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Liver fibrosis is associated with cutaneous inflammation in the imiquimod-induced murine model of psoriasiform dermatitis.

Running head: Liver fibrosis in psoriasis.

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What's already known?

- Psoriasis is a systemic disease.
- Severe liver fibrosis is more frequent in psoriatic patients.

What does this study add?

- Long-term maintenance of the imiquimod-induced murine model of psoriasiform dermatitis recapitulates the main features of psoriasis.
- Mice with psoriasiform dermatitis display portal hepatitis characterized by T cell, monocyte and polymorphonuclear cell infiltrates.
- Hepatitis observed in imiquimod-treated mice evolves towards liver fibrosis

What is the translational message?

- Psoriasis may be a co-factor of liver fibrosis.

Summary

Background. Psoriasis associates several extra-cutaneous manifestations. Little is known about hepatic parameters specifically associated with psoriasis.

Objective. To study whether psoriasiform dermatitis is associated with liver injury.

Methods. We studied liver parameters of inflammation and fibrosis in a murine model of psoriasiform dermatitis induced by topical application of imiquimod for nine weeks.

Results. Topical treatment with imiquimod induced a form of psoriasiform dermatitis reminiscent of the human disorder, characterized by thickened and scaly skin, psoriasiform epidermal hyperplasia, altered keratinocyte differentiation, and cutaneous overexpression of interleukin-17A. Mice with dermatitis displayed hepatitis, as shown by elevation of plasma transaminase levels, as well as portal and periportal hepatitis characterized by T lymphocyte (CD3 ϵ^+) and polymorphonuclear cell (Gr1 $^+$) infiltrates. The hepatitis progressed toward liver fibrogenesis, as shown by excessive Sirius red staining, consistent with the expression of alpha-smooth muscle actin by hepatic stellate cells.

Conclusions. These results indicate that liver inflammation and fibrosis is associated with experimental psoriasiform dermatitis. Our results suggest that psoriatic inflammation may be associated with specific liver injury.

Introduction

Psoriasis is a complex inflammatory condition, affecting 2% of the population worldwide¹. Its pathophysiology involves chronic dysregulation of T helper (Th)1 and Th17 lymphocytes^{1,2}. Beyond the obvious skin lesions, psoriatic patients suffer from several extracutaneous manifestations and have a reduced lifespan³. Psoriasis is now known to be a risk factor for myocardial infarction⁴, for which cardiovascular mortality is higher in patients with severe psoriasis than those with mild disease⁵. Hepatic parameters that are specifically associated with psoriasis are poorly defined, apart from case reports of neutrophilic cholangitis associated with generalized pustular psoriasis^{6,7}. Indeed, psoriatic patients share a high prevalence of confounding factors for the analysis of liver pathology (obesity and type 2 diabetes⁸, excessive alcohol consumption⁹ and hepatotoxic drugs) and studies that have evaluated liver fibrosis in biopsy specimens focused on methotrexate (MTX)-induced

hepatotoxicity¹⁰⁻¹². Limited data suggest that psoriasis itself may induce liver damage. The comparison of MTX toxicity in patients with psoriasis and those with rheumatoid arthritis has shown that both elevation of serum transaminases levels and the development of severe liver fibrosis, attributed to the regimen, are twice as likely in psoriatic subjects^{13,14}. Factors independently associated with liver test abnormalities in a cohort of patients admitted for severe psoriasis flare were not only underlying liver disease, as expected, but also plaque-type and pustular psoriasis¹⁵. The frequency of nonalcoholic fatty liver disease (NAFLD) in psoriatic patients is markedly higher than that in non-psoriatic control populations^{16,17} and psoriasis has been shown to be an additional predictor of NAFLD beyond common risk factors, such as alcohol consumption or metabolic syndrome¹⁸. Overall, these clinical data raise the possibility that psoriasis causes liver injury. We investigated this possibility more directly by developing an experimental approach consisting of adapting the murine model of psoriasiform dermatitis induced by repeated topical application of imiquimod (IMQ), a toll like receptor (TLR) 7 agonist¹⁹. In the short-term, this model recapitulates central features of psoriasis, such as the macroscopic aspect of plaques, the histological changes of the epidermidis, and a dependence on T cells and the IL-17/IL-23 axis²⁰. Psoriasis is a chronic disease. Thus, we subjected the mice to the IMQ treatment for nine weeks before evaluating liver injury. Liver inflammation and fibrosis were assessed by histological, immunofluorescent and transcriptomic studies.

