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Slfn2 mutation-induced loss of T-cell quiescence leads to elevated *de novo* sterol synthesis

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Summary

Acquisition of a 'quiescence programme' by naive T cells is important to provide a stress-free environment and resistance to apoptosis while preserving their responsiveness to activating stimuli. Therefore, the survival and proper function of naive T cells depends on their ability to maintain quiescence. Recently we demonstrated that by preventing chronic unresolved endoplasmic reticulum (ER) stress, Schlafen2 (Slfn2) maintains a stress-free environment to conserve a pool of naive T cells ready to respond to a microbial invasion. These findings strongly suggest an intimate association between quiescence and stress signalling. However, the connection between ER stress conditions and loss of T-cell quiescence is unknown. Here we demonstrate that homeostasis of cholesterol and lipids, is disrupted in T cells and monocytes from Slfn2-mutant, *elektra*, mice with higher levels of lipid rafts and lipid droplets found in these cells. Moreover, *elektra* T cells had elevated levels of free cholesterol and cholesteryl ester due to increased *de novo* synthesis and higher levels of the enzyme HMG-CoA reductase. As cholesterol plays an important role in the transition of T cells from resting to active state, and ER regulates cholesterol and lipid synthesis, we suggest that regulation of cholesterol levels through the prevention of ER stress is an essential component of the mechanism by which Slfn2 regulates quiescence.

Keywords: cholesterol synthesis; Slfn2; T cell quiescence.

Introduction

An immune system must be capable of defending the host while sparing its tissues. To fulfil this task the immune system generates and maintains a vast repertoire of immunologically naive lymphocytes that are maintained in a quiescent state until encountering their specific antigen. This quiescent state is characterized by small size, lack of spontaneous proliferation, and low metabolic rate with a catabolic signature.[1–3] Acquisition of a 'quiescence programme' is important to provide a stress-free environment and resistance to apoptosis while preserving naive T cells responsive to activating stimuli.[1,3–6] Therefore, the survival and proper function of naive T cells depends on their continued quiescence.[1] A growing number of factors, including Forkhead box O1,[7,8] Krüppel-like factor 2,[9] Transducer of ErbB-2 (TOB),[10] Tuberous Sclerosis Protein (TSC)1,[11,12] TSC2,[13] Forkhead box protein P1[14,15] and Schlafen2 (Slfn2),[16] are associated with the regulation of quiescence in immune cells. However, lymphocyte quiescence is poorly understood.

The Schlafen genes (*Slfn*) were first described in mice as a family that is transcribed during thymocyte maturation.[17] Members of the *Slfn* family are expressed in tissues of the immune system, and their expression levels vary during T-cell and macrophage development as well as in response to infections.[17,18] Previously, we described a mouse strain bearing the *Slfn2*-mutated allele, *elektra*.[16] The *elektra* mutation causes an isoleucine-to-asparagine substitution of residue 135 of the 278-amino-acid Slfn2 protein leading to its loss of function. In the *elektra* mutant mouse ($Slfn2^{eka/eka}$), naive newly thymic emigrant ($CD44^{lo}$) cells fail to maintain quiescence and instead acquire a semi-activated phenotype characterized by activation of Jun

N-terminal kinase and p38 and a higher propensity to enter the cell cycle. Consequently, upon maturation or encountering activation signals, *elektra* T cells fail to acquire a memory-like phenotype and to engage pro-survival machinery, leading to premature apoptosis. Inflammatory monocytes are affected by the *elektra* mutation as well, exhibiting similar fragility in the presence of signals that induce their maturation or activation.[16] Following that study, we recently demonstrated that *elektra* monocytes and T cells exhibit unresolved endoplasmic reticulum (ER) stress, which leads to their impaired proliferation and survival.[19] This study revealed a connection between the loss of quiescence in Slfn2 deficiency and chronic ER stress.

Cholesterol is an essential constituent in mammalian cell membranes and is crucial for cell growth, proliferation, signalling and intracellular trafficking.[20] Cholesterol homeostasis is preserved by two main nuclear receptor systems: sterol regulatory element binding proteins (SREBPs) and liver X receptors (LXRs). SREBP-2 resides in the ER under conditions of sufficient sterol.[21] When cells are depleted of sterols, SREBP-2 is cleaved and transported into the nucleus to activate genes controlling sterol synthesis and uptake, such as 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase (HMGCR), HMG-CoA synthase and low-density lipoprotein receptor (LDLR).[21] Alternatively, under conditions of excess cellular cholesterol, the LXR pathway is activated to induce cholesterol efflux through the up-regulation of the lipid transporters, ATP-binding cassette A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1).[22]

Naive T cells undergo metabolic reprogramming upon their priming by antigen-presenting cells. Rapid up-regulation of lipid-biosynthesis pathways and down-regulation of LXR target genes are key components in this metabolic reprogramming.[23] Cholesterol derivatives (such as oxysterols) or ligand activation of LXR have been shown to inhibit mitogen-driven T-cell expansion by altering cellular sterol content.[23,24] In addition, loss of LXR or cholesterol transporter ABCG1 expression confers a proliferative advantage to lymphocytes, resulting in enhanced homeostatic and antigen-driven responses.[23,25,26]

These and other studies provide strong evidence that sterol and lipid levels play a critical role during the transition from a quiescent lymphocyte to an activated lymphocyte. However, the molecular mechanisms that link the lipid/sterol anabolic programme to quiescence disruption remain poorly defined. Moreover, the question of whether there is a functional connection between previously identified T-cell quiescence factors and sterol homeostasis has yet to be addressed.

In the present study, we demonstrate that the loss of function mutation, *elektra*, in the quiescence factor Slfn2 leads to accumulation of cholesterol due to increased *de novo* synthesis in T cells and inflammatory monocytes. Our results establish a potential role for the quiescence factor Slfn2 in maintaining quiescence through regulating sterol homeostasis.

