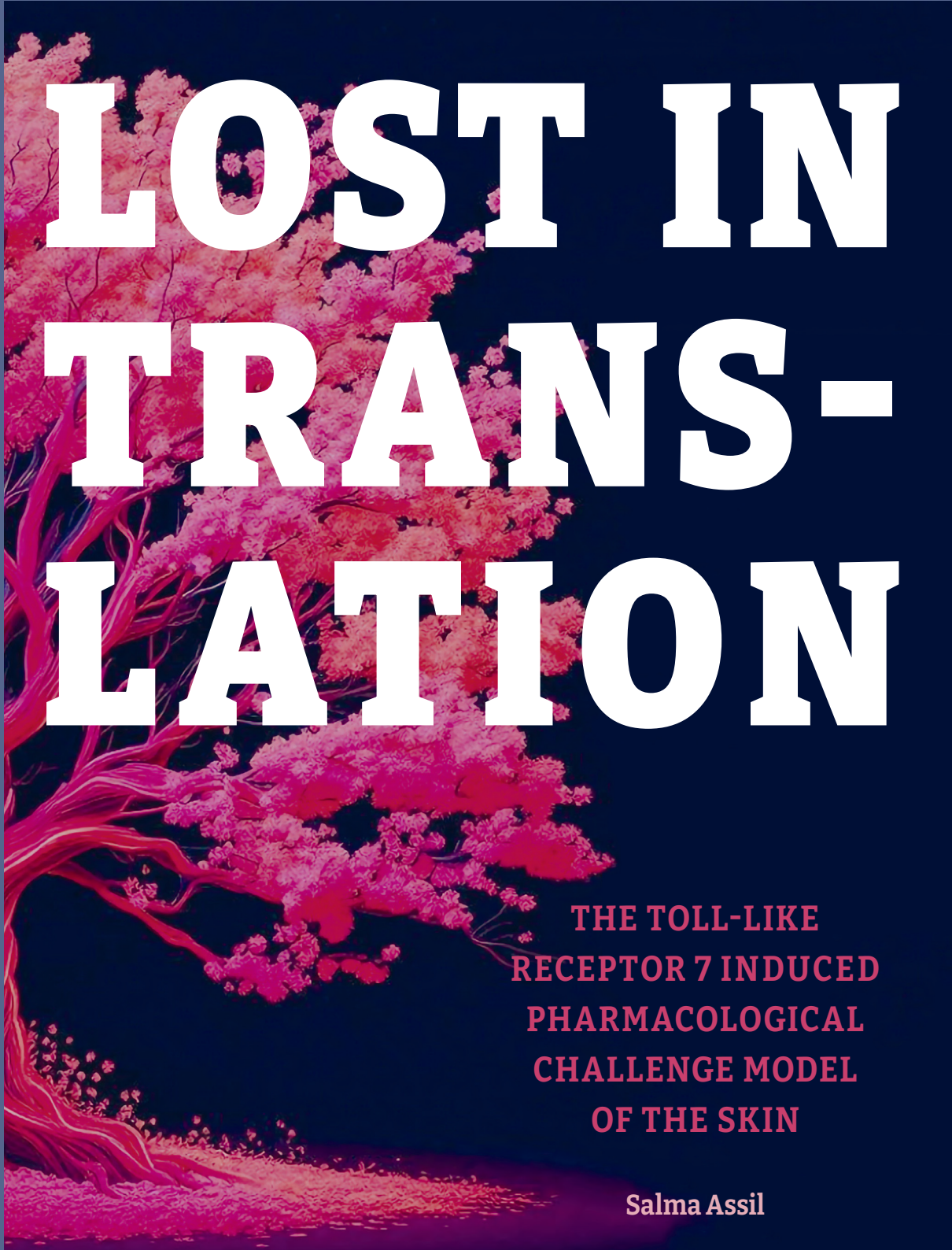
LOST IN TRANS-LATION
THE TOLL-LIKE RECEPTOR 7 INDUCED PHARMACOLOGICAL CHALLENGE MODEL OF THE SKIN
Salma Assil

LOST IN TRANSLATION

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LOST IN TRANSLATION THE TOLL-LIKE RECEPTOR 7 INDUCED PHARMACOLOGICAL CHALLENGE MODEL OF THE SKIN

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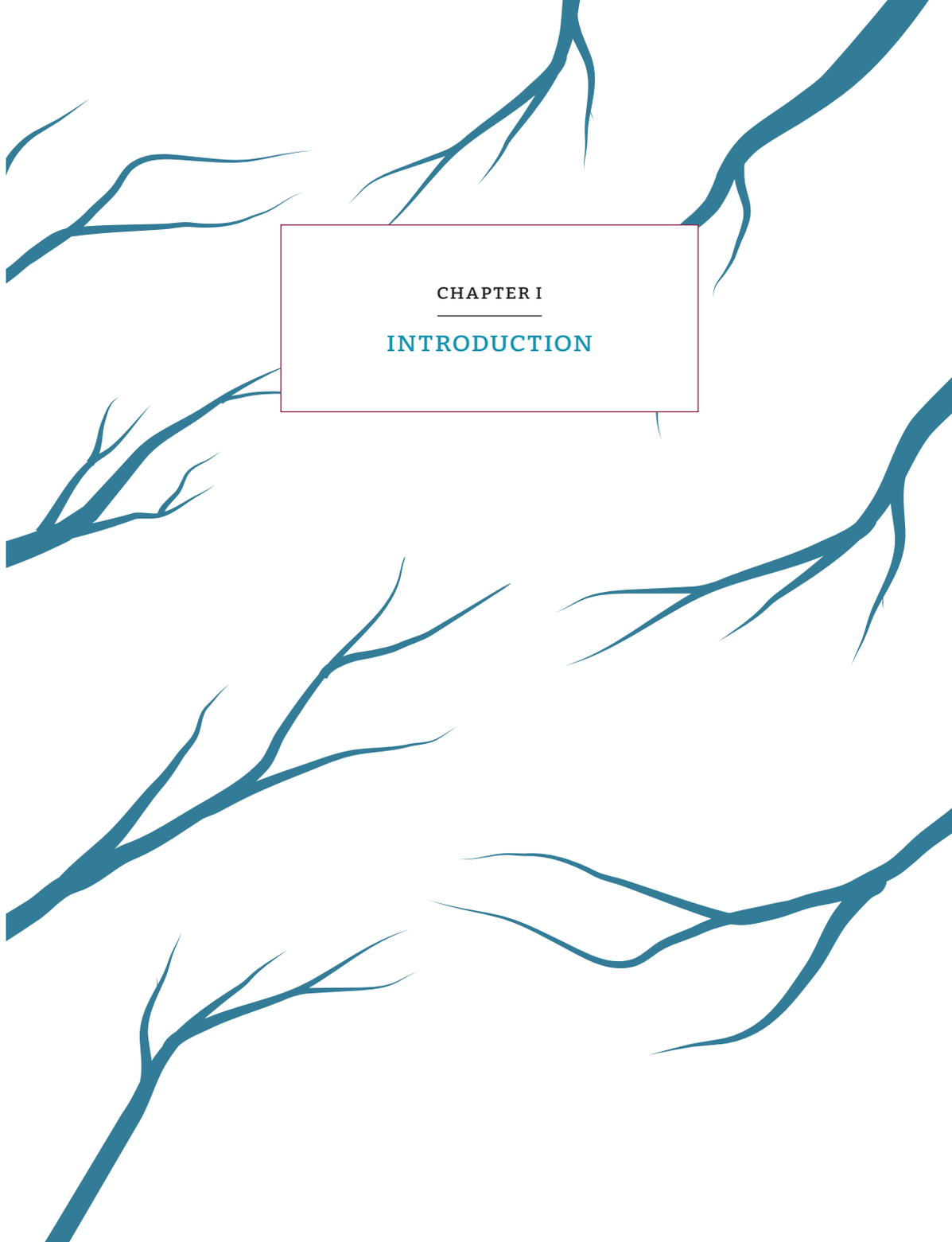


CHAPTER I

INTRODUCTION

EARLY PHASE CLINICAL DRUG DEVELOPMENT

Drug development is an extensively well-established but resource-demanding, and lengthy process. The gap between drug discovery and market availability is generally estimated to span between 10 to 15 years.[1,2] Generally, the final stage in these programs is centred around clinical trials testing drug candidates. The conventional approach for clinical trials that is used by pharmaceutical industry, was established in the early 1960's as a guideline by FDA and divides clinical trials into four phases, i.e., phase I-III and a post- marketing phase (IV).[3] In this approach, pharmacokinetics, safety, and tolerability are generally prioritized as primary objectives in phase I/II trials.[4] However, with the current approach, insufficient or delayed early information is obtained on the pharmacodynamics and efficacy of the drug that is studied. This limitation can lead to high attrition rates reaching as high as 90% across all phases, with 55% attributed to lack of efficacy in phase 2/3, *Figure 1*.

As a consequence, drug candidates fail to progress to the market resulting in high losses of investment.[5-7] Implementing biomarkers into clinical trials, with the goals of patient selection, enhancing safety, and serving as a surrogate clinical endpoint, has resulted in a twofold increase in the probability of success (POS) compared to trials that do not utilize biomarkers (POS 10.3% versus 5.5%). While the use of biomarkers improves the POS in all phases, the effects were more pronounced in phase I and II which is also the case in the field of autoimmune and inflammation therapeutics.[7] Nonetheless, despite the numerous advantages of incorporating a biomarker into a clinical trial, it also comes with certain drawbacks. Making a go/no-go decision regarding a biomarker at the end of a phase I trial is only reasonable when the biomarker's predictive accuracy is established at 93.4%.[8] Hence, improvement is necessary, e.g., by augmenting the primary study objectives with research goals that specifically examine fundamental aspects of the drug's properties, such as its ability to reach the intended site of action and its target engagement, commonly known as proof-of-mechanism studies, which can be integrated into phase I/II trials.[9,10]

Proof-of-mechanism studies focus on the initial validation of the appropriateness of a target in a specific population (volunteers or patients), the most effective dosage regimen, and the duration of treatment allowing to minimize the resource wastage.[11] Selecting an appropriate patient population is vital for this type of studies. However, the absence of a disorder, e.g. an inflammatory condition, in healthy volunteers may impede the examination of these hallmarks. To overcome this problem, pharmacological challenge models or experimental models in humans can be established that temporarily mimic components of physiological and pathophysiological conditions. Such models are currently minimally available but can potentially be of great importance, e.g., in the field of immune-mediated inflammatory diseases (IMID).

INFLAMMATION AND PHARMACOLOGICAL CHALLENGE MODELS

Inflammation is the first response of our body to pathogens and ancient physicians practicing Ayurvedic medicine in the Indian peninsula, as early as 1500 BC, possessed prior knowledge of this phenomenon.[12,13] Aulus Celsus described the four characteristics of inflammation around 30 BC namely rubor (erythema), calor (increased heat), dolor (pain) and tumor (swelling), while Galen subsequently, in the third century, introduced the fifth sign, functio laesa (loss/disturbance of function) in the affected tissue/organ. Altogether, the four characteristics served as fundamental hallmarks referring to an acute inflammatory response while loss of function is an universal sign that accompanies all inflammatory processes.[14-16] Currently, inflammation is defined as the physiological reaction of the body to injury or infection, wherein the release of chemical mediators initiates an immune response aimed at contesting infections or enabling restoration of the impaired tissue.[17,18]

The prolonged continuation of inflammation over an extended period frequently contributes to the development of diverse chronic inflammatory conditions including auto-inflammatory diseases and IMID, which very often have cutaneous manifestations. The incidence of these kind of (skin) diseases in Europe is rising and the prevalence remains high with poor quality of life and major impact on socioeconomic burden.[19-21] The prevalence for diseases such as atopic dermatitis (5.5%), alopecia (5.8%), psoriasis (2.9%), chronic urticaria (1.4%) and cutaneous lupus erythematosus (0.065 - 0.85%) is quite high, whilst therapeutic options are still limited.[22-25] This highlights that there is a high medical need for novel therapeutics. Currently, a growing array of therapeutic agents is being explored in this field, with e.g., over 70 novel compounds specifically for atopic dermatitis in the development phase.[26] Despite this increase, being a direct result of improved understanding regarding the fundamental mechanism of the disease, drug development still is a lengthy process often spanning up to a decade and beyond from bench to bedside.[1,2] Therefore, innovative strategies are required to optimize and accelerate the process of drug development for bringing clinically effective therapies to the patients in need. Hence, the development of a robust challenge model for skin inflammation can be an important step in the optimization and acceleration process.

One of the pharmacological skin challenge models that has demonstrated high potential in preclinical mice studies is a model with imiquimod (IMQ) application. This model shows clinical features similar to psoriasiform lesions, but also activates immunological pathways that are of importance for various other auto-inflammatory/immunity diseases, such as type I interferonopathies (e.g., cutaneous lupus erythematosus).[27-31] A fully characterized translation of this model to humans has not yet been accomplished. Therefore, the objective of this thesis is I) to set up an experimental imiquimod challenge model in healthy volunteers, based on murine models, II) to suppress the IMQ-induced response with the anti-inflammatory prednisolone and III) to test applicability of the model with a novel investigational drug.

IMIDAZO[4,5-C]QUINOLINES

IMIQUIMOD Imiquimod or 1-(2-methylpropyl)-1H-IMIDazo[4,5-c]quinolin-4-amine (*Figure 2A*) is a synthetic, small molecule with a molecular weight of 240.3 g/mol, logP (2.65 – 2.83) that functions as an immunomodulating agent.[32] This small sized, highly hydrophobic nucleoside analogue is the active ingredient in Aldara® 5%, a topical cream marketed by 3M Pharmaceuticals in 1995 for the treatment of external genital warts and perianal warts and in 1997 for actinic keratosis, and superficial basal cell carcinoma.[33-37] IMQ is available on the market as a 5% cream, however it is also available in lower concentrations, 2.5% and 3.75% (Zyclara). IMQ acts as a Toll like receptor (TLR)7 agonist (TLR7, EC50=10.7 µM) and primarily activates the innate immune pathways followed by adaptive upon topical application, leading to the production of cytokines.[38,39] Whilst imiquimod's potential antiviral, antitumor, and immunoregulatory effects make it a compelling choice for the treatment of a diverse range of dermatologic conditions, it has also been used as a challenge agent in mice and rats to induce inflammation and psoriasiform lesions.[28,31,40]

RESIQUIMOD Resiquimod or 4-amino-2-ethoxymethyl-α,α-dimethyl -1H-IMIDazo[4,5-c]quinolin-1-ethanol (*Figure 2B*) is a synthetic small molecule with molecular weight of 314.4 g/mol, logP (1.72-2.24) and was also developed by 3M Pharmaceuticals. While belonging to the same chemical family as imiquimod, resiquimod can activate both TLR7 (EC50=1.5 ± 0.3 µM) and TLR8 (EC50=4.5 ± 3.2 µM) and therefore also being a good candidate for the treatment of genital herpes and actinic keratosis.[39,41] However, unlike imiquimod, resiquimod has never reached market approval due to inconsistent results in clinical trials.[42]

MECHANISM OF ACTION TLR7/8 AGONISTS Currently, there has been significant progress made in understanding the mechanism of action of imiquimod (Aldara®) and resiquimod. Recent evidence suggests that the main pathway is TLR dependent for both IMQ and resiquimod. The

other two inferior pathways following IMQ application are related to inflammasome activation and inhibition of the adenosine receptor.[27,41,43-45] The main pathway which is TLR-dependent leads to IMQ or resiquimod engaging with the pathogen recognition receptors (PRR) such as TLR7 and 8 respectively, that are predominantly expressed among other cells in humans on plasmacytoid dendritic cells (PDCS) and myeloid dendritic cells (MDCS), thereby activating the signal transduction cascade downstream of these receptors.[46,47] These receptors belong to the family of transmembrane glycoproteins containing an ectodomain of leucine- rich motifs allowing involvement in recognition of certain components of microbes. Furthermore, TLRs also have a transmembrane domain and a cytoplasmic tail domain that is primarily responsible for the initiation of intracellular cascades.[48-54] Upon binding of IMQ or resiquimod to TLR7 and TLR8, the TLR dimerizes and undergoes conformational changes resulting in recruitment of the adapter protein called myeloid differentiation primary response 88 (MYD88) which in turn interacts with members of interleukin (IL) 1 receptor associated kinases (IRAK) protein kinase family via death domain. This leads to phosphorylation of IRAK4 which activates IRAK1.[55] Upon phosphorylation of these two kinases, dissociation from MYD88 occurs leading to interaction with tumour necrosis factor (TNF) receptor associated factor (TRAF)6 with E3 ubiquitin ligase activity.[56] This formed scaffold phosphorylates the inhibitor of κB (IκB) – protein with a primarily function of keeping nuclear factor kappa B (NF- κB) in the cytoplasm - and therewith degrades IκB promoting translocation of NF- κB to the nucleus.[57] Activation of this downstream pathway is important in an early immune response such as secretion of pro-inflammatory cytokines including, TNF, IL-1, IL-1RA, IL-6, and IL-8[58-61] , *Figure 3.* Furthermore, the formed scaffold activates the AP-1 family transcription factors via phosphorylation of the mitogen- activated protein kinase (MAPK) pathway leading to production of interferon (IFN)β and TNF. Additionally, IRAK1 via TRAF3 interacts with interferon regulatory factor (IRF) 7 which is highly expressed in specific cell subsets among which PDCs. Phosphorylation of IRF7 shifts into the nucleus and triggers the production of type 1 IFNs, such as IFN-α and IFN-β.[62-64]

The second pathway is TLR-independent and presumably Aldara indirectly activates the inflammasome via NLRP3, which also triggers activation of caspase 1 and leads to pyroptosis accompanied by the secretion of IL-1β and IL-18.[43,65] An alternative pathway of Aldara® is also TLR- independent and involves the activation of adenosine receptors. Imiquimod selectively binds to the A_1 and A_2A subtypes of adenosine receptors and potentially exerts an antagonistic effect on these receptors.[44,45,66] This results in the inhibition of adenyl cyclase activity, which, in turn, prevents the conversion of adenosine monophosphate to cyclic adenosine monophosphate, and allows for the unimpeded transcription of proinflammatory cytokines, such as TNF, IL-4 and IFN-γ. This cascade potentially contributes to augmentation of inflammation synergistically of the TLR-mediated proinflammatory activity.

IMIQUIMOD IN (PRE)CLINICAL STUDIES

Initially imiquimod was proposed as a challenge agent to drive psoriasis-like inflammation in mice, in addition to its marketed applications in the treatment of basal cell carcinoma and actinic keratosis, by *van der Fits et al.* In this research, IMQ was applied to the shaved back and the right ear for 5 to 6 consecutive days resulting in a daily dose of 62.5 mg. Within 2-3 days of application, the first clinical inflammation symptoms such as erythema, scaling and skin thickening became apparent. The epidermal thickening was also confirmed with Hematoxylin and Eosin (H&E) stained sections, which also showed increased numbers of dermal DCs, PDCs, neutrophils, and T cells. On a molecular level, elevated levels of IL-17/IL-23 measured by quantitative polymerase chain reaction (QPCR) were observed. Furthermore, a significant 2-fold spleen enlargement was observed after mice have been subjected to 5 to 6 days of IMQ application with increased percentages of macrophages, DCs and PDCs.[31] These findings indicate that topical IMQ application in mice leads to systemic effects.

Subsequently the mouse model of psoriasis-like skin inflammation gained popularity and has been utilized in more than 150 preclinical published studies.[29,67] This model offers several benefits, including its

ease of implementation, cost-effectiveness, and ability to induce acute skin inflammation.[68-70] However, it is not without drawbacks. Some of the disadvantages include unintended systemic effects from topical treatment, its excessive usage with limited validation studies, histological misinterpretation, and its limited representation of various aspects of human psoriasis.[29,70-75] Another challenge is the difficulty in translatability to humans given the presence of interspecies differences. In general, human and murine skin share similar cellular compositions in the dermis and epidermis, but they primarily differ in thickness. Murine skin is characterized by thin (less than 25 µM) and loose structure while human skin is firm and thick (100 µM) and is firmly adhering to the underlying tissues with 5-10 layers of the epidermis, compared to murine which has only 2-3 layers.[76-80] Beyond the morphological distinctions, there are also significant immunological differences between human and murine skin. For instance, human skin contains the cytokine IL-8, which is absent in murine skin.[81] This particular cytokine plays a crucial role in neutrophil attraction in humans and is secreted upon activation of the TLR7 receptor and involvement of the NF-κB pathway. However, since mice lack this cytokine, its absence, as well as the absence of potentially other cytokines, may lead to discrepancies between the expected mode of action of IMQ and the observed response in mice. This highlights the necessity for achieving translatability of the IMQ model, ensuring that the findings from mouse studies can be effectively translated and applied to humans.

IMQ has not earlier been tested as a challenge compound in humans, however the safety and efficacy were tested extensively in healthy volunteers and in patients. Furthermore, it has proven to be effective for the treatment of certain viral infections such as perianal and genital warts, superficial basal cell carcinoma, superficial squamous cell carcinoma, actinic keratosis, and certain superficial malignant melanomas.[82] Application of IMQ depends on the skin disease, however in general, patients are exposed to 5% IMQ at least three to four times per week for a minimal period of four weeks.[83] While topically applied IMQ has shown to be locally effective, neglectable amounts (<0.9% of the dose) of the compound after single dose reaching systemic circulation were reported.[84] Following topical application of IMQ local skin reactions were observed including local pruritus, burning sensation, erythema, excoriation, and oedema. In general, the drug appears to be well-tolerated.[85-90]

OBJECTIVE CHARACTERIZATION OF (SKIN) INFLAMMATION

The use of validated biomarkers in the context of clinical drug development has the potential to yield significant time and cost saving by obtaining early validation of pharmacological effects or "proof- of-pharmacology". We define a biomarker as objectively measurable characteristics that serve as indicators of normal biological processes, pathological processes, or pharmacological response to an intervention.[91]

In the fields of immunology and dermatology, many biomarkers and endpoints that are used in clinical settings provide only one-dimensional information. For instance, in the context of skin inflammation, biopsies are considered the conventional method for obtaining information about the cellular and molecular process within the skin.[92,93] Moreover, the impact on the skin's surface is frequently visualized using imaging techniques to gain valuable insights into erythema.[94] Whilst these assessments are commonly used in clinical settings they offer only limited, one-dimensional information on skin inflammation, despite its multifaceted nature as a complex biological process. Hence, there is need for a multimodal characterization of skin inflammation. To address this need, we introduce a novel and comprehensive approach covering different domains, i.e. imaging, biophysical, molecular and cellular and physicians score, *Figure 4*. The goal of this approach is to have a more complete profile regarding skin inflammation that can be used to characterize drug specific effects in early phase clinical trials.

AIMS AND OUTLINE OF THIS THESIS

The primary aim of this thesis is developing and characterizing a mechanistic model to investigate skin inflammation on a mechanistic basis in healthy volunteers by applying imiquimod, for utilization in drug development programs. Extensive characterization of the inflammatory response to imiquimod is performed using an array of assessments focusing on imaging based, biophysical, cellular, and molecular changes.

In **chapter 2**, we broaden the scope and present a comprehensive overview of other challenge agents for mechanistical models in healthy volunteers studying inflammation, pruritus and models that target the adaptive immune response.

Chapter 3 describes the development of a temporary skin inflammation model in healthy volunteers. Cutaneous inflammation in this model is induced by the topical TLR7 agonist IMQ (Aldara®).

In **chapter 4** of this thesis, a clinical study is performed to investigate whether the immune response driven by IMQ could be suppressed. This study involves the administration of a registered anti-inflammatory drug, i.e. oral prednisolone compared with a placebo. In addition, a technique called suction blistering is implemented in this study to further characterize the molecular and cellular aspects of the immune response.

The objective of **chapter 5** is to conduct a translational study that further studies the IMQ-induced skin inflammation in healthy volunteers by comparing short and long IMQ exposure. The study aims, amongst other objectives, to determine whether the complement system played a role in the underlying mechanisms associated with the induced skin inflammation as earlier described in preclinical studies and if prolonged IMQ exposure enhances the cellular effect. Also, this chapter attempts understanding the role of neutrophil in this pharmacological model. Furthermore, we aim to gain a deeper comprehension of the potential translational gap that exists in this model.

Chapter 6 predominantly centres on the application of the IMQ model, in combination with a novel drug candidate called omiganan, belonging to the group of cathelicidins, to examine if application of both compounds results in synergy of the IMQ-induced inflammatory response, as evidenced from in vitro studies.[95]

Lastly, **chapter 7** provides a synthesis of the main findings of this thesis, accompanied by a general discussion and recommendations regarding suitability of the model for future drug development programs.

Figure 1 Process of conventional drug discovery and the failure rate at each step. *Created with BioRender.com*

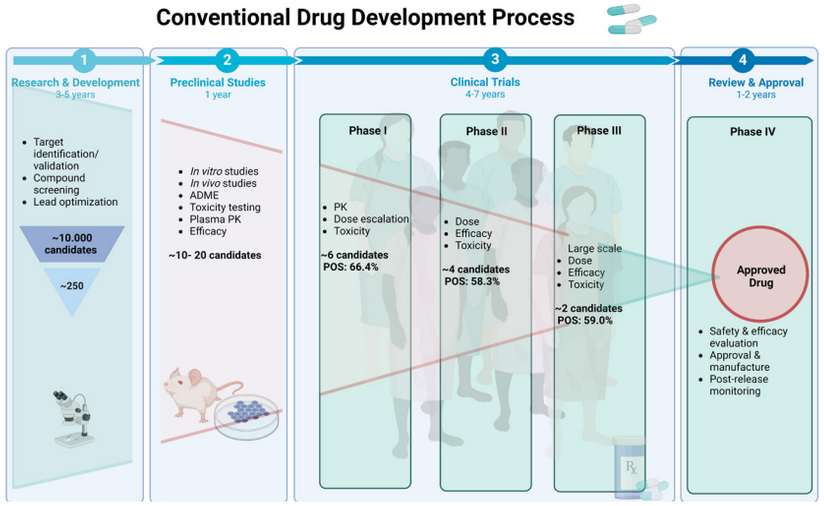
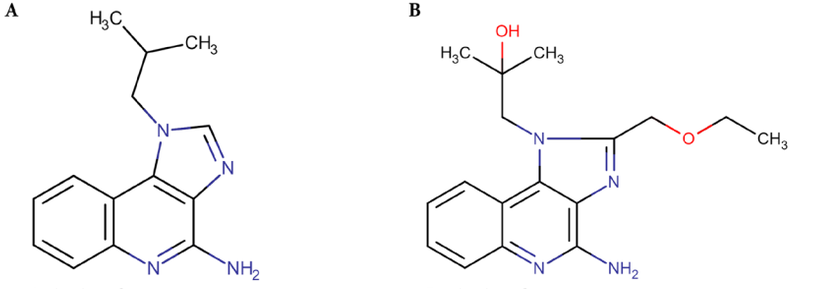


Figure 2 Chemical properties of imiquimod and resiquimod. *Created with BioRender.com*



Chemical properties:
- Molecular weight: 240.3 g/mol
- Target: TLR7 (EC50 = 10.7 µM)
- logP: 2.65 – 2.83

Chemical properties:
- Molecular weight: 314.4 g/mol
- Target: TLR7 (EC50=1.5 ± 0.3 µM) and TLR8 (EC50=4.5 ± 3.2 µM)
- logP: 1.72 - 2.24

Figure 3 Potential mechanism of TLR7 agonists: IMQ and resiquimod. (I) MY-D88 dependent pathway. (II) Via NLRP3. (III) MY-D88 independent pathway via adenosine receptor. *Created with BioRender.com.*

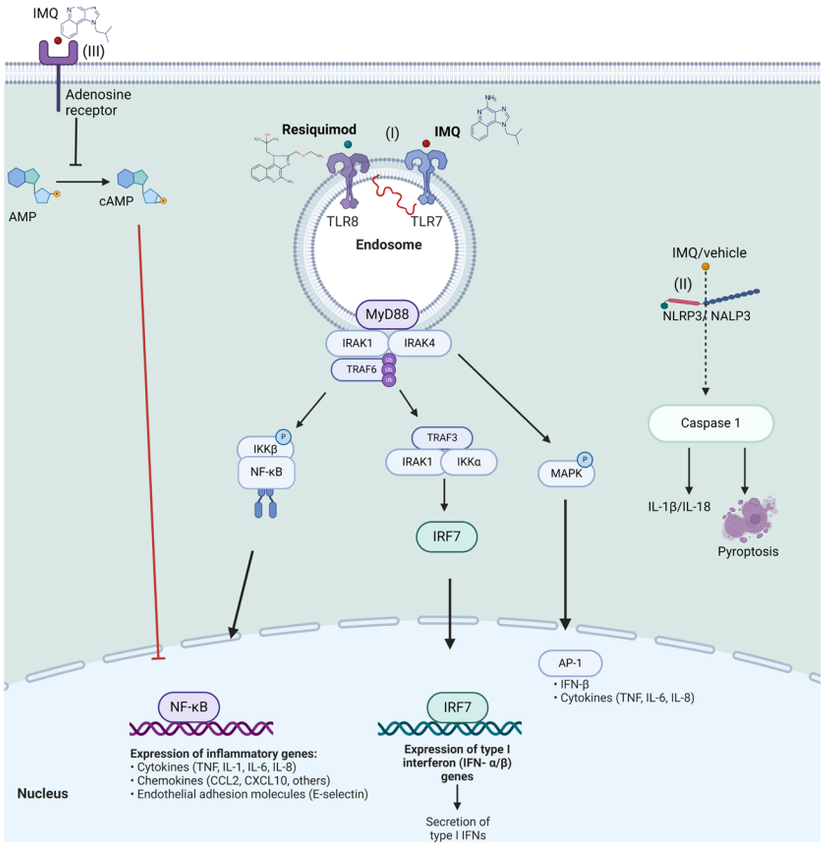
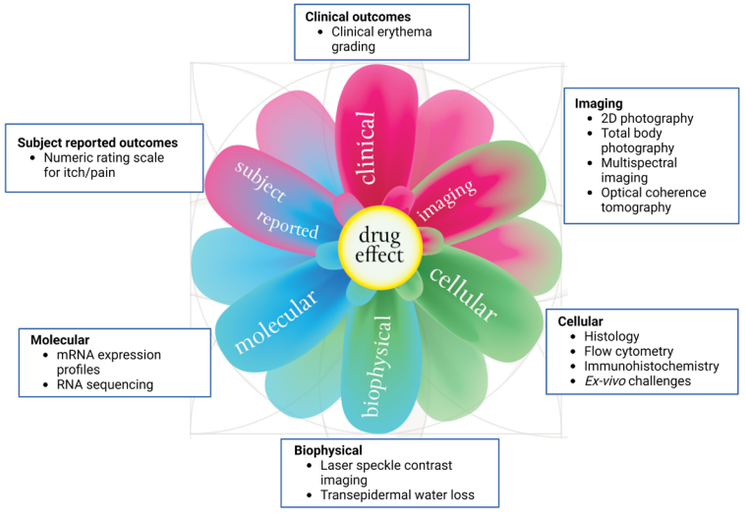


Figure 4 Multimodal approach to characterise drug effect. Derma flower created by F. van Meurs, adapted for this chapter with BioRender.com



REFERENCES

1 Hinkson, I. V., Madej, B. & Stahlberg, E. A. Accelerating Therapeutics for Opportunities in Medicine: A Paradigm Shift in Drug Discovery. Front. Pharmacol. 11, 1–7 (2020).

2 Sun, D., Gao, W., Hu, H. & Zhou, S. Why 90% of clinical drug development fails and how to improve it? Acta Pharm. Sin. B 12, 3049–3062 (2022).

3 Stuart J. Pocock. Clinical trials. Cancer vol. 33 (1989).

4 Umscheid, C. A., Margolis, D. J. & Grossman, C. E. Key concepts of clinical trials: A narrative review. Postgrad. Med. 123, 194–204 (2011).

5 Avorn, J. The $2.6 Billion Pill — Methodologic and Policy Considerations. N. Engl. J. Med. 372, 1877–1879 (2015).

6 Hwang, T. J. et al. Failure of investigational drugs in late-stage clinical development and publication of trial results. JAMA Intern. Med. 176, 1826–1833 (2016).

7 Wong, C. H., Siah, K. W. & Lo, A. W. Estimation of clinical trial success rates and related parameters. Biostatistics 20, 273–286 (2019).

8 Hurko, O. & Jones, G. K. Valuation of biomarkers. Nat. Rev. Drug Discov. 10, 253–254 (2011).

9 Cohen, A. F., Burggraaf, J., van Gerven, J. M. A., Moerland, M. & Groeneveld, G. J. The Use of Biomarkers in Human Pharmacology (Phase I) Studies. Annu. Rev. Pharmacol. Toxicol. 55, 55–74 (2015).

10 Hijma, H. J. & Groeneveld, G. J. Analgesic drug development: proof-of-mechanism and proof-of-concept in early phase clinical studies. Med. Drug Discov. 10, 100083 (2021).

11 Campbell, C. M., Gilron, I., Doshi, T. & Raja, S. Designing and conducting proof-of-concept chronic pain analgesic clinical trials. Pain Reports 4, (2019).

12 Pole, S. Ayurvedic Medicine. Ayurvedic Med. 86, 75–89 (2006).

13 Garodia, P., Ichikawa, H., Malani, N., Sethi, G. & Aggarwal, B. B. From ancient medicine to modern medicine: Ayurvedic concepts of health and their role in inflammation and cancer. J. Soc. Integr. Oncol. 5, 25–37 (2007).

14 Granger, D. N. & Senchenkova, E. Inflammation and the Microcirculation. (2010).

15 Silva, M. R. e. A brief Survey of the History of Linguistics. Course Gen. Linguist. 8, 86–90 (1978).

16 Chen, L. et al. Oncotarget 7204 www.impactjournals.com/oncotarget Inflammatory responses and inflammation-associated diseases in organs. Oncotarget 9, 7204–7218 (2018).

17 Scott, A., Khan, K. M., Cook, J. L. & Duronio, V. What is 'inflammation'? Are we ready to move beyond Celsus? Br. J. Sports Med. 38, 248–249 (2004).

18 Bennett, J. M., Reeves, G., Billman, G. E. & Sturmberg, J. P. Inflammation-nature's way to efficiently respond to all types of challenges: Implications for understanding and managing 'the epidemic' of chronic diseases. Front. Med. 5, 1–30 (2018).

19 Pärna, E., Aluoja, A. & Kingo, K. Quality of life and emotional state in chronic skin disease. Acta Derm. Venereol. 95, 312–316 (2015).

20 Lundberg, L., Johannesson, M., Silverdahl, M., Hermansson, C. & Lindberg, M. Health-related quality of life in patients with psoriasis and atopic dermatitis measured with SF-36, DLQI and a subjective measure of disease activity. Acta Derm. Venereol. 80, 430–434 (2000).

21 Hong, J., Koo, B. & Koo, J. The psychosocial and occupational impact of chronic skin disease. Dermatol. Ther. 21, 54–59 (2008).

22 Flohr, C. & Hay, R. Putting the burden of skin diseases on the global map. Br. J. Dermatol. 184, 189–190 (2021).

23 Richard, M. A. et al. Prevalence of most common skin diseases in Europe: a population-based study. J. Eur. Acad. Dermatology Venereol. 36, 1088–1096 (2022).

24 Bieber, T. Mechanisms of disease: Atopic dermatitis. N. Engl. J. Med. 358, 1483–1494 (2008).

25 Jarukitsopa, S. et al. Epidemiology of systemic lupus erythematosus and cutaneous lupus erythematosus in a predominantly white population in the United States. Arthritis Care Res. (Hoboken). 67, 817–828 (2015).

26 Bieber, T. Atopic dermatitis: an expanding therapeutic pipeline for a complex disease. Nat. Rev. Drug Discov. 21, 21–40 (2022).

27 Flutter, B. & Nestle, F. O. TLRs to cytokines: Mechanistic insights from the imiquimod mouse model of psoriasis. Eur. J. Immunol. 43, 3138–3146 (2013).
28 Gudjonsson, J. E., Johnston, A., Dyson, M., Valdimarsson, H. & Elder, J. T. Mouse models of psoriasis. J. Invest. Dermatol. 127, 1292–1308 (2007).
29 Hawkes, J. E., Gudjonsson, J. E. & Ward, N. L. The Snowballing Literature on Imiquimod-Induced Skin Inflammation in Mice: A Critical Appraisal. J. Invest. Dermatol. 137, 546–549 (2017).
30 Schon, M. P. Animal models of psoriasis: a critical appraisal. Exp. Dermatol. 17, 703–712 (2008).
31 van der Fits, L. et al. Imiquimod-Induced Psoriasis-Like Skin Inflammation in Mice Is Mediated via the IL-23/IL-17 Axis. J. Immunol. 182, 5836–5845 (2009).
32 Chollet, J. L. et al. Development of a topically active imiquimod formulation. Pharm. Dev. Technol. 4, 35–43 (1999).
33 Flutter, B. & Nestle, F. O. TLRs to cytokines: Mechanistic insights from the imiquimod mouse model of psoriasis. Eur. J. Immunol. 43, 3138–3146 (2013).
34 Caperton, C. & Berman, B. Safety, efficacy, and patient acceptability of imiquimod for topical treatment of actinic keratoses. Clin. Cosmet. Investig. Dermatol. 35–40 (2011).
35 Edwards, L. et al. Self-administered topical 5% imiquimod cream for external anogenital warts. Arch. Dermatol. 134, 25–30 (1998).
36 Lebwohl, M. et al. Imiquimod 5% cream for the treatment of actinic keratosis: results from two phase iii, randomized, double-blind, parallel group, vehicle-controlled trials. J. Am. Acad. Dermatol. 50, 714–721 (2004).
37 Tambunlertchai, S., Geary, S. M. & Salem, A. K. Topically Applied Resiquimod versus Imiquimod as a Potential Adjuvant in Melanoma Treatment. Pharmaceutics 14, (2022).
38 Bhagchandani, S., Johnson, J. A. & Irvine, D. J. Evolution of Toll-like receptor 7/8 agonist therapeutics and their delivery approaches: From antiviral formulations to vaccine adjuvants. Adv. Drug Deliv. Rev. 175, (2021).
39 Hemmi, H. et al. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat. Immunol. 3, 196–200 (2002).
40 Gangwar, R. S., Gudjonsson, J. E. & Ward, N. L. Mouse Models of Psoriasis: A Comprehensive Review. J. Invest. Dermatol. 142, 884–897 (2022).
41 Jurk, M. et al. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat. Immunol. 3, 499 (2002).
42 Fife, K. H., Meng, T.-c., Ferris, D. G. & Liu, P. Effect of resiquimod 0.01% gel on lesion healing and viral shedding when applied to genital herpes lesions. Antimicrob. Agents Chemother. 52, 477–482 (2008).
43 Köllisch, G. et al. Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. Immunology 114, 531–541 (2005).
44 Schön, M. P., Schön, M. & Klotz, K. N. The small antitumoral immune response modifier imiquimod interacts with adenosine receptor signaling in a TLR7- and TLR8-independent fashion. J. Invest. Dermatol. 126, 1338–1347 (2006).
45 Schön, M. P. & Schön, M. Immune modulation and apoptosis induction: two sides of the antitumoral activity of imiquimod. Apoptosis 9, 291–298 (2004).
46 Petes, C., Odoardi, N. & Gee, K. The toll for trafficking: toll-like receptor 7 delivery to the endosome. Front. Immunol. 8, 1075 (2017).
47 Doxsee, C. L. et al. The immune response modifier and Toll-like receptor 7 agonist S-27609 selectively induces IL-12 and TNF-α production in CD11c+ CD11b+ CD8− dendritic cells. J. Immunol. 171, 1156–1163 (2003).
48 Takeuchi, O. & Akira, S. Pattern recognition receptors and inflammation. Cell 140, 805–820 (2010).
49 Kawai, T. & Akira, S. TLR signaling. Cell Death Differ. 13, 816–825 (2006).
50 Kawagoe, T. et al. Essential role of IRAK-4 protein and its kinase activity in Toll-like receptor–mediated immune responses but not in TCR signaling. J. Exp. Med. 204, 1013–1024 (2007).
51 Leulier, F. & Lemaitre, B. Toll-like receptors—taking an evolutionary approach. Nat. Rev. Genet. 9, 165–178 (2008).
52 Kaushik, D., Kaur, A., Petrovsky, N. & Salunke, D. B. Structural evolution of toll-like receptor 7/8 agonists from IMIDazoquinolines to IMIDazoles. RSC Med. Chem. 12, 1065–1120 (2021).
53 Janssens, S. & Beyaert, R. A universal role for MyD88 in TLR/IL-1R-mediated signaling. Trends Biochem. Sci. 27, 474–482 (2002).
54 Akira, S., Takeda, K. & Kaisho, T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol. 2, 675–680 (2001).
55 Patinote, C. et al. Agonist and antagonist ligands of toll-like receptors 7 and 8: Ingenious tools for therapeutic purposes. Eur. J. Med. Chem. 193, (2020).
56 Adhikari, A., Xu, M. & Chen, Z. J. Ubiquitin-mediated activation of TAK1 and IKK. Oncogene 26, 3214–3226 (2007).
57 Kawai, T. & Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Immunol. 11, 373–384 (2010).
58 Karin, M. Nuclear factor-κB in cancer development and progression. Nature 441, 431–436 (2006).
59 Reiter, M. J., Tester man, T. L., Miller, R. L., Weeks, C. E. & Tomai, M. A. Cytokine induction in mice by the immunomodulator imiquimod. J. Leucoc. Biol. 55, 234–240 (1994).
60 Gibson, S. J. et al. Cellular requirements for cytokine production in response to the immunomodulators imiquimod and S-27609. J. Interf. cytokine Res. Off. J. Int. Soc. Interf. Cytokine Res. 15, 537–545 (1995).
61 Megyeri, K. et al. Stimulation of interferon and cytokine gene expression by imiquimod and stimulation by Sendai virus utilize similar signal transduction pathways. Mol. Cell. Biol. 15, 2207–2218 (1995).
62 Bell, E. Intracellular trafficking, IRF7 and type-I-IFN responses. Nat. Rev. Immunol. 5, 361 (2005).
63 Urosevic, M. et al. Disease-independent skin recruitment and activation of plasmacytoid predendritic cells following imiquimod treatment. J. Natl. Cancer Inst. 97, 1143–1153 (2005).
64 Sauder, D. N. Imiquimod: modes of action. Br. J. Dermatol. 149, 5–8 (2003).
65 Kanneganti, T.-D. et al. Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. Nature 440, 233–236 (2006).
66 Schön, M. et al. Tumor-selective induction of apoptosis and the small-molecule immune response modifier imiquimod. J. Natl. Cancer Inst. 95, 1138–1149 (2003).
67 Schön, M. P., Manzke, V. & Erpenbeck, L. Animal models of psoriasis—highlights and drawbacks. J. Allergy Clin. Immunol. 147, 439–455 (2021).
68 Grine, L. et al. Topical imiquimod yields systemic effects due to unintended oral uptake. Sci. Rep. 6, 20134 (2016).
69 Grine, L., Dejager, L., Libert, C. & Vandenbroucke, R. E. Dual inhibition of TNFR1 and IFNAR1 in imiquimod-induced psoriasiform skin inflammation in mice. J. Immunol. 194, 5094–5102 (2015).
70 Walter, A. et al. Aldara activates TLR7-independent immune defence. Nat. Commun. 4, 1560 (2013).
71 Luo, D.-Q., Wu, H.-H., Zhao, Y.-K., Liu, J.-H. & Wang, F. Different imiquimod creams resulting in differential effects for imiquimod-induced psoriatic mouse models. Exp. Biol. Med. 241, 1733–1738 (2016).
72 Lebre, M. C. et al. Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9. J. Invest. Dermatol. 127, 331–341 (2007).
73 Amberg, N., Holcmann, M., Stulnig, G., Glitzner, E. & Sibilia, M. Effects of depilation methods on Imiquimod-induced skin inflammation in mice. J Invest Dermatol 137, 528–531 (2017).
74 Matos, T. R. et al. Clinically resolved psoriatic lesions contain psoriasis-specific IL-17–producing αβ T cell clones. J. Clin. Invest. 127, 4031–4041 (2017).
75 Swindell, W. R. et al. Genome-wide expression profiling of five mouse models identifies similarities and differences with human psoriasis. PLoS One 6, e18266 (2011).

76 Pasparakis, M., Haase, I. & Nestle, F. O. Mechanisms regulating skin immunity and inflammation. Nat. Rev. Immunol. 14, 289–301 (2014).
77 Mestas, J. & Hughes, C. C. W. Of mice and not men: differences between mouse and human immunology. J. Immunol. 172, 2731–2738 (2004).
78 Eming, S. A., Martin, P. & Tomic-canic, M. Wound repair and regeneration: mechanisms, signaling, and translation. Sci. Transl. Med. 6, 265sr6-265sr6 (2014).
79 Wong, V. W., Sorkin, M., Glotzbach, J. P., Longaker, M. T. & Gurtner, G. C. Surgical approaches to create murine models of human wound healing. Biomed Res. Int. 2011, (2011).
80 Abdullahi, A., Amini-Nik, S. & Jeschke, M. G. Animal models in burn research. Cell. Mol. life Sci. 71, 3241–3255 (2014).
81 Hu, Y. et al. The role of interleukin-1 in wound biology. Part II: In vivo and human translational studies. Anesth. Analg. 111, 1534–1542 (2010).
82 Wagstaff, A. J. & Perry, C. M. Topical imiquimod: A review of its use in the management of anogenital warts, actinic keratoses, basal cell carcinoma and other skin lesions. Drugs 67, 2187–2210 (2007).
83 EMA. Bijlage I samenvatting van de productkenmerken 1 Apidra. 1–24 (2017).
84 Monograph, P. Aldara ® P. 1–39 (2013).
85 Beutner, K. R. et al. Treatment of genital warts with an immune-response modifier (imiquimod). J. Am. Acad. Dermatol. 38, 230–239 (1998).
86 Korman, N. et al. Dosing with 5% imiquimod cream 3 times per week for the treatment of actinic keratosis: results of two phase 3, randomized, double-blind, parallel-group, vehicle-controlled trials. Arch. Dermatol. 141, 467–473 (2005).
87 Schulze, H. J. et al. Imiquimod 5% cream for the treatment of superficial basal cell carcinoma: results from a randomized vehicle-controlled phase iii study in Europe. Br. J. Dermatol. 152, 939–947 (2005).
88 Geisse, J. et al. Imiquimod 5% cream for the treatment of superficial basal cell carcinoma: results from two phase iii, randomized, vehicle-controlled studies. J. Am. Acad. Dermatol. 50, 722–733 (2004).
89 Geisse, J. K. et al. Imiquimod 5% cream for the treatment of superficial basal cell carcinoma: a double-blind, randomized, vehicle-controlled study. J. Am. Acad. Dermatol. 47, 390–398 (2002).
90 Szeimies, R. M. et al. Imiquimod 5% cream for the treatment of actinic keratosis: results from a phase iii, randomized, double-blind, vehicle-controlled, clinical trial with histology. J Am Acad Dermatol 51, 547–555 (2004).
91 Aronson, J. K. Biomarkers and surrogate endpoints. Br. J. Clin. Pharmacol. 59, 491 (2005).
92 Harvey, N. T., Chan, J. & Wood, B. A. Skin biopsy in the diagnosis of inflammatory skin disease. Aust. Fam. Physician 46, 283–288 (2017).
93 Berekméri, A. et al. Non-invasive approaches for the diagnosis of autoimmune/autoinflammatory skin diseases—a focus on psoriasis and lupus erythematosus. Front. Immunol. 10, 1931 (2019).
94 Ruccia, F., Zoccali, G., Cooper, L., Rosten, C. & Nduka, C. A three-dimensional scar assessment tool for keloid scars: Volume, erythema and melanin quantified. Ski. Res. Technol. 27, 1007–1016 (2021).
95 Grievink, H. W. et al. Antimicrobial Peptide Omiganan Enhances Interferon Responses to Endosomal Toll-Like Receptor Ligands in Human Peripheral Blood Mononuclear Cells. Clin. Transl. Sci. 13, 891–895 (2020).

