VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

Imiquimod-Induced Psoriasis-Like Skin Lesions Do Not Accelerate Atherosclerosis in Low-Density Lipoprotein Receptor—Deficient Mice

Marie Madsen,^{*} Peter R. Hansen,^{*†} Lars B. Nielsen,^{*‡} Renata M. Cardoso,[§] Miranda van Eck,[§] and Tanja X. Pedersen^{*}

From the Department of Biomedical Sciences,^{*} University of Copenhagen, Copenhagen N, Denmark; the Department of Cardiology,[†] Herlev-Gentofte University Hospital, Hellerup, Denmark; the Department of Clinical Biochemistry,[‡] Rigshospitalet, Copenhagen University Hospital, Copenhagen Ø, Denmark; and the Cluster BioTherapeutics,[§] Leiden Academic Centre for Drug Research, Leiden University, Leiden, the Netherlands

Accepted for publication
February 13, 2018.

Address correspondence to
Tanja X. Pedersen, M.Sc., Ph.D.,
Gubra, In Vivo Pharmacology,
Hørsholm Kongevej 11B,
DK-2970 Hørsholm,
Denmark. E-mail: txp@gubra.dk.

Psoriasis is a chronic inflammatory skin disorder associated with several comorbidities, including atherosclerosis. Disease mechanisms that may affect both psoriasis and atherosclerosis include activation of T helper 1 and T helper 17 cells. Imiquimod application is an established mouse model of psoriasis-like skin inflammation. The cardiac glycoside digoxin inhibits the master transcription factor of T helper 17 differentiation, retinoid acid receptor—related orphan nuclear receptor γ t, and attenuates IL-17—dependent pathologies in mice. We investigated whether cyclic imiquimod-induced psoriasis-like skin inflammation affects atherosclerosis in low-density lipoprotein receptor—deficient mice and whether digoxin modifies either disease. Topical imiquimod application increased ear thickness, keratinocyte proliferation, and accumulation of CD3⁺ T cells in the skin of low-density lipoprotein receptor—deficient mice. Also, imiquimod affected the mice systemically with induction of splenomegaly as well as increased plasma levels of IL-17A and serum amyloid A. Overall, imiquimod reduced atherosclerosis in the aortic arch *en face*, but it did not affect atherosclerosis in the aortic root. Digoxin significantly reduced the imiquimod-induced ear thickening, had divergent effects on imiquimod-induced systemic inflammation, and did not affect atherosclerosis. In conclusion, cyclic imiquimod applications can be used for long-term induction of psoriasis-like skin lesions, but they attenuate atherosclerosis in low-density lipoprotein—deficient mice. In this model, digoxin reduces skin inflammation, but it has no effect on atherosclerosis. (*Am J Pathol* 2018, 188: 1486–1496; <https://doi.org/10.1016/j.ajpath.2018.02.005>)

Psoriasis is a chronic systemic inflammatory disease that primarily affects the skin, but it is associated with several comorbidities, including atherosclerotic disease (eg, myocardial infarction and stroke).^{1–3} A mechanistic link between psoriasis and atherosclerosis may be that the two diseases are dependent on similar immunoinflammatory pathways [eg, with activation of T helper (Th) 1 and Th17 cells and involvement of respective cytokines, including tumor necrosis factor- α and IL-17].^{3,4} Another shared pathogenic feature is the activation of endothelial cells with up-regulation of adhesion molecules (eg, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1), stimulating leukocyte migration into the psoriatic lesion or the atherosclerotic plaque.⁵ To gain further insights

into potential shared disease mechanisms, an animal model combining psoriasis-like skin inflammation and atherosclerosis would be highly valuable.

Previously, few experimental studies have demonstrated that psoriasis-like skin inflammation (eg, by transgenic dermal overexpression of IL-17) can promote vascular inflammation and thrombosis.^{6–8} However, these studies

Supported by an unrestricted grant from the LEO foundation, Laurtrupsgade 7, DK-2100 (P.R.H.)

Disclosures: None declared.

Current address of L.B.N., Faculty of Health Sciences, Aarhus University, Aarhus C, Denmark; of T.X.P., Gubra, In Vivo Pharmacology, Hørsholm, Denmark.

were performed in normocholesterolemic mice without atherosclerosis.^{6–8} The most commonly used hypercholesterolemic mouse models of atherosclerosis include apolipoprotein E-deficient (apoE^{−/−}) and low-density lipoprotein receptor-deficient (LDLr^{−/−}) mice.^{9,10} In a recent study, we found that skin inflammation induced by topical application of the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in apoE^{−/−} mice resulted in systemic inflammatory effects, but it did not affect atherogenesis.¹¹ In a similar study with LDLr^{−/−} mice, we observed a significant TPA-induced increase in the relative amount of macrophages in the aortic root (M.M., P.R.H., L.B.N., K. Hartvigsen, A. Aarup, and T.X.P., unpublished data). Skin inflammation induced by TPA resembles some, but not all, aspects of psoriatic skin inflammation. In recent years, topical application of imiquimod (IMQ), a ligand of toll-like receptor 7, has emerged as a well-established mouse model of psoriasis-like skin inflammation that is dependent on the IL-23/IL-17 axis.^{12,13} In these studies, IMQ application has primarily been used as an acute model, with typical application duration of 1 week; frequently, mice treated with daily IMQ for >2 weeks die, hitherto hindering use of this model for the study of psoriasis comorbidities (eg, atherosclerosis) that develop over longer periods of time.¹⁴

