Endogenous versus exogenous fatty acid availability affects lysosomal acidity and MHC class II expression

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Abstract **Although the immune system, inflammation, and cellular metabolism are linked to diseases associated with dyslipidemias, the mechanism(s) remain unclear. To determine whether there is a mechanistic link between lipid availability and inflammation/immune activation, we evaluated macrophage cell lines incubated under conditions of altered exogenous and endogenous lipid availability. Limiting exogenous lipids results in decreased lysosomal acidity and decreased lysosomal enzymatic activity. Both lysosomal parameters are restored with the addition of oleoyl-CoA, suggesting that fatty acids play a role in the regulation of lysosomal function. Cell surface expression of major histocompatibility complex (MHC)-encoded molecules is also decreased in the absence of exogenous lipids. Additionally, we observe decreased γ-interferon stimulation of cell surface MHC class II. Using cerulenin to limit the endogenous synthesis of fatty acids results in decreased cell surface expression of MHC class II but does not appear to alter lysosomal acidity, suggesting that lysosomal acidity is dependent on exogenous, but not endogenous, fatty acid availability. Testing these conclusions in an in vivo mouse model, we observed statistically significant, diet-dependent differences in lysosomal acidity and MHC class II cell surface expression. Collectively, these data demonstrate a mechanistic link between lipid availability and early events in the immune response.**—Schweitzer, S. C., A. M. Reding, H. M. Patton, T. P. Sullivan, C. E. Stubbs, E. Villalobos-Menuey, S. A. Huber, and M. K. Newell. **Endogenous versus exogenous fatty acid availability affects lysosomal acidity and MHC class II expression.** ***J. Lipid Res.*** **2006.** 47: **2525–2537.**

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Lipids are essential components of all cells. A cell's lipid needs are usually met by a mixture of endogenous synthesis, exogenous (dietary) availability, stored excess, and reuse through salvage pathways (1). Not surprisingly, defects in lipid metabolism are commonly accepted components of many diseases, including heart disease (2–5), diabetes (6, 7), obesity (8, 9), lysosomal storage disorders (10–12), alcoholism (13), cancer (14–16), aging (8), autoimmune diseases (17, 18), and infectious diseases (19, 20). The diversity of these diseases has made the identification of a mechanistic link between lipids and the pathogenesis of disease difficult (21).

Recent work suggests that the immune system (22, 23), inflammation (24), and oxidative damage (25) are key components of many diseases associated with dyslipidemias. The immune system is a complex organization of cells, tissues, and organs serving to protect the body from harm. Centrally important players include macrophages, lymphocytes, and the chemical mediators produced by these cells (26). An important function of macrophages is to engulf and degrade antigens in the endosomal/lysosomal compartment (27, 28). In the lysosome, the processed antigens are loaded onto major histocompatibility complex (MHC) class II molecules and trafficked to the cell surface, allowing T-lymphocytes to recognize and respond to MHC class II and antigen. This recognition is the first of two requisite steps (29, 30) leading to T-cell activation, cytokine production, and cytokine release. The cytokines released by activated T-lymphocytes, macrophages, and other immune cells are important mediators of inflammation (31).

Because lysosomes play an important role in fatty acid storage and in the early cellular events of antigen processing and presentation (32, 33), we hypothesized that the lysosome represents a "common denominator" linking lipids (particularly fatty acids) to immunological events. We reasoned that altering the availability of exogenous and/or endogenous lipids would affect antigen processing and/or presentation and potentially the extent and nature of T-lymphocyte activation. Our rationale is supported by the established influence of diet on diseases associated with dyslipidemias, inflammation, and the immune response (34–36).

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To explore our hypothesis, we chose to use both an in vitro cell culture model and a long-term (16 week) in vivo mouse study. Results from our in vitro model, in which we limit either exogenous availability or the endogenous synthesis of fatty acids, are consistent with the interpretation that fatty acids are important in both maintaining lysosomal acidity and determining the level of expression of cell surface MHC class II molecules. Our in vivo dietary model suggests that dietary lipids influence lysosomal acidity and also supports a diet-dependent effect on the level of cell surface MHC class II expression.

METHODS

Cell culture and adaptation to low lipid conditions

Cell lines were maintained in RPMI medium supplemented with 5% FBS, 2 mM L-glutamine, 10 mM HEPES buffer, 10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, 0.04 μg/ml gentamycin, and penicillin/streptomycin (500 U/ml and 500 μg/ml, respectively) (all reagents were from Gibco BRL). Cell suspensions were incubated at 37°C under 5% CO_2 humidified atmospheric conditions. To adapt cells to growth in low-lipid conditions, log growth cells ($0.2–0.8 \times 10^6$ cells/ml) were grown in medium containing increasing concentrations of delipidized bovine calf serum (Pel-Freez Biologicals) as a replacement for FBS. A second source of low-lipid additive, Completely Processed Serum Replacement-1 (Sigma), was also used to establish low-lipid conditions and gave similar results (data not shown). Low-lipid cell lines derived from HL60 cells included 60-7/low lipid and 60-15/low lipid. Low-lipid cell lines derived from U-937 cells included U-15/low lipid and U-16/low lipid.

Flow cytometry

For cytometric analysis, stained cells were washed and resuspended in 0.5 ml of PBS containing 2.5% FBS (PBS/FBS). Fluorescence was detected on a Coulter Excel flow cytometer, and unless specified otherwise, results are expressed as the geometric mean fluorescence of stained samples minus unstained controls or isotype. Flow cytometry data were analyzed using Cell Quest (Becton Dickinson) or FlowJo software. Percentage cell death was calculated based on forward versus side scatter profiles of cytometrically gated populations of live and dead cells. Cell size was determined by analysis of the forward scatter profiles of gated populations of live cells.

LysoSensor™ staining of acidic compartments

Lysosomal acidity was determined by staining 100 μl of log growth cells with LysoSensor Green DND-189 (34) (Molecular Probes) according to the manufacturer's instructions. Briefly, 100 μl of washed cells was incubated with 0.5–1.0 μl of LysoSensor reagent in the dark at 37°C for 20 min. Cells were washed in PBS/FBS, and fluorescence was detected by flow cytometry.

Lysosomal glucocerebrosidase assay

The functional activity of lysosomes was determined using a flow cytometric assay for lysosomal glucocerebrosidase as described by van Es et al. (35). Briefly, fluorescein β-D-glucopyranoside (FDGlu; Sigma) was added to 100 μl of cells ($10^5–10^6$ cells) to give a final substrate concentration of 1.05 mM. Cells were incubated with substrate for 45 min at 37°C. Lysosomal glucocerebrosidase activity produces a fluorescent substrate that is detected by flow cytometry. As a control, 5–40 μM concentrations of chloroquine (Sigma) were incubated with cells for 1 h before the addition of FDGlu substrate.

Cell surface immunofluorescence staining

Intact cells were washed and resuspended in PBS/FBS to a cell density of $5–10 \times 10^6$ cells/ml. Suspensions of human cells were blocked for nonspecific staining by incubating washed cells on ice for 25–30 min with an equal volume of 10% normal mouse serum (Sigma) diluted in PBS/FBS. Mouse cells were Fc-blocked by the addition of 1 μl of anti-mouse Fc_γ clone 24G2 (Pharmingen) to 100 μl of washed cells, followed by incubation on ice for 15–20 min. Staining was done in microtiter plates using 100 μl of cell suspension and FITC- or phycoerythrin (PE)-conjugated antibodies, according to the manufacturer's recommended procedures and concentrations. The one exception was MHC class I staining of human cells, which used 10 μl of antibody instead of the recommended 20 μl. Fluorescence was detected by flow cytometry. Fluorochrome-conjugated antibodies included mouse anti-human HLA-DR, -DP, and -DQ (Pharmingen), mouse anti-human HLA-DR (BD Diagnostics), mouse anti-human HLA-A,B,C (*e*Biosciences), mouse anti-mouse M5114 antibody detecting mouse I-A and I-E (BD Bioscience), mouse anti-human CD11b (Pharmingen), and mouse anti-human CD14 (Pharmingen).

