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Oxidized Lipids Block Antigen Cross-Presentation by Dendritic Cells in Cancer

Wei Cao,*1 Rupal Ramakrishnan,*1 Vladimir A. Tuyrin,† Filippo Veglia,‡ Thomas Condamine,‡ Andrew Amoscato,* Dariush Mohammadzayed,† Joseph J. Johnson,*† Lan Min Zhang,†† Judith Klein-Seetharaman,* Esteban Celis,* Valerian E. Kagan,† and Dmitry I. Gabrilovich*8,‡

Cross-presentation is one of the main features of dendritic cells (DCs), which is critically important for the development of spontaneous and therapy-inducible antitumor immune responses. Patients, at early stages of cancer, have normal presence of DCs. However, the difficulties in the development of antitumor responses in patients with low tumor burden raised the question of the mechanisms of DC dysfunction. In this study, we found that, in differentiated DCs, tumor-derived factors blocked the cross-presentation of exogenous Ags without inhibiting the Ag presentation of endogenous protein or peptides. This effect was caused by intracellular accumulation of different types of oxidized neutral lipids: triglycerides, cholesterol esters, and fatty acids. In contrast, the accumulation of nonoxidized lipids did not affect cross-presentation. Oxidized lipids blocked cross-presentation by reducing the expression of peptide–MHC class I complexes on the cell surface. Thus, this study suggests the novel role of oxidized lipids in the regulation of cross-presentation. The Journal of Immunology, 2014, 192: 2920–2931.

Antitumor immune responses, either spontaneous or induced by immune therapy, depend on the adequate function of host dendritic cells (DCs) (1–3). Defects in DC function in tumor-bearing (TB) patients or mice, with advanced disease, are well documented. They manifest in the expansion of immature DCs, unable to properly present Ag, and the generation of cells with immune-suppressive activity, including regulatory DCs and myeloid-derived suppressor cells (4). Together with other immune-suppressive factors, those changes contribute to the inability of CTLs to mount antitumor immune responses (5–8).

Cross-presentation of Ags is a unique feature of DCs, which is critically important for antitumor immunity. Cross-presentation is the process where exogenous Ags are ingested and processed to generate peptide T cell epitopes that are presented by MHC class I (MHC-I) molecules (9, 10). Currently, two main pathways of cross-presentation have been described: cytosolic and vacuolar. After uptake, exogenous Ags are internalized into phagosomes or endosomes (11, 12). The cytosolic pathway involves the transfer of exogenous Ag from the endosome/phagosome into the cytosol for proteasomal degradation. Similar to direct presentation, this pathway is dependent on the TAP. In contrast, the vacuolar pathway is TAP independent and suggests that exogenous proteins are degraded into peptides by lysosomal proteases within the phagosome (or endosome). These peptides are then loaded onto MHC-I molecules that recycle through the endocytic compartments by peptide exchange. The use of each pathway may depend on the type of Ag and the mechanism of its uptake.

The main paradigm of tumor immunology stipulates that the efficient CTL priming requires an uptake of tumor Ags by DCs, their migration to draining lymph nodes, and a cross-presentation of the Ags to CD8⁺ T cells in the context of MHC-I (13). DCs from TB mice are able to cross-present tumor Ag to CTLs (14–17). DC infiltration of solid tumors is well documented in TB patients and mice (18–21). Tumor growth is associated with tumor cell apoptosis, necrosis, and DCs have access to a large amount of tumor Ags via numerous mechanisms such as phagocytosis/endocytosis of cell-associated or soluble Ag bound to heat shock proteins, as well as via gap junction transfer, through the capture of exosomes, or via “cross-dressing” (acquisition of peptide–MHC-I complexes from contact with necrotic cells) (22, 23). The tumor milieu contains soluble mediators such as type-I IFN and endogenous “danger signals” (DNA, HMGB1, S100), which are able to activate DCs. Taken together, all of these factors induce DC differentiation and activation. However, this does not result in the development of potent antitumor immune responses. Moreover, the induction of strong immune responses to cancer vaccines...
is a difficult task, even in patients with a relatively small tumor burden.

In this study, we tried to address this question by studying the effect of tumor-derived factors (TDFs) on partially differentiated DCs. We found that TDFs inhibited the cross-presentation of exogenous proteins and long peptides, requiring Ag processing in DCs, without affecting the presentation of endogenous proteins and directly loaded short/minimal MHC-I binding peptides.

Recently, lipid droplets or lipid bodies (LBs) were implicated in cross-presentation via their association with endoplasmic reticulum (ER)–resident 47-kDa immune-related GTPase, Igpt (IgRm3) (24). LBs are neutral lipid storage organelles present in all eukaryotic cells. It is now established that LBs perform functions beyond lipid homeostasis. In addition, LBs were implicated in the regulation of immune responses via PGs and leukotrienes and, possibly, in IFN responses (reviewed in Ref. 25). Under physiological conditions in most cells, LBs are rather small with a diameter ranging from 0.1 to 0.2 μm (26). In the tumor microenvironment, DCs accumulate lipids and form large LBs (27), which we hypothesized could directly interfere with cross-presentation. We present the results indicating that cross-presentation is directly regulated by the oxidized lipids that accumulate in DCs.

Materials and Methods

Human cells, mice, and tumor models

Donors’uffy coat blood was purchased from the local blood bank. Animal experiments were approved by the University of South Florida Institutional Animal Care and Use Committee. BALB/c or C57BL/6 mice were obtained from the National Cancer Institute. OVA transgenic (Tg) mice C57BL/6-Tg(CAG-OVA)916JulI (Cat: 001545), CD204 knockout mice B6.Cg-Mertm1(Coa/Jul) (Cat: 006096), mice on high-fat diet (60 kcal% fat), and the same-age control mice (10 kcal% diet) were all purchased from The Jackson Laboratory. OT-I TCR-Tg mice (B6.Cg Thi1-1Tg[TcrTcrb]8Rest/J) were kindly provided by Dr. N. Restifo.