It is well established that Th17 cells secrete, for example, IL-17, and are essential players in psoriasis pathogenesis and in several other immunoinflammatory diseases (eg, rheumatoid arthritis and systemic lupus erythematosus).¹⁵ Accordingly, the IL-23/IL-17 pathway is a central target for new biologics (eg, monoclonal antibodies against IL-17) that recently have shown high efficacy in treatment of psoriasis.¹⁶ Moreover, emerging small-molecule therapeutics target the retinoic acid-related orphan nuclear receptor γ t, a transcription factor regulating differentiation of Th17 cells, and consequently reduce their IL-17 production.¹⁷ Interestingly, the cardiac glycoside digoxin, which has been used for decades in treatment of heart failure and atrial fibrillation through inhibition of the sodium-potassium pump in cardiomyocytes,¹⁸ was more recently shown to inhibit retinoic acid-related orphan nuclear receptor γ t.¹⁹ Indeed, digoxin can attenuate a range of IL-17-dependent pathologies in mice (eg, experimental autoimmune encephalomyelitis, autoimmune arthritis, and abdominal aortic aneurysms).^{19–21} In addition, we recently found that digoxin was capable of suppressing short-term IMQ-induced ear inflammation in wild-type mice.²²

In the present study, we tested whether IMQ-induced psoriasis-like skin inflammation increased atherosclerosis in hypercholesterolemic LDLr^{−/−} mice. Also, the effects of digoxin on skin inflammation and atherosclerosis were analyzed. Because atherosclerosis is a slowly progressing disease, which is unlikely to be affected by a 1-week application of IMQ, we examined whether IMQ may be applied cyclically for an extended period of time (ie, three 5-day rounds of IMQ application interspersed with pauses during a 7- to 8-week period).

Materials and Methods

Mice and Diet

Female LDLr^{−/−} mice (aged 6 to 7 weeks old) were purchased from the Jackson Laboratory (002207; B6.129S7-LdlrtmHer/J; Bar Harbor, ME), and they were housed in single-ventilated cages in a temperature- and humidity-controlled 12-hour light/dark room and given water and diet ad libitum. After 1 week of acclimatization, the mice were put on a high-cholesterol semisynthetic diet with 4% fat (wt/wt) and 0.5% cholesterol (Clinton/Cybulsky Rodent Diet, product D00083101C; Research Diets, New Brunswick, NJ). All animal experiments were performed according to the principles stated in the Danish law on animal experiments and were approved by the Animal Experiment's Inspectorate, Ministry of Justice, Denmark (permit number 2014-15-0201-00410). The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* set forth by the European Parliament (EU directive 2010/63/EU). The ethical policy of the University of Copenhagen (Copenhagen, Denmark) complies with that of the NIH (A5846-01).

Experimental Setup

At 14 to 15 weeks of age, mice received daily topical application of 7.5 mg of either 5% IMQ cream (Aldara; Meda, Solna, Sweden) or vehicle cream (Skanderborg Apotek, Skanderborg, Denmark) on both ears for 5 days. All applications were performed under anesthesia maintained at 1% isoflurane (IsoFlo vet 100%; Abbott Laboratories, Sittingbourne, UK) administered via a facemask. All mice received neutral eye ointment (paraffin oil/Vaseline; Ophtha; Tubilux Pharma, Pomexia, Italy) to avoid cream from the ears getting into the eyes. Two hours after cream application, mice were treated with i.p. 20 μ g digoxin (SAD solution for injection; Amgros, København Ø, Denmark) or saline on 3 days of each 5-day application round. The 5-day application scheme was repeated for a total of three rounds, with 2 to 3 weeks of pause in between. The mice were stratified into four groups (with or without topical IMQ and with or without i.p. digoxin; $n = 10$ to 13 per group) according to baseline plasma cholesterol levels taken 1 week before initiation of IMQ application. The study was terminated approximately 24 hours after the last IMQ application/digoxin treatment in the third application round. In all three application rounds, mice were weighed daily and ear thicknesses of both ears were measured with a digimatic thickness gauge (Mitutoyo, Kawasaki, Japan). At termination, mice were anesthetized subcutaneously with a 0.1 mL/10 g mouse dose of a mixture of tiletamine (1.63 mg/mL), zolazepam (1.63 mg/mL), xylazine (2.61 mg/mL), and butorphanol tartrate (0.065 mg/mL). Adequacy of anesthesia was checked by withdrawal reflex testing by applying forceps pressure to the paw before mice were bled out, euthanized by cervical dislocation, and perfused with ice-cold saline.

Skin Histology

Half of the right ear was fixed in 10% neutral-buffered formalin (Lillie formaldehyde solution 4%; Hounisen, Skanderborg, Denmark) for 3 days and kept in 70% ethanol until paraffin embedding. Sections (4 μm thick) were stained with Mayer’s hematoxylin and eosin (Rigshospitalet, København, Denmark), and the epidermal thickness was estimated by counting the average number of epidermal cell layers. Keratinocyte proliferation was assessed with immunohistochemical staining using an anti-Ki-67 primary antibody [rabbit monoclonal (SP6) 1:400; ab16667; Abcam, Cambridge, UK], a horseradish peroxidase-labelled secondary antibody (EnVision⁺, labeled polymer anti-rabbit kit; Dako, Glostrup, Denmark), visualization with diaminobenzidine (Dako), and counterstain with eosin. T cells were stained with anti-CD3 primary antibody [rabbit monoclonal (SP7) 1:250, RM-9107-S; Thermo Fisher Scientific, Boston, MA] and visualized with donkey anti-rabbit Alexa Flour 568 (ab175693; Abcam). The immunohistochemical staining was quantified by counting the number of epidermal Ki-67⁺ cells at five distinct locations on each tissue section or by counting the number of CD3⁺ cells in the epidermis per 700 μm as an average number of cells on the two sides of the ear. Representative images were obtained with an Axio Scan.Z1 (Zeiss, Oberkochen, Germany). The investigator (M.M.) quantifying the histochemical and immunohistochemical staining was blinded during the entire quantification process.

