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Mass-spectrometric characterization of peroxidized and hydrolyzed lipids in plasma and dendritic cells of tumor-bearing animals

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ABSTRACT

Dendritic cells are the most potent antigen presenting cells responsible for the development of immune responses in cancer. However, it is known that the function of dendritic cells in tumor-bearing hosts is severely compromised. Our previous studies demonstrated that the defects in dendritic cell function are due, to a large extent, to the accumulation of high amounts of lipids – predominantly triglycerides – in a substantial proportion of dendritic cells in tumor-bearing mice and patients with cancer. The dendritic cells accumulation of lipids is likely associated with their up-regulation of a scavenger receptor A. This receptor is primarily responsible for uptake of modified lipids. Here, by using different versions of liquid chromatography–mass spectrometry, we identified several molecular species of oxygenated lipids in plasma of tumor-bearing animals that may be responsible for their uptake and accumulation by dendritic cells via scavenger receptor A-dependent pathway – the effect that may be associated with the loss of dendritic cell's immune surveillance function in cancer.

1. Introduction

Dendritic cells (DC) are most potent antigen presenting cells responsible for the development of immune responses against cellular antigens and various pathogens. In cancer, DC's function is to "instruct" the ensuing immune response through their ability to acquire, process, and present tumor-associated antigens to T cells for the induction of antigen-specific tumor immune responses. It appears, however, that this function of both circulating and tumor infiltrating DC is substantially affected [1]. Complex interactions of cancer cells with the host organism result in the beneficial for tumor growth tolerant microenvironment and activation of an immunosuppressive network in which erroneous functions of DC may play a prominent role in counteractive immune responses [2]. Our previous studies revealed that the defects in DC function are due, to a large extent, to the accumulation of high amounts of lipids – predominantly triacylglycerols (TAG) – in a substantial proportion of DC in tumor-bearing mice and patients with cancer [3]. The DC accrual of lipids is likely associated with their preferential up-regulation of a scavenger receptor A (SRA1, CD204 or MSR1) [3]. This multifunctional receptor has been shown to bind a broad variety of negatively-charged ligands, including oxidized LDL, anionic phospholipids (e.g., phosphatidylserine), oxidized phospholipids as well as products of their hydrolysis by phospholipases A_2 (PLA_2) (e.g., lysophosphatidylcholines (lyso-PC)), fatty acids and oxygenated fatty acids [4]. Based on these considerations, we hypothesized that peroxidized lipids are essential for the SRA1-driven lipid accumulation in DC of tumor-bearing animals. To the best of our knowledge, assessments and characterization of peroxidation products in major classes of lipids in plasma of tumor-bearing animals, particularly with respect to their interactions with SRA1 and translocation to DC, has not been previously addressed. Here, by using different versions of liquid chromatography–mass spectrometry (LC/MS), we identified several molecular species of oxygenated lipids in plasma of tumor-bearing animals that may be responsible for their uptake and accumulation by DC via SRA1-dependent pathway – the effect that may be associated with the loss of DC's immuno-surveillance function in cancer.

2. Materials and methods

2.1. Mice and tumor models

C57BL/6 mice were obtained from Harlan Laboratories (Indianapolis, IN, USA). *Msr1*$^{-/-}$ mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). For *in vivo* experiments naïve C57BL6 and EL-4 tumor-bearing C57BL6 mice were used. For tumor

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explant supernatants (TES) generation, EL-4 lymphoma cell line was used. Mice were housed and sacrificed according to University of South Florida Institutional Animal Care and Use Committee guidelines.