Methods

Animal and treatment protocol

After seven days acclimation, eight-week old male C56BL/6J mice (Janvier Labs, Le Genest-Saint-Isle, France) were randomly assigned to receive topical application of 62 mg 5% IMQ cream (Aldara 5% cream, Meda, Solna, Sweden) (IMQ mice) or control cream (Vaseline,

Cooper, Melun, France) (control mice) on their shaved back and right ear (n =10 mice/group), five days per week, every other week, for nine weeks. Mice received food and water *ad libitum* and were maintained in a temperature- and light-controlled facility. Mice received an intraperitoneal injection of 300 μ L PBS each day of treatment because IMQ treatment has been reported to induce dehydration. All animal procedures were approved by the local ethics committee and were in accordance with the French laws and the institution guidelines for animal welfare.

Scoring of experimental skin inflammation

Inflammation of the skin was evaluated using a score adapted from the clinical PASI, and which is described elsewhere²¹. The cumulative score (0-12) consisted of the sum of three criteria (erythema, scaling, and thickening) ranging from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked.

Histological analysis

Skin from the back, right ear, and liver were fixed in 10% buffered formalin and embedded in paraffin for hematoxylin & eosin (HE) and Sirius red staining to evaluate liver fibrosis. The skin was analyzed by measuring epidermal thickening every 50 μ m for the entire length of four random sections. Liver inflammation was evaluated using the modified hepatic activity index (HAI) developed by Ishak and colleagues to grade necroinflammatory changes in various forms of chronic hepatitis²². Briefly, this semi-quantitative score grades portal inflammation (0-4), interface hepatitis (0-4), and focal inflammation (0-4). Quantification of the Sirius red stained surface was carried out using MacBiophotonics (ImageJ). All slides were blindly analyzed (PV).

Immunofluorescence

Immunofluorescent staining for alpha-smooth muscle actin (SMA) (Novus Biologicals, Littleton, CO, 1:200), keratin (KRT)-10, Gr1, CD3 ϵ and F4/80 were performed as published elsewhere²³. Confocal microscopy was carried out using an Olympus FV1000 (ImageUP microscopy platform, University of Poitiers). The number of hepatic F4/80⁺, CD3 ϵ ⁺ and Gr1⁺ cells per high power field (hpf) was determined from four randomly chosen hpf per mouse.

Real-time quantitative PCR analysis

Total skin and liver RNA was isolated and quantified as published elsewhere²⁴. Gene expression was normalized to the *GAPDH* housekeeping gene.

Mouse biochemistry

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in plasma were measured using a clinical calibrated analyzer (Cobas, Roche diagnostics).

Statistical analysis

The parameter of primary interest was the presence of liver injury in the histological samples. Preliminary results from short-term treatment showed that all treated mice displayed hepatitis. We assumed a frequency of 10 and 90% for mice with hepatitis for the control and IMQ groups, respectively. Thus, we chose to study 10 mice per group (alpha risk 0.05, power 0.9, chi-square test on proportions). Secondary objectives were quantification of liver inflammation and liver fibrosis. Data are presented as the median \pm SD. We performed the non-parametric Mann-Whitney test for the statistical evaluation of the secondary objectives when comparing two groups (control vs IMQ mice), or the Kruskal-Wallis test with Dunns's multiple comparison test when comparing three groups (control vs IMQ week 1 and IMQ

week 9 mice) (Prism software version 5.01, GraphPad Software, San Diego, CA). The two-tailed p values were as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

