Macrophages Deficient in CTP:Phosphocholine Cytidylyltransferase-α Are Viable under Normal Culture Conditions but Are Highly Susceptible to Free Cholesterol-induced Death

MOLECULAR GENETIC EVIDENCE THAT THE INDUCTION OF PHOSPHATIDYLCHOLINE BIOSYNTHESIS IN FREE CHOLESTEROL-LOADED MACROPHAGES IS AN ADAPTIVE RESPONSE

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Macrophages in atherosclerotic lesions accumulate excess free cholesterol (FC) and phospholipid. Because excess FC is toxic to macrophages, these observations may have relevance to macrophage death and necrosis in atheroma. Previous work by us showed that at early stages of FC loading, when macrophages are still healthy, there is activation of the phosphatidylcholine (PC) biosynthetic enzyme, CTP:phosphocholine cytidylyltransferase (CT), and accumulation of PC mass. We hypothesized that this is an adaptive response, albeit transient, that prevents the FC:PC ratio from reaching a toxic level. To test this hypothesis directly, we created mice with macrophage-targeted disruption of the major CT gene, CTα, using the Cre-lox system. Surprisingly, the number of peritoneal macrophages harvested from CTα-deficient mice and their overall health under normal culture conditions appeared normal. Moreover, CT activity and PC biosynthesis and in vitro CT activity were decreased by 70–90% but were not absent. As a likely explanation of this residual activity, we showed that CTβ2, a form of CT that arises from another gene, is induced in CTα-deficient macrophages. To test our hypothesis that increased PC biosynthesis is an adaptive response to FC loading, the viability of wild-type versus CTα-deficient macrophages under control and FC-loading conditions was compared. After 5 h of FC loading, death increased from 0.7% to only 2.0% in wild-type macrophages but from 0.9% to 29.5% in CTα-deficient macrophages. These data offer the first molecular genetic evidence that activation of CTα and induction of PC biosynthesis in FC-loaded macrophages is an adaptive response. Furthermore, the data reveal that CTβ2 in macrophages is induced in the absence of CTα and that a low level of residual CT activity, presumably due to CTβ2, is enough to keep the cells viable in the peritoneum in vivo and under normal culture conditions.

Cholesterol-loaded macrophages, or foam cells, play critical roles in atherogenesis, including the conversion of relatively benign early lesions into advanced lesions that lead to acute vascular occlusion (1, 2). An important event related to macrophage foam cells in advanced atherosclerosis is the death of these cells (3–6), which likely plays an important role in lesional necrosis and perhaps plaque rupture and acute thrombotic vascular occlusion (2, 3, 6). Because advanced lesional macrophages are known to accumulate large amounts of free cholesterol (FC) (7–10), and because excess cellular FC is known to lead to macrophage death (11, 12), FC-induced death in macrophages in advanced atherosclerotic lesions may be a critically important event in advanced lesional complications (13, 14).

In this context, our laboratory has studied cellular events that occur during FC loading of macrophages, and we discovered that the cells respond initially by an increase in PC biosynthesis and cellular FC mass, which also appears to occur in lesional macrophages in vivo (15, 16). The mechanism of this response is post-translational activation of CTP:phosphocholine cytidylyltransferase (CT; also known as CCT), which catalyzes the conversion of choline-phosphate to CDP-choline (15, 17). We have hypothesized that activation of CT in response to FC loading in macrophages is an adaptive response (12). Cellular FC excess is known to be toxic to cells primarily by altering the physical properties of cellular membranes, leading to dysfunction of integral membrane proteins (18). We therefore reasoned that the induction of PC biosynthesis in FC-loaded cells would help keep the FC:phospholipid ratio in cellular membrane from reaching cytotoxic levels. Observations of macrophages after prolonged periods of FC loading, where cell death follows a decline in the PC biosynthetic response, provided evidence in support of this hypothesis, but direct molecular support has been lacking (12).