Total cellular HLA-DR staining

Total cellular pools of HLA-DR were measured by permeabilizing cells with CytopermCytofix™ (Pharmingen) using the manufacturer's recommended procedure. Permeabilized human cells were blocked by incubation with 10% normal mouse serum diluted in CytopermCytofix wash buffer and then stained with fluorochrome-conjugated antibodies. Intracellular HLA-DR was determined by subtracting cell surface HLA-DR fluorescence detected in intact cells from total cellular HLA-DR fluorescence detected in permeabilized cells.

Fatty acid reconstitution assays

For the time course, low-lipid cells were incubated with and without 50 μM oleoyl-CoA at 37°C under 5% CO_2 for 24 h. At select time points, cells were removed from the culture, washed in PBS/FBS, and assayed with LysoSensor (described above). Reconstitution assays with specific fatty acids used log growth cultures incubated for 20–24 h in medium with and without 75–100 μM oleoyl-CoA, palmitoleoyl-CoA, palmitoyl-CoA, stearoyl-CoA, or linoleoyl-CoA (all from Sigma). Glucocerebrosidase activity, LysoSensor staining, and/or HLA-DR cell surface staining (described above) were done after incubation on cells treated and untreated with fatty acids. Alternatively, studies used 100 μM oleic acid, linoleic acid, or linolenic acid with or without 0.5 mM CoA. Acylated fatty acid-CoA derivatives were diluted in sterile deionized water; free fatty acids were solubilized in 100% ethanol, and the results were compared with those from an ethanol control.

Vitamin D_3 treatment of cells

Vitamin D_3 (Sigma) was solubilized in isopropanol. Cells were incubated in low-lipid medium with or without 10^{-6} M vitamin D_3 for 36–38 h at 37°C under 5% CO_2. Cells were washed, blocked, and stained for cell surface expression of CD11b and CD14 as described above.

γ-Interferon stimulation of HLA-DR expression

Log growth cultures were incubated for 30 h at 37°C under 5% CO_2 in either FBS or low-lipid medium with or without 25 ng/ml

γ-interferon (Gibco Invitrogen). Cells were washed, blocked, and stained for both total cellular and cell surface HLA-DR as described above.

Cerulenin treatment of cells

Cerulenin (Sigma) was solubilized in 100% ethanol. For acute response experiments, cell cultures were incubated in appropriate medium containing no additive, ethanol vehicle control, or 5, 7.5, or 10 μg/ml cerulenin for 20–24 h at 37°C under 5% CO_2. Cells were washed, blocked, and stained as described above. U-937 cells were adapted for continuous growth in FBS medium containing either 3 or 5 μg/ml cerulenin by slowly increasing the concentration of cerulenin in the medium from 1 to 5 μg/ml. These cell lines were designated U-C3 and U-C5, respectively.

In vivo mouse study

Female, age-matched Balb/c and C57/B6 mice were fed either a low-fat or moderately high-fat diet for 16 weeks. Each mouse received 5 g of food per day. Consumption of food per cage was determined by collecting and weighing uneaten food. Each mouse was weighed three times during the 16 week period. Mice were monitored daily for observable effects. After the 16 week feeding regimen, mice were euthanized, and their spleens were processed using the procedure of Leanderson and Julius (36). Splenocytes were stained with LysoSensor and the M5114 (37) antibody to mouse MHC class II I-A and I-E.

Gating of mouse splenocyte subpopulations

Cytometric analysis of LysoSensor staining was performed on a specific population of large cells based on cell size and granularity as determined by cytometric analysis. The large cell gate likely represents macrophages and/or other probable antigen-presenting cells (26). Mouse MHC class II cell surface expression was detected on MHC class II-positive large cells (likely antigen-presenting cells) or small cells (likely B-cells) as identified by positive class II staining and forward scatter.

Statistical significance

Error bars represent SEM. Statistical significance defined by Student's *t*-test ($P < 0.05$), was determined using Graph Pad software.

RESULTS

Lysosomes are established sites for fatty acid accumulation (2, 32, 33, 38), and MHC class II molecules must traffic through the acidic lysosomal/endosomal compartment (27, 28, 39). Thus, we reasoned that fatty acid availability might have a direct impact on lysosomal acidity and consequent antigen processing and presentation via MHC class II molecules. To determine the effect(s) of limiting exogenous lipids, we adapted the well-characterized human macrophage cell lines HL60 (40) and U-937 (41) for continuous growth in medium containing 5% delipidized bovine calf serum as a replacement for 5% FBS (see Methods). We then compared the properties of the "low-lipid cells" with those of cells grown in traditional FBS medium.

To date, nine different cell lines have been successfully adapted for growth under low-lipid conditions. Characterization of newly adapted low-lipid cells reveals several distinctive properties, including increased cell death (**Fig. 1A**) and an increase in cell size (Fig. 1B), as cells are exposed to increasing concentrations of delipidized bovine calf serum. Bright-field microscopy (Fig. 1C) of HL60 cells maintained in FBS and low-lipid media revealed a tendency for low-lipid cells to clump in cell suspensions. Individual cell types differed in their ability to adapt to low-lipid conditions and with respect to their stabilized phenotypes (viability, size, and morphologic appearance) with long-term incubation in low-lipid medium.

Notably, the most distinctive feature of low-lipid cells was a decrease in lysosomal acidity. Comparison of LysoSensor (34) staining of HL60/FBS and U-937/FBS cells and their low-lipid counterparts revealed a decrease in LysoSensor fluorescence (**Fig. 2A**), and hence a decrease in lysosomal acidity, in the low-lipid cell lines. Mouse L1210 (42) cells showed a similar low-lipid effect (Fig. 2B). A sustained decrease in LysoSensor staining was seen in every cell line we adapted to low-lipid conditions and represents a stable hallmark of the low-lipid phenotype.

To determine whether we could restore lysosomal acidity by adding back lipids, we compared LysoSensor staining of cells in low-lipid medium with staining from cells in low-lipid medium reconstituted with a specific lipid. We chose oleic acid for the reconstitution assay because fatty acids accumulate in lysosomes as a result of cholesteryl ester hydrolysis (2, 38) and oleic acid is easily transported into cells (1, 38, 43). Preliminary experiments using free oleic acid, oleic acid plus CoA, and oleoyl-CoA showed that the CoA derivative is a more effective modifier of lysosomal acidity than free fatty acid or fatty acid plus CoA (**Table 1**). The addition of oleoyl-CoA to low-lipid medium resulted in time-delayed (Fig. 2C) and dose-dependent (Fig. 2D) increases in LysoSensor staining. Subsequent reconstitution experiments were done by incubating low-lipid cells for 20–24 h in medium with or without the addition of 100 μM oleoyl-CoA (Fig. 2E).

To determine whether other fatty acids would have the same effect on lysosomal acidity, low-lipid cells from both HL60 and U-937 cell lines were incubated in low-lipid medium reconstituted with 100 μM oleoyl-CoA, palmitoyl-CoA, palmitoleoyl-CoA, stearoyl-CoA, or linoleoyl-CoA. Oleoyl-CoA, palmitoleoyl-CoA, and linoleoyl-CoA consistently showed significant increases in LysoSensor staining compared with cells in low-lipid medium with no fatty acid additive (**Tables** 1, **2**). Notably, the addition of the saturated fatty acids palmitoyl-CoA and stearoyl-CoA at 100 μM concentrations resulted in significant cell death. Seventy percent to 80% of the cells treated with 100 μM palmitoyl-CoA died as a result of the treatment, and >95% of cells treated with 100 μM stearoyl-CoA died. The unsaturated fatty acid derivatives, oleoyl-CoA, palmitoleoyl-CoA, and linoleoyl-CoA, did not significantly affect cell viability at 100 μM. The increases in lysosomal acidity seen in our reconstitution experiments suggest that exogenous fatty acids are involved in establishing or maintaining lysosomal acidity.