Results

Topical treatment with imiquimod for nine weeks chronic psoriasiform dermatitis

The model of IMQ-induced psoriasiform dermatitis has been developed on short-term kinetics¹⁹. We attempted to reproduce the features of chronic psoriasis by treating mice every other week for nine weeks. From week 1 onward the dorsal skin showed psoriasiform changes with erythema and thickening, with scales becoming macroscopically visible thereafter. Scaling was as severe at week 9 as week 1, but the erythema was more severe (Figure 1A and 1C). IMQ treatment induced epidermal psoriasiform hyperplasia within one week, characterized by downward elongation of rete ridges that was more severe after nine weeks (Figure 1B and 1D). IMQ induced altered keratinocyte differentiation, a key feature of psoriasis, as shown by the retention of nuclei within the stratum corneum (parakeratosis) (Figure 1B) and the loss of KRT 10 expression in the epidermis at week 9 (Figure 1E). IMQ-treated mice showed inflammatory infiltrates in the dermis (Figure 1B), composed of CD3ε⁺ T lymphocytes and Gr1⁺ granulocytes at week 9 (Figure 1E). Psoriasiform plaques at week 9 displayed overexpression of the Th17-related cytokines IL-23 and IL-17A, of the Th17-recruiting chemokine CCL20 and of the Th1-related chemokine CXCL9 (Figure 2). KRT 6 and the antimicrobial peptide S100A9, two markers of keratinocyte activation, were overexpressed in the skin of IMQ mice (Figure 2).

IMQ-induced chronic dermatitis is associated with portal hepatitis

We studied histological parameters of liver inflammation after nine weeks of topical treatment with IMQ and graded them according to the modified Ishak score²². Control

Vaseline-treated mice did not show features of liver inflammation, whereas analysis of HE-stained liver sections from IMQ mice revealed hepatitis. We observed moderate infiltrates composed of mononuclear cells and polymorphonuclear neutrophils (PMNs), in most portal areas, as well as mild interface hepatitis accompanied by a small number of acidophilic bodies, indicative of apoptotic hepatocytes. The biliary ductules were normal. There were rare focal mononuclear infiltrates within the lobules and around the centrilobular veins. IMQ mice did not show hepatocyte steatosis (Figure 3A and B). Liver weight was higher in the IMQ mice than control mice (7.8 ± 0.5 vs $4.8 \pm 0.7\%$ of total body weight, $p = 0.0002$), consistent with the frequency of portal infiltrates, whereas the livers were comparable by gross examination in both groups (Figure 3C and 3D). Plasma levels of AST (70 ± 22 vs 38 ± 19 UI/L) and ALT (14 ± 16 vs 6 ± 4 UI/L) were moderately higher in the IMQ than control mice ($p = 0.001$ and $p = 0.002$, respectively) (Figure 3E), reflecting the presence of hepatitis.

Portal infiltrates of IMQ mice are enriched for T cells, monocytes, and polymorphonuclear cells

We then characterized the liver infiltrates by immunofluorescence staining. The periportal infiltrates observed in IMQ mice were enriched in Gr1⁺ PMN and Gr1⁺ monocytes, as well as CD3ε⁺ T cells (Figure 4A and 4B). These results were further confirmed by overexpression of Gr1 and CD3ε transcripts in the liver of IMQ mice (data not shown). Immunostaining for F4/80, the macrophage/Kupffer cell marker, revealed that IMQ mice had two-fold more lobular macrophages compared than controls (115 ± 8 vs 50 ± 6 stained cells per hpf, $p = 0.006$). F4/80-stained cells in IMQ mice were enlarged, with more intense labelling (Figure 4A and 4B), consistent with the overexpression of its transcript in IMQ mice (data not shown).

We quantified the expression of transcription factors associated with Th lymphocyte lineage to better characterize the T cell infiltrates. The mRNA for T-bet, the Th1-related transcription factor, was strongly overexpressed in the livers of IMQ mice, as well as that of IFN- γ , the archetypal Th1 cytokine. FoxP3 (forkhead box P3) and the anti-inflammatory cytokine IL-10, two markers of T regulatory lymphocytes, were also strongly overexpressed in the livers of IMQ mice (Figure 4C). We did not detect overexpression of the Th17 markers ROR γ -t (retinoid-related orphan receptor gamma-t), IL-17A, or IL-22, nor that of the Th2 markers GATA-3, IL-4, or IL-13 (data not shown).