To address this issue, we sought to create a mouse model whose macrophages had deficient CT activity and compromised PC biosynthetic capacity. Our hypothesis predicts that macrophages from such a mouse would be more susceptible to FC-

1 The abbreviations used are: FC, free cholesterol; acetyl-LDL, acetylated low-density lipoprotein; CT, CTP:phosphocholine cytidylyltransferase; CTα, CTβ, CTβ2, CTα genomic construct containing flanking loxp sites; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LysMCα, Cre recombinase gene in the murine lysozyme locus; PBS, phosphate-buffered saline; PC, phosphatidylcholine; kb, kilobase pairs; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair.

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induced cytotoxicity. Our strategy was to manipulate genetically CT in macropages using the Cre-lox system (19). By way of background, at least two genes in mammalian cells encode CT enzymes as follows: one that gives rise to CTα, which is ubiquitous and the most active form of CT, and another gene called CTβ, which gives rise to an enzyme called CTβ2 and, at least in humans, to a truncated form arising by alternative splicing called CTβ1 (20, 21). CTα is subject to regulation by both lipid binding and phosphorylation (22, 23). Both forms of CTβ have catalytic and lipid-binding domains that are homologous to CTα, and CTβ2 also has a homologous phosphorylation domain (21), but little is known about the regulation or function of either isoform of CTβ.

We now report the creation of a mouse in which the CTα gene has been disrupted in macropages using the Cre-lox system. We show that peritoneal macropages obtained from this mouse model have no detectable CTα protein and markedly decreased, although not absent, CT activity and PC biosynthesis. Interestingly, CTβ2 is induced in these macropages, and this finding likely explains the residual CT activity in CTα-deficient macropages, the normal use of peritoneal macropages present in the mouse, and the ability of the CTα-deficient macropages to remain healthy under normal culture conditions. Most importantly, CTα-deficient macropages are markedly more sensitive to FC-induced death, thus providing important molecular genetic evidence in support of the hypothesis described above.

**EXPERIMENTAL PROCEDURES**

**Materials**—The restriction endonucleases and other enzymes were purchased from either New England Biolabs (Beverly, MA) or Life Technologies, Inc. [α-32P]dCTP, [methyl-3H]-hochrome, and phospho [meth] (see above) were purchased from PerkinElmer Life Sciences. DNA preparation kit was from Qiagen (Chatsworth, CA). The random primer labeling kit and synthetic primers were from Life Technologies, Inc. The pBluescript II KS+ vector was from Stratagene (La Jolla, CA). Goat anti-rabbit IgG and the Super Signal-enhanced chemiluminescence immunoblot detection kit were purchased from Pierce. The Falcon tissue culture plasticware used in these studies was purchased from Fisher. Tissue culture media and other tissue culture reagents were obtained from Life Technologies, Inc. Fetal bovine serum (FBS) was obtained from HyClone Laboratories (Logan, UT) and was heat-inactivated for 1 h at 65 °C (HI-FBS). Compound 58035 (3-[decyldimethylsilyl]-4-methylpheny)-1-phenylethyl propanamide (24), an inhibitor of acyl-CoA cholesterol acyltransferase, was generously provided by Dr. John Heider of Sandoz, Inc. (East Hanover, NJ); a 10 mg/ml stock solution was prepared in dimethyl sulfoxide, and the final dimethyl sulfoxide concentration in both treated and control cells was 0.05%. All other chemicals and reagents were from Sigma, and all organic solvents were from Fisher.

**Construction of the CTαflα Replacement Vector**—The CTαflα replacement vector was constructed by manipulation of a 12.5-kb fragment of the murine CTα gene containing exons 4–8, the intervening introns, and parts of introns 3 and 8 (clone 4 in Ref. 25). As depicted in Fig. 1A, the “short arm” included a 5.5-kb BamHI fragment containing the 34-bploxP sequence (5′-ATAACTCTTGATGACATGATACGAGTTAAT-3′) inserted at the NsiI site, which is 330 bp upstream of exon 4. The “long arm” consisted of a 5.5-kb BamHI/NheI fragment that included exons 6 and 7. These two arms plus a 1.9-kb neomycin resistance cassette (Neo) flanked byloxP sites (“flxed” Neo) and a 1.8-kb thymidine kinase gene cassette were assembled in the order shown in Fig. 1A, and the final construct was embedded into pBluescript II KS+ vector.