CHAPTER II

PHARMACOLOGICAL CHALLENGE MODELS IN CLINICAL DRUG DEVELOPMENTAL PROGRAMS

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ABSTRACT

Early phase clinical research for drug development requires the investigation of safety, tolerability and efficacy. The latter is hampered by the absence of the disorder in healthy volunteers which is why challenge models are often applied in order to demonstrate 'proof of pharmacology' of novel compounds. These challenge models can often be translatable from animal work and can inform the drug developer which dose, dosing regimen or application frequency should be selected prior to phase 2 studies in the target population. Furthermore, these challenge models represent well controlled settings to perform activity screening of the compound. The following topical skin challenge models are included in this book chapter: inflammation induced by Toll-like receptor (TLR)4 agonist such as lipopolysaccharide (LPS), keyhole limpet hemocyanin (KLH) challenge, ultraviolet (UV)-B radiation and agents inducing itch.

INTRODUCTION

Translational research centers on translating insights from animal models to applications in humans, with determining the first-in-human dose being the most crucial part. In general, safety, tolerability and efficacy are examined in early phases of clinical research.[1-3] However, the absence of the disorder in healthy volunteers may hamper investigation of the hallmarks of drug development. In preclinical animal research this problem is solved by using challenge models. These challenge models induce a pathophysiological condition that mimic certain aspects of a disease and are widely used in the areas of asthma, cancer, central nervous system (CNS) diseases and immune mediated inflammatory (skin) diseases such as rheumatoid arthritis, systemic lupus erythematosus and psoriasis. The latter one has gained growing interest leading to the development of numerous psoriasis murine models including spontaneous mutation model, genetically engineered model, cytokine injection model and transplantation model.[4] While these animal models replicate psoriasis-like cutaneous characteristics, they also present limitations such as the requirement for specialized experimental facilities and the lack of effectiveness of anti-psoriatic drugs.[4-7] In general, animal models are far from perfect especially in terms of pharmaco- and toxicokinetics, highlighting the need for human (pharmacological) challenge models.[8]

The use of human challenge models is not entirely novel, dating back to the 18th century when Edward Jenner developed the smallpox vaccine by inoculating humans with material from cowpox lesions.[9] Nowadays, this method of viral inoculation remains common in the field of infectiology for vaccine testing.

A similar approach could be applied to evaluate the pharmacological effects of compounds on immune-mediated inflammatory skin diseases and to gain a deeper understanding of their underlying mechanisms. These skin diseases are often characterized by inflammation, pruritus, and involvement of the innate and adaptive immune response.[10]

Therefore, this chapter will provide an overview reflecting the four aspects by reviewing four different (local) inflammation models that have been developed in immuno- dermatology. The models include inflammation by Toll-like receptor agonists such as LPS (inflammation), UV-B irradiation (inflammation and pain), histamine provocation (itch) and KLH challenge (delayed type hypersensitivity).

TLR4 AGONIST (LPS)

Skin inflammation is a common response of our immune system to penetrating pathogens, skin trauma, exposure of xenobiotics, microbes and parasites.[11,12] Inflammation is physiological recognizable by erythema, pain, heat and swelling.[13] Generally, in inflamed skin, different immune cell, of both innate and adaptive system, are involved to combat the pathogens. However, imbalance of these immune cells may lead various inflammatory disorders. Currently, many investigations are addressing the biomolecular mechanisms of inflammation, however, the pathophysiology of the skin remains complex and needs further investigation.[14,15]

To study human inflammation and the effects of potential anti-inflammatory drugs, several challenge models have been developed, including systemic lipopolysaccharide (LPS) administration, that has been known for decades. LPS is a well-characterized, pathogen- associated molecular pattern (PAMP) which is a major component of the outer membrane of Gram- negative bacteria such as Escherichia coli and Salmonella.[16-18] Upon recognition of LPS by TLR4, MYD88- and TRIF- dependent signalling pathways are activated resulting in a transient release of proinflammatory cytokines and interferons including IFN-α and IFN-β in a dose dependent manner.[19,20] Systemic exposure to LPS in humans leads to presence of clinical manifestations such as headache, fever, myalgia and tachycardia making this model suitable for studying the pathophysiology of human host response to infection.[21] Despite extensive clinical testing and consequent dose reductions of LPS, the systemic model continues to present several limitations, which could be solved by development of a model resembling local inflammatory reactions. A recent study examined neutrophil recruitment induced by intradermal application of different doses of LPS in healthy individuals. Rapid, localized inflammation was observed accompanied by release of CXCL8, IL-1α and IL-1β.[22] *Buters et al.* went a step further and characterized the cutaneous response following intradermal LPS administration more thoroughly. Administration of 5 NG LPS intradermally resulted in an acute, localized, and transient inflammatory response characterized by an increase in skin perfusion, erythema and local skin temperature. Furthermore, on cellular readouts LPS response was evident, showing a classical inflammation pattern illustrated by a rapid increase of neutrophils peaking at 3h post injection, followed by an increase of classical, intermediate and nonclassical monocytes, and dendritic cells.[23] The rise in neutrophils together with local inflammation of the skin was concordant with the findings observed by *Basran et al.* LPS not only triggered a cellular response but also induced a rapid increase in cytokines IL-6, IL-8, IL-1β, and TNF in blister exudate. The transient nature of the model is confirmed as the skin response, along with the cellular and cytokine responses, returned to baseline within 24-48 hours. The applicability of the model was examined by investigating local effects of two corticosteroids, oral prednisolone and cutaneous applied clobetasol following LPS administration. *Buters et al.*, demonstrated that topical and systemic corticosteroid pretreatment effectively suppressed the classical hallmarks of LPS-induced dermal inflammation-erythema, heat, and perfusion. Additionally, the suppressed inflammatory response is quantifiable by a reduction in inflammatory cell attraction.[23,24] These findings endorse the use of the intradermal LPS model for future proof-of-mechanism studies and for profiling novel anti-inflammatory compounds. An interesting finding in both studies involving intradermal LPS administration was the discrepancy in timing of peak responses between vascular and cellular aspects. While the peak of neutrophils, monocytes, and dendritic cells, occurs within 3 to 10 hours post-injection, the vascular response, characterized by increased perfusion and erythema, peaks only after 24 hours. This suggests that the cellular response might not drive the vascular response.

Besides the role of being a model for systemic and local inflammation, LPS is frequently used to initiate the alternative complement pathway in preclinical studies, which plays a crucial role in the first line of defense against pathogens and the clearance of apoptotic cells.[25] However, when hyperactivated, the alternative complement pathway becomes a key driver of various autoimmune and inflammatory diseases, rendering it a compelling target for therapeutic intervention in the industry. LPS is known to activate the complement system and induce TLR signalling.[26,27] Systemic administration of LPS has been linked to complement system activation in both animal models and humans. Although plasma complement proteins are primarily synthesized in the liver, they can also be produced by other cell types, such as endothelial cells, epithelial cells, and immune cells. Additionally, leukocytes and endothelial cells can locally express complement receptors.[26,28,29] Thus, a complement response can be triggered by both local and systemic stimuli. Currently a study is ongoing that evaluates activation of complement after local, intradermal LPS challenge in healthy volunteers. The results of this study, combined with the established characteristics of the LPS model, will provide a foundation for developing this model for future clinical evaluation of anti-inflammatory drugs, including those targeting the complement system.

MODELS FOR ITCH: HISTAMINE AND COWHAGE PROVOCATION

Itch, interchangeably used as pruritus, is a common skin sensation and together with pain are crucial symptoms in many chronic and allergic skin diseases. Itch can be induced by mechanical, thermal and chemical stimuli and can additionally lead to impairment of the skin therewith affecting a person's quality of life. Four types of pruritus were defined that are involved in chronic itch including pruriceptive, neuropathic, neurogenic and psychogenic itch.[30,31] Skin inflammation, dryness, or other skin damage are the main factors causing pruriceptive itch and are found in diseases such as scabies, urticaria and reactions to insect bite.[31] Neuropathic itch is caused most of the times due to nerve injury and can arise at any point along the afferent pathway of the neurons. This itch is observed after Varicella Zoster infection or nerve trauma. Itch that is originated from activation of the central nervous system is called the neurogenic itch. The underlying mechanism is complex since it involves pruriceptive itch as well. This type is most of the times observed in visceral disease states, renal diseases, and kidney failures. The last subtype of itch, that is termed, is psychogenic itch. This type of itch arises with somatization and delusional state of parasitophobia.[31-33] In this chapter, more attention will be paid to the pruriceptive itch and the translational model for it.

Generally, theories have been proposed that explained the relation between itch and pain. Itch is mediated through weak activation of nociceptors and stronger activation would result in weak pain.[34,35] This is also called, the intensity theory. However, further research has elucidated new aspects that explain pruriceptive sensory mechanism in the nervous system. This resulted in two main pathways including specificity and pattern theories. The specificity theory explains that there are different sets of neuron fibers transferring information to the central nervous system which sends responsive signals including itch and pain. The pattern theory stipulates that many sensory receptors and spinal cord neurons are involved in sensation of itch.[36] Although, the neural mechanism of pruritus has been investigated there remains much to be learned. Therefore, studies that use chemical agents to induce itch have been developed to study the sensory pattern of itch and pain in humans.

One of the most frequently and widely used pruritic agent, that evokes itch, is histamine.[37] Originally, histamine is a neurotransmitter that is associated with pathological processes such as inflammation, pruritus and vascular leak. Histamine is stored in several immune cells, basophils and mast cells and is quickly released after stimulation. Stimulation with histamine, triggers the unmyelinated nerve fibers, also known as C-fibers. Subset of C-fibers (CMi or CMh) are stimulated according to the intensity of the stimulus. In case of histamine stimulation, sustained response of CMi occurs.[38] Histidine decarboxylase (HDC), an enzyme that is responsible for histamine production, increases through stimulation with certain

mediators that are found in skin lesions of patients with atopic dermatitis.[39] Hence, this enhancement is associated with upregulated histamine release and thus with increased itch sensitivity.[37,40]

Histamine has been used in literature as an important inflammatory mediator that is responsible for vascular and inflammatory effects.[41] In the early 1900s the first studies were conducted regarding the potential vascular role of histamine in vivo, however, only a couple of years ago a clinical study was conducted that investigated cutaneous inflammatory response in human skin.[42] *Falcone et al.* has developed an easy-to-use model to study early stage of skin inflammation. Eighteen (18) subjects with Fitzpatrick skin type II and III received topically applied histamine after performing histamine iontophoresis. The subject had to rate their perceived itch on visual analog scale (VAS) with 3 being the threshold for willingness to scratch the skin. Additionally, different skin assessments were performed including trans-epidermal water loss, skin redness and punch biopsy for processing of immunohistochemistry. Itch was observed up to 30 minutes after stimulation with histamine iontophoresis and was above the itch threshold (VAS>3). Immunohistochemistry has resulted in an increase of the epidermal thickness, after 72h of histamine iontophoresis challenge. Summarized, this model, can be used as an in vivo model to provoke local and acute skin inflammation, without having an impact on the barrier function. However, no data is available on cell level or on cytokine expression.[42]

Increased production of histamine has been related to several skin diseases including atopic dermatitis.[38] In addition, histamine has been the main prototypical pruritogen that has been used for experimental purposes. The working mechanism of histamine is going via G- protein coupled receptors: H1-H4. It appears that the H1 and H4 receptors are involved in the itch response caused by histamine in mice.[41] In humans, the role of other receptor subtypes (H2 and H3) in itch is not well- examined. Generally, the classical antihistamines bind to H1 receptor and are prescribed in patients suffering from atopic dermatitis. However, recent research clarifies that histamine pathway is not playing a major role in atopic dermatitis. Also, the clinical use of antihistamines in atopic dermatitis population has been ineffective and questionable which corroborates these findings.[34,43,44]

Therefore, there was a need to establish an alternative itch model, relating to another pathway. The pruritus pathway that has physiological functions such as skin barrier homeostasis, inflammation, itch and pain, is the protease-activated receptor (PAR) pathway. PARs are classified as G-protein-coupled receptors and consist of four members, PAR-1, PAR-2, PAR-3 and PAR-4, whereas PAR-2 is mainly associated with skin diseases such as atopic dermatitis.[45] *Papoiu et al.* established a simple human model based on exogenously stimulation of the PAR-2 pathway in order to provoke itch by applying Cowhage spicules. Additionally, the Cowhage model was compared to the traditional histamine iontophoresis model and the effect of the combined model (histamine iontophoresis and Cowhage) was observed. VAS rating was increased in both atopic dermatitis patients and healthy volunteers, the Cowhage and combination model compared to the histamine model, resulting in no synergy between the Cowhage and the combined model.[35] This finding suggests, that Cowhage was the major contributor of itch after stimulation of both pathways simultaneously. The Cowhage model is simple and easy to use and could serve to study itch related skin diseases such as atopic dermatitis. On the other hand, less is known about this pathway and more research is required to examine the mechanism behind this model.

In summary, two main challenge models, histamine and Cowhage, were discussed to provoke itch, each with different underlying mechanisms. The PAR-2 pathway, more prominently involved in atopic dermatitis pruritus, suggesting that drugs targeting this pathway could be effective for chronic itch treatment. Since the cellular mechanisms of Cowhage are less understood, further research should focus on biomarker expression, skin photography assessments, and vascularity flow. Advanced studies involving both healthy individuals and atopic dermatitis patients are required to monitor skin responses in both models, as well as to evaluate the efficacy of novel anti-pruritic drugs.

MODEL FOR INFLAMMATION: UV-B SKIN IRRADIATION

Ultraviolet (UV) irradiation is classified as a carcinogenic since it has the ability to initiate and promote a tumor. Additionally, increased exposure to UV irradiation can lead to various skin malignancies including inflammation and degenerative aging.[46] Generally, UV energy is divided into three main classes based on physical properties and emission: UV-A, UV-B, and UV-C, characterised by differences in wavelength, with UV-C having the shortest, but the highest energy, UV-A having the longest but lowest energy photons and UV-B being in between. Longer wavelengths of UV-A penetrate deeper into the skin, reaching the dermis, whereas UV-B irradiation is mainly absorbed by the epidermis, with minimal exposure to the dermis. This suggests that the penetration of UV irradiation is wavelength dependent.[47]

UV-B can cause physiological changes in the skin, leading to a cascade of cytokine activation and resulting in an inflammatory response, known as "sunburn".[48] In addition, UV-B exposure is associated with the accumulation of epidermal keratinocytes, thereby increasing epidermal thickness. UV-B irradiation has an additional effect on the skin, it is able to upregulate the production and the accumulation of melanin in the skin and is also linked to cancer susceptibility.[49]

In well-controlled clinical settings, exposure to UV-B exposure is widely used as an animal and human challenge model to induce local cutaneous hyperalgesia (pain) and inflammation. Primary hyperalgesia is induced after 24h of UV-B exposure and persists for more than 48h, making the model suitable for multiple dosing studies. The amount of UV-B irradiation applied to the skin must be adapted to the subject's skin type, according to the Fitzpatrick skin type classification.[50,51]

This UV model is one of the pain models that can be used as a screening tool for early-stage clinical drug development. However, the UV model is also used in research to study the effects of anti-analgesics or local anaesthetics. Research coducted by Okkerse et al used the UV-B model, among others, to compare the effects of several analgesics with placebo. The analgesics included fentanyl, phenytoin, (S)-ketamine and placebo. The second part of the study examined the effect of imipramine, pregabalin, ibuprofen and placebo. Ibuprofen and fentanyl were the only two compounds able to increase the heat pain detection threshold on UV-B-exposed skin.[52]

A different research group investigated the effects of UV-A (150J/cm^2), UV-B (0.5, 1 and 3 MED) and UV-C (1 MED) irradiation on skin blood flow and barrier function using laser- Doppler flowmeter and evaporimeter, respectively. Irradiation with different UV light resulted in skin inflammation characterised by erythema, assessed visually, but the scores correlated with the observed increase in blood flow assessed by laser- Doppler flowmeter. UV irradiation did not damage the skin barrier function, as no increase in the trans- epidermal water loss was observed, with the exception of the 3 MED.[53]

These two studies are examples of examining the effects of UV-B irradiation as a model for studying the effects of analgesic compounds and measuring the effects on the skin. However, no studies in humans have attempted to investigate the inflammatory effect of UV-B irradiation on the skin by fully characterising it. From in vitro work we know that UV-B irradiation generally induces the production of pro-inflammatory cytokines in the human keratinocyte cell line HaCaT, including IL-1, IL-6, IL-8, IL-10 and TNF, leading to changes in skin immune cells, but the involvement of immune cells in skin inflammation after UV-B irradiation has not yet been investigated and monitored in healthy volunteers.[54]

Future research should aim to apply the UV-B challenge model to induce temporary skin inflammation and objectively characterise the response through a comprehensive methodological approach.

KLH CHALLENGE

The challenge models described in this chapter have mostly focused on induction of an innate response. However, in auto-immune skin diseases, activation of the adaptive immune system is crucial, as is the involvement

of the T cells.[55] It is quite challenging to evaluate the efficacy of novel drugs targeting T lymphocytes in healthy volunteers as they are in the resting phase. Therefore, challenge models may provide the desired solution by activating autoreactive T cell pathways in healthy volunteers. Previous research has investigated KLH as a potential immunisation candidate to study the cell- mediated immune response.[56] KLH is a large molecule (~8000 KDA) consisting of several subtypes and has been widely used in animal and human research for more than 40 years to outline cellular and humoral responses.[57-59] Additionally, KLH is considered as a carrier protein for cancer vaccines and as an immunotherapy for bladder cancer.[60,61] Due to its xenogeneic properties to the human immune system, KLH is able to promote a reliable primary response. The following routes of administration are known and have been used in previous research: intradermal, subcutaneous, intramuscular and inhalation.[62-68] Furthermore, KLH is considered to be clinically safe, with no reports of significant adverse according to the comprehensive review by *Harris et al.* Only mild adverse events have been reported including pruritus, rash and soreness at the injection site.[69,70] A single dose immunisation with KLH induces a predictable primary T cell mediated immune response. An additional intradermal dose of KLH elicits an additional immune response resulting in a delayed type IV hypersensitivity reaction around the injection site.[56] The presence of erythema and induration are features of a cell-mediated response and are most often scored by visual inspection, which is a subjective method and may result to interrater variability.

Therefore, *Saghari et al.* established a challenge model to activate T cells in healthy volunteers after immunisation with KLH followed by intradermal injection with KLH, with both cellular response and delayed type hypersensitivity objectively quantified. Humoral immunity was measured by anti-KLH IGM and IGG blood serum level titers. In addition, skin perfusion, erythema and oedema were objectively measured by laser speckle contrast imaging (LSCI), and multispectral imaging.[71] An increase in anti-KLH IGM and IGG was observed after intramuscular KLH administration compared to placebo. Additionally, imaging and biophysical readouts yielded consistent results. To date, no studies have quantitatively assessed oedema and erythema responses using non-invasive methodologies after intradermal KLH challenge. Nonetheless, investigating the cellular and molecular characteristics following intradermal KLH administration would add value to the model. Fortunately, such a study has been conducted, and the results are currently pending.

In conclusion, the delayed-type hypersensitivity model may serve as a candidate for studying the pharmacological and pharmacodynamic effects of immunomodulators in healthy volunteers.

An overview on the human challenge models is provided in *Table 1.*

CONCLUSION

Generally, in vivo mice models are a crucial part in pre-clinical drug development programs, for assessing safety and efficacy. However, these animal models often lack the specific disease or differ in morphological and physiological properties from humans. Therefore, challenge models that temporarily mimic certain aspects of a disease, could provide a possible solution and act as translational models. This chapter has provided an overview of challenge models that are known to initiate skin inflammation or other characteristics of immune mediated inflammatory skin diseases. First, the human intradermal LPS challenge model was introduced as a safe and well- tolerated model to study temporarily induced skin inflammation by targeting the TLR4 receptor. This model was followed by description of two models for pruritus focusing on two different mechanisms. The first model used histamine as pruritogen to evoke itch via a subset of C-fibers. An upregulation of HDC is associated with an increase in histamine release and is found in the lesions of patients suffering from atopic dermatitis. Antihistamines are often prescribed against itch in patients with atopic dermatitis even though they are ineffective. Therefore, an alternative model was developed targeting the PAR-2 pathway by applying Cowhage spicules to the forearm of healthy volunteers and patients with atopic dermatitis.

Another model described in this chapter is the UV-B irradiation model, which is generally used to induce pain stimuli in healthy volunteers. Couple of studies elucidate the occurrence of skin inflammation after UV-B irradiation. However, specific research focusing on UV-B-induced skin inflammation in humans remains lacking.

The final model discussed is the neo-antigen challenge model with KLH in healthy volunteers. Administration of KLH induced a delayed type IV immune response, characterized by presence of antibody titers in the blood circulation and by increase in cutaneous blood perfusion, erythema and swelling. All of the aforementioned models can be employed for future proof-of-mechanism and proof-of-pharmacology studies.

Table 1 Overview of challenge agents applied in healthy volunteers.

Challenge	Application	Mode of action	Condition induced	Immune response
INFLAMMATION				
BCG	Intradermal	TLR4, 9 agonist	Local inflammation, systemic immune response	Adaptive
Imiquimod	Local under occlusion	agonist	Local inflammation	Innate
LPS	Intra dermal	TLR4 agonist	Inflammatory response	Innate
Cantharidin	Paper disc with cantharidin	Neutrophils	Local inflammation	Innate
Injected UV Killed E.COLI	Intradermal	Neutrophils	Erythema, heat, swelling and pain	Innate
KLH	Intradermal, Intramuscular	Neo-antigen	Local inflammation, systemic immune response	Adaptive
ITCH				
Capsaicin	Intradermal, intra muscular, topical	TRPV 1 receptor	Itch	N/A
Histamine	Intradermal, epicutaneous	H1,2,3,4 receptor CMIA fibers	Itch	N/A
Cowhage	Cutaneous	CMH-fibers	Itch Burning	N/A
UV-EXPOSURE				
UV-B irradiation	LOCAL Thermode	PI3K/AKT/mTOR-upregulation	Pain, pigmentation, erythema, inflammation	Not reported

REFERENCES

1 Pocock, S. J. Clinical Trials: A practical Approach. (Wiley & Sons , 2013).
2 Rubio, D., Schoenbaum, E.E., Lee, L.S., Schteingart, D.E., Marantz, P.R., Anderson, K.E., Platt, L.D., Baez, A., Esposito, K. NIH Public Access. Acad Med. 85, 470–475 (2011).
3 Sedgwick, P. What are the four phases of clinical research trials? BMJ 348, 1–2 (2014).
4 Wagner, E. F., Schonthaler, H. B., Guinea-Viniegra, J. & Tschachler, E. Psoriasis: what we have learned from mouse models. Nat. Rev. Rheumatol. 6, 704–714 (2010).
5 Gudjonsson, J. E., Johnston, A., Dyson, M., Valdimarsson, H. & Elder, J. T. Mouse models of psoriasis. J. Invest. Dermatol. 127, 1292–1308 (2007).
6 Schon, M. P. Animal models of psoriasis: a critical appraisal. Exp. Dermatol. 17, 703–712 (2008).
7 Schön, M. P., Manzke, V. & Erpenbeck, L. Animal models of psoriasis—highlights and drawbacks. J. Allergy Clin. Immunol. 147, 439–455 (2021).
8 Suckling, K. Animal research: too much faith in models clouds judgement. Nature 455, 460 (2008).
9 Riedel, S. Edward Jenner and the history of smallpox and vaccination. Proc. (Bayl. Univ. Med. Cent). 18, 21–25 (2005).
10 Pezzolo, E. & Naldi, L. Epidemiology of major chronic inflammatory immune-related skin diseases in 2019. Expert Rev. Clin. Immunol. 16, 155–166 (2020).
11 Leung, D. Y. M. & Soter, N. A. Cellular and immunologic mechanisms in atopic dermatitis. J. Am. Acad. Dermatol. 44, S1–S12 (2001).
12 Grabbe, S. & Schwarz, T. Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity. Immunol Today 19, 37–44 (1998).
13 Tracy, R. P. The five cardinal signs of inflammation: Calor, dolor, rubor, tumor... and penuria (apologies to Aulus Cornelius Celsus, De medicina, c. AD 25). Journals Gerontol. Ser. a-biological Sci. Med. Sci. 61, 1051–1052 (2006).
14 Bieber, T. Mechanisms of disease: Atopic dermatitis. N. Engl. J. Med. 358, 1483–1494 (2008).
15 Pasparakis, M., Haase, I. & Nestle, F. O. Mechanisms regulating skin immunity and inflammation. Nat. Rev. Immunol. 14, 289–301 (2014).
16 Akira, S. & Takeda, K. Toll-like receptor signalling. Nat. Rev. Immunol. 4, 499–511 (2004).
17 Raetz, C. R. H. & Whitfield, C. Lipopolysaccharide endotoxins. Annu. Rev. Biochem. 71, 635–700 (2002).
18 Cohen, J. The immunopathogenesis of sepsis. Nature 420, 885–891 (2002).
19 Seeley, J. J. & Ghosh, S. Molecular mechanisms of innate memory and tolerance to LPS. J. Leukoc. Biol. 101, 107–119 (2017).
20 Millischer, V. et al. Intravenous administration of LPS activates the kynurenine pathway in healthy male human subjects: a prospective placebo-controlled cross-over trial. J. Neuroinflammation 18, 158 (2021).
21 van Poelgeest, E. P. et al. Characterization of immune cell, endothelial, and renal responses upon experimental human endotoxemia. J. Pharmacol. Toxicol. Methods 89, 39–46 (2018).
22 Basran, A. et al. Roles of neutrophils in the regulation of the extent of human inflammation through delivery of IL-1 and clearance of chemokines. J. Leukoc. Biol. (2013) doi:10.1189/jlb.0512250.
23 Buters, T. P. et al. Intradermal lipopolysaccharide challenge as an acute in vivo inflammatory model in healthy volunteers. Br. J. Clin. Pharmacol. 88, 680–690 (2022).
24 Buters, T. P. et al. Clinical, Cellular, and Molecular Effects of Corticosteroids on the Response to Intradermal Lipopolysaccharide Administration in Healthy Volunteers. Clin. Pharmacol. \& Ther. 111, 964–971 (2022).
25 Walport, M. J. Complement. First of two parts. N. Engl. J. Med. 344, 1058–1066 (2001).
26 Zhang, X. et al. Regulation of Toll-like receptor-mediated inflammatory response by complement in vivo. Blood 110, 228–236 (2007).
27 Hajishengallis, G. & Lambris, J. D. Crosstalk pathways between Toll-like receptors and the complement system. Trends Immunol. 31, 154–163 (2010).
28 Zhang, Q.-Y. et al. Salvianolic acid A alleviates lipopolysaccharide-induced disseminated intravascular coagulation by inhibiting complement activation. BMC Complement. Med. Ther. 22, 245 (2022).
29 Maibaum, J. et al. Small-molecule factor D inhibitors targeting the alternative complement pathway. Nat. Chem. Biol. 12, 1105–1110 (2016).
30 Yosipovitch, G., Greaves, M. W. & Schmelz, M. Itch. Lancet 361, 690–694 (2003).
31 Yosipovitch, G. & Samuel, L. S. Neuropathic and psychogenic itch. Dermatol. Ther. 21, 32–41 (2008).
32 Binder, A., Koroschetz, J. & Baron, R. Disease mechanisms in neuropathic itch. Nat. Clin. Pract. Neurol. 4, 329 (2008).
33 Potenzieri, C. & Undem, B. J. Basic mechanisms of itch. Clin. Exp. Allergy 42, 8–19 (2012).
34 Sandilands, E. A. et al. Histamine-induced vasodilatation in the human forearm vasculature. Br. J. Clin. Pharmacol. 76, 699–707 (2013).
35 Papoiu, A. D. P., Tey, H. L., Coghill, R. C., Wang, H. & Yosipovitch, G. Cowhage-Induced Itch as an Experimental Model for Pruritus. A Comparative Study with Histamine-Induced Itch. PLoS One 6, (2011).
36 Shim, W. S. & Oh, U. Histamine-induced itch and its relationship with pain. Mol. Pain 4, (2008).
37 Schmelz, M., Schmidt, R., Bickel, A., Handwerker, H. O. & Torebjork, H. E. Specific C-receptors for itch in human skin. J. Neurosci. 17, 8003–8008 (1997).
38 Yarbrough, K. B., Neuhaus, K. J. & Simpson, E. L. The effects of treatment on itch in atopic dermatitis. Dermatol. Ther. 26, 110–119 (2013).
39 Gutowska-Owsiak, D. et al. The histamine-synthesizing enzyme histidine decarboxylase is upregulated by keratinocytes in atopic skin. Br. J. Dermatol. 171, 771–778 (2014).
40 Schmelz, M. Itch and pain. Neurosci. Biobehav. Rev. 34, 171–176 (2010).
41 Criado, P. R., Criado, R. F., Maruta, C. W. & Machado Filho, C. Histamine, histamine receptors and antihistamines: new concepts. An Bras Dermatol 85, 195–210 (2010).
42 Falcone, D., Uzunbajakava, N., Richters, R., Van De Kerkhof, P. C. M. & Van Erp, P. E. J. Histamine Iontophoresis as in vivo Model to Study Human Skin Inflammation with Minimal Barrier Impairment: Pilot Study Results of Application of the Model to a Sensitive Skin Panel. Skin Pharmacol. Physiol. 30, 246–259 (2017).
43 Hanifin, J. M. The Role of Antihistamines in Atopic-Dermatitis. J. Allergy Clin. Immunol. 86, 666–669 (1990).
44 Rossbach, K. et al. Histamine H-1, H-3 and H-4 Receptors Are Involved in Pruritus. Neuroscience 190, 89–102 (2011).
45 Akiyama, T., Lerner, E. A. & Carstens, E. Protease-activated receptors and itch. Handb Exp Pharmacol 226, 219–235 (2015).
46 Elwood, J. M. & Diffey, B. L. A Consideration of Ambient Solar Ultraviolet-Radiation in the Interpretation of Studies of the Etiology of Melanoma. Melanoma Res. 3, 113–122 (1993).
47 Elwood, J. M. et al. UV Radiation and the Skin. Int. J. Mol. Sci. 14, 12222–12248 (2013).
48 Bayerl, C., Taake, S., Moll, I. & Jung, E. G. Characterization of sunburn cells after exposure to ultraviolet light. Photodermatol. Photoimmunol. Photomed. 11, 149–154 (1995).
49 Coelho, S. G. et al. Short-and long-term effects of UV radiation on the pigmentation of human skin. in Journal of Investigative Dermatology Symposium Proceedings vol. 14 32–35 (Elsevier, 2009).
50 Sayre, R. M., Desrochers, D. L., Wilson, C. J. & Marlowe, E. Skin type, minimal erythema dose (MED), and sunlight acclimatization. J Am Acad Dermatol 5, 439–443 (1981).
51 Oertel, B. G. & Lotsch, J. Clinical pharmacology of analgesics assessed with human experimental pain models: bridging basic and clinical research. Br J Pharmacol 168, 534–553 (2013).
52 Okkerse, P. et al. The use of a battery of pain models to detect analgesic properties of compounds: a two-part four-way crossover study. Br. J. Clin. Pharmacol. 83, 976–990 (2017).

53 Frodin, T., Molin, L. & Skogh, M. Effects of Single Doses of Uva, Uvb, and Uvc on Skin Blood-Flow, Water-content, and Barrier Function Measured by Laser-Doppler Flowmetry, Optothermal Infrared Spectrometry, and Evaporimetry. Photodermatology 5, 187–195 (1988).
54 Kim, Y. et al. The anti-inflammatory effect of alloferon on UVB-induced skin inflammation through the down-regulation of pro-inflammatory cytokines. Immunol. Lett. 149, 110–118 (2013).
55 Chow, S., Rizzo, C., Ravitskiy, L. & Sinha, A. A. The role of T cells in cutaneous autoimmune disease. Autoimmunity 38, 303–317 (2005).
56 Swaminathan, A., Lucas, R. M., Dear, K. & McMichael, A. J. Keyhole limpet haemocyanin - a model antigen for human immunotoxicological studies. Br. J. Clin. Pharmacol. 78, 1135–1142 (2014).
57 Curtis, J. E., Hersh, E. M., Harris, J. E., McBride, C. & Freireich, E. J. The human primary immune response to keyhole limpet haemocyanin: interrelationships of delayed hypersensitivity, antibody response and in vitro blast transformation. Clin Exp Immunol 6, 473–491 (1970).
58 Weigle, W. O. IMMUNOCHEMICAL PROPERTIES OF HEMOCYANIN. Immunochemistry 1, 295–302 (1964).
59 Swanson, M. A. & Schwartz, R. S. Immunosuppressive therapy. The relation between clinical response and immunologic competence. N Engl J Med 277, 163–170 (1967).
60 Jurincic-Winkler, C. D., Metz, K. A., Beuth, J. & Klippel, K. F. Keyhole limpet hemocyanin for carcinoma in situ of the bladder: a long-term follow-up study. Eur Urol 37 Suppl 3, 45–49 (2000).
61 Perabo, F. G. & Muller, S. C. Current and new strategies in immunotherapy for superficial bladder cancer. Urology 64, 409–421 (2004).
62 Miller, J. S. et al. Diminished neo-antigen response to keyhole limpet hemocyanin (KLH) vaccines in patients after treatment with chemotherapy or hematopoietic cell transplantation. Clin Immunol 117, 144–151 (2005).
63 Spazierer, D. et al. T helper 2 biased de novo immune response to Keyhole Limpet Hemocyanin in humans. Clin Exp Allergy 39, 999–1008 (2009).
64 Grant, R. W. et al. Cardiovascular exercise intervention improves the primary antibody response to keyhole limpet hemocyanin (KLH) in previously sedentary older adults. Brain Behav Immun 22, 923–932 (2008).
65 Schuyler, M., Lyons, C. R., Masten, B. & Bice, D. Immunoglobulin response to intrapulmonary immunization of asthmatics. Immunology 91, 167–175 (1997).
66 Kantele, A. et al. Humoral immune response to keyhole limpet haemocyanin, the protein carrier in cancer vaccines. Clin Dev Immunol 2011, 614383 (2011).
67 Boelens, P. G. et al. Primary immune response to keyhole limpet haemocyanin following trauma in relation to low plasma glutamine. Clin. Exp. Immunol. 136, 356–364 (2004).
68 Boulton, C., Meiser, K., David, O. J. & Schmouder, R. Pharmacodynamic effects of steady-state fingolimod on antibody response in healthy volunteers: a 4-week, randomized, placebo-controlled, parallel-group, multiple-dose study. J Clin Pharmacol 52, 1879–1890 (2012).
69 Harris, J. R. & Markl, J. Keyhole limpet hemocyanin (KLH): a biomedical review. Micron 30, 597–623 (1999).
70 Bingham 3rd, C. O. et al. Immunization responses in rheumatoid arthritis patients treated with rituximab: results from a controlled clinical trial. Arthritis Rheum 62, 64–74 (2010).
71 Saghari, M. et al. A randomized controlled trial with a delayed-type hypersensitivity model using keyhole limpet haemocyanin to evaluate adaptive immune responses in man. Br. J. Clin. Pharmacol. 87, 1953–1962 (2021).

CHAPTER III

COMPREHENSIVE, MULTI-MODAL CHARACTERIZATION OF AN IMIQUIMOD-INDUCED HUMAN SKIN INFLAMMATION MODEL FOR DRUG DEVELOPMENT

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ABSTRACT

Imiquimod (IMQ) is often used as topical challenge agent to provoke local skin inflammation. The objective of this study was to develop and refine a rapid, temporary and reversible human skin inflammation model with IMQ for application in clinical drug development. A randomized, vehicle-controlled, open-label, dose-ranging study was conducted in 16 healthy male subjects. IMQ (5 mg) was applied once daily for 72h under occlusion to intact skin (n=8) or tape stripped (TS) skin (n=8). Although IMQ alone induced limited effects, TS+IMQ treatment showed larger responses in several domains including erythema and perfusion ($p<0.0001$), mRNA expression of inflammatory markers ($p<0.01$) and inflammatory cell influx compared to vehicle. In conclusion, a rapid, human IMQ skin inflammation challenge model was successfully developed with a clear benefit of TS prior to IMQ application. Future interaction studies will enable *proof-of-pharmacology* of novel compounds targeting the innate immune system.

INTRODUCTION

Skin inflammation is a physiological immune response to various stimuli including skin trauma, physical challenge, exposure to xenobiotics, microbes and parasites. Dysregulation of this immune response is involved in chronic inflammatory skin diseases e.g. psoriasis vulgaris, acne vulgaris and atopic dermatitis.[1,2] Although much mechanistic insight has been gained, including involvement of the innate immune system via Toll-like receptors (TLRS) and the adaptive immune system, the pathophysiology of skin inflammation is complex and remains to be elucidated further.[3]

Different models that mirror aspects of chronic inflammation have been developed to study skin inflammation. For instance, rapid, acute skin inflammation can be induced by topical, cutaneous application of imiquimod cream (IMQ; Aldara®). IMQ application leads to agonistic activation of TLR7 and TLR8-mediated MYD88 signalling, activation of NF-κB and the induction and release of pro-inflammatory cytokines, type-1 interferons, chemokines and other mediators. Ultimately, this leads to an innate and TH1 and TH17-weighted cellular immune activation and enhancement of pro-inflammatory effects.[4]

Although the use of IMQ appears to be safe and reasonably tolerated, disease exacerbation can occur in psoriasis patients and even the development of psoriasis in individuals without a prior history of the disease is reported.[5-10] Based on the initial findings, the first IMQ-induced skin inflammation mouse model was successfully developed.[11] This model has become widely accepted for preclinical studies of psoriasis because of its straightforward approach, the inexpensiveness and the fast acute inflammatory response.[11,12] Nevertheless the murine model has some crucial disadvantages the major ones being immediate systemic effects and the limited extrapolation of murine findings to humans due to differences in the immunology and TLRS.[12,13]

Therefore, several skin inflammation models with IMQ have been evaluated in humans. A short, 7-day model in psoriasis patients using IMQ and tape stripping (TS), showed that psoriasis-like skin inflammation occurred but typical psoriasis did not develop.[5] Contact dermatitis driven

by plasmacytoid dendritic cells (pDC) was observed in an extended model of 4 weeks, both in healthy volunteers and in patients with inflammatory skin diseases. Only limited aspects of the molecular signature of psoriasis were observed.[14] Other studies in healthy volunteers characterized the model with either solely focussing on biopsy biomarkers or only systemic effects after high topical doses of IMQ.[15,16] Acute and rapid IMQ-induced inflammation models in healthy volunteers with detailed characterization have not yet been reported. A well-characterized, comprehensive human model to study skin inflammation would open opportunities for understanding the pathogenesis of several skin diseases and for the profiling of novel drugs in development. With this study we aimed to develop a skin inflammation challenge model with

I topical IMQ application for 24, 48 and 72 hours on a fully competent skin barrier, and
II topical IMQ application for 24, 48 and 72 hours on TS-perturbed skin barrier to enhance drug delivery.

Cetomacrogol cream, an indifferent neutral emollient, was used as control. Skin inflammation was assessed by measurement of erythema, perfusion and using biopsy material (mRNA expression, histology, immunohistochemistry). In the future, these models may be used in drug development programs for *proof-of-pharmacology*, drug profiling or interactions studies of novel compounds targeting the innate immune system and translational research of inflammatory skin diseases.

MATERIALS AND METHODS

The protocol of this randomized, open-label, vehicle-controlled, parallel-cohort, dose ranging study was approved by the independent Medical Ethics Committee 'Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek' (Assen, the Netherlands). The study was conducted according to the Dutch Act on Medical Research involving Human Subjects (WMO). Subjects were recruited throughout the Netherlands via advertisement campaigns on the internet and in the newspaper. All subjects gave informed consent prior to any study procedure. The study was conducted from May 2016 to June 2016 at the Centre for Human Drug Research, Leiden, The Netherlands.

STUDY POPULATION Sixteen (16) healthy male Caucasian (Fitzpatrick skin type I-II) volunteers, aged 18-45 years, were included in the study. Health status was verified with a medical history, physical examination, laboratory tests and 12-lead electrocardiodiagrams (ECG). Subjects with a medical history or family history of psoriasis or any disease associated with immune system impairment were excluded from the study. Previous use of imiquimod, resiquimod or gardiquimod was not allowed.

IMQ TREATMENT Prior to the first dose administration, 4 treatment areas (squares) were identified on the upper back and marked with a dermatological marker. During the treatment period a standard daily dosage containing 5 mg IMQ (100 mg Aldara®) was applied under occlusion by a 12 mm Finn chamber (Smart Practice, Phoenix, USA), meaning the Finn chamber was replaced with a new dose after 24h. Treatment area 1 was treated 1 day (5 mg IMQ, 24h), treatment area 2 was treated 2 days (cumulative 10 mg IMQ, 48h) and treatment area 3 was treated for 3 days (cumulative 15 mg IMQ, 72h). Treatment area 4 was treated with 100 mg cetomacrogol (indifferent) cream for 72h (negative control), *Figure 1A*. The sample size and dose were selected based on a previous study with imiquimod in healthy volunteers. Given the exploratory character of the study no formal power calculation was performed. Subjects were randomized 1:1 to receive these treatments either over a fully competent skin barrier or over a disrupted skin barrier by tape stripping (TS) of the skin. Tape stripping enhances drug delivery over the skin barrier. It is known from literature that only a limited amount of drug can be delivered over a fully competent barrier. Given the short treatment duration in this study it was decided to tape strip one group before application to ensure drug delivery. The tape stripping procedure was performed as follows: tape (D-Squame, CUDERM, Dallas,

US) was applied to the marked treatment area and a roller was used to press the tape to the skin to avoid furrows and wrinkles by a single operator. After this the tape was removed at a constant velocity. The procedure was repeated for at least 15 times until the trans epidermal water loss (TEWL) by Aquaflux (BioX, London, UK) was between 20-25 g/m²h (partial removal of the stratum corneum). Safety and tolerability were monitored by recording adverse events.

CLINICAL SCORES Clinical assessments were performed daily of treatment area 3 and 4, and included visual erythema grading (Clinician Erythema Assessment (CEA) scale, 0 represents absence of erythema, 4 very severe), colorimetry (a value (DSM II ColorMeter, Cortex Technology, Hadslund, Denmark) with a total of 3 repeats on each treatment area, 2D photography erythema index analysis and perfusion by laser speckle contrast imaging (LSCI; PeriCam PSI System, Perimed Jäfälla, Sweden).

TRANSDERMAL ANALYSIS PATCH (TAP) Skin surface biomarkers were collected pre-dose and after 1, 2 and 3 days of treatment by with TAP (FibroTx, Estonia). TAP consists of a multiplex capture-antibody micro-array that is supported by a dermal adhesive bandage for fixture to skin and can measure up to 6 markers per TAP. IFN-β, IFN-γ, IL-8, IL-6, HBD-2, IL-1β were chosen and captured from skin were qualitatively and quantitatively analysed by spot-ELISA.

SKIN PUNCH BIOPSIES Three millimetre skin biopsies were collected pre-dose (of tape stripped skin in the TS cohort), 24h after end-of-treatment of each treatment area and from a distant site with a total of six biopsies per subject. After harvest, the biopsies were placed in RNA later medium and stored at 4°C. The biopsy samples were analysed at the Immunology Laboratory at Erasmus MC, Rotterdam, The Netherlands. RNA extraction and real-time quantitative PCR analysis was performed for the following biomarkers: IP10/CXCL10, IFN-β, IFN-γ, TNF-α, IL-1β, IL-6, HBD-2, MX1, MX-A and ICAM-1, that were chosen based on the murine model.

Additionally, a histopathological score was obtained of haematoxylin and eosin (H&E) stained tissue by two blinded persons for the following characteristics of psoriasis and dermatitis: general infiltration (all type of inflammatory cells present), parakeratosis, acanthosis, papillomatosis and spongiosis. The histopathological score for each characteristic was graded based on fold increase or decrease compared to a reference biopsy of a healthy subject not related to the clinical trial (1; equal to the reference biopsy, 2; 2-fold increase compared to the reference biopsy etc.). Furthermore, immunohistochemical staining was performed to obtain scoring of markers CD11C, CD14, CD1a, CD4, CD8 and HLA-DR. This was also performed by two blinded persons and graded the same way as for the histopathological characteristics.

STATISTICS All calculations were performed using SAS for windows V9.4 (SAS Institute, INC., Cary, NC, USA). Treatments effects were analysed with a mixed model analysis of variance with fixed factors treatment, cohort, time, treatment by cohort, treatment by time, cohort by time and treatment by cohort by time, random factors subject, subject by treatment and subject by time and the baseline measurement as covariate. To determine the differences among the treatments, contrasts on all measurements were calculated. Analysis results per variable were generated with estimates of the difference of the different contrasts and a back transformed estimate of the difference in percentage for log transformed parameters, 95% confidence intervals (in percentage for log-transformed parameters) and Least Square Means (geometric means for log transformed parameters), and the p-value of the contrasts.

RESULTS

In total thirty-five (35) subjects were screened of whom twenty-six (26) subjects were eligible for participation in the study. All of the sixteen (16) included subjects completed the study and the treatments of IMQ or TS+IMQ were administered as depicted in *Figure 1a*. The study participants

were all Caucasian and had a mean age of 22.3 (18-33) years. The treatments were in general well-tolerated. The most frequent occurring treatment- emergent adverse event was application site pruritus, observed in 25% of the participants of both IMQ-treatments. No increase in IFN-α or IFN-ɣ was detected in the systemic circulation.

IMQ APPLICATION INDUCED REVERSIBLE ERYTHEMATOUS HYPERPERFUSED SKIN LESIONS

A clear exposure-dependent increase in erythema was observed with IMQ and TS+IMQ for all erythema measurements compared to the control as determined by erythema index (EI) photo analysis, erythema by colorimetry and erythema by visual grading (*Figure 1b*, *Figure 2*). Upon 48h IMQ treatment, the difference with vehicle was statistically significant ($p<0.05$) (EI; 11.55, 95%CI 1.00-22.10, $p=0.03$; colorimetry; 2.16, 95%CI 0.66-3.65, $p=0.006$, *Table 1*). TS+IMQ resulted in more significant contrasts ($p<0.01$) compared to vehicle and these were already achieved after 24h (EI; 18.64, 95%CI 7.89-29.38, $p = 0.001$, colorimetry; 3.22, 95%CI 1.72-4.73, $p = 0.0001$, *Table 1*). An exposure-dependent increase in perfusion that plateaued after 48h was only observed with TS+IMQ (*Figure 2c*). Concordant with the erythema, this increase was already statistically significant 24h after application (21%, 95%CI 7.0%-36.9%, $p=0.003$, *Table 1*). TS itself did not induce significant changes in erythema and perfusion (*Figure S1*). The skin clinically recovered after end-of-treatment (not shown).

IMQ-INDUCED ACTIVATION OF INNATE IMMUNE SYSTEM

Expression of CXCL10, HBD-2, ICAM-1, IFN-β, IFN-γ, IL-1β, IL-6, Mx-1, Mx-A and TNF-α in punch biopsies was investigated by real-time quantitative qPCR analysis, and normalized for the housekeeping gene ABL. As shown in *Figure 3*, CXCL10, Mx-A, ICAM-1 and HBD-2 showed a statistically significant increase after 48h and 72h in the TS+IMQ treatment group compared to the untreated area ($p<0.01$). This was only observed for HBD-2 in the IMQ treatment group versus untreated. In addition, an increased expression of both TNF-α and IL-1β was observed with 48h and 72h treatment, compared to the untreated site, this was significant for IL-1β ($p<0.05$), and only observed for the conditions without tape stripping, *Figure 3*. TAP data was highly variable and not significantly different from untreated skin (data not shown).

INFILTRATION AND ACANTHOSIS INDUCED BY IMQ TREATMENT

H&E stained skin punch biopsies were independently analysed by two investigators blinded to treatment. In the IMQ treatment group, no changes compared to the reference biopsy were observed. TS+IMQ treatment demonstrated ≥ 2-fold increase in general infiltration in 31% and 44% of the subjects after 48h and 72h, respectively, compared to the reference. Acanthosis was compared to the reference two times more frequent in 19% of these subjects after 48h (*Figure 3*, *Figure 4*). Moreover, parakeratosis, papillomatosis and spongiosis were scored. These parameters were not different from the reference biopsy in both cohorts. No histological skin changes were noted in the vehicle controls.