Atherosclerosis Quantification

The aortic arch from the heart to the seventh rib was opened longitudinally, and digital images were acquired. The amount of atherosclerosis *en face* was quantified by a blinded investigator, who measured the area of plaques relative to the area of the aortic arch (Visiomorph software version 4.6; Visiopharm, Hørsholm, Denmark). The aortic

root was fixed in formalin overnight and snap frozen in ice-cold Tissue-Tek O.C.T. (Sakura Finetek, AV Alphen aan den Rijn, the Netherlands), and sections (10 μm thick) were collected. The sections were stained biochemically for lipids with oil-red-O (Sigma-Aldrich, St. Louis, MO). Monocytes and macrophages were stained with MOMA-2 primary antibody (monoclonal rat anti-mouse macrophages/monocytes MCA519, 1:500; BioRad, Hercules, CA). Corresponding antibody isotype control was run with monoclonal rat IgG2b (MAB0061, 1:500; R&D Systems, Minneapolis, MN). For detection, a biotinylated secondary rabbit anti-rat antibody (E0468, 1:2000; Dako) and a horseradish peroxidase approach (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) were used, followed by staining with diaminobenzidine. Both stainings were counterstained with Mayer’s hematoxylin. Masson’s trichrome staining was performed according to the manufacturer’s instructions (Sigma-Aldrich). Images of sections were acquired on a light microscope (Leica Microsystems, Wetzlar, Germany) or a slide scanner (Axio Scan.Z1; Zeiss). The relative content of specific components in relation to the area of the atherosclerotic lesions on the same slides was quantified with the Visiomorph software version 4.6 (Visiopharm) or Leica Qwin Imaging software version 3.5.0 (Leica Microsystems) by a blinded investigator (M.M.). Quantification of necrotic core area in percentage of plaque area was performed on trichrome-stained sections, and necrotic core was defined as acellular regions in the atherosclerotic lesion.

RNA and Real-Time PCR

After *en face* images had been acquired, the aortic arch was snap frozen. Using a TissueLyser, the aorta was homogenized in TRIzol (Invitrogen, Nærum, UK), and RNA was precipitated with isopropanol. cDNA was made from 200 ng total RNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

Table 1 Primer Sequences

Gene name (symbol)	Primer sequences
β2-Microglobulin (<i>B2m</i>)	F: 5'-TCTCACTGACCGGCCTGTAT-3' R: 5'-TTTCAATGTGAGGCGGGTGG-3'
Vascular cell adhesion molecule 1 (<i>Vcam-1</i>)	F: 5'-CTTCATCCCCACCATTGAAG-3' R: 5'-TGAGCAGGTCAGGTTACAG-3'
Intercellular adhesion molecule 1 (<i>Icam-1</i>)	F: 5'-ATGCCGACCCAGGAGAGCACAA-3' R: 5'-TCGACGCCGCTCAGAAGAACCA-3'
Inducible nitric oxide synthase (<i>iNos</i>)	F: 5'-CACCTTGAGTTACACCCAGT-3' R: 5'-ACCACTCGTACTTGGGATGC-3'
Cluster of differentiation 206 (<i>Cd206</i>)	F: 5'-TCGCCCACCAGAGCCCA-3' R: 5'-ATGCTCGCCAGCTCTCCACCT-3'
<i>F4/80</i>	F: 5'-GCCTTCTGGATCCATTTGAA-3' R: 5'-TGCATCTAGCAATGGACAGC-3'

F and R primers used for gene expression analyses of the indicated genes.
F, forward; R, reverse.

Real-time quantitative PCR was run on a TaqMan (Applied Biosystems), with each gene measured in duplicate. cDNA (1 ng) and 0.5 μ L primers were used for each 10- μ L PCR mixture made with Fast SYBR Green Master Mix (Applied Biosystems). Quantification of mRNA expression levels was made from standard curves of serial dilutions from a pool of all cDNAs. The mRNA expressions of selected genes were normalized against the housekeeping gene β 2-microglobulin (*B2m*). All primer sequences can be found in Table 1.

Plasma Analysis

Blood samples were taken at baseline (ie, 1 week before application start in the first round from submandibular veins), midway through the study (between the second and third application round from submandibular veins), and at study termination (retro-orbital blood) in EDTA-coated tubes, and plasma was acquired by centrifugation at $1000 \times g$ for 10 minutes at 4°C. Unless otherwise specified, plasma analyses were run on termination samples as single measurements. Plasma IL-17A, serum amyloid A (SAA), and digoxin concentrations were measured with commercial ELISAs, according to the manufacturers' instructions (R&D Systems, Tridelta, Ireland; and Monobind, Lake Forest, CA). Digoxin concentrations were measured in both midway and termination samples. Plasma carbamide was measured on a Cobas autoanalyzer with commercial reagents (Roche Diagnostics, Rotkreuz, Switzerland). Total protein levels were measured with the Pierce BCA protein assay (Thermo Fisher Scientific). Plasma cholesterol was measured in duplicate at baseline, midway, and at termination with the CHOD-PAP reagent (Roche Diagnostics). Fast protein liquid chromatography was performed on plasma pools, essentially as described previously,²³ with the following exception: plasma pools from all mice in each of the four treatment groups were made, and 400 μ L of each pool was mixed with 400 μ L phosphate-buffered saline before loading on the Superose 6 column (Amersham Pharmacia Biotech, Hørsholm, Denmark). Subsequently, cholesterol and triglyceride content was measured in the fast protein liquid chromatography fractions using standard enzymatic assays in an automated Cobas analyzer (Roche Diagnostics).

Statistical Analysis

Statistics were performed with the software GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA). Data are presented as means $\pm$ SEM in all figures. Unless otherwise specified in figure legends, the numbers of mice in each group were as follows: vehicle-control and IMQ-control, $n = 13$ per group; vehicle-digoxin, $n = 11$; and IMQ-digoxin, $n = 10$. Data were analyzed with two-way analysis of variance, followed by Tukey multiple comparison test. $P < 0.05$ was considered statistically significant.

