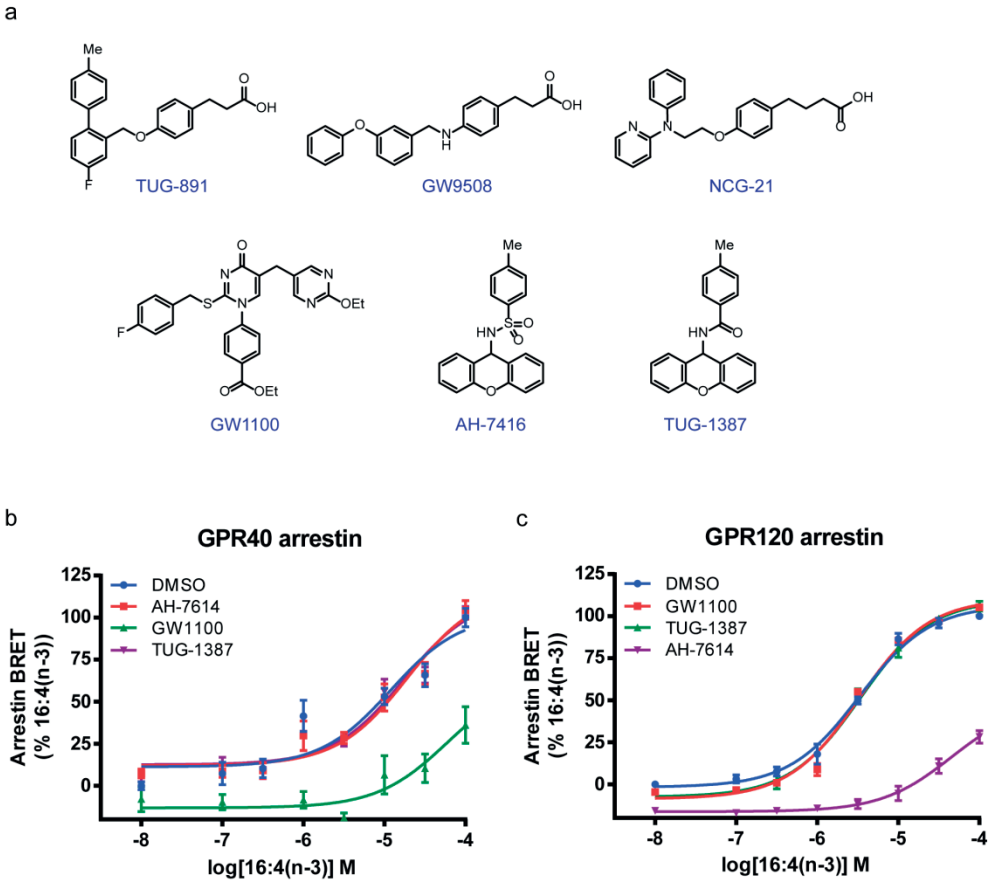
Funding

This research was supported by the Dutch Cancer Society (KWF, grant number: UU 2009-4534), Biotechnology and Biological Sciences Research Council (grant number BB/K019864/1), a University of Glasgow Leadership Fellowship (BDH) and Danish Council for Strategic Research (grant number 11-1161-96).

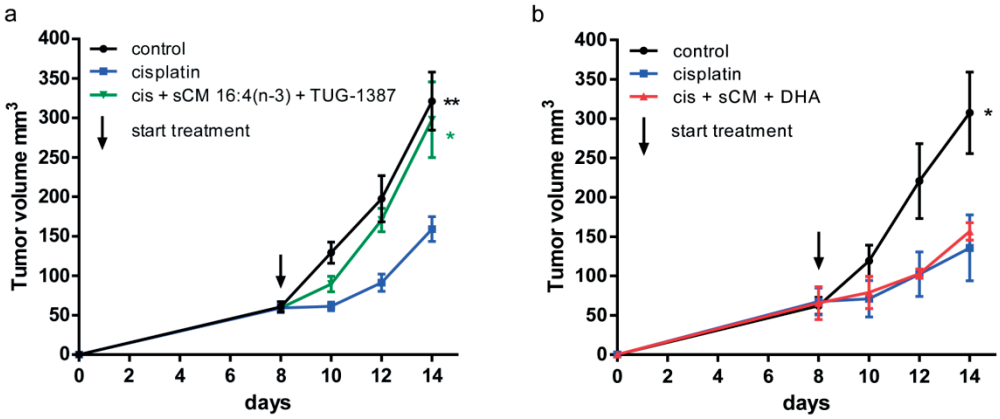
Supplementary information:

Compounds				
Agonists	***pEC_{50} GPR40***	***pEC_{50} GPR120***	***Formula***	***Ref.***
TUG-891	4.19	7.7	$C_{23}H_{21}FO_3$	10,11
GW9508	6.6-7.3	5.5	$C_{22}H_{21}NO_3$	11,12
NCG21	4.7	5.9	$C_{23}H_{23}N_2O_3$	11,13
Antagonists	***pIC_{50} GPR40***	***pIC_{50} GPR120***		
GW1100	6.0	<5.0	$C_{27}H_{25}FN_4O_4S$	12
AH-7614	<4.6	7.1	$C_{20}H_{17}NO_3S$	14
Control compound				
TUG-1387	-	-	$C_{21}H_{17}NO_2$	-

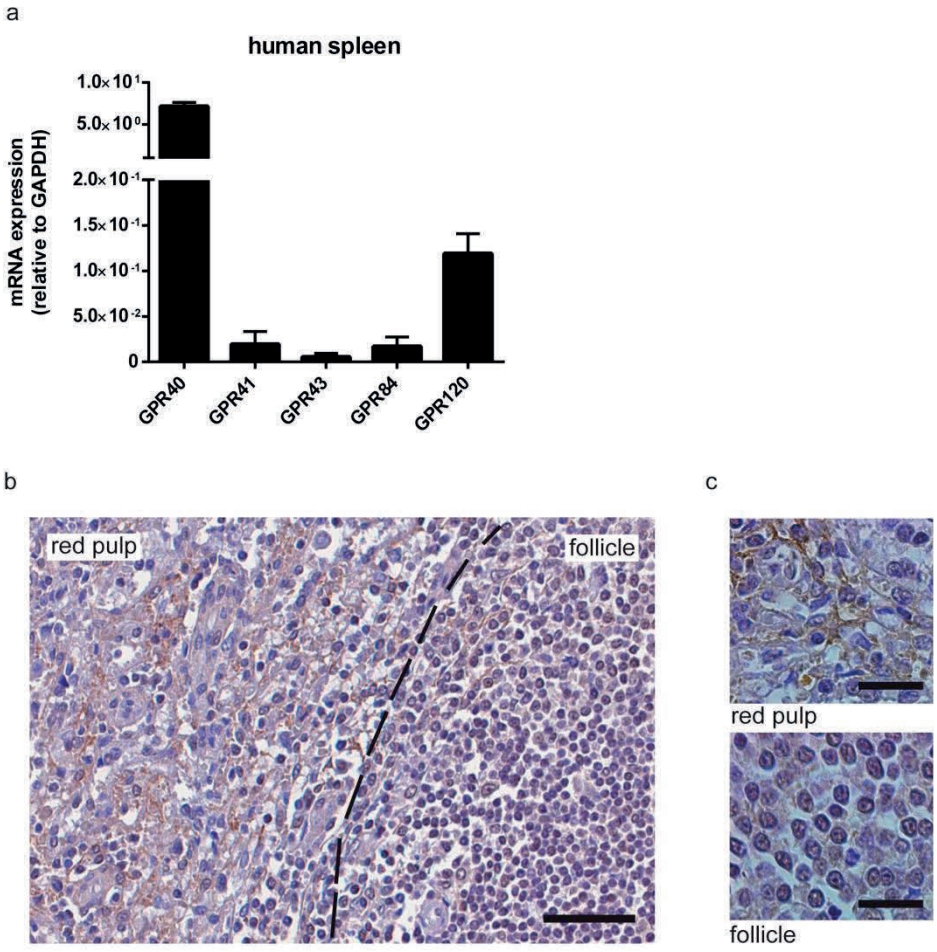
Supplemental table 1: GPR40 and GPR120 agonists and antagonists used in the study including their pEC_{50} or pIC_{50} on mouse GPR40 and GPR120, chemical formula and references.



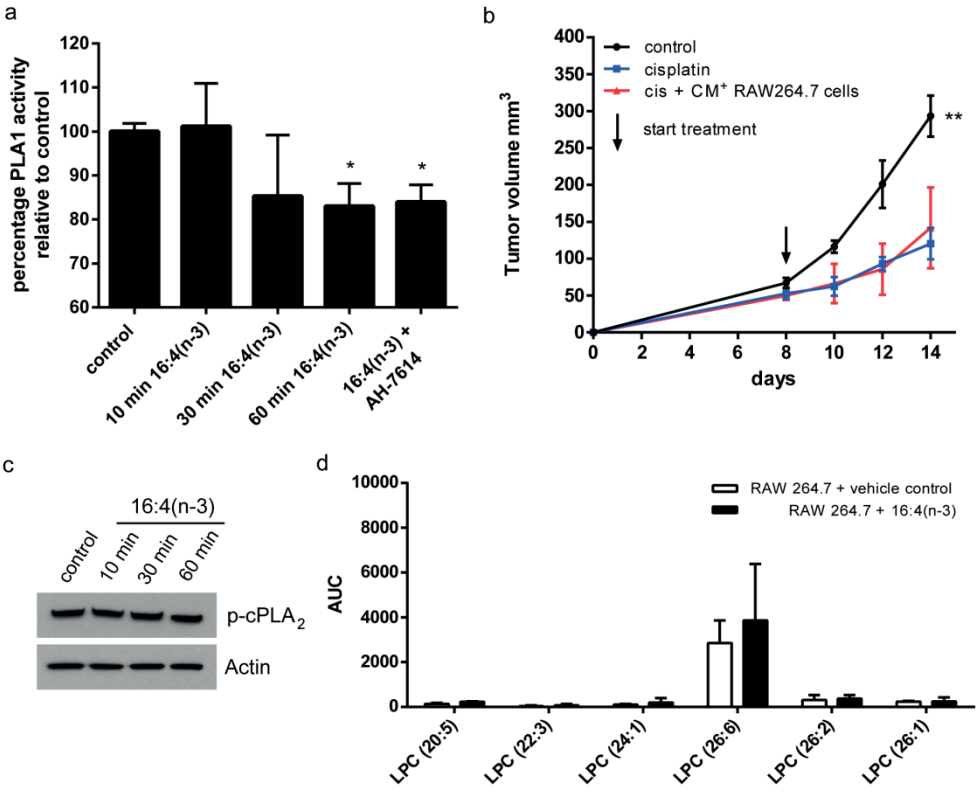
Supplemental figure 1: β-arrestin recruitment to GPR40 and GPR120 upon 16:4(n-3) stimulation
Panel a shows the chemical structures of the agonists and antagonists used in the study. β-arrestin2 is recruited to both GPR40 and GPR120 receptor after stimulation with increasing concentrations of 16:4(n-3) (panel b and c respectively). The recruitment to GPR40 was blocked by addition of a GPR40 selective antagonist (GW1100) but not by the GPR120 selective antagonist (AH-7614). Recruitment of β-arrestin2 to GPR120 upon stimulation with 16:4(n-3) can be inhibited by AH-7614 but not GW1100. The structurally related but inactive compound TUG-1387 does not inhibit recruitment to either GPR40 or GPR120. AH-7614, GW1100 and TUG-1387 were all used at a 10 μM concentration. Graphs in panel b and c show grouped results of three independent experiments with similar outcome. Data are represented as mean ± SEM.



Supplemental figure 2: sCM derived from DHA treated splenocytes does not induce chemotherapy resistance. Co-administration of cisplatin with sCM derived from splenocytes incubated with TUG-1387 does not induce chemoresistance in BALB/c mice (panel a). BALB/c mice treated with cisplatin and sCM derived from splenocytes incubated with DHA (100 μM) did not induce chemotherapy resistance (panel b). Graphs show results of two independent experiments (n=8 per group). Data are represented as mean ± SEM. Statistical significance was determined by one-way ANOVA. All compared to cisplatin alone * $P<0.05$, ** $P<0.01$.



Supplemental figure 3: human splenocytes express GPR120. Relative expression of GPR40, GPR41, GPR43, GPR84 and GPR120 in human spleen was determined by q-PCR. GPR40 and GPR120 are expressed by human splenocytes (panel a). Immunohistochemical staining of GPR120 on human splenic tissue reveals GPR120 positive cells in the red pulp of the spleen (panel b and c, scale bar in b:100 μm, scale bars in c: 20 μm). Graph in panel a shows result of two independent experiments. Data are represented as mean ± SEM.



Supplemental figure 4: RAW264.7 cells cannot induce chemotherapy resistance in response to 16:4(n-3). Phospholipase A1 activity is not enhanced in splenic macrophages derived from BALB/c mice stimulated for the indicated time points with 16:4(n-3) or 16:4(n-3) and AH-7614 (10 min pre-incubation of 10 μM AH-7614 before addition of 16:4(n-3)) (panel a). CM prepared from RAW264.7 cells stimulated with 16:4(n-3) does not induce chemoresistance in BALB/c mice (panel b). No increase in phospho-cPLA2 was seen in a western blot analysis of RAW264.7 cells stimulated with 16:4(n-3) (25 nM) for indicated time points (panel c). LPC measurements in the CM from RAW264.7 cells incubated with either vehicle control or 16:4(n-3) showed overall low LPC levels and no significant changes between control and 16:4(n-3) stimulation (panel d). Graphs show results of two independent experiments (panel a and d n=4 per group, panel b n=8 per group). Data are represented as mean ± SEM. Statistical significance was determined by two-tailed Students T-test (panel a, d) or one-way ANOVA (panel b). Panel a and d: all compared to vehicle control, panel b: all compared to cisplatin alone * $P<0.05$ ** $P<0.01$.

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CHAPTER 4


HEMA
KIND
Vloeibare Visolie
Omega-3-6-9
opgroeiende en actieve kinderen;
voor betere leerprestaties.
TOBIS
Omega-
Visolie
KINDER OMEGA
Vloeibaar
BANAAN / SINAASAPPEL
Optimax
aov
SOLIE FORTE
LIQUID

Increased Plasma Levels of Chemoresistance-Inducing Fatty Acid 16:4(n-3) After Consumption of Fish and Fish Oil

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+: these authors contributed equally to this work

JAMA Oncology **1**: 350-358 (2015)

Abstract

***Importance*: Our research group previously identified specific endogenous platinum-induced fatty acids (PIFAs) that, in nanomolar quantities, activate splenic macrophages leading to resistance to chemotherapy in mouse models. Fish oil was shown to contain the PIFA 16:4(n-3) (hexadeca-4,7,10,13-tetraenoic acid) and when administered to mice neutralized chemotherapy activity.**

***Objective*: Because patients with cancer frequently use fish oil supplements, we set out to determine exposure to 16:4(n-3) after intake of fish or fish oil.**

***Design, Setting, and Participants*: (1) In November 2011, 400 patients with cancer undergoing treatment at the University Medical Center Utrecht were surveyed to determine their use of fish oil supplements; 118 patients responded to the questionnaire (30%); (2) pharmacokinetic analysis of the 16:4(n-3) content of 6 fish oils and 4 fishes was carried out; (3) from April through November 2012, a healthy volunteer study was performed to determine 16:4(n-3) plasma levels after intake of 3 different brands of fish oil or 4 different fish species. Thirty healthy volunteers were randomly selected for the fish oil study; 20 were randomly selected for the fish study. These studies were supported by preclinical tumor experiments in mice to determine chemoresistance conducted between September 2011 and December 2012.**

***Primary Outcomes and Measures*: (1) Rate of use of fish oil supplements among patients undergoing cancer treatment at our institution; (2) levels of 16:4(n-3) present in 3 brands of fish oil and 4 species of fish; and (3) plasma levels of 16:4(n-3) present in healthy volunteers after consuming fish oil or fish.**

***Results*: Eleven percent of respondents reported using omega-3 supplements. All fish oils tested contained relevant amounts of 16:4(n-3), from 0.2 to 5.7 μM. Mouse experiments showed that addition of 1 μL of fish oil to cisplatin was sufficient to induce chemoresistance, treatment having no impact on the growth rate of tumors compared with vehicle-treated controls (estimated tumor volume difference, 44.1 mm^3; $P > .99$). When the recommended daily amount of 10 mL of fish oil was administered to healthy volunteers, rises in plasma 16:4(n-3) levels were observed, reaching up to 20 times the baseline levels. Herring and mackerel contained high levels of 16:4(n-3) in contrast to salmon and tuna. Consumption of fish with high levels of 16:4(n-3) also resulted in elevated plasma levels of 16:4(n-3).**

***Conclusions and Relevance*: All tested fish oils and herring and mackerel fishes contained relevant levels of fatty acid 16:4(n-3), a fatty acid with chemotherapy-negating effects in preclinical models. After ingestion of these fish oils or fishes, 16:4(n-3) was rapidly taken up in the plasma of human volunteers. Until further data become available, fish oil and fish containing high levels of 16:4(n-3) may best be avoided on the days surrounding chemotherapy.**

Introduction

With the intention to influence and improve their health status, patients with cancer often adopt lifestyle changes[1]. Burstein *et al* documented a 3-fold increase in food supplement use after people were diagnosed with cancer[2]. However, there is a growing concern that simultaneous use of supplements and anticancer drugs may negatively influence treatment outcome.

Omega-3 fatty acids are popular supplements estimated to be used by 20% of US patients with cancer, most often in the form of fish oil[3]. Fish oil is a highly complex, nonstandardized mixture of fatty acids produced from various fish species. In general, product information is limited to total omega-3 content and the concentrations of the 2 most abundant fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Although the US Food and Drug Administration (FDA) has advised not to use more than 3 g of EPA and DHA per day, 2 g of which may be from a dietary supplement[4], omega-3 supplement production does not require FDA review or approval. As such, fish oil content may vary between supplements and sometimes even from batch to batch. It remains unclear which other biologically active molecules are present in the mixtures.

Previous research from our laboratory identified 2 fatty acids, 12-S-HHT (12-S-hydroxy-5,8,10-heptadecatrienoic acid) and 16:4(n-3) (hexadeca-4,7,10,13-tetraenoic acid), that in minute quantities induced resistance to chemotherapy in mice[5,6]. Human and murine mesenchymal stem cells secrete these fatty acids upon platinum stimulation, hence the term platinum-induced fatty acids (PIFAs). Recently, our group has shown that 1 of these PIFAs, 12-S-HHT, can stimulate $F4/80^{+}/CD11b^{low}$ macrophages residing in the spleen to produce lysophospholipids, which are responsible for this systemic form of resistance[6]. Interestingly, relevant levels of PIFA 16:4(n-3), but not 12-S-HHT, were detected in fish oil, and the antitumor activity of chemotherapy was neutralized by fish oil in various mouse models[5]. These unexpected findings stirred up a lively discussion about the benefits and risks of fish oil supplementation in patients undergoing active cancer treatment[7].

To address these concerns we analyzed PIFA content in a panel of fish oils and found relevant levels of 16:4(n-3) in all tested fish oils. To assess the consequences of our preclinical findings for patients with cancer taking fish oil, a healthy volunteer study was designed. Different doses of fish oil were administered to volunteers followed by repeated measurements of 16:4(n-3) in plasma. Finally, we explored 16:4(n-3) levels in fish species and determined the effects of fish consumption on 16:4(n-3) pharmacokinetics and supported our findings with preclinical studies.

Results

Cisplatin-Neutralizing Activity in Mice of Fish Oil Containing 16:4(n-3)

To determine how prevalent PIFAs are in fish oil products, we analyzed 6 commercially available fish oils for 16:4(n-3) and 12-S-HHT content. No 12-S-HHT could be detected in the fish oils, but all fish oils contained substantial levels of 16:4(n-3), with averages ranging from 0.2 to 5.7 µM (Table). We therefore focused on 16:4(n-3). Next, purified 16:4(n-3) was tested in mice with subcutaneous C26 tumors. When 6 mg/kg of cisplatin was administered intraperitoneally, tumor growth was suppressed. However, the combination of cisplatin with as little as 2.5 pmol of orally administered 16:4(n-3) induced virtually complete chemoresistance. For vehicle vs cisplatin, there was an estimated tumor volume difference of 142.4 mm^3 (P = 0.001); for cisplatin vs cisplatin plus 16:4(n-3), there was an estimated tumor volume difference of 95.5 mm^3 (P = 0.04) (Figure 1a). One hundred microliters of fish oil A, which contained high levels of 16:4(n-3), also induced resistance to cisplatin therapy (t = −2.79; P = 0.01) (Figure 1b), whereas fish oil monotherapy did not alter tumor volume compared with vehicle-treated mice. The addition of fish oil also reduced the activity of other chemotherapeutic agents compared with the agents alone. For irinotecan, there was an estimated tumor volume difference of 166.3 mm^3 (95% CI, 13.31-319.36 mm^3) (P =0 .03); for oxaliplatin, there was an estimated tumor volume difference of 147.1 mm^3; 95% CI, 0.07-294.11) (P = 0.05) (Figure 1c).

Table. Levels of 16:4(n-3) Found in Tested Fish Oils

Fish Oil[a]	Level of 16:4(n-3), Mean (SD), µM	Source
A	5.7 (0.7)	Anchovy and/or sardine
B	2.7 (1.7)	Anchovy and/or sardine
C	4.1 (3.0)	Anchovy and/or sardine
D	1.0 (0.1)	Unknown
E	0.4 (0.1)	Sand eel
F	0.2 (0.03)	Unknown

Abbreviation: 16:4(n-3), hexadeca-4,7,10,13-tetraenoic acid.

[a] Three batches of each of 6 brands of fish oil were analyzed on independent occasions by triple-quadruple mass spectrometry.

To determine the relationship between 16:4(n-3) levels and chemoresistance, a dose response study was performed. Using fish oil A, 100 µL, 10 µL, 1 µL, or 0.1 µL was diluted with sunflower oil to a total volume of 100 µL. The sunflower oil did not contain any 16:4(n-3) and did not affect chemotherapy activity. For vehicle vs cisplatin plus sunflower oil, there was an estimated tumor volume difference of 82.5 mm^3 (P = 0.03) (Figure 1d). Tumors in mice treated with cisplatin plus 1 µL of fish oil A did not grow significantly differently from vehicle-treated tumors. This roughly corresponds to a dose of 3 mL for an average-sized patient, a dose well below the recommended daily amount of 10 mL (Figure 1d).