Materials and methods

Mice

Slfn2$^{eka/eka}$ mice were previously generated as described in Berger *et al.* [16] C57BL/6J (wild-type) mice were from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained and bred under specific pathogen-free conditions in the Hebrew University's animal facilities according to the Institutional Animal Care and Use Committee's regulations. All mice were maintained on the C57BL/6 background and used for experiments between 8 and 12 weeks.

Inflammatory monocytes and T-cell isolation

For monocyte isolation, total bone marrow cells were harvested and inflammatory monocytes were isolated using the EasySep mouse monocyte isolation kit (Stem Cell Technologies, Vancouver, Canada). For further purification, Cd11b^{+} Ly6C^{hi} monocytes were sorted by flow cytometry. Total T cells were isolated from spleen by EasySep Mouse CD90.2/Thy-1.2 Positive Selection Kit (Stem Cell Technologies). CD4^{+} T cells were enriched using an EasySep Mouse CD4^{+} T-Cell Enrichment Kit (Stem Cell Technologies).

Real-time PCR

Total RNA from purified monocytes or T cells was extracted with Direct-zol RNA MiniPrep Plus (Zymo Research, Irvine, CA). cDNA was synthesized using ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA). Quantitative real-time PCR was then performed using QuantStudio 12K Flex Real Time PCR system with a Power SYBR green PCR master mix kit (Applied Biosystems, Foster City, CA). The reaction was performed as follows: one cycle of 2 min at 50°; one cycle of 10 min at 95°; 40 cycles of 15 seconds at 95° followed by 1 min at 60°; one cycle of 15 seconds at 95°; one cycle of 1 min at 60°; and one cycle of 15 seconds at 95°.

Data were analysed and presented on the basis of the relative expression method. The formula for this calculation is as follows: relative expression = $2^{-(S\Delta Ct\ -C\Delta Ct)}$ where ΔCt is the difference in the threshold cycle between the gene of interest and the housekeeping gene (*mUBC*), S is the *elektra* mouse, and C is the C57BL/6J control mouse. In Fig. 1(b), the mean value of the C group was returned to 1 in each gene tested.

Flow cytometry

To visualize lipid rafts, splenocytes were labelled with conjugated monoclonal antibody against CD4 and CD8

Quantitative real-time PCR primers used in this study

Gene	Forward (5′–3′)	Reverse (5′–3′)
UBC	CAGCCGTATA TCTTCCCAGACT	CTCAGAGGGATGCCAGT AATCTA
ALOX15	GGCTCCAACAACG AGGTCTAC	AGGTATTCTGACACATC CACCTT
ACAT2	ACAAGACAGACCT CTTCCCTC	ATGGTTCGGAAATGT TGCACC
AGPAT9	GGCCTTCGGA TTATCCCTGG	CTTGGGGGCTCC TTTCTGAA
HPGD	GTGAACGGCAA AGTGGCTCT	TCCAATCCACCAATGC TACCT
FABP4	AAGGTGAAGAG CATCATAACCCT	TCACGCCTTTCATAACAC ATTCC
METRNL	CTGGAGCAGGG AGGCTTATTT	GGACAACAAAGTCAC TGGTACAG
SREBP-2	GCGTTCTGGA GACCATGGA	ACAAAGTTGCTCTGAA AACAAATCA
LDLR	GTCAGTCCAATC AATTCAGC	TCTCGGTCTCCATCACAC
HMG Red	CTTGTGGAATGCC TTGTGATTG	AGCCGAAGCAGC ACATGAT
HMG Syn	GCCGTGAAC TGGGTCGAA	GCATATATAGCAATGTCT CCTGCAA
ABCG1	CTTTCCTACTCT GTACCCGAGG	CGGGGCATTCCATTGAT AAGG
ABCA1	GGTTTGGAGATGGTT ATACAATAGTTGT	CCCGGAAACGCAAGTCC
SREBP-1c	GGAGCCATGGA TTGCACATT	GGCCCGGGAAGTCACTGT

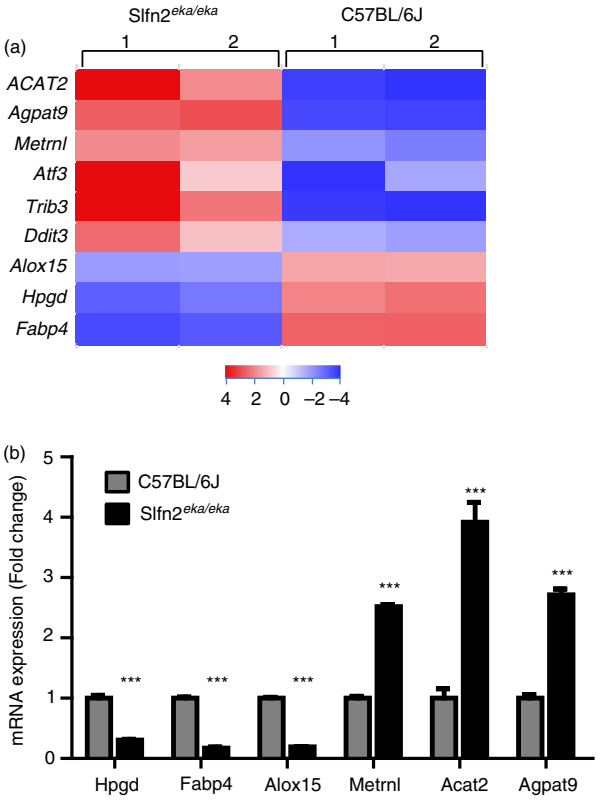

Figure 1. Altered expression of lipid- and sterol-related genes in *elektra* inflammatory monocytes. (a) Heat map of cDNA expression array shows differentially expressed genes in C57BL/6J and Slfn2$^{eka/eka}$ bone marrow CD11b^{+} ly6C^{hi} sorted monocytes. (b) Gene expression analysed by quantitative RT-PCR of lipid- and sterol-related genes in C57BL/6J and Slfn2$^{eka/eka}$ bone marrow CD11b^{+} ly6c^{hi} monocytes ($n = 4$). *$P < 0{\cdot}05$ (two-tailed Student's *t*-test). Error bars are SEM. [Colour figure can be viewed at wiley onlinelibrary.com]