Results

IMQ Induces Psoriasis-Like Skin Lesions that Are Attenuated by Digoxin

To enable sufficient time for development of atherosclerosis during the study period, IMQ or vehicle was applied daily to both ears of $LDLr^{-/-}$ mice for 5 days, and this cycle was repeated three times with 2 to 3 weeks of pause in between (Figure 1A). IMQ progressively increased the thickness of both ears during the application periods (data not shown) (Figure 1B). These findings were corroborated by measurements of epidermal thickness on hematoxylin and eosin-stained sections at study termination (Figure 1, C and D). Further morphological characterization of the ear skin revealed signs of increased keratinocyte proliferation, as judged from Ki-67 staining (Figure 1, C and E), and increased accumulation of CD3⁺ T cells (Figure 1, C and F) in mice treated with IMQ compared with vehicle. During each 5-day application round, all mice received three i.p. injections of either digoxin or saline (control). Digoxin significantly reduced the IMQ-induced ear thickening macroscopically and microscopically (Figure 1, B and D), and it attenuated the induced keratinocyte proliferation (Figure 1E). There was no effect of digoxin on the accumulation of T cells in the epidermis (Figure 1F).

IMQ Application Increases Systemic Inflammation, Which Is Somewhat Affected by Digoxin

At study termination, there were no differences in body weight between the different groups of mice (Table 2). IMQ application increased the relative weight of the spleen, and this effect was significantly attenuated by digoxin (Table 2). Plasma concentrations of IL-17A were increased in mice with topical IMQ and were unaffected by digoxin injections (Table 2). The acute-phase reactant SAA was induced by both IMQ application and digoxin treatment, and there was a strong additive effect of IMQ and digoxin combined on plasma SAA concentrations (Table 2). Approximately 24 hours after the last i.p. injection, mice that had received digoxin had means $\pm$ SEM plasma digoxin concentration of 24.6 ± 2.4 ng/mL. More important, plasma digoxin was not detectable in midway samples taken 10 days after the last digoxin injection in application round 2. Plasma concentrations of carbamide and total protein suggested that neither IMQ nor digoxin had affected the kidney function or the hydration status of the mice (Table 2). At study termination, plasma total cholesterol concentrations were higher in mice treated with digoxin compared with control mice, and, interestingly, IMQ attenuated the digoxin-induced increase in plasma cholesterol levels (Figure 2A). More important, fast protein liquid chromatography analyses of pooled plasma at study termination showed no effect of IMQ or digoxin on the content and distribution of cholesterol- or triglyceride-containing lipoproteins (Figure 2B and

Table 2 Effects of Imiquimod and Digoxin on Body Weight, Spleen Weights, and Plasma Concentrations at Study Termination of IL-17A, Serum Amyloid A, Total Protein, and Carbamide

Criterion	Veh	IMQ
BW, g ($n = 47$)		
Control	21.19 $\pm$ 1.53	21.16 $\pm$ 1.38
Digoxin	20.84 $\pm$ 1.37	20.34 $\pm$ 1.88
Spleen weight, mg/g BW ($n = 47$)		
Control	4.6 $\pm$ 1.1	14.6 $\pm$ 3.5*
Digoxin	4.9 $\pm$ 1.6	8.1 $\pm$ 1.3*†
P-IL-17A, pg/mL ($n = 45$)		
Control	4.87 $\pm$ 2.64	24.65 $\pm$ 7.80*
Digoxin	6.43 $\pm$ 1.63	32.43 $\pm$ 16.21*
P-SAA, μ g/mL ($n = 46$)		
Control	16.77 $\pm$ 5.77	314.03 $\pm$ 359.38
Digoxin	175.40 $\pm$ 275.69	1160.34 $\pm$ 746.87*††
P-total protein, μ g/mL ($n = 47$)		
Control	4.92 $\pm$ 0.28	5.13 $\pm$ 0.56
Digoxin	5.13 $\pm$ 0.38	5.12 $\pm$ 0.42
P-carbamide, mmol/L ($n = 47$)		
Control	8.5 $\pm$ 1.22	7.8 $\pm$ 1.3
Digoxin	8.3 $\pm$ 1.8	9.8 $\pm$ 2.8

Where n was <47 , technical challenges led to exclusion of samples. Data are expressed as means $\pm$ SD. Statistics were performed with two-way analysis of variance using Tukey multiple comparison.

* $P < 0.0001$ Veh-control versus IMQ-control or Veh-digoxin versus IMQ-digoxin.

† $P = 0.01$, †† $P < 0.001$ Veh-control versus Veh-digoxin or IMQ-control versus IMQ-digoxin.

BW, body weight; IMQ, imiquimod; P, plasma; SAA, serum amyloid A; Veh, vehicle.

Supplemental Figure S1). There were no differences in plasma total cholesterol concentrations between the different mouse groups, neither at baseline nor in plasma samples taken in the pause between the second and last IMQ application round (Figure 2A).

IMQ Attenuates Atherosclerosis in the Aortic Arch, but Not in the Aortic Root, and There Are No Effects of Digoxin on Atherogenesis

Contrary to our hypothesis, data indicated that IMQ overall reduced ($P = 0.01$, two-way analysis of variance) rather than increased the *en face* area of atherosclerotic lesions in the aortic arch, although the effect was not statistically

significant when comparing the individual groups with Tukey multiple comparison test ($P = 0.10$, IMQ-control vs IMQ-digoxin) (Figure 2C). This finding was supported by reduced mRNA expression of the proinflammatory genes *Vcam1* and inducible nitric oxide synthase (*iNos*) in the aortic arch of IMQ- compared with vehicle-treated mice (Figure 2D). In contrast, IMQ increased mRNA expression of the macrophage marker *F4/80* and did not affect the expression of the proinflammatory gene *Icam1* or the macrophage marker *Cd206* compared with vehicle mice (Figure 2D). Apart from *F4/80*, for which mRNA expression was reduced in digoxin versus control IMQ mice, there were no effects of digoxin on gene expression in the aortic arch (Figure 2D). Likewise, digoxin did not affect atherosclerosis in the aortic arch *en face* (Figure 2C). We further quantified atherosclerotic plaque size and composition by histochemical staining of aortic root cross sections. In the aortic root, we found no effects of IMQ or digoxin on atherosclerotic plaque size (oil-red-O staining), on the relative amount of MOMA-2⁺ macrophages, or on the necrotic core area (Figure 3, A–C and F). As determined by Masson's trichrome staining, IMQ application increased the relative amount of collagen and concordantly reduced the amount of nonspecified cells in the aortic root plaque (Figure 3, D–F). This effect was neutralized with digoxin treatment (Figure 3, D and E). Digoxin did not otherwise affect plaque composition or size (Figure 3, A–E).