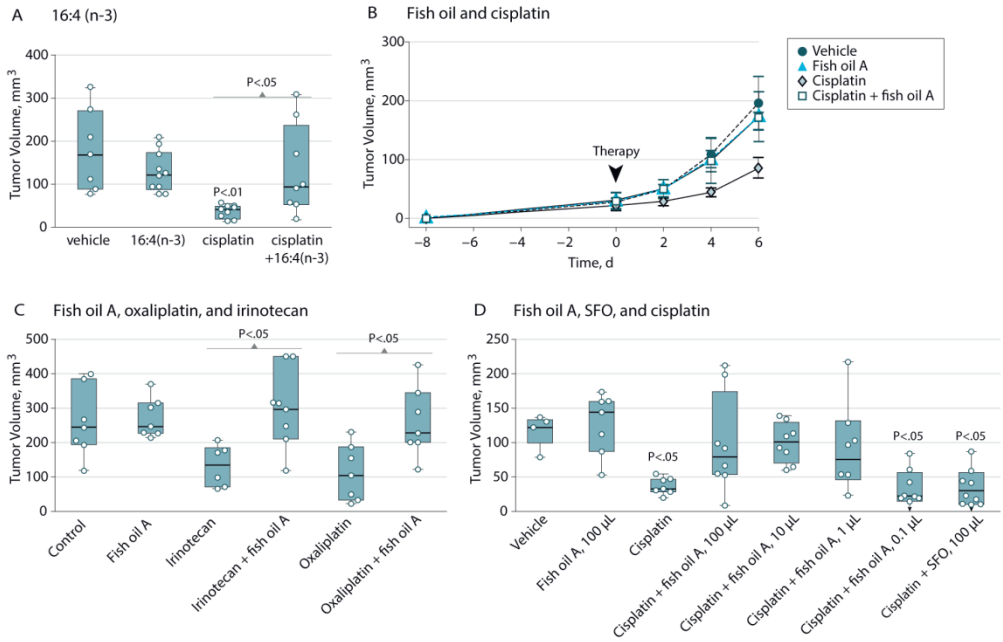

Figure 1: fish oil effects on cisplatin activity in mice. A, Dose of 2.5 pmol of 16:4(n-3) (hexadeca-4,7,10,13-tetraenoic acid) was administered to tumor-bearing mice alone or combined with cisplatin; all depicted tumor volumes were measured on day 4 after therapy. B, Fish oil A (100 µL) and cisplatin were administered to tumor-bearing mice. Tumor growth was monitored; volumes were log transformed; and differences between the intervention groups were calculated using repeated measurement analyses; error bars indicate standard error of the mean (SEM). C, Fish oil A (100 µL), oxaliplatin, and irinotecan were administered to tumor-bearing mice; all depicted tumor volumes were measured on day 6 after therapy. D, Tumor-bearing mice were treated with cisplatin, pure fish oil A (100 µL), or fish oil A diluted 1:10, 1:100, or 1:1000 in sunflower oil (SFO). Fish oil A (100 µL) and the combination of SFO (100 µL) and cisplatin were administered as controls; all depicted tumor volumes were measured on day 4 after therapy. All P values represent the comparison with vehicle unless otherwise indicated by group-spanning brackets. All whiskers and box plot points represent the distribution of the parameters at fixed times.

Patient Questionnaire on Use of Nutritional Supplements

Since all tested fish oils contained detectable levels of 16:4(n-3), our preclinical findings suggested a potential adverse effect for patients undergoing cancer treatment who were taking supplements containing fish oils. Of 118 patients who responded to the questionnaire, 35 (30%) reported regular use of nutritional supplements (eTable 2 in the Supplementals). Although this percentage is lower than reported in the United States, [2,3,8] still 11% of our responders (n = 13) used nutritional supplements containing omega-3 fatty acids, most often in the form of fish oil (38% of omega-3 users [n = 5]). Eleven of the respondents (84% of omega-3 users) reported continuing omega-3 intake during chemotherapy, and 6 of these reported notifying their physician.

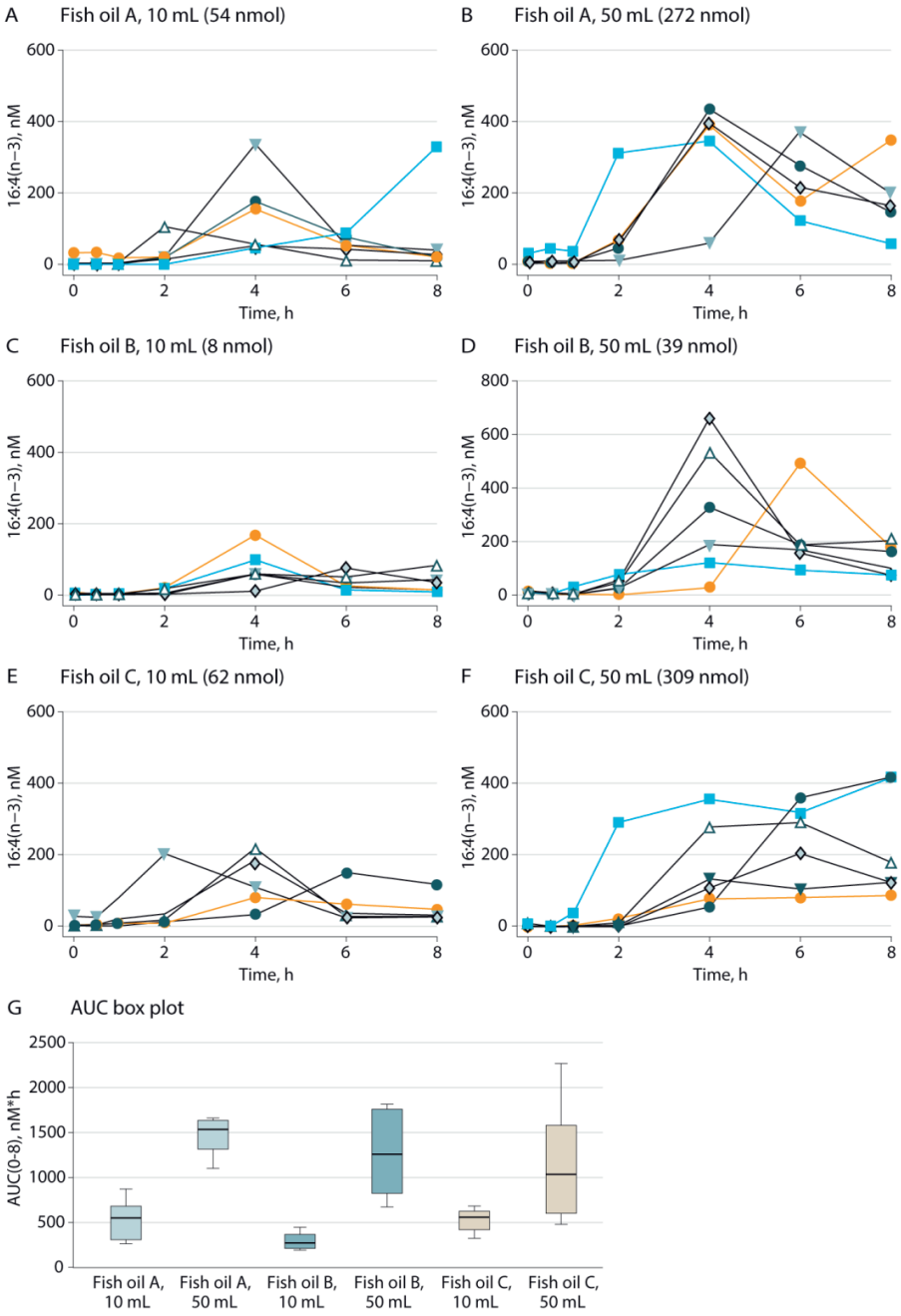

Figure 2: effects of fish oil ingestion on plasma levels of 16:4(n-3) in healthy human volunteers. Three fish oils were administered to 6 volunteers each in either 10- or 50-mL doses; each graph line represents 1 volunteer. Calculated quantities of ingested 16:4(n-3) are shown in parentheses in the panel labels. The average area under the curve (AUC) (0-8 hours) was calculated for each fish oil; whiskers and box plots represent the distribution of the parameters at fixed times.

Fish Oil Intake and Increased Plasma Levels of 16:4(n-3) in Healthy Volunteers

With fish oil use being so widespread, we aimed to study 16:4(n-3) uptake in the plasma of healthy volunteers after ingestion of either 10 mL (the recommended daily amount) or 50 mL of fish oil. Three different fish oils were used. Fish oil A contained 5.4 µM of 16:4(n-3); fish oil B, 0.8 µM; and fish oil C, 6.27 µM. Per fish oil, 1 batch was used for the study; therefore, the contents differ from the average values reported in the Table. In total, 30 volunteers were included, 53% men and 47% women. Although the fish oil was generally well tolerated, mild bloating and nausea were observed following intake of the 50-mL fish oil dose, a dose below that safely administered in a previous study[9]. The mean (SD) baseline level of 16:4(n-3) in plasma was 11.4 (8.1) nM. In all volunteers, plasma levels increased following fish oil intake, most reaching maximum levels 4 hours after consumption (Figure 2a-f). The recommended daily amount of 10 mL was sufficient to induce clear increases in 16:4(n-3) levels in plasma (Figure 2a, c, and e). An almost complete normalization was observed 8 hours after the 10-mL fish oil dose intake, whereas a prolonged 16:4(n-3) level elevation was present after the 50-mL dose (Figure 2b, d, and f). For all fish oils, the magnitude of the plasma peak was discrepantly high compared with the calculated 16:4(n-3) intake. For instance, 50 mL of fish oil A contained 272 nmol of 16:4(n-3), whereas the plasma levels after ingestion of fish oil A rose to 400 nM. Furthermore, although fish oil B contained considerably lower 16:4(n-3) levels than fish oils A and C, plasma level elevations and areas under the curve were comparable (Figure 2g). This suggests that other fatty acids in fish oil are metabolized to 16:4(n-3) in the body.

Other Fatty Acids in Fish Oil Metabolized to 16:4(n-3) in Mice

Next, we aimed to examine whether metabolism of fatty acids into 16:4(n-3) can take place. Therefore, 100 µL of fish oil A or its purified components 16:4(n-3) or EPA were administered to mice. Oral administration of 100 µL of fish oil A containing 543 pmol of 16:4(n-3) induced a rapid rise in 16:4(n-3) plasma levels in mice up to 1350 nM, which is higher than expected based on an average plasma volume of 1.5 mL. When lower amounts of fish oil were administered, plasma levels of 16:4(n-3) decreased accordingly (Figure 3a). Following administration of 1 µL of fish oil, 16:4(n-3) levels were too close to the detection limit of the assay to draw conclusions.

Purified components of 100 µL of fish oil were administered to mice in a total volume of 100 µL; 543 pmol of purified 16:4(n-3) induced a trend toward a modest peak in 16:4(n-3) plasma levels (Figure 3b). The most abundant fatty acid in fish oil is EPA; 16:4(n-3) plasma levels after ingestion of 69 µmol of EPA, the amount present in 100 µL of fish oil A, remained lower than after fish oil ingestion (Figure 3c). In addition, 69 µmol of purified EPA was able to neutralize cisplatin effects in mice. For cisplatin vs vehicle, there was an

estimated tumor volume difference of 137.2 mm^3 (95% CI, 17.49-256.87 mm^3; $P = 0.02$); for cisplatin plus EPA vs vehicle, there was an estimated tumor volume difference of 51.7 mm^3 (95% CI, −67.97 to 171.41; $P = 0.64$) (Figure 3d). Since the purified EPA was found to contain 213.4 nmol/L of 16:4(n-3) when analyzed by triple-quadruple mass spectrometry, which theoretically corresponds to 17.8 pmol of 16:4(n-3) in the administered dose of EPA, we cannot distinguish between metabolization of EPA into 16:4(n-3) and a direct effect of 16:4(n-3) on chemoresistance.

However, since 1 µL of fish oil A was sufficient to induce chemoresistance in mice, the purified EPA content of 1 µL of fish oil (690 nmol) was administered together with cisplatin. This low EPA dose, containing less than 0.2 pmol of 16:4(n-3), did not influence chemotherapy activity. Both cisplatin and cisplatin plus low-dose EPA significantly suppressed tumor growth ($t = 2.48$; $P = 0.02$) (Figure 3e). Thus, in clinically relevant amounts, 16:4(n-3) was responsible for chemoresistance, but EPA was not. However, we cannot rule out the possibility that metabolism of the large amount of unspecified fatty acids in fish oil may contribute to the observed plasma levels of 16:4(n-3).

Consumption of Fish Containing 16:4(n-3) and Plasma Level Elevations in Healthy Volunteers

We questioned whether consumption of fish, rather than fish oil, would have similar effects on 16:4(n-3) plasma levels. First, the 16:4(n-3) content was analyzed in fish homogenates purchased on 3 independent occasions. Smoked mackerel and cured herring contained high amounts of 16:4(n-3), up to 1200 nM/100 g (eTable 3 in the Supplementals). We next measured 16:4(n-3) levels in the plasma of healthy human volunteers after fish ingestion. We determined the exact amount of 16:4(n-3) present in the fish used for the study, which differs from the average 16:4(n-3) content of these fishes mentioned in eTable 3 in the Supplement. In total, 20 volunteers were included, 12 men and 8 women. Intake of 100 g of mackerel or herring resulted in substantial plasma elevations (Figure 4a, b, and e). Importantly, the fish used in the clinical study contained very high levels of 16:4(n-3). In contrast, intake of 100 g of salmon resulted in a small, short-lived peak, whereas consumption of tuna, which contained the lowest levels of 16:4(n-3), did not affect plasma levels (Figure 4c-e). Thus, consumption of fish rich in 16:4(n-3) enhanced plasma levels of this PIFA.

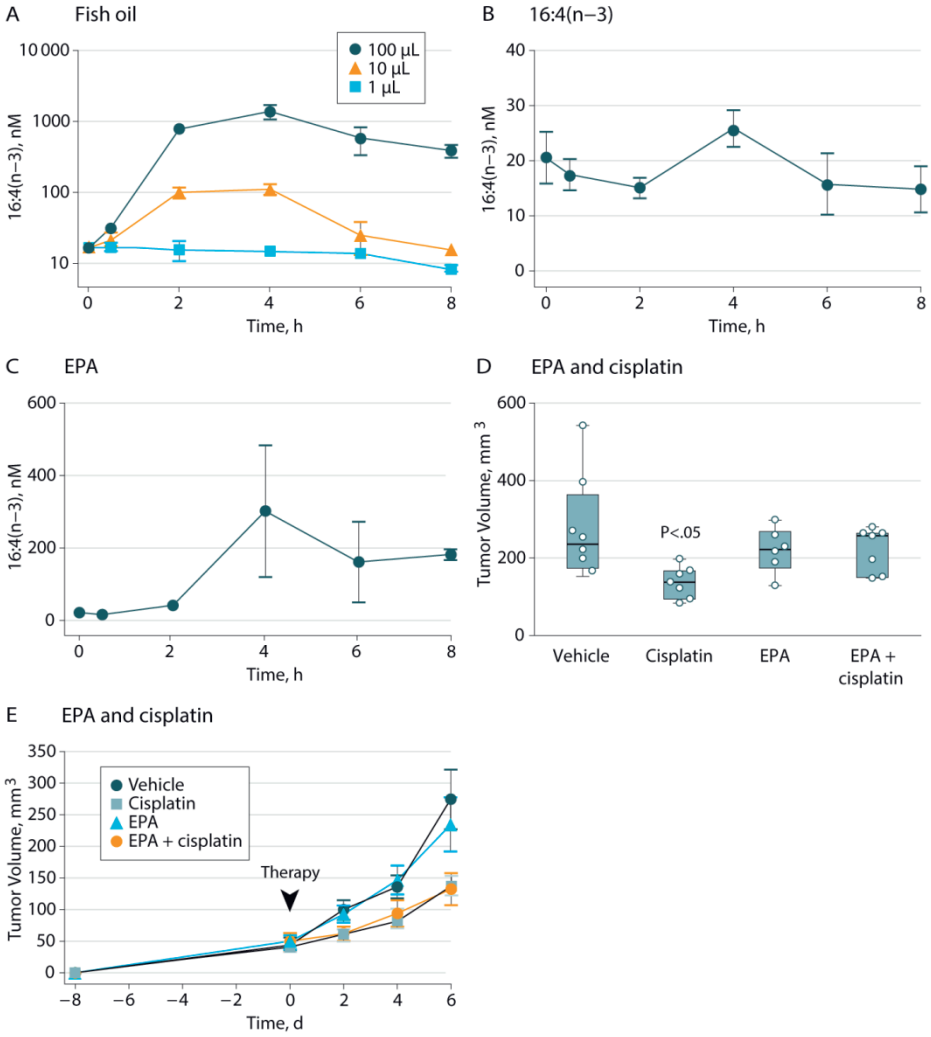

Figure 3: metabolization in mice of other fatty acids in fish oil to 16:4(n-3). A, Undifferentiated fish oil A administered to mice; plasma withdrawn and evaluated for 16:4(n-3) levels. B, Dose of 543 pmol of 16:4(n-3) administered to mice; plasma withdrawn and evaluated for 16:4(n-3) levels. C, Dose of 69 µmol of EPA administered to mice; plasma withdrawn and evaluated for 16:4(n-3) levels. D, Dose of 69 µmol of EPA and cisplatin administered to tumor-bearing mice and tumor volume plotted; whiskers and box plots represent the distribution of the parameters at fixed times (P value represents comparison with vehicle). E, Dose of 690 nmol of EPA and cisplatin administered to tumor-bearing mice; tumor growth monitored. Error bars in panels A, B, C, and E represent standard error of the mean (SEM).

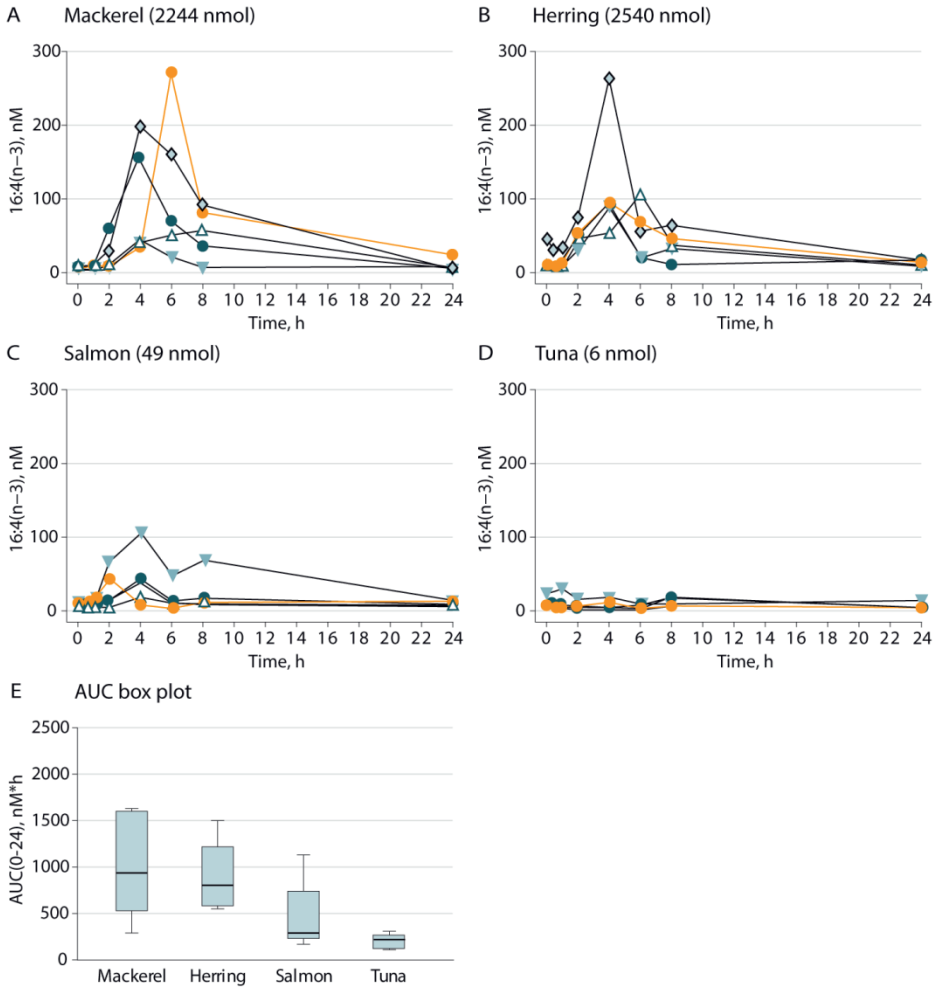

Figure 4: effects of consumption of fish containing 16:4(n-3) on plasma levels of 16:4(n-3) in healthy human volunteers. As can be seen by the quantities of 16:4(n-3) contained in the ingested fish portions (shown in parentheses in the panel labels), mackerel and herring contained high levels, and salmon and tuna contained low levels. Each fish portion of 100 g was ingested by 5 volunteers; plasma samples were then taken, evaluated, and plotted. In the box plot, the average area under the curve (AUC) (0-24 hours) was calculated; whiskers and box plots represent the distribution of the parameters at fixed times.

Discussion

Herein we show that fish oil contains substantial levels of 16:4(n-3), a fatty acid with potent chemotherapy-negating effects in preclinical models, and that intake of low doses of fish oil interferes with chemotherapy activity in mice. Ingestion of the recommended daily amount of fish oil by healthy volunteers rapidly increased 16:4(n-3) plasma levels.

Since low concentrations of 16:4(n-3) were still active in mice, and since 11% of patients undergoing cancer therapy in our center used omega-3 supplements (and reports in the literature indicate even more frequent use), these findings may have important clinical implications.

Extending the fish oil findings to fish, 100 g of herring and mackerel increased 16:4(n-3) plasma levels in contrast to consumption of tuna, which contained very little 16:4(n-3). Salmon intake resulted in a short-lived 16:4(n-3) peak. Intriguingly, plasma level increase in 16:4(n-3) after fish consumption were mild compared with those occurring after fish oil ingestion, although the portions of mackerel and herring contained higher levels of 16:4(n-3) than 50 mL of fish oil produced from sardines and anchovies. Pharmacokinetic experiments in mice cannot rule out the possibility that a large part of the 16:4(n-3) plasma peak after fish oil intake was due to metabolism of unspecified components of fish oil. Apparently, this type of metabolism is much less abundant after fish consumption. Part of the 16:4(n-3) peak after fish oil consumption could be explained by EPA metabolism. Conversion of EPA to 16:4(n-3) by peroxisomal β-oxidation has been shown in human fibroblasts[10]. It is likely that 16:4(n-3) produced from EPA accumulates as further β-oxidation requires reduction and 2,4-dienoyl-coenzyme A reductase-catalyzed isomerization, which has been suggested to be rate limiting[11,12].