To demonstrate that the decrease in lysosomal acidity alters enzymatic function, we used an assay for lysosomal

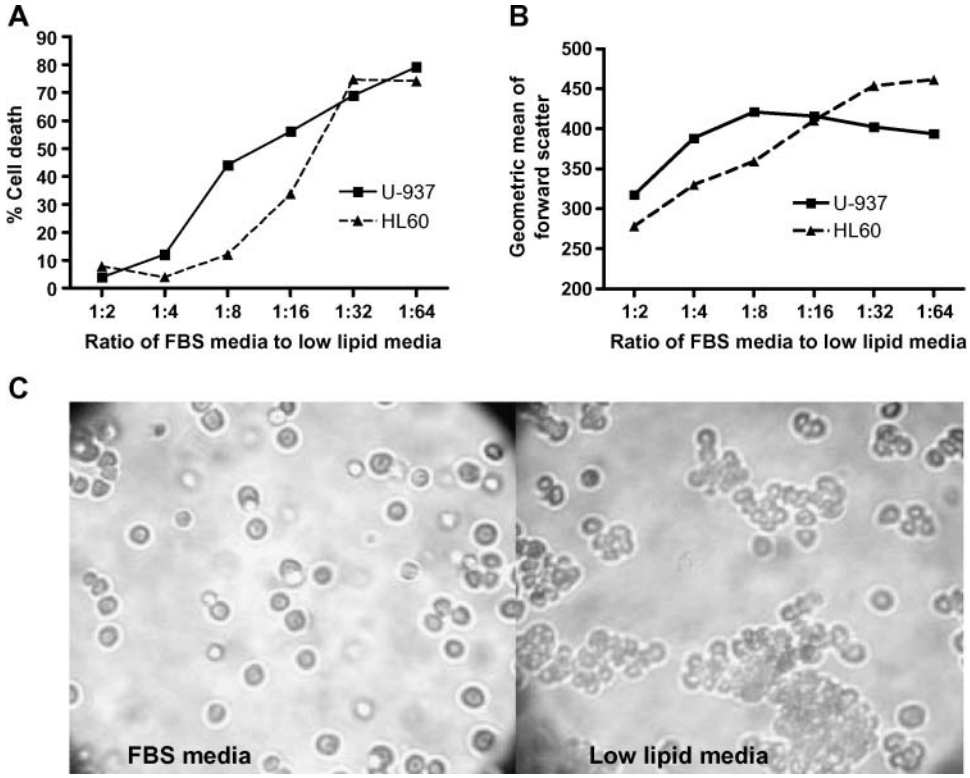

Fig. 1. HL60 and U-937 human macrophage cells were incubated in medium containing increasing concentrations of delipidized bovine calf serum. The effects of the low-lipid conditions on cell viability, cell size, and cell morphology were observed. A: Percentage cell death was calculated based on forward versus side scatter profiles of cytometrically gated populations of live and dead cells. Manual cell counts using trypan blue gave comparable patterns. B: Cell size was determined by cytometric analysis of forward scatter profiles from gated populations of live cells. C: Bright-field microscopy of HL60 cells maintained in medium containing 5% FBS or 5% delipidized bovine calf serum (low-lipid medium). Magnification, 60×. Photographs were taken to illustrate cell morphology and are not normalized for absolute cell number.

glucocerebrosidase activity (35). For this assay, cells were incubated with 1.05 mM FDGlu at 37°C for 45 min. In the presence of active enzyme, this substrate was converted to a fluorescent product detected by flow cytometry. Experiments measuring both enzyme activity and lysosomal acidity in 60-15/low lipid and HL60/FBS cells showed decreases in both fluorescent enzyme product (**Fig. 3A**) and LysoSensor staining (Fig. 3B). When oleoyl-CoA was added to low-lipid medium, staining revealed both an increase in LysoSensor and an increase in enzyme activity (Fig. 3A, B). As a control, enzyme activity was shown to be sensitive to chloroquine (Fig. 3C), a weak base known to decrease lysosomal acidity (44).

The presentation of exogenous antigen to T-lymphocytes is a critical step in T-lymphocyte activation (27) and a recognized contributor to inflammation (23, 45). Effective antigen presentation involves the degradation of antigen by acid hydrolases in the lysosomal/endosomal system, insertion of the processed antigen into MHC class II molecules, and transport of peptide-loaded MHC molecules to the cell surface of antigen-presenting cells (39, 46). Given the link between lysosomal acidity and MHC class II expression, we used our cell culture system to determine the impact of low lipid conditions on the level of HLA-DR expression. We observed a decrease in HLA-DR cell surface expression in human HL60 and U-937 low-lipid cell lines (**Fig. 4A**) and a decrease in MHC class II molecules I-A and I-E in mouse L1210 cells adapted to low-lipid conditions (Fig. 4B). Because HLA-DR represents only one of three MHC class II isoforms in humans, we repeated the experiments with an anti-HLA-DR, -DP, and -DQ antibody. A similar decrease in the cell surface expression of MHC class II was seen in low-lipid cells with this antibody to all three isoforms (data not shown).

If the observed decrease in lysosomal acidity is responsible for the decrease in MHC class II cell surface expression, then fatty acid reconstitution experiments would be expected to cause both an increase in lysosomal acidity and an increase in cell surface HLA-DR expression. Reconstitution experiments adding back 100 μM oleoyl-CoA, palmitoyl-CoA, linoleoyl-CoA, stearoyl-CoA, or palmitoleoyl-CoA did not consistently demonstrate statistically significant ($P < 0.05$) increases in HLA-DR cell surface expression (data not shown). Our results suggest that simply restoring lysosomal acidity with the addition of specific fatty acids to low-lipid medium is not sufficient to restore HLA-DR cell surface expression.

To determine whether low-lipid conditions also affect MHC class I cell surface expression, we stained FBS and low-lipid cell lines with antibody to the combination HLA-

