Supplementary Material
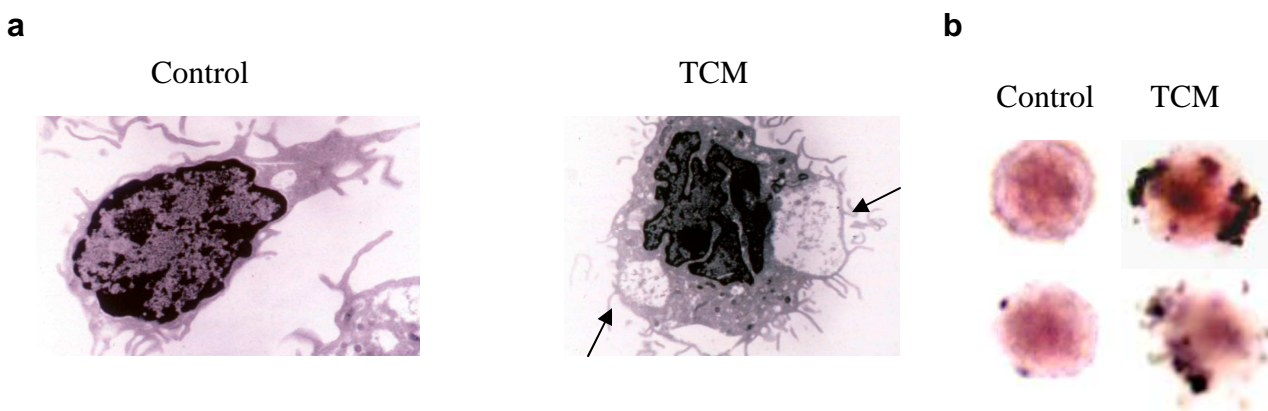
a

Control   TCM

b

Control   TCM

Supplementary Figure 1. Accumulation of lipids in DCs

a. DCs were generated from human CD34$^+$ hematopoietic progenitor cells by 7-day culture with GM-CSF and IL-4 in the presence of 25% of conditioned medium from fibroblasts (control) or HTB-22 breast tumor cells. DCs were washed in PBS prior to fixation in 4% glutaraldehyde. Samples were then enrobed in 1.5% low gelling temperature agarose solution. Post fixation was done in 1% OsO4. Pellets were then incubated in 2% uranyl acetate, dehydrated, infiltrated with araldite resin, embedded, and sections were mounted on mesh copper grids. Grids were stained using Reynold's lead citrate for 2–3 min followed by carbon coating of the grids. For each sample, sections were viewed on grids on the electron microscope. Large "holes" in the cytoplasm (arrows) are typically a result of lipid deposits either extracted during fixation of the cells or reflect saturated lipid that does not take up the osmium stain. **b.** CD34$^+$ hematopoietic progenitor cells were cultured for 7 days with GM-CSF and IL-4 in the presence of control conditioned medium **(Control)** or tumor cell (HTB-22) conditioned medium **(TCM)**. Cells were collected, placed on slides using cytospin centrifugation, stained with sudan black, which detects neutral lipids and counterstained with safranin. Cells were evaluated under light microscope (Mag.x 900).