Our results add to the growing awareness that not all dietary supplements are beneficial or harmless: some may interfere with treatment outcome. A convincing example is the influence of St John's wort on the pharmacokinetics of a large number of drugs[12]. However, not all examples are so straightforward. In the 1980s and early 1990s, numerous retrospective and prospective studies showed a significant association between β-carotene in the diet and lower incidence of lung cancer, which was supported by preclinical studies from that period[13-15]. Subsequently, 2 large trials showed that β-carotene actually enhanced the number of lung cancer cases and overall mortality, reversing the opinion on these supplements[16,17]. Will fish oil be the new β-carotene? In cardiovascular disease, enthusiasm regarding omega-3 supplements is waning after 2 recent meta-analyses, representing tens of thousands of patients, showed no beneficial effects of these supplements[18,19]. The assessment of fish oil might be even more complex than that of β-carotene, since its composition is largely unknown and it is made from numerous fish species. We found large differences in 16:4(n-3) content between fish oils and between batches of the same fish oil.

The major limitation of the current study is the difficulty to directly translate our preclinical data to the clinic. This would require a controlled clinical trial to show that 16:4(n-3)-containing fish oil inactivates chemotherapy. We consider this unethical,

thereby limiting the information on the relationship of chemotherapy and fish oil to reports in the existing literature. Unfortunately, clinical studies are scarce, and their interpretation is difficult in the absence of a well-defined product. A recent study suggested that fish oil augmented the tolerability of cytotoxic therapy, but this study was underpowered, and statistical significance was not reached[20]. Only 1 trial, published in 2011, has addressed fish oil supplementation on chemotherapy outcome. Murphy *et al*[21] assigned patients with stage IIIB/IV non–small-cell lung cancer to platinum-based chemotherapy with or without fish oil supplementation. The product used contained 2.2 g of EPA and 500 mg of DHA per day. In contrast to our findings, this study showed a higher response rate and a trend toward a higher 1-year survival when fish oil was added as an adjuvant to chemotherapy[21]. However, patient numbers were limited to 15 patients treated in the combined treatment arm. To obtain more clinical evidence, retrospective analysis of fish oil use and response to chemotherapy in large cohort studies would be interesting. However, dose and type of fish oil were not registered in relation to chemotherapy in such cohorts, and it remains unclear whether fish oil use was continued during chemotherapy. This makes interpretation of PIFA exposure impossible.

Conclusions

Taken together, our findings are in line with a growing awareness of the biological activity of various fatty acids and their receptors and raise concern about the simultaneous use of chemotherapy and fish oil. Based on our findings, and until further data become available, we advise patients to temporarily avoid fish oil from the day before chemotherapy until the day thereafter. This advice was adopted by the Dutch Cancer Society, and by the Dutch National Working Group for Oncologic Dieticians[22]. Although further evidence on the relation between fish consumption and chemotherapy activity is desired, we would currently also recommend to avoid herring and mackerel in the 48 hours surrounding chemotherapy exposure.

Material and Methods

Volunteer study

The study was approved by the University Medical Center Utrecht ethics committee and conducted from April through November 2012 in accordance with the Declaration of Helsinki. Since this was an explorative study, no sample size calculation was performed. Volunteers were recruited via advertisements that had been approved by the ethics committee and were enrolled if they met the inclusion criteria (age ≥18 years, no comorbidities, no pregnancy or breast feeding, no allergy to fish or related products, no ingestion of fish oil products within 2 weeks before the start of the study, and no ingestion of fish within 1 week before the start of the study). After giving informed consent, volunteers ingested a single dose of 10 or 50 mL of 3 commercially available fish oils (n = 6 per group; total n = 30), and then blood samples were taken. Three fish oils were selected containing variable levels of 16:4(n-3), but all were produced from anchovies and sardines so that fatty acid intake was standardized as much as possible. Different vials from the same fish oil batch were pooled and analyzed for 16:4(n-3) content before intake. Two volunteers withdrew from the study owing to technical problems taking repeated blood samples. For the fish study, volunteers received 100 g of raw salmon or tuna, smoked mackerel, or cured herring (n = 5 per group, total n = 20; 11 volunteers in the fish study also took part in the fish oil study). Fish was obtained fresh from a local fish trader and kept on ice at all times. Samples of the fish were homogenized and analyzed, and 16:4(n-3) concentrations were determined. There was a 2-week follow-up period. Blood was collected from the volunteers in BD Vacutainer CPT cell preparation tubes with sodium citrate (Becton Dickinson) before any fish or fish oil ingestion and then one-half, 1, 2, 4, 6, and 8 hours after ingestion. In the fish study, blood was also collected 24 hours after ingestion, since normalization to baseline plasma levels of 16:4(n-3) was not reached within 8 hours in the fish oil study. After centrifugation (2300 g, 10 minutes), plasma was stored at −80°C until analysis.

Quantification of 16:4(n-3)

To 100 µL of plasma, 20 µL of internal standard d6-16:3(n-3) (7,8,10,11,13,14-^{2}H6)7,10,13-hexadecatrienoic acid) (0.078 µM) in 400 µL of methanol was added, followed by liquid-liquid extraction, reconstitution in 100 µL of chloroform, and solid-phase extraction (SPE) on Sep-Pak 500-mg aminopropyl (NH_2) SPE cartridges (Waters Corporation). The eluate containing fatty acids was evaporated and dissolved in 100 µL of acetonitrile. Chromatographic separation was achieved on a ACQUITY UPLC BEH C18 column (2.1 × 100 mm) (Waters), and detection by triple-quadruple mass spectrometry (TQMS Xevo; Waters). The data acquisition was performed with the electrospray source operating in negative mode with multiple reaction monitoring settings: 16:4(n-3): 247.23 > 203.23 and d6-16:3(n-3): 255.38 > 211.27. A calibration curve (0-500 nmol/L 16:4(n-3) in plasma) was

included in each analytical run. Samples containing high 16:4(n-3) levels were diluted and reanalyzed. This assay was extensively validated (eTable 1 in the Supplement)[23]: coefficients of variation at concentrations of 5 nmol/L and 80 nmol/L were 7.7% (n = 10) and 1.8% (n = 10), respectively. The 16:4(n-3) detection limit was 1.1 nmol/L; the limit of quantification was 3.0 nmol/L. The analysis was linear (<5% deviation) in plasma in concentration ranges of 0 to 500 nmol/L. For oil and fish quantification of 16:4(n-3), samples were diluted 1:50 in chloroform; 1.56 pmol of internal standard d6-C16:3(n-3) was added; and NH_2 SPE cleanup was performed. Accuracy was tested by adding different amounts of C16:4 to the oil samples. Recovery of the added amounts is detailed in eTable 1 in the Supplement. After establishment of this method, 6 randomly selected fish oils were analyzed for 16:4(n-3) content. For all fish oils, 3 independent batches were measured.

Mouse experiments

Mouse experiments were conducted as reported previously,[5] in agreement with Dutch Law on Animal Experiments and approved by the University Medical Center Utrecht Animal Care Ethics Committee. The C26 cells were maintained in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) containing fetal calf serum–penicillin-streptomycin; 1×10^6 cells were implanted subcutaneously into BALB/c mice. Tumor size was determined by calipers; volume was calculated $0.5 \times \text{length} \times \text{width}^2$. When tumors reached a volume of 50 to 100 mm^3, therapy was started using 6 mg/kg of cisplatin, 10 mg/kg of oxaliplatin, or 100 mg/kg of irinotecan intraperitoneally (PCH Pharmachemie). Commercially available fish oil, obtained via internet stores; pure sunflower oil (Sigma-Aldrich); EPA (Cayman Chemicals); 16:4(n-3); or vehicle were orally administered by gavage. The 16:4(n-3) was purified from algae[24]. In preparation for pharmacokinetic analysis, cardiac punctures were performed, and blood was collected in EDTA (ethylenediaminetetraacetic acid) tubes, centrifuged (1800g, 10 minutes), and stored at −80°C until analysis. Experiments in which tumors were measured and plotted were performed at least twice with at least 8 mice in each treatment group. For pharmacokinetic studies, at least 3 mice were used per time point per treatment group.

Questionnaire

An anonymous, voluntary, nonvalidated questionnaire was approved by the medical ethical authority and handed out to patients undergoing active cancer treatment who visited the medical oncology outpatient ward at University Medical Center Utrecht in November 2011. Our aim was to determine supplement use among the patients. Every patient undergoing active cancer treatment was eligible; 400 questionnaires were handed out, of which 118 were returned and analyzed (30% response rate).

Statistical analysis

Statistical analysis was performed using SPSS software, version 22 (IBM Corporation). Analysis of variance (ANOVA) was performed using the Tukey procedure to control the type I error rate, or a nonparametric Kruskal-Wallis test was performed followed by Mann-Whitney for pairwise comparisons. Bonferroni correction of *P* values for multiple testing was applied. For graphs depicting mouse tumor growth over time after therapy, volumes were log transformed, and differences between the intervention groups were calculated using repeated measurement analyses. All *P* values reported are 2-sided.

Supplementary Information

	Concentration in nM	**% C16:4(n-3) added**
Standard 7	31,25	
Standard 9	125,0	
Oil 1	19,2	
Oil 1 + standard 7	56,3	118,9
Oil 1 + standard 9	162,4	114,6
Oil 2	6,2	
Oil 2 + standard 7	39,2	105,7
Oil 2 + standard 9	120,3	91,3
Oil 3	3,5	
Oil 3 + standard 7	36,4	105,3
Oil 3 + standard 9	146,2	114,1
Oil 4	121,0	
Oil 4 + standard 7	164,2	138,2
Oil 4 + standard 9	264,2	114,5
Oil 5	196,4	
Oil 5 + standard 7	228,4	102,9
Oil 5 + standard 9	330,7	107,4
Average		111,3
Standard deviation		12,3

eTable 1: Accuracy of 16:4(n-3) measurements in fish oil was tested by adding different amounts of C16:4 to diluted oil samples with 1.56 pmol of internal standard d6-C16:3(n-3). Samples were subjected to normal phase SPE clean-up.

			n	%	95% CI	p*
General						
Response rate			118	30	-	
Gender	Male		59	52	-	0.45
	Female		49	44	-	
	Unknown		4	4	-	
Age	Mean (years)	58			55-60	
	SD	14				
Supplement use			**35**	**30**		
Gender	Male		11	31	-	0.06
	Female		22	63	-	
	Unknown		2	6	-	
Age	Mean (years)	58			53-62	0.99
	SD	13				
Type of supplements	Omega-3		13	37	-	
	Vitamins		28	80	-	
	Herbs		8	23	-	
	Accupuncture		4	11	-	
	Other		29	83	-	
Omega-3 use			**13**	**11**		
Gender	Male		4	31	-	0.23
	Female		8	61	-	
	Unknown		1	8	-	
Age	Mean (years)	54			45-63	0.35
	SD	14				
Omega-3 products used	Fish oil		5	38	-	
	Cod liver oil		2	15	-	
	Margarine		3	23	-	
	Not specified		3	23	-	

		n	%	95% CI	p*
Frequency of use	Daily	9	69	-	
	Multiple times per week	3	23	-	
	Weekly	0	0	-	
	Monthly	0	0	-	
	Unknown	1	8	-	
Physician awareness	Yes	6	46	-	
	No	7	54	-	
Use during therapy	Yes	11	84	-	
	No	1	8	-	
	Unknown	1	8	-	

eTable 2: 400 anonymous questionnaires regarding the use of food supplements were handed out to patients treated with chemotherapy at the department of Medical Oncology at the UMC Utrecht. Results were analyzed and significance was calculated.

Fish species	**16:4(n-3) nmol per 100 grams Mean ± SD**
Mackerel	1224 ± 895
Herring	1244 ± 1128
Salmon	24 ± 22
Tuna	6 ± 4

eTable 3: A panel of commonly consumed fishes were homogenized and 16:4(n-3) content was measured on three independent occasions. Mean 16:4(n-3) levels and standard deviations are shown.

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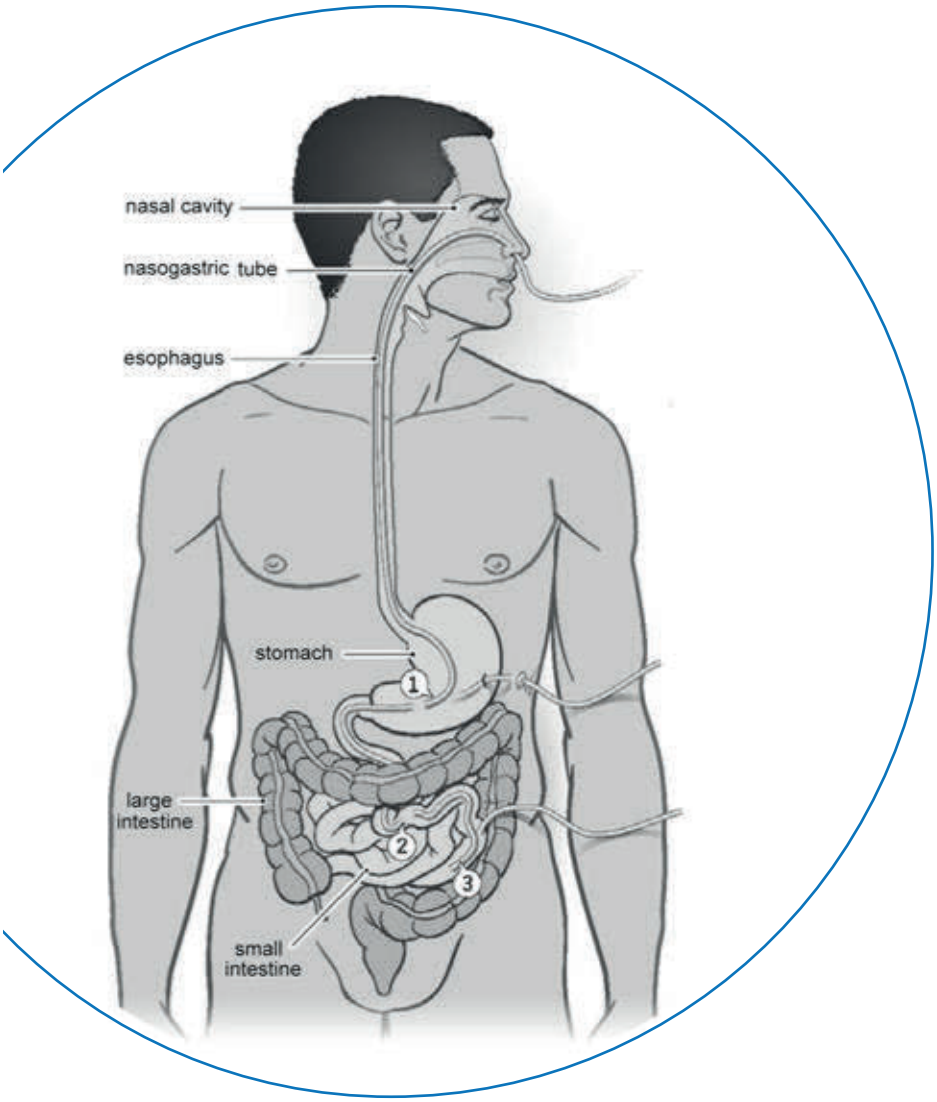
Appendix


nasal cavity
nasogastric tube
esophagus
stomach
1
large intestine
2
3
small intestine

Screening of (par)enteral nutrition for presence of 16:4(n-3)

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Abstract

Cancer patients often experience significant weight loss. Cancer-associated weight loss may hamper continued effective anti-cancer treatments and therefore undernourished cancer patients have less favorable outcomes compared to well-nourished patients. To prevent malnutrition and cachexia additional nutrients can be administered via different routes. Tube feeding (enteral nutrition) or intravenous injection (parenteral nutrition) of essential nutrients can aid help. Recently we showed that fish oil contains 16:4(n-3), a platinum-induced fatty acid that induces resistance against a range of DNA-damaging chemotherapeutics. A healthy volunteers study showed that ingestion of the daily recommended dose of fish oil can lead to increased plasma levels of 16:4(n-3) and that these levels are within the range found to induce chemoresistance in mice. In the present study we measured the 16:4(n-3) levels of various enteral and parenteral formulations used for the treatment of underweight cancer patients. 16:4(n-3) was present in five out of the nine measured nutrition preparations. However, the levels were, based on preclinical data, not likely to induce chemotherapy resistance. Interestingly, the parenteral nutrition containing the highest level of 16:4(n-3) (29,4 nmol/L) contained fish oil, further establishing fish oil as a natural source of 16:4(n-3). In summary, we have shown that enteral and parenteral nutritions are not a main source of 16:4(n-3) and there are no direct implications preventing its use for cancer patients.

Maintaining a healthy and well-balanced diet is of importance to cancer patients. Patients who prevent weight loss throughout anti-cancer therapy have favorable outcomes compared to patients who are undernourished or underweight[1,2]. Critically ill cancer patients are at risk of developing cachexia, a multifactorial syndrome characterized by massive loss of body fat, skeletal muscle deterioration and even systemic inflammation[3]. Cancer cachexia is most prevalent in patients suffering from pancreatic cancer or gastric cancer, whereas patients with non-Hodgkin's lymphoma, acute non-lymphocytic leukemia and sarcomas show the lowest frequency of cachexia[4]. When ingesting sufficient nutrition becomes difficult due to, for instance, nausea, treatment-induced loss of taste or surgeries involving the upper gastrointestinal tract other methods can be used to maintain a healthy body weight. These methods include enteral and parenteral nutrition. Enteral nutrition can be administered via a nasogastric tube running via the nasal cavity into the stomach. Alternatively a tube can be inserted directly into the stomach of small intestine via a small incision in the abdomen (gastric tube or jejunal tube respectively)[5].

Our recent findings that the platinum-inducible fatty acid (PIFA) 16:4(n-3) is also present in certain fish oils and fresh fatty fish[6] and that fish oil is sometimes added to (par)enteral nutrition led us to investigate the levels of 16:4(n-3) in various (par)enteral nutrition preparations. Since enteral and parenteral nutrition can be given to cancer patients who receive chemotherapy, these patients could potentially be at risk for developing 16:4(n-3)-mediated chemotherapy resistance if they would receive high levels of 16:4(n-3). We therefore measured nine different types of commonly used enteral and parenteral nutritions for the presence of 16:4(n-3) (Table 1). Four out of nine preparations contained no or extremely low levels of 16:4(n-3). Another four contained some 16:4(n-3) (between 5-10 nmol/L). Interestingly, these were all preparations containing extra fibers. From our preclinical data we know that the levels in these supplements are insufficient to induce chemotherapy resistance in mice. However one preparation contained relatively high levels of 16:4(n-3) (29,42 nmol/L) compared to others. This parenteral nutrition is composed of three compartments containing amino acids, glucose and a lipid emulsion. Interestingly, 15% of the lipids present in the lipid compartment are derived from fish oil.

The addition of fish oil to enteral and parenteral nutrition is based on the multiple studies that show that fish oil-derived omega-3 fatty acids like DHA or EPA can alleviate cachexia symptoms[7-10]. The anti-inflammatory properties of DHA and EPA have been shown to reduce cachexia-associated inflammation. In addition, body weight maintenance and preservation of muscle mass has also been attributed to omega-3 fatty acids DHA and EPA[11]. However, fish oil also contains other omega-3 fatty acids of which there beneficial effects have not been proven or even investigated. Depending on the source and production process, fish oil contains 2-20% of other omega-3 fatty acids than EPA or DHA.

16:4(n-3) is one of those other omega-3 fatty acids that can induce chemotherapy resistance at extremely low concentrations in various preclinical tumor models. The fraction of other omega-3 fatty acids is rarely specified on the product information which with new biological insights emerging should be reconsidered.

(Par)enteral nutrition brand and type	16:4(n-3) levels in nmol/L
Enteral nutrition	
Nutrison concentrated	0,58
Nutrini Peptisorb	0,10
Nutrison Advanced Protison	0,29
Nutrison Soya	0,27
Nutrison 800 Complete Multi Fibre	8,70
Nutrison Multi Fibre	10,81
Nutrison Energy Multi Fibre	5,32
Nutrison Protein Plus Multi Fibre	8,15
Parenteral nutrition	
Smofkabiven	29,42
12-S-HHT was not detectable in any of the samples	

Table 1: 16:4(n-3) levels in various enteral and parenteral nutritions. 16:4(n-3) levels were quantified by triple-quadruple mass spectrometry as described previously[6].

Potential health benefits from fish oil have been widely investigated in the context of various diseases including cardiovascular disease, cancer, Crohn's disease, ulcerative colitis, diabetes and even depression[12-18]. The outcomes of these studies vary significantly. One of the problems when interpreting the data from these studies is the variation in fish oil products used. While most studies document EPA and DHA levels in the supplements used, the fraction of other omega-3 fatty acids is often forgotten. Also the variation in dosage and contributions of other ingested foods or food supplements is often being ignored. Despite these difficulties, one of the largest misconceptions is that omega-3 fatty acids are being interpreted as being all the same. Various studies show that different omega-3 fatty acids can have different, sometimes even opposing functions, both *in vitro* and *in vivo* [16,19-23]. Therefore it would be naïve to presume that using a mixture of multiple omega-3-fatty acids like fish oil would give consistent results. In order to investigate the contribution of various omega-3 fatty acids a more systematic approach is desirable where patients are exposed to purified doses of particular fatty acids.

Taken together we have shown that various commonly used (par)enteral nutritions contain little or no 16:4(n-3). Interestingly, the parenteral nutrition containing the highest 16:4(n-3) levels contained fish oil. Our findings do not have immediate consequences for

the use of these par(enteral) nutritions in cancer patients since a small fraction of cancer patients receiving chemotherapy use parenteral nutrition[24], but it may be advisable to use purified lipids in enteral and parental nutrition to avoid unwarranted biological effects of (unknown) fatty acids present in fish oil.

