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Role of Fatty-Acid Synthesis in Dendritic Cell Generation and Function

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Dendritic cells (DC) are professional APCs that regulate innate and adaptive immunity. The role of fatty-acid synthesis in DC development and function is uncertain. We found that blockade of fatty-acid synthesis markedly decreases dendropoiesis in the liver and in primary and secondary lymphoid organs in mice. Human DC development from PBMC precursors was also diminished by blockade of fatty-acid synthesis. This was associated with higher rates of apoptosis in precursor cells and increased expression of cleaved caspase-3 and BCL-XL and downregulation of cyclin B1. Further, blockade of fatty-acid synthesis decreased DC expression of MHC class II, ICAM-1, B7-1, and B7-2 but increased their production of selected proinflammatory cytokines including IL-12 and MCP-1. Accordingly, inhibition of fatty-acid synthesis enhanced DC capacity to activate allogeneic as well as Ag-restricted CD4+ and CD8+ T cells and induce CTL responses. Further, blockade of fatty-acid synthesis increased DC expression of Notch ligands and enhanced their ability to activate NK cell immune phenotype and IFN-γ production. Because endoplasmic reticulum (ER) stress can augment the immunogenic function of APC, we postulated that this may account for the higher DC immunogenicity. We found that inhibition of fatty-acid synthesis resulted in elevated expression of numerous markers of ER stress in humans and mice and was associated with increased MAPK and Akt signaling. Further, lowering ER stress by 4-phenylbutyrate mitigated the enhanced immune stimulation associated with fatty-acid synthesis blockade. Our findings elucidate the role of fatty-acid synthesis in DC development and function and have implications to the design of DC vaccines for immunotherapy. The Journal of Immunology, 2013, 190: 4640–4649.

Fatty-acid synthesis is an essential element of cellular metabolism. However, its role in DC development and function is uncertain. A study by Zeyda et al. (2) investigated the effects of exogenous administration of polyunsaturated fatty acids (PUFA) to DC and found that PUFA block DC immunogenic function independent of NF-κB activation. In particular, DC capacity for T cell activation was markedly inhibited in DC treated with PUFA. Similarly, a more recent report by Herber et al. (3) found that DC acquire exogenous lipids within the tumor microenvironment in both mice and humans, which renders them poorly functional, accounting for their inability to generate a potent antitumor immune response. The diminished DC immunogenicity facilitates the cancer’s capacity to evade immune recognition. Nevertheless, whereas exogenous fatty-acids, either directly administered or accumulated in tumor bearing hosts, appear to lessen the immunogenic potential of DC, the role of endogenous fatty-acid synthesis on dendropoiesis in vitro and in vivo and on DC functional properties is uncertain. In this study, we found that blocking fatty-acid synthesis using either inhibitors of acetyl CoA carboxylase or fatty-acid synthase diminished dendropoiesis from bone marrow or PBMC precursors. However, surprisingly, inhibition of fatty-acid synthesis upregulated DC expression of TLRs and markedly augmented DC capacity to stimulate Ag-restricted CD4+ and CD8+ T cells, induce CTL, and activate innate immune effector cells. Our mechanistic studies revealed that blockade of fatty-acid synthesis enhances MAPK, PI3K/Akt, and Notch signaling in DC and leads to higher endoplasmic reticulum (ER) stress. These findings suggest an important role for fatty-acids synthesis in modulating basic DC biology and have implications for the design of more effective immunotherapy regimens.

Dendritic cells (DC) have emerged over the past two decades as the most specialized professional APCs, which initiate adaptive and innate immune responses (1). As a consequence, DC have an important role in immune surveillance against developing cancer or invading pathogens and have potential to serve as vehicles for immunotherapy. Hence, elucidating the cellular biochemistry of DC has implications both for understanding immunity and for the design of immunotherapy regimens.

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Abbreviations used in this article: BMDC, bone marrow–derived dendritic cell; DC, dendritic cell; eIF2α, eukaryotic translation initiation factor 2α; ER, endoplasmic reticulum; MHC II, MHC class II; moDC, monocyte-derived dendritic cell; PB, PIPES buffer; PPAR-γ, peroxisome proliferator–activated receptor γ; PUFA, polyunsaturated fatty acid; T-BMDC, tall oil fatty acid–treated bone marrow–derived dendritic cell; TOFA, tall oil fatty acid.