$CD4^+$, $CD8^+$, $CD11^+$ AND HLA-DR^+ CELLS INFILTRATED THE DERMIS FOLLOWING IMQ TREATMENT

Immunohistochemical staining was performed to further explore cell infiltration and showed an infiltration of $CD4^+$ T-cells, $CD8^+$ T-cells, $CD11^+$ dendritic cells and HLA-DR^+ macrophages, mostly in the TS+IMQ treatment group. $CD8^+$ cells were ≥1.5 times more present in 56% of the subjects in the TS^+IMQ treatment group after 48h and 72h, compared to the reference biopsy (*Figure 4*, *Figure 5*). In 37.5% of the subjects $CD4^+$ cells were ≥1.5 times more present examined after 48h and 72h of treatment in the TS+IMQ treatment group. Moreover, $CD11c^+$ infiltration was slightly apparent (2 times more present in 21.4% of the subjects after 48h, 12.5% of the subjects after 72h in TS). HLA-DR^+ cells 12.5% of the tape stripped subjects developed a 3-fold increase of HLA-DR^+ cells after 48h of IMQ treatment, while 18.8% was observed after 72h of IMQ treatment, compared to the reference biopsy. However, HLA-DR was already more present at baseline in this group of subjects, compared to the reference biopsy (*Figure 5*).

DISCUSSION

COMPREHENSIVE CHARACTERIZATION OF THE SKIN INFLAMMATION MODEL

This is the first study that comprehensively characterized an acute inflammatory model combining IMQ and TS in healthy volunteers with the purpose to apply in drug development programs. We utilized different, complementary assessment modalites to monitor the effects thouroughly, including clinical (physician scoring), biophysical (LSCI), imaging (colorimetry), molecular (mRNA expression) and cellular (IHC) aspects. The synthesis of this multi-modal assessment is presented in *Figure 6* and clearly shows concordant effects on complementary modalities and a clear dose-dependency, exposure-response relationship. While previously models were developed in psoriasis patients or with a lengthy treatment-period of 28 days in healthy volunteers without TS,[5,14] our study shows a rapid and reproducible way of inducing short-term inflammatory skin lesions with effects on all the different domains as discussed in detail in the next paragraphs.

STRONG AGREEMENT OF MEASUREMENTS ASSESSING THE CLINICAL PHENOTYPE

IMQ induced a dose-dependent increase in erythema in all measurements, which occured much quicker and more pronouced when combined with TS. Statistically significant effects on EI, colorimetry and LSCI were already observed 24h after TS+IMQ treatment, versus 48h without TS. There were no clear differences in erythema intensity between 48h and 72h of treatment for the TS+IMQ treatment which is also seen in the murine model.[11] Increased skin perfusion as a result of IMQ application was only observed when it was combined with TS and also no clear differences between 48h and 72h appeared. In a recent study where a human model with 4 weeks of IMQ treatment was developed to study psoriasis, maximal effects occurred at day 4 or later, but TS of the skin was not performed to enhance drug delivery of IMQ.[14] Importantly, in our study we showed that within 24-48h of the last dose, the skin fully recovered clinically (data not shown), which is in agreement with previous reports.[5] The similar clinical observations with different methods and reversibility of effects are two strong points of our approach.

FULLY COMPREHENSIVE MECHANISTIC INSIGHTS OF MOLECULAR PATTERNS

CXCL10, MX-A, ICAM-1 and HBD-2 were all statistically significant upregulated in the skin of subjects treated with IMQ for 48h and 72h in combination with TS and to a lesser extent in the non-TS cohort (only for HBD-2) compared to vehicle, which is concordant with the molecular findings of *Dickson et al.*[15] This reflects the intermediate phase response of IMQ (24-72h) where activation of the innate as well as adaptive immune system occurs, featuring infiltration of neutrophils, lymphocytes and macrophages, as described in a recent review of the murine translational IMQ skin inflammation models.[17] CXCL10, a chemokine which is highly expressed when keratinocytes are activated in inflamed skin, is regulated by T-cells and found in psoriasis and other autoimmune diseases, which corresponds with the findings in our clinically induced skin inflammation.[1,15,18] Upregulation of MX-A, a down-stream mediator of interferons, reflects the activation of plasmacytoid dendritic cells (pDCs), which play a major role in the pathophysiology of psoriasis (1, 19, 20) . Furthermore, this refects the anti-viral response by IMQ, which was expected since IMQ is effective against several HPV-induced skin diseases.[21,22] The mRNA expression of adhesion molecule ICAM-1 was observed to be upregulated which corroborates previous findings showing induction by TNF-α.[23] ICAM-1 facilitates leukocyte endothelial transmigration and enhancement of skin inflammation. Upon IMQ treatment alone no statistically significant differences in upregulation were observed between 48h and 72h in the biopsy markers which confirms the better suitability of TS+IMQ combination for further application in drug development. Interestingly, in the biopsies of the non-TS cohort, we found an upregulation of TNF-α and IL-1β compared to the untreated site while this was not present in the TS+IMQ cohort. Presumably, the initial phase (within 24h), where

the innate immune system is activated as a consequence of release of inflammatory mediators including IFN-α, IL-1α, IL-1β, IL-6 and TNF-α and where cellular changes such as accumulation of neutrophils and proliferation of keratinocytes occur, is at a later time point because of less drug delivery without tape stripping.[17] This would also explain why TNF-α and IL-1β are not upregulated in the TS cohort after 48h; due to enhanced penetration by TS of the skin, the initial upregulation might already be passed. This matches with the fact that erythema was observed in the TS cohort already after 24h, but not in the non-TS cohort, where it appeared after 48h. Moreover, the presence of ICAM-1 in the TS cohort confirms the presence of TNF-α in an earlier stage, and therewith the initial phase as also seen in the murine model, since it is a downstream marker of TNF-α.[23] *Vinter et al.* did still find TNF-α and IL-1β after 48h, but tape stripped less extensively (10 times *vs.* ± 15 times in our study). Hereafter, both markers also normalized while the downstream markers increased.[5]

CONCORDANT CELLULAR OBSERVATIONS Histologically, a general infiltration with little acanthosis was seen only in the TS cohort. Classical dermatitis and psoriasis characteristics such as spongiosis, acanthosis and parakeratosis were not observed. Although some of those characteristics were reported in literature after longer treatment, the treatment duration in this study of maximally 72h is presumably too short.[5,14] In psoriasis patients, exacerbations after IMQ treatment also occur only after a prolonged period of application (average 9 weeks).[7] In addition, $CD11c^+$, HLA-DR, $CD4^+$ and $CD8^+$ cells infiltrated the dermis, more in the TS cohort than in the non-TS cohort and with no clear difference between 48h and 72h. $CD11c^+$ cells reflect the inflammatory myeloid DCs. These are highly increased in the psoriatic dermis and are known to stimulate the production of type 1 helper (TH1) cytokines [1]. Likewise, the macrophages (HLA-DR) are involved in this process which are mediated via the TLR-7 response.[24-26] Infiltration of DCs, macrophages and T-cells indicate activation of both an innate and adaptive immune response. All histological effects are consistent with the intermediate and late phase response of IMQ. The late phase is characterized by expression of both IL-17 and IL-22 as a result of IL-23 production, and infiltration of T-cells. It normally occurs after 72h[17]. However, due to the enhanced drug delivery by TS of the skin this was already observed in the 48h biopsies.

TRANSLATIONAL VALUE OF THE INFLAMMATION MODEL FOR DRUG DEVELOPMENT Taken together, the IMQ-induced histological changes are highly similar to those that were observed in the murine model and have features of both psoriasis and contact dermatitis with activation of the innate and adaptive immunesystem. Concordant with findings of others no complete phenotype induction such as psoriasiform histology was observed.[5,14] However, since the purpose of the study was to develop a model for drug profiling and interaction studies, and not primarily to study disease pathophysiology this is not considered a limitation. A limitation of the study is the open-label design which could have lead to an observer bias of the clinical erythema grading. However, since clinical scores are highly concordant with the objective erythema measures, EI and colorimetry, the bias is presumably negligible.

In conclusion, we succesfully translated the murine IMQ skin inflammation model to a fully characterized safe, rapid and reversible human model in healthy volunteers. Clinical and histological phenotypes were fully concordant in the TS+IMQ cohort. Therefore, TS of the skin to enhance drug delivery of IMQ is required to induce a quicker and stronger inflammatory response. No significant differences in effects of IMQ were observed between 48h and 72h of application suggesting that 48h of treatment is the most suitable for this model. Future interaction studies with the model in drug development programs will enable *proof of pharmacology* of novel compounds targeting the innate immune system.

REFERENCES

1 Nestle, F.O., Kaplan, D.H. & Barker, J. Psoriasis. *The New England journal of medicine* **361**, 496-509 (2009).
2 Bieber, T. Atopic dermatitis. *The New England journal of medicine* **358**, 1483-94 (2008).
3 Pasparakis, M., Haase, I. & Nestle, F.O. Mechanisms regulating skin immunity and inflammation. *Nat Rev Immunol* **14**, 289-301 (2014).
4 Schon, M.P. & Schon, M. Imiquimod: mode of action. *The British journal of dermatology* **157 Suppl 2**, 8-13 (2007).
5 Vinter, H., Iversen, L., Steiniche, T., Kragballe, K. & Johansen, C. AldaraR-induced skin inflammation: studies of patients with psoriasis. *The British journal of dermatology* **172**, 345-53 (2015).
6 Wu, J.K., Siller, G. & Strutton, G. Psoriasis induced by topical imiquimod. *The Australasian journal of dermatology* **45**, 47-50 (2004).
7 Patel, U., Mark, N.M., Machler, B.C. & Levine, V.J. Imiquimod 5% cream induced psoriasis: a case report, summary of the literature and mechanism. *The British journal of dermatology* **164**, 670-2 (2011).
8 Fanti, P.A., Dika, E., Vaccari, S., Miscial, C. & Varotti, C. Generalized psoriasis induced by topical treatment of actinic keratosis with imiquimod. *International journal of dermatology* **45**, 1464-5 (2006).
9 Rajan, N. & Langtry, J.A. Generalized exacerbation of psoriasis associated with imiquimod cream treatment of superficial basal cell carcinomas. *Clinical and experimental dermatology* **31**, 140-1 (2006).
10 Smith, W.A., Siegel, D., Lyon, V.B. & Holland, K.E. Psoriasiform eruption and oral ulcerations as adverse effects of topical 5% imiquimod treatment in children: a report of four cases. *Pediatric dermatology* **30**, e157-60 (2013).
11 van der Fits, L. *et al.* Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *Journal of immunology (Baltimore, Md : 1950)* **182**, 5836-45 (2009).
12 Hawkes, J.E., Gudjonsson, J.E. & Ward, N.L. The Snowballing Literature on Imiquimod-Induced Skin Inflammation in Mice: A Critical Appraisal. *The Journal of investigative dermatology* **137**, 546-9 (2017).
13 Mestas, J. & Hughes, C.C. Of mice and not men: differences between mouse and human immunology. *Journal of immunology (Baltimore, Md : 1950)* **172**, 2731-8 (2004).
14 Garzorz-Stark, N. *et al.* TLR7/8 agonists stimulate plasmacytoid dendritic cells to initiate a TH17-deviated acute contact dermatitis in humans. *The Journal of allergy and clinical immunology*, (2017).
15 Dickson, M.C., Ludbrook, V.J., Perry, H.C., Wilson, P.A., Garthside, S.J. & Binks, M.H. A model of skin inflammation in humans leads to a rapid and reproducible increase in the interferon response signature: a potential translational model for drug development. *Inflammation research : official journal of the European Histamine Research Society.et al]* **64**, 171-83 (2015).
16 Pasmatzi, E. *et al.* Topical application of imiquimod induces alterations in peripheral blood lymphocytes in healthy individuals. *Acta dermato-venereologica* **89**, 134-9 (2009).
17 Flutter, B. & Nestle, F.O. TLRs to cytokines: mechanistic insights from the imiquimod mouse model of psoriasis. *European journal of immunology* **43**, 3138-46 (2013).
18 Ottaviani, C., Nasorri, F., Bedini, C., de Pita, O., Girolomoni, G. & Cavani, A. CD56(bright)CD16(-) NK cells accumulate in psoriatic skin in response to CXCL10 and CCL5 and exacerbate skin inflammation. *European Journal of Immunology* **36**, 118-28 (2006).
19 Nestle, F.O. *et al.* Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. *The Journal of experimental medicine* **202**, 135-43 (2005).
20 Boehncke, W.H. & Schon, M.P. Psoriasis. *Lancet (London, England)* **386**, 983-94 (2015).
21 van Seters, M. *et al.* Treatment of vulvar intraepithelial neoplasia with topical imiquimod. *The New England journal of medicine* **358**, 1465-73 (2008).
22 Beutner, K.R., Spruance, S.L., Hougham, A.J., Fox, T.L., Owens, M.L. & Douglas, J.M., Jr. Treatment of genital warts with an immune-response modifier (imiquimod). *Journal of the American Academy of Dermatology* **38**, 230-9 (1998).
23 Burke-Gaffney, A. & Hellewell, P.G. Tumour necrosis factor-alpha-induced ICAM-1 expression in human vascular endothelial and lung epithelial cells: modulation by tyrosine kinase inhibitors. *British journal of pharmacology* **119**, 1149-58 (1996).
24 Racz, E. & Prens, E.P. Molecular pathophysiology of psoriasis and molecular targets of antipsoriatic therapy. *Expert reviews in molecular medicine* **11**, e38 (2009).
25 Ueyama, A. *et al.* Mechanism of pathogenesis of imiquimod-induced skin inflammation in the mouse: a role for interferon-alpha in dendritic cell activation by imiquimod. *The Journal of dermatology* **41**, 135-43 (2014).
26 Gregorio, J. *et al.* Plasmacytoid dendritic cells sense skin injury and promote wound healing through type I interferons. *The Journal of experimental medicine* **207**, 2921-30 (2010).

TABLE 1 Analysis results of erythema and perfusion measurements.

	IMQ vs vehicle			TS + IMQ vs TS + vehicle		
	24h	**48h**	**72h**	**24h**	**48h**	**72h**
Erythema index	9.18 (-1.37, 19.73) p=0.09	11.55 (1.00, 22.10) p=0.03	16.84 (6.29, 27.38) p=0.003	18.64 (7.89, 29.38) p=0.001	29.76 (19.01, 40.50) p=<.0001	36.53 (25.78, 47.28) p=<.0001
Colorimetry	1.16 (-0.34, 2.66) p=0.12	2.16 (0.66, 3.65) p=0.006	3.66 (2.16, 5.16) p=<.0001	3.22 (1.72, 4.73) p=0.0001	5.13 (3.6, 6.64) p=<.0001	5.64 (4.13, 7.15) p=<.0001
Basal Flow (% change)	-3.1% (-13.5%, 8.5%) p=0.57	1.2% (-9.6%, 13.4%) p=0.83	6.6% (-4.9%, 19.4%) p=0.26	21.0% (7.0%, 36.9%) p=0.003	44.0% (28.5%, 61.4%) p=<.0001	38.8% (23.8%, 55.5%) p=<.0001

The differences between IMQ vs vehicle and TS + IMQ vs. TS + vehicle are shown. Data is presented as mean, 95% confidence interval and p-value.

FIGURE 1A Treatment schedule of the study. Treatment area 1 and 2 served as biopsy sites for biopsies after 1 (24h) and 2 (48h) days of treatment. Treatment site 3 and 4 served as sites for all erythema and perfusion measurements and biopsies after the longest treatment duration (3 days, 72h, 5 mg imiquimod (IMQ) application at 0h, 24h and 48h). In 8 subjects the local treatment area was tape stripped before IMQ application. All treatments were applied under occlusion by a 12 mm Finn chamber.

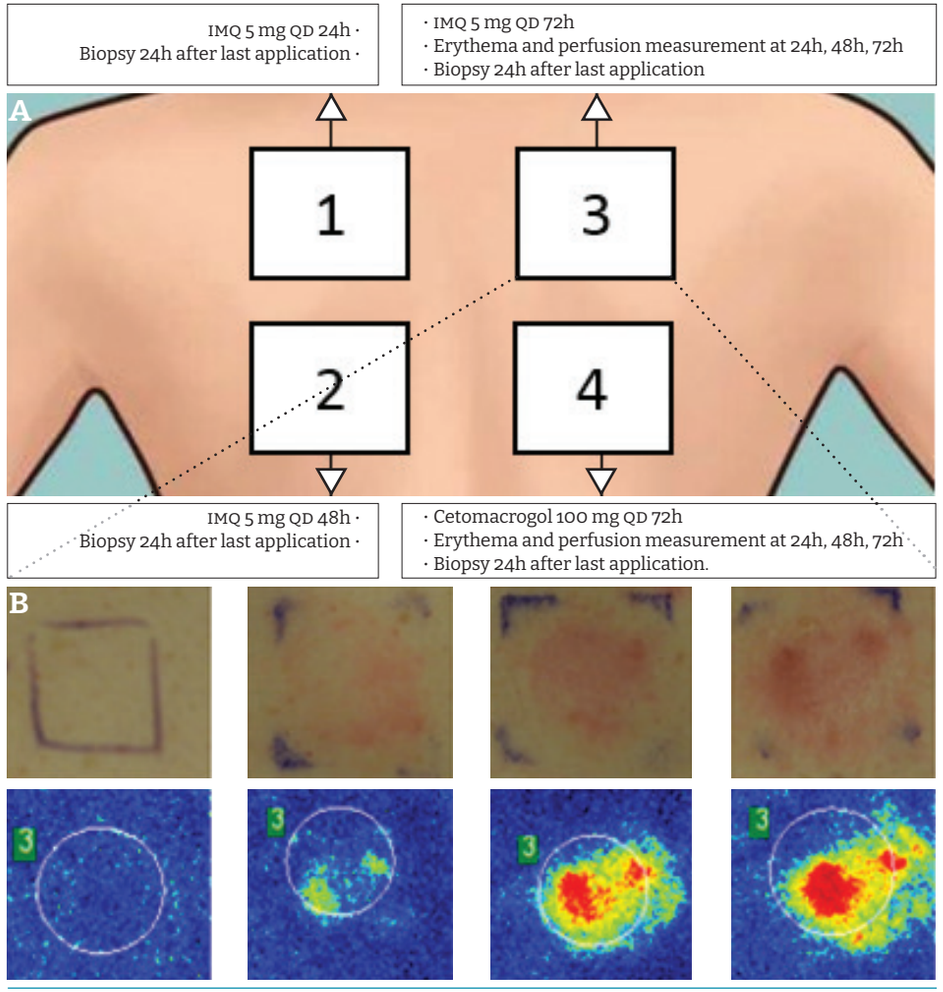


FIGURE 1B Clinical impression of site 3, tape stripping +IMQ 72h. An increase of erythema and perfusion is observed.

FIGURE 2 Erythema and perfusion induced by imiquimod (IMQ) and tape stripping +IMQ application. Error bars are defined as LSM +/- upper and lower limit. A) LSM change from baseline in erythema index, B) LSM change from baseline in erythema by colorimetry, C) LSM change from baseline in % by laser speckle contrast imaging, D) erythema by visual grading displayed as % presence per time point.

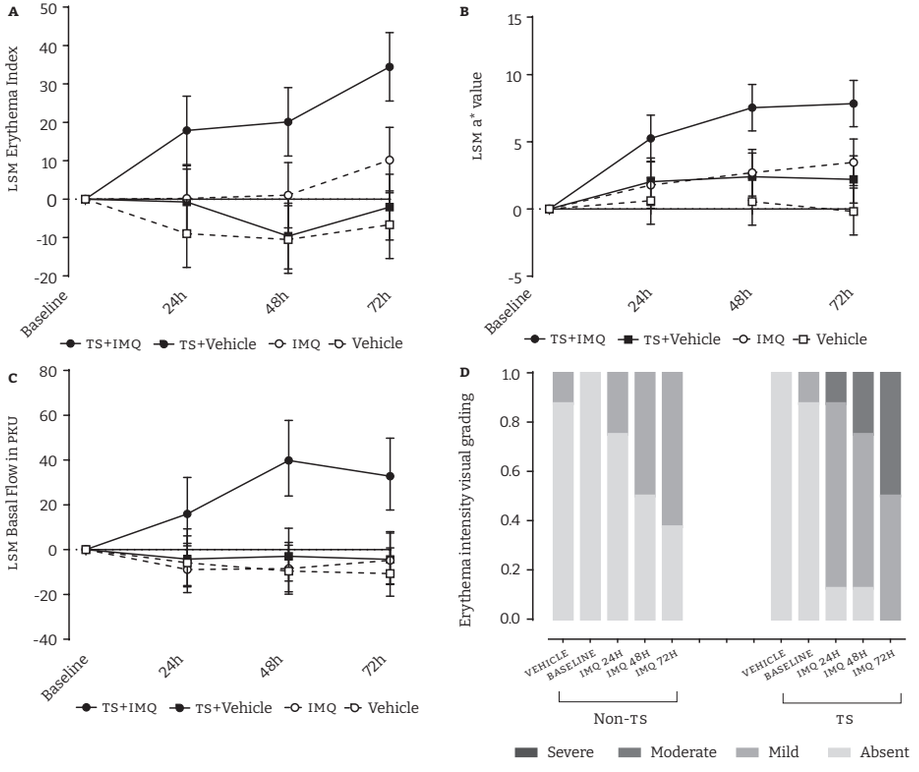


FIGURE 3 mRNA expression in skin over time in imiquimod (IMQ) and tape stripping +IMQ treated skin of CXCL10, HBD-2, Mx-A, ICAM1, TNF-α and IL-1β. *Statistical significance is indicated as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

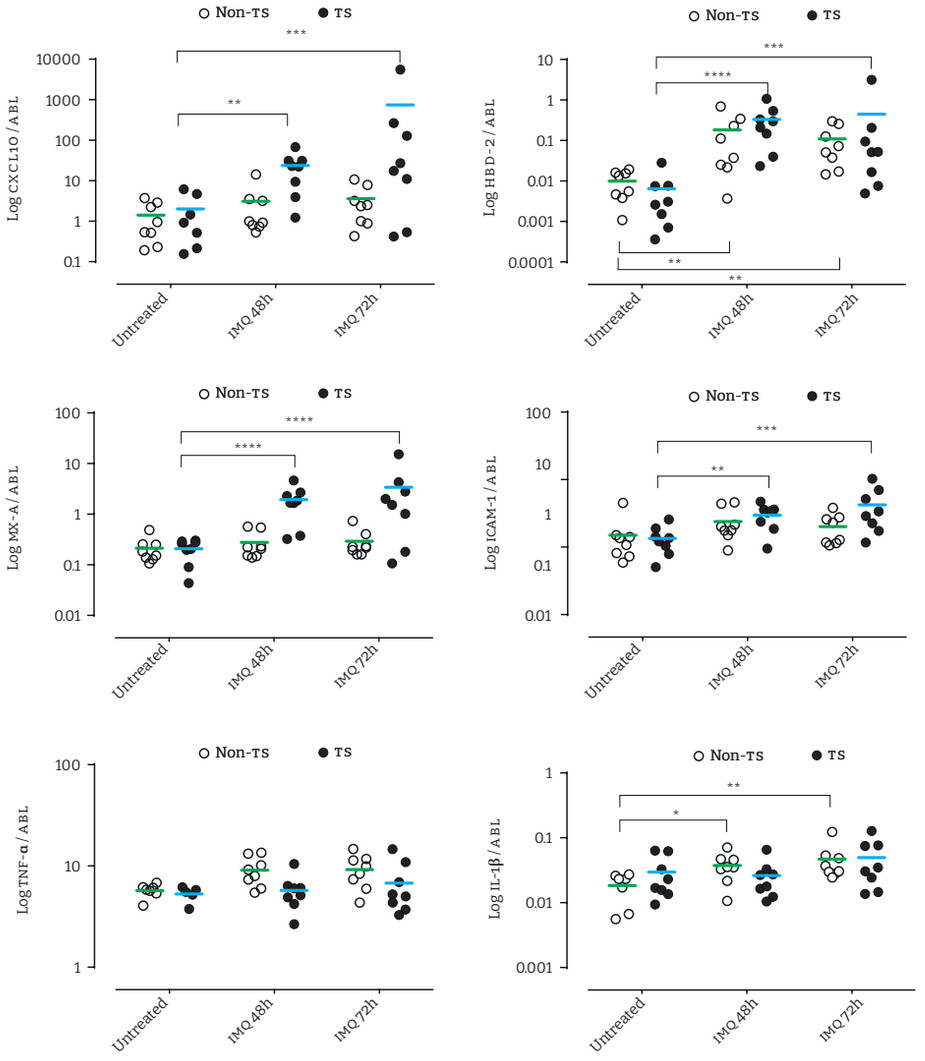
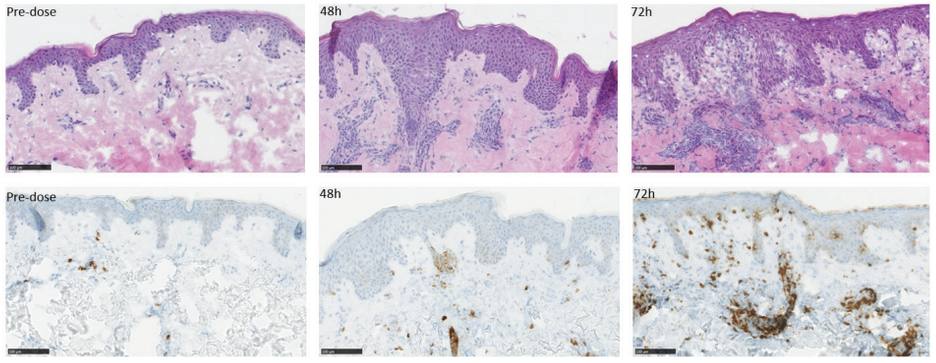


FIGURE 4 H&E (upper row) and CD8$^+$ staining (lower row) over time of subject 15 treated with TS+IMQ.



Scale bars indicate 100µm.

FIGURE 5 Histology and immunohistochemistry in skin punch biopsies of imiquimod (IMQ) and tape stripping +IMQ treated skin compared to untreated skin, displayed in % fold increase compared to the reference biopsy. A) general infiltration, B) acanthosis, C) CD4$^+$ and CD8$^+$ infiltration, D) CD11c$^+$ and HLA-DR infiltration. Fraction of subjects is depicted.

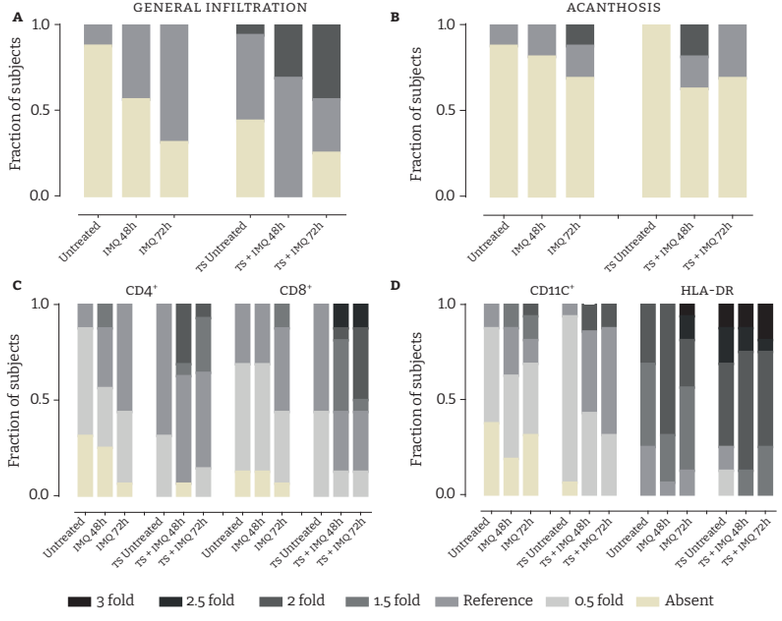
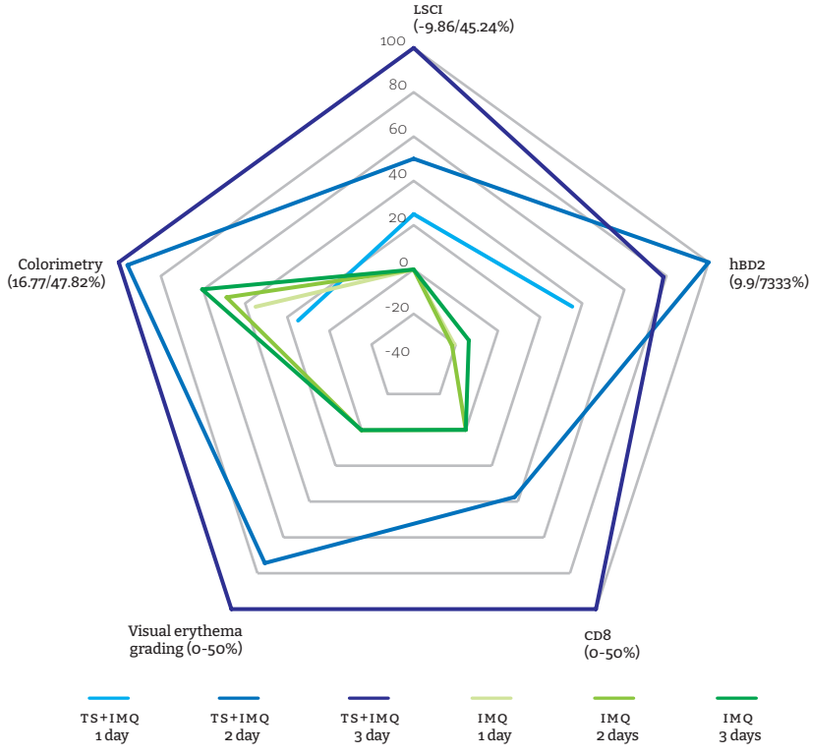
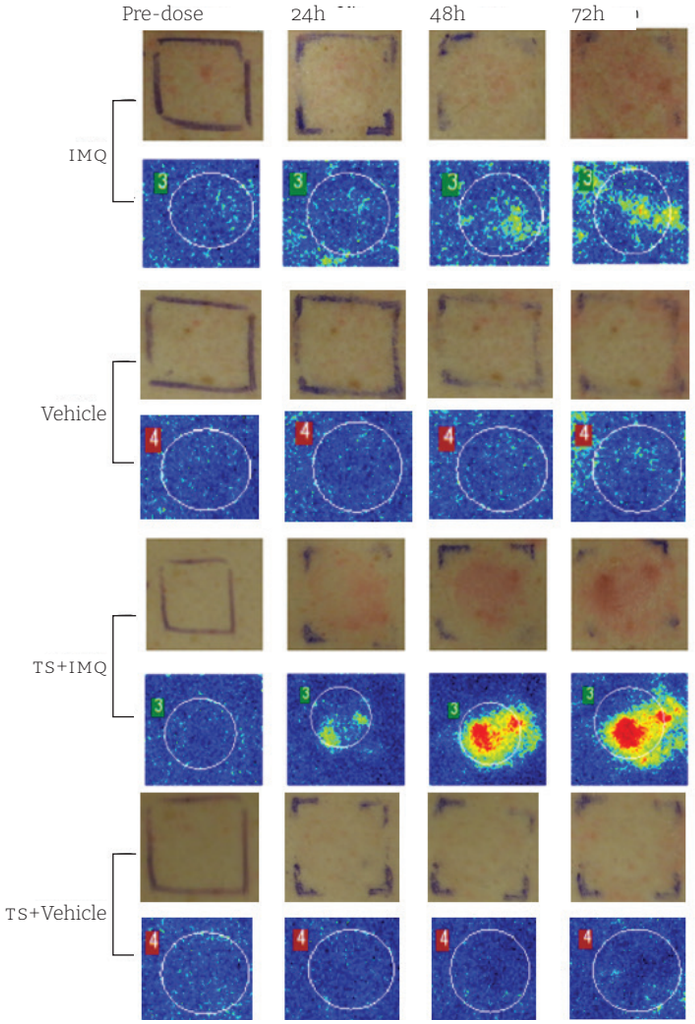


FIGURE 6 Multi-modal assessment of the model including clinical (physician scoring), biophysical (laser speckle contrast imaging), imaging (colorimetry), molecular (mRNA expression, hBD2) and cellular (IHC, CD8$^+$) aspects. The observed maximal effect is used for normalization of the respective axes. For the imiquimod alone group, only increased erythema by colorimetry is observed without a dose dependent relationship. In the tape stripping+imiquimod group it is clearly visible that the effects on all domains spread over the spiderplot in a dose dependent manner.



SUPPLEMENTAL MATERIAL

FIGURE S1 Clinical impression and heat maps of laser speckle contrast imaging of the skin inflammation models in one subject with IMQ treatment and another subject upon TS+IMQ treatment. Topical application of IMQ induces mild erythema and a slight increase in perfusion. When combined with TS prior to dose administration, moderate erythema with papules and marked increase in perfusion is observed. No increase in erythema and perfusion occurred in the vehicle controls.



CHAPTER IV

ORAL PREDNISOLONE SUPPRESSES SKIN INFLAMMATION IN A HEALTHY VOLUNTEER IMIQUIMOD CHALLENGE MODEL

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ABSTRACT

Imiquimod (IMQ) is a registered topical agent that induces local inflammation via the Toll-like receptor 7 pathway. Recently, an IMQ-driven skin inflammation model was developed in healthy volunteers for application in proof-of-pharmacology trials. The aim of this study was to profile the cellular, biochemical and clinical effects of the marketed anti-inflammatory compound prednisolone on the IMQ model.

A randomized, double-blind, placebo-controlled study was conducted in 24 healthy volunteers. Oral prednisolone (0.25 mg/kg/dose) or placebo (1:1) was administered twice daily for 6 consecutive days. Two days after treatment initiation with prednisolone or placebo, 5 mg imiquimod (IMQ) once daily for two following days was applied under occlusion on tape stripped skin of the back for 48 hours in healthy volunteers. Non-invasive (imaging and biophysical) and invasive assessments – skin punch biopsies and blister induction – were performed as well as IMQ *ex vivo* stimulation on whole blood.

Prednisolone reduced blood perfusion and skin erythema following 48h of IMQ application (95% CI [-26.4%, -4.3%], p=0.0111 and 95% CI [-7.96, -2.13], p=0.0016). Oral prednisolone suppressed the IMQ-elevated total cell count (95% CI [-79.7%, -16.3%], p=0.0165), NK and dendritic cells (95% CI [-68.7%, -5.2%], p=0.0333, 95% CI [-76.9%, -13.9%], p=0.0184) and classical monocytes (95% CI [-76.7%, -26.6%], p=0.0043) in blister fluid. Of note, TNF, IL-6, IL-8 and Mx-A responses in blister exudate were also reduced by prednisolone compared to placebo.

Oral prednisolone suppresses IMQ-induced skin inflammation, which underlines the value of this cutaneous challenge model in clinical pharmacology studies with novel anti-inflammatory compounds. In such studies, prednisolone can be used as benchmark.

INTRODUCTION

Early phase clinical research commonly evaluates the safety, tolerability, and pharmacological activity of novel compounds.[1-3] For anti-inflammatory and immunomodulatory compounds, immune challenge models are becoming increasingly popular to demonstrate 'proof of pharmacology' at an early clinical stage, thereby giving us insights in the mechanism of action and providing target engagement.[4] These pharmacological challenge models are often translated from animal work and can guide the drug developer on dose selection and dosing regimen for subsequent phase II studies in the target population.

A widely used preclinical model to study (modulation of) inflammation, also applied in healthy volunteers (HV), is the topical imiquimod (IMQ) challenge, driving a toll like receptor (TLR) 7-mediated response. The TLR dependent pathway activates nuclear factor kappa B (NF-κB) signaling and IRF via MY-D88, which is important in an early immune response such as secretion of pro-inflammatory cytokines including interferon (IFN)-α, interleukin (IL)-1, IL-1RA, IL-6, and IL-8.[5] IMQ, applied under occlusion for 48 hours, drives a transient local reversible inflammatory response indicated by an increase in blood perfusion, erythema, and cytokine production.[6] The IMQ model was valuable in the evaluation of the potential combined effect of IMQ and omiganan (a synthetic indolicidin) in HV.[7] The IMQ challenge can be valuable for proof-of-mechanism studies with compounds that target TLR7-mediated responses. However, formal benchmarking of the topical IMQ challenge model in man, using a registered anti-inflammatory drug, has not been performed yet.

An alternative human innate immune challenge model is the intradermal LPS challenge, driving TLR4-mediated responses. Corticosteroid treatment (oral prednisolone and topical clobetasol propionate) suppressed the characteristics of the dermal inflammatory reaction, also reflected by a reduction in inflammatory cell attraction in blister fluid.[8,9] Moreover, for the topical IMQ model, profiling of the effects of a known strong anti-inflammatory compound that is widely used in dermatology,

such as oral prednisolone, would be valuable to benchmark the model for future evaluations of novel anti-inflammatory or immunomodulatory compounds.

Therefore, in this study, we aimed to profile the cellular, biochemical and clinical effects of oral prednisolone on the IMQ skin inflammation model in healthy volunteers with the goal to:

I benchmark the IMQ model for future novel anti-inflammatory compounds
II expand the mechanistic insights into the IMQ-driven skin response by a more thorough molecular and cellular evaluation
III gain insight into the immunomodulatory mechanism of prednisolone in TLR7-mediated tissue inflammation.

MATERIAL AND METHODS

This randomized, double-blind, placebo-controlled, investigator-initiated study was conducted according to the Dutch Act on Medical Research involving Human Subjects (WMO) and the study protocol was approved by a Medical Ethics Committee (Stichting Beoordeling Ethiek Biomedisch Onderzoek, Assen, The Netherlands) prior to the start of the clinical phase. Subjects gave written informed consent before any study related procedures were undertaken.

STUDY DESIGN AND SUBJECTS

A total of 24 healthy male and female Caucasian (Fitzpatrick skin type I-III) volunteers, aged between 18 and 45 years, were enrolled in this study. Health status was confirmed with a medical history, physical examination, laboratory tests, and 12-lead electrocardiograms (ECG). All participants had no family history of psoriasis, no pathological skin conditions at the treatment area, no history of hypertrophic scarring or keloid, no prior use of imiquimod and no known hypersensitivity to prednisolone.

TREATMENT AND IMQ CHALLENGE

Participants were equally randomized into two groups to receive orally prednisolone (0.25 mg/kg/dose) or placebo, twice daily with a 10-12 hours interval between doses, over a period of six consecutive days. On the sixth day, the volunteers received only one prednisolone or placebo dose in the morning. Treatments were administered under supervision at the clinical research unit to ensure treatment compliance. After two days of pre-treatment with oral prednisolone or placebo, challenge with IMQ commenced.[6] For this purpose, the upper back was divided into seven rectangles of 4x3 CM each. Each treatment area was tape stripped 20 times (D-Squame, CuDerm, Dallas, TX) to induce mild barrier skin disruption whereafter the trans-epidermal water loss (TEWL; AquaFlux, Biox Systems) was measured to quantify skin permeability.[6,7] A TEWL between 15-20 G/M^2/h was considered as mild barrier skin disruption. No IMQ was applied to the first two areas since these represented the non-treated areas, while to the other three to five areas, 5 mg IMQ (100 mg Aldara®) was applied for either 24h or 48h (depending on the cohort) under occlusion by a 12 mm Finn chamber (Bipharma, Almere, The Netherlands) to initiate an inflammatory skin reaction. Subjects were randomized on (pre-) treatment (prednisolone or placebo) and timepoint of invasive measurements (blister and biopsy), illustrated in *Figure 1*.

IMAGING-BASED ENDPOINTS

Subjects underwent multiple assessments to evaluate the inflammatory skin response; before the IMQ challenge and 24h, 48h,72h, 168h and 216h after IMQ application. One single treatment site was selected to evaluate non-invasive endpoints throughout the study period (*Figure 1*) resulting in N=12 per time point. An overview of the number of samples per time point is presented in Table S1. Erythema was graded in two ways: by a physician using a 4-point scale ranging from 0 (absent) to 3 (severe) and by multispectral photo analysis (Antera 3D, Miravex, Ireland). Perfusion was quantified by laser speckle contrast imaging (LSCI; PeriCam PSI System, Perimed Jäfälla, Sweden) and by Optical Coherence Tomography (OCT; VivoSight, Michelson Diagnostics

Maidstone, UK). The latter was also used to measure the epidermal thickness. All skin assessments were performed in a room under standardized conditions with a room temperature between 20-24 degrees Celsius.

BIOPSY AND BLISTER EXUDATE ASSESSMENTS

Suction blisters and 3 mm biopsy samples were taken from the IMQ treated areas and control areas at indicated time points, depending on the cohort and randomization (*Figure 1*). In total 3 biopsies and 3 blisters were collected from each healthy volunteer. Suction blisters were induced according to the method described by *Buters et al*.[8] Biopsies were placed in RNAlater medium directly after harvest and stored at 4 degrees Celsius until analysis at the Immunology Laboratory of Erasmus Medical Center, Rotterdam, The Netherlands. Immunohistochemical staining was performed to obtain scoring of markers CD11c (Clone 5D11; Cell Marque), CD14 (Clone EPR3653; Cell Marque), CD1a (Clone EP3622; Cell Marque), CD4 (Clone SP35; Ventana), CD8 (Clone SP57; Ventana), and HLA-DR (CR3/43; Dako) using a 6-point rating scale; 0=negative, 1=minimal, 2=few, 3=moderate, 4=many, 5=excessive.

Blister fluid was collected in a V-bottom plate containing 50 µL 3% sodium citrate (Sigma) in PBS (Gibco) and kept on ice. The plate was centrifuged, and supernatant was weighed to estimate the volume and then frozen at -80°C for cytokine analysis (Meso Scale Discovery, Rockville, Maryland, USA), the following cytokines were analysed: TNF, ASC, IL-1β, IL-6, IL-10, IL-8, IFN-γ, and downstream marker for type 1 interferon Mx-A (V-plex proinflammatory panel of MSD and Mx-A protein ELISA kit of BioVendor). The cell pellet was resuspended in RoboSep buffer (Stemcell). A cocktail of fluorescent antibodies for cell surface markers was added to the cells and incubated for 30 minutes on ice. Stained samples were washed with PBS and measured with a MACSQuant 16 (Miltenyi Biotec GmbH). Flow cytometry data was analysed with Flowlogic 7.2 (Inivai). Parallel to the blister fluid, peripheral blood was collected by venipuncture in a sodium heparin vacutainer (BD). 100 µL whole blood was treated with red blood cell lysis buffer (eBioscience) and washed with PBS and resuspended in RoboSep buffer. Staining was similar to previously mentioned blister cells and served as template for the gating strategy. The following antibodies were used: CD56-PE (cat# 130-113-312, Miltenyi Biotec), CD14-PE-Vio615 (cat# 130-110-526, Miltenyi Biotec), CD16-VioBrighT FITC (cat# 130-119-616, Miltenyi Biotec), CD66b-AF700 (cat# 305114, Biolegend), CD19-BV650 (cat# 302238, Biolegend), CD20-BV650 (cat# 302336, Biolegend), HLA DR-APC (cat# 130-111-790, Miltenyi Biotec), CD4-VioBlue (cat# 130-114-534, Miltenyi Biotec), CD8-BV570 (cat# 301038, Biolegend), CD45-VioGreen (cat# 130-110-638, Miltenyi Biotec), CD1c-PE-Vio770 (cat# 130-110-538, Miltenyi Biotec), CD3-APC-Vio770 (cat# 130-113-136, Miltenyi Biotec), 7AAD (cat# 130-111-568, Miltenyi Biotec). An overview of the gating strategy is provided in *Figure S1*. Cell populations (single live cells) were classified based on the following profile: $CD45^+$ $HLA\text{-}DR^-$ $CD66b^+$ $CD16^+$ neutrophils, $CD45^+$ $HLA\text{-}DR^+$ $CD14^+$ $CD16^-$ classical monocytes, $CD45^+$ $HLA\text{-}DR^+$ $CD14^+$ $CD16^+$ intermediate monocytes, $CD45^+$ $HLA\text{-}DR^+$ $CD14^-$ $CD16^+$ non-classical monocytes, $CD45^+$ $HLA\text{-}DR^+$ $CD19^-$ $CD20^-$ $CD14^-$ $CD16^-$ $CD1c^+$ dendritic cells, $CD45^+$ $HLA\text{-}DR^-$ $CD56^+$ NK Cells, $CD45^+$ $HLA\text{-}DR^-$ $CD3^+$ $CD4^+$ $CD8^-$ T helper cells, $CD45^+$ $HLA\text{-}DR^-$ $CD3^+$ $CD4^-$ $CD8^+$ cytotoxic T cells, and $CD45^+$ $HLA\text{-}DR^+$ $CD19^+$ $CD20^+$ B cells.

EX VIVO WHOLE BLOOD STIMULATION

To investigate the extent of the systemic immune suppression with prednisolone (*ex vivo* drug activity), an IMQ whole blood stimulation was used with cytokine release as readout. Blood was drawn from healthy volunteers at four timepoints: pre-dose, 48h, 52h and 96h after initial prednisolone/placebo dose. The blood samples of 48h and 96h were drawn before the morning prednisolone/placebo dose. The sample at 52h after first administration was taken 4 hours after the previous prednisolone/placebo dose. On these time points, sodium heparinized whole blood was stimulated with 20 µG/ml IMQ (cat# tlrl-imq, Invivogen) for 24 hours. After incubation, cultures were spined down and supernatant was collected and frozen at -80°C for cytokine analysis. Samples were shipped to Ardena (Assen, the Netherlands) for analysis. The following cytokines were analysed:

IL-1β, IL-6, IFN-γ (V-plex proinflammatory panel of MSD), IP-10 (V-plex chemokine panel of MSD) and Mx-A (human Mx-A protein ELISA kit of BioVendor).

STATISTICS All repeatedly measured PD endpoints were summarised (n, mean, SD, MIN and MAX values) by treatment and time. Repeatedly measured continuous PD endpoints were analysed using a mixed model analysis of covariance with fixed factors treatment, time and treatment by time with a random factor subject as covariate. Baseline is the pre prednisolone/placebo treatment measurement. A summary table of the analysis results per variable was generated with estimates of the difference of the different contrasts and a back transformed estimate of the difference in percentage for log transformed parameters, 95% confidence intervals (in percentage for log-transformed parameters) and Least Square Means (geometric means for log transformed parameters), and the p-value of the contrasts. Statistical analysis was performed using SAS for windows V9.4 M6 (SAS Institute, INC., Cary, NC).

RESULTS

Twenty-one female (87.5%) and three male (12.5%) Caucasian subjects participated in the study and completed without withdrawal. The mean age was 26.3 ± 4.6 years. Headache was the most frequent occurring adverse event, however, was of mild nature and was probably related to administration of prednisolone. No serious adverse events were reported throughout the study.

ORAL CORTICOSTEROIDS SUPPRESS THE CLINICAL RESPONSE INDUCED BY TOPICAL IMQ APPLICATION Oral prednisolone or placebo was administered at 0.25 mg/kg per dose B.I.D. for a period of six consecutive days, and the effects on IMQ-driven clinical responses were evaluated. IMQ was applied for a maximum of 48 hours under occlusion after the skin was tape stripped twenty times. Maximal responses were observed after 48 hours of IMQ application in the placebo group (*Figure 2A,B*). Treatment with prednisolone resulted in a reduction of the IMQ-driven response from 24 hours until 72 hours, quantified by imaging of blood perfusion (estimated difference: -16.1%, 95% confidence interval (CI) [-26.4%, -4.3%], p=0.0111), erythema (estimated difference: -5.04, 95% CI [-7.96, -2.13], p=0.0016) and epidermal thickness (estimated difference: -0.018, 95% CI [-0.029, -0.006], p=0.0044) compared to placebo, (*Figure 2A-C*). A visual overview of blood perfusion and erythema is provided in Figure 2D. Application of IMQ (for 24h and 48h) on the skin resulted in thickened epidermis, disappearance of the rete ridges and dilatation of the blood vessels (*Figure 2E*). Furthermore, prednisolone treatment shifted the clinically scored erythema response from moderate to mild (at 48h) and reduced the fraction of clinically scored erythema, *Figure S2*. All quantified responses (perfusion, erythema, and epidermal thickness) were reversible and returned to baseline during the follow-up phase (168h and 216h).

ORAL CORTICOSTEROIDS REDUCE THE IMQ-DRIVEN CELL INFILTRATION IN BLISTER EXUDATE AND BIOPSY In earlier skin challenge research, cellular responses were studied using invasive techniques including suction blisters and skin punch biopsies.[6-9] In this study, we implemented the same approaches by inducing suction blisters and taking skin punch biopsies at indicated timepoints resulting in a total of three blisters and three biopsies per subject (*Figure 1)*. Biopsy samples were stained for dermal immune cell infiltration and scored by an independent investigator who was blinded for treatment.