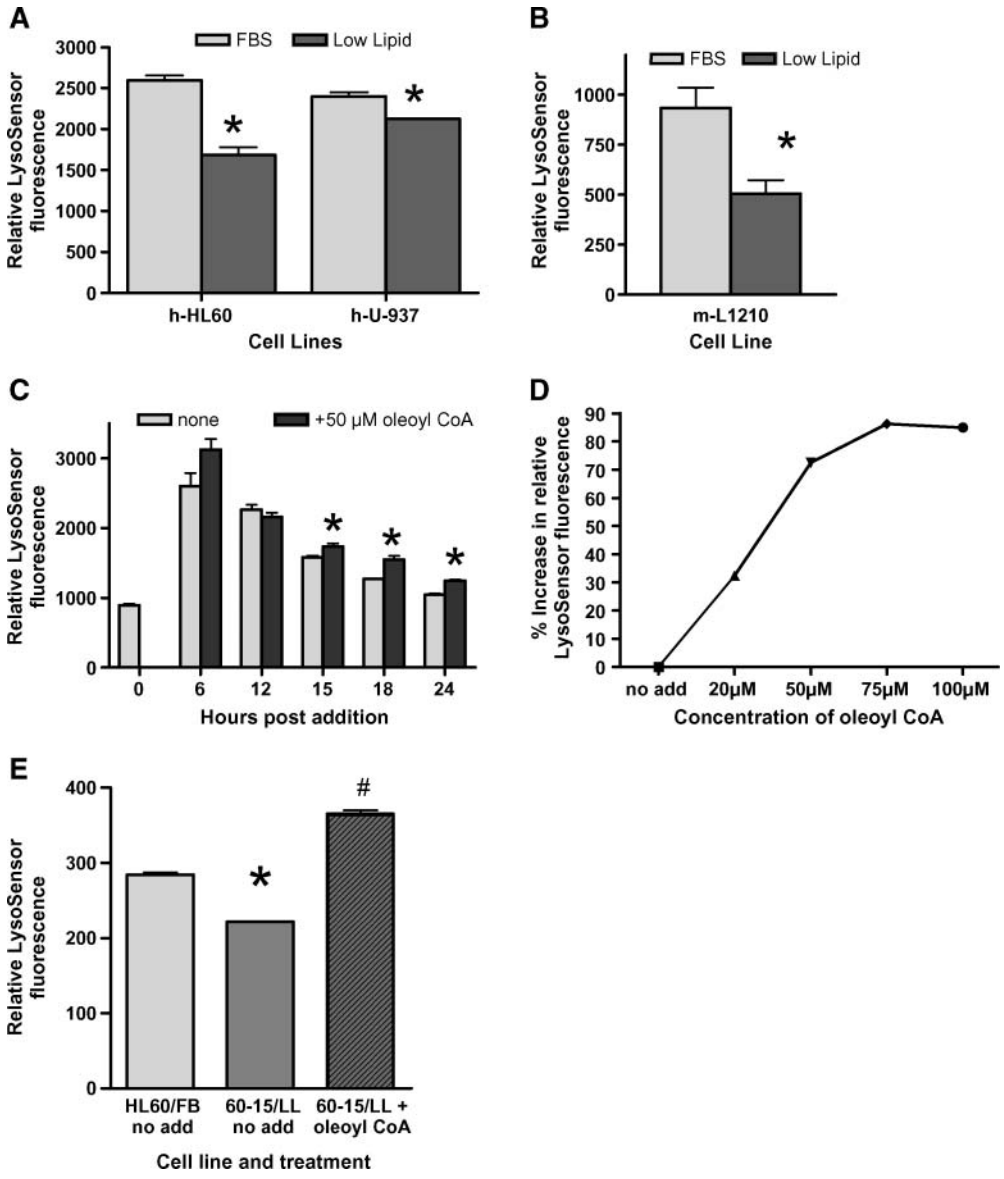

Fig. 2. FBS and low-lipid cells were stained with LysoSensor as a measure of lysosomal acidity and analyzed by flow cytometry. A decrease in fluorescence indicates a decrease in lysosomal acidity. A, B: Comparisons of human HL60 and U-937 cells (A) and mouse L1210 cells (B) maintained in FBS or low-lipid medium and stained with LysoSensor. Bars represent triplicate assays and are representative of at least three separate experiments. C: 60-7/low lipid cells were incubated with 50 μM oleoyl-CoA; at select time points, cells were removed and stained with LysoSensor. * Statistically significant difference between low-lipid and reconstituted media. D: LysoSensor staining of U-16/low lipid cells incubated for 20–24 h in low-lipid medium containing no fatty acid or 20, 50, 75, or 100 μM oleoyl-CoA. E: LysoSensor staining of HL60/FBS cells, 60-15/low lipid cells, and 60-15/low lipid cells in low-lipid medium containing 100 μM oleoyl-CoA. Bars represent triplicate assays and are representative of at least three separate experiments. * Statistically significant difference between FBS and low-lipid cells; # statistically significant difference between low-lipid and reconstituted media. Statistical significance was defined by Student's *t*-test ($P < 0.05$). Error bars represent SEM.

A,B,C (Fig. 4C). The results demonstrate that low-lipid conditions also led to decreased cell surface expression of MHC class I. Because MHC class I and class II molecules are processed and presented on the surface of cells differently (39, 47, 48), and because low-lipid conditions decrease the cell surface expression of both MHC class I and class II molecules, these studies suggest that exogenous lipid availability may affect more than one event in MHC cell surface expression.

To rule out the possibility that low-lipid conditions nonspecifically decrease all cell surface molecules, we examined the cell surface expression of CD11b (Fig. 4D) and CD14 (Fig. 4E), macrophage-specific cell surface molecules. In addition, we examined the ability of low-lipid

TABLE 1. Effects of free fatty acids, free fatty acids with CoA, or CoA-derivatized fatty acids on lysosomal acidity and cell death

Variable	Oleic Acid	Linoleic Acid	Oleic Acid + CoA	Linoleic Acid + CoA	Oleoyl-S-CoA	Linoleoyl-S-CoA
Percentage increase, LysoSensor	5.6 (3)	2.8 (1)	5.0 (5)	9.2 (2)	72.8 (10)	55.8 (6)
Percentage increase, cell death	45.8 (4)	240.0 (1)	96.8 (6)	150.3 (3)	22.2 (12)	20.1 (6)

All data are expressed as percentage increases over controls. Oleoyl-S-CoA and linoleoyl-S-CoA values include data from both HL60 and U-937 low-lipid cells; all other values are from HL60/low lipid cells. Numbers in parentheses represent the number of experiments; all experiments represent triplicate assays from each treatment group.

cells to respond to a known stimulus, vitamin D_3 (49), that increases cell surface expression of CD11b and CD14. Vitamin D_3 treatment of 60-15 cells resulted in a substantial increase in CD11b but not CD14 (Fig. 4D, E). These experiments demonstrate that low-lipid cells maintain the ability to selectively increase cell surface molecules in response to stimuli, thus indicating that decreases in cell surface MHC molecules are not a generalized phenomenon of the low-lipid conditions.

To determine whether low-lipid cells respond to MHC class II-inducing stimuli, specifically γ-interferon (50), we treated both FBS and low-lipid cells with 25 nM γ-interferon for 30 h. Predictably, in both HL60/FBS (**Fig. 5A**) and U-937/FBS (data not shown) cells, treatment with γ-interferon resulted in a substantial increase in cell surface expression of HLA-DR. However, there was little to no γ-interferon-induced increase in cell surface HLA-DR expression under low-lipid conditions (Fig. 5A). To determine whether low-lipid cells have decreased amounts of total cellular HLA-DR, we modified our staining procedure by gently permeabilizing the cells (Cytoperm-Cytofix) to allow antibodies access to both extracellular and intracellular pools of HLA-DR. The data (Fig. 5B) suggest that the amount of total cellular HLA-DR in FBS and low-lipid cells is similar and that incubation with γ-interferon does not induce significant changes in total cellular HLA-DR. These data are consistent with the interpretation that cells maintained under low-lipid conditions cannot transport intracellular HLA-DR to the cell surface and correlate with findings that HLA-DR is transported via lipid rafts (51–54).

In addition to the pool of exogenous fatty acids, nonessential fatty acids can be synthesized endogenously (1). To evaluate the effects of inhibiting endogenous fatty acid synthesis, we added the fatty acid synthase inhibitor cerulenin (55, 56) to FBS medium. Pizer et al (57) have shown that HL60 cells, as well as other tumor cells (58–60), die in the presence of cerulenin. In that study with HL60 cells, this sensitivity to cytotoxity was overcome by the addition of exogenous fatty acids, and the authors showed that "cerulenin toxicity is mediated through fatty acid starvation" (57). For these reasons, we monitored the effect of cerulenin by quantifying the percentage of cell death resulting from cerulenin treatment. We observed dose- and time-dependent increases in cell death with the addition of cerulenin to FBS and low-lipid medium in both acute (**Table 3**) and long-term (**Fig. 6E**) treatments with cerulenin. Predictably, the highest levels of cell death were seen in long term incubations in low-lipid medium containing cerulenin, conditions that limit the availability of both exogenous and newly synthesized fatty acids.

In contrast to limiting exogenous lipids (low-lipid conditions), incubating U-937/FBS cells with 5–10 μg/ml cerulenin for 20–24 h did not result in a decrease in lysosomal acidity in live cells (Fig. 6A), suggesting that maintenance of lysosomal acidity is more dependent on exogenous fatty acid availability than on endogenous fatty acid synthesis. However, our experiments do not exclude the possibility that other sources of endogenous fatty acids (phospholipids or triacylglycerides) may be involved in regulating lysosomal acidity.