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Materials and Methods

Animals

Male C57BL/6 (H-2k), BALB/c (H-2k), OT-I (B6.Cg-RAG2tm1Fwa-TgN), and OT-II (B6.Cg-RAG2tm1Alt-TgN) mice were purchased from Taconic Farms (Germantown, NY). Age-matched 6–8-wk-old mice were used in experiments. Animals were housed in a clean vivarium and fed standard mouse chow. In selected experiments, mice were injected three times weekly for 4 wk with saline or C75 (250 μg, i.p.; Sigma-Aldrich, St. Louis, MO), an inhibitor of fatty-acid synthase. Animal procedures were approved by the New York University School of Medicine Institutional Animal Care and Use Committee.

Murine bone marrow DC and human monocye-derived DC generation

Bone marrow–derived DC (BMDC) were generated as described (4). Briefly, bone marrow aspirates were cultured for 8 d in complete RPMI (RPMI 1640 with 10% heat-inactivated FBS, 2 mM L-glutamine, and 0.05 mM 2-ME) supplemented with GM-CSF (20 ng/ml). To generate human monocytederived DC (moDC), leukocyte-enriched buffy coats were obtained from the New York Blood Center. PBMCs were separated by density gradient centrifugation on Ficoll-Hypaque (GE Healthcare, Piscataway, NJ). Cells were cultured for 5–7 d in complete RPMI supplemented 10% human serum, 800 U/ml GM-CSF, and 1000 U/ml IL-4 (R&D Systems, Minneapolis, MN). In selected experiments, acetyl CoA carboxylase was inhibited in murine BMDC or human moDC cellular suspensions using tall oil fatty acid (TOFA; 5 μg/ml, Cayman Chemical, Ann Arbor, MI) beginning on day 2 of culture (5–7). In selected experiments, a lower dose of TOFA was used (1 μg/ml). Ethanol (0.5%) was used as a solvent for TOFA. In additional experiments, staurosporine (10 μM) was employed to induce DC apoptosis (8, 9).

Leukocyte isolation from liver and spleen

Murine hepatic nonparenchymal cells were isolated as described (10). Briefly, the portal vein was infused with Collagenase IV (Sigma-Aldrich) followed by hepatocyte and mechanical digestion. Hepatocytes were excluded by serial low-speed (300 rpm) centrifugation. Nonparenchymal cells were further enriched over an Optiprep (Sigma-Aldrich) density gradient. Hepatocytes were further enriched over an Optiprep (Sigma-Aldrich) gradient. Hepatocytes were isolated by the Folsch extraction method, and C-14 uptake was determined by scintigraphy as described (11).

Flow cytometry and cytokine analysis

Flow cytomtery was performed using the FACSCalibur (BD Biosciences, Franklin Lakes, NJ) after incubating 5 × 10^6 cells/tube with 1 μg anti–FcyRIII/II Ab (2.4G2, Fc block; mAb Core, Sloan-Kettering Institute, New York, NY) and then labeling with 1 μg fluorescently conjugated mAb against MHC class II (MHC II: I-A^b), CD4 (RM4-5), CD8 (53-6.7), CD11b (M1/70), CD11c (HL3), CD19 (ID3), CD25 (3C7), CD40 (H44-21), CD45 (30-F11), CD54 (YN1/17.4), B7-1 (1-61A10), B7-2 (GL1), Foxp3 (FJK-16s; all from eBioscience, San Diego, CA), CD3e (145-2C11; BioLegend, San Diego, CA), and TLR2 (T2.5) (Ingenex, San Diego, CA). Alternatively, cells were labeled with unconjugated Abs against Jagged-1 (Santa Cruz Biotechnology, Santa Cruz, CA), TLR4, TLR7, and TLR9 (all from Imgenex) and subsequently stained with fluorescently labeled secondary Abs. Human moDC were measured using mAbs directed against HLA-DR and CD11c (BD Biosciences). For cytokine analysis, cells suspensions were cultured in complete RPMI at a concentration of 1 × 10^6 cells/ml for 24 h before supernatant harvest and analysis either using either a cytometric bead array (BD Biosciences) or the Milliplex Immunoassay bead array (BD Biosciences) or the Milliplex Immunoassay bead array (BD Biosciences) or the Milliplex Immunoassay bead array (BD Biosciences) or the Milliplex Immunoassay bead array (BD Biosciences) or the Milliplex Immunoassay bead array (BD Biosciences).