Discussion

Epidemiological data have associated psoriasis with cardiovascular disease independent of traditional risk factors, although the association has not been confirmed in all studies.^{1–3,24,25} An animal model that recapitulates key characteristics of human psoriasis, while on an atherosclerosis-prone background, would be highly valuable to study both potential shared disease mechanisms and effects of treatment on both diseases. In the present study, long-term cyclic IMQ application induced psoriasis-like skin inflammation and led to significant systemic inflammation in hypercholesterolemic LDLr^{−/−} mice. Surprisingly, the data suggested that topical IMQ somewhat inhibited, rather than promoted, atherosclerosis. Treatment with digoxin reduced some of the IMQ-induced effects on

Figure 1 Digoxin delays imiquimod (IMQ)—induced skin inflammation in low-density lipoprotein receptor—deficient mouse ears. **A:** Timeline detailing the experimental setup in the study. All mice received a high-cholesterol (HC) diet starting 7 weeks before the first IMQ application and until study termination. In each of the three experimental rounds (round 1 to 3), all mice received 5 consecutive days of topical application of either IMQ or vehicle (Veh) and simultaneously three i.p. injections of digoxin. **B:** Ear thickness (mm). **Vertical dotted lines** indicate pauses between the three IMQ application rounds. P values are shown only for IMQ-control versus IMQ-digoxin. **C:** Representative images of ear cross sections: hematoxylin and eosin (HE; epidermal layer delineated with **brackets**), proliferation (Ki-67; brown), and T cells (CD3, red fluorophore; **white dashed lines** delineate epidermis). **D:** Epidermal thickness (HE, number of cell layers). **E:** Keratinocyte proliferation (Ki-67⁺, number of cells per mm basal membrane). **F:** Epidermal T-cell infiltration (CD3⁺, number of cells per 700 μ m). **Horizontal lines** indicate means (**D–F**). Two-way analysis of variance (Tukey multiple comparison test) was used. Data are expressed as means $\pm$ SD (**B**). $n = 47$ (**A–F**). ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ versus IMQ-digoxin; † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$, and †††† $P < 0.0001$. Scale bars: 500 μ m (**C**, HE and Ki-67, **left column**); 50 μ m (**C**, HE and Ki-67, **right column**, and CD3). + and −, days with or without, respectively, topical IMQ/Veh and i.p. digoxin/saline.

Figure 2 Imiquimod (IMQ) does not accelerate atherosclerosis or increase mRNA expression of inflammatory genes in the aorta. **A:** Total plasma cholesterol (mmol/L) measured at baseline, midway, and at termination [vehicle (Veh)]. **B:** Cholesterol gel filtration profiles of pools of plasma samples at study termination for each of the four groups of mice. **C:** Amount of atherosclerosis in the aorta *en face* on unstained tissue presented as percentage plaque area relative to the entire aortic arch area. Horizontal lines indicate means. Representative images are shown from control Veh and IMQ mice. **D:** Aortic arch mRNA expression of five selected genes [ie, vascular cell adhesion protein-1 (*Vcam1*), intercellular adhesion molecule-1 (*Icam1*), inducible nitric oxide synthase (*iNos*), *Cd206*, and *F4/80*], as determined by real-time quantitative PCR. The expression of each gene was normalized to $\beta 2$ -microglobulin (*B2m*) (seven mice omitted from analyses because too little RNA amount was extracted from these samples). Statistics were performed with two-way analysis of variance (Tukey multiple comparison test). Data are expressed as means $\pm$ SD (**A**); data are expressed as means $\pm$ SEM (**D**). $n = 47$ (**C**); $n = 40$ (**D**). * $P < 0.05$, *** $P < 0.001$, and **** $P < 0.0001$. AU, arbitrary unit.

the ear skin, and it either attenuated (reduced spleen weight) or increased (augmented plasma SAA concentrations) systemic effects of IMQ. Irrespective of these effects, treatment with digoxin did not affect atherosclerosis in the present study aside from an interaction with IMQ-induced increase in aortic root collagen content, neither in mice applied with vehicle nor in mice applied with IMQ.

Only a few studies have previously investigated the effects of psoriasis on the vasculature.^{6–8} These studies showed direct effects of skin inflammation on vascular pathology, including endothelial dysfunction, vascular inflammation, and thrombosis, but they were all performed in mice models that did not develop atherosclerosis.^{6–8} We, and others, have previously investigated the effect of topical application of either TPA or croton oil (from which TPA is derived) on atherosclerosis in apoE^{−/−} mice and found no effect of skin inflammation induced by TPA/croton oil on atherosclerosis.^{11,26} Although TPA/croton oil is used for induction of acute or chronic dermatitis, the IMQ model is recognized as a more appropriate model for induction of psoriasis-like skin lesions, although it does not reflect all aspects of human psoriasis.^{12,27} Furthermore, the IMQ-induced phenotype is strain dependent, and interestingly, it was recently concluded in an RNA-sequencing expression profiling that the C57Bl/6 strain seems to best replicate the human disease.²⁸ The LDLr^{−/−} mice used in the present study were on the C57Bl/6 background, suggesting that these mice had the best possible genetic makeup to allow for reflection of human psoriasis in the IMQ model. The lack of effects of IMQ on atherosclerosis in the present study suggests that the combination of topical IMQ application and hypercholesterolemia associated with LDL receptor deficiency does not represent a feasible mouse model to study shared disease mechanisms in psoriasis and atherosclerosis. IMQ stimulates toll-like receptor 7, and functional inactivation of toll-like receptor 7 in apoE^{−/−} mice appears to accelerate atherosclerosis.²⁹ Furthermore, stimulation of human atheroma cultures with IMQ may suppress production of key proatherogenic factors (eg, monocyte chemoattractant protein-1).²⁹ In addition, it was recently shown that the IMQ model is dependent on unintended IMQ ingestion owing to the grooming behaviors of mice.³⁰ If topically applied IMQ was ingested and entered the circulation and affected the vasculature directly, this might hypothetically have led to the inhibitory effect of IMQ on atherogenesis observed in our study. Interestingly, in a recent study with apoE^{−/−} mice topically applied with IMQ, the authors found that in mice receiving IMQ, total cholesterol levels in plasma were decreased.³¹ In comparison, it was found that IMQ application was not in itself associated with decreased plasma cholesterol (vehicle-control vs IMQ-control). These results emphasize the potential for strain-dependent effects of IMQ.²⁸