We then studied the expression of pro-inflammatory cytokines known to be produced by activated Kupffer cells and PMNs, and contribute to hepatocellular damage. Tumor necrosis factor (TNF)- α and IL-1 β were overexpressed in the livers of IMQ mice. Moreover, PMNs are known to produce chemokines that promote and sustain inflammatory infiltrates. Indeed, CXCL1, a PMN-recruiting chemokine, was overexpressed in the livers of IMQ mice, along with CCL3, a T-cell and PMN-recruiting chemokine (Figure 4C).

We determined IMQ plasma concentrations by MS/MS to distinguish the role of psoriasiform inflammatory phenomena from systemic exposure to IMQ. Plasma concentrations were measured 1, 6, 12, and 24 hours after a single topical application of 62 mg 5% Aldara™ to assess systemic IMQ absorption. Systemic absorption was weak and progressive, peaking at 266 ± 113 ng/mL 12 hours after treatment. The IMQ plasma trough level at the time of sacrifice of mice treated with IMQ for nine weeks was low (38 ± 9 ng/mL), similar to levels 24 hours after application in the pharmacokinetic assay (77 ± 4 ng/mL). The unbound free fraction in mice treated for nine weeks represented only $20 \pm 3\%$ of the entire fraction.

IMQ-induced chronic dermatitis is associated with liver fibrosis

We further assessed whether the liver infiltrates observed in IMQ mice, along with overexpression of pro-inflammatory cytokines, such as CCL3, TNF- α , and IL-1 β , were associated with liver fibrosis. Sirius red staining showed excessive deposition of collagen around the portal tracts at the site of infiltration previously highlighted by HE staining. The percentage of Sirius red-stained surface was higher in IMQ mice than control mice (0.54 ± 0.09 vs $0.42 \pm 0.06\%$ of the surface per field, $p = 0.02$) (Figure 5A). We performed immunostaining of α -SMA since myofibroblasts derived from hepatic stellate cells (HSC) are the major source of collagen deposition during liver fibrosis. Alpha-SMA expression was restricted to the liver vessels in control mice, whereas we detected α -SMA labeling in the periportal areas that extended to the lobules in IMQ mice, corresponding to activated HSCs. Moreover, α -SMA transcripts were overexpressed in the livers of IMQ mice (Figure 5B and 5C). Transforming growth factor (TGF)- β 1, a key cytokine implicated in the activation of HSC and the production of collagen, was also overexpressed (Figure 5C) consistent with the expression of α -SMA.

Discussion

Main results

We used the murine model of psoriasiform dermatitis induced by topical application of Aldara¹⁹, which is primarily dependent on the activation of the TLR7/Myd88 pathway in dendritic cells by IMQ²⁸. Short-term treatment induces macroscopic and histological features reminiscent of human psoriasis. IMQ-induced dermatitis relies on an initial wave of innate mediators, followed by the activation of lymphoid cells and the IL-23/17 axis²⁰. Here, we

showed that long-term treatment with IMQ also induced psoriasiform dermatitis. The macroscopic psoriasiform phenotype was maintained, and psoriasiform epidermal hyperplasia, a histological hallmark of psoriasis, was more severe after nine weeks treatment than after one. Chronic treatment with IMQ induced cutaneous infiltration of PMNs and T cells and overexpression of the Th17-related cytokines IL-23, IL-17A, and CCL20, as also observed in psoriatic patients. The lower expression of KRT-10 in the stratum corneum and the presence of parakeratosis indicated disturbed keratinocyte differentiation, another key feature of psoriasis.