together with 10 ng Alexa Fluor 488-labelled cholera toxin B (Invitrogen, Carlsbad, CA) for 30 min at 4°. For lipid droplet staining, cells were first labelled with CD4 and CD8 for 30 min at 4°, and then rapidly stained with 1 μg/ml BODIPY 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene) (Molecular Probes, Eugene, OR) for 30 min at room temperature. LDL uptake was measured by incubation of T cells with the fluorescently labelled LDL from plasma, BODIPY FL complex (Molecular Probes) (10 μg/ml) in RPMI-1640 medium supplemented with 2% BSA for 2 hr in 37°, and cells were then stained for surface markers. Stained cells were analysed by Gallios flow cytometer with KALUZA software (Beckman Coulter, Brea, CA). The following antibodies were used for flow cytometry: anti-CD8α (53-6.7), anti-CD4 (L3T4), anti-CD11b (M1/70) and anti-Ly6C (AL-21). All antibodies were from BioLegend (San Diego, CA).

Cholesterol content in T cells

This procedure was performed as previously described:[26] $2{\cdot}5 \times 10^6$ positively isolated total T cells were pelleted by low-spin centrifugation. After several washes with PBS, the cell pellet was extracted with isopropanol containing 5-cholestane as an internal standard. Total and free cholesterol contents were determined by gas–liquid chromatography and normalized to cellular protein, as previously described.[27] Cholesteryl ester was calculated as (total cholesterol – free cholesterol) × 1·67. Multiplying by 1·67 corrects for the average fatty acid mass that is lost during saponification.

T-cell cholesterol and cholesteryl ester biosynthesis rate

Cellular cholesterol biosynthesis was assayed after incubation of 5×10^6 cells (purified T cells) for 3 hr at 37° with [^{3}H]acetate (3·3 μCi/ml) in serum-free medium (RPMI-1640) supplemented with 0·2% BSA.[28]Cellular lipids were extracted with hexane/isopropanol (3 : 2, v/v) and the upper phase was dried under nitrogen. The lipids were then separated by thin-layer chromatography on silica gel plates and developed in hexane/ether/acetic acid

(130/30/1·5, v/v/v). Un-esterified cholesterol spots were visualized by iodine vapour (using an appropriate standard for identification) and ^{3}H labels were quantified by β-counter (Packard Tri Carb 2100TR; PerkinElmer, Waltham, MA). Results were expressed as cpm/mg cell protein.

High-density lipoprotein-mediated cholesterol efflux from T cells

Cells (3×10^{6}) were incubated with ^{3}H-labelled cholesterol (2 μCi/ml) in serum-free medium supplemented with 0·2% BSA for 1 hr at 37°. After washing with PBS, the cells were further incubated with RPMI-1640 in the presence of high-density lipoprotein (HDL) (100 μg HDL protein/ml) or in the absence of HDL for 3 hr at 37°. Cellular and medium ^{3}H labels were quantified by β-counter (Packard Tri Carb 2100TR, PerkinElmer), and the basal or HDL-mediated cholesterol efflux was calculated as the ratio of ^{3}H label in the medium/(^{3}H label in the medium + ^{3}H label in the cells). Net HDL-mediated cholesterol efflux data were corrected for the basal level.

Western blot analysis

Purified naive CD4 T cells (5×10^{6} to 10×10^{6}) were lysed in radioimmune precipitation assay (RIPA) buffer; 20 μg protein from each sample was separated by SDS–PAGE, and immunoblotted with goat anti-HMGCR C-18 (Santa Cruz Biotech, Dallas, TX; sc-27578) followed by peroxidase bovine anti-goat IgG (Jackson Laboratory; 805-035-180). Blotting with anti AMPKα antibody (Cell Signalling, Danvers, MA; 2532) followed by peroxidase donkey anti-rabbit IgG (Jackson Laboratory; 711-005-152) were used as a loading control.

Results

Lipid and sterol-related genes are altered in Slfn2$^{eka/eka}$ monocytes

Our previous research revealed the essential role that Slfn2 plays in the defence against multiple pathogens as it maintains quiescence in immune cells.[16] Following this study, we demonstrated a further essential role for Slfn2 in the progression of diseases involving aberrant T-cell development, such as T-cell acute lymphoblastic leukaemia.[29] Moreover, most recently we established for the first time a functional connection between the loss of quiescence in Slfn2 deficiency and chronic, unresolved ER stress.[19] These findings strongly indicate that there is an intimate association between quiescence and stress signalling. Hence, we hypothesize that the development of stress conditions will accompany the loss of cellular quiescence, which will be countered by compensatory responses mediated by Slfn2. This balance may substantially influence decisions that determine the fates of T cells.