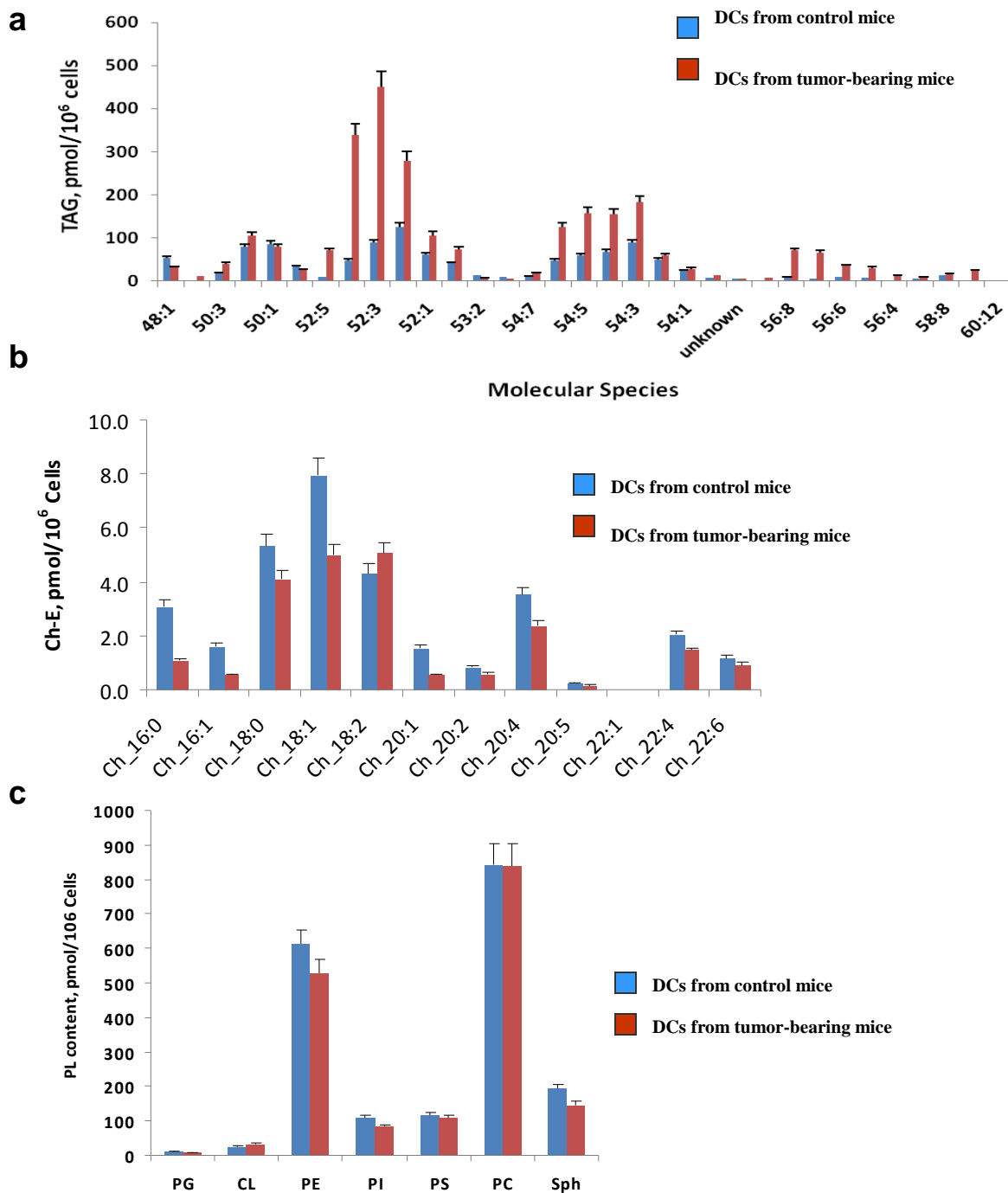
Supplementary Figure 2. Profile of lipids in DCs from naïve and tumor-bearing mice. CD11c^{+} DCs were isolated from spleens of naïve control BALB/c mice and CT26 TB mice. Different types of lipids were evaluated by ESI-MS. Molecular species of **a.** – trigrlycerides (triacylglycerol, TAG), **b.** cholesteryl-esters, and **c.** – phospholipids are shown. Experiments were performed in triplicate. Phospholipid species: PG- phosphatidylglycerol, CL- cardiolipin or (diphosphatidylglycerol), PE- phosphatidylethanolamine, PI- phosphatidylinositol, PS- phosphatidylserine, PC- phosphatidylcholine, Sph- sphigomyelin.
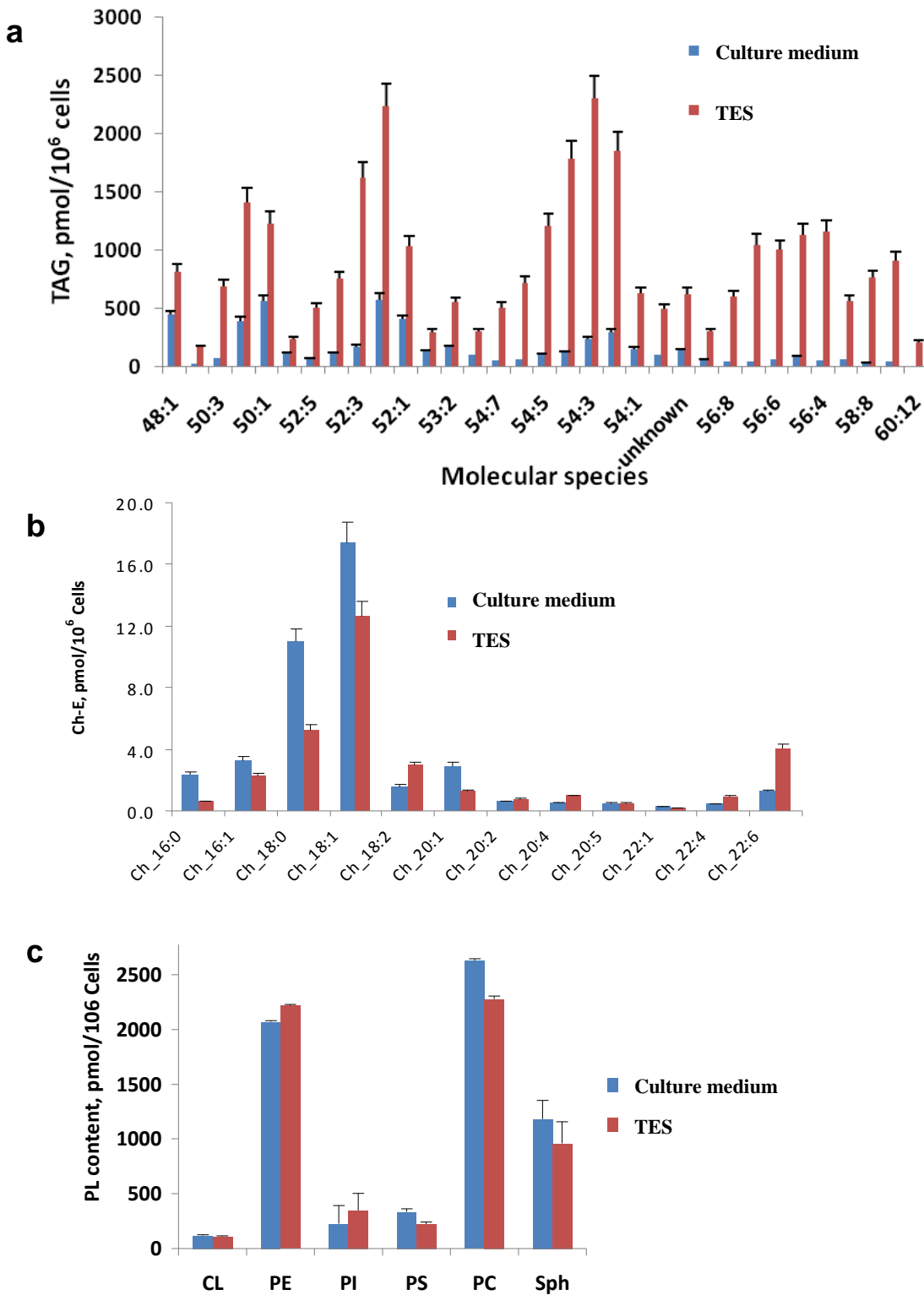
Supplementary Figure 3. Profile of lipids in DCs generated from progenitors. Enriched bone marrow progenitors were cultured with GM-CSF for 5 days in the presence of TES from CT26 tumor. CD11c^{+} DCs were isolated and different types of lipids were evaluated by ESI-MS. Molecular species of **a.** –TAG, **b.** cholesteryl-esters, and **c.** – phospholipids are shown. Experiments were performed in triplicate. Phospholipid species: PG- phosphatidylglycerol, CL- cardiolipin or (diphosphatidylglycerol),

