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Keyhole limpet hemocyanin challenge model for studying adaptive immune system responses in early-phase clinical drug development

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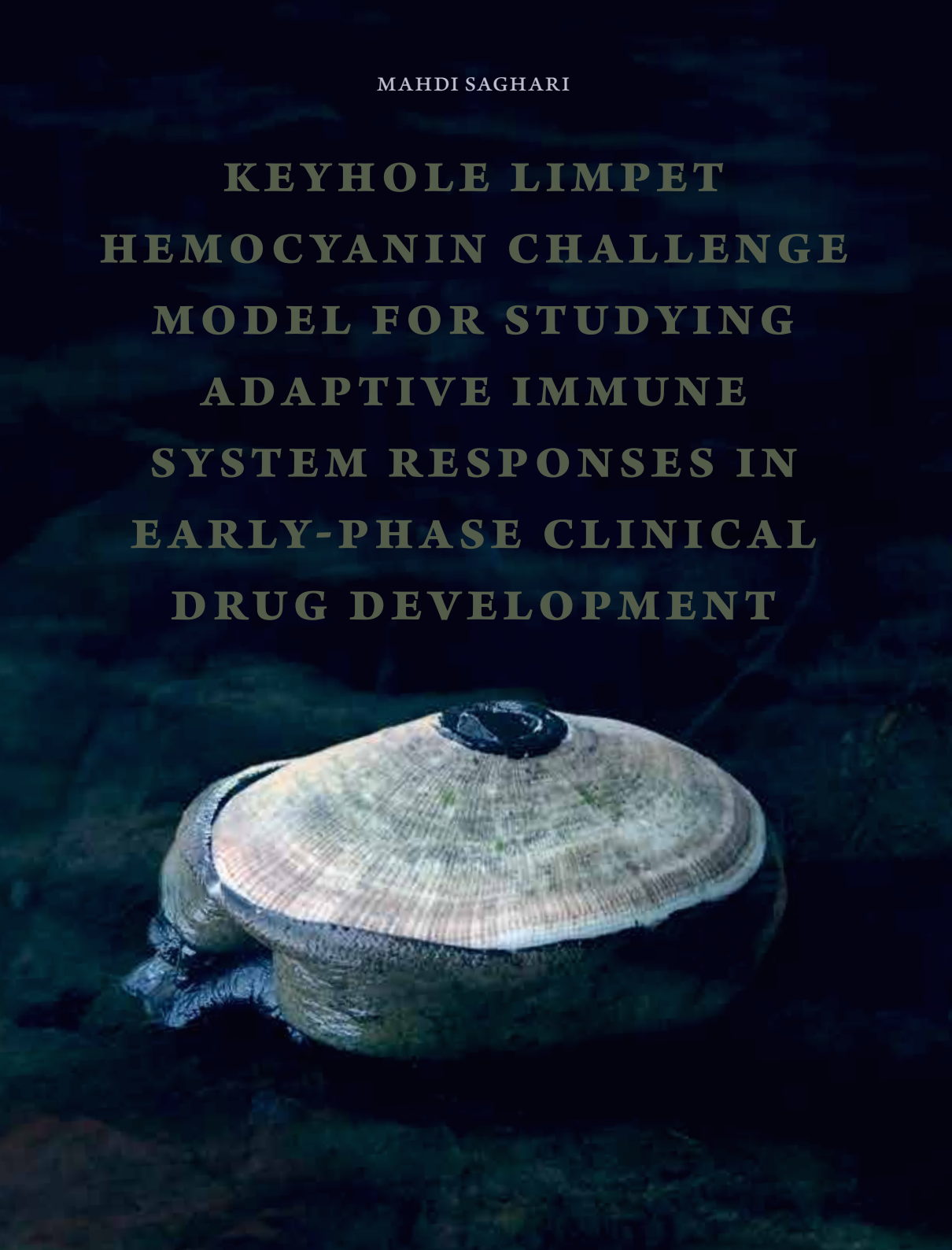
MAHDI SAGHARI

KEYHOLE LIMPET HEMOCYANIN CHALLENGE MODEL FOR STUDYING ADAPTIVE IMMUNE SYSTEM RESPONSES IN EARLY-PHASE CLINICAL DRUG DEVELOPMENT



KEYHOLE LIMPET HEMOCYANIN CHALLENGE MODEL FOR STUDYING ADAPTIVE IMMUNE SYSTEM RESPONSES IN EARLY-PHASE CLINICAL DRUG DEVELOPMENT

For Mira Eflin, my beacon of light

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DESIGN Caroline de Lint, Den Haag (caro@delint.nl)
COVER IMAGE *Megathura crenulata*, © Andrew Harmer, 2016

Protection of Megathura crenulata and its habitat is of vital importance, especially since keyhole limpet hemocyanin cannot be produced synthetically.

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KEYHOLE LIMPET HEMOCYANIN CHALLENGE MODEL FOR STUDYING ADAPTIVE IMMUNE SYSTEM RESPONSES IN EARLY-PHASE CLINICAL DRUG DEVELOPMENT

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CHAPTER I

GENERAL INTRODUCTION

Autoimmune diseases affect around 5–8% of the global population. At present over 80 different disorders have been identified, categorized as either organ specific or systemic.[1] They cause significant patient distress and pose a substantial socioeconomic challenge. Although our understanding of the intricate mechanisms behind certain autoimmune diseases has grown, the different factors driving them remain poorly comprehended, including environmental triggers and pathogenesis. Most currently used immunomodulatory drugs for the treatment of autoimmune diseases lack specificity and are associated with side effects due to broad-spectrum effects, including malignant disease and infection.[1] Additionally, a significant proportion of patients either do not respond optimally or do not respond at all to these treatments. Therefore, there is an urgent need to develop new drugs based on a molecular and clinical comprehension of specific autoimmune diseases.

The immune system, and more specifically the adaptive immune system, is a key area for the development of such new treatment strategies, particularly for managing infections, tumors, and autoimmune diseases that are resistant to conventional therapies. However, due to the lack of biological understanding only 13.8% of all drug development programs advance from phase I clinical trials to market registration across all therapeutic areas and for inflammatory and autoimmune treatments merely 6.3% reach commercial approval.[2] Currently the prices for new drugs are significantly influenced by high costs during drug development (approximately 2.7 billion USD for each novel immunomodulatory drug approved by the US Food and Drug Administration).[3] Nearly 60% of the developmental costs of investigational medicinal products (IMPs) is attributed to failure, with 60% to 80% of this failure due to insufficient efficacy observed at later stages during drug development. Hence, there is a necessity for more rational strategies in early drug development, such as adopting question-based drug development including biomarkers or utilizing a quantitative model-based methodology.[4] It is potentially valuable to provide proof-of-mechanism in humans through the pharmacokinetic and pharmacodynamic relationship before beginning phase II clinical trials. The success rate in phase II clinical trials appears to be significantly higher when proof-of-mechanism is established by the end of phase I (29%) compared to when it is not established (0%).[5]

Assessing the pharmacological activity of immunomodulatory investigational drugs during early-phase clinical trials can be difficult due to the lack of biomarker expression in healthy volunteers. However, one potential solution is to challenge the immune system of healthy volunteers by activating T cells and/or B cells, allowing for the evaluation and quantification of the effects of an investigational compound on the adaptive immune system.[6]

THE HUMAN IMMUNE SYSTEM

The human immune system is a vast and complex network of proteins, cells, and organs, that defends the body against foreign invaders and protects the body's own cells. The immune system can roughly be divided into 2 parts distinguished by the speed and specificity of their reactions: the innate and the adaptive immune response.[7] The innate immune response encompasses physical, microbiological, and chemical barriers, as well as the components of the immune system that provide immediate defense against potential pathogens, including the complement system, neutrophils, macrophages, and monocytes. This response is essential for survival and is highly conserved among animals.[8] In contrast, the adaptive immune response is a characteristic of the immune system of more evolved animals. It involves the activation of T and B lymphocytes to produce specific, antigen-targeted reactions. While the innate immune response is rapid, it lacks specificity and sometimes causes damage to normal tissues. In contrast, the adaptive response is highly specific but takes longer to develop. Additionally, the adaptive response has the ability to retain a "memory" of past infections, allowing for a more robust and efficient response to subsequent exposures.[9,10]

B and T lymphocytes originate from precursor cells in the bone marrow. T cells migrate to the thymus during their development. Both B and T cells produce antigen-specific receptors through a random DNA rearrangement and splicing process. These DNA segments encode the binding regions of the receptors. This process takes place before the cells are exposed to antigens, resulting in a wide range of T cell receptor and antibody specificities that can recognize and respond to potential pathogens.[11] This allows the immune system to protect an individual from a wide range of potential infections throughout their lifetime.

New clones of T and B cells are continuously produced throughout life, although this process slows down after the mid-20s. This may make it more difficult for people in this age range to regenerate their immune system after a bone marrow transplant or the use of antiretroviral therapy to treat HIV-related immunodeficiency.[12] Generally, aging causes a decrease in the amount of immune cells as well as their regenerative capacity and functionality known as immunosenescence.[13]

T AND B LYMPHOCYTES

After T and B cell receptor rearrangement, these cells can respond to the presence of an antigen and trigger an immune response. However, the activation of these cells is regulated to ensure that only harmful antigens are recognized and responded to.

There are two major types of effector T cells: T helper (Th) cells, which have cluster of differentiation 4 (CD4) molecules on their surface, and T cytotoxic (Tc) cells, which have CD8 molecules on their surface. $CD4^+$ Th cells play a crucial role in coordinating the immune response, recognizing foreign antigens and activating several other components of the cell-mediated immune response to eliminate pathogens. They also help to activate B cells. $CD8^+$ Tc cells, on the other hand, play a role in antiviral and potentially antitumor activity. Both types of cells are important in controlling intracellular pathogens. Another significant subpopulation of T cells are the regulatory T (Treg) cells. Many Treg forms exist, but the most well-characterized are the Treg cells that express CD4, CD25, and FOXP3.[14] Treg cells are an essential part of the immune system because they can suppress the immune responses of other cells. After elimination of invading organisms Treg cells help in controlling immune responses, and they are also involved in prevention of autoimmunity.[15]

The combination of $CD4^+$ T cell cytokines, which activate macrophages to eliminate intracellular pathogens, and $CD8^+$ T cells, which kill virally infected cells, is the basis of the adaptive immune system to effectively control intracellular infections that the innate immune system cannot achieve on its own.

B cells produce antibodies that help to neutralize toxins, prevent organisms from attaching to mucous membranes, activate complement, opsonize phagocytosis-ready bacteria, and make infected or tumor cells more vulnerable for antibody-dependent cytotoxic attack by immune cells. Thus, antibodies enhance the functions of the innate immune system. During the early stages of B cell development, the antibody is a membrane-bound molecule acting as the B cell receptor. In this role, it internalizes antigens and presents them to T cells to initiate an immune response. Once the B cell is activated, it produces secreted antibodies with the aforementioned functions.

Different types of antibodies are found in different parts of the body. For example, IgM is found in the intravascular space, IgG is the primary antibody found in the tissues and blood, and IgA is found in secretions. Mucosa-associated lymphoid tissue refers to lymphoid tissue found at various mucous membrane sites, such as the bronchi, digestive tract, and urogenital tract. These sites are interconnected in their function, as certain subpopulations of B cells are specifically attracted to these tissues. An immune response triggered at one site will provoke immune responses at other sites to the same antigen. This effect can be therapeutically exploited, as generalized mucosal immunity can be induced *via* vaccination at a single mucosal site.[16]

KEYHOLE LIMPET HEMOCYANIN

Keyhole limpet hemocyanin (KLH) is considered a model antigen for use in immunization studies involving the adaptive immune system.[6,17] KLH is a protein found in the hemolymph of the keyhole limpet (*Megathura crenulata*), a marine mollusk. Keyhole limpets are primarily found in the Pacific coastal waters of California and Mexico. KLH is responsible for the transport of oxygen within many mollusk species.[17,18] Hemocyanins are similar to hemoglobin found in the blood of vertebrates, but instead of containing iron to bind oxygen, they contain copper. KLH is a large protein, with a molecular weight of approximately 4–8 MDa, and it is composed of multiple subunits of approximately 350–390 kDa each.[6,17-19] It was clinically introduced in 1967 to study the immunocompetence of humans[20] and it exhibited outstanding immunostimulatory properties in experiments with animals and man.[21-24] The innate as well as the adaptive immune response, including both the humoral and cellular response, are activated by KLH (Figure 1).[25] It is a useful antigen to study the adaptive immune response in man because the human immune system is typically naïve to KLH prior to immunization. This allows researchers to control the degree, duration, and timing of exposure to KLH, unlike other natural or therapeutic antigens commonly used for human immune challenge studies, such as varicella zoster or Bacille Calmette-Guérin (BCG), to which the human immune system is not naïve. Currently, subunit KLH is registered as an effective immunotherapeutic treatment modality for bladder cancer.[26,27] KLH is also used as a hapten carrier protein for small molecules, or as an adjuvant in vaccine therapy or along with immunomodulatory drugs.[17,18,28-31]

HUMORAL AND CELL-MEDIATED IMMUNITY

KLH exerts a robust systemic primary humoral response upon immunization in man. After KLH antigen processing by antigen-presenting cells, naïve $CD4^+$ T cells are activated which in turn help B cells to activate, proliferate and differentiate to plasma cells (Figure 1). The response is characterized by production of initially anti-KLH IgM antibodies followed by an increase in more specific anti-KLH IgG antibodies.[25] Maximal responses are reached approximately 3 weeks after KLH immunization.[25] Enzyme-linked immunosorbent assay is the most widely used assay for quantification of KLH-specific antibodies.[25]

The "memory" function of the adaptive immune system can be evaluated by monitoring the cell-mediated immune response following a KLH challenge. Rechallenging the skin with an intradermal KLH administration after initial immunization evokes dendritic cell-mediated antigen presentation which causes KLH-specific $CD4^+$ and $CD8^+$ T cells within the draining lymph nodes to activate and proliferate (Figure 1).[32] Effector T cells migrate into the skin after priming and imprinting of homing molecules by dendritic cells. The cutaneous inflammatory response that follows consists of local immune cell infiltration and increased vascular permeability, which can be clinically observed as erythema and oedema.[33,34]

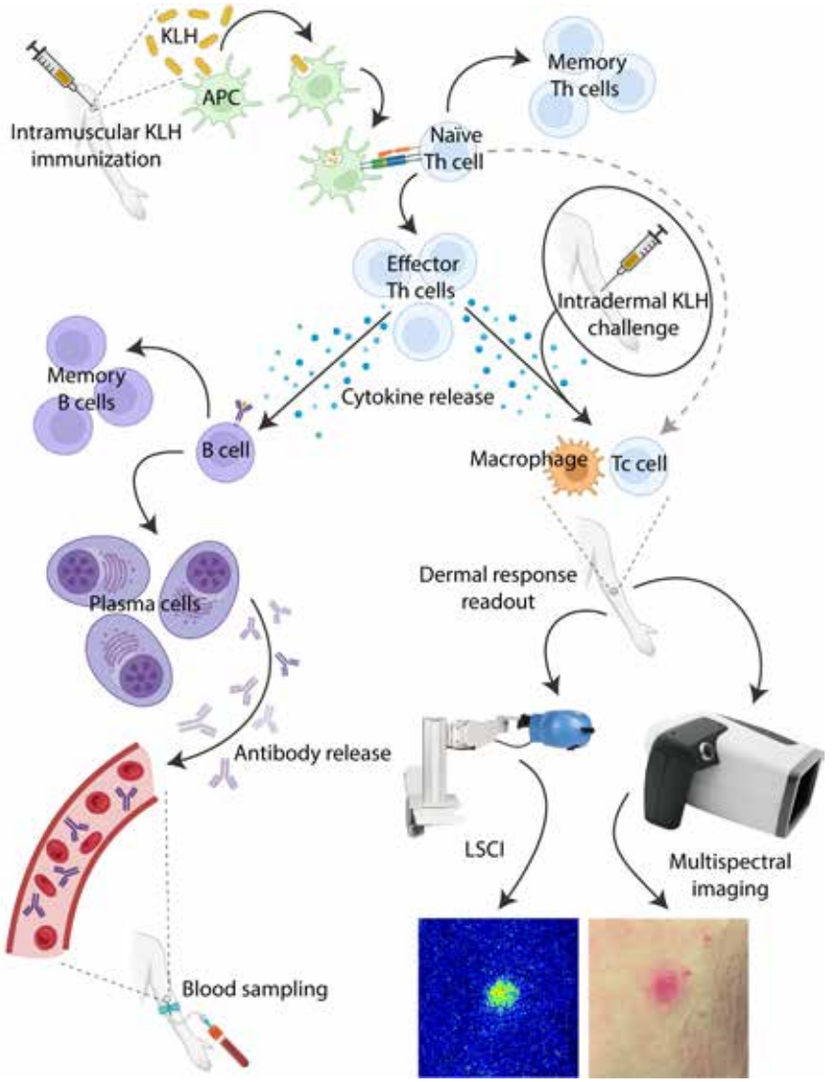
SKIN IMAGING

Although the skin response can be measured subjectively by visual inspection of the skin for erythema and oedema, this method is often scored categorically and is variable when scored by multiple assessors.[35] Objective quantification of the skin response on a continuous scale is therefore preferred.

Laser speckle contrast imaging (LSCI) provides real-time non-invasive monitoring of cutaneous microvascular perfusion.[36] LSCI's theoretical foundation can be traced back to the late 1960s when dynamic light scattering was developed.[37,38] In the 1970s, the correlation between speckle temporal dynamics and particle dynamics was established,[39] which led to the introduction of LSCI for blood flow imaging in the 1980s.[40] Originally, LSCI was used to image blood flow in the retina, but in the 1990s, it was further developed for skin imaging.[41] Since then, LSCI has been widely used in both preclinical and clinical studies, such as in pediatric burn wounds healing times assessment[42] and investigating microvasculature in coronary artery disease patients.[43] The technique is based on analysis of speckle contrast, calculated as the ratio of its standard deviation to its mean intensity, providing an blood flow index. Illumination of tissue with a laser generates a random interference (speckle) pattern. This pattern decorrelates after scattering of laser light by moving red blood cells. The intensity of each speckle fluctuates according to the velocity of the moving red blood cells, resulting in a decrease in time-integrated speckle contrast. LSCI measurements are often reported as arbitrary units as the technique does not provide a precise measurement of blood flow in mL/min. Although susceptible to motion artefacts, LSCI measurements can be performed continuously. Combined with a good temporal and spatial resolution and reproducibility, it is a useful tool in objective quantification of cutaneous blood perfusion following an intradermal challenge.[6]

FIGURE 1 – *NEXT PAGE* Schematic overview of keyhole limpet hemocyanin (KLH) challenge model. Antigen presenting cells (APCs) act as phagocytes and are activated after KLH immunization. Presentation of KLH antigens *via* MHC molecules on APCs activate naïve T helper cells (Th cells) which then proliferate further to effector Th cells and memory Th cells. Effector Th cells release cytokines activating B cells that have already been exposed to KLH antigens to proliferate further to plasma cells and memory B cells. Plasma cells produce antibodies against KLH which can be measured in the blood *via* enzyme-linked immunosorbent assay. Cytokines released by effector Th cells also activate macrophages and T cytotoxic cells (Tc cells), though Tc cells can also be directly activated by naïve Th cells. Upon intradermal KLH challenge these immune cells migrate to the skin injection site causing a local inflammatory response. This response consists of local immune cell infiltration and increased vascular permeability, which can be clinically observed as erythema and oedema. The increased vascular permeability can be objectively measured using laser speckle contrast imaging (LSCI) and the local skin erythema can be measured using multispectral imaging.

FIGURE 1



PeriCam PSI NR LSCI image source: https://www.perimed-instruments.com/content/pericam-psi-nr/; Miravex Antera 3D multispectral camera image source: https://miravex.com/pharma-cosmetics-biotech/. KLH, keyhole limpet hemocyanin; APC, antigen-presenting cell; Th cell, T helper cell; Tc cell, T cytotoxic cell; LSCI, laser speckle contrast imaging.

Multispectral computer-assisted 3D imaging of the skin can be acquired using the Antera 3D® camera. This novel camera utilizes multi-directional light in a closed chamber to reconstruct the skin surface in 3D.[44] It uses reflectance mapping of seven different wavelengths of light that cover the complete visible spectrum, enabling more accurate analysis of cutaneous colorimetric properties, including erythema. Spectral images are obtained and transformed into reflectance maps. The data is then converted to skin absorption coefficients, and mathematical correlations with known spectral absorption data of hemoglobin are used to quantify erythema.

A main focus of this thesis is the use of KLH as an *in vivo* immunization antigen for studying and objectively quantifying immune responses in humans with abovementioned skin imaging techniques.

AIMS AND OUTLINE OF THIS THESIS

The main objective of this thesis was to develop and characterize a human immune challenge model in healthy volunteers using KLH, and to subsequently apply this challenge model in healthy volunteers to evaluate the effects of immunomodulatory IMPs in drug development programs.

Section I provides background information by means of a systematic literature review on the KLH challenge model used in healthy volunteers as well as in several patient populations, including the effect of pharmacological interventions on the KLH response (Chapter 2). Section II focuses on the characterization and optimization of the KLH challenge model with objective quantification of the cell-mediated immune response in healthy volunteers (Chapter 3). In section III the KLH challenge model is implemented in early-phase clinical trials that included novel immunomodulatory IMPs (Chapters 4–6). Finally, the translation of preclinical effects of an IMP to early-phase clinical effects, based on KLH challenges, and eventually to patients, is discussed (Chapter 7).

SECTION I – KLH IMMUNE CHALLENGE MODEL BACKGROUND

In this thesis, the characterization and standardization of the KLH challenge model is described, with subsequent application of the methodology for evaluation of the activity of novel investigational compounds. Although this model has previously been used in multiple clinical trials, the precise immunological processes are still relatively unidentified. Moreover, KLH dosages, use of adjuvants, and immunization and rechallenge routes and regimens are not standardized, and the outcomes to characterize immune responses following the KLH challenge have not been optimized. A systematic review performed by Swaminathan et al. presented an outline of KLH doses, administration routes, and topline response monitoring based on 16 clinical studies.[17]

As sequel, Chapter 2 provides a more thorough systematic review examining the KLH response in clinical studies. This review expands upon the work of Swaminathan et al. by including a larger number of clinical studies and focuses on 3 main areas: the different methods used to study the systemic and local immune responses triggered by KLH, identifying the most reliable biomarkers for KLH response monitoring, and evaluating the impact of diseases and pharmacological interventions on the KLH challenge response.

SECTION II – KLH IMMUNE CHALLENGE MODEL IN HEALTHY VOLUNTEERS

A major shortcoming of most studies implementing a KLH challenge in immunopharmacological studies is the use of subjective quantification to assess the local cell-mediated response upon KLH skin challenge. Chapter 3 describes the use of a KLH challenge model in healthy volunteers with highly sensitive and objective techniques quantifying the local KLH recall response, overcoming the shortcomings of subjective evaluations such as inter-rater variability. Apart from characterization of the skin response upon dermal KLH challenge with various imaging techniques, correlations and power calculations were also performed on the humoral and cell-mediated responses to predict sample sizes for future studies involving IMPs.

SECTION III – KLH IMMUNE CHALLENGE MODEL IN EARLY-PHASE CLINICAL TRIALS

This section describes the evaluation of novel immunomodulatory IMPs in clinical trials, based on the KLH challenge model as performed in Chapter 3. Chapter 4 describes the first-in-human study of an OX40 ligand (also known as CD134) inhibitor (KY1005, currently amlitelimab) and its effects on the adaptive immune system. In Chapter 5, 3 formulations of a single-strain microbial intervention prepared from *Lactococcus lactis* spp. *cremoris* (EDP1066) were investigated in a clinical study. The aim of the trial was to characterize pharmacodynamic effects of EDP1066 on the adaptive immune system. Chapter 6 details the effects of a second single-strain microbe prepared from *Prevotella histicola* (EDP1815) on the immune system in a clinical trial. All 3 studies were performed in healthy volunteers. Lastly, Chapter 7 summarizes and translates the immunological effects of EDP1815 in preclinical trials to an early-phase clinical trial in healthy volunteers, and to clinical trials performed in patients with atopic dermatitis and psoriasis.

GENERAL DISCUSSION AND SUMMARY

Finally, a summary and general discussion of the thesis are provided in Chapter 8, and a summary conclusion in Dutch is given in Chapter 9.

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SECTION I

KLH IMMUNE CHALLENGE MODEL BACKGROUND

CHAPTER II

CHARACTERIZATION OF KLH-DRIVEN IMMUNE RESPONSES IN CLINICAL STUDIES: A SYSTEMATIC REVIEW

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ABSTRACT

Introduction: The pharmacological activity assessment of novel immunomodulatory drugs in early-stage drug development is challenging as healthy volunteers do not express relevant immune biomarkers. Alternatively, the immune system can be challenged with keyhole limpet hemocyanin (KLH), a suitable antigen for studying adaptive immune responses. This report systemically reviews the KLH challenge in clinical studies focusing on the characterization of the KLH-driven systemic and local immune responses, identification of the KLH-induced biomarkers, and the evaluation of the effect of pharmacological interventions and diseases on the KLH response.

Methods: A systematic literature review was carried out in PubMed spanning from 1967 to 2022.

Results: The systemic humoral KLH responses could be characterized by ELISA after 3 weeks following immunization. For the systemic cellular and molecular immune responses multiple KLH immunizations and the use of novel techniques such as flow cytometry and ELISpot yield optimal results. The objective evaluation of dermal KLH rechallenge allows for more accurate and sensitive quantification of the local response compared to subjective scoring. For the local cellular and molecular assays after KLH dermal rechallenge we also advocate the use of multiple KLH immunizations. Furthermore, oral KLH feeding, age, physical activity, alcohol consumption, stress, as well as certain auto-immune diseases also play a role in the KLH-induced immune response. Importantly, based on the KLH challenges, the effect of (novel) immunomodulatory drugs could be demonstrated in healthy volunteers, providing valuable information for the clinical development of these compounds.

Conclusion: This review underlines the value of KLH challenges in clinical studies, but also the need for standardized and well-controlled methodology to induce and evaluate KLH responses.

INTRODUCTION

The evaluation of pharmacological activity of immunomodulatory investigational drugs in the early-phase of clinical development is challenging as healthy volunteers do not express biomarkers related to immunological disorders. A workaround is to challenge the immune system by activating T cells and/or B cells in healthy volunteers.[1] Subsequently, the effect of these investigational drugs on the adaptive immune system can be quantified.

Keyhole limpet hemocyanin (KLH) is a suitable immunization antigen for studying the adaptive immune system.[1,2] KLH is derived from the hemolymph of the marine mollusk, *Megathura crenulata*, which can be found in the Pacific coastal waters of California and Mexico. Hemocyanins are metalloproteins (copper-containing molecules) with as main function the transport of oxygen within many mollusk species.[2,3] KLH is used extensively as an immunostimulant in clinical research as it drives a strong humoral and cell-mediated immune response, is harmless to human subjects, and is available as a clinical grade product. KLH induces a T cell-dependent response, which makes it an effective agent for studying the effect of novel immunomodulatory drugs on T cell-mediated immunity.[4] KLH was first clinically introduced in 1967 to study the immunocompetence of humans.[5] KLH has proven to be effective in bladder cancer immunotherapy[6,7] and is also registered as a treatment modality for the disease. KLH is also used as a carrier protein, as an immunostimulatory challenge agent driving an immune response, or as an adjuvant in cancer vaccines or along with immunomodulatory drugs against autoimmune disorders.[2,8-11] However, the exact immunological actions are still unknown, KLH doses and regimens are not standardized, and the endpoints to characterize KLH responses have not been optimized. Swaminathan et al. have systematically reviewed the use of KLH in 16 clinical studies.[2] This review provided an overview of KLH doses, routes of administration, and high-level response monitoring. As sequel, we performed an in-depth systematic review focusing on the KLH response characterization in clinical studies, extending the scope of Swaminathan's review by inclusion of a significantly larger number of clinical studies, and focusing on 1) the various approaches for characterization of the systemic (humoral and cellular/molecular) and local (planimetric and cellular/molecular) immune response driven by KLH, 2) identification of the most robust biomarkers for monitoring of a KLH response, based on response size and variability, and 3) evaluation of the effect of pharmacological interventions and diseases on the KLH response.

METHODS

A systematic literature review was carried out spanning a period from 1967 up to the 20th of February 2022 in PubMed. The systematic review was registered on PROSPERO (identifier CRD42022335419). No systematic review protocol was prepared. Figure 1 provides a schematic outline based on the PRISMA 2020 statement flow diagram for systematic reviews of the search steps for the identification, screening, and inclusion process. The PRISMA 2020 checklist for the systematic review report can be found in the Supplementary Table S1. The execution of the database search, screening and data extraction were all performed by a single author (MS). The search query contained the keywords *keyhole limpet hemocyanin*, *immunotherapy*, and *response* and it encompassed any derivatives from these keywords. The exact search strategy is given in Supplementary Table S2. The outcomes were defined as any immune system response following a KLH challenge subdivided into 4 categories: systemic humoral response, systemic cellular/molecular response, local planimetric response, and local cellular/molecular response. The outcomes for the evaluation of the effect of pharmacological interventions and diseases on the KLH response were similarly approached and included the presence of at least 2 groups for comparison (e.g., treatment *vs.* no treatment or disease *vs.* no disease). The KLH immunization and the local rechallenge strategy was tabulated per article and included data on the KLH formulation, the use of an adjuvant, the immunization dose, the immunization route, the number of immunizations, the interval between immunizations, and the skin rechallenge dose. The immune system response following KLH immunization was also tabulated per article subdivided into the 4 categories (systemic humoral response, systemic cellular/molecular response, local planimetric response, and local cellular/molecular response) and included the measurement assay/technique used and the response size and variability per category. Lastly, the outcomes for evaluation of the effect of (pharmacological) interventions and/or diseases on the KLH challenge were also tabulated and included data on the intervention and/or patient population, the comparison between groups examined, and the differences in immune system responses observed between groups.

The initial search resulted in a total of 1,605 records. The titles and abstracts of those records were screened for eligibility. Only the records where KLH was studied in humans were included. The records in which no KLH immunization was performed or no clinical use of KLH was mentioned, no original trial was reported, or only KLH-pulsed cells were used were excluded. Other exclusion criteria were non-English articles and KLH used as a conjugate. A total of 142 records remained, of which 11 articles were not retrievable. The full-text reports of the remaining 131 records were screened for eligibility using the same in- and exclusion criteria. A total of 57 studies were included in this systematic review of which 45 studies were relevant for objectives 1 and 2, and 43 studies for objective 3.

As no validated and widely adopted reference material was available for many of the outcomes the effects were analyzed as the mean fold change compared to baseline. The variability was also reported as the fold change compared to baseline mean. A few outcomes in a couple of studies had no baseline measurement, in these cases the outcomes were analyzed as the mean fold change compared to untreated or placebo instead. For some outcomes, e.g., the local planimetric induration and erythema responses, it was possible to keep the reporting similar to the original article as the methods and reference material used were similar across studies. Furthermore, not many studies had included numbers of the analyses performed in a tabulated form or within the article text, but only had a graphical presentation of the analyses. In these cases, the mean fold change over baseline was estimated from the graphical presentations using graphical software tools. In the majority of the cases only partial information from selected studies was needed for this systematic review, namely the KLH challenge outcomes, therefore the GRADE guidelines could not be implemented and the individual articles could not be graded in their entirety, including the risk of bias.

RESULTS

KLH IMMUNIZATION AND RECHALLENGE

KLH has been used in the clinical studies in various formulations, doses, routes of administration, and immunization regimens (number of immunizations and interval). Table 1 displays the use of the KLH challenge model for human studies in which healthy volunteers were immunized with KLH without any other interventions. Figure 2 summarizes the KLH challenge in terms of the formulation, the immunization dose, the immunization route, and the number of immunizations across the studies displayed in Table 1. There are currently 2 clinical grade KLH formulations available: High Molecular Weight (HMW) and subunit KLH.[1-3,12] HMW KLH is the native KLH protein consisting of multiple subunits with a size of approximately 4–8 MDa. Each subunit has a size of approximately 350–390 kDa. HMW KLH was used in 30 out of 45 studies and subunit KLH in 14 out of 45 studies (Table 1 and Figure 2). A study performed by Miller et al. used both KLH formulations.[13] They compared 3 different KLH formulations; HMW KLH, subunit KLH, and subunit KLH with Montanide ISA-51 as an adjuvant. HMW KLH and subunit KLH with Montanide ISA-51 showed a comparable immune response in healthy participants, and were more potent compared to subunit KLH alone. The immunogenicity of KLH is presumably related to the carbohydrate

and peptide epitopes.[3,14] Because of the lower immunogenicity, subunit KLH is often used concurrently with an adjuvant, such as aluminum hydroxide.[15] Out of the 9 studies that used an adjuvant together with subunit KLH, participants were immunized with subunit KLH adsorbed to aluminum hydroxide in 7 studies.[1,16-21]

FIGURE 1 PRISMA 2020 flow diagram of the PubMed search query for the systematic review.

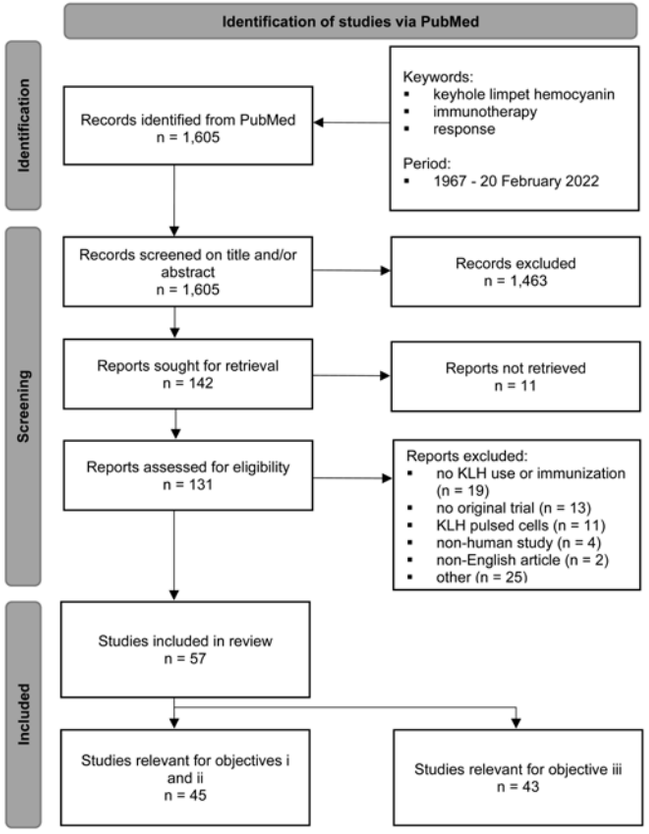


TABLE 1 Study design in relation to KLH challenge

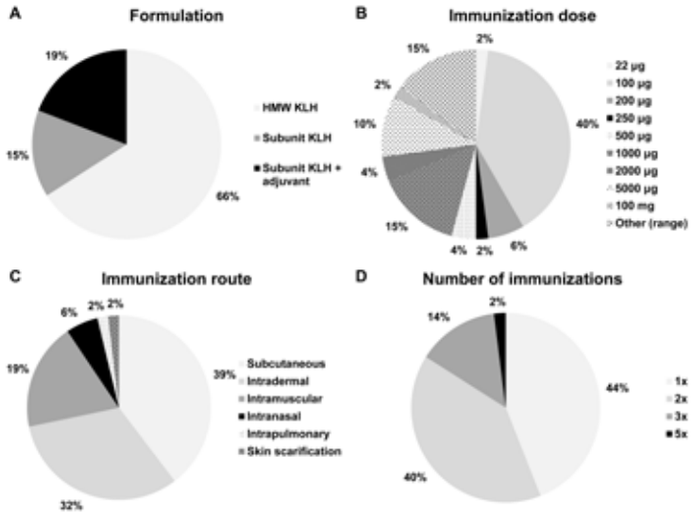
Author article, year	No. sub.	Formulation	Adjuvant	Immunization dose	Route	Frequency	Interval	Rechallenge skin dose**
Saghari M, et al. 2022[16]	10	Subunit KLH	ALUM	100 µg	I.M.	1×		1 µg
Yang J, et al. 2021[35]	16	Subunit KLH		1,000 µg	S.C.	1×		NA
Otterhaug T, et al. 2021[22]	12	Subunit KLH	Hiltonol	100 µg	I.D.	2×	2 weeks	NA
Saghari M, et al. 2020[1]	12	Subunit KLH	ALUM	100 µg	I.M.	1×		1 µg
Giesecke C, et al. 2018[46]	5	Subunit KLH		10–1,000 µg	S.C. and/or I.D.	2–3×	7 days–18 months	NA
Poirier N, et al. 2016[17]	8	Subunit KLH	ALUM	100 µg	I.M.	1×		NA
Belson A, et al. 2015[34]	13	Subunit KLH		5,000 µg	S.C.	1×		100 µg
Hostmann A, et al. 2015[56]	8	Subunit KLH		1,000 µg	S.C. and I.D.	3×	1–3 weeks	100 µg
Ferbas J, et al. 2013[47]	8	HMW KLH		1,000 µg	I.D.	2×	4 weeks	NA
Boulton C, et al. 2012[18]	24	Subunit KLH	ALUM	1,00 µg	I.M.	3×	1 week	10 µg
Kantele A, et al. 2011[23]	5	HMW KLH		100 µg	S.C.	2×	10 days	NA
Milgrom H, et al. 2010[48]	24	Subunit KLH		50–250 µg	I.D. or SS	2×	3 weeks	1–10 µg
Kapp K, et al. 2010[49]	6	Subunit KLH		1,000 µg	S.C. and I.D.	2×	1 week	NA
Spazierer D, et al. 2009[19]	16	Subunit KLH	ALUM	100 µg	I.M.	3×	2 weeks	10 µg
Miller JS, et al. 2005[13]	37	HMW KLH or Subunit KLH	Montanide ISA-51	1,000 µg	S.C.	1×		NA
Moldoveanu Z, et al. 2004[24]	8	HMW KLH		100 µg	I.M.	1×		10 µg
Smith TP, et al. 2004[20]	19	Subunit KLH	ALUM	100 µg	I.M.	1×		1 µg
Smith A, et al. 2004[21]	23	Subunit KLH	ALUM	100 µg	I.M.	1×		1 µg
Kraus TA, et al. 2004[25]	8	HMW KLH		100 µg	S.C.	2×	10 days	NA
Boelens PG, et al. 2004[36]	17	HMW KLH		500 µg	I.M.	1×		1–10 µg
Lange CG, et al. 2003[26]	10	HMW KLH		100 µg	I.D.	2×	4 weeks	100 µg
Rentenaar RJ, et al. 2002[37]	10	HMW KLH		1,000 µg	S.C.	1×		100 µg
Valdez H, et al. 2000[50]	5	HMW KLH		1,000 µg	I.D.	2×	6 weeks	1,000 µg
Diaz-Sanchez D, et al. 1999[57]	10	HMW KLH		100–1,000 µg	I.N.	3×	2 weeks	NA
Kantele A, et al. 1999[27]	5	HMW KLH		100 µg	S.C.	2×	10 days	NA
Schuyler M, et al. 1997[38]	9	HMW KLH		500 µg	I.P.	1×		NA
Kondratenko I, et al. 1997[39]	6	HMW KLH		200 µg	I.D.	1×		NA
de Fijter JW, et al. 1996[58]	18	HMW KLH		250 µg	S.C.	3×	2 weeks	NA
Waldo FB, et al. 1994[28]	4	HMW KLH		100 µg	S.C.	1×	2 weeks	10 µg
				100 mg	I.N.	3×		
Husby S, et al. 1995[29]	8	HMW KLH		100 µg	S.C.	2×	10 days	10 µg
Snyder BK, et al. 1993[30]	89	HMW KLH		100 µg	S.C.	1×		NA
Falconer AE, et al. 1992[40]	7	HMW KLH		200 µg	I.D.	1×		NA
Ward MM, et al. 1990[41]	6	HMW KLH		5,000 µg	S.C.	1–2×	5 years	NA
Bird P, et al. 1990[51]	23	HMW KLH		200 µg	S.C. or I.D.	2×	1 year	NA
Ochs HD, et al. 1988[31]	26	HMW KLH		100 µg	I.D.	2×	6 weeks	NA

CONTINUATION TABLE 1

Author article, year	No. sub.	Formulation	Adjuvant	Immunization dose	Route	Frequency	Interval	Rechallenge skin dose**
Ashorn RG, et al. 1986[42]	2	HMW KLH		2,000 µg	I.D.	1×		5 µg
Palestine AG, et al. 1985[43]	5	HMW KLH		5,000 µg	I.M.	1×		50 µg
Birdsall HH, et al. 1983[32]	20	HMW KLH		100 µg	I.D.	2×	1 month	NA
Ford D, et al. 1983[44]	3	HMW KLH		10–2,000 µg	S.C. and/or I.D.	1–2×	3 weeks	NA
Volkman DJ, et al. 1981[52]	6	HMW KLH		5,000 µg	S.C.	2×	2 weeks	NA
Powell AE, et al. 1978[53]	2	HMW KLH		10–100 µg	I.D.	2×	5–9 months	NA
Paty JG, et al. 1975[33]	13	HMW KLH		100 µg	S.C.	1×		NA
Brunner CM, et al. 1973[45]	1	HMW KLH		2,000 µg	S.C.	1×		1–100 µg
Curtis JE, et al. 1972[54]	13	HMW KLH		1–5,000 µg	S.C. or I.D.	2×	1–3 weeks	100 µg
Salvaggio J, et al. 1969[55]	35	HMW KLH		22 µg	I.D.	2×	1 week–2.5 months	22 µg
				300–600 µg	I.N.	5×		

** Number of healthy subjects without any other interventions other than KLH challenge. ** NA indicates no dermal KLH rechallenge performed. HWM, high molecular weight; KLH, keyhole limpet hemocyanin; Alum, aluminum hydroxide; i.m., intramuscular; s.c., subcutaneous; i.d., intradermal; SS, skin scarification; i.n., intranasal; i.p., intrapulmonary.*

FIGURE 2 Ratios of (A) KLH formulation, (B) immunization dose, (C) immunization route, and (D) number of immunizations across all studies with a KLH challenge in healthy subjects without any other immunomodulatory interventions.



HWM, high molecular weight; KLH, keyhole limpet hemocyanin.

Immunizations with doses of 1–5,000 µg have been reported, with 100 µg being the most frequently used dose (Figure 2).[1,16-33] Higher KLH immunization doses have been used in earlier studies and in studies using subunit KLH alone compared to studies using HMW KLH or subunit KLH with an adjuvant (Table 1). Notably, Belson et al. used a high KLH immunization dose of 5,000 µg causing a participant withdrawal rate of approximately 38% due to large local reactions following a single subcutaneous administration.[34] Overall, there seems to be a dose-effect relationship following KLH immunization as the maximum response sizes appear to be greater when higher immunization doses are used (Table 2 and Table 3). The number of KLH immunizations also appears to increase the maximum response size. Out of 45 studies, 22 studies immunized participants once,[1,13,16,17,20,21,24,28,30,33-45] 20 studies immunized participants twice,[22,23,25-27,29,31,32,41,44,46-55] and 8 studies immunized participants more than 2 times (Table 1 and Figure 2).[18,19,28,46,55-58] Spazierer et al. demonstrated that both the systemic humoral as well as the cell-mediated immune response increase in strength after a subsequent KLH immunization.[19] The response was already observed 14 d after the initial immunization, however, the response size on antigen-specific antibodies and proliferation of peripheral blood mononuclear cells (PBMCs) increased and peaked on day 57 (4 weeks after the third and last immunization). The maximum systemic humoral response had increased 24-fold for anti-KLH immunoglobulin M (IgM, range 8–38-fold), 10,000-fold for anti-KLH IgG1 (range 4,000–19,000-fold) and 40-fold for anti-KLH IgG4 (range 10–110-fold) compared to the pre-immunization anti-KLH antibody titers (Table 2). Similarly, Giesecke et al. demonstrated an increase in anti-KLH antibody responses after a secondary immunization up to 18 months after the primary immunization, though the sample size was small (n = 3).[46] Moreover, they also found that the secondary immune response occurred faster with an increase of the anti-KLH IgG antibodies 1 week after the secondary immunization, compared to an increase of the anti-KLH IgG antibody responses 2 weeks after the primary immunization.

TABLE 2 Maximum systemic responses to KLH challenge.

Author article, year	Systemic humoral		Systemic cellular/molecular	
	Technique	Response size (variability)*	Technique	Response size (variability)*
Saghari M, et al. 2022[16]	ELISA	IgG 4.9× (1.3–9.3×)		
		IgM 1.4× (1–2.4×)		
Yang J, et al. 2021[35]	ELISA	IgG 45× (1–90×)**		
		IgM 40× (1–100×)**		
Otterhaug T, et al. 2021[22]	ELISA	IgG 13,000× (variability unclear)**		
Saghari M, et al. 2020[1]	ELISA	IgG 6.8× (4.4–10.4×)		
		IgM 2.2× (1.5–3.2×)		
Giesecke C, et al. 2018[46]	ELISA	IgG 5.5× (2–12×)**	ELISpot***	Plasmablasts 44× (variability unclear)
		IgM 76.9× (4.3–240×)**		

CONTINUATION TABLE 2

Author article, year	Systemic humoral		Systemic cellular/molecular	
	Technique	Response size (variability)*	Technique	Response size (variability)*
		IgA 14.9× (2–32×)**		
Poirier N, et al. 2016[17]	ELISA	IgG 12× (7–17×)**		
Hostmann A, et al. 2015[56]	ELISA	IgG 500× (50–900×)**	FC***	$CD4^+$ proliferated T cells 16× (8–41×)**
		IgG1 300× (100–750×)**		$CD4^+CD154^+$ T cells 19× (1–29×)**
		IgG2 2× (1–5×)**		$CD4^+$ T cells IL-2 140× (90–170×)**
		IgG3 60× (10–120×)**		$CD4^+$ T cells IL-4 36× (22–40×)**
		IgG4 6× (2–40×)**		$CD4^+$ T cells IL-10 12× (5–17×)**
		IgM 30× (4–55×)**		$CD4^+$ T cells IL-17 3× (1–7×)**
		IgA 300× (1–700×)**		$CD4^+$ T cells IFN-γ 20× (10–30×)**
				$CD4^+$ T cells TNF 7× (5–10×)**
Ferbas J, et al. 2013[47]	CBA	IgG 260× (35–700×)**	ELISpot***	B cells 1,250× (1–2,600×)
		IgM 15× (2–35×)**		
Boulton C, et al. 2012[18]	ELISA	IgG 16× (variability unclear)**		
		IgM 19× (variability unclear)**		
Kantele A, et al. 2011[23]			ELISpot***	IgA plasmablasts 18× (10–36×)**
				IgG plasmablasts 34× (13–55×)**
				IgM plasmablasts 8× IgM (4–12×)**
Milgrom H, et al. 2010[48]	ELISA	IgG 2.2× (0–4.7×)		
		IgA 2.4× (0–5.2×)		
		IgM increased (variability unclear)		
Kapp K, et al. 2010[49]	ELISA	IgG 500× (1–1,300×)**	FC***	$CD4^+$ proliferated T cells 21× (2–44×)**
		IgG1 250× (50–900×)**		$CD4^+CD154^+$ T cells 40× (15–60×)**
		IgG2 1× (1–5×)**		$CD4^+CD154^+$ T cells IL-2 30× (10–50×)**
		IgG3 20× (2–80×)**		$CD4^+CD154^+$ T cells IL-4 10× (4–16×)**
		IgG4 3× (1–20×)**		$CD4^+CD154^+$ T cells IL-10 2× (1–10×)**
		IgM 50× (1–150×)**		$CD4^+CD154^+$ T cells IFN-γ 21× (5–45×)**
		IgA 1,100× (100–2,900×)**		$CD4^+CD154^+$ T cells TNF 30× (5–50×)**
Spazierer D, et al. 2009[19]	ELISA	IgG1 10,000× (4,000–19,000×)**	TIA***	Proliferation 4× (3.3–4.7×)**
		IgG4 40× (10–110×)**	Multiplex***	IL-5 15× (1–80×)**
		IgM 2.4× (8–38×)**		IL-10 12× (1–20×)**
				IL-13 120× (10–500×)**
				IFN-γ 15× (1–30×)**
Miller JS, et al. 2005[13]	ELISA	Intracel KLH IgG1 37.6× (−11.8 to 87×)	TIA***	Proliferation response size unclear (6.5–32.3×)
		Intracel KLH IgG2 6.0× (1.3–10.7×)	ELISpot***	IFN-γ 10× (5–20×)**
		Intracel KLH IgM 2.9× (0.1–5.6×)		
		Biosyn KLH + adj. IgG1 67.4× (8.5–126.3×)		
		Biosyn KLH + adj. IgG2 7.4× (2.4–12.5×)		
		Biosyn KLH + adj. IgM 5.9× (0.8–11×)		
Moldoveanu Z, et al. 2004[24]	ELISA	IgA 30× (23–37×)**	TIA***	Proliferation 18× (10–23×)**
		IgG 48× (45–50×)*		
Smith TP, et al. 2004[20]	ELISA	IgG 5× (4.7–5.3×)**		
		IgM 1.7× (1.6–1.8×)**		

CONTINUATION TABLE 2

Author article, year	Systemic humoral		Systemic cellular/molecular	
	Technique	Response size (variability)*	Technique	Response size (variability)*
Smith A, et al. 2004[21]	ELISA	IgG 5.1× (0.9–9.3×)	TIA***	Proliferation 1.5× (0.6–2.4×)
Kraus TA, et al. 2004[25]	ELISA	IgG + IgM 1.9× (1.8–2.4×)**	TIA***	Proliferation 24.4× (4–48×)**
Boelens PG, et al. 2004[36]	ELISA	IgG 12× (3–16×)**	TIA***	Proliferation 2.5× (1–18×)**
		IgG1 1.6× (1.1–1.9×)**		
		IgG2 5× (3–11×)**		
		IgG3 3× (1.5–33×)**		
		IgM 10× (4–28×)**		
		IgA 24× (10–36×)**		
Lange CG, et al. 2003[26]	ELISA	IgG 32× (20–73×)**	TIA***	Proliferation 25× (16–80×)**
Rentenaar RJ, et al. 2002[37]	ELISA	IgG 3× (1–900×)**	TIA***	Proliferation 5× (0.6–20×)**
Valdez H, et al. 2000[50]			TIA***	Proliferation 10× (5–20×)**
Kantele A, et al. 1999[27]			TIA***	Proliferation 8.8× (0.3–29.4×)**
Schuyler M, et al. 1997[38]	ELISA	IgG1 35× (21–49×)**		
		IgG4 1.5× (1.3–1.7×)**		
		IgM 175× (125–225×)**		
		IgA1 22× (8–36×)**		
Kondratenko I, et al. 1997[39]	ELISA	IgG increased (variability unclear)	TIA***	Proliferation 6.1× (variability unclear)
		IgM increased (variability unclear)		
de Fijter JW, et al. 1996[58]	ELISA	IgG 5× (3–7×)**	ELISpot***	IgG ASC 10× (5–15×)**
		IgA 20× (13–27×)**		IgM ASC 30× (20–40×)**
				IgA ASC 70× (45–95×)**
Waldo FB, et al. 1994[28]	ELISA	IgG 10× (9–11×)**		
		IgA 4.5× (3.2–5.8×)**		
Husby S, et al. 1995[29]	ELISA	IgG 5× (3.8–6.3×)**	TIA***	Proliferation 7.7× (1.6–16.8×)**
		IgM 1.8× (1.4–2.2×)**	ELISpot***	IgG ASC 10× (4–16×)**
		IgA 35× (23–47×)**		IgM ASC 4× (1–7×)**
				IgA ASC 4× (3–5×)**
Snyder BK, et al. 1993[30]			TIA***	Proliferation 3.8× (−1.7 to 9.5×)
Falconer AE, et al. 1992[40]	ELISA	IgG1 17× (2–35×)**		
		IgG2 6× (1–27×)**		
		IgG3 15× (1–40×)**		
		IgG4 8× (1–22×)**		
Ward MM, et al. 1990[41]	ELISA	IgG 76× (variability unclear)**		
		IgM 8.5× (variability unclear)**		
Bird P, et al. 1990[51]	ELISA	IgG 23× (15–40×)**		
		IgG1 58× (30–120×)**		
		IgG2 4× (1.8–12.5×)**		
		IgG3 2× (1.5–3×)**		
		IgG4 78× (40–100×)**		
Ochs HD, et al. 1988[31]	HA	IgG 64× (2–128×)		
Ashorn RG, et al. 1986[42]			TIA***	Proliferation 7.5× (4.9–10×)
Palestine AG, et al. 1985[43]	ELISA	IgM 4.4× (3.4–5.5×)	TIA***	Proliferation 5.1× (2.9–9.8×)**
Birdsall HH, et al. 1983[32]	RIA	IgG 5.7× (0.9–12.3×)		

CONTINUATION TABLE 2

Author article, year	Systemic humoral		Systemic cellular/molecular	
	Technique	Response size (variability)*	Technique	Response size (variability)*
		IgM 1.8× (0.7–3.8×)		
Ford D, et al. 1983[44]			TIA***	Proliferation 9.7× (single subject)
Volkman DJ, et al. 1981[52]	ELISA	IgG + IgM 20–50× (variability unclear)		
Powell AE, et al. 1978[53]			LAI***	Adherence inhibition 35× (26–45×)**
Paty JG, et al. 1975[33]	HA	Total Ig increased (variability unclear)	TIA***	Proliferation 14.4× (8.2–18.6×)
Brunner CM, et al. 1973[45]	HA	Total Ig 64× (single subject)**	TIA***	Proliferation 26× (single subject)
Curtis JE, et al. 1972[54]	HA	Total Ig 5.3× (2–13.9×)	TIA***	Proliferation 1.5× (0.2–8.7×)
Salvaggio J, et al. 1969[55]	HA	IgG 310× (32–1,024×)**		
		IgM 2× (1–32×)**		

** Fold change compared to baseline unless stated otherwise. ** Estimated from graphical presentation. *** In presence of KLH-coated plates or after ex vivo KLH stimulation and subsequent incubation. ELISA, enzyme-linked immunosorbent assay; HA, hemagglutination assay; CBA, cytometric bead array; RIA, radioimmunoassay; Ig, immunoglobulin; KLH, keyhole limpet hemocyanin; adj., adjuvant; ELISpot, enzyme-linked immune absorbent spot; FC, flow cytometry; TIA, thymidine incorporation assay; LAI, leucocyte adherence inhibition; CD, cluster of differentiation; IL, interleukin; IFN-γ, interferon gamma; TNF, tumor necrosis factor; ASC, antibody secreting cell.*

TABLE 3 Maximum local responses to KLH challenge.