Atherosclerosis is a cholesterol-driven disease that is believed to be accelerated by increased systemic inflammation.⁴ Although IMQ applications led to clear systemic

effects, as measured by increased plasma SAA and IL-17A concentrations as well as marked splenomegaly, these inflammatory changes did not promote atherosclerosis in the present study. Downstream of toll-like receptor 7, induction of skin inflammation with IMQ is dependent on the IL-23/IL-17 axis.¹² There are conflicting results as to whether IL-17 acts as a proatherogenic or an antiatherogenic mediator, and findings in hypercholesterolemic mice models are not consistent.³² In view of the current sum of evidence, the present study suggests that immune components perturbed by IMQ application predominantly induce antiatherogenic effects. It is a limitation to the present study that mice received a high-cholesterol diet to promote plaque formation before IMQ application and digoxin treatment. It cannot be excluded that the vascular response to IMQ and/or digoxin would have been different if either of these was initiated before or simultaneously with the high-cholesterol diet. Furthermore, aortic root vascular smooth muscle cells were not examined and, thus, the effects of IMQ and digoxin on this central cellular determinant of plaque biology in the current model clearly require further study.

Psoriasis is a chronic disease, and the lack of chronicity in the IMQ model has hitherto been a major limitation for studies of psoriasis comorbidities.¹⁴ In the present study, the progressive increase in ear skin thickness after repeated cycles of IMQ application opens up for use of IMQ to induce chronic psoriasis-like skin inflammation. Indeed, while our study was ongoing, it was reported that daily IMQ applications for 2 weeks led to skin lesions resembling more closely the chronic or stable phase of human psoriasis.³³ A potential drawback of such a prolonged exposure to IMQ is the induction of adverse systemic effects. For example, extended topical IMQ application has been described to represent a model of systemic lupus erythematosus-like disease, in which application of IMQ three times per week generated systemic lupus erythematosus symptoms starting only 4 weeks after study initiation.³⁴ In that study, IMQ application led to renal dysfunction, with increased serum creatinine levels, proteinuria, and pathological changes in the kidney. In our study, plasma carbamide concentrations suggested no effects on kidney function. Collectively, our findings, together with the aforementioned data,³³ therefore suggest that with use of specific application regimens, IMQ may be used as a more chronic psoriasis model. However, long-term topical IMQ may require that applications are directed to the ears alone because hair on the back skin of some mice strains may grow out in patches because of hair follicles being in different phases, which markedly hampers the practical feasibility to chronically apply IMQ to the back skin of such mice.³⁵

Digoxin is primarily known as a digitalis drug that inhibits the cardiac sodium-potassium adenosine triphosphatase, but several studies have shown that digoxin inhibits retinoic acid-related orphan nuclear receptor γ t function and thereby suppresses Th17 differentiation and IL-17 production.^{19,21} In line with these effects, IMQ-induced

Figure 3 Minor effects of imiquimod (IMQ) and digoxin on atherosclerotic plaque morphology in the aortic root. **A:** Atherosclerotic plaque area (mm²) measured on oil-red-O (ORO)—stained sections of the aortic root (two mice omitted from analyses because of technical challenges). **B:** Macrophage infiltration determined by staining for the monocyte and macrophage marker MOMA-2 (brown) and quantified as percentage of plaque area (one mouse omitted because of fold in section). **C–E:** Relative amounts (percentage) of necrotic/acellular area (**C**), collagen (blue; **D**), and cytoplasm/muscle representing unspecified cells (red; **E**) of plaque area in the aortic root stained with Masson's trichrome. Horizontal lines indicate means (**A–E**). **F:** Representative images of aortic root sections stained for lipids (ORO), macrophages (MOMA-2), or collagen (trichrome). Statistics were performed with two-way analysis of variance (Tukey multiple comparison test). $n = 45$ (**A**); $n = 46$ (**B**); $n = 47$ (**C–E**). * $P < 0.05$, ** $P < 0.01$. Scale bars = 500 μ m (**F**). Veh, vehicle.

skin inflammation was reduced by digoxin in the present study, whereas there was no effect of digoxin on atherosclerosis. This is in contrast to a recent study, in which digoxin attenuated atherosclerosis progression in apoE^{-/-} mice.³⁶ This discrepancy may be caused, in part, by the use of different atherosclerosis models that may respond dissimilarly to digoxin (ie, apoE^{-/-} mice in their study versus LDLr^{-/-} mice in our study). Notably, because total plasma cholesterol in nonfasted mice was measured, it is unclear whether the increase in plasma cholesterol levels at study termination in mice treated with digoxin reflects a direct effect of digoxin on plasma cholesterol or rather an effect on the eating behavior of the mice, albeit that the body weight of animals was similar across the groups. Furthermore, in our study, the LDLr^{-/-} mice received digoxin injections at a dose of 20 µg per mouse in three cycles of three times per week, with 2 to 3 weeks of treatment pause in between, whereas Shi et al³⁶ injected approximately 20 or 40 µg per mouse daily for 12 weeks. It is possible that the more lengthy and consistent exposure to digoxin during the entire study period resulted in the anti-atherogenic effect observed by Shi et al,³⁶ including decreased plasma cholesterol levels, compared with the effects of cyclic exposure to digoxin over shorter treatment periods in our study. Also, despite that, Shi et al³⁶ used daily injections of the same or a higher dose of digoxin than were used in this study. Plasma concentrations of digoxin 24 hours after the last injection in the LDLr^{-/-} mice in this study were up to 22 times higher than those found in their study with apoE^{-/-} mice, possibly suggesting an impairment of digoxin excretion in LDLr^{-/-} mice. Digoxin is excreted primarily in a nonmetabolized form by the kidneys, with estimated half-lives of approximately 36 to 48 hours in humans and 3 to 6 hours in normocholesterolemic Swiss white and BALB/c mice, respectively.^{37–39} Mice are more resistant to digoxin toxicity than humans,⁴⁰ and the present observations, including analyses of plasma carbamide levels, comparable weight status, and absence of considerable mortality, suggest that there were no marked toxic effects of digoxin in the LDLr^{-/-} mice.