To better describe stress signals activated by the loss of function of Slfn2, we compared the transcriptome profiling of Slfn2$^{eka/eka}$ to wild-type cells. To avoid possible secondary defects, such as activation of apoptotic signalling pathways mediated by the *elektra* mutation, we analysed monocyte precursors (CD11b^{+}/ly6C^{hi}) from the bone marrow. These cells are phenotypically normal and viable in *elektra* mice; however, they undergo rapid cell death upon their maturation or activation.[16]

The gene expression profile (Fig. 1a) show that in addition to ER stress related genes (our published data[19]), Slfn2$^{eka/eka}$ bone marrow monocytes have altered expression levels of lipid/sterol-related genes in comparison to bone marrow monocytes from wild-type mice.

Among the genes found to be elevated in Slfn2$^{eka/eka}$ in comparison to wild-type monocytes are: *ACAT2*, which codes for the ER membrane-bound sterol *O*-acyltransferase 2, which produces intracellular cholesteryl esters from long-chain fatty acyl CoA and cholesterol;[30] *AGPAT9* (1-acylglycerol-3-phosphate *O*-acyltransferase 9, the gene encoding for GPAT3), which regulates triacylglycerol biosynthesis and cytosolic lipid droplet formation[31,32]; meteorin-like (*METRNL*), which is highly expressed in activated monocytes and during adipogenesis and encodes for a protein that promotes lipid metabolism and inhibits adipose inflammation.[33,34] Furthermore, our gene profile analysis showed that Slfn2$^{eka/eka}$ monocytes have highly reduced levels of the genes *ALOX15*, *FABP4* and *HPGD*, which have regulatory roles in fatty acid and cholesterol metabolism,[35,36] fatty acid uptake/transport[37] and lipid/prostaglandin metabolism,[38] respectively. Microarray results were validated by real-time PCR (Fig. 1b). These findings suggest that the *elektra* mutation in *Slfn2* leads to disruption in both lipid and sterol homeostasis in bone marrow monocytes.

Elevated lipid droplets and lipid rafts in bone marrow monocytes and T cells from Slfn2$^{eka/eka}$ mice

To test whether the *elektra* mutation in *Slfn2* leads to impaired intracellular balance of cholesterol and lipids, we compared general components of lipid and sterol between wild-type and Slfn2$^{eka/eka}$ monocytes and T cells. Cholesterol is an important component of the membrane's highly ordered ganglioside-rich platform lipid rafts. Therefore, we quantified the membrane lipid raft content in wild-type and Slfn2$^{eka/eka}$ monocytes (Fig. 2a) and T cells (Fig. 2b) using fluorescently labelled cholera toxin-B, which binds to the pentasaccharide chain of ganglioside GM1, a raft-associated lipid.[39] We observed a significant increase in the lipid rafts in the plasma membrane of Slfn2$^{eka/eka}$ monocytes (Fig. 2a) and in both CD4 and CD8 resting T cells (Fig. 2b). Additionally, we

measured lipid droplet levels in wild-type and Slfn2$^{eka/eka}$ cells. Lipid droplets provide reservoirs of lipids (such as sterols, fatty acids and phospholipids) for membrane synthesis and energy sources.[40] Flow cytometry analysis revealed dramatically increased lipid droplet levels in both Slfn2$^{eka/eka}$ monocytes (Fig. 2c) and T lymphocytes in comparison with wild-type cells (Fig. 2d). Our data demonstrate that loss of function mutation in Slfn2 leads to aberrant content of lipids and membrane sterol in T cells.

Elevated cholesterol and cholesteryl ester content in T lymphocytes from Slfn2$^{eka/eka}$ mice

Modulating intracellular levels of cholesterol can alter the membrane structure and lipid droplet content.[20,21] Therefore, it is possible that the increased content in lipid droplets and lipid rafts observed in Slfn2$^{eka/eka}$ cells is due to accumulation of intracellular levels of cholesterol. To test this hypothesis we measured cholesterol content using gas–liquid chromatography in resting wild-type and Slfn2$^{eka/eka}$ T cells isolated from the spleens. Indeed, total (Fig. 3a), free cholesterol (Fig. 3b) and cholesteryl ester (Fig. 3c) were all highly elevated in Slfn2$^{eka/eka}$ T cells. Notably, the observed increased cholesterol levels in Slfn2$^{eka/eka}$ T cells is robust in comparison to the elevation previously reported in other aberrant cholesterol accumulation phenotypes in T cells.[26]

These results demonstrate an excess of cellular cholesterol in Slfn2$^{eka/eka}$ T cells. Under such conditions the LXR pathway is expected to be activated. Therefore, we compared the expression level of the main LXR target genes in Slfn2$^{eka/eka}$ versus wild-type T cells. This analysis revealed a slightly increased expression of *SREBP1-c* in Slfn2$^{eka/eka}$ T cells with no change in the expression of *ABCA1* and *ABCG1*, which increases cholesterol efflux through the lipid transporters (Fig. 3d).

These results demonstrate that the *elektra* mutation in *Slfn2* leads to accumulation of cholesterol in T cells with only minimal activation of the compensatory, LXR pathway.

Accumulation of cholesterol in Slfn2$^{eka/eka}$ T cells is caused by elevated *de novo* synthesis

Next, we aimed to find the cause of the increased cholesterol in *elektra* T cells. Cellular cholesterol homeostasis is maintained by the balance between uptake, efflux and endogenous *de novo* synthesis.[41] Therefore, initially, as a proxy for cholesterol uptake we compared the LDL uptake by *elektra* and wild-type T cells using LDL-FITC followed by flow cytometry analysis. In comparison to wild-type cells, *elektra* CD4 and CD8 T cells show, respectively, slightly elevated or equal uptake of LDL (Fig. 4a). Next, excess cholesterol efflux measurement unexpectedly revealed no significant differences between T cells from Slfn2$^{eka/eka}$ and wild-type mice (Fig. 4b). Finally we measured the *de novo* synthesis of free cholesterol, which reflects the activity of the enzyme HMG-CoA and found a fivefold increase in Slfn2$^{eka/eka}$ T cells (Fig. 4c). In addition, we observed a 2·7-fold increase in cholesteryl ester synthesis in *elektra* cells compared with naive wild-type cells (Fig. 4d). This *de novo* synthesis is supported by elevated HMG-CoA reductase protein levels in Slfn2$^{eka/eka}$ T cells in comparison to wild-type cells (Fig. 4e). Interestingly, SREBP-2 target genes, including HMG-CoA reductase mRNA, were not elevated in *elektra* T cells (Fig. 4f), so excluding SREBP-2 activation as the cause for the elevated cholesterol *de novo* synthesis.