Author article, year	Local planimetric		Local cellular/molecular	
	Technique	Response size (variability)*	Technique	Response size (variability)*
Saghari M, et al. 2022[16]	LSCI	Basal flow 1.2× (1–1.5×)** *vs.* placebo		
	MI	Erythema 1.2× (1–1.5×)** *vs.* placebo		
Saghari M, et al. 2020[1]	LSCI	Basal flow 1.4× (1.0–1.9×)** *vs.* placebo		
	MI	Erythema 1.4× (1.2–1.9×)** *vs.* placebo		
	Colorimetry	Erythema 1.1× (1.0–1.5×)** *vs.* placebo		
	Photography	EI 1.0× (0.9–1.2×)** *vs.* placebo		
Belson A, et al. 2015[34]	BPP diameter	Induration 51.84 mm (35.8–75.1 mm)	Biopsy	$CD3^+$ T cells 16× (14–19×)** *vs.* untreated
	Ruler	Erythema 73.4 mm (57.4–93.9 mm)		$LAG3^+$ T cells 20× (12–28×)** *vs.* untreated
	LDI	Flare area 29.49 cm2 (20.6–42.3 cm2)	SB	Leucocytes 60× (15–130×)** *vs.* untreated
		Flare intensity 355.1 PU (313.9–401.7 PU)		Lymphocytes 280× (70–600×)** *vs.* untreated
				$CD3^+$ T cells 350× (50–650×)** *vs.* untreated
				$LAG3^+$ T cells 17× (1–70×)** *vs.* untreated
				$CD4^+$ CM T cells 100× (30–300×)** *vs.* untreated
				$CD4^+$ naïve T cells 25× (1–60×)** *vs.* untreated
				$CD4^+$ E T cells 5× (1–25×)** *vs.* untreated
				$CD4^+$ EM T cells 50× (25–180×)** *vs.* untreated
				$CD8^+$ CM T cells 25× (1–200×)** *vs.* untreated
				$CD8^+$ naïve T cells 20× (1–120×)** *vs.* untreated
				$CD8^+$ E T cells 25× (1–100×)** *vs.* untreated
				$CD8^+$ EM T cells 30× (1–180×)** *vs.* untreated
Boulton C, et al. 2012[18]	Diameter	Induration >5 mm 8%		
Milgrom H, et al. 2010[48]	Diameter	Induration >5 mm 25%		

CONTINUATION TABLE 3

Author article, year	Local planimetric		Local cellular/molecular	
	Technique	Response size (variability)*	Technique	Response size (variability)*
Spazierer D, et al. 2009[19]	Diameter	Induration 11 mm (2–45 mm)**	Biopsy	Eosinophils 70× (1–140×)** *vs.* placebo
				IgE^+ cells 75× (1–180×)** *vs.* placebo
				IL-1β 2× (1–9×)** *vs.* placebo
				IL-4 9× (5–12×)** *vs.* placebo
				IL-13 19× (8–50×)** *vs.* placebo
				IL-17 10× (3–24×)** *vs.* placebo
				IL-22 15× (1–45×)** *vs.* placebo
				IL-23 p19 4× (1–6×)** *vs.* placebo
				IFN-γ 5× (2–8×)** *vs.* placebo
Moldoveanu Z, et al. 2004[24]	Diameter	Induration 14.5 mm (1–30 mm)		
Smith TP, et al. 2004[20]	Diameter	Induration 10 mm (7.5–12.5 mm)**		
Smith A, et al. 2004[21]	Diameter	Induration 5.6 mm (1.0–10.2 mm)		
Boelens PG, et al. 2004[36]	Diameter	Induration 10 mm (0–50 mm)**		
		Erythema 28 mm (14–65 mm)**		
Lange CG, et al. 2003[26]	Diameter	Induration 30 mm (5–75 mm)**		
Rentenaar RJ, et al. 2002[37]	Diameter	Induration 18 mm (9–42 mm)**		
Valdez H, et al. 2000[50]	Diameter	Induration >5 mm 80%		
Diaz-Sanchez D, et al. 1999[57]			ELISA	IgG 16.5× (5–29.5×)**
			(nasal fluid)	IgG4 2.3× (1–5×)**
				IgA 5× (1–8×)**
				IgE 1× (1–1×)**
				IL-4 1× (−0.3 to 2.3×)**
				IFN-γ 1.1× (0.8–1.8×)**
Waldo FB, et al. 1994[28]	Diameter	Induration 11.9 mm (0–23 mm)		
Husby S, et al. 1995[29]	Diameter	Induration 11.9 mm (0–23 mm)		
Ashorn RG, et al. 1986[42]	Diameter	Induration 17.5 mm (10–25 mm)		
Palestine AG, et al. 1985[43]	Diameter	Induration 15.4 mm (8–20 mm)**		
Brunner CM, et al. 1973[45]	Diameter	Induration 15 mm (single subject)		
Curtis JE, et al. 1972[54]	Diameter	Induration 8.7 mm (0–18 mm)		
Salvaggio J, et al. 1969[55]	Diameter	Induration 6.1 mm (0–20 mm)		
		Erythema 14.7 mm (3–32 mm)		

** Fold change compared to baseline unless stated otherwise. ** Estimated from graphical presentation. LSCI, laser speckle contrast imaging; MI, multispectral imaging; BPP, ball point pen; LDI, laser doppler imaging; PU, perfusion units; SB, suction blister; ELISA, enzyme-linked immunosorbent assay; CD, cluster of differentiation; LAG3, lymphocyte-activation gene 3; CM T cells, central memory T cells; E T cells, effector T cells; EM T cells, effector memory T cells; IL, interleukin; IFN-γ, interferon gamma.*

Subcutaneous KLH injection is the most frequently used administration route (21 out of 45 studies, Table 1 and Figure 2).[13,23,25,27-30,33-35,37,41,44-46,49,51,52,54,56,58] Other frequently used routes of administration include intramuscular and intradermal injections.[1,16-22,24,26,31,32,36,39,40,42-44,46-51,53-56] Intranasal KLH inhalation has also been reported, however, sufficient penetration of KLH through the mucosal tissue likely requires higher (cumulative) KLH doses in order to exert measurable systemic immune responses.[28,55,57] Intradermal KLH administration is the preferential administration route when analyzing the skin response after KLH rechallenge.[1,16,18-21,24,26,28,29,34,36,37,42,43,45,48,50,54-56] The arm is most often used for KLH administration as it is easily accessible and convenient for the (subjective) evaluation of the skin rechallenge.

ASSAYS FOR QUANTIFICATION OF SYSTEMIC HUMORAL RESPONSES FOLLOWING KLH IMMUNIZATION

The systemic humoral response after immunization with KLH was investigated in 36 out of 45 studies. Analysis methods varied from enzyme-linked immunosorbent assay (ELISA) to hemagglutination assay (HA), radioimmunoassay (RIA), and cytometric bead array (CBA) (Table 2). The majority of the studies had used ELISA to quantify KLH-specific antibodies.[1,13,16-22,24-26,28,29,35-41,43,46,48,49,51,52,56,58]

Earlier studies used HA or RIA to identify antibodies. A disadvantage of HA is the difficulty to distinguish between the different types of antibodies, therefore often the total anti-KLH antibody response was measured.[31,33,45,54,55] Birdsall et al. used RIA to quantify the humoral immune response which is a more specific method compared to HA and also based on the binding of antibodies in the sample sera to a known concentration of antigen.[32] Similar to ELISA, RIA also allows for the quantification of the various subtypes of KLH-specific antibodies, however, the antigen is radiolabeled as opposed to an enzyme linked color change in ELISA. The simplicity, practicality, and no need for special equipment or radioactive labels have made ELISA the gold standard for detection and quantification of protein biomarkers.[59,60]

A more recent study performed by Ferbas et al. showed the course of anti-KLH IgG and IgM production by B cells in serum with a CBA method.[47] With CBA, beads with various fluorescence intensities are used and conjugated to human Ig subclasses. Subsequently, the samples are analyzed with a flow cytometer.[61]

The KLH-specific antibody responses were analyzed differently across the studies included in this review. The comparison of optical density (OD) values of experimental sera in precalculated dilutions to negative control and OD values of a positive control included on the same ELISA plate was used in several studies.[1,20,21,25,38,47,48,51] Other studies analyzing the anti-KLH antibody production in sera also prepared standard curves for each studied antibody isotype with established KLH antibody concentrations in mg/L, thereby being able to calculate KLH antibody levels.[13,17,19,22,36,39,43,46,50,57,58] Another method used was to compare the OD values of the sample sera to a reference serum.[41,49,56] This reference serum contained high-antibody titer sera from immunized subjects defined to contain 1,000 arbitrary units.

SYSTEMIC HUMORAL KLH RESPONSE SIZE AND VARIABILITY

Various anti-KLH antibody subtypes (e.g., IgG, IgM, IgA, IgE) were studied (Table 2). Anti-KLH IgG antibodies were measured in all studies characterizing the systemic humoral immune response, of which 8 studies also included the IgG subtypes (IgG1–4).[13,19,36,38,40,49,51,56] Anti-KLH IgM antibodies were analyzed in 20 studies[1,13,18-20,24,25,28,29,32,36,38,39,41,43,45-49,52,55,56,58] and anti-KLH IgA antibodies were analyzed in 10 studies.[28,29,32,36,38,46,48,49,56-58] Some older studies only analyzed the total, non KLH-specific Ig response.[33,45,54] The response sizes as well as the variability of the antibody subtypes varied between studies. This could be attributed to differences in the analytical and statistical methodology and the study setup. However, it is evident that the KLH response size increases with increasing immunization dose and the number of immunizations. All studies tested anti-KLH antibodies at baseline. The antibody titer was consistently lower compared to post dose values. KLH is a neoantigen and as such little to no background signal is expected to occur. However, as no validated reference material is available for the KLH-specific antibody assessment in humans, it is impossible to state with certainty that the baseline anti-KLH antibody titers are undetectable. Given the increases observed in the KLH-specific antibody assays across the studies that used calibration curves or defined study specific reference sera containing a high anti-KLH antibody titer, it is possible to suggest that the KLH-specific antibody titers were at the very least low in baseline samples. 3 weeks after immunization was the most frequently used interval for antibody assessment, ranging from 1 to 8 weeks with some also analyzing antibodies after 1 or 5 years.[41,46,51]

It is difficult to say which anti-KLH antibody shows the strongest response as comparison between studies is complex due to variations in the immunization dose, the interval between immunization and sampling, and the differences in endpoints and analytical methods between studies. Overall, the maximum anti-KLH IgG response increases to a greater extent from baseline values than the IgM response. We previously showed that the anti-KLH IgG response is stronger than the IgM response after one intramuscular immunization with KLH.[1] Anti-KLH IgG increased 4.9-fold (range 1.3–9.3-fold) compared to a 1.4-fold (range 1–2.4-fold) increase in IgM. Both antibody titers started to increase from 7 to 14 d after immunization and remained constant until day 28. Similarly, Smith et al. showed a 5-fold (range 4.7–5.3-fold) increase in

anti-KLH IgG titers compared to a 1.7-fold (range 1.6–1.8-fold) increase in anti-KLH IgM titers.[20] These changes were observed 3 weeks post KLH immunization. Both studies used a 100 µg subunit KLH formulation with alum as adjuvant for the intramuscular immunization. Miller et al. used 3 different formulations of KLH.[13] Both HMW KLH and subunit KLH with Montanide ISA-51 as adjuvant showed a stronger increase in antigen specific IgG compared to IgM. HMW KLH induced an increase of 37.6-fold in IgG1 (range 11.8–87-fold), 6-fold in IgG2 (range 1.3–10.7-fold) and only 2.9-fold in IgM (range 0.1–5.6-fold). Subunit KLH with Montanide ISA-51 induced comparable responses with an increase of 67.4-fold in IgG1 (range 8.5–126.3-fold), 7.4-fold IgG2 (range 2.4–12.5-fold) and 5.9-fold IgM (range 0.8–11-fold).

IgE does not seem to be produced after immunization with KLH. A study performed by Schuyler et al. did not detect anti-KLH IgE antibody levels after immunization with KLH.[38] The anti-KLH antibody response was analyzed between atopic asthmatics and non-atopic asthmatics after KLH immunization by instillation into a subsegment of the lingula of the left lung. Anti-KLH IgG1, IgG4, IgA1 and IgM antibodies were detected in serum. The levels of IgG1 (38 IU/mL), IgM (280 IU/mL) and IgA1 (25 IU/mL) peaked after 12 d and decreased thereafter for IgM (200 IU/mL) and IgA1 (18 IU/mL) displaying a difference in the peak time for each (sub)type of anti-KLH antibody as the IgM and IgA antibody response increased early followed by the IgG antibody response. Overall, the response size increased 175-fold for anti-KLH IgM (range 125–225-fold), 35-fold for anti-KLH IgG1 (range 21–49-fold), 1.5-fold for anti-KLH IgG4 (range 1.3–1.7-fold), and 35-fold for anti-KLH IgA (range 23–47-fold). A study by Ward et al. also demonstrated differences in the peak times of various anti-KLH antibodies.[41] Peak anti-KLH IgM responses (8.5-fold increase) were observed 7 d after immunization and peak anti-KLH IgG responses (76-fold increase) 21 d after immunization. Spazierer et al. showed that the anti-KLH IgM antibody reaction was higher at 2–4 weeks after immunization, plateauing in the 2 weeks after that, whereas IgG1 continued to increase until day 57.[19] IgM increased 24-fold (range 8–38-fold), IgG1 increased 10,000-fold (range 4,000–19,000-fold) and IgG4 increased 40-fold (range 10–110-fold) compared to baseline.

Boelens et al. found a difference in the anti-KLH IgG titers between the IgG subtypes.[36] An increase of 12-fold in total IgG (range 3–16-fold), 1.6-fold in IgG1 (range 1.1–1.9-fold), 5-fold in IgG2 (range 3–11-fold), and 3-fold in IgG3 subtypes (range 1.5–33-fold) was reported compared to baseline. No IgG4 anti-KLH antibodies were detected. This increase of IgG1, IgG2 and IgG3 anti-KLH antibodies and no change in the anti-KLH IgG4 antibodies was also described by Bird et al.[51] Total anti-KLH IgG showed a 23-fold increase in serum antibody (range 15–40-fold), of which an increase of 58-fold (range 30–120-fold) in IgG1, 4-fold (range 1.8–12.5-fold) in IgG2, 3-fold (range 1.5–3-fold) in IgG3 and undetectable IgG4 was observed. After a secondary immunization a year later, the anti-KLH IgG4 antibody response showed an increase of 78-fold (range 40–100-fold). The rise in anti-KLH IgG4 titers after the secondary immunization could be attributed to an increase in T helper cells after a secondary immunization with KLH. This could lead to the class switching of B cells and possibly the proliferation of more IgG4-producing plasma cells. Potentially, these IgG4-producing plasma cells were only able to mature during the secondary response as the primary anti-KLH IgG4 antibody response might have been insufficient to stimulate B cell differentiation.[51]

ASSAYS FOR QUANTIFICATION OF SYSTEMIC CELLULAR AND MOLECULAR RESPONSES FOLLOWING KLH IMMUNIZATION

A total of 26 studies characterized aspects of the systemic cell-mediated immunity following KLH immunization, using *ex vivo* restimulation of immune cells isolated from KLH-immunized volunteers (Table 2).[13,19,21,23-27,29,30,33,36,37,39,42-47,49,50,53,54,56,58] These studies used conventional *in vitro* lymphocyte proliferation assays, where PBMCs were incubated with KLH to induce the proliferation of T cells and the release of cytokines. The incubation time with KLH varied from 4 to 8 d. 3 different assays were used, a thymidine incorporation assay (TIA, 19 studies),[13,19,21,24-28,30,33,36,37,39,42-45,50,54] an enzyme-linked immunosorbent spot assay (ELISpot, 6 studies),[13,23,29,46,47,58] and flow cytometry (FC, 2 studies).[49,56] Although TIA is the most widely used assay to measure T cell proliferation after KLH rechallenge, a main disadvantage is the use of radioactive labels, therefore more modern techniques are now used.[62] ELISpot is often used for the detection of cytokine secreting cells, however, it can also be used for the determination of antibody secreting cells.[63] FC is currently widely used for rapid specific protein characterization analyses and phosphorylation states of individual cells.[62]

Spazierer et al. used the multiplex Luminex method for the quantification of cytokines secreted in cell culture.[19,64] They found an increase in IL-5 secretion (15-fold, range 1–80-fold), IL-10 secretion (12-fold, range 1–20-fold), IL-13 secretion (120-fold, range 10–500-fold), and IFN-γ secretion (15-fold, range 1–30-fold). Importantly, most cytokines are released by Type 2 T helper (Th2) cells.

An older technique used to quantify the systemic cellular response is the leukocyte adherence inhibition assay (LAI).[65] Powell et al. reported a LAI response of 35-fold (range 26–45-fold) in subjects immunized with KLH compared to baseline indicative of cell-mediated immunity.[53]

SYSTEMIC CELLULAR AND MOLECULAR KLH RESPONSE SIZES AND VARIABILITY

The proliferative responses of PBMCs after KLH immunization were all increased by 1.5- to 26-fold from baseline (Table 2). The *ex vivo* sample workup plays a role in the variability observed between studies. Factors such as the PBMC or T cell isolation, the incubation time, and the *ex vivo* KLH stimulation protocol were expectedly variable between studies. However, within single studies there was also a rather moderate to large variability of the proliferative responses. Spazierer et al. observed a mean proliferation response of 4-fold with limited variability (min–max 3.3–4.7-fold),[19] whereas Lange et al. observed a stronger proliferation response size of 25-fold with a substantially higher variability (min–max 16–80-fold).[66]

KLH-driven B cell responses by ELISpot were evaluated in multiple studies. Giesecke et al. and Ferbas et al. showed a 44-fold (variability unclear) increase in plasmablasts and a 1,250-fold (range 1–2,600-fold) increase in B cells, respectively.[46,47] Several studies characterized the B cell response by analyzing the antibody type produced by the cells (IgG, IgM, IgA).[23,29,58] KLH responses were detected, but there was no consistency between the studies in which antibody producing cell type was the most or least increased.

Kapp et al. analyzed the cytokine production by $CD4^+CD154^+$ T cells.[49] Immunization with KLH resulted in the induction of a T cell subset secreting IL-2 (30-fold increase, range 10–50-fold), IL-4 (10-fold increase, range 4–16-fold), IL-10 (2-fold increase, range 1–10-fold), IL-17 (3-fold increase, range 1–7-fold), TNF (30-fold increase, range 5–50-fold), and IFN-γ (21-fold increase, range 5–45-fold) compared to baseline. The induction of T cells secreting IL-2, IL-4, IL-10, TNF and IFN-γ after KLH immunization was reported by Hostmann et al,[56] reporting T cell responses for IL-2 of 140-fold (range 90–170-fold), IL-4 of 36-fold (range 22–40-fold), IL-10 of 12-fold (range 5–17-fold), TNF of 7-fold (range 5–10-fold), and IFN-γ of 20-fold (range 10–30-fold).

Miller et al. analyzed IFN-γ release by ELISpot and showed an increase of 10-fold (range 5–20-fold) compared to baseline indicating activation of the adaptive immune system.[13]

The systemic cellular responses upon KLH immunization seem to be heavily dependent on the number of immunizations and, to a lesser extent, on the immunization dose. All but one study that had used ELISpot, FC, and/or Multiplex analyses had immunized subjects with KLH at least twice.[19,23,29,46,47,49,56,58] Miller et al. immunized subjects with KLH only once before ELISpot analysis of IFN-γ release, however, the 1,000 μg KLH dose was rather high when compared to the most frequently used dose of 100 μg.[13] Notably, the observed IFN-γ release was lower compared to the results reported by Spazierer et al. (10-fold increase *vs.* 15-fold increase) where subjects were immunized with 100 μg KLH 3 times (cumulative dose of 300 μg).[19] Though, it should be noted that the IFN-γ analysis method differed between the studies (ELISpot *vs.* Multiplex).

Although a little less evident, similar findings can be observed for TIA across studies supporting the hypothesis that multiple KLH immunizations are more effective for increased systemic cellular responses compared to higher doses of KLH. Several studies that immunized subjects with 100 μg KLH twice showed larger increases in TIA responses (7.7–25-fold increase)[25-27,29] compared to Kondratenko et al. where subjects were immunized with 200 μg KLH only once (6.1-fold increase).[39] Other studies used even higher single KLH immunization doses (500 μg up to 5,000 μg), however, the TIA responses were nevertheless lower overall (2.5–7.5-fold increase).[36,37,42,43]

TECHNIQUES FOR EVALUATION OF SKIN RESPONSES FOLLOWING INTRADERMAL KLH ADMINISTRATION

The antigen-specific cell-mediated immunity can be studied locally by challenging the skin with intradermal KLH, after an initial immunization. This T cell-driven inflammatory response usually takes more than 12 hours to develop, and the maximal response time usually occurs between 24 and 72 hours. The effects induced by the intradermal KLH rechallenge are likely to be driven by a mixed reaction of innate immune responses,[19] T cell-driven delayed-type hypersensitivity (DTH),[1,16,19,34] and partially Th2 cell-type late-phase skin response effects.[19]

A total of 20 studies evaluated a skin rechallenge to investigate the local cell-mediated immune response (Table 3).[1,18-21,24,26,28,29,34,36,42,43,45,48,50,54,55] The studies varied in the initial KLH immunization dose/regimen, as well as the rechallenge timing and KLH dose (Table 1). KLH skin rechallenge doses ranging from 1 to 1,000 μg have been reported, with 10 μg being most frequently used for the skin rechallenge. Most studies evaluated the KLH skin responses induced by an intradermal injection 2–3 weeks post initial immunization. Subsequently, the response was evaluated at 24–72 hours post challenge, commonly at 48 hours. The skin response was predominantly evaluated by (subjective) planimetric scoring and measurement of induration (18 studies) and/or erythema (3 studies) with either a ruler or ballpoint pen technique (Table 3).[67] A positive response was sometimes scored categorically with a positive reaction defined as induration with a mean diameter of greater than 5 mm.[18,48,50]

The method of measuring diameter index of the skin rechallenge response and the ballpoint technique or ruler technique[67] both suffer from a lot of inter-rater

variability.[1,68] An objective, non-invasive method for evaluation of induration and erythema would be favored. A few studies used objective methods to quantify the skin rechallenge response, such as laser speckle contrast imaging (LSCI), laser doppler imaging (LDI), multispectral imaging (MI), colorimetry and erythema index calculated from photographs (Table 3).[1,16,34] LSCI and LDI both measure the cutaneous blood flow by using different laser techniques.[69] MI captures images of a defined location without exposure to ambient light and illuminates this region with multidirectional light.[70] MI-based endpoints can include wrinkles, erythema, elevations, and depressions assessments. Colorimetry captures reflected light from the skin and measures the light intensity, usually utilizing the CIELab color space coding system.[71]

LOCAL PLANIMETRIC KLH RESPONSE SIZES AND VARIABILITY

Induration was observed following the intradermal KLH rechallenge with a mean diameter of 5.6–51.8 mm across studies (Table 3). Importantly, higher intradermal rechallenge KLH doses lead to larger induration reactions. Smith et al. observed a mean induration response of 5.6 mm (range 1.0–10.2 mm) in subjects immunized with 100 µg KLH and rechallenged with 1 µg intradermally[21] whereas Belson et al. showed a mean induration response of 51.8 mm (range 35.8–75.1 mm) in subjects immunized with 5,000 µg KLH and rechallenged with 100 µg intradermally.[34] Furthermore, the erythema response after skin rechallenge was always larger compared to the induration response.[34,36,55] This finding is consistent with literature, however, as induration is a widely accepted measure of skin rechallenge response it is advantageous to at least use the induration index as outcome when assessing the skin responses following an intradermal KLH rechallenge.[72]

Several studies objectively scored the KLH skin rechallenge response by imaging techniques (Table 3). We used LSCI, MI, colorimetry and photography to score cutaneous blood perfusion and erythema in 2 separate studies following an intradermal KLH rechallenge.[1,16] Interestingly, in the initial study we were unable to detect a positive skin rechallenge response using subjective evaluation, since we used a single KLH immunization and a low KLH rechallenge dose.[1] However, due to the increased sensitivity of the applied imaging techniques, significant KLH-dependent changes in cutaneous blood flow (1.4-fold increase, range 1.0–1.9-fold) and erythema (1.4-fold increase, range 1.2–1.9-fold) were detected compared to placebo-treated subjects. Belson et al. used LDI to evaluate the skin rechallenge response.[34] They demonstrated that LDI measurements showed increased inter-subject variability in the area of flare, compared to the results of induration diameter and erythema. The LDI measurements of the area of flare were 29.5 cm^2 (range 20.6–42.3 cm^2) after 48 hours and 2.2 cm^2 (range 0.7–9.7 cm^2) after 120 hours. These imaging devices could become important measurement instruments for objectively studying the skin reactions in future clinical trials. Notably, 2 out of 3 studies that performed categorical scoring of induration (>5 mm) after the intradermal KLH rechallenge had poor responder rates (8% and 25%).[18,48] The third study had a responder rate of 80%, however, the intradermal KLH dose of 1,000 µg was rather high compared to the most frequently used dose of 10 µg. Considering both the high non-responder rate and increased inter-rater variability when scoring the skin response subjectively, imaging techniques are preferred for analysis of the skin reactions as they provide more sensitive, objective quantification of the skin reactions.

EVALUATION OF LOCAL CELLULAR AND MOLECULAR RESPONSES FOLLOWING KLH ADMINISTRATION

The local cellular and molecular responses following a local KLH rechallenge have been rarely studied: only 3 out of 45 studies evaluated these responses (Table 3).[19,34,57] The challenged skin can be harvested by performing skin (punch) biopsies, the subsequent sample can be subjected to a multitude of analyses such as immunohistochemistry, immunofluorescence, qPCR and more. Another method is to assess the local cellular and molecular skin response by inducing suction blisters. The suction blister exudate can be aspirated and analyzed for the presence of immune cells by FC and cytokine concentrations by ELISA.[34]

Diaz-Sanchez et al. analyzed the local molecular immune response in nasal fluid samples after intranasal KLH immunizations.[57] They did not find any significant KLH-induced changes in IL-4 and IFN-γ concentrations in nasosorption samples. Moreover, the increase in the systemic humoral response was small which could possibly indicate that aerosol immunization induces a weaker immune response compared to an intramuscular, subcutaneous, or intradermal immunization with KLH.

LOCAL CELLULAR AND MOLECULAR KLH RESPONSE SIZES AND VARIABILITY

Belson et al. evaluated the local KLH responses in both skin punch biopsies and skin suction blisters.[34] The skin biopsies were examined by single chromogenic immunohistochemical staining, to quantify the number and activation of T cells. Following the KLH rechallenge, $CD3^+$ and $LAG3^+$ cells were detected in the biopsies at larger numbers compared to control skin (treated with PBS) or unchallenged skin. There was a 16-fold (range 14–19-fold) increase in $CD3^+$ cells and 20-fold (range 12–39-fold) more $LAG3^+$ T cells compared to the control skin after 48 hours. In parallel, the suction blisters were induced at the site of the KLH skin rechallenge for the harvesting

of immune cells. Multi-color FC showed a 60-fold (range 15–130-fold) increase in leucocyte numbers in KLH treated compared to untreated skin. The cells within the suction blister exudate were dominated by lymphocytes (mean lymphocyte percentage of 72.6%). Additionally, FC showed that the lymphocytes in the blister fluid were predominantly $CD4^+$ T helper cells, of which 42% had a central memory phenotype and 44% had an effector memory phenotype (indicated by $CD4^+CCR7^+CD45RA^-$). A shift in the absolute mean cell numbers from central memory $CD4^+$ T cells towards the effector memory $CD4^+$ T cells was observed between 48 hours and 120 hours after the skin rechallenge.

Spazierer et al. examined the local cellular and molecular immune response by skin biopsies.[19] Eosinophils and IgE positive cells were analyzed in skin biopsies with immunohistochemical staining. They showed a 70-fold (range 1–140-fold) increase in eosinophils and a 75-fold (range 1–180-fold) increase in IgE positive cells compared to placebo. The eosinophilic and IgE cell positive infiltrate in KLH rechallenged skin is indicative of a local Th2 response.[73-76] Furthermore, they also observed increased cytokines in the rechallenged skin compared to placebo, including a 2-fold IL-1β increase (range 1–9-fold), 9-fold IL-4 increase (range 5–12-fold), 19-fold IL-13 increase (range 8–50-fold), 10-fold IL-17 increase (range 3–24-fold), 15-fold IL-22 increase (range 1–45-fold), 4-fold IL-23 P19 increase (range 1–6-fold), and 5-fold IFN-γ increase (range 2–8-fold). The high local levels of IL-4 and IL-13 suggest a Th2 response driven largely by a late-phase skin reaction rather than a DTH response demonstrated by the reaction peak observed at 24 hours post intradermal rechallenge whereas a DTH reaction peak is expected 48–72 hours after induction.[77,78] Based on the low IL-33 levels, a known Th2 response promoter, it seems unlikely that the Th2 response was induced by this cytokine. Moreover, the importance of increased IL-17 and IL-22 levels compared to placebo remain to be elucidated as their role in the KLH-induced late-phase skin reactions are unknown.

EFFECT OF (PHARMACOLOGICAL) INTERVENTIONS AND DISEASE ON KLH RESPONSES

KLH challenges have been used extensively to study the influences of environmental, psychological, and physical factors as well as the effect of diseases and (immunomodulatory) drugs on the adaptive immune system. A total of 43 studies were identified in which the KLH challenges were utilized in intervention studies and/or patient populations (Table 4). Out of these studies, 26 focused primarily on the effect of interventions on the KLH challenge model.[16-18,20-22,24,25,27-29,30,35,38,43,49,56,57,79-86] The remaining 17 studies evaluated the KLH responses in various patient populations.[13,19,26,31,33,36,37,39,40,47,50,55,58,87-90]

TABLE 4 Effect of (pharmacological) interventions and disease on KLH response.

Author article, year	Intervention / Patient population	Outcome
Saghari M, et al. 2022[16]	Intervention: Amlitelimab in HVs	↓ humoral response, ↓ skin rechallenge response
Yang J, et al. 2021[35]	Intervention: Acazicolcept in HVs	↓ humoral response
Otterhaug T, et al. 2021[22]	Intervention: Fimaporfin + light in HVs	↑ humoral response
Swaminathan A, et al. 2019[79]	Intervention: Solar UVR in HVs	= humoral response, ↓ skin rechallenge response, ↑ Th17/$CD4^+$ T cell ratio
Poirier N, et al. 2016[17]	Intervention: VEL-101 in HVs	↓ humoral response
Hostmann A, et al. 2015[56]	Intervention: Oral KLH feeding in HVs	Primed/Immunized subjects: = humoral response, = skin rechallenge response, ↓ $CD4^+$ T cell, IL-2, IL-17, CLA, IFN-γ, ↑ $CD4^+$ T cell, IL-10
Kaufman M, et al. 2014[80]	Intervention: Natalizumab in RRMS	= humoral response (compared to no treatment)
Gallegos A, et al. 2013[81]	Intervention: MBSR in HVs	↓ humoral response (compared to HVs)
Ferbas J, et al. 2013[47]	Patient population: SLE *vs.* HVs	Predominance of IgG2 followed by IgG1 after 2nd immunization (compared to predominance of IgG1 in HVs), = B cell ELISpot response
Boulton C, et al. 2012[18]	Intervention: Fingolimod in HVs	↓ humoral response (compared to no treatment)
Kapp K, et al. 2010[49]	Intervention: Oral KLH feeding in HVs	Non primed/immunized subjects: faster and ↑ humoral response, = skin rechallenge response, ↑ $CD4^+$ T cells, ↓ $CD4^+CLA^+$ T cells, faster ↑ in $CD4^+$ T cells (including IL-2, IL-4, IFN-γ, TNF, and integrin β7 producing cells) and proliferated $CD4^+$ T cells after immunization Primed/Immunized subjects: ↓ $CD4^+$ T cell IL-2, IFN-γ, and TNF and ↑ $CD4^+$ T cell IL-4 and IL-10, ↓ $CD4^+CLA^+$ T cells
Bingham C, et al. 2010[82]	Intervention: Rituximab + MTX in RA	↓ humoral response (compared to only MTX)
Weide B, et al. 2009[83]	Intervention: mRNA immunotherapy in metastatic melanoma	↓ $FOXP3^+CD4^+$ regulatory T cells
Spazierer D, et al. 2009[19]	Patient population: Allergic rhinitis *vs.* HVs	↑ immediate flare skin rechallenge response, mild ↑ IL-17 and IL-22 in biopsies of challenged skin (compared to strong ↑ in HVs)
Grant R, et al. 2008[87]	Patient population: Physical exercise *vs.* stretching in sedentary older adults	↑ humoral response (compared to stretching)
Miller J, et al. 2005[13]	Patient population: HCT or cancer	↓ humoral response, ↓ $CD4^+$ T cells
Moldoveanu Z, et al. 2004[24]	Intervention: Oral KLH feeding in HVs	Immunized subjects: = humoral response, = skin rechallenge response, = T cell proliferation, IL-2, IL-4, IL-10, IFN-γ, and TGF-β responses
Smith A, et al. 2005[84]	Intervention: HLA genotype + distress in HVs	↓ skin rechallenge response in HLA-$DQ2^+$ genotype, ↑ skin rechallenge response in HLA-$DQ5^+$ genotype
Smith A, et al. 2004[85]	Intervention: Alcohol + distress in HVs	↓ skin rechallenge response (when distressed during KLH immunization), ↓ skin rechallenge response (when alcohol use during skin rechallenge induction)
Smith TP, et al. 2004[20]	Intervention: Age + physical activity in HVs	↓ humoral response in older sedentary men, ↓ skin rechallenge response in older sedentary men
Kraus T, et al. 2004[25]	Intervention: Oral KLH feeding in IBD *vs.* HVs	Non primed/immunized subjects: ↓ T cell proliferation in HVs, ↑ T cell proliferation in IBD, faster humoral response in IBD
Boelens P, et al. 2004[36]	Patient population: Trauma *vs.* HVs	= humoral response, ↓ skin rechallenge response, ↓ PBMC proliferation
Smith A, et al. 2004[21]	Intervention: Distress in HVs	= humoral response, ↓ skin rechallenge response when distressed during KLH immunization, = lymphocyte proliferation

CONTINUATION TABLE 4

Author article, year	Intervention / Patient population	Outcome
Lange C, et al. 2003[26]	Patient population: HIV *vs.* HVs	↓ humoral response, ↓ skin rechallenge response, ↓ lymphocyte proliferation
Rentenaar R, et al. 2002[37]	Patient population: Immunosuppression in renal transplant *vs.* HVs	↓ humoral response in prednisone + cyclosporine A + mycophenolate mofetil compared to other groups, ↓ skin rechallenge response (compared to HVs), = lymphocyte proliferation
Valdez H, et al. 2000[50]	Patient population: HIV *vs.* HVs	↓ skin rechallenge response, ↓ lymphocyte proliferation
Diaz-Sanchez D, et al. 1999[57]	Intervention: DEPs + intranasal KLH in atopics	↑ mucosal humoral response including anti-KLH IgE, ↑ mucosal IL-4, = nasal IFN-γ (compared to no DEPs)
Kantele A, et al. 1999[27]	Intervention: Oral KLH feeding in HVs	↑ α4β7 T cells after oral KLH feeding (compared to no feeding), difference disappears after subsequent subcutaneous KLH administration
Abrams J, et al. 1999[86]	Intervention: Abatacept in psoriasis vulgaris	↓ humoral response (compared to no treatment)
Schuyler M, et al. 1997[38]	Intervention: Intrapulmonary KLH in atopics *vs.* HVs	= humoral response (compared to non-atopics)
Kondratenko I, et al. 1997[39]	Patient population: CVID *vs.* HVs	↓ humoral response, ↓ T cell proliferation
de Fijter JW, et al. 1996[58]	Patient population: IgAN *vs.* HVs	= humoral response
Wishahi M, et al. 1995[88]	Patient population: Cystic TCC	↓ cystic TCC recurrence rate after KLH immunization and KLH instillations treatment into bladder
Waldo F, et al. 1994[28]	Intervention: Intranasal KLH in HVs	↓ humoral response, ↓ skin rechallenge response after subsequent KLH immunization (compared to no intranasal KLH)
Husby S, et al. 1994[29]	Intervention: Oral KLH feeding in HVs	↑ humoral response, ↓ skin rechallenge response, ↓ T cell proliferation after subsequent KLH immunization (compared to no oral KLH feeding)
Snyder B, et al. 1993[30]	Intervention: Stress in HVs	↓ lymphocyte proliferation (compared to 'good' stress)
Falconer A, et al. 1992[40]	Patient population: Atopics *vs.* HVs	↑ humoral anti-KLH IgG4 response, ↓ humoral anti-KLH IgG1 response (compared to HVs)
Sidell N, et al. 1990[89]	Patient population: Isotretinoin in cystic acne	↑ humoral response (compared to no treatment)
Ochs H, et al. 1988[31]	Patient population: HIV and PGL *vs.* HVs	↓ humoral response (compared to HVs)
Palestine A, et al. 1985[43]	Intervention: Cyclosporine in uveitis	= humoral response, ↓ skin rechallenge response, = lymphocyte proliferation (compared to no treatment)
Berd D, et al. 1984[90]	Patient population: Cyclophosphamide in cancer	↑ humoral response, ↑ skin rechallenge response (compared to no treatment)
Paty J, et al. 1975[33]	Patient population: SLE *vs.* HVs	↓ humoral response, ↓ lymphocyte proliferation (compared to HVs)
Salvaggio C, et al. 1969[55]	Patient population: Atopics *vs.* HVs	= humoral response, ↑ skin rechallenge response (compared to HVs)

HVs, healthy volunteers; UVR, ultraviolet radiation; Th, T helper; CD, cluster of differentiation; KLH, keyhole limpet hemocyanin; IL, interleukin; CLA, cutaneous lymphocyte antigen; IFN-γ, interferon gamma; RRMS, relapsing remitting multiple sclerosis; MBSR, mindfulness based stress reduction; SLE, systemic lupus erythematosus; Ig, immunoglobulin; TNF, tumor necrosis factor; MTX, methotrexate; RA, rheumatoid arthritis; mRNA, messenger ribonucleic acid; HCT, hematopoietic cell transplantation; TGF, transforming growth factor; HLA, human leukocyte antigen; IBD, inflammatory bowel disease; PBMC, peripheral blood mononuclear cell; HIV, human immunodeficiency virus; DEP, diesel exhaust particle; CVID, common variable immunodeficiency; IgAN, IgA nephropathy; TCC, transitional cell carcinoma; PGL, persistent generalized lymphadenopathy.

Several clinical trials investigated whether oral KLH feeding would affect subsequent immunization and skin rechallenge response outcomes (Table 4).[24,25,27,29,49,56] Immune tolerance after oral KLH administration was inconsistent across studies: some showed systemic T cell tolerance development after oral KLH administration[29,49,56] whereas others did not.[24,25] Kapp et al. included both orally primed and non-primed healthy volunteers that were subsequently immunized with KLH.[49] Oral KLH priming induced immune tolerance (decreased $CD4^+$ T cell IL-2, IFN-γ, and TNF cytokine secretion, increased $CD4^+$ T cell IL-4 and IL-10 secretion, and decreased $CD4^+CLA^+$ T cells). The KLH-specific systemic $CD4^+$ T cell response shifted from a Th type 1 toward a Th type 2 cytokine pattern and the B cell response was amplified after immunization. Their findings are largely consistent with Hostmann et al. showing a decreased pro-inflammatory phenotype in KLH-specific $CD4^+$ T cells (decreased $CD4^+$ T cell IL-2, IL-17, and IFN-γ cytokine secretion, and $CD4^+CLA^+$ T cells, increased $CD4^+$ T cell IL-10 cytokine secretion, skin rechallenge and humoral response unaltered).[56] The differences observed between the oral KLH studies may be attributed to KLH doses used for oral and parenteral administration.[49] Studies where higher oral and lower parenteral doses of KLH were used displayed decreased skin rechallenge responses and reduced PBMC proliferation,[29] possibly confirming an oral KLH dose-dependent effect. Low doses of oral KLH induced systemic T cell responses and modulated the systemic immune responses induced by parenteral KLH.[49,56]

The immune response as evoked by the KLH challenge diminishes with increased age,[20,87] decreased physical activity,[20,87] increased alcohol consumption,[85] and increased stress (Table 4).[21,81,84,85] Physically fit older adults have increased humoral and skin rechallenge responses after KLH challenge compared to sedentary older adults,[20] but interestingly, the humoral response can be restored in previously sedentary older adults when physical exercise is introduced compared to stretching exercises.[87] Distress during KLH immunization impairs the skin rechallenge response to KLH, but not the humoral or lymphocyte proliferation response[21,85] whereas alcohol consumption during the intradermal KLH skin rechallenge decreases the skin rechallenge response[85] hinting toward different mechanisms and targets for stress and alcohol consumption to alter the KLH challenge response. Furthermore, Smith et al. concluded that a distress phenotype together with $HLA\text{-}DQ2^+$ or $HLA\text{-}DQ5^+$ genotype possibly contributes to the skin rechallenge response as they found a decreased skin rechallenge response in subjects with $HLA\text{-}DQ2^+$ genotype and an increased skin rechallenge response in subjects with $HLA\text{-}DQ5^+$ genotype.[84]

KLH challenge responses have been evaluated in various patient populations, compared to healthy volunteers. Patients with atopic characteristics tend to have increased responses following KLH immunization and subsequent intradermal KLH skin

rechallenge.[19,40,55] The humoral response is overall not upregulated. Falconer et al. observed increased anti-KLH IgG4 and decreased anti-KLH IgG1 compared to healthy volunteers[40], however, Spazierer et al. was not able to find this discrepancy in the IgG1 and IgG4 subclasses between healthy controls and atopic patients.[19] Patients with systemic lupus erythematosus (SLE), human immunodeficiency virus (HIV), and common variable immunodeficiency disorder (CVID) all have a decreased humoral and cell-mediated response following KLH challenge.[26,31,33,39,47,50] This is explained by the immunodeficiencies (polyclonal B cell activation with a shift toward immature B cells in SLE, decrease in $CD4^+$ T cells in HIV, and decreases in antibody levels in CVID) in all these patient populations.

A couple of investigational medicinal products and registered drugs have been evaluated for their modulatory effect on the KLH-driven immune responses (Table 4).[16-18,22,35,43,80,82,83,86,89,90] Palestine et al. showed that cyclosporine administration in uveitis patients suppressed the KLH skin rechallenge response, but did not alter the humoral and lymphocyte proliferation response.[43] Weide et al. investigated whether mRNA immunotherapy therapy consisting of Melan-A, Tyrosinase, gp100, Mage-A1, Mage-A3, and Survivin would have an effect on the KLH challenge in metastatic melanoma patients.[83] They showed a decrease in $FOXP3^+CD4^+$ regulatory T cells indicating that the mRNA mixture was able to inhibit the regulatory T cell signals induced by KLH immunization. Boulton et al. administered fingolimod, a S1PR modulator present on lymphocytes, in healthy volunteers and observed a decreased humoral response following KLH immunization.[18] Otterhaug et al. performed an innovative study in which they gave fimaporfin, a synthetic light-activated compound that localizes to endosomes and lysosomes and induces endosomal content release into the cell cytosol after activation, intradermally to healthy volunteers and exposed them to a light source thereafter.[22] Interestingly, subjects treated with fimaporfin exhibited an increased humoral response to KLH immunization. The increase of antibody production may possibly be explained by an enhancement of $CD4^+$ T cell responses, potentially stimulating antibody production by plasma cells.[91] Several other studies used targeted therapies in combination with a KLH challenge to evaluate the immune response. These compounds included monoclonal antibodies against CD28 (VEL-101),[17] and CD20 (rituximab),[82] and chimeric proteins against CD28 and ICOS (acazicolcept)[35] and CD80 and CD86 (abatacept).[86] We demonstrated that a novel monoclonal antibody targeted against CD134/OX40 ligand (amlitelimab) was able to suppress the immune response following a KLH challenge, both on a systemic as well as a local level.[16] Subsequently, this compound also proved effective in inadequately controlled moderate-to-severe atopic dermatitis patients[92] and was sold to Sanofi S.A. for $1.5 billion[93] underlining the potential predictive value of the KLH challenge in healthy volunteers for future clinical efficacy.

DISCUSSION

KLH has been shown to be an effective immunostimulatory antigen for human clinical studies. Swaminathan et al. has previously set up a framework including the various formulations, doses and routes of administration.[2] The aim of this systematic review was to expand this framework and to characterize the local and systemic immune response of the immunization with KLH, and to define the optimal biomarkers for KLH immunization readout based on the response size and variability. Furthermore, we have also summarized the effect of a multitude of interventions and diseases on the *in vivo* KLH challenge in humans.