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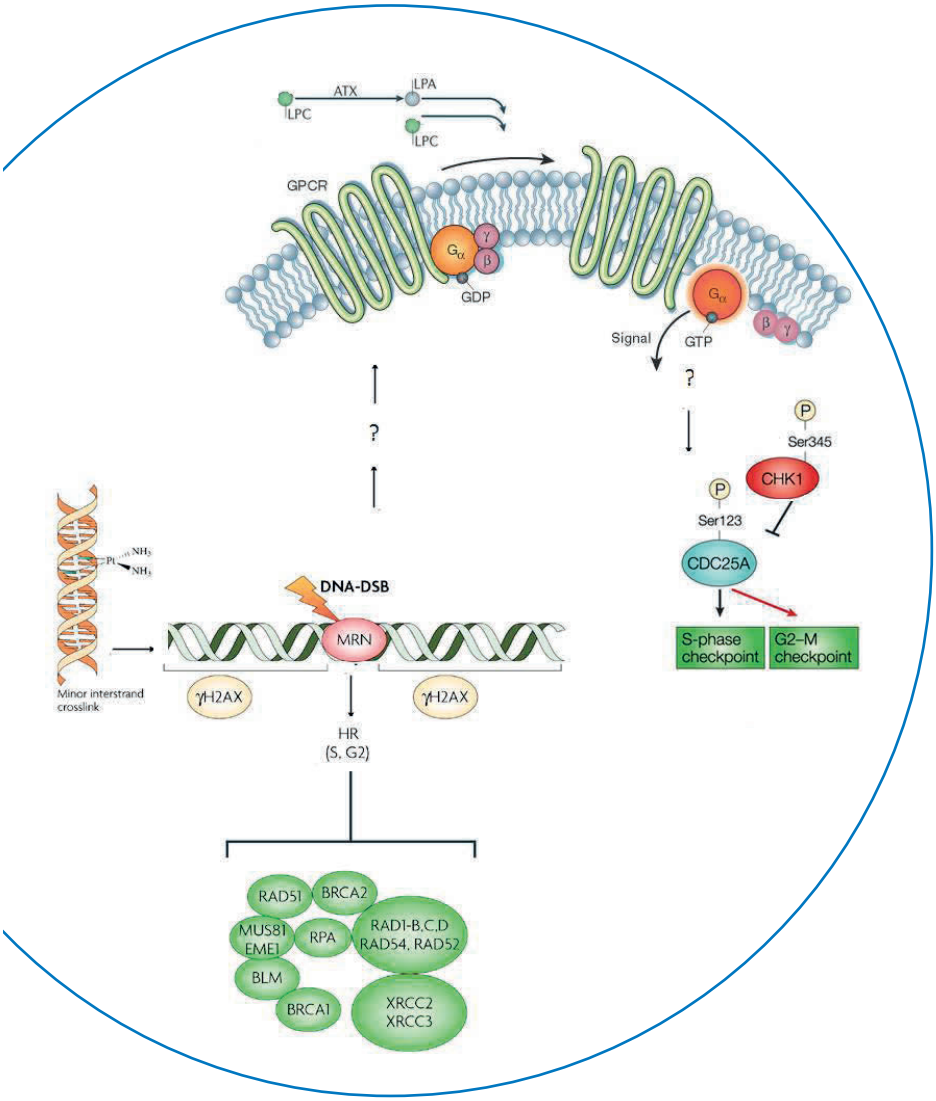
CHAPTER 5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MUS81 EME1
RPA
RAD1-B,C,D RAD54, RAD52
BLM
BRCA1
XRCC2 XRCC3

PIFA-mediated enhancement of DNA damage repair leads to chemotherapy resistance

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Abstract

We recently identified two platinum-induced fatty acids (PIFAs) that, at minute concentrations, induced resistance to chemotherapy. Here, we set out to determine how PIFAs 16:4(n-3) and 12-S-HHT protect tumors from chemotherapy-induced cell death. Co-administration of cisplatin and PIFAs to tumor-bearing mice reduced tumor DNA damage levels as measured by γH2AX, leading to decreased efficacy of cisplatin and continued tumor growth. The lack of differences in intratumoral cisplatin burden between cisplatin or cisplatin and PIFA-treated mice at early time points and the finding that the PIFAs can still induce chemoresistance even after early establishment of DNA damage are suggestive of altered DNA damage repair. Increased levels of phospho-CHK1 and RAD51 and decreased levels of CDC25a in tumors of mice that received cisplatin and PIFAs compared to cisplatin alone treatment support this hypothesis. In addition, we found that PIFAs were unable to induce resistance in mice bearing BRCA1$^{-/-}$;p53$^{-/-}$ tumors, a model for loss of homologous recombination. Partial restoration of homologous recombination by additional loss of 53BP1 was sufficient to reinstate PIFA-mediated chemoresistance. These results indicate that PIFAs protect tumors against DNA-damaging chemotherapeutics via early DNA damage repair.

Introduction

Despite the introduction of targeted therapies, the majority of patients with disseminated tumors are still treated with chemotherapy. Unfortunately, the response to therapy is often hampered by the development of resistance and continued tumor growth. Currently, platinum-based chemotherapeutics are an essential for the treatment of ovarian, colorectal, esophageal, lung and head-and-neck cancer, either as monotherapy or in combination with other anti-cancer agents. The binding of cisplatin to DNA and subsequent DNA damage are generally accepted as key events for its anti-tumor activity. Upon binding, cisplatin forms adducts leading to intrastrand crosslinks and, to a lower degree, interstrand crosslinks. Subsequently, these crosslinks inhibit DNA replication and transcription, leading to single and double strand DNA breaks (DSBs) and eventually cell death[1]. However, genomic integrity is vital to the survival of cells and various mechanisms protect and repair the DNA. The adducts formed by cisplatin can be removed from the cell by nucleotide excision repair (NER). Even after DSBs are established the cell repairs these lesions via either non-homologous end joining (NHEJ) or homologous recombination (HR)[1,2]. Which method of repair is being employed depends, amongst others on the stage of the cell cycle. Tumor cells can also use these mechanisms to generate resistance against platinum-analogues[2,3]. In addition to enhanced activation of DNA damage repair pathways other mechanisms of resistance include altered drug uptake or efflux, tolerance to the adducts, defects in downstream apoptotic signaling and sequestering of cisplatin by glutathione and metallothioneins[2].

Chemotherapy resistance does not always arise within tumor cells themselves, but can also be the results of signals from the tumor microenvironment[4]. Recently, we found that MSCs become activated by cisplatin to release specific fatty acids, 12-hydroxy-5,8,10-heptadecatrienoic acid (12-S-HHT) and hexadeca-4,7,10,13-tetraeonic acid (16:4(n-3)). These so-called platinum-induced fatty acids (PIFAs) interfere with the activity of a broad range of chemotherapeutic agents including cisplatin. Co-administration of PIFAs and cisplatin or other DNA-damaging chemotherapeutics can lead to chemotherapy resistance in various mouse tumor models[5]. The PIFAs do not induce resistance directly to the tumor cells but function via splenic macrophages. These macrophages become activated by the PIFAs and subsequently release lysophosphatidylcholines (LPCs), like LPC(24:1), which convers resistance to the tumor cells[6]. The mechanism of action through which PIFAs and LPCs prevent chemotherapy-induced tumor cell death is presently unclear. Here, we show that PIFAs specifically protect tumor cells against DNA damaging chemotherapeutic agents by inducing early DNA damage repair. These findings provide a novel link between systemic lipid signaling and protection of the integrity of cellular DNA.

Results

PIFAs and LPC(24:1) decrease γH2AX levels in tumors

To determine the kinetics of DNA damage *in vivo* we analyzed γH2AX (a marker for double strand DNA breaks) over time in tumors of mice treated with cisplatin or cisplatin and PIFAs. We found that cisplatin caused a rapid increase in γH2AX with a peak at 4 hours after treatment. Mice treated with cisplatin and PIFAs showed a similar increase at early time points (30 min and 1 hour), however after 4 hours γH2AX levels were significantly lower compared to cisplatin monotherapy (Figure 1a, c). Similar results were found when we co-administered cisplatin and LPC(24:1), one of the specific resistance-inducing molecules secreted by PIFA-stimulated splenic macrophages (Figure 1b, d).

Previously, we found that the intratumoral cisplatin burden did not differ 1 hour after cisplatin or cisplatin and PIFAs exposure, excluding the possibility of enhanced drug efflux or impaired drug influx as a mechanism of action by the PIFAs[6]. We also know from previous experiments that PIFAs are still able to induce chemoresistance when administered up to 3 hours after cisplatin[5]. However, as the time course experiment in figure 1 shows a substantial degree of DNA damage is already present at this time point, suggesting that the PIFAs have the ability to actively reduce γH2AX levels or induce DNA damage repair ultimately leading to chemoresistance and continued tumor growth. To show that the observed chemoresistance is indeed accompanied by a decrease in γH2AX-positive cells, cisplatin was administered to tumor-bearing mice followed 3 hours later by the PIFAs, and another 4 hours later, γH2AX levels were analyzed. PIFA administration was found to significantly reduce the number of γH2AX-positive cells (Figure 1c). Taken together, this substantiated the hypothesis that PIFAs induce DNA repair in these tumors.

PIFAs and LPC(24:1) induce early DNA-damage repair via CHK1

The two major pathways to repair double strand DNA breaks (DSBs) are homologous recombination (HR) and non-homologous end joining (NHEJ). During the process of NHEJ the damaged nucleotides are removed by DNA-PK and ligases join the DNA ends together, resulting in small deletions, frameshifts, insertions or translocations. NHEJ is a fast repair mechanism mainly employed in G1 phase of the cell cycle. HR on the other hand, is error free but slower. It requires the sister chromatid as a template for homology-driven repair and restoration of genomic integrity. HR requires a homologous DNA strand to facilitate repair, therefore it can only take place during G2/S phase of the cell cycle[7]. Since cisplatin-induced DSBs are predominantly repaired by homologous HR[8,9], we analyzed various proteins involved in HR-mediated DNA damage repair and found an upregulation of phosphorylated CHK1 (S345) and RAD51 in tumors of mice treated with cisplatin and PIFAs compared to the tumors of mice treated with cisplatin alone (Figure 2a).

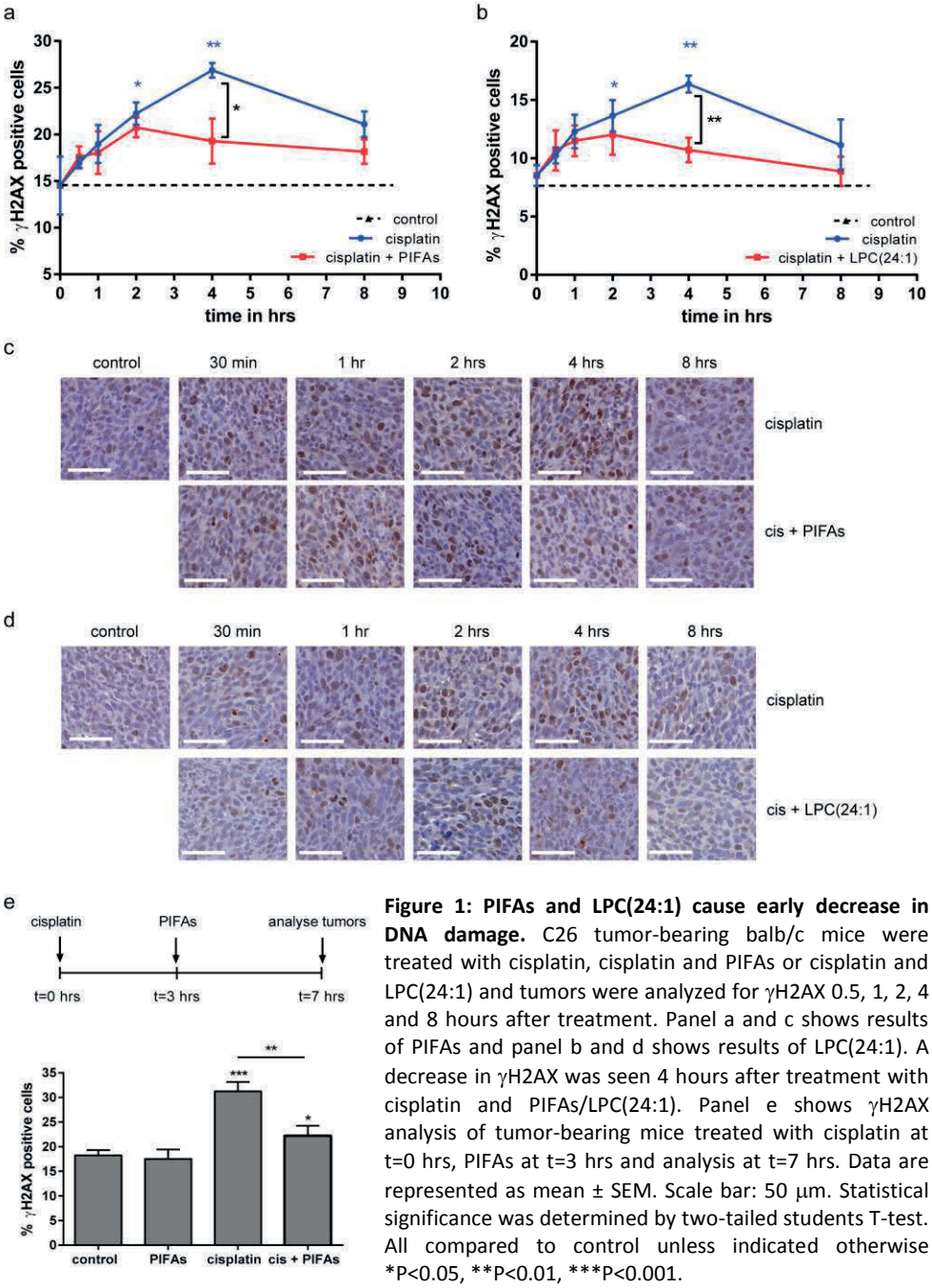


Figure 1: PIFAs and LPC(24:1) cause early decrease in DNA damage. C26 tumor-bearing balb/c mice were treated with cisplatin, cisplatin and PIFAs or cisplatin and LPC(24:1) and tumors were analyzed for γH2AX 0.5, 1, 2, 4 and 8 hours after treatment. Panel a and c shows results of PIFAs and panel b and d shows results of LPC(24:1). A decrease in γH2AX was seen 4 hours after treatment with cisplatin and PIFAs/LPC(24:1). Panel e shows γH2AX analysis of tumor-bearing mice treated with cisplatin at t=0 hrs, PIFAs at t=3 hrs and analysis at t=7 hrs. Data are represented as mean ± SEM. Scale bar: 50 μm. Statistical significance was determined by two-tailed students T-test. All compared to control unless indicated otherwise *P<0.05, **P<0.01, ***P<0.001.

In line with the upregulation of phospho-CHK1 we also found decreased CDC25a levels (Fig 2a). In addition, we found similar effects on phospho-CHK1 and CDC25a when administering cisplatin and LPC(24:1), however RAD51 does not appear to be increased (Figure 2b). CHK1, CDC25a and RAD51 are important and well-known regulators in cell cycle progression upon DNA damage, however little is known about phospholipids that influence their functions. Interestingly, PIFA treatment did not alter phospho-p53 levels (Supplemental figure 1a). This is in line with our findings that PIFAs induce chemoresistance in tumors with both wildtype (C26) and mutated (LLC, MDA-MB-231) p53. Since PIFA-mediated chemoresistance is lipid-mediated and mTOR has recently been shown to become activated by lipids and plays a role in DNA damage signaling we investigated if two mTOR inhibitors, temsirolimus and PP242, could block chemoresistance *in vivo*. Neither temsirolimus nor PP242 were able to block chemoresistance as quantified by γH2AX (Supplemental figure 1b).

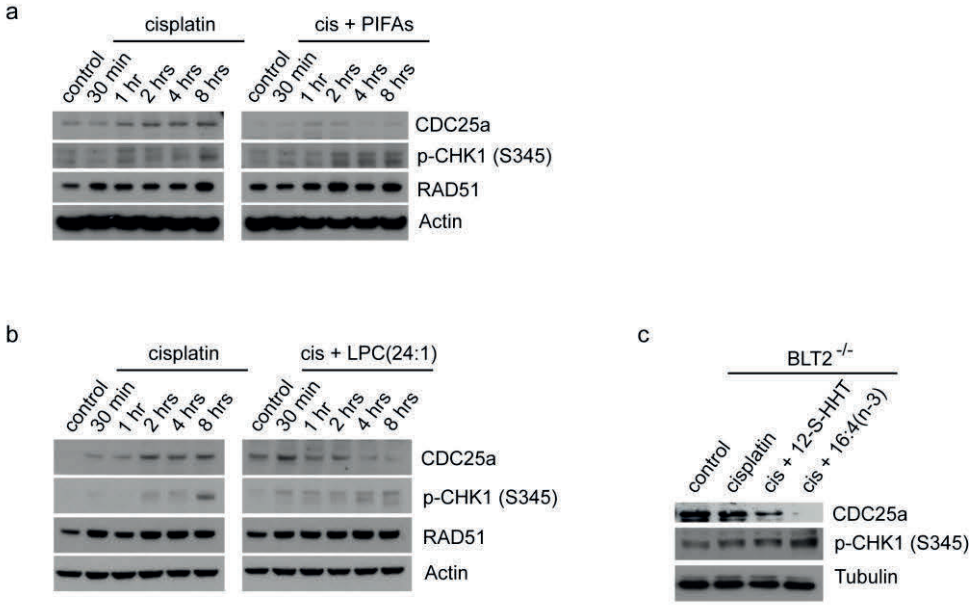


Figure 2: PIFA treatment enhances phospho-CHK1 signaling and RAD51. Snap frozen C26-tumors isolated from cisplatin, cisplatin and PIFAs or cisplatin and LPC(24:1) treated mice at indicated time points were analyzed by western blot for the expression of phosho-CHK1(S345), RAD51, CDC25a and beta-actin. Panel a shows results of co-administration of cisplatin and PIFAs and panel b shows results of co-administration of cisplatin and LPC(24:1). Increased expression of phospho-CHK1 (S345) and RAD51 were found in mice treated with cisplatin and PIFAs or LPC(24:1) compared to cisplatin treated mice. CDC25a was decreased in mice receiving co-treatment of cisplatin and PIFAs or LPC(24:1) compared to mice receiving cisplatin monotherapy. Panel c shows results of western blot analysis of LLC-tumors harvested from BLT2$^{-/-}$ mice 4 hours after treatment with either cisplatin alone, cisplatin and 12-S-HHT or cisplatin and 16:4(n-3). Data show representative result of 3 (PIFAs) or 2 (LPC24:1 and BLT2$^{-/-}$) independent experiments.

Previously, we found that 12-S-HHT-mediated signaling in splenic macrophages depends on leukotriene B4 receptor 2 (BLT2) and that 12-S-HHT-mediated chemotherapy resistance was lost in $BLT2^{-/-}$ mice. In addition, sCM derived from $BLT2^{-/-}$ splenocytes incubated with 12-S-HHT failed to induce resistance in wildtype mice. Tumors from these mice are well suitable to investigate if the upregulated proteins found in wildtype mice treated with both PIFAs are a direct result of PIFA-mediated chemoresistance. We found that the upregulation of phospho-CHK1 was lost in $BLT2^{-/-}$ mice treated with 12-S-HHT, but still present in $BLT2^{-/-}$ mice treated with 16:4(n-3) 4 hours after treatment. Furthermore, decreased levels of CDC25a were seen in $BLT2^{-/-}$ mice treated with 16:4(n-3) but not with 12-S-HHT (Figure 2c).

Inhibition of CHK1 in vivo prevents chemoresistance

In order to investigate the contribution of CHK1 to PIFA-mediated chemotherapy resistance *in vivo* we treated tumor-bearing mice with a CHK1 inhibitor (MK-8776). To determine correct timing of administration of MK-8776 to ensure complete inhibition of CHK1 at the time of PIFA and cisplatin treatment we treated mice with a single dose of MK-8776 and harvested tumors 1, 2 or 4 hours after treatment. MK-8776 can give an increase in phospho-CHK1 as a cellular response to the inhibitor causing a futile loop of phosphorylation, so in order to establish if sufficient inhibition was reached we analyzed CDC25a stabilization and found that during the first two hours after MK-8776 treatment CDC25a was stabilized, slowly returning back to baseline at 4 hours (Figure 3a). Based on these results we treated mice with MK-8776 one hour before and one hour after cisplatin and PIFA treatment. As expected, co-administration of cisplatin, PIFAs and MK-8776 blocked PIFA-mediated chemotherapy resistance (Figure 3b).

Loss of HR prevents PIFA-mediated chemoresistance in vivo

Although CHK1, CDC25a and RAD51 have distinct functions in HR-mediated DNA damage repair additional mechanisms could not yet be excluded. To further support the role of HR in PIFA-mediated chemoresistance we performed experiments in a validated genetically engineered mouse model lacking DNA repair by HR due to loss of BRCA1 and p53. The $BRCA1^{-/-}p53^{-/-}$ tumors from K14cre;$Brca1^{F/F}$;$p53^{F/F}$ mice, which are highly sensitive to cisplatin, were implanted into wild type FVB/N mice. The combination of cisplatin and PIFAs did not induce resistance in this model and tumor endpoints were reached after 29 days in the presence or absence of PIFAs (Figure 3c). Concurrent loss of 53BP1, which partially restores HR, was sufficient to restore PIFA-induced chemoresistance. Tumor endpoints were reached on average 17 days after therapy in the mice receiving the combination treatment as opposed to 22 days in the cisplatin treated tumors ($p<0.001$, Figure 3d). This indicates that PIFAs can only induce resistance to cisplatin when HR is at

least partially functional. Taken together, our results indicate that PIFAs induce chemotherapy resistance via interference with HR-mediated DNA damage repair.

Discussion

Here we show that PIFAs and their effector molecule LPC(24:1), a phospholipid secreted by PIFA-activated splenic macrophages, interfere with the early effects of DNA damaging chemotherapeutics. Co-administration of cisplatin with PIFAs or LPC24:1 reduced DNA damage *in vivo* compared to cisplatin monotherapy as early as 4 hours after treatment. Interestingly, PIFAs can reduce DNA damage even when administered after cisplatin-induced DNA damage had already been established, suggestive of enhanced DNA damage repair. In line with this hypothesis we found increased phopho-CHK1 levels in tumors of mice treated with cisplatin and PIFAs or LPC(24:1). CHK1 is an important cell cycle regulator and its functions dependent on the stage of the cell cycle the cell is in. Its main functions are activating DNA damage repair and induction of cell cycle arrest in G2. Downstream targets of CHK1 are amongst others p53, CDC25 proteins and RAD51. CDC25a is a positive regulator of the cell cycle that functions by activating cyclin dependent kinases (CDKs) that drive cell cycle progression. Inhibition of CDC25a by phospho-CHK1 functions as an important cell cycle checkpoint upon DNA damage, allowing the cell to repair the damage before continuing cell cycle progression[7-10]. Various studies show that inhibition of CHK1 functions as a sensitizer for chemotherapy and radiotherapy[11-14]. In addition, enhanced activation of CHK1 has been shown to mediate drug resistance in prostate cancer stem cell-like cells and in circulating breast tumor cells[15,16]. RAD51 plays an important role in homologous recombination as it binds the DNA at the site of the break to protect it and provides a docking site for other DNA damage repair proteins like BRCA1 and BRCA2. Interestingly, RAD51 overexpression in cancer cells has been described as a mechanism of therapy resistance in both preclinical and clinical studies[17-20]. In addition, we found that loss of HR *in vivo* prevented PIFA-mediated resistance and restoration of HR by concurrent loss of 53BP1 restored PIFA-mediated chemoresistance. These results suggest that the PIFAs function via early enhancement or acceleration of HR-mediated DNA damage repair. However, due to the complexity of DNA damage repair and the lack of an *in vitro* model for PIFA-mediated chemoresistance we cannot exclude contributions by other repair mechanisms and our results are merely scratching the surface of the full mechanism of PIFA and LPC(24:1)-mediated chemotherapy resistance.