In addition, immune cells in blister exudate were evaluated by flow cytometry. A full overview of the analysed immune cell subsets is presented in *Figure 3*. The average time for blister induction was (estimated mean, 95% CI) 86.7 minutes, 95% CI [71.9, 101.4] in the placebo group and 84.1 minutes, 95% CI [69.4, 98.8] in the prednisolone group (data not shown). No significant difference in time required for blister formation was observed between groups (estimated difference: -2.5, 95% CI [-23.4, 18.3], p=0.8034). The total number of cells ($CD45^+$) increased mildly

following IMQ application with a peak of 624.6 ± 469.5 cells in blister exudate at 48h (after 2x IMQ application), *Figure 3A*. IMQ increased the numbers of CD45$^+$ HLA-DR$^-$ CD56$^+$ (NK cells), reaching a maximum of 84.2 ± 89.76 cells also at 48h (after 2x IMQ application), *Figure 3B*. The infiltration was followed by other innate immune cells such as CD45$^+$ HLA-DR$^+$ CD19$^-$ CD20$^-$ CD14$^-$ CD16$^-$ CD1c$^+$ (dendritic cells) and CD45$^+$ HLA-DR$^+$ CD14$^+$ CD16$^-$ ("classical monocytes") peaking at 72h (24 hours after the second IMQ application) with means ± SD of 26.0 ± 21.06 cells and 25.3 ± 27.7 cells respectively, however, resulting in a weaker response compared to influx of NK cells (*Figure 3C, D*). Although neutrophils are strongly involved in innate immune responses, no infiltration of CD45$^+$ HLA-DR$^-$ CD66b$^+$ CD16$^+$ (neutrophils) was observed after IMQ application, resulting in cell counts of <5 cells (data not shown). Similar low cell counts were observed for CD45$^+$ HLA-DR$^+$ CD14$^+$ CD16$^+$ ("intermediate monocytes"), CD45$^+$ HLA-DR$^+$ CD14$^-$ CD16$^+$ ("non-classical monocytes") and CD45$^+$ HLA-DR$^+$ CD19$^+$ CD20$^+$ (B cells), data not shown. IMQ treatment did not result in a significant B cell increase in blister fluid (data not shown), nor a substantial attraction of CD45$^+$ HLA-DR$^-$ CD3$^+$ CD4$^+$ CD8$^-$ (T helper cells) and CD45$^+$ HLA-DR$^-$ CD3$^+$ CD4$^-$ CD8$^+$ (cytotoxic T cells), *Figure 3 E, F*. Prednisolone reduced the number of immune cells in blister fluid. This reduction, compared to placebo, was observed for total cells, NK cells, dendritic cells and classical monocytes; estimated difference: -58.8%, 95% CI [-79.7%, -16.3%], p=0.0165, estimated difference: -45.5%, 95% CI [-68.7%, -5.2%], p=0.0333, estimated difference: -55.4%, 95% CI [-76.9%, -13.9%], p=0.0184 and estimated difference: -58.6%, 95% CI [-76.7%, -26.6%], p=0.0043, respectively. Although no substantial changes were seen following IMQ challenge, prednisolone significantly reduced the number of T cell subsets (estimated difference: -76.0%, 95% CI [-92.4%, -4.7%], p=0.0168 for T helper cells and estimated difference: -70.5%, 95% CI [-89.6%, -16.0%], p=0.0242) for cytotoxic T cells.

Immune cell subsets in biopsies, quantified by IHC, showed a comparable picture as the cells analysed by flow cytometry in blister fluid. Administration of prednisolone reduced HLA-DR cells and the infiltrating CD11c (dendritic cells), CD4$^+$ (T helper cells), CD1a (Langerhans cells), and CD8$^+$ cells (cytotoxic T cells), *Figure 4*. Time courses of IMQ effects and prednisolone effects were comparable between biopsy and blister derived immune cells.

ORAL CORTICOSTEROIDS SUPPRESS IMQ-DRIVEN CYTOKINE RESPONSES IN BLISTER EXUDATE AND WHOLE BLOOD CULTURES

In addition to the dermal cellular response, cytokines levels were analysed in blister exudate for evaluation of NF-κB- and IRF7-driven responses. IMQ application induced mild IL-6 response at 24h and 48h (*Figure 4A, B*) but had no clear effect on the levels of NF-κB-driven cytokines IL-1β IL-8 and IL-10 (*Figure 4C-E*). IMQ increased Mx-A concentration in blister fluid, with a peak at 48h and 72h (*Figure 4F*) indicating activation of the IRF7 pathway. Prednisolone treatment reduced the levels of NF-κB-driven cytokines (IL-6, IL-8, and TNF), IL-1β and Mx-A (*Figure 4A,B,C,F*). No formal statistical analysis for cytokines IL-6, IL-1β and Mx-A was conducted for the contrast placebo versus prednisolone because the immune suppression by prednisolone was so strong that most cytokine levels were below LLOQ. ASC and IFN-γ concentrations in blister fluid were very low and are therefore not reported.

Whole blood samples, drawn from study participants at predefined timepoints, were stimulated with IMQ for evaluation of *ex vivo* prednisolone activity. Stimulation with IMQ led to an increase in IL-1β, IFN-γ and IL-6 (*Figure 6A-C*), but not in a detectable Mx-A response (data not shown). An overview of the *ex vivo* results, including unstimulated control conditions, is provided in Table S2. Overall the effect of prednisolone compared to placebo had a significant effect on IFN-γ and IL-1β concentrations (estimated difference: -86.8%, 95% CI [-94.1%, -70.3%, p<0.0001), -55.8%, 95% CI [-78.8%, -8.3%], p=0.0301 respectively), yet not on IL-6 (estimated difference: -44.1%, 95% CI [-69.1%, 1.0%], p=0.0537). However, prednisolone treatment resulted in a statistically significant reduction of IMQ-driven cytokines (IL-1β, IL-6 and IFN-γ), with a maximum inhibitory effect at 52h post administration, estimated difference:-92.9%, 95% CI [-96.8%,

-84.3%], p<0.0001, estimated difference: -87.1%, 95% CI [-93.2%, -75.7%], p<0.0001 and estimated difference: -99.0%, 95% CI [-99.6%, 97.4%], p<0.0001, respectively. Of interest, prednisolone had no effect on IMQ-induced response at timepoints 48h and 96h. No statistical analysis was performed on IP-10 concentration given the fact that more than 60% of the samples were above ULOQ.

DISCUSSION

This study aimed to characterize the effects of orally administered prednisolone on TLR7-driven immune responses, using *in vivo* skin and *ex vivo* whole blood IMQ challenges. Prednisolone significantly suppressed the objectified transient clinical response to IMQ (imaging-based perfusion and erythema) as well as clinically graded erythema. An interesting finding was the inhibitory effect of prednisolone on the epidermal thickness measured by optical coherence tomography, a non- invasive technique generating 2D images of tissue microstructure. In a psoriasiform murine model, an abundance of infiltrating cells resulted in a significant increase of epidermal thickness .[10] This is in line with our study, showing an IMQ-driven increase in epidermal thickness at 48h, suggesting initiation of an inflammatory response.

To date, this is the first clinical study to examine the effect of prednisolone on TLR7-driven cellular and cytokine response in vivo. Earlier studies evaluated the local immune response following topical IMQ application. In these studies, the dermal cellular and cytokine responses to IMQ were characterized by immunohistochemistry and QPCR. IMQ treatment resulted in upregulated chemokines (IP-10), pro-inflammatory cytokine (IL-6) and interferons (Mx-A and IFN-γ). Elevated CD14, CD1a, CD11c, $CD4^+$ and $CD8^+$ cell numbers were observed following IMQ application on tape stripped skin, peaking at 48- and 72-hours post-dose, fully comparable to IHC results in this study.[6,7] However, current study also evaluated IMQ-driven cellular responses in the skin by flow cytometric analysis of suction blister exudate, giving a more quantitative impression of the inflammatory response. Upon IMQ treatment, a mild influx of NK cells, dendritic cells and classical monocytes was observed. Orally administered prednisolone fully suppressed this cell infiltration, confirming the compound's strong anti-inflammatory activity. Of note, no traces of neutrophils were found in blister fluid, indicating that the applied IMQ regimen did not drive neutrophil attraction, which contradicts earlier preclinical findings, showing topical IMQ treatment for 5-6 consecutive days in mice.[11] This resulted in an influx of neutrophils, accumulating beneath the stratum corneum. Possibly, the absence of neutrophil infiltration in our study can be explained by the duration of IMQ application. The current duration of 48h IMQ application might not be sufficient to initiate a neutrophil influx and therefore a prolonged application is suggested to align more with the duration in preclinical studies. In contrast to IMQ, LPS drives an acute and strong innate immune response characterized by influx of neutrophils (peaking at 10h), monocytes and NK cells peaking at 24h post injection.[8]

TLR7 activation generally drives IRF and NF-κB signalling, playing an important role in recruitment of immune cells to the dermis.[12] Topical application of IMQ resulted in a clear increase in Mx-A concentrations in blister exudate at 48 and 72 hours, indicative of IRF-mediated production of type 1 interferons. Whilst it is expected that IMQ initiates NF-κB signalling and cytokine production,[12-14] only a mild IL-6 response was observed, whereas other NF-κB-driven cytokine responses (IL-8, IL-1β, and IL-10) were limited or absent. The relatively mild inflammatory response at the molecular level may be explained by a potentially limited IMQ exposure, related to the only partial delivery of applied IMQ to the dermal tissue. Alternatively, the low NF-κB responses may be explained by the timing of the sampling: the innate immune system is activated in phases, with the initial phase resulting in the secretion of proinflammatory cytokines generally occurring within 24 hours.[5,15,16] Possibly, our first post-IMQ blister time point was already too late to detect the early innate immune response driven by IMQ. Lastly, it is well possible that IMQ in this formulation and at the current regimen, for these types of

innate immune challenges is simply a weak immune agonist compared to the LPS. This is also supported by the relatively mild cellular responses that were observed. Despite the small cytokine responses, a clear effect of prednisolone treatment was observed: Mx-A, IL-6, TNF, and IL-1β responses to IMQ were significantly lower in the prednisolone-treated volunteers (though for Mx-A, IL-6, and IL-1β no formal p value could be calculated given the substantial number of samples with cytokine levels below the limit of quantification in the prednisolone-treated group). For future studies, the abovementioned readouts could be used to evaluate the effect of IMQ.

Interestingly, oral prednisolone and topical clobetasol treatment did not significantly suppress dermal cytokine responses in an earlier human challenge study applying intradermal LPS injections, driving TLR4-mediated responses.[8] This contrasts with the prednisolone effects on IMQ-driven cytokine responses observed in the current study. The difference in treatment response might be explained by the much more pronounced dermal cytokine response driven by LPS (~ 5 to 100-fold) compared to IMQ. This difference in challenge response size, also reflected at the cellular level, may be explained by the different routes of administration - intradermal for LPS versus topical for IMQ leading to difference in intradermal concentrations. Furthermore, LPS may also be a more potent immune agonist. The argument that a stronger immune response is more difficult to counteract by a corticosteroid is contradicted by the efficient suppression of LPS-driven skin perfusion, erythema, and local cell attraction by prednisolone and clobetasol. Therefore, most likely, the successful suppression of IMQ-driven cytokine responses by prednisolone, versus the poor suppression of LPS-driven responses of the same cytokines, should be searched at the physiological level: the difference between TLR4 and TLR7 signalling.

Finally, *ex vivo* pharmacological activity of prednisolone was monitored by means of whole blood IMQ challenges. Prednisolone treatment resulted in a significant reduction of IFN-γ, IL-1β and IL-6 release, but only when measured at 52 hours after initiation of prednisolone treatment, which was 4 hours after the previous prednisolone intake. Cytokine release was not suppressed at timepoints 48 and 96 hours following first prednisolone administration. This may be explained by the pharmacokinetic profile of prednisolone which is considerably complex in humans.[17] Prednisolone is rapidly absorbed and is available between 80%-100% after an oral intake. The plasma concentration peaks 1 to 2 hours after administration and the corresponding half- lives vary between 2.5h and 6.6h and are dose dependent.[18,19] At 48 and 96 hours, shortly before the next prednisolone dose, the suppressive effect of prednisolone is neglectable since the systemic concentration of the drug is considered low. Of interest, the reductions seen in cytokine levels in blister fluid are less dependent on the pharmacokinetic profile of prednisolone, which is concordant with literature describing that no relationship has been demonstrated between prednisolone concentration in blood and therapeutic effect.[20,21] This discrepancy between the systemic drug activity measured in whole blood cultures *ex vivo*, and the peripheral drug effect evaluated in skin *in vivo*, underlines the value of *in vivo* human immune challenges such as the topical IMQ challenge for evaluation of drug effects.

In this clinical study, we successfully demonstrated that orally administered prednisolone, at a conventional clinical dose, suppresses IMQ-induced skin inflammation in healthy volunteers, which underlines the potential value of this cutaneous challenge model for future clinical pharmacology studies with novel anti-inflammatory compounds targeting the TLR7 pathway. In such studies, prednisolone can be used as benchmark.

Figure 1 Overview of the treated sites on the back and study schedule. A) A total of three biopsies and three blisters were obtained from each subject, with the timing and location of each procedure determined by their assigned cohort. One site on the back was used solely for non-invasive measurements throughout the study period. The skin responses were evaluated using a multi-modal approach and are represented in the 'derma flower.' B) A schematic overview of all assessments performed relative to dosing. *Created with BioRender.com. Derma flower created by F. van Meurs, adapted for this manuscript.*

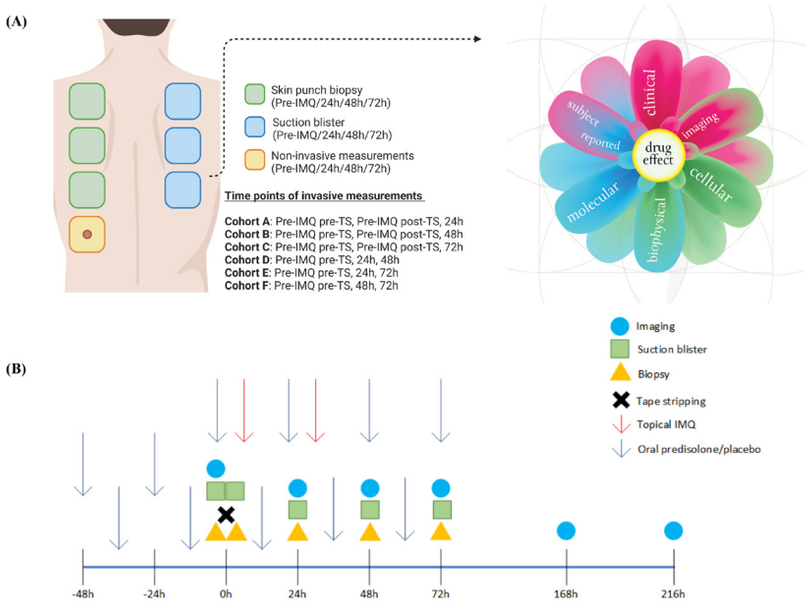
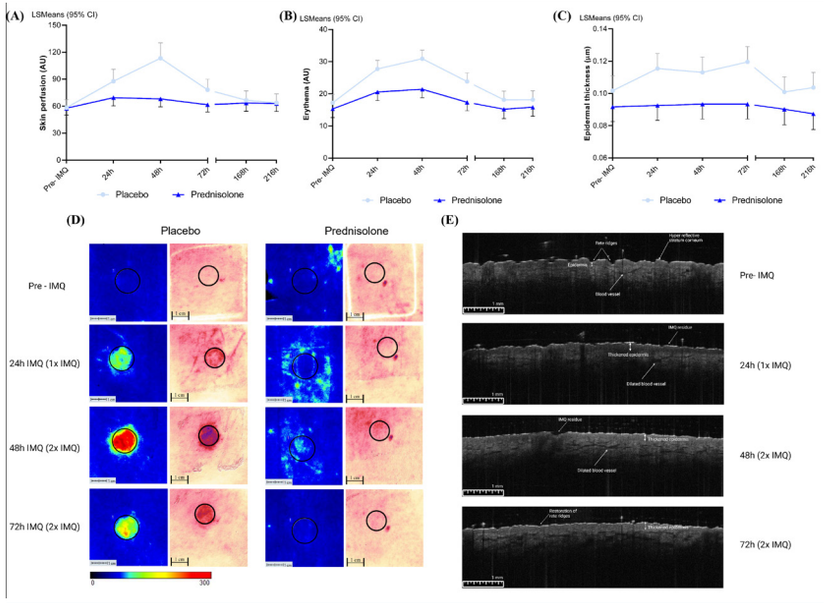
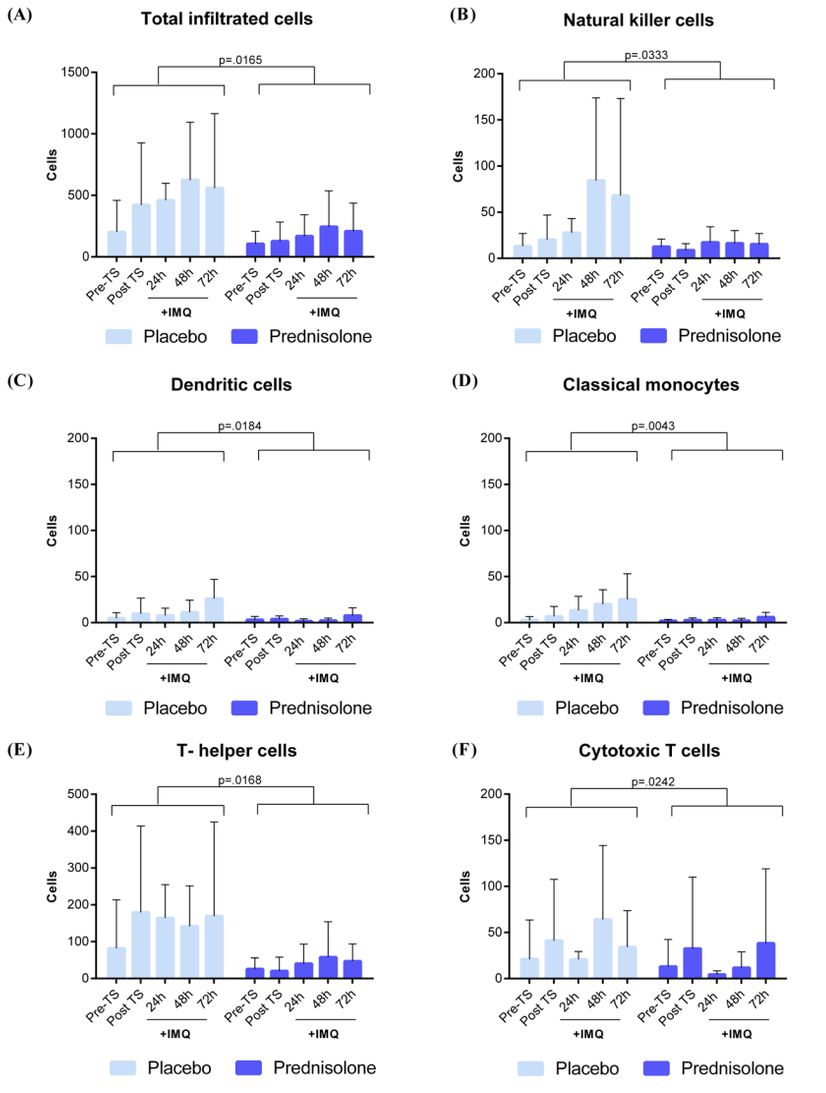


Figure 2 Imaging based and clinical erythema grading assessment. Pre-dose timepoint refers to assessment prior to IMQ application, whereas 24h refers to 1x IMQ application and 48h to 2x IMQ application. 72h refers to 2x IMQ application however measured 24 hours post last IMQ application. A) Skin perfusion by LSCI, estimated difference: -16.1%, 95% CI [-26.4%, -4.3%], p=0.0111. B) Erythema measured by multispectral camera, estimated difference: -5.04, 95% CI [-7.96, -2.13], p=0.0016. C) Epidermal thickness by OCT, estimated difference: -0.018, 95% CI [-0.029, -0.006], p=0.0044. D) Overview of objective assessments including LSCI and multispectral imaging in the prednisolone and placebo group. A ROI of 12 mm is selected for quantification. E) OCT image of one subject of the placebo group after application of IMQ over time.



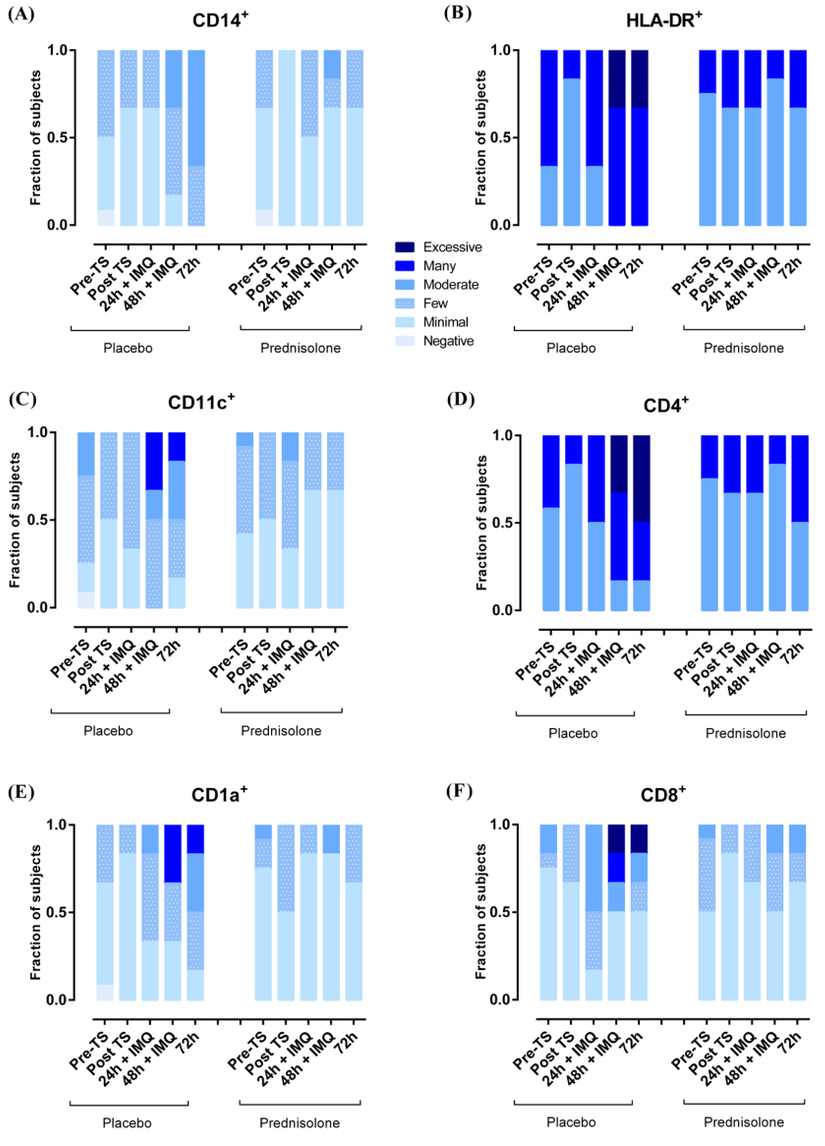
Abbreviations: CI=confidence interval, IMQ=Imiquimod, LSCI=Laser speckle contrast imaging, LSMEANS=Least Squares Mean, OCT=Optical coherence tomography.

Figure 3 Oral prednisolone reduces IMQ- driven immune cell infiltration. A) Total cells, B) Natural killer cells, C) Dendritic cells, D) Classical monocytes, E) T helper cells, F) Cytotoxic T cells. Immune cells in blister exudate were quantified by flow cytometry at different time points, pre and post IMQ application. Data are presented as mean ± SD.



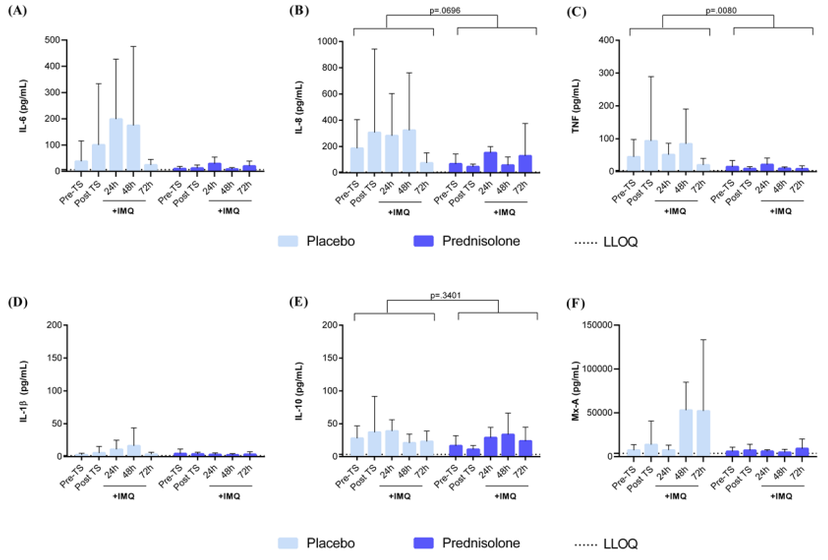
Abbreviations: TS = tape stripped.

Figure 4 Prednisolone reduces infiltration of IMQ-driven immune cells as measured by IHC in skin punch biopsies. A) Macrophages, B) HLA-DR, C) myeloid dendritic cells, D) T helper cells, E) Langerhans cells, F) Cytotoxic T cells.



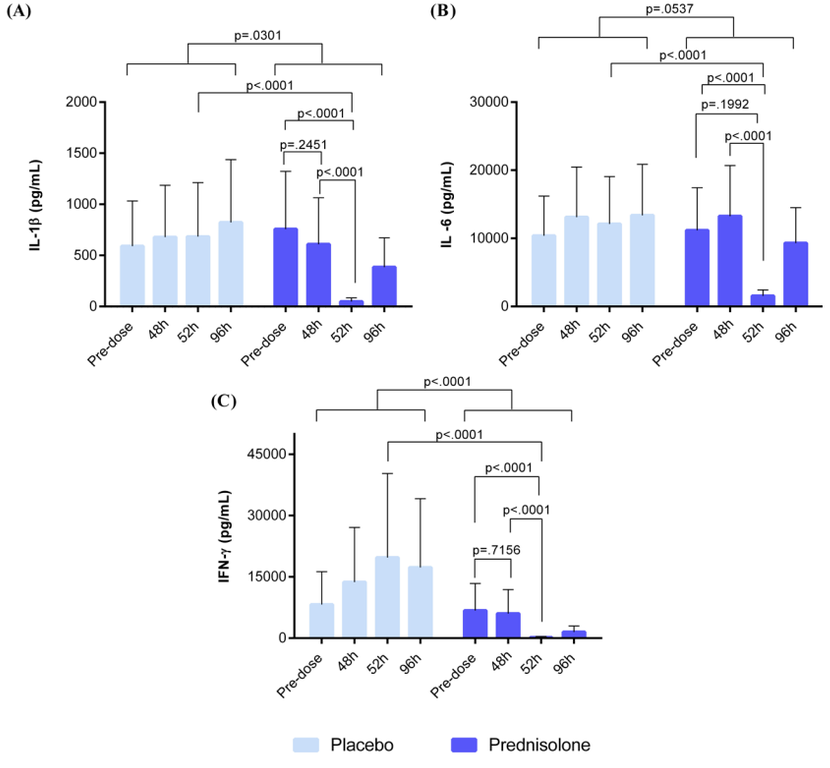
Abbreviations: TS = tape stripped.

Figure 5 Prednisolone suppresses thE NF-κB Driven cytokines and IRF7 driven response in blister fluid. A) IL-6, B) IL-8, C) TNF, D) IL-1β, E) IL-10, F) Mx-A. Cytokine concentrations in blister fluid were analysed by MSD and Mx-A by ELISA. Data are presented as mean ± SD, for IL-6, IL-1β and Mx-A no statistical model was applied as the majority of the values were <LLOQ in the prednisolone group.



Abbreviations: IL=interleukin, TNF=tumor necrosis factor, MSD=meso scale discovery, ELISA=enzyme- linked immunosorbent assay, LLOQ=lower limit of quantification, TS=tape stripped.

Figure 6 Ex-vivo whole blood stimulated with 20 µg/ml IMQ. A) IL-1β, B) IL-6, C) IFN-γ. Cytokine concentrations in blister fluid were analysed by V-plex and MSD. Pre-dose time point refers to sample take prior to prednisolone/placebo dosing. The time point 48h refers to sample taken at 48h after initial dose but before the 5th dose of prednisolone/placebo. Sample taken at 52h refers to time point 4 hours after 5th dose. At 96h after prednisolone/placebo, the sample was taken before the 8th dose).



Abbreviations: IL=interleukin, IFN=interferon, MSD=meso scale discovery.

SUPPLEMENTAL MATERIAL

Table S1 Overview of number of samples per assessment.

Assessment	Prednisolone (N)	Placebo (N)	Reference
Imaging	12		*Figure 2*
SUCTION BLISTERS			*Figure 3 + 4*
Pre tape stripping	12	12	
Post tape striping	6	6	
24h	6	6	
48h	6	5	
72h	6	6	
BIOPSY			*Figure 5*
Pre tape stripping	12	12	
Post tape striping	6	6	
24h	6	6	
48h	6	6	
72h	6	6	
WHOLE BLOOD STIMULATION			*Figure 6*
Pre- dose	12	12	
48h	12	12	
52h	12	10	
96h	12	12	

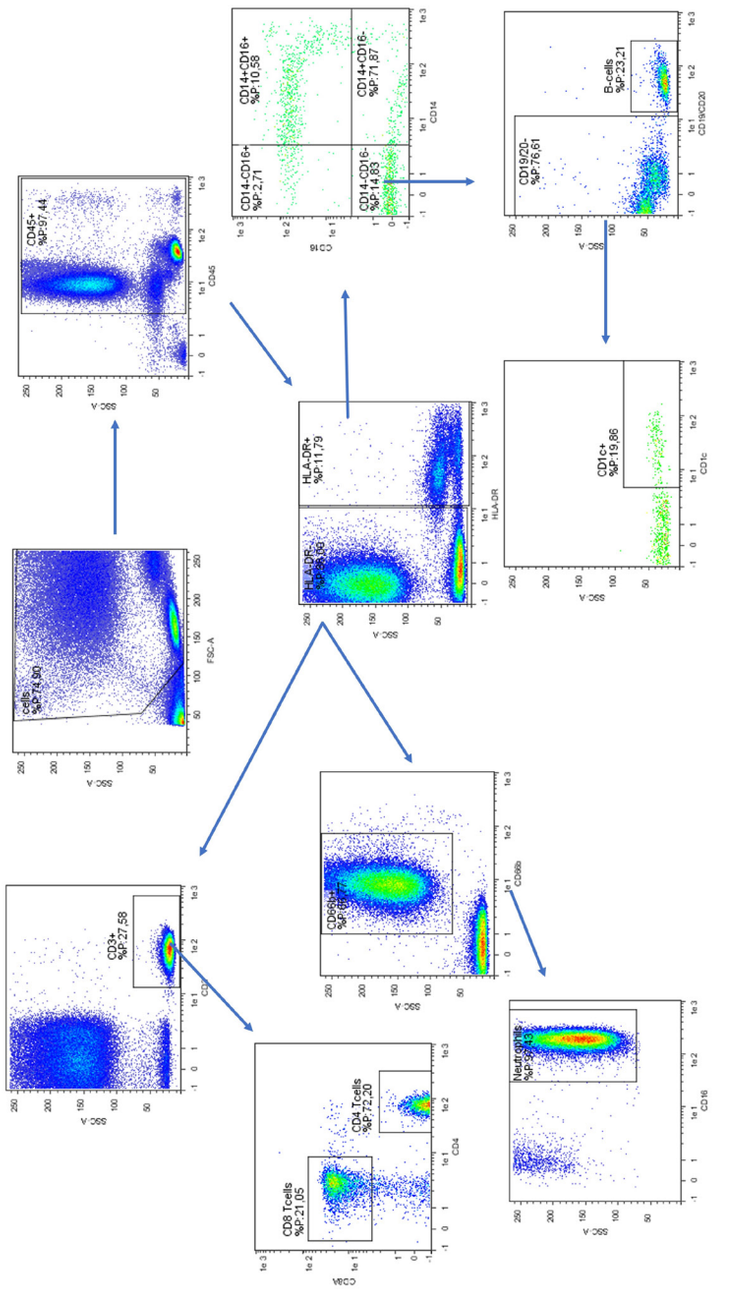


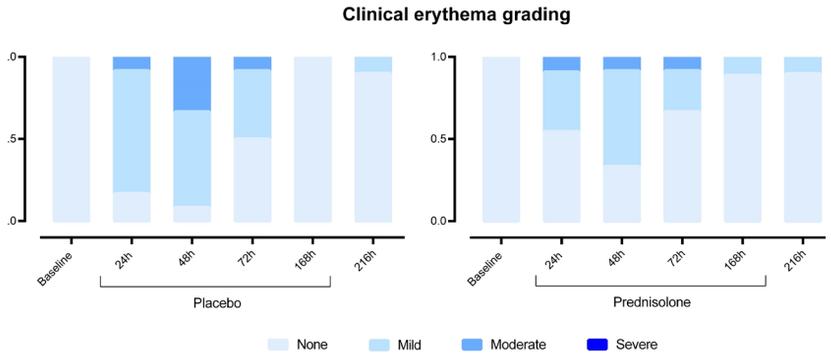
Figure S1 Gating strategy used for flow cytometry analysis of the blister exudate.

Table S2 Overview of whole blood ex vivo results stimulated and unstimulated with 20µg/mL IMQ.

	IFN-γ		IL-1β		IP-10		IL-6		Mx-A	
	St. with IMQ	UnSt.	St. with IMQ	UnSt.	St. with IMQ	UnSt.	St. with IMQ	UnSt.	St. with IMQ	UnSt.
	Mean ± SD (pg/mL)									
PLACEBO										
Pre-dose	14426 ± 18648	38 ± 39	806 ± 712	2 ± 1.7	97133 ± 24778	900 ± 1536	13780 ± 10395	7.0 ± 6.6	15885 ± 32982	14219 ± 33578
48h	22984 ± 21880	37 ± 41	1016 ± 888	1.6 ± 1.0	941912 ± 29086	1037 ± 1813	17669. ± 13116	6.1 ± 5.0	15705 ± 33037	14006 ± 33669
52h	28545 ± 26669	55 ± 106	1052 ± 1611	1.8 ± 1.3	89270 ± 24812	1529 ± 3561	13727 ± 10750	6.9 ± 7.2	17144 ± 36339	16256 ± 36808
96h	25919 ± 23784	49 ± 58	1220 ± 1191	1.7 ± 1.0	97108 ± 25927	1079 ± 1445	17050 ± 13378	11.6 ± 17.9	15686 ± 33042	13658 ± 33757
PREDNISOLONE										
Pre-dose	10801 ± 6778	24 ± 9	1083 ± 865	1.6 ± 0.8	94683 ± 25500	344 ± 378	14147 ± 11184	16 ± 4	5576 ± 3871	2674 ± 1914
48h	12362 ± 6030	23 ± 7	853 ± 700	2.1 ± 1.2	102675 ± 17272	388 ± 675	16422 ± 13266	10.7 ± 13.0	5619 ± 3911	2802 ± 2186
52h	317 ± 193	21 ± 0	90 ± 99	1.6 ± 0.9	40970 ± 30494	115 ± 111	2293 ± 1558	2.8 ± 0	5051 ± 3928	2772 ± 2195
96h	3346 ± 1509	21 ± 0	499 ± 330	1.9 ± 1.3	91975 ± 28295	166 ± 169	11130 ± 9302	5.6 ± 9.9	5004 ± 3867	2768 ± 2154

St. = Stimulated

Figure S2 Clinical erythema assessment by physician.



REFERENCES

1 Borcherding, S. M. Drugs: From Discovery to Approval. Journal of Pharmacy Technology vol. 20 (John Wiley \& Sons, 2004).
2 Pocock, S. J. Clinical Trials: A practical Approach. (Wiley & Sons , 2013).
3 Sedgwick, P. What are the four phases of clinical research trials? BMJ 348, 1–2 (2014).
4 Cohen, A. F., Burggraaf, J., van Gerven, J. M. A., Moerland, M. & Groeneveld, G. J. The Use of Biomarkers in Human Pharmacology (Phase I) Studies. Annu. Rev. Pharmacol. Toxicol. 55, 55–74 (2015).
5 Flutter, B. & Nestle, F. O. TLRs to cytokines: Mechanistic insights from the imiquimod mouse model of psoriasis. Eur. J. Immunol. 43, 3138–3146 (2013).
6 van der Kolk, T. et al. Comprehensive, Multimodal Characterization of an Imiquimod-Induced Human Skin Inflammation Model for Drug Development. Cts-clinical Transl. Sci. 11, 607–615 (2018).
7 Niemeyer-van der Kolk, T. et al. Omiganan Enhances Imiquimod-Induced Inflammatory Responses in Skin of Healthy Volunteers. Clin. Transl. Sci. 13, 573–579 (2020).
8 Buters, T. P. et al. Intradermal lipopolysaccharide challenge as an acute in vivo inflammatory model in healthy volunteers. Br. J. Clin. Pharmacol. 88, 680–690 (2022).
9 Buters, T. P. et al. Clinical, Cellular, and Molecular Effects of Corticosteroids on the Response to Intradermal Lipopolysaccharide Administration in Healthy Volunteers. Clin. Pharmacol. \& Ther. 111, 964–971 (2022).
10 Silver, R. et al. Using optical coherence tomography for the longitudinal non-invasive evaluation of epidermal thickness in a murine model of chronic skin inflammation. Ski. Res. Technol. Off. J. Int. Soc. Bioeng. Ski. [and] Int. Soc. Digit. Imaging Ski. [and] Int. Soc. Ski. Imaging 18, 225–231 (2012).
11 van der Fits, L. et al. Imiquimod-Induced Psoriasis-Like Skin Inflammation in Mice Is Mediated via the IL-23/IL-17 Axis. J. Immunol. 182, 5836–5845 (2009).
12 Schön, M. P. & Schön, M. Imiquimod: Mode of action. Br. J. Dermatol. 157, 8–13 (2007).
13 Megyeri, K. et al. Stimulation of interferon and cytokine gene expression by imiquimod and stimulation by Sendai virus utilize similar signal transduction pathways. Mol. Cell. Biol. 15, 2207–2218 (1995).
14 Gibson, S. J. et al. Cellular requirements for cytokine production in response to the immunomodulators imiquimod and S-27609. J. Interf. cytokine Res. Off. J. Int. Soc. Interf. Cytokine Res. 15, 537–545 (1995).
15 Olesen, C. M. et al. Advancement through epidermis using tape stripping technique and Reflectance Confocal Microscopy. Sci. Rep. 9, 12217 (2019).
16 Heib, V. et al. Mast cells are crucial for early inflammation, migration of Langerhans cells, and CTL responses following topical application of TLR7 ligand in mice. Blood 110, 946–953 (2007).
17 Bashar, T., Apu, M. N. H., Mostaid, M. S., Islam, M. S. & Hasnat, A. Pharmacokinetics and bioavailability study of a prednisolone tablet as a single oral dose in bangladeshi healthy volunteers. Dose-Response 16, 1–6 (2018).
18 Tanner, A., Bochner, F., Caffin, J., Halliday, J. & Powell, L. Dose-dependent prednisolone kinetics. Clin. Pharmacol. Ther. 25, 571–578 (1979).
19 Al-Habet, S. & Rogers, H. J. Pharmacokinetics of intravenous and oral prednisolone. Br. J. Clin. Pharmacol. 10, 503–508 (1980).
20 Pickup, M. E. Clinical pharmacokinetics of prednisone and prednisolone. Clin. Pharmacokinet. 4, 111–128 (1979).
21 Kauh, E. et al. Prednisone affects inflammation, glucose tolerance, and bone, turnover within hours of treatment in healthy individuals. Eur. J. Endocrinol. 166, 459–467 (2012).

CHAPTER V

EXTENDING THE IMQ MODEL: DEEP CHARACTERIZATION OF THE HUMAN TLR7 RESPONSE FOR EARLY DRUG DEVELOPMENT

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ABSTRACT

Imiquimod (IMQ; brand name Aldara®) is a registered topical agent that has been proven to induce local inflammation via the Toll-like receptor (TLR)7 pathway. The purpose of this study was to characterize TLR7-mediated inflammation following 7 days (168h) of topical IMQ exposure in healthy volunteers, and to compare the effects of short exposure (48h-72h) with prolonged exposure (120h-168h).

IMQ (100 mg) was applied under occlusion to 5 different tape-stripped treatment sites on the back of 10 healthy participants for a maximum of 7 consecutive days. Erythema and skin perfusion were measured daily up to 168h. Biopsies for immunohistochemical staining and RNA sequencing were collected at 0h, 48h, 72h, 120h and 168h post IMQ application.

IMQ triggered an inflammatory response starting at 48h after application, including erythema and perfusion of the skin. At the transcriptomic level, IMQ induced TLR7 signalling, IRF involvement and activation of TNF signalling via NF-κB. Furthermore, an enhanced inflammatory response at the cellular level was observed after prolonged IMQ exposure, with cellular infiltration of dendritic cells, macrophages and T cells which was also corroborated by transcriptomic profiles. No difference was found in the erythema and perfusion response after 168h of IMQ exposure compared to 72h.

Prolonged IMQ exposure revealed enhanced cellular responses and additional pathways with modulated activity compared to short exposure and can therefore be of interest as a model for investigational compounds targeting innate and adaptive immune responses.

INTRODUCTION

Mouse models are the backbone of the preclinical development of investigational compounds, but in the field of inflammatory diseases, species differences can be pronounced and can hamper the translational step from animals to humans.[1,2] In this context, human innate immune challenge models are a valuable tool to mimic components of the pathophysiology of a disease state in a healthy individual. Innate immune challenge models can thereby unravel underlying human physiological processes and facilitate the evaluation of pharmacological effects of investigational compounds in early clinical development.

Imiquimod (IMQ) is primarily an agonist of the Toll-like receptor (TLR)7, an endosomal TLR which recognizes single stranded RNA from viruses. IMQ is marketed as a 5% cream (Aldara®) for the treatment of (pre)malignant and HPV-induced skin lesions because of its antiviral and tumoricidal effects.[3] The antiviral and tumoricidal effects are attributed to the attraction of TLR7-bearing monocytes, macrophages and plasmacytoid dendritic cells (pDCs), which subsequently produce proinflammatory cytokines and chemokines and attract other immune cells to the application site.[4] Topical IMQ has been used as a challenge agent to induce psoriasis-like skin inflammation in mice.[4-6] In these studies, mice were exposed to IMQ for 120h to 168h, resulting in a dose-dependent clinical inflammation (i.e. increased ear thickness, erythema and scaling) for the entire duration of the treatment.[4,5] The clinical inflammation was accompanied by a substantial influx of T cells, conventional dendritic cells (DCs) and pDCs, with an essential role for the interleukin (IL) 23/IL-17 axis.[4,6] Additionally, research showed that the murine IMQ response was driven by neutrophil influx and complement factor C3.[5]

IMQ was previously used as a human pharmacological challenge agent in multiple studies, where it was topically applied for 48h or 72h.[7,8] In these studies, IMQ application resulted in a transient, mild to moderate local skin inflammation with a significant increase in skin erythema and perfusion peaking 48h after the first application. Consistent with

the working mechanism of IMQ, the response was accompanied by a clearly increased expression of Mx-A, an interferon-driven protein, suggesting engagement of Interferon Regulatory Factor (IRF)7 signalling.[7,8] Interestingly, the cellular and molecular responses after 48h-72h were relatively mild with a moderate influx of monocytes, natural killer (NK) cells and DCs, mild IL-6 production, and no significant deposition of complement. There was almost no involvement of neutrophils in this model after 48h-72h of IMQ application.[8] The lack of neutrophil involvement is surprising given the molecular signalling of TLR7,[4] and contrasts the preclinical findings in mice, in which neutrophils play a more prominent role. Notably, the duration of the exposure to IMQ in clinical studies has never exceeded 72h, whilst preclinical studies usually span up to 6 days. Characterization of IMQ-induced skin inflammation in healthy participants following extended exposure may therefore elucidate valuable novel aspects of the model and the underlying human immune response.

The purpose of this study was to characterize TLR7-mediated inflammation following 7 days (168h) of IMQ exposure in healthy volunteers. We aimed to provide deeper insights into the translational value of the IMQ model for future early-stage clinical studies, particularly for the investigation of the pharmacological activity of innate immune-targeting compounds.

MATERIALS AND METHODS

This clinical study was a single-centre, open-label, investigator-initiated inflammatory challenge study executed in accordance with the Dutch Act on Medical Research involving Human Subjects (WMO). The study protocol was approved by a Medical Ethics Committee (Stichting Beoordeling Ethiek Biomedisch Onderzoek, Assen, the Netherlands). Written informed consent was obtained from all subjects prior to any study-related procedures.

STUDY DESIGN AND INCLUSION

We recruited ten healthy male and female volunteers between the ages of 18 and 45 and with Fitzpatrick skin types I-III. Their health status was assessed by means of medical history, physical examination, laboratory tests, and 12-lead electrocardiograms (ECG). Participants were excluded if they had a familial history of psoriasis, pathological skin conditions in the treatment area, prior experience with hypertrophic scarring or keloid, or if they were exposed to IMQ within three months of enrolment.

TREATMENT

IMQ was topically applied to five treatment sites on the back for a maximum of seven consecutive days (168h). The back was marked with six squares: one untreated area and five treatment areas (*Figure 1)*. Each treatment area was tape stripped (D-Squame, CuDerm, Dallas, TX) to induce mild skin barrier disruption until a trans-epidermal water loss (AquaFlux, Biox Systems) value of 20g/m^2*h was reached. After tape stripping, a standard daily dose of 5 mg IMQ (100 mg Aldara® 5%) was applied under occlusion using a 12mm Finn chamber (Bipharma, Almere, the Netherlands), which was renewed once every 24h. In this article, we define "short exposure" as the application of IMQ for 48h-72h, and "long exposure" as IMQ application in the duration of 120h-168h.

SKIN ASSESSMENTS

To evaluate the inflammatory skin response, subjects underwent sequential assessments prior to IMQ challenge (0h) and at 48h, 72h, 120h and 168h post IMQ challenge, as well as during follow up (14 days after first IMQ application). One treatment site was selected to evaluate endpoints of non-invasive procedures only, throughout the duration of the study (*Figure 1).* Erythema was assessed by a physician using a 4-point scale ranging from 0 (absent) to 3 (severe). Additionally, erythema and skin perfusion were assessed by means of multispectral imaging analysis (Antera 3D, Miravex, Ireland) and laser speckle contrast imaging (LSCI, PeriCam PSI System, Perimed Jäfälla, Sweden), respectively.

All skin assessments were performed under standardized conditions with a room temperature between 20-24 degrees Celsius (°C).

At specified time intervals (*Figure 1)*, 4-millimetre biopsy samples were obtained from the IMQ-treated regions as well as from the untreated region, resulting in a total of 5 biopsies per volunteer. One part of the biopsy was fixed in 4% formaldehyde at 4°C for 24-48h and subsequently transferred to 70% ethanol at room temperature. The other part was rapidly frozen in gelatine capsules containing Tissue Tek OCT medium (Sakura Finetek USA, Inc., Torrance, USA) and stored in liquid nitrogen until immunohistochemistry (IHC) staining at the pathology Laboratory of Erasmus Medical Centre, Rotterdam, the Netherlands.