PE- phosphatidylethanolamine, PI- phosphatidylinositol, PS- phosphatidylserine, PC-phosphatidylcholine, Sph- sphigomyelin.
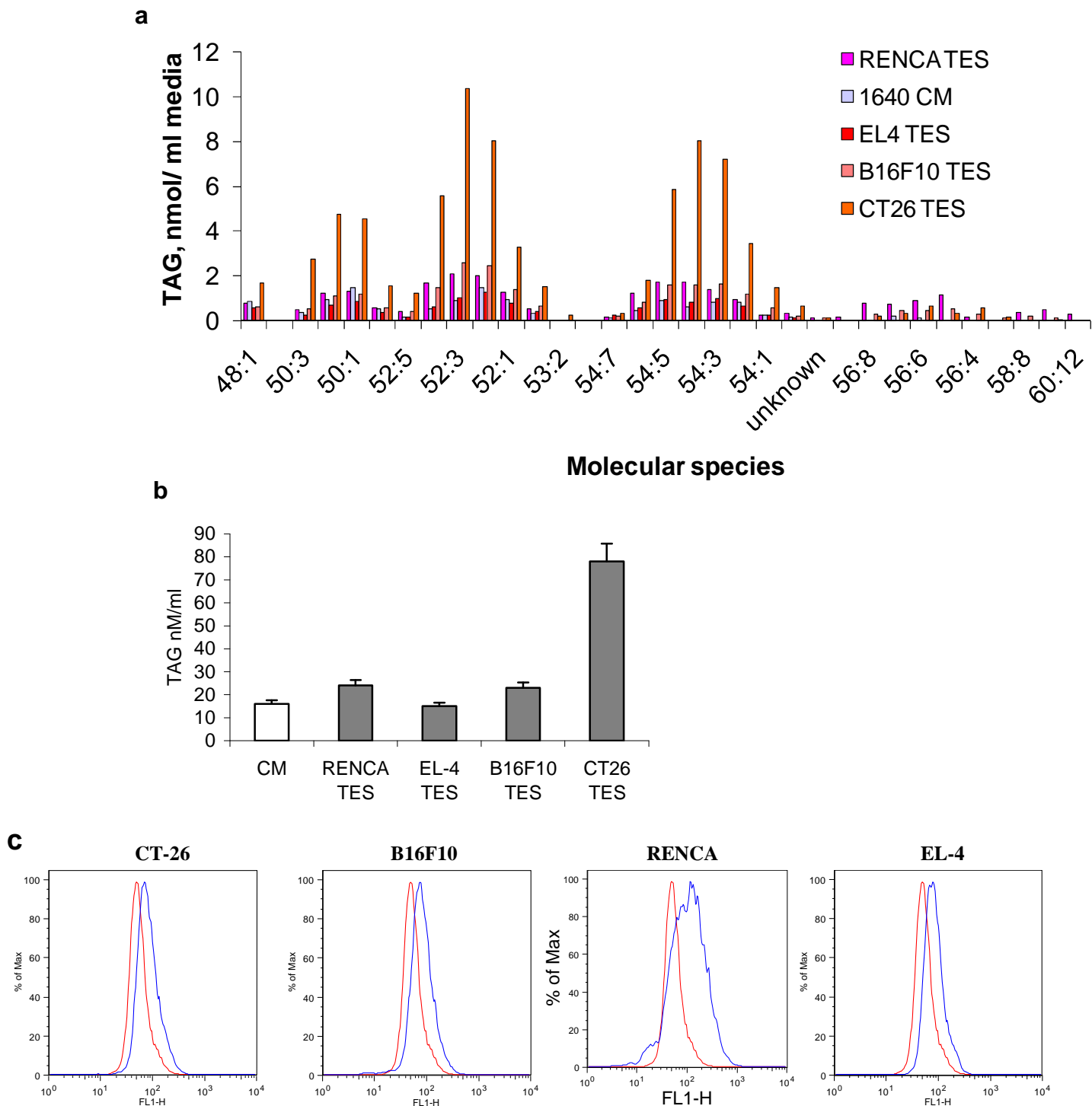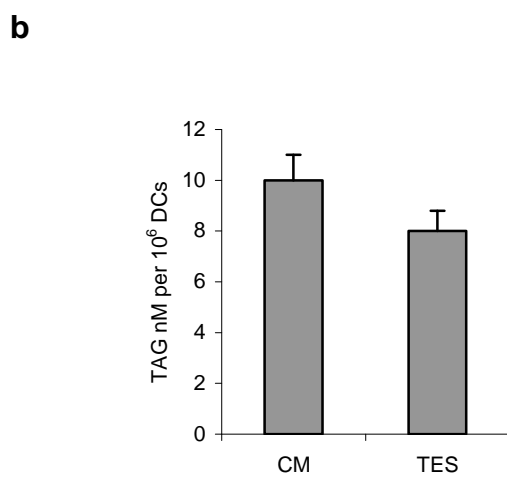
Supplementary Figure 4. The presence of TAG in TES and their effect on lipid accumulation in DCs. a,b. molecular species and total TAG level in TES from different tumors: 1640 CM – control medium (RPMI 1640); CT26 – colon carcinoma (Balb/c mice), RENCA – renal cell cancer (Balb/c mice), B16F10 – melanoma (C57BL/6 mice), EL-4 - thymoma (C57BL/6 mice). TAG were evaluated as described in Methods. **c.** Effect of different TES on accumulation of lipids in DCs. DCs were generated from HPC by 5-day incubation with GM-CSF and IL-4 in the presence of indicated TES. Lipid levels were measured using Bodipy 493/503 within the population of CD11c$^+$ DCs.