KLH immunization drives a robust systemic humoral response. All studies included in this systematic review report an anti-KLH IgG response, whereas the anti-KLH IgM and IgA were also increased after KLH immunization. The maximal systemic humoral response is reached approximately 3 weeks after KLH immunization. Anti-KLH IgM antibody levels rise first, anti-KLH IgG antibody levels rise thereafter and in time overtake the anti-KLH IgM response. The systemic cellular response is commonly examined with lymphocyte proliferation assays to assess the T cell responses and cytokine production. FC and ELISpot assays are used more frequently over the past years and give more specific and accurate results. The *ex vivo* proliferation of KLH-specific T cells, most commonly $CD4^+$ cells, and cytokine production (IFN-γ, IL-10 and IL-4) have been studied most often. Importantly, the number of KLH immunizations and, less evidently, the immunization dose profoundly influences the systemic cellular responses. Taken together, we recommend implementing KLH-specific antibody assessments using ELISA when performing a KLH challenge in clinical trials. This humoral assay should be performed on samples collected at least before and 3 weeks after KLH immunization. When analyzing systemic cellular and molecular immune responses we advise multiple KLH immunizations and the preferential use of novel techniques such as FC and ELISpot over TIA. As too few studies have performed KLH-specific systemic immune response analyses it is hard to make suggestions as to which immune cell types and specific cytokines should be analyzed.

The local skin response upon the KLH rechallenge was quantified mostly subjectively, by measuring the induration diameter. Although subjective evaluation of induration diameter index of the skin rechallenge response is widely used throughout literature, this technique suffers from a lot of inter-rater variability.[1,68] When studying the skin rechallenge response with objective imaging techniques, the local KLH response can be quantified more accurately and with higher sensitivity. Although the local cellular and molecular immune responses upon KLH rechallenge have not been investigated in many studies, this approach has provided valuable mechanistic

insight into the local KLH response. Local activation of T cells after KLH cutaneous rechallenge was observed by the increased presence of $CD3^+$ and $LAG3^+$ T cells.[34] Furthermore, increased eosinophils and IgE positive cells in KLH rechallenged skin indicate a local Th2 response.[19] This Th2 response is likely driven by a late-phase skin reaction as high local levels of IL-4 and IL-13 were detected and the reaction peak was observed at 24 hours after rechallenge, whereas a DTH reaction peak is usually observed after 48–72 hours. Between 48 and 120 hours after the skin rechallenge, a shift from central memory towards effector memory $CD4^+$ T cells was observed.[34] Based on the literature discussed in this review, we recommend performing both a subjective evaluation of the dermal KLH rechallenge (current gold standard) as well as objective measurement of the local inflammation by using imaging techniques. Evaluations should be performed at least before, 24 hours and 48 hours after dermal KLH rechallenge. If possible, the addition of multiple time windows after rechallenge could prove valuable, including 2 and 6 hours (innate response) and possibly 72 and 120 hours (late DTH response) after intracutaneous KLH administration. For the local cellular and molecular assays after KLH dermal rechallenge we also advocate the use of multiple KLH immunizations in order to increase the systemic KLH-specific immune cell pool. Too few studies have analyzed local cellular and molecular immune responses in order to make recommendations on which cell types and cytokines to analyze.

Lastly, we have summarized the effect of environmental, psychological, and physical factors and (investigational) drugs on the KLH response as well as the impact of disease. Oral KLH feeding induced immune tolerance when administered orally and parenterally depending on the KLH dosing regimen used.[29,49,56] Factors such as age, physical activity, alcohol consumption, and stress all play a role in the immune response following KLH challenge. Atopy seems to partially increase the immune responses following KLH immunization and a subsequent intradermal KLH skin rechallenge, possibly due to a stronger immune response after repeated contact with an antigen. In contrast, SLE, HIV, and CVID patients showed a decreased humoral and cell-mediated KLH response. KLH challenges have proven their value in healthy volunteer trials evaluating the effects of immunomodulatory drugs. Based on the implementation of KLH challenges in the earliest stages of drug development, pharmacologically active doses of the investigational drugs could be identified, which facilitated dose selection for the subsequent phase 1B/phase 2A studies in patient populations. The best example of this is the recent success of Kymab's OX40 ligand blocker amlitelimab, which was effective in suppressing KLH-driven responses in healthy volunteers,[16] and subsequently showed improvements in symptoms of moderate-to-severe atopic dermatitis patients.[92] In the meanwhile, the compound has been acquired by Sanofi.[93]

A limitation of this systematic review was the difficulty in generalizing the response sizes and variabilities due to the differences in analytical and statistical methods, or reference material in the studies. Some studies reported the range as the standard deviation from the mean whereas others reported the range as the 95% confidence interval. A few studies reported the range as the standard error of the mean. Another constraint of this review was that the response sizes and variabilities in many studies were based on estimations from graphical presentations as no concrete numbers were mentioned within the article text or tables. We analyzed these data as best as we were able to from the data at hand. Finally, it is important to stress that most KLH responses have been presented as fold-increase compared to baseline. Conceptually, this approach is questionable since KLH is regarded to be a neoantigen to most study participants, which means that at baseline no KLH-specific immunoglobulins or immune cells should be detected. However, for the authors this was the only way to systematically present the KLH responses over the wide range of clinical studies.

In conclusion, this systematic review provides an overview of the systemic and local responses to immunization with KLH and discusses the preferred imaging, planimetric, cellular and molecular biomarkers for the KLH response characterization. Whereas the circulating KLH-specific immunoglobulins are a very common endpoint for KLH challenge studies, the systemic KLH-specific immune cells have been evaluated less frequently. Importantly, these KLH-specific cells are rare in the circulation, so from a methodological point-of-view the detection of these cells is challenging. Evaluation of the skin response to a local KLH rechallenge yields important information since it is a measure for a specific T cell response at the tissue level. Although subjective evaluation of skin responses to KLH is already being done for decades, our review shows the importance of state-of-the-art imaging techniques to capture the often-mild perfusion increase, erythema and induration caused by KLH. Only a few studies evaluated the cellular and molecular responses to a dermal KLH rechallenge. Since blister exudate- or biopsy-based response characterization provides mechanistic insight into the immune responses driven by KLH, we advocate the implementation of invasive KLH response evaluation in future clinical trials. Based on the KLH challenges, the effect of immunomodulatory drugs could be demonstrated already in healthy volunteers, providing valuable information for the clinical development of these compounds. Moreover, based on the KLH challenge responses the functional immune status of different patient populations could be discriminated from healthy controls, providing novel insight into the pathophysiology of these diseases. Taken together, our review underlines the potential value of KLH challenges in clinical studies, but also the need for standardized and well-controlled methodology to induce and evaluate KLH responses.

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SECTION II

KLH IMMUNE CHALLENGE MODEL IN HEALTHY VOLUNTEERS

CHAPTER III

A RANDOMIZED CONTROLLED TRIAL WITH A DELAYED-TYPE HYPERSENSITIVITY MODEL USING KEYHOLE LIMPET HEMOCYANIN TO EVALUATE ADAPTIVE IMMUNE RESPONSES IN MAN

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ABSTRACT

Introduction: Keyhole limpet hemocyanin (KLH) immunization is a clinical model for the evaluation of human antibody responses. The current study evaluated the anti-KLH antibody response after KLH immunization and the delayed-type hypersensitivity response following intradermal KLH administration, using objective imaging techniques.

Methods: Healthy male subjects aged 24.5 ± 5.4 years were randomized to intramuscular immunization with 100 µg KLH (n = 12) or placebo (n = 3). Anti-KLH antibody (Ig)M and IgG titers were determined before and every 7 days after KLH immunization for a total of 28 days. 21 days after the immunization, all subjects received 1 µg KLH intradermally. Prior to and 2 days after intradermal KLH administration, skin blood perfusion, erythema and oedema were quantified using noninvasive imaging tools. Repeated measures ANCOVAs were used to analyze data.

Results: Anti-KLH IgM and IgG titers increased after KLH immunization compared to placebo (estimated difference (ED): 37%, 95% confidence interval (CI): 19–51% and ED: 68%, 95% CI: 56–76% respectively). Upon intradermal KLH administration an increase in skin blood perfusion (ED: 10.9 arbitrary units (AU), 95% CI: 1.4–20.4 AU) and erythema (ED: 0.3 AU, 95% CI: 0.1–0.5 AU) was observed in KLH-immunized subjects compared to placebo.

Conclusion: KLH immunization followed by intradermal KLH administration resulted in increased anti-KLH IgM and IgG titers and a delayed-type hypersensitivity response quantified by an increase in skin blood perfusion and erythema. Using noninvasive imaging tools, the KLH model has the potential to serve as an objective tool to study the pharmacodynamics of T cell-directed immunomodulatory drugs.

INTRODUCTION

Autoreactive T cells play an essential role in immune-mediated diseases including type 1 diabetes mellitus,[1] autoimmune arthritis,[2] multiple sclerosis[3,4] and psoriasis.[5] Novel immunomodulatory drugs targeting the adaptive immune system and specifically T cells are often investigated in healthy subjects as part of the development program. However, evaluation of the pharmacological activity of such immunomodulatory drugs is challenging since a target engagement biomarker is not constitutively expressed in a healthy population. An *in vivo* immune challenge in which T cells are activated could serve as an alternative approach. By inducing an antigen-specific T cell response in healthy subjects, the effect of investigational drugs targeting the adaptive immune system could be quantified. However, i*n vivo* challenges for evaluation of the adaptive immune response in humans are currently not well-characterized.

Keyhole limpet hemocyanin (KLH) is a metalloprotein considered to be a suitable immunization antigen for studying cell-mediated immune responses.[6] It has been used in many clinical trials and found to be safe.[7-16] KLH is available in 2 different formulations. High molecular weight KLH consists of native KLH, predominantly a didecamer of roughly 4–8 MDa. Subunit KLH is dissociated native KLH known as immunocyanin, each subunit is approximately 400 kDa. As the immunogenicity of subunit KLH is lower compared to native KLH,[17] subunit KLH has been combined with an adjuvant such as aluminum hydroxide to provide a more potent immune response.[18,19] KLH was found to elicit a T cell-dependent immune response following 1–3 KLH immunizations.[7-16] Immunization doses of 8 µg up to 5,000 µg KLH have been reported, with 100 µg KLH being most frequently used.[20,21] Commonly, the KLH-specific immune response is measured by quantifying the anti-KLH antibody response by enzyme-linked immunosorbent assay.[8-11,13-16,19,21-24] However, the mechanism of the KLH-induced immune response is not fully understood. KLH drives an innate immune response through activation of nuclear factor κB, partially mediated *via* spleen tyrosine kinase and extracellular-signal-regulated kinase pathways, both associated with inflammatory responses, apoptosis and phagocytosis.[25] In parallel, KLH is recognized as a neoantigen driving an adaptive immune response. This cellular immune response can be evoked and studied *in vivo* by intradermal administration of a second KLH dose, 2–3 weeks after the initial intramuscular KLH immunization. The intradermal KLH administration induces a delayed-type IV hypersensitivity (DTH) response at the intradermal injection site.[8,9,11,13-16,26] This KLH-induced DTH response may serve as a model for the clinical evaluation of future drugs that modulate the adaptive immunity. In previous studies the KLH-mediated DTH response was only measured subjectively by visual inspection of the skin to assess the presence of

induration and erythema[7-11,13-16,26,27] and none of the studies has objectively quantified the erythema and induration response using noninvasive instruments. A positive skin reaction is often defined as an induration of ≥5 mm. This method of reporting DTH skin response is subject to inter-rater variability leading up to 12% reclassification of the skin response[28] and is often scored categorically. Furthermore, measurement of small distances with a ruler can easily provide imprecise results and observer bias. Objective, noninvasive quantification of skin blood perfusion, induration and erythema using a continuous numerical scale would be preferred.

Therefore, a clinical trial was designed to objectively quantify KLH-specific DTH responses, in relation to KLH-specific circulating antibody responses. As such, this study aimed to evaluate KLH immunization with a subsequent intradermal KLH administration as a challenge model for characterization of the adaptive immune responses in man implementing objective measures. This model could potentially be used in future clinical pharmacology studies with drugs targeting the immune system in healthy subjects.

METHODS

This was a randomized, double blind, placebo-controlled study in 15 healthy subjects. The study was conducted at the Centre for Human Drug Research, Leiden, The Netherlands. The Declaration of Helsinki was the principle for trial execution. The study protocol was approved by the Medical Ethics Committee *Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek* (Assen, the Netherlands). All subjects provided written informed consent prior to any study activity.

SUBJECTS

Healthy male subjects aged 18–45 years with a body mass index between 18 and 35 kg/m^2 were included in the trial. The health status was verified by a detailed medical history, a complete physical examination, vital signs, 12-lead electrocardiogram and laboratory test (including hepatic and renal panels, complete blood count, virology and urinalysis). Subjects were not eligible if they had any disease associated with immune system impairment, or received immunomodulatory medication within 30 days of enrolment. Subjects with known previous exposure to KLH were excluded.

STUDY DESIGN AND TREATMENTS

A timeline overview of the study design is shown in Figure 1. Subjects were randomized to intramuscular KLH immunization (n = 12) or placebo (n = 3). On the first study day, 100 µg of subunit KLH (Immucothel, Biosyn Corporation, Carlsbad, CA, USA), adsorbed to 900 µg aluminum hydroxide (Alhydrogel, Brenntag AG, Essen, Germany), was used for immunization in the deltoid muscle of the left arm. The KLH-specific immune response was monitored for 28 days by quantification of blood serum titers of anti-KLH antibodies. In addition, all participants received an intradermal KLH administration (1 µg Immucothel, no adjuvant), 21 days after intramuscular KLH immunization in the left ventral forearm for induction of DTH. Prior to and 2 days after the intradermal KLH administration, the skin DTH response was quantified as described in more detail below. Matching areas on the right ventral forearm were used as untreated control.

FIGURE 1 Study timeline.

Numbers represent visit days; i.m., intramuscular; i.d., intradermal; KLH, keyhole limpet hemocyanin; DTH, delayed type hypersensitivity.

ANTIBODY RESPONSES

Anti-KLH IgM and IgG titers in serum were quantified by enzyme-linked immunosorbent assay (Ardena Bioanalytical Laboratory, Assen, the Netherlands). Microwell plates were precoated with KLH (BCI-ImmuneActivator, Intracel Resources LLC. Rockville, MD, USA). For human antibodies specifically raised against KLH, no species-specific (polyclonal) reference material was available. Therefore, a pool of positive serum samples was used as positive control and a pool of human serum samples naive for KLH was used as negative control as well as a negative reagent control. Bound human anti-KLH IgM antibodies were detected by adding an anti-human IgM-horseradish peroxidase conjugate. Ratios relative to baseline were calculated based on mean optical density for all samples collected after KLH immunization. The lower and upper limits of quantification for anti-KLH IgG and IgM were baseline-corrected optical densities of 0.060 and 3.900, respectively.

SKIN BLOOD PERFUSION

Skin blood perfusion measurements were performed with laser speckle contrast imaging (LSCI; PeriCam PSI System, Perimed AB, Järfälla, Sweden) in a temperature-controlled room with a temperature around 22 °C, after subjects were accommodated to the temperature for at least 15 minutes. The camera to forearm distance was standardized to 12.5 cm. An area of 7 × 7 cm was measured with a frame rate of 21 images/s. Dedicated software (PimSoft, Perimed AB, Järfälla, Sweden) was used to capture LSCI recordings of at least 30 seconds. The recording with the strongest response was used to define a circular region of interest at the intradermal injection site. Area size-matched regions of interest of the intradermal injection sites and untreated control sites were identified in all other recordings and skin blood perfusion (indicated as basal flow) was quantitatively measured and expressed in arbitrary units (AUs). The homogeneity of skin blood perfusion in the region of interest (indicated as flare) was expressed as values that are +1 standard deviation from the mean basal flow within the region. The flare was also quantitatively assessed and expressed in AUs.

ERYTHEMA AND OEDEMA

Erythema was quantified with several modalities: multispectral imaging (Antera 3D, Miravex, Dublin, Ireland), colorimetry (DSM II ColorMeter, Cortex Technology, Hadslund, Denmark) and automated 2D photography (FotoFinder Bodystudio ATBM, FotoFinder Systems GmbH, Bad Birnbach, Germany). Quantification of target site oedema was performed with multispectral imaging. Induration, erythema, tenderness and pain were also assessed using a validated toxicity grading scale (TGS),[29] which was performed by an experienced physician (MS).

The multispectral imaging camera captures images of 5 × 5 cm without exposure to ambient light. The image with the strongest response in average redness was used to define a circular region of interest at the intradermal injection site. Area size-matched regions of interest of the intradermal injection sites and untreated control sites were identified in all other recordings. Erythema (indicated as CIELab color space a* value and average redness) was quantitatively assessed and expressed in AUs. Oedema height, area and volume were also quantitatively assessed and expressed in mm, mm^2 and mm^3 , respectively using Antera 3D software.

Colorimetry was performed using the DSM II ColorMeter, measuring erythema 3 consecutive times and reported as the CIELab a* value. The average of 3 measurements was used for statistical analysis.

Automated 2D photographs of both forearms were obtained using the FotoFinder bodystudio ATBM. Intradermal injection sites on both forearms were identified in the captured images and erythema index was calculated using color correction software (QPcolorsoft 501, QPcard AB, Everöd, Sweden) and image processing software (ImageJ, National Institutes of Health, Bethesda, MD, USA).

STATISTICS

All statistical programming was conducted with SAS 9.4 for Windows (SAS Institute Inc., Cary, NC, USA). The randomization code was generated using SAS by a study-independent statistician. Subjects were randomized to intramuscular KLH immunization or placebo in a 4:1 ratio in a consecutive order starting with the lowest number. The randomization code was only made available for data analysis after study completion. Demographic and baseline variables were summarized by allocation to intramuscular KLH or placebo immunization. Anti-KLH antibody and cell-mediated immunity endpoints were analyzed with a mixed model analysis of covariance (ANCOVA) with treatment, time and treatment by time as fixed factors and subject, subject by treatment and subject by time as random factors and the (average) baseline measurement as covariate. The Kenward–Roger approximation was used to estimate denominator degrees of freedom and model parameters were estimated using the restricted maximum likelihood method. Anti-KLH IgM and IgG titers, erythema index quantified from ATBM captured photographs and oedema area and volume quantified by multispectral imaging required log transformation. The general treatment effect and specific contrasts were reported with the estimated difference (ED) and the 95% confidence interval (CI), the least square mean (LSM) estimates and the *P* value. For anti-KLH IgM and IgG titers additional contrasts were calculated per time point. Graphs of the LSM estimates over time by treatment were presented with 95% CI as error bars, as well as change from baseline LSM estimates. To correlate the antibody with the DTH responses the following ratios were calculated: anti-KLH IgM and IgG antibody titers at week 3 *vs.* baseline, LSCI (basal flow and flare) and multispectral imaging (average redness and CIELab a*) levels 2 days post intradermal KLH administration *vs.* baseline. Spearman rank correlations between anti-KLH antibodies and DTH responses were performed. Based on the generated data, power calculations were performed supporting future trials investigating the effect of immunomodulatory drugs based on this KLH model. Sample sizes were calculated for anti-KLH antibodies, skin blood perfusion by LSCI and erythema by multispectral imaging assuming similar variability, based on an anticipated drug-dependent inhibition of the KLH-induced response of 75%.

RESULTS

BASELINE CHARACTERISTICS

The study was conducted between February and May 2017. Fifteen healthy, male subjects were enrolled in the study, 12 receiving an immunization with KLH and 3 receiving placebo. All subjects received an intradermal administration of KLH and all subjects completed the study and were included in the analysis population. Baseline characteristics were comparable between the treatment groups (Table 1). No serious adverse events or deaths occurred during the study. 1 subject reported mild discomfort upon touch after intradermal KLH administration at the injection site which resolved within 2 days. No other adverse events occurred that were considered related to KLH immunization or intradermal KLH administration.

TABLE 1 Baseline characteristics.

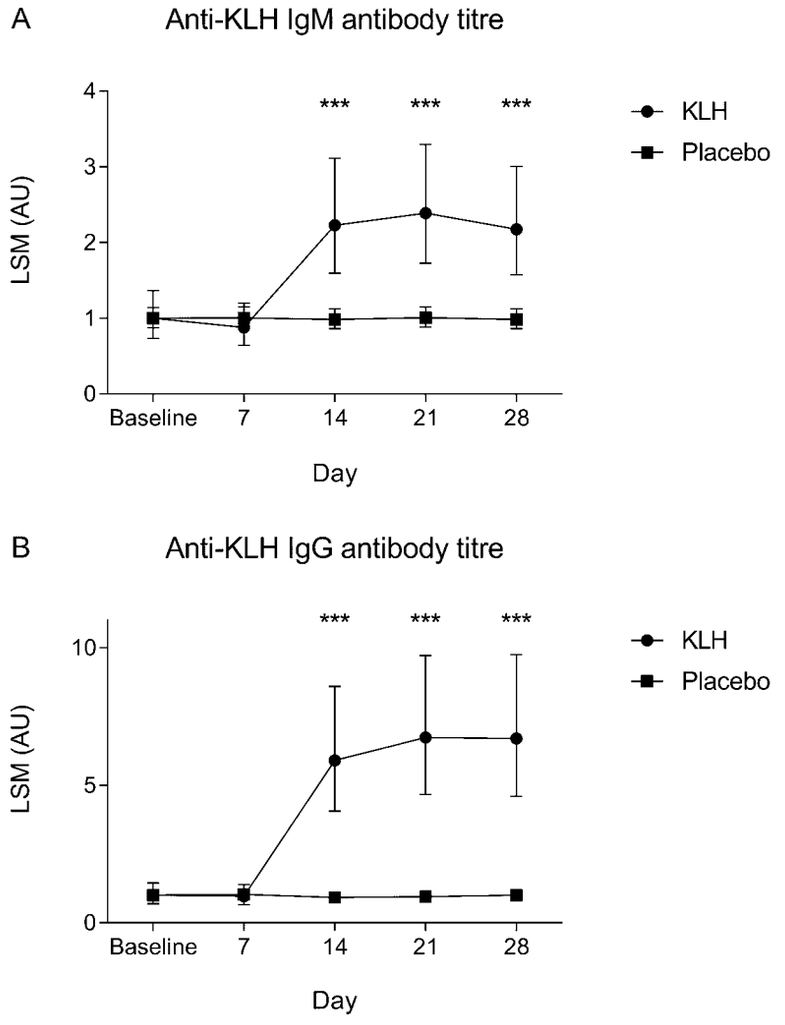
	All subjects *n* = 15	**KLH** *n* = 12	**Placebo** *n* = 3
Age (years)	24.5 (5.4)	24.7 (6.1)	23.7 (0.6)
Weight (kg)	80.8 (8.5)	82.2 (8.7)	75.6 (5.8)
Height (cm)	181.7 (9.0)	183.1 (8.9)	175.9 (8.2)
BMI (kg/m^2)	24.6 (2.9)	24.6 (2.8)	24.6 (4.1)
Hemoglobin (mmol/L)	9.37 (0.46)	9.44 (0.45)	9.07 (0.47)
Leucocytes (× 10^9/L)	6.48 (1.71)	6.35 (1.90)	6.99 (0.53)
Eosinophils (× 10^9/L)	0.26 (0.60)	0.30 (0.67)	0.13 (0.03)
Basophils (× 10^9/L)	0.05 (0.04)	0.05 (0.04)	0.03 (0.01)
Neutrophils (× 10^9/L)	3.71 (1.14)	3.60 (1.25)	4.15 (0.43)
Lymphocytes (× 10^9/L)	1.94 (0.41)	1.90 (0.42)	2.09 (0.40)
Monocytes (× 10^9/L)	0.52 (0.18)	0.51 (0.18)	0.58 (0.15)

Parameters are shown as mean (standard deviation). BMI, body mass index.

ANTIBODY RESPONSES

Intramuscular KLH immunization resulted in an increase in circulating anti-KLH IgM and IgG titers (Figure 2A and B). Titers started to rise after day 7 and reached a plateau at day 14–21. The increase in antibody titers was significant compared to placebo (ED: 37%, 95% CI: 19–51%, $P = .002$ and ED: 68%, 95% CI: 56–76%, $P < .0001$ for IgM and IgG, respectively; Table 2). Also at individual time points, treatment group contrasts reached a statistically significant difference at 2, 3 and 4 weeks after vaccination (Figure 2A and 1B).

FIGURE 2 Figure 2. (A) Anti-KLH IgM and (B) IgG antibody titers over time by treatment group. Data are shown as LSM with 95% confidence interval.



*Asterisks indicate significance between groups, *** $P < .001$. KLH, keyhole limpet hemocyanin; LSM, least square means; AU, arbitrary unit.*

TABLE 2 Summary statistics for pharmacodynamic endpoints.

Pharmacodynamic parameter	LSM			ED (95% CI)		
	KLH	Placebo		KLH *vs.* Placebo		
ANTI-KLH ANTIBODIES						
Anti-KLH IgM (% change)	1.59	1.00		37.3% (19.4–51.2%)[b]		
Anti-KLH IgG (% change)	3.03	0.98		67.7% (56.3–76.1%)[d]		
	LSM			ED (95% CI)		
	i.m. KLH i.d. KLH (n = 12)	i.m. Placebo i.d. KLH (n = 3)	i.m. KLH or Placebo i.d. untreated (n = 15)	i.m. KLH i.d. KLH *vs.* i.m. Placebo i.d. KLH	i.m. KLH i.d. KLH *vs.* i.m. KLH or Placebo i.d. untreated	i.m. Placebo i.d. KLH *vs.* i.m. KLH or Placebo i.d. untreated
SKIN BLOOD PERFUSION						
LSCI						
Basal flow (AU)	42.89	31.97	31.14	10.92 (1.41–20.44)[a]	11.75 (6.59–16.91)[c]	−0.83 (−9.75 to 8.10)
Flare (AU)	79.57	74.72	74.84	4.86 (1.24–8.48)[a]	4.74 (2.59–6.88)[c]	0.12 (−3.33 to 3.58)
ERYTHEMA						
MULTISPECTRAL IMAGING						
Average redness (AU)	1.17	0.91	0.97	0.26 (0.05–0.47)[a]	0.21 (0.09–0.32)[b]	0.05 (−0.14 to 0.25)
CIELab a* (AU)	14.88	11.79	12.88	3.09 (0.84–5.34)[b]	2.00 (0.79–3.22)[b]	1.09 (−1.04 to 3.22)
COLORIMETRY						
CIELab a* (AU)	13.05	11.37	11.45	1.69 (−0.29 to 3.66)	1.61 (0.57–2.65)[b]	0.08 (−1.72 to 1.88)
ERYTHEMA INDEX						
Erythema index (% change)	62.58	61.39	57.45	1.9% (−13.6 to 15.3%)	8.2% (1.5–14.4%)[a]	−6.8% (−21.8 to 6.3%)
OEDEMA						
MULTISPECTRAL IMAGING						
Oedema height (mm)	0.17	0.06	0.04	0.11 (−0.01 to 0.22)	0.12 (0.06–0.19)[b]	−0.02 (−0.13 to 0.09)
Oedema area (% change)	8.28	4.15	4.74	49.9% (-1.85×10^6 to 100%)	42.8% (-7.00×10^4 to 100%)	12.4% (-5.32×10^7 to 100%)
Oedema volume (% change)	1.45	0.41	0.88	71.4% (-1.46×10^7 to 100%)	39.2% (-1.68×10^5 to 100%)	53.0% (-4.47×10^8 to 100%)

[a] $P < .05$, [b] $P < .01$, [c] $P < .001$, [d] $P < .0001$. *LSM, least square means; KLH, keyhole limpet hemocyanin; ED, estimated difference; i.m., intramuscular; i.d., intradermal; AU, arbitrary unit.*

DTH RESPONSE

A statistically significant increase in skin blood perfusion as determined by LSCI basal flow (ED: 10.9 AU, 95% CI: 1.4–20.4 AU, P = .026) and flare (ED: 4.9 AU, 95% CI: 1.2–8.5 AU, P = .011) was observed at the KLH intradermal injection site on the left ventral forearm in the KLH immunized group compared to placebo (Table 2 and Figure 3A and B). When compared to the untreated control area (on the right ventral forearm), there was also a significant increase in basal flow and flare at the KLH intradermal injection site (on the left ventral forearm) in the KLH immunized group (ED: basal flow 11.8 AU, 95% CI: 6.6–16.9 AU, P < .001, ED: flare 4.7 AU, 95% CI: 2.6–6.9 AU, P < .001) whilst no statistically significant difference was observed in the placebo treated group (ED: −0.8 AU, 95% CI: −9.8 to 8.1 AU, P = .85 and ED: 0.1 AU, 95% CI: −3.3 to 3.6 AU, P = .94). LSCI basal flow illustrations 2 days after intradermal KLH administration of a single subject per group are shown in Figure 4.

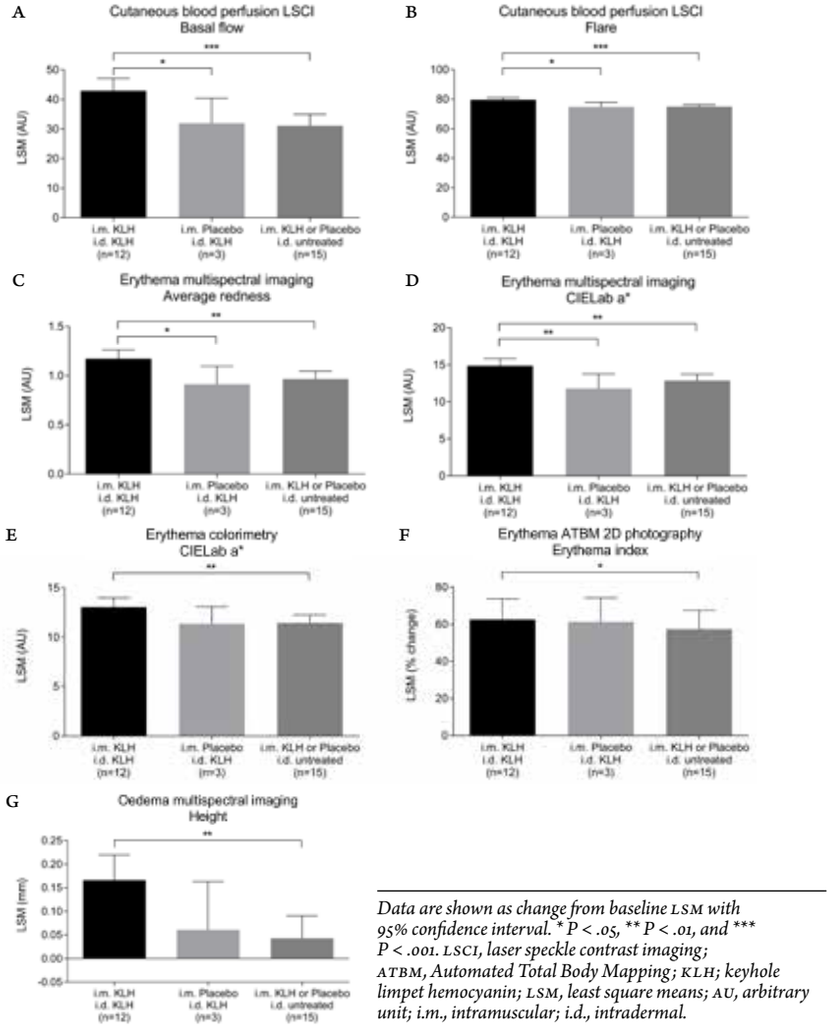
Erythema quantified with multispectral imaging was significantly increased at the KLH intradermal injection site on the left ventral forearm in the KLH immunized group compared to placebo, both for average redness (ED: 0.3 AU, 95% CI: 0.1–0.5 AU, P = .017) as well as CIELab color space a* value (ED: 3.1 AU, 95% CI: 0.8–5.3 AU, P = .009; Table 2 and Figure 3C and D). A significant increase in erythema, expressed as average redness and as CIELab a* value was also observed when comparing the KLH intradermal injection site to the untreated control area (ED: 0.2 AU, 95% CI: 0.1–0.3 AU, P = .002 and ED: 2.0 AU, 95% CI: 0.8–3.2 AU, P = .003, respectively). No differences were observed in average redness and CIELab a* value between the KLH intradermal injection site and the untreated control area in the placebo treated group (ED: 0.1 AU, 95% CI: −0.1 to 0.3 AU, P = .58 and ED: 1.1 AU, 95% CI: −1.0 to 3.2 AU, P = .30, respectively). Illustrations of erythema quantified as CIELab a* with multispectral imaging 2 days after intradermal KLH administration of a single subject per group are shown in Figure 4.

Erythema quantified by colorimetry (CIELab a* value) and by color-corrected automated photography (erythema index) showed statistically significant differences between the KLH intradermal injection site and the untreated control area in the KLH immunized group (ED: 1.6 AU, 95% CI: 0.6–2.7 AU, P = .005 and ED: 8%, 95% CI: 2–14%, P = .021, respectively; Table 2 and Figure 3E and F). However, differences between the KLH intradermal injection site in the KLH immunized group and the placebo group were not statistically significant (ED: 1.7 AU, 95% CI: −0.3 to 3.7 AU, P = .09 and ED: 2%, 95% CI: −14 to 15%, P = .78, respectively).

Similarly, oedema height quantified by multispectral imaging was significantly increased at the KLH intradermal injection site compared to the untreated control area in the KLH immunized group (ED: 0.1 mm, 95% CI: 0.1–0.2 mm, P = .002; Table 2 and Figure 3G). However, no statistically significant difference was observed at the KLH intradermal injection site between the KLH immunized group and placebo (ED: 0.1 mm, 95% CI: −0.01 to 0.2 mm, P = .07). No differences were observed in oedema area and volume between the treatment groups (Table 2).

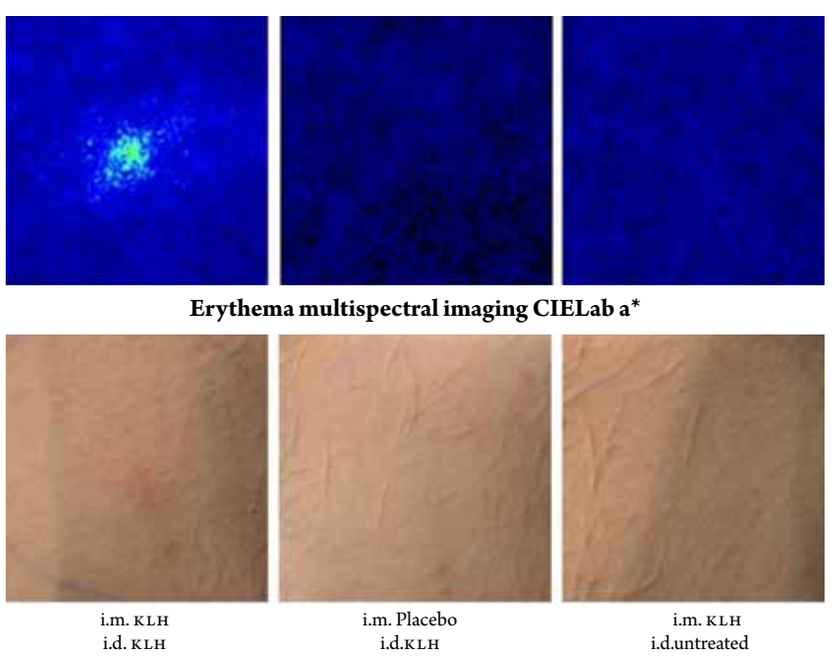
Based on the TGS, only 1 subject reported tenderness directly after intradermal KLH administration categorized as mild discomfort upon touching, which had disappeared within 2 days. There were no visual changes in erythema and tactile examination showed no induration based on the TGS at the intradermal injection sites during DTH readout.

FIGURE 3 Skin blood perfusion assessed as (A) LSCI basal flow and (B) flare, erythema assessed as (C) average redness, (D) CIELab a* with multispectral imaging, (E) CIELab a* with colorimetry, and (F) erythema index with ATBM 2D photography, and (G) oedema height with multispectral imaging by treatment group. Treatment groups are defined as subjects receiving i.m. KLH immunization and i.d. KLH administration ($n = 12$), i.m. placebo immunization and i.d. KLH administration ($n = 3$) and both immunization groups combined (KLH or placebo) and no i.d. administration (untreated arm; $n = 15$).



*Data are shown as change from baseline LSM with 95% confidence interval. * $P < .05$, ** $P < .01$, and *** $P < .001$. LSCI, laser speckle contrast imaging; ATBM, Automated Total Body Mapping; KLH; keyhole limpet hemocyanin; LSM, least square means; AU, arbitrary unit; i.m., intramuscular; i.d., intradermal.*

FIGURE 4 Illustrations of LSCI basal flow and erythema assessed as CIELab a* with multispectral imaging 2 days after intradermal KLH administration of a subject treated with i.m. KLH immunization and i.d. KLH administration (left images), i.m. placebo immunization and i.d. KLH administration (middle images) and i.m. KLH immunization and untreated control arm (right images).



LSCI, laser speckle contrast imaging; KLH, keyhole limpet hemocyanin; i.m., intramuscular; i.d., intradermal.

CORRELATIONS AND POWER CALCULATION

Spearman nonparametric rank correlation between anti-KLH IgM and LSCI flare showed a statistically significant positive correlation $r = 0.67$ ($P = .033$). No other statistically significant correlations between anti-KLH antibodies and DTH responses (LSCI and multispectral imaging) were observed.

Based on the observed KLH responses and observed variability, a sample size of at least 12 per group would be required to detect a 75% inhibition of the KLH-induced anti-KLH IgM and IgG antibody response, the DTH skin blood perfusion response quantified by LSCI (basal flow and flare) and the DTH erythema response quantified

by multispectral imaging (average redness and CIELab a*) using a parallel study design, with an α of .05 and a power of 80%. To detect a 75% inhibition of only the anti-KLH IgM and IgG antibody response following KLH immunization a sample size of at least 4 per group would be required. The most sensitive readout based on the KLH responses is the anti-KLH IgG antibody response requiring a sample size of at least 2 per group and the least sensitive readout is the LSCI basal flow requiring a sample size of at least 12 per group to detect a 75% inhibition of the responses using a parallel study design, with an α of .05 and a power of 80%.

DISCUSSION

In this study we evaluated the response of healthy subjects to KLH immunization, by the quantification of anti-KLH IgM and IgG and the DTH response of the skin upon intradermal KLH administration. Our study confirms that KLH immunization and intradermal KLH administration are well-tolerated and result in a primary antibody response against KLH. Intradermal KLH administration resulted in a DTH response in KLH-immunized subjects that was quantified as increased skin blood perfusion and erythema by imaging, but that remained undetected by visual inspection. Based on our findings, KLH immunization followed by an intradermal KLH administration may serve as a model for quantification of adaptive immune responses in healthy subjects, potentially for future use in clinical pharmacology studies with drugs targeting the adaptive immune system.

Multiple studies have used a KLH challenge model including a DTH response to evaluate the pharmacodynamic effects of immunomodulatory drugs such as cyclosporine treatment in bone marrow transplant patients,[30] methotrexate and rituximab treatment in patients with rheumatoid arthritis[10] and multiple immunomodulatory drugs in renal transplant patients.[14] Recent studies investigating novel targets of immunomodulatory drugs in healthy subjects used a KLH challenge to show a significant decrease in anti-KLH antibody response of >90% compared to placebo;[31,32] however, the cell-mediated immune response using either *in vivo* (DTH response) or *ex vivo* (lymphocyte proliferation assays) testing was not evaluated in these studies.

The DTH response has been reported to be primarily induced by a type IV hypersensitivity reaction in the skin involving antigen-presenting cells that display antigens using major histocompatibility complex class II molecules to dermal cluster of differentiation 4 ($CD4^+$) T cells.[33] This causes activation of and an increase in dermal $CD4^+$ T cells, which usually takes up to 48–72 hours to reach a maximum response.[8,9,11,13-16,26] Subsequent cytokine secretion, such as interleukin (IL)-2 and interferon-γ, by activated $CD4^+$ T cells primarily causes proliferation of $CD8^+$ T cells and attraction of macrophages that migrate and infiltrate the affected area.[33] Tumor necrosis factor α secretion by activated T cells induces prostacyclin release from endothelial cells that promote vasodilation and increased permeability, resulting in increased blood perfusion, erythema and oedema.[34] Previous studies have measured the DTH response to intradermal KLH administration as induration[7-11,13,14,16,26,27] and erythema[13,27] expressed as the largest diameter of the skin reaction or the average of orthogonal diameters measured with either a ruler or the ball point pen technique.[35] In line with our results, previous studies were also unable to detect a DTH response subjectively by visual inspection[9,15] or the response was only positive in a portion of the treated population.[13,26] This might be attributed to a low immunization dose and/or a low subsequent intradermal administration dose.[6] The devices used in the present study were able to detect small changes in the DTH response compared to the traditional categorical scale for erythema and induration suggesting a higher sensitivity of the imaging techniques. Importantly, the DTH response in the current study was quantified objectively on continuous numerical scales, which makes the impact of inter-rater variability minimal, which is inherent to subjective DTH scoring approaches. LSCI and multispectral imaging have, to our knowledge, not yet been used before in the investigation of DTH skin reactions. Based on the results observed in this study, these techniques may acquire a prominent role in objectively evaluating DTH skin reactions in future clinical trials.

The systemic cell-mediated immune response to KLH can be quantified *ex vivo* by lymphocyte proliferation assays, although the intra-assay and interindividual variability is high.[8,11-14,19,22,24] Also, other techniques, such as ELISpot, *ex vivo* cytokine production assays or cell activation based on L-selectin expression suffer from the same limitations.[19,22,36] Although we performed cell-based assays in the present study to quantify the systemic cellular response to KLH, we were unable to detect systemic and significant antigen-specific circulating T cell responses in KLH immunized subjects. This may be explained by the aforementioned bioanalytical variability and the number of antigen-specific T cells in the circulation, which was assumed to be very low at the KLH dose that we selected (single vaccination, 100 μg of subunit KLH). However, the KLH-driven skin responses after intradermal rechallenge of KLH-immunized volunteers proves a KLH-specific T cell response upon KLH immunization. The skin response after intradermal KLH administration contains both a type IVa DTH component as a result of increased interferon-γ secretion by T helper 1 (Th1) cells, as well as a type IVb DTH component characterized by increased IL-5, IL-4 and IL-13 production in Th2 cells involved in KLH immunization.[37]

The fact that we did not observe a clear correlation between the anti-KLH antibody response and the DTH response suggests that the DTH response to intradermal KLH administration is unlikely to be driven by the antibody response to the initial KLH immunization. This underlines the value of the antibody response and the DTH response as 2 KLH-driven but mechanistically independent phenomena, 1 reflecting B cell-mediated responses and the other reflecting T cell-mediated responses.

As stated earlier, we administered a relatively low dose of KLH (100 µg), vaccinated only once and used a KLH subunit, which is less immunogenic than high-molecular weight KLH. Therefore, the elicited immune responses were relatively mild (no apparent systemic KLH-specific T cells, no positive DTH response by visual inspection). Future research including multiple primary and booster KLH formulations could shed light on the KLH exposure versus effect relationship. Furthermore, such studies could include specific pharmacological interventions modulating T and B cell responses (e.g., corticosteroids, compounds resulting in suppression of nuclear factor of activated T cells, modulators of co-stimulatory molecules) to establish a benchmark for testing pharmacodynamic effects of novel immunomodulatory drugs in healthy subjects.

We were unable to detect an *ex vivo* systemic cell-mediated immune response to KLH and an *in vivo* positive DTH response by visual inspection in the current study, probably due to the selection of a KLH monomer with aluminum adjuvant, immunization dose and the dosing regimen. Future research including multiple primary and booster KLH formulations and various intervals is needed to characterize and optimize the outcome measures with noninvasive instruments in the current trial. Furthermore, a pharmacological intervention modulating the adaptive immune response should be included in order to establish a benchmark for testing pharmacodynamic effects of novel immunomodulatory drugs in healthy subjects.

CONCLUSION

In this study, KLH immunization resulted in the release of anti-KLH antibodies and intradermal KLH administration following initial KLH immunization produced an objectively measured and quantifiable increase in skin blood perfusion and erythema as DTH response. Importantly, these effects remained undetected upon visual inspection, underlining the importance of sensitive and objective imaging techniques for evaluation of dermal responses. Our KLH model has the potential to serve as an objective measurement tool to study the pharmacodynamic effects of B or T cell-directed immunomodulatory drugs.

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SECTION III

KLH IMMUNE CHALLENGE MODEL IN EARLY-PHASE CLINICAL TRIALS

CHAPTER IV

OX40L INHIBITION SUPPRESSES KLH-DRIVEN IMMUNE RESPONSES IN HEALTHY VOLUNTEERS: A RANDOMIZED CONTROLLED TRIAL DEMONSTRATING PROOF-OF-PHARMACOLOGY FOR KY1005

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ABSTRACT

Introduction: The safety, tolerability, immunogenicity, and pharmacokinetic (PK) profile of an anti-OX40L monoclonal antibody (KY1005, currently amlitelimab) were evaluated. Pharmacodynamic (PD) effects were explored using keyhole limpet hemocyanin (KLH) and tetanus toxoid (TT) immunizations.

Methods: 64 healthy male subjects (26.5 ± 6.0 years) were randomized to single doses of 0.006, 0.018, or 0.05 mg/kg, or multiple doses of 0.15, 0.45, 1.35, 4, or 12 mg/kg KY1005, or placebo (6:2). Serum KY1005 concentrations were measured. Antibody responses upon KLH and TT immunizations and skin response upon intradermal KLH administration were performed. PD data were analyzed using repeated measures analysis of covariances (ANCOVAs) and post hoc exposure-response modeling.

Results: No serious adverse events occurred and all adverse events were temporary and of mild or moderate severity. A nonlinear increase in mean serum KY1005 concentrations was observed (median time to maximum concentration (T_{max}) ~ 4 hours, geometric mean terminal half-life ($t_{½}$) ~ 24 days). Cutaneous blood perfusion (estimated difference (ED) −13.4 arbitrary unit (AU), 95% confidence interval (CI) −23.0 to −3.8 AU) and erythema quantified as average redness (ED −0.23 AU, 95% CI −0.35 to −0.11 AU) decreased after KY1005 treatment at doses of 0.45 mg/kg and above. Exposure-response analysis displayed a statistically significant treatment effect on anti-KLH antibody titers (IgG maximum effect (E_{max}) −0.58 AU, 95% CI −1.10 to −0.06 AU) and skin response (erythema E_{max} −0.20 AU, 95% CI −0.29 to −0.11 AU).

Conclusion: Administration of KY1005 demonstrated an acceptable safety and tolerability profile and PK analyses displayed a nonlinear profile of KY1005. Despite the observed variability, skin challenge response after KY1005 treatment indicated pharmacological activity of KY1005. Therefore, KY1005 shows potential as a novel pharmacological treatment in immune-mediated disorders.

INTRODUCTION

The significance of the T cell costimulatory molecule OX40 and its ligand OX40L in immunoregulation is increasing, especially as therapeutic targets. OX40 is predominantly expressed on activated memory and regulatory cluster of differentiation 4 ($CD4^+$) T cells and in lower levels on activated $CD8^+$ T cells,[1] natural killer cells[2,3] and neutrophils.[4] OX40 agonism has been shown to result in an increase of the antigen-specific T cell pool[5,6] and prolonged activation.[7] Similar to OX40, the expression of OX40L is upregulated after antigen presentation on various antigen-presenting cells such as B cells,[8] dendritic cells,[9] macrophages[10] and specific cell types outside the immune system.[11-14] Activation of this costimulatory OX40-OX40L pathway may contribute to resistance of T lymphocytes to regulatory signals.[15]

OX40-OX40L signaling may be a target for the treatment of auto-immune diseases.[16-21] Several animal models confirmed that OX40L is involved in diabetes,[22] colitis,[23] rheumatoid arthritis,[24] uveitis,[25] and multiple sclerosis.[26,27] In human studies, OX40 inhibition using an anti-OX40 monoclonal antibody has been shown to improve the Eczema Area and Severity Index (EASI) score in patients with atopic dermatitis with up to 56% reduction from baseline EASI score compared with 38% reduction in placebo-treated subjects.[20] Another study showed that although OX40L inhibition with an anti-OX40L monoclonal antibody in patients with mild allergic asthma had no effect on airway hyperresponsiveness or allergen-induced airway responses, total serum immunoglobulin E (IgE) decreased 16.5% from baseline compared with a 14% increase in placebo-treated subjects and sputum eosinophils decreased 75% from baseline compared with a 14% decrease in placebo-treated subjects.[28] Blockade of the OX40-OX40L pathway seems a scientifically plausible approach to modulate persistent inflammation caused by autoreactive memory T effector cell populations. This blockade may possibly also induce or restore immune tolerance to autoantigens (e.g., in autoimmune disease) or alloantigens (e.g., following transplants).

KY1005 (also known as SAR445229 and currently as amlitelimab) is a novel non-depleting IgG4 human anti-OX40L monoclonal antibody that binds OX40L and thereby prevents persistent inflammation by blocking the interaction with OX40. *In vitro*, KY1005 inhibited interleukin-2 (IL-2), IL-13, and tumor necrosis factor α release in human mixed lymphocyte reaction (MLR) tests (Supplementary Materials and Methods S1). *In vivo* studies in rhesus monkeys with acute graft-versus-host disease showed prolonged median survival time (MST) > 100 days when KY1005 was co-administered with sirolimus compared with KY1005 monotherapy (MST 19.5 days), sirolimus monotherapy (MST 14 days), or no prophylaxis (MST 8 days).[29] The synergistic effect of KY1005/sirolimus was possibly induced by sustained T regulatory cell reconstitution as well as suppression of T effector activity. Based on these experiments,

KY1005 could be a treatment modality to inhibit the activation of the immune system as a result of high OX40-OX40L expression and consequently restore the homeostasis between proinflammatory T effector and anti-inflammatory T regulatory cells in immune-mediated diseases.