**Generation and Identification of Gene-targeted ES Clones**—The replacement vector was transfected into 129/Sv ES cells, and G418-resistant clones were selected and screened by Southern blot analysis using a 1-kb NheI/EcoRI exon 6-containing probe (Fig. 1A) as follows. Genomic DNA (3–5 μg) was digested overnight in 0.5% agarose gel. After transferring to the nylon membrane, the hybridization was performed using Quickhyb solution (Stratagene) containing 1 × 106 cpm/ml of [32P]-labeled probe for 2 h and exposed overnight. ES cells undergoing homologous recombination with the replacement vector would show a 6.5-kb band in addition to the 12.5-kb wild-type band (see Fig. 1A and “Results”).

To demonstrate that the recombinant ES cells retained the firstloxP site (i.e. 5′ to exon 4), PCR was conducted using the CT382 primer (5′-CTTGGGCTGTAGCATTAGA-3′) and theloxP-5–16 primer (5′-CTCTGATAGAA-3′), with an expected PCR product of 440 bp. Another PCR was conducted to exclude the possibility that a positive result of first PCR was due to the insertional contamination of the tail cell vector in ES cell chromosomal DNA. This was done using the CT382 primer (above) and M13 reverse primer, which is located on pBluescript II KS+ vector, upstream of the short arm. Homologous recombination would fail to produce a PCR product, whereas random insertion would yield a product of 3.2 kb (see Fig. 1A and “Results”).

**Generation and Identification of CTαflα Mice and CTαflα/LysMCre Mice**—Cells from an ES cell colony containing homologously recombined CTαflα were injected into C57BL/6J host blastocysts, which were then implanted into pseudopregnant female mice by the Animal Core Facility of Rockefeller University. Male offspring with 75–90% agouti color, the coat color contributed by the ES cells, were bred with C57BL/6J females. Pups that were 100% agouti, indicating germ line transmission, were screened as follows. Tail clips were digested for 18 h at 55 °C in a buffer containing 0.3 mg/ml proteinase K, 0.1 μM Tris-HCl, pH 8.0, 2.5 mM EDTA, and 0.5% SDS; the DNA was extracted with phenol/chloroform, purified by ethanol precipitation, and dissolved at 65 °C in 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA. The DNA was then subjected to Southern analysis using the normal 34-bploxP cassette described above as a probe. Heterozygous CTαflα mice were identified and ablated, and homozygous CTαflα mice resulting from this mating (~25% of the offspring) were identified by PCR using the CT382 primer (above) and a primer called CT3UL (5′-GAATAGTGGCAGCTGAATCAGG-3′) just upstream of the 3′loxP site (see Fig. 2A and “Results”). These homozygous CTαflα mice were bred with homozygous LysMCre mice (obtained from Dr. Irungard Forster, Technical University of Munich), in which the Cre recombinase is driven by the lysosome promoter via gene targeting into the lysosome locus (26). The resulting pups, which were heterozygous for both CTαflα and LysMCre, were then bred with homozygous CTαflα mice. By PCR screening tail DNA from the pups for both CTαflα (above) and Cre (26), mice homozygous for CTαflα and heterozygous for LysMCre (i.e. mice having CTα-deficient macropages) were identified. DNA samples from both tail clips and macropages were further analyzed by the PCR assay described above (CT382-CT3UL) to distinguish the wild-type allele, the CTαflα allele, and Cre-mediated recombination (see “Results” and Fig. 2A).

**Harvesting, Culturing, and Incubations of Mouse Peritoneal Macropages**—Macropages were harvested from the peritoneum of mice 3–4 days following injection of 40 μg of concanavalin A in 0.5 μl of PBS and then cultured as described previously (27). On the day of the experiment, the cells were washed three times with warm PBS and incubated for the indicated times in 0.2 ml of DMEM, 1% FBS (w/v) alone or containing 100 μg of acetyl-LDL/ml plus 10 μg of compound 58035/ml, or each compound separately, as described previously (15). At the end of the incubation period, the cells were assayed for cell death as described above.