These results demonstrate that the *elektra* mutation in Slfn2 leads to disruption of cholesterol homeostasis in T cells, mainly through elevating the *de novo* synthesis of cholesterol with no influence on cholesterol uptake and efflux.

Discussion

Our results demonstrate that a loss of function mutation in the quiescence factor Slfn2 caused impaired cholesterol homeostasis in T cells as a consequence of induced *de novo* cholesterol synthesis and elevated protein expression of the enzyme HMGCR.

Although Slfn2$^{eka/eka}$ T cells had higher *de novo* cholesterol synthesis levels, no substantial elevation in the efflux or reduction in uptake of cholesterol was observed. In addition, lipid droplets and lipid rafts accumulated in these cells. These results suggest that there is no activation of compensatory mechanisms to oppose the higher *de novo* synthesis in Slfn2$^{eka/eka}$ T cells. Therefore, we consider that the impaired cholesterol homeostasis in Slfn2$^{eka/eka}$ T cells stems from a defect in sensing excess amounts of cholesterol or from activation of a cellular programme that sets the default cholesterol content at higher levels than in wild-type T cells.

T-cell quiescence is an actively enforced cell programme aimed at maintaining a stress-free environment to allow long-term survival while maintaining the ability to enter and progress through the cell cycle upon stimulation. Retaining this programme requires induction of cell cycle arrest and enforcement of low metabolism rate with a catabolic signature, part of which is the inhibition of sterol/lipid synthesis. Upon activation, T cells reprogramme their metabolism to meet the extraordinary demands of cell division by the induction of pathways involved in cell growth.[42] Among these anabolic pathways is the lipid/sterol biogenesis pathway, which is activated following T-cell receptor stimulation, enabling the activated cells to meet the dramatic requirements for membrane synthesis. Our current findings suggest that inhibition of cholesterol's *de novo* synthesis anabolic pathway is one of the mechanisms by which Slfn2 maintains quiescence in T cells.