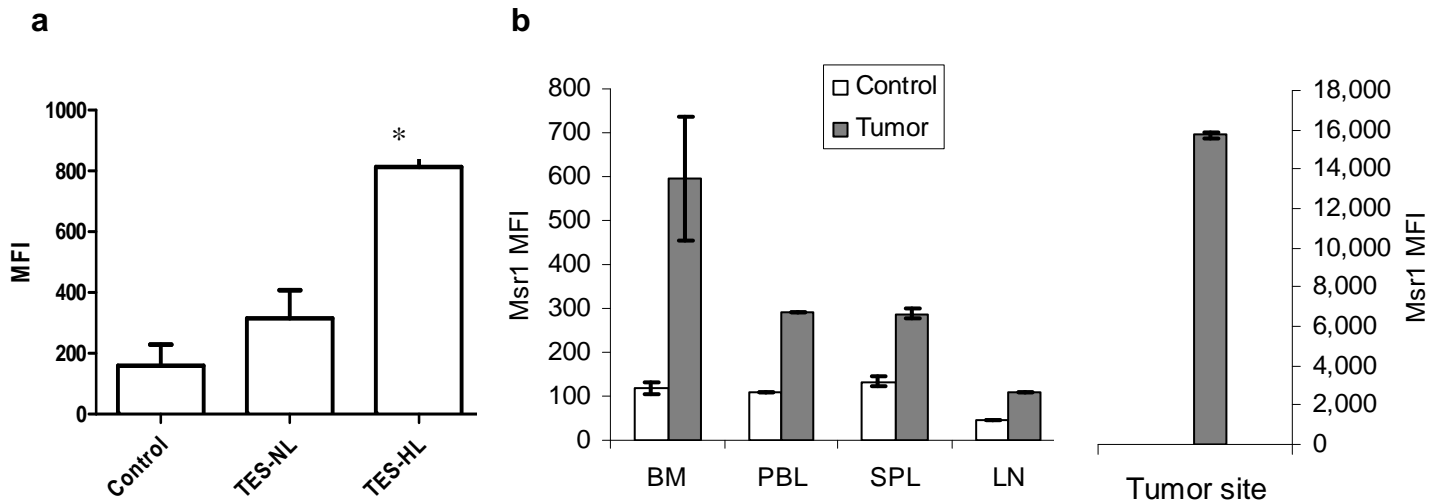
Supplementary Figure 5. **Expression of Msr1 on DCs. a.** Expression of Msr1 (CD204) was evaluated in DCs generated from HPC of naïve BALB/c mice with GM-CSF and IL-4. CT-26 TCM was added for 3 days. Cells were stained with PE-Cy7 conjugated anti-CD11c antibody, Bodipy and APC conjugated antibody to Msr1. The level of Msr1 expression was evaluated among DCs with normal (DC-NL) or high (DC-HL) lipid content. Control – DCs incubated without the presence of TES. Mean ± SD from 3 performed experiments are shown. * - statistically significant differences (p<0.05) from values in control DCs. Fluorescence of cells labeled with isotype control IgG was less than 20 in all samples. **b.