The livers of mice with psoriasiform dermatitis showed inflammatory foci in most portal tracts, composed of T cells, PMNs and inflammatory monocytes, whereas hepatic lobules were enriched for macrophages, similar to autoimmune hepatitis. These infiltrates were not accompanied by significant necrotic bodies, consistent with the moderate increase in plasma transaminases levels. Alpha-SMA immunostaining and Sirius red coloration revealed a clear evolution of the periportal hepatitis towards liver fibrogenesis. Myofibroblastic transition of HSCs, induced by cytokines²⁹, is the main cause of extracellular matrix deposition during liver injury. Both IL-1 β and TNF- α , mainly produced by macrophages/Kupffer cells, have pro-fibrotic effects on HSCs^{30,31} and were overexpressed in the livers of IMQ mice. Hepatic overexpression of the chemokines CCL3 and CXCL1 may also have been indirectly pro-fibrogenic in our study. CXCL1 participates in the recruitment of PMNs and can induce collagen I production by human HSCs³² and CCL3 promotes experimental liver fibrosis by attracting T cells and stimulating the proliferation and migration of HSCs³³. Conversely, we detected hepatic overexpression of IFN- γ and IL-10, which have been reported to exert anti-fibrotic activity on HSCs³¹, possibly reflecting a counterbalancing phenomenon.

Other studies

The liver is a target organ in numerous autoimmune or systemic disorders²⁵ and the presence of putative “psoriatic hepatitis” must be carefully considered. Investigation of systemic inflammation in psoriatic patients using FDG-PET/CT in two studies showed hepatic hypermetabolism relative to matched healthy controls^{26,27}. In the latter study, which enrolled patients with mild psoriasis, only arterial and liver inflammation were observed²⁷, whereas patients with severe psoriasis showed additional joint and skin inflammation by FDG-PET/CT²⁶. Thus, the liver may be involved during the early inflammatory processes of psoriasis. The clinical significance of this observation is difficult to determine as most studies that evaluated liver injury during psoriasis were conducted in patients either treated with MTX or in those with other risk factors of liver disease, such as alcohol over-consumption, diabetes mellitus, or obesity¹⁰⁻¹². The increased prevalence of NAFLD in psoriatic patients¹⁶, possibly consistent with a high prevalence of metabolic syndrome⁸, may add another confounder to identifying a specific link between psoriasis and liver disease.

Our experimental results corroborate those of a recent population-based study that enrolled 1,535 participants, aged 55 years and older, in which liver fibrosis was indirectly evaluated by transient elastography (Fibroscan). The authors showed that subjects with psoriasis were twice as likely to have severe liver fibrosis, independently of the usual risk factors for liver disease (age, sex, alcohol consumption, presence of metabolic syndrome and steatosis)³⁴. The link between dermatitis and liver injury is yet to be identified. The number of circulating Th1 and Th17 lymphocytes in untreated psoriatic patients is elevated³⁵. Thus, these inflammatory cells may have deleterious activity; for example IL-17 can stimulate hepatocytes, HSCs or Kupffer cells to secrete various pro-inflammatory cytokines³⁶. Psoriasis is also characterized by a low-grade chronic inflammatory state³⁷, similar to that observed in obesity/NAFLD, which may trigger liver injury. Various mechanisms can lead to liver injury in other models

of autoimmune and auto-inflammatory diseases. Systemic production of auto-antibodies is associated with peri-portal hepatitis in a model of systemic lupus erythematosus³⁸. Steatohepatitis appears as a consequence of altered microbiota and bacterial translocation in models of ulcerative colitis^{39,40}. Mice with collagen-induced arthritis display increased levels of oxidation products in both the serum and liver⁴¹.

Limitations

Hepatitis observed in our mice with psoriasiform dermatitis may not result from systemic activation of TLR7-expressing cells by IMQ. First, our pharmacokinetic study performed after a single cutaneous application showed that the IMQ peak plasma concentration was significantly lower than the threshold necessary to induce cytokine secretion by murine macrophages⁴². Second, in our study, IMQ trough levels of the unbound active fraction in mice treated for nine weeks were approximately 10 times lower than this threshold. Third, repeated systemic administration of large amounts of IMQ (125 µg) for several weeks does not induce significant histological changes in the liver⁴³.