In vitro T cell assays and CTL assays

For CD4^+ or CD8^+ T cell proliferation assays, BMDC were pulsed with the appropriate Ova peptide (10 μg/ml; Abcam, Cambridge, Massachusetts) for 90 min before washing and plating, respectively, with CD4^+OT-II TCR-transgenic T cells (1 × 10^5) specific for Ova257–264 peptide or CD8^+OT-I TCR-transgenic T cells specific for Ova257–264 peptide for 72 h in 96-well plates as described (10). In selected experiments, non–peptide-pulsed DC were used to stimulate allogeneic BALB/c T cells in an MLR as described (10). For cross-presentation experiments, DC were loaded with OVA (1 mg/ml; Sigma-Aldrich) and used to stimulate OT-I T cells as described (10). For the last 24 h, 1 μCi [ ^3H]thymidine was added to wells and proliferation measured using a MicroBeta counter (PerkinElmer, Waltham, MA). Alternatively, T cell activation was assessed by measuring Th1, Th2, and Th17 cytokine production using a cytometric bead array or by examination of T cell surface phenotype. In selected experiments, soluble inhibitors of PI3K (50 μM; LY294002) and MAPK (100 μM; PD98059) were used in experiments. Animals were homogenized on ice. Alternate experiments were performed without inhibitors. For the last 24 h, 100 ng/ml IL-2 was added to co-cultures. In selected experiments, the ability of DC to produce a CTL response in vivo, naive mice were immunized i.p. twice at weekly intervals with DC-Ova257–264 (1 × 10^6) or mock immunized. One week later, splenocytes were harvested from immunized mice, restimulated in vitro with Ova257–264, and cell–cell culture supernatant assayed for IFN-γ and IL–10 as described (4).

NK cell assays

DC–NK cell cocultures were performed as described with slight modifications (4). Briefly, splenic NK cells (1 × 10^5) were plated with BMDC (1 × 10^5) in a 1:1 ratio in 96-well plates for 24 h. IFN-γ was measured in cell–culture supernatant using a cytometric bead assay (BD Biosciences). In addition, NK cell expression of CD25 was analyzed by flow cytometry.

Ag capture assays

To assess DC capacity for Ag uptake, BMDC were incubated with FITC-dextran, FITC-albumin, or FITC-mannose albumin (1 mg/ml; all from Sigma-Aldrich) at 37°C for various time intervals. Ag uptake was determined by flow cytometry. For in vivo Ag uptake, control or C75-treated mice were injected i.p. with 1 mg FITC-albumin. Mice were then sacrificed at 30 min and splenic DC fluorescence determined by flow cytometry.

Microscopy and lipid analysis

For light and fluorescent microscopic analysis, cells were spun onto slides and stained with H&E, Giemsa, HCS LipidTOX Red specific for neutral lipids, and HCS LipidTOX Green specific for phospholipids (Invitrogen, Grand Island, NY). Light microscopic images were captured using an Axiovert 40 microscope (Zeiss, Thornwood, NY). Fluorescent light images were captured on an Axiovert 200M (Zeiss). Cells were also tested by flow cytometry using BODIPY (Invitrogen) as described (11). For electron microscopy analysis, BMDC suspensions were incubated with 4% formaldehyde and 2% glutaraldehyde in 0.1 M (pH 7.5) PIPES buffer (PB) for 30 min at room temperature. Cells were further fixed with 4% glutaraldehyde and 0.4% tannic acid in PB for 30 min followed by 2% osmium tetroxide in PB for 1 h. The samples were then counterstained with 2% uranylacetate for 12 h at 4°C before exchanging the solution with ethanol. Samples were then infused with epoxy resin and polymerized at 60°C. The sample blocks were sectioned to a thickness of 50–70 μm, collected on electron microscope grids, and stained with uranylacetate and Sato Lead Stain. The samples were then imaged using a Philips CM12 microscope (Philips, Eindhoven, The Netherlands). For each section, the cellular, nuclear, and cytosolic areas were estimated by assuming the cell and nucleus sections are ellipses and measuring the long and short axes. To measure the rate of intracellular fatty-acid synthesis in BMDC, C-14–labeled acetate was added to BMDC cultures (2 μCi/well) for 6 h. More than 4 × 10^5 cells from each treatment group were analyzed. Intracellular lipids were isolated by the Folsch extraction method, and C-14 uptake was determined by scintigraphy as described (11).