IHC AND DIRECT IMMUNOFLUORESCENCE (DIF)

IHC staining was performed for the following targets: CD11c (Clone EP157, Bio SB), CD14 (Clone EPR3653, Ventana), CD20 (Clone L26, Ventana), CD1a (Clone EP3622, Cell Marque), CD3 (Clone 2GV6, Ventana), CD4 (Clone SP35, Ventana), CD8 (Clone C8/144B, DAKO), HLA-DR (Clone CR3142, Ventana), MPO (Polyclonal, Ventana), and NF-κB (Clone d14e12, Cell Signaling). Biopsies were scored by a clinical pathologist using a 6-point nominal scale: negative (0), minimal (1), few (2), moderate (3), many (4), excessive (5). DIF was applied for complement C3c (rabbit polyclonal, DAKO, Glostrup, Denmark) and C4d (rabbit polyclonal, Biomedica, Wien, Austria). DIF intensity was scored by a clinical pathologist on a nominal scale of 0–3: none (0), weak (1), moderate (2) and strong (3). l RNA isolation, sequencing, and data preprocessing l The remaining snap frozen tissue was lysed using RLT lysis buffer with β-mercaptoethanol and extracted using the RNeasy micro plus kit (Qiagen, cat no. 74034). The extracted RNA concentration was assessed using the Molecular Probes Quant iT RNA HS Assay Kit (ThermoFisher Scientific, cat no. Q32852). A set of 45 samples yielded sufficient amounts of RNA for sequencing at Genomescan BV, Leiden. RNA libraries were constructed using the NEBNext Ultra II Directional RNA Library Prep Kit from Illumina (New England BioLabs, Ipswich, MA, USA, cat. no. E7760S/L). Samples were prepared using the NEBNext® Poly(A) mRNA Magnetic Isolation Module. mRNA was isolated using oligo-dT magnetic beads, followed by RNA fragmentation, cDNA synthesis, adapter ligation and PCR amplification of the cDNA library. Bulk RNA sequencing was performed to obtain 40 million reads per sample using the Illumina NovaSeq 6000, yielding 150 bp paired end reads. Raw sequencing reads were processed as follows: adapter trimming and filtering of low-quality bases using fastpv0.23.2, alignment to GRCh38.p13 human reference using STAR2 v2.7.10 and gene level raw count quantification using HTSeq version 2.0.2.

RNA SEQUENCING DATA ANALYSES

Data visualization and statistical analyses of the RNA sequencing data were performed using R statistical software (v4.3.1).[9] An overview of samples and associated information is provided in *Supplemental Table S1*. t-distributed Stochastic Neighbour Embedding (t-SNE) was used as an unsupervised dimensionality reduction approach to visualize the intrinsic structure of the dataset. The algorithm was applied to DESeq2 variance-stabilized (vst) counts of 2000 most variably expressed genes using the Rtsne package (v0.16).[10-12] Based on transcriptomic profiles, a set of 15 IMQ-treated samples co-clustering with the untreated samples were designated as putative (partial or) non-responders. The molecular response to IMQ was investigated in the transcriptomes of responders, using differential gene expression and subsequent pathway analyses. Differential gene expression analysis was performed using the DESeq2 package (v1.40.2) with subsequent LFC shrinkage using the 'apeglm' estimator.[13,14] Gene set enrichment analysis (GSEA) was performed on a curated subset of the Molecular Signatures Database (MSigDB) v2023.2.Hs using the clusterProfiler package (v4.8.2).[15-18] Databases referenced included: hallmark gene sets, oncogenic signatures Gene Ontology, 3CA, miRNA and transcription factor targets, curated gene sets from Wikipathways, PID, Reactome, Biocarta and KEGG/KEGG Medicus. For the MSigDB hallmark gene set collection,[18] pathway activity was additionally assessed at the single-sample level using Gene Set Variation Analysis (GSVA) applied with the GSVA package (v1.48.3).[19] Hierarchically clustered gene expression heatmaps were generated using

the pheatmap package (v1.0.12).[20] Plots linking core enrichment genes and enriched pathways were drawn using the cnetplot function of the enrichplot package (v1.20.3).[21] Gene expression boxplots were produced using the DESeq2 plotCounts function and further modified with ggplot2 (v3.5.0). CIBERSORTx[22] was used to quantify the abundances of 22 immune cell type populations across all samples in the RNA sequencing dataset. The LM22 signature matrix was used as the reference profile and B-mode batch correction was applied. The Skillings-Mack test was used to assess the differences in absolute scores for each cell type across five sample groups (untreated, IMQ48h, IMQ72h, IMQ120h, IMQ168). The Conover's all-pairs test was applied with the PMCMRplus package (v1.9.10) as the post hoc test using data from subjects with complete observations. Unless otherwise specified, the statistical significance threshold across all analyses was set at 0.05 and a Benjamini-Hochberg correction was applied to account for multiple testing.

COMPLEMENT ANALYSIS IN BLOOD SAMPLES Blood was collected in a 4 mL Clot activation Tube (CAT) and plasma in a 4 mL K2EDTA collection tube. Concentrations of the complement components C3, C3d, C3d/C3 ratio and the soluble membrane attack complex C5B-9 were measured in plasma at the laboratory of the Department of Nephrology, University Medical Centre Groningen as described earlier.[23]

STATISTICS All repeatedly measured pharmacodynamic (PD) endpoints were summarised (n, mean, standard deviation (SD)) by area and time. Repeatedly measured continuous PD endpoints were analysed using a mixed model analysis of covariance (ANCOVA) with area (48h IMQ, 72h IMQ, 120h IMQ, 168h IMQ and untreated), hours (0h, 48h,72h,120h and 168h) and area by hours as the fixed factors and subject as the random factor and the covariate baseline measurement (when applicable). A summary table of the analysis results per variable was generated with estimates of the differences between the contrasts and a back transformed estimate of the differences in percentage for log transformed parameters, 95% confidence intervals (in percentages for log-transformed parameters) and Least Square Means (geometric means for log transformed parameters) with corresponding p-values.

RESULTS

STUDY POPULATION AND ADVERSE EVENTS Ten healthy volunteers were enrolled, 7 of whom were female and 3 were male. General subject characteristics are provided in *Supplemental Table S2*. All subjects had Fitzpatrick skin type I-III and a mean age of 25.6 (SD ± 6.7) years. The most frequently reported adverse event was application site pruritus, which was generally transient and disappeared spontaneously after IMQ application was stopped. No serious adverse events were reported.

IMQ TREATMENT DRIVES ERYTHEMA AND SKIN PERFUSION, BUT LONG EXPOSURE DOES NOT ENHANCE THESE RESPONSES A visual representation of all imaging and biophysical assessments is provided in *Figure 2A*. IMQ application under occlusion for 168h led to a significant increase in erythema (estimated difference (ED): 7.69, 95% CI[5.51, 9.86], p<0.0001) and blood perfusion (ED: 25.1%, 95% confidence interval (CI)[13.5%, 37.9%], p<0.0001) compared to untreated (*Figure 2A-C*). Comparison of the response after 168h of IMQ versus 0h revealed a similar significant difference for erythema (11.48, 95% CI[6.75, 16.21], p<0.0001) and for perfusion (ED: 41.6%, 95% CI[14.2%, 75.6%], p<0.0018). The erythema and perfusion response peaked at 48h with subsequent decline over time. No significant differences in erythema (ED: 1.07, 95% CI[-3.67, 5.80], p=0.6567) or perfusion (estimated difference: -12.8%, 95% CI[-29.7%, 8.1%], p=0.2093) were observed upon long IMQ exposure (168h) compared to short exposure (48h; *Figure 2B-C*).

IMQ TRIGGERS AN INFLAMMATORY RESPONSE AT THE TRANSCRIPTOMIC LEVEL Analysis of the RNA sequencing dataset using t-SNE revealed three major clusters predominantly enriched in samples from

the untreated group, short exposure time points (48h-72h) and long exposure time points (120h-168h) (*Figure 3A*). Based on transcriptomic profiles, we grouped samples from the short exposure time points (48h-72h) and samples from the long exposure time points (120h-168h) for downstream analysis. The short exposure group (48h-72h) was compared to the long exposure group (120h-168h), and both groups were also compared to the untreated group. Pathway activity for Hallmarks gene sets demonstrated that IMQ application leads to an activation of the following inflammatory pathways: TNF signalling via NF-κB, IFN-α and IFN-γ responses, and complement pathways, which were most prominently activated after long exposure to IMQ (*Figure 3B, Supplemental Figure S3*).

IMQ EXPOSURE INDUCES TLR7 SIGNALLING AND ACTIVATION OF TNF SIGNALLING VIA NF-κB

We further investigated the biological processes involved in the different stages of exposure to IMQ using GSEA (*Figure 3C*). Short IMQ exposure led to activation of TLR signalling pathways, interferon-driven responses and TNF signalling via NF-κB (*Figure 3C*). Upon longer IMQ exposure, activation of TLR-induced IRF7 signalling was revealed and TNF signalling via NF-κB became more prominent (*Figure 3C*). Additionally, downstream effects representative of the TLR pathway including induction of type I and type II interferons leading to the activation of the JAK-STAT pathway were identified after long exposure (*Figure 3C*). When comparing long to short IMQ exposure, a positive enrichment of gene sets relating to IFN-α and -γ was found, as well as additional activated pathways including IL-2 signalling (*Figure 3C*). A network plot was generated displaying the linkages between the biological pathways involved in the IMQ response (*Figure 4A*). We next focused on a selection of relevant differentially expressed genes (DEGs) involved in TLR-induced IRF7 signalling, NF-κB signalling and complement activation pathways (*Figure 4B*; full pathways shown in *Supplemental Figure 4*). For IRF signalling, a time-dependent increase was found in the expression of transcripts encoding for IRF7, Mx-1 and CXCL10 in IMQ-treated samples compared to untreated samples (*Figure 4B*). RNA sequencing-based NF-κB responses were confirmed by immunohistochemical staining of skin punch biopsies: NF-κB (total) staining was elevated between 120h and 168h post IMQ application, compared to baseline (*Supplemental Figure S5C*). Downstream of NF-κB, we found a similar increase in expression for IL-6 and CCL2 (*Figure 4B*). CXCL8 expression was upregulated after IMQ application, but the difference in expression was not significant between long and short exposure. An overview of expression for markers corresponding to the IHC staining is available in *Supplemental Figure S6*.

IMQ-DRIVEN EXPRESSION OF COMPLEMENT GENES

Transcriptomic analysis at the pathway level showed a positive enrichment of gene sets related to the complement cascade, with stronger enrichment upon prolonged exposure (*Figure 3C*). Complement genes elevated in expression by IMQ application included C1QA, C3 and CFB (*Figure 4B*). Results for the transcripts encoding for complement proteins downstream of C3 were inconsistent, with mixed expression levels between samples (*Supplemental Figure S4B*). IHC staining for complement revealed traces of dermal C4d at baseline, but no deposition of C3c or C4d after 168h of exposure to IMQ. Complement proteins showed no systemic elevation of C3, C3d, C3d/C3 or C5B-9 in plasma after 168h of IMQ-exposure (not shown).

INCREASED CELLULAR INFILTRATION AFTER LONG IMQ EXPOSURE

Histologically, the general inflammation pattern after IMQ exposure was a lymphohistiocytic perivascular dermatitis with an increasing degree and deeper extension of inflammation over time (*Figure 5A*). In 9/10 individuals the infiltrate showed peri-adnexal (peri-follicular and/or peri-eccrine) involvement and epidermal interface dermatitis (6/10). A rise in general cell infiltration accompanied by a mild increase in acanthosis as well as lymphocytic exocytosis (*Supplemental Figure S5A,B*) was evident upon prolonged IMQ exposure (Figure 5B). Immunohistochemical staining showed infiltration of monocytes, DCs and macrophages (*Figure 5B*) but

no presence of neutrophils (*Supplemental Figure S5E*). The cells were present after 48h-72h, with infiltration more elevated after 120h-168h post IMQ application. This pattern was mirrored by the T cell response, which showed a rise in T helper cells and cytotoxic T cells until 168h. (Figure 5B). A minimal number of Langerhans cells and B cells was present (*Supplemental Figure S5D,F)*.

The CIBERSORTx algorithm identified a total of 19 out of 22 immmune cell subpopulations (*Figure 6*). IMQ application generally led to increased absolute scores of several immune cell types compared to the untreated samples. A globally significant difference (using Skillings-Mack test) between groups is observed, among others, for resting NK cells (p=0.0292), naïve B cells, (p=0.0051,), M1 Macrophages (p=0.0003), M2 macrophages (p=0.0403), activated DCs (p=0.001,), resting $CD4^+$ memory T cells (p=0.0003) and activated $CD4^+$ memory T cells (p=0.0241), and $CD8^+$ T cells (p=0.0463) depicted in *Supplemental Figure S7*. Of these, naïve B cells, M1 macrophages, resting NK cells and both resting and activated $CD4^+$ memory T cells showed significant increase in abundance at 168h vs. 72h after IMQ exposure in the post-hoc analysis. Additionally, a statistically significant reduced abundance was observed in both resting and activated DCs at 168h vs. 72h. M2 macrophages and $CD8^+$ T cells did not show statistically significant changes at individual timepoints. Remarkably, overall low abundance of neutrophils was observed (*Supplemental Figure S7*).

DISCUSSION

This study is the first to elucidate the acute effects of long-term IMQ exposure using a multimodal approach in healthy volunteers. We have shown that compared to short exposure, long exposure to IMQ results in a stronger immunological response as evidenced by additional enriched pathways such as TLR-induced IRF7 signalling, more prominent TNF signalling via NF-κB along with downstream effects such as induction of type I and type II interferons leading to activation of the JAK-STAT pathway. Furthermore, increased complement gene expression was identified upon long exposure to IMQ.[7, 22] Although imaging and biophysical measurements showed no significantly enhanced response after long IMQ application compared to short exposure, a strong cellular infiltration boost was observed. At the transcriptomic level, this was demonstrated by an increased abundance of M1 macrophages, resting

NK cells, and resting and activated $CD4^+$ memory T cells. While a statistically significant increase was observed in M2 macrophages and $CD8^+$ T cells following IMQ application, no difference was observed between long and short exposure. The increased abundance of naïve B cells and the appearance of T cells is indicative of both innate and adaptive immune responses involvement. The transcriptomic profile partially aligns with the IHC-based cellular infiltration of macrophages, NK cells and $CD4^+$ T cells, demonstrating clear time-dependent effects with increased infiltration after long IMQ exposure. Additionally, IMQ increased the expression of type II interferon-related genes, which aligns with the IHC observation of $CD8^+$ cell influx. These cellular findings are consistent with classical TLR signalling. Activation of TLRs is also known to trigger MyD88, IRAK1 and IRAK4, leading to IRF7 and NF-κB signalling, which is in line with our findings. These pathways result in upregulation of transcription factors for several cytokines including type I interferons, TNF, IL-2, IL-6, IL-8, IL-12, IFN-α and chemokines such as macrophage inflammatory protein (MIP)-1α, MIP-1β and monocyte chemotactic protein-1.[24]

Another challenge agent that we use to effectively induce an in vivo TLR response in men is lipopolysaccharide (LPS).[25-28] Intradermal injection of LPS triggers an acute inflammatory response via TLR4, leading to increased innate immune cell populations including neutrophils, monocytes and dendritic cells. Furthermore, LPS elicits an adaptive immune response, as evidenced by the presence of B and T cells. Elevated levels of IL-6, IL-8, IL-1β and TNF following LPS injection indicate NF-κB involvement. The current study showed moderate activation of NF-κB signalling after long IMQ application, supported by upregulated expression of NF-κB1, NF-κB2, IL-6, CXCL8, CCL2, IL-17C and IL-23A at the transcriptomic level. This contradicts previous studies, as no significant IL-6 and IL-8

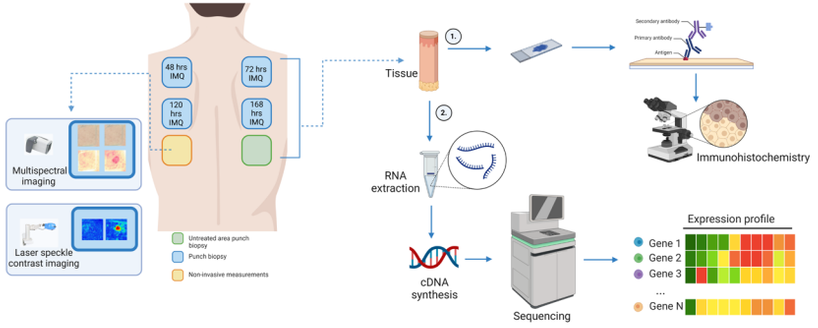
responses were observed 72 hours after IMQ application, suggesting only mild NF-κB involvement. However, the release of Mx-A (a downstream marker indicative of IFN-α activation through IRF7) was evident.[7,8] Our current data reinforces this finding, as it suggests both MY-D88 transcript expression and downstream IRF7 and Mx-1 activation, which increases upon prolonged IMQ application. The activation of interferons leads to the engagement of their respective receptors, which in turn triggers the JAK-STAT pathway culminating in the release of proinflammatory cytokines.[29,30] The JAK-STAT pathway was more enriched, with chemokines such as CXCL9, CXCL10 and CXCL11 significantly overexpressed after prolonged IMQ exposure. Recently, *Chen et al*. provided an overview of studies examining DNA and RNA specific profiles in cutaneous lupus erythematosus (CLE) patients, which indicates an upregulation of innate immune response functions including JAK-STAT signalling, TLR signalling, and pattern recognition receptors. Furthermore, there was a notable increase in the expression of type 1 interferons, along with an upregulated expression of chemokines CXCL9, CXCL10, and CXCL11, which are recognized as characteristic indicators for CLE.[31-34] Although a direct comparison of our data with the existing RNA datasets of CLE patients was not conducted, analysis of pathway activity and overexpressed genes detected upon prolonged IMQ application allows us to conclude that our current model aligns more closely with CLE characteristics than with psoriasiform lesions.[7] In addition, the histopathological changes of a vacuolar interface dermatitis with adnexal involvement were also reminiscent of CLE. These observations differ from the prevalent use of the model in preclinical studies, where the murine IMQ model is typically used to investigate psoriasis-like conditions.[35]

In contrast to our cellular observations, imaging and biophysical measurements showed no significantly enhanced response after long IMQ application compared to short exposure. This may be because Aldara (besides its role as a TLR7 agonist) may also act as an exogenous mediator by enhancing transient receptor potential vanilloid 1 channel activity on the primary afferent sensory neuron.[36] Activation of this channel leads to the release of bioactive substances such as nitric oxide (NO). NO can then interact with target cells in the surrounding tissue, including vascular smooth muscle cells. The interaction of NO with smooth muscle cells leads to vasodilation, resulting in increased blood perfusion and erythema[6, 35]. Our observations suggest that the vascular response is independent of the inflammatory process. The role of bioactive, vasodilating substances in the IMQ-induced erythema and perfusion response remains to be further elucidated.

Our second objective was to explore the translational value of the IMQ model. In contrast to the mouse data, IHC staining did not show involvement of complement in the human IMQ response. We hypothesize that this may be due to the difference in severity of the hit, as in mice, the entire surface area of the back is challenged, whereas in humans IMQ is applied to a much smaller relative surface area. Therefore, it is currently unknown whether the observed differences are a result of the magnitude of TLR activation or can be contributed to species differences.[1,2] However, at the transcriptome level, classical, and alternative pathway genes were enriched, particularly after prolonged exposure to IMQ, suggesting complement involvement in the human IMQ response. It is unclear how these sequencing results translate to the protein level, or if the transient nature of complement explains the lack of IMQ-driven complement responses in IHC analysis. The same holds true for the observed lack of neutrophils and CXCL8 expression, which emphasizes that the role of neutrophils in the human IMQ response needs to be elucidated further.[6] The observed differences between preclinical animal models and the human response may partially be explained by species differences in TLR7 expression.[37] For instance, *Bhagchandani et al*. described that the expression of TLR7 on neutrophils is higher in mouse than in men.[37,38] Moreover, expression patterns of TLR7 within a given cell type may differ across tissues and across activation status of the cell,[39] further complicating the translational interpretation both across and within species. These findings highlight the complementary value of human challenge models in the development of immune-targeting compound development. In conclusion,

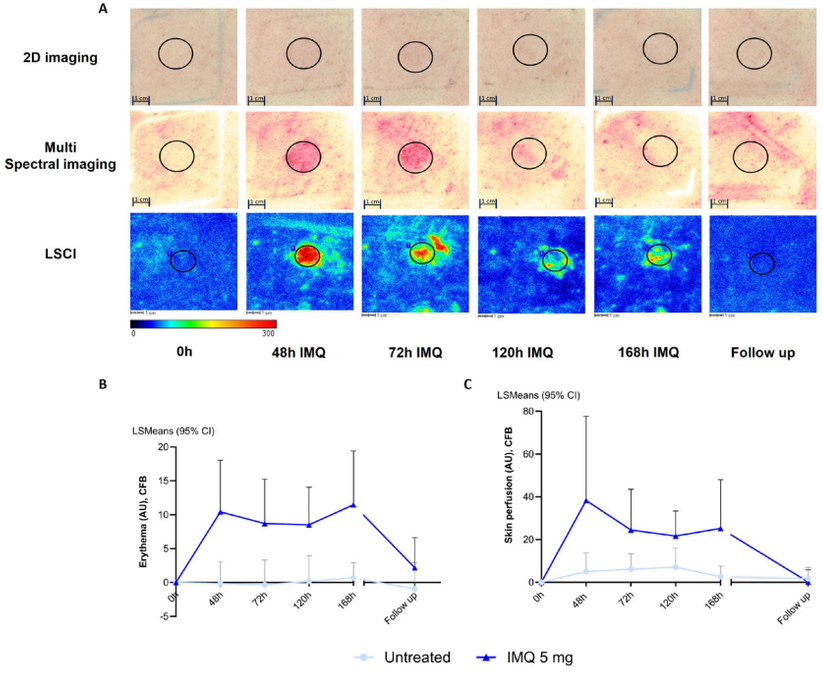
our study provides a comprehensive characterization of the cutaneous response to both short and prolonged IMQ exposure in healthy volunteers by using a multimodal approach. We have demonstrated that prolonging the IMQ exposure has added value by enhancing cellular responses and increasing abundance of specific immune cell types along with stronger activation of a diverse set of pathways, particularly those driven by IRF and related to complement. We also argue that prolonged IMQ application results in a CLE-like cutaneous inflammation, both at the transcriptomic level and from a histopathological perspective. Our results suggest that biophysical and vascular responses are not exclusively driven by cutaneous inflammation. The described discrepancies between preclinical and clinical results, most notably the neutrophil response, illustrate the complementary value of human challenge models in the development of compounds targeting the immune system. This *in vivo* immune challenge model is of value for future early clinical evaluation of topically or systemically applied anti-inflammatory or immunomodulatory compounds, particularly compounds targeting IRF and JAK-STAT signalling.

Figure 1 Overview of the study design. IMQ was topically applied to five treatment sites on the back of healthy participants for a maximum of seven consecutive days (168h). At specified time intervals (48h, 72h, 120h and 168h), 4-millimetre biopsy samples were obtained from the IMQ-treated regions as well as from the untreated region, resulting in a total of 5 biopsies per volunteer. Endpoints included non-invasive imaging, immunohistochemical staining and RNA sequencing of biopsy material.



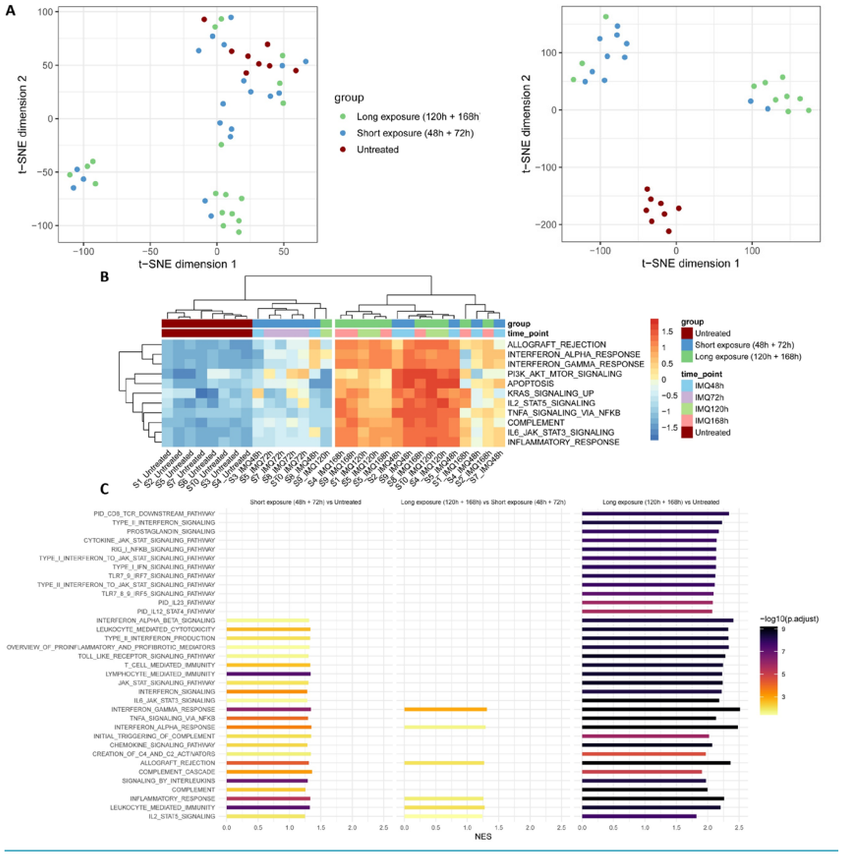
Abbreviations: CDNA=complement DNA, h=hours, IMQ=imiquimod. Created with BioRender.com

Figure 2 Clinical impression and quantification of inflammatory skin response by multi-spectral imaging and LSCI. A) Overview of 2D imaging, LSCI and multispectral imaging. B) Erythema measured by multispectral camera, illustrated as CFB (n=10 for 0-168h, n=9 for follow up, imaging data of 1 subject were missing at follow up (D14) as D14 visit took place outside of the allowed time window). No difference observed between short (48h) and long (168h) IMQ exposure, estimated difference: 1.07, 95% CI [-3.67, 5.80], p=0.6567. C) Skin perfusion by LSCI, illustrated as CFB (n=10 for 0-168h, n=9 for follow up). No difference observed between short (48h) and long (168h) IMQ exposure, estimated difference: -12.8%, 95% CI [-29.7%, 8.1%], p= 0.2093.



Abbreviations: CFB= change from baseline, CI= confidence interval, IMQ = imiquimod, LSCI= laser speckle contrast imaging, LSMEANS= Least Squares Mean.

Figure 3 Transcriptomic profiling of imiquimod response in tissue biopsies. A) T-SNE plot visualizing the clusters present in the full dataset (n=45, top) and a reduced subset of the dataset (n=30) excluding the treated samples which showed similarity to the untreated group (putative non-responders based on transcriptomic profiles). Top figure represents the full dataset (n=45), while the bottom plot excludes the samples (n=15) that show similarity to untreated group based on their transcriptomic profiles. B) Heatmap of GSVA single-sample pathway enrichment scores on 11 representative MSIGDB Hallmark gene sets (n=30). IMQ application leads to activation of pathways involved in inflammatory and immune responses, including TNF signalling via NF-κB, IFN-α and IFN-γ responses, and complement pathways. C) Barplots displaying GSEA normalized enrichment scores of 35 representative pathways upregulated upon IMQ exposure for the following comparisons: short exposure versus untreated (left), long exposure versus short exposure (middle) and long exposure versus untreated (right), based on the selected dataset (n=30).



Abbreviations: GSEA= gene set enrichment analysis, GSVA= gene set variation analysis, IFN= interferon, IMQ= imiquimod, NES= normalized enrichment score, NF-κB= nuclear factor kappa-light-chain-enhancer of activated B cells, TNF= tumour necrosis factor, T-SNE= t-distributed Stochastic Neighbour Embedding.

Figure 4 Key pathways activated after long imiquimod exposure. A) Network plot displaying selected biological pathways involved in the IMQ response and their associated core enrichments genes. Colours of network edges correspond to each biological pathway including complement, TNF-signalling via NF-κB, TLR7/9/IRF7 Signalling, Type I interferon to JAK-STAT signalling and TLR signalling. Colours of nodes correspond to the log2 fold change values for individual genes based on the late exposure versus untreated group comparison. B) Boxplots of DESeq2 normalized counts with an added pseudocount of 0.5 shown for 9 selected DEGs involved in the TLR-induced IRF7 signalling (top row), NF-κB signalling (middle row) and complement activation pathways (bottom row). Statistical significance corresponds to adjusted p-values from DESeq2 differential expression analysis for each given comparison.

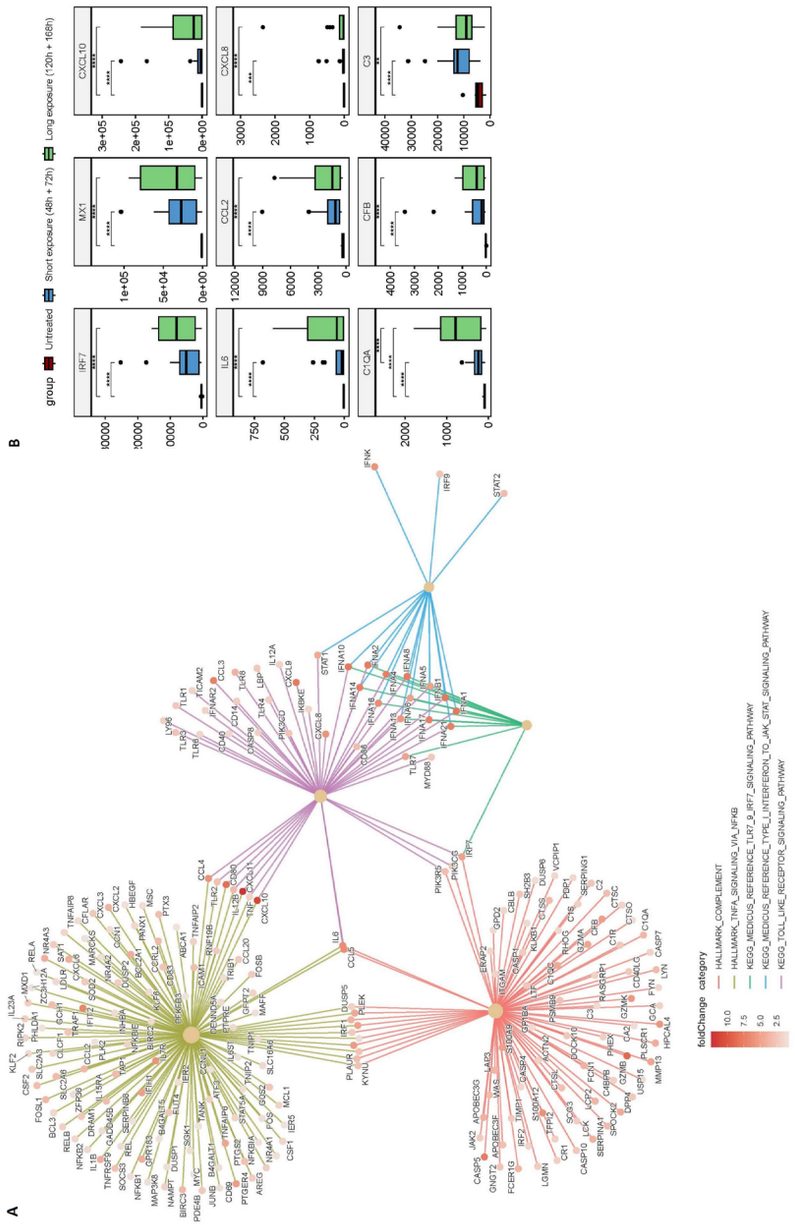
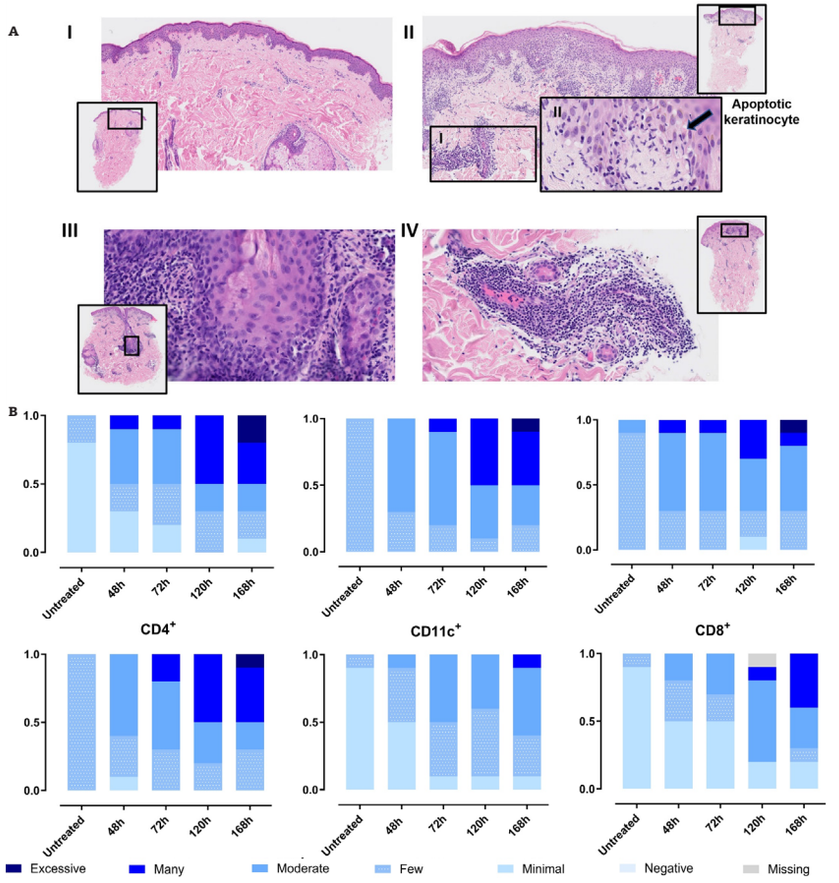
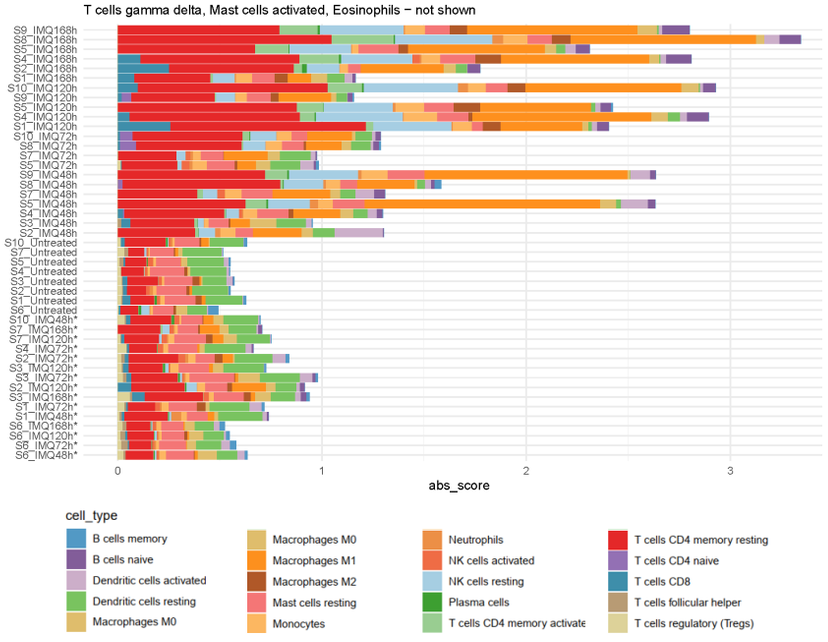


Figure 5 Enhanced inflammatory responses at histological and cellular levels observed upon prolonged IMQ exposure. A) histological sections. Section I) Untreated skin, shows healthy skin without any signs of inflammation (zoom factor 5). II) Section of 72h post IMQ application showing an abundant lymphocytic exocytosis in the epidermis and superficial lymphohistiocytic perivascular infiltrate (zoom factor 5). Inset I shows exocytosis of lymphocytes in a sweat gland (zoom factor 5). Inset II shows lymphocytic exocytosis in the epidermis accompanied by an apoptotic/necrotic keratinocyte (arrow, zoom factor 40). III) Section of 120h of IMQ application showing profound perifollicular inflammation and influx of lymphocytes in the hair follicle epithelium (zoom factor 20). IV) Section of 168h of IMQ application resulting in cuffing/lymphocytic vasculopathy of the infiltrate surrounding the deep vascular plexus at the levels of the sweat glands (zoom factor 20). B) General infiltration and IMQ-driven immune cells (macrophages, monocytes, T helper cells, dendritic cells and cytotoxic T cells) measured by IHC in skin punch biopsies.



Abbreviations: IHC= immunohistochemistry, IMQ= imiquimod.

Figure 6 Overview of immune cell type infiltration based on CIBERSORTX. Stacked barplot displaying the absolute scores for 19 cell types detected by CIBERSORTX across all samples (n=45). An asterisk (*) refers to putative molecular non-responders based on transcriptomic profile similarity to untreated samples.



Abbreviations: IMQ=imiquimod, NK=natural killer.

SUPPLEMENTAL MATERIAL

Table S1 Overview of samples analysed using RNA sequencing.

Sample ID	Sample	Subject number	Timepoint	Group	Responder (molecular)
105669-001-004	S6_Untreated	6	Untreated	Untreated	
105669-001-009	S1_Untreated	1	Untreated	Untreated	
105669-001-014	S2_Untreated	2	Untreated	Untreated	
105669-001-019	S3_Untreated	3	Untreated	Untreated	
105669-001-024	S4_Untreated	4	Untreated	Untreated	
105669-001-029	S5_Untreated	5	Untreated	Untreated	
105669-001-034	S7_Untreated	7	Untreated	Untreated	
105669-001-049	S10_Untreated	10	Untreated	Untreated	
105669-001-001	S6_IMQ48h*	6	IMQ48h	Short exposure (48h + 72h)	no
105669-001-006	S1_IMQ48h*	1	IMQ48h	Short exposure (48h + 72h)	no
105669-001-011	S2_IMQ48h	2	IMQ48h	Short exposure (48h + 72h)	yes
105669-001-016	S3_IMQ48h	3	IMQ48h	Short exposure (48h + 72h)	yes
105669-001-021	S4_IMQ48h	4	IMQ48h	Short exposure (48h + 72h)	yes
105669-001-026	S5_IMQ48h	5	IMQ48h	Short exposure (48h + 72h)	yes
105669-001-031	S7_IMQ48h	7	IMQ48h	Short exposure (48h + 72h)	yes
105669-001-036	S8_IMQ48h	8	IMQ48h	Short exposure (48h + 72h)	yes
105669-001-041	S9_IMQ48h	9	IMQ48h	Short exposure (48h + 72h)	yes
105669-001-046	S10_IMQ48h*	10	IMQ48h	Short exposure (48h + 72h)	no
105669-001-002	S6_IMQ72h*	6	IMQ72h	Short exposure (48h + 72h)	no
105669-001-007	S1_IMQ72h*	1	IMQ72h	Short exposure (48h + 72h)	no
105669-001-017	S3_IMQ72h*	3	IMQ72h	Short exposure (48h + 72h)	no
105669-001-020	S2_IMQ72h*	2	IMQ72h	Short exposure (48h + 72h)	no
105669-001-022	S4_IMQ72h*	4	IMQ72h	Short exposure (48h + 72h)	no

(Continuation Table S1)

Sample ID	Sample	Subject number	Timepoint	Group	Responder (molecular)
105669-001-027	S5_IMQ72h	5	IMQ72h	Short exposure (48h + 72h)	yes
105669-001-032	S7_IMQ72h	7	IMQ72h	Short exposure (48h + 72h)	yes
105669-001-037	S8_IMQ72h	8	IMQ72h	Short exposure (48h + 72h)	yes
105669-001-047	S10_IMQ72h	10	IMQ72h	Short exposure (48h + 72h)	yes
105669-001-003	S6_IMQ120h*	6	IMQ120h	Long exposure (120h + 168h)	no
105669-001-008	S1_IMQ120h	1	IMQ120h	Long exposure (120h + 168h)	yes
105669-001-013	S2_IMQ120h*	2	IMQ120h	Long exposure (120h + 168h)	no
105669-001-018	S3_IMQ120h*	3	IMQ120h	Long exposure (120h + 168h)	no
105669-001-023	S4_IMQ120h	4	IMQ120h	Long exposure (120h + 168h)	yes
105669-001-028	S5_IMQ120h	5	IMQ120h	Long exposure (120h + 168h)	yes
105669-001-033	S7_IMQ120h*	7	IMQ120h	Long exposure (120h + 168h)	no
105669-001-043	S9_IMQ120h	9	IMQ120h	Long exposure (120h + 168h)	yes
105669-001-048	S10_IMQ120h	10	IMQ120h	Long exposure (120h + 168h)	yes
105669-001-005	S6_IMQ168h*	6	IMQ168h	Long exposure (120h + 168h)	no
105669-001-010	S1_IMQ168h	1	IMQ168h	Long exposure (120h + 168h)	yes
105669-001-012	S3_IMQ168h*	3	IMQ168h	Long exposure (120h + 168h)	no
105669-001-015	S2_IMQ168h	2	IMQ168h	Long exposure (120h + 168h)	yes
105669-001-025	S4_IMQ168h	4	IMQ168h	Long exposure (120h + 168h)	yes
105669-001-030	S5_IMQ168h	5	IMQ168h	Long exposure (120h + 168h)	yes
105669-001-035	S7_IMQ168h*	7	IMQ168h	Long exposure (120h + 168h)	no
105669-001-040	S8_IMQ168h	8	IMQ168h	Long exposure (120h + 168h)	yes
105669-001-045	S9_IMQ168h	9	IMQ168h	Long exposure (120h + 168h)	yes

Table S2 Overview of subject demographics.

All subjects	
AGE (YEARS)	
N	10
Mean (SD)	25.6 (6.7)
Median	24
Min, Max	18, 37
HEIGHT (CM)	
N	10
Mean (SD)	176.57 (7.48)
Median	176.0
Min, Max	164.9, 192.0
WEIGHT (KG)	
N	10
Mean (SD)	77.290 (13.677)
Median	77.68
Min, Max	57.40, 95.50
BMI (KG/M^2)	
N	10
Mean (SD)	24.68 (3.33)
Median	24.7
Min, Max	20.1, 30.0
SEX	
Female	7 (70.0%)
Male	3 (30.0%)

Figure S1 Heatmap of GSVA single-sample pathway enrichment scores on 11 representative MSIGDB Hallmark gene sets, scaled by row and shown across the full data set (n=45 samples).

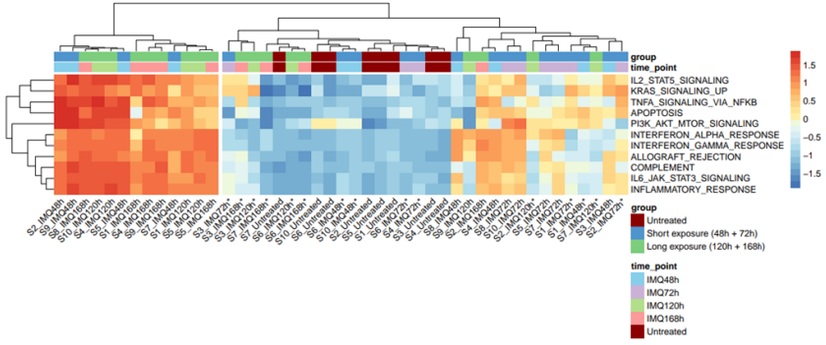
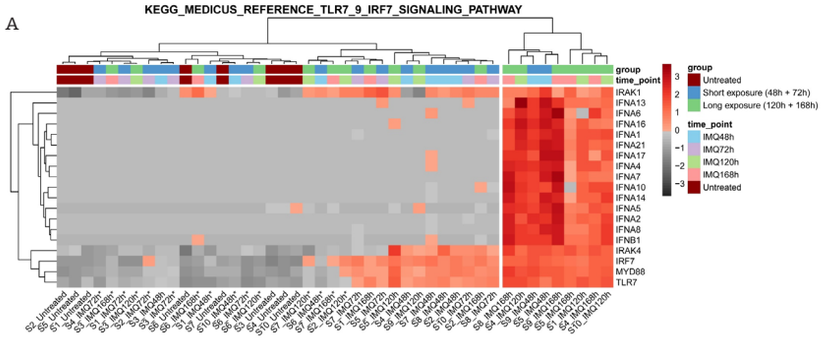
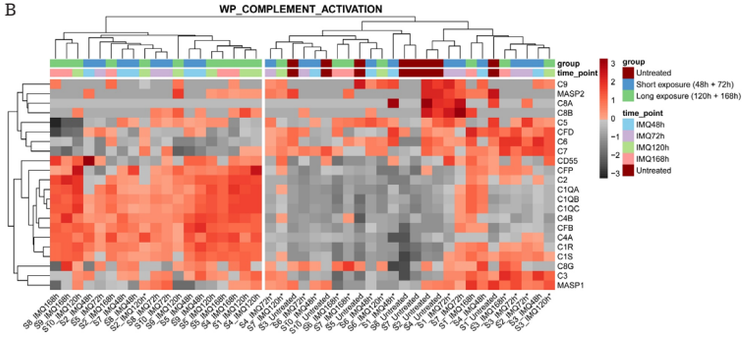


Figure S2 Key pathways involved in the IMQ response. Gene expression for members of the A) TLR7/9 signalling pathway (KEGG Medicus), B) complement activation pathway (WP; WikiPathways), C) a subset of the TNF signalling via NF-κB pathway (Hallmark gene sets); scaled by row across the full dataset (n=45 samples).

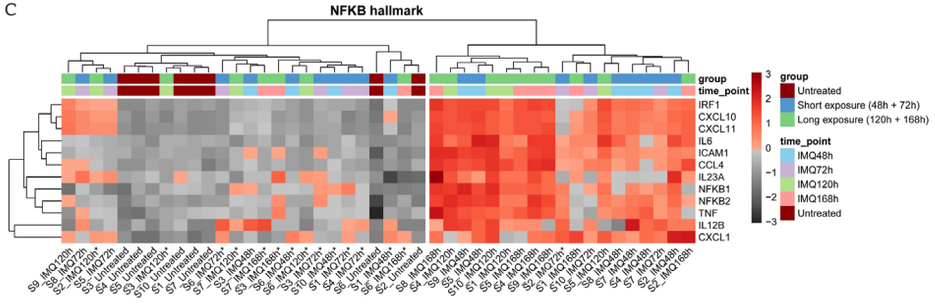
A



B



C



NF-κB= nuclear factor kappa-light-chain-enhancer of activated B cells

Figure S3 Staining of biopsies for A) acanthosis, B) lymphocytic exocytosis, C) NF-κB, D) B cells, E) neutrophils, F) Langerhans cells.

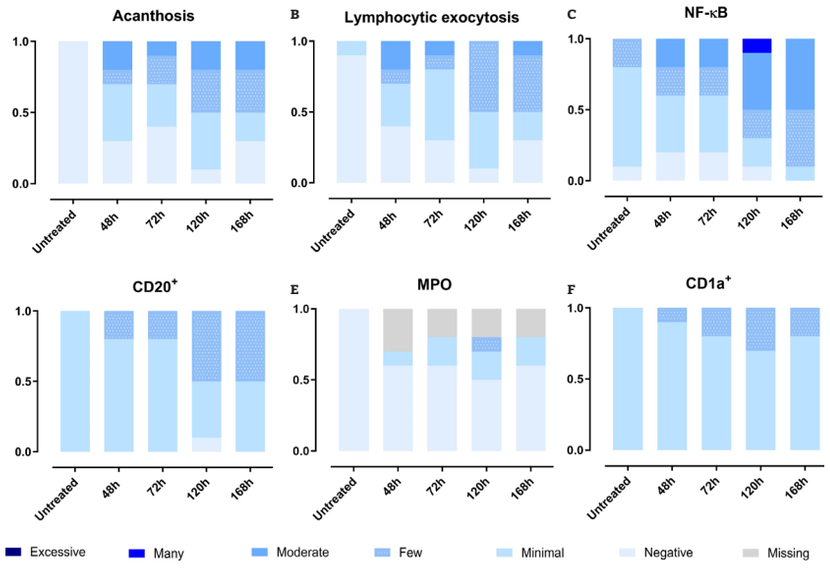


Figure S4 Expression of transcripts corresponding to the markers used for IHC scaled by row and shown across the full data set (n=45 samples).