2.2. Generation and isolation of dendritic cells

In vivo, DC were purified from spleen of C57BL/6 naïve tumor-free and EL-4 tumor-bearing mice using biotinylated CD11c specific antibody (BD Pharmingen) and magnetic beads (Miltenyi Biotec). For *in vitro* generation of DC, hematopoietic progenitor cells (HPC) were isolated from bone marrow of tumor-free mice using lineage cell depletion kit (Miltenyi Biotec) and cultured for 3 days in completed RPMI 1640 medium supplemented 10% FCS, antibiotics and 10 ng/mL granulocyte macrophage colony stimulated factor (GM-CSF) (Peprotech). After that time, the medium was replaced with the one containing 20% v/v TES. After 2 additional days of incubation DC were isolated using CD11c antibody and magnetic beads. TES were prepared by excising unulcerated EL-4 tumors approximately 2 cm in diameter. Tumors were bathed in 70% isopropanol for 30 s and minced into pieces <3 mm in diameter and digested in 2 mg/mL collagenase Type D/IV at 37 °C for 1 h. The digested tissue pieces were then pressed through a 70 μm mesh screen to create a single cell suspension. Cells were washed with PBS and resuspended in RPMI 1640 supplemented with 20 mM *N*-2-hydroxylpiperazine-*N*′-2-ethanesulfonic acid, 2 mM L-glutamine, 200 U/mL penicillin plus 50 μg/mL streptomycin, and 10% FBS. Cells were cultured overnight at 10^7 cells/mL and the cell free supernatant collected.

2.3. Analysis of lipids

Total lipids were extracted from plasma and DC by Folch procedure [5] and analyzed by using a Dionex Ultimate™ 3000 HPLC

Table 1
Content of free fatty acid oxidation products in plasma and DC from C57BL/6 naïve tumor-free and EL-4 tumor-bearing mice.

Name	Plasma (FFAox, nmol/mL)		DC (FFAox, pmol/10^6 cells)	
	Naïve	EL-4 tumor bearing	Naïve	EL-4 tumor bearing
HODE	0.16 ± 0.07	0.25 ± 0.02*	0.67 ± 0.05	1.38 ± 0.17*
12-HETE	3.68 ± 0.44	6.76 ± 0.94*	0.06 ± 0.01	0.23 ± 0.07*
Tetranor-12-HETE	0.21 ± 0.08	0.31 ± 0.08	3.90 ± 0.35	6.03 ± 0.72*
16-HDoHE	0.05 ± 0.02	0.12 ± 0.03	0.15 ± 0.02	0.17 ± 0.02

Data are mean ± SD.
* $p < 0.05$ vs naïve mice.