Reagents and cell lines

Tumor cell lines including CT26 and MC38 colon carcinomas, EL4 lymphoma, LLC (Lewis Lung Carcinoma), and B16F10 melanoma were maintained in RPMI 1640 medium supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO) at 37°C, 5% CO2. Tumors were injected s.c. at 5 × 105 cells/mouse. SIINFEKL peptide (derived from OVA), and (Pam)2-KMFV-KVPRNQDWL were purchased from The Jackson Laboratory. OV A transgenic (Tg) mice were purchased from the National Cancer Institute. OV A transgenic (Tg) mice C57BL/6-Tg(CAG-OVA)916JulI (Cat: 001545), CD204 knockout mice B6.Cg-Mertm1(Coa/Jul) (Cat: 006096), mice on high-fat diet (60 kcal% fat), and the same-age control mice (10 kcal% diet) were all purchased from The Jackson Laboratory. OT-I TCR-Tg mice (B6.Cg Thi1-1Tg[TcrTcrb]8Rest/J) were kindly provided by Dr. N. Restifo.

Preparation of tumor explant supernatant

Tumor explant supernatants (TES) were prepared from excised nonulcerated tumors ~1.5 cm in diameter. Tumor tissues were bathed in 70% isopropanol for 30 s and then transferred to a Petri dish. Tumors were minced into pieces <3 mm in diameter and digested in 2 mg/ml collagenase Type D/V 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-hydroxy piperazine-N’-2-ethanesulfonic acid, 100 U/ml penicillin plus 50 μg/ml streptomycin, and 10% FBS. Cells were cultured overnight at 105 cells/ml, and the cell free supernatants were collected and kept at −80°C.

Analysis of cell phenotype by flow cytometry

Cell-surface labeling was performed on ice for 20 min and analyzed by a FACScalibur or LSRII flow cytometer and CellQuest program. For lipid cell-surface labeling was performed on ice for 20 min and analyzed by a FACScalibur or LSRII flow cytometer and CellQuest program. For lipid

Functional assays

Mouse DCs were generated from bone marrow (BM) progenitor by a 3-d culture with 10 ng/ml GM-CSF (20). On day 3, medium was replaced with the one containing 20% v/v, and the cells were cultured for an additional 48 h. Cells were labeled with biotinylated CD11c Ab (BD Biosciences, San Jose, CA) followed by incubation with magnetic beads, coupled to streptavidin and positive selection on magnetic column according to the manufacturer’s protocol (Miltenyi Biotec). In some experiments, DCs were generated with 20 ng/ml GM-CSF and 10 ng/ml IL-4 for 5 d. At that time, CD11c+ DCs were isolated using magnetic columns according to the manufacturer’s protocol. Forty-eight hours later, the nonadherent and loosely adherent cells were collected.

Human DCs were generated from mononuclear cells, isolated from donors’ buffy coat blood by Ficoll gradient centrifugation. Cells were cultured for 3 d with 40 ng/ml recombinant human GM-CSF and 20 ng/ml IL-4. On day 3, media were replaced with the one containing 20% v/v, and the cells were cultured for an additional 48 h. Cells were labeled with biotinylated CD11c Ab (BD Biosciences, San Jose, CA) followed by incubation with magnetic beads, coupled to streptavidin and positive selection on magnetic column according to the manufacturer’s protocol (Miltenyi Biotec). In some experiments, DCs were generated with 20 ng/ml GM-CSF and 10 ng/ml IL-4 for 5 d. At that time, CD11c+ DCs were isolated using magnetic beads and incubated with 20% v/v, and the cells were cultured for an additional 48 h. DCs were also directly isolated from mouse spleen, using the same method.

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18 h of incubation, followed by cell harvesting and a radioactivity count on liquid scintillation counter.

Confocal microscopy
Cells were fixed and permeabilized with Fixation & Permeabilization Buffers (BD) for 20 min at 4°C, washed with PBS, and then blocked with PBS containing 1% FBS for 1 h. Cells were incubated with different Abs overnight at 4°C and then stained with HCS LipidTOX red lipid stain or BODIPY, to detect LBs for 30 min at 4°C. The cells were imaged with a Leica TCS SP5 laser-scanning confocal microscope through a 63×/1.40NA Plan Achromat oil immersion objective lens (Leica Microsystems, Wetzlar, Germany). Both 405-nm and 555-nm diode lasers were applied to excite the samples. An acousto-optical beam splitter was used to collect peak emission photons, sequentially, to minimize cross talk between fluorochromes.

Protein and peptide liquid chromatography–multiple reaction monitoring mass spectrometry
Cells (n = 10^5) were lysed in aqueous 8M urea/100 mM ammonium bicarbonate buffer on ice. After lysis, the cell supernatants were clarified by centrifugation and decanted. Lysates were loaded for SDS-PAGE separa-
tion (Criterion XT 4–12%) and visualized with colloidal Coomassie brilliant blue G-250 (Bio-Rad, Hercules, CA). Gel regions, determined by comparison with OVA standards, were excised. Proteins were reduced, alkylated, and digested in gel overnight digestion at 37°C with sequencing grade trypsin (Promega, Madison, WI). The resulting tryptic peptides were concentrated by vacuum centrifugation and resuspended in 2% acetoni-trile, with 0.1% formic acid for mass analysis. A digest of OVA standard protein (Sigma, St. Louis, MO) was used for assay development to select peptides between 7 and 25 aa in length, for both specificity and sensitivity of detection. Liquid chromatography–multiple reaction monitoring mass spectrometry (MS) was performed, as previously described (29).

