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

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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 γt, 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 receptor–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)
were performed in normocholesterolemic mice without atherosclerosis.\textsuperscript{6–8} The most commonly used hypercholesterolemic mouse models of atherosclerosis include apolipoprotein E−deficient (apoE\textsuperscript{−/−}) and low-density lipoprotein receptor−deficient (LDLr\textsuperscript{−/−}) mice.\textsuperscript{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\textsuperscript{−/−} mice resulted in systemic inflammatory effects, but it did not affect atherogenesis.\textsuperscript{11} In a similar study with LDLr\textsuperscript{−/−} 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.\textsuperscript{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.\textsuperscript{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).\textsuperscript{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.\textsuperscript{16} Moreover, emerging small-molecule therapeutics target the retinoic acid−related orphan nuclear receptor γ\textsubscript{T}, a transcription factor regulating differentiation of Th17 cells, and consequently reduce their IL-17 production.\textsuperscript{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,\textsuperscript{18} was more recently shown to inhibit retinoic acid−related orphan nuclear receptor γ\textsubscript{T}.\textsuperscript{19} Indeed, digoxin can attenuate a range of IL-17−dependent pathologies in mice (eg, experimental autoimmune encephalomyelitis, autoimmune arthritis, and abdominal aortic aneurysms).\textsuperscript{19–21} In addition, we recently found that digoxin was capable of suppressing short-term IMQ-induced ear inflammation in wild-type mice.\textsuperscript{22}

In the present study, we tested whether IMQ-induced psoriasis-like skin inflammation increased atherosclerosis in hypercholesterolemic LDLr\textsuperscript{−/−} 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\textsuperscript{−/−} 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/Cybulsy 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 (parafin 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 μg 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/macrophages 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

<table>
<thead>
<tr>
<th>Gene name (symbol)</th>
<th>Primer sequences</th>
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| β2-Microglobulin (B2m) | F: 5’-TTCCTACTGCCGCGCTGTAT-3’  
R: 5’-TTTCCAGTGGGCGGAGTGG-3’ |
| Vascular cell adhesion molecule 1 (Vcam-1) | F: 5’-CTTCTATCCCCACCACTTGAG-3’  
R: 5’-TGAGCAGGTCAATTCACAG-3’ |
| Intercellular adhesion molecule 1 (Icam-1) | F: 5’-ATGCCGACCCAGGACCAAC-3’  
R: 5’-TCGACGCCGCTCAGAAGAACCA-3’ |
| Inducible nitric oxide synthase (iNos) | F: 5’-CACCCTTGGAGTTCACCCCATG-3’  
R: 5’-ACACCTTGCTATTGGGTGAC-3’ |
| Cluster of differentiation 206 (Cd206) | F: 5’-TGGGCCCCACACAGGACCCA-3’  
R: 5’-ATGCTTGCCAGCTTGCCACT-3’ |
| F4/80 | F: 5’-GCTCTTGATCCATTTGAA-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 µL primers were used for each 10-µL 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 β2-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 × 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 µL of each pool was mixed with 400 µL 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 ± 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<sup>−/−</sup> 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 CD<sup>3+</sup> 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 ± 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...
Contrary to our hypothesis, data indicated that IMQ overall decreased the amount of collagen and concordantly reduced the relative amount of MOMA-2+ macrophages, or on the necrotic core area (Figure 2D). In contrast, IMQ increased mRNA expression of the proinflammatory gene Vcam1 or the macrophage marker Cd206 compared with vehicle mice (Figure 2D). Apart from F-4/80, 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 nonspecific 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 skin inflammation in low-density lipoprotein receptor−/− mouse ears.

**Figure 1**  Digoxin delays imiquimod (IMQ)−induced skin inflammation in low-density lipoprotein receptor−/− 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 third 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 F-4/80 and did not affect the expression of the proinflammatory gene Icam1 or the macrophage marker Cd206 compared with vehicle mice (Figure 2D). Apart from F-4/80, 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 nonspecific 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).

**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

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

Where n was <47, technical challenges led to exclusion of samples. Data are expressed as means ± 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).
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 β2-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 ± SD (A); data are expressed as means ± 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.11,25 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.39 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.12 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 γt function and thereby suppresses Th17 differentiation and IL-17 production.10,21 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 (mm$^2$) 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 μm (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. 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 al 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 antiatherogenic effect observed by Shi et al, 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 al 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. More resistant to digoxin toxicity than humans, and the present observations, including analyses of plasma carbamid 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.

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Supplemental Data

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References