The aim of this first-in-human study was to evaluate the safety and tolerability, immunogenicity, and pharmacokinetic (PK) profile of KY1005 in healthy volunteers. Intramuscular immunizations with a neoantigen (keyhole limpet hemocyanin; KLH) and a recall antigen (tetanus toxoid; TT) were used to explore pharmacodynamic (PD) effects of KY1005, including measurements of serum anti-KLH and anti-TT antibody titers and objective quantification of skin challenge response following an intradermal KLH administration.

METHODS

This was a phase I, randomized, placebo-controlled, double-blind, single ascending dose (SAD) and multiple ascending dose (MAD) study in 64 healthy volunteers performed at the Centre for Human Drug Research (CHDR), Leiden, The Netherlands. The Declaration of Helsinki was the principle for trial execution. The independent Medical Ethics Committee *Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek* (Assen, The Netherlands) approved the study prior to any clinical study activity. All subjects provided written informed consent before participation. The trial was registered on ClinicalTrials.gov (NCT03161288).

SUBJECTS

Main inclusion criteria were male gender, 18–45 years of age, with a body mass index between 18 and 30 kg/m^2, and previous immunization with TT more than 6 months prior to screening and no known previous exposure to KLH. Health status was verified by recording a detailed medical history, a complete physical examination, vital signs, a 12-lead electrocardiogram (ECG), and laboratory testing (including hepatic and renal panels, complete blood count, virology, and urinalysis). Subjects were excluded in case of any disease associated with immune system impairment, or use of prescription medication within 2 weeks prior to enrollment.

DOSE SELECTION AND REGIMEN

The starting dose of 0.006 mg/kg KY1005 was based on a minimal anticipated biological effect level (MABEL) principle using *in vitro* data obtained in human MLR experiments. The maximum dose of 12 mg/kg KY1005 was based on the maximal effects observed in these experiments and predicted exposure equivalent to that at which maximum *in vivo* inhibition of the IgG response to KLH immunization occurred in monkeys. Detailed information on dose selection can be found in Supplementary Materials and Methods S1. The interval of 4 weeks between the loading dose and 2 maintenance doses was based on scaling of the KY1005 terminal half-life (t½) of 23 ± 1 day observed in cynomolgus monkeys. 3-fold dose increments between cohorts were based on a 30-fold difference in concentration between the lowest effects and maximal effects observed in the MLR experiments, a modest slope of the dose response and theoretical risks related to OX40L blocking.

STUDY DESIGN AND TREATMENTS

An overview of the study design is shown in Figure S1. Subjects were enrolled into 8 cohorts. In each cohort, subjects were randomized to either 30 minutes of intravenous administration of KY1005 or placebo (6:2). 2 subjects per cohort started as a sentinel group and if no safety issues arose within 48 hours after dosing, the remaining 6 subjects were dosed. The first 3 cohorts received single doses of 0.006, 0.018, or 0.05 mg/kg KY1005, respectively. The 5 subsequent cohorts received multiple doses starting with an initial loading dose of 0.15, 0.45, 1.35, 4, or 12 mg/kg KY1005, respectively, followed by 2 maintenance doses of 50% of the loading dose, administered at 4 and 8 weeks after the initial administration. Intramuscular KLH and TT immunizations were performed in the deltoid muscles 1 week after the last (third) KY1005/placebo dose in the MAD cohorts. KLH was administered in a formulation of 0.1 mg of subunit KLH (Immucothel, Biosyn, Fellbach, Germany) adsorbed in 0.9 mg aluminum hydroxide (Alhydrogel, Brenntag Biosector A/S, Frederikssund, Denmark) into 0.5 mL NaCl 0.9%, as described previously.[30] TT was administered in the marketed formulation of ≥ 40 IU TT (Bilthoven Biologicals, Bilthoven, The Netherlands) in 0.5 mL NaCl 0.9%.[31,32] 21 days after intramuscular KLH administration, all subjects received an intradermal KLH administration in the left ventral forearm and placebo administration in the right ventral forearm. The formulation of 0.001 mg subunit KLH in 0.1 mL NaCl 0.9% used for intradermal administration was based on a previously conducted trial.[30] The interval of 21 days between intramuscular KLH immunization and intradermal KLH administration and the interval of 48 hours between baseline and follow-up skin challenge assessment has been used in previous other studies.[30,31,33-36] Prior to and 2 days after the intradermal KLH administration, the skin challenge response was quantified.

SAFETY AND TOLERABILITY

Safety and tolerability were monitored by physical examination, assessment of vital signs, laboratory parameters (i.e., full blood count, biochemistry, and urinalysis) and ECG data from 12-lead and 24-hour Holter ECGs at regular intervals. Subjects were monitored continuously for adverse events (AEs).

KY1005 PHARMACOKINETICS AND IMMUNOGENICITY

Serum concentrations of KY1005 for PK profiling and serum concentrations of anti-drug antibodies (ADAs) were measured by Eurofins Pharma Bioanalysis Services UK Ltd. (Abingdon, UK) using validated bioanalytical assay methods. The PK samples were analyzed using a luminescent enzyme-linked immunosorbent assay with a lower limit of quantification (LLOQ) of 9.77 ng/mL. ADAs were measured using an electrochemiluminescence solid-phase extraction with acid dissociation method. In both ADA screening and confirmatory formats, the assay tolerated up to 100 µg/mL KY1005 at positive control anti-KY1005 antibody concentrations of 100 and 250 ng/mL.

OX40 AND OX40L EXPRESSION

OX40 and OX40L expression was measured by CHDR (Leiden, The Netherlands) on cell subsets of whole blood samples using flow cytometry. Red blood cell lysis was performed on heparinized whole blood using RBC lysis buffer (Thermo Fisher, Waltham, MA, USA). Leukocytes were stained with fluorochrome labelled antibodies at 4 °C for 30 minutes, see Table S1 for a complete list. After staining, the cells were washed with PBS (Thermo Fisher). Samples were measured on a MACSQuant 10 analyzer, and analyzed using MACSQuantify software (both Miltenyi Biotec, Bergisch-Gladbach, Germany). See Figure S2 for the gating strategy. OX40 expression was assessed in $CD4^+$ and $CD8^+$ T cells, regulatory T cells and Th17 cells and OX40L expression was assessed in $CD19^+$ and $CD14^+$ monocytes in all cohorts. In addition, expression of OX40 and OX40L in $CD4^+$ and $CD8^+$ effector memory and central memory cells was assessed in cohorts 4–8.

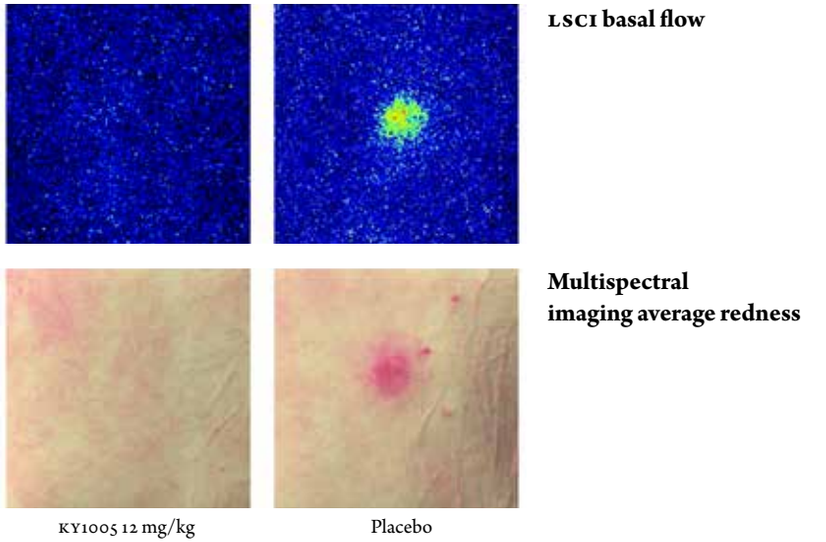
HUMORAL IMMUNITY TO KLH AND TT

The humoral response to intramuscular KLH and TT immunization was measured by anti-KLH and anti-TT IgM and IgG blood serum titers 21 days after immunization. Serum samples were assessed by quantitative enzyme-linked immunosorbent assay for anti-KLH and anti-TT IgM and IgG levels, as previously described.[30] In KLH-immunized subject blood samples, mean optical density of baseline samples was set to 1.00 and ratios relative to baseline were calculated for all subsequent samples. The LLOQ and the upper limit of quantification (ULOQ) for anti-KLH IgM and IgG were a baseline corrected optical density of 0.060 and 3.900, respectively. The LLOQ and ULOQ for anti-TT IgM were 10.0 and 100 IU/mL, respectively, and the LLOQ and ULOQ for anti-TT IgG were 0.100 and 5.00 IU/mL, respectively.

CUTANEOUS BLOOD PERFUSION

Cutaneous blood perfusion quantification was performed with laser speckle contrast imaging (LSCI; PeriCam PSI System, Perimed AB, Järfälla, Sweden), as previously described.[30] In short, assessments were performed in a temperature-controlled room (22 °C) after acclimatization of the subjects. LSCI recordings of the target area on the left and right ventral forearms were captured with the use of dedicated software (PimSoft, Perimed AB). Circular regions of interest at the intradermal injection sites were defined and cutaneous blood perfusion (indicated as basal flow) was quantitatively assessed and expressed in arbitrary units (AUs). The homogeneity of cutaneous blood perfusion in the region of interest (indicated as flare), expressed as values that are +1 standard deviation (SD) from the mean basal flow within the region, was also quantitatively assessed and expressed in AUs. Illustrations of cutaneous blood perfusion measured with LSCI are depicted in Figure 1.

FIGURE 1 Illustrations of LSCI basal flow and erythema assessed as average redness with multispectral imaging. Images were taken at intradermal KLH injection site 2 days after intradermal KLH administration of a subject treated with an initial KY1005 12 mg/kg dose (left images) and a subject that received placebo (right images).



KLH, keyhole limpet hemocyanin; LSCI, laser speckle contrast imaging.

ERYTHEMA

Erythema quantification was performed with multispectral imaging (Antera 3D, Miravex, Dublin, Ireland), as previously described.[30] In short, the camera was placed on the target area on the ventral forearms and images were captured using dedicated software (Antera 3D software, Miravex). Circular regions of interest at the intradermal injection sites were defined and erythema was quantified using the average redness and CIELab a* Antera 3D software modalities expressed as AUs. The average redness modality displays the distribution of redness using an internal software algorithm and the CIELab a* value, which is part of the CIELab color space, expresses color as a numerical value on a green–red color scale.[37] Illustrations of erythema measured with multispectral imaging are depicted in Figure 1.

STATISTICS

Detailed statistical procedures used in the current study are provided in the Supplementary Materials and Methods S1. Subjects were randomized to KY1005 or placebo in a 3:1 ratio. Demographic and baseline variables were summarized by treatment. For safety and tolerability endpoints, summary statistics for observed values were calculated for all continuous parameters. For every KY1005 dose, the peak serum concentration (C_{max}), t½, area under the curve from zero to the last measurable concentration (AUC_{0-last}) and clearance (CL) were reported as mean (coefficient of variation percentage; CV%) and the time at which C_{max} is observed (T_{max}) was reported as median (range). PD endpoints measured at multiple timepoints post baseline were analyzed with a mixed effect repeated measures model. Endpoints with one post dose measurement were analyzed with an analysis of covariance (ANCOVA) model. Skin challenge endpoints were analyzed with ANCOVA with the change from the saline-injected control (right forearm) added as covariate. The general treatment effect and specific contrasts were reported with the estimated difference (ED), 95% confidence interval (CI), and *P* value, and graphically as ED, 95% CI, and *P* value or mean change from baseline, SD, and *P* value. Negative change from baseline values for skin challenge endpoints were possible due to measurement variability and the dynamic nature of the measurements. Nonlinear mixed effects analysis of the exposure-response relationship was performed for anti-KLH and anti-TT antibody titers and skin challenge endpoints.

RESULTS

BASELINE CHARACTERISTICS

The study was conducted between May 2017 and March 2018. 24 subjects were enrolled in the SAD part and 40 subjects in the MAD part of the study. 4 subjects did not complete the study: 1 subject was withdrawn due to a suspected hypersensitivity reaction consisting of pruritus, swelling of the palate and gums, and slurred speech lasting ~ 2 hours after the first dose, 3 subjects withdrew consent for reasons unrelated to the study treatment. Baseline characteristics of all treatment groups are presented in Table 1.

TABLE 1 Baseline characteristics.

	SAD				MAD					
	KY1005			Placebo	KY1005					Placebo
Loading dose	**0.006 mg/kg**	**0.018 mg/kg**	**0.05 mg/kg**	**NA**	**0.15 mg/kg**	**0.45 mg/kg**	**1.35 mg/kg**	**4 mg/kg**	**12 mg/kg**	**NA**
Maintenance doses	**NA**	**NA**	**NA**	**NA**	**0.075 mg/kg**	**0.225 mg/kg**	**0.675 mg/kg**	**2 mg/kg**	**6 mg/kg**	**NA**
	***n* = 6**	***n* = 6**	***n* = 6**	***n* = 6**	***n* = 6**	***n* = 6**	***n* = 6**	***n* = 6**	***n* = 6**	***n* = 10**
DEMOGRAPHICS										
Age (years)	24.3 (4.5)	23.8 (1.5)	25.3 (3.7)	26.2 (7.1)	28.0 (9.2)	23.3 (3.9)	24.8 (3.9)	24.7 (4.1)	34.2 (6.3)	28.5 (5.6)
BMI (kg/m²)	22.9 (1.3)	21.7 (1.5)	23.5 (2.8)	23.5 (2.2)	22.9 (2.2)	23.1 (3.1)	22.8 (2.3)	23.1 (1.8)	24.9 (3.0)	24.5 (3.1)
VITAL SIGNS										
Systolic blood pressure (mmHg)	119 (8)	115 (11)	126 (11)	121 (16)	118 (3)	120 (11)	123 (13)	118 (11)	127 (7)	122 (9)
Diastolic blood pressure (mmHg)	72 (5)	68 (8)	77 (6)	68 (12)	72 (9)	75 (10)	68 (9)	70 (13)	78 (8)	72 (13)
Heart rate (BPM)	57 (13)	59 (7)	68 (11)	63 (17)	57 (8)	59 (8)	63 (14)	59 (10)	67 (9)	61 (8)
Temperature (°C)	36.7 (0.3)	36.9 (0.1)	36.8 (0.6)	36.7 (0.3)	36.4 (0.3)	36.9 (0.2)	36.5 (0.4)	36.5 (0.2)	36.6 (0.5)	36.6 (0.2)
LABORATORY TESTS										
Leucocytes ($\times 10^9$/L)	6.61 (1.05)	6.10 (1.58)	5.89 (1.06)	5.94 (0.96)	5.21 (1.16)	5.66 (0.86)	6.75 (1.52)	6.59 (1.59)	6.57 (1.19)	6.05 (2.42)
Thrombocytes ($\times 10^9$/L)	244.5 (26.4)	279.2 (68.1)	267.3 (43.0)	215.3 (40.1)	254.4 (69.8)	270.7 (53.8)	229.2 (29.6)	216.3 (53.3)	269.6 (65.7)	253.2 (53.7)
ALT (IU/L)	18.0 (5.8)	13.7 (4.2)	16.5 (3.6)	16.2 (7.1)	23.2 (14.1)	20.2 (11.7)	31.3 (20.1)	24.8 (19.0)	30.3 (11.7)	22.2 (7.0)
AST (IU/L)	23.2 (9.1)	20.7 (5.3)	19.2 (3.6)	18.2 (5.1)	22.7 (6.0)	24.0 (4.8)	28.5 (9.4)	25.3 (8.7)	27.0 (8.7)	21.2 (5.8)

Parameters are shown as mean (SD). ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; MAD, multiple ascending dose; NA, not applicable; SAD, single ascending dose.

SAFETY AND TOLERABILITY

No serious AEs occurred during the study. 1 subject in the MAD part of the study (12 mg/kg cohort) did not receive the second and third KY1005 doses due to a possible hypersensitivity reaction (mild palatal pruritus and swelling and slurred speech) after the first dose. No medication was administered based on the mild nature of the AEs and all symptoms resolved within 2 hours. Additional blood chemistry and hematology, including complement activation markers and tryptase, were all within normal ranges. No other AE-related discontinuations occurred during the study. The most frequently occurring treatment emergent AE was headache (Tables S2, S3). All treatment emergent AEs were of mild ($n = 190$) or moderate severity ($n = 16$) and self-resolving without sequalae. Treatment did not result in any clinically significant changes in any safety laboratory parameters, physical examination, vital signs measures, 12-lead ECG recordings, and Holter ECG recordings (data not shown).

KY1005 PHARMACOKINETICS AND IMMUNOGENICITY

A dose-dependent increase in mean serum concentrations of KY1005 was observed after single administrations (cohorts 1–3) and after multiple administrations (cohorts 4–8; Figure 2). KY1005 reached T_{max} ~ 4 hours after the start of infusion (median across cohorts from 0.5–24 hours) and had a t½ of ~ 24.3 days (mean across cohorts from 7.1–43.1 days with CV% of 15.5–51.8%; Table 2). Overall, single or multiple doses of KY1005 as measured by noncompartmental PK analysis appeared to be nonlinear (Table 2). KY1005 clearance remained relatively stable at concentrations > 2 mg/mL approximately (data not shown).

The number of subjects with ADAs increased with increasing KY1005 dose in cohorts 1–3 (2, 2, and 5 subjects, respectively). In cohorts 4–8, however, the largest number of subjects positive for ADAs (4 subjects) was observed at the lowest dose regime (0.15 mg/kg KY1005 cohort), with no subjects developing detectable ADAs at the highest dose (12 mg/kg KY1005 cohort). There was no correlation between ADAs and any of the PK parameters, including CL (data not shown).

OX40 AND OX40L EXPRESSION

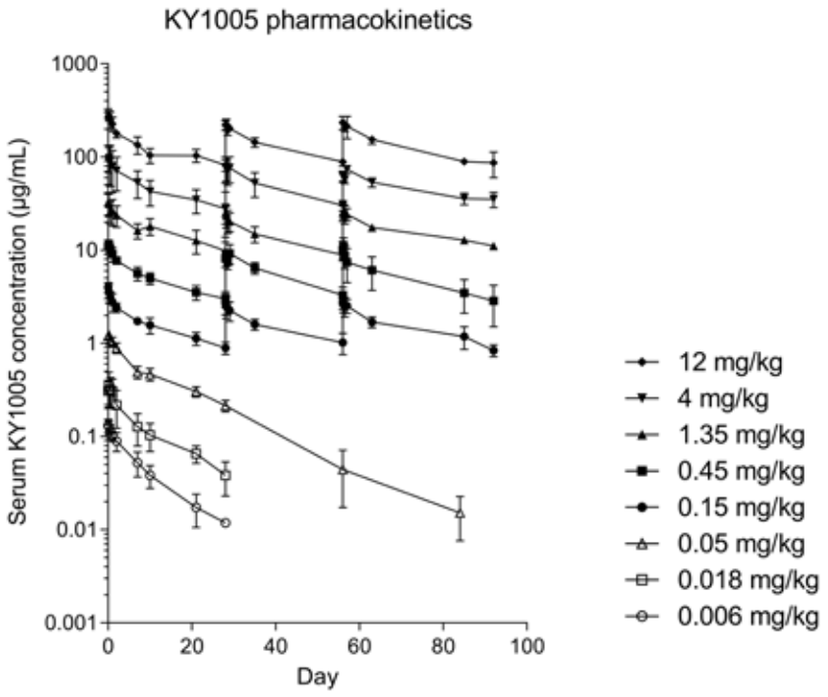
No consistent OX40 and OX40L expression profile trends were observed across the groups, although some P values < .05 compared with placebo were noted (Table S4). The differences compared with placebo in OX40 and OX40L expression on a variety of immune cells did not induce any clinically relevant observations.

TABLE 2 Summary of pharmacokinetic parameters of KY1005 per dose level.

Parameter	SAD			MAD				
FIRST KY1005 DOSE	**0.006 mg/kg (*n* = 6)**	**0.018 mg/kg (*n* = 6)**	**0.05 mg/kg (*n* = 6)**	**0.15 mg/kg (*n* = 6)**	**0.45 mg/kg (*n* = 6)**	**1.35 mg/kg (*n* = 6)**	**4 mg/kg (*n* = 6)**	**12 mg/kg (*n* = 6)**
T_{max} (hours)	0.5 (0.5–4.0)	4.0 (0.5–12.0)	2.3 (0.5–4.0)	0.5 (0.5–4.0)	4.0 (0.5–12.0)	2.3 (0.5–24.0)	2.3 (0.5–4.5)	4.0 (0.5–24.0)
C_{max} (µg/mL)	0.1 (16.2%)	0.4 (40.4%)	1.3 (6.7%)	4.0 (12.4%)	11.8 (10.3%)	34.8 (24.0%)	112.2 (33.8%)	289.7 (17.5%)
t½ (days)	7.1 (33.3%)	13.4 (41.3%)	12.1 (18.6%)	20.8 (26.9%)	23.1 (26.2%)	23.2 (44.7%)	20.3 (17.9%)	22.7 (21.9%)
$AUC_{(0\text{-last})}$ (µg × day/mL)	0.9 (33.9%)	3.1 (31.1%)	17.3 (15.4%)	43.0 (8.9%)	138.5 (11.5%)	453.3 (18.2%)	1263.7 (30.3%)	3337.7 (7.5%)
CL (mL/minutes)	0.30 (33.2%)	0.27 (58.7%)	0.15 (15.5%)	0.11 (19.7%)	0.10 (19.5%)	0.10 (26.5%)	0.12 (25.8%)	0.12 (19.1%)
SECOND KY1005 DOSE	**Not applicable**	**Not applicable**	**Not applicable**	**0.075 mg/kg (*n* = 6)**	**0.225 mg/kg (*n* = 5)**	**0.675 mg/kg (*n* = 6)**	**2 mg/kg (*n* = 6)**	**6 mg/kg (*n* = 5)**
T_{max} (hours)				2.5 (0.5–12.5)	24.0 (4.6–24.0)	2.5 (0.5–24.0)	12.5 (0.5–24.0)	4.6 (0.5–24.0)
C_{max} (µg/mL)				2.9 (20.9%)	9.8 (21.3%)	26.1 (19.3%)	89.6 (31.1%)	229.9 (11.7%)
t½ (days)				27.0 (51.8%)	30.1 (NA)	25.1 (18.3%)	23.4 (32.0%)	24.7 (15.5%)
$AUC_{(0\text{-last})}$ (µg × day/mL)				41.2 (12.2%)	157.6 (12.5%)	380.1 (18.6%)	1314.5 (22.4%)	3833.9 (10.8%)
CL (mL/minutes)				0.11 (27.9%)	0.08 (NA)	0.10 (2.2%)	0.09 (11.6%)	0.11 (21.4%)
THIRD KY1005 DOSE	**Not applicable**	**Not applicable**	**Not applicable**	**0.075 mg/kg (*n* = 6)**	**0.225 mg/kg (*n* = 5)**	**0.675 mg/kg (*n* = 6)**	**2 mg/kg (*n* = 5)**	**6 mg/kg (*n* = 5)**
T_{max} (hours)				4.5 (0.5–12.5)	4.5 (4.5–24.0)	4.5 (0.5–12.5)	24.0 (24.0–24.0)	0.5 (0.5–24.0)
C_{max} (µg/mL)				3.0 (15.7%)	11.5 (16.6%)	28.8 (18.3%)	73.8 (9.9%)	249.6 (14.4%)
$t_{1/2}$ (days)				27.7 (20.5%)	28.3 (24.6%)	43.1 (24.1%)	41.7 (25.9%)	24.3 (17.3%)
$AUC_{(0\text{-last})}$ (µg × day/mL)				53.0 (15.2%)	195.5 (9.9%)	551.9 (8.1%)	1674.8 (10.6%)	4505.2 (9.7%)
CL (mL/minutes)				0.09 (20.3%)	0.07 (21.0%)	0.06 (13.1%)	0.06 (22.9%)	0.10 (35.7%)

Data displayed as mean (coefficient of variation %) and for T_{max} as median (range). $AUC_{0\text{-last}}$, area under the curve from zero point to the last measurable concentration; CL, clearance; C_{max}, peak serum concentration; MAD, multiple ascending dose; NA, no regression line could be fitted; SAD, single ascending dose; t½, half-life; T_{max}, time at which the C_{max} is observed.

FIGURE 2 KY1005 serum concentrations (μg/mL).



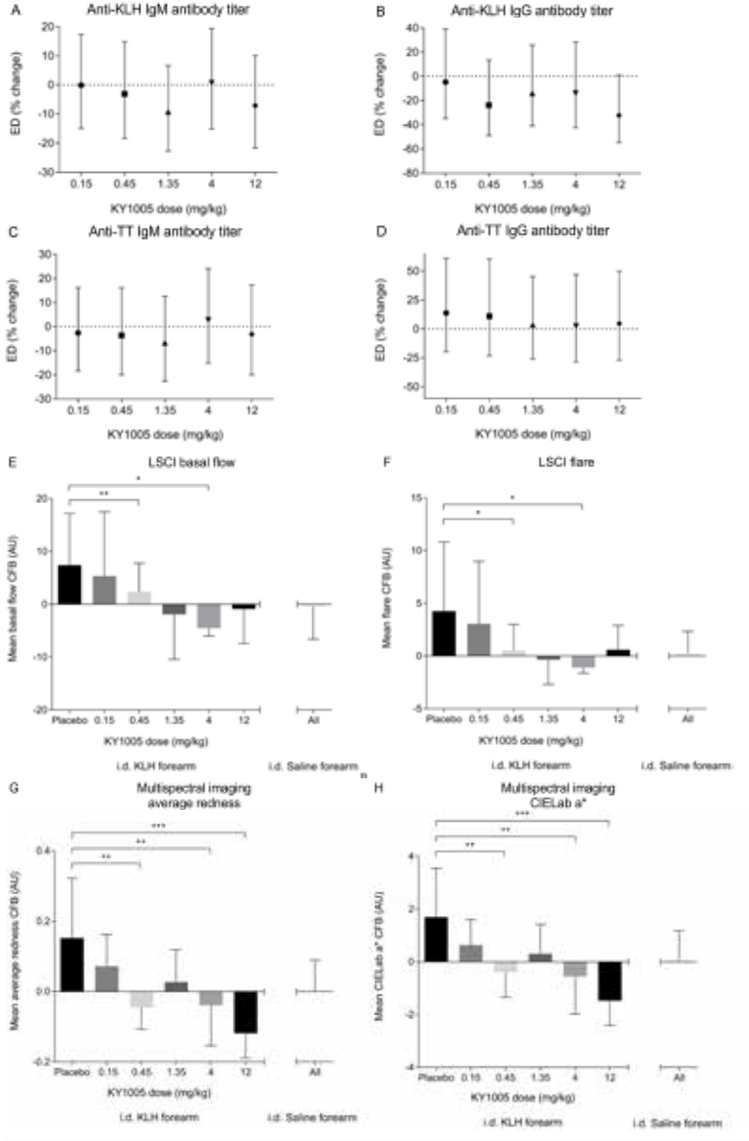
Data displayed on log10 scale as mean (SD)

HUMORAL IMMUNITY TO KLH AND TT

Although no statistical significance was reached, KY1005 treatment seemed to suppress the anti-KLH IgM and IgG antibody response after intramuscular KLH immunization (Figure 3A,B).

FIGURE 3 (A) Anti-KLH IgM and (B) IgG antibody titers, (C) anti-TT IgM, and (D) IgG antibody titers 21 days after KLH and TT immunizations, cutaneous blood perfusion by LSCI (E) basal flow and (F) flare, erythema by multispectral imaging (G) average redness, and (H) CIELab a* 2 days after intradermal KLH administration by treatment group. Data are shown as estimated difference percentage change (95% confidence interval) for A–D and as mean CFB (SD) for E–H. The *P* values are based on estimated differences between groups with correction for baseline measurements and saline administration.

FIGURE 3



** P < .05, ** P < .01, *** P < .001. AU, arbitrary unit; CFB, change from baseline; ED, estimated difference; i.d., intradermal; KLH, keyhole limpet hemocyanin; LSCI, laser speckle contrast imaging; TT, tetanus toxoid.*

KY1005 appeared to have a PD effect from doses of 0.45 mg/kg and above based on the anti-KLH IgG response. The ED between KY1005 and placebo-treated subjects was maximally −32.4% (95% CI −54.7 to 0.9%, $P = .06$) observed for anti-KLH IgG at the highest KY1005 dose of 12 mg/kg (Table 3). No consistent effect of KY1005 on anti-TT IgM and IgG antibodies was observed (Table 3, Figure 3C,D).

EXPOSURE-RESPONSE MODELING OF HUMORAL IMMUNITY TO KLH AND TT

Given the small sample size, post hoc KY1005 exposure-response modeling was performed. This analysis showed a modest treatment effect of KY1005 (Akaike's Information Criteria maximum effect (AIC)E_{max} model < AIC$_{no\text{-}effect\ model}$) on anti-KLH IgM ($E_{max}$ −0.22 AU, 95% CI −0.46 to 0.02 AU) and IgG antibody titers (E_{max} −0.58 AU, 95% CI −1.10 to −0.06 AU), whereas no exposure-response was observed on anti-TT IgM and anti-TT IgG antibody titers (Figure 4A–D), based on the exposure-response model. The 50% of the maximal effect (EC_{50}) could not be reliably determined for any of the variables, likely due to the high variability of the measurements as well as the small sample size.

TABLE 3 Summary statistics for pharmacodynamic endpoints.

	KY1005 dose level				
Pharmacodynamic parameter	**0.15 mg/kg (*n* = 6)**	**0.45 mg/kg (*n* = 5)**	**1.35 mg/kg (*n* = 6)**	**4 mg/kg (*n* = 5)**	**12 mg/kg (*n* = 5)**
Anti-KLH IgM (% change)	−0.1% (−14.9 to 17.4%)	−3.1% (−18.3 to 14.9%)	−9.2% (−22.7 to 6.6%)	0.7% (−15.1 to 19.4%)	−7.1% (−21.7 to 10.1%)
Anti-KLH IgG (% change)	−4.8% (−34.8 to 39.0%)	−23.9% (−49.0 to 13.5%)	−13.9% (−41.0 to 25.7%)	−14.0% (−42.4 to 28.3%)	−32.4% (−54.7 to 0.9%)
Anti-TT IgM (% change)	−2.6% (−18.4 to 16.2%)	−3.6% (−20.0 to 16.1%)	−6.7% (−22.6 to 12.5%)	2.7% (−15.1 to 24.1%)	−3.2% (−20.0 to 17.2%)
Anti-TT IgG (% change)	13.8% (−19.6 to 61.2%)	11.0% (−23.2 to 60.5%)	3.6% (−26.1 to 45.2%)	2.4% (−28.6 to 46.9%)	4.6% (−27.0 to 49.9%)
LSCI basal flow (AU)	−3.3 (−11.3 to 4.7)	−13.4 (−23.0 to −3.8)**	−6.2 (−14.2 to 1.8)	−11.0 (−19.8 to −2.3)*	−5.9 (−14.4 to 2.5)
LSCI flare (AU)	−2.2 (−6.9 to 2.4)	−7.5 (−13.2 to −1.8)*	−4.5 (−9.0 to 0.1)	−5.8 (−10.8 to −0.9)*	−3.1 (−7.9 to 1.8)
Multispectral imaging average redness (AU)	−0.04 (−0.16 to 0.09)	−0.20 (−0.32 to −0.07)**	−0.11 (−0.23 to 0.01)	−0.17 (−0.29 to −0.05)**	−0.23 (−0.35 to −0.11)***
Multispectral imaging CIELab a* (AU)	−0.7 (−2.1 to 0.6)	−2.1 (−3.5 to −0.8)**	−1.2 (−2.5 to 0.1)	−2.0 (−3.4 to −0.7)**	−2.6 (−4.0 to −1.3)***

*Data displayed as estimated difference (95% confidence interval). * P < .05, ** P < .01, *** P < .001. AU, arbitrary unit; KLH, keyhole limpet hemocyanin; LSCI, laser speckle contrast imaging; TT, tetanus toxoid.*

CUTANEOUS BLOOD PERFUSION

Overall, KY1005 reduced the intradermal KLH-driven increase in cutaneous blood perfusion quantified by LSCI basal flow and flare (Table 3, Figure 3E,F). Although a clear dose dependence was absent, pharmacological KY1005 effects on LSCI basal flow and flare based on suppression of skin challenge response were observed at intermediate dose levels of 0.45 mg/kg (ED −13.4 AU, 95% CI −23.0 to −3.8 AU, $P < .01$ and ED −7.5 AU, 95% CI −13.2 to −1.8 AU, $P < .05$, respectively), 4 mg/kg (ED −11.0 AU, 95% CI −19.8 to −2.3 AU, $P < .05$ and ED −5.8 AU, 95% CI −10.8 to −0.9 AU, $P < .05$, respectively), and 12 mg/kg (ED −5.9 AU, 95% CI −14.4 to 2.5 AU, $P = .16$ and ED −3.1 AU, 95% CI −7.9 to 1.8 AU, $P = .21$, respectively; Figure 3E,F). All groups showed a reduced cutaneous blood perfusion response compared with placebo.

ERYTHEMA

KY1005 treatment also reduced erythema quantified by multispectral imaging as average redness and as CIELab a* (Table 3, Figure 3G,H). Similar to the observations with LSCI, multispectral imaging average redness and CIELab a* were decreased in the groups that received KY1005 as initial dose of 0.45 mg/kg (ED −0.20 AU, 95% CI −0.32 to −0.07 AU, $P < .01$ and ED −2.1 AU, 95% CI −3.5 to −0.8 AU, $P < .01$, respectively), 4 mg/kg (ED −0.17 AU, 95% CI −0.29 to −0.05 AU, $P < .01$ and ED −2.0 AU, 95% CI −3.4 to −0.7 AU, $P < .01$, respectively), and 12 mg/kg (ED −0.23 AU, 95% CI −0.35 to −0.11 AU, $P < .001$ and ED −2.6 AU, 95% CI −4.0 to −1.3 AU, $P < .001$, respectively) compared with placebo (Table 3, Figure 3G,H).

EXPOSURE-RESPONSE MODELING OF SKIN CHALLENGE ENDPOINTS

Exposure-response modeling showed a treatment effect of KY1005 (AICE_{max} model < AIC$_{no\text{-}effect\ model}$) on LSCI basal flow ($E_{max}$ −7.09 AU, 95% CI −13.23 to −0.96 AU), LSCI flare (E_{max} −4.77 AU, 95% CI −8.06 to −1.48 AU), multispectral imaging average redness (E_{max} −0.20 AU, 95% CI −0.29 to −0.11 AU) and CIELab a* (E_{max} −2.10 AU, 95% CI −3.05 to −1.15 AU; Figure 4E–H), based on the exposure-response model. The EC_{50} could not be reliably determined for any of the variables, likely due to the high variability of the measurements as well as the small sample size.

DISCUSSION

In this first-in-human study, we showed that KY1005 was safe and well-tolerated and we demonstrated proof-of-pharmacology for KY1005 as the drug suppressed the KLH-driven neoantigen immune response *via* OX40-OX40L signaling interference, despite the observed variability in the skin challenge response.

Importantly, KY1005 treatment in the current study had an unremarkable safety and tolerability profile. 1 hypersensitivity reaction was observed in this study in 1 subject in the 12 mg/kg group that was possibly related to KY1005. An allergy to KY1005 or any excipients was considered unlikely, because this was the first KY1005 exposure and the subject had never received an intravenous administration of any kind.

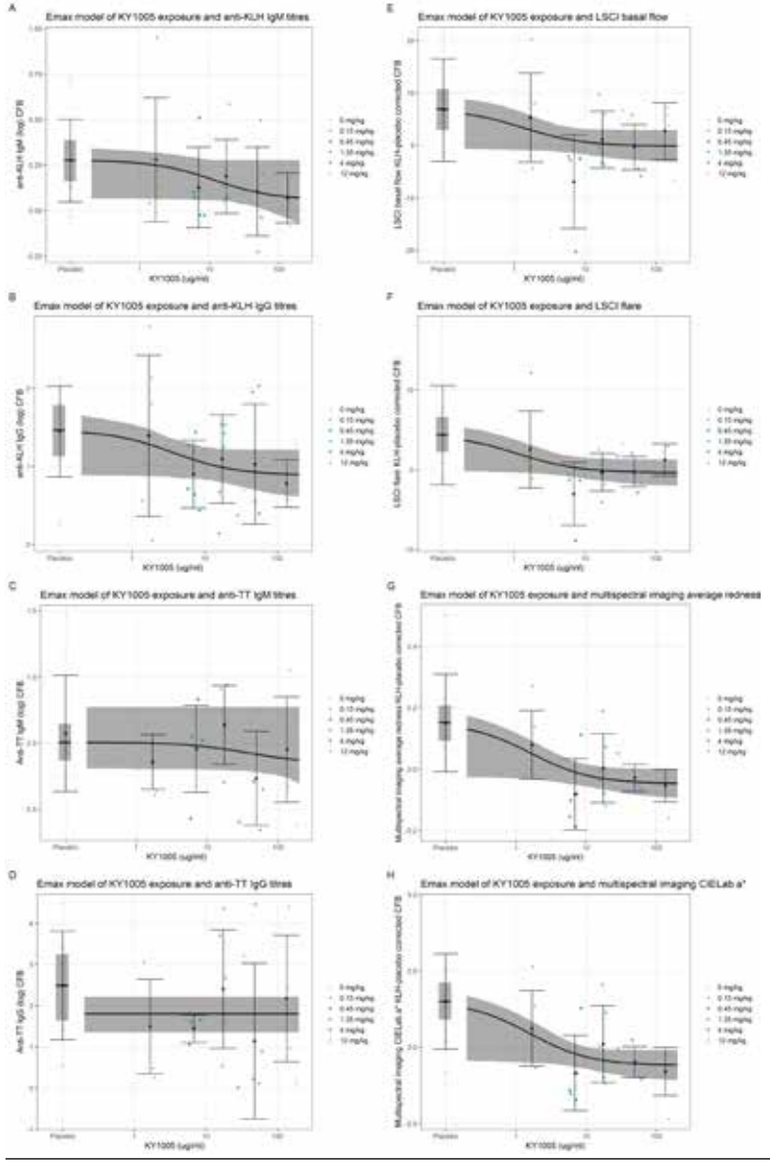
A pseudoallergy might have been the cause of the AEs. This pseudoallergy was classified as grade 1, since no medication was administered and the symptoms resolved spontaneously within 2 hours. No other AE-related discontinuations of KY1005 treatment occurred. AEs observed after monoclonal antibody administration are usually related to infection and immunomodulation.[38] No increase in infection rate after KY1005 treatment was observed compared with placebo, possibly explained by the fact that the OX40-OX40L pathway is primarily involved in sustaining T cell activation and not in the initial stimulation.[7]

PK analyses displayed a nonlinear increase in mean serum concentrations of KY1005. The PK profile of KY1005 displayed nonlinear target-mediated drug disposition (TMDD)[39] as is commonly observed for monoclonal antibodies.[40] At low KY1005 concentrations, a high CL was observed as a large portion of the drug is likely cleared *via* drug-target binding and subsequent degradation of the drug-target complex. Saturation of TMDD presumably led to lower observed CL at higher KY1005 doses. The mean KY1005 t½ of 24.3 days was similar to the expected predicted mean t½ of 26 ± 7 days based on preclinical experiments. ADAs may influence the clearance of monoclonal antibodies. We did not find evidence for ADA-mediated clearance of KY1005, which may reflect no such effect, or an insufficient number of subjects exposed. At approximate concentrations of > 2 mg/mL, KY1005 clearance remained relatively stable, which might indicate TMDD saturation and possibly 100% target binding.

Between 25% and 50% of KY1005-treated participants had positive ADA responses, but this was not associated with unexpected changes in the serum PK indicating, where present, the ADAs were non or only weakly neutralizing. The number of subjects with ADAs increased with increasing KY1005 dose in SAD cohorts. In MAD cohorts, however, the largest number of subjects positive for ADAs was observed at the lowest dose regimen (0.15 mg/kg), with no subjects developing detectable ADAs at the highest dose regimen (12 mg/kg). This observation is in keeping with the pattern expected with increasing suppression of ADA development at higher doses reaching saturation of the target and suppression of antibody response to KY1005.

FIGURE 4 E_{max} model of KY1005 exposure and (A) anti-KLH IgM, (B) anti-KLH IgG, (C) anti-TT IgM, (D) anti-TT IgG, (E) LSCI basal flow, (F) LSCI flare, (G) multispectral imaging average redness, and (H) multispectral imaging CIELab a*. Dots represent individual data points. Black squares (error bars) represent mean (SD) of observed data per dose level. Black line (grey area) represents model predicted mean (90% confidence interval).

FIGURE 4



Data are shown as log10 change from baseline ratios vs. KY1005 concentration. CFB, change from baseline; E_{max}, maximum effect; KLH, keyhole limpet hemocyanin; LSCI, laser speckle contrast imaging; TT, tetanus toxoid.

No statistically significant reduced antibody titers against KLH and TT were observed after KY1005 treatment compared with placebo. The lack of effect and consistency by dose group most possibly reflects the small sample size and normal variability observed with respect to the PD markers. Despite this the observed data indicated moderate pharmacological activity of KY1005 at loading doses of 0.45 mg/kg and above. Importantly, combined individual data of all KY1005 serum concentrations plotted against anti-KLH IgM and IgG antibody titers revealed a modest treatment effect of KY1005. This treatment effect was stronger on anti-KLH IgG compared with IgM possibly explained by the time window of 21 days between baseline and post immunization measurements and class switching between the isotypes. Our results are translationally confirmed by a previously published study performed in mice, which showed that blockade of the OX40-OX40L signaling pathway inhibited T cell-dependent antibody production after KLH immunization.[41] Based on these results, inhibition of OX40L may possibly interfere with T cell-dependent antibody production. Furthermore, maximum effects of KY1005 on anti-KLH antibody titers seem to have been reached based on the concentration-effect models. The recall antigen response to TT is probably not sufficiently suppressed as memory B cells are able to differentiate to plasma cells in the absence of T cells[42] and other pathways besides OX40-OX40L can still be stimulated. Although it is known that T cell-dependent B cell activation requires CD40-CD40L costimulatory factors following T cell receptor-Major Histocompatibility Complex II-peptide binding,[43,44] the exact mechanism and pathways underlying T cell-dependent B cell activation and the role of OX40-OX40L signaling remain to be elucidated. *Ex vivo* antigen rechallenges of lymphocytes isolated from KY1005-exposed volunteers may provide additional insight and improved characterization of immune pathways modulated by OX40-OX40L inhibition.

The KLH skin challenge model used in the present study was previously validated in healthy volunteers using multispectral imaging and LSCI, similar to the methods used in the current study.[30] Various clinical studies have demonstrated that KLH is a potent immunostimulatory antigen, producing a robust immune response, and having an excellent safety profile.[45] Formally, the study was not powered for detection of KY1005 effects on the skin challenge response;[30] the sample size used is common in first-in-human trials and the KLH-based PD skin challenge endpoints were exploratory in nature only. Despite being underpowered, we found that KY1005 suppressed the skin challenge response following intradermal KLH administration as cutaneous blood perfusion and erythema were lower in KY1005-treated subjects compared with placebo. The half maximal inhibitory concentration (IC_{50}) of KY1005 in preclinical *in vitro* experiments was 0.30 ± 0.01 nM (mean ± standard error of the mean). The KY1005 starting dose of 0.006 mg/kg corresponds to a concentration of 1.0 nM and was based on the MABEL principle using *in vitro* data obtained in human MLR experiments. Substantial decreases in cutaneous blood perfusion and erythema as a result of skin challenge response were initially observed at a KY1005 dose of 0.45 mg/kg, which corresponds to a concentration of 75 nM, a 250-fold higher dose compared to the IC_{50}. In contrast to KY1005's effects on anti-KLH antibodies, the effects on the skin challenge response were seen to be dose-dependent. Accordingly, exposure-response analyses displayed a treatment effect of KY1005 on all skin challenge endpoints (LSCI basal flow and flare and multispectral imaging average redness and CIELab a* values).

T cell-dependent immune responses are complex to monitor and to modulate. Therefore, a successful translation of the observed PD effects of KY1005 (suppression of the KLH-driven responses in healthy volunteers) to clinical effects in patients with immune-mediated diseases is challenging. However, the unremarkable safety and tolerability profile of KY1005 combined with the observed immunomodulatory properties support the potential of KY1005 as a novel compound targeting the OX40-OX40L signaling pathway for immune-mediated disorders. Based on the data generated in the present study, a successful phase IIa trial of KY1005 has recently been completed in patients with atopic dermatitis and a phase IIb trial is planned.

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CHAPTER V

IMPACT OF ORAL ADMINISTRATION OF SINGLE-STRAIN *LACTOCOCCUS LACTIS* SPP. *CREMORIS* ON IMMUNE RESPONSES TO KEYHOLE LIMPET HEMOCYANIN IMMUNIZATION AND GUT MICROBIOTA: A RANDOMIZED PLACEBO-CONTROLLED TRIAL IN HEALTHY VOLUNTEERS

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ABSTRACT

Introduction: *Lactococcus lactis* spp. *cremoris* has been associated with promising immunomodulatory results in preclinical trials. The aim of this study was to investigate the pharmacodynamic (PD) effects of 3 monoclonal microbial formulations of *L. lactis* spp. *cremoris* (EDP1066) on the immune response to keyhole limpet hemocyanin (KLH). Potential effects on the gut microbiota were also investigated.

Methods: The trial was registered on Netherlands Trial Register (trial ID NL7519, https://trialsearch.who.int). 81 healthy subjects (median 28, range 18–59 years) were randomized to 28 days of enteric-coated capsules at 5 doses (n = 13) (1.5×10^{12} total cells daily), freeze-dried powder at 1 dose (n = 12) (3.0×10^{11} total cells daily) or 5 doses (n = 12), minitablets at 1 dose (n = 12) or 5 doses (n = 12), or placebo (n = 20) prior to KLH immunization. Antibody responses and circulating regulatory T cells (Tregs) were measured after KLH immunization, and skin responses were evaluated after a KLH rechallenge by laser speckle contrast imaging and multispectral imaging. *Ex vivo* lymphocyte (phytohemagglutinin) and monocyte (lipopolysaccharide; LPS) cytokine release assays were explored in the minitablet-treated groups only. The prevalence of *L. lactis* spp. *cremoris* in the gastrointestinal tract and the impact on the fecal microbiota were assessed by qPCR and 16S rRNA sequencing, respectively.

Results: Repeated-measures analysis of covariances revealed no significant treatment effects on the antibody responses to KLH, number of Tregs, or KLH skin rechallenge outcomes. *Ex vivo* LPS-driven cytokine responses in whole blood were lower in the low dose minitablet group compared to placebo: tumor necrosis factor (estimated difference (ED) from placebo: −44.2%, 95% confidence interval (CI) −65.3 to −10.3%), interleukin (IL)-1β (ED −41.4%, 95% CI −63.5 to −5.8%), and IL-6 (ED −39.2%, 95% CI −56.8 to −14.5%). The fecal presence of *L. lactis* spp. *cremoris* increased during treatment by all EDP1066 formulations and normalized 5 days after the last dose. Microbiome α-diversity did not change by the treatments compared to placebo.

Conclusion: The EDP1066 formulations did not affect the immune response to KLH immunization in healthy individuals. However, exposure to *L. lactis* spp. *cremoris* in minitablet formulation impacted *ex vivo* whole blood LPS cytokine response. The clinical impact of these effects awaits further investigations.