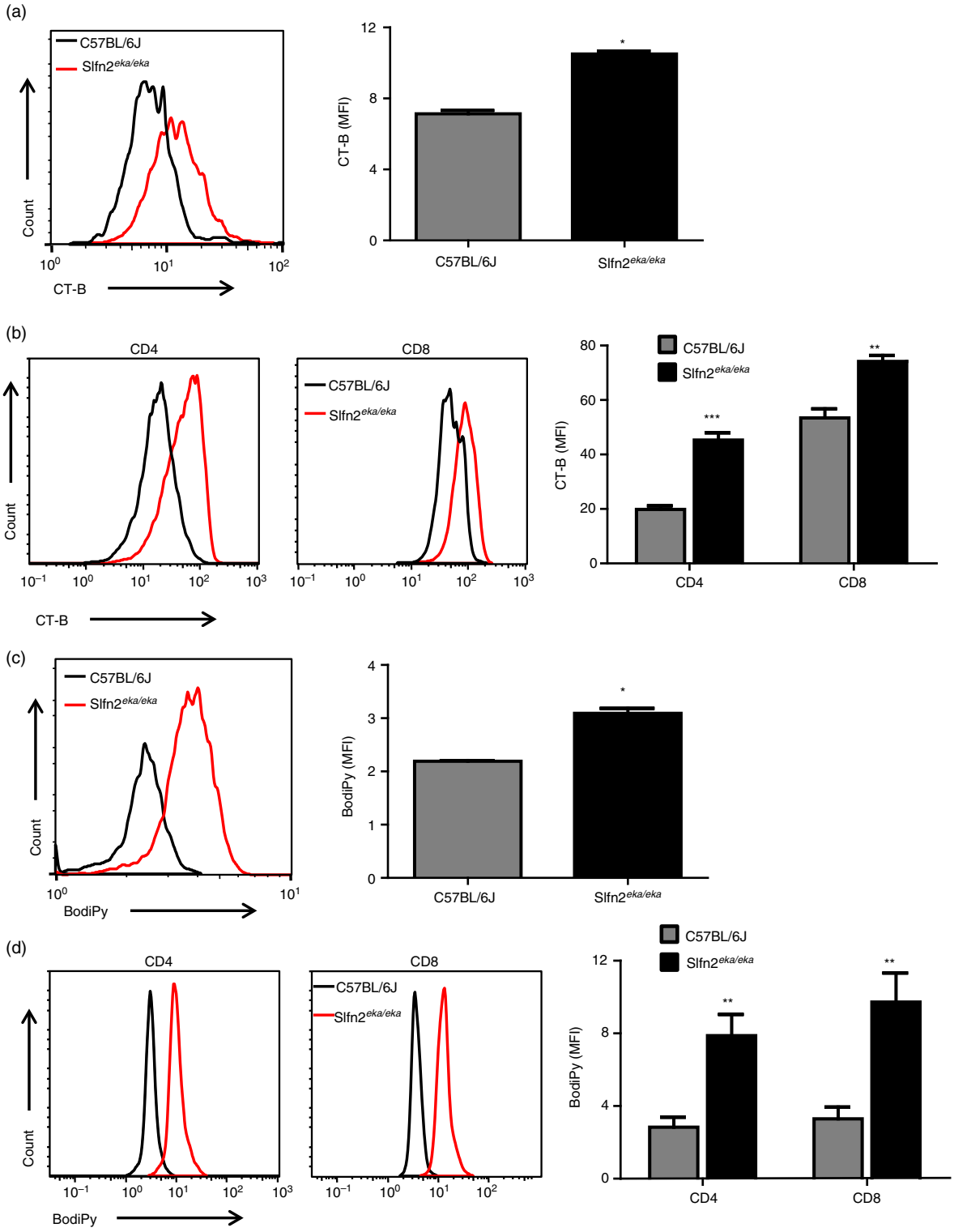

Figure 2. Elevated lipid droplets and lipid rafts in bone marrow monocytes and T cells from Slfn2$^{eka/eka}$ mice. (a, b) Lipid rafts were measured by flow cytometry using fluorescently labelled cholera toxin B subunit (CT-B). Flow cytometry analysis of the CT-B-Alexa-Fluor 488 staining of Slfn2$^{eka/eka}$ (red) and C57BL/6J (black) on bone marrow monocytes, CD11b^{+}, Ly6C^{hi} (a, left panel) and on CD4 or CD8 T cells (b, left panel). Bar graphs summarizing the flow cytometry results shown on the left (a and b, right panels, $n = 4$ mice). (c, d) Lipid droplets were measured by flow cytometry using the fluorescent dye BODIPY. Flow cytometry analysis of the BODIPY staining of Slfn2$^{eka/eka}$ (red) and C57BL/6J (black) bone marrow monocytes, CD11b^{+}, Ly6C^{hi} (c, left panel), and on CD4 or CD8 T cells (d, left panel). Bar graphs summarizing the flow cytometry results shown on the left (c and d, right panels, $n = 4$ mice). MFI, mean fluorescence intensity. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$ and ***$P < 0{\cdot}001$ (two-tailed Student's *t*-test). Error bars are SEM. [Colour figure can be viewed at wileyonlinelibrary.com]

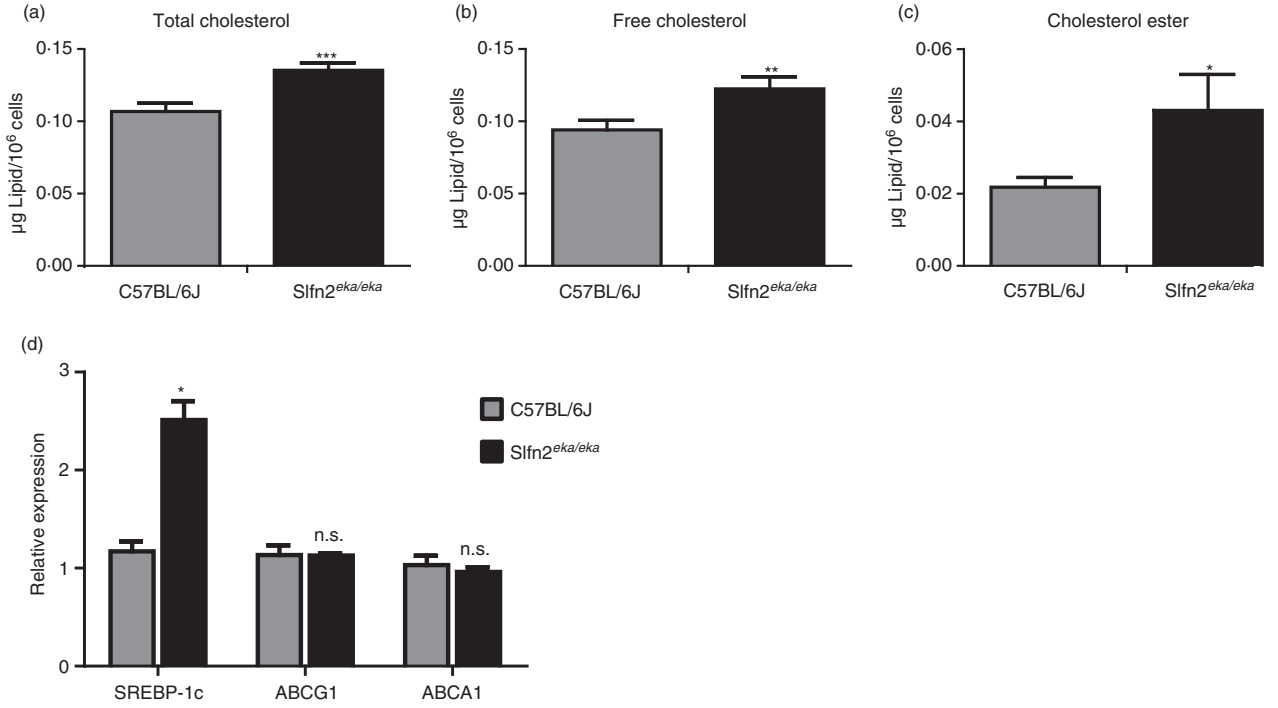

Figure 3. Elevated cholesterol and cholesteryl ester content in T lymphocytes from Slfn2$^{eka/eka}$ mice. (a) Total cholesterol, (b) free cholesterol and (c) cholesteryl ester content measured in positively selected naive T cells from Slfn2$^{eka/eka}$ (black) and C57BL/6J (grey) by liquid–gas chromatography per million cells (n = 10 mice). (d) Relative expression of LXR target genes measured in naive T cells from Slfn2$^{eka/eka}$ (black) and C57BL/6J (grey) using quantitative RT-PCR (n = 4 mice). Not significant (n.s.) > 0·05, *P < 0·05, **P < 0·01 and ***P < 0·001 (two-tailed Student's t-test). Error bars are SEM.