Cellular responses to DNA damage are intensively studied however, little is known about systemic signaling mechanisms to protect genomic integrity. PIFAs are quickly released from MSCs after they are exposed to platinum analogues, and PIFAs rapidly activate splenic macrophages to produce LPCs like LPC(24:1) leading to a reduction in DNA damage

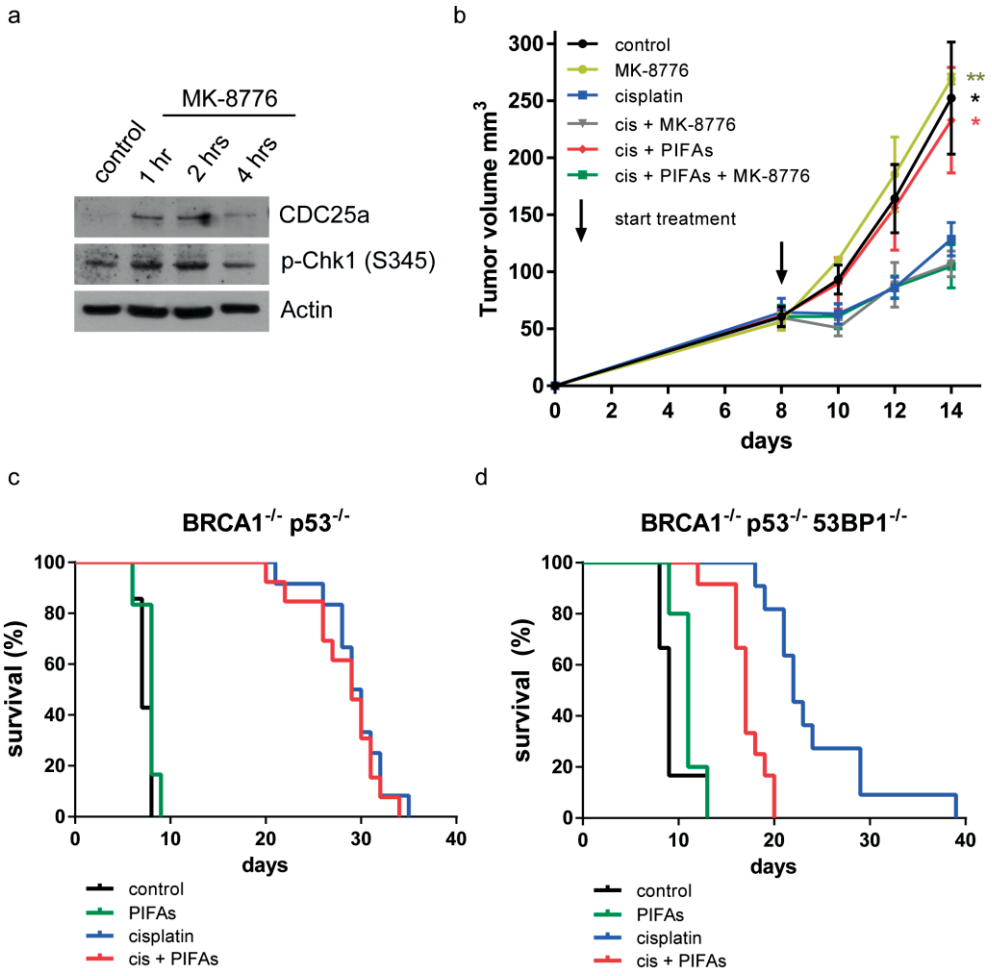


Figure 3: Inhibition of CHK1 or loss of HR prevents PIFA-mediated chemoresistance. C26 tumor-bearing balb/c mice received a single injection of MK-8776 and tumors were harvested after indicated timepoints to assess extent of CHK1 inhibition by western blot (panel a). Tumor-bearing balb/c mice were either left untreated or treated with MK-8776, cisplatin, cisplatin and MK-8776, cisplatin and PIFAs or cisplatin, PIFAs and MK-8776 and tumor volumes were assessed every two days. MK-8776 was administered one hour before and one hour after the additional treatments. Co-administration of cisplatin, PIFAs and MK-8776 prevented PIFA-mediated chemotherapy resistance (panel b). Brca1-/-;p53-/- and (panel c) Brca1-/-;p53-/-;53BP1-/- (panel d) mammary tumors were orthotopically transplanted into wild-type FVB/N. Therapy with either vehicle, PIFA, cisplatin or cisplatin and PIFA was started when tumors reached 100-150 mm3 and tumor growth was monitored until tumor endpoints were reached (tumor volume >1000mm^3). Loss of HR (panel c) prevented PIFA-mediated resistance whereas additional loss of 53bp1 (panel d), partially restoring HR reinstated PIFA-mediated chemoresistance. Data are represented as mean ± SEM. All graphs show results of 2 independent experiments (n=8-12 per group). Statistical significance was determined by one-way ANOVA. All compared to cisplatin unless indicated otherwise * P<0.05, ** P<0.01.

This mechanism could represent a physiological stress response of the body to protect itself from DNA-damaging agents which backfires on us in case of anti-cancer treatment. This hypothesis is supported by observations that dietary polyunsaturated fatty acids can lower DSBs in the context of diabetes and cancer[21-23]. Interestingly, these findings are also in line with our results that 16:4(n-3)-containing fish oils can interfere with chemotherapy induced cell death[5,24]. However, it is important to realize that the class of fatty acids and phospholipids consist of a wide variety of molecules, each with specific biological functions.

Taken together, our findings have uncovered a systemic signaling network, mediated by lipids, which targets cellular DNA damage repair functions. Further research is needed to elucidate the mechanism of PIFA-mediated chemoresistance in more detail and find potential drug targets that will provide opportunities to improve anti-cancer therapy.

Materials and Methods

Reagents

12-S-HHT was purchased at Cayman Chemical. 16:4(n-3) was isolated from *Ulva Pertusis* and kindly provided by Dr. K. Ishihara[25]. LPC(24:1) was synthesized as described previously (see chapter 3 of this thesis). MK-8776 was purchased at SelleckChem. For immunohistochemical staining the following antibodies were used: anti-γH2AX (Cell Signaling, 2577) and poly-HRP goat-anti-Rabbit/Rat/Mouse (Immunologic). For western blot the following antibodies were used: rabbit anti-RAD51 (Cell signaling, 8875), rabbit anti-phospho-CHK1 (S345) (Cell signaling, 2341), rabbit anti-CDC25a (Cell signaling, 3652), rabbit anti-phospho p53 (S20) (ThermoScientific, PA5-35804), mouse anti-vinculin (Sigma-Aldrich, V9131), mouse anti-beta actin (Novus Biologicals, NB600-501), goat-anti-rabbit HRP (life technologies) and goat-anti-mouse HRP (Santa Cruz).

Animal experiments

C26 tumor cells were implanted in balb/c (Charles River) mice as described previously[6]. For all experiments 8-10 week old male mice were used. At day 0 the mice were injected subcutaneously with 1*106 (for C26) tumor cells. At day 8 when the tumors reached a size of 50-100 mm3 animals were randomized into groups and treatment was started. The mice received an intraperitoneal injection of cisplatin 6mg/kg alone or in combination with a subcutaneous injection of 100 μl 16:4(n-3) (25 nM) and 100 μl 12-S-HHT (20 nM) or 100 μl of LPC(24:1) (10 nmol). Mice treated with MK-8776 received an intraperitoneal injection of MK-8776 (10mg/kg) one hour before and one hour after cisplatin and PIFAs treatment. Blinded tumor volume measurements were done once every two days using a digital caliper. Tumor volume was determined as length*width^2*0.5. Control mice received the appropriate vehicles. All experimental animal procedures conducted in Utrecht (the Netherlands) were approved by the University Medical Center Animal Ethics Committee and were in agreement with the current Dutch Laws on animal experiments. All experimental animal procedures conducted in the BLT2-/- mice in Louisville (KY, USA) were approved by the University of Louisville Animal Care and Use Committee[6]. In order to show a difference of 20% in tumor volume with a standard deviation of 10% and a type I error (alfa) of 5% using a power of 90% a minimal of 8 mice per treatment group are required.

Brca1$^{-/-}$;p53$^{-/-}$ and Brca$^{-/-}$;p53$^{-/-}$;53bp1$^{-/-}$ mammary tumors

Experiments with Brca1$^{-/-}$;p53$^{-/-}$ mammary tumors were performed at the Netherlands Cancer Institute and all experimental procedures on animals were approved by the Animal Ethics Committee of Netherlands Cancer Institute. Brca1$^{-/-}$;p53$^{-/-}$ mammary tumors were generated in K14cre;Brca1$^{F/F}$;p53$^{F/F}$;Abcb1a/b$^{-/-}$ (KB1PM) female mice. In this study we used KB1PM8 (53BP1 wild-type) and KB1PM8-ol-res (53BP1 deficient). The latter lost

53BP1 due to a heterozygous truncating mutation and silencing of the other allele[26]. Expression of 53BP1 in KB1PM8 and its loss in KB1PM8-ol-res was reconfirmed by IHC in the outgrowing control tumors (data not shown). Orthotopic transplantations into wild-type FVB/N mice were performed as described[26]. Brca1$^{-/-}$;p53$^{-/-}$;53BP1$^{-/-}$;Abcb1a/b$^{-/-}$ tumors were previously described[27]. Therapy was started when tumors reached 100-150 mm3 and tumor growth was monitored as described above. Endpoints were determined as a tumor volume of >1000 mm^3.

Western blot

Snap frozen tumors were homogenized in protein lysis buffer (1% Triton-X100, 50 mM Tris pH 8.0, 150 mM NaCl, 1x complete protease inhibitor (Roche), 0,25% Sodium deoxycholate, 0.05M sodiumfluoride and 0.1 μM Calyculin A) and subjected to SDS-PAGE and western blotting. The membranes were blocked in 5% BSA in TBST and incubated overnight in the antibodies diluted 1:1000 in 5% BSA in TBST according to the manufacturers protocol. Secondary antibodies used for detection were diluted 1:7500 in 5% BSA in TBST.

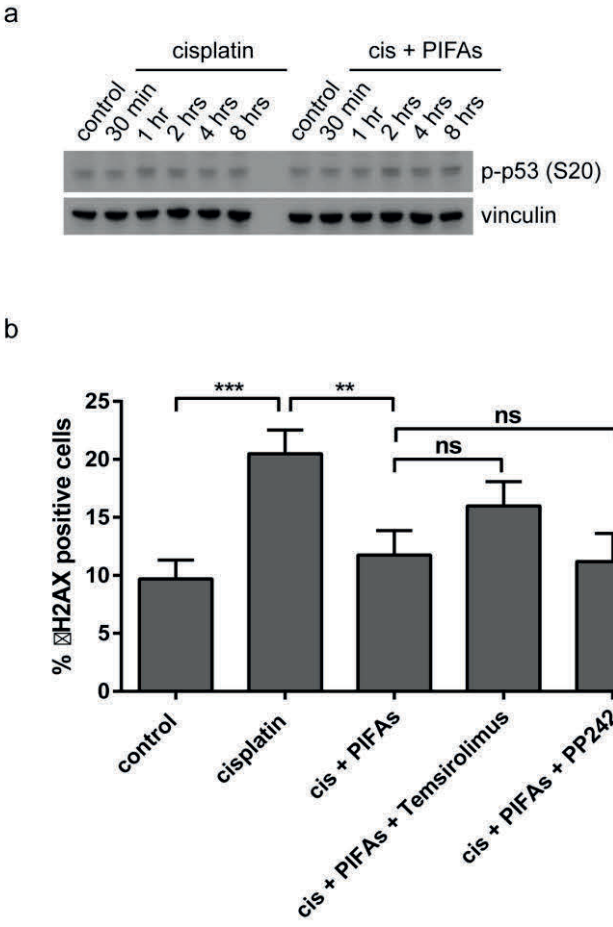
Immunohistochemistry

Formaldehyde-fixed paraffin-embedded tissue sections were deparaffinated and rehydrated and incubated in endogenous peroxidase blocking buffer containing 5% H2O2. Antigen retrieval was performed by cooking slides in citrate buffer. Tissues were blocked in 5% goat serum (Life Technologies) in TBST. The slides were incubated overnight at 4°C with rabbit anti-γH2AX 1:200 in 5% goat serum. Poly-HRP goat-anti-Rabbit/Rat/Mouse (Immunologic) was used as secondary antibody, followed by DAB staining. Slides were counterstained with hematoxyline. Each slide was scanned using a Leica Aperio Scanscope and 8 to 10 images per tumor were analyzed. To determine the percentage of positive cells a grid with 54 intersection points was used. A second examiner, blinded to the treatment groups, also quantified the γH2AX stainings.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical significance for the animal experiments in which tumor volumes was assessed was determined by one-way ANOVA with Tukey correction for multiple testing. A value of $P<0.05$ was considered to be statistically significant. All other data was analyzed using a two-tailed Students T-test. A P value <0.05 was considered statistically significant. A Levene's test was used to determine if variance between groups was comparable. Animals were excluded from analysis if two or more tumor measurements were significant outliners compared to the rest using the Grubbs outlier test (alfa: 0.05).

Supplementary information



Supplemental figure 1: p53 and mTOR signaling are not involved in PIFA-mediated chemoresistance. Snap frozen tumors isolated from cisplatin and cisplatin and PIFAs treated mice at indicated time points were analyzed by western blot for the expression of phosho-p53 (S20) and vinculin (loading control, panel a). Tumor-bearing balb/c mice were either left untreated or treated with cisplatin, cisplatin and PIFAs, cisplatin, PIFAs and temsirolimus or cisplatin, PIFAs and PP242 and tumors were harvested 4 hours after treatment to assess γH2AX by immunohistochemistry. No significant differences in γH2AX were found when comparing cisplatin and PIFA treated mice with cisplatin, PIFA and temsirolimus or PP242 treated mice (panel b). Data are represented as mean ± SEM. Data show representative result of 2 (panel a) or 3 (panel b) independent experiments. Statistical significance was determined by two-tailed students T-test. All compared to cisplatin alone unless indicated otherwise * P<0.05, ** P<0.01.

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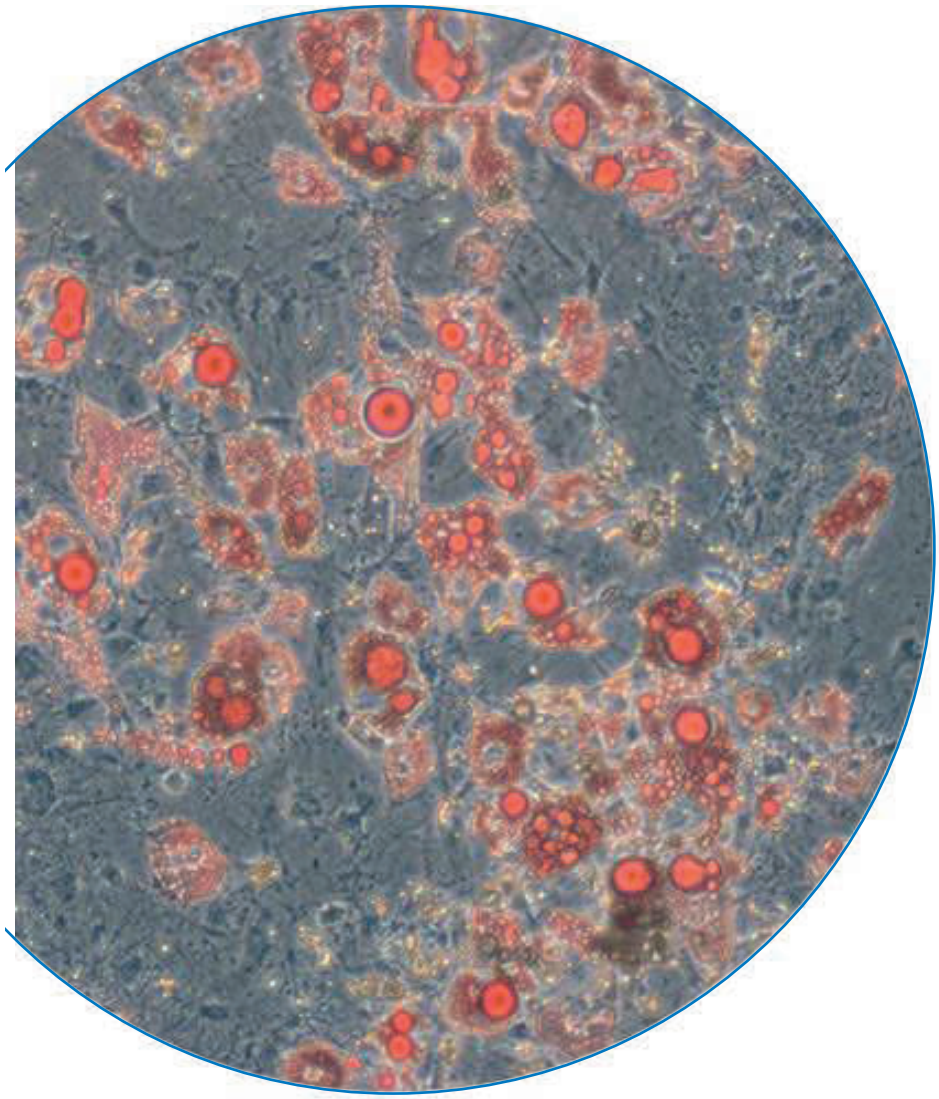
CHAPTER 6



Platinum-Induced Fatty Acid (PIFA) production depends on $cPLA_2$ and PLAA signaling and is restricted to mesenchymal stem cells

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Abstract

Resistance against chemotherapy is a major obstacle in the continued effective treatment of cancer patients. Increasing evidence shows that mesenchymal stem cells (MSCs) play a role in resistance against anti-cancer drugs. We found that MSCs induce chemotherapy resistance via the release of platinum-induced fatty acids (PIFAs). PIFAs induce resistance at nanomolar concentrations and are specifically produced in response to platinum-containing chemotherapeutics. However, once the PIFAs are present they can protect against multiple other DNA-damaging agents. Here, we show that PIFA production in MSCs depends on phospholipase A2-activating protein (PLAA) and cytosolic phospholipase A2 ($cPLA_2$) activity and that cisplatin can increase PLAA and $cPLA_2$ activity in a dose-dependent manner. This phenomenon is seen only in MSCs and not in their differentiated progeny. In addition, the cellular lipid content of MSCs changes drastically over the course of differentiation into either adipocytes or osteoblasts, however the cellular content of arachidonic acid (AA) and eicosapentaenoic acid (EPA), the main precursors of PIFAs, do not change significantly during differentiation. Taken together, we have shown that PIFA production is restricted to MSCs and depends on $cPLA_2$ and PLAA activity.

Introduction

Increasing evidence shows that non-neoplastic cells in the tumor microenvironment contribute to tumor growth and anti-cancer therapy resistance. MSCs reside in adipose tissue and bone marrow and they can give rise to various cell types including osteoblasts, adipocytes, chondrocytes, fibroblasts and myocytes[1,2]. Their strong immune regulatory functions and ability to home to damaged tissues affects tumor progression and therapy efficacy in multiple tumor types like leukemia, head-and-neck cancer and ovarian cancer[2-5]. We previously found that MSCs can induce resistance against multiple types of DNA-damaging chemotherapeutics via the release of two PIFAs called 12S-hydroxy-5,8,10-heptadecatrienoic acid (12-S-HHT) and hexadeca-4,7,10,13-tetraenoic acid (16:4(n-3)). 12-S-HHT and 16:4(n-3) are omega-6 and omega-3 derived fatty acids, respectively[6]. The body's ability to form these fatty acids depends on essential omega-3 and omega-6 precursors derived from our diet. Once the essential dietary lipids are converted to phospholipids they are incorporated into the membranes of cells. These phospholipids are important for membrane fluidity, the formation of lipid rafts and they function as signaling molecules[7]. Phospholipases can hydrolyze phospholipids thereby generating lysophospholipids and free fatty acids, which can both serve as signaling molecules. Arachidonic acid (AA, omega-6) and eicosapentaenoic acid (EPA, omega-3) are two important free fatty acids that through a series of enzymatic conversions can form eicosanoids, signaling molecules involved in various processes like vasodilation and constriction, platelet aggregation and neutrophil chemotaxis[8-10]. Generally omega-6 derived eicosanoids have a pro-inflammatory effect, whereas omega-3 derived fatty acids have an anti-inflammatory effect[9,10]. $cPLA_2$ is a calcium-dependent phospholipase that hydrolyses phospholipids in the membrane and has specificity for phospholipids that contain either AA or EPA at the sn-2 position, making $cPLA_2$ the rate-determining enzyme in the cascade leading to the production of eicosanoids[11,12].

Here we show that the production of PIFAs depends on $cPLA_2$ and PLAA activity and that cisplatin induces a concentration-dependent rise in $cPLA_2$ and PLAA in MSCs but not in differentiated osteoblasts and adipocytes. Furthermore, the lipid content of MSCs changes during differentiation. Gaining detailed insight in how MSCs produce PIFA-mediated chemoresistance and why this process is platinum-dependent could provide new targets for intervention to improve outcome of treatment with chemotherapy.

Results

$cPLA_2$ expression is lost upon differentiation of MSCs

Based on our previous mouse studies that showed that $cPLA_2$ inhibition prevents chemotherapy resistance[6], and the fact that $cPLA_2$ is the rate-determining step in eicosanoid and PIFA production (Figure 1a), we investigated the role of $cPLA_2$ in PIFA-

mediated chemoresistance. We previously found that only MSCs or MEFs can induce chemoresistance via the release of PIFAs, whereas differentiated progeny of MSCs or other stem cells like hematopoietic stem cells (HSCs) could not produce PIFAs[6]. One hypothesis for this difference is that expression levels of $cPLA_2$ differ within tissues and cell types. In order to test this we performed western blot analysis on bone marrow-derived mature hematopoietic cells (defined as positive for Lineage markers (Lin^+)), hematopoietic stem cells (HSCs, $Lin^-/CD45^+$) and MSCs ($Lin^-/CD45^-$) and found that $cPLA_2$ and its active form phospho-$cPLA_2$ (S505) are only expressed by the $Lin^-/CD45^-$ fraction which contains MSCs (Figure 2a-b). In line with these findings we found that MSCs downregulated phospho-$cPLA_2$ upon differentiation. As early as three days after switching MSCs to differentiation medium phosho-$cPLA_2$ levels are dramatically reduced in both adipocytes and osteoblasts (Figure 2c-d). At this time point only few mature adipocytes and osteoblasts were present as shown by oil red O staining for triglycerides and alizarin red staining for calcium deposits (Figure 2e).