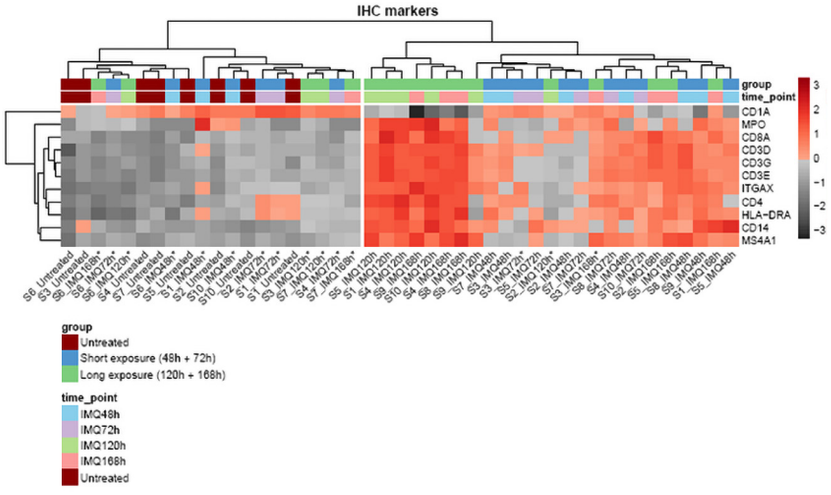
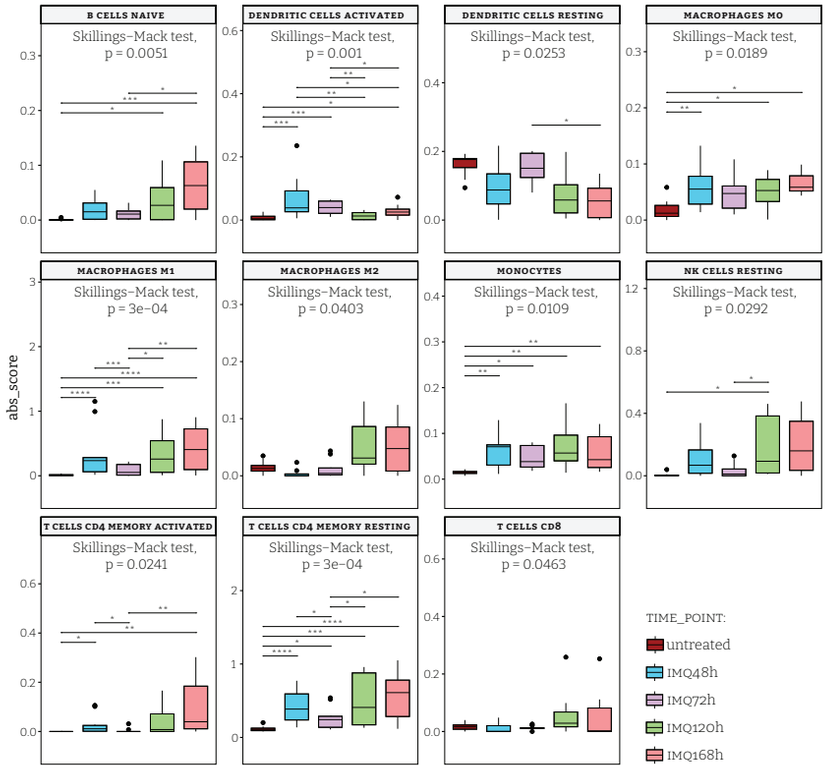


Figure S5 Overview of cell types profiled using CIBERSORTX per time point. Boxplots depicting absolute scores for 11 cell types with statistically significant changes across time points (Skillings-Mack test). The Conover's all-pairs test with FDR correction was applied as the post hoc test using data from subjects with complete observations. Three subjects were excluded from the analysis due to incomplete observations.



conover's all–pairs test for subjects with complete observations, p.adjust method = FDR

REFERENCES

1 Junhee Seok, H. Shaw Warren, Alex GC, et al (2013) Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci U S A 110:3507–3512

2 Mestas J, Hughes CCW (2004) Of Mice and Not Men: Differences between Mouse and Human Immunology. The Journal of Immunology 172:2731–2738

3 Hanna E, Abadi R, Abbas O (2016) Imiquimod in dermatology: an overview. Int J Dermatol 55:831–844

4 van der Fits L, Mourits S, Voerman JSA, et al (2009) Imiquimod-Induced Psoriasis-Like Skin Inflammation in Mice Is Mediated via the IL-23/IL-17 Axis. The Journal of Immunology 182:5836–5845

5 Giacomassi C, Buang N, Ling GS, Crawford G, Cook HT, Scott D, Dazzi F, Strid J, Botto M (2017) Complement C3 Exacerbates Imiquimod-Induced Skin Inflammation and Psoriasiform Dermatitis. Journal of Investigative Dermatology 137:760–763

6 Flutter B, Nestle FO (2013) TLRs to cytokines: Mechanistic insights from the imiquimod mouse model of psoriasis. Eur J Immunol 43:3138–3146

7 van der Kolk T, Assil S, Rijneveld R, et al (2018) Comprehensive, Multimodal Characterization of an Imiquimod-Induced Human Skin Inflammation Model for Drug Development. Clin Transl Sci 11:607–615

8 Assil S, Buters TP, Hameeteman PW, et al (2023) Oral prednisolone suppresses skin inflammation in a healthy volunteer imiquimod challenge model. Front Immunol 14:1–11

9 Team RC (2023) R: A Language and Environment for Statistical Computing. www.r-project.org/.

10 Maaten L Van Der (2014) Accelerating t-SNE using Tree-based Algorithms. 15:3221–3245

11 Van Der Maaten L, Hinton G (2008) Visualizing data using t-SNE. Journal of Machine Learning Research 9:2579–2625

12 Krijthe J (2015) Rtsne: T-Distributed Stochastic Neighbor Embedding using Barnes-Hut Implementation_. R package version 0.16.

13 Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:1–21

14 Zhu A, Ibrahim JG, Love MI (2019) Heavy-tailed prior distributions for sequence count data: removing the noise and preserving large differences. Bioinformatics 35:2084–2092

15 Wu T, Hu E, Xu S, et al (2021) clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. Innovation (Cambridge (Mass)) 2:100141

16 Yu G, Wang L-G, Han Y, He Q-Y (2012) clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS 16:284–287

17 Subramanian A, Tamayo P, Mootha VK, et al (2005) Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 102:15545–15550

18 Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP, Tamayo P (2015) The molecular signatures database hallmark gene set collection. Cell Syst 1:417–425

19 Hänzelmann S, Castelo R, Guinney J (2013) GSVA: Gene set variation analysis for microarray and RNA-Seq data. BMC Bioinformatics. https://doi.org/10.1186/1471-2105-14-7

20 Kolde R, Kolde MR (2015) Package 'pheatmap.' R package 1:790

21 Yu G (2023) enrichplot: Visualization of Functional Enrichment Result. R-package

22 Newman AM, Steen CB, Liu CL, et al (2019) Determining cell type abundance and expression from bulk tissues with digital cytometry. Nat Biotechnol 37:773–782

23 Prens LM, Ardon CB, van Straalen KR, van der Zee HH, Seelen MAJ, Laman JD, Prens EP, Horváth B, Damman J (2021) No Evident Systemic Terminal Complement Pathway Activation in Hidradenitis Suppurativa. Journal of Investigative Dermatology 141:2966-2969.e1

24 Gorden KB, Gorski KS, Gibson SJ, Kedl RM, Kieper WC, Qiu X, Tomai MA, Alkan SS, Vasilakos JP (2005) Synthetic TLR Agonists Reveal Functional Differences between Human TLR7 and TLR8. The Journal of Immunology 174:1259–1268

25 Buters TP, Hameeteman PW, Jansen IME, et al (2022) Clinical, Cellular, and Molecular Effects of Corticosteroids on the Response to Intradermal Lipopolysaccharide Administration in Healthy Volunteers. Clin Pharmacol Ther 111:964–971
26 Buters TP, Hameeteman PW, Jansen IME, et al (2022) Intradermal lipopolysaccharide challenge as an acute in vivo inflammatory model in healthy volunteers. Br J Clin Pharmacol 88:680–690
27 Dillingh MR, Van Poelgeest EP, Malone KE, Kemper EM, Stroes ESG, Moerland M, Burggraaf J (2014) Characterization of inflammation and immune cell modulation induced by low-dose LPS administration to healthy volunteers. Journal of Inflammation (United Kingdom) 11:1–9
28 van Poelgeest EP, Dillingh MR, de Kam M, Malone KE, Kemper M, Stroes ESG, Burggraaf J, Moerland M (2018) Characterization of immune cell, endothelial, and renal responses upon experimental human endotoxemia. J Pharmacol Toxicol Methods 89:39–46
29 Majoros A, Platanitis E, Kernbauer-Hölzl E, Rosebrock F, Müller M, Decker T (2017) Canonical and non-canonical aspects of JAK-STAT signaling: Lessons from interferons for cytokine responses. Front Immunol. https://doi.org/10.3389/fimmu.2017.00029
30 Mahjoor M, Mahmoudvand G, Farokhi S, Shadab A, Kashfi M, Afkhami H (2023) Double-edged sword of JAK/STAT signaling pathway in viral infections: novel insights into virotherapy. Cell Communication and Signaling 21:1–17
31 Nickles MA, Huang K, Chang YS, Tsoukas MM, Sweiss NJ, Perkins DL, Finn PW (2020) Gene Co-expression Networks Identifies Common Hub Genes Between Cutaneous Sarcoidosis and Discoid Lupus Erythematosus. Front Med (Lausanne) 7:1–8
32 Solé C, Gimenez-barcons M, Ferrer B, Ordi-Ros J, Cortés-Hernández J (2016) Microarray study reveals a transforming growth factor-β-dependent mechanism of fibrosis in discoid lupus erythematosus. British Journal of Dermatology 175:302–313
33 Blomberg S, Rönnblom L, Eloranta ML, Cederblad B, Alm G V., Nordlind K, Nordlind K (2001) Presence of cutaneous interferon-α producing cells in patients with systemic lupus erythematosus. Lupus 10:484–490
34 Wongpiyabovorn J, Ruchusatsawat K, Ongnantapong Y, Sintupak W, Hirankarn N (2011) Interferon Alpha mRNA level and subtypes in lesion and non-lesion from discoid lupus erythematosus patients without systemic lupus erythematosus. Asian Biomedicine 5:643–647
35 Flutter B, Nestle FO (2013) TLRs to cytokines: Mechanistic insights from the imiquimod mouse model of psoriasis. Eur J Immunol 43:3138–3146
36 Kittaka H, Tominaga M (2017) The molecular and cellular mechanisms of itch and the involvement of TRP channels in the peripheral sensory nervous system and skin. Allergology International 66:22–30
37 Bhagchandani S, Johnson JA, Irvine DJ (2021) Evolution of Toll-like receptor 7/8 agonist therapeutics and their delivery approaches: From antiviral formulations to vaccine adjuvants. Adv Drug Deliv Rev. https://doi.org/10.1016/j.addr.2021.05.013
38 Hayashi F, Means TK, Luster AD (2003) Toll-like receptors stimulate human neutrophil function. Blood 102:2660–2669
39 Trinchieri G, Sher A (2007) Cooperation of Toll-like receptor signals in innate immune defence. Nat Rev Immunol 7:179–190

CHAPTER VI

OMIGANAN ENHANCES IMIQUIMOD-INDUCED INFLAMMATORY RESPONSES IN SKIN OF HEALTHY VOLUNTEERS

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ABSTRACT

Omiganan (omiganan, a synthetic cationic peptide) and imiquimod (imiquimod, a TLR7 agonist) have synergistic effects on interferon responses *in vitro*. The objective of this study was to translate this to a human model for *proof-of-concept*, and to explore the potential of omiganan add-on treatment for viral skin diseases. Sixteen (16) healthy volunteers received topical imiquimod, omiganan or a combination of both for up to 4 days on tape stripped skin. Skin inflammation was quantified by laser speckle contrast imaging and 2D photography, and molecular and cellular responses were analyzed in biopsies. Imiquimod treatment induced an inflammatory response of the skin. Co-treatment with omiganan enhanced this inflammatory response to imiquimod, with increases in perfusion (+17.1%, 95% CI 5.6-30%, P<0.01) and erythema (+1.5, 95% CI 0.25-2.83, P=0.02). IRF- and NFκβ-driven responses following TLR7 stimulation were enhanced by omiganan (increases in IL-6, IL-10, Mx-A, and IFNɣ), and more immune cell infiltration was observed (in particular $CD4^+$, $CD8^+$ and $CD14^+$ cells). These findings are in line with the earlier mechanistic *in vitro* data, and support evaluation of imiquimod/omiganan combination therapy in HPV-induced skin diseases.

INTRODUCTION

Cathelicidins are a family of antimicrobial (cationic) peptides that play an important role in the first line immune defence of the skin, related to their broad antimicrobial activity against bacteria, viruses and fungi.[1] LL-37 is the only human member of the cathelicidin family.[1] Besides its antimicrobial effects, this peptide also has direct immunomodulatory activity. LL-37 affects the response of neutrophils to viruses, and modulates interferon (IFN) responses induced by viral triggers.[2] LL-37 converts self-RNA into a ligand for Toll Like Receptor (TLR) 7 and TLR8 in human dendritic cells, thereby enhancing IFNα production in human skin.[3]

Omiganan is a synthetic indolicidin (a cathelicidin isolated from bovine neutrophils), currently under development as topical gel for several clinical indications. Omiganan is known to have activity against a wide variety of microorganisms such as gram-positive and gram-negative bacteria and fungi.[4,5] Moreover, omiganan enhances IFN responses induced by TLR3 (POLY:IC), TLR7 (imiquimod), TLR8 (ssRNA) and TLR9 (CPG) in human immune cells, comparable but not similar to the effects observed for LL-37 (unpublished data, *Grievink et al.*). These observations support the future application of omiganan as co-treatment with endosomal TLR ligands for viral skin disease in humans.

Imiquimod is the only registered endosomal TLR ligand, as Aldara® topical cream. The mechanism of action of imiquimod is based on TLR7-dependent MYD88-signalling.[6,7] This results in two responses: a tumoricidal effect by the release of several pro-inflammatory cytokines (e.g. TNF-α, IL-6 and IL-8, via NFκβ) and an anti-viral response by the induction of IFNα and IFN-inducible genes (e.g. Mx1 and Mx-A, via IRF7).[8] Based on these mechanisms imiquimod is widely used in clinical practice for human papilloma virus (HPV)-induced anogenital warts and high grade squamous intraepithelial lesions of the vulva (vulvar HSIL), actinic keratosis (AK), and basal cell carcinoma (BCC).[9] In most of these conditions, drug efficacy is suboptimal, and lesions may reoccur after treatment discontinuation.[10] Therefore, a treatment enhancing the

efficacy of imiquimod in these dermatological conditions would be of great benefit. Based on its observed preclinical activity, omiganan may be a good candidate for combination treatment with imiquimod.

We recently developed an *in vivo* challenge model with transient local skin inflammation, induced by 48h imiquimod (Aldara® cream) application under occlusion by a 12mm Finn Chamber to tape stripped skin.[11] This model was used in the current study to explore the potential of combined imiquimod and omiganan treatment as novel therapeutic modality for HPV-induced skin diseases, e.g. genital warts and vulvar HSIL. Omiganan was applied topically to imiquimod-primed skin, and the clinical, biophysical, cellular and molecular responses to this combined treatment were investigated.

METHODS

STUDY DESIGN AND SUBJECTS

This was a randomized, open-label, evaluator-blinded, vehicle controlled, parallel-cohort, dose ranging study. The study was conducted from February 2017 to March 2017 at the Centre for Human Drug Research, Leiden, the Netherlands, and was approved by the independent Medical Ethics Committee 'Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek' (Assen, the Netherlands). The study was conducted according to the Dutch Act on Medical Research involving Human Subjects (WMO). Before study procedures started, all subjects gave informed consent. Sixteen (16) healthy male and female Caucasian (Fitzpatrick skin type I-II) volunteers, aged 18 to 45 years, were included. Subjects with a (family) history of psoriasis or any disease associated with immune system impairment were excluded.

TREATMENTS AND RANDOMIZATION

To explore the effect of omiganan and the combination of omiganan and imiquimod on tape stripped skin, treatment combinations were applied and randomized over different treatment sites on the back (*Table 1*). All 4 treatment combinations were explored in each study participant. A standard daily dosage containing either 100 mg Aldara® 5% (5 mg imiquimod, IMQ), 100 mg omiganan 1% (1 mg omiganan, OMN), 100 mg omiganan 2.5%, 100 mg omiganan vehicle (VehO) or cetomacrogol (which served as imiquimod vehicle, VehI) was applied under occlusion by a 12 mm Finn chamber (Smart Practice, Phoenix, U.S.A.). The tape stripping procedure included 20 times stripping with tape (D-Squame, CuDerm, Dallas, US) to induce mild barrier disruption.

It should be noted that within the same clinical study, alternative regimens and control conditions were explored, within the same group of 16 volunteers. These additional conditions included the reverse treatment sequence (first imiquimod, then omiganan) and partial control groups vehicle/imiquimod or vehicle/omiganan (1% or 2.5%). To increase the readability of this manuscript, it was decided to not present data related to these conditions.

SKIN ASSESSMENTS

The skin was assessed daily for 5 days for signs of inflammation (erythema and hyper perfusion) by 2D photography erythema index analysis, visual erythema grading (Clinician Erythema Assessment (CEA) scale; 0 represents absence of erythema, 4 very severe), colorimetry (a value; DSM II ColorMeter, Cortex Technology, Hadslund, Denmark), and perfusion by laser speckle contrast imaging (LSCI; PeriCam PSI System, Perimed Jäfälla, Sweden). TAP (FibroTx, Estonia) were used to quantify skin surface biomarkers (IL-8, IFNα, IL-6, IL-10, CCL20 and HBD-2) by spot-ELISA at pre-dose and after end-of-treatment. Skin swabs were collected for microbiome analysis.

Three-millimetre punch biopsies were collected pre-dose (after tape stripping) and at end-of-treatment. For all 16 subjects, a biopsy of the VehO+VehI, IMQ+OMN1% and IMQ+OMN2.5% treated areas was collected. For only 8 subjects the IMQ+VehO treated area was biopsied, to limit the number of biopsies per subject. Biopsies were snap frozen using liquid nitrogen and stored at -80°C until analysis at the Immunology Laboratory of Erasmus Medical Center, Rotterdam, The Netherlands for determination of IFNα, IFN-γ, IL-1β, IL-6, IL-8, HBD-2, Mx1, Mx-A, CCL20 and IL-10 mRNA expression relative to the housekeeping gene ABL by quantitative

PCR. In addition, all biopsies were haematoxylin and eosin (H&E) stained to obtain histopathological scores of psoriasis and dermatitis; general infiltration, parakeratosis, acanthosis, papillomatosis and spongiosis. The histopathological score for each characteristic was graded based on fold increase or decrease compared to a reference biopsy of a healthy subject not participating in the clinical trial (1; equal to the reference biopsy, 2; 2-fold increase compared to the reference biopsy etc.). Furthermore, immunohistochemical staining was performed to obtain scoring of markers CD11c (Clone 5D11, Cell Marque), CD14 (Clone EPR3653, Cell Marque), CD1a (Clone EP3622, Cell Marque), CD4 (Clone SP35, Ventana), CD8 (Clone SP57, Ventana) and HLA-DR (CR3/43, Dako).

SAFETY ENDPOINTS Safety and tolerability were monitored by tracking adverse events, performing physical examination, measuring vital signs, 12-lead electrocardiograms, and laboratory tests (i.e. hematology, chemistry and urinalysis) at multiple time points throughout the study. IFNα, IFN-β and IFNɣ were measured in blood samples to detect a possible systemic effect of the interventions.

STATISTICS Treatment effects were analysed with a mixed model analysis of variance with the baseline measurement as covariate. To determine the differences between the treatments, contrasts were calculated for all measurements. All calculations were performed using SAS for windows V9.4 (SAS Institute, Inc., Cary, NC, USA). Evaluation window for non-invasive measures was 0-96 hours (day 4), whereas biopsies were collected at 120 hours (day 5).

RESULTS

12 female (75%) and 4 male (25%) Caucasian subjects participated in the study. All 16 included subjects completed the study according to the schedule in *Table 1*. The mean age was 24.6 (SD ±5.8 years). Application site pruritus was the most frequent occurring Adverse Event (AE) in 14/16 subjects (87.5%). This can be related to the tape stripping procedure, occlusion procedure or one of the treatments or vehicles. No serious adverse events (SAEs) or discontinuations due to AEs occurred. No systemic effects of any of the treatment in terms of elevated circulating cytokines (serum IFNα, IFN-β of IFNɣ) were observed (data not shown).

Imiquimod treatment resulted in a modest inflammatory response, observed as enhanced erythema (quantified by 2D photograph, *Figure 1* top panel) and perfusion (quantified by laser speckle contrast imaging, *Figure 1* bottom panel). The maximal imiquimod response was reached after 1-2 days treatment (*Figure 2*). After 48 hours of imiquimod/vehicle exposure, the target areas were treated with omiganan (or vehicle) for an additional 2 days. Omiganan treatment enhanced the imiquimod-driven increase in skin perfusion and erythema, without an indication of omiganan dose-dependency (*Figure 2*). Omiganan treatment significantly enhanced perfusion (profile 0-96h) (*Figure 2A*: imiquimod+vehicle versus imiquimod+omiganan; +17.1%, 95% CI 5.6-30%, p<0.01 and +15.1%, 95% CI 3.8-27.7%, p<0.01, for 1% and 2.5% omiganan, respectively). For erythema, a statistically significant omiganan effect was observed (profile 0-96h;for colorimetry, but only at the 1% omiganan dose (*Figure 2B*: imiquimod+vehicle versus imiquimod+omiganan +1.5, 95% CI 0.25-2.83, p=0.02 and +0.92, 95% CI 0.37-2.21, p=0.16, for 1% and 2.5% omiganan, respectively). Omiganan treatment did not significantly alter imiquimod-related increases in erythema index (profile 0-96h; +0.8, 95% CI -1.62-3.25, p=0.51 and +2.21, 95% CI -0.23-4.64, p=0.08 for 1% and 2.5% omiganan, respectively). The enhanced inflammatory responses were observed during the omiganan treatment period (day 3 and 4, 48-96h). Hereafter, perfusion and erythema returned within one day to levels as observed for the imiquimod + vehicle treatment within one day (*Figure 2*, 120h).

In addition to the above non-invasive assessments, skin punch biopsies were taken from the target areas. Biopsies were stained for dermal immune cell infiltration, and independently analyzed by two investigators blinded to treatment compared to a reference biopsy (healthy unaffected skin). Imiquimod treatment resulted in an influx of immune cells in the skin, reflected by an increase in macrophages, HLA-DR cells, myeloid

dendritic cells, Langerhans cells, and CD4$^+$ and CD8$^+$ T cells (*Figure 3A-F*, second bars versus first bars). Consistent with the observations for perfusion and erythema, omiganan treatment enhanced the imiquimod-driven inflammatory response as quantified in skin punch biopsies. When imiquimod-exposed skin was treated with omiganan, this resulted in a strong increase of infiltrating immune cells (*Figure 3A-F*, third and fourth bars versus second bars). There was no indication of a clear omiganan dose-dependency, although the response to the 1% omiganan formulation appeared slightly higher.

Subsequently, the effects of imiquimod and omiganan add-on treatment on local cytokine responses were investigated. As expected, imiquimod treatment resulted in an NFκβ-driven increase in IL-6 and IL-10 (*Figure 4A*, IL-6 imiquimod/vehicle versus vehicle/vehicle +120.9%, 95% CI 2.6%-375.6%, p=0.04, IL-10 imiquimod/vehicle versus vehicle/vehicle +132.1%, 95% CI 40.8%-282.8%, p=0.001). In line with this, imiquimod increased the expression of type I interferon-driven Mx-A (*Figure 4B* left panel, imiquimod/vehicle versus vehicle/vehicle +213.3%, 95% CI 50.7%-551%, p=0.002) and IFNɣ (*Figure 4B* right panel, imiquimod/vehicle versus vehicle/vehicle +542.4%, 95% CI 132.1%-1678.3%, p<0.001). No treatment effect was observed for Mx1 expression. Subsequently, omiganan was applied for two days to the target areas. Though omiganan did not significantly alter any of the imiquimod-driven responses, a higher level of cytokines was consistently found in the imiquimod/omiganan treatment group when compared with the imiquimod/vehicle treatment group (*Figure 4A*, IL-6 and IL-10: for 1% omiganan +26.3%, 95% CI -41.6%-173.1%, p=0.55, and +36.1%, 95%CI -17.7%-125.1%, p=0.23, for IL-6 and IL-10 respectively; *Figure 4B*: +88.4%, 95% CI -9.4%-291.5%, p=0.09, and +44.4%, 95%CI -48.1%-302.4%, p=0.48, for Mx-A and IFNɣ, respectively). Overall, the response induced by 1% omiganan was more outspoken than the response to 2.5% omiganan. IL-8 was induced by imiquimod but no enhancement was seen with omiganan addition (data not shown). No effects of imiquimod and omiganan add-on treatment were observed for the skin surface biomarkers by transdermal analysis patch (TAP), or on skin microbiome (data not shown).

DISCUSSION

In human peripheral blood mononuclear cells, omiganan enhances inflammatory responses driven by endosomal TLRS (unpublished data, *Grievink et al.*). Omiganan strongly increased type 1 IFN responses when cells were incubated with ligands for TLR3 (Poly:IC), TLR7 (imiquimod), TLR8 (ssRNA) or TLR9 (CpG). IRF (interferon regulatory factor) and NFκβ pathways, induced by these endosomal TLRS, drive tumoricidal and antiviral responses. Therefore, enhancement of endosomal TLR signalling in the skin may be of therapeutic interest for a variety of pathophysiological conditions. To investigate the clinical translation of omiganan's enhancement of endosomal TLR signalling, a healthy volunteer study was designed exploring the effects of imiquimod combined with omiganan add-on treatment. This combination was well tolerated by the study participants, the main adverse event being mild application site pruritus which was equal to the imiquimod alone and omiganan alone treatment groups. The clinical skin response was evaluated with laser speckle contrast imaging (perfusion) and erythema assessments (colorimetry, erythema, and visual grading by the physician). Two days of imiquimod treatment induced an inflammatory response similar as previously described,[11] with erythema, increased perfusion and increased inflammatory cell infiltration on histopathology lasting for at least 5 days. This effect was enhanced when imiquimod was combined with omiganan treatment. The influx of immune cells coincided with an increased cytokine response. Imiquimod induces an inflammatory response via TLR7-driven IRF and NFκβ signaling (Guiducci et al, 2009), which plays a role in a variety of dermal cells (T cells, keratinocytes, macrophages, Langerhans cells, dendritic cells). In this study, omiganan treatment increased the imiquimod-driven production of IL-6 and IL-10, reflecting NFκβ activity. Also IRF-driven pathways were enhanced: after application of omiganan, elevated expression levels Mx-A were observed. Mx-A is a downstream mediator of interferons; its expression indicates an IFNα response.[12] Moreover, omiganan treatment increased type II interferon (IFNɣ) levels, which is mainly produced by T cells. Importantly, cellular

and molecular responses were quantified in skin biopsies collected at day 5 (120 hours), where omiganan (or vehicle) was applied at 0, 24, 48 and 72 hours. It could be contemplated that at earlier (uninvestigated) time points, the additive effect of omiganan on immune responses was more outspoken, as observed for laser speckle and 2D photography data at time points 72 en 96 hours.

Our results relate to experimental conditions where skin of healthy human volunteers was first primed with imiquimod, and subsequently treated with omiganan. The reverse sequence was also studied, with omiganan pretreatment for 2 days followed by 2 days application of imiquimod. With this treatment sequence, the enhenced effects of omiganan on imiquimod responses were not observed (data not shown). This is in line with mechanistic in vitro experiments on human PBMCs, which suggest that coinciding exposure to omiganan and endosomal TLR ligands result in the strongest immune response (unpublished data, *Grievink et al.*). Furthermore, omiganan treatment alone did not induce any clinical, molecular or cellular immune response (data not shown), which also corroborates with earlier PBMC-based experiments. It is hypothesized that the immune enhancing effects of omiganan on endosomal TLR signaling requires a complex formation between the cationic peptide and the TLR ligand. Such complex formation has been demonstrated earlier, for example between TLR9 ligand CPG and the bovine host defense peptide indolicidin, thereby enhancing innate and adaptive immune responses.[13]

The potentiating effect of omiganan on imiquimod induced responses, and potentially on the effect of other endosomal TLR ligands that are currently under development as immunostimulatory compounds, may be interesting from a drug development perspective. The effectiveness of imiquimod treatment for HPV-induced skin disease is suboptimal. In anogenital warts for example, the estimated complete clearance is approximately 50%, with a recurrence rate of 13-19%. For HSIL, effectiveness of imiquimod is estimated to be 58% with a 16% recurrence rate (10, 14-16). These data underline the need for enhanced treatment modalities. The combination treatment of imiquimod with omiganan may be considered as such. Although omiganan's effect size on top of imiquimod-induced responses was relatively small in our study, and no clear dose-dependency for omiganan was observed, our findings support the mechanistic concept of omiganan-dependent enhancement of endosomal TLR signaling. Thus, optimization of combined omiganan/imiquimod treatment appears to be a rational way forward.

For practical reasons, imiquimod and omiganan could only be administered as alternating treatments. Since a plausible mechanistic basis for omiganan-enhanced imiquimod effects is the complex formation between TLR ligand and cationic peptide, it is not likely that pharmaceutical adjustments can be made to increase the desired effects. This may consist of optimization of the formulation containing a mixture of both compounds, or application of treatment regimens with rapid alternation of omiganan and imiquimod. Importantly, the observed enhanced imiquimod responses by omiganan co-treatment also support further exploration of treatments combining omiganan with other endosomal TLR ligands. The limitation is that currently no other endosomal TLR ligands besides imiquimod are available for clinical application in the EU. Rintatolimod, a TLR3 ligand, is only accessible via an Early Access Program for chronic fatigue syndrome. Other interesting candidates for combined treatment with omiganan include resiquimod, a TLR7/8 agonist, or one of the TLR9 agonists that are currently being evaluated in phase III clinical programs.

In summary, omiganan enhanced the inflammatory skin response to imiquimod, as studied in healthy volunteers with laser speckle contrast imaging (perfusion), 2D photography (colorimetry, erythema, visual grading), and analysis of molecular and cellular responses in skin biopsies. *Figure 5* provides a graphical summary of key biomarkers, and underlines the omiganan-induced increase of imiquimod-driven responses. These findings are in line with the observations of enhanced endosomal TLR responses by omiganan in *in vitro* experiments on primary human immune cells, and are supporting evaluation of imiquimod/omiganan combination therapy in HPV-induced skin diseases such as anogenital warts or HSIL.

REFERENCES

1 Kosciuczuk EM, Lisowski P, Jarczak J, Strzalkowska N, Jozwik A, Horbanczuk J, et al. Cathelicidins: family of antimicrobial peptides. A review. Mol Biol Rep. 2012;39(12):10957-70.

2 Takiguchi T, Morizane S, Yamamoto T, Kajita A, Ikeda K, Iwatsuki K. Cathelicidin antimicrobial peptide LL-37 augments interferon-beta expression and antiviral activity induced by double-stranded RNA in keratinocytes. The British journal of dermatology. 2014;171(3):492-8.

3 Ganguly D, Chamilos G, Lande R, Gregorio J, Meller S, Facchinetti V, et al. Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8. J Exp Med. 2009;206(9):1983-94.

4 Fritsche TR, Rhomberg PR, Sader HS, Jones RN. Antimicrobial activity of omiganan pentahydrochloride against contemporary fungal pathogens responsible for catheter-associated infections. Antimicrob Agents Chemother. 2008;52(3):1187-9.

5 Fritsche TR, Rhomberg PR, Sader HS, Jones RN. Antimicrobial activity of omiganan pentahydrochloride tested against contemporary bacterial pathogens commonly responsible for catheter-associated infections. The Journal of antimicrobial chemotherapy. 2008;61(5):1092-8.

6 Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, et al. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol. 2002;3(2):196-200.

7 Lee J, Chuang TH, Redecke V, She L, Pitha PM, Carson DA, et al. Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: activation of Toll-like receptor 7. Proc Natl Acad Sci U S A. 2003;100(11):6646-51.

8 Schon MP, Schon M. Imiquimod: mode of action. The British journal of dermatology. 2007;157 Suppl 2:8-13.

9 Wagstaff AJ, Perry CM. Topical imiquimod: a review of its use in the management of anogenital warts, actinic keratoses, basal cell carcinoma and other skin lesions. Drugs. 2007;67(15):2187-210.

10 Grillo-Ardila CF, Angel-Muller E, Salazar-Diaz LC, Gaitan HG, Ruiz-Parra AI, Lethaby A. Imiquimod for anogenital warts in non-immunocompromised adults. The Cochrane database of systematic reviews. 2014(11):CD010389.

11 van der Kolk T, Assil S, Rijneveld R, Klaassen ES, Feiss G, Florencia E, et al. Comprehensive, multi-modal characterization of an imiquimod-induced human skin inflammation model for drug development. Clin Transl Sci. 2018.

12 Haller O, Kochs G. Human MxA protein: an interferon-induced dynamin-like GTPase with broad antiviral activity. J Interferon Cytokine Res. 2011;31(1):79-87.

13 Kovacs-Nolan J, Mapletoft JW, Lawman Z, Babiuk LA, van Drunen Littel-van den Hurk S. Formulation of bovine respiratory syncytial virus fusion protein with CpG oligodeoxynucleotide, cationic host defence peptide and polyphosphazene enhances humoral and cellular responses and induces a protective type 1 immune response in mice. J Gen Virol. 2009;90(Pt 8):1892-905.

14 Wiley DJ, Douglas J, Beutner K, Cox T, Fife K, Moscicki AB, et al. External genital warts: diagnosis, treatment, and prevention. Clin Infect Dis. 2002;35(Suppl 2):S210-24.

15 Moore RA, Edwards JE, Hopwood J, Hicks D. Imiquimod for the treatment of genital warts: a quantitative systematic review. BMC Infect Dis. 2001;1:3.

16 Lawrie TA, Nordin A, Chakrabarti M, Bryant A, Kaushik S, Pepas L. Medical and surgical interventions for the treatment of usual-type vulval intraepithelial neoplasia. The Cochrane database of systematic reviews. 2016(1):CD011837.

TABLE 1 Treatment combinations.

	DAY 0	DAY 1	DAY 2	DAY 3
1	imiquimod	imiquimod	vehicle(omiganan)	vehicle(omiganan)
2	imiquimod	imiquimod	omiganan 1%	omiganan 1%
3	imiquimod	imiquimod	omiganan 2.5%	omiganan 2.5%
4	vehicle(omiganan)	vehicle(omiganan)	vehicle(imiquimod)	vehicle(imiquimod)

FIGURE 1 Clinical impression of imiquimod (IMQ) response (left panel) and imiquimod + omiganan (OMN, middle and right panel) of one subject at day 4, 24 hours after the last application of omiganan or vehicle.

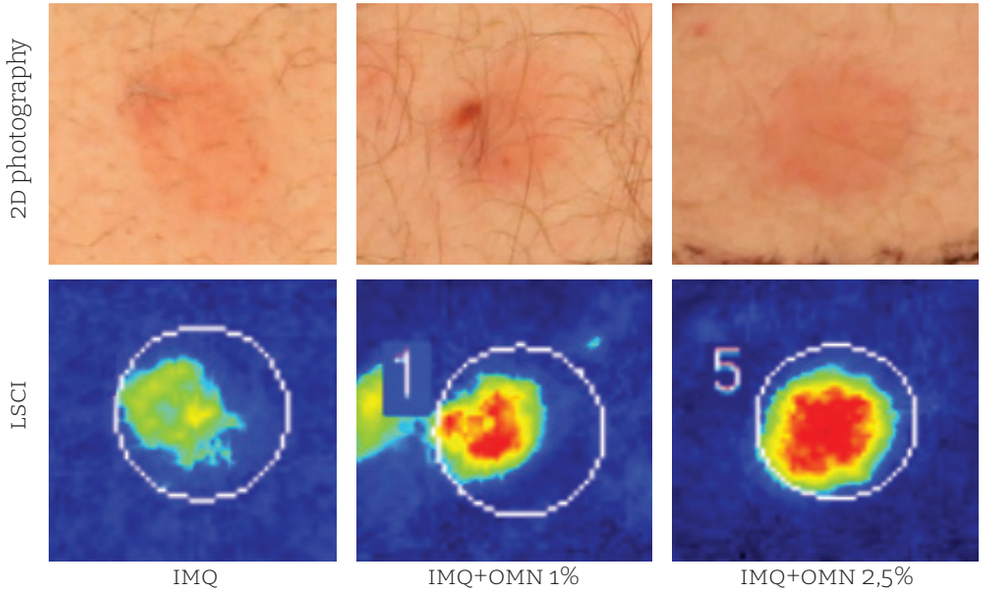

FIGURE 2 Skin inflammation induced by imiquimod (IMQ) and omiganan (OMN), as quantified by LSCI (perfusion/basal flow, A), and erythema assessments (B: colorimetry, C: erythema, D: visual grading).

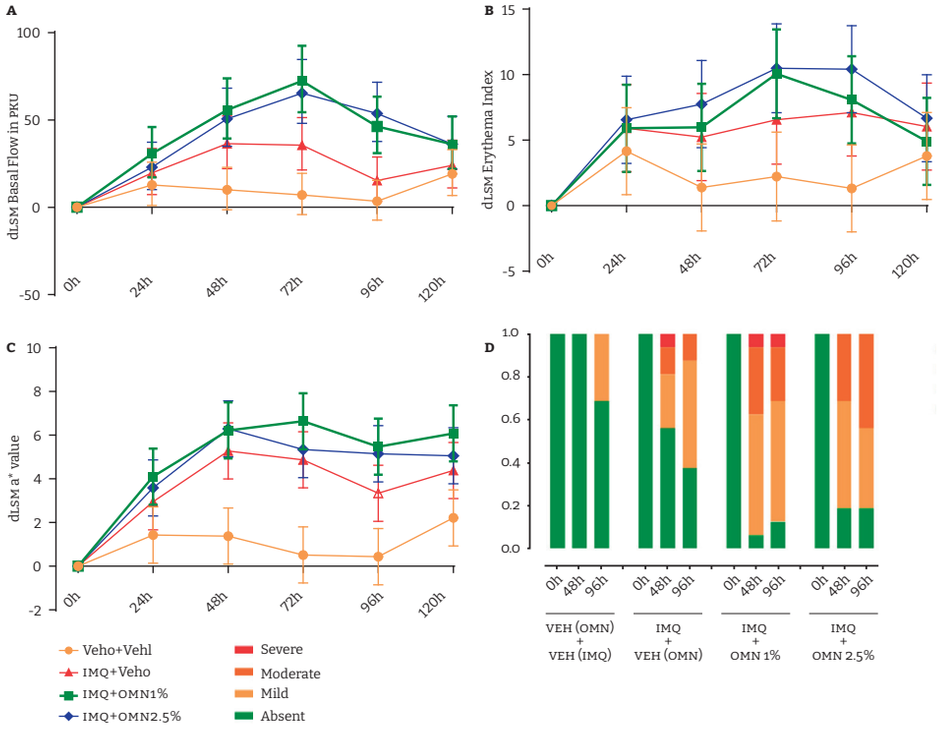

FIGURE 3 Skin inflammation induced by imiquimod (IMQ) and omiganan (OMN) on day 5 (scored compared to a reference biopsy), as quantified by immune cell influx. A) $CD14^+$ macrophages, B) HLA-DR cells, C) $CD11c^+$ myeloid dendritic cells, D) $CD1a^+$ Langerhans cells, E) $CD4^+$ T cells, F) $CD8^+$ T cells.

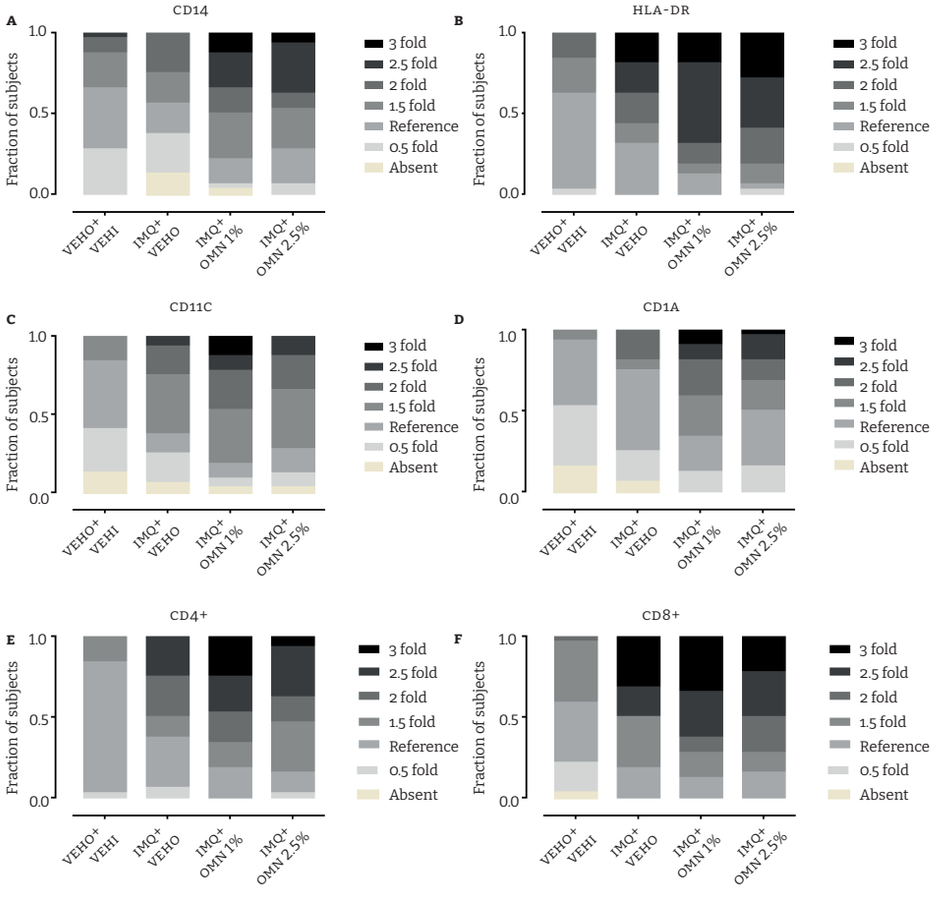

FIGURE 4 Skin inflammation induced by imiquimod (IMQ), omiganan (OMN), vehicle imiquimod (VI) and vehicle omiganan (VO) on day 5, as quantified by cytokine production (qPCR) relative to ABL. A) IL-6 (left panel) and IL-10 (right panel), B) Mx-A (left panel) and IFNγ (right panel). N=8 for the IMQ+VehO contrast and N=16 for the other contrasts.

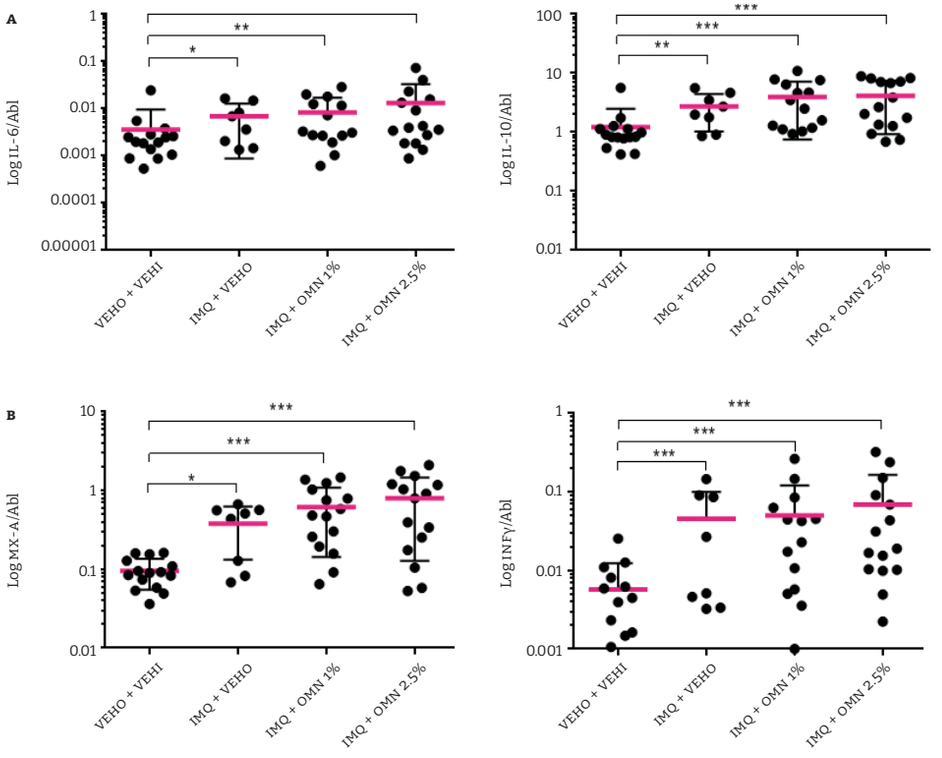

FIGURE 5 Graphical summary of key biomarkers. NF-κB-driven immune response (IL-6), IRF-driven immune response (Mx-A), perfusion (LSCI), colorimetry (erythema), and immune cell infiltration (CD1a Langerhans cells). Responses were normalized to the maximal effects. Category labels indicate the actual minimum and maximum response.

CHAPTER VII

SUMMARY AND PERSPECTIVES

Adapted from: Lost in translation: A Commentary on TLR7 agonistic response between mice and humans

Submitted

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Over the past decades, increased attention has been directed towards the improvement of the drug development process. The conventional approach of categorizing clinical trials into phase I-III and a post- marketing phase (IV) tends to be replaced by a more integral, biomarker-enriched, question based drug development approach in which answers to the most relevant questions, I.E., does the drug reach the site of action, does the compound have a pharmacological effect etc., are obtained to gain as much information as possible on the mechanism of action already in the early phase of drug development.[1]

The introductory section of this thesis outlines the strategies to meet this integral approach by obtaining more information about the mechanism of action in the earliest phase of drug development. Firstly, implementing biomarkers with acceptable predictive accuracy into clinical trials is needed to increase the probability of success (POS) compared to trials not using biomarkers. Secondly, implementing proof-of-mechanism studies targeted at a specific population is encouraged, aiming for an effective dosage regimen and duration. These studies seem to provide loads of information about the mechanism of action of the investigated drug in early phase drug development programs. Pharmacological challenge models or experimental models in humans that temporarily mimic components of physiological and pathophysiological conditions are vital for this type of studies, as the absence of a disorder, E.G. an inflammatory condition, in healthy volunteers may impede the examination of these hallmarks. Proof-of-mechanism models have been previously successfully introduced in the field of neurology, especially to gain a more in-depth understanding of pain. These models, generally referred to as human evoked pain models, have proved to be pivotal in bridging the gap between animal research and patients with pain.[2-4] In infectiology, the so called controlled human infection models (CHIMS) gain growing interest, especially aftermath of the SARS-COV-2 pandemic, with the aim of expediting vaccine development.[5-7] Within the field of psychiatry, inhalation of elevated CO2 concentrations in healthy individuals can induce panicogenic symptoms similar to those experienced during a panic attack by patients.[8-10]

One of the pharmacological skin challenge models that has demonstrated high potential in preclinical mice studies is a model with imiquimod application.[11] This model shows clinical features similar to psoriasiform lesions, but also activates immunological pathways that are of importance for various other auto-inflammatory/immunity diseases, such as type I interferonopathies (E.G., cutaneous lupus erythematosus). However, a translation of this model to humans has not yet been accomplished. The model should fulfil two criteria:

I elicit an inflammatory response comparable to the one observed in murine models
II should fit for purpose for proof-of mechanism in healthy volunteers.

The studies described in this thesis address the outlined strategies to obtain more information about the mechanism of action in the earliest phase of drug development by implementing biomarkers into clinical trials and implementing proof-of-mechanism studies. The primary aim of this thesis was to develop and characterize a mechanistic model to investigate skin inflammation on a mechanistic basis in healthy volunteers by applying imiquimod, for utilization in drug development programs. By using an array of assessments focused on imaging-based, biophysical, cellular, and molecular changes, this thesis describes the extensive characterization of the inflammatory response.