Western blotting

Western blotting was performed as described (11, 12). Briefly, BMDC were homogenized in RIPA buffer, and proteins were separated from larger fractions by centrifugation at 14,000 × g. Samples were equiloaded onto 10% polyacrylamide gels (NuPage; Invitrogen), electrophoresed at 200 V, electrotransferred to polyvinylidene difluoride membranes, and probed with mAbs to GRP-78, eukaryotic translation initiation factor 2α (eIF2α), p-eIF2α, XBP-1, peryxosome proliferator–activated receptor γ (PPAR-γ), caspase-3, BCL-xl, cyclin B1, Jagged-1, α, Akt, p-Akt, p-ERK1/2, phosphorylated phosphoinositol-3-kinase, β-actin (all from Santa Cruz Biotechnology). Blots were developed by ECL (Thermo Scientific, Asheville, NC).
Statistics
Statistics were calculated using GraphPad Prism V5.00 (GraphPad Software, San Diego, CA). Data are presented as mean ± SEM. Statistical significance (p < 0.05) was determined using the Student t test and the log-rank test.

Results
Blockade of fatty-acid synthesis inhibits dendropoiesis

To determine whether blockade of fatty-acid synthesis in vivo affects dendropoiesis in lymphoid and nonlymphoid organs, mice were serially administered C75, an inhibitor of fatty-acid synthase (13, 14), and the number of CD11c+ cells was measured in the bone marrow, spleen, and liver. Treatment for 4 wk resulted in an 80% reduction in the fraction and total number of CD11c+ cells in the liver (Fig. 1A, 1B) and an ~20% reduction in the spleen and bone marrow (Fig. 1B). Other cell types, including B cells, T cells, neutrophils, and macrophages, were not affected (Fig. 1C).

To investigate the effects of inhibition of fatty-acid synthesis on DC generation in vitro from bone marrow precursors, we isolated bone marrow cells and cultured them in GM-CSF-supplemented media for 8 d to drive dendropoiesis, as described (4). In parallel, for the duration of in vitro culture, bone marrow cells were coincubated with TOFA, which inhibits acetyl CoA carboxylase (15, 16). The number of nonviable propidium iodide+ cells was increased on day 8 of culture (Fig. 1D) as well as at earlier time points (not shown) in cellular suspensions incubated with TOFA. Further, there was increased expression of cleaved caspase-3 and BCL-xL in TOFA-treated BMDC (T-BMDC), consistent with increased rates of apoptosis (Fig. 1E). Accordingly, cyclin B1, an anti-apoptotic gene, was downregulated in T-BMDC (Fig. 1E).

The total number and fraction of CD11c+ cells produced per mouse femur (Fig. 1F) and BMDC cellular proliferation (Fig. 1G) were also lower in TOFA-treated bone marrow cultures. Generation of human moDC was similarly hindered by TOFA (Fig. 1H). Furthermore, serial in vivo administration of C75 resulted in less-efficient generation of BMDC after bone marrow harvest (Supplemental Fig. 1A). Taken together, these data show that blockade of fatty acid synthesis inhibits dendropoiesis in vitro and in vivo and in both mice and humans.

FIGURE 1. Blockade of fatty-acid synthesis inhibits dendropoiesis in mice and humans. (A–C) Mice were treated for 4 wk with C75 or saline. (A) Live CD45+ liver leukocytes were gated using flow cytometry, and the subfraction of hepatic CD11c+ cells was determined. (B) The percentage decrease in the number of liver, spleen, and bone marrow DC was calculated. (C) The fraction of splenocytes expressing CD3, CD19, and CD11b in saline- or C75-treated mice was tested. (D–G) BMDC were grown alone or with TOFA. (D) The fraction of propidium iodide (PI)+ cells was calculated on day 8 of culture. (E) Day 8 BMDC and T-BMDC were also tested for expression of caspase-3, cleaved caspase-3, BCL-xL, cyclin B1, and β-actin by Western blotting. (F) In addition, the total number and fraction of CD11c+ cells was calculated in day 8 BMDC and T-BMDC cultures. (G) Cellular proliferation was compared in day 8 BMDC and T-BMDC by pulsing with [3H]thymidine. (H) moDC grown in control media and TOFA-enriched media were tested for HLA-DR and CD11c expression. Median fluorescence intensity is indicated for each respective histogram (*p < 0.05, **p < 0.01, ***p < 0.001).
Inhibition of fatty-acid synthesis alters DC morphology and surface phenotype

As anticipated, bone marrow–derived cells grown in TOFA exhibited a decreased rate of fatty-acid synthesis (Fig. 2A). Accordingly, on both electron microscopy and light microscopy, T-BMDC exhibited decreased vacuolization and numbers of lipid droplets (Fig. 2B, 2C, Supplemental Fig. 1B). Similarly, HCS LipidTOX Red staining revealed a substantial reduction in total neutral lipids (Fig. 2D, Supplemental Fig. 1C), and HCS LipidTOX Green staining revealed decreased phospholipid levels in T-BMDC (Fig. 2E, Supplemental Fig. 1D). Further, T-BMDC had diminished staining for BODIPY, which binds total neutral lipids (Supplemental Fig. 1E).