INTRODUCTION

Over the past decades, evidence has emerged for an interplay between the systemic immune system and the intestinal microbiome.[1-3] The epithelium of the intestinal wall contains immune cells throughout, including in aggregated lymphoid nodules (Peyer's patches), and the lamina propria and linked mesenteric lymph nodes.[1,4] Regional specialization of the gut immune network has been thoroughly studied in mice with differences found in antigenic composition, leukocyte populations, and gut-associated lymphoid tissue (GALT).[1] Although less evident, similar observations have been made in humans. The mucosa of the intestinal wall is also home to an abundance of microorganisms, and the composition and distribution of the microbial populations are dependent on the location within the gastrointestinal (GI) tract.[1] Alterations in either the intestinal immune system or the gut microbiome can lead to various ailments such as celiac disease and inflammatory bowel disease.[1,5-7] Importantly, there is a growing body of evidence that hypothesizes that the effects of intestinal dysbiosis are not limited to local immunity and can also modify the immune response more distally as observed in systemic lupus erythematosus,[8] rheumatoid arthritis,[9] psoriasis,[10] and more.[11,12] Altering the intestinal microbiota in these patient populations with intestinal dysbiosis, therefore, seems a plausible approach to evoke systemic immune modulation and consequently treat diseases associated with dysregulated immune responses. This hypothesis has been tested in more recent trials with orally administered probiotics (live microorganisms, when administered in adequate amounts, confer a health benefit on the host),[13,14] prebiotics (non-digestible carbohydrates used as nutrients for probiotics), and/or synbiotics (blend of probiotics and prebiotics), which seem to have beneficial effects on dysregulated systemic immune responses,[15-19] with some exceptions.[15] Intake of certain probiotics has also been found to increase the responses to certain vaccinations (e.g., influenza) in humans depending on the choice, strain, dose, etc., of probiotics and vaccine type, dose, timing, and route.[20] Interestingly, oral probiotics have also been demonstrated to be effective for the treatment of topical skin conditions, such as atopic dermatitis, acne, and rosacea,[21] indicating induction of immune regulators. How oral administration of probiotic bacteria can modulate systemic immune responses and T cell-mediated inflammation in remote skin tissue is however unclear. Furthermore, studies using microbial strain mixtures suggest different immunomodulatory effects or even antagonism between species when compared with single-strain microbes, complicating the understanding of the underlying mechanisms.[22-25]

One such single-strain microbial intervention is EDP1066, prepared from *Lactococcus lactis* spp. *cremoris* identified from powders used in dairy product

manufacturing. Preclinical data of EDP1066 on both *in vitro* immune cell cultures and *in vivo* murine immune challenge and disease models show promising results; however, these data are not currently available in the public domain. In separate independent research, *L. lactis* spp. *cremoris* restored T cell impairment in aged mice,[26] and coadministration of *L. lactis* spp. *cremoris* with *Lactobacillus paracasei* spp. *paracasei* relieved atopic dermatitis symptoms, decreased serum immunoglobulin (Ig)E concentration, and rebalanced the population of T helper 1 (Th1)/Th2 cells in an atopic dermatitis mouse model.[27]

Keyhole limpet hemocyanin (KLH) is a metalloprotein derived from the hemolymph of the marine mollusk, *Megathura crenulata*, which can be found in the Pacific coastal waters of California and Mexico. As the human body is unfamiliar with KLH, an *in vivo* immune response to this protein can be used to "mimic" an immune response to a pathogen or allergen in healthy volunteers (such as KLH-specific antibody formation and increased T cell response after intradermal KLH rechallenge), providing essential information on proof-of-pharmacology during early-phase drug development.[28-34] KLH was clinically introduced in 1967 to study the immunocompetence of humans[35] and since then is proven to be safe and widely used in clinical trials.[28-31,36-41]

The primary aim of the present study was to characterize the pharmacodynamic (PD) effects of EDP1066 on the systemic immune response to an intramuscular immunization with KLH and secondary to evaluate the effects on a subsequent KLH skin rechallenge. Because the exposure sites within the GI tract for ingested microbes may depend on the formulation and therefore be important for the immunomodulatory effect,[1,42] we also aimed at comparing different EDP1066 formulations (enteric-coated capsules, free freeze-dried powder, and minitablets) having different expected peak exposure sites. Furthermore, EDP1066 effects on numbers of circulating regulatory T cells (Tregs) were evaluated, and the *ex vivo* immunomodulatory activity of EDP1066 was explored by whole blood stimulation with the Toll-like receptor 4 ligand lipopolysaccharide (LPS) and phytohemagglutinin (PHA) for monocyte and lymphocyte stimulation, respectively. Finally, we aimed at assessing the impact of EDP1066 on the fecal microbiota, next to routine safety and tolerability assessments.

METHODS

ETHICS

The independent Medical Ethics Committee *Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek* (Assen, the Netherlands) approved the study prior to any clinical study activity. All subjects provided written informed consent before participation. The trial was registered on the Netherlands Trial Register, currently available for consultation at the International Clinical Trial Registry Platform (trial ID NL7519, https:// trialsearch.who.int).

SUBJECTS

Healthy male and female participants were recruited *via* media advertisements and from the subjects' database of the Centre for Human Drug Research (CHDR), Leiden, the Netherlands. Enrolled participants were 18 to 60 years of age with a body mass index between 18 and 35 kg/m^2 and without previous exposure to KLH. Health status was verified by recording a detailed medical history, a complete physical examination, vital signs, a 12-lead electrocardiogram (ECG), and laboratory testing (including hepatic and renal panels, complete blood count, fecal calprotectin, virology, and urinalysis). Subjects were excluded in case of any disease associated with immune (e.g., active infection, auto-immune disease, primary or acquired immune deficiency, and clinically profound allergies) or GI system impairment (e.g., short bowel syndrome, diarrhea, inflammatory bowel disease, irritable bowel syndrome, and celiac disease) or use of prescription medication within 4 weeks prior to the first dose. Other exclusion criteria were antibiotic treatment within 42 days prior to initial dosing and during the course of the study and the use of probiotic capsules within 14 days of screening and during the course of the study.

DOSE SELECTION AND REGIMEN

All EDP1066 and placebo formulations were manufactured and provided by Evelo Biosciences Inc. (Cambridge, MA, USA). The doses tested were based on the results of a separate first-in-man study.[43] The highest dose tested contained 1.5×10^{12} total cells per dose, approximately 5 times the predicted therapeutic dose level, calculated from allometric scaling of the preclinically efficacious dose level based on conversion between mouse and human gut surface area. This dose was well tolerated in humans. Three different formulations of the investigational drug were investigated: enteric-coated capsules containing EDP1066 freeze-dried powder, EDP1066 as free freeze-dried powder, and noncoated capsules containing enteric-coated EDP1066 minitablets. For each EDP1066 formulation, matching placebo formulations were used in order to preserve the blinding. The 3 placebo formulations contained similar excipients as their active treatment counterparts, without the EDP1066 microbes. The excipients present in the 3 EDP1066/placebo formulations (e.g., microcrystalline cellulose, magnesium stearate, mannitol, citric acid, and sodium hydroxide) are widely used in drug product manufacturing, and none of the excipients were expected to elicit immune system modulation.

STUDY DESIGN AND TREATMENTS

This was a phase 1, randomized, placebo-controlled, double-blind, multiple-dose study in 80 healthy volunteers performed at the CHDR based on the principles of the Declaration of Helsinki. An overview of the study design is shown in Table 1. Participants were randomized to 1 out of the 5 groups of EDP1066 or placebo (12:4 per group) in a consecutive order starting with the lowest number. The randomization code was computer-generated by a study-independent statistician and was only made available for data analysis after study completion. 1 group received EDP1066 freeze-dried powder in enteric-coated capsules, supplied as 1.5×10^{11} total cells per capsule, administered orally at a dose of 10 capsules daily (5× Capsules). 2 other groups received EDP1066 as free freeze-dried powder with an achlorhydria regimen administered orally at a dose of 3.0×10^{11} (1× Powder) and 1.5×10^{12} (5× Powder) total cells daily. The achlorhydria regimen consisted of omeprazole 40 mg and aluminum hydroxide/magnesium hydroxide 200/400 mg administration 3 hours prior to each EDP1066 dose. Both drugs increase the gastric pH[44-46] and were expected to improve the transition of EDP1066 through the stomach and into the duodenum. Omeprazole and aluminum hydroxide/magnesium hydroxide are not known to induce immune system modulation. Another 2 groups received noncoated capsules containing enteric-coated EDP1066 minitablets, supplied as 1.5×10^{11} total cells per capsule, administered orally at a dose of 2 (1× Minitablets) and 10 (5× Minitablets) capsules daily. Participants were dosed once daily for 28 consecutive days. Compliance was confirmed by the supervised administration of the study treatment during the in-clinic period. Administration at home was recorded by an electronic diary. Intramuscular KLH immunization was performed in the left deltoid muscle after the completion of the third administration of EDP1066/placebo. KLH immunization was administered as 0.1 mg of Immucothel (Biosyn Corporation, Carlsbad, CA, USA) adsorbed in 0.9 mg of aluminum hydroxide (Alhydrogel, Brenntag AG, Essen, Germany) into 0.5 mL of NaCl 0.9% as previously described.[47] All subjects were administered KLH (0.001 mg of Immucothel) and saline in 0.1 mL of NaCl 0.9% intradermally in the left and right ventral forearms, respectively, 23 days after KLH immunization. The skin challenge response was quantified prior to and 2 days after intradermal KLH administration. These are similar intervals between assessments as in our previous studies, which also detail the methodology.[29,32,36,38,41,47,48] To account for ambient and environmental factors, the responses observed at the intradermal KLH administration site were corrected against the intradermal saline administration site on the contralateral forearm. A follow-up visit 5 days after the last EDP1066/placebo dose and a study discharge visit 12 days after the last EDP1066/placebo dose were included in order to assess EDP1066 stool persistence and prevalence and EDP1066 effects on the gut microbiome.

TABLE 1 Study timeline.

Timepoint	Treatment									FU
Activity	D −1	D 1	D 3	D 5	D 10	D 17	D 26	D 28	D 33	D 40
EDP1066 / placebo administration	<---- ---------- ONCE DAILY---------->									
KLH immunization			×							
Anti-KLH IgM and IgG			×		×	×	×			×
Tregs + *ex vivo* stimulation assays		×	×	×	×		×			×
Intradermal KLH administration							×			
Intradermal KLH readout (LSCI, MI)								×		
Fecal EDP1066 concentration	×						×		×	
Fecal microbiome	×								×	×
Admission	<---------->									

× *indicates performed activity. D, day; KLH, keyhole limpet hemocyanin; Tregs, regulatory T cells; LSCI, laser speckle contrast imaging; MI, multispectral imaging; FU, follow-up.*

HUMORAL IMMUNITY TO KEYHOLE LIMPET HEMOCYANIN

The humoral response to KLH immunization was measured by anti-KLH IgM and IgG serum titers. Serum samples for the analysis of anti-KLH IgM and IgG were obtained in non-additive tubes by venipuncture at the time points indicated in Table 1. Samples were centrifuged at 2,000 g for 10 minutes with a temperature of 2–8°C, and the serum was aliquoted. The aliquots were stored at a temperature of −40 °C until shipment and analysis. Samples were assessed by quantitative enzyme-linked immunosorbent assay (ELISA) for anti-KLH IgM and IgG as previously described (ELISA developed in-house by Ardena Bioanalytical Laboratory (Assen, the Netherlands)).[47] For the analysis of human antibodies raised against KLH, no reference material was available for the preparation of calibration standards and quality checks. Quantitative measurement of human anti-KLH IgG and IgM (in µg/mL) using a standard curve was not an option. Therefore, the mean optical density of baseline samples was set to 1.00, and relative ratios were calculated for all subsequent samples.

CUTANEOUS BLOOD PERFUSION AND ERYTHEMA

Cutaneous blood perfusion quantification was performed with laser speckle contrast imaging (LSCI; PeriCam PSI System, Perimed AB, Järfälla, Sweden), and erythema quantification was performed with multispectral imaging (Antera 3D, Miravex, Dublin, Ireland) as previously described.[47] Circular regions of interest at the intradermal injection sites were defined. Cutaneous blood perfusion (indicated as basal flow) was quantitatively assessed and expressed in arbitrary units (AUs). The homogeneity of cutaneous blood perfusion in the region of interest (indicated as flare), expressed as

values that are +1 standard deviation (SD) from the mean basal flow within the region, was also quantitatively assessed and expressed in AUs. Erythema was quantified using the average redness and CIELab a* Antera 3D software modalities expressed as AUs. The average redness modality displays the distribution of redness using an internal software algorithm, and the CIELab a* value, which is part of the CIELab color space, expresses color as a numerical value on a green–red color scale.

CIRCULATING REGULATORY T CELLS AND EX VIVO STIMULATION ASSAYS

The percentage of circulating Tregs was evaluated by flow cytometry. Venous blood was collected in sodium heparin tubes by venipuncture at the time points indicated in Table 1. Red blood cell (RBC) lysis was performed on heparinized whole blood using RBC lysis buffer (Thermo Fisher, Waltham, MA, USA). Leukocytes were stained with fluorochrome-labeled antibodies CD4-VioBlue, CD25-APC, and CD127-PE; propidium iodide was used as viability dye (all Miltenyi Biotec, Bergisch-Gladbach, Germany). Samples were analyzed on a MACSQuant 16 analyzer (Miltenyi Biotec) using FlowLogic software (Inivai, Mentone, VIC, Australia). Tregs were defined as $CD4^+CD25^+CD127^-$; see Figure S1 for the gating strategy. *Ex vivo* lymphocyte and monocyte cytokine release assays were incorporated later in the study to examine nuclear factor (NF)-κB-driven responses and only performed in the minitablet-treated groups in which the most optimal immunomodulatory results were expected based on preclinical data. Sodium heparinized whole blood was incubated with 10 μg/mL of PHA (Sigma-Aldrich, Deisenhofen, Germany) or 2 ng/mL of LPS (strain O111:B4 from *Escherichia coli*, Sigma-Aldrich) for 24 hours at 37 °C, 5% CO_2. After 24 hours, the supernatant was collected, and cytokines were measured using qualified ELISA-based assays by Ardena Bioanalytical Laboratory. Interferon gamma (IFN-γ) and interleukin (IL)-2 were measured in the PHA-stimulated samples; tumor necrosis factor (TNF), IFN-γ, IL-1β, IL-6, and IL-8 were measured in the LPS-stimulated samples.

EDP1066 STOOL PERSISTENCE AND GUT MICROBIOME

Fecal concentrations of EDP1066 for stool persistence and prevalence and the gut microbiome were measured by Diversigen Inc. (Houston, TX, USA) using validated bioanalytical assay methods. In short, fecal microbial DNA was extracted based on the Zymo Research (Irvine, CA, USA) fecal DNA extraction methodology. EDP1066-specific primers and probes had been developed to enable the detection of the *L. lactis* spp. *cremoris* strain. The fecal samples were analyzed using a qPCR with a lower limit of quantification of 5.0 copies/5 ng DNA. For gut microbiome analyses, extracted DNA was prepared for Illumina sequencing *via* PCR amplification of the variable region 4 of the bacterial 16S rRNA gene. After PCR purification using AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, IN, USA), sample-specific barcodes using Illumina Nextera XT Index kit (Illumina Inc., San Diego, CA, USA) were appended to the PCR products during a second PCR. The PCR products were purified for a second time, and lastly, the PCR products were equimolarly pooled and sequenced on the Illumina MiSeq platform using the MiSeq v3 sequencing kit.

SAFETY AND TOLERABILITY

Safety and tolerability were monitored by physical examination, assessment of vital signs, laboratory parameters (i.e., full blood count, biochemistry, serology, immunophenotyping, circulating cytokines, fecal calprotectin, and urinalysis), and ECG data from 12-lead ECGs at regular intervals. Subjects were monitored continuously for adverse events (AEs). Participants were also asked to daily complete the Bristol Stool Scale (BSS) and questions regarding defecation patterns using an electronic diary app in order to obtain insight into the participants' stool patterns at the time of fecal sample collection.

STATISTICS

The sample size was based on previously performed power calculations on KLH challenge endpoints.[47] In order to detect a 75% inhibition of the KLH-specific antibody response, cutaneous blood perfusion response (LSCI), and erythema response (multispectral imaging), a sample size of 12 per group was required using a parallel study design, with an α of .05 and a power of 80%. It was deemed appropriate to pool the placebo-treated participants for analyses in order to increase the statistical power. Demographic and baseline variables were summarized by treatment. PD endpoints measured at multiple time points after baseline were analyzed with a mixed-effects repeated-measures model with fixed factors treatment, time and treatment by time, random factor subject, and the baseline value as covariates. Endpoints with one post dose measurement were analyzed with a linear model with treatment as a fixed factor. Anti-KLH antibody parameters were analyzed without baseline as a covariate. Skin re-challenge endpoints were analyzed with an analysis of covariance with treatment as a fixed factor and the baseline and the change from baseline (CFB) of the saline-injected control added as covariates. Anti-KLH IgM and IgG titers and *ex vivo* monocyte cytokine release assays required log transformation. The general treatment effect and specific contrasts were reported as the estimated difference (ED) with a 95% confidence interval (CI) and graphically as ED with 95% CI, as least squares mean (LSM) with 95% CI, or as mean with SD. Fecal EDP1066 concentration was reported graphically as median with range. Fecal microbiome endpoints were analyzed using Python (Python

Software Foundation, Wilmington, DE, USA) by Diversigen Inc. Read count and relative abundance tables were calculated at the genus level and retrieved using custom Python scripts and the One Codex Python library, an in-house curated database of bacterial marker genes including 16S rRNA. The relative abundances of all microorganisms at the genus level were calculated to present the occurrence of the *Lactococcus* genus relative to all microbial DNA in the samples. Diversity trend analysis was performed using the Shannon diversity index. The Shannon diversity index was calculated for all samples using the One Codex Python library. Results were aggregated and plotted using custom Python scripts. To determine whether some genera were more or less abundant in placebo *vs.* EDP1066 treated individuals, read count tables were fed to ANCOM, a statistical framework for the analysis of microbiomes. Fecal microbiome diversity was reported graphically as median with an interquartile range.

RESULTS

BASELINE CHARACTERISTICS

The study was conducted between February 2019 and January 2020. 95 subjects were enrolled in the study of which 81 were treated (Figure 1). A total of 76 subjects completed the treatment and the follow-up period. 5 subjects did not complete the study. 1 subject was withdrawn due to a possible hypersensitivity reaction to EDP1066. Due to very limited EDP1066 exposure (2 doses) and collected data, it was decided to replace this subject. The withdrawal in the other 4 was unrelated to the study drug or procedures (emergency dental procedure (1), tetanus vaccination and antibiotics treatment (1), and consent withdrawal (2)). The baseline characteristics of all treatment groups are presented in Table 2. Treatment compliance was 99.4% in subjects who completed the treatment and follow-up period (range number of days EDP1066 intake 26–28 days). 9 subjects missed 1 dosing day, and 2 subjects missed 2 dosing days.

HUMORAL IMMUNITY TO KEYHOLE LIMPET HEMOCYANIN AND CUTANEOUS BLOOD PERFUSION AND ERYTHEMA

No statistically significant treatment or formulation effects were observed on the humoral KLH challenge outcomes. Observations closest to the desired treatment effect were lower anti-KLH IgG (Figure 2, ED −16.8%, 95% CI −35.5 to 7.3%, $P = .15$) and IgM (Figure 2, ED −16.8%, 95% CI −31.8 to 1.4%, $P = .07$) levels in the 5× Minitablets group compared to placebo, not reaching a level of statistical significance. No statistically significant treatment or formulation effects were observed on the KLH skin rechallenge outcomes (Figure 3).

FIGURE 1 CONSORT 2010 flow diagram of the progress through the phases of the trial.

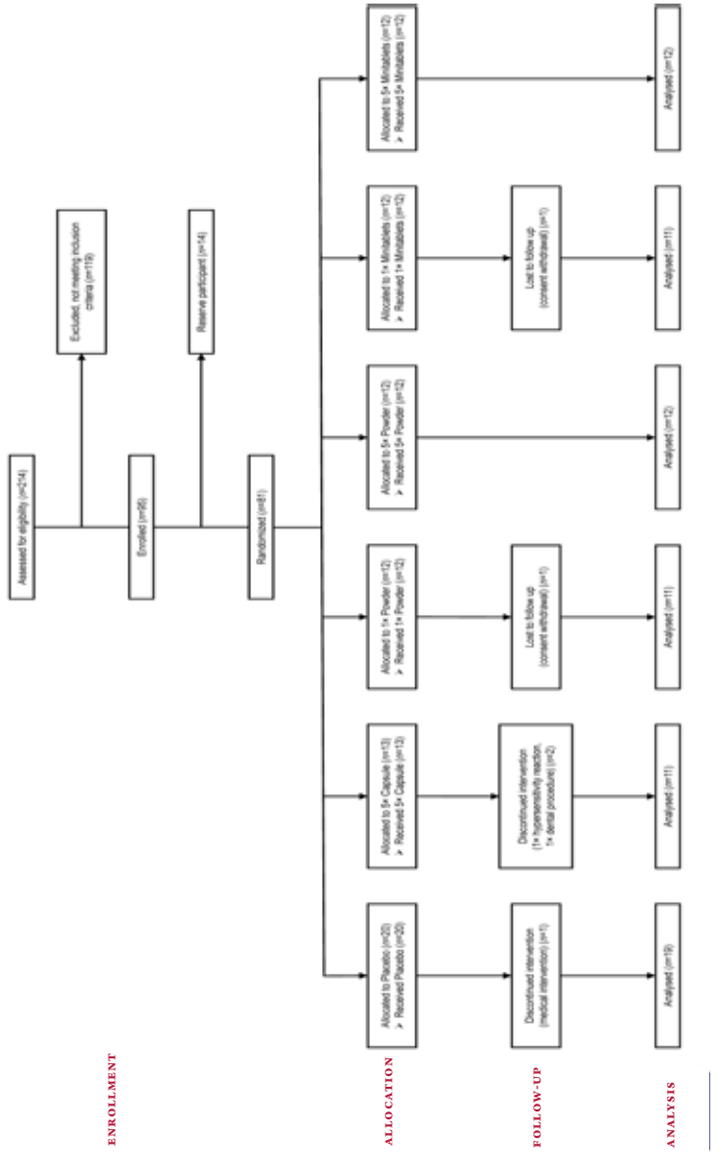
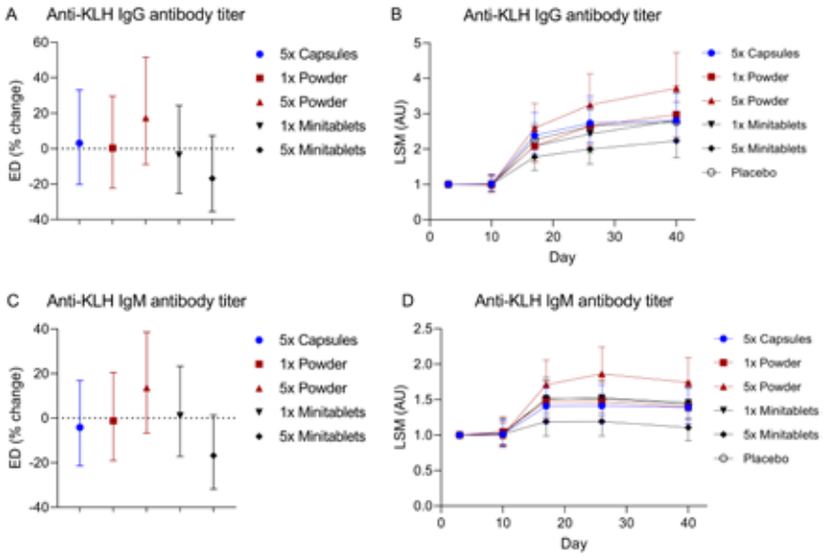


FIGURE 2 Anti-keyhole limpet hemocyanin (A,B) IgG and (C,D) IgM antibody titers by EDP1066 treatment group. Data are shown as estimated difference with 95% confidence interval expressed as percentage of placebo in panels (A,C) and as least square means with 95% confidence interval in panels (B,D) The estimated difference was calculated with a mixed-effects repeated-measures model with fixed factors treatment, time and treatment by time, and random factor subject as covariate.



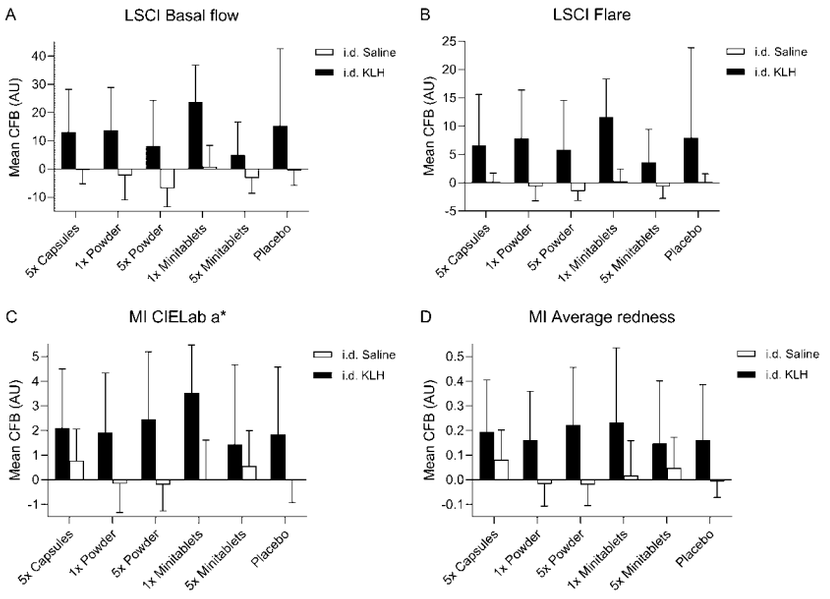
KLH, keyhole limpet hemocyanin; ED, estimated difference; LSM, least square means.

TABLE 2 Baseline characteristics.

EDP1066 formulation	Enteric-coated capsules	Free powder	Minitablets			Placebo
Daily dose	1.5×10^{12} (5×) total cells	3.0×10^{11} (1×) total cells	1.5×10^{12} (5×) total cells	3.0×10^{11} (1×) total cells	1.5×10^{12} (5×) total cells	
	n = 13	*n* = 12	*n* = 12	*n* = 12	*n* = 12	*n* = 20
DEMOGRAPHICS						
Age (years)	30 (18–59)	26 (19–58)	29 (20–59)	25 (18–56)	51 (22–56)	26 (19–59)
BMI (kg/m^2)	24.5 (3.8)	23.7 (2.7)	26.0 (4.4)	21.9 (3.0)	22.0 (2.6)	24.3 (3.7)
Male gender (*n*)	9 (69.2%)	6 (50.0%)	8 (66.7%)	6 (50.0%)	6 (50.0%)	10 (50.0%)
VITAL SIGNS						
Systolic blood pressure (mmHg)	117 (13)	111 (9)	118 (11)	109 (13)	110 (10)	110 (8)
Diastolic blood pressure (mmHg)	68 (10)	65 (9)	69 (9)	64 (10)	66 (8)	64 (6)
Heart rate (BPM)	60 (12)	56 (9)	59 (8)	62 (8)	61 (5)	56 (7)
Temperature (°C)	36.4 (0.3)	36.5 (0.3)	36.5 (0.3)	36.6 (0.3)	36.4 (0.5)	36.5 (0.5)
LABORATORY TESTS						
Leucocytes (×10^9/L)	7.47 (2.00)	7.00 (1.50)	7.55 (1.79)	7.27 (2.42)	7.35 (1.34)	6.71 (1.53)
Lymphocytes (×10^9/L)	2.25 (0.49)	2.22 (0.55)	2.46 (0.50)	2.38 (0.81)	2.55 (0.73)	2.35 (0.77)
Thrombocytes (×10^9/L)	276.7 (61.3)	254.8 (49.3)	251.9 (35.4)	230.8 (48.7)	253.5 (64.0)	259.3 (46.4)
ALT (IU/L)	21.4 (6.7)	24.4 (8.9)	24.6 (10.4)	20.6 (13.0)	25.6 (11.3)	19.8 (7.7)
AST (IU/L)	20.3 (3.3)	22.4 (7.4)	23.9 (9.2)	20.1 (4.6)	25.7 (5.2)	20.4 (5.9)
CRP (mg/L)	1.05 (1.34)	1.56 (2.36)	1.41 (1.43)	1.58 (2.05)	0.53 (0.50)	1.61 (1.70)
Fecal calprotectin (µg/g)	34.0 (36.2)	11.0 (12.9)	9.0 (11.9)	17.4 (14.6)	16.8 (11.9)	18.0 (15.3)

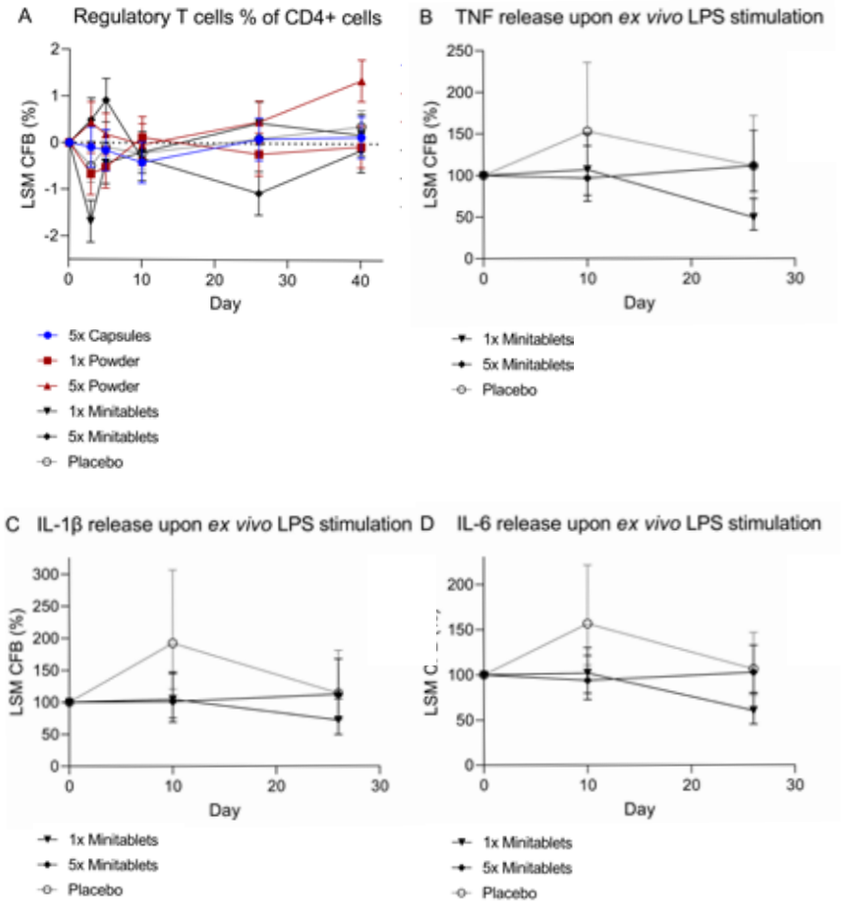
Parameters are shown as mean (standard deviation), age as median (range), and male gender as count (percentage). ALT, alanine transaminase; AST, aspartate transaminase; CRP, C-reactive protein; BMI, body mass index.

FIGURE 3 Cutaneous blood perfusion by LSCI (A) basal flow and (B) flare, erythema by multispectral imaging (C) CIELab a*, and (D) average redness after intradermal KLH and saline administration by 3 EDP1066 formulations of *Lactococcus lactis* spp. *cremoris*: i) enteric-coated capsules, ii) freeze-dried powder (dose 1× and 5×), and iii) minitablets (dose 1× and 5×). The average redness modality displays the distribution of redness using an internal software algorithm and the CIELab a* value, which is part of the CIELab color space, expresses color as a numerical value on a green–red color scale.



Data are shown as mean change from baseline with standard deviation. LSCI, laser speckle contrast imaging; MI, multispectral imaging; KLH, keyhole limpet hemocyanin; CFB, change from baseline; AU, arbitrary unit; i.d., intradermal.

FIGURE 4 (A) Circulating regulatory T cells as percentage of $CD4^+$ T cells from heparinized blood. Monocyte cytokine release assay of (B) tumor necrosis factor, (C) interleukin-1β, and (D) interleukin-6 release from whole blood cultures after *ex vivo* lipopolysaccharide stimulation. X-axis represents number of days after initial EDP1066 dose.



Data are shown as least squares mean change from baseline with 95% confidence interval. LSM, least squares mean; CFB, change from baseline; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin.

EDP1066 STOOL PERSISTENCE AND GUT MICROBIOME

L. lactis spp. *cremoris* was detected in all actively treated groups in 64–73% of subjects on study day 26. Levels returned toward baseline 5 days after the last EDP1066 dose (Figure 5). Dosing by 5× Capsules formulation resulted in the detection of fecal *L. lactis* spp. *cremoris* in all subjects on study day 26 (Figure 5). *Lactococcus* genera were represented only in trace amounts in all samples (Figure S2). The maximum number of *Lactococcus* reads detected in any of the subjects was approximately 500, which corresponds to 0.6% of the total classified 16S reads. These results suggest that EDP1066 did not colonize the gut of any of the participants. Microbiome diversity (Shannon diversity index) was comparable among time points and treatment groups, albeit some changes could be observed on individual levels for a subset of the participants (data not shown). Overall microbiome diversity seemed to be slightly lower in EDP1066-treated samples; however, many of these differences probably occurred due to the small sample size when calculations were performed for individual groups. When Shannon diversity indices were aggregated across all the groups, the mean Shannon diversity was very stable between time points and treatment groups (Figure 6). The 10 most abundant genera were very stable between EDP1066- and placebo-treated subjects (Figure S3). There was some variation in relative abundance, but no large or consistent shifts were seen across all groups. Variation was most likely due to individual differences in microbiome composition between subjects and not dependent on treatment.

SAFETY AND TOLERABILITY

Overall, no major safety concerns were observed during the study. No serious adverse events occurred. Most AEs were related to the GI tract (93 AEs in 46 subjects) with no distinction between EDP1066 and placebo treatment (Table S1). 1 subject was withdrawn from further treatment after the second EDP1066 dose due to a possible hypersensitivity reaction to EDP1066 consisting of a mild burning sensation and itch of the throat lasting approximately 6 hours. No abnormalities were found upon physical examination and additional vital sign measurements. Due to the mild and limited nature of the AEs, no further diagnostics were conducted. No earlier hypersensitivity AEs after EDP1066 administration had been reported. The subject also did not report any allergies to cheese or other dairy products. Allergic reactions to excipients used in the 5× Capsule formulation (microcrystalline cellulose, magnesium stearate, and colloidal silicon dioxide) have been reported before; however, these are very rare.[49-51] Placebo-treated subjects had slightly fewer AEs (75%) compared to EDP1066-treated subjects (83.3–91.7%). No clinically significant changes were observed in laboratory parameters, vital signs, ECG recordings, and the BSS and feces questionnaire.

FIGURE 5 Fecal concentration of *Lactococcus lactis* spp. cremoris measured by quantitative polymerase chain reaction. Data are shown as median with range. qPCR, quantitative polymerase chain reaction.

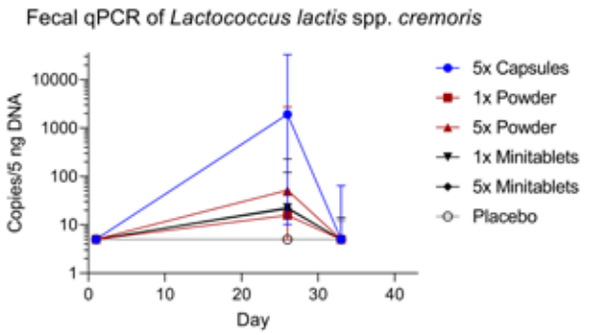
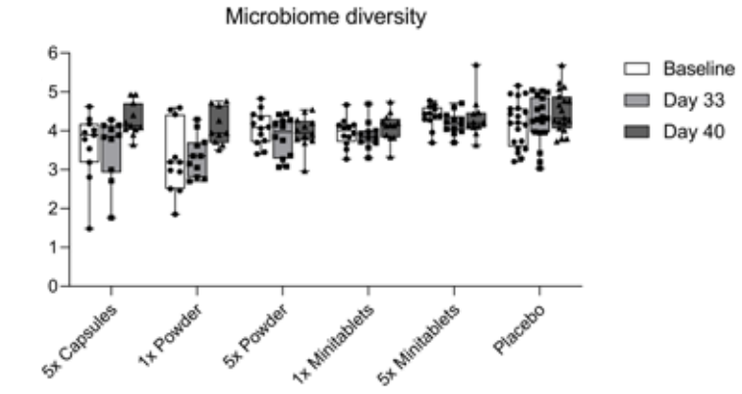


FIGURE 6 Fecal microbiome diversity calculated using the Shannon diversity index by treatment group per time point. Data are shown as median with interquartile range. AU, arbitrary unit.



DISCUSSION

In this study, we showed that daily EDP1066 treatment in encapsulated, powdered, and minitablet formulations and daily doses up to 1.5×10^{12} total cells, 5 times the expected therapeutic dose, did not result in consistent significant effects on KLH challenge responses and LPS- and PHA-driven cytokine release in whole blood cultures. We demonstrated that *L. lactis* spp. *cremoris* was detected in the fecal samples and increased during the 28-day treatment period for all EDP1066 formulations tested. However, the fecal levels returned to baseline levels 12 days after the end of treatment, indicating no prolonged persistence. Overall, EDP1066 was considered safe and well-tolerated. To the best of our knowledge, the current trial is the first to investigate the effects of orally administered *L. lactis* spp. *cremoris* in high doses on systemic immune responses and the gut microbiome.

EDP1066 did not show a consistent immunomodulatory effect on KLH-driven responses in the present study. Though no statistical significance was reached, decreased anti-KLH antibody titers and cutaneous blood perfusion and erythema were observed in the 5× Minitablets group compared to placebo. Although circulating Tregs as a percentage of $CD4^+$ T cells were significantly increased in subjects treated with 5× Powder compared to placebo, it should be noted that these percentages remained within the general range of Tregs in the CD4 population as reported in the literature (5–10%).[52,53] The PD results observed in this study are in contrast with preclinical data where EDP1066 induced IL-10 production in *in vitro* human dendritic cell (DC) cultures, without significant induction of proinflammatory cytokines (unpublished data), and EDP1066 significantly reduced KLH- and ovalbumin-induced ear inflammation in mice and improved intestinal pathology and weight loss in an acute dextran sulfate sodium-induced colitis mouse model (unpublished data). Also in contrast to our results, other preclinical trials reported that *L. lactis* spp. *cremoris* restored T cell impairment in aged mice[26] and that coadministration of *L. lactis* spp. *cremoris* with *L. paracasei* spp. *paracasei* showed promising results in an atopic dermatitis mouse model.[27] Probiotics in general have been shown to be effective in (the prevention of) multiple diseases.[20,25] Multiple studies have reported enhanced responses to influenza vaccination after the intake of probiotics.[54-58] Another study showed an enhanced response to hepatitis A vaccination after probiotic intake.[59] Single strains of both *Lactobacillus rhamnosus* GG and *Lactobacillus helveticus* R52 have been shown to reduce the risk of developing antibiotic-associated diarrhea.[25] *L. rhamnosus* GG single-strain treatment was also effective in the prevention of necrotizing enterocolitis.[25] Furthermore, *Bifidobacterium animalis* spp. *lactis* BB12 prevented upper respiratory tract infections, indicating distally evoked immune system effects.[25]

EDP1066 treatment suppressed KLH-driven increases in LPS-driven cytokine release *ex vivo* in both the 1× and the 5× Minitablets groups, reaching statistical significance for IL-1β, IL-6, and TNF in the 1× Minitablets group, which may indicate innate immune system inhibition.[60] The observed increase in LPS-driven cytokine release by monocytes in placebo-treated subjects may be attributed to KLH immunization, priming the innate immune response for subsequent stimulation. Although KLH is primarily recognized as an agent that induces cell-mediated responses, there is evidence that KLH immunization and rechallenge most likely cause a mixed reaction of innate, late-phase skin reaction and delayed-type hypersensitivity.[61] Furthermore, similar to LPS, KLH induces innate immunity *via* the activation of NF-κB.[60]

In the present study, we evaluated the immunomodulatory activity of EDP1066 as powder formulation (free and in enteric-coated capsules) and as minitablets in non-coated capsules. The minitablets in noncoated capsule formulations were expected to achieve the highest concentration of relatively intact EDP1066 bacteria in the duodenum. Noncoated capsules were used to ease the intake of relatively large numbers of enteric-coated minitablets and to preserve the blinding. Based on *in vitro* experiments, the minitablet formulation was predicted to release in the proximal small intestine (unpublished data). Duodenal EDP1066 exposure was hypothesized to be important for the immunomodulatory effect, as immune cell subsets are found at the highest concentrations in the duodenum and jejunum, particularly the $CD103^+CD11B^+$ DCs, which are thought to play distinct roles in intestinal immune homeostasis.[1] The small intestine is the most likely point where luminal contents can access GALT and have a pronounced immune-regulatory effect.[1] However, we did not observe any differences between the formulations on the humoral KLH challenge and subsequent skin KLH rechallenge, circulating Tregs, gut microbiome, and safety and tolerability outcomes. We did observe increased fecal detection of EDP1066 in the capsule formulation compared to powder and minitablet formulations. This can possibly be explained by the fact that the enteric-coated capsules dissolve lower in the GI tract leading to postponed EDP1066 release and higher EDP1066 exposure toward the end of the GI tract.

The current human trial did not confirm previous findings from preclinical trials that oral administration of EDP1066 had immunomodulatory effects as measured on antibody response to KLH immunization or skin immune responses to KLH rechallenge. There are several potential explanations for the suboptimal translation of EDP1066 activity between mice and humans. Firstly, it was impossible to do conventional allometric scaling between mice and humans. Other than for most medicinal products, the exposure to EDP1066 was considered to remain restricted to the GI tract. This was hypothesized to be sufficient, since the mechanism of action of EDP1066 only requires local interaction with cells of the GI mucosa, driving subsequent systemic

effects. Under these conditions, assumptions of traditional allometric scaling may not hold true. For this reason, relative GI mucosal surface area and stool mass are key parameters for allometric scaling. The relative GI mucosal surface area has been estimated as a function of body mass to the ¾ power.[62] As the dose selection rationale was mainly hypothetical, the actual EDP1066 doses administered might have been too low to exert significant PD effects. For practical reasons, the administration of higher EDP1066 doses was not explored since this would require a daily intake of >10 capsules. Secondly, differences in diet and also differences in microbial composition are likely to introduce highly variable individual responses to microbial exposure. As *L. lactis* spp. *cremoris* is used by the dairy industry, it is likely that participants have developed at least some intestinal tolerance to this microbe,[63] possibly explaining the differences observed between the current trial and preclinical data. Furthermore, apart from the well-known immunological differences between rodents and humans, EDP1066 activity is dependent on relatively unknown physiological systems or principles such as the GI microbiome and the interplay between the local and systemic immune systems. The exact molecular target and target location for EDP1066 are unknown, as are the exact EDP1066 components required for biological effect, further complicating interspecies translation. As intestinal dysbiosis can cause altered local as well as systemic immune system changes, we hypothesized that live EDP1066 would be required to interact with the gut microbiome and local immune system. However, based on the results observed in this study, we cannot exclude the possibility that dead EDP1066 could potentially also provoke immune system responses. Finally, the *in vitro* prediction of the release criteria of the enteric-coated capsules and minitablets may underestimate the time to release, suggesting that the true release of EDP1066 was in the distal small intestine or colon rather than in the proximal small intestine.

In conclusion, oral EDP1066 treatment for healthy volunteers did not consistently result in significant immune modulation. Future clinical studies should build onto the insights obtained in this study and further investigate formulation *vs.* local release and dose–effect relationships, which will ultimately be beneficial not only for EDP1066 but also for the field of therapeutic human commensals in general.

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CHAPTER VI

EVALUATION OF SINGLE-STRAIN PREVOTELLA HISTICOLA ON KLH-DRIVEN IMMUNE RESPONSES IN HEALTHY VOLUNTEERS: A RANDOMIZED CONTROLLED TRIAL WITH EDP1815

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ABSTRACT

Introduction: EDP1815 is a single-strain of *Prevotella histicola* with preclinical immunomodulatory properties. The aim of this study was to evaluate pharmacodynamic effects of EDP1815 on the immune response following immunization with keyhole limpet hemocyanin (KLH) and dermal rechallenge.

Methods: Thirty-two healthy subjects (median 30, range 18–59 years) were randomized over two cohorts to EDP1815 or placebo (12:4). Both cohorts received 8.0×10^{11} total cells daily for 28 days, reconstituted in 10 (A formulation) or 5 (B formulation) capsules. KLH-specific antibodies and circulating regulatory T cells were evaluated. Skin response after rechallenge was assessed with imaging. Immune cell subsets from blister exudates were assessed in the B cohort only. *Ex vivo* phytohemagglutinin and lipopolysaccharide whole blood challenges were performed to evaluate cytokine release. Gastrointestinal tract persistence, prevalence, and colonization of EDP1815 were assessed by fecal qPCR and microbiome assays. Data were analyzed using repeated measures analysis of covariances.

Results: There was a trend toward a treatment effect on the KLH-induced skin rechallenge response (CIELab a* estimated difference (ED) −1.50 arbitrary unit (AU), 95% CI −3.47–0.47 AU, $P = .13$ in A cohort, and average redness ED −0.14 AU, 95% CI −0.31–0.03 AU, $P = .10$ in B cohort) and, to a lesser extent, on the humoral KLH response. No notable EDP1815 effects were observed on gut persistence, microbiome, and other safety parameters.

Conclusion: Based on our findings and the clinical benefit observed in the phase 2 study in psoriasis, further investigation of the immunomodulatory effects and potential clinical benefit of EDP1815 is warranted.

INTRODUCTION

Several lines of evidence have suggested that microbes within the gastrointestinal (GI) tract interact with the systemic immune system.[1-3] Throughout the epithelium of the small intestines, immune cells can be found in both the lamina propria and associated mesenteric lymph nodes, and in aggregated lymphoid nodules also known as Peyer's patches.[1,4] The intestinal immune network in mice contains specialized regions differing in gut-associated lymphoid tissue (GALT), leukocyte populations, and antigenic composition. Similarities between murine and human intestinal immune systems have been observed.[1] Commensal microorganisms colonize the mucosal wall of the small and large intestine with variable local distribution and composition within the GI tract.[1] Conditions such as celiac disease and inflammatory bowel disease are caused by an altered intestinal immune system or gut microbiome.[1,5-7] Notably, recent advances in this field of research have suggested that intestinal disruptions to microbiota homeostasis seem not to be limited to local immunity, but can also affect more distal immune responses as observed in psoriasis,[8] rheumatoid arthritis,[9] systemic lupus erythematosus,[10] and more.[11,12] More recent trials have investigated the interaction between the intestinal microbiota of the large intestine and the systemic immune system, evaluating potentially beneficial effects of probiotics, prebiotics, and/or synbiotics.[13-17] The involved underlying mechanisms are not fully understood and are further complicated by diverse or even contrasting immunomodulatory effects when comparing administration of microbial strain mixtures to single-strain microbes.[18-21] The potential of using single strains of microbes as treatments are favored in order to advance the understanding of the interactions between the systemic immune system and the small intestine which drive tolerance of foreign food antigens.[22,23]

EDP1815 is one such single-strain microbe prepared from *Prevotella histicola*, a gram-negative, obligate, non-sporulating, commensal anaerobe isolated from a duodenal biopsy of a human donor. To date, *Prevotella* species have been identified in oral, nasopharyngeal, GI, and genitourinary mucosa in humans[24] and stool abundance ranges from <1% up to approximately 50% of total fecal microbial amount.[25] *In vitro*, EDP1815 stimulated the secretion of anti-inflammatory cytokines interleukin (IL)-10 and IL-27 from human macrophages, proinflammatory M1-biased antigen presenting cells, and dendritic cells (DCs), whilst not inducing significant levels of proinflammatory cytokines including tumor necrosis factor (TNF) and interferon gamma (IFN-γ) (unpublished data). EDP1815 reduced ear inflammation in murine keyhole limpet hemocyanin- (KLH), MC903- (vitamin D_3 analog), and imiquimod-induced skin challenge models.[26] Furthermore, *ex vivo* immunophenotyping in these mouse models showed increased regulatory T cells (Tregs).[27,28] In disease mouse models,

P. histicola decreased incidence and severity of arthritis in collagen-induced arthritis,[27] suppressed disease in experimental autoimmune encephalomyelitis,[28] delayed the onset of type 1 diabetes,[29] and suppressed T cell responses to gliadin indicating a possible future treatment modality in celiac disease.[30] Clinically, EDP1815 was considered to be safe and well tolerated in a first-in-human study and a phase 2 study in psoriasis (ClinicalTrials.gov identifier NCT04603027).[26,31]

In this clinical study the immune system of healthy volunteers was challenged with KLH immunization, a safe challenging antigen widely used for evaluation of cell-mediated responses in man.[27-38] KLH is recognized as a neoantigen that drives a cellular immune response. *In vivo,* this response can be studied by an intradermal KLH rechallenge. The resulting local inflammatory response can serve as a functional biomarker for clinical evaluation of (novel) immunomodulatory drugs in early-phase drug development.[32,34-36,43-45]

The aim of this study was to evaluate the effects of EDP1815 on the immune response following intramuscular immunization with KLH. The response to KLH immunization was evaluated by serum KLH-specific antibody concentrations and circulating Tregs, and by characterization of the skin response following intradermal KLH rechallenge, by objective imaging techniques and at the level of tissue cytokines and immune cell subsets. EDP1815 effects were also explored *ex vivo* by whole blood stimulation with Toll-like receptor 4 ligand lipopolysaccharide (LPS) and phytohemagglutinin (PHA) for lymphocyte stimulation. EDP1815 GI tract persistence, prevalence, and colonization were assessed by quantitative polymerase chain reaction assay (qPCR) using EDP1815 specific primers and 16S ribosomal RNA (rRNA) sequencing of fecal material, next to routine safety and tolerability assessments.

METHODS

This was a phase 1, randomized, placebo-controlled, double-blind, multiple dose study in thirty-two (32) healthy volunteers performed at the Centre for Human Drug Research (CHDR), Leiden, The Netherlands, based on principles of the Declaration of Helsinki. The independent Medical Ethics Committee *Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek* (Assen, the Netherlands) approved the study prior to any clinical study activity. All subjects provided written informed consent before participation. The trial was registered on the Netherlands Trial Register, currently available for consultation at the International Clinical Trial Registry Platform (trial ID NL8676, https:// trialsearch.who.int). Materials and methods were executed as described previously.[46]

SUBJECTS

Healthy male and female participants were enrolled, 18 to 60 years of age with a body mass index between 18 and 35 kg/m^2, without previous exposure to KLH. Health status was verified by recording a detailed medical history, a complete physical examination, vital signs, a 12-lead electrocardiogram (ECG) and laboratory testing (including hepatic and renal panels, complete blood count, fecal calprotectin, virology and urinalysis). Subjects were excluded in case of any disease associated with immune or GI system impairment, or use of prescription medication within four weeks prior to first dose.