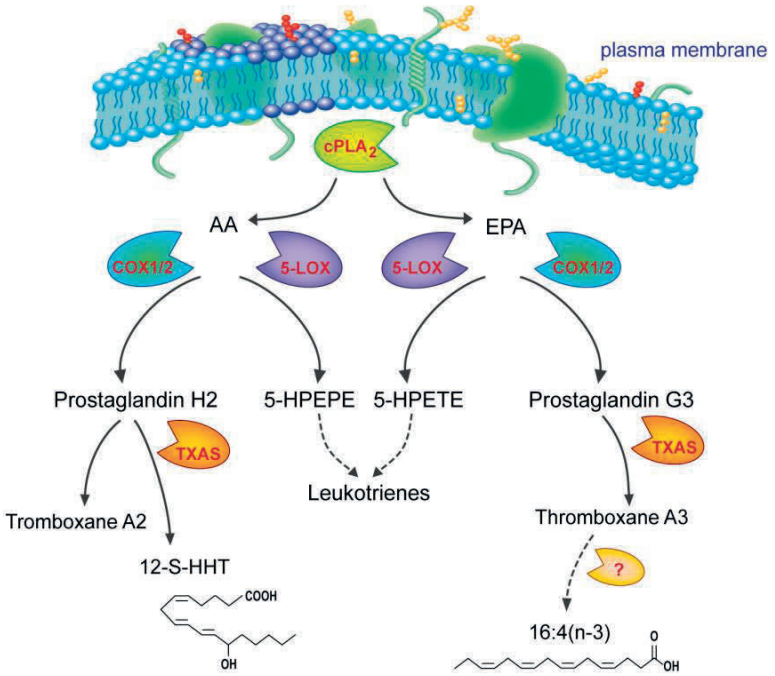

Figure 1: schematic overview of the enzymes involved in PIFA production. A schematic overview of omega-3 and omega-6 fatty acid-derived eicosanoid and PIFA production. In black the lipids, in red the enzymes mediating the conversion. Dashed lines indicate that multiple steps and enzymes are involved in the conversion leading to depicted end product.

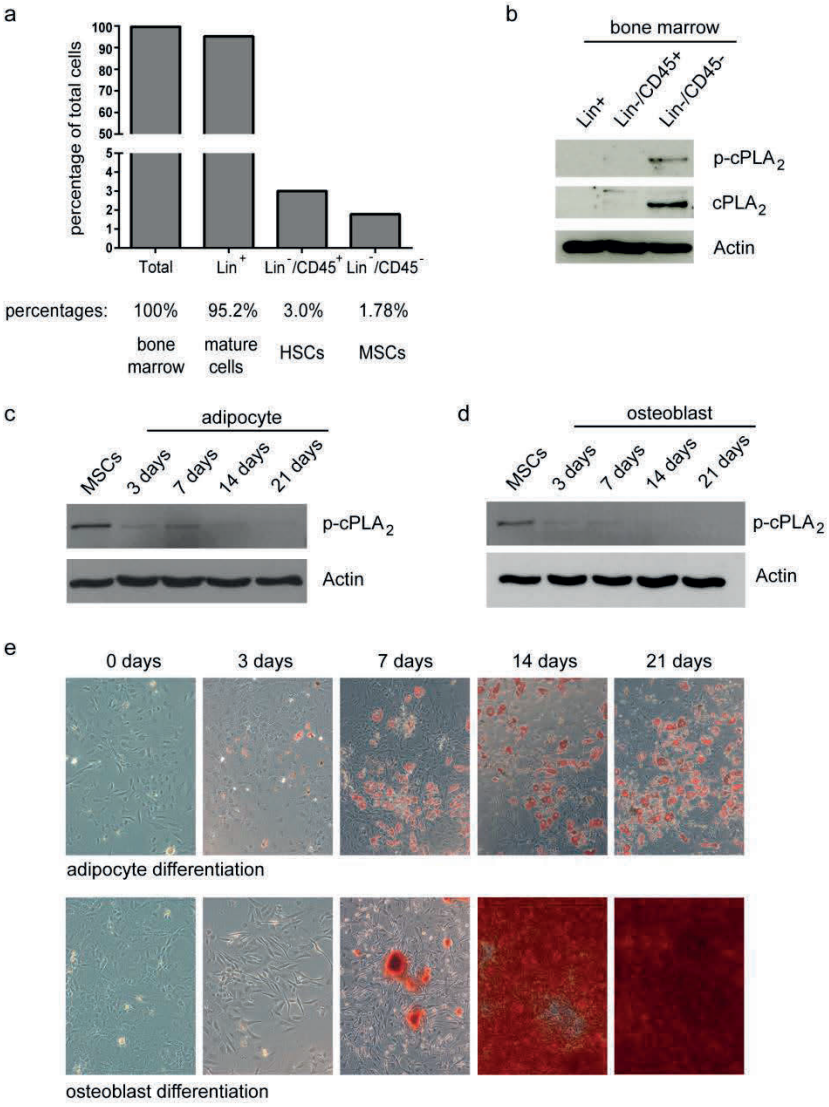

Figure 2: $cPLA_2$ and phospho-$cPLA_2$ are specifically expressed by MSCs *in vivo* and *in vitro*. Distribution of cell types in the bone marrow as determined by magnetic bead sorting of mature Lin^+, $Lin^-/CD45^+$ and $Lin^-/CD45^-$ cells (panel a). Western blot analysis sorted bone marrow for the presence $cPLA_2$, phospho-$cPLA_2$ (S505) and actin shows specific expression of $cPLA_2$ and phospho-$cPLA_2$ in the $Lin^-/CD45^-$ fraction containing the MSCs (panel b). Actin was used as a loading control. *In vitro* differentiation of MSCs into adipocytes (panel c) or osteoblasts (panel d) shows strong downregulation of phospho-$cPLA_2$ already 3 days after switching to differentiation medium. Panel e shows differentiation of MSCs into adipocytes and osteoblasts. The presence of adipocytes is shown by an Oil Red O triglyceride staining and the presence of osteoblasts is shown by an Alizarin red staining for calcium deposits. All panels show representative results of 2 independent experiments with similar outcome.

MSCs change their lipid profile during differentiation

A second hypothesis that could explain the MSC-specific production of PIFAs is that the lipids needed to fuel the enzymatic cascade leading to PIFAs differ between MSCs and their differentiated progeny. To test validity of this hypothesis we analyzed the lipid content of MSCs and adipocytes and osteoblasts harvested 0, 3, 7, 14 or 21 days after switching them to either adipocyte or osteoblast differentiation medium. A total lipid extraction, mass spectrometry analysis and principal component analysis (PCA) revealed large changes in overall lipid content over the course of differentiation towards either adipocytes or osteoblasts in two independent experiments with similar outcome (Figure 3 a). Since the precursors of PIFAs are AA and EPA we determined if the content of AA and EPA changes within the MSCs as they differentiate into adipocytes and osteoblasts. Interestingly, no apparent changes occur in EPA levels upon induction of differentiation into adipocytes or osteoblasts (Figure 3 b and c). AA levels increase in adipocytes during differentiation whereas they decrease during osteoblast differentiation. Despite these differences fully differentiated osteoblasts and adipocytes still contain AA and EPA making it less likely that the absolute levels of these fatty acids are a determining factor in PIFA production.

Cisplatin induces $cPLA_2$ and PLAA expression in MSCs

Previous studies showed that MSCs only produce PIFAs in response to platinum-containing chemotherapeutics like cisplatin. How cisplatin stimulates PIFA production in MSCs is yet unknown. A study by Zhang *et al* showed that phospholipase A2-activating protein (PLAA) is upregulated by cisplatin treatment in HeLa cells[13]. PLAA functions as an activator of $cPLA_2$, in addition to its dependence on calcium and phosphorylation at serine 505 to gain activity. We therefore performed an experiment in which we analyzed PLAA expression in MSCs stimulated with various concentrations or for various time points with cisplatin. Cisplatin caused a concentration- and time dependent increase in both phospho-$cPLA_2$ and PLAA in MSCs (Figure 4 a,b), which was not seen in differentiated adipocytes (Figure 4 c,d) or osteoblasts (Figure 4 e,f). Interestingly, the upregulation of phospho-$cPLA_2$ and PLAA was not seen in MSCs stimulated with SN-38, the active metabolite of irinotecan, which does not contain platinum, proving that the effect is platinum-dependent (Figure 5 a,b). In order to confirm that the cisplatin-induced increase in phospho-cPLA2 and PLAA is restricted to MSCs we also tested RAW264.7 cells, a macrophage cell line with high basal expression of phospho-cPLA2 and PLAA, which cannot induce PIFA-mediated chemoresistance (see chapter 3 of this thesis). RAW264.7 cells do not show a time- and concentration- dependent increase in PLAA or phospho-cPLA2 despite the presence of both proteins within the cell, suggesting that the response is specific to MSCs (Figure 5c,d).

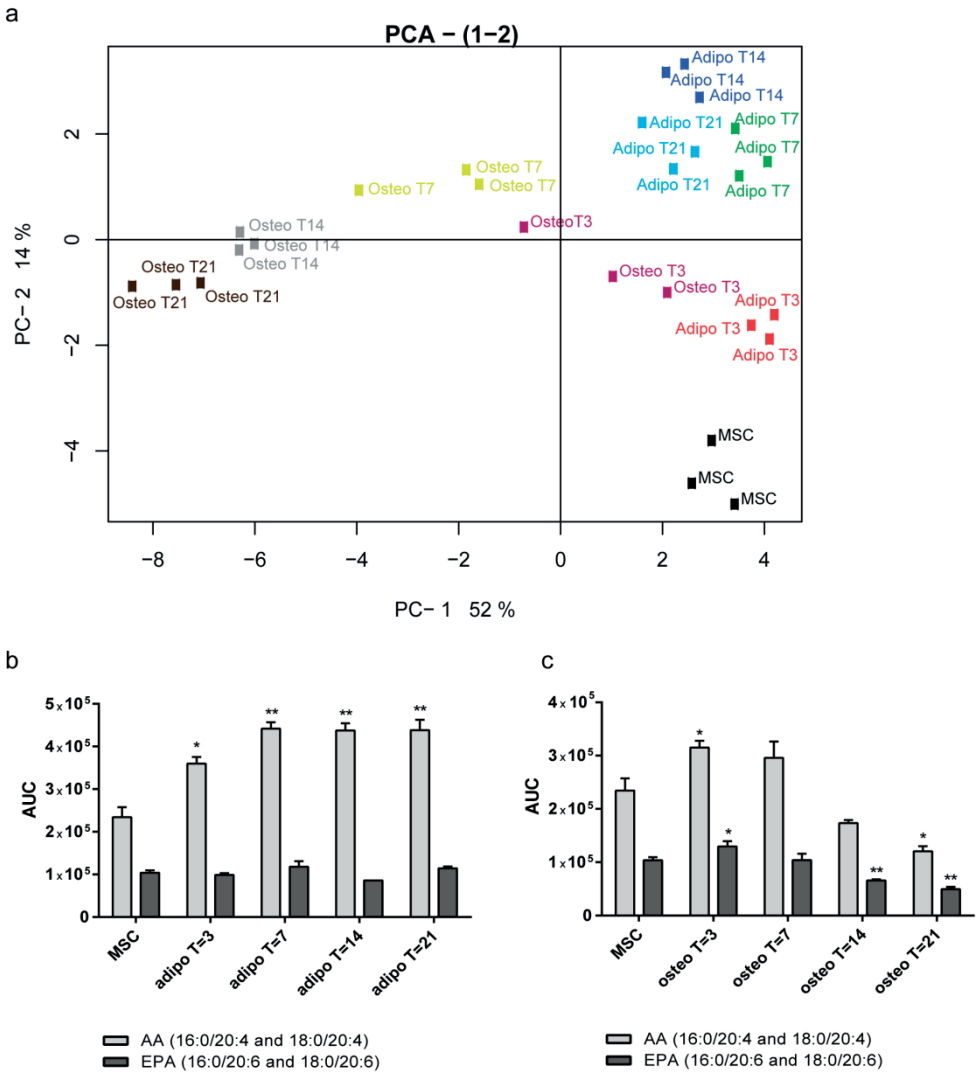

Figure 3: The total lipid content of MSCs changes during differentiation. Mesenchymal stem cells were harvested 0, 3, 7, 14 or 21 days after induction of differentiation into either adipocytes or osteoblasts and the lipid content was analyzed by mass spectrometry. A principle component analysis was used to show differences occurring over time after induction of differentiation (a). Each square represents an individual sample, for instance: Osteo T14 = osteoblasts harvested 14 days after induction of differentiation. Panel b and c show AA and EPA content in MSCs, adipocytes (b) and osteoblasts (c) during differentiation. AA increases in adipocytes over the course of differentiation. Osteoblasts show a decrease in AA during differentiation, only minor changes are seen in EPA content. Data shows representative images of 2 independent experiments with similar outcome and with 3 samples per time point.

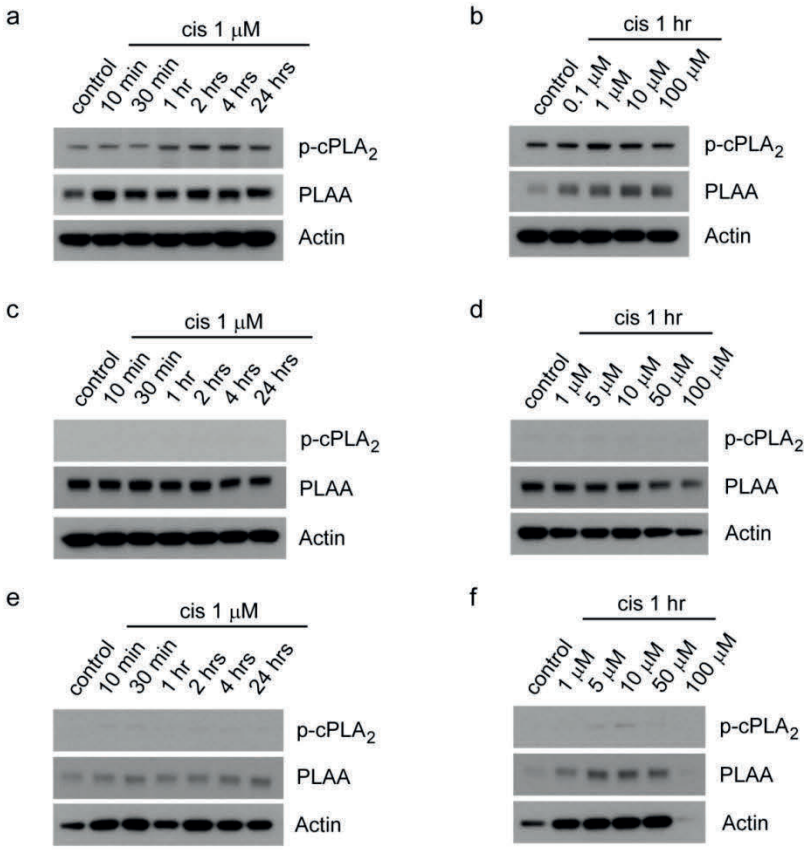

Figure 4: cisplatin induces upregulation of phospho-cPLA$_2$ and PLAA in MSCs but not in adipocytes or osteoblasts. MSCs stimulated for indicated time points with 1 μM cisplatin (panel a) or with indicated concentrations of cisplatin for 1 hour (panel b) were analyzed by western blot for phospho-cPLA$_2$ and PLAA levels. Actin was used as a loading control. Increased phospho-cPLA$_2$ and PLAA levels were found after incubation with cisplatin. This time and dose dependent increase of phospho-cPLA$_2$ and PLAA was not seen in differentiated adipocytes (panel c and d) or osteoblasts (panel e and f). All panels show representative result of 2 independent experiments with similar outcome.

Discussion

Despite the current achievements in targeted therapies for cancer patients a large portion of the patients still benefit from chemotherapy[14]. Unfortunately, resistance against chemotherapy poses a significant problem and strategies to improve chemotherapy efficacy are desirable[15, 16]. Here we showed that the production of PIFAs in MSCs depends on the activity of cPLA$_2$ and PLAA. Inhibition of cPLA$_2$ or other components in the eicosanoid pathway like TXAS, iCa^{2+} or COX-1 prevented MSC-mediated chemoresistance *in vivo*[6]. MSCs showed a time and dose-dependent increase in phospho-cPLA$_2$ and PLAA upon cisplatin treatment but not upon SN-38 treatment. These findings are in line with

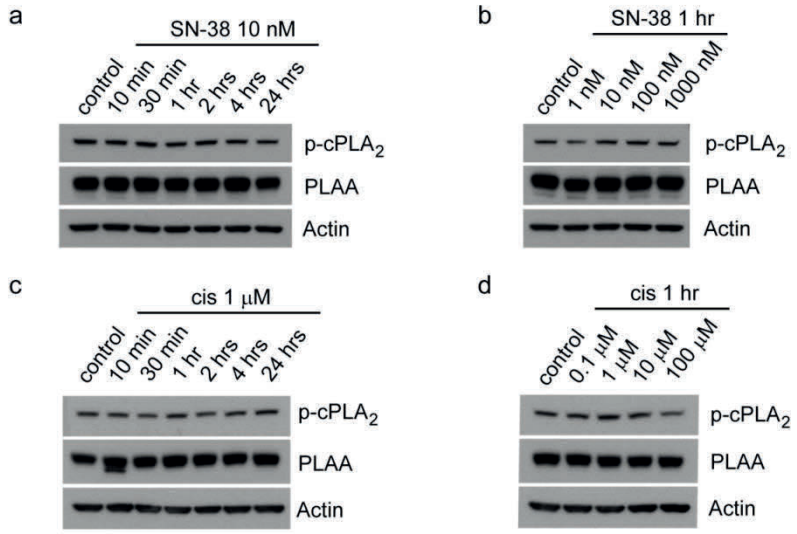

Figure 5: enhanced PLAA-cPLA2 signaling is restricted to MSCs and does not occur upon stimulation with a non-platinum containing chemotherapeutic agent. MSCs (panel a and b) or RAW264.7 cells (panel c and d) were stimulated for indicated time points with 1 μM cisplatin or with indicated concentrations of cisplatin for 1 hour and were analyzed by western blot for phospho-$cPLA_2$ and PLAA levels. Actin was used as a loading control. SN-38 is unable to enhance PLAA and phospho-cPLA2 levels in MSCs. RAW264.7 cells despite relatively high basal levels of both phospho-cPLA2 and PLAA cannot enhance PLAA-cPLA2 signaling in response to cisplatin. All panels show representative result of 2 independent experiments.

our data showing that only platinum-containing chemotherapeutics induce PIFA production. The production of PIFAs was restricted to MSCs since differentiated adipocytes and osteoblasts did not express $cPLA_2$ and did not show a time and concentration dependent increase in phospho-$cPLA_2$ or PLAA in response to cisplatin treatment. The molecular mechanism leading to platinum-induced activation of $cPLA_2$ and PLAA is currently unknown. In addition to its effects on DNA cisplatin is known to bind to various intracellular proteins, so potentially cisplatin binds to PLAA leading to increased activity. However, if this process would be passive other cell types that would express eicosanoid enzymes like $cPLA_2$, PLAA, COX-1 and TXAS should in theory also be able to produce PIFA although our results indicate that is not the case, suggesting active induction of $cPLA_2$ and PLAA upon cisplatin treatment.

Despite the lack of apparent changes in AA and EPA content within MSCs and their differentiated progeny, we did find overall changes in lipid content within these cells. Several studies show lipid and metabolome changes when comparing stem cells to differentiated progeny[17, 18]. Interestingly, Yanes *et al* found that inhibition of eicosanoid production promoted embryonic stem cell pluripotency[18], whereas we show that $cPLA_2$,

an important enzyme in eicosanoid production, is strongly downregulated upon differentiation. Signaling via lipid rafts controls the differentiation of dental pulp stem cells, which are stem cells with various MSC-like features[19]. In addition, resistance against doxorubicin has been linked to changes in lipid profiles of breast cancer cells[20,21]. The difference in lipid profile combined with the difference in enzyme expression and activation may provide insight in the MSC-specific function of PIFA production and chemotherapy resistance. Importantly, insight into the mechanism of PIFA production by MSCs provides potential targets to improve chemotherapy efficacy.

Materials and Methods

Reagents

The following antibodies were used for western blotting: rabbit anti-phospho-$cPLA_2$ (serine 505, Cell Signaling), rabbit anti-$cPLA_2$ (Cell Signaling), rabbit anti-PLAA (Sigma-Aldrich), mouse anti-beta-actin (Novus Biologicals), goat-anti-rabbit HRP (life technologies) and goat-anti-mouse HRP (Santa Cruz). Cisplatin was purchased from Accord Healthcare, SN-38 was purchased from SelleckChem. RAW264.7 cells originated from ATCC and were maintained at 37°C, 5% CO_2 in DMEM (4,5 gr glucose/L) + 5% FCS.

Isolation, culturing and differentiation of MSCs

BALB/c mice were used as bone marrow donors. In brief, bone marrow cells were harvested by flushing femurs and tibias with Mesencult basal medium (StemCell technologies). Bone marrow was either used for magnetic bead sorting (see section below) or for culturing of mesenchymal stem cells (MSCs) using the Mesencult proliferation kit with Mesenpure for mouse (StemCell technologies). MSCs were maintained at 37°C, 3% O_2, 5% CO_2 and medium was refreshed every three days. To induce differentiation MSCs were plated at a density of 40.000 cells/well (for adipocytes) or 80.000 cells/well (for osteoblasts) in a 12-wells dish at day 0 and after 2 days the MSC medium with Mesenpure was replaced by either osteogenic differentiation medium or adipogenic differentiation medium (both from StemCell technologies).

Magnetic bead sorting

A MACS lineage cell depletion kit mouse (Miltenyi Biotec) was used for depletion of mature cells from mouse bone marrow or blood. The Lin^- cells (stem/progenitor cells) were stained with anti-mouse CD45-FITC antibody (eBioscience), incubated with Goat anti-FITC microbeads (Miltenyi Biotec) and magnetically separated using LS columns (Miltenyi Biotec) according to the manufacturers protocol. Thereby three populations were obtained: the lineage positive, mature haematopoietic cells; the lineage negative CD45 positive immature, haematopoietic stem cells and the lineage negative CD45 negative MSCs. These populations were lysed in protein lysisbuffer (1% Triton-X100, 50 mM Tris pH 8.0, 150 mM NaCl, 1x complete protease inhibitor (Roche), 0,25% Sodium deoxycholate and 0.05M sodiumfluoride) and analyzed by western blot.

