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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 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 variability⁷ 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 pre-clinical 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 Ib 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 $\times$ 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 an 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 μ g.

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 μ g.

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.

REFERENCES

- Wong CH, Siah KW, Lo AW. Corrigendum: Estimation of clinical trial success rates and related parameters. *Biostatistics*. 2019;20(2):366-366. doi:10.1093/biostatistics/kxy072
- Wouters OJ, McKee M, Luyten J. Estimated Research and Development Investment Needed to Bring a New Medicine to Market, 2009-2018. *JAMA*. 2020;323(9):844-853. doi:10.1001/JAMA.2020.1166
- Wu SS, Fernando K, Allerton C, Jansen KU, Vincent MS, Dolsten M. Reviving an R&D pipeline: a step change in the Phase II success rate. *Drug Discov Today*. 2021;26(2):308-314. doi:10.1016/j.drudis.2020.10.019
- Saghari M, Gal P, Ziagos D, et al. A randomized controlled trial with a delayed-type hypersensitivity model using keyhole limpet haemocyanin to evaluate adaptive immune responses in man. *Br J Clin Pharmacol*. 2021;87(4):1953-1962. doi:10.1111/bcp.14588
- Swaminathan A, Lucas RM, Dear K, McMichael AJ. Keyhole limpet haemocyanin - a model antigen for human immunotoxicological studies. *Br J Clin Pharmacol*. 2014;78(5):1135-1142. doi:10.1111/bcp.12422
- White KL. Plaque Versus ELISA Assays. Evaluation of Humoral Immune Responses to T-Dependent Antigens. In: *Encyclopedic Reference of Immunotoxicology*. Springer-Verlag; :505-508. doi:10.1007/3-540-27806-0_1172
- Palestine AG, Roberge F, Charous BL, Lane HC, Fauci AS, Nussenblatt RB. The effect of cyclosporine on immunization with tetanus and keyhole limpet hemocyanin (KLH) in humans. *J Clin Immunol*. 1985;5(2):115-121. doi:10.1007/BF00915009
- Auli M, Domènech A, Andrés A, et al. Multiparametric immunotoxicity screening in mice during early drug development. *Toxicol Lett*. 2012;214(2):200-208. doi:10.1016/j.toxlet.2012.08.020
- Bouchez C, Gervais F, Fleurance R, Palate B, Legrand JJ, Descotes J. Development of a Delayed-Type Hypersensitivity (DTH) Model in the Cynomolgus Monkey. *J Toxicol Pathol*. 2012;25(2):183-188. doi:10.1293/tox.25.183
- Herzyk DJ, Holsapple M. Immunotoxicity Evaluation by Immune Function Tests: Focus on the T-Dependent Antibody Response (TDAR) [Overview of a Workshop Session at the 45th Annual Meeting of the Society of Toxicology (SOT) March 5-9, 2006 San Diego, CA]. *J Immunotoxicol*. 2007;4(2):143-147. doi:10.1080/15476910701337308
- Gore ER, Gower J, Kurali E, et al. Primary antibody response to keyhole limpet hemocyanin in rat as a model for immunotoxicity evaluation. *Toxicology*. 2004;197(1):23-35. doi:10.1016/j.tox.2003.12.003
- ICH. 2006. International Conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. ICH Harmonised Tripartite Guideline Immunotoxicity Studies For Human Pharmaceuticals S8.
- Saghari M, Jansen MAA, Grievink HW, Rissmann R, Moerland M. Characterization of KLH-driven immune responses in clinical studies: A systematic review. *Front Drug Discov*. 2022;2. doi:10.3389/fddsv.2022.992087