**Cell Death Assays**—The binding of Alexa-488 annexin V was conducted using a modification (28) of the method of Vermes et al. (29). After the indicated incubations, the cells were incubated in 100 μl of 1× Annexin-binding buffer (25 mM HEPES, 140 mM NaCl, 1 mM EDTA, pH 7.4, 0.1% BSA) containing 5 μl of Alexa-488 annexin V and 1 μl of 100 μg/ml propidium iodide for 15 min at room temperature, according to the manufacturer’s instructions (Molecular Probes). Cells were immediately viewed with a 40× objective using an Olympus IX-70 inverted fluorescence microscope equipped with filters appropriate for fluorescein (Alexa-488 annexin) and rhodamine (propidium iodide). Five fields of cells for each condition (~1000 cells) were counted.

**Immunoblot Analysis**—Cells were scraped on ice in PBS, and the total amount of cellular protein was determined by the method of Lowry et al. (30). The cellular homogenates were mixed with concentrated Laemmli sample buffer (31) to give a final protein concentration of 2 μg/ml and then boiled for 5 min. Proteins were separated by 5–20% gradient SDS-polyacrylamide gel electrophoresis using 30 μg of protein/ lane and then electrotransferred to nitrocellulose membranes. These blots were blocked in Tris-buffered saline containing 5% nonfat dry milk for 1 h and then incubated with the indicated antibodies in buffer A (Tris-buffered saline containing 0.1% bovine serum albumin and 0.1% Tween 20 for 3 h at room temperature). The three rabbit polyclonal antibodies used in this study were as follows: an antibody directed against the N terminus of CTα that is specific for this form of CT (Ref. 32; kindly supplied by Dr. Claudia Kent, University of Michigan); an antibody directed against the B3 domain of CTβ2 that is
FC-induced Death in CTα-deficient Macrophages

To carry out this strategy, we created a replacement vector in which exons 4 and 5, which encode a major portion of the catalytic domain of CTα (25), were flanked by loxP sites (Fig. 1A). A Neo cassette was also included so that ES cell transfec-
tants could be positively selected with G-418. Cre-mediated scission at the loxP sites would not only lead to deletion of exons 4 and 5, but the partially deleted gene would also encode a new stop codon at the site of scission. 129/Sv ES cells were transfected with the replacement vector, and Neo-containing clones (i.e. those able to grow in G-418) were isolated and expanded into individual colonies. To distinguish homologous recombination from random insertion, the colonies were subject-
ted to Southern analysis using a probe containing exon 8, which lies external to the 5’ end of the replacement vector (refer to Fig. 1A). As shown in Fig. 1B, mock-transfected colony 4 showed only the presence of the 12.5-kb wild-type EcoRI fragment (i.e. containing exon 8 but not the Neo cassette), whereas the other colonies shown in this figure demonstrated both the wild-type allele and the recombinant allele, as evidenced by the 6.8-kb EcoRI fragment that indicates the presence of both the Neo cassette and exon 8. To verify that the loxP site 5’ to exon 4 was still present and in the correct location, the colonies were subjected to PCR as depicted in the bottom scheme in Fig. 1A. The data in Fig. 1C show that all four positive colonies and the vector control, but not the negative colony, yielded the predicted 440-bp PCR product. Finally, to rule out further the possibility of random vector insertion, the colonies were subjected to PCR in which the 5’ primer was directed to an area of the vector that was external to the 5’ portion of the CTα replacement vector (refer to middle scheme in Fig. 1A). As predicted, none of the colonies yielded the predicted PCR product, which is shown for the vector control (Fig. 1D). Thus, several ES cell transfec-
tants show evidence of successful homologous recombination of the loxP-containing CTα replacement vector (CTαloxp).