Because we found that inhibition of fatty-acid synthesis prevents dendropoiesis, we postulated that it may also affect BMDC maturation. To test this, bone marrow–derived CD11c+ cells were analyzed for expression of MHC II, costimulatory, and adhesion molecules. As anticipated, T-BMDC exhibited decreased expression of MHC II, ICAM-1, B7-1, and B7-2 (Fig. 2F). However, CD40 and CD11b were consistently upregulated in BMDC grown in TOFA (Fig. 2F). Similar phenotypic differences between T-BMDC and controls were seen when gated exclusively on CD11c+ MHC II+ cells (not shown). Surprisingly, despite a diminished maturation phenotype, blockade of fatty-acid synthesis upregulated DC surface expression of TLR2 and TLR4 and intracellular expression of TLR7 and TLR9 (Fig. 2G). Conversely, in contrast to the effects of TOFA, staurosporine, which also induced BMDC apoptosis (Supplemental Fig. 2A), upregulated MHC II expression on BMDC (Supplemental Fig. 2B), and did not increase BMDC TLR expression (Supplemental Fig. 2C), suggesting that effects of TOFA are specific to fatty-acid synthesis inhibition.

TOFA increases ER stress, PPAR-γ expression, and cytokine production in BMDC

ER stress can have marked affects on the immune-stimulatory capacity of APCs (17–19). Because inhibition of fatty-acid synthesis induces ER stress in neoplastic cells (20), we postulated that TOFA-grown BMDC would exhibit high ER stress. In consort with our hypothesis, we found that GRP-78, eIF2α, p-eIF2α, and XBP-1, all markers of ER stress (21), were more highly expressed in T-BMDC compared with controls (Fig. 3A). Human moDC generated in TOFA also expressed markedly elevated p-eIF2α (Fig. 3B). Higher PPAR-γ expression has been linked to increased ER stress and is associated with enhanced DC capacity to present Ag (22–25). Accordingly, we found substantial upregulation of PPAR-γ expression in murine T-BMDC at both the protein (Fig. 3C) and mRNA levels (Fig. 3D). TOFA-treated human moDC also expressed higher PPAR-γ (Fig. 3E).

The respective roles of ER stress or endogenous fatty-acid synthesis on DC production of immune-modulatory cytokines and
chemokines are uncertain. Because DC regulate immunity by production of soluble inflammatory mediators, we tested T-BMDC cytokine and chemokine production. BMDC production of an array of inflammatory mediators, including IL-1α, IL-1β, IL-6, IL-10, IFN-γ, IP-10, keratinocyte chemotactrant, LIF, MCP-1, M-CSF, MIG, MIP-2, and G-CSF, were higher in T-BMDC compared with controls (Fig. 3F, 3G). However, the CC chemokines MIP-1α, MIP-1β, and RANTES were expressed at markedly lower levels in T-BMDC (Fig. 3H). Lower-dose TOFA (1 mg/dl) also increased BMDC cytokine production; however, 0.5% ethanol alone had no effect nor did staurosporine (Fig. 3I).

Blockade of fatty-acid synthesis enhances DC capacity for Ag capture

Ag uptake is a primary function of DC and a critical consideration in constructing DC vaccines for cancer immunotherapy (26, 27). To determine the role of fatty-acid synthesis in DC capacity to capture Ag, BMDC were grown alone or in media supplemented with TOFA, as above. Consistent with their relative immaturity, T-BMDC exhibited enhanced ability to capture Ag via generalized macropinocytosis (Fig. 4A) or using specialized mannose receptors (Fig. 4B, 4C). Similarly, serial treatment of mice with C75 resulted in markedly enhanced spleen DC capacity to capture Ag in vivo (Fig. 4D). Low-dose TOFA was similarly effective at enhancing DC capacity for Ag capture as high-dose TOFA (Supplemental Fig. 3A). Conversely, ethanol or staurosporine did not enhance DC ability to capture soluble Ag (Supplemental Fig. 3B). These data imply that blockade of fatty-acid synthesis enhances DC capacity for Ag capture in multiple contexts.