- Harris JR, Markl J. Keyhole limpet hemocyanin: Molecular structure of a potent marine immunoactivator. In: *European Urology*. Vol 37. S. Karger AG; 2000:24-33. doi:10.1159/000052389
- Swanson MA, Schwartz RS. Immunosuppressive Therapy. *N Engl J Med*. 1967;277(4):163-170. doi:10.1056/NEJM196707272770401
- Jurincic-Winkler CD, Metz KA, Beuth J, Klippel KF. Keyhole Limpet Hemocyanin for Carcinoma in situ of the Bladder: A Long-Term Follow-Up Study. *Eur Urol*. 2000;37(Suppl. 3):45-49. doi:10.1159/000052392
- Schumacher K. Keyhole limpet hemocyanin (KLH) conjugate vaccines as novel therapeutic tools in malignant disorders. *J Cancer Res Clin Oncol*. 2001;127(S2):R1-R2. doi:10.1007/BF01470991
- Gandhi RT, O'Neill D, Bosch RJ, et al. A randomized therapeutic vaccine trial of canarypox-HIV-pulsed dendritic cells vs. canarypox-HIV alone in HIV-1-infected patients on antiretroviral therapy. *Vaccine*. 2009;27(43):6088-6094. doi:10.1016/j.vaccine.2009.05.016
- Jia T, Pan Y, Li J, Wang L. Strategies for active TNF- α vaccination in rheumatoid arthritis treatment. *Vaccine*. 2013;31(38):4063-4068. doi:10.1016/j.vaccine.2013.06.101
- Perabo FGE, Müller SC. Current and new strategies in immunotherapy for superficial bladder cancer. *Urology*. 2004;64(3):409-421. doi:10.1016/j.urolgy.2004.04.026
- Lammers RJM, Witjes WPJ, Janzing-Pastors MHD, Caris CTM, Witjes JA. Intracutaneous and Intravesical Immunotherapy With Keyhole Limpet Hemocyanin Compared With Intravesical Mitomycin in Patients With Non-Muscle-Invasive Bladder Cancer: Results From a Prospective Randomized Phase III Trial. *J Clin Oncol*. 2012;30(18):2273-2279. doi:10.1200/JCO.2011.39.2936
- Siripassorn K, Charoenwongse P, Ruxrungtham K. Three suitable antigens for delayed-type hypersensitivity skin testing in a tropical country like Thailand. *Asian Pacific J allergy Immunol*. 2006;24(2-3):143-152. http://www.ncbi.nlm.nih.gov/pubmed/17136880
- Yates AB, DeShazo RD. Delayed Hypersensitivity Skin Testing. *Immunol Allergy Clin North Am*. 2001;21(2):383-397. doi:10.1016/S0889-8561(05)70213-7
- Drennan PG, Karponis D, Richards D, Coles M, Fullerton JN. In vivo human keyhole limpet hemocyanin challenge in early phase drug development: A systematic review. *Clin Transl Sci*. 2022;00(1):1-26. doi:10.1111/cts.13457
- Saghari M, Gal P, Gilbert S, et al. OX40L Inhibition Suppresses KLH-driven Immune Responses in Healthy Volunteers: A Randomized Controlled Trial Demonstrating Proof-of-Pharmacology for KY1005. *Clin Pharmacol Ther*. 2022;111(5):1121-1132. doi:10.1002/cpt.2539
- Webb GJ, Hirschfield GM, Lane PJJ. OX40, OX40L and Autoimmunity: a Comprehensive Review. *Clin Rev Allergy Immunol*. 2016;50(3):312-332. doi:10.1007/s12016-015-8498-3
- Blum JS, Wearsch PA, Cresswell P. Pathways of Antigen Processing. *Annu Rev Immunol*. 2013;31(1):443-473. doi:10.1146/annurev-immunol-032712-095910
- Crotty S. A brief history of T cell help to B cells. *Nat Rev Immunol*. 2015;15(3):185-189. doi:10.1038/nri3803
- Weidinger S, Bieber T, Cork MJ, et al. Safety and efficacy of amlitelimab, a fully human nondepleting, noncytotoxic anti-OX40 ligand monoclonal antibody, in atopic dermatitis: results of a phase IIa randomized placebo-controlled trial. *Br J Dermatol*. 2023;189(5):531-539. doi:10.1093/bjd/ljad240
- Sanofi Amlitelimab Pipeline. Published 2024. Accessed March 24, 2024. https://www.sanofi.com/en/our-science/our-pipeline
- Mowat AM, Agace WV. Regional specialization within the intestinal immune system. *Nat Rev Immunol*. 2014;14(10):667-685. doi:10.1038/nri378