Cells from colony 124 were injected into C57BL/6J blastocysts, which were then implanted into pseudopregnant female mice. Nine males with 75–90% agouti color were born and were eventually bred with C57 female mice. All litters except one were 100% agouti, indicating germ line transmission of the recombinant allele. These pups were screened by Southern analysis (above), and those showing both the 12.5- and 6.5-kb bands (~50% of the pups), indicating heterozygosity for the

RESULTS

Creation of Mice Whose Macrophages Are Depleted in CTα—To obtain mouse peritoneal macrophages with decreased PC biosynthesis, the overall strategy was to create a mouse with the endogenous CTα locus replaced by a CTα gene with loxP sites flanking one or more exons critical for CTα function. This mouse would then be crossed with a previously described mouse in which the Cre recombinase is driven by the endoge-

nous lysosome promoter (LysMCre) and thus is expressed pri-
marily in differentiated macrophages (as well as in neutro-
phils) (26). The progeny from this cross should therefore have macrophages with a dysfunctional CTα gene (19, 26).

specific for this form of CT (21); and an antibody directed against the B2 epitope of CTβ that recognizes both CTβ1 and CTβ2 (21). After washing four times (10 min each) with buffer A, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:15,000) for 1 h in buffer A at room temperature. The blots were then washed extensively with buffer A, incubated with SuperSignal chemi-

luminescence reagent for 7 min, and exposed to x-ray film for up to 10 min. Detection of CTβ2 mRNA by RT-PCR—CTβ2 mRNA was detected by RT-PCR (21) using primers based on the sequence of rat CTβ, which is 98% identical in amino acid sequence to human CTβ2 (33). The forward primer was 5’-CCAAGGAGCTGAAATGTCAGC-3’ (corresponding to nu-
cleotides 889–909 of the rat sequence) and the reverse primer was 5’-GCCATTGTGAACCCGCAC-3’ (corresponding to nucleotides 1141–1121 of the rat sequence); the reverse primer should be specific for CTβ2 based on comparison with the human CTβ1 and CTβ2 sequences (21). The PCR product in rats is 452 base pairs, and a similar size product was found in mice (see “Results”).
culture conditions, the cells appeared healthy (see below). Macrophage DNA from heterozygous and wild-type CTαflox mice without Cre generated the PCR product expected in the absence of scission (lanes 6 and 7), but macrophage DNA from heterozygous CTαflox mice with LysMCre generated no detectable PCR product from the CTαflox allele (lanes 9 and 10), indicating disruption of the loxP-exon 4 portion of this allele. Macrophage DNA from homozygous CTαflox mice without Cre generated the expected CTαflox product (lane 11), whereas macrophage DNA from homozygous CTαflox mice with LysMCre generated no detectable PCR products at all (not displayed). In summary, the CTαflox allele in macrophage DNA, but not tail DNA, was successfully disrupted in the presence of LysMCre but not in its absence.

To determine if disruption of the CTα gene resulted in decreased CTα protein expression, homogenates of macrophages from wild-type and homozygous CTαflox/LysMCre mice were subjected to immunoblot analysis using an antibody specific for CTα. As demonstrated in Fig. 2B, the CTαflox/LysMCre macrophages expressed no detectable CTα in comparison with wild-type macrophages (lane 1) or macrophages with LysMCre alone (not shown). As expected from these data, the homogenates from the CTαflox/LysMCre macrophages also demonstrated very little CT activity (Fig. 2C). Thus, Cre-mediated disruption of the macrophage CTα gene results in no detectable CTα expression and a marked decrease in in vitro CT activity.

Living macrophages from wild-type and CTαflox/LysMCre mice were examined for their ability to convert [3H]choline to PC and biosynthetic intermediates. The cells were pulse-labeled with [3H]choline and then chased without label for 3 h. The data in Fig. 3A show that the cellular content of [3H]choline itself was relatively low in both types of macrophages (compare the y axes of A with those of the other three panels in Fig. 3), indicating that the majority of intracellular label was utilized for PC biosynthesis over the 3-h chase period. The cellular content of [3H]choline-phosphate was approximately 2-fold higher in the CTαflox/LysMCre macrophages (Fig. 3B) and that of CDP-[3H]choline was approximately 3-fold lower (Fig. 3C) in comparison with wild-type macrophages. These data are consistent with a partial block in the CT reaction. Finally, as shown in D, [3H]PC content was decreased to a similar relative degree as CDP-[3H]choline content in CTαflox/LysMCre macrophages. Thus, disruption of the CTα gene results in disruption of choline flux at the CT step, and this results in decreased PC synthesis.