Liquid chromatography and mass-spectral analysis of lipids
Lipids were extracted by Folch procedure (30) with slight modifications, under nitrogen atmosphere, at all steps. Liquid chromatography–electro-spray ionization–mass spectrometry (LC-ESI-MS) analysis was performed on a Dionex HPLC system (using the Chromel software), consisting of a Dionex UltiMate 3000 mobile phase pump, equipped with an UltiMate 3000 degassing unit and UltiMate 3000 autosampler (sample chamber temperature was set at 4°C). The Dionex HPLC system was coupled to an LXQ ion trap mass spectrometer or to a hybrid quadrupole-orbitrap mass spectrometer, Q-Exactive (ThermoFisher, San Jose, CA) with the Xcalibur operating system. The instrument was operated in both the negative and the positive ion modes (at a voltage differential of −3.5 to 5.0 V/LV, source temperature was maintained at 150°C). For phospholipid MS analysis, spectra were acquired in negative ion mode using a full-range zoom (200–1600 mass-to-charge ratio [m/z]) or ultra-zoom (SIM) scans. Tandem MS analysis of individual phospholipid species was used to determine the fatty acid (FA) composition. Spectra of free fatty acids (FFAs) were also obtained in negative ion mode. For triglyceride (TAG) MS analysis, spectra were acquired in positive ion mode using range zoom (600–1200 m/z). TAG cations were formed through molecular ammonium adduction ([M+NH4]⁺). MS² analysis was carried out with relative collision energy, ranged from 20–40%, with activation q value at 0.25 for collision-induced dissociation, and q value at 0.7 for pulsed-Q dissociation technique. The spectra of cholesterol esters (CEs) were also acquired in positive ion mode on a hybrid quadrupole-orbitrap mass spectrometer (Q-Exactive; ThermoFisher; San Jose, CA).

Statistical analysis
Statistical analysis was performed using unpaired two-tailed Student t test with significance determined at a p value < 0.05.

Results
TDFs inhibit Ag cross-presentation by DCs
To evaluate the effect of TDF on the ability of differentiated DCs to present Ags, we generated DCs in vitro from BM progenitors in culture with GM-CSF for 3 d. After that time, TESs, from different tumors (EL-4 lymphoma, MC38 colon carcinoma, and CT-26 colon carcinoma), were added for an additional 48 h. Under these conditions, TES did not affect the expression of MHC-I or MHC-II molecules, and caused a slight increase in the expression of costimulatory molecules on DCs (Supplemental Fig. 1A). Analysis of the expression of markers specific for different myeloid cells (CD11c, CD11b, F4/80, Gr-1) showed that under these conditions, TES did not affect the phenotype of differentiated DCs (Supplemental Fig. 1B). Little differences were observed in the effect of LPS on DC activation (Supplemental Fig. 1C). After TES treatment, the DCs were loaded with an H-2Kb binding peptide (SIINFEKL), and no defect in their ability to stimulate peptide-specific OT-1 Tg CD8⁺ T cells was observed (Fig. 1A). The expression of SIINFEKL/H-2Kb complexes (peptide MHC [pMHC]) was evaluated by flow cytometry, using the 25-D1.16 Ab, which specifically recognizes OVA-derived peptide SIINFEKL bound to H2-Kb of MHC-I. The results showed that TES treatment of DCs did not significantly affect the cell-surface expression of pMHC, generated by the direct loading of MHC-I with exogenous peptide (Fig. 1B). To assess the effect of TES on Ag cross-presentation, we loaded DCs with OVA protein, during the last 24 h of culture, and then used them for stimulating OT-1 T cells and for the presence of pMHC on the surface of DCs. TES, from all tested tumors, significantly (p < 0.01) reduced the ability of DCs to stimulate peptide-specific OT-1 T cells (Fig. 1C) and the expression of pMHC on DCs (Fig. 1D). To confirm these observations, we used a long OVA-peptide construct (Pam2-KMFVESIINFEKL) that cannot bind directly to H2Kb but is very effective in generating pMHC and stimulating CD8 T cell responses (E. Celis, unpublished observations). Loading of DCs with Pam2-KMFVESIINFEKL generated a higher density of pMHC than loading of DCs with OVA protein, which allowed for better visualization of these complexes by microscopy. Treatment of DCs with TES resulted in a substantial decrease of pMHC on the DC surface (Fig. 1E, 1F). Similar results were obtained using DCs differentiated from BM progenitors with GM-CSF and IL-4 for 5 d (Supplemental Fig. 2A, 2B).

To confirm inhibition of cross-presentation in a different experimental system, we used another long peptide, (Pam)2-KMFV-KVPRNQDWL, which contains a CD8 T cell epitope from the melanoma-associated gp100 protein. Peptide-epitope–specific pmel-1 Tg CD8⁺ T cells, which recognize the minimal gp100 epitope KVPRNQDWL, were used as responders (31). Similar to the results obtained with OVA, TES inhibited the cross-presentation of the Ag derived from the gp100 long peptide (Fig. 1G).