- 32 Meresse B, Malamut G, Cerf-Bensussan N. Celiac Disease: An Immunological Jigsaw. *Immunity*. 2012;36(6):907-919. doi:10.1016/j.immuni.2012.06.006
- 33 Chassaing B, Darfeuille-Michaud A. The Commensal Microbiota and Enteropathogens in the Pathogenesis of Inflammatory Bowel Diseases. *Gastroenterology*. 2011;140(6):1720-1728.e3. doi:10.1053/j.gastro.2011.01.054
- 34 Nguyen HTT, Dalmasso G, Müller S, Carrière J, Seibold F, Darfeuille-Michaud A. Crohn's Disease-Associated Adherent Invasive *Escherichia coli* Modulate Levels of microRNAs in Intestinal Epithelial Cells to Reduce Autophagy. *Gastroenterology*. 2014;146(2):508-519. doi:10.1053/j.gastro.2013.10.021
- 35 Zhang L, Qing P, Yang H, Wu Y, Liu Y, Luo Y. Gut Microbiome and Metabolites in Systemic Lupus Erythematosus: Link, Mechanisms and Intervention. *Front Immunol*. 2021;12:686501. doi:10.3389/fimmu.2021.686501
- 36 Gupta VK, Cunningham KY, Hur B, et al. Gut microbial determinants of clinically important improvement in patients with rheumatoid arthritis. *Genome Med*. 2021;13(1):149. doi:10.1186/s13073-021-00957-0
- 37 Sikora M, Stec A, Chrabaszcz M, et al. Gut Microbiome in Psoriasis: An Updated Review. *Pathogens*. 2020;9(6):463. doi:10.3390/pathogens9060463
- 38 Belkaid Y, Naik S. Compartmentalized and systemic control of tissue immunity by commensals. *Nat Immunol*. 2013;14(7):646-653. doi:10.1038/ni.2604
- 39 Gupta VK, Hand TW. Role of the Microbiota in Immunity and Inflammation. *Cell*. 2014;157(1):121-141. doi:10.1016/j.cell.2014.03.011
- 40 Liu Y, Alokaran J, Rhoads J. Probiotics in Autoimmune and Inflammatory Disorders. *Nutrients*. 2018;10(10):1537. doi:10.3390/nu10101537
- 41 Askari G, Ghavami A, Shahdadian F, Moravejolahkami AR. Effect of synbiotics and probiotics supplementation on autoimmune diseases: A systematic review and meta-analysis of clinical trials. *Clin Nutr*. 2021;40(5):3221-3234. doi:10.1016/j.clnu.2021.02.015
- 42 Bock PM, Telo GH, Ramalho R, et al. The effect of probiotics, prebiotics or synbiotics on metabolic outcomes in individuals with diabetes: a systematic review and meta-analysis. *Diabetologia*. 2021;64(1):26-41. doi:10.1007/s00125-020-05295-1
- 43 Chang YS, Trivedi MK, Jha A, Lin YF, Dimaano L, García-Romero MT. Synbiotics for Prevention and Treatment of Atopic Dermatitis. *JAMA Pediatr*. 2016;170(3):236. doi:10.1001/jamapediatrics.2015.3943
- 44 Morshedi M, Hashemi R, Moazzen S, Sahebkar A, Hosseini-fard ES. Immunomodulatory and anti-inflammatory effects of probiotics in multiple sclerosis: a systematic review. *J Neuroinflammation*. 2019;16(1):231. doi:10.1186/s12974-019-1611-4
- 45 Saghari M, Gal P, Grievink HW, et al. 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. *Front Immunol*. 2022;13. doi:10.3389/fimmu.2022.1009304
- 46 Saghari M, Gal P, Grievink HW, et al. Evaluation of single-strain *Prevotella histicola* on KLH-driven immune responses in healthy volunteers: A randomized controlled trial with EDP1815. *Med Microbiol*. 2024;19:100088. doi:10.1016/j.medmic.2023.100088
- 47 Itano A, Maslin D, Ramani K, et al. Clinical translation of anti-inflammatory effects of *Prevotella histicola* in Th1, Th2, and Th17 inflammation. *Front Med*. 2023;10. doi:10.3389/fmed.2023.1070433
- 48 Waldman AD, Fritz JM, Lenardo MJ. A guide to cancer immunotherapy: from T cell basic science to clinical practice. *Nat Rev Immunol*. 2020;20(11):651-668. doi:10.1038/s41577-020-0306-5
- 49 Madley R, Nauman G, Danzl N, et al. Negative selection of human T cells recognizing a naturally-expressed tissue-restricted antigen in the human thymus. *J Transl Autoimmun*. 2020;3:100061. doi:10.1016/j.jtauto.2020.100061
- 50 Hostmann A, Meyer T, Maul J, et al. Preexisting antigen-specific immune responses are modulated by oral KLH feeding in humans. *Eur J Immunol*. 2015;45(7):1991-1996. doi:10.1002/eji.201445024
- 51 Kapp K, Maul J, Hostmann A, et al. Modulation of systemic antigen-specific immune responses by oral antigen in humans. *Eur J Immunol*. 2010;40(11):3128-3137. doi:10.1002/eji.201040701
- 52 Spazierer D, Skvara H, Dawid M, et al. T helper 2 biased de novo immune response to Keyhole Limpet Hemocyanin in humans. *Clin Exp Allergy*. 2009;39(7):999-1008. doi:10.1111/j.1365-2222.2008.03177.x
- 53 Matsuda S, Koyasu S. Mechanisms of action of cyclosporine. *Immunopharmacology*. 2000;47(2-3):119-125. doi:10.1016/S0162-3109(00)00192-2
- 54 in 't Veld AE, Jansen MAA, Huisman BW, et al. Monitoring of Ex Vivo Cyclosporin A Activity in Healthy Volunteers Using T Cell Function Assays in Relation to Whole Blood and Cellular Pharmacokinetics. *Pharmaceutics*. 2022;14(9):1958. doi:10.3390/pharmaceutics14091958
- 55 Poirier N, Blanco G, Hiance M, et al. First-in-Human Study in Healthy Subjects with FR104, a Pegylated Monoclonal Antibody Fragment Antagonist of CD28. *J Immunol*. 2016;197(12):4593-4602. doi:10.4049/jimmunol.1601538
- 56 Shi R, Honczarenko M, Zhang S, et al. Pharmacokinetic, Pharmacodynamic, and Safety Profile