Cerulenin treatment of U-937/FBS cells resulted in statistically significant decreases in HLA-DR cell surface expression (Fig. 6B), indicating that endogenous synthesis of fatty acids, as well as exogenous fatty acids, are involved in the effective expression of cell surface HLA-DR. Cerulenin treatment of HL60/FBS cells gave results similar to those with U-937/FBS cells for both lysosomal acidity and HLA-DR cell surface expression (data not shown).

To compare the long-term effects of blocking fatty acid synthesis with acute cerulenin treatment, we cultured

TABLE 2. Percentage increase in LysoSensor staining in low-lipid cells after 20–24 h of incubation with fatty acid-CoA derivatives

Cell Line	Oleoyl-CoA	Palmitoyl-CoA	Palmitoleoyl-CoA	Stearoyl-CoA	Linoleoyl-CoA
HL60 low lipid	++ to +++[a] (2)	++++[a] (1), ++[b] (1)	++[a] (1)	+++[b] (1)	++ to ++++[a] (2)
U-937 low lipid	+++ to ++++[a] (3)	0 to +++[a] (3), significant cell death (2)	+++[a] (2)	*0*[a] (3), significant cell death	++ to +++[a] (3)

Numbers in parentheses represent the number of experiments; each experiment represents quadruplicate assays. +, 5–25% increase; ++, 25–50% increase; +++, 50–75% increase; ++++, 75–>100% increase in LysoSensor staining with the addition of fatty acid to low-lipid medium.
[a] 100 μM concentration of fatty acid-CoA derivative.
[b] 75 μM concentration of fatty acid-CoA derivative.

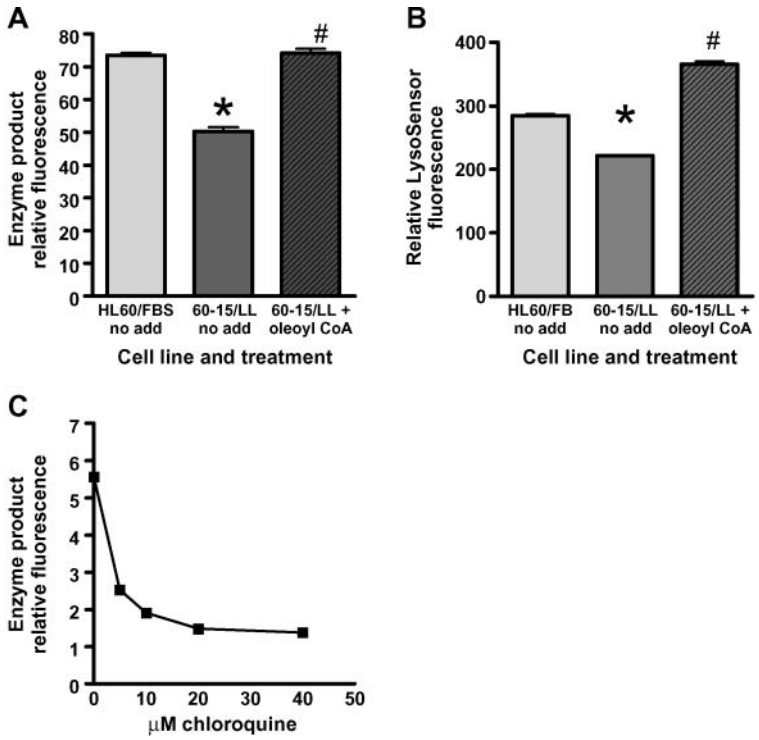

Fig. 3. Lysosomal enzymatic glucocerebrosidase activity was determined to assess lysosomal function relative to changes in acidity. A, B: HL60/FBS, 60-15/low lipid, and 60-15/low lipid cells treated with 100 μM oleoyl-CoA were assayed for lysosomal glucocerebrosidase activity (A) and for lysosomal acidity (B) using LysoSensor staining. Bars represent triplicate assays and are representative of at least three separate experiments. * Statistically significant difference between HL60/FBS and 60-15/low lipid cells; # statistically significant difference between 60-15/low lipid and 60-15/low lipid cells with 100 μM oleoyl-CoA. Statistical significance was defined by Student's *t*-test ($P < 0.05$). C: HL60/FBS cells were incubated with no additive or 5, 10, 20, or 40 μM chloroquine at room temperature for 60 min and subsequently assayed for lysosomal glucocerebrosidase activity. Error bars represent SEM.

U-937 cells continuously for 3 months in FBS medium containing either 3 or 5 μg/ml cerulenin. These cell lines were designated U-C3 and U-C5, respectively. LysoSensor and HLA-DR cell surface staining of U-C3 and U-C5 cells revealed no decrease in lysosomal acidity (Fig. 6C) but a decrease in the expression of HLA-DR (Fig. 6D). These results are consistent with those observed in acutely treated cells.

To compare our cell culture results with an analogous dietary program in vivo, we did an extended 16 week dietary study using Balb/c and C57/B6 female mice (for details, see Methods and **Table 4**). Data from LysoSensor staining of mouse splenocytes demonstrate diet-dependent, statistically significant changes in lysosomal acidity (**Fig. 7A**). Splenocytes from mice on the low-lipid diet had a lower lysosomal acidity than cells from mice fed a high-fat diet, a pattern identical to that seen in the in vitro cell culture system.

MHC class II staining of mouse splenocytes revealed diet-dependent, statistically significant differences in the cell surface expression of MHC class II molecules in female Balb/c mice but not in female C57/B6 mice (Fig. 7B). The results in C57/B6 mice correlate with the absence of MHC class II I-E molecules (the mouse equivalent of human HLA-DR) in this strain of mice. This mouse strain was selected for this study specifically to address the contribution of different MHC class II isoforms.

As noted in Methods, we determined MHC class II expression in a subpopulation of MHC class II-positive large cells. Large splenocytes that are MHC class II-positive are widely accepted to be capable of antigen presentation (26). When we compared the effects of the high- and low-fat mouse diets on MHC class II expression between the large and small positive populations in Balb/c mice, we observed a statistically significant, diet-dependent difference in the levels of expression of MHC class II molecules on large, but not on small, cells (Fig. 7C).

The diet-induced decrease in MHC class II expression in Balb/c female mice fed a high-fat diet is the opposite of the results in the cell culture system. In the in vitro model, HLA-DR cell surface expression was lower in cells maintained under low-lipid conditions. Recognizing the complexity of lipid metabolism in a whole animal, the difference may represent the animal's ability to adapt to, or use, multiple fuel sources (i.e., fat stores, salvage pathways, or endogenously synthesized fatty acids). Nonetheless, it is significant that we observed statistical differences in MHC class II cell surface expression as a function of diet.

DISCUSSION

Using in vitro cell cultures and an in vivo mouse model, we demonstrate that limiting the availability of lipid affects lysosomal acidity and MHC class II cell surface expression. The reproducibility of our results in different cell lines and in a mouse model suggests that the observed changes represent a cellular response to the removal of lipid. Our results have important implications relative to diet and early events in inflammation and in the immune response.