CTβ2 Is Present in Macrophages and Induced in CTα-deficient Macrophages—Although CTαflox/LysMCre macrophages showed a marked decrease in CT activity and PC biosynthesis (above), these values were clearly not zero. Indeed, the residual CT activity in these macrophages undoubtedly...
accounts for the normal number of peritoneal macrophages and their overall good health under normal culture conditions (see below). In light of the recent findings that a second gene can give rise to another form of CT, called CTβ (21), we sought evidence for the existence of this enzyme in the CTα-deficient macrophages. Through alternative splicing, the CTβ gene gives rise to two transcripts encoding CTβ1 and CTβ2. By using an antibody that specifically recognizes CTβ2 via its unique B3 epitope (21), we were able to detect this protein in homogenates of wild-type macrophages obtained from wild-type mice or from homozygous (Homozyg.) CTαfoxflox mice expressing LysMCre; a CTα-specific antibody was used at 1:1000 dilution. C, in vitro CT assay using 20 µg of cell homogenate from peritoneal macrophages obtained from wild-type or CTαfoxflox/LysMCre mice.

FIG. 2 Cre-mediated recombination of the CTαfoxflox gene in peritoneal macrophages. A, PCR assay of DNA from tail clips and from peritoneal macrophages from wild-type (wt), heterozygous, or homozygous CTαfoxflox (flx) mice with no or one copy of the LysMCre allele. B, immunoblot of homogenates of peritoneal macrophages obtained from wild-type mice or from homozygous (Homozyg.) CTαfoxflox mice expressing LysMCre; a CTα-specific antibody was used at 1:1000 dilution. C, in vitro CT assay using 20 µg of cell homogenate from peritoneal macrophages obtained from wild-type or CTαfoxflox/LysMCre mice.

accounts for the normal number of peritoneal macrophages and their overall good health under normal culture conditions (see below). In light of the recent findings that a second gene can give rise to another form of CT, called CTβ (21), we sought evidence for the existence of this enzyme in the CTα-deficient macrophages. Through alternative splicing, the CTβ gene gives rise to two transcripts encoding CTβ1 and CTβ2. By using an antibody that specifically recognizes CTβ2 via its unique B3 epitope (21), we were able to detect this protein in homogenates of wild-type macrophages (not shown) and in control macrophages from mice that express LysMCre but not CTαfoxflox (1st lane in Fig. 4A). Importantly, in CTα-deficient macrophages (i.e., those obtained from homozygous CTαfoxflox/LysMCre mice), there was increased expression of CTβ2 (Fig. 4A). By using PCR primers that are able to detect murine CTβ2 mRNA by RT-PCR, we compared RNA isolated from macrophages obtained from control mice (i.e., expressing only LysMCre) and from CTαfoxflox/LysMCre mice. As shown in Fig. 4B, the CTβ2 RT-PCR product was below the limit of detection in the control macrophages (lane 1) but was clearly visible in the CTαfoxflox/LysMCre macrophages (lane 2); for comparison, the PCR products for liver (lane 3) and brain (lane 4) from wild-type mice are also shown. In summary, the data in Fig. 4 indicate that CTα-deficient macrophages have an increased expression of CTβ2 mRNA and protein.3

3 By using another antibody that recognizes both human CTβ1 and
PC biosynthesis is induced in wild-type macrophages by FC loading via post-translational activation of CT (15, 17), which, as we have seen above, is almost entirely CTα. The availability of macrophages with CTβ2 but no CTα afforded us the unique opportunity to determine whether such cells could also respond to FC loading with an increase in incorporation of [3H]choline into [3H]PC.4 As shown in Fig. 4C, [3H]choline incorporation in control macrophages was increased 2-fold after 5 h of FC loading. Although total [3H]PC in CTα-deficient macrophages was much lower than that in the control cells, it was clearly induced by FC loading. In fact, this value in the FC-loaded CTα-deficient macrophages might be as much as 30% higher given the substantial number of dead cells in this population (see below). These data suggest that the signaling involved in activation of CTα in control macrophages (17) may also work on CTβ2 in the CTα-deficient macrophages. The data also show that the level of [3H]choline incorporation into [3H]PC after a 5-h period of FC loading, the precise conditions used in the cell death studies below, is substantially less in CTα-deficient macrophages versus control macrophages.