In conclusion, we have shown that induction of psoriasis-like skin inflammation by prolonged cyclic IMQ application is feasible, but it attenuates atherosclerosis in hypercholesterolemic LDLr^{-/-} mice. Digoxin decreased key features of the IMQ-induced skin inflammation, but it had no effect on atherosclerosis in this model. Thus, a mouse model that supports a causal link between psoriasis and atherosclerosis remains to be defined.

Acknowledgments

We thank Claus Johansen for kindly providing the vehicle cream; Birgitte S. Nielsen, Anne-Sofie Østergaard Gadsbøll, and Christina Christoffersen for excellent handling of the mice; Charlotte Wandel for technical assistance;

Heidi M. Paulsen for histological processing; and the Core Facility for Integrated Microscopy, Faculty of Health and Medical Sciences, University of Copenhagen.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.02.005>.

References

- Gelfand JM, Neimann AL, Shin DB, Wang X, Margolis DJ, Troxel AB: Risk of myocardial infarction in patients with psoriasis. *JAMA* 2006, 296:1735–1741
- Ahlehoff O, Gislason GH, Jørgensen CH, Lindhardsen J, Charlott M, Olesen JB, Abildstrøm SZ, Skov L, Torp-Pedersen C, Hansen PR: Psoriasis and risk of atrial fibrillation and ischaemic stroke: a Danish Nationwide Cohort Study. *Eur Heart J* 2012, 33:2054–2064
- Armstrong EJ, Krueger JG: Lipoprotein metabolism and inflammation in patients with psoriasis. *Am J Cardiol* 2016, 118:603–609
- Libby P: Inflammation in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2012, 32:2045–2051
- Armstrong AW, Voyles SV, Armstrong EJ, Fuller EN, Rutledge JC: A tale of two plaques: convergent mechanisms of T-cell-mediated inflammation in psoriasis and atherosclerosis. *Exp Dermatol* 2011, 20:544–549
- Wang Y, Gao H, Loyd CM, Fu W, Diaconu D, Liu S, Cooper KD, McCormick TS, Simon DI, Ward NL: Chronic skin-specific inflammation promotes vascular inflammation and thrombosis. *J Invest Dermatol* 2012, 132:2067–2075
- Karbach S, Croxford AL, Oelze M, Schüler R, Minwegen D, Wegner J, Koukes L, Yogev N, Nikolaev A, Reißig S, Ullmann A, Knorr M, Waldner M, Neurath MF, Li H, Wu Z, Brochhausen C, Scheller J, Rose-John S, Piotrowski C, Bechmann I, Radsak M, Wild P, Daiber A, von Stebut E, Wenzel P, Waisman A, Münzel T: Interleukin 17 drives vascular inflammation, endothelial dysfunction, and arterial hypertension in psoriasis-like skin disease. *Arterioscler Thromb Vasc Biol* 2014, 34:2658–2668
- Golden JB, Wang Y, Fritz Y, Diaconu D, Zhang X, Debanne SM, Simon DI, McCormick TS, Ward NL: Chronic, not acute, skin-specific inflammation promotes thrombosis in psoriasis murine models. *J Transl Med* 2015, 13:382
- Zhang SH, Reddick RL, Piedrahita JA, Maeda N: Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 1992, 258:468–471
- Ishibashi S, Brown MS, Goldstein JL, Gerard RD, Hammer RE, Herz J: Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J Clin Invest* 1993, 92:883–893
- Madsen M, Hansen PR, Nielsen LB, Hartvigsen K, Pedersen AE, Christensen JP, Aarup A, Pedersen TX: Effect of 12-O-tetradecanoylphorbol-13-acetate-induced psoriasis-like skin lesions on systemic inflammation and atherosclerosis in hypercholesterolaemic apolipoprotein E deficient mice. *BMC Dermatol* 2016, 16:9
- van der Fits L, Mourits S, Voerman JS, Kant M, Boon L, Laman JD, Cornelissen F, Mus A-M, Florencia E, Prens EP, Lubberts E: Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *J Immunol* 2009, 182:5836–5845
- Flutter B, Nestle FO: TLRs to cytokines: mechanistic insights from the imiquimod mouse model of psoriasis. *Eur J Immunol* 2013, 43: 3138–3146
- Hawkes JE, Gudjonsson JE, Ward NL: The snowballing literature on imiquimod-induced skin inflammation in mice: a critical appraisal. *J Invest Dermatol* 2017, 137:1–4