A decrease in lysosomal acidity is the hallmark of cells maintained under conditions that limit the availability of exogenous lipids, both in vitro and in vivo. Low-lipid cell culture results demonstrating an increase in lysosomal acidity with the addition of exogenous oleoyl-CoA (and other fatty acid-CoA derivatives) support a cause-and-effect

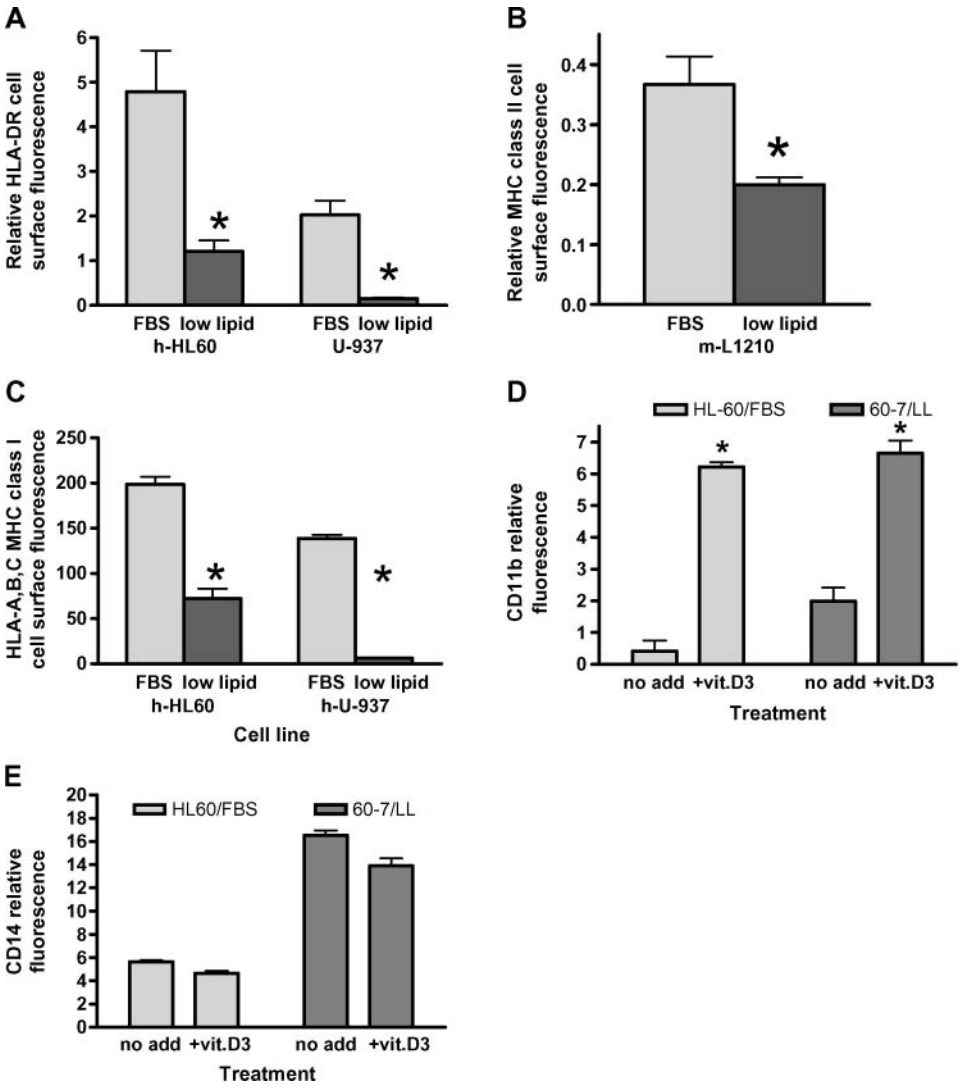

Fig. 4. Human HL60, human U-937, mouse L-1210 cells, and their respective low-lipid counterparts were stained with fluorochrome-conjugated antibodies to major histocompatibility complex (MHC) molecules and analyzed by flow cytometry. A, B: Cell surface expression of MHC class II isoform HLA-DR in human macrophage cell lines (A) and mouse MHC class II complexes I-A and I-E in L-1210 mouse cells lines (B). C: Cell surface expression of the HLA-A,B,C MHC class I complex in human macrophage cell lines was determined by staining with an antibody against combined HLA-A,B,C. D, E: Cell surface expression of CD11b (D) and CD14 (E) was determined in 60-15/low lipid cells with and without treatment with 10^{-6} M vitamin D_3. Bars represent triplicate assays and are representative of at least three separate experiments. * Pairings that are statistically different as determined by Student's *t*-test ($P < 0.05$). Error bars represent SEM.

relationship between exogenous fatty acid availability and lysosomal acidity. Notably, we observed increases in lysosomal acidity at oleoyl-CoA concentrations as low as 20 μM. However, fatty acids serve many functions within the cell, and reconstituting low-lipid medium with derivatized fatty acids will have pleotrophic effects. We used a high concentration of fatty acids (100 μM) in our reconstitution assays to provide saturating conditions. Given the inherent complexity of fatty acid transport and metabolism, the statistically significant ($P < 0.05$), sustained, and dose-dependent changes in lysosomal acidity seen with the addition of oleoyl-CoA (and other fatty acids) provide substantial evidence for a cause-and-effect relationship.

Although the exact role of fatty acids in the regulation/maintenance of lysosomal acidity cannot be discerned from this study, we propose that fatty acids act as a regulator of lysosomal acidity. Mellman and colleagues (61) demonstrated changes in lysosomal acidity in dendritic cells (antigen-presenting cells) during maturation. These reported changes in lysosomal acidity may represent a situation in which lysosomal acidity requires a high level of control, possibly mediated by fatty acid. Future work is aimed at elucidating the mechanism by which exogenous fatty acids influence lysosomal acidity.

Lysosomal acidity is unaffected when endogenous fatty acid synthesis is blocked by cerulenin. In contrast, in-

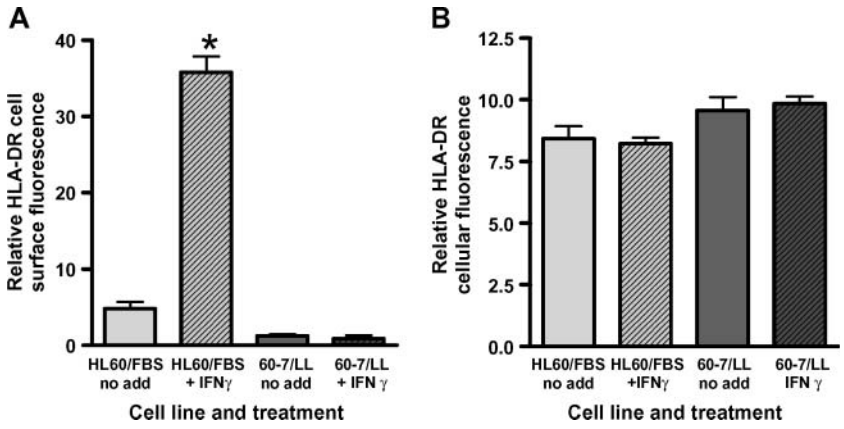

Fig. 5. HL60/FBS and 60-7/low lipid cells were incubated with 25 nM γ-interferon (IFNγ) for 30 h followed by staining with antibodies to HLA-DR. A: Cell surface HLA-DR levels of expression were determined by antibody staining of intact, nonpermeabilized cells. Bars represent triplicate assays and are representative of at least three experiments. B: Total cellular HLA-DR levels were determined by gently permeabilizing cells before staining with antibodies to HLA-DR. * Statistically significant difference as determined by Student's *t*-test ($P < 0.05$). Error bars represent SEM.

hibiting endogenous fatty acid synthesis and limiting the exogenous availability of fatty acids result in decreased cell surface expression of MHC class II. Therefore, fatty acids may be involved in more than one aspect of MHC class II cell surface expression. Lysosomal antigen processing into MHC class II molecules is followed by transport of the peptide-loaded molecule to the cell surface (27, 39). Lipid rafts are known to be key components of this process (52, 54), and current research has strengthened the link between rafts and fatty acids: polyunsaturated fatty acids are involved in membrane raft formation (62, 63), fatty acids are transported on lipid rafts (64), and altered lipid conditions affect the trafficking of lipid rafts (65–67). Disruption of lipid rafts by our low-lipid conditions may represent one explanation for the decrease in both MHC class I and class II.