Conclusion

Overall, our work shows that liver fibrosis is associated with IMQ-induced psoriasiform dermatitis. This result reinforces the concept of psoriasis as a systemic disease and should encourage dermatologists to investigate psoriatic patients for liver fibrosis, even in the absence of conventional risk factors, to prevent evolution towards cirrhosis and hepatocellular carcinoma.

List of abbreviations

Th: T helper, MTX: methotrexate, NAFLD: nonalcoholic fatty liver disease, BMI: body mass index, IMQ: imiquimod, TLR: toll-like receptor, SMA: smooth muscle actin, KRT: keratin, PASI: psoriasis area severity index, IL: interleukin, IFN: interferon, PMN: polymorphonuclear neutrophil, TNF: tumor necrosis factor, HSC: hepatic stellate cell, TGF: transforming growth factor, FDG-PET/CT: ¹⁸F-fluorodeoxyglucose positron emission tomography/computed tomography.

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Figure Legends

Figure 1. Topical treatment with IMQ for nine weeks induces chronic psoriasiform dermatitis. (A) Representative pictures of the dorsal skin of mice treated with IMQ or control Vaseline. (B) HE-stained representative dorsal skin sections at weeks 1 and 9. Arrows show dermis infiltrates. Parakeratosis foci are shown in the inset. Original magnification is 100x, scale bar represents 50 μm . (C) Cumulative clinical score over the nine weeks of treatment. (D) Histological analysis of epidermal thickness in mice treated with Vaseline or IMQ. Measurements were made every 50 μm on four random sections per mouse. (E) Representative immunostaining of the dorsal skin for the differentiation marker KRT 10, the granulocyte antigen Gr1 and the T cell antigen CD3 ϵ . Dotted lines delineate the limits of the epidermis. Immunostained cells are shown in the inset. Original magnification is 400x. All values are presented as the median and were compared using the Kruskal-Wallis and Dunn's tests. * $p < 0.05$, *** $p < 0.001$.

Figure 2. Nine weeks of topical IMQ treatment induces a psoriasiform transcriptomic profile. Relative skin mRNA expression of IL-23, IL-17A, CCL20, CXCL9, KRT-6, and S100A9 between control and IMQ-treated mice. Results were normalized to GAPDH expression. All values are presented as the median and were compared using the Mann-Whitney U test. **p < 0.01, ***p < 0.001.

Figure 3. IMQ-induced chronic dermatitis is associated with portal hepatitis. (A) HE-stained representative liver sections from control and IMQ mice, 40x (scale bar represents 100 μ m) and 100x (scale bar represents 50 μ m) magnifications. The arrow shows portal infiltrates spilling into the lobule corresponding to interface hepatitis. Infiltrating mononuclear and polymorphonuclear cells are shown in the inset. (B) Semi-quantitative Ishak score for IMQ mice. (C,D) Macroscopic aspect of livers and liver weight at week 9 for control and IMQ mice. (E) Plasma levels of AST and ALT at week 9. All values are presented as the median and were compared using the Mann-Whitney U test. **p < 0.01, ***p < 0.001.

Figure 4. Livers from IMQ mice are enriched for macrophages, PMNs, and T cells. (A) Representative immunostaining of livers from control and IMQ mice with the macrophage marker F4/80, the PMN marker Gr1 and the T cell marker CD3 ϵ . Original magnifications are 200x and 400x. Dotted lines highlight portal areas. (B) Quantification of stained cells were made from four randomly chosen high power fields per mouse. (C) Relative hepatic levels of T-Bet, IFN- γ , FOXP3, IL-10, TNF- α , IL-1 β , CCL3, and CXCL1 mRNAs. Results were normalized to GAPDH. All values are presented as the median and were compared using the Mann-Whitney U test. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 5. IMQ-induced chronic dermatitis is associated with liver fibrogenesis. (A)

Representative Sirius red-stained sections from the livers of control and IMQ mice at week 9.

(B) Immunostaining for the marker of activated HSCs, α -SMA. (C) Relative hepatic levels of α -SMA and TGF- β 1 mRNAs. Results were normalized to GAPDH expression. All values are presented as the median and were compared using the Mann-Whitney U test. * $p < 0.05$, *** $p < 0.001$.