CTα-deficient Macrophages Are Markedly More Susceptible to FC-induced Death—We have hypothesized that the increase in PC biosynthesis and cellular PC mass that occurs in FC-loaded macrophages is part of an adaptive response (12). We propose that this response helps protect cells, albeit transiently, from FC-induced death by preventing a lethal rise in the FC:PC ratio in critical cellular membranes (12). By having access to macrophages that have deficient CT activity and PC biosynthesis but that are able to remain healthy under normal culture conditions allowed us to test this hypothesis directly. Therefore, peritoneal macrophages from control mice (expressing only LysMCre) and from CTαflox/LysMCre (CTα-deficient) mice were incubated under control or FC-loading conditions for 5 h. To detect apoptotic changes, the cells were stained with the phosphatidylserine-binding protein, annexin V, which in the assay used here is conjugated to Alexa-488 (green) (29). We have recently shown that after 9 h of FC loading, peritoneal macrophages undergo apoptotic changes, including externalization of phosphatidylserine (28). To detect more advanced cell death, including necrotic death, the cells were also exposed to the membrane-impermeable nucleic acid fluorophore, propidium iodide (red-orange). As shown in Fig. 5A, most of the control macro-

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4 In a previous study (15), we showed that FC loading of macrophages affected neither [3H]choline uptake by macrophages nor the size of the intracellular unlabeled choline pool. If these points apply to the current model, incorporation of [3H]choline into [3H]phosphatidylcholine would reflect PC biosynthesis per se.
phages under non-loading conditions showed no signs of death, and after 5 h of FC loading, the number of dead cells increased only slightly (Fig. 5B). CTα-deficient macrophages under non-loading conditions also appeared healthy, and the vast majority demonstrated neither Alexa-488 annexin nor propidium iodide staining (Fig. 5C). In striking contrast, there were many annexin and/or propidium iodide-stained cells among the FC-loaded CTα-deficient macrophages (Fig. 5D).

Quantification of data from a large number of cells verified that 5 h of FC loading in CTα-deficient macrophages resulted in much more cell death than that observed in similarly treated control macrophages (Fig. 6A). To rule out the possibility that
CTα deficiency enhanced the effect of cell death inducers in general, we treated control and experimental macrophages with the protein kinase C inhibitor staurosporine, which is a known inducer of apoptotic death in mononuclear leukocytes (36). As shown in the first set of bars in Fig. 6B, we chose a concentration of staurosporine that causes only partial cell death in control macrophages so that any enhancing effect could be readily detected in the experimental macrophages. As shown by the data in Fig. 6B, however, CTα-deficient macrophages show no significant increase in staurosporine-induced death compared with the control cells. In summary, the absence of CTα, leading to a deficiency of PC biosynthesis, renders macrophages much more susceptible to FC-induced death. This finding strongly supports the hypothesis that the activation of CTα and the increase in PC biosynthesis seen in FC-loaded macrophages is a cell-survival adaptive response.

**DISCUSSION**

A major impetus for the project described in this report was related to our previous finding that macrophages respond to FC loading by increasing CT activity, PC biosynthesis, and cellular PC mass (15). Several observations have led us to propose that these events represent an adaptive response to prevent, at least initially, the FC:phospholipid ratio from reaching cytotoxic levels (18). For example, after prolonged FC loading of macrophages, CT activity and PC biosynthesis decline, and shortly thereafter the cells begin to die (18). The most direct test of this hypothesis, however, required studying macrophages with defective PC biosynthesis via molecular genetic manipulation of CT. The data in this report clearly demonstrate that such macrophages are markedly more susceptible to FC-induced death, thus providing strong support to the hypothesis. We propose that in FC-loaded wild-type macrophages, the excess PC, which accumulates mostly in intracellular whorl-like membrane structures (15), acts as a sink for the excess FC and thus prevents accumulation of FC in cellular membranes.