SUMMARY OF FINDINGS

In **chapter 2** we provided a summary of clinical studies in which cutaneous pharmacological challenge models are used. These models offer a controlled and appropriate way to assess a drug candidate's effects before proceeding to the next phase where the compound is tested in patients, therewith potentially reducing the attrition rate. The chosen challenge agents represent processes within the skin and target innate immune responses. Introduction was made to the LPS challenge and different examples have been provided such as UV-B model, assessing itch by histamine and cowhage. Furthermore, the delayed type of hypersensitivity

model using KLH has also been elucidated. Interestingly, notable achievement was reached with the KLH challenge, implemented to evaluate the efficacy of amlitelimab, a novel non-depleting IGG4 human anti- OX40L monoclonal antibody in healthy volunteers.[12-14] Based on the data that was generated and the selected dose from the first-in-human study, the compound has successfully been tested in patients with atopic dermatitis, and the results were presented at the American Academy of Dermatology meeting in March 2024.[15] Of note, the dose-selection for the patient studies was based on the effects observed with amlitelimab on the KLH challenge. This serves as an excellent example of the contemporary shift in drug development paradigms. Rather than conducting a first-in-human trial solely focused on safety and tolerability, an integrated approach has been adopted to assess the effect of a new drug candidate at an early stage, aiding in dose determination for trials in subsequent phases of drug development.

A similar integrated approach has been utilized in **chapter 3**, which described a randomized, open-label, vehicle-controlled clinical study to develop a temporary skin inflammation model. Cutaneous inflammation in this model was induced by 5 mg of the topical TLR7 agonist, I.E. imiquimod (IMQ). IMQ was applied under occlusion on the upper back of 16 healthy male volunteers in an open labelled fashion for 24h, 48h and 72h. Healthy volunteers were randomized to undergo either tape-stripping (TS) of the region of interest before IMQ application or application of IMQ to the intact skin. The results showed that while IMQ alone induced limited effects, the combination of tape stripping and IMQ application led to more significant and measurable skin inflammatory responses. These responses included increased erythema and skin perfusion, elevated MRNA expression of inflammatory markers, and an influx of inflammatory cells compared to the control group treated with a vehicle. Activation of the innate immune system was evidenced by significantly elevated expression of chemokines CXCL10, ICAM-1, HBD-2 and MX-A of the TS group compared to untreated. These findings were further amplified by infiltration of cells such as T helper cells, cytotoxic T cells, macrophages, and dendritic cells after 48h to 72h of IMQ application. The effects were more pronounced in the tape stripped group. In general, there was no additional effect observed after 72h of IMQ application compared to 48h on all readouts, indicating that a 48h application period appears to be the most suitable duration.

The next step was to test if the clinical response and immunological response by IMQ could be reversed using an anti-inflammatory compound that could serve as a benchmark. For this purpose, in **chapter 4**, we randomly administered oral prednisolone/placebo at a clinically conventional dose of 0.25 mg/kg/dose in 24 healthy volunteers for six consecutive days. In this study, IMQ was applied on TS skin for 48h. The clinical, cellular, and molecular responses were characterized using a multimodal approach and a different technique was introduced to examine the TLR7 cellular and cytokine responses by inducing suction blisters. Oral prednisolone suppressed the TLR7-driven response on almost every readout including skin perfusion, erythema, epidermal thickness, immune cells (i.e. NK, classical monocytes, T helper, cytotoxic cells) and cytokines (i.e. IL-6, IL-8, TNF and MX-A). In previous chapters we have demonstrated that IMQ does not enter the systemic circulation. However, there was increased interest to investigate the difference in effect of prednisolone between blood and peripheral tissue. As a result, we have tested the *ex vivo* drug activity via whole-blood IMQ stimulation with cytokine release as a readout. We have proved that suppression on cytokines by prednisolone is less dependent on pharmacokinetic profile of the compound. In both compartments, blood and tissue, prednisolone showed anti-inflammatory characteristics, yet in blood these effects were of transient nature.

Furthermore, this was the first study that compared the pharmacodynamic biomarkers obtained from the conventional skin punch biopsies with those in blister exudate in IMQ- induced inflammation. Already back in the mid 1960's suction blisters were described as a method for dermo-epidermal separation, providing added value for studying inflammation in tissue.[16] This technique has also been used in multiple

studies in which inflammatory responses to different intradermally applied challenge agents, such as tuberculin purified protein derivative (PPD), UV-killed *Escherichia coli* (UVKEC), KLH and LPS, were studied.[17-21] Cells and cytokines in blister exudate following LPS challenge were fully concordant with the measured cellular response in biopsies, with minimal invasive inflammatory cell influx and neglectable cytokine concentrations related to the blister procedure itself. While cellular responses studied in blister exudate focus on intradermally administered challenge agents, topical applications using this technique have also been reported. Blister exudates taken from participants who were subjected to topical application of 0.075% capsaicin, an extract of chili peppers, have not revealed any differences in inflammatory markers such as IL-1α and TNF.[22] In our study, despite the low number of total cells in blister exudate, comparable immune cell subsets were seen in IHC staining. Although we have not identified cytokines through QPCR in this study for a direct comparison between blister exudate and biopsy, we can infer the expression of specific cytokines from the research that has been previously conducted. Comparable cytokine patterns were observed, suggesting that the blister technique is both effective and straightforward for investigating inflammation in a minimally invasive manner. As opposed to intradermal administration of challenge agents, the readout of blister exudate for topically applied challenge agents is not robust across studies. Research in which immunomodulatory effects of a single-strain probiotic were evaluated on the TLR7 driven IMQ model, resulted in a more prominent cell infiltration of different cell types in blister exudate.[23] Larger numbers of total cells were found as well as infiltration of neutrophils, NK cells, granulocytes, monocytes (classical, intermediate, and non-classical), MDCs, T helper cells and cytotoxic T cells. The majority of these cells are in line with findings observed in murine models and **chapters 3 and 4** of this thesis. However, one significant contrast exists between murine models, the research conducted by *Eveleens-Maarse et al.,* and our findings. This difference lies in the notable recruitment of neutrophils observed in murine models and the work of *Eveleens-Maarse et al.,* which was not observed in our studies. Moreover, IMQ activated the complement cascade in mice, however this process was not yet evident from clinical studies.

The absence of neutrophils in human IMQ model was surprising and markedly different from the murine IMQ model, where neutrophils are typically present. A potential explanation was presented to be the relatively short duration of IMQ exposure in our clinical studies so far. Therefore, in **chapter 5** we characterized the TLR7-mediated inflammatory response after 7 days (168h) of IMQ exposure in healthy volunteers and compared this response to the already established short duration 2-3 days (48h and 72h). As a secondary objective, we aimed to test if the complement cascade could be activated following prolonged IMQ exposure. For this purpose, 10 healthy volunteers participated in a randomized, open-label study. Prolonged IMQ exposure resulted in amplification of the IMQ-induced inflammatory response. Transcriptomic analysis revealed activation of TNF signalling, complement and predominantly strong interferon responses. These findings were especially feasible after prolonged IMQ exposure. Furthermore, we have observed that secretion of interferons leads to enrichment of the JAK-STAT pathway, with an increase in the expression of CXCL9, CXCL10 and CXCL11 chemokines. Additionally, in this study, absence of neutrophils as a driving factor in the response was confirmed, which was not surprising given the minimal presence of IL-8, leading to a significant translational gap between mice and humans.

Interestingly, prolonged IMQ exposure did not lead to increased skin perfusion or erythema, revealing a discrepancy between cellular and vascular responses. This suggests that the observed imaging/biophysical changes are not mediated by cellular inflammation. Hence, to elucidate the mediators driving perfusion and erythema, future clinical studies should prioritise investigating the vascular response mechanisms rather than cellular aspects.

To examine the applicability of our model in drug development, we have conducted a clinical study presented in **chapter 6**. In this chapter we investigated the effects of omiganan (OMN) on the inflammatory responses induced by imiquimod (IMQ) and vice versa in the skin of 16

healthy individuals. OMN, a synthetic cathelicidin, shows broad antimicrobial activity and enhances IFN responses, resembling LL-37 effects. Cathelicidins, including LL-37, are vital antimicrobial peptides in skin immunity, combating various pathogens. LL-37 also modulates immune responses, enhancing IFN production induced by TLR3 ligand polyI:C in keratinocytes.[24] However, *in vitro* studies have demonstrated that this effect also applies for TLR7 ligand, IMQ.[25] IMQ treatment induced modest inflammatory responses, characterized by increased erythema and perfusion, peaking after 24h and 48h. Combining IMQ and OMN further enhanced these responses, particularly at a 1% concentration, which was also applicable for cellular response. An increase in macrophages/monocytes, DCs and T helper cells was evident, however this effect was limited in the corresponding cytokine response. IMQ treatment increased mRNA levels of IL-6, IL-10, MX-A, and IFN-γ, with OMN showing a trend towards amplification of these cytokine responses. In a recently conducted study, 2.5% OMN was applied in patients with anogenital warts and with high-grade squamous intraepithelial lesions, however, clinical efficacy was not demonstrated, despite reductions in viral load.[26] This further indicates that a combination therapy of IMQ and OMN might be beneficial for HPV-induced skin diseases.

CRITICAL EVALUATION OF THE IMQ-INDUCED INFLAMMATION MODEL

IS THERE A TRANSLATIONAL GAP? The introductory section of this thesis outlined the inflammatory immune response to IMQ in mice. In mice, clinical manifestations including thickening of the skin, erythema and scaling already appear within 2-3 days of IMQ application.[11] In humans, similar clinical findings were present, evidenced by an increase in skin perfusion, erythema, and epidermal thickness following 2-3 days of IMQ application.

In mice, exposure to IMQ triggers the activation and attraction of neutrophils, along with increased number of PDCs and γδ T cells. This was further reinforced by elevated cytokine levels of TNF, IL-1β and IL-6 coupled with a transient rise in IL-23, IL-17A, IL-17F, and IL-22 suggesting involvement of adaptive immune response, resembling psoriasiform lesions. In line with preclinical findings, in humans, infiltration of monocytes/macrophages, NK cells, DCs, T helper cells, and cytotoxic T cells was evident. At the cytokine level, there was notable IRF signalling, evidenced by increased MX-A, IFN-γ, and IP-10. Nevertheless, differences existed among species at the cellular and cytokine levels (*Figure 1)*. While increased levels of IL-6 and TNF were observed, their expression was less than that of IFN signalling, indicating that IMQ serves more as a model for IRF signalling rather than NF-κB.

A second notable difference between murine and human IMQ model is the lack of IL-8 and therewith neutrophils in healthy individuals. Neutrophils, acting as the first line of defence are rapidly recruited to the sites of inflammation in humans.[27,28] Therefore, it was first hypothesized that the window of opportunity to measure neutrophils with an IMQ challenge might have been missed, given that neutrophils are considered short-lived, with an estimated half-life of 13-19h.[29] However, the difference in neutrophilic response between mice and humans is attributable to the absence of TLR3 expression and the limited expression of TLR7 in human neutrophils.[30-32] They do respond to TLR4 ligands, such as LPS, which induces a more immediate and classical inflammatory response that complements the effects of IMQ.

Lastly, the difference in cellular and cytokine profiles in response to IMQ between mice and humans also affects the relevance of this model to disease. In preclinical research IMQ is often used to resemble psoriasis-like inflammation given the involvement of IL-23/IL-17A/IL-22 axis, while in humans the expression of these cytokines is limited. In the human IMQ studies, strong activation of IFN response is evident, leading to enrichment of the JAK-STAT pathway. These findings together with histopathological changes of a vacuolar interface dermatitis with adnexal involvement are indicative that the IMQ model in humans is more representative of lupus characteristics rather than psoriasis.

USE OF ACCURATE READ-OUTS In the field of clinometry, physicians typically perform lesion scoring for the features like erythema and or swelling. Researchers often strive to incorporate many biomarkers and endpoints, however, these happen to provide only one-dimensional information. In clinical research, a multi-dimensional, in-depth understanding of skin processes can be obtained by using a multimodal approach, outlined in the introduction section of this thesis. The pillars of this approach include (Figure 2):

I Clinical scores
II Subject- reported outcomes
III Imaging
IV Biophysical assessments
V Molecular analyses in biosamples E.G. biopsies, and blister exudate
VI Cellular assessments

In the chapters of this thesis, different combinations of the mentioned pillars have been thoroughly discussed. Moreover, imaging methods and biophysical evaluations, such as laser speckle contrast imaging, have been examined in other clinical studies involving challenge agents like LPS and KLH, in which they were suitable to objectively quantify erythema and skin perfusion.[13,21,33] While these assessments predominantly center on characterizing superficial skin reactions, there are also non-invasive techniques available that facilitate instant readouts on epidermal morphology up to 1-2 MM depth.[34] Optical coherence tomography, generally used in clinical research with patients, does recognize major histological structures, however it lacks high resolution to identify individual cellular changes as well as to determine specific proteins in the epidermis.[35] Nevertheless, within this thesis, optical coherence tomography has been utilized to quantify epidermal thickness, a measure that was effectively evaluated. This underscores the feasibility of substituting conventional biopsy methods with this technique in skin challenge studies. This multimodal approach has also been successfully implemented in phenotyping atopic dermatitis and seborrheic dermatitis, with the latter employing an integrated quantification of specific characteristics.[36–38] The techniques applied in this thesis offer promising grounds in characterizing the inflammatory response following IMQ application. Future research should aim to integrate diverse results and visualize the data after examining the most relevant hallmarks to achieve a more comprehensive understanding of the biological function of cells of interest and consequently, the mechanism or pathology of the disease.

CHALLENGES & PERSPECTIVES

IMQ serves as a valuable challenge agent, primarily targeting the interferon pathway, with a consistent immune response observed across studies despite variations in individual cellular responses. One of the limitations of IMQ is its topical application. While all subjects receive the same dosage, the extent of absorption through the skin has not been evaluated. It could be argued that topical administration of IMQ may not be the optimal approach for this model. Previous attempts, to activate and mobilize DCs through intradermal application of the TLR7 ligand on *ex vivo* skin, were unsuccessful, whereas topical administration of IMQ proved effective.[39] To refine the model, it is crucial to analyse the variability in dosing. *Wind et al.* introduced a methodology in her research employing matrix-assisted laser desorption/ionization mass spectrometry imaging to measure pharmacokinetic concentrations from skin biopsies following topical treatment with bimiralisib[40]. The same approach could be adapted for evaluating the pharmacokinetic properties of IMQ in the skin model. While the IMQ model underwent rigorous testing with novel drug candidates targeting their specific signalling cascade,[41] it is interesting to explore alternative fields where IMQ can be effectively applied.

The discoveries described in this thesis not only demonstrate successful translation but also indicate consistency across studies, suggesting the repeatability of the IMQ response in healthy individuals while a formal test-retest study has not yet been conducted. Another translational step will be set to patients. Recently the next step, I.E. translation to patients with cutaneous lupus erythematosus has been initiated by means of a clinical study.[42]

Lately, increased attention has been directed towards elucidating the function of TLRs and their potential as adjuvants in vaccine development.[43–45] IMQ was applied topically as skin adjuvant and showed enhanced responses to intradermal influenza vaccine[46]. A more recent illustration involves the evaluation of synthetic TLR7/8 ligands by Inimmune CORP. *in vivo* co-administered with SARS-COV-2 vaccine, which enhanced T cell responses as well as neutralizing antibody titres[47]. Given these encouraging outcomes, these molecules are presently evaluated in phase I clinical trials.

In addition to their potential use as adjuvants, synthetic TLR7/8 ligands have gained significant interest in cancer research[48]. Immunostimulatory molecules targeting TLRS 7/8 can activate both innate and adaptive immune responses by inducing cytokine production and activating cytotoxic cells. Through this immune response it is hypothesized that 'cold' tumours could be converted to "hot" tumours, which respond more favourably to immune checkpoint inhibitors.[49,50] Consequently, these TLR agonists are promising candidates for novel monotherapies or combination therapies. While some research suggests that TLR activation could be advantageous in cancer treatment, other studies indicate that TLR signalling might contribute to tumour development. Fully understanding the role of TLRs across different cancer cell types remains a significant challenge, necessitating extensive current clinical trials (Table 1). Given the promising signals stemming from preliminary analyses of some clinical trials, more comprehensive data is anticipated from ongoing studies of TLR agonists being developed for the treatment of solid tumours.

OVERALL CONCLUSIONS

This thesis encourages for implementation of challenge models in early phases of drug development to efficiently conduct clinical trials and facilitate timely go/no-go decisions. The use of a TLR7 agonist as challenge agent has been effectively translated from animals to human individuals, showing similarities and differences between species. The imiquimod response was comprehensively characterized through an integrated approach of multimodal biomarkers. The successful model development was achieved through a systematic process involving model optimization, benchmarking with an anti-inflammatory compound, and by implementing this model in a proof-of-mechanism study to demonstrate the *in vivo* pharmacological activity of a novel drug candidate. With this thesis we established a framework of studies that can be conducted prior to commercial application of a novel challenge model. This strategy employs multiple approaches to gather crucial data, assisting in making informed decisions about advancing to later stages or discontinuing product development early. Herewith, we anticipate that our approach will revolutionize the traditional paradigm of drug development by streamlining clinical research processes, thereby enhancing efficiency and reducing costs.

REFERENCES

1. Visser, S. J. de. Drug development project management by a new question based approach and decision analysis support. (2003).
2. Hijma, H. J. & Groeneveld, G. J. Analgesic drug development: proof-of-mechanism and proof-of-concept in early phase clinical studies. *Med. Drug Discov.* **10**, 100083 (2021).
3. Okkerse, P. *et al.* The use of a battery of pain models to detect analgesic properties of compounds: a two-part four-way crossover study. *Br. J. Clin. Pharmacol.* **83**, 976–990 (2017).
4. Siebenga, P. S. *et al.* Reproducibility of a battery of human evoked pain models to detect pharmacological effects of analgesic drugs. *Eur. J. Pain (United Kingdom)* **23**, 1129–1140 (2019).
5. Morrison, H., Jackson, S. & McShane, H. Controlled human infection models in COVID-19 and tuberculosis: current progress and future challenges. *Front. Immunol.* **14**, 1–10 (2023).
6. Choy, R. K. M. *et al.* Controlled Human Infection Models To Accelerate Vaccine Development. *Clin. Microbiol. Rev.* **35**, e0000821 (2022).
7. Vi, C. O. Controlled Human Infection Studies in the Netherlands ROADMAP.
8. Leibold, N. K. *et al.* CO2 exposure as translational cross-species experimental model for panic. *Transl. Psychiatry* **6**, e885 (2016).
9. Salvadore, G. *et al.* Translational evaluation of novel selective orexin-1 receptor antagonist JNJ-61393215 in an experimental model for panic in rodents and humans. *Transl. Psychiatry* **10**, 308 (2020).
10. A Trial of the Effect of CVL-865 on Panic Symptoms Induced by Carbon Dioxide Inhalation in Healthy Subjects. *ClinicalTrials.gov* Updated 11 January 2022, Accessed 11 June 2024 https://clinicaltrials.gov/study/NCT04592536.
11. van der Fits, L. *et al.* Imiquimod-Induced Psoriasis-Like Skin Inflammation in Mice Is Mediated via the IL-23/IL-17 Axis. *J. Immunol.* **182**, 5836–5845 (2009).
12. M. Saghari D. Ziagkos, M.B.A. van Doorn, J. Burggraaf, R. Rissman, M. Moerland, P. G. Evaluation of delayed-type hypersensitivity (DTH) in healthy volunteers using innovative imaging techniques. (2019).
13. Saghari, M. *et al.* A randomized controlled trial with a delayed-type hypersensitivity model using keyhole limpet haemocyanin to evaluate adaptive immune responses in man. *Br. J. Clin. Pharmacol.* **87**, 1953–1962 (2021).
14. Saghari, M. *et al.* OX40L inhibition suppresses KLH-driven immune responses in healthy volunteers: a randomized controlled trial demonstrating proof-of-pharmacology for KY1005. *Clin. Pharmacol. Ther.* **111**, 1121–1132 (2022).
15. Parliament, E. *et al.* Press release Press release. *Presws Release* **1**, 32–33 (2011).
16. Kiistala, U. & Mustakallio, K. K. In-vivo separation of epidermis by production of suction blisters. *Lancet* **283**, 1444–1445 (1964).
17. Akbar, A. N. *et al.* Investigation of the cutaneous response to recall antigen in humans in vivo. (2013).
18. Belson, A. *et al.* Characterisation of the clinical and activated T cell response to repeat delayed-type hypersensitivity skin challenges in human subjects, with KLH and PPD, as a potential model to test T cell-targeted therapies. *Inflamm. Res.* **65**, 389–404 (2016).
19. Pitzalis, C. *et al.* Selective migration of the human helper-inducer memory T cell subset: confirmation by in vivo cellular kinetic studies. *Eur. J. Immunol.* **21**, 369–376 (1991).
20. Motwani, M. P. *et al.* Novel translational model of resolving inflammation triggered by UV-killed E. coli. *J Pathol Clin Res* **2**, 154–165 (2016).
21. Buters, T. P. *et al.* Intradermal lipopolysaccharide challenge as an acute in vivo inflammatory model in healthy volunteers. *Br. J. Clin. Pharmacol.* **88**, 680–690 (2022).
22. Reilly, D. M. & Green, M. R. Eicosanoid and cytokine levels in acute skin irritation in response to tape stripping and capsaicin. *Acta Derm. Venereol.* **79**, (1999).
23. Eveleens Maarse, B. C. *et al.* Immunomodulating effects of the single bacterial strain therapy EDP1815 on innate and adaptive immune challenge responses—a randomized, placebo-controlled clinical trial. *Immunol. Res.* 1–12 (2024).
24. Lee, B. L. & Barton, G. M. Trafficking of endosomal Toll-like receptors. *Trends Cell Biol.* **24**, 360–369 (2014).
25. Grievink, H. W. *et al.* Antimicrobial Peptide Omiganan Enhances Interferon Responses to Endosomal Toll-Like Receptor Ligands in Human Peripheral Blood Mononuclear Cells. *Clin. Transl. Sci.* **13**, 891–895 (2020).
26. Rijsbergen, M. *et al.* Results of phase 2 trials exploring the safety and efficacy of omiganan in patients with human papillomavirus-induced genital lesions. *Br. J. Clin. Pharmacol.* **86**, 2133–2143 (2020).
27. Basran, A. *et al.* Roles of neutrophils in the regulation of the extent of human inflammation through delivery of IL-1 and clearance of chemokines. *J. Leukoc. Biol.* (2013) doi:10.1189/jlb.0512250.
28. Maini, A. A. *et al.* A Comparison of Human Neutrophils Acquired from Four Experimental Models of Inflammation. *PLoS One* **11**, e0165502 (2016).
29. Prince, L. R., Whyte, M. K., Sabroe, I. & Parker, L. C. The role of TLRs in neutrophil activation. *Curr. Opin. Pharmacol.* **11**, 397–403 (2011).
30. Tamassia, N. *et al.* Activation of an immunoregulatory and antiviral gene expression program in poly (I: C)-transfected human neutrophils. *J. Immunol.* **181**, 6563–6573 (2008).
31. Janke, M. *et al.* Selective and direct activation of human neutrophils but not eosinophils by Toll-like receptor 8. *J. Allergy Clin. Immunol.* **123**, 1026–1033 (2009).
32. Hattermann, K. *et al.* The Toll-like receptor 7/8-ligand resiquimod (R-848) primes human neutrophils for leukotriene B4, prostaglandin E2 and platelet-activating factor biosynthesis. *FASEB J.* **21**, 1575–1585 (2007).
33. Buters, T. P. *et al.* Clinical, Cellular, and Molecular Effects of Corticosteroids on the Response to Intradermal Lipopolysaccharide Administration in Healthy Volunteers. *Clin. Pharmacol. \& Ther.* **111**, 964–971 (2022).
34. Silver, R. *et al.* Using optical coherence tomography for the longitudinal non-invasive evaluation of epidermal thickness in a murine model of chronic skin inflammation. *Ski. Res. Technol. Off. J. Int. Soc. Bioeng. Ski. [and] Int. Soc. Digit. Imaging Ski. [and] Int. Soc. Ski. Imaging* **18**, 225–231 (2012).
35. Mokbul, M. I. Optical Coherence Tomography: Basic Concepts and Applications in Neuroscience Research. *J. Med. Eng.* **2017**, 3409327 (2017).
36. Niemeyer–van der Kolk, T. *et al.* Topical antimicrobial peptide omiganan recovers cutaneous dysbiosis but does not improve clinical symptoms in patients with mild to moderate atopic dermatitis in a phase 2 randomized controlled trial. *J. Am. Acad. Dermatol.* **86**, 854–862 (2022).
37. Niemeyer-van der Kolk, T. *et al.* Pharmacodynamic effects of topical omiganan in patients with mild to moderate atopic dermatitis in a randomized, placebo-controlled, phase II trial. *Clin. Transl. Sci.* **13**, 994–1003 (2020).
38. Rousel, J. *et al.* Lesional skin of seborrheic dermatitis patients is characterized by skin barrier dysfunction and correlating alterations in the stratum corneum ceramide composition. *Exp. Dermatol.* **33**, e14952 (2024).
39. Fehres, C. M. *et al.* Topical rather than intradermal application of the TLR7 ligand imiquimod leads to human dermal dendritic cell maturation and CD8+ T-cell cross-priming. *Eur. J. Immunol.* **44**, 2415–2424 (2014).
40. Wind, S. S. *et al.* Topical bimiralisib shows meaningful cutaneous drug levels in healthy volunteers and mycosis fungoides patients but no clinical activity in a first-in-human, randomized controlled trial. *Cancers (Basel).* **14**, 1510 (2022).
41. Jodl, S. J. *et al.* The oral IRAK4 inhibitors zabedosertib and BAY1830839 suppress local and systemic immune responses in a randomized trial in healthy male volunteers. *Clin. Transl. Sci.* **17**, e13771 (2024).
42. Deep Phenotyping of Cutaneous Lupus Erythematosus. *ClinicalTrials.gov* Updated 13 May 2024. Accessed 12 June 2024 https://clinicaltrials.gov/study/NCT06411106.
43. Oladejo, M., Tijani, A. O., Puri, A. & Chablani, L. Adjuvants in cutaneous vaccination: A comprehensive analysis. *J. Control. Release* **369**, 475–492 (2024).
44. Chen, X. Emerging adjuvants for intradermal vaccination. *Int. J. Pharm.* **632**, 122559 (2023).

45. Dowling, D. J. Recent Advances in the Discovery and Delivery of TLR7/8 Agonists as Vaccine Adjuvants. *ImmunoHorizons* **2**, 185–197 (2018).
46. Li, C. *et al.* Co-stimulation With TLR7 Agonist Imiquimod and Inactivated Influenza Virus Particles Promotes Mouse B Cell Activation, Differentiation, and Accelerated Antigen Specific Antibody Production. *Front. Immunol.* **9**, 2370 (2018).
47. Siram, K. *et al.* Co-Delivery of Novel Synthetic TLR4 and TLR7/8 Ligands Adsorbed to Aluminum Salts Promotes Th1-Mediated Immunity against Poorly Immunogenic SARS-CoV-2 RBD. *Vaccines* **12**, 1–23 (2024).
48. Patinote, C. *et al.* Agonist and antagonist ligands of toll-like receptors 7 and 8: Ingenious tools for therapeutic purposes. *Eur. J. Med. Chem.* **193**, (2020).
49. Rolfo, C., Giovannetti, E., Martinez, P., McCue, S. & Naing, A. Applications and clinical trial landscape using Toll-like receptor agonists to reduce the toll of cancer. *npj Precis. Oncol.* **7**, 26 (2023).
50. Khosravi, G.-R. *et al.* Immunologic tumor microenvironment modulators for turning cold tumors hot. *Cancer Commun. (London, England)* **44**, 521–553 (2024).

Table 1 Overview of TLR7 and TLR7/8 agonists in clinical development for the treatment of tumours. Adapted from: Applications and clinical trial landscape using Toll-like receptor agonists to reduce the toll of cancer (2023).[49]

Agent	Clinical study phase	Route of administration	Monotherapy or combination therapy	Tumour types	Identifier
TLR 7 AGONISTS					
TQ-A3334	I/II	Oral	Monotherapy & Combined with anti- VEGF	NSCLC	NCT04273815
SHR2150	I/II	Oral	Combined with chemotherapy and anti–PD-1 or anti-CD47	Solid tumours	NCT04588324
RO7119929	I	Oral	Monotherapy	HCC, Biliary tract cancer Solid tumours with liver metastases	NCT04338685
DSP-0509	I/II	IV	Monotherapy & Combined with pembrolizumab	Solid tumours	NCT03416335
BNT411	I/IIA	IV	Monotherapy & Combined with atezolizumab, carboplatin, and etoposide	Solid tumours & ES-SCLC	NCT04101357
APR003	I	Oral	Monotherapy	CRC with liver metastases	NCT04645797
Imiquimod (UGN-201)	I	Intravesical	Combined with UGN-301	Non- muscle invasive bladder cancer	NCT05375903
CAN1012	I	IT	Monotherapy	Solid tumours	NCT05580991
Imiquimod	I/II	Cutaneous	Monotherapy & Combined with cyclophosphamide and radiotherapy	Skin metastases from breast cancer	NCT01421017
Imiquimod	II	Cutaneous	Monotherapy	Skin metastases from breast cancer	NCT00899574
TLR 7/8 AGONISTS					
BDB001	I/II	IASC	Monotherapy & Combined with pembrolizumab, atezolizumab and atezolizumab and RT	Solid tumours	NCT03486301 NCT04196530 NCT03915678 NCT04819373
BDC-1001	I/II	IV	Monotherapy & Combined with nivolumab	HER2-positive solid tumours	NCT04278144

(Continuation Table 1)

Agent	Clinical study phase	Route of adminis-tration	Monotherapy or combination therapy	Tumour types	Identifier
CV8102	I	IT	Monotherapy & Combined with anti-PD-1	Melanoma, cSCC, SCCHN, ACC	NCT03291002
TransCon TLR7/8	I/II	IT	Monotherapy & Combined with pembrolizumab	Solid tumours	NCT04799054
MBS-8	I	IV	Monotherapy	Solid tumours	NCT04855435
BDB-018	I	IV	Monotherapy	Solid tumours	NCT04840394
MEDI9197	I	IT	Monotherapy & Combined with durvalumab	Solid tumours	NCT02556463

ACC = adenoid cystic carcinoma, CD47 = cluster differentiation 47; CRC = colorectal cancer, CRPC = castrate-resistant prostate cancer, cSCC = cutaneous squamous cell carcinoma; ES-SCLC = extensive-stage small-cell lung cancer, HCC = hepatocellular carcinoma, HER2 = human epidermal growth factor receptor 2, IASC = immune-stimulating antibody conjugate, IT = intratumoral; IV = intravenous, NSCLC = non-small-cell lung cancer, PD-1 = programmed cell death protein-1, RT = radiotherapy, SCCHN = squamous cell carcinoma of the head and neck, TLR = Toll-like receptor, VEGF = vascular endothelial growth factor.

Figure 1 Difference in inflammatory response between mice and human to IMQ.

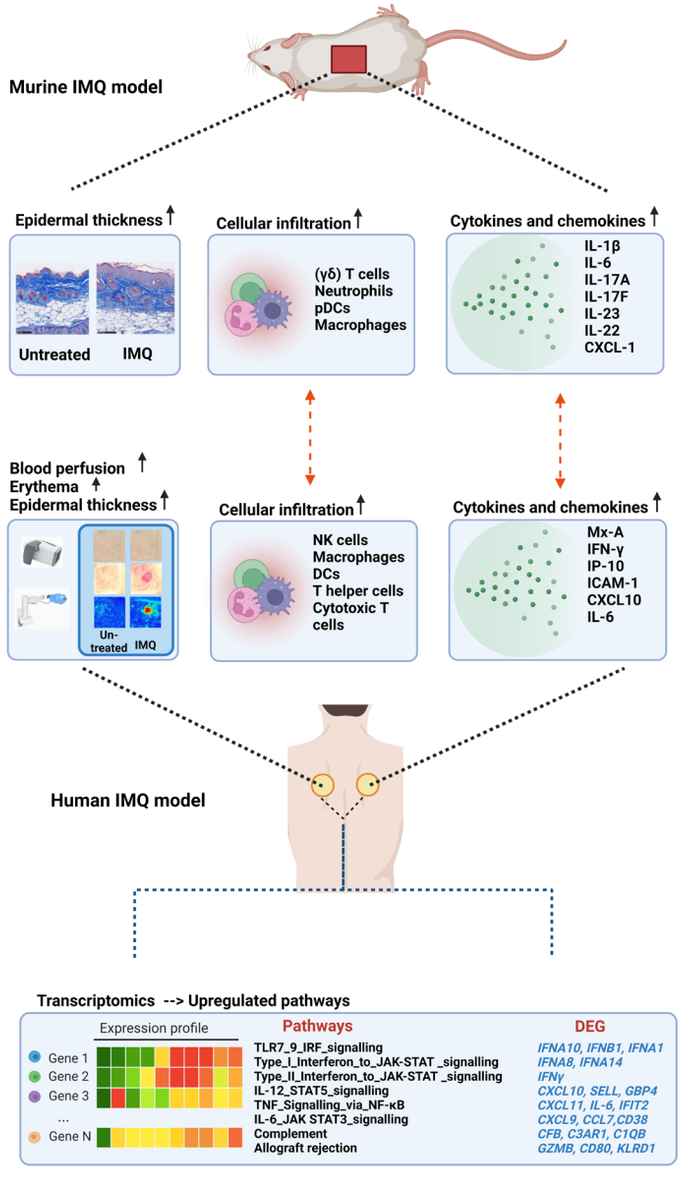
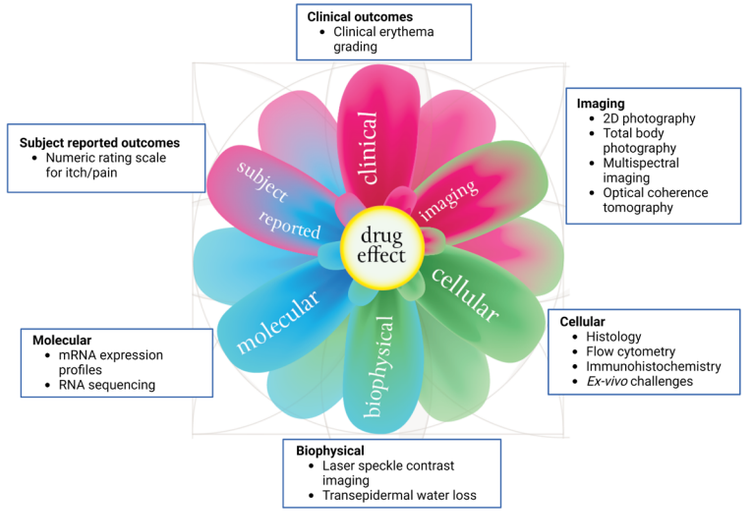


Figure 2 Multimodal approach in characterizing the effect of drug.



APPENDICES

APPENDICES

NEDERLANDSE SAMENVATTING

Het ontwikkelen van geneesmiddelen is een zeer uitgebreid, langdurig en kostbaar proces. Het kan wel 10 tot 15 jaar duren voordat het kandidaat-geneesmiddel geregistreerd wordt en beschikbaar komt voor gebruik bij patiënten. Het geneesmiddelenontwikkelingsproces richt zich op preklinische en klinische proeven, waarbij klinische proeven de traditionele fase I-IV structuur volgen gericht op informatie verzamelen over farmacokinetiek, veiligheid en verdraagbaarheid. Door gebrek aan gegevens over de effectiviteit van kandidaat-geneesmiddelen in de vroege fase van geneesmiddelenonderzoek is het uitvalpercentage hoog (~90%). Dit is vooral aan de orde in fase II/III, waarbij een uitvalpercentage oploopt tot 55% en te wijten is aan het uitblijven van tekenen van effectiviteit in (grote) patiëntenstudies. Dit heeft als gevolg dat de beslissing om niet door te gaan met het kandidaat-geneesmiddel vrij laat in het traject wordt genomen wat leidt tot hoge kosten. Om deze redenen is er in de afgelopen decennia steeds meer aandacht besteed aan de verbetering van het vroege geneesmiddelenontwikkelingsproces. De traditionele procedure, waarbij klinische proeven worden gecategoriseerd in vier fasen, wordt steeds vaker vervangen door een meer integrale, biomarker-verrijkte en vraaggerichte benadering. Hierin worden antwoorden gezocht op de meest relevante vragen voor het geneesmiddelenontwikkelingsproces, bijvoorbeeld: of het geneesmiddel de plaats waar het moet werken bereikt, of het middel een farmacologisch effect heeft en of het middel gunstige effecten op de ziekte of de pathofysiologie ervan heeft. Het doel hierbij is om al in een vroeg stadium van de geneesmiddelenontwikkeling zoveel mogelijk informatie over het werkingsmechanisme te verzamelen.

Het verbeteren van het geneesmiddelenontwikkelingsproces kan op verschillende manieren. In literatuur wordt de nadruk gelegd op het gebruik van biomarkers in klinische onderzoeken om zo de kans op succes van geneesmiddelen te vergroten. In dit proefschrift ligt de focus op het uitvoeren van 'proof-of-mechanism' onderzoeken om zo tot een effectief dosis en duur van een nieuw te onderzoeken geneesmiddel te komen. Hierbij zijn farmacologische challenge-modellen in gezonde vrijwilligers zeer waardevol. Deze challenge-modellen bootsen tijdelijk componenten van fysiologische en pathofysiologische processen na. 'Proof-of-mechanism'-modellen zijn eerder met succes geïntroduceerd op het gebied van neurologie en pijn, vooral om de achterliggende mechanismes beter in kaart te brengen. Deze modellen worden over het algemeen 'human evoked pain-tests' genoemd en zijn van meerwaarde gebleken bij studies met gezonde vrijwilligers. De human evoked pain-tetst helpen bij vroege fase geneesmiddel ontwikkeling om de stap van dieren naar patienten met pijn te verkleinen. Enkele voorbeelden van deze testen zijn het UV-B hitte pijn model, koude pijn test en de Von Frey pin prick test. Binnen de infectieziekten krijgen de gecontroleerde humane infectiemodellen (controlled human infection models) steeds meer aandacht. Door gezonde vrijwilligers bloot te stellen aan een virus, zoals rhinovirus of SARS-COV-2 kan het effect van een vaccin vroeg in de ontwikkeling worden geëvalueerd met als doel het versnellen van de vaccinontwikkeling. Ook op het gebied van psychiatrie worden gecontroleerde modellen gebruikt door gezonde personen verhoogde CO2-concentraties te laten inhaleren. Dit zorgt bij gezonde personen voor paniek klachten die vergelijkbaar zijn met de symptomen die patiënten ervaren tijdens een paniekaanval.

In deze thesis hebben wij ons voornamelijk gericht op de ontwikkeling van een model binnen het onderzoeksveld van immuno-dermatologie. Een van de farmacologische challenge modellen die in preklinische (muizen) studies succesvol bleek te zijn, is een model waarbij imiquimod (IMQ) op de huid wordt gesmeerd om lokale inflammatie op te wekken. IMQ is de actieve stof van een goedgekeurd geneesmiddel genaamd Aldara®. Dit middel werkt op de Toll-like receptor (TLR) 7 en wordt in de praktijk voorgeschreven aan patiënten met huidaandoeningen zoals actinische keratose, basaalcelcarcinoom en genitale wratten. Hoewel dit middel al geregistreerd is, wordt het in het dieronderzoek gebruikt om klinische kenmerken van de huidziekte psoriasis na te bootsen. IMQ activeert in dieren immunologische routes (interferon route en NF-κB) die belangrijk zijn voor verschillende andere auto-inflammatoire/afweerziekten, zoals type I interferonopathieën (bijvoorbeeld cutane lupus erythematosus). Er bestaat echter nog geen vertaling van dit model naar de mens. Om een

ontstekingsmodel succesvol te transleren naar de mens moet het voldoen aan twee criteria: 1) een reactie opwekken die vergelijkbaar is met de reactie die in dieronderzoeken wordt waargenomen en 2) moet geschikt zijn voor proof-of-mechanism onderzoeken bij gezonde vrijwilligers.

Het hoofddoel van deze thesis is het opzetten van een humaan IMQ-model om zo het onderliggende ontstekingsmechanisme te bestuderen en dit verder te kunnen gebruiken in vroege fase van de geneesmiddelenontwikkeling. Deze thesis omvat daarom een aantal klinische studies met IMQ die zijn uitgevoerd in gezonde vrijwilligers. Daarnaast besteden we aandacht aan diverse technieken om de ontsteking van de huid goed in kaart te brengen. Hiervoor gebruiken we een multimodale benadering die verschillende soorten uitleesmaten omvat zoals beeldvorming, biofysische, moleculaire en cellulaire analyses, evenals de beoordeling door artsen, *Figuur 1*.

Voordat de klinische studies met imiquimod worden besproken, worden een aantal challenge modellen in kaart gebracht in **hoofdstuk 2**. De gekozen challenge agents richten zich op de aangeboren afweerreactie van de huid en het aanzetten van bepaalde immunologische processen in de huid. Zo is de challenge met lipopolysaccharide (LPS) beschreven die de aangeboren afweersysteem aanzet gericht op het TLR4. Verder is het UV-B model uitgelicht dat preklinisch ingezet wordt om inflammatie op te wekken en in de mensen toegepast wordt om inflammatoire pijn te bestuderen. Naast de eerdergenoemde modellen met betrekking tot het bestuderen van het afweersysteem, worden in dit hoofdstuk ook twee methoden beschreven voor het opwekken van jeuk, namelijk via toediening van histamine en cowhage. Een model voor het aanzetten van het adaptieve afweersysteem door de toediening van KLH wordt ook in **hoofdstuk 2** genoemd. Interessant om te vermelden was dat dit model is ingezet om het effect van een nieuw kandidaat-geneesmiddel genaamd amlitelimab, een humane IgG4 anti OX40L monoklonale antilichaam, in gezonde vrijwilligers te bestuderen. Gebaseerd op de gegenereerde data en de geselecteerde dosis uit de first-in-human studie, is het onderzoeksmiddel succesvol getest bij patiënten met atopische dermatitis. Dit vormt een uitstekend voorbeeld van de huidige verschuiving in geneesmiddelontwikkeling. In plaats van een traditionele first-in-human studie die primair gericht is op veiligheid en verdraagbaarheid, is een geïntegreerde benadering toegepast die het effect van een nieuw kandidaat-geneesmiddel al in een vroeg ontwikkelingsstadium succesvol evalueert.

Een vergelijkbare geïntegreerde benadering is toegepast in **hoofdstuk 3**, waarin een gerandomiseerde, open-label, vehicle-gecontroleerde klinische studie werd uitgevoerd gericht om tijdelijk cutane ontsteking op te wekken. Cutane ontsteking in dit model werd geïnduceerd door 5 mg van TLR7-agonist, namelijk IMQ te smeren op de huid. IMQ werd onder occlusie aangebracht op de bovenkant van de rug van 16 gezonde mannelijke vrijwilligers in een open-label setting gedurende 24 uur, 48 uur en 72 uur. Gezonde vrijwilligers werden gerandomiseerd om ofwel tape-stripping (TS) van de huid te ondergaan voorafgaand aan de IMQ-toediening, of om IMQ op de intacte huid aan te brengen. De resultaten toonden aan dat, hoewel IMQ alleen beperkte ontstekingsreacties veroorzaakte, de combinatie van TS en IMQ-toediening leidde tot een sterkere en meetbare cutane ontstekingsreacties. Vergeleken met de controlegroep, werd er in de IMQ-groep verhoogd erytheem en doorbloeding van de huid, verhoogde mRNA-expressie van ontstekingsmarkers en een influx van ontstekingscellen waargenomen. Activatie van het aangeboren immuunsysteem werd aangetoond door significante verhoging in de expressie van chemokinen zoals CXCL10, ICAM-1, HBD-2 en MX-A bij de TS-groep ten opzichte van onbehandelde gebieden (geen TS en geen IMQ). Deze activatie van het immuunsysteem werden verder onderbouwd door het vaststellen van immuun infiltraat zoals T-helpercellen, cytotoxische T-cellen, macrofagen en dendritische cellen na 48 tot 72 uur IMQ-toediening. De ontstekingsreacties waren duidelijker in de TS-groep. Over het algemeen werd er na 72 uur toedienen van IMQ geen extra effect waargenomen ten opzichte van 48 uur bij alle metingen, wat suggereert dat een toedieningsperiode van 48 uur de optimale duur lijkt te zijn. Hiermee is er een model opgezet waarbij 48 uur blootstelling zorgt voor een goed aantoonbare rectie die gebruikt kan worden voor ontwikkeling van geneesmiddel.

De volgende stap was om te testen of de klinische en immunologische respons door IMQ ook kon worden omgekeerd met behulp van een ontstekingsremmend middel dat als benchmark zou kunnen dienen. Hiervoor hebben we in **hoofdstuk 4** willekeurig orale prednisolon/placebo toegediend aan 24 gezonde vrijwilligers gedurende zes opeenvolgende dagen. Prednisolon is een geregistreerd en ontstekingsremmend middel dat voornamelijk op de glucocorticoïd receptor werkt. In deze studie werd IMQ gedurende 48 uur op TS-huid aangebracht. De klinische, cellulaire en moleculaire reacties werden gekarakteriseerd door de technieken afgebeeld in *Figuur 1*. Daarbij is er een andere techniek geïntroduceerd om de TLR7-cellulaire en cytokineresponsen te onderzoeken door blaren te trekken. Vergeleken met placebo, onderdrukte prednisolon de TLR7-gedreven respons op bijna elke meting, waaronder doorbloeding van de huid, erytheem, epidermale dikte, immuuncellen (zoals NK, klassieke monocyten, T-helper-, cytotoxische cellen) en cytokinen (zoals IL-6, IL-8, TNF en Mx-A). In een eerder onderzoek hebben we aangetoond dat IMQ niet in de systemische circulatie terechtkomt. Echter, werd het relevant geacht om het verschil in effect van prednisolon in het bloed en in perifeer weefsel te onderzoeken. Daarom hebben we de *ex vivo* geneesmiddelactiviteit getest via IMQ-stimulatie in volbloed met cytokine-afgifte als uitleesmaat. We hebben aangetoond dat onderdrukking van cytokinen door prednisolon minder afhankelijk is van het farmacokinetische profiel van de stof. Zowel in bloed als in weefsel toonde prednisolon ontstekingsremmende eigenschappen, hoewel deze effecten in het bloed reversibel waren.

Dit was het eerste onderzoek dat de uitkomsten van de biopten vergeleek met die van blaren na IMQ-toediening. Het trekken van blaren is niet een geheel nieuwe procedure; al in de jaren zestig van de vorige eeuw werd dit techniek toegepast om de dermis van de epidermis te scheiden. Dit zorgde ervoor dat het bestuderen van ontstekingsreacties op een niet invasieve manier kon plaatsvinden. Deze techniek is daarom ook gebruikt in meerdere (pre-)klinische studies waarin ontstekingsreacties op verschillende intradermaal toegediende challenges werden bestudeerd, zoals tuberculine gezuiverd eiwitderivaat (PPD), UV-dode Escherichia coli (UVKEC), KLH en LPS. Na intradermale toediening van LPS, waren de cellen en cytokinen waargenomen in blaarvocht volledig in lijn met de gemeten cellulaire respons in biopten. In dit hoofdstuk, hebben we vergelijkbare immuuncellen waargenomen in blaarvocht en in de IHC-kleuringen. Dit suggereert dat de blaartechniek zowel effectief als eenvoudig is voor het onderzoeken van ontsteking op een minimaal invasieve manier.

Echter, waren er ook belangrijke verschillen tussen het IMQ-diermodel en het humane IMQ-model. In het diermodel waren de neutrofielen doorgaans aanwezig terwijl in het humane IMQ-model deze geheel ontbraken. Een mogelijke verklaring was de relatief korte duur van de IMQ-blootstelling in onze klinische studies tot nu toe. Daarom hebben we in **hoofdstuk 5** de TLR7-gemedieerde ontstekingsreactie gekarakteriseerd na 7 dagen (168 uur) IMQ-toediening bij gezonde vrijwilligers en deze respons vergeleken met de respons van de al vastgestelde kortere IMQ-toediening van 2-3 dagen (48 uur en 72 uur). Daarnaast werd het ontstekingsreactie in dieren aangedreven door complement C3. Als gevolg hiervan was onze secundaire doel om te onderzoeken of de complementcascade geactiveerd kon worden na verlengde IMQ-blootstelling. Hiervoor namen 10 gezonde vrijwilligers deel aan een gerandomiseerde, open-label studie. Verlengde IMQ-blootstelling resulteerde in versterking van de IMQ-geïnduceerde ontstekingsreactie op zowel moleculair als cellulair niveau. Verder hebben we in dit onderzoek transcriptoom analyse toegepast die de activering van TNF-signaaltransductie, complement en vooral sterke interferonreacties aantoonde. Deze bevindingen waren vooral duidelijk na een verlengde IMQ-blootstelling. Daarbij hebben we waargenomen dat secretie van interferonen leidt tot de inductie van de JAK-STAT-route, met een toename in de expressie van de chemokinen CXCL9, CXCL10 en CXCL11. Als laatste bevestigde deze studie het ontbreken van neutrofielen als een bepalende factor in de respons, wat leidt tot een aanzienlijk translationeel verschil tussen muizen en mensen.