Next, we asked whether the earlier described defects were specifically associated with the exogenous cross-presentation pathway or would also be observed with the presentation of endogenous Ags. To address this question, we used OVA-Tg mice with a constitutive expression of OVA. Treatment of DCs, generated from these mice, with TES did not affect their ability to stimulate OT-1 T cells (Fig. 1H) and did not decrease pMHC expression on these DCs (Fig. 1I). It was possible that in OVA-Tg DCs, pMHC, formed before exposure to TES, were still present on the surface of DCs, thus negating the possible effect of TES on de novo Ag processing. To address this concern, we stripped peptides from MHC-I on the DC surface, before TES application, using a mild acid treatment (Fig. 1J). Under these conditions, TES still did not affect the formation of new surface pMHC on DCs (Fig. 1K). It was possible that DCs in OVA-Tg mice present greater levels of OVA because of a constitutive OVA expression, which could compensate for the effect of TES. We have compared, side-by-side, during the same experiment, the expression of pMHC in DCs loaded with OVA-derived peptide (SIINFEKL) and isolated from OVA-Tg mice. OVA-Tg DCs had lower expression of pMHC than peptide-loaded DCs (Supplemental Fig. 2C, 2D). To confirm the inhibitory effect of tumor-derived factors on DC cross-presentation, we cultured DCs with supernatant from splenocytes prepared exactly the same way as TES. Supernatant from splenocytes did not affect DC cross-presentation, whereas TES caused a profound inhibitory effect in
DCs loaded with long peptide (Supplemental Fig. 2C). Thus, taken together, these data indicate that TES does not affect the presentation of Ags derived from endogenous proteins and that the effects seem to be circumscribed to the cross-presentation pathway.

We proceeded to evaluate the ability of DCs to process Ags and express pMHC in vivo. EL-4 tumors were established in wild-type and OVA-Tg mice. Spleens were isolated, and the pMHC expression was evaluated in CD11c+IAb+ total population of DCs and in CD8+ subset known to be primarily involved in cross-presentation. DCs isolated from TB mice and loaded with long OVA-derived peptide (SIINFEKL) showed significantly reduced presentation of pMHC on the surface (Fig. 2A). In contrast, no decrease in pMHC expression was found in DCs isolated from TB OVA-Tg mice (Fig. 2B). These data support the conclusion that DCs in TB hosts are not able to effectively cross-present exogenous Ags but continue to be effective in processing and presenting endogenous Ags.

Mechanisms of inhibition of cross-presentation in cancer

We had previously observed an accumulation of lipids in DCs, from cancer patients and TB mice, and the association of this accumulation to defects in DC function (27). Therefore, we investigated the possible role of lipids in the defective Ag cross-presentation by...
DCs in cancer. As expected, a 2-d exposure of DCs to TES caused the accumulation of lipids in DCs (Fig. 3A). DCs, treated with TES and loaded with OVA, were gated based on the level of lipids: cells with normal lipid level (NL) and cells with high lipid content.

FIGURE 2. Cross-presentation in DCs isolated from TB mice. (A) Expression of pMHC on the surface of CD11c+ DCs isolated from spleens of naive or EL-4 TB (tumor ≈1 cm in diameter) mice and loaded with 5 μg/ml OVA long peptide. (Left) Typical example of staining. (Right) Cumulative results of four experiments in indicated populations of DCs. (B) Expression of pMHC on the surface of DCs isolated from spleens of naive or EL-4 TB (tumor ≈1 cm in diameter) OVA-Tg mice. (Left) Typical example of staining. (Right) Cumulative results of four experiments in indicated populations of DCs. *p < 0.05, statistically significant differences versus control in all experiments.

FIGURE 3. Effect of TES on Ag processing in DCs. (A) Typical example of lipid level in gated CD11c+ DCs treated with EL-4 TES for 48 h after staining with BODIPY. Seven experiments with similar results were performed. (B) Cross-presentation of Ags by DCs with different levels of lipids. DCs treated with TES and loaded with OVA were gated with BODIPY. (Left panel) Example of gates set for discriminating DCs with NLs (NL-DCs) from DCs with HLs (HL-DCs). DCs were considered NL-DCs when their fluorescence overlapped the fluorescence of the control DCs. Control DCs are in red; DCs treated with TES are in blue. Three experiments, with the same results, were performed. (C) Cross-presentation of long OVA peptide by DCs with different levels of lipids. Left panels show the gate of NL-DCs and HL-DCs, and pMHC expression is shown in the right panel. (D) TES does not affect cross-presentation in CD204-deficient DCs. DCs generated from Msr1−/− mice were treated with TES, loaded with OVA, and used for stimulation of OT-1 T cells as described in Fig. 1C. Proliferation of OT-1 T cells was measured in triplicate. Typical results of four performed experiments are shown. (E) Typical examples of pMHC (left panel) and H2Kb (right panel) expression in CD204-deficient DCs. Gray shaded line represents DCs without OVA treatment; blue line represents DCs loaded with OVA in CM; red, green, and orange lines represent DCs loaded with OVA and pretreated with TES from EL-4, MC38, and CT26 tumors, respectively.
Although no differences were observed in the overall H-2Kb expression between NL and HL DCs, the NL DCs had a substantially higher expression level of pMHC as compared with the HL DCs (Fig. 3B). Similar results were obtained when DCs were loaded with OVA-derived long peptide and exposed to TES from different tumors (Fig. 3C). There was some overlap in pMHC expression between DCs with control and HLs because only 50–60% of TES-treated DCs had increased lipid level.

To further assess the possible involvement of lipids in the decreased generation of pMHC via cross-presentation, we used DCs deficient for the scavenger receptor CD204 (Msr1). This receptor was previously found to be primarily responsible for lipid accumulation in DCs in cancer (27). CD204-deficient DCs were generated from BM of Msr1−/− mice and cultured with TES and OVA, as described earlier. In contrast with wild-type DCs (Fig. 1), TES did not affect the ability of CD204-deficient DCs to stimulate OT-1 T cell proliferation (Fig. 3D) or to express pMHC (Fig. 3E), indicating that lipid accumulation may be directly involved in the defective cross-presentation in DCs in cancer.