Inhibition of fatty-acid synthesis enhances BMDC capacity to activate allogeneic and Ag-restricted CD4+ and CD8+ T cells

Because blockade of fatty-acid synthesis augments BMDC ER stress and increases their production of inflammatory mediators, we postulated it would enhance their immune stimulatory function. In consort with our hypothesis, T-BMDC induced higher proliferation of allogeneic T cells in an MLR compared with controls (Fig. 5A). To determine the effect of inhibiting fatty-acid synthesis on DC capacity to stimulate Ag-restricted CD4+ T cell, control or T-BMDC were loaded with Ova 323–339 and then cocultured in various concentrations with CD4+ OT-II T cells. Peptide-pulsed BMDC grown in TOFA induced more vigorous proliferation of Ag-restricted CD4+ T cells (Fig. 5B) and induced higher CD4+ T cell production of Th1 and Th17 cytokines (Fig. 5C) compared with peptide-pulsed control BMDC stimulators. Conversely, Th2 cytokines were uniformly expressed at low levels after stimulating OT-II cells using either BMDC or T-BMDC. There was similarly no significant difference between control and T-BMDC in their propensity to generate CD4+CD25+Foxp3+ regulatory T cells in
production of IFN-\(\gamma\) with CD8\(^+\) OT-I T cells. Peptide-pulsed T-BMDC induced (Fig. 6E) and production of IFN-\(\gamma\) presentation of OVA as evidenced by higher T cell proliferation at weekly intervals with Ova\(257–264\) peptide–pulsed control BMDC.

To determine the requirement for fatty-acid synthesis during DC generation on their ability to generate CTL in vivo, mice were immunized twice every 3 wk after the second immunization and were restimulated in vitro with Ova\(257–264\) peptide. On day 5, CTL cultures were tested for production of IFN-\(\gamma\) and IL-10. Consistent with our previous findings, in vivo immunization using T-BMDC induced elevated production of IFN-\(\gamma\) in CTL supernatant (Fig. 6G). Moreover, T-BMDC immunization resulted in decreased production of IL-10, an inhibitory cytokine, in CTL cultures compared with immunization using peptide-pulsed control BMDC (Fig. 6H). Taken together, these data suggest that blockade of fatty-acid synthesis is an attractive strategy to enhance DC capacity for induction of immunogenic CTL responses.

**Blockade of fatty-acid synthesis enhances MAPK and PI3K/Akt signaling in BMDC**

Because DC immune-stimulatory capacity has been linked to the MAPK, NF-\(\kappa\)B, and PI3K/Akt signaling pathways (29), we tested the effect of inhibition of fatty-acid synthesis on the cellular activation of these pathways. Consistent with their enhanced CD4\(^+\) and CD8\(^+\) T cell stimulatory capacity, we found that T-BMDC expressed elevated levels of pERK-1, an activated MAPK signaling intermediate (Supplemental Fig. 4A). We also found that T-BMDC expressed elevated levels of pAkt as well as p70 S6 kinase, which acts downstream of phosphatidylinositol 3,4,5-triphosphate, suggesting activation of the PI3K/Akt signaling pathway in BMDC in the context of fatty-acid synthesis blockade (Supplemental Fig. 4B). Phosphatase and tensin homolog, which negatively regulates PI3K/Akt signaling, was equally expressed in TOFA-treated and control BMDC (Supplemental Fig. 4B). However, activated NF-\(\kappa\)B intermediates were expressed at lower levels after TOFA treatment, which is consistent with their elevated intracellular ER stress (30) (Supplemental Fig. 4C).

**Enhanced T cell stimulatory capacity of TOFA-treated BMDC is contingent on their higher ER stress**

Elevated ER stress has been linked to enhanced Ag presentation by APC (31). Because the chaperone 4-phenylbutyrate inhibits adipogenesis by modulating the unfolded protein response and decreasing ER stress (32), we postulated this may mitigate the increased immunogenicity of TOFA-treated BMDC. Accordingly, we found that the preincubation with 4-phenylbutyrate significantly reduced the CD4\(^+\) and CD8\(^+\) T cell stimulatory capacity of T-BMDC (Fig. 7A, 7B). T cell activation by control BMDC was affected to a lesser extent by the chaperone. These data imply that the increased immunogenicity of T-BMDC may be related to their increased ER stress. Blockade of MAPK or PI3K/Akt signaling did not mitigate the augmented capacity of T-BMDC to induce T cell proliferation (Fig. 7C). However, MAPK inhibition lessened T cell activation (Fig. 7D). PI3K blockade had no effect (data not shown).