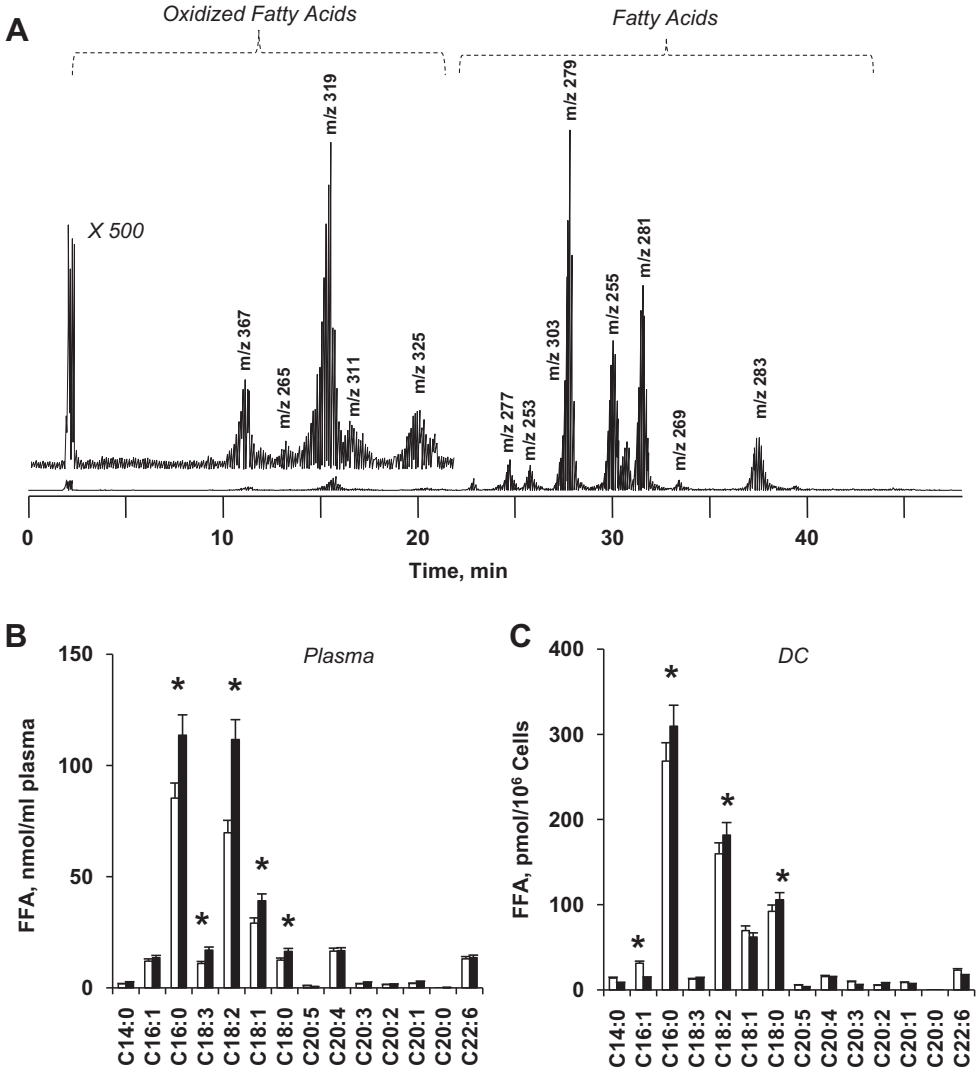

Fig. 1. LC/ESI-MS analysis of FFA molecular species isolated from plasma and DC from C57BL/6 naïve tumor-free and EL-4 tumor-bearing mice. Typical LC/ESI-MS profile of FFA (A) and oxidized FFA (FFAox) (A, inset) from plasma of EL-4 tumor-bearing mice. Content of non-oxidized FFA in mouse plasma (B) and DCs (C). Open bars – naïve mice, closed bars – tumor bearing mice. Data are mean ± SD of at least three experiments. *$p < 0.05$ in samples from tumor bearing mice vs naïve mice.

coupled on-line to ESI and a linear ion trap mass spectrometer (LXQ Thermo-Fisher). Simultaneous LC/ESI-MS analysis of free fatty acids (FFA) and their oxidation products was performed as follows. Aliquots of extracted lipids (5 μL) were injected into a C_{18} reverse phase column (Luna, 3 μm, 150 × 2 mm). Gradient solvents (A – tetrahydrofuran/methanol/water/CH_3COOH, 25:30:50:0.1 (v/v/v/v) and B – methanol/water 90:10 (v/v)) containing 5 mM ammonium acetate at a flow rate of 0.2 mL/min were used. The column was eluted during first 3 min isocratically at 50% B, from 3 to 23 min with a linear gradient from 50% solvent B to 98% solvent B, then 23–40 min isocratically using 98% solvent B, 40–42 min with a linear gradient from 98% solvent B to 50% solvent B, 42–48 min isocratically using 50% solvent B for equilibration of the column. MS spectra were acquired in negative ion mode using full range zoom (200–400 *m*/*z*).

The LC/ESI-MS analysis of TAG was performed using gradient solvents as previously described [3]. The analysis was performed using gradient solvents (A – methanol and B – 2-propanol) containing 0.1% ammonium hydroxide. The column was eluted during the first 6 min linear gradient 0–3% solvent B, from 6 to 18 min isocratic at 3% solvent B, from 18 to 35 min with a linear gradient from 3% to 40% solvent B, then 35–40 min isocratic using 40% solvent B, 40–80 min with a linear gradient from 40% to 55% solvent B, 80–83 min isocratic using 55% solvent B, then 83–85 min with linear gradient 55–0% solvent B, 85–90 min isocratic at 0% solvent B for equilibration of the column. MS spectra were acquired in positive ion mode using range zoom (500–1000 *m*/*z*). TAG cations were formed through molecular ammonium adduction (TAG + NH_4).