Om de toepasbaarheid van ons model met een nieuw kandidaat-geneesmiddel te onderzoeken, hebben we een klinische studie uitgevoerd, beschreven in **hoofdstuk 6**. In dit hoofdstuk zijn de effecten

van omiganan (OMN) en IMQ op ontstekingsreacties in de huid van 16 gezonde deelnemers onderzocht. OMN is een synthetisch cathelicidine met antimicrobiële eigenschappen vergelijkbaar met LL-37 die de IFN-responsen versterkt. Cathelicidinen, waaronder LL-37, zijn essentiële antimicrobiële peptiden in huidimmuniteit en bestrijden verschillende pathogenen. LL-37 moduleert ook immuunreacties en versterkt de IFN-productie die geïnduceerd worden door TLR3-ligand polyI:C in keratinocyten. Er is echter in *in vitro* studies aangetoond dat dit effect ook geldt voor de TLR7-ligand, zoals IMQ. Net zoals in hoofdstuk 3, Toediening met IMQ induceerde een matige ontstekingsreacties, gekenmerkt door verhoogd erytheem en doorbloeding, met een piek na 24 en 48 uur. Combinatie van IMQ en OMN versterkte deze fysiologische reacties verder alsmede de cellulaire response, vooral bij de hoogste concentratie van 1%. Een toename van macrofagen/monocyten, DC's en T-helpercellen was evident, maar dit effect was beperkt in de corresponderende cytokinerespons. IMQ-behandeling verhoogde de mRNA-niveaus van IL-6, IL-10, MX-A en IFN-ɣ, waarbij OMN een trend toonde naar versterking van deze cytokineresponsen. In een eerder onderzoek, waarbij 2.5% OMN werd aangebracht bij patiënten met anogenitale wratten en hooggradige plaveiselcel intra-epitheliale laesies, werd geen klinische effectiviteit aangetoond. Dit wijst erop dat een combinatietherapie van IMQ en OMN mogelijk gunstig kan zijn voor HPV-geïnduceerde huidaandoening.

KRITISCHE EVALUATIE VAN HET IMQ-GEÏNDUCEERD INFLAMMATIEMODEL

IS ER EEN TRANSLATIONELE KLOOF? De inleidende sectie van dit proefschrift beschreef de inflammatoire immuunrespons op IMQ bij muizen. De bevindingen kenmerken zich bij muizen door klinische verschijnselen, zoals verdikking van de huid, erytheem en schilfering die binnen 2-3 dagen na IMQ-toediening optreden. Bij mensen waren vergelijkbare, maar meer beperkte, klinische bevindingen aanwezig, zoals een toename in huiddoorbloeding, erytheem en epidermale dikte na 2-3 dagen IMQ-toediening.

Bij muizen activeert blootstelling aan IMQ, neutrofielen en trekt deze aan, samen met een verhoogd aantal PDC's en γδ T-cellen. Dit werd verder ondersteund door verhoogde niveaus van de cytokinen TNF, IL-1β en IL-6, in combinatie met een tijdelijke toename van IL-23, IL-17A, IL-17F en IL-22, wat wijst op de betrokkenheid van de adaptieve immuunrespons en lijkt op psoriasiforme laesies. In lijn met preklinische bevindingen was bij mensen infiltratie van monocyten/macrofagen, NK-cellen, DC's, T-helpercellen en cytotoxische T-cellen duidelijk zichtbaar. Op cytokineniveau was er duidelijke IRF-signalisatie, wat bleek uit verhoogde MX-A, IFN-γ en IP-10. Desondanks waren er verschillen tussen de soorten op cellulair en cytokineniveau (*Figuur 2*). Hoewel verhoogde niveaus van IL-6 en TNF werden waargenomen, was de expressie minder dan die van IFN, wat aangeeft dat IMQ meer als model voor IRF-stimulatie dient dan voor NF-κB. Een tweede belangrijk verschil tussen het muizen- en mensenmodel met IMQ is het gebrek aan IL-8 en daarmee neutrofielen bij gezonde individuen. Neutrofielen, die als eerste verdedigingslinie fungeren, worden bij mensen snel naar ontstekingsplaatsen gerekruteerd. Daarom werd aanvankelijk verondersteld dat het mogelijk was dat het moment om neutrofielen te meten bij een IMQ-challenge gemist was, gezien de korte levensduur van neutrofielen, met een geschatte halfwaardetijd van 13-19 uur. Het verschil in neutrofiele respons tussen muizen en mensen is echter te wijten aan de afwezigheid van TLR3-expressie en de beperkte expressie van TLR7 in menselijke neutrofielen. Zij reageren wel op TLR4-liganden, zoals LPS, wat een meer directe en klassieke inflammatoire respons induceert die de effecten van IMQ aanvult.

Tot slot beïnvloedt het verschil in cellulaire en cytokineprofielen in respons op IMQ tussen muizen en mensen ook de relevantie van dit model voor ziektes. In preklinisch onderzoek wordt IMQ vaak gebruikt als model voor psoriasis-achtige ontsteking, gezien de betrokkenheid van de IL-23/IL-17A/IL-22-as, terwijl de expressie van deze cytokinen bij mensen beperkt is. In menselijke IMQ-studies is er sterke activatie van de IFN-respons zichtbaar, wat leidt tot verrijking van de JAK-STAT-route. Deze bevindingen, samen met histopathologische veranderingen wijzen erop dat het IMQ-model bij mensen mogelijk beter representatief is voor lupus dan voor psoriasis.

GEBRUIK VAN ACCURATE UITLEESMATEN Op het gebied van klinimetrie scoren de artsen aangedane huid meestal op kenmerken zoals erytheem en zwelling. Onderzoekers proberen vaak enkele biomarkers en eindpunten te integreren, maar deze leveren doorgaans slechts eendimensionale informatie. In klinisch onderzoek kan een multidimensionaal, dieper inzicht in huidprocessen worden verkregen door gebruik te maken van een multimodale benadering, zoals beschreven in de inleiding van dit proefschrift. De pijlers van deze aanpak omvatten het volgende (*Figuur 1*):

I Klinische scores
II Door de patiënt gerapporteerde uitkomsten
III Beeldvorming
IV Biofysische metingen
V Moleculaire analyses in biologische monsters, zoals biopten en blaarvocht
VI Cellulaire uitleesmaten

In de hoofdstukken van dit proefschrift zijn verschillende combinaties van de genoemde pijlers uitvoerig uitgelicht en toegepast. Daarnaast zijn beeldvormingstechnieken en biofysische metingen, zoals laser speckle contrast imaging, eerder onderzocht in andere klinische studies met challenges zoals LPS en KLH, waarbij ze geschikt bleken om erytheem en huiddoorbloeding objectief te kwantificeren. Hoewel deze evaluaties voornamelijk gericht zijn op het karakteriseren van oppervlakkige huidreacties, zijn er ook niet-invasieve technieken beschikbaar die directe metingen van de epidermale morfologie tot een diepte van 1-2 MM mogelijk maken. Optical coherence tomography, doorgaans gebruikt in klinisch onderzoek met patiënten, herkent grote histologische structuren, maar mist de hoge resolutie die nodig is om individuele cellulaire veranderingen te identificeren en specifieke eiwitten in de epidermis te bepalen. Desondanks is optical coherence tomography in dit proefschrift ingezet om de epidermale dikte te kwantificeren. Dit benadrukt de haalbaarheid om conventionele biopten te vervangen door deze techniek in huid-challenge-studies. De technieken dien in dit proefschrift zijn toegepast bieden veelbelovende mogelijkheden om de inflammatoire respons na IMQ-toediening te karakteriseren. Toekomstig onderzoek zou zich moeten richten op het combineren van verschillende resultaten en het visualiseren van data om een beter begrip te krijgen van de functie van de cellen en daarmee het mechanisme of de pathologie van een bepaalde ziekte.

UITDAGINGEN & PERSPECTIEVEN

IMQ is waardevol gebleken als een challenge agent, voornamelijk gericht op het aanzetten van het interferonrespons. In verschillende studies laat het een consistente immuunrespons zien ondanks de waargenomen variaties in individuele cellulaire reacties. Echter, een van de beperkingen van IMQ is de topische formulering. Hoewel alle deelnemers dezelfde dosis ontvangen is de absorptie door de huid onvoldoende gekarakteriseerd tussen verschillende proefpersonen, wat met het oog op de verbeterde response na tape-strippen een deel van de variabiliteit van de respons zou kunnen verklaren. Men zou kunnen stellen dat de topische toediening van IMQ mogelijk niet de optimale toedieningsvorm is voor dit model. Eerdere pogingen om DC's te activeren en te mobiliseren via intradermale toediening van de TLR7-ligand op *ex vivo*-huid waren niet succesvol gebleken, terwijl topische toediening van IMQ effectief bleek te zijn. Om het model te verfijnen, is het cruciaal om de variabiliteit van de dosering te definiëren. In een onderzoek door *Wind et al.* introduceerden de groep een methodologie in hun onderzoek waarbij matrix-assisted laser desorption/ionization massaspectrometrie imaging werd gebruikt om concentraties van bimiralisib te meten in huidbiopten na een topische behandeling. Dezelfde benadering zou kunnen worden aangepast om de farmacokinetische eigenschappen van IMQ in het huidmodel te evalueren. Hoewel het IMQ-model werd getest met nieuwe geneesmiddelkandidaten die zich richten op hun specifieke signaalcascade, is het interessant om alternatieve toepassingsgebieden te verkennen waar IMQ effectief kan worden toegepast.

De bevindingen die in dit proefschrift worden beschreven, tonen niet alleen een succesvolle vertaling aan dier naar mens, maar duiden ook de consistentie tussen studies, wat suggereert dat de IMQ herhaalbaar is in gezonde individuen, hoewel er nog geen formele test-hertest studie is uitgevoerd. Een volgende translatiestap zal naar patiënten worden gezet. Onlangs is er een klinische studie gestart die zich richt op de translatie van IMQ naar patiënten met cutaan lupus erythematosus.

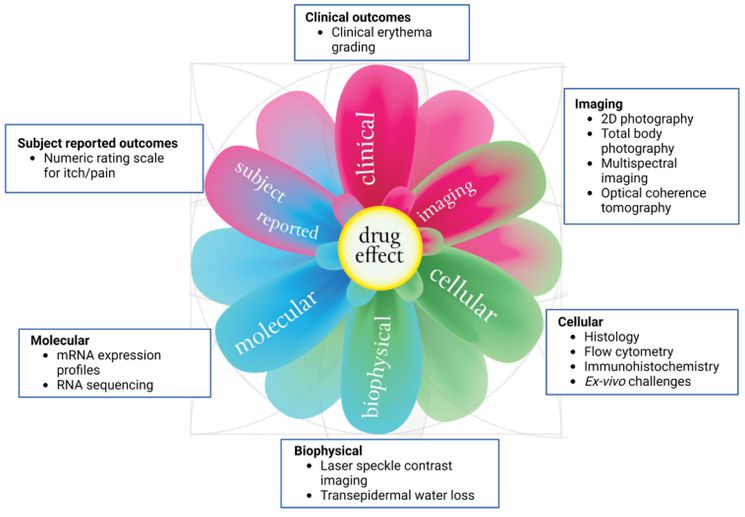
Daarnaast, is er recent meer aandacht gekomen voor het verduidelijken van de functie van TLR-agonisten en hun potentieel als adjuvanten in vaccinontwikkeling. IMQ werd topisch toegepast als huidadjuvans en toonde verbeterde reacties op intradermale influenzavaccins. Een recent voorbeeld betreft de evaluatie van synthetische TLR7/8-liganden door Inimmune Corp., die in vivo werden toegediend samen met het SARS-COV-2-vaccin, wat de T-cel reacties en neutraliserende antilichaamtiters verhoogde duidend op een mogelijk effectiever vaccin. Gezien deze veelbelovende resultaten, worden deze moleculen momenteel geëvalueerd in fase I klinische onderzoeken.

Naast hun potentieel als adjuvanten, hebben synthetische TLR7/8-liganden aanzienlijke belangstelling gekregen binnen de oncologie. Immunostimulerende moleculen die zich richten op TLR7/8 kunnen zowel aangeboren als adaptieve immuunresponsen activeren door cytokineproductie en het activeren van cytotoxische cellen. Via deze immuunrespons wordt verondersteld dat 'cold' tumoren omgezet kunnen worden in 'hot' tumoren, die gunstiger reageren op immuun-checkpointremmers. Als gevolg hiervan worden deze TLR-agonisten als veelbelovende kandidaten gezien voor nieuwe monotherapieën of combinatietherapieën. Hoewel sommige onderzoeken suggereren dat TLR-activatie voordelig kan zijn bij tumorbehandeling, wijzen andere studies erop dat TLR-signaaltransductie mogelijk bijdraagt aan tumorgroei. Het volledig begrijpen van de rol van TLR's in verschillende tumorceltypes blijft een aanzienlijke uitdaging, die uitgebreide lopende klinische onderzoeken vereist. Gezien de veelbelovende signalen die voortkomen uit voorlopige analyses van enkele klinische onderzoeken, worden er meer uitgebreide gegevens verwacht van de lopende studies naar TLR-agonisten die worden ontwikkeld voor de behandeling van solide tumoren.

CONCLUSIE

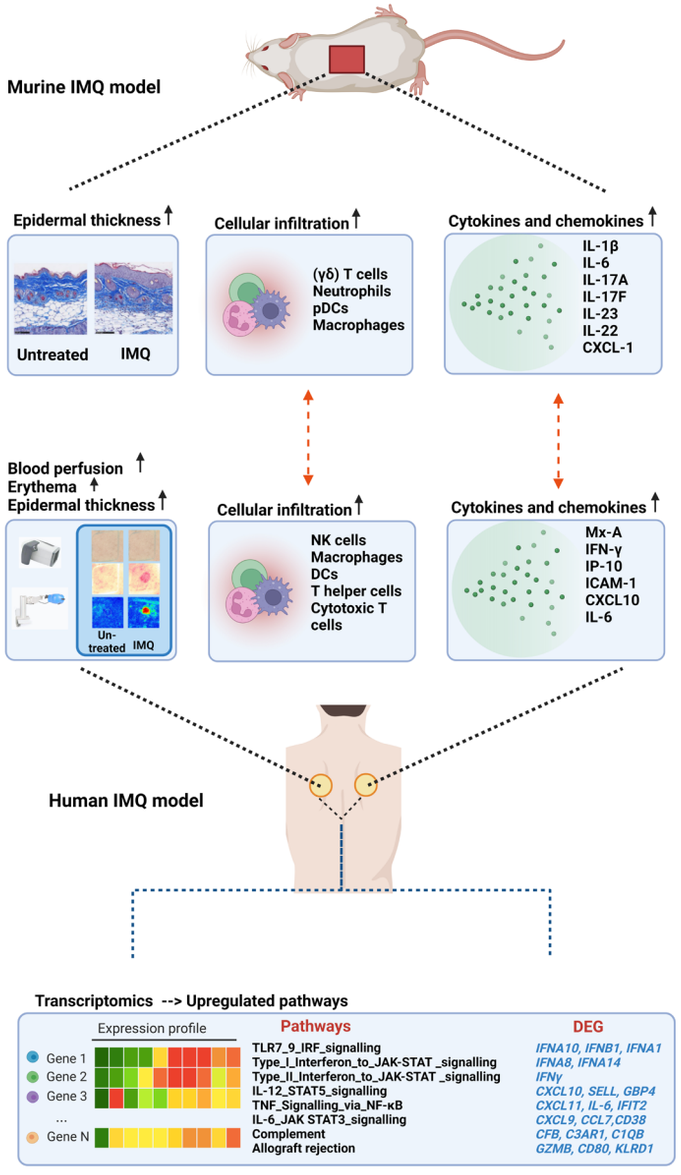
Dit proefschrift moedigt de implementatie van challenge-modellen in de vroege fasen van geneesmiddelontwikkeling aan om klinische onderzoeken efficiënt uit te voeren en tijdige go/no-go-beslissingen te vergemakkelijken. Het gebruik van een TLR7-agonist als challenge-agent is succesvol vertaald van dieren naar mensen, waarbij overeenkomsten en verschillen tussen soorten zichtbaar werden gemaakt. De imiquimod-respons werd grondig gekarakteriseerd door een geïntegreerde benadering van multimodale biomarkers. Het model werd succesvol ontwikkeld door een systematische aanpak van modeloptimalisatie, benchmarking met een ontstekingsremmend middel, en door het model te gebruiken in een 'proof-of-mechanism' studie om de in vivo farmacologische werking van een nieuw geneesmiddel te tonen. Met dit proefschrift hebben we een raamwerk van studies opgezet die uitgevoerd kunnen worden voorafgaand aan de commerciële toepassing van een nieuw challenge-model. Het gebruik van verschillende complementaire benaderingen zorgt voor een alomvattend beeld waardoor beslissingen naar latere fase of het vroegtijdig beëindigen van de productontwikkeling sneller genomen kunnen worden. Met deze aanpak verwachten we het traditionele paradigma van geneesmiddelontwikkeling te transformeren door de klinische onderzoeksprocessen te vereenvoudigen, leidend tot hogere efficiëntie en lagere totale kosten.

Figuur 1 Multimodale benadering voor het karakteriseren van geneesmiddel effect.



Derma bloem is gecreëerd door F. van Meurs, en is aangepast met BioRender.com voor dit hoofdstuk.

Figuur 2 Verschil in inflammatoir respons op IMQ tussen muis en mens.



APPENDICES

краткое содержание на русском

Разработка лекарственных препаратов — это сложный, длительный и дорогостоящий процесс. Может пройти от 10 до 15 лет, прежде чем тестируемый препарат будет зарегистрирован и станет доступным к применению дляпациентов. Процесс разработки лекарств включает доклинические и клинические исследования, где клинические исследования следуют традиционной структуре фаз I-IV, ориентированной на сбор информации о фармакокинетике, безопасности и переносимости.

Из-за недостатка данных об эффективности тестируемого препарата на ранних этапах исследований процент прекращения их дальнейшей разработки крайне высок (~90%). Это особенно заметно на этапах II/III, где уровень отказов от дальнейшего тестирования достигает 55% и связан с отсутствием признаков эффективности в (крупных) исследованиях с участием пациентов. В результате, решения о прекращении разработки тестируемого препарата принимаются на более поздних этапах, что приводит к значительным финансовым затратам.

По этим причинам в последние десятилетия всё больше внимания уделяется совершенствованию процесса ранней разработки лекарств. Традиционная процедура, в рамках которой клинические испытания классифицируются на четыре фазы, всё чаще заменяется более интегративным подходом, ориентированным на биомаркеры и спрос . В рамках этого подхода акцент делается на ответах на наиболее важные вопросы, связанные с разработкой лекарственных средств, например: достигает ли препарат целевого участка воздействия, оказывает ли он фармакологическое действие и имеет ли он положительное влияние на заболевание или его патофизиологию. Основной целью является получение как можно большего объема информации о механизме действия препарата на ранних этапах разработки.

Совершенствование процесса разработки лекарств возможно разными способами. В литературе акцент делается на использовании биомаркеров в клинических исследованиях для увеличения шансов на успех препаратов. В этой диссертации основное внимание уделяется проведению исследований типа «доказательство механизма» (proof-of-mechanism), чтобы определить эффективную дозировку и продолжительность действия нового лекарственного средства. В этих исследованиях фармакологические модели стимуляции у здоровых добровольцев имеют высокую ценность. Такие модели временно воспроизводят компоненты физиологических и патофизиологических процессов.

Модели «доказательства механизма» с успехом были внедрены в области неврологии и боли, особенно для лучшего понимания основных механизмов. Эти модели, известные как «тесты на вызванную боль у человека» (human evoked pain-tests), оказались полезны в исследованиях с участием здоровых добровольцев. Они помогают на ранних этапах разработки лекарств сократить разрыв между экспериментами на животных и пациентами с болевыми синдромами. Примеры таких тестов включают модель боли от теплового воздействия (UV-B), тест на боль от холода и тест с использованием игольчатых стимулов (Von Frey pin prick test).

В области инфекционных заболеваний всё больше внимания привлекают контролируемые модели инфекций у человека (controlled human infection models). В рамках этих исследований здоровые добровольцы подвергаются воздействию вируса, например риновируса или SARS-CoV-2, что позволяет оценить эффективность вакцины на ранних этапах её разработки и ускорить процесс создания вакцин. В области психиатрии контролируемые модели используются для воссоздания панических симптомов у здоровых добровольцев путём ингаляции повышенных концентраций CO2, что вызывает ощущения, схожие с паническими атаками у пациентов.

В этой диссертации основное внимание уделено разработке модели в исследовательской области иммунной дерматологии. Одной из фармакологических моделей стимуляции, которая показала успешные результаты в доклинических (мышиных) исследованиях, является модель, в рамках которой имихимод (IMQ) наносится на кожу для вызова локального воспаления. IMQ — это активное вещество зарегистрированного лекарственного средства под названием Aldara®. Это средство действует через Toll-подобный рецептор (TLR) 7 и применяется на

практике для лечения кожных заболеваний, таких как актинический кератоз, базально-клеточная карцинома и генитальные бородавки. Хотя это средство уже зарегистрировано, в исследованиях на животных его используют для моделирования клинических признаков псориаза. IMQ активирует у животных иммунные пути (интерфероновый путь и NF-κB), которые важны для ряда других аутоиммунных и воспалительных заболеваний, например, интерферонопатий первого типа (таких как кожная форма красной волчанки). Однако до сих пор эта модель не была применена на человеке

Для успешного применения воспалительной модели на человеке, она должна соответствовать двум критериям: 1) вызывать реакцию, сходную с той, которая наблюдается в исследованиях на животных, и 2) быть пригодной для исследований типа «доказательство механизма» с участием здоровых добровольцев. Основной целью данной диссертации является создание человеческой модели с использованием IMQ для изучения основного механизма воспаления и его дальнейшего применения на ранних этапах разработки лекарств. Эта диссертация включает серию клинических исследований с IMQ, проведённых на здоровых добровольцах. Также уделено внимание различным методам оценки воспаления кожи. Для этого используется мультидисциплинарный подход, включающий различные параметры оценки, такие как визуализация, биофизический, молекулярный и клеточный анализ, а также клиническая оценка, как показано на Рисунке 1.

Прежде чем обсуждать клинические исследования с использованием имихимода, в главе 2 рассматриваются несколько моделей испытаний (challenge models). Выбранные тестовые агенты нацелены на врожденную имунную реакцию кожи) и активацию определенных иммунологических процессов. Например, описана модель с использованием липополисахарида (LPS), который активирует врожденную иммунную систему через TLR4. Также выделена модель воздействия УФ-В излучения, которая используется в доклинических исследованиях для индукции воспаления и применяется у людей для изучения воспалительной боли.

Помимо упомянутых моделей, связанных с изучением иммунной системы, в этой главе описаны два метода для вызова зуда: с использованием гистамина и cowhage (бархатного боба). Также упоминается модель активации адаптивной иммунной системы с помощью введения KLH (ключевого лимфоцитарного антигена). Интересно отметить, что эта модель была использована для изучения эффекта нового препарата под названием амлителимаб — человеческого IgG4 моноклонального антитела против OX40L, на здоровых добровольцах. Из первой фазы исследования на людях и на основании полученных данных, а также правильно выбранной дозы, препарат успешно протестирован на пациентах с атопическим дерматитом. Это является отличным примером современного подхода к разработке лекарств. Вместо традиционных исследований первой фазы, направленных главным образом на оценку безопасности и переносимости, был применен интегрированный подход, позволяющий успешно оценить эффект нового тестируемого препарата на раннем этапе разработки.

Аналогичный интегрированный подход был применен в главе 3, где было проведено рандомизированное, открытое клиническое исследование с контролем на транспортном средстве, направленное на временное индуцирование кожного воспаления. В этой модели воспаление кожи вызывалось нанесением 5 мг агониста TLR7, имихимода (IMQ), на кожу. IMQ наносился под окклюзией на верхнюю часть спины 16 здоровых мужчин-добровольцев в открытом формате на 24, 48 и 72 часа. Добровольцы были рандомизированы на две группы: одна группа подвергалась процедуре снятия верхнего слоя кожи (tape-stripping, TS) перед нанесением IMQ, а другая группа получала IMQ на интактную кожу.

Результаты показали, что, хотя IMQ сам по себе вызывал лишь ограниченные воспалительные реакции, комбинация TS и нанесения IMQ привела к более выраженной и измеримой кожной воспалительной реакции. По сравнению с контрольной группой в группе IMQ наблюдались усиленная эритема и кровообращение в коже, повышенная экспрессия мРНК воспалительных маркеров и приток воспалительных

клеток. Активация врожденной иммунной системы была подтверждена значительным увеличением экспрессии хемокинов, таких как CXCL10, ICAM-1, hBD-2 и Mx-A, в группе TS по сравнению с необработанными участками (без TS и без IMQ).

Эта активация иммунной системы была дополнительно подтверждена идентификацией инфильтрата иммунных клеток, таких как Т-хелперы, цитотоксические Т-клетки, макрофаги и дендритные клетки, через 48–72 часа после нанесения IMQ. Воспалительные реакции были более выраженными в группе TS. В целом, после 72 часов нанесения IMQ не было обнаружено дополнительных эффектов по сравнению с 48 часами для всех измерений, что позволяет предположить, что оптимальная продолжительность нанесения составляет 48 часов. Таким образом, была разработана модель, при которой 48-часовое воздействие обеспечивает четкую воспроизводимую реакцию, подходящую для разработки лекарственных средств.

Следующим шагом было проверить, может ли клинической аий и иммунологической аий реакция на IMQ быть обращена с помощью противовоспалительного средства, которое могло бы служить эталоном. Для этого в главе 4 24 здоровым добровольцам случайным образом назначали пероральный преднизолон или плацебо в течение шести последовательных дней. Преднизолон — это зарегистрированное противовоспалительное средство, которое действует главным образом на глюкокортикоидные рецепторы.

В данном исследовании IMQ наносился на кожу, обработанную методом TS (tape-stripping), в течение 48 часов. Клинические, клеточные и молекулярные реакции характеризовались с использованием методов, изображенных на рисунке 1. Кроме того, была введена другая методика для исследования клеточных и цитокиновых ответов TLR7 — создание волдырей.

По сравнению с плацебо преднизолон подавлял реакцию , вызванную TLR7, почти по всем параметрам, включая кровообращение в коже, эритему, толщину эпидермиса, количество иммунных клеток (таких как NK-клетки, классические моноциты, Т-хелперы, цитотоксические клетки) и уровни цитокинов (таких как IL-6, IL-8, TNF и Mx-A).

В предыдущем исследовании мы продемонстрировали, что IMQ не попадает в системный кровоток. Однако важным было изучить различие в эффектах преднизолона в крови и периферических тканях. Поэтому мы протестировали ex vivo активность препарата с помощью стимуляции IMQ в цельной крови, используя выделение цитокинов в качестве измеряемого показателя. Мы показали, что подавление цитокинов преднизолоном в меньшей степени зависит от фармакокинетического профиля вещества. Как в крови, так и в тканях преднизолон демонстрировал противовоспалительные свойства, хотя эти эффекты в крови были обратимыми.

Это было первое исследование, которое сравнило результаты биопсии с данными, полученными из содержимого волдырей после применения IMQ. Создание волдырей не является абсолютно новой процедурой; еще в 1960-х годах этот метод использовался для разделения дермы и эпидермиса. Это позволило изучать воспалительные реакции неинвазивным способом. Эта техника также применялась во многих (пере-)клинических исследованиях, в которых изучались воспалительные реакции на различные интрадермальные воздействия, такие как очищенный производный белок туберкулина (PPD), ультрафиолетовая обработка убитой Escherichia coli (UVKEC), KLH и LPS. После интрадермального введения LPS клетки и цитокины, обнаруженные в содержимом волдырей, полностью соответствовали клеточным реакциям, измеренным в биопсиях. В данной главе мы обнаружили аналогичные иммунные клетки как в содержимом волдырей, так и при иммуногистохимическом окрашивании (IHC). Это свидетельствует о том, что техника создания волдырей является как эффективной, так и простой для минимально инвазивного изучения воспалительных процессов.

Однако существовали и важные различия между моделью IMQ на животных и моделью IMQ на людях. В модели на животных нейтрофилы обычно присутствовали, тогда как в человеческой модели IMQ они полностью отсутствовали. Одним из возможных объяснений этого является относительно короткая продолжительность воздействия IMQ в наших клинических исследованиях до настоящего времени. Поэтому в главе 5

мы охарактеризовали TLR7-опосредованную воспалительную реакцию после 7 дней (168 часов) воздействия IMQ у здоровых добровольцев и сравнили эту реакцию с реакцией, наблюдаемой после более короткого воздействия IMQ в 2-3 дня (48 и 72 часа). Кроме того, воспалительная реакция у животных была опосредована комплементом C3. Следовательно, нашей вторичной целью было исследовать, может ли быть активирован каскад комплемента после продолженного воздействия IMQ. Для этого 10 здоровых добровольцев участвовали в рандомизированном, открытом исследовании. Продленное воздействие IMQ привело к усилению IMQ-индуцированного воспаления как на молекулярном, так и на клеточном уровне. Кроме того, в этом исследовании мы применили анализ транскриптома, который продемонстрировал активацию сигнальной передачи TNF, комплемента и, особенно, сильные интерфероновые реакции. Эти результаты были особенно выражены после продолженного воздействия IMQ. Мы также наблюдали, что секреция интерферонов приводит к индукции пути JAK-STAT, с увеличением экспрессии хемокинов CXCL9, CXCL10 и CXCL11. Наконец, это исследование подтвердило отсутствие нейтрофилов как определяющий фактор в ответной реакции, что приводит к значительному трансляционному различию между мышами и людьми.

Для того чтобы исследовать применимость нашей модели у тестируемого препарата , мы провели клиническое исследование, описанное в главе 6. В этой главе были изучены эффекты оминанана (OMN) и имиквимода (IMQ) на воспалительные реакции в коже 16 здоровых участников. OMN — это синтетический кателицидин с антимикробными свойствами, схожими с LL-37, который усиливает интерфероновые ответы. Кателицидины, включая LL-37, являются важными антимикробными пептидами в иммунитете кожи и борются с различными патогенами. LL-37 также модулирует иммунные реакции и усиливает продукцию IFN, индуцированную лигандами TLR3, такими как полила:C, в кератиноцитах. Однако в инвитро-исследованиях показано, что этот эффект также относится к лиганду TLR7, таким как IMQ. Как и в главе 3, применение IMQ вызывало умеренные воспалительные реакции, характеризующиеся повышением эритемы и кровообращения, с пиком через 24 и 48 часов. Комбинация IMQ и OMN еще больше усилила эти физиологические реакции, а также клеточную реакцию, особенно при самой высокой концентрации 1%. Было очевидное увеличение макрофагов/моноцитов, дендритных клеток и Т-хелперных клеток, но этот эффект был ограничен в соответствующей цитокиновой реакции.. Лечение IMQ повысило уровни мРНК IL-6, IL-10, Mx-A и IFN-γ, при этом OMN показал тенденцию к усилению этих цитокиновых ответов. В предыдущем исследовании, в котором 2,5% OMN наносили на пациентов с аногенитальными бородавками и высоко градиентными плоскими клеточными интраэпителиальными поражениями, не было продемонстрировано клинической эффективности. Это указывает на то, что комбинированная терапия IMQ и OMN может быть полезной для лечения кожных заболеваний, вызванных ВПЧ (HPV).

Критическая оценка модели воспаления, индуцированного IMQ

Есть ли трансляционный разрыв? В вступление этой диссертации была описана воспалительная иммунная реакция на IMQ у мышей. Эти результаты характеризуются клиническими признаками, такими как утолщение кожи, эритема и шелушение, которые появляются через 2-3 дня после применения IMQ. У людей наблюдались схожие, но более ограниченные клинические результаты, такие как увеличение кровообращения в коже, эритема и утолщение эпидермиса через 2-3 дня после применения IMQ.

У мышей воздействие IMQ активирует нейтрофилы и привлекает их, а также увеличивает количество пDC и γδ Т-клеток. Это также подтверждается повышенными уровнями цитокинов TNF, IL-1β и IL-6, в сочетании с временным увеличением IL-23, IL-17A, IL-17F и IL-22, что указывает на участие адаптивного иммунного ответа и напоминает псориазоподобные поражения. В соответствии с предклиническими данными у людей наблюдается инфильтрация моноцитов/макрофагов, NK-клеток,

DC, Т-хелперных клеток и цитотоксических Т-клеток. На уровне цитокинов наблюдается явная сигнальная активация IRF, что выражается в повышении Mx-A, IFN-γ и IP-10. Тем не менее, были выявлены различия между видами на клеточном и цитокиновом уровнях (Рисунок 2). Хотя были зафиксированы повышенные уровни IL-6 и TNF, их экспрессия была ниже, чем у IFN, что указывает на то, что IMQ в большей степени служит моделью стимуляции IRF, чем NF-κB.

Вторым важным различием между моделью IMQ у мышей и людей является отсутствие IL-8 и нейтрофилов у здоровых людей. Нейтрофилы, которые являются первой линией защиты , быстро привлекаются к местам воспаления. Изначально считалось, что момент для измерения нейтрофилов в модели IMQ может быть упущен из-за короткого времени их жизни, с предполагаемым временем полураспада 13-19 часов. Однако различие в нейтрофильном ответе между мышами и людьми связано с отсутствием экспрессии TLR3 и ограниченной экспрессией TLR7 у человеческих нейтрофилов. Они, однако, реагируют на лиганды TLR4, такие как LPS, что вызывает более прямую и классическую воспалительную реакцию, которая дополняет эффекты IMQ.

Наконец, различия в клеточных и цитокиновых профилях в ответ на IMQ между мышами и людьми также влияют на релевантность этой модели для заболеваний. В предклинических исследованиях IMQ часто используется как модель псориазоподобного воспаления, учитывая участие оси IL-23/IL-17A/IL-22, в то время как экспрессия этих цитокинов у людей ограничена. В клинических исследованиях IMQ у людей наблюдается сильная активация IFN-ответа, что ведет к обогащению пути JAK-STAT. Эти результаты, вместе с гистопатологическими изменениями, указывают на то, что модель IMQ у людей может быть более репрезентативной для красной волчанки, чем для псориаза.

Использование точных методов В области клиниметрии врачи обычно оценивают пораженную кожу по таким характеристикам, как эритема и отек. Исследователи часто пытаются интегрировать некоторые биомаркеры и конечные точки, но они обычно предоставляют только одномерную информацию. В клинических исследованиях можно получить многомерное, более глубокое понимание процессов в коже, используя мультимодальный подход, как описано во введении к данной диссертации. Опорные элементы этого подхода включают следующее (Рисунок 1):

3 Клинические оценки
4 Результаты, сообщаемые пациенту
5 Визуализация
6 Биофизические измерения
7 Молекулярные анализы биологических образцов, таких как биопсии и жидкость из пузырей
8 Клеточные показатели

В главах данной диссертации подробно рассматриваются различные комбинации этих элементов, а также их применение. Кроме того, такие методы визуализации и биофизические измерения, как лазерная спекл-контрастная визуализация, были ранее исследованы в других клинических испытаниях с применением таких моделей, как LPS и KLH, и показали свою пригодность для объективной количественной оценки эритемы и кожного кровообращения. Хотя эти оценки в основном направлены на характеристику поверхностных кожных реакций, существуют также неинвазивные методы, которые позволяют проводить прямые измерения эпидермальной морфологии на глубину 1-2 мм. Оптическая когерентная томография, обычно используемая в клинических исследованиях с пациентами, позволяет выявить крупные гистологические структуры, но ей не хватает высокой разрешающей способности для определения индивидуальных клеточных изменений и выявления специфических белков в эпидермисе. Тем не менее, оптическая когерентная томография была использована в этой диссертации для количественной оценки толщины эпидермиса. Это подчеркивает возможность замены традиционных биопсий этой техникой в исследованиях, связанных с кожными реакциями. Методы, примененные в данной диссертации, открывают многообещающие возможности для характеристики воспалительной реакции после применения IMQ. Будущие исследования должны быть направлены на

сочетание различных результатов и визуализацию данных для лучшего понимания функции клеток и, следовательно, механизма или патологии конкретного заболевания.

Проблемы и перспективы IMQ оказался ценным препаратом для моделирования воспаления, в первую очередь для активации интерферонного ответа. В различных исследованиях он демонстрирует стабильный иммунный ответ, несмотря на наблюдаемые вариации в клеточных реакциях у отдельных пациентов. Однако одной из ограничений IMQ является его топическая форма. Несмотря на то, что все участники получают одинаковую дозу, поглощение препарата через кожу недостаточно охарактеризовано между различными испытуемыми, что, с учетом улучшенной реакции после использования метода ленты, может частично объяснять вариативность результата или реакции?(посмотри что лучше подходит). Можно предположить, что топическое применение IMQ может и не быть оптимальной формой для данной модели. Ранее попытки активировать и мобилизовать дендритные клетки через интрадермальное введение лиганда TLR7 на экс-виво коже не увенчались успехом, в то время как топическое введение IMQ оказалось эффективным. Для уточнения модели важно определить вариативность дозировки. В одном из исследований, проведенных Винд и соавторами, была представлена методология, при которой использовалась масс-спектрометрия с лазерной десорбцией/ионизацией в матрице для измерения концентрации бимирализига в кожных биопсиях после топической обработки. Тот же подход можно было бы адаптировать для оценки фармакокинетических свойств IMQ в модели кожи.

Хотя модель IMQ была протестирована в данном исследуемом препарате , направленным на их специфическую сигнальную каскаду, интересно также исследовать альтернативные области применения, где IMQ может быть эффективно использован.

Результаты, описанные в данной диссертации, показывают не только успешный перенос от животных к человеку, но и консистентность между исследованиями, что предполагает, что модель IMQ воспроизводима у здоровых людей, хотя еще не проводилось официальное исследование с повторными тестами. Следующий шаг в трансляции будет направлен на пациентов. Недавно было начато клиническое исследование, которое сосредоточено на переносе IMQ на пациентов с кожным красным волчаном.

Кроме того, недавно было уделено больше внимания прояснению роли агонитов TLR и их потенциала как адъювантов при разработке вакцин. IMQ применялся топически как кожный адъювант и показал улучшение реакции на интрадермальные вакцины против гриппа. Недавний пример касается оценки синтетических лигандов TLR7/8 компанией Inimmune Corp., которые вводились в организм совместно с вакциной против SARS-CoV-2, что приводило к усилению Т-клеточных реакций и повышения титров нейтрализующих антител, что указывает на возможность более эффективной вакцины. Учитывая эти многообещающие результаты, эти молекулы в настоящее время будут оценены в клинических исследованиях фазы I.

Помимо их потенциала как адъювантов, синтетические лиганды TLR7/8 также привлекли значительный интерес в онкологии. Иммуностимулирующие молекулы, направленные на TLR 7/8, могут активировать как врожденные, так и адаптивные иммунные ответы путем продукции цитокинов и активации цитотоксических клеток. Считается, что через этот иммунный ответ «холодные» опухоли могут быть превращены в «горячие», которые будут более эффективно реагировать на ингибиторы иммунных контрольных точек. Таким образом, эти агониты TLR рассматриваются как многообещающие кандидаты для новых монотерапий или комбинированных терапий. Хотя некоторые исследования предполагают, что активация TLR может быть полезной при лечении опухолей, другие исследования указывают, что сигнализация через TLR может способствовать росту опухолей. Полное понимание роли TLR в различных типах опухолевых клеток остается значительной проблемой, требующей проведения масштабных клинических исследований. Учитывая многообещающие сигналы, полученные в ходе

предварительных анализов некоторых клинических исследований, ожидается больше данных от продолжающихся исследований агонитов TLR, которые разрабатываются для лечения солидных опухолей.

Заключение

Данная диссертация способствует внедрению моделей испытаний на ранних этапах разработки лекарств для эффективного проведения клинических исследований и упрощения принятия своевременных решений о продолжении или остановке разработки. Использование агонистов TLR7 в качестве агента-испытания успешно переведено с животных моделей на людей, при этом были выявлены сходства и различия между видами. Ответ на имикимод был тщательно охарактеризован с использованием интегрированного подхода многомодальных биомаркеров. Модель была успешно разработана с помощью систематического подхода к оптимизации модели, бенчмаркинга с противовоспалительным средством и использована в исследовании доказательства механизма для демонстрации in vivo фармакологического действия нового лекарства. В этой диссертации мы создали структуру исследований, которые могут быть проведены перед коммерческим применением новой модели испытаний. Использование различных дополнительных подходов дает всестороннее представление, что позволяет быстрее принимать решения о переходе на следующий этап или преждевременном прекращении разработки продукта. С помощью такого подхода мы надеемся преобразовать традиционную парадигму разработки лекарств, упростив процессы клинических исследований, что приведет к повышению эффективности и снижению общих затрат.

Рисунок 1 Мультимодальный подход к характеристике эффекта лекарственных средств. «Дерма-цветок» создан F. van Meurs и адаптирован с помощью BioRender.com для этой главы.

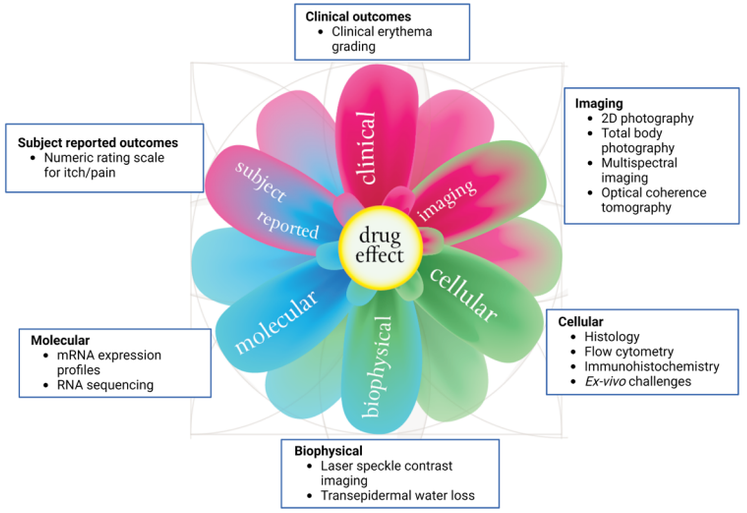
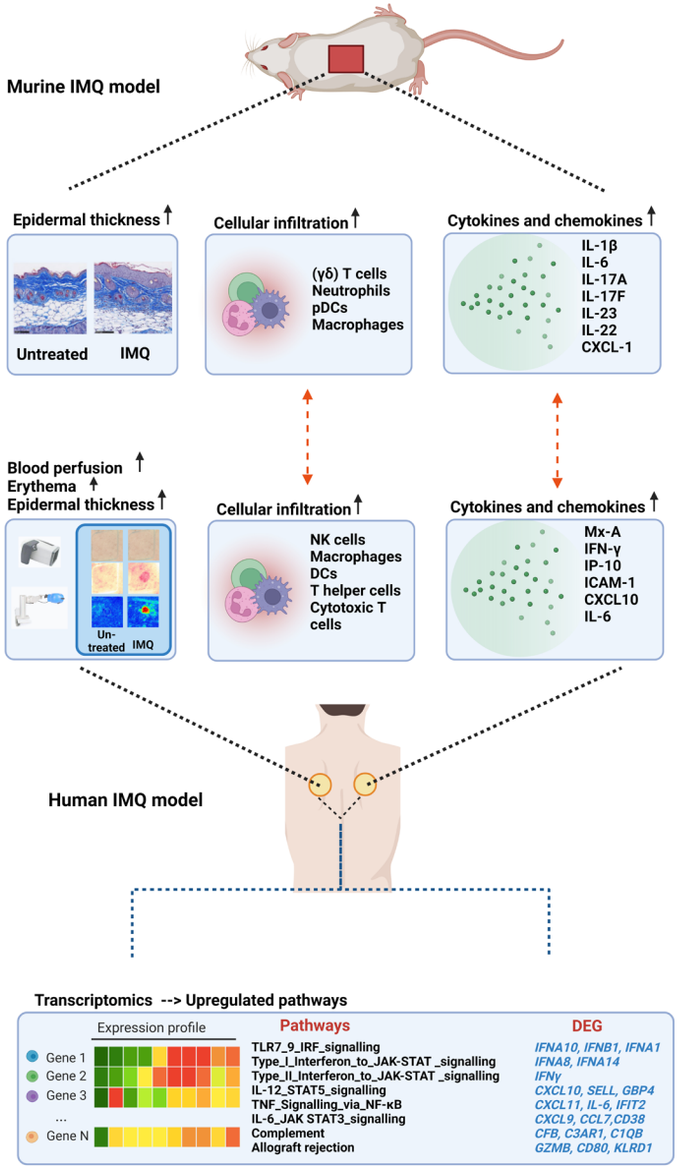


Рисунок 2 Различия в воспалительной реакции на IMQ у мышей и людей.



LIST OF PUBLICATIONS

Van der Meulen L.W.J., Bergmans M.E., **Assil S.**, Abdisalaam I., *Rijneveld R., et al.* No clinical improvement after short term topical anti-staphylococcal endolysin SA.100 therapy in patients with mild to moderate atopic dermatitis; results of a randomized, vehicle-controlled trial. *Submitted.*

Assil S., van Noort J.A., Rissmann R., Moerland M., Niemeyer-Van der Kolk T. Lost in translation: A Commentary on TLR7 agonistic response between mice and humans. *Submitted.*

Assil S.*, van Noort J.A.*, Ronner M.N., Osse M., *Pot I., et al.* (2024). Extending the IMQ model: deep characterization of the human TLR7 response for early drug development. *Inflammation,* doi.org/10.1007/s10753-024-02127-x.

Meertens M.A., Luijf T., van Lindonk E.A., Soegiharto R., ***Assil S.***, *et al.* (2023). Age and fast initial response predict omalizumab retreatment in chronic urticaria. The Journal of Allergy and Clinical Immunology: In Practice, 11(11). doi.org/10.1016/j.jaip.2023.07.030.

Assil S., Buters T.P., Hameeteman P.W., Hallard C., *Treijtel N., et al.* (2023). Oral prednisolone suppresses skin inflammation in a healthy volunteer imiquimod challenge model. *Frontiers in Immunology*, 14. doi.org/10.3389/fimmu.2023.1197650.

Assil S.*, Niemeyer- Van der Kolk T.*, Rijsbergen M., Buters T.P., *Klaassen E.S., et al.* (2019). Omiganan enhances imiquimod-induced inflammatory response in a human skin challenge model. *Clinical and Translational Science*. Doi.org/10.1111/cts.12741.

Assil S., Rissmann R., and Van Doorn M.B.A. (2019) Pharmacological Challenge Models in Clinical Drug Developmental Programs. *Inflammation-From Molecular Basis to Clinical Outcomes. IntechOpen.* doi.org/10.5772/intechopen.85352.

Van der Kolk T., **Assil S.**, Rijneveld R., Klaassen E.S., *Feiss G. et al. (2018).* Comprehensive, Multimodal Characterization of an Imiquimod-Induced Human Skin Inflammation Model for Drug Development. *Clinical Translational Science.* doi.org/10.1111/cts.12563

CURRICULUM VITAE

Salma Assil was born on 5 april 1995, in Moscow. She completed her gymnasium education at Sint- Maartenscollege in Voorburg in 2013 and started her bachelor's degree 'Bio-Farmaceutische Wetenschappen' at the Leiden University the same year. After graduating with her bachelor's degree in 2016, she pursued a master's degree in 'Bio-Pharmaceutical Sciences' specializing in the track 'Science based business'. During her master's program, she completed two internships: the first at the Centre for Human Drug Research (CHDR) in Leiden and a six-month marketing internship at Sanofi. After obtaining her master's degree in December 2018, Salma advanced her scientific career as PhD student and Clinical Scientist at CHDR and LACDR, under the supervision of prof. dr. Robert Rissmann, Dr. Martijn van Doorn and Dr. Tessa Niemeyer-Van der Kolk. In 2023, Salma was promoted to the role of Clinical Study Manager at the CHDR. During her PhD, Salma fell for the charms of the fellow PhD student and ski instructor, Wouter Bakker, with whom she is now engaged. Salma lives with Wouter and Lucas in Sassenheim.