** Expression of Msr1 in DCs isolated from different sites of control or CT-26 tumor-bearing mice. Cumulative results of 3 performed experiments are shown.
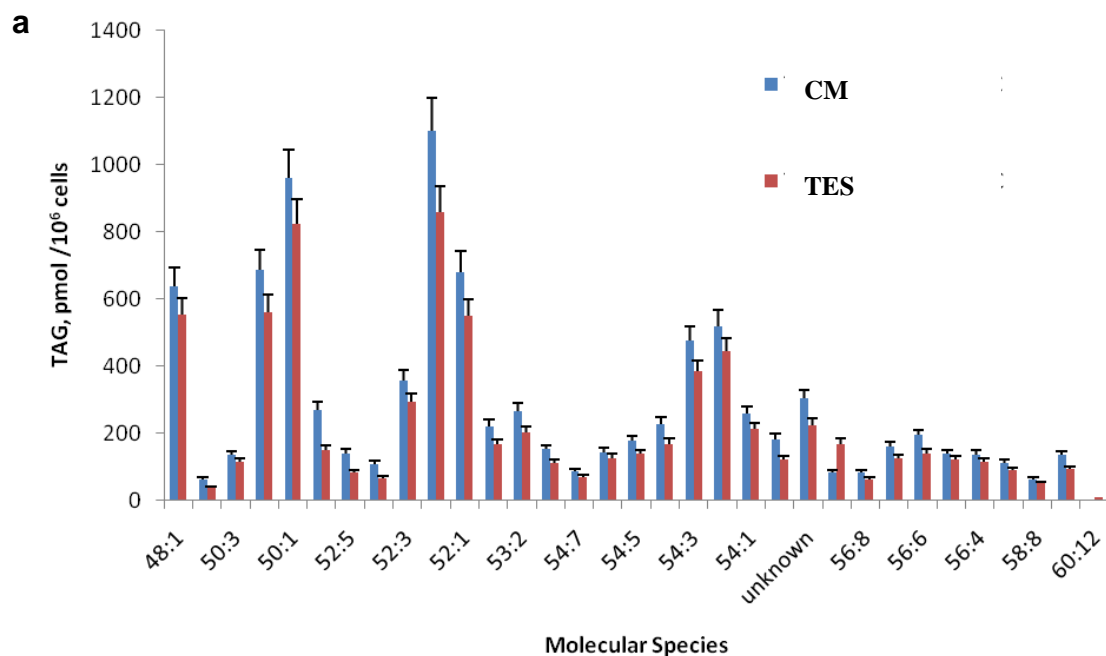
Supplementary Figure 6. TAG in DCs generated from Msr1-deficient HPCs in the presence of TES. HPCs were enriched from bone marrow of Msr1-deficient mice and cultured for 5 days with GM-CSF and IL-4 culture medium alone (CM) or in the presence of 20% EL-4 TES (TES). CD11c^{+} DCs were isolated and TAG were analyzed by EIS-MS. **a** – molecular species of TAG, **b** – the total amount of TAG calculated per 10^6 cells. Measurements were performed in triplicate.
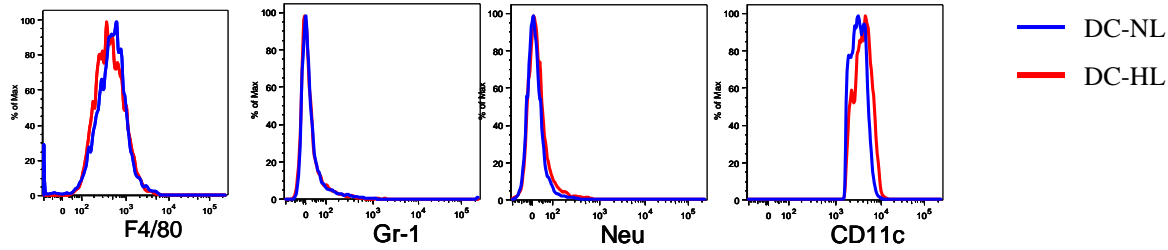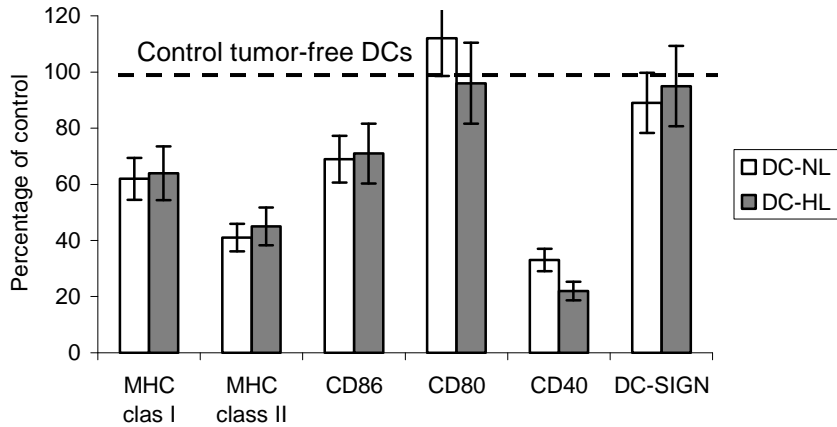
Supplementary Figure 7. Phenotype of lipid-laden DCs