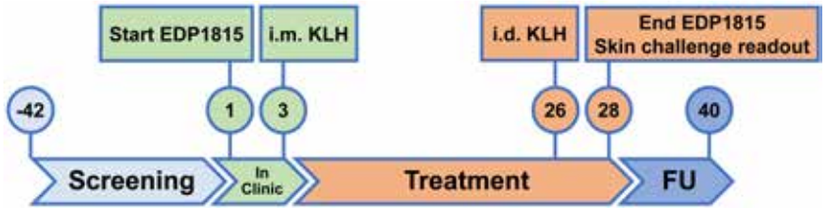
DOSE SELECTION AND REGIMEN

The doses tested were based on the results of a separate first-in-man study.[26,31] The highest dose tested contained 8.0×10^{11} total cells per dose, approximately 5 times the predicted therapeutic dose level, calculated from allometric scaling the preclinically efficacious dose level based on conversion between mouse and human gut surface area. This dose was well tolerated in humans. Participants were dosed once daily for 28 consecutive days. Participants in the second cohort received a similar dose level as the previous cohort, however, a manufacturing process change approximately doubled the EDP1815 concentration per capsule reducing the number of capsules required from 10 to 5.

STUDY DESIGN AND TREATMENTS

An overview of the study design is shown in Figure 1. Subjects were enrolled into two cohorts and randomized to either EDP1815 or placebo (12:4). The first cohort received EDP1815 freeze-dried powder in enteric-coated capsules, supplied as 8.0×10^{10} total cells per capsule, administered orally at a dose of 10 capsules daily (A formulation). The second cohort received EDP1815 freeze-dried powder in enteric-coated capsules supplied as 1.6×10^{11} total cells per capsule, administered orally at a dose of 5 capsules daily (B formulation). Compliance was confirmed by supervised administration during the in-clinic period. Administration at home was recorded by an electronic diary. Intramuscular KLH immunizations were performed in the left deltoid muscle after completion of the third EDP1815/placebo administration. KLH immunization was administered as 0.1 mg Immucothel (Biosyn Corporation, Carlsbad, CA, USA) adsorbed in 0.9 mg aluminum hydroxide (Alhydrogel, Brenntag AG, Essen, Germany) into 0.5 mL NaCl 0.9% as previously described.[42] All subjects were administered KLH (0.001 mg Immucothel) and saline in 0.1 mL NaCl 0.9% intradermally in the left and right ventral forearms, respectively, 23 days after KLH immunization. Dermal imaging was done prior to and two days after intradermal KLH administration, as in previous KLH studies.[33,34,38,41-43,47]

FIGURE 1 Study timeline. Numbers represent visit days.



i.m., intramuscular; i.d., intradermal; KLH, keyhole limpet hemocyanin; FU, follow-up.

SYSTEMIC IMMUNITY: HUMORAL RESPONSE TO KLH AND CIRCULATING REGULATORY T CELLS

The humoral response to KLH immunization was measured by anti-KLH IgM and IgG serum titers. Serum samples were analyzed by quantitative enzyme-linked immunosorbent assay (ELISA) for anti-KLH IgM and IgG as previously described.[42] Mean optical density of baseline samples was set to 1.00 and relative ratios were calculated for all subsequent samples. The percentage of circulating Tregs was evaluated by flow cytometry. Red blood cell (RBC) lysis was performed on heparinized whole blood using RBC lysis buffer (Thermo Fisher, Waltham, Massachusetts, USA). Leukocytes were stained with fluorochrome labelled antibodies (see Table S1), propidium iodide was used as viability dye (Miltenyi Biotec, Bergisch-Gladbach, Germany). Samples were measured on a MACSQuant 16 analyzer (Miltenyi Biotec), and analyzed using Flowlogic software (Inivai, Mentone, Australia). Tregs were defined as $CD4^+CD25^+CD127^-$, see Figure S1 for gating strategy.

TISSUE IMMUNITY: DERMAL RESPONSE UPON KLH INJECTION

Cutaneous blood perfusion was evaluated by laser speckle contrast imaging (LSCI; PeriCam PSI System, Perimed AB, Järfälla, Sweden) and erythema was quantified by multispectral imaging (Antera 3D, Miravex, Dublin, Ireland) as previously described.[42] Circular regions of interest at the intradermal injection sites were defined. Cutaneous blood perfusion (indicated as basal flow) was quantitatively assessed and expressed in arbitrary units (AUs). The homogeneity of cutaneous blood perfusion in the region of interest (indicated as flare), expressed as values that are +1 standard deviation (SD) from the mean basal flow within the region, was also quantitatively assessed and expressed in AUs. Erythema was quantified using the average redness and CIELab a* Antera 3D software modalities expressed as AUs. The average redness modality displays the distribution of redness using an internal software algorithm and the CIELab a* value, which is part of the CIELab color space, expresses color as a numerical value on a green–red color scale. Blister exudate was collected after blister induction and puncture in the second cohort (B formulation/placebo). Blister exudate supernatants were evaluated for immune cells ($CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, Tregs, B cells, classical monocytes, intermediate monocytes, nonclassical monocytes, DCs, natural killer (NK) cells, and neutrophils) on a MACSQuant 16 analyzer and analyzed using Flowlogic software, see Figure S2 for gating strategy. Leukocytes were stained with fluorochrome labelled antibodies (see Table S1 for a complete list), 7AAD (Miltenyi Biotec) was used as viability dye.

EX VIVO STIMULATION ASSAYS

Sodium heparinized whole blood was incubated with 10 µg/mL PHA (Sigma-Aldrich, Deisenhofen, Germany) or 2 ng/mL LPS (strain O111:B4 from *Escherichia coli*, Sigma-Aldrich) for 24 hours at 37 °C, 5% CO_2. After 24 hours, supernatant was collected and cytokines were quantified using qualified ELISA based assays (V-PLEX Proinflammatory Panel 1 Human Kit, Meso Scale Diagnostics, Rockville, Maryland, USA) by Ardena Bioanalytical Laboratory (Assen, the Netherlands). IFN-γ and IL-2 were measured in the PHA stimulated samples, TNF, IL-1β, IL-6, and IL-8 were measured in the LPS stimulated samples.

EDP1815 STOOL PERSISTENCE AND GUT MICROBIOME

Fecal concentrations of EDP1815 for stool persistence and prevalence and gut microbiome were measured by BaseClear B.V. (Leiden, the Netherlands) using validated bioanalytical methods. In short, fecal microbial DNA was extracted based on the Zymo Research (Irvine, CA, USA) fecal DNA extraction methodology. EDP1815 specific-primers and probe had been developed enabling the detection of the *P. histicola* strain. The fecal samples were analyzed using a qPCR with a lower limit of quantification of 26,400 copies/g feces. For gut microbiome analyses, extracted DNA was prepared for Illumina sequencing *via* PCR amplification of the variable region 3 and 4 of the bacterial 16S rRNA gene. After PCR purification, sample specific barcodes using Illumina Nextera XT Index kit (Illumina Inc., San Diego, CA, USA) were appended to the PCR products during a second PCR. PCR products were purified for a second time and lastly, the PCR products were equimolarly pooled and sequenced on the Illumina MiSeq platform using the MiSeq v3 sequencing kit.

SAFETY AND TOLERABILITY

Safety and tolerability were monitored by physical examination, assessment of vital signs, laboratory parameters (i.e., full blood count, biochemistry, serology, immuno-

phenotyping, circulating cytokines, fecal calprotectin, and urinalysis) and ECG data from 12-lead ECGs at regular intervals. Subjects were monitored continuously for adverse events (AEs). Participants were also asked to daily complete the Bristol Stool Scale (BSS) and questions regarding defecation pattern using an electronic diary app, to get insight in the participant's stool patterns at the time of feces sample collection.

STATISTICS

Subjects were randomized to EDP1815 or placebo in a 3:1 ratio. The placebo-treated subjects were pooled to achieve a sample size of 8 and a sample size of 12 was achieved on active treatment per group. Demographic and baseline variables were summarized by treatment. Pharmacodynamic (PD) endpoints measured at multiple time points after baseline were analyzed with a mixed effect repeated measures model with fixed factors treatment, time and treatment by time, random factor subject and the baseline value as covariate. Endpoints with one post dose measurement were analyzed with a linear model with treatment as fixed factor. Anti-KLH antibody parameters were analyzed without baseline as covariate. Skin rechallenge endpoints were analyzed with an analysis of covariance with treatment as fixed factor and the baseline and the change from baseline of the saline-injected control added as covariates. Anti-KLH IgM and IgG titers, *ex vivo* monocyte cytokine release, and immune cells in blister exudate assays required log transformation. The general treatment effect and specific contrasts were reported as estimated difference (ED) with 95% confidence interval (CI), and graphically as ED with 95% CI, as least squares mean (LSM) with 95% CI, or as mean with SD. The error terms of the mixed models are tested for normality by Shapiro-Wilk test. Fecal EDP1815 concentration was reported graphically as median with range. Fecal microbiome endpoints were analyzed using Python (Python Software Foundation, Wilmington, DE, USA). The relative abundances of all microorganisms at genus level were calculated to present the occurrence of all microorganisms relative to *Prevotella* genus in the samples. Diversity trends were analyzed using alpha diversity (Inverse Simpson's) and beta diversity (Bray-Curtis dissimilarity). The beta diversity analysis was performed solely on the ten most prevalent microorganisms based on their counts. Fecal microbiome endpoints were reported graphically as median with interquartile range for *Prevotella* relative abundance and microbiome alpha diversity. To calculate the difference between the different days (within a treatment) and between treatments for the *Prevotella* relative abundance and the alpha diversity index, independent t-tests were used. To calculate those differences for the beta diversity, a Mann-Whitney U test was used. Principal Coordinate Analysis (PCoA) was also implemented, a multidimensional scaling method, to analyze and compare the distance metrics across different days and treatments.

RESULTS

The study was conducted between December 2019 and February 2021. Thirty-two (32) subjects were enrolled and treated. All subjects completed the treatment (12 subjects EDP1815 A formulation, 12 subjects EDP1815 B formulation, and 8 subjects placebo) and the follow-up period. Baseline characteristics of all treatment groups are presented in Table 1. Treatment compliance as recorded *via* an electronic diary was 100%, no dosing days were missed.

TABLE 1 Baseline characteristics.

EDP1815 formulation	**A formulation**	**B formulation**	**Placebo**
Daily dose	**10 capsules of 8.0×10^{10} (5×) total cells**	**5 capsules of 1.6×10^{11} (5×) total cells**	
	***n* = 12**	***n* = 12**	***n* = 8**
		DEMOGRAPHICS	
Age (years)	45 (20–59)	27 (20–48)	24 (18–53)
BMI (kg/m^2)	24.5 (4.2)	23.5 (3.5)	23.1 (2.6)
Male gender (*n*)	6 (50%)	5 (41.7%)	2 (25%)
		VITAL SIGNS	
Systolic blood pressure (mmHg)	116 (11)	116 (10)	111 (9)
Diastolic blood pressure (mmHg)	70 (8)	66 (11)	65 (7)
Heart rate (BPM)	58 (9)	69 (9)	62 (13)
Temperature (°C)	36.3 (0.3)	36.9 (0.2)	36.9 (0.4)
		LABORATORY TESTS	
Leucocytes ($\times 10^9$/L)	4.85 (0.97)	5.42 (1.51)	6.07 (1.69)
Lymphocytes ($\times 10^9$/L)	1.74 (0.37)	1.75 (0.37)	1.90 (0.45)
Thrombocytes ($\times 10^9$/L)	216.1 (44.4)	243.4 (52.3)	264.0 (57.3)
ALT (IU/L)	19.2 (7.4)	15.2 (3.0)	21.4 (12.7)
AST (IU/L)	21.9 (6.1)	19.1 (5.1)	22.4 (6.0)
CRP (mg/L)	1.16 (1.21)	1.15 (1.70)	1.12 (0.96)
Fecal calprotectin (µg/g)	16.6 (9.7)	8.5 (4.8)	12.9 (13.2)

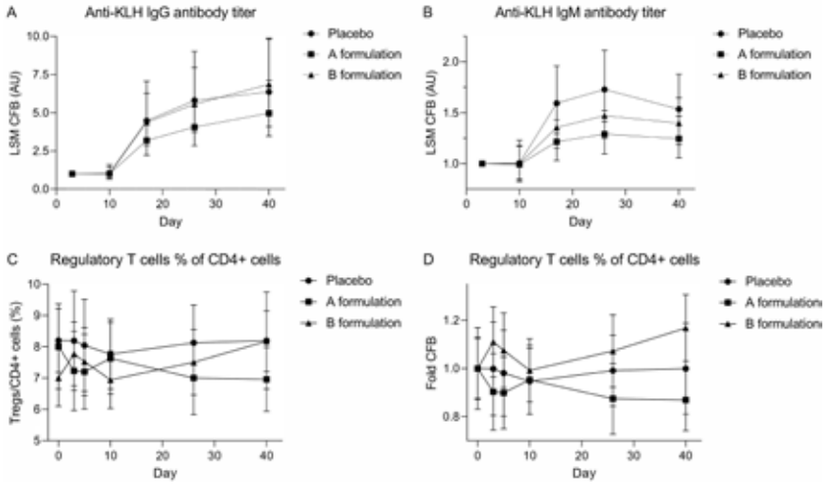
Parameters are shown as mean (standard deviation), for age as median (range), and for male gender as count (percentage). BMI, body mass index; ALT, alanine transaminase; AST, aspartate transaminase; CRP, C-reactive protein.

SYSTEMIC IMMUNITY: HUMORAL RESPONSE TO KLH AND CIRCULATING REGULATORY T CELLS

Although anti-KLH antibody levels were consistently lower in EDP1815-treated groups compared to placebo, no statistically significant EDP1815 effects were observed on

KLH-driven endpoints. Lower anti-KLH IgM (ED −17.8%, 95% CI −34.2–2.7%, $P = .08$) and IgG (ED −21.5%, 95% CI −52.1–28.7%, $P = .33$) were observed in EDP1815 A-treated participants as well as lower anti-KLH IgM (ED −10.0%, 95% CI −28.0–12.5%, $P = .34$) in EDP1815 B-treated participants compared to placebo (Table S2 and Figure 2). Although circulating Tregs as percentage of $CD4^+$ T cells was significantly decreased in the A group (ED −0.69%, 95% CI −1.23–−0.14%, $P < .05$) and significantly increased in the EDP1815 B group (ED 0.62%, 95% CI 0.05–1.19%, $P < .05$) compared to placebo (Table S2 and Figure 2), it should be noted that these percentages remained within the general range of Tregs in the CD4 population as reported in literature (5-10%).[48,49]

FIGURE 2 Anti-keyhole limpet hemocyanin (A) IgG and (B) IgM antibody titers, (C) circulating regulatory T cells as percentage of $CD4^+$ T cells, and (D) change from baseline circulating regulatory T cells as percentage of $CD4^+$ T cells over time by EDP1815 treatment group.



Data are shown as least square means change from baseline with 95% confidence interval for antibody titers and as mean with standard deviation for regulatory T cells. KLH, keyhole limpet hemocyanin; LSM, least square means; CFB, change from baseline; Tregs, regulatory T cells.

TISSUE IMMUNITY: DERMAL RESPONSE UPON KLH INJECTION

The KLH-driven skin response was overall lower in the EDP1815-treated groups compared to placebo, but none of the endpoints reached a level of significant difference (LSCI basal flow ED −8.0 AU, 95% CI −33.3–17.2 AU, $P = .52$ in A group, ED −8.4 AU, 95% CI −32.4–15.6 AU, $P = .48$ in B group and flare ED −2.4 AU, 95% CI −18.2–13.4 AU, $P = .76$ in A group, ED −4.6 AU, 95% CI −20.7–11.5 AU, $P = .56$ in B group; multispectral imaging: CIELab a* ED −0.66 AU, 95% CI −2.67–1.35 AU, $P = .50$ in A group, ED −1.50 AU, 95% CI −3.47–0.47 AU, $P = .13$ in B group and average redness ED −0.06 AU, 95% CI −0.23–0.11 AU, $P = .46$ in A group, ED −0.14 AU, 95% CI −0.31–0.03 AU, $P = .10$ in B group; Table S2 and Figure 3). The immune cells in blister exudates of placebo-treated subjects after KLH skin rechallenge consisted mainly of T cells (mean $CD3^+$ T cell count 397, SD 319, mean $CD4^+$ T cell count 288, SD 262, mean $CD8^+$ T cell count 81, SD 68, and mean Treg cell count 194, SD 189), NK cells (mean cell count 149, SD 77), and DCs (mean cell count 149, SD 77) (Table S2 and Figure 4). Scant amounts of B cells (mean cell count 12, SD 10), monocytes (mean classical monocyte count 78, SD 34, mean intermediate monocyte count 3, SD 3, and mean non-classical monocyte count 2, SD 1), and neutrophils (mean cell count 23, SD 21) were found in blister exudates of placebo-treated subjects (Table S2 and Figure 4). Although there was no clear EDP1815 treatment effect on immune cell subsets in blister exudates (Table S2 and Figure 4), definitive conclusions cannot be drawn due to very low number of cells.

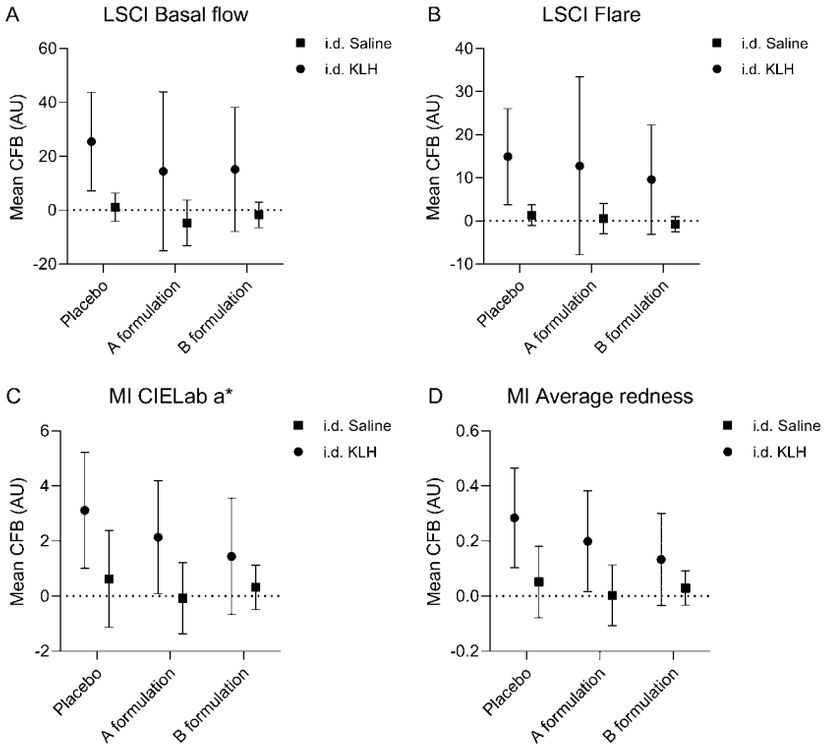
EX VIVO STIMULATION ASSAYS

EDP1815 did not significantly impact LPS- and PHA-driven cytokine responses *ex vivo*. All cytokines in supernatants from LPS-stimulated (TNF, IL-1β, IL-6, and IL-8) or PHA-stimulated (IFN-γ and IL-2) whole blood cultures were comparable across treatment groups (Table S2 and Figure 5).

EDP1815 STOOL PERSISTENCE AND GUT MICROBIOME

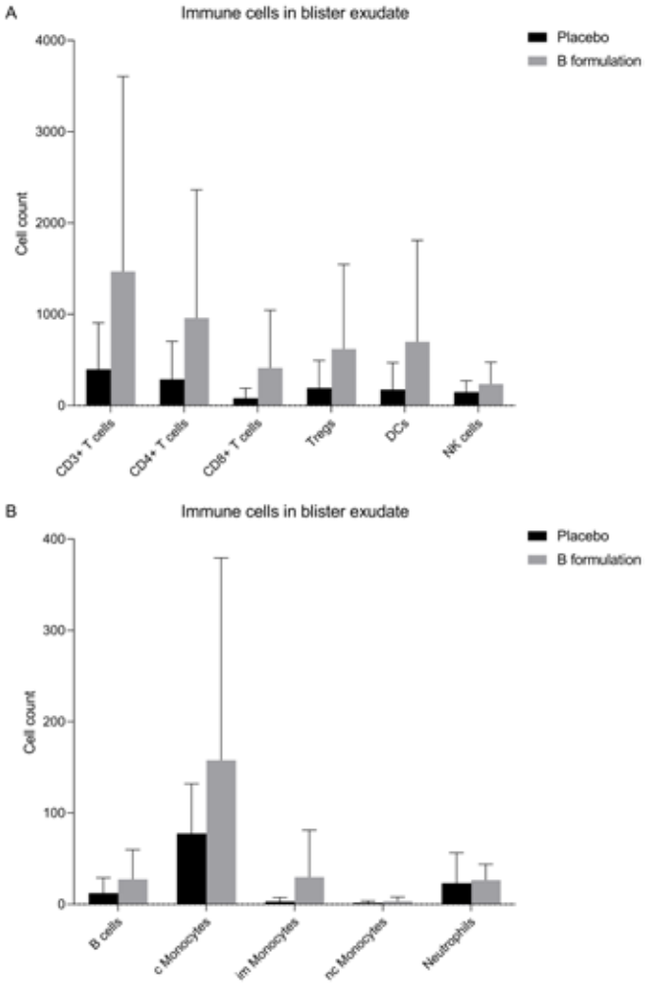
EDP1815 was detected between 18–25% on study day 26 in subjects on active treatment, returning to below the limit of quantification levels 5 days after the last EDP1815 dose (Table S3 and Figure 6). *Prevotella* was the second most abundant genus found in the top ten most abundant microbial populations over all samples combined (Table S4). *Prevotella* abundance was comparable across all treatment groups (Table S5 and Figure 6). Although a statistically significant difference was observed in microbiome alpha diversity between placebo and EDP1815 B formulation groups ($P = .02$), no differences were found within the EDP1815 B formulation group over time when compared to baseline (Table S6 and Figure 6). Furthermore, the two principal components used for the beta diversity PCoA plots combined contributed between 72% to 88% to the total score weights and no statistically significant differences were found in microbiome beta diversity (Table S7 and Figure 7).

FIGURE 3 Cutaneous blood perfusion by LSCI (A) basal flow and (B) flare, erythema by multispectral imaging (C) CIELab a* and (D) average redness after intradermal KLH and saline administration by EDP1815 treatment group.



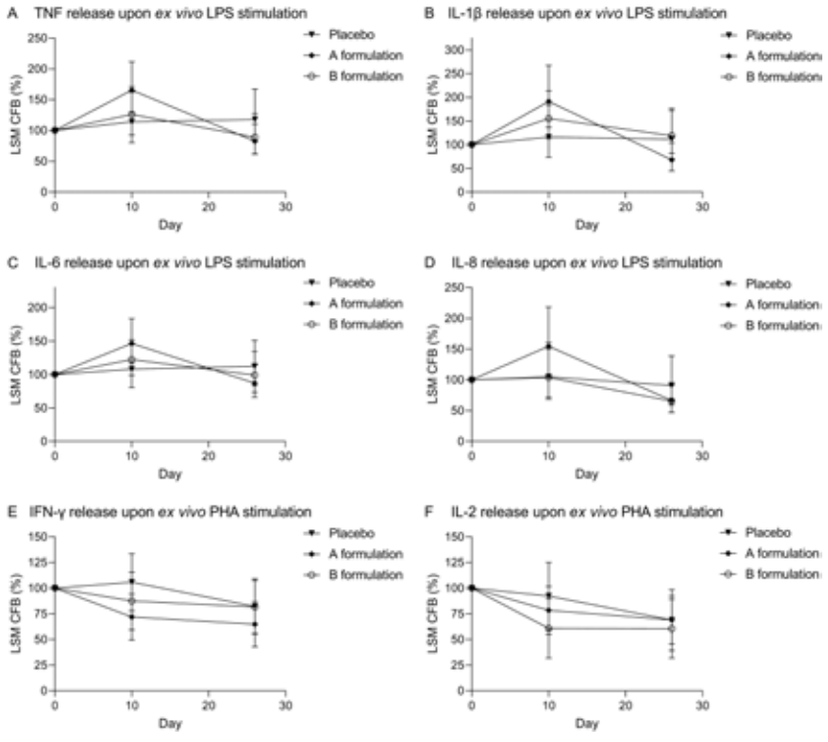
Data are shown as mean change from baseline with standard deviation. LSCI, laser speckle contrast imaging; MI, multispectral imaging; KLH, keyhole limpet hemocyanin; CFB, change from baseline; AU, arbitrary unit; i.d., intradermal.

FIGURE 4 Immune cell counts of (A) T cell subsets, dendritic cells, and natural killer cells, and (B) B cells, monocyte subsets, and neutrophils in blister exudate after intradermal KLH administration by EDP1815 treatment group.



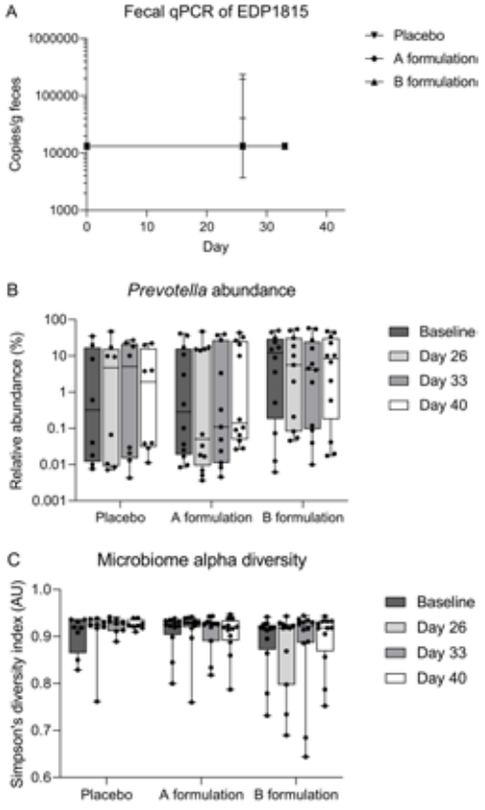
Data are shown as mean with standard deviation. c, classical; im, intermediate; nc, non-classical; DCs, dendritic cells; NK, natural killer.

FIGURE 5 Monocyte cytokine release assay of (A) tumor necrosis factor, (B) interleukin-1β, (C) interleukin-6, and (D) interleukin-8 release from whole blood cultures after *ex vivo* lipopolysaccharide stimulation and lymphocyte cytokine release assay of (E) interferon gamma and (F) interleukin-2 release from whole blood cultures after *ex vivo* phytohemagglutinin stimulation. X-axis represents number of days after initial EDP1815 dose.



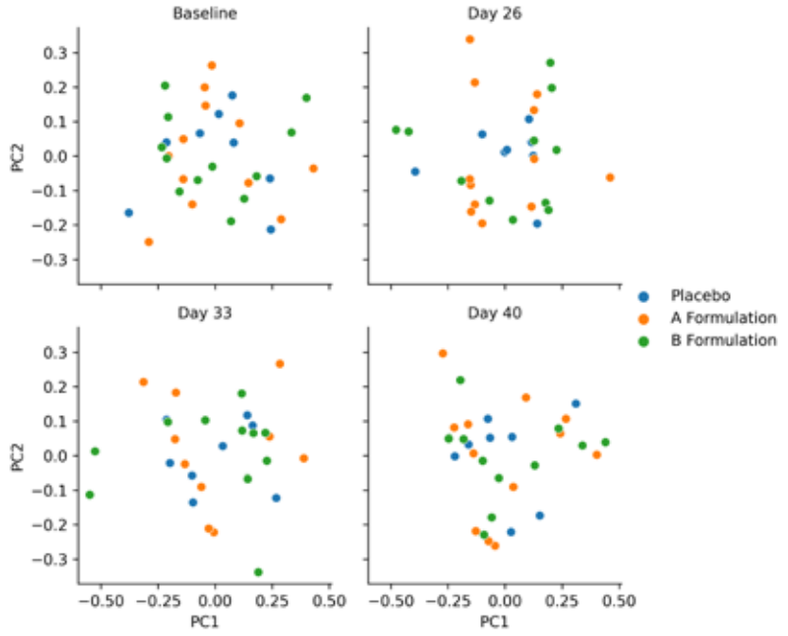
Data are shown as least squares mean change from baseline with 95% confidence interval. LSM, least squares mean; CFB, change from baseline; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin; PHA, phytohemagglutinin; IFN-γ, interferon gamma.

FIGURE 6 (A) Fecal concentration of EDP1815 over time on log10 scale measured by quantitative polymerase chain reaction, (B) *Prevotella* relative abundance expressed on log10 scale as percentage composition of total microbiome per time point, and (C) microbiome diversity calculated using the Simpson's alpha diversity index per timepoint.



Data are shown as median with range for fecal EDP1815 concentration and as median with interquartile range for Prevotella abundance and microbiome alpha diversity. qPCR, quantitative polymerase chain reaction; AU, arbitrary unit.

FIGURE 7 Microbiome diversity calculated using a principal coordinate analysis plot of the top ten most abundant microbial populations based on the Bray-Curtis dissimilarity beta diversity index per timepoint per group.



PC, *Principal Component.*

SAFETY AND TOLERABILITY

Overall, EDP1815 treatment did not raise safety concerns. No serious AEs occurred. Most AEs were related to the GI tract (20 AEs in 11 subjects, Table S8) and all were mild and transient in nature and considered to be possibly related to treatment. The most frequently reported GI tract AEs were upper abdominal pain (5 AEs in 4 subjects) and nausea (4 AEs in 3 subjects). Subjects treated with B formulated EDP1815 also had overall slightly fewer AEs (66.7%) compared to A formulated EDP1815 and placebo-treated subjects (91.7–100%). No clinically significant changes were observed in laboratory parameters, vital signs, ECG recordings. The assessments and observations in clinical laboratory values, vital signs, and ECG recordings were similar across treatment groups. Some participants showed one or more laboratory parameters outside the normal range. These observations were incidental, not treatment-related, and judged to be of no clinical relevance. No effect of EDP1815 was observed on fecal calprotectin or the BSS and feces questionnaire.

DISCUSSION

In this study we show that various KLH-driven immune responses were overall lower in groups treated daily with EDP1815 in a dose of 8.0×10^{11} total cells, but that for none of the individual PD endpoints a level of statistical significance was reached. There was no prolonged EDP1815 persistence or colonization within the GI tract as indicated by qPCR and microbiome analyses, respectively. Treatment compliance was 100% which is in line as previously reported using a similar e-diary app.[50] Overall, EDP1815 was considered safe and well-tolerated.

In order to detect a 75% inhibition of the KLH-specific antibody response, cutaneous blood perfusion response (LSCI), and erythema response (multispectral imaging) a sample size of 12 per group was required using a parallel study design, with an α of .05 and a power of 80%.[42] Although our study was sufficiently powered for the detection of anti-KLH IgM and IgG antibody responses (minimum sample size of 4 per group required), we were underpowered to detect differences in skin rechallenge outcomes with LSCI and multispectral imaging modalities (minimum sample size of 12 per group required). We have previously shown that the T cell-dependent antibody response (TDAR) following KLH immunization is not correlated with the skin rechallenge response quantified with LSCI and multispectral imaging, suggesting that the KLH-induced humoral response likely does not drive the skin response after intradermal KLH administration.[42] In a separate study we further displayed a discrepancy between TDAR and subsequent skin rechallenge response in healthy volunteers receiving amlitelimab, an OX40L inhibitor, and subsequent KLH immunization and skin rechallenge.[43] The inhibition of the TDAR response was less pronounced compared to the inhibition of the skin rechallenge response indicating that these two responses are possibly mechanistically independent, the TDAR reflecting a B cell-induced response and the skin rechallenge reflecting a T cell-induced response.

The circulating Tregs comprised between 5-10% of detected $CD4^+$ T cells which is consistent with data described in literature.[48,49] The inconsistent but statistically significant changes in circulating Tregs observed between the treatment groups are all within the 5-10% range of total $CD4^+$ T cells. Therefore, we believe the observed variability is not clinically relevant and is most likely attributed to normal variation.

Immune cell subsets present in blister exudates after intradermal KLH rechallenge and e*x vivo* stimulation assays included substantial proportions of T cells and DCs as

anticipated. Delayed-type hypersensitivity reactions usually occur 48–72 hours after antigen rechallenge as antigen presenting cells such as DCs display antigens using major histocompatibility complex class II molecules to dermal $CD4^+$ T cells.[33,34,36,38-43,47] Proinflammatory cytokine secretion by activated $CD4^+$ T cells, such as IL-2 and IFN-γ, induces cytotoxic $CD8^+$ T cell activation and proliferation to help aid in elimination of the antigen.[51] The immune cell subsets present in blister exudates were comparable between EDP1815 and placebo-treated participants, however, the total cell count from the blister exudates was very low. For comparison, total cell counts obtained from human PBMC isolation contain roughly 1.3×10^6 cells[52] whereas we found a maximum mean total cell count of merely 2,914 cells (EDP1815 B-treated participants).

Traditional immunomodulatory therapies such as corticosteroids, non-steroidal anti-inflammatory drugs, and anti-histamines have been well-described and are successful in suppression of inflammation. However, due to lack of tissue or cell specificity they can cause a wide range of side effects, particularly after prolonged use.[53] More novel antibody-based therapies overcome this lack of specificity, however, the primary obstacle predominantly revolves around their immunogenicity and production of antidrug antibodies, leading to decreased clinical efficacy.[53] Single-strain microbes are emerging novel therapies and have been shown to be effective in preventing and ameliorating several diseases.[21] *Lactobacillus rhamnosus* GG is a single-strain microbe that has been reported to reduce the risk of antibiotic-associated diarrhea (AAD) and to prevent necrotizing colitis. *Lactobacillus helveticus* R52 has also been shown to be effective in AAD. Notably, *Bifidobacterium animalis* spp. *lactis* BB12 is a single-strain microbe that has been reported to prevent upper respiratory tract infections, indicating a systemic immune response after local GI tract exposure. Although the underlying mechanisms involved in induction of systemic immunomodulatory effects after local exposure are not fully elucidated,[22] targeting the GI tract with microbial therapies seems a plausible approach. Investigating immunomodulatory effects of compounds such as EDP1815 aids in elucidating the poorly described link between the systemic and enteric immune systems. This connection between the systems holds potential for harnessing pharmacological effects and introduces an innovative strategy for treating inflammatory diseases, possibly overcoming some of the limitations observed in traditional as well as antibody-based immunomodulatory therapies.

This clinical study did not confirm the profound and consistent immunomodulatory effects of EDP1815 on KLH-driven responses as seen in preclinical *in vitro* and *in vivo* mouse studies. There are different potential explanations for the absent translation of convincing immunomodulatory effect of EDP1815 between mice and man.

Firstly, conventional allometric scaling between mouse *vs.* human was challenging as EDP1815 exposure was believed to be restricted to the GI tract compared to systemic uptake as commonly observed in other medicinal products. The immunomodulatory action of EDP1815 was hypothesized to be solely dependent on local interplay with cells in the GI tract mucosa followed by systemic immune modulation. Stool mass and relative mucosal surface area of the GI tract are also important factors that need to be considered for allometric scaling.[54] The daily EDP1815 dose of 8.0×10^{11} total cells was possibly insufficient to generate significant immune system inhibition as the justification for dose finding was primarily hypothetical. Higher doses were not explored as daily administration of >10 capsules was impractical. Secondly, release of EDP1815 enteric-coated capsules early in the proximal small intestine is thought to be important for the mechanism of action as described before.[46] Furthermore, the EDP1815 target site, exact molecular target, as well as which EDP1815 element(s) exert immunomodulatory properties are still being elucidated. A recent publication has described an orally ingestible microdevice that entraps GI microbiota and biomarkers whilst passing through the GI tract.[55] This method of capturing GI tract biomarkers and microbiota is non-invasive and could potentially allow for site-specific sampling. Lastly, relatively unknown physiological systems and factors such as the interaction between the local and systemic immune system and the GI microbiome likely play important roles in EDP1815-induced immunomodulatory effects.

Importantly, immunosuppression with amlitelimab, an OX40L inhibitor, efficiently translates from *in vivo* animal models to a first-in-human trial including a similar KLH challenge model, and eventually to patients with atopic dermatitis.[43] Incorporating the KLH challenge model in early-phase clinical trials with novel compounds targeting the immune system is a suitable approach to evaluate desired treatment effects early on during clinical development.

Microbial interventions are used for prevention and treatment of several immune related diseases. The interaction between the systemic and GI immune system is currently not fully understood. Although oral EDP1815 did not evoke consistent significant systemic immune modulation, there was a trend toward a treatment effect on the KLH-induced skin rechallenge response and, to a lesser extent, on the humoral KLH response. Based on our clinical data and data from other EDP1815 trials,[26] it is premature to conclude that EDP1815 is not effective in inhibiting (KLH-driven) immune responses in man. We advocate more extensive and mechanistic EDP1815 research, including facilitation of higher doses of EDP1815. Currently, both EDP1815 in early-release enteric-coated capsule formulation as well as a microbial extracellular vesicle are being investigated in a similar setup as in our study.

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CHAPTER VII

CLINICAL TRANSLATION OF ANTI-INFLAMMATORY EFFECTS OF *PREVOTELLA HISTICOLA* IN TH1, TH2, AND TH17 INFLAMMATION

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ABSTRACT

Introduction: EDP1815 is a non-colonizing pharmaceutical preparation of a single stain of *Prevotella histicola* isolated from the duodenum of a human donor. We report here preclinical and clinical studies showing that the action of EDP1815, an orally delivered and gut-restricted single strain of commensal bacteria can regulate inflammatory responses throughout the body.

Methods: Supported by evidence for anti-inflammatory activity in three preclinical mouse models of Th1-, Th2-, and Th17-mediated inflammation, EDP1815 was tested clinically in three phase 1b studies in patients with psoriasis, patients with atopic dermatitis, and healthy volunteers in a KLH skin challenge model.

Results: Preclinically, EDP1815 was efficacious in all three mouse models of inflammation, showing reduction in skin inflammation as well as related tissue cytokines. In the phase 1b studies, EDP1815 was found to be well-tolerated by participants, with a safety profile comparable to placebo, including no severe or consistent side-effects reported, and no evidence of immunosuppression with no opportunistic infection occurring in these studies. In psoriasis patients, signs of clinical efficacy were seen after 4 weeks of treatment, which continued beyond the treatment period in the higher-dose cohort. In atopic dermatitis patients, improvements were seen throughout the key physician- and patient-reported outcomes. In a healthy volunteer study of a KLH-induced skin inflammatory response, consistent anti-inflammatory effects were seen in two cohorts through imaging-based measures of skin inflammation.

Discussion: This is the first report demonstrating clinical effects from targeting peripheral inflammation with a non-colonizing gut-restricted single strain of commensal bacteria, providing proof-of-concept for a new class of medicines. These clinical effects occur without systemic exposure of EDP1815 or modification of the resident gut microbiota, and with placebo-like safety and tolerability. The breadth of these clinical effects of EDP1815, combined with its excellent safety and tolerability profile and oral administration, suggests the potential for a new type of effective, safe, oral, and accessible anti-inflammatory medicine to treat the wide range of diseases driven by inflammation.

INTRODUCTION

The small intestine (SI) is an immunological window on the environment. Its mucosal surfaces must tolerate required foreign antigens that are absorbed as nutrients while protecting against toxic antigens and pathogens. Immune cells are found throughout the SI epithelial lining, both in specialized tertiary immune structures called Peyer's patches and within the lamina propria and associated mesenteric lymph nodes.[1] These mucosal surfaces are also colonized by a low density of commensal microorganisms which are distinct from the bulk of the colonic microbiota in their abundance, microenvironment, and taxonomic range.[2,3] Individual strains of microbes sampled from the mucosal surface of the SI have been shown to alter the phenotype of antigen presenting and immune effector cells in human *in vitro* cell experiments and to have anti-inflammatory effects in murine *in vivo* models of inflammation.[4] The *in vivo* effects are not dependent on mucosal colonization; indeed, non-viable bacteria can induce these systemic effects, suggesting that signals generated by intestinal cells upon recognition of structural features on the surface of the microbes initiate the anti-inflammatory effect.[4] Bacterial surface structures, such as capsular Polysaccharide A from *Bacteroides fragilis* have been previously described and shown to modulate local immune cell responses as well as systemic inflammatory responses.[5,6] The ability to modify systemic inflammation without systemic exposure confirms a link between mucosal and systemic immunology. Harnessing this link through pharmacological modulation offers the potential to create a new class of therapeutics which can modify systemic immunology without the need and risk of systemic exposure.

EDP1815 is prepared from a single strain of *Prevotella histicola* (*P. histicola*), which is a gram-negative, non-sporulating, obligate commensal anaerobe isolated from a duodenal biopsy of a human donor. *Prevotella* species have been found in the oral, nasopharyngeal, gastrointestinal (GI), and genitourinary mucosal surfaces of all human populations tested to date.[7] Abundance in stool can range from <1% to nearly 50% of total fecal microbial load.[8]

EDP1815 drug product is manufactured from a master cell bank by fermentation and subsequent lyophilization and encapsulation. The drug substance is a lyophilized powder which is rendered essentially non-viable and non-colonizing during the manufacturing process after fermentation, with a cell viability of <0.02%. It has not been genetically modified.

This specific *P. histicola* strain was selected for its pharmacological anti-inflammatory properties using *in vitro* and *in vivo* models in a similar way to the discovery of conventional small molecule or biologic drugs. Preclinical studies in models of rheumatoid arthritis (CIA)[9] and experimental acute encephalomyelitis (EAE)[10] have

shown that oral treatment with this strain of *P. histicola* has immunomodulatory effects leading to reduced inflammation development and severity. Furthermore, its beneficial effects in murine models of celiac disease,[11] and of type 1 diabetes[12] have also previously been published. Mechanistic studies in mice have been conducted to show that these effects occur without systemic exposure and are due to the connectivity between mucosal and systemic immune networks.[4]

To investigate whether the immunomodulatory activity of a non-colonizing microbial therapeutic can drive pharmacological effects which translate from mice to humans, we tested orally administered EDP1815 for its ability to modulate inflammation in a range of preclinical and clinical studies. We describe broad and potent anti-inflammatory effects of EDP1815 in preclinical studies which capture Th1, Th2 and Th17 biology and in three corresponding clinical studies that determined the safety and efficacy of EDP1815 in, (1) a T cell-mediated skin challenge model in healthy volunteers; (2) patients with atopic dermatitis, predominantly Th2-driven; and (3) patients with psoriasis, predominantly Th17-driven, demonstrating translation from mice to humans for a broadly acting, oral, immunomodulatory, non-colonizing microbial strain targeting the SI.

The delayed-type hypersensitivity (DTH) reaction, also known as type IV hypersensitivity reaction, is a common model of T cell-mediated inflammation in mice and other mammals. It is used for evaluating cell-mediated immune responses associated with $CD4^+$ or $CD8^+$ T cell reactivity, studying the mechanisms of skin inflammation, and evaluating therapeutic efficacy. Multiple effector mechanisms are involved but it is generally considered to be predominantly driven by Th1 cells[13] with some Th2 cell involvement.[14] A similar T cell-mediated response can also be induced in humans using a neoantigen skin challenge, with a resulting DTH response quantified by an increase in skin blood perfusion and erythema.[15,16] Therapeutic interventions that target T cells inhibit both the mouse and human keyhole limpet hemocyanin (KLH) DTH response by a similar mechanism, and therefore the mouse model can be used to predict T cell-mediated responses in humans.[17,18]

Atopic dermatitis has a prevalence of 11–30% in children[19,20] and 2–10% in adults[20] with the majority of patients having mild to moderate disease. Genetic predisposition, disruption of the epidermal barrier and immune dysregulation are components in the development of atopic dermatitis.[21] Barrier disruption leads to skin inflammation and allergic sensitization driven by activation of T cell subsets, predominantly Th2 immune responses. Th2 cytokines interleukin-4 (IL-4) and IL-13 drive chemokine production, further epidermal barrier dysfunction and allergic inflammation.[22] Clinical data from studies with monoclonal antibodies including dupilumab and lebrikizumab have validated the role of anti-IL-4 and anti-IL-13 therapy in moderate to severe atopic dermatitis[23] though these therapies are limited to use in patients with moderate to severe disease due to challenges related to safety, convenience, and high cost. Another Th2 cytokine, IL-31, has been reported to increase production of cytokines and chemokines from skin cells, thereby inducing itch and pruritic skin lesions.[24,25] EDP1815 was tested in patients with atopic dermatitis to confirm its potential in treating Th2 diseases such as atopic dermatitis, allergy, and asthma.

Psoriasis is a chronic immune-mediated inflammatory disease with predominant pathological effects in the skin and musculoskeletal tissue with an adult prevalence of up to 2%.[26] Similar to atopic dermatitis, most patients with psoriasis suffer from mild to moderate disease. It is characterized by psoriatic plaques and acanthosis due to uncontrolled keratinocyte proliferation. Disruptive cutaneous immune responses are responsible for the sustained inflammation seen in the psoriatic skin.[27] As well as cutaneous features, it is associated with nail disease, arthritis, and metabolic syndrome.[25] Infiltration of inflammatory dendritic cells drives the initial stages of disease followed by activation of Th17 cells. Th17, Th2, and Th1 cells have been noted in psoriatic lesions. The immune-pathophysiology associated with psoriasis involves overexpression of interferon (IFN), tumor necrosis factor (TNF), IL-17, IL-20, and IL-22.[28] Clinical data with therapeutic monoclonal antibodies have validated the role of anti-TNF, anti-IL-17 and anti-IL-23 therapy in moderate to severe psoriasis.[27]

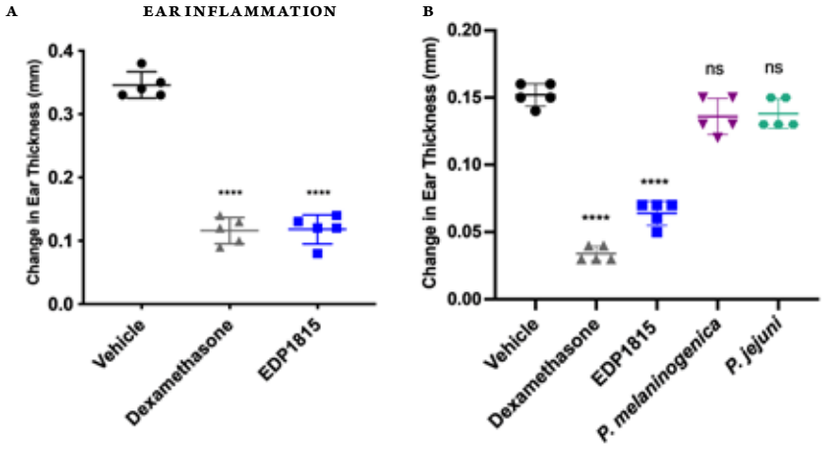
Here we describe for each of the Th1, Th2, and Th17 inflammation subtypes the translation from mice to an equivalent human model or disease with the aim of providing proof-of-concept for a novel treatment approach to resolve systemic inflammation through the small intestinal axis.

RESULTS

EDP1815 IS EFFECTIVE IN A PRECLINICAL MODEL OF TH1-PREDOMINANT INFLAMMATION

To determine the therapeutic potential of orally delivered EDP1815 in Th1-driven inflammation a murine DTH was performed. Mice were sensitized subcutaneously with KLH on the back and subsequently dosed with EDP1815 by oral gavage for 4 weeks and given an ear challenge with KLH on day 29. 24 hours later, ear swelling was measured as a marker of inflammation. Treatment with EDP1815 significantly reduced ear swelling compared with the vehicle-treated group (Figure 1A). Furthermore, in a shorter model, mice were dosed with EDP1815 by oral gavage for 8 days, given a KLH challenge on the ear on the ninth day and ear swelling was measured 24 hours post challenge. EDP1815 was the most efficacious strain in lowering ear inflammation compared to strains of other closely related *Prevotella* species (Figure 1B).

FIGURE 1 EDP1815 resolves Th1-driven inflammation *in vivo*. (A) In a DTH model, C57BL/6 mice were immunized with KLH and CFA on day 0 and challenged in the ear 4 weeks later with KLH. Mice were orally dosed for 5 days per week from the day after immunization through ear challenge with vehicle or EDP1815 (TCC 4.69×10^9) or with dexamethasone systemically. Ear inflammation was measured 24 hours post ear challenge. Data shown as change in ear thickness ($n = 5$ mice/group). (B) DTH model was set up as previously described. Mice were challenged in the ear 9 days after sensitization. Ear inflammation was measured 24 hours post ear challenge. Data shown as change in ear thickness ($n = 5$ mice/group) for groups dosed with EDP1815 and other *Prevotella* strains (*P. jejuni* TCC 6.29×10^9, *P. melaninogenica* TCC 2.48×10^9). All experiments were performed twice.



*Data shown are representative and results are expressed as mean ± standard error of the mean. **** $P < .0001$, ns: not significant as determined by ordinary One-Way ANOVA.*

EDP1815 IS EFFECTIVE AND WELL-TOLERATED IN A CLINICAL MODEL OF TH1-PREDOMINANT INFLAMMATION

EDP1815-102 was a phase 1b, single-center, randomized placebo-controlled study investigating the potential of EDP1815 to modulate Th1-driven inflammation in healthy human volunteers. Participants were dosed with EDP1815 for 28 days. Immunological sensitization to KLH was induced by intramuscular injection on day 3 of dosing followed by intradermal KLH challenge on day 26. Inflammation was assessed using specialized imaging techniques to measure antigen specific responses to KLH challenge comparing drug-treated and placebo participants, expressed in arbitrary units (AU) Laser Speckle Contrast Imaging (LSCI) of basal flow and flare. Skin color and average redness was assessed by multispectral imaging. These quantitative endpoints were measured just before and then 2 days following the intradermal KLH challenge. Thirty-two subjects were enrolled in 2 cohorts. In each cohort, 12 subjects received EDP1815, and 4 received matching placebo for 28 days. Active subjects in both cohorts were administered with 8.0×10^{11} cells of EDP1815, once daily as either 10 (cohort 1) or 5 capsules (cohort 2). Participants in cohort 2 were fasted 2 hours pre-dose.