Phospholipids and their hydrolysis products, lysophospholipids, were analyzed as previously described [6]. The lipids were separated on a normal phase column (Luna 3 μm Silica 100A, 150 × 2 mm, (Phenomenex, Torrance CA)) with flow rate 0.2 mL/min using gradient solvents containing 5 mM CH_3COONH_4 (A – *n*-hexane:2-propanol:water, 43:57:1 (v/v/v) and B – *n*-hexane: 2-propanol:water, 43:57:10 (v/v/v)). MS spectra were acquired in negative ion mode using full range zoom (200–1600 *m*/*z*).

To quantitatively assess lipids contents the following standards were used: oxidized fatty acids – 13S-OOH-9Z,11E-octadecadie-

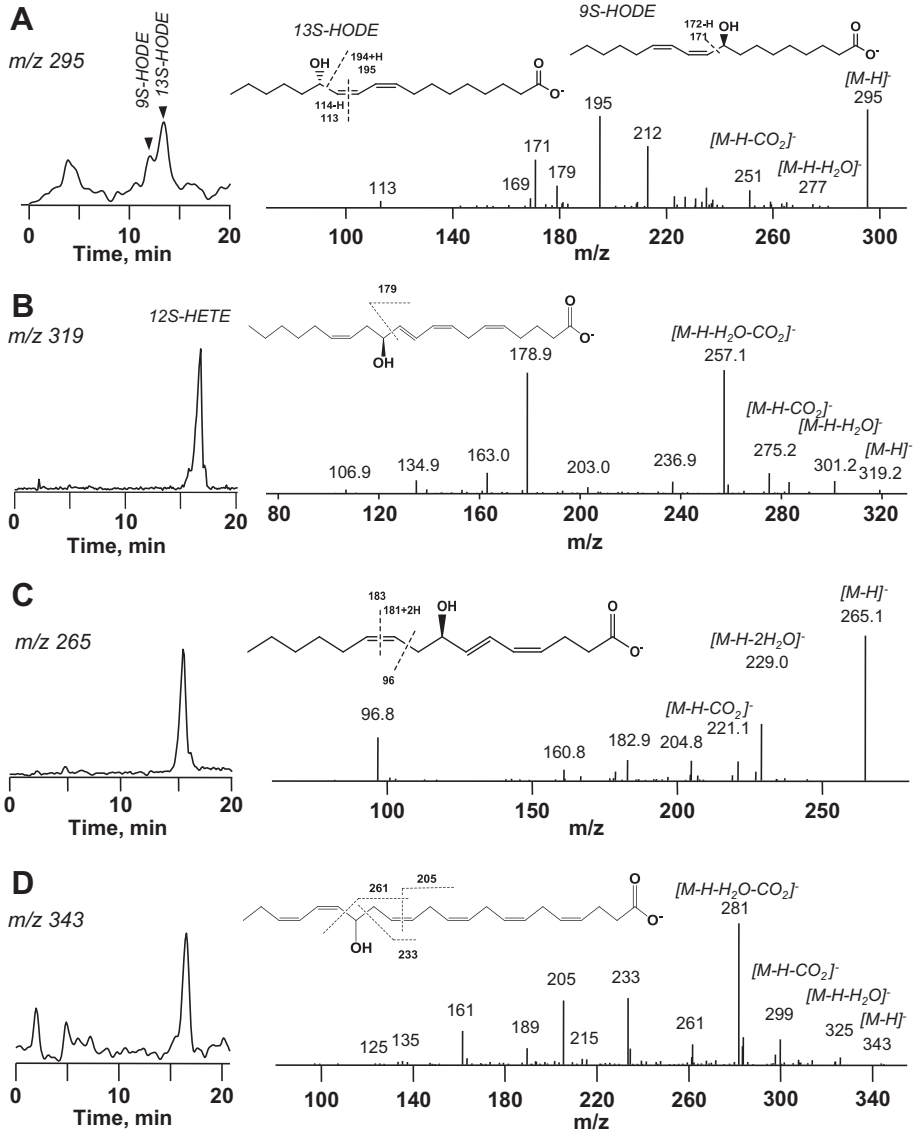

Fig. 2. Identification of oxidized FFA. LC/ESI-MS reconstructed profiles of hydroxy-FFA isolated from plasma of EL-4 tumor-bearing mice (left panels) and their MS/MS spectra (right panels). Possible structures of hydroxy-FFA are presented as inserts. (A) Molecular ion with *m*/*z* 295 corresponding to $C_{18:2}$-OH (a mixture of 13S-HODE and 9S-HODE). (B) Molecular ion with *m*/*z* 319 corresponding to $C_{20:4}$-OH (12S-HETE). (C) Molecular ion with *m*/*z* 265 corresponding to tetranor-$C_{20:4}$-OH (tetranor-12S-HETE). (D) Molecular ion with *m*/*z* 343 corresponding to $C_{22:6}$-OH (16-HDoHE).

noic acid, 9-OH-10E,12Z-octadeacadienoic acid, 15S-OOH-5Z,8Z,11Z13E-eicosatetraenoic acid, 12S-OH-5Z,8Z,10E,14Z-eicosatetraenoic acid, 8S-OH-4Z,6E,10Z-hexadecatrienoic acid, 16-OH-4Z,7Z,10Z,13Z,17E,19Z-docosahexaenoic acid from Cayman chemicals (Ann Arbor, MI); TAG internal and reference standards were from Supelco (Bellefonte, PA), lysophospholipid (1-octadecanoyl-*sn*-glycero-3-phosphocholine) was from Avanti Polar Lipids Inc (Alabaster, AL).

2.4. Statistics