a. Spleens were isolated from CT-26 tumor-bearing mice and stained with bodipy, PE-Cy7 conjugated anti-CD11c antibody and APC conjugated antibodies against indicated molecules. **b.** Expression of the indicated surface molecules was measured in spleen DCs from naïve tumor-free mice and tumor-bearing mice (CT26, EL-4, RENCA). Expression in DCs from tumor-free mice was set up as 100%. Expression of indicated surface molecules in DC-NL and DC-HL from tumor-bearing mice is shown as percentage from the control level. Five independent experiments were performed and cumulative results are shown. Cells labeled with isotype control IgG showed fluorescence less than 20.

Supplementary Methods and Discussion

Human subjects. Several cohorts of person with cancer have been analyzed. All patients signed University of South Florida IRB approved consent forms. The first cohort included 12 individuals (47-78 years old) with resectable stage of III/IV head and neck cancer (HNC). No patient had received radiation or chemotherapy for at least 3 months prior to sample collection.

The second cohort included 6 subjects with kidney cancer (ages 58-80) with no medical anticancer treatment for at least 6 months, and no recent corticosteroid use.

A third cohort of patients included 6 subjects with non-small lung cancer (NSCLC) (ages 48-78) with no radiation or chemotherapy for at least 3 months prior to sample collection. In cohorts 2 and 3 only DCs from peripheral blood were evaluated. As a control, peripheral blood DCs from 11 healthy volunteers (ages 34-49) were used for cohorts 1-3. All patients in cohorts 1-3 were treated at H. Lee Moffitt Cancer Center.

A fourth cohort of patients included 6 subjected treated at the University of Pittsburgh. Lymph node samples from these previously untreated patients were used to compare the level of lipids in DCs from individuals with cancer and subjects with benign tumors. Three subjectes (ages 39-81) were diagnosed with HNC (2) or metastatic papillary thyroid cancer (1). Three other subjects (ages 35-71) underwent surgery for benign diseases, including a parotid tumor, a benign thyroid tumor, and benign/reactive lymphadenopathy.

Peripheral blood, tumor tissues and draining lymph nodes were collected at the time of planned surgery. All cell samples obtained at H. Lee Moffitt Cancer Center were analyzed within 3 h following collection. All samples from patients treated at the University of Pittsburgh were shipped overnight and analyzed separately.