15. Zhu S, Qian Y: IL-17/IL-17 receptor system in autoimmune disease: mechanisms and therapeutic potential. *Clin Sci (Lond)* 2012, 122: 487–511
16. Kim J, Krueger JG: Highly effective new treatments for psoriasis target the IL-23/type 17 T cell autoimmune axis. *Annu Rev Med* 2017, 68: 255–269
17. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelletier A, Lafaille JJ, Cua DJ, Littman DR: The orphan nuclear receptor ROR- γ directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 2006, 126:1121–1133
18. Pincus M: Management of digoxin toxicity. *Aust Prescr* 2016, 39: 18–20
19. Huh JR, Leung MWL, Huang P, Ryan DA, Krout MR, Malapaka RR, Chow J, Manel N, Ciofani M, Kim SV, Cuesta A, Santori FR, Lafaille JJ, Xu HE, Gin DY, Rastinejad F, Littman DR: Digoxin and its derivatives suppress TH17 cell differentiation by antagonizing ROR γ t activity. *Nature* 2011, 472:486–490
20. Wei Z, Wang Y, Zhang K, Liao Y, Ye P, Wu J, Wang Y, Li F, Yao Y, Zhou Y, Liu J: Inhibiting the Th17/IL-17A-related inflammatory responses with digoxin confers protection against experimental abdominal aortic aneurysm. *Arterioscler Thromb Vasc Biol* 2014, 34: 2429–2438
21. Lee J, Baek S, Lee J, Lee J, Lee DG, Park MK, Cho ML, Park SH, Kwok SK: Digoxin ameliorates autoimmune arthritis via suppression of Th17 differentiation. *Int Immunopharmacol* 2015, 26:103–111
22. Madsen M, Pedersen TX, Nielsen LB, Johansen C, Hansen PR: Differential effects of digoxin on imiquimod-induced psoriasis-like skin inflammation on the ear and back. *Ann Dermatol* 2017, (in press)
23. Bosteen MH, Svarrer EMM, Bisgaard LS, Martinussen T, Madsen M, Nielsen LB, Christoffersen C, Pedersen TX: Effects of apolipoprotein M in uremic atherosclerosis. *Atherosclerosis* 2017, 265:93–101
24. Wakke M, Herings RMC, Nijsten T: Psoriasis may not be an independent risk factor for acute ischemic heart disease hospitalizations: results of a large population-based Dutch cohort. *J Invest Dermatol* 2010, 130:962–967
25. Stern RS, Huibregtse A: Very severe psoriasis is associated with increased noncardiovascular mortality but not with increased cardiovascular risk. *J Invest Dermatol* 2011, 131:1159–1166
26. Ko KWS, Corry DB, Brayton CF, Paul A, Chan L: Extravascular inflammation does not increase atherosclerosis in apoE-deficient mice. *Biochem Biophys Res Commun* 2009, 384:93–99
27. Petersen TK: In vivo pharmacological disease models for psoriasis and atopic dermatitis in drug discovery. *Basic Clin Pharmacol Toxicol* 2006, 99:104–115
28. Swindell WR, Michaels KA, Sutter AJ, Diaconu D, Fritz Y, Xing X, Sarkar MK, Liang Y, Tsoi A, Gudjonsson JE, Ward NL: Imiquimod has strain-dependent effects in mice and does not uniquely model human psoriasis. *Genome Med* 2017, 9:24
29. Salagianni M, Galani IE, Lundberg AM, Davos CH, Varela A, Gavril A, Lyytikäinen LP, Lehtimäki T, Sigala F, Folkersen L, Gorgoulis V, Lenglet S, Montecucco F, Mach F, Hedin U, Hansson GK, Monaco C, Andreaskos E: Toll-like receptor 7 protects from atherosclerosis by constraining inflammatory macrophage activation. *Circulation* 2012, 126:952–962
30. Grine L, Steeland S, Van Ryckeghem S, Ballegeer M, Lienenklaus S, Weiss S, Sanders NN, Vandenbroucke RE, Libert C: Topical imiquimod yields systemic effects due to unintended oral uptake. *Sci Rep* 2016, 6:20134
31. Xie X, Zhang L, Lin Y, Wang Y, Liu W, Li X, Li P: Imiquimod induced ApoE-deficient mice might be a composite animal model for the study of psoriasis and dyslipidaemia comorbidity. *J Dermatol Sci* 2017, 88:20–28
32. Taleb S, Tedgui A, Mallat Z: IL-17 and Th17 cells in atherosclerosis: subtle and contextual roles. *Arterioscler Thromb Vasc Biol* 2014, 35: 1–8
33. Terhorst D, Chelbi R, Wohn C, Malosse C, Tamoutounour S, Jorquera A, Bajenoff M, Dalod M, Malissen B, Henri S: Dynamics and transcriptomics of skin dendritic cells and macrophages in an imiquimod-induced, biphasic mouse model of psoriasis. *J Immunol* 2015, 195:4953–4961
34. Yokogawa M, Takaishi M, Nakajima K, Kamijima R, Fujimoto C, Kataoka S, Terada Y, Sano S: Epicutaneous application of toll-like receptor 7 agonists leads to systemic autoimmunity in wild-type mice: a new model of systemic lupus erythematosus. *Arthritis Rheumatol* 2014, 66:694–706
35. Plikus MV, Chuong C-M: Complex hair cycle domain patterns and regenerative hair waves in living rodents. *J Invest Dermatol* 2008, 128: 1071–1080
36. Shi H, Mao X, Zhong Y, Liu Y, Zhao X, Yu K, Zhu R, Wei Y, Zhu J, Sun H, Mao Y, Zeng Q: Digoxin reduces atherosclerosis in apolipoprotein E-deficient mice. *Br J Pharmacol* 2016, 173:1517–1528
37. Eichhorn EJ, Gheorghiade M: Digoxin. *Prog Cardiovasc Dis* 2002, 44: 251–266
38. Griffiths NM, Hewick DS, Stevenson IH: The effect of immunization with digoxin-specific antibodies on digoxin disposition in the mouse. *Biochem Pharmacol* 1984, 33:3041–3046
39. Kong L-L, Zhuang X-M, Yang H-Y, Yuan M, Xu L, Li H: Inhibition of P-glycoprotein gene expression and function enhances triptolide-induced hepatotoxicity in mice. *Sci Rep* 2015, 5:11747
40. Schinkel AH, Wagenaar E, Van Deemter L, Mol CA, Borst P: Absence of the *mdr1a* P-glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J Clin Invest* 1995, 96:1698–1705