The results are presented as means ± SD values from at least three experiments, and statistical analyses were performed by either paired/unpaired Student's *t*-test or one-way ANOVA. The statistical significance of differences was set at $p < 0.05$.

3. Results and discussion

To identify possible sources of lipids taken-up by the DC of tumor-bearing animals, we performed lipidomics/oxidative lipidomics analysis via LC/MS characterization of the likely candidates – peroxidized fatty acids, TAG and lysophospholipids. As a part of this task, we developed a protocol for simultaneous detection of non-oxidized and oxidized FFA using reverse-phase liquid chromatography (Fig. 1A). By using this protocol we comparatively quantified the contents of FFA in plasma and DC of control and EL-4 tumor-bearing mice (3–4 weeks after tumor inoculation with tumor size around 1.5 cm in diameter). We found significant amounts of highly oxidizable polyunsaturated FFA which were represented mostly by $C_{18:2}$, $C_{18:3}$, $C_{20:4}$ and $C_{22:6}$ species (Fig. 1B and C). Their contents were slightly but significantly higher in EL-4 tumor-bearing animals than in control mice. Both plasma and DC contained oxygenated species derived from these polyunsaturated FFA whereby their levels were significantly higher in EL-4 tumor-bearing mice than in controls (Table 1). To identify the oxygenated FFA species we performed their MS/MS analysis and, based on their characteristic fragmentation profiles, established that they were represented by 13-HODE and 9-HODE (*m*/*z* 295), 12-HETE (*m*/*z* 319), tetranor-12-HETE (*m*/*z* 265), and 16-HDoHE (*m*/*z* 343). In addition, oxygenated fatty acids with *m*/*z* 311, 325 and 335, 367 corresponding to $C_{18:2}$ containing two and three oxygens and $C_{20:4}$ with two and four oxygens, respectively, were also detected in plasma and DC from EL-4 tumor bearing mice but their contents were comparable to those found in normal mice (Fig. 2).