In order to obtain single cell suspensions from tumors and LN, solid tissue was subjected to 1 h enzymatic digestion using hyaluronidase (0.1 mg/ml; Sigma) collagenase (2 mg/ml; Sigma), DNase (600 U/ml; Sigma) and protease (0.2 mg/ml; Sigma) in RPMI 1640. The digested tissue was then passed through a 70 µm mesh, erythrocytes removed by hypotonic lysis, and washed thoroughly to remove debris. Mononuclear cell suspensions were obtained from whole blood using a density gradient centrifugation. Cells were labeled with a cocktail of PE-conjugated lineage-specific antibodies (CD3, CD14, CD16, and CD19), as well as APC conjugated anti-CD4 antibody (all from BD Pharmingen) and stained with Bodipy 493/503 (500 µL of Bodipy 493/503 at 0.5µg/mL in PBS for 15 min at RT). Cells were then washed once with PBS and re-suspended with DAPI for viability staining and the phenotype of the cells was evaluated by multi-color flow cytometry using an LSRII flow cytometer (BD Biosciences, Mountain View, CA). At least 100,000 live cells (DAPI negative) were analyzed for each sample in order to obtain reliable data. To standardize the data, every experiment included control sample of mononuclear cells obtained from a single donor and kept in small aliquots in liquid nitrogen.

Preparation of tumor conditioned medium. EL-4 or CT-26 tumors, without ulceration, approximately 2 cm in diameter were removed under sterile conditions after euthanizing the mouse. Tumors were bathed in 70% isopropanol for 30 seconds and then minced into pieces <3mm in diameter and digested in 2mg/mL collagenase Type D/IV at 37°C for one hour. Similarly, spleens from naïve mice were collected for generation of control supernatant, but were only digested for 30 min. The digested tissue pieces were then pressed through a 70µm mesh screen to create a single cell suspension. Cells were washed with PBS and resuspended in RPMI 1640 supplemented with 20 mM N-2-hydroxyl piperazine-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 10^7cells/mL and the cell free supernatant collected.

Lipid profiling by thin layer chromatography. *LC/ESI-MS analysis* of lipids was performed on a Dionex HPLC system (utilizing the Chromeleon software). Dionex HPLC system was coupled to ion trap mass spectrometer (LXQ^{TM} with the Xcalibur operating system, Thermo Electron, San Jose, CA). For optimization of MS conditions and preparation of tune files, lipid standards (2 pmol/µL) were injected by direct infusion through a syringe pump (flow rate 10 µL/min) into the HPLC solvent flow (flow rate 0.2 mL/min). The electrospray probe was operated at a voltage differential of –3.5-5.0 kV in the negative or positive ion mode. Source temperature was maintained at 150°C. For phospholipid (PL) MS analysis spectra were acquired in negative ion mode using full range zoom (200-1600 m/z) or ultra zoom (SIM) scans. Tandem mass spectrometry (MS/MS analysis) of individual PL species was used to determine the fatty acid composition. For TAG and cholesteryl esters (CE) MS analysis spectra were acquired in positive ion mode using range zoom (500 - 1000 m/z). TAG and CE cations were formed through molecular ammonium adduction (TAG+NH$_4$)$^+$ and (CE+NH$_4$)$^+$, respectively. For CE MS analysis an instrument method is created to detect specified SRM pairs which included parent ion (full range) and fragment ion regardless CE moiety- with m/z 369 (SRM range) *e.g.* 690/369; 666/369; 668/369, which corresponded cholesteryl arachidonate, cholesteryl linoleate and cholesteryl oleoate, respectively. MS^n analysis was carried out with relative collision energy ranged from 20-40% and with activation q value at 0.25 for collision-induced dissociation (CID) and q value at 0.7 for pulsed-Q dissocation (PQD) technique.

Normal phase column separation of phospholipids. Phosholipids were separated on a normal phase column (Luna 3 µm Silica (2) 100A, 150x2 mm, (Phenomenex, Torrance, CA)) with flow

rate 0.2 mL/min, at 30°C. The analysis was performed using gradient solvents (A and B). Solvent A was chloroform/methanol/28% ammonium hydroxide, 80:19.5:0.5 (v/v). Solvent B was chloroform/methanol/water/28% ammonium hydroxide, 60:34:5:0.5 (v/v). The column was eluted during the first 3 min isocratic at 0% solvent B, 3– 20 min with a linear gradient from 0% solvent B to 100% solvent B, and then 20–23 min isocratic at 100% solvent B, 23–35 min linear gradient to 0% solvent B, 35-40 min isocratic at 0% solvent B for equilibrium column [1].

Reverse phase column separation of TAG and CE. Prior to LC-ESI-MS analysis, neutral lipids were separated by 1D-HPTLC on silica G plates in the system containing hexane/diethyl ether/glacial acetic acid, 85:15:1 (v/v). Spots corresponding to triglycerides and cholestryl esters were scraped, transferred into tubes. TAG and CE were extracted by chloroform/methanol/water (20:10:2, v/v), evaporated under N_2, re-suspended in methanol and separated on a reverse phase column (Luna 3 µm C18 (2) 100A, 150x2 mm, (Phenomenex)) at a flow rate of 0.2 mL/min. The column was maintained at 30°C. The analysis was performed using gradient solvents (A and B) containing 0.1% ammonium hydroxide. Solvent A was methanol. Solvent B was 2-propanol. 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.