**Blockade of fatty-acid synthesis enhances BMDC capacity to activate NK cells**

We and others have demonstrated that BMDC are powerful activators of innate immune effector cells such as NK and NKT cells (33, 34). To examine the role of fatty-acid synthesis in BMDC capacity to activate NK cells, we cocultured T-BMDC and controls with equal numbers of NK cells before NK cell harvest and measurement of their phenotypic activation and production of IFN-\(\gamma\). T-BMDC induced elevated NK cell expression of CD25 (Fig. 8A) and induced 4-fold higher production of IFN-\(\gamma\) compared with control BMDC (Fig. 8B). Because DC capacity to activate NK cells has recently been linked to their expression of Notch ligands (35), we tested whether blockade of

**FIGURE 4.** Blockade of fatty-acid synthesis enhances DC capacity for Ag capture in vitro and in vivo. BMDC and T-BMDC were tested at various time points for uptake of fluorescent albumin (A), dextran (B), and mannosylated albumin (C, D). Splenic CD11c\(^+\) cells from control or C75-treated mice were tested for uptake of FITC-albumin at 30 min after in vivo administration. Data are representative of experiments performed three times. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\). MFI, Median fluorescence intensity.
acetyl CoA carboxylase secondarily increases Notch ligand expression in BMDC. As postulated, T-BMDC expressed higher Jagged-1 and Δ4 compared with control BMDC on analysis by Western blotting (Fig. 8C) and flow cytometry (Fig. 8D).

Discussion
DC are a specialized population of APCs that link innate and adaptive immunity (1). DC can influence immune responses by both direct interaction with effector cells, such as T cells and NK cells, and via production of a wide array of inflammatory mediators. In this study, we found that blockade of fatty-acid synthesis markedly inhibits DC development from bone marrow or PBMC precursors in mice and humans, respectively, and induces apoptosis in DC precursors, which is associated with elevated cellular expression of cleaved caspase-3, BCL-xL, and downregulation of cyclin B1. For our in vivo experiments, we employed C75 in

FIGURE 5. T-BMDC induce enhanced alloge neic and Ag-restricted CD4+ T cell stimulation. (A) Various concentrations of BMDC and T-BMDC were tested for their ability to induce proliferation of allogeneic T cells in an MLR. BMDC and T-BMDC pulsed with Ova257–264 peptide were tested for their ability to induce Ag-restricted CD4+ T cell proliferation (B) and Th1, Th2, and Th17 cytokine production (C) in OT-I T cells. (D) CD4+ T cell coexpression of CD25 and Foxp3 was tested at 96 h after splenocytes were cocultured in a 1:1 ratio with BMDC or T-BMDC. *p < 0.05, **p < 0.01, ***p < 0.001. FSC, Forward light scatter.

FIGURE 6. T-BMDC induce enhanced CD8+ T cell activation. (A–D) BMDC and T-BMDC were loaded with Ova257–264 peptide and plated in various ratios with CD8+ OT-I T cells. (A) OT-I proliferation was measured by incorporation of [3H]thymidine. (B) OT-I T cell expression of CD44 was measured on flow cytometry (median fluorescence intensity is indicated). IFN-γ (C) and TNF-α (D) production by CD8+ OT-I T cells was measured in cell-culture supernatant. (E and F) To test DC capacity for cross-presentation, BMDC and T-BMDC were loaded with OVA and used in various ratios to stimulate CD8+ OT-I T cells. OT-I cellular proliferation (E) and production of IFN-γ (F) were measured. Restimulated CTL cultures from mice twice immunized by adoptive transfer of Ova257–264 peptide–pulsed BMDC or T-BMDC were tested for production of IFN-γ (G) and IL-10 (H). *p < 0.05, **p < 0.01, ***p < 0.001.
lieu of TOFA, as TOFA is highly toxic when administered systemically (7, 36). We found that in vivo blockade of fatty-acid synthesis hinders DC generation in peripheral tissues as well as primary and secondary lymphoid organs. Fatty-acid synthesis inhibition also has variable effects on DC surface phenotype including suppression of MHC II expression but increased CD40 expression. Further, T-BMDC express higher levels of selected MAPK and PI3K/Akt signaling intermediates and produce markedly elevated levels of numerous cytokines and chemokines (37, 38). Interestingly, the CC chemokines MIP-1α, MIP-1β, and RANTES, which are purported to play a role in granulocytic lineage proliferation or differentiation (39), are suppressed by fatty-acid synthesis inhibition. Understanding the mechanistic regulation of BMDC cytokine and chemokine production by fatty-acid synthesis requires more exact study; however, there are likely to be autocrine effects of specific cytokines on further BMDC production of additional inflammatory mediators. For example, Stober et al. (40) reported that IL-12 can influence IFN-γ production by BMDC. Taken together, our data suggest that the capacity for fatty-acid synthesis is important for DC generation and expression of their distinct immune phenotype.