**Accumulation of oxidized lipids in DCs**

Treatment of DCs with TES caused an accumulation of enlarged LBs in DCs that were easily observed inside the cells (Fig. 4A, red fluorescence). The proportion of DCs with enlarged LBs has been calculated by counting 100 cells. It increased from 9.2% in control to 43.5% in DCs treated with TES. We tested the possibility that LBs colocalized with the cellular compartments involved in Ag processing and the formation of pMHC and, thus, might interfere with this process. However, no colocalization of LBs with lysosomes (Fig. 4A),
trans-Golgi network (Fig. 4B), early endosomes (Fig. 4C), or ER (Fig. 4D) was found. These data suggest that enlarged LBs in DCs is unlikely to cause a direct physical disruption of Ag processing.

In a previous study, we observed an accumulation of TAGs and FFAs in DCs from TB mice treated with TES via CD204 receptor (27). However, it is known that TAGs are not directly taken up by DCs but are synthesized inside the cells from FAs, and scavenger receptor A (CD204) binds primarily modified lipoproteins (32). To better understand the nature of lipid accumulation in DCs that affects Ag cross-presentation, we used liquid chromatography/MS to quantitatively characterize different types of lipids and their oxidation products. In control DCs, most FAs were represented by monosaturated and polyunsaturated FAs (PUFA), with C18:2 linoleic acid (LA) being the most abundant PUFA (Fig. 4E). In the presence of TES, DCs accumulate large amounts of PUFA, primarily LA (Fig. 4F). Because unsaturated FFAs are highly susceptible to oxidation, we evaluated the oxidative status of lipids. DCs isolated from spleens of naive mice contained very little oxidized LA (oxLA) whereas DCs from TB mice had a markedly higher concentration of oxLA (Fig. 4G). Similar results were obtained with oxidized arachidonic acid (AA), although its level in DCs was substantially lower than that of oxLA. No differences between naive and TB mice were seen in the levels of oxLA or oxidized AA in sera (Fig. 4G). Similarly, DCs cultured without TES contained a practically undetectable level of oxLA, whereas DCs cultured with TES had a dramatically higher amount of oxLA (Fig. 4H). A dramatic accumulation of mono-oxygenated TAGs was seen in DCs treated with TES (Fig. 4I). This was associated with a large increase in the presence of oxidatively truncated TAGs (TAGs that were oxidized with subsequent shortening of the oxygenated FA residues; Fig. 4J). Culture of DCs with TES also resulted in the accumulation of oxidized CEs (oxCEs; Fig. 4K).

No detectable amounts of oxidized phospholipids were observed in different classes of DC phospholipids from either TB animals or DCs cultured in the presence of TES (data not shown). Typical fragmentation analysis of oxidized LA species in oxidized TAGs (oxTAGs) in DCs is shown in Supplemental Fig. 3.

The proportion of oxLA among total LA was substantially increased in DCs from TB mice as compared with control DCs. In control DCs, the content of oxidized free LA constituted ~0.2 mol%, whereas in DCs from TB mice it was 2.5 mol%. TAGs containing LA residues underwent massive oxidative modification in DCs from TB mice as compared with naive animals. For example, the content of oxTAGs at m/z 920 corresponding to oxLA molecular species C18:2/C18:2+3O/C16:0 in DCs from TB mice was 59.3 ± 3.8 versus 1.2 ± 0.1 pmol/10⁶ cells in control. Thus, disproportionally

**FIGURE 5.** Accumulation of oxidized lipids in human DCs. (A) Total lipids in donor’s DCs treated with SK-MEL TCM. Typical example of BODIPY staining. (B) Major FFAs detected in DCs; (C) oxLA in DCs. MS² spectrum of parent ions at m/z 295 (left panel), its possible structure (middle panel), the amount of oxLA (9-HODE) in DCs (right panel); (D) CE levels in human DCs cultured with and without TCM; (E) oxCE in DCs. Possible structure of CE 18:2-OOH (upper left panel); MS1 and MS2 spectra of CE 18:2-OOH (lower left panel); amount of CE 18:2-OOH in DCs (right panel). (F) Accumulation of individual molecular species of TAGs in DCs. (G) OxTAGs in DCs. Typical LC-ESI-MS profile (left panel); MS² spectrum of TAG at m/z 932 (middle panel); amount of oxTAGs in DCs (right panel). Fold increase over values in DCs cultured in CM are shown. (B–G) Two experiments were performed. *p < 0.05, statistically significant differences versus control in all experiments.
higher accumulation of oxidation products versus increase of the respective neutral lipids was observed in TB mice versus naive animals.

Human DCs, cultured with tumor cell–conditioned medium (TCM), had higher lipid levels than control DCs (Fig. 5A). The predominant type of FFA observed in DCs, generated with TCM, was poorly oxidizable monounsaturated C₁₈:₁ oleic FA (Fig. 5B). However, the concentration of oxLA in these cells was >2-fold higher than in DCs cultured in control medium (CM; Fig. 5C). Although the total amount of CEs showed no difference between DCs cultured with and without TCM (Fig. 5D), DCs exposed to TCM had a dramatically higher presence of oxCE (Fig. 5E). DCs cultured with TCM had a modestly higher amount of TAGs than DCs cultured without TCM (Fig. 5F). In contrast, TCM caused a substantial accumulation of oxTAG in DCs (Fig. 5G). The detailed structural analysis of oxidized lipids in human DCs is provided in Supplemental Fig. 3.