A recent study from our group illustrated that Slfn2$^{eka/eka}$ T cells display chronic ER stress and die as a result of ER stress-induced apoptosis.[19] The connection between ER stress and accumulation of cholesterol is well established. Tabas and colleagues showed that an excess of cellular cholesterol in macrophages induces unfolded protein response (UPR) activation and ER stress-induced apoptosis.[43] Free cholesterol enrichment of the normally cholesterol-poor ER membrane has been shown to inhibit the ER calcium pump, sarcoplasmic–endoplasmic reticulum calcium ATPase-2b (SERCA2b), resulting in depletion of ER calcium stores. This may be a critical event in the induction of UPR and free-cholesterol-induced apoptosis.[44] These studies suggest that the ER stress is a consequence of cholesterol accumulation. However, as ER is the major site for synthesis and sensing of phospholipids and sterols, it is possible that ER stress directly leads to sterol accumulation. Although UPR was initially identified as a mechanism to maintain protein homeostasis and proper folding in the ER, it has also been shown to be involved in the control of lipid/sterol homeostasis. For example, improving the ER protein folding capacity by overexpression of the chaperone glucose-regulated protein 78 (GRP78 or BiP) in the livers of the leptin-deficient, *ob/ob* mice led to reduced hepatic triglyceride and cholesterol content.[45] Furthermore, the processing enzymes site-1 protease (S1P) and site-2 protease (S2P) that cleave and activate the UPR-induced transcription factor ATF6, are also responsible for the cleavage and activation of the transcription factors SREBP-1c and SREBP-2 that regulate the synthesis of lipid and cholesterol.[46] Finally, the UPR-induced transcription factor, XBP1, was identified as a critical regulator of lipid metabolism in the liver by directly regulating lipogenic genes including Dgat2, Scd1 and Acc2.[47] Selective deletion of XBP1 in the liver caused hypocholesterolaemia and hypotriglyceridaemia, secondary to a decreased production of lipids from the liver.[47]

In lymphocytes, as opposed to macrophages, cholesterol accumulation has been shown to provide several advantages.[26,48] Primarily, isotopomer-enrichment studies showed that activation of lymphocytes results in a rapid increase in new biosynthesis of cholesterol and fatty acids.[49] In addition, pharmacological inhibition of the enzyme HMGCR by statins inhibited anti-CD3-induced T-cell mitogenesis in a dose-dependent manner.[24] Finally, Hedrick and colleagues demonstrated that robust cholesterol accumulation by genetic depletion of the cholesterol transporter, ABCG1, not only did not cause ER stress-induced apoptosis, it actually provided a proliferative advantage for naive T cells.[26] Based on these studies, we believe that the observed cholesterol accumulation in Slfn2$^{eka/eka}$ T cells is most likely secondary to the chronic