Quantitative real-time polymerase chain reaction (qRT-PCR). RNA was extracted with an RNase Mini kit and cDNA was synthesized using SuperScript III Reverse Transcriptase kit (QIAGEN, Valencia, CA). PCR was performed as described earlier [2] with 2.5 µl cDNA, 12.5 µl SYBR Master Mixture (Applied Biosystems, Foster City, CA), and target gene-specific primers

(see supplementary material). Amplification of endogenous β-actin was used as an internal control.

Primers used for evaluation of scavenger receptors.

Cd36: AK052825.1
Forward: 5' GACATTTGCAGGTCTATCTAC 3'
Reverse: 5' CACAGGCTTTCCTTCTTTGC 3'

Cd68: AK002264.1
Forward: 5' GGTGGAAGAAAGGCTTGGG 3'
Reverse: 5' ATGTAGGTCCTGTTTGAATCC 3'

Msr1: AF203781.1
Forward: 5' GTGCTCCAGGAATAAGAGGT 3'
Reverse: 5' TGTCTTAAGGGGGGTTGATC 3'

Marco: AK169230.1
Forward: 5' CAAAATCCAGAGTTGTTCCAG 3'
Reverse: 5' CCTCTCCCTTGCTGCCTG 3'

β-actin:
Forward: 5' TGAGAGGGAAATCGTGCGTGACAT 3'
Reverse: 5' ACCGCTCGTTGCCAATAGTGATGA 3'

Discussion

Effect of dietary lipids on DC function. A number of studies have shown the negative effect of dietary lipids on DC function. This includes diminished antigen presentation activity and expression of several adhesion molecules on DCs[3], decreased density of DCs in the skin[4], inhibited lipopolysaccharide (LPS)-induced maturation of DCs resulting in a reduced capability to stimulate T cells[5,6], reduced splenic DC mobilization, and reduced IL-12 responses[7]. The n-3 polyunsaturated fatty acid (PUFA), docosahexaenoic acid, inhibited TLR4 agonist (LPS)-induced up-regulation of the co-stimulatory molecules, MHC class II, and cytokine production. In contrast, the saturated fatty acid lauric acid up-regulated the expression of co-stimulatory molecules, MHC class II, and cytokines (IL-12p70 and IL-6) in DCs[8]. A high-fat/cholesterol diet

caused inhibition of TLR-induced production of pro-inflammatory cytokines as well as up-regulation of co-stimulatory molecules by DCs *in vivo* [9]. Hypercholesterolemic conditions associated with atherosclerosis resulted in inhibition of DC migration from peripheral tissues to draining lymph nodes[10]. In contrast, a recent study by Packard et al. demonstrated that DCs remain functional under the hypercholesterolemic conditions present in atherosclerosis[11]. These data indicate that fatty acids could be an important factor regulating DC function, although their role in DC activity remains unclear.

Scavenger receptors. There are several cell surface receptors grouped under the general classification of scavenger receptors. Class A scavenger receptors (Msr1 (CD204), Marco) are expressed on DCs as well as macrophages and bind acetylated and oxidized low-density lipoproteins (LDL)[12]. Msr1 was expressed in a subset of macrophages and DCs that infiltrated prostatic tissues and the number of Msr1-positive cells was significantly increased in prostatic intraepithelial neoplasia as compared with normal prostatic tissue[13]. Msr1–deficient DCs exhibited a more mature phenotype after LPS stimulation[14,15], were more responsive to inflammatory stimuli and display a more effective antigen-presenting capability compared with wild-type cells[15]. Class B (CD36) and class D (CD68) scavenger receptors are also expressed on both macrophages and DCs[16].

Potential role of M-CSF and GM-CSF in up-regulation of scavenger receptors on DCs. Many tumors including those used in our study produce high levels of M-CSF and GM-CSF[17,18]. Moreover, M-CSF was previously directly implicated in abnormal DC differentiation and function[19,20]. The increase in Msr1 expression on DCs could be caused by these two cytokines, especially taking into account the fact that they have been shown up-regulate the expression of SRA in macrophages[21-23]. To clarify the role of these two cytokines in the up-regulation of Msr1

expression in DCs, we generated DCs from HPC using FLT3 ligand instead of GM-CSF. TES caused accumulation of lipids in DCs comparable to the level seen in cells generated with GM-CSF (data not shown). The presence of different concentrations of neutralizing anti-GM-CSF and anti-M-CSF antibodies alone or in combination did not prevent the up-regulation of Msr1 expression on DCs caused by TES (data not shown). More studies are necessary to determine the nature of the factors responsible for up-regulation of Msr1 on DCs in tumor-bearing hosts.

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