Athanassakis, Ranella, and Vassiliadis (68) reported that, in trophoblast cells, γ-interferon facilitates the release of MHC class II from intracellular pools independent of protein synthesis. Consistent with these results, we did not see a significant increase in total cellular HLA-DR in either FBS or low-lipid cells incubated with γ-interferon for 30 h. Furthermore, increased amounts of HLA-DR are detected only on the cell surface of FBS cells and not low-lipid cells. These data are consistent with the interpretation that low-lipid conditions disrupt the transport of intracellular stores of MHC class II to the cell surface, thus explaining the failure of γ-interferon to increase the cell surface expression of MHC class II under low-lipid conditions.

An alternative, albeit more complex, interpretation of the data demonstrating a requirement for both endogenous and exogenous fatty acids for the cell surface expression of MHC class II could be that our low-lipid conditions create such a great need for fatty acids that the cell cannot keep up with the synthesis demands. If this is the case, the in vitro low-lipid conditions (limited exogenous availability) could represent a decreased availability of both exogenous and endogenous fatty acids. The results of experiments examining cell death as a function of growth in low-lipid medium versus growth in low-lipid medium containing cerulenin argue against this explanation: if the low-lipid environment also depleted endogenous supplies of fatty acids, we would expect substantial increases in cell death with continuous incubation in low-lipid medium. This is not what we observed; rather, cells maintained long term under low-lipid conditions stabilized with respect to cell death. Under experimental conditions in which cells were deprived of both endogenous synthesis and exogenous availability of fatty acids (low-lipid medium plus cerulenin), we observed a dramatic increase in cell death. Therefore, the in vitro low-lipid con-

TABLE 3. Percentage cell death in U-937 cell lines treated with cerulenin for 20–24 h or incubated continuously in medium containing cerulenin

Exposure to Cerulenin	Cell Line	Cerulenin	Cell Death
20–24 h	U-937/FBS	None 5 μg/ml 7 μg/ml 10 μg/ml	3.4% 11.9% 24.5% 27.5%
	U-16/low lipid	None 5 μg/ml 7 μg/ml 10 μg/ml	2.3% N/A 4.8% 9.8%
Continuous incubation in cerulenin	U-C3	3 μg/ml	5.6%
	U-C5	5 μg/ml	16.4%

N/A, not applicable.

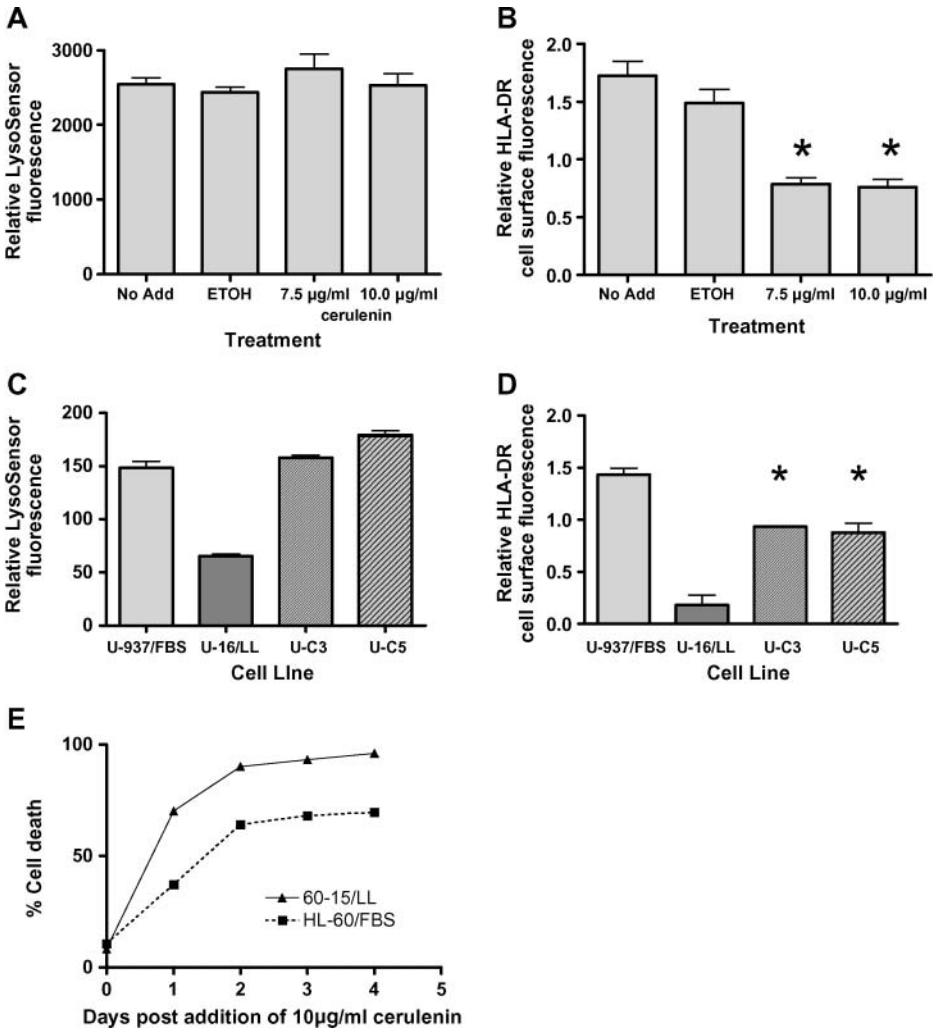

Fig. 6. U-937/FBS cells were incubated in FBS medium with no addition, ethanol (ETOH; vehicle control), 7.5 μg/ml cerulenin, or 10 μg/ml cerulenin for 20–24 h. A, B: Cerulenin-treated and untreated U-937/FBS cells were stained with LysoSensor (A) and antibodies to MHC class II isoform HLA-DR (B). Statistical significance was determined by comparing cerulenin-treated cells with the ethanol control. C, D: LysoSensor (C) and HLA-DR (D) cell surface staining of U-937/FBS, U-16/low lipid, and two cell lines adapted for continuous incubation in FBS medium containing 3 and 5 μg/ml cerulenin (U-C3 and U-C5, respectively). Statistical significance was determined by comparing the staining of U-C3 and U-C5 cells with U-16/low lipid cells. * Pairings that are statistically different as determined by Student's *t*-test ($P < 0.05$). E: HL60/FBS and 60-15/low lipid cells were incubated in medium containing 10 μg/ml cerulenin; percentage cell death was calculated based on forward versus side scatter profiles of cytometrically gated populations of live and dead cells. Error bars represent SEM.

ditions (limited exogenous availability) used in this study do not appear to reflect significant alterations in endogenous synthesis or availability as determined by cell death.

In contrast to the in vitro results, in the mouse model, we observed a statistically significant decrease in MHC class II in mice fed the high-fat diet. In the cell culture model, in which low-lipid conditions resulted in a decrease in MHC class II, the medium represents the only source of noncellular exogenous fatty acids. However, in vivo, the low-fat diet represents only one possible source of exogenous fatty acids. Fatty acids stored as fat (triacylglycerides) and/or retrieved from salvage pathways provide additional sources of nonsynthesized fatty acids. Thus, exogenous lipid availability in mice fed a low-fat diet may not represent the same low-lipid conditions present in the cell culture system, and MHC class II cell surface expression in vivo may not be affected in a manner analogous to the in vitro system.