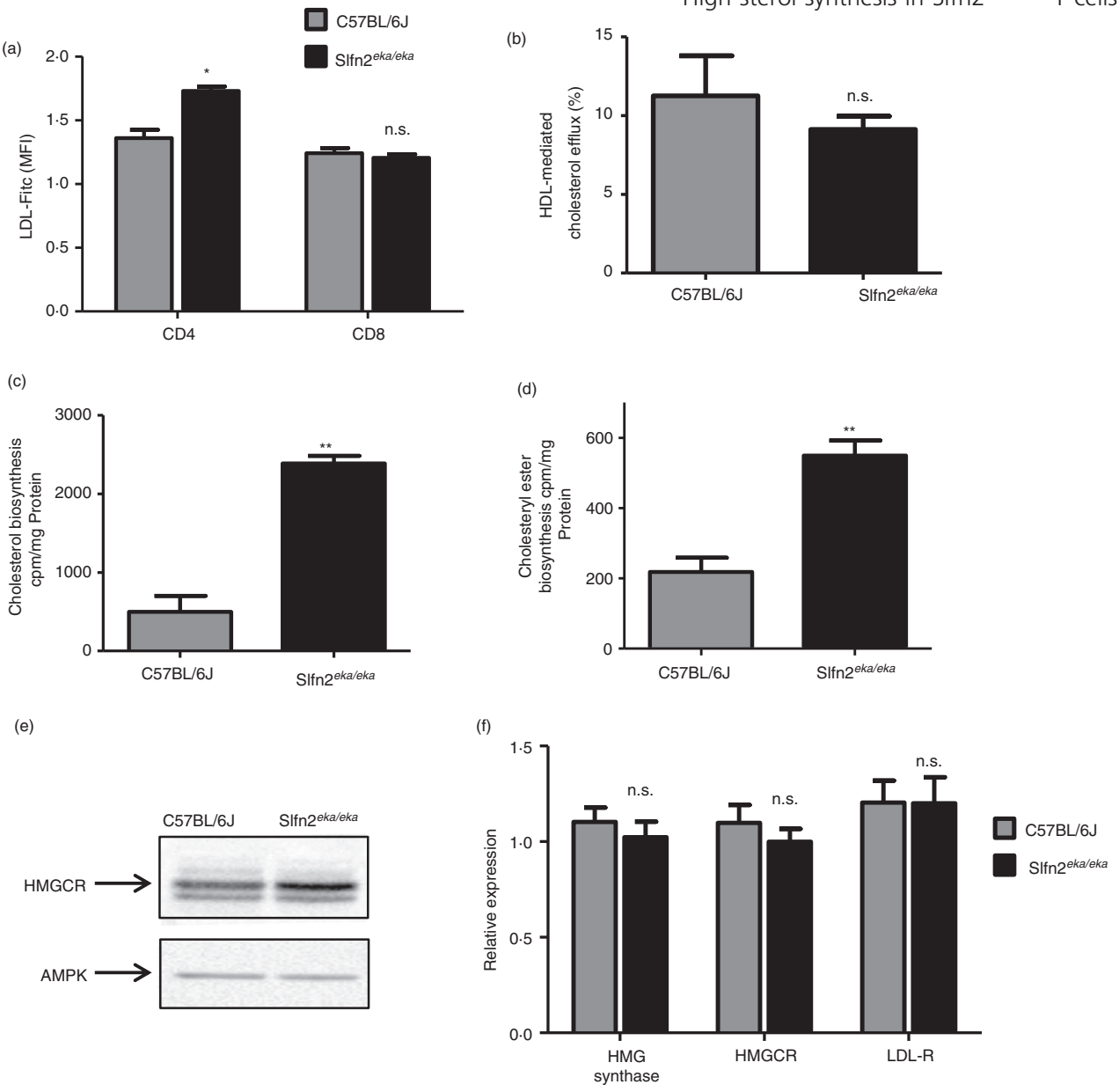

Figure 4. Accumulation of cholesterol in Slfn2$^{eka/eka}$ T cells is caused by elevated *de novo* synthesis. (a) Low-density lipoprotein (LDL) uptake was measured by incubation of splenocytes with fluorescently labelled LDL (10 μg/ml) in RPMI-1640 medium supplemented with 2% BSA for 2 hr at 37°, after surface marker staining, cells were analysed by flow cytometry. (b) Cholesterol efflux in Slfn2$^{eka/eka}$ or wild-type purified T cells. T cells were incubated with ^{3}H-labelled cholesterol (2 μCi/ml) in RPMI-1640 medium containing 0·2% BSA for 1 hr at 37° followed by a cell wash with PBS. The cells were then further incubated in the presence of high-density lipoprotein (HDL) protein (100 μg/ml) for 3 hr at 37°. Cellular and media [^{3}H] radiolabels were quantified and HDL-mediated cholesterol efflux was calculated as the ratio of [^{3}H] radiolabel in the medium to [^{3}H] radiolabel in the medium plus [^{3}H] radiolabel in cells. (c, d) Cellular cholesterol (c), and cholesteryl ester (d) biosynthesis was assayed by incubation of positively selected T cells with ^{3}H-radiolabeled acetate for 3 hr, after which cellular lipids were extracted in hexane/isopropanol, separated by thin-layer chromatography on silica gel plates and developed in hexane/ether/acetic acid (130/30/1·5; v/v/v). Cholesterol spots were visualized using iodine vapour (using an appropriate cholesterol standard), scraped into scintillation vials and counted in a β-counter. Rate of cholesterol biosynthesis was calculated as cpm per mg cellular protein. (e) Cell lysates from negatively selected Slfn2$^{eka/eka}$ and wild-type T cells were separated by SDS–PAGE and immunoblotted with the indicated antibodies. Blot is representative of three different experiments. (f) SREBP-2 target genes measured in purified T cells by quantitative RT-PCR (n = 4). Not significant (n.s.) > 0·05, *P < 0·05, **P < 0·01 (two-tailed Student's t-test). Error bars are SEM.

ER stress. Therefore, our results suggest that similar to hepatocytes, ER stress plays an important role in regulating cholesterol and lipid synthesis in T lymphocytes.

In our previous study we showed that by genetically inhibiting the ER stress response we could partially rescue the 'loss of quiescence phenotype' resulting from the loss-

of-function mutation in the quiescence factor Slfn2. In line with the current study's results, we hypothesize that one optional mechanism by which ER stress disrupts quiescence of T cells is the up-regulation of cholesterol and cholesteryl ester synthesis. Recently, it was demonstrated that activated CD8 T cells rapidly re-programme their sterol metabolism towards cholesterol accumulation through the activity of SREBP and LXR to promote cholesterol synthesis and uptake, while inhibiting efflux.[23,48] Furthermore, disrupting the activity of SREBP signalling in CD8 T cells has been shown to prevent their blasting and attenuated clonal expansion during viral infection.[48] Expression of the cholesterol esterification genes, acyl-CoA cholesterol, acyltransferase (ACAT), in particular ACAT1, is up-regulated in activated CD8 T cells.[50] ACAT1 and ACAT2 reside in the ER and convert free cholesterol to cholesteryl esters to be stored in the lipid droplets. Overall, these studies and others indicate that intracellular cholesterol levels provide a threshold for T-cell activation, a threshold that, if passed, may result in a spontaneous loss of quiescence.

Finally, our study shows that the enzyme HMGCR is up-regulated in Slfn2$^{eka/eka}$ cells. This enzyme catalyses the rate-limiting formation of mevalonate, which is the first committed precursor for cholesterol synthesis.[51] The level of HMGCR is negatively regulated by cholesterol both transcriptionally and post-translationally.[52] In Slfn2$^{eka/eka}$ cells, we observed elevation in the protein level of HMGCR with no elevation of *HMGCR* mRNA and other *SREBP2* target genes. These results may suggest that the HMGCR is controlled at the protein level by Slfn2. Therefore, it would be interesting to study the molecular mechanism by which Slfn2 deficiency causes this elevation and determine whether there is a direct interaction between Slfn2 and HMGCR. Additionally, it would be interesting to study whether the *elektra* phenotype can be reverted by inhibiting HMGCR activity by statin treatment. This will allow dissection of the overall contribution of the elevated HMGCR levels and cholesterol biosynthesis to the survival and development of *elektra* T cells.

Authorship Contributions

IO and MB conceived and designed the experiments and wrote the paper. IO, OR, LCD and AKG performed the experiments. IO, MB, OR, AKG and JSP analysed the data. MA and JSP contributed reagents/materials/analysis tools.

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Disclosures

The authors declare no conflict of interest.

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