Taken together, these data indicate that DCs, in the presence of TDF, have a substantial accumulation of oxFA, which apparently incorporated into TAG and CE, generating an increased amount of different oxidized lipid species.

Effect of intracellular oxidized lipids on cross-presentation

In view of the earlier text, we next asked whether the accumulation of nonoxidized or oxidized lipids in DCs could have an effect on Ag cross-presentation. Based on the fact that oxLA was the most abundant FA accumulated in DCs in TB mice, we assessed whether the defect in cross-presentation could be reproduced by loading control DCs with LA and its subsequent intracellular oxidation. To induce intracellular peroxidation of neutral lipids, we used a lipophilic peroxyl radical initiator, azobis-(2,4-dimethylvaleronitrile) (AMVN), which partitions into hydrophobic phases (32). AMVN decomposes in lipids at a constant rate to yield carbon-centered alkyl radicals, which react with molecular oxygen to generate reactive peroxyl radicals leading to lipid peroxidation (33). DCs were loaded for 24 h with LA in serum-free medium, treated with AMVN for 2 h, washed, and then used in experiments. Loading of DCs with LA or in combination with AMVN resulted in an accumulation of large LBs, similar to the effect of TES (Fig. 6A). At a concentration of 0.2–0.5 mM, AMVN did not affect DC viability or expression of MHC-I, MHC-II, or costimulatory molecules (Supplemental Fig. 4A, 4B). In contrast, AMVN induced oxidation of LA in DCs (Fig. 6B). In addition to oxLA, we also observed accumulation of oxTAGs and oxCE in DCs loaded with LA and treated with AMVN (Fig. 6C, 6D). In contrast with robust AMVN-driven accumulation of peroxidation products in major neutral lipid components of LBs (peroxidized species of TAGs, FFA, and CEs were increased up to 244.6 ± 7.5, 74.8 ± 12.4, and 1.2 ± 0.2 pmol/10⁶ cells, respectively), only...
minor peroxidation of membrane phospholipids was detected (9.2 ± 1.8 pmol/10^6 cells), which constitutes ~3% of the total increase in the content of oxidized lipids). Thus, these experimental conditions recapitulated accumulation of ox-lipids in DCs observed on DCs exposed to TES or TCM, and were used in further experiments.

After loading with LA, with or without subsequent treatment with AMVN, DCs were incubated overnight with OVA and then used for stimulation of OT-1 T cells. DCs, treated with LA or AMVN alone, showed no defect in Ag cross-presentation function, whereas a combination of AMVN and LA significantly reduced the ability of the DCs to stimulate Ag-specific T cells (Fig. 6E). In contrast, if DCs were directly loaded with SIINFEKL peptide, the combination of AMVN and LA did not affect DC stimulation of OT-1 T cells (Fig. 6F). Treatment of DCs with LA alone did not affect the pMHC surface expression after loading of the cells with long OVA peptide, whereas AMVN by itself caused a small decrease in the levels of surface pMHC (Fig. 6G). In contrast, the combination of AMVN and LA resulted in a 2-fold decrease of pMHC surface expression (Fig. 6G), confirming that the accumulation of ox-lipids in DCs specifically inhibits Ag cross-presentation but does not affect DC function if the peptide is directly loaded onto the MHC-I on the surface. To confirm these observations in a different experimental system, we loaded DCs with long peptide derived from gp100 protein and used pmel-1 T cells as responders. The combination of LA and AMVN, but not each of them separately, significantly (p < 0.05) inhibited the cross-presentation function of the DCs (Fig. 6H).

To verify the specific role of lipid oxidation in defective cross-presentation in our experimental system, we treated DCs with a higher concentration of AMVN (0.5 mM). At this dose, AMVN directly inhibited the ability of OVA-loaded DCs to stimulate OT-1 T cells without need to preload DCs with LA (Fig. 7A). To block lipid peroxidation, we pretreated cells with α-tocopherol (vitamin E), which is known to partition into hydrophobic phases of lipid structures and has been shown to effectively inhibit lipid peroxidation in biomembranes and lipoproteins (34). Tocopherol abrogated the inhibitory effect of AMVN on cross-presentation of OVA to T cells (Fig. 7A), and abrogated the inhibitory effect of AMVN on the expression of pMHC, after DC loading with either OVA or long OVA-derived peptide (Fig. 7B). The negative effect of AMVN on surface pMHC levels affected only cross-presentation, because it was not observed in DCs obtained from OVA-Tg mice (Fig. 7C). Likewise, treatment of DCs with AMVN inhibited the stimulation of OT-1 T cells by DCs loaded with OVA, but did not affect DCs from OVA-Tg mice (Fig. 7D). Thus, ox-lipids can directly inhibit cross-presentation in DCs without affecting the presentation of endogenous Ag.

**Mechanism of inhibition of cross-presentation by oxidized lipids**

The endogenous pathway of presentation invariably depends on proteasomes, whereas the pathway of cross-presentation (cytosolic or vacuolar) depends on the type of Ag and the mechanism of its uptake (12, 35). In our experiments, inhibition of proteasomes with lactacystin almost completely abrogated the surface expression of pMHC in DCs isolated from OVA-Tg mice (Fig. 8A). In contrast, lactacystin had little effect on cross-presentation OVA by DCs (Fig. 8A, 8B) and a modest effect on cross-presentation of long OVA-derived peptide (Fig. 8A), suggesting that a vacuolar mechanism could be the main pathway involved in cross-presentation under these circumstances.