The properties of DC generated in the context of fatty-acid synthesis inhibition are relevant not only to understanding basic DC immunobiology but also for the development of vaccines for immunotherapy. In particular, TOFA-treated DC exhibited increased capacity to activate CD4+ and CD8+ T cells, which can be exploited in the construction of DC cancer vaccines. T-BMDC induction of Ag-restricted CD8+ T cells led to increased production of IFN-γ and TNF-α, and decreased production of IL-10. DC immunotherapy regimens in cancer and benign diseases have largely been of limited clinical efficacy because of the modest adaptive immune responses and CTLs induced (28, 41). Varied cytokine cocktails and methods of exogenous DC stimulation have been employed to bolster the host’s Ag-restricted and innate immunogenic responses to DC vaccines (28); hence, our data suggest that inhibiting fatty-acid synthesis may be an attractive adjuvant in experimental immunotherapy.

The mechanism for the enhanced immune-stimulatory capacity of DC generated in the context of fatty-acid synthesis inhibition appears to be related in part to their elevated ER stress. ER stress is generated in response to an accumulation of unfolded or misfolded proteins in the lumen of the endoplasmic reticulum (42). ER stress attempts to restore normal cellular function by halting protein translation and activating signaling pathways, leading to produc-
tion of molecular chaperones that facilitate protein folding. This process has been found to be conserved among all mammalian species and can result in cellular apoptosis if not resolved (43). There is an emerging role for ER stress in the function of APCs. Goodall et al. (44) reported that activation of ER stress, in combination with TLR ligation, markedly enhances DC expression of selected cytokines. Additionally, Oh et al. (45) recently reported that ER stress is a functional switch regulating M2 macrophage differentiation and phenotype including cellular cholesterol content. Our observations of elevated ER stress in TOFA-treated BMDC were made in both our murine and human models and were further adduced by higher DC expression of PPAR-γ after TOFA treatment. Further, our finding of increased expression of certain activated MAPK signaling intermediates in T-BMDC is consistent with a recent report showing involvement of Erk MAPK in ER stress in human neuroblastoma cells (46). Notably, Hayakawa et al. (30) recently found that ER stress depresses NF-κB activation, which is in consort with our finding of diminished levels activated NF-κB intermediates in T-BMDC. These findings are also consistent with the observation by Zeyda et al. (2), who reported that exogenous administration of PUFAs lessened DC immune-stimulatory capacity independent of NF-κB signaling. However, our findings of enhanced Akt activation are surprising in this context because ER stress has been reported to negatively regulate the Akt/mTOR pathway (47). Further, PPAR-γ can also negatively regulate Akt phosphorylation (48). These data suggest that alternate mechanisms may be responsible for the elevated levels of pAkt in T-BMDC. It is also notable that MAPK inhibition but not PI3K/Akt signaling blockade mitigated the enhanced T cell immune-stimulatory capacity in developing human moDC (2). However, taken in the context of our recent work, there appears to be a dichotomy between the effects of fatty-acid synthesis inhibition on DC developing from cellular precursors versus blockade of fatty-acid synthesis on fully mature DC populations. In particular, we recently reported that fully mature liver DC can be divided into two distinct populations based on intracellular lipid content, including triglycerides and phospholipids (11). Further, we found that liver DC immunogenicity is determined by their lipid content, as lipid-rich liver DC were more immunogenic in comparison with lipid-poor liver DC. This was demonstrated by their higher secretion of cytokines and activation of Ag-restricted CD4+ and CD8+ T cells as well as NK cells and NKT cells. Moreover, blockade of fatty-acid synthesis in terminally differentiated lipid-rich liver DC using TOFA diminished their capacity for T cell and NK cell activation (11). Therefore, the effects of fatty acids, or blockade of their production, on DC properties in the current study appears to be limited to developmental effects rather than applicable to mature fully differentiated DC.

Disclosures

The authors have no financial conflicts of interest.

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