To determine the extent to which SRA1 might be involved in the uptake and intracellular transport of peroxidized FFA we assessed their content in DC generated from wild-type and *Msr1*$^{-/-}$ HPC *in vitro* in complete culture medium supplemented with GM-CSF or TES. As shown in Fig. 3, markedly reduced levels of all four characterized oxygenated FFA were found in DC from k/o mice vs those detected in wild-type animals. These data indicate that SRA1, indeed, was significantly involved in the uptake of oxidatively modified FFA by DC in the presence of tumor-derived factors contained in TES.

Further, we estimated whether oxygenated FFA in DC were esterified into the most abundant class of neutral lipids accumulating in DC of EL-4 tumor bearing animals, TAG. One of molecular species of TAG with *m*/*z* 848 – $C_{16:1}/C_{16:0}/C_{18:1}$ – was present in DC from both control and EL-4-bearing mice (Fig. 4A) (its content was 1.5 higher in the latter, Fig. 4B). Detailed analysis of TAG species with this *m*/*z* (848) using reversed phase LC revealed the presence of an oxygenated TAG species containing $C_{18:2}$-OH – $C_{16:1}/C_{18:2}$-OH/$C_{15:0}$ (Fig. 4A). The structure of this oxygenated TAG was confirmed by MS/MS analysis with a typical fragment $C_{18:2}$-OH (HODE with *m*/*z* 295) (Fig. 4A, inset). Notably, the peroxidized TAG species was present only in DC from tumor-bearing mice (Fig. 4B).

Oxygenated lipids, particularly phospholipids, can be avidly hydrolyzed by specific PLA_2 (type VIIA) – known to be secreted by monocytic cells [7,8]. Assuming that peroxidized phospholipids, particularly phosphatidylcholine (PC), may be present in plasma, we were interested to examine the levels of lyso-PC in plasma and DC of EL-4 tumor-bearing mice. LC–MS analysis did not reveal significant differences in the levels of lyso-PC species in plasma and DC from EL-4 tumor-bearing hosts and control mice (data not shown).

One of the means by which tumor cells implement the host immune-surveillance evasion is through suppression of DC recognition and antigen-presenting function [1]. We demonstrated that this is due, at least in part, to the DC's enrichment with neutral lipids, specifically TAG [3]. The accumulation of lipids might be due to their increased synthesis or stem from increased lipid uptake from the circulation. The dependence of this effect on the expression of