IFN-γ is known to upregulate MHC-I expression and increase cross-presentation in DCs. We assessed whether pretreatment of DCs with IFN-γ would abrogate the inhibitory effect of TES on cross-presentation. As expected, in DCs cultured in CM, IFN-γ caused a marked upregulation of overall H-2Kb and a >2-fold increase in the content of oxidized lipids. Thus, these experimental conditions recapitulated accumulation of ox-lipids in DCs observed on DCs exposed to TES or TCM, and were used in further experiments.
increase in the expression of pMHC, after DC loading with long OVA peptide (Supplemental Fig. 4C). IFN-γ substantially upregulated the expression of H-2Kb on DCs cultured with TES from three different tumors (Fig. 8C, top panels). However, IFN-γ did not completely overcome the block in pMHC expression (Fig. 8C, bottom panels). Treatment of DCs with IFN-γ did not rescue DCs inhibited by TES or affect DCs ability to stimulate OT-1 T cell proliferation after cross-presentation of OVA protein (Fig. 8D).

We also tested the effect of IFN-γ on cross-presentation of DCs loaded with LA and treated with AMVN. Similar to the effect observed in the presence of TES, IFN-γ upregulated the H-2Kb expression, but failed to abrogate the defect in pMHC expression (Fig. 8E). Despite the presence of a small population of DCs with increased expression of pMHC, we did not find an improvement in the ability of DCs to stimulate Ag-specific T cells after cross-presentation of OVA-derived long peptide (Fig. 8F).

**Discussion**

In this article, we report that oxidized lipids inhibit cross-presentation in DCs. This may have an implication on therapeutic efforts in patients with low tumor burden, who are considered optimal candidates for cancer vaccines. Accumulation of lipids in DCs, from TB hosts, is mediated via upregulation of scavenger receptor (Msr1 or CD204). This receptor binds various acetylated and oxidized lipids, including low-density lipoproteins (36). Msr1-deficient DCs showed a more effective Ag-presenting capability as compared with wild-type cells, but this was primarily linked with their more mature phenotype (37). In the absence of CD204 (in Msr12/2 mice), TDF failed to inhibit the Ag cross-presentation function in DCs, supporting the possible role of extracellular lipids in the negative effects of TDF on DCs. Extracellular lipids may affect DC function via various receptor-mediated mechanisms (38–41). However, our data indicated that the effect of lipids on cross-presentation was primarily intracellular. When DCs treated with TDF were separated based on their level of intracellular lipids, the defect in cross-presentation was associated only with high lipid-laden DCs. We previously have shown that although TES obtained from different tumor cell lines had variable levels of TAGs, they caused comparable upregulation of lipids in DCs (27). It is known that TAGs are not directly taken up by DCs but are synthesized inside the cells from FAs. CD204 binds modified primarily oxidized lipoproteins (36).
Small LBs are present in all DCs and have been implicated in cross-presentation via interaction with Irgm3, an IFN-related GTPase. Irgm3 is required for IFN-γ-induced efficient cross-presentation (24). Using high-resolution confocal microscopy, van Manen et al. (42) have demonstrated the transient association of LBs with latex bead-containing phagosomes in neutrophils and macrophages. If LBs are important for cross-presentation and could be transiently associated with phagosomes, then it was conceivable that large LBs could disrupt cross-presentation. However, our data argue against this possibility. Because we could not detect colocalization of large LBs with any cellular compartment associated with cross-presentation or with pMHC. Treatment of DCs with IFN-γ did not rescue the defect of cross-presentation caused by TDF, despite the substantial upregulation of MHC-I. Loading of DCs with LA, which resulted in formation of large LBs, did not cause the defect in cross-presentation by DCs. Thus, it appears that the mere accumulation of lipids in DCs is not sufficient to affect cross-presentation.

Our data demonstrated a substantial accumulation of different classes of neutral ox-lipids in DCs in TB hosts, which is consistent with the important role of CD204 in this process (43). To directly test the hypothesis that ox-lipids are involved in regulation of cross-presentation, we could not use a treatment with extracellular oxidized lipoproteins or lipids. Oxidized and nonoxidized lipids and lipoproteins differ in the nature of the receptors they bind and signaling they cause, as well as in the kinetic of their uptake by the cells (44). DCs are lacking the machinery for lipid peroxidation and TDF did not cause the upregulation of myeloperoxidase or ROS needed for this process. Therefore, in our model experiments, we used an LA and lipophilic generator of peroxyl radicals, AMVN. Our data directly implicated intracellular ox-lipids in the inhibition of cross-presentation in DCs.

Why do oxidized lipids affect only cross-presentation and not presentation of endogenous Ags? In contrast with endogenous presentation, which involves the proteasomal degradation of proteins, the transfer of peptides to the ER by TAP, and the formation of pMHC in the ER with the subsequent transport to the membrane (45), these components are not essential for the vacuolar pathway in Ag cross-presentation (46). This pathway largely involves the recycling of MHC-I from the surface and formation of new pMHC in endosomes and lysosomes via peptide exchange. The cytoplasmic domain of MHC-I can direct the protein to both the endosomal and lysosomal compartments of DCs, where loading of peptides derived from exogenous proteins can occur (47, 48). In addition to recycling MHC-I, transport of MHC-I, from ER to the endocytic compartment, has also been demonstrated (49, 50).