Based on the data of Yeagle (18) and others (14), this process would be expected to promote cell survival by protecting enzymes and transport proteins in the plasma membrane and other membranes from an abnormally rigid lipid environment. The eventual failure of this adaptive response even in wild-type macrophages (above) may be due to direct inactivation of CT itself by an overwhelming load of FC. Moreover, this process may be accelerated by apoptosis-induced inhibition of choline phosphotransferase (37). In FC-loaded CTα-deficient macrophages, the failure of adaptation is accelerated by the low level of PC biosynthetic activity in these cells.

The unique model described in this report has allowed us to address another important issue related to macrophage PC metabolism, namely the overall response of macrophages to CTα deficiency. Our data suggest that an increased level of expression of CTβ2 in CTα-deficient macrophages provides sufficient CT activity and PC biosynthesis to allow the cells to survive under normal conditions both in vivo, as evidenced by the overall health of the mice and the normal number of peritoneal macrophages, and in cell culture (Fig. 5C). The unmasking of a phenotype in these macrophages under FC-loading conditions, however, suggests that other “stressors” might also uncover interesting deficiencies in these cells. For example, macrophage proliferation (38), an event known to occur in atherosclerotic lesions (39–41), as well as other cellular events associated with membrane remodeling, such as phagocytosis and exocytosis (42, 43), might be deficient in CTα-deficient macrophages.

The functions of CTβ are still under investigation. In our study with murine macrophages, we were only able to detect the presence of CTβ2, which could be due to either suboptimal reagents for murine tissue (i.e. antibodies and PCR primers based on human or rat CTβ) or to the possibility that mice have no or very low levels of CTβ1. CTβ2 lacks the N-terminal nuclear localization domain present in CTα but has regions
homologous to the catalytic, membrane-binding, and C-terminal phosphorylation domains of CTα (21). Most importantly, CTβ demonstrates catalytic activity, although its specific activity appears to be less than that of CTα when expressed in COS-7 cells (20). This latter point may account for our finding that PC biosynthesis was still markedly decreased in CTβ-deficient macrophages even in the face of induced expression of CTβ2. Another interesting result from our studies was that [3H]choline incorporation into [3H]PC was increased by FC loading in CTα-deficient macrophages (Fig. 4C). Pending further work in this area, we hypothesize that CTβ2, like CTα (15, 17), can be activated by FC loading. In this context, we previously found that FC-induced CT activation in wild-type macrophages was associated with dephosphorylation of CTα (17), and so the presence of a phosphorylation domain in CTβ2 may be important in the increase in [3H]PC biosynthesis in FC-loaded CTα-deficient macrophages.

The current study has focused on cultured peritoneal macrophages obtained from the unique model described herein. However, in future studies we plan to address the role in vivo of PC biosynthesis in the function of macrophages as well as neutrophils, which are also targeted by LysMCre (26). To carry out these studies, it will first be necessary to remove the “floxed” Neo cassette from CTαfloxed gene in our mice (44).2 Once this has been accomplished, we will be able to test specific hypotheses in vivo using the LysMCre system. For example, we and others (13, 14, 45) have proposed that FC-induced macrophage death is an important cause of necrosis in advanced atherosclerotic lesions. Based on this proposal and the cell culture data reported here, we would predict that lesional necrosis would be increased in CTαfloxed/LysMCre mice with an atherosclerotic background (e.g. after crossing into the apolipoprotein E knock-out background). Because lesional necrosis is a likely contributor to acute vascular events (46), such a finding might have important implications related to the clinical complications of atherosclerotic vascular disease. Similarly, CTαfloxed/LysMCre mice can be used to study other PC-dependent functions of macrophages in vivo (e.g. in host defense), and the CTαfloxed mice can be crossed with mice expressing Cre in other tissues to address specific questions related to CTα, CTβ, and PC biosynthesis in these tissues.

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