Western blot

After indicated treatments cells were lysed in protein lysis buffer (1% Triton-X100, 50mM Tris pH 8.0, 150mM NaCl, 1x complete protease inhibitor (Roche), 0,25% Sodium deoxycholate and 0.05 M sodiumfluoride) and subjected to SDS-PAGE and western blotting. The membranes were blocked in 5% BSA in TBST (for incubation with anti-phospho-$cPLA_2$, anti-$cPLA_2$ and anti-actin antibodies) or 5% non-fat milk in TBST (for

incubation with anti-PLAA antibody) and incubated overnight in the antibodies diluted in 5% BSA in TBST (phospho-cPLA$_2$: 1:1000, cPLA$_2$: 1:1000 and actin: 1:50.000) or 5% non-fat milk in TBST (PLAA: 1:1000). Secondary antibodies goat-anti mouse HRP (Santa Cruz) or goat-anti rabbit (Life Technologies) used for detection were diluted 1:7500 in 5% BSA in TBST or 5% non-fat milk in TBST.

Lipid content analysis

MSCs, adipocytes and osteoblasts were harvested 0, 3, 7, 14 or 21 days after induction of differentiation. In brief, cells were washed twice in cold PBS, scraped from the culture dish in ice cold PBS and spun for 5 minutes at 300 g at 4°C. PBS was aspirated and the cell pellets were snap frozen at -80°C and stored until processing. Total lipids from the cell pellets were extracted using a modified Bligh & Dyer extraction according to Retra *et al*[23]. the lipids were fractionation by HPLC using a C18 HALO column and analyzed by mass spectrometry. Data were recorded with Analyst v1.4.2 software (MDS Sciex, Concord, ON, Canada) and exported to mzXML format. Principle component analysis (PCA) was performed using the PCA methods package (pcaMethods)[24].

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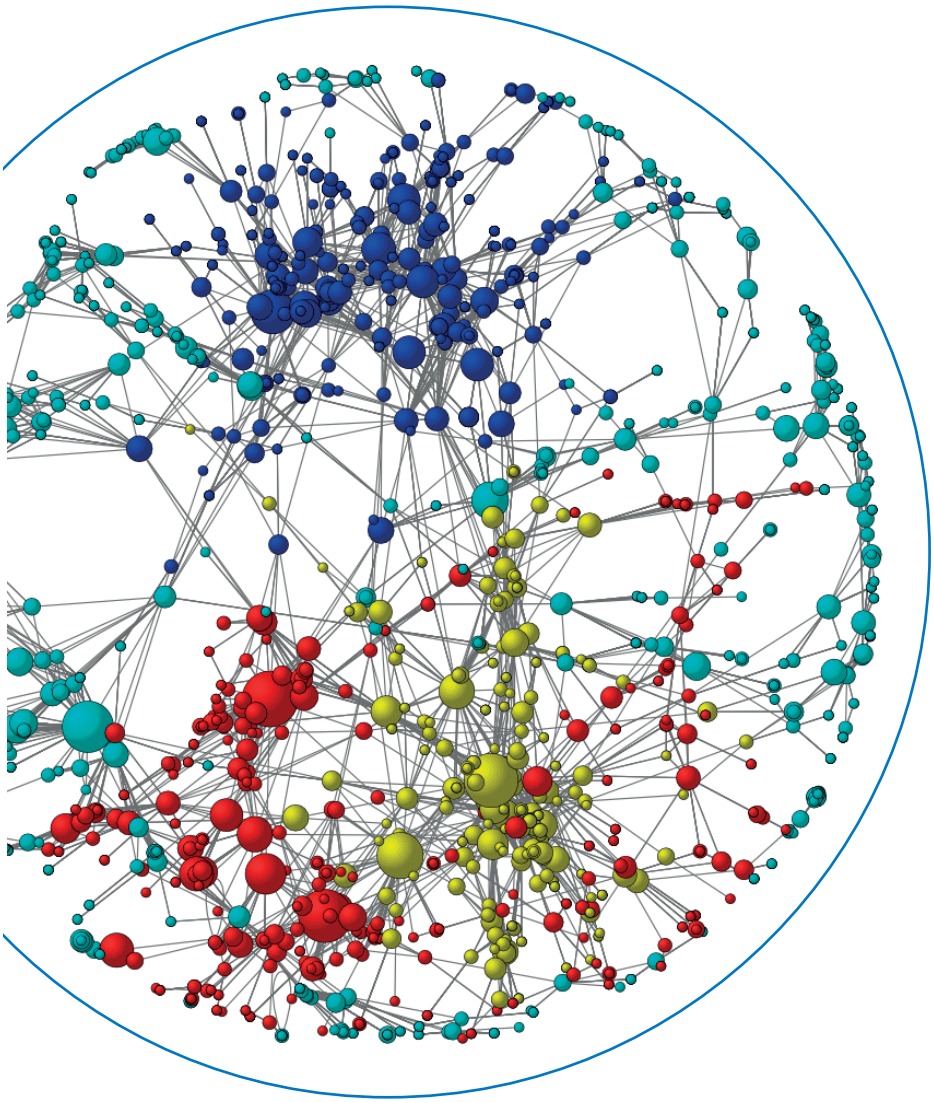
CHAPTER 7



General Discussion

Chemotherapy remains one of the most used anti-cancer treatments. Resistance against chemotherapy is a major hurdle in the effective treatment of cancer patients. Whether resistance will occur or not is difficult to assess, since numerous resistance mechanisms have been described. Various prognostic and predictive biomarkers for resistance have been described but often they are not applicable to a wide variety of tumors or are present in a small fraction of patients. Most of these markers are expressed or presented by tumor cells, for example, KRAS or EGFR mutations in lung and colon cancer, BRCA1 and BRCA2 mutations in breast and ovarian cancer, ADAMTS mutations in ovarian cancer and microsatellite instability in colorectal tumors[1-6]. However non-neoplastic host cells in the tumor microenvironment and even at distant sites can also affect the response of the tumor to chemotherapy[7].

Chapter 1 summarizes research by multiple other groups that show how non-neoplastic cells can affect the response to anti-cancer drugs, with a focus on mesenchymal stem cells (MSCs) and macrophages. MSCs are notorious for their ability to home to damaged tissues and their immune regulatory properties. These functions can also impact a developing tumor since the distorted structure of a tumor often mimics damaged tissue and can attract a variety of immune cells. Various cytokines like SDF-1α, VEGF and IL-6 are key players in the crosstalk between MSCs and tumor cells. In addition, macrophages also home to tumors, where they are called tumor-associated macrophages (TAMs). Large infiltrates of macrophages have been correlated with decreased anti-cancer drug response and decreased overall survival in multiple tumor types like breast cancer, lung cancer, head-and-neck cancer and colon cancer[8-15]. Unlike macrophages, which are often accepted as undesirable in developing tumors, research in the field of 'MSCs in cancer' shows more disagreement as MSCs can have positive and negative effects on tumor progression and therapy resistance[16, 17]. Most likely this difference can be attributed to the different tumor types investigated, the source and amount of MSCs used and other cell types in the microenvironment.

The research described in this thesis displays a novel mechanism in which the signaling between MSCs and macrophages leads to decreased efficacy of DNA-damaging chemotherapeutics. There are two main characteristics that set our research apart from the current literature. First, the mechanism by which the MSCs and splenic macrophages induce resistance depends solely on lipid signaling rather than cytokines and second, the MSCs and macrophages do not require direct contact with each other or the developing tumor but can establish chemoresistance in a systemic way. In chapter 2 we showed that the PIFAs produced by MSCs in response to platinum-containing chemotherapeutics do not induce resistance directly to tumor cells but function via a distinct population of red pulp splenic macrophages, the F4/80^{+}/CD11b^{low} macrophages. The spleen harbors four

distinct populations of macrophages each with their own function and localization in the spleen[18-21]. The red pulp macrophages (F4/80 positive) are, as their name suggests, located in the red pulp of the spleen, this is the tissue located around the T- and B-cell harboring follicles (also called white pulp). They are important scavenging cells involved in the elimination of foreign particles and aged erythrocytes from the blood[19]. The marginal zone macrophages (MARCO and SIGNR1 positive) and metallophilic marginal zone macrophages (MOMA-1/CD169 positive) are both located on the border of red and white pulp (marginal zone) and are important in trapping antigens from the circulation and presenting them to the T- and B-cells in the white pulp[20-21]. And finally the white pulp macrophages (CD68 positive), they are important in the phagocytosis of apoptotic B-cells that arise during somatic hypermutation and isotype switching[20-21]. The PIFAs function specifically on the $F4/80^{+}/CD11bl^{ow}$ macrophages located in the red pulp, as MOMA-1/CD169 positive macrophages or $F4/80^{+}/CD11b^{high}$ macrophages cannot induce chemoresistance upon stimulation with PIFAs. Only a one-hour stimulation with PIFA is sufficient to activate the macrophages leading to secretion of six unsaturated LPCs that induce chemoresistance. For one of the PIFAs, 12-S-HHT, we found that the signaling leading to the LPCs and resistance is dependent on the BLT2 receptor. Genetic loss of BLT2 or inhibition of BLT2 signaling using a small molecule inhibitor was sufficient to prevent 12-S-HHT mediated chemoresistance. In the literature BLT2 signaling has been shown to be of importance in tumor progression and even cisplatin resistance in ovarian cancer patients[22-25]. However these effects are attributed to BLT2 overexpression on tumor cells whereas we show that BLT2 signaling on normal cells at a distant site from the tumor can also affect chemotherapy treatment. Additional research is needed to determine if PIFA-mediated chemoresistance specifically requires splenic red pulp macrophages or if other macrophages can have similar effects. Our findings that splenectomy is sufficient to prevent chemoresistance *in vivo* would indicate that tissue resident macrophages of other tissues cannot take over the function of the splenic macrophages. Preliminary data shows that tumor-associated macrophages (TAMs) derived from K14cre;$Cdh1^{F/F}$;$Trp53^{F/F}$ mice could not induce PIFA-mediated chemotherapy resistance, indicating that at least these TAMs do not respond like splenic macrophages. Due to the diversity and plasticity of macrophages further research will be needed to assess if other TAMs can generate PIFA-mediated chemoresistance.

In chapter 3 we found that 16:4(n-3)-mediated chemoresistance depends on GPR120 signaling. A specific GPR120 inhibitor could prevent 16:4(n-3)-mediated resistance, whereas a specific GPR40 inhibitor could not. Furthermore, genetic loss of GPR120 confirmed our findings obtained with GPR120 inhibitor. GPR120 has been shown to bind medium-to-long chain fatty acids including omega-3 fatty acids. It is expressed on adipose tissue, macrophages and taste buds and is involved in insulin signaling, taste perception

and anti-inflammatory functions[26-30]. Aberrant GPR120 signaling has been linked with obesity and diabetes[26,30]. Despite the lack in structural homology between GPR120 and GPR40, another medium- to long-chain fatty acid receptor linked to insulin signaling, they share various ligands of which alfa-linolenic acid (αLA), EPA and DHA are the most familiar ones. In cancer biology GPR40 and GPR120 seem to have opposing functions. Fukushima *et al*. showed that loss of GPR40 in pancreatic cancer cells promoted migration whereas loss of GPR120 inhibited migration[31]. GPR120 has been shown to promote angiogenesis and metastasis in colorectal cancer but seems to inhibit tumor growth in prostate cancer cells[32-33]. Furthermore, Mobraten *et al*. showed that EPA, AA and DHA could elicit similar signaling events in GPR120 expressing cells but with different potency[34]. These seemingly contradictory results could be explained by the ligands and concentrations used, the cell types studied, availability of downstream signaling molecules, splice variants of the receptor or even the presence of other fatty acid receptors with affinity for medium-to-long chain fatty acids[35]. To add to the complexity, differences are also seen when comparing *in vitro* versus *in vivo* studies. We showed that 16:4(n-3) could bind and activate both GPR40 and GPR120 *in vitro* with similar efficiency. However, only signaling via GPR120 was relevant for 16:4(n-3)-mediated chemoresistance *in vivo* despite the fact that both GPR40 and GPR120 are expressed by splenic macrophages. These findings indicate that we are just beginning to understand the complexity of fatty acid signaling and that further research is necessary to determine ligand and receptor specificity.

Previously we identified 6 unsaturated LPCs in the conditioned medium derived from splenic macrophages stimulated with both PIFAs. In chapter 3 we found that one of these LPCS, LPC(24:1) is specifically released from splenic macrophages upon 16:4(n-3) exposure and that administration of nanomolar concentrations of LPC(24:1) induced chemoresistance *in vivo*. Additional clinical research could provide insight into whether the presence of LPC(24:1) in patient plasma can predict chemotherapy response.

Little is known about the function of LPCs and in particular LPC(24:1) in tumor progression and anti-cancer drug resistance. Sneider *et al*. showed that LPC and lysophosphatidic acid (LPA) induce migration and metastasis of rhabdomyosarcoma *in vitro* and *in vivo*. In addition, LPC and LPA levels increased when mice were treated with either vincristine or radiotherapy postulating the hypothesis that the treatment-induced increase in LPC and LPA would facilitate metastasis[36]. On the contrary, Raynor *et al.* showed that LPCs are rapidly metabolized by cancer cells and that they can prevent metastasis[37]. A great deal of controversy has marked the field of LPC research, especially in the context of receptors for LPC. Originally, GPR4 was identified as a receptor for LPC, however this paper was retracted 4 years later[38]. Similarly, the paper describing the pH-sensitive receptor G2A (GPR132) as a receptor for LPC was also retracted because findings could not be

reproduced and no direct evidence exists that LPCs actually bind to G2A[39]. Nevertheless, in recent years multiple research groups found that many LPC functions depend on the presence of G2A. G2A-LPC signaling has been implicated in the polarization of macrophages to a M1 phenotype and the chemotaxis of T-cells, macrophages and natural killer (NK) cells[40-42]. Interestingly, Weng *et al*. showed that DNA damage and cellular stress induces G2A expression in B-cells leading to a block in the G2/M phase of the cell cycle[43]. Our findings show that PIFAs and LPC(24:1) can reduce DNA damage when combined with cisplatin treatment (chapter 5). However, baseline levels of DNA damage as measured by γH2AX do not change when the PIFAs are administered alone. Potentially, DNA damaging agents are needed to enhance G2A expression in tumor cells making them susceptible LPC-mediated chemoresistance. Despite a lack of evidence that G2A is expressed in tumors, it has been shown that G2A overexpression in fibroblasts can lead to oncogenic transformation like anchorage independent growth, decreased dependence on serum and tumor formation in mice[44]. Additional research is needed to determine if the tumor cells in our models express G2A and if G2A signaling is required for LPC-mediated chemoresistance.

In addition to its production in MSCs 16:4(n-3) is also present in the seaweed *Ulva pertusa* and we found that extracts of this seaweed could also induce chemoresistance in tumor-bearing mice. The widespread distribution of *Ulva pertusa* along the coasts of Asia, West-Africa, Australia and New Zealand makes it an important food source for various fish species and crustaceans[45, 46]. In addition, humans in various Asian countries consume *Ulva pertusa*[46]. In chapter 4 we found that 16:4(n-3) is also present in certain fish oils, and the concentrations are within the range known to induce chemoresistance in mice. In a healthy volunteer study we examined the effects of ingesting 10 ml (daily recommended dose) or 50 ml of 16:4(n-3)-containing fish oils on the 16:4(n-3) plasma levels over time (chapter 4). We found elevated 16:4(n-3) levels in the plasma of humans 4 hours after ingestion of fish oil. These levels returned to baseline after 8 hours in most volunteers. Interestingly, the fish oils containing relatively high levels of 16:4(n-3) (5-6 μM) were able to increase plasma 16:4(n-3) levels to values known to induce chemoresistance in mice even when they were ingested at the daily recommended dose. Furthermore, we determined that EPA, one of the main components of fish oil and a precursor molecule for 16:4(n-3), did not contributed to elevated 16:4(n-3) plasma levels and chemoresistance in mice when administered in the daily recommended dose. Only ingestion of large quantities of EPA could lead to sufficient conversion of EPA into 16:4(n-3) to induce chemoresistance in mice. The use of fish oil and other omega-3 fatty acid-containing products is being promoted as healthy. Despite the various studies describing positive effects of fish oil on cancer progression and therapy outcome[47-49], several meta-analyses shown only marginal benefits or no benefit at all[50-53]. Furthermore, a study recently

published showed that Greenlandic Inuits display genetic and physiological adaptations in order to cope with their fish and omega-3 polyunsaturated fatty acid (n-3 PUFAs)-based diet. The highest level of adaption was found in a cluster for fatty acid desaturases, which include enzymes that alter and metabolize the n-3 PUFAs[54].

A few important points need to be kept in mind when placing our study in the context of other studies. First, fish oil is a mixture of omega-3 fatty acids. The main components are EPA and DHA, for which multiple studies have indeed shown that they have anti-inflammatory properties and can inhibit tumor growth[55-58]. Depending on the production and purification process most fish oils contain 2-20% of other omega-3 fatty acids, which are not specified on the label. 16:4(n-3) is one of these other omega-3 fatty acids for which we have showed in various tumor mouse models that it induces chemoresistance. 16:4(n-3), EPA and DHA are all omega-3 fatty acids but can have different functions so attributing health benefits to omega-3 fatty acids in general is a misconception and exceptions do apply. In addition, various studies show that different omega-3 fatty acids can have different functions *in vitro* than *in vivo* and their effects can be different depending on the tissues types investigated, indicating that a more systemic approach to investigate the contribution of various omega-3 fatty acids is desirable to distinguish their effects on tumor cells[58-61]. Second, our healthy volunteer study reveals that the timing of intake is also important. 16:4(n-3) levels peaked around 4 hours after intake and returned to baseline after 8 hours when a single daily recommended dose was ingested. Combining these findings with our previous observations that PIFAs can only induce chemoresistance in mice when administered no later than 3 hours after chemotherapy clearly show that timing is of the essence. Additional research is needed to determine if the pharmacodynamics and kinetics of 16:4(n-3)-containing fish oil is comparable upon repeated use and in cancer patients.

In chapter 5 we focused on the mechanism leading to PIFA-mediated chemoresistance in the tumor cells. We found decreased γH2AX levels in tumors of mice treated with cisplatin and PIFAs compared to tumors of mice treated with cisplatin alone. In addition, the increased phospho-CHK1 levels in PIFA treated mice and the absence of resistance in mice lacking homologous recombination (HR) hints towards a mechanism in which PIFA signaling interferes with the DNA damage response and repair pathway. However, the complexity of DNA damage repair mechanisms and the lack of a proper *in vitro* model hinder us in investigating PIFA-mediated resistance in more detail. Enhancing DNA damage repair has been described as a resistance mechanism for tumor cells to overcome chemotherapy-induced cell death. For instance, overexpression of RAD51, enhanced CHK1 signaling and restoration of loss of HR by concomitant loss of 53BP1 are resistance mechanisms described for various tumor types like breast cancer, colorectal cancer,

leukemia, pancreatic cancer and lung cancer[62-67]. However, all of these mechanisms depend on tumor cells changing their genetic profile to accommodate their needs for anti-cancer drug survival. Little is known about extracellular lipids actively changing the DNA damage response and repair to generate chemotherapy resistance. One of the lipids that has been shown to influence chemotherapy sensitivity of tumor cells is lysophosphatidic acid (LPA). Mechanisms underlying LPA-induced anti-cancer drug resistance include upregulation of multidrug resistance transporters and anti-oxidant genes, normalization of mitotic spindles and release from G2/M arrest and the upregulation of pro-survival factors like TRIP-6 and sphingosine 1-phosphate and the downregulation of pro-apoptotic factors like Siva-1[68-70]. Interestingly, we found that the resistance molecules secreted by splenic macrophages are LPCs and LPC can be converted into LPA by autotaxin (ATX). Preliminary data indicate that inhibition of ATX does not inhibit LPC(24:1)-mediated chemoresistance. Nevertheless, more research is needed to investigate this in more detail, for instance testing LPC(24:1) and the other unsaturated LPCs in $ATX^{-/-}$ mice or chemically converting LPC(24:1) into LPA(24:1) and testing its chemoresistance inducing potential.

In chapter 6 we investigated the determinants of PIFA production in MSCs. Previous research by our lab showed that only MSCs and mouse embryonic fibroblasts (MEFs) were able to generate PIFAs and subsequent chemoresistance, whereas differentiated adipocytes, osteoblasts and other stem cells could not[71]. PIFA production depends on $cPLA_2$, COX-1, TXAS and iCa^{2+}, since inhibitors of these enzymes and co-factors inhibited chemoresistance[71]. Since $cPLA_2$ is the rate-determining step in the cascade leading to PIFA (and eicosanoid) production we focused on this enzyme. Analysis of bone marrow fractions and cultured MSCs showed that $cPLA_2$ and its active form phospho-$cPLA_2$ is only expressed in MSCs and not in mature hematopoietic cells, hematopoietic stem cells or differentiated adipocytes and osteoblasts. In addition, cisplatin treatment was able to induce an increase in phospho-$cPLA_2$ as well as the activator of $cPLA_2$, PLAA, in MSCs but not in adipocytes or osteoblasts. Treatment of MSCs with a non-platinum based chemotherapeutic SN-38 did not induce phospho-$cPLA_2$ or PLAA levels, confirming our findings that PIFA production is activated by platinum. The significant downregulation of $cPLA_2$ upon differentiation and the lack of induction upon cisplatin treatment could explain the specificity of MSCs in PIFA production. In addition, the lipid profile of MSCs changes over the course of differentiation into adipocytes and osteoblasts, however no apparent changes were seen in EPA and AA content, the precursors of PIFAs. It has been shown before that cisplatin can induce PLAA expression and subsequent $cPLA_2$ activation, however it is not yet known exactly how cisplatin enhances PLAA expression[72] . It is likely to be an active process since multiple cell types express PLAA (like osteoblasts and adipocytes) but not all cells respond to cisplatin treatment by increasing PLAA. Additional research is needed to dissect the exact mechanism leading to cisplatin-induced PLAA and

$cPLA_2$ activity and PIFA production. The findings that $cPLA_2$ is essential in PIFA production in the MSCs as well as in LPC(24:1) production in 16:4(n-3)-stimulated splenic macrophages makes this enzyme a potential therapeutic target to enhance chemotherapy efficacy. Inhibition of $cPLA_2$ does not only prevent endogenous PIFA production but can also potentially prevent the effects of exogenous intake of 16:4(n-3) like in fish oil, by stopping the LPC(24:1) production in splenic macrophages. Inhibition of cPLA2 in colorectal cancer cells has been shown to suppress proliferation and can function as a radiosensitizer in an animal model for lung cancer and in ovarian cancer cells *in vitro*[73-75].