A second possible explanation for the differences observed between the in vitro and in vivo systems involves the complexity of metabolism in the animal model. Mice may either preferentially use, or preferentially store, a particular source of fatty acids. If mice preferentially use endogenously synthesized fatty acids, the high-fat diet could represent ample exogenous fatty acids but limited endogenous fatty acids. Similar in vitro conditions resulted in a decrease in MHC class II cell surface expression. Given the dependence of lysosomal acidity on exogenous but

TABLE 4. Nutritional information and selected fatty acid contents of low-fat and moderately high-fat mouse diets

Diet Contents	Low-Fat Diet	Moderately High-Fat Diet
Protein (w/w) Casein DL-Methionine	17.8% 200.0 g/kg 3.0 g/kg	17.8% 200.0 g/kg 3.0 g/kg
Carbohydrate (w/w) Sucrose Corn starch Maltodextrin Cellulose (fiber)	66.3% 200.00 g/kg 336.49 g/kg 150.00 g/kg 50.00 g/kg	57.7% 200.00 g/kg 238.68 g/kg 150.00 g/kg 50.00 g/kg
Fat (w/w) Safflower oil Coconut oil	1.27% 10.00 g/kg	10.25% 50.00 g/kg 50.00 g/kg
Fatty acid by weight Lauric Palmitic Oleic Linoleic	0% 0.055% 0.137% 0.762%	2.33% 0.69% 0.995% 3.885%
kcal/g/diet	3.48	3.94
Percentage kcal from fat	3.3	23.40

The low-fat diet was Harlan Teklad diet TD.03120; the moderately high-fat diet was Harlan Teklad diet TD.03121. Additional ingredients were as follows: Mineral Mix, AIN-93G-MX (TD 94046) (35.0 g/kg in the low-fat diet, 40 g/kg in the moderately high-fat diet); $CaHPO_4$ (3.0 g/kg in the low-fat diet, 4.0 g/kg in the moderately high-fat diet); Vitamin Mix, AIN-93-VX (TD 94947) (10.0 g/kg in the low-fat diet, 11.5 g/kg in the moderately high-fat diet); choline bitartrate (2.5 g/kg in the low-fat diet, 2.8 g/kg in the moderately high-fat diet); and Tert-butylhydroquinone (TBHQ) (antioxidant) (0.01 g/kg in the low-fat diet, 0.02 g/kg in the moderately high-fat diet).

not endogenous fatty acids, and the observation that decreased lysosomal acidity was seen in both the in vitro and in vivo systems, our results confirm the importance of fatty acid availability for the cell surface expression of MHC class II.

A statistically significant, diet-dependent decrease in MHC class II expression was observed in the large, class II-positive cell gate in Balb/c mice but not in C57/B6 mice. This was not unexpected; C57/B6 mice lack the mouse MHC class II antigen I-E (69) (the analog to human HLA-DR). The M5114 antibody used for the detection of mouse MHC class II molecules detects both I-A and I-E (37); thus, M5114 staining in C57/B6 mice represents I-A staining only (the analog to human HLA-DP and -DQ), whereas staining of cells from Balb/c mice represents the detection of all (I-A and I-E) mouse MHC class II isoforms. MHC class II staining in the human cell culture system was performed using two antibodies: one directed against the single HLA-DR isoform, and one that reacts with all three human class II isoforms, HLA-DR, -DP, and -DQ. Staining with both of these antibodies showed decreased cell surface expression of MHC class II molecules under low-lipid conditions. Hence, the antibody used for staining Balb/c mouse MHC isoforms in vivo is analogous to the antibody used to stain human MHC class II isoforms in vitro.

The lack of statistically significant diet-dependent changes in C57/B6 mice (expressing I-A only) supports

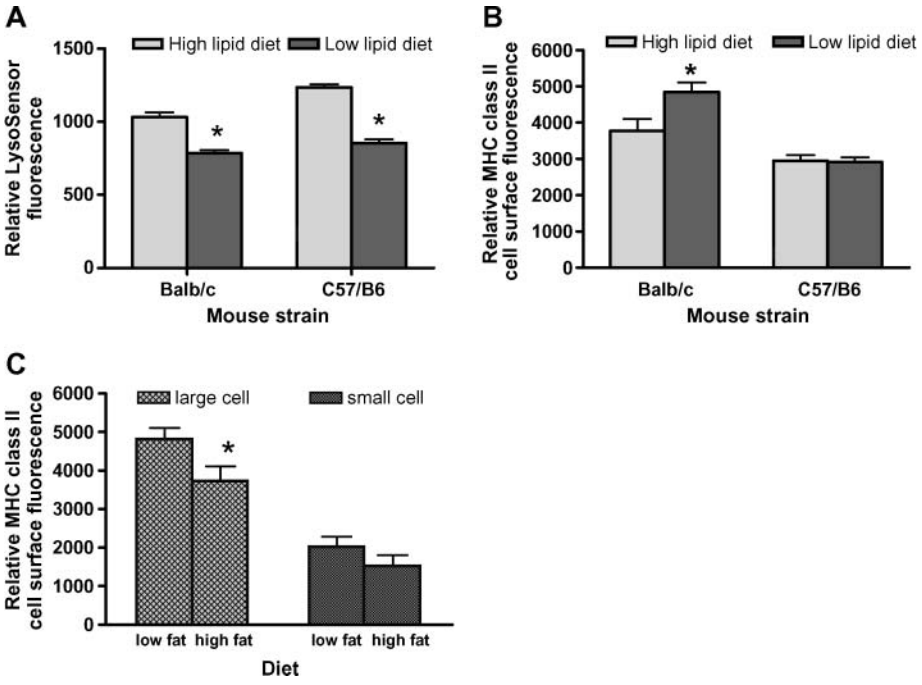

Fig. 7. Balb/c and C57/B6 female mice were fed either a low-fat or moderately high-fat diet for 16 weeks (diets are described in detail in Table 4). A, B: Mouse splenocytes were stained with LysoSensor (A) or M5114 (B), a fluorochrome-conjugated antibody with specificity for both mouse MHC class II I-A and I-E molecules. C: Mouse MHC class II cell surface expression was analyzed on large cells identified as MHC class II-positive (probable antigen-presenting cells) and on MHC class II-positive small cells. Bars represent averages of all assays from all mice in a given category. * Pairings that are statistically different as determined by Student's *t*-test ($P < 0.05$). Error bars represent SEM.

the possibility of a functional, lipid-dependent distinction between MHC class II I-E/HLA-DR and MHC class II I-A/HLA-DP and -DQ. In this study, female mice lacking I-E did not respond to differences in lipid availability in the same manner as mice bearing both I-A and I-E isoforms. Functional distinctions between the MHC class II isoforms are not unprecedented. Previous work in our laboratories has shown differential susceptibility to coxsackievirus-induced cardiomyopathies and early atherogenesis as a function of the presence or absence of I-E in transgenic mouse models of disease (70). Together, our data suggest a potential functional or mechanistic link between lipid availability and the expression of cell surface MHC class II isoforms.

This interpretation could have important implications for explaining the well-established genetic correlation between HLA-DR and HLA-DP and -DQ and between susceptibility and resistance to many diseases. Diseases with established genetic links to one or the other, HLA-DR or HLA-DP and -DQ, include heart disease (71), autoimmune diseases (72), infectious diseases (73), and inflammatory conditions (74). Notably, many autoimmune diseases, such as multiple sclerosis (75), systemic lupus erythematosus (76), and rheumatoid arthritis (77), show strong associations specifically with HLA-DR expression, and these diseases are more common in women than in men. Our observed effects of lipid availability on the level of cell surface expression of HLA-DR in human cell culture and MHC class II I-E in female mice may suggest a possible mechanistic link between lipid availability, autoimmune diseases, and HLA-DR. Our current work is aimed at exploring the relationships between gender, lipid availability, and HLA-DR cell surface expression.

In conclusion, the changes we have observed in lysosomal acidity and MHC class II cell surface expression in response to varying lipid conditions, both in vitro and in vivo, represent important cellular responses. These changes may affect antigen processing and presentation, T-lymphocyte activation, inflammation, and potentially the nature and production of cytokines by T-lymphocytes. We observed a preferential use of specific fatty acid pools (exogenous vs. endogenous fatty acids), and these data suggest that diet and/or pharmacologic intervention may be used to influence early events in inflammation and the immune response.

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