Although the study was not powered to detect statistically significant differences across treatment groups, notable trends corresponding to a reduction in inflammation as measured by dermal imaging were observed in the groups treated with EDP1815 in comparison to placebo. This was observed for all measurements: LSCI basal flow and flare (Figure 2A), and multispectral imaging skin color and average redness (Figure 2B). The effects were consistent across measures and reproduced in the two cohorts. Given subject numbers the effect size did not reach statistical significance.

EDP1815 was safe and well-tolerated with no overall difference in adverse events (AEs) from placebo. There were no severe AEs in any participants (Table 1).

FIGURE 2 EDP1815 leads to reductions in inflammation measurements in a randomized double-blind trial of healthy volunteers administered KLH challenge. The effect of EDP1815 on the systemic immune system was evaluated in a double-blind, randomized, placebo-controlled trial using a KLH challenge. Two cohorts of 16 patients randomized 3:1 active ($n = 12$) to placebo ($n = 4$) were treated once daily for 28 days, with intramuscular administration of KLH on day 3, followed by intradermal KLH rechallenge on day 26. On the contralateral arm, placebo was administered intradermally on day 26 to account for local inflammation due to the injection. (A) Cutaneous microcirculation as a marker of inflammation was assessed by LSCI to measure flare, expressed in AUs, at baseline and day 26. (B) Cutaneous erythema as a marker of inflammation was assessed by multispectral imaging to quantify redness, expressed in AUs, at baseline and day 26.

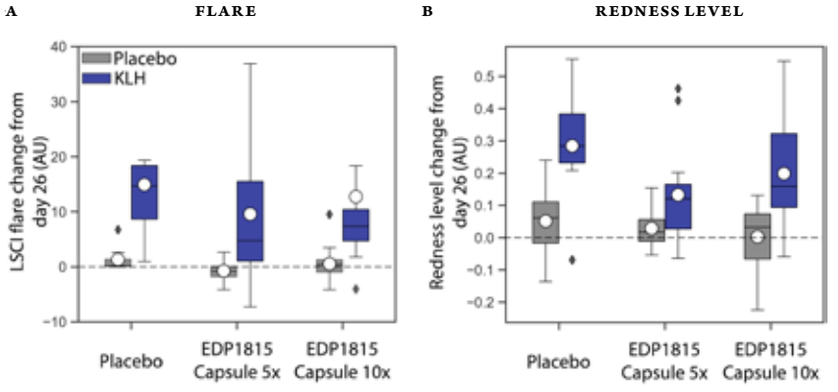


TABLE 1 EDP1815 combined safety data summary healthy volunteers, psoriasis, and atopic dermatitis.

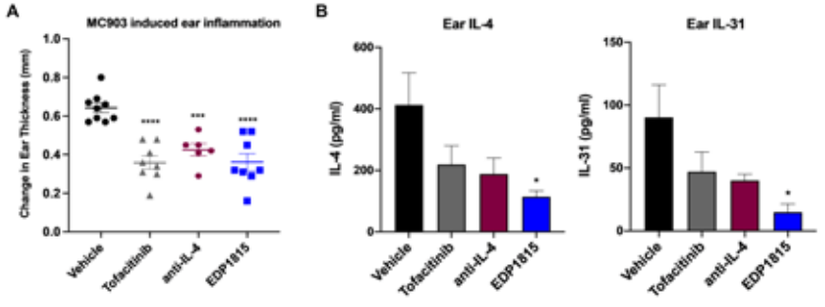
Adverse event	Skin challenge healthy volunteers			Psoriasis			Atopic dermatitis	
	Placebo (*n* = 8)	Active cohort 1 (*n* = 12)	Active cohort 2 (*n* = 12)	Placebo (*n* = 10)	Active low-dose (*n* = 8)	Active high-dose (*n* = 12)	Placebo (*n* = 8)	Active (*n* = 16)
ANY TEAE								
Mild	8 (100%)	11 (92%)	8 (67%)	5 (50%)	4 (50%)	9 (75%)	6 (75%)	14 (88%)
Moderate	0	1 (8%)	0	5 (50%)	1 (13%)	3 (25%)	3 (13%)	3 (19%)
Severe	0	0	0	0	0	0	0	0
Serious	0	0	0	0	0	0	0	0
RELATED TEAE								
Mild	4 (50%)	7 (58%)	2 (17%)	1 (10%)	3 (38%)	2 (17%)	1 (13%)	5 (31%)
Moderate	0	0	0	2 (20%)	0	1 (13%)	0	0
Severe	0	0	0	0	0	0	0	0
Serious	0	0	0	0	0	0	0	0
Death	0	0	0	0	0	0	0	0
ADVERSE EVENT REPORTED BY ≥ 3 PATIENTS IN EITHER GROUP								
Headache	2 (25%)	7 (58%)	6 (50%)	3 (30%)	1 (13%)	2 (17%)	2 (25%)	8 (50%)
Fatigue	3 (38%)	1 (8%)	0	0	0	0	0	0
Myalgia	2 (25%)	4 (33%)	2 (17%)	0	0	0	0	0
Viral upper respiratory tract infection	3 (38%)	3 (25%)	1 (8%)	0	0	0	0	0
Rash	0	3 (25%)	0	0	0	0	0	0
Abdominal pain upper	0	3 (35%)	0	0	0	1 (8%)	0	0
Abdominal pain	1 (13%)	0	0	0	0	0	0	3 (19%)
Diarrhea	0	0	0	0	1 (13%)	0	0	6 (38%)
TREATMENT MODIFICATION								
Discontinuation	-	-	-	0	0	0	0	0
Dose Interruption	-	-	-	0	0	0	0	1 (6%)
Dose Reduction	-	-	-	0	0	0	0	0

The preferred term was used to summarize the adverse events across the 3 groups. Different dictionaries may have been used between the healthy volunteer and psoriasis and atopic dermatitis studies.

EDP1815 IS EFFECTIVE IN A PRECLINICAL MODEL OF TH2-PREDOMINANT INFLAMMATION

The vitamin D3 analog, MC903, can be used in mice to generate Th2-driven epidermal inflammation with increased dermal cell infiltrates consisting of eosinophils, T cells, neutrophils, and mast cells. Following application of MC903, skin shows increased levels of a range of Th2 cytokines including IL-4, IL-5, IL-13, and IL-31.[29] BALB/C mice were sensitized on the ears with MC903 for 14 days and dosed orally daily with EDP1815 for 14 days. Treatment with EDP1815 resulted in significantly lower ear inflammation in comparison with vehicle-treated animals, on par with tofacitinib, an oral JAK-1/3 inhibitor, and a systemic antibody blocking IL-4 (Figure 3A). In animals treated with EDP1815, *ex vivo* analysis revealed a reduction in levels of IL-4, a central Th2 cytokine, as well as of IL-31 (Figure 3B).

FIGURE 3 EDP1815 is protective in Th2-driven model of allergic inflammation. BALB/C mice were topically sensitized daily on the ear with 45 nM MC903 from day 1 to 14. Mice were dosed orally daily with vehicle or EDP1815 (TCC 3.13×10^9). Ear inflammation was measured on day 14. (A) Change in ear thickness ($n = 5$ mice/group). (B) Upon termination of study, ears were homogenized, and protein levels of IL-4 and IL-31 were measured by MSD. All experiments were performed twice.



*Data shown are representative and results are expressed as mean ± standard error of the mean. * $P < .05$, *** $P < .001$, **** $P < .0001$ as determined by Ordinary one-way ANOVA.*

EDP1815 IS EFFECTIVE AND WELL-TOLERATED IN ATOPIC DERMATITIS, A CONDITION WITH TH2-PREDOMINANT INFLAMMATION

To test the potential of EDP1815 to treat Th2-driven inflammatory disease, it was evaluated in a phase 1b clinical study (EudraCT # 2018-002807-32). A cohort of 24 participants with mild and moderate atopic dermatitis was randomized 2:1 active:placebo.

8.0×10^{11} total cells of EDP1815 was administered once daily for 56 days, with a follow-up visit after 14 days off drug on day 70. The primary endpoint was safety and tolerability of EDP1815. Secondary endpoints included physician-rated scales of atopic dermatitis severity (Eczema Area and Severity Index (EASI), Investigator's Global Assessment (IGA), Body Surface Area (BSA), IGA×BSA, and Scoring Atopic Dermatitis (SCORAD)); as well as patient-reported outcomes (pruritus numerical rating scale (NRS), Dermatology Life Quality Index (DLQI), and Patient-Oriented Eczema Measure (POEM)). Baseline mean EASI and IGA scores were 8.31 and 2.63 respectively, for the 16 patients receiving EDP1815, and 9.31 and 2.7, respectively for the 8 patients receiving placebo.

EDP1815 had a placebo-like safety and tolerability profile with no treatment-related AEs of moderate or severe intensity, and no serious adverse events (SAEs) (Table 1).

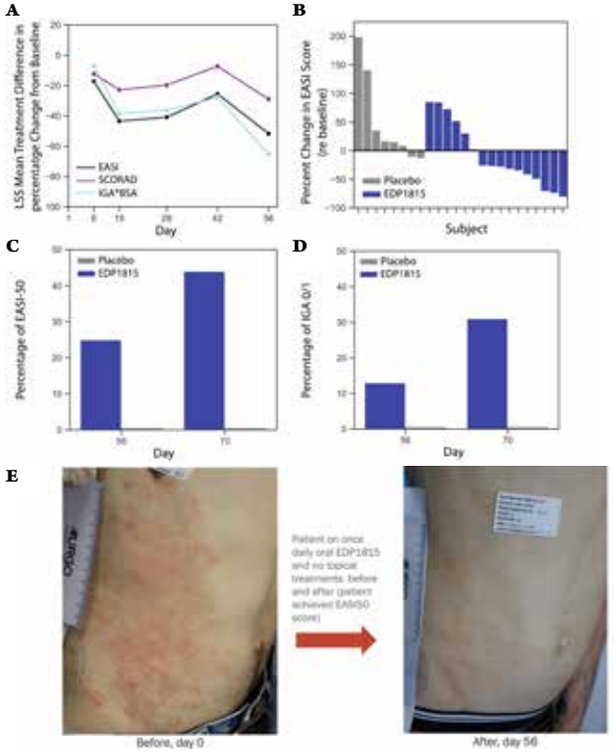
The differences in percentage decrease from baseline in EASI, IGA×BSA and SCORAD between the EDP1815-treated group and the placebo group were 52% (P = .062), 65% (P = .022), and 35% (P = .068), respectively (Figure 4A). 10 of 16 patients receiving EDP1815 saw improvements in their EASI score at day 56, compared to only 2 out of 8 patients receiving placebo (Figure 4B).

At the day 70 follow-up visit, further clinical improvements were observed in the EDP1815-treated group. The percentage of patients receiving EDP1815 achieving EASI50 was 44% compared with 0% in the placebo group (Figure 4C); and the proportion achieving an IGA score of 0 or 1 was 31%, with 0% again in the placebo group (Figure 4D). Figure 4E shows a representative clinical improvement of skin condition in a participant receiving EDP1815, and no other oral or topical treatments, for 56 days in this trial. This was an EASI50 response, with an improvement from an EASI score of 9.8 at baseline to 4.9 at day 56. In addition to the clinical improvements in the physician rating scales, this participant's patient-reported outcomes also improved with the DLQI score moving from 13 (severe impact) to 1 (no impact), and the POEM score from 22 (self-rating of 'severe eczema') to 5 ('mild eczema') at day 56.

The mean individual patient-reported outcomes improvement from baseline in the DLQI (−3.6) and POEM (−4.1) in EDP1815-treated patients at day 56 exceeded the minimally clinically important difference thresholds[30,31] and exceeded the placebo group changes (−0.3 and +1.6, respectively). Mean improvements in itch and sleep were seen within all scales measuring these parameters (pruritis NRS, DLQI, SCORAD, and POEM) at the end of the treatment period.

These results provide proof-of-concept that EDP1815 can resolve Th2-driven inflammation with a placebo-like safety and tolerability profile in patients with atopic dermatitis.

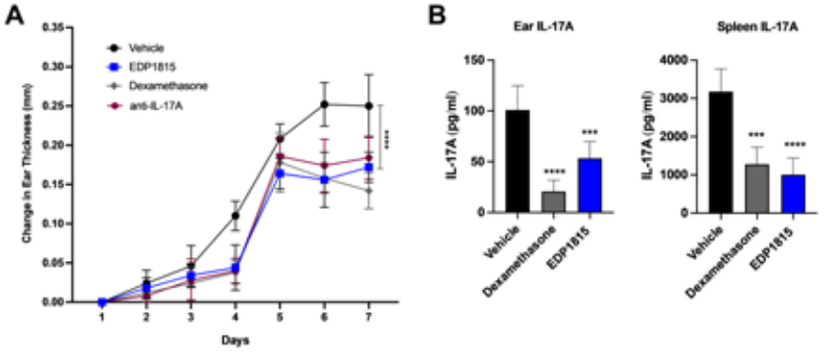
FIGURE 4 EDP1815 leads to clinical improvements in a randomized double-blind trial of patients with atopic dermatitis. A phase 1b cohort of 24 patients with mild and moderate atopic dermatitis were randomized to EDP1815 (n = 16) or placebo (n = 8) and treated once daily for 56 days, with follow-up off treatment at day 70. (A) Clinical parameters of atopic dermatitis were measured at baseline and treatment days 8, 15, 28, 42, and 56. The treatment difference was calculated by subtracting the mean percentage change from baseline in placebo patients from that in active patients at each time point, and for each of the key clinical scores quantifying disease severity: EASI, IGA×BSA, and SCORAD. At day 56, the treatment difference for EASI was 52% (P = .062), for IGA×BSA was 65% (P = .022), and for SCORAD was 35% (P = .068). (B) Waterfall plot, with each participant's percentage change from baseline in the EASI score at day 56 represented by each bar. Two placebo patients saw improvement, compared to ten patients randomized to EDP1815, with 4 patients achieving EASI50 or better at this timepoint. (C) Proportion of patients achieving EASI50 threshold or better, in active versus placebo group patients at day 56 (25% *vs.* 0%, respectively) and day 70 (44% *vs.* 0%, respectively). (D) Proportion of patients achieving IGA0/1 threshold in active versus placebo group patients at day 56 (13% *vs.* 0%, respectively) and day 70 (31% *vs.* 0%, respectively). (E) Photographs taken of a subject receiving EDP1815 and no topical or other active atopic dermatitis treatment in this study, at baseline and after 56 days of treatment. Significant improvements in erythema, papulation and excoriations are visible. The patient achieved an EASI improvement of 50%, from 9.8 at baseline to 4.9 at day 56.



EDP1815 IS EFFECTIVE IN A PRECLINICAL MODEL OF TH17-PREDOMINANT INFLAMMATION

A toll-like receptor-7 (TLR7) agonist, imiquimod, induces clinical and histological changes characteristic of human psoriasis, including epidermal thickening, scaling and erythema.[32] Mice were sensitized on the ear and back with imiquimod cream daily for 7 consecutive days and dosed daily with oral EDP1815, vehicle, dexamethasone, or anti-IL-17A antibody. Ear thickness was a measure of inflammation. Treatment effects in animals dosed with EDP1815 were seen as early as 4 days after the start of imiquimod application and were comparable to those observed in animals treated with dexamethasone or anti-IL-17A in reducing ear thickness as well as back inflammation (Figure 5A). At termination on day 8, IL-17A protein levels in the ear tissue were reduced by treatment with EDP1815 in comparison to vehicle (Figure 5B). Imiquimod is known to also induce an increase in IL-17A production in splenocytes.[32] *Ex vivo* re-stimulation of splenocytes with phorbol myristate acetate (PMA)/Ionomycin showed decreased production of IL-17A in mice treated with EDP1815 (Figure 5B).

FIGURE 5 EDP1815 alleviates skin pathology in a Th17 model of cutaneous inflammation. BALB/C mice were topically treated with 5% imiquimod, a TLR7 agonist, for 7 days on the back skin and ear. Mice were dosed orally daily from day 1 through 7 with vehicle, dexamethasone (1 mg/kg i.p.) or EDP1815 (TCC 3.13×10^9). (A) Time course of change in ear inflammation over 7 days. (B) At termination, splenocytes were *ex vivo* re-stimulated with PMA/Ionomycin for 48 hours. Protein levels of IL-17A were measured from supernatants by MSD. IL-17A protein levels were also measured in ear tissue homogenates. All experiments were performed twice.



*Data shown are representative and results are expressed as mean ± standard error of the mean. *** P < .001, **** P < .0001 as determined by Ordinary one-way ANOVA.*

EDP1815 IS EFFECTIVE AND WELL-TOLERATED IN PSORIASIS, A CLINICAL DISEASE OF TH17-INFLAMMATION

To determine the potential of EDP1815 to treat Th17-driven inflammatory disease it was evaluated in two parallel cohorts of a phase 1b clinical study in patients with psoriasis (EudraCT # 2018-002807-32). Adult patients with mild to moderate chronic plaque psoriasis were randomized 2:1 to receive EDP1815 or matching placebo capsules. Doses were 1.6×10^{11} (cohort 3) and 8.0×10^{11} (cohort 4) bacterial cells, once daily for 28 days, with follow-up after 14 days off treatment at day 42. 12 patients were dosed with the lower dose, and 18 with the higher dose. Placebo subjects were pooled across both cohorts. The primary endpoint was safety and tolerability of EDP1815. Secondary endpoints included physician-rated scales of psoriasis: Psoriasis Area and Severity Index (PASI) and Lesion Severity Score (LSS). Baseline mean PASI scores were 9.5 (cohort 3), 6.2 (cohort 4), and 6.7 (pooled placebo cohorts). Mean LSS scores at baseline were 8.1 (cohort 3), 7.8 (cohort 4), and 7.8 (pooled placebo cohorts).

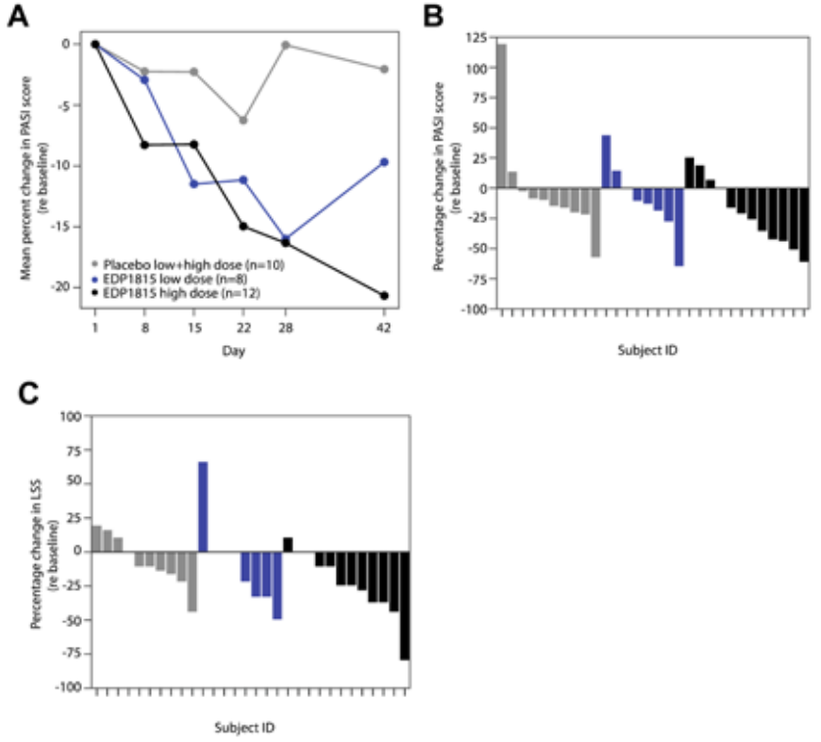
The primary endpoint safety data showed EDP1815 to have a safety and tolerability profile comparable to placebo (Table 1). As for the other studies reported here, there were no SAEs, and no AEs of severe intensity.

The PASI score is a composite measure of psoriasis plaque severity, and body coverage.[33] Following 28 days of treatment, the mean percentage reduction in PASI for EDP1815 cohorts was 16%, compared to 0.1% for placebo. At day 42, the percentage improvement from baseline in cohort 2 active participants increased further to 21% (Figure 6A). In this cohort, 6 of the 12 patients achieved a 25% improvement in PASI or better at day 42, compared to 1 of 10 in placebo (Figure 6B).

LSS measures the severity of a target plaque using scaling, erythema, and plaque elevation, giving a maximum total score of 12. The mean percentage reductions in LSS scores at day 28 were 23 and 15% in the low- and high-dose cohorts respectively, compared to a 1% increase from baseline in the placebo group. Figure 6C shows the individual percentage changes in LSS from baseline at day 42, with 7 of the 12 patients in the high-dose cohort achieving 25% improvement or greater, compared to 1 of 10 in the placebo group.

These two comparable sets of clinical data provide proof-of-concept that EDP1815 can drive clinical improvements and resolve inflammation in the skin of patients with psoriasis with a placebo-like safety and tolerability profile. As seen in atopic dermatitis, responses were continuing to improve and had not reached peak effect at the end of the dosing period.

FIGURE 6 EDP1815 leads to clinical improvements in two randomized double-blind cohorts of patients with psoriasis. Two parallel phase 1b cohorts of 12 and 18 patients with mild and moderate psoriasis were randomized to EDP1815 or placebo in a 2:1 ratio, and treated once daily for 28 days, with follow-up off treatment at day 42. (A) Mean percentage change from baseline in PASI score from baseline to final follow-up visit. At the end of treatment visit on day 28, the mean percentage reduction in PASI for EDP1815 cohorts was 16%, compared to 0.1% for placebo. (B) Waterfall plot, with each participant's percentage change from baseline in the PASI score at day 42 represented by each bar. EDP1815 low- and high-dose compared to pooled placebo. A 25% or greater improvement in PASI was observed in 1 of 10 placebo patients, 2 of 8 participants receiving low-dose EDP1815, and 6 of 12 participants receiving high-dose EDP1815. (C) Waterfall plot, with each participant's percentage change from baseline in the LSS score at day 42 represented by each bar. EDP1815 low- and high-dose compared to pooled placebo. A 25% or greater improvement in LSS was observed in 1 of 10 placebo patients, 3 of 8 participants receiving low-dose EDP1815, and 7 of 12 participants receiving high-dose EDP1815. EDP1815 is gut-restricted with no systemic absorption and no impact on background microbiome.



To determine the biodistribution of EDP1815 following oral dosing in mice, strain-specific primers were designed to differentiate EDP1815 from other *P. histicola* strains. This enabled sensitive tracking of EDP1815 in mouse experiments in the potential presence of alternate species of *P. histicola* in the background GI microbiome.

Following oral administration of a single dose, EDP1815 was transiently detected in the GI tract and stool. EDP1815 was detected in the intestine and stool for up to 8 hours and not at 16 hours post administration, showing that the lyophilized microbes did not colonize the gut. Importantly, EDP1815 was not detected outside of the GI tract at any time point. These data demonstrate that EDP1815 is luminally restricted with undetectable systemic exposure following oral dosing in mice (Supplementary Figure S1).

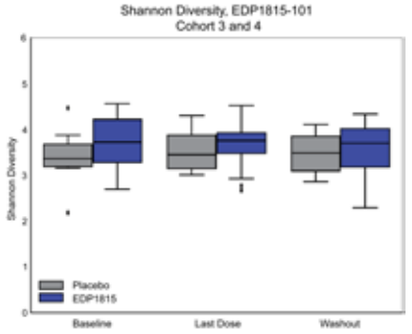
Evidence of systemic exposure of EDP1815 in humans was evaluated in the phase 1b clinical cohorts of psoriasis patients described previously. EDP1815 was not detected in blood by polymerase chain reaction (PCR) or by culture at any time-point during the dosing period, through completion of the 28 days of dosing.

In these psoriasis cohorts EDP1815 was not detected by quantitative PCR (qPCR) in stool samples taken 14 days after completion of dosing. This experiment was repeated in the healthy volunteer DTH study. Again fecal concentrations of EDP1815 were below the limit of quantification pre-dose and 5 days post last dose in all treatment groups, confirming a lack of gut colonization.

Finally, 16S ribosomal ribonucleic acid (rRNA) sequencing of stool samples confirmed that the pharmacodynamic activity observed with EDP1815 was not due to secondary alterations in the colonic microbiome. Samples taken at baseline, on drug and at follow-up showed no significant changes in the Shannon Index (diversity) or composition from baseline either during or following cessation of dosing (Figure 7). Furthermore, no significant changes in fecal microbe abundance at the genera level were detected for either placebo or EDP1815 dosed subjects when comparing between time points (Supplementary Figure S2). Microbes from the *Bacteroides* and *Blautia* genera were the most abundant in the fecal microbiome for all groups, and for all groups the 10 most abundant genera made up more than 90% of the microbiome by percent composition.

These data confirm that the resolution of inflammation observed with EP1815 is not due to systemic exposure, gut-colonization, nor indirect effects on the colonic microbiome.

FIGURE 7 EDP1815 transits through the GI tract between 8 and 16 hours. 16S PCR analysis of stool samples taken from patients at baseline, day 28 and day 42. The top 10 genera by percentage composition are given for EDP1815 and placebo across the two cohorts at each timepoint. There are no significantly differentiated genera for EDP1815-treated individuals, or significant differences between EDP1815 and placebo.



DISCUSSION

Here we describe the translational development of EDP1815, a single strain of *P. histicola*, and demonstrate that it can modify systemic inflammation in humans across Th1-, Th2- and Th17-driven inflammation through its action in the gut. The ability of gut-restricted EDP1815 in the mouse DTH study (Figure 1) to reduce a distal inflammatory response to the same degree as systemic administered dexamethasone is remarkable. It demonstrates a link between the enteric and systemic immune systems which is effective at a level which has not been previously described and can be harnessed for pharmacological effects. It suggests an entirely novel approach to the treatment of a wide range of inflammatory diseases.

This is the first report of a single microbe that is able to pharmacologically modulate multiple distinct inflammatory pathways in preclinical models and in human studies. In a healthy volunteer study of a KLH-induced skin inflammatory response, consistent anti-inflammatory effects were seen in two cohorts through imaging-based measures of skin inflammation. In psoriasis patients, signs of clinical efficacy were seen after 4 weeks of treatment, which continued beyond the treatment period in the higher-dose cohort. In atopic dermatitis patients, improvements were seen throughout the key physician- and patient-reported outcomes. In all these studies, EDP1815 was found to be well-tolerated by participants, with a safety profile comparable to placebo, including no severe or consistent side-effects reported. Of note is the lack of any evidence of immunosuppression with no opportunistic infection occurring in these studies.

The mechanism by which EDP1815 impacts these pathways leading to inflammation resolution is under investigation. We recently published data using another orally delivered non-live microbial product, EDP1867, showing induction of peripheral T cells with an anti-inflammatory phenotype generated by the action of EDP1867 within the gut and the cellular trafficking mechanism that enables this.[4] We have similar data for EDP1815 (manuscript in preparation).

Previously published studies have demonstrated that the strain of *P. histicola* from which EDP1815 was developed can resolve both Th1- and Th17-mediated inflammation in mouse CIA and EAE.[9,10] Efficacy of oral treatment of mice with *P. histicola* was dependent on regulation by $CD103^+$ dendritic cells and by generation of regulatory T cells in the gut, resulting in suppression of pro-inflammatory Th1 and Th17 responses and increased transcription of IL-10. And recently, oral administration of *P. histicola* was reported to delay the onset of type 1 diabetes in non-obese diabetic mice.[34] This effect correlated with a significant increase in regulatory T cells and decrease in $NKP46^+$ cells in the pancreatic lymph nodes. These findings are in line with the preclinical studies described here, where treatment with EDP1815 led to a similar down-regulation of Th1 and Th17 cytokines, as well as Th2 cytokines in a model of atopic skin inflammation. Commensal microbes have been shown to interact with and modulate cells of the GI tract to influence local inflammation.[35] These mechanisms include production of molecules such as bacterial metabolites or bioactive lipids and require colonization by the bacteria to exert their effects. However, data from the clinical studies described here show that the systemic effects of EDP1815 occur with no systemic absorption, no colonization, and no impact on the gut microbiome. Therefore, it is likely that direct interactions between EDP1815 and microbial pattern recognition receptor-expressing cells, such as intestinal epithelial cells and immune cells that can sample contents of the lumen, lead to the downstream systemic inflammation resolving effects described here. A regulatory mechanism that relies solely on direct interactions between the microbe and the cells of the intestine after oral administration would explain how EDP1815 can exert its effects with only transient occupancy of the GI tract without systemic exposure.

One limitation of the study is that the psoriasis and atopic dermatitis cohorts comprised a relatively small number of patients. While the primary goal of these first in human studies was to establish safety and tolerability, the magnitude and consistency of the clinical effects of EDP1815 are encouraging. Another limitation is that the clinical studies were of relatively short duration. The mechanism of action proposed by the preclinical studies would predict that deeper responses would develop over time, as more regulatory T cells accumulate at sites of inflammation and continue to down-modulate effector Th1 and Th17 cells.

In conclusion, the preclinical activity of EDP1815 and the clinical proof-of-concept results demonstrate that EDP1815 has the potential to be an effective, safe, and well-tolerated oral anti-inflammatory therapy. The data presented here led to the further clinical development of EDP1815: in a phase 2 dose-ranging study in mild and moderate psoriasis (NCT04603027), and a phase 2 study of mild to severe atopic dermatitis (NCT05121480). The data from the phase 1b studies described here also suggest the potential of EDP1815 for the treatment of a wide range of inflammatory conditions, introducing a new class of medicines to the medical armamentarium.

MATERIALS AND METHODS

MOUSE STUDIES

MICE Female BALB/C and C57BL/6 mice (6–8 weeks old) were purchased from Taconic Farms. Animals were housed in specific pathogen-free conditions in a vivarium (5 mice per cage), and all experiments were performed under Institutional Animal Care and Use Committee (IACUC) approved protocols and guidelines at Avastus Preclinical Services (Cambridge, MA, USA). Mice were allowed to acclimate in the vivarium for 1–2 weeks prior to the start of experiments. Mice were monitored daily, provided PicoLab Rodent Diet 20 and autoclaved water *ad libitum*.

DOSING WITH EDP1815 AND CONTROLS *IN VIVO* For each *in vivo* study, EDP1815 aliquots were distributed into plastic test tubes with caps and stored at 4°C. Mice were treated orally with EDP1815 (specific total cell count (TCC) is noted in each figure legend) or vehicle control (anaerobic sucrose, per os) for duration of different models as described in figure legends. Dexamethasone (1 mg/kg, intraperitoneally (i.p.), Sigma) was used as a positive control unless otherwise specified.

DELAYED-TYPE HYPERSENSITIVITY MOUSE MODEL Mice were immunized with 50 µL of emulsion of KLH in Complete Freund's Adjuvant (CFA) on four sites on the back. In a longer duration model, mice were dosed for 4 weeks and on day 29, mice were challenged with KLH (10 µg/10 µL) intradermally in the ear. In a shorter duration model, mice were dosed for 8 days and on day 9, mice were challenged with KLH (10 µg/10 µL) intradermally in the ear. Ear measurements were recorded 24 hours post ear challenge using digital calipers. Change in ear thickness was expressed as ear thickness at 24 hours post challenge minus ear thickness at baseline.

IMIQUIMOD-INDUCED PSORIASIS-LIKE SKIN INFLAMMATION PROTOCOL Mice were sensitized topically with 20 mg imiquimod cream (Aldara; 3M Pharmaceuticals, St Paul, MN, USA) on ears daily for 7 consecutive days. Ear measurements were taken daily using digital calipers and scores were reported as change in ear thickness calculated as ear score on day 8 minus baseline ear score on day 1.

MC903-DRIVEN ATOPIC DERMATITIS Mice were sensitized daily for 14 consecutive days with 45 nmol of MC903 (calcipotriol; Tocris Bioscience) in 20 µL of 100% ethanol on ears. Baseline ear measurements were taken prior to the first ear sensitization on day 1 using Digital Calipers (Fowler). On day 14, ear thickness was measured. Delta change in ear thickness was expressed as ear thickness at day 14 minus ear thickness at baseline.

MOUSE *EX VIVO* RE-STIMULATION ASSAYS Spleens were harvested at terminal time points and collected into 0.5 mL of cold, complete-RPMI (10% FBS, 1x Glutamax, 1 mM sodium pyruvate, 100 mM HEPES, 1x non-essential amino acids, 1x beta-mercaptoethanol, 1x antibiotic-antimycotic) (all reagents from Gibco). Single cell suspensions were prepared and 200,000 cells/well were plated. Cells were stimulated *ex vivo* with either lipopolysaccharide (200 ng/ml, Invivogen) or Poly I:C (Invivogen) for 48 hours at 37°C and 5% CO_2. Supernatants were collected at the end of stimulations and used for multiplex enzyme-linked immunosorbent assays of cytokine levels using Meso Scale Discovery (MSD) kits. Ear tissues were dissociated in 250 µL Tissue Protein Extraction Reagent buffer (Thermo Scientific) containing Halt Protease (Thermo Scientific) and protein was quantified with BCA kit (Thermo Scientific). 100 µg of protein was used to measure cytokine levels using MSD kits.

CLINICAL STUDIES

EDP1815 PRODUCTION AND FORMULATION EDP1815 drug substance is freeze-dried *P. histicola* bacterial cells. EDP1815 drug product is manufactured as enteric-coated hydroxypropyl methylcellulose hard capsules in two strengths, 80 billion (8.0×10^{10}) and 160 billion (1.6×10^{11}) total cells per capsule. The capsule formulations of EDP1815 consist of drug substance, mannitol, magnesium stearate and colloidal silicon dioxide. Both dose strengths are enteric coated to protect EDP1815 from stomach pH degradation and designed for release at pH ≥ 5.5. Corresponding placebo capsule formulation was manufactured using microcrystalline cellulose and magnesium stearate.

HEALTHY VOLUNTEER KLH STUDY DESIGN This was a phase 1, randomized, placebo-controlled, double-blind, multiple dose study in thirty-two healthy volunteers performed at the Centre for Human Drug Research, Leiden, The Netherlands. The Declaration of Helsinki was the principle for trial execution. The independent Medical Ethics Committee "*Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek*" (Assen, the Netherlands) approved the study prior

to any clinical study activity. All subjects provided written informed consent before participation. The trial was registered on trialregister.nl (trial identifier NL8676, currently available for consultation at the International Clinical Trial Registry Platform, https://trialsearch.who.int).

SUBJECTS Main inclusion criteria were healthy light skinned (Fitzpatrick skin type I–III) participants, 18 to 60 years of age with a body mass index between 18 and 35 kg/m^2, and no known previous exposure to KLH. Health status was verified by recording a detailed medical history, a complete physical examination, vital signs, a 12-lead electrocardiogram (ECG) and laboratory testing (including hepatic and renal panels, complete blood count, fecal calprotectin, virology, and urinalysis). Subjects were excluded in case of any disease associated with immune or GI system impairment or use of prescription medication within 4 weeks prior to first dose.

DOSE SELECTION AND REGIMEN The dose, 8.0×10^{11} total cells once daily, was based on the results of study EDP1815-101.

STUDY DESIGN AND TREATMENTS Subjects were enrolled into two cohorts. In each cohort, subjects were randomized to either EDP1815 or placebo (12:4). The first cohort received EDP1815 powder in enteric-coated capsules, supplied as 8.0×10^{10} TCC per capsule, administered orally at a dose of 10 capsules daily for 28 days. The second group also received EDP1815 powder in enteric-coated capsules, however this was supplied as 1.6×10^{11} TCC per capsule, administered orally at a dose of 5 capsules daily for 28 days. Intramuscular KLH immunization was performed in the deltoid muscle after three subsequent doses of study drug. KLH was administered in a formulation of 0.1 mg of subunit KLH (Immucothel®) adsorbed in 0.9 mg aluminum hydroxide (Alhydrogel®) into 0.5 mL sodium chloride 0.9%. Twenty-three days after intramuscular KLH administration, all subjects received an intradermal KLH administration in the left ventral forearm and placebo administration in the right ventral forearm. The formulation of 0.001 mg subunit KLH in 0.1 ml sodium chloride 0.9% used for intradermal administration and interval of twenty-three days between intramuscular KLH immunization and intradermal KLH administration and the interval of 48 hours between baseline and follow-up skin challenge response assessment was based on previous other studies.[15,36-40] Prior to, and 2 days after the intradermal KLH administration, the skin hypersensitivity response was quantified.

SAFETY AND TOLERABILITY Safety and tolerability were monitored by physical examination, assessment of vital signs, laboratory parameters (i.e., full blood count, biochemistry, serology, immunophenotyping, fecal calprotectin, and urinalysis) and ECG data from 12-lead ECGs at regular intervals. Subjects were monitored continuously for AEs.

STUDY TREATMENT COMPLIANCE Compliance was assured by supervised administration of the study treatment during the in-clinic period. Administration at home was recorded by an electronic diary by means of photography of the capsules taken and recording the date and time.

SKIN CHALLENGE RESPONSE CUTANEOUS BLOOD PERFUSION Cutaneous blood perfusion quantification was performed with LSCI (PeriCam PSI System, Perimed AB, Järfälla, Sweden) as previously described.[15] In short, assessments were performed in a temperature-controlled room (22°C) after acclimatization of the subjects. LSCI recordings of the target area on the left and right ventral forearms were captured with the use of dedicated software (PimSoft, Perimed AB, Järfälla, Sweden). Circular regions of interest at the intradermal injection sites were defined and cutaneous blood perfusion (indicated as basal flow) was quantitatively assessed and expressed in AUs. The homogeneity of cutaneous blood perfusion in the region of interest (indicated as flare), expressed as values that are +1 standard deviation (SD) from the mean basal flow within the region, was also quantitatively assessed and expressed in AUs.

KLH SKIN CHALLENGE ERYTHEMA Erythema quantification was performed with multispectral imaging (Antera 3D®, Miravex, Dublin, Ireland) as previously described.[15] In short, the camera was placed on the target area on the ventral forearms and images were captured using dedicated software (Antera 3D® software, Miravex, Dublin, Ireland). Circular regions of interest at the intradermal injection sites were defined and erythema was quantified using the average redness and CIELab a* Antera 3D® software modalities expressed as AUs. The average redness modality displays the distribution of redness using an internal software algorithm and the CIELab a* value, which is part of the CIELab color space and expresses color as a numerical value on a green–red color scale.

STATISTICS Subjects were randomized to EDP1815 or placebo in a 3:1 ratio. KLH skin challenge endpoints were analyzed with an analysis of covariance (ANCOVA) with treatment as fixed factor and the baseline and the change from baseline of the saline-injected control (right forearm) added as covariates. The general treatment effect and specific contrasts were reported with the mean change from baseline and SD. Fecal microbiome endpoints were analyzed using Python (Python Software Foundation,

Wilmington, DE, USA). The relative *Prevotella* abundance was calculated separately per treatment arm and over time. For microbiome diversity a diversity trend analysis was performed using Simpson's diversity index.

PSORIASIS AND ATOPIC DERMATITIS STUDY DESIGN (EDP1815-101) This clinical trial is a first-in-human study of EDP1815 in healthy volunteers, patients with psoriasis, and patients with atopic dermatitis. This randomized, double-blind, placebo-controlled study with dose escalations was designed to assess the safety, tolerability and pharmacodynamic effect of various doses and formulations of EDP1815. The primary objective was safety and tolerability of EDP1815 treatment in each cohort, the secondary objectives were clinical efficacy measures of either psoriasis or atopic dermatitis. As a phase 1 study investigating dose escalations and safety of the investigational medicinal product, the study was not powered for detection of statistical significance of clinical efficacy, but the sample size was selected to determine the initial safety profile of a range of doses of EDP1815, while informing sample size for a subsequent phase 2 study in both psoriasis and atopic dermatitis. Both participants and investigators were blinded to treatment allocation until study completion. 10 cohorts were assessed in this study: cohorts 1–4 and 7 assessed the enteric-coated capsule formulation. Cohorts 5–6 and 8–10 assessed alterations in drug substance or drug product, and therefore results of these cohorts are not presented in this manuscript. Cohorts 1 and 2 were performed in healthy human volunteers, cohorts 3–4 in patients with mild and moderate psoriasis, and cohort 7 in patients with mild and moderate atopic dermatitis. The data from these patient cohorts using enteric-coated capsules are presented in this manuscript.

STUDY OVERSIGHT This trial was reviewed and approved by the Medicines and Healthcare products Regulatory Agency as a Clinical Trial Application (EudraCT #2018-002807-32) and registered on ClinicalTrials.gov (NCT03733353). The protocol and all patient facing materials including the informed consent form were approved by the Health Research Authority Research Ethics Committee (London-Chelsea). Written and signed informed consent was obtained from all participants prior to their enrollment in the study.

SUBJECTS Main inclusion criteria were healthy participants, other than having the inflammatory skin disease under question in the respective cohort. Subjects were required to be 18 to 65 years of age with a body mass index between 18 and 35 kg/m^2 and have no known previous exposure to EDP1815. Health status was verified by recording a detailed medical history, a complete physical examination, vital signs, a 12-lead ECG and laboratory testing (including hepatic and renal panels, complete blood count and urinalysis). Participants were excluded in case of any active infection, any GI tract disease that could interfere with drug delivery or GI transit time or having received medications other than paracetamol or antihistamine within 14 days of baseline.

DOSE SELECTION The starting dose for the clinical study is based on the predicted therapeutic range based on preclinical *in vitro* and *in vivo* experiments. This expected range is based on the TCC of microbes given by oral gavage to the mice in the preclinical animal model experiments. This has been adjusted using allometric scaling approaches and converted to a milligram equivalent dose providing an estimate of the likely therapeutic range.

STATISTICAL ANALYSIS OF CLINICAL DATA Subjects were randomized to EDP1815 or placebo in a 2:1 ratio. Randomization for each cohort was created using a simple block design generated by an unblinded statistician who had no other involvement in the study. The randomization was administered centrally, with the next available randomization number used for each new participant. Investigators and participants were blinded to treatment assignment and all containers and study medication for placebo were identical to those for EDP1815. For cohorts 3 and 4, a sentinel pair was used to dose one EDP1815 and one placebo participant; safety data for the first 3 days of multiple dosing for the sentinel pair was reviewed prior to the opening of the cohort for further participants. For cohort 7, the same dose of EDP1815 as was used in cohort 4 was administered and as such no sentinel dosing was required.

The sample sizes were chosen to explore the tolerability and safety of this new treatment and no formal power calculations were performed. All participants taking at least one dose of study medication were included in the safety analyses. For the efficacy analyses, all randomized participants were included. The protocol did prespecify that any participants who had an important protocol deviation affecting psoriasis-related efficacy variables would be excluded, but no such deviations occurred.

For cohorts 3 and 4, where a single dose was administered before being followed up with daily dosing on day 3, Baseline for efficacy endpoints was assessed as the measurement taken at the day 3 (start of daily dosing) visit. For cohort 7, no sentinel dose was used and baseline was assessed as the measurement taken on day 1.

Incidences of AEs and SAEs were produced by treatment and severity with separate summaries of study drug-related events. For the continuous efficacy endpoints for skin assessment (LSS and PASI for psoriasis; EASI, IGA, affected BSA, and SCORAD for atopic dermatitis), data was analyzed with a mixed model for repeated measures, including terms for treatment, visit, baseline score and treatment-by-visit interaction. Waterfall plots showing individual percentage changes from baseline were also produced. Patient-reported outcomes (DLQI, POEM and pruritis NRS) were summarized using mean,

median, standard deviation, and range. Responder endpoints were summarized using the number and percentage of participants to meet the relevant response definition.

PSORIASIS: STUDY EDP1815-101, COHORTS 3 AND 4

Two cohorts of 12 and 18 patients with mild to moderate psoriasis, both randomized 2:1 active to matching placebo.

EDP1815 was administered as a single dose (day 1), and after confirming safety, as once daily for 28 days (days 3–30), with follow-up at day 42. The dose was 1.6×10^{11} (cohort 3) or 8.0×10^{11} (cohort 4) bacterial cells per day. Placebo subjects were pooled across both cohorts in the analysis.

SUBJECTS The psoriasis-specific inclusion criteria were as follows: patient has a confirmed diagnosis of plaque psoriasis for at least 6 months, and a BSA of 10% or less (excluding the scalp), with at least two psoriatic lesions. Patients were excluded if they had received systemic non-biologic psoriasis therapy within 4 weeks prior to screening, biologic therapy within 12 months prior to screening, or topical agents that could affect psoriasis within 2 weeks of dosing (unmedicated emollient was permitted if the subject was already using this as part of their standard care). Pharmacologically active treatments for psoriasis or atopic dermatitis were not permitted at any timepoint.

SAFETY AND TOLERABILITY DATA The primary endpoint was safety and tolerability. Measurements were AEs, laboratory assessments (biochemistry including C-reactive protein (CRP), hematology, urinalysis), physical examination, vital signs, and ECG readings at multiple timepoints, including end of treatment and follow-up. AEs were monitored continuously from screening to follow-up visit.

EFFICACY DATA The secondary endpoints of LSS, BSA, PGA and the PASI score were measured at baseline, weekly until day 28, and at day 42.

MICROBIOME SEQUENCING Stool samples were taken at three time points: baseline, day 28, and day 42. Stool was collected in DNA/RNA Shield Fecal Collection tube (Zymo Research) and stored at −80°C until processing. DNA extraction, qPCR and 16S sequencing were performed at and by Baseclear (Leiden, Netherlands) according to their standard operating procedures.

For 16S sequencing, the V4 region of bacterial 16S rRNA was amplified using universal primer set 515F and 806R.[41] Resulting products were sequenced through the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) on a 2 × 250 paired-end run. Reads were demultiplexed and quality filtered before being uploaded to the One Codex platform.[42] Paired-end reads were merged and then characterized using One Codex's in-house Targeted Loci Database, a curated database of bacterial marker genes including 16S rRNA. Read count and relative abundance tables were calculated at the genus level and retrieved using custom Python scripts and the One Codex Python library.

To determine whether some genera were abundant in placebo- *vs.* EDP1815-treated individuals, read count tables were fed to ANCOM, a statistical framework for the analysis of microbiomes.[43] Genera were determined to be significantly different between comparators if mean relative abundance was >1% in either comparator, or they passed the significance threshold identified by ANCOM.

16S sequencing reads were classified at the genera level using the One Codex Platform.[42] Reads were grouped by subject ID, treatment and time point using custom Python scripts and the Pandas library.[44] Figure was generated using custom python scripts and the python libraries Matplotlib and Seaborn.[45,46]

ATOPIC DERMATITIS: STUDY EDP1815-101 COHORT 7

Twenty-four participants with mild and moderate atopic dermatitis were randomized 2:1 active to matching placebo. EDP1815 was administered as 8.0×10^{11} bacterial cells once daily for 56 days, with follow-up at day 70.

SUBJECTS The atopic dermatitis-specific inclusion criteria were as follows: patient has a confirmed diagnosis of atopic dermatitis for at least 6 months, an IGA score of 2 or 3, and a BSA involvement of 5–40%. Patients were excluded if they had received systemic non-biologic atopic dermatitis therapy within 4 weeks prior to screening, biologic therapy within 12 months prior to screening, or topical agents that could affect atopic dermatitis within 2 weeks of dosing although unmedicated emollient and low potency steroids were permitted if the subject was already using this as part of their standard care.

SAFETY AND TOLERABILITY DATA The primary endpoint was safety and tolerability. Measurements were AEs, laboratory assessments (biochemistry including CRP, hematology, urinalysis), physical examination, vital signs, and ECG readings at multiple timepoints, including end of treatment and follow-up. AEs were monitored continuously from screening to follow-up visit.

EFFICACY DATA Efficacy was assessed using the clinician-reported outcomes of EASI, SCORAD, IGA, BSA, and IGA×BSA, and the patient-reported outcomes of DLQI, POEM, and pruritus NRS.

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CHAPTER VIII

SUMMARY AND GENERAL DISCUSSION

Less than 15% of all drug development programs advance from early-phase clinical trials to registration on the market.[1] Close to 60% of the expenses related to the development of investigational medicinal products (IMPs) are ascribed to failure, with up to 80% of these failures attributed to inadequate efficacy at later stages.[2] Notably, the success rate of phase II clinical trials is higher (29%) when proof-of-mechanism is confirmed by the end of phase I, compared to when it's not confirmed (0%).[3] This may be due to the difficulty to establish proof-of-mechanism for immunomodulatory investigational drugs during early-phase clinical trials because of the absence of biomarker expression in healthy volunteers. However, a potential solution entails the use of antigens that stimulate T cells and/or B cells in healthy volunteers to challenge the immune system, facilitating the assessment and quantification of an investigational compound's impact on the adaptive immune system.[4]

An optimal antigen for adaptive immune system stimulation should be available as a clinical grade and pure homogenous product, have little or no adverse effects to the recipient, immunogenic across the entire population without genetic restrictions, lacking cross-reactive antibodies, capable of inducing predictable primary immune responses following administration, and able to generate a quantifiable immune response with a high sensitivity for detecting subtle changes through validated immune assays.[5] Conventional antigens for evaluating a T cell-dependent antibody response (TDAR) include sheep red blood cells (SRBC) and tetanus toxoid (TT).[6,7] The primary drawback of the SRBC challenge lies in its dependence on SRBC as the T cell-dependent antigen. As SRBC is not commercially available, it necessitates screening of sheep to verify the ability of the SRBC to generate a robust immune response and to ensure that the sheep are not subjected to excessive bleeding for SRBC acquisition.[6] Furthermore, since clinical grade SRBC is not commercially available it is hard to assure reproducibility, consistency and quality of the antigen. On the other hand TT is readily available as a clinical grade product, however, due to national immunization programs TT is introduced to the human immune system early on during childhood causing more variability[7] and making it impossible to utilize TT as a neoantigen in clinical trials. Other regularly used antigens include commercially available vaccines such as influenza and hepatitis B, however, a significant disadvantage of these vaccines is also previous exposure of clinical trials participants *via* wild-type infection or immunization.[5]

Keyhole limpet hemocyanin (KLH) is a well-established immunostimulant driving a TDAR and fulfills the aforementioned optimal antigen criteria.[5,8-13] KLH is an oxygen-transporting metalloprotein found within the hemolymph of the giant keyhole limpet *Megathura crenulata*. This species of keyhole limpet inhabits the Pacific coastal regions of California and Mexico. The two genes responsible for encoding keyhole limpet hemocyanin are known as KLH1 and KLH2.[14] These genes exhibit approximately 60% similarity in their protein sequences. Each gene encodes a glycosylated protein composed of roughly 3,400 amino acids, with a molecular weight of approximately 390 kDa. These proteins assemble into a didecameric complex, i.e. 20 individual monomers. The KLH protein possesses high immunogenicity and consequently, it is highly regarded as a model antigen in immunization studies.[13] Due to its substantial size and glycosylation, it cannot be synthesized artificially; it is exclusively obtainable as a purified biological product derived from the keyhole limpet *Megathura crenulata*.