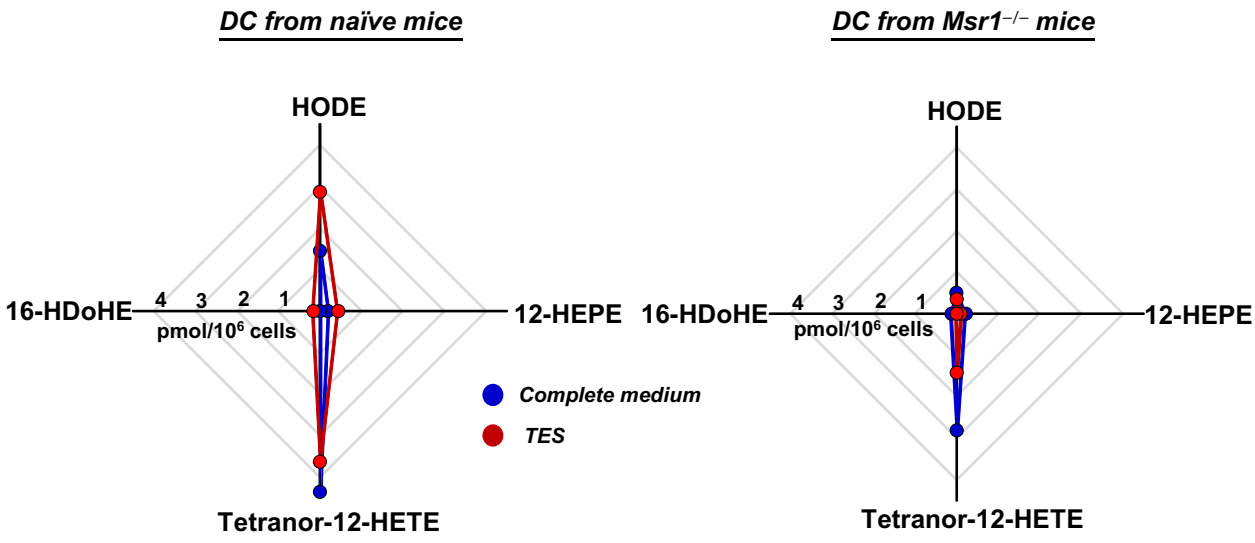

Fig. 3. Levels of oxygenated FFA in DC isolated from C57BL/6 naïve and C57BL6/*Msr1*$^{-/-}$ mice. DCs were maintained either in complete medium supplemented with GM-CSF or in the same medium containing TES as described in Section 2.

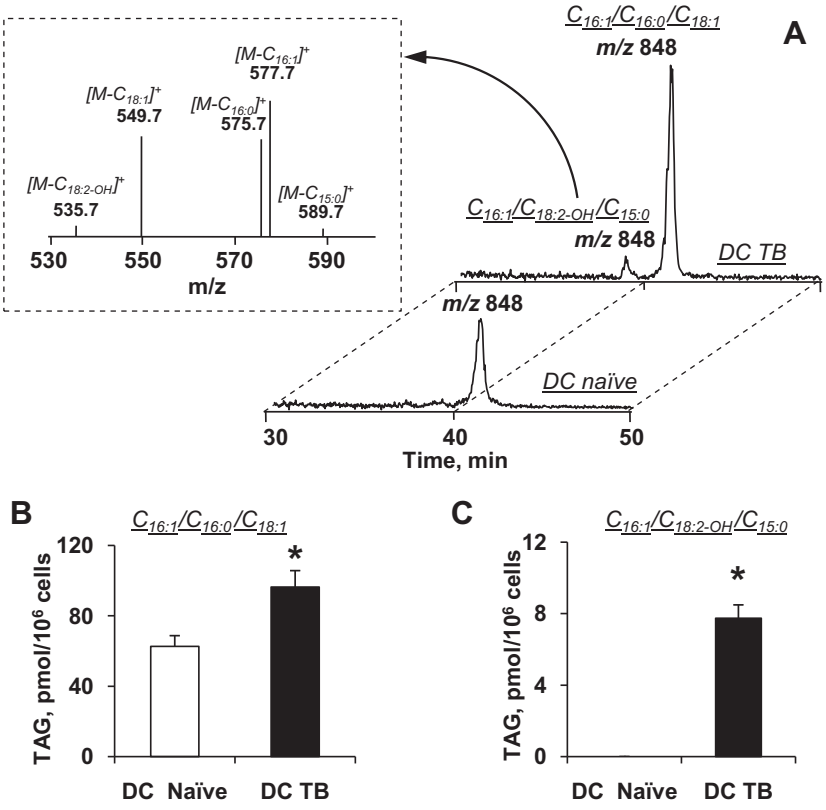

Fig. 4. LC/ESI-MS analysis of TAG molecular species in DC isolated from C57BL/6 naïve tumor-free and EL-4 tumor-bearing mice. LC/ESI-MS reconstructed profiles (A) of TAG molecular species with *m*/*z* 848 from naïve and EL-4 tumor bearing (TB) mice. MS/MS spectrum (in the range of *m*/*z* from 530 to 600) of TAG with *m*/*z* 848 from DC isolated from TB (inset). Quantitative assessment of non-oxidized (B) and oxidized (C) TAG with *m*/*z* 848 in DC. Note that oxidized TAG molecular species with *m*/*z* 848 corresponding to $C_{16:1}/C_{18:2}$-OH/$C_{15:0}$ were detected in DC from EL-4 tumor bearing mice only. Data are mean ± SD. $^{*}p < 0.05$ vs DC from naïve mice.

SRA1 suggests that the second explanation is more likely. Overall, our findings indicate that the presence of oxygenated species of lipids in plasma of EL-4 tumor-bearing animals may be responsible for their uptake by DC possibly resulting in the loss of their immuno-surveillance function.

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