Taken together our research uncovered a systemic lipid signaling cascade that reduces chemotherapy sensitivity leading to continued tumor growth. Interestingly, the process is rapid and reversible and requires only nanomolar concentrations of the effector molecules. Additional research will provide more insight in the mechanism of LPC-induced resistance in the tumor cells and ongoing studies will determine if the amount of circulating MSCs, PIFAs or LPC(24:1) can function as markers for therapy response. The insights provided into the mechanism leading to PIFA and LPC production could, in the future, provide potential targets to influence chemotherapy efficacy.

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CHAPTER 8

Nederlandse samenvatting

Het onderzoek beschreven in dit proefschrift is gericht naar het identificeren van specifieke vetzuren geproduceerd door lichaamseigen cellen die de effectiviteit van chemotherapie kunnen verlagen.

Het eerste hoofdstuk betreft een algemene introductie waarin beschreven wordt hoe verschillende gezonde cellen in ons lichaam de groei van een tumor kunnen beïnvloeden. Een groot deel van de kankerpatiënten wordt behandeld met chemotherapie. Chemotherapie is erop gebouwd om de tumorcellen te doden en de gezonde cellen te sparen. Zoals de vele bijwerkingen van chemotherapie doen verwachten hebben ook de gezonde cellen in ons lichaam last van de behandeling. De reactie van de gezonde cellen op de chemotherapie kan voor een deel bepalen hoe de tumorcellen op de behandeling reageren. Dit noemen we de gastheer-reactie.

In hoofdstuk 2 wordt beschreven hoe specifieke cellen in de milt genaamd macrofagen (Grieks: grote eters, makros=groot, phagein=eten) resistentie kunnen opwekken tegen chemotherapie. In eerder onderzoek hebben we laten zien dat specifieke stamcellen in het beenmerg genaamd mesenchymale stamcellen (MSCs), als reactie op chemotherapie twee vetzuren produceren. Deze vetzuren hebben wij platinum-geïnduceerde vetzuren (platinum-induced fatty acids, PIFAs) genoemd omdat deze alleen door de MSCs gemaakt worden als ze in aanraking komen met platinum-houdende chemotherapie. De PIFAs 12-S-HHT en 16:4(n-3) kunnen de werking van chemotherapie teniet doen. Echter de PIFAs werken niet direct op de tumorcellen maar functioneren via de milt. De milt bestaat uit verschillende soorten immuuncellen en de voornaamste functie van de milt is het controleren van het bloed op de aanwezigheid van bacteriën en andere ziekteverwekkers. Daarnaast zorgt de milt ook voor het verwijderen van oude rode bloedcellen. In de milt bevinden zich onder andere macrofagen. Deze cellen zijn in staat resten van dode of beschadigde cellen en lichaamsvreemde deeltjes zoals bacteriën op te eten. Het zijn deze cellen in de milt die zorgen voor de recycling van oude rode bloedcellen. Met behulp van verschillende tumormuismodellen hebben we kunnen laten zien dat de macrofagen aanwezig in de milt geactiveerd kunnen worden door de PIFAs en als gevolg daarvan een zestal onverzadigde lysophosphatidylcholines (LPCs) uitscheiden. Deze LPCs zijn ook lipiden (net als de PIFAs) en zorgen ervoor dat de tumorcellen ongevoelig worden voor de chemotherapie. Van één van de PIFAs, 12-S-HHT, hebben we in dit hoofdstuk laten zien dat deze functioneert via activatie van de leukotriene B4 receptor 2 (BLT2). Dit is een receptor die op de miltmacrofagen zit. Receptoren op cellen zorgen ervoor dat signalen van buitenaf herkent en verwerkt worden en zijn te vergelijken met onze zintuigen. Muizen die de BLT2 receptor niet hebben zijn ongevoelig voor de effecten van 12-S-HHT.

In hoofdstuk 3 beschrijven we de werking van PIFA 16:4(n-3) op de miltmacrofagen. Door genexpressie analyse en studies met verschillende remmers hebben we gevonden dat de receptor voor 16:4(n-3) GPR120 is. Door deze receptor te remmen in muizen kan er geen 16:4(n-3)-gemedieerde chemoresistentie plaatsvinden. Activatie van GPR120 door 16:4(n-3) zorgt ervoor dat de macrofagen een enzym ($cPLA_2$) aanzetten wordt waardoor LPCs geproduceerd worden. Deze LPCs worden uitgescheiden door de macrofagen en gaan naar de tumorcellen waar ze chemoresistentie bewerkstelligen.

Naast de lichaamseigen productie van PIFAs door MSCs kan 16:4(n-3) ook geproduceerd worden door zeewier genaamd *Ulva Pertusa*. Deze zeesla komt veel voor rond Azië, Australië en Nieuw-Zeeland. Het vormt een belangrijke voedselbron voor vissen, schaal- en schelpdieren en in Azië wordt het ook door mensen gegeten. In hoofdstuk 4 laten we zien dat 16:4(n-3) ook aanwezig is in verschillende soorten visolie en dat de hoeveelheden op basis van muizenstudies voldoende zijn om chemotherapie resistentie te bewerkstelligen. Deze bevinding heeft ertoe geleidt dat we een gezonde vrijwilligers-studie hebben opgezet om te bepalen wat er gebeurd met de 16:4(n-3) in visolie na inname. Dertig vrijwilligers hebben 50 ml of 10 ml (dagelijks aanbevolen hoeveelheid) visolie geconsumeerd waarna er op verschillende tijdspunten bloed werd afgenomen om de concentratie 16:4(n-3) in het bloedplasma te meten, met de rationale dat, als 16:4(n-3) in ongemetaboliseerde vorm in het bloed kan worden opgenomen deze ook in theorie de tumor (en milt) van een patiënt kan bereiken. De geteste visoliën waren in staat de bloedplasma 16:4(n-3) waarden te verhogen met een maximum 4 uur na inname. Bij de meeste gezonde vrijwilligers gingen de 16:4(n-3) waarden terug naar het beginniveau binnen 8 uur. In de groep vrijwilligers die 50 ml visolie hadden gedronken duurde dit langer. De gevonden waarden waren in theorie voldoende voor chemotherapie resistentie. Naast visolie hebben we ook verse vis geanalyseerd en vonden dat 16:4(n-3) aanwezig is in vette vis zoals makreel en haring maar niet of nauwelijks in zalm en tonijn. Ook de inname van 100 gram haring of makreel leidde tot verhoogde 16:4(n-3) waarden in het bloed van de vrijwilligers, terwijl een dergelijke stijging uitbleef na consumptie van 100 gram zalm of tonijn. Op basis van onze data raden we aan dat kankerpatiënten een dag voor en na toediening van de chemotherapie geen visolie of vette vis zoals haring en makreel consumeren. Ons advies is overgenomen door het KWF en de Landelijke Werkgroep Diëtisten Oncologie (LWDO).

In hoofdstuk 5 duiken we in het mechanisme van resistentie op de tumorcellen. De PIFAs geven resistentie tegen chemotherapeutica die DNA schade induceren. Als een cel getroffen wordt door DNA schade kan het twee dingen doen, de cel kan de schade herstellen of kan dood gaan (apoptose). Als de DNA schade herstelt kan worden treedt de DNA schade respons op. Dit is een complex proces waarbij veel verschillende eiwitten bij

betrokken zijn. Als een cel DNA schade detecteert worden de reparatie-eiwitten erheen gestuurd om de schade te herstellen. γH2AX vormt als het ware een herkenningspunt op het stuk beschadigde DNA zodat de reparatie-eiwitten weten waar de schade zich bevindt en deze kan herstellen. De PIFAs en LPCs zorgen voor een verlaging van γH2AX in de tumor binnen 4 uur na toediening van chemotherapie en PIFAs. Deze verlaging en de daaruit voorvloeiende resistentie kan op twee manier verklaard worden: de schade wordt niet herkend of genegeerd of de schade wordt sneller herstelt. Analyse van andere eiwitten betrokken bij de reparatie van DNA schade zoals CHK1 waren verhoogd aanwezig in tumoren behandelt met chemotherapie en PIFAs (of LPCs) ten opzichte van tumor behandelt met alleen chemo. Daarnaast is PIFA-gemedieerde chemoresistentie afwezig in muizen behandeld met een CHK1 remmer. Dit wijst dus op herstel van de schade. Ook experimenten gedaan met muizen die geen DNA schade respons hebben lieten zien ongevoelig te zijn voor de werking van de PIFAs. Het lijkt er dus op dat PIFAs en LPCs interfereren met de DNA schade respons met als gevolg dat de schade sneller hersteld kan worden waardoor de tumorcellen de chemotherapie overleven. Meer onderzoek is nodig om andere spelers in dit proces te identificeren en een completer plaatje te krijgen van het mechanisme van PIFA- en LPC-geïnduceerde chemoresistentie.

Hoofdstuk 5 brengt ons terug naar het begin van het onderzoek waarin we hebben laten zien dat de PIFAs gemaakt worden door MSCs. Verschillende experimenten laten zien dat de PIFAs alleen gemaakt kunnen worden door MSCs en niet door andere celtypes. De vraag is; wat maakt een MSC zo speciaal dat alleen zij PIFAs kan maken? De productie van PIFAs is afhankelijk van een aantal enzymatische stappen die lipiden aanwezig in de cel op een specifieke manier knippen zodat 16:4(n-3) en 12-S-HHT gevormd worden. Het eerste en belangrijkste enzym in dit proces is $cPLA_2$. Dit is een fosfolipase die de vetmoleculen waaruit het celmembraan bestaat kan knippen in een vrij vetzuur en een lysofosfolipide. Het zo gevormde vrije vetzuur is nodig voor de volgende stappen die uiteindelijk leiden tot PIFAs. Analyse van MSCs en hun gedifferentieerde dochtercellen zoals vetcellen en botcellen demonstreert dat alleen MSCs $cPLA_2$ tot expressie brengen. Stimulatie van MSCs met het platinum-houdende chemotherapeuticum cisplatine zorgt voor activatie van $cPLA_2$ terwijl de niet-platinum houdende chemotherapie irinotecan dit niet doet. Dit bevestigt onze eerdere data dat PIFAs alleen gemaakt worden als de MSCs blootgesteld worden aan platinum-houdende chemotherapie. De experimenten beschreven in dit hoofdstuk geven meer inzicht in de specifiek functie van MSCs in PIFA productie. Meer onderzoek is nodig om te bepalen waarom dit proces afhankelijk is van platinum en wat de fysiologische rol van de PIFAs is.

Het onderzoek beschreven in dit proefschrift geeft inzicht in hoe lipiden chemotherapie resistentie kunnen opwekken. Naast de lichaamseigen productie van 12-S-HHT en 16:4(n-

3) als reactie op platinum-houdende chemotherapie bevindt 16:4(n-3) zich ook in vette vis en visolie. Onderzoek naar de exacte werking van de PIFAs, de miltmacrofagen, LPCs en de DNA schade respons in tumoren geeft ons mogelijkheden om dit proces te beïnvloeden en chemotherapie beter te laten functioneren.

CHAPTER 9

Dankwoord
List of publications
Curriculum vitae

Dankwoord

Daar is het dan eindelijk! Mijn proefschrift. Wetenschappelijk onderzoek doe je nooit alleen en daarom wil ik iedereen die een bijdrage geleverd heeft hartelijk bedanken. Een aantal van hen verdienen een bijzondere vermelding:

Prof. dr. E.E. Voest, beste Emile, dank je wel dat je mij de mogelijkheid hebt gegeven aan het PIFA-project te mogen deelnemen. Toen ik mijn open sollicitatie stuurde gaf je aan alleen plek te hebben op een zeker next-generation tumor sequencing project. Aangezien 4 jaar lang tumoren sequencen me helemaal niets leek ging ik daarom ook zeer relaxed het sollicitatiegesprek in. Toen je tijdens dit gesprek aangaf toch ook wel een plekje op het MSC project te hebben wist ik dat ik me daar wel in wilde vastbijten! Zo gezegd, zo gedaan. En nu ruim 5 jaar later zijn we toch een behoorlijk stuk opgeschoten met onze gekke vetzuren ☺. Je klinische blik op fundamenteel onderzoek heeft me altijd geïnspireerd en je wist altijd de rode draad van het onderzoek in het vizier te houden. De vrijheid die je mij gegeven hebt in mijn onderzoek heeft mij nog zelfstandiger gemaakt en is voor mij van onschatbare waarde geweest.

Prof. dr. R.H. Medema, beste René, dank je wel dat je mij en Laura als adoptie-PhD studenten hebt opgenomen in je groep in de periode dat Emile op sabbatical was. Je scherpe en kritische blik op het onderzoek werd door ons beide zeer gewaardeerd. Dank voor begeleiding en het plaatsnemen in mijn leescommissie.

Prof. dr. P.J. van Diest, beste Paul, de werkbesprekingen van de moleculaire pathologie staan me nog helder voor de geest. Meestal kwam je te laat, zat dan ook nog 20% van de tijd op je telefoon te kijken maar wist toch altijd de million-dollar-question te stellen aan degene die stond te presenteren. Ik zat vaak vol bewondering naar je commentaren en suggesties te luisteren en wist als ik jouw vragen kon beantwoorden ik het goed gedaan had. Daarom heb ik geen moment getwijfeld om jou te vragen voor mijn leescommissie. Hartelijk dank voor kritisch doorlezen van mijn proefschrift en het deelnemen aan mijn commissie.

Dr. K. de Visser, lieve Karin, al vroeg in mijn PhD traject zat ik bij je in het NKI om te brainstormen over hoe onze miltmacrofagen via vetzuren chemotherapie resistentie konden veroorzaken. Dank je voor je talloze suggesties en kritische vragen. Jouw enthousiasme voor de wetenschap en bereidheid te helpen waar je kan zijn voor mij heel belangrijk geweest. Jouw glas-is-half-vol mentaliteit en gezelligheid zijn zeer aanstekelijk

en zijn vaak een goede peptalk voor mij geweest. Dank je wel dat je erbij bent op mijn verdediging en voor het beoordelen van mijn proefschrift.

Prof. dr. B. Haribabu, dear Hari, thank you very much for your hospitality when I came to your lab for the $BLT2^{-/-}$ mice. It has been a wonderful experience for me both scientifically and personally and I really appreciated the welcoming atmosphere in the lab. It is a pleasure to have you in my thesis committee and I really appreciate you coming over to the Netherlands to participate in my defense.

Prof. dr. P.O. Witteveen, beste Els, ik wil je hartelijk bedanken voor het beoordelen van mijn proefschrift en het plaatsnemen in mijn commissie.

Mijn lieve paranimfen, Ilse, we zijn rond dezelfde tijd aan onze PhD-carrière begonnen en wat hebben we toch een hoop meegemaakt! Vanaf het moment dat we kamergenoten zijn geworden zijn we begonnen met kletsen en niet meer opgehouden ☺. We hebben lief en leed gedeeld en behoorlijk wat late uurtjes in het GDL. Je doorzettingsvermogen is bewonderenswaardig en daar kunnen veel mensen nog wat van leren! Onze vriendschap is niet in een paar regels te omschrijven. Dank je wel dat je aan mijn zijde wilt staan als paranimf. Lieve Nicolle, ik weet nog goed de eerste barbecue bij Emile waar wij als nieuwelingen tussen de gevestigde orde zaten. Onze vriendschap is voor mij heel belangrijk en ik vind het super dat je straks naast me staat als mijn paranimf. Op nog heel veel gezellige etentjes die altijd weer eindigen in nog een uur nakletsen in de gang met ons jas al aan.

Alle Voest-labgenoten (oude en nieuwe garde), beste Jeanine, jouw doortastendheid en harde werk heeft het PIFA-project al op vroeg stadium tot grote hoogte gebracht. De solide basis die jij hebt gelegd is essentieel geweest voor mijn onderzoek. Dank je wel voor je betrokkenheid en veel succes in de kliniek. Beste Laura, jouw onvermoeibare energie en go-getter mentaliteit zijn een inspiratie voor mij geweest. Je hebt prachtige resultaten behaald binnen het PIFA/visolie project en daarbuiten. Veel succes met je carrière in de kliniek! Krijn, (aka Boss-man) dank voor je input betreffende mijn onderzoek en veel succes met je immuno-project, als er iemand is die het tot een succes kan maken, ben jij het! Dear Chelsea, your love for research and enthusiasm is contagious. I wish you a lot of luck with your project, I'm sure you'll rock it! I will definitely drop by for a coffee and a chat in May. Dear Sovann (aka Grumpy cat), thanks for your help and sharing the frustrations of PhD-life. I will miss your sense of humor and straight-forward mentality. Good luck with finishing your thesis (I expect a copy, obviously!). Salo (aka Diesel), nog nooit heb ik iemand zoveel zien eten op een dag (en ik dacht dat ik wel wat gewend was met Krijn ☺). Veel succes met je fusiegenen project en alles wat er nog op je pad mag

komen. Het gaat je goed en dank voor de gezelligheid op onze Voest-kamer. Daphne, het bleek toch nog wel even een uitdaging, 40 patienten FACSen op zoek naar circulerende MSCs met nog maar een maandje de tijd, maar het is ons gelukt! Dank voor je gezelligheid en de goede samenwerking en veel succes met de verdere projecten. Fleur, dank voor de gouden tip voor als je Emile wilt spreken (aan Alice vragen wanneer hij in de auto zit en dan bellen, dan neemt hij altijd op). Of had ik deze tip hier niet mogen blootgeven? Anyway, dank je wel voor je input tijdens werkbesprekingen en de gezelligheid. We zien elkaar nog op de AACR! Myriam, heel veel succes met je PhD-traject en natuurlijk je werkzaamheden als arts in het NKI. Marijn, dank je wel voor je versterking aan het PIFA-team en al onze gezellige uitjes en tripjes naar het kuuroord. Ilse en ik komen je snel een keer opzoeken in Berlijn. Sahar, dank je wel voor de nodige q-PCR ondersteuning aan mijn project en de gezelligheid op onze meiden-kamer in het UMC.

En natuurlijk niet te vergeten Marco, Geert, Christa, Marlous, Stef, Martijn, Wijnand, en alle collega's uit het Medema, Kops, Lens, Derksen en Peeper-lab. Dank voor jullie hulp en gezelligheid.

De studenten die mij geholpen hebben: Lizet van der Waals, Clara Oudenaarden en Kevin Kos. Kevin, dank je voor al je hulp bij het afronden van hoofdstuk 5. Ik wens je heel veel succes in je verder carrière.

Alle GDL medewerkers, en in het bijzonder Paul, Helma, Anja en Yvonne. Dank voor jullie goede zorgen voor mijn muizen. Paul, dank voor de afleiding en gezelligheid als ik weer eens om 19.00 uur in het GDL verscheen om mijn muizen te meten. En natuurlijk dank aan alle muizen die onderdeel zijn geweest van mijn studies, zonder jullie had dit proefschrift hier nooit gelegen.

Alle collaborators: Chris, zonder jouw hulp had ik nooit factor X kunnen vinden! Je hulp en expertise op het gebied van lipiden en massa spectrometry zijn essentieel geweest voor onze gezamelijke Nature Communications publicatie. Proost op onze vruchtbare samenwerking! Akira, thank you very much for your kindness, hospitality and all your help with the GPR120$^{-/-}$ mice, they were the missing link in my manuscript. Kenji, many thanks for supplying us with numerous batches of 16:4(n-3) for our studies. Without this, our research would have been a lot more complicated. Dear Graeme, your critical view on my research and manuscript has been of great importance. Please make sure to also pass on my gradidute to Brian, how performed all the in vitro studies with 16:4(n-3). It was very nice to meet you in Amsterdam and I look forward to our continued collaboration. Trond, thank you so much for supplying us with the selective GPR120 and GPR40 agonists and

antagonists for testing. They have been proven vital in our understanding of 16:4(n-3)-mediated chemoresistance and will hopefully soon lead to a nice joined publication.
Nanda en Johan, dank voor het doormeten van de talloze samples op de aanwezigheid van de PIFAs. Saskia, dank je voor je hulp met de traffic light reporter en heel veel succes met je verdere carrière.

Lieve Kelly, mijn sushi-maatje ☺, dank je wel voor je steun, humor en gezelligheid door de jaren heen.

Lieve paps en mams, jullie kunnen natuurlijk niet ontbreken in dit dankwoord. Jullie hebben als geen ander de highs en lows van het onderzoek gezien. Dank jullie wel voor jullie onvoorwaardelijke steun, niet alleen gedurende mijn PhD maar ook daarbuiten. Ik hou van jullie!

En last but not least…mijn lieve verloofde Ronald. Wie had gedacht dat we elkaar zouden vinden op zo'n suffe schietvereniging ☺. Dank je wel voor je steun en begrip als ik weer eens om 21.00 uur thuiskwam. Je relativeringsvermogen en frisse blik op dingen als niet-onderzoeker hebben vaker geholpen dan je denkt. Ik hou zielsveel van je! WBWY! X

Julia

List of publications

JM Houthuijzen, I Oosterom, BD Hudson, A Hirasawa, LGM Daenen, CM McLean, SVF Hansen, MTM van Jaarsveld, DS Peeper, S Jafari Sadatmand, JML Roodhart, CHA van de Lest, T Ulven, K Ishihara, G Milligan, EE Voest. GPR120/FFAR4 activation by fatty acid 16:4(n-3) plays a key role in resistance to chemotherapy. *Under review at Nature Communications*

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JML Roodhart, LGM Daenen, EC Stigter, HJ Prins, J Gerrits, JM Houthuijzen, MG Gerritsen, HS Schipper, MJ Backer, M van Amersfoort, JS Vermaat, P Moerer, K Ishihara, E Kalkhoven, JH Beijnen, PW Derksen, RH Medema, AC Martens, AB Brenkman, EE Voest. (2011) Mesenchymal stem cells induce resistance to chemotherapy through the release of platinum-induced fatty acids. *Cancer Cell* 2011; 20(3)

Curriculum Vitae

Julia Houthuijzen was born on the 6th of August 1987 in Heemskerk. She attended primary school in Heemskerk and Gorinchem. She obtained her VWO diploma from C.S.G Oude Hoven in Gorinchem. Afterwards she studied Biomedical Sciences at the University of Utrecht where she obtained her Bachelor's degree. During her Master program Biology of Disease she worked for 9 months in the laboratory of Prof. Marc Vooijs at the Pathology Department at the University Medical Center Utrecht. Under the supervision of Dr. Iordanka Ivanova she investigated the role of Fer-kinase in breast cancer metastasis. Her second internship was in the group of Dr. Louk Vanderschuren at the Rudolf Magnus Institute in Utrecht. Together with Dr. Heidi Lesscher she investigated the role of 14-3-3ζ in alcoholism using animal models. Julia wrote her Master thesis entitled 'The role of polyunsaturated fatty acids in cancer and chemotherapy resistance' under the supervision of Dr. Laura Daenen in the group of Prof. Emile Voest. In 2010 Julia obtained her Master's degree and started her PhD in the group of Prof. Emile Voest. The results obtained during this project are described in this thesis.