We propose the concept of disrupted DC Ag cross-presentation in cancer. Under physiological conditions, DCs, in contrast with neutrophils and macrophages, produce and accumulate very low levels of peroxidized lipids. In TB hosts, TDFs induce the upregulation of Mrsl (27), which facilitates an uptake of peroxidized lipids present in plasma or TCM. Inside DCs, oxidized lipid moiety can be directly trafficked to endosomes and localized in the lysosomal compartment (51). In addition, the accumulation of lipids in DCs manifests in the formation of large LBs. The presence and size of LBs is defined by the accumulation of FA precursors and their esterification into TAGs and CEs, the major constituents of the hydrophobic core of LBs (26). The significant uptake of oxF-FAs, in particular, oxLA, facilitates its integration into ox-LA–containing TAGs and CEs. The presence of very polar groups in these oxidized lipid species, or oxidatively truncated TAGs, causes their translocation and integration into the less hydrophobic surface area of LBs—as has been confirmed by our computational coarse-grained molecular dynamic simulations (52), where they can be further metabolized (e.g., hydrolyzed) by the respective hydrolyzing enzymes. These peroxidized lipid species could be located in close molecular proximity to the contact sites with neighboring organelles, such as lysosomes. As a result of this proximity, the direct transfer of peroxidized lipids from LBs into lysosomes becomes possible. The exact mechanisms and pathways through which these peroxidized lipids affect the cross-presentation process remain to be elucidated.

Our data describe a novel mechanism of inhibition cross-presentation in DCs, associated with oxidized lipids, which may play an important role in the negative regulation of cross-presentation in cancer and, possibly, in other pathological processes associated with the accumulation of oxidized lipids, and may suggest potential targets for therapeutic regulation of cross-presentation. It is possible that this mechanism may affect recycling of other receptors, and thus affect function of the cells.

Disclosures
The authors have no financial conflicts of interest.

References


Corrections


The author list was published incorrectly. The corrected author line is shown below. In addition, an author’s last name was misspelled. The correct spelling is Vladimir A. Tyurin.


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Figure S1. Effect of tumor explant supernatants on the expression of MHC class I, II and co-stimulatory molecules on DCs and cross-presentation of OVA. A. DCs were generated from bone marrow progenitor with GM-CSF. On day 3 medium was replaced with one containing 20% v/v tumor explant supernatants (TES) from three different tumors: CT 26, EL4, or MC38. Two days later, cells were collected and analyzed. Expression of MHC class I, II, and co-stimulatory molecules was analyzed in gated CD11c⁺ cells. Typical example of 4 experiments is shown. B. The phenotype of cells cultured in the presence of TES. DCs were generated as described above in complete medium during 3–day culture with GM-CSF. On day 3 CD11c⁺ cells were isolated using magnetic beads and cultured for additional 48 hr in the presence of TES from EL-4 tumors. The phenotype of the cells was evaluated by flow cytometry. C. DCs generated as described above were cultured with 500 ng/ml LPS overnight. Expression of indicated molecules within gated CD11c⁺ cells was evaluated.
Figure S2

A

OVA

OVA derived Long Peptide

SIINFEKL

DC no antigen

DC no TES

DC TES

B

DCs loaded with OVA long Peptide

DC loaded with SIINFEKL

C

D

No Pept.

TES

SES

No TES

Isot.

SES

No TES

TES
Figure S2. Effect of tumor-derived factors and control spleen explant supernatant (SES) on pMHC and cross-presentation in DCs. **A.** DCs were differentiated from BM progenitors with GM-CSF and IL-4. At day 5, CD11c+ cells were isolated and cultured for 2 days with TES from EL4 tumors. DCs were loaded with OVA or long peptide during the last 24 hours of culture. **A.** Expression of pMHC and MHC class I (H2Kb) on DCs. **B.** Stimulation of OT-1 CD8+ T-cell proliferation. T-cell proliferation was measured in triplicates. * - p<0.05. **C.** DCs from WT mice cultured for 48 hr with either TES or explants from control splenocytes (SES). During last 24 hr in culture cells were loaded with long OVA-derived peptide and pMHC complexes were evaluated using 25-D1 antibody. **D.** DCs from OVA-Tg mice. Experiments were performed as above. Three experiments with the same results were performed.
Figure S3
Figure S3. Profile of oxidized lipids in DCs. A-C. DCs isolated from TB mice. A. Suggested structure of TAGs at m/z 872 and its oxygenated metabolite at m/z 920. B. Typical LC-MS spectra of TAG (C_{18:2}/C_{18:2}/C_{16:0}) at m/z 872 (left panel) and its oxygenated species (C_{18:2}/C_{18:2+3O}/C_{16:0}) at m/z 920 in control (middle panel) and tumor-derived (left panel) DCs. C. MS² spectra of TAGs at m/z 872 (left panel) and m/z 920 (right panel). D-G. Fragmentation LC-MS analysis of oxidized lipids in human DCs. D. Typical MS¹ spectrum of TAGs from human DCs cultured with TCM. E. Typical LC-MS profiles of oxidized TAGs at m/z 764 and 766. F. MS² spectra of oxidized TAGs with m/z 764 and 766, respectively, containing molecular species C_{16:1}/C_{18:1}/C_{9:0}+O and C_{16:0}/C_{18:1}/C_{9:0}+O with truncated 9-oxo-nonanoic acid from DCs. G. Possible structures of oxTAGs m/z 764 and 766.
Figure S4
**Figure S4. Effect of AMVN on the expression of surface molecules on DCs and IFN-γ on cross-presentation of long OVA-derived peptide.**  
**A.** DCs were generated from BM progenitors and treated for 2 hr with 0.5 mM AMVN. Cells were then washed and cultured in complete medium supplemented with GM-CSF for additional 24 hr. Cell phenotype was evaluated within CD11c+ cell population. Red line – control, blue line cells treated with AMVN. Three experiments with the same results were performed. Similar results were obtained with AMVN concentration 0.2 mM.  
**B.** Viability of DCs treated with 0.5 mM AMVN.  
**C.** DCs were cultured with IFN-γ (250 ng/ml) for 1 day, followed by loading with long OVA-derived peptide. Left panel – pMHC expression; right panel –MHC class I (H2Kb) expression.