The use of KLH in clinical research dates back to 1967 and has been recognized as an immunostimulatory agent driving a robust humoral as well as cell-mediated immune response.[13,15] Other uses of KLH include serving as a hapten carrier protein for small molecules, as an immunostimulant in bladder cancer immunotherapy, or functioning as an adjuvant for cancer vaccines or together with immunomodulatory drugs targeting autoimmune disorders.[5,16-21] Cyclosporine, a calcineurin inhibitor with a main effect to lower T cell activity, inhibits the TDAR response evoked by KLH in rodents exhibiting the importance of T cell driven B cell activation following KLH immunization.[8] KLH is also frequently used to study *in vivo* local cell-mediated immunity such as delayed-type hypersensitivity (DTH),[5,9,13] however, these methods are less established and more variable compared to TDAR. Various antigens are currently used to study local skin cell-mediated immunity, of which *Candida albicans* extract, *Trichophyton mentagrophytes* extract, purified protein derivative (PPD), and TT are the most well-known.[22,23] Each of these antigens has factors associated with a positive DTH skin test, mostly based on previous exposure to the antigen.[22,23] Tuberculosis, prior Bacillus Calmette-Guérin (BCG) vaccination, or working in health care are associated with a positive DTH response following PPD exposure. History of candidiasis or a history of dermatophyte infection is associated with a positive DTH skin test after *C. albicans* or *T. mentagrophytes* extract injection, respectively. Previous TT vaccination is associated with a positive DTH response after a TT skin challenge. In theory, all these antigens can be used clinically to drive cell-mediated immune responses for evaluation of immunomodulatory compounds. Notably, a prerequisite to achieve a successful local KLH skin challenge response is prior sensitization in the form of initial KLH immunization.[4,5,13] Therefore, the use KLH as an *in vivo* neoantigen to study cell-mediated immunity is advantageous compared to other antigens as it also easily allows evaluation of preventive properties and characteristics of immunomodulators.

Although KLH has been extensively studied and multiple systematic reviews have been published, the exact immunological actions and pathways driven by KLH remain to be elucidated.[5,13,24] Therefore, the aim of this thesis was the development and characterization of an *in vivo* human keyhole limpet hemocyanin (KLH) challenge model,

and subsequent application of the model in pharmacological healthy volunteer studies to evaluate the effects of immunomodulatory investigational medicinal products.

In Chapter 2 we have provided a comprehensive systematic review of KLH as an immunostimulant in clinical trials.[13] The systematic review focused on different methods to measure the systemic and local immune responses triggered by KLH, on identifying the most reliable biomarkers for monitoring KLH responses, taking into account the size and variability of the response, and on assessing how pharmacological treatments and diseases impact the KLH response. The majority of studies analyzed the systemic immune response by assessing anti-KLH antibodies characterized by enzyme-linked immunosorbent assay (ELISA). Since KLH is xenogeneic to the human immune system it induces a primary immune response after the initial KLH immunization that could be detected three weeks after immunization. A few studies also analyzed systemic cellular and molecular responses. These responses are greatly dependent on the number of KLH immunizations and, to a lesser extent, the KLH immunization dosage. Local immune recall responses can be evoked by a dermal KLH challenge where objective quantification using imaging tools is preferred over subjective quantification. Local cellular and molecular responses following KLH immunization and dermal challenge were rarely studied. The KLH-induced immune response can be influenced by factors such as age, physical activity, alcohol intake, stress, and specific autoimmune diseases. Moreover, antigen tolerance after oral KLH feeding has been described. Immunomodulatory drugs such as cyclosporine, fingolimod, and monoclonal antibodies targeting CD28 (VEL-101), CD20 (rituximab), CD28/ICOS (azizolcept), CD80/CD86 (abatacept), and CD134 (KY1005, currently amlitelimab) effectively suppressed the immune response triggered by a KLH challenge. Conclusively, our review emphasizes the significance of implementing KLH challenges in early-phase clinical research, while also highlighting the necessity for established and rigorously controlled methodologies to induce and assess KLH responses.

Prior to implementing a KLH challenge in early-phase clinical research involving immunomodulatory drugs we performed a randomized controlled trial with a KLH challenge in healthy volunteers as detailed in Chapter 3 to validate objective quantification of systemic humoral as well as local immune responses following KLH immunization and dermal KLH challenge.[4] KLH immunization and subsequent intradermal KLH administration were well-tolerated. KLH immunization led to elevated levels of KLH-specific antibodies after three weeks, which was in line with previous literature.[13] To date, there is unfortunately no species-specific reference material available for human antibodies targeted against KLH. In our studies we therefore compared optical density (OD) values of experimental sera in precalculated dilutions to negative control and to OD values of a positive control included on the same ELISA plate. A different method is to prepare standard curves for each analyzed antibody isotype using established KLH antibody concentrations measured in mg/L. Although more time-consuming, this approach does enable the quantitative determination of KLH antibody levels. Another approach is to compare OD values of sample sera to a reference serum from immunized subjects which contains a high-antibody titer (defined as 1,000 arbitrary units). A subsequent intradermal KLH challenge resulted in objectively quantified increased skin blood perfusion and erythema as analyzed by laser speckle contrast imaging (LSCI) and multispectral imaging, respectively. In this study the dermal KLH challenge response was objectively measured using continuous numerical scales, thereby minimizing the influence of inter-rater variability, a factor inherent in subjective scoring methods. By employing noninvasive imaging techniques, the KLH challenge model holds promise as an objective approach for investigating the pharmacodynamics of immunomodulatory drugs in early-phase clinical research.

The human *in vivo* KLH challenge model was implemented in several clinical studies to study the immunomodulatory potency of KY1005 (an OX40 ligand inhibitor), EDP1066, and EDP1815 (single-strain microbial preparations of *Lactococcus lactis* spp. *cremoris*, and *Prevotella histicola*, respectively), as described in Chapters 4, 5, and 6. Preclinically, weekly KY1005 doses of 5, 25, and 100 mg/kg all substantially attenuated the anti-KLH IgG responses to 3 mg KLH immunizations on day 30 (primary response) and day 60 (recall response) in cynomolgus monkeys when compared to the vehicle control group, whereas anti-KLH IgM responses seemed comparable between the control and treatment groups (unpublished data). Maximum effects were reached at 5 mg/kg KY1005 (human equivalent dose of 1.6 mg/kg) as no significant differences were observed between the actively treated groups. In Chapter 4 pharmacological activity of KY1005 was observed at loading doses of 0.45 mg/kg and higher.[25] Exposure-response modeling revealed a KY1005 treatment effect on anti-KLH antibody titers which was more profound for anti-KLH IgG compared to anti-KLH IgM. KY1005 clearance remained relatively stable from groups treated with loading doses of 0.45 mg/kg and higher which suggests target-mediated drug disposition and possibly 100% target binding. This finding re-enforces the preclinical findings where maximum KY1005 effects in cynomolgus monkeys were already observed at 5 mg/kg. Importantly, KY1005 dose-dependently inhibited the response to the KLH skin challenge, further supporting the development and use of KY1005 in future studies. It is unclear why the effect of KY1005 on anti-KLH antibody titers was less pronounced compared to the local KLH skin challenge response. Although OX40-OX40L signaling effects are not fully elucidated, it is possible that inhibition of this pathway is of less importance in B cell signaling and subsequent antibody production compared to

immune cells involved in the dermal challenge response (such as macrophages and effector T cells). Notably, for TDAR, B cell activation is dependent on both antigen binding to the B cell receptor as well as CD40-CD40L interaction with activated T cells, whereas OX40-OX40L binding only plays a co-stimulatory role.[26-28] Since the completion of our first-in-man trial with KY1005, a follow-up phase IIa trial including atopic dermatitis patients has been successfully completed.[29] KY1005 was overall well-tolerated and did not exhibit any remarkable safety concerns. Patients were treated intravenously with either 200 mg KY1005 (low-dose group), 500 mg KY1005 (high-dose group), or placebo followed by 3 maintenance doses (50% of initial dose) every 4 weeks. Although in total four KY1005 doses were administered in this trial compared to three KY1005 doses in our trial, the dose levels per timepoint as well as the cumulative dose were overall in the same range as the two highest dose groups of our trial (initial doses of 4 mg/kg and 12 mg/kg and maintenance doses of 2 mg/kg and 6 mg/kg, respectively).[25] A cumulative dose of 500 mg and 1250 mg KY1005 was administered in the low-dose and high-dose groups, respectively, compared to 560 mg and 1680 mg in the 4 mg/kg and 12 mg/kg groups of our trial, respectively, assuming an average body weight of 70 kg. Notably, clinical improvements in EASI scores of atopic dermatitis patients were observed in both the low-dose and high dose groups. These findings support and signify the implementation of challenge models such as KLH in early-phase clinical drug development to provide proof-of-mechanism before advancing to late phase trials. As OX40-OX40L signaling occurs relatively early on within the adaptive immunity cascade and its effects are wide-ranging, it can be targeted for many inflammatory and immune-mediated disorders. Currently, KY1005 is being investigated in atopic dermatitis (phase 3), hidradenitis suppurativa (phase 2), and asthma (phase 2).[30]

Intestinal dysbiosis is hypothesized to have modifying effects on the local (intestinal) as well as the systemic immune system.[31-39] Altering the intestinal microbiome with orally administered probiotics, prebiotics, and/or synbiotics seems to have favorable effects on dysregulated systemic immune responses.[40-44] EDP1066 and EDP1815 are single-strain microbial preparations of *Lactococcus lactis* spp. *cremoris* and *Prevotella histicola*, respectively, and both have demonstrated promising preclinical results in *in vitro* immune cell cultures and *in vivo* murine immune challenge and disease models, including KLH challenges. Unfortunately, we were unable to achieve similar clinical results following daily EDP1066 treatment as described in Chapter 5.[45] In contrast to KY1005 treatment, no consistent significant treatment effects on the KLH challenge model in healthy volunteers were observed. The unsuccessful translation of preclinical to clinical EDP1066 findings could potentially be attributed to the impossibility of conventional allometric scaling and subsequent possibility of too low EDP1066 dosing, high individual response variability due to differences in dietary intake and gastrointestinal microbial composition, and the uncertainty whether EDP1066 was released at the target site within the gastrointestinal tract. Importantly, these findings also highlight some limitations of the KLH challenge model as implemented in our trials since we had only focused on merely two late-stage aspects of the challenge, namely the anti-KLH antibody response and the dermal KLH challenge response. In-depth molecular and cellular analyses during several phases (encounter, activation, effector, and memory phase) of the adaptive immune response following the KLH challenge were not the primary objective of this thesis. However, optimization and characterization of the challenge could possibly elucidate why no clinical effects were observed. Although no statistically significant outcomes were observed on the humoral KLH response and the KLH skin challenge response following EDP1815 administration in healthy volunteers as described in Chapter 6, there was a trend toward a treatment effect.[46] Possible explanations for the preclinical to clinical translational absence of immunomodulatory EDP1815 effects are similar to the examples given above for EDP1066. However, EDP1815 was further tested in a phase 1b clinical trial including patient populations of atopic dermatitis and psoriasis detailed in Chapter 7.[47] Notably, the sample size for the atopic dermatitis and psoriasis patients trial was selected to determine the EDP1815 safety profile and was not powered nor tested for statistical significance of clinical efficacy. Similar to our findings in healthy volunteers, a convincing treatment effect was absent, but possible signs of clinical EDP1815 efficacy were observed in atopic dermatitis patients (based on the Eczema Area and Severity Index, Investigator's Global Assessment × Body Surface Area, Scoring Atopic Dermatitis, Dermatology Life Quality Index, Patient-Oriented Eczema Measure, and pruritus numerical rating scale outcomes) and psoriasis patients (based on Psoriasis Area and Severity Index and Lesion Severity Score outcomes) when compared to patients treated with placebo.

We successfully developed and characterized a human KLH immune challenge model in healthy volunteers and subsequently applied this challenge model in several early-phase studies with healthy volunteers receiving (potentially) immunomodulatory investigational medicinal products. Based on the studies performed in this thesis, the underlying immunological pathways and molecular and cellular involvement are not fully elucidated. Although the KLH challenge model is a valuable addition in early-phase clinical trials and we achieved our primary objective of this thesis, further optimization and characterization of the challenge would be warranted. In our KLH challenge studies the primary endpoints were ELISA-based systemic anti-KLH antibody assessments and imaging-based local KLH skin response evaluation. A limitation of our KLH challenge studies is therefore the lack of possible valuable data on pathways,

mechanisms, cells, and molecules between KLH immunization up to antibody and dermal response readout. Two of five cardinal signs of inflammation can be captured with the imaging tools used in our KLH challenge studies: heat (calor) indirectly with laser speckle contrast imaging and redness (rubor) with multispectral imaging. Heat sensation arises from increased blood flow into environmentally cooler areas through dilation of blood vessels. This response also induces redness by augmenting the circulation of erythrocytes in the affected region. These signs are generally accepted to be present in inflammatory responses, including after a KLH skin challenge. However, we did not collect cellular or molecular data following the KLH skin challenge and as a result we are unable to correlate perfusion and erythema data gathered with imaging with cellular and cytokine data.

This thesis provides evidence that KLH can be used to assess the immunological response in the absence and presence of immunomodulators. Nevertheless, it should be noted that KLH is an exogenous antigen, and most immunotherapies are directed to self-antigens or neoantigens. We noted a broad generalized immunological response including adaptive, T cell-mediated and antibody responses against KLH, and it has been shown by us and others that it is possible to specifically block distinct (disease) specific components of the immunological response. If this also applies to all self- or neoantigens is at present unclear. Indeed, these antigens may show abnormal expression in malignancies or are only produced during specific stages of differentiation and T cells specialized for neoantigens can bypass negative selection effects due to the highly antigenic neoantigens acquired through somatic tumor mutations.[48,49] If and how this will hamper the application of KLH as a tool to assess drug effects in specific diseases is still unclear and should be explored further.

Systemic molecular and cellular responses following KLH immunization has only been described by a few studies. These studies had immunized subjects with KLH at least twice or used a much higher KLH immunization dose compared to the KLH immunization regimen and dose in our trials.[13] Local molecular and cellular responses after KLH dermal challenge can be analyzed following skin punch biopsies or induction of suction blisters of challenged skin.[13] In the few studies where these responses were characterized again either multiple KLH immunizations and/or higher KLH immunization and rechallenge doses were used. Notably, Hostmann et al. and Kapp et al. demonstrated that T helper cells (CD4$^+$ T cells) and activated T helper cells (CD4$^+$CD154$^+$ T cells), respectively, secreted primarily interleukin-2 (IL-2), tumor necrosis factor (TNF), and interferon-γ (IFN-γ) indicating a systemic T helper cell type-1 (Th1) response.[50,51] To a lesser extent IL-4 secretion by the same cell types was also observed suggesting a less pronounced systemic Th2 response. Spazierer et al. demonstrated a moderate systemic Th2-skewed response based on increased levels of IL-5, IL-10, and IL-13 and to a lesser extent IFN-γ.[52] These findings were further supported by high local IL-4 and IL-13 cytokine levels after a intradermal KLH challenge and by increased eosinophils at the injection site. Moreover, the skin challenge response peaked at around 24h post skin challenge indicative of a Th2-driven late-phase skin reaction whereas a Th1-driven DTH response is usually strongest at 48h to 72h post skin challenge.

Future clinical trials with the KLH challenge model should include currently available well-established immunomodulatory drugs with different modes of action targeting various parts of the immune system which will likely improve our current understanding of the model and provide more insight in how these drugs can affect the KLH response. Cyclosporine is one such drug that is highly specific in inhibiting T cell activation and proliferation by targeting calcineurin and blocking JNK and p38 signaling pathways which are involved in antigen recognition.[53,54] Cyclosporine administration in autoimmune uveitis patients suppressed the KLH skin challenge response, but treatment did not affect the humoral and lymphocyte proliferation response.[7] In contrast, cyclosporine reduced the anti-KLH IgM and IgG response in rats after a single KLH administration in the footpad by 60% and 95%, respectively.[11] Naturally the immune system between rats and humans cannot be extrapolated 1:1 which might explain the differences. Also, the immune system of autoimmune uveitis patients is altered compared to healthy humans. With KY1005 we have shown that more specific therapies targeting the interaction between antigen-presenting cells and T cells can modulate KLH responses.[25] Other monoclonal antibodies were also shown to modulate the immune response triggered by a KLH challenge.[55-62]

Rituximab on the other hand is a highly specific B cell inhibitor and causes apoptosis through binding to the B cell specific surface protein CD20.[63] Bingham et al. investigated the effect of KLH immunization in rheumatoid arthritis patients receiving either methotrexate or a combination of methotrexate and rituximab.[64] Anti-KLH IgG antibody titers were 3-fold lower in patients receiving both methotrexate and rituximab compared to patients receiving methotrexate monotherapy suggesting an enhanced B cell suppressive effect of rituximab treatment. To the best of our knowledge, no KLH immunization study has yet been performed in healthy volunteers receiving rituximab treatment.

Another potential candidate drug for benchmarking the KLH challenge model is fingolimod, a sphingosine 1-phosphate receptor (S1PR) modulator.[65] Boulton et al. showed that fingolimod administered to healthy volunteers dose-dependently suppressed the anti-KLH IgM and IgG response after multiple KLH immunizations.[66] Unfortunately, they were unable to evoke significant DTH responses following an intradermal KLH challenge which they related to a low KLH skin test dose of 10 µg.

Importantly, they defined a positive DTH response as a diameter of induration of ≥ 5 mm. We have previously demonstrated that categorizing the intradermal KLH skin response in induration categories is an inaccurate analysis method leading to a few to none responders.[4] We showed that more sensitive methodology such as LSCI and multispectral imaging can capture small changes of the dermal KLH challenge response on a continuous scale with an even lower dose of 1 µg.

Lastly, benchmarking the KLH challenge model with unspecific immunosuppressants, such as corticosteroids, can potentially also provide additional insight on the possible innate immune effects of a KLH dermal challenge. Several preclinical trials have shown immunosuppressive effects of these drugs on the KLH skin challenge response.[67-69] Based on available information we were not able to find any clinical trials investigating the effect of prednisolone or dexamethasone on KLH challenge responses in healthy volunteers.

The KLH challenge model can potentially also be implemented in studies with immunostimulants of the adaptive immune response, such as immune checkpoint inhibitors targeting CTLA-4 (ipilimumab and tremelimumab), PD-1 (nivolumab, pembrolizumab, cemiplimab, and dostarlimab), and PD-L1 (atezolizumab, avelumab, and durvalumab). These rather novel types of immunotherapeutic drugs are becoming increasingly important in cancer immunotherapy and have even led to the Nobel prize for immunologists James P. Allison and Tasuku Honjo.[70] Preclinically, KLH-induced IFN-γ production was significantly increased upon *ex vivo* KLH re-stimulation in mice treated with anti-CTLA-4 compared to vehicle control.[71] To our knowledge, no clinical trials combining an *in vivo* KLH challenge with anti-CTLA-4 therapy have been conducted to date. For studies with immune checkpoint inhibitors targeting PD-1 and PD-L1 including a KLH challenge no preclinical or clinical work has been performed as far as we know.

In conclusion, this thesis shows that the *in vivo* human KLH challenge model is a valuable methodological tool in early-phase drug development trials. Thanks to well-established methodology and experience with KLH and relatively easy implementation in clinical trials the KLH challenge model can aid in understanding the pharmacology of novel compounds and reduce the costs and failure rate of drug development programs by establishing proof-of-mechanism during early phases of clinical research. This thesis has provided a means for early pharmacodynamic testing of novel drugs targeting the adaptive immune response, however, further optimization and characterization of the KLH challenge model and benchmarking the model with well-known immunomodulatory drugs could provide useful information for implementation in future clinical research.

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CHAPTER IX

NEDERLANDSE SAMENVATTING

INTRODUCTIE

Auto-immuunziekten treffen ongeveer 5–8% van de wereldbevolking. De meeste immunomodulerende geneesmiddelen die momenteel worden gebruikt voor de behandeling van auto-immuunziekten missen specificiteit en gaan gepaard met bijwerkingen vanwege hun brede werking, waaronder het risico op kwaadaardige tumoren en infecties. Daarnaast reageert een aanzienlijk deel van de patiënten niet of niet optimaal op deze behandelingen. Daarom is er een dringende behoefte aan de ontwikkeling van nieuwe geneesmiddelen op basis van een goed moleculair en klinisch begrip van specifieke auto-immuunziekten. Het immuunsysteem, en meer specifiek het adaptieve immuunsysteem, is een belangrijk aandachtsgebied voor de ontwikkeling van dergelijke nieuwe behandelingsstrategieën, vooral voor de behandeling van infecties, tumoren en auto-immuunziekten die resistent zijn tegen conventionele therapieën.

Vanwege het gebrek aan kennis over onderliggende biologische werkingsmechanismen van nieuwe onderzoeksmiddelen komt echter slechts 13,8% van alle geneesmiddelontwikkelingsprogramma's over alle therapeutische gebieden van fase I klinische onderzoeken tot aan marktregistratie. Slechts 6,3% van de anti-inflammatoire en auto-immuun geneesmiddelen wordt uiteindelijk geregistreerd voor klinisch gebruik. Bijna 60% van de ontwikkelingskosten van geneesmiddelen wordt toegeschreven aan falen, waarvan 60% tot 80% te wijten is aan onvoldoende werkzaamheid in latere stadia van de geneesmiddelontwikkeling. Er is daarom een noodzaak voor rationelere strategieën in vroege geneesmiddelontwikkeling. Het kan waardevol zijn om 'proof-of-mechanism' in mensen aan te tonen (bewijs van gewenste farmacologische activiteit) voordat fase II klinische onderzoeken beginnen. Het slagingspercentage in fase II klinische onderzoeken blijkt aanzienlijk hoger wanneer 'proof-of-mechanism' is vastgesteld aan het einde van fase I (29%) in vergelijking met wanneer dit niet is vastgesteld (0%).

Het beoordelen van de farmacologische activiteit van nieuwe immunomodulerende onderzoeksmiddelen tijdens vroege fase klinisch onderzoek kan bemoeilijkt worden door het gebrek aan relevante biomarkers bij gezonde vrijwilligers; het immuunsysteem is immers in rust. Een mogelijke oplossing hiervoor is het gebruik van farmacologische challenge modellen om T-cellen en/of B-cellen van het immuunsysteem van gezonde vrijwilligers te activeren, waardoor de effecten van een onderzoeksmiddel op het adaptieve immuunsysteem kunnen worden geëvalueerd en gekwantificeerd.

KEYHOLE LIMPET HEMOCYANINE

Keyhole limpet hemocyanine (KLH) wordt algemeen beschouwd als een modelantigeen voor gebruik in immunisatiestudies. KLH is een eiwit dat voorkomt in de hemolymfe van de Californische reuzengathoren (*Megathura crenulata*), een weekdier dat voornamelijk voorkomt in de kustwateren van Californië en Mexico. Het is verantwoordelijk voor zuurstoftransport binnen weekdiersoorten. Hemocyaninen zijn vergelijkbaar met hemoglobine dat wordt gevonden in het bloed van gewervelde dieren, maar in plaats van ijzer om zuurstof te binden, bevatten ze koper. KLH is een groot eiwit met een molecuulgewicht van ongeveer 4–8 MDa, samengesteld uit meerdere subeenheden van ongeveer 350–390 kDa elk. Het werd voor het eerst klinisch geïntroduceerd in 1967 om de immunocompetentie van mensen te bestuderen. KLH vertoont uitstekende immunostimulerende eigenschappen in experimenten met dieren en mensen. Zowel de aangeboren als de adaptieve immuunrespons, waaronder de humorale en cellulaire respons, worden geactiveerd door KLH. Het is een waardevol challenge agens voor evaluatie van de adaptieve immuunrespons bij de mens, omdat het menselijke immuunsysteem meestal naïef is voor KLH vóór immunisatie. Dit is niet het geval voor andere antigenen die vaak worden gebruikt in immuun challenge studies bij mensen, zoals varicella zoster of Bacille Calmette-Guérin. Momenteel is subunit KLH geregistreerd als een effectieve immunotherapeutische behandelingsmodaliteit voor blaaskanker. KLH wordt ook gebruikt als hapteen-dragereiwit voor kleine moleculen, of als adjuvans in vaccintherapie of samen met immunomodulerende medicijnen.

HUMORALE EN CELGEMEDIEERDE IMMUNITEIT

KLH wekt een sterke systemische primaire humorale respons op na immunisatie bij de mens. Na verwerking van het KLH-antigeen door antigeen-presenterende cellen worden naïeve $CD4^+$ T-cellen geactiveerd, die op hun beurt B-cellen activeren, en aanzetten tot proliferatie en differentiatie tot plasmacellen. De respons wordt gekenmerkt door de aanvankelijke productie van anti-KLH-IgM antilichamen, gevolgd door een toename van meer specifieke anti-KLH-IgG antilichamen.

De 'geheugen' functie van het adaptieve immuunsysteem kan worden geëvalueerd door de celgemedieerde immuunrespons te volgen na een initiële KLH-sensitisatie. Wanneer KLH vervolgens intradermaal wordt toegediend, worden de antigenen gepresenteerd door dendritische cellen, waardoor KLH-specifieke $CD4^+$ en $CD8^+$ T-cellen in de lymfeklieren worden geactiveerd zodat die gaan prolifereren. Effector T-cellen migreren naar de huid na het primen en imprinten van homing-moleculen door

dendritische cellen. De daaropvolgende cutane ontstekingsreactie bestaat uit lokale infiltratie van immuuncellen en verhoogde vasculaire permeabiliteit, wat klinisch kan worden waargenomen als roodheid (erytheem) en zwelling (oedeem).

BEELDVORMING VAN DE HUID

Hoewel de huidreactie subjectief kan worden gemeten door visuele inspectie op erytheem en oedeem, wordt deze methode vaak categorisch beoordeeld, met variabiliteit in de score tussen verschillende beoordelaars. Daarom heeft objectieve kwantificatie van de huidreactie op een continue schaal de voorkeur.

Laser speckle contrast imaging (LSCI) biedt live non-invasieve monitoring van de cutane microvasculaire perfusie. De techniek is gebaseerd op de analyse van speckle-contrast, berekend als de verhouding van de standaardafwijking tot de gemiddelde intensiteit, die een bloedstroomindex oplevert. Oplichting van weefsel met een laser genereert een willekeurig interferentiepatroon (speckle). Dit patroon verandert na verstrooiing van laserlicht door bewegende rode bloedcellen. De intensiteit van elke speckle fluctueert afhankelijk van de snelheid van de bewegende rode bloedcellen, wat resulteert in een afname van het tijd-geïntegreerde speckle-contrast. Vanwege een goede temporele en ruimtelijke resolutie en reproduceerbaarheid is het een nuttig hulpmiddel voor de objectieve kwantificatie van cutane bloedperfusie.

Multispectrale computerondersteunde 3D-beeldvorming van de huid kan worden verkregen met behulp van de Antera 3D® camera. Deze innovatieve camera maakt gebruik van multidirectioneel licht in een gesloten ruimte om het huidoppervlak in 3D te reconstrueren. Het brengt de weerkaatsing van licht in kaart van zeven verschillende golflengten licht die het volledige zichtbare spectrum beslaan. Daardoor is het mogelijk een nauwkeurigere analyse te maken van de colorimetrische eigenschappen van de huid (waaronder erytheem). Spectrale beelden worden verkregen en omgezet in een reflectiekaart. De gegevens worden vervolgens omgezet in huidabsorptie-coëfficiënten, en wiskundige correlaties met bekende spectrale absorptiegegevens van hemoglobine worden gebruikt om erytheem te kwantificeren.

SAMENVATTING PROEFSCHRIFT

Het hoofddoel van dit proefschrift was het ontwikkelen en karakteriseren van een humaan challenge model bij gezonde vrijwilligers met behulp van KLH, en het toepassen van het model om de effecten van nieuwe immunomodulerende geneesmiddelen te evalueren.

SECTIE I. ACHTERGRONDINFORMATIE KLH-CHALLENGE MODEL

In dit proefschrift wordt de karakterisering en standaardisatie van het KLH-challenge model beschreven, met de daaropvolgende toepassing van de methodologie voor de evaluatie van de activiteit van nieuwe onderzoeksmiddelen. Hoewel dit model eerder is gebruikt in meerdere klinische onderzoeken, zijn KLH-doseringen, de combinatie met adjuvantia en de toedieningsroutes en regimes voor immunisatie en rechallenge niet gestandaardiseerd. Bovendien zijn de biomarkers voor karakterisatie en kwantificatie van KLH-gedreven immuunreacties niet geoptimaliseerd. Een systematische review uitgevoerd door Swaminathan et al. (2014, DOI: 10.1111/bcp.12422) gaf een overzicht van KLH-doseringen, toedieningsroutes en karakterisatie van de belangrijkste responsen op basis van 16 klinische studies.

Als vervolg biedt hoofdstuk 2 een grondigere systematische review van de KLH-reactie in klinische studies. Dit onderzoek breidt het werk van Swaminathan et al. uit door een groter aantal klinische studies op te nemen, gericht op drie hoofdgebieden: de verschillende methoden die worden gebruikt om de systemische en lokale immuunreacties na KLH-toediening te bestuderen, het identificeren van de meest betrouwbare biomarkers voor analyse van de KLH-respons en het evalueren van de impact van ziekten en farmacologische interventies op de KLH-reactie.

Een overgroot deel van de onderzoeken evalueerde de systemische immuunrespons door anti-KLH-antilichamen te kwantificeren door middel van enzyme-linked immunosorbent assay (ELISA). Aangezien KLH xenogeen is voor het menselijke immuunsysteem, induceert het een primaire immuunrespons na de initiële KLH-immunisatie, die veelal drie weken na immunisatie een plateau bereikt. Enkele studies analyseerden ook systemische cellulaire en moleculaire reacties. Deze reacties zijn sterk afhankelijk van het aantal KLH-immunisaties en, in mindere mate, van de dosering van de KLH-immunisatie. Lokale 'recall'-immuunresponsen kunnen worden opgewekt door een dermale KLH-challenge, waarbij objectieve kwantificatie met behulp van beeldvormingstechnieken de voorkeur heeft boven subjectieve kwantificatie. Lokale cellulaire en moleculaire reacties na KLH-immunisatie en dermale challenge zijn zelden bestudeerd. De KLH-geïnduceerde immuunrespons kan worden beïnvloed door factoren zoals leeftijd, lichamelijke activiteit, alcoholconsumptie, stress en specifieke auto-immuunziekten. Bovendien is antigeen-tolerantie na orale KLH-inname beschreven. Immunomodulerende geneesmiddelen zoals ciclosporine, fingolimod en monoklonale antilichamen gericht tegen CD28 (VEL-101), CD20 (rituximab), CD28/ICOS (azizolcept), CD80/CD86 (abatacept) en CD134 (KY1005, ook bekend als amlitelimab) onderdrukken effectief de immuunrespons die wordt getriggerd door een KLH-challenge. Concluderend benadrukt onze review het belang van

het implementeren van een KLH-challenge in vroege fase klinisch onderzoek, terwijl het ook de noodzaak onderstreept van gestandaardiseerde en sensitieve methode om KLH-responsen op te wekken en te beoordelen.

SECTIE II. KLH-CHALLENGE MODEL BIJ GEZONDE VRIJWILLIGERS

Een groot gebrek van de meeste studies die gebruik maakten van een KLH-challenge in immunofarmacologische studies is het gebruik van subjectieve kwantificatie om de lokale celgemedieerde reactie te beoordelen na een dermale KLH-challenge. Hoofdstuk 3 beschrijft het gebruik van een KLH-challenge model bij gezonde vrijwilligers met gevoelige en objectieve technieken om de lokale KLH 'recall'-respons te kwantificeren, waardoor de tekortkomingen van subjectieve beoordelingen zoals variabiliteit tussen beoordelaars worden overwonnen. KLH-immunisatie en daaropvolgende intradermale KLH-toediening werden goed verdragen. KLH-immunisatie leidde na drie weken tot verhoogde niveaus van KLH-specifieke antilichamen, wat in overeenstemming was met eerdere literatuur. Een daaropvolgende intradermale KLH-challenge resulteerde in objectief gekwantificeerde verhoogde perfusie van de huid en erytheem, geanalyseerd met behulp van LSCI en multispectrale beeldvorming (Antera 3D® camera). In deze studie werd de dermale KLH-challenge respons objectief gemeten met behulp van continue numerieke schalen, waardoor de invloed van variabiliteit tussen beoordelaars, een factor die inherent is aan subjectieve beoordelingsmethoden, werd geminimaliseerd. Door gebruik te maken van non-invasieve beeldvormingstechnieken kan de lokale KLH-respons objectief en kwantitatief geëvalueerd worden, wat van belang is bij het onderzoeken van de farmacodynamiek van immunomodulerende geneesmiddelen in vroege fase klinisch onderzoek.

SECTIE III. KLH-CHALLENGE MODEL IN VROEGE FASE KLINISCH GENEESMIDDELENONDERZOEK

Deze sectie beschrijft de evaluatie van nieuwe immunomodulerende geneesmiddelen in klinische onderzoeken, gebaseerd op het KLH-challenge model zoals uitgevoerd in hoofdstuk 3.

Hoofdstuk 4 beschrijft de eerste toediening aan de mens van een remmer van OX40-ligand (CD134) (KY1005, inmiddels bekend als amlitelimab) en de effecten ervan op het adaptieve immuunsysteem bij gezonde vrijwilligers. Farmacologische activiteit van KY1005 werd waargenomen bij initiële doseringen van 0,45 mg/kg en hoger, gebaseerd op suppressie van KLH-gedreven immuunresponsen. KY1005 toediening resulteerde in een suppressie van de anti-KLH-antilichaamtiters, die meer uitgesproken was voor anti-KLH-IgG dan voor anti-KLH-IgM. KY1005 behandeling leidde tot een dosisafhankelijke remming van de respons op de dermale KLH-challenge, wat de ontwikkeling en het gebruik van KY1005 in toekomstig onderzoek ondersteunt. Sinds de voltooiing van ons 'first-in-man'-onderzoek met KY1005 is een vervolgonderzoek (fase 2a) in patiënten met atopisch eczeem met succes afgerond. KY1005 werd over het algemeen goed verdragen en vertoonde geen opmerkelijke bijwerkingen. Klinische verbeteringen in Eczema Area and Severity Index (EASI) scores van patiënten met atopisch eczeem werden waargenomen in zowel de lage als hoge dosisgroepen. Deze bevindingen tonen de meerwaarde van gebruik van challenge modellen zoals KLH in de vroege klinische fase van geneesmiddelenontwikkeling. Aangezien OX40-OX40L signalering relatief hoog in de cascade van de adaptieve immuunrespons plaatsvindt en de effecten wijdverspreid zijn, zou KY1005 effectief kunnen zijn voor een breed scala van inflammatoire en immuungemedieerde aandoeningen. Momenteel wordt KY1005 onderzocht in atopisch eczeem (fase 3), hidradenitis suppurativa (fase 2) en astma (fase 2).

Intestinale dysbiose wordt verondersteld modificerende effecten te hebben op zowel het lokale immuunsysteem in de darmen als het systemische immuunsysteem. Het wijzigen van het darmmicrobioom met oraal toegediende probiotica, prebiotica en/of synbiotica lijkt gunstige effecten te hebben op verstoorde systemische immuunresponsen. Hoofdstuk 5 beschrijft de klinische evaluatie van drie formuleringen van een 'single-strain'-microbiële interventie, bereid uit *Lactococcus lactis* spp. *cremoris* (EDP1066). Het doel van het onderzoek was het karakteriseren van de farmacodynamische effecten van EDP1066 op het adaptieve immuunsysteem in gezonde vrijwilligers. Hoofdstuk 6 beschrijft de effecten van een tweede 'single-strain'-microbe, bereid uit *Prevotella histicola* (EDP1815), op het immuunsysteem in gezonde vrijwilligers in een klinische studie. Preklinisch werden er met EDP1066 en EDP1815 veelbelovende resultaten behaald in *in vitro* immuuncelculturen en *in vivo* op basis van immuunchallenges en ziektemodellen in muizen, inclusief het KLH-challenge model. Helaas werden vergelijkbare klinische resultaten niet behaald na dagelijkse EDP1066 toediening, zoals beschreven in hoofdstuk 5. In tegenstelling tot de behandeling met KY1005 werden er geen consistente significante behandelingseffecten op KLH-gedreven responsen in gezonde vrijwilligers waargenomen. Deze bevindingen met EDP1066 kunnen mogelijk worden toegeschreven aan de onmogelijkheid van conventionele allometrische schaling en de daaruit voortvloeiende mogelijkheid van een te lage EDP1066 dosering, hoge individuele responsvariabiliteit door verschillen in dieet en gastro-intestinale microbiële samenstelling en de onzekerheid of EDP1066 voldoende op de doelplaats binnen het gastro-intestinale stelsel terecht kwam. Belangrijk is dat deze bevindingen ook enkele beperkingen van het KLH-challenge model benadrukken, zoals geïmplementeerd in onze onderzoeken, aangezien we ons alleen hadden gefocust op twee eindaspecten van de challenge, namelijk de anti-KLH-antilichaamrespons en de dermale

KLH-challenge respons. Moleculaire en cellulaire analyses tijdens verschillende fasen (presentatie-, activatie-, effector- en geheugenfase) van de adaptieve immuunrespons na de KLH-challenge waren geen onderdeel van de gepresenteerde onderzoeken, en hadden mogelijk wel immunomodulerende effecten van de onderzoeksmiddelen kunnen tonen. Mogelijke verklaringen voor de afwezigheid van significante immunomodulerende effecten van EDP1815 in onze klinische proeven, in tegenstelling tot de preklinische experimenten, zijn gelijk aan de eerder gegeven voorbeelden voor EDP1066.

EDP1815 werd echter verder getest in een fase 1b klinisch onderzoek in patiëntenpopulaties met atopisch eczeem en psoriasis, beschreven in hoofdstuk 7. Belangrijk om te vermelden is dat de steekproefgrootte voor het onderzoek in patiënten met atopisch eczeem en psoriasis was gebaseerd op evaluatie van het veiligheidsprofiel van EDP1815, niet om statistische significantie van klinische werkzaamheid aan te tonen. Vergelijkbaar met onze bevindingen bij gezonde vrijwilligers ontbrak een overtuigend behandelingseffect, maar mogelijke tekenen van klinische werkzaamheid van EDP1815 werden waargenomen bij patiënten met atopisch eczeem (gebaseerd op de EASI, Investigator's Global Assessment × Body Surface Area, Scoring Atopic Dermatitis, Dermatology Life Quality Index, Patient-Oriented Eczema Measure en pruritus Numerical Rating Scale uitkomsten) en psoriasispatiënten (gebaseerd op de Psoriasis Area and Severity Index en Lesion Severity Score uitkomsten) vergeleken met patiënten behandeld met placebo.

DISCUSSIE

We hebben met succes een humaan KLH-challenge model ontwikkeld en gekarakteriseerd bij gezonde vrijwilligers en hebben dit challenge model vervolgens toegepast in verschillende vroege fase klinische studies in gezonde vrijwilligers die (potentieel) immunomodulerende experimentele geneesmiddelen ontvingen. Op basis van de onderzoeken die in dit proefschrift beschreven worden, zijn de onderliggende immunologische routes en de moleculaire en cellulaire betrokkenheid in het model nog niet volledig opgehelderd. Hoewel het KLH-challenge model een waardevolle aanvulling is in klinische proeven in een vroege fase en het primaire doel van dit proefschrift is bereikt, zou verdere optimalisatie en karakterisering van de KLH-gedreven immuunrespons waardevol zijn. In onze KLH-onderzoeken waren de primaire eindpunten ELISA-gebaseerde systemische anti-KLH-antilichamen en beeldvorming-gebaseerde dermale KLH-respons. Twee van de vijf kardinale tekenen van ontsteking konden worden waargenomen: warmte (calor) indirect met LSCI en roodheid (rubor) met multispectrale beeldvorming. De warmteperceptie ontstaat door een verhoogde bloedstroom naar koudere omgevingsgebieden door verwijding van de bloedvaten.

Deze respons induceert ook roodheid door de circulatie van erytrocyten in het aangetaste gebied te vergroten. Deze tekenen worden algemeen erkend als aanwezig bij inflammatoire responsen. Een limitatie van onze KLH-onderzoeken is het gebrek aan informatie over betrokken moleculaire en cellulaire routes.

Systemische moleculaire en cellulaire responsen na KLH-immunisatie zijn slechts in enkele onderzoeken beschreven. In deze onderzoeken werden proefpersonen met KLH ten minste twee keer geïmmuniseerd of werd een veel hogere KLH-immunisatiedosis gebruikt dan in onze onderzoeken. Lokale moleculaire en cellulaire responsen na een dermale KLH-challenge kunnen worden geanalyseerd op huidbiopten of in opgewekte blaren. Hostmann et al. (2015, DOI: 10.1002/eji.201445024) en Kapp et al. (2010, DOI: 10.1002/eji.201040701) toonden aan dat $CD4^+$ T-helpercellen en geactiveerde $CD4^+CD154^+$ T-cellen voornamelijk interleukine-2 (IL-2), tumornecrosefactor en interferon-γ (IFN-γ) produceerden, wat wijst op een systemische T-helpercel type-1 (Th1) respons. In mindere mate werd ook IL-4 secretie door dezelfde celtypes waargenomen, wat wijst op een minder uitgesproken systemische Th2 respons. Spazierer et al. (2009, DOI: 10.1111/j.1365-2222.2008.03177.x) toonde een gematigde systemische Th2-verschoven respons aan. Bovendien bereikte de dermale respons een piek ongeveer 24 uur na de challenge, wat wijst op een Th2-gedreven 'late-phase'-huidreactie, terwijl een Th1-gedreven 'delayed-type hypersensitivity' (DTH)-respons doorgaans het sterkst is tussen 48 en 72 uur na de dermale challenge.

Toekomstige klinische proeven met het KLH-challenge model zouden huidig beschikbare, goed beschreven immunomodulerende geneesmiddelen met verschillende werkingsmechanismen moeten omvatten die verschillende onderdelen van het immuunsysteem moduleren. Dit zal onze kennis van het model verbeteren en meer inzicht bieden in hoe deze geneesmiddelen de KLH-respons beïnvloeden. Ciclosporine is zo'n medicijn dat T-celactivatie en -proliferatie remt door calcineurine te remmen en de moleculaire JNK- en p38-routes te blokkeren die betrokken zijn bij antigeenherkenning. Toediening van ciclosporine aan patiënten met auto-immuun uveïtis onderdrukt de KLH-challenge respons, maar de behandeling heeft geen invloed op de humorale en lymfocytenproliferatierespons. Daarentegen vermindert ciclosporine de antilichaamrespons tegen KLH bij ratten na een enkele KLH-toediening.

Rituximab is een zeer specifieke B-celremmer die apoptose drijft door te binden aan het B-cel-specifieke oppervlakte-eiwit CD20. Bingham et al. (2010, DOI: 10.1002/art.25034) onderzocht het effect van KLH-immunisatie bij patiënten met reumatoide artritis die ofwel methotrexaat of een combinatie van methotrexaat en rituximab ontvingen. De anti-KLH-IgG antilichaamtiters waren drie keer lager bij patiënten die zowel methotrexaat als rituximab ontvingen in vergelijking met patiënten die alleen methotrexaat kregen, wat wijst op een versterkt suppressief effect van rituximab op

B-cellen. Voor zover ons bekend is, is er nog geen KLH-immunisatieonderzoek uitgevoerd bij gezonde vrijwilligers die rituximab kregen toegediend.

Een andere potentiële kandidaat voor het benchmarken van het KLH-challenge model is fingolimod, een modulator van de sphingosine-1-fosfaatreceptor (S1PR). Fingolimod remde dosisafhankelijk de antilichaamrespons tegen KLH in gezonde vrijwilligers. Helaas werden geen significante DTH-responsen waargenomen na een intradermale KLH-challenge, wat door de auteurs werd toeschreven aan een lage dermale KLH-dosis van 10 µg. Een positieve DTH-respons werd gedefinieerd als een induratie diameter van ≥ 5 mm. Wij hebben met ons onderzoek aangetoond dat het categoriseren van de intradermale KLH-respons in induratiecategorieën onnauwkeurig en ongevoelig is en dat gevoeligere methodologieën, zoals LSCI en multispectrale beeldvorming, kleine dermale KLH-responsen kunnen detecteren, zelfs bij een lagere KLH-dosis van 1 µg.

Ten slotte kan het benchmarken van het KLH-challenge model met niet-specifieke immunosuppressiva, zoals corticosteroïden, ook informatief zijn. Verschillende preklinische proeven hebben immunosuppressieve effecten van dit soort geneesmiddelen op de dermale KLH-respons aangetoond. We konden in de literatuur echter geen klinische proeven vinden die het effect van prednisolon of dexamethason op KLH-responsen bij gezonde vrijwilligers hadden beschreven.

Het KLH-challenge model kan ook worden toegepast in studies met immunostimulantia op de adaptieve immuunrespons, zoals immune checkpoint inhibitors die CTLA-4 (ipilimumab en tremelimumab), PD-1 (nivolumab, pembrolizumab, cemiplimab en dostarlimab) en PD-L1 (atezolizumab, avelumab en durvalumab) raken. Deze relatief nieuwe immunotherapeutische geneesmiddelen worden steeds belangrijker in de immunotherapie bij kanker en hebben zelfs geleid tot de Nobelprijs voor immunologen James P. Allison en Tasuku Honjo. Preklinisch wordt de KLH-geïnduceerde IFN-γ-productie significant verhoogd na *ex vivo* KLH-herstimulatie bij muizen die met anti-CTLA-4 waren behandeld in vergelijking met de controlegroep. Voor zover ons bekend zijn er tot nu toe geen klinische proeven uitgevoerd die een *in vivo* KLH-challenge model combineren met anti-CTLA-4 therapie. Wat betreft immune checkpoint inhibitors die PD-1 en PD-L1 targeten is er, voor zover ons bekend, helemaal geen KLH-gebaseerd onderzoek uitgevoerd.

CONCLUSIE

De onderzoeken in dit proefschrift tonen aan dat het *in vivo* humane KLH-challenge model een waardevol methodologisch hulpmiddel kan zijn in vroege fase geneesmiddelontwikkeling. Dankzij de uitgebreid beschreven en toegepaste methodologie en klinische ervaring met KLH en de relatief gemakkelijke implementatie in klinische onderzoeken kan het KLH-challenge model helpen bij het begrijpen en kwantificeren van de farmacologie van nieuwe geneesmiddelen. Het model kan mogelijk bijdragen aan efficiëntere geneesmiddelontwikkelingsprogramma's doordat het mogelijk wordt om in een vroege fase 'proof-of-mechanism' vast te stellen en daarmee farmacologisch actieve doses te selecteren. Deze thesis biedt een raamwerk voor KLH-gebaseerde farmacodynamische evaluatie in een vroege klinische fase voor geneesmiddelen die ontwikkeld zijn om de adaptieve immuunrespons te moduleren. Verdere optimalisatie en karakterisering van het KLH-challenge model en benchmarking van het model met geregistreerde immunomodulerende geneesmiddelen zijn logische vervolgstappen.

APPENDICES

CURRICULUM VITAE

Mahdi Saghari was born on the 11th of October 1989 in Herat, Afghanistan. After completing bilingual VWO at secondary school CVO Farelcollege (Ridderkerk, the Netherlands) in 2008, he studied Medicine at Erasmus University Rotterdam (Rotterdam, the Netherlands). He obtained his bachelor's degree in Medicine in 2012. Due to his interest in both academic research and medicine he simultaneously studied Neuroscience and Medicine, for which he obtained a Master's degree in 2014 and 2017, respectively. His primary focus during Neuroscience was the preclinical development of novel antisense oligonucleotide (ASO) therapy for Angelman syndrome in the lab of prof. dr. Ype Elgersma at the Erasmus Medical Center (Rotterdam, the Netherlands). His research served as the starting point for further evaluation of ASO therapy in Angelman syndrome, with promising results (Milazzo et al. 2021, DOI: 10.1172/jci.insight.145991).

Upon obtaining his medical degree in 2017, he started his career as a research physician and project leader at the Centre for Human Drug Research (CHDR, Leiden, the Netherlands). Here Mahdi began his PhD trajectory as described in this thesis under supervision of dr. M. Moerland, prof. dr. R. Rissmann and prof. dr. J. Burggraaf. In addition to performing clinical research described in this PhD thesis, he also contributed to many other clinical studies across different therapeutic areas including dermatology, immunology and cardiology. Whilst working at CHDR, he became a board-certified Clinical Pharmacologist in 2022.

In 2023 Mahdi worked as a medical intern at the dermatology department of the Admiraal de Ruyter ziekenhuis (ADRZ, Goes, the Netherlands). Since April 2024 he works as a medical intern in occupational health medicine at HumanCapitalCare B.V. (Capelle aan den IJssel, the Netherlands), with the aim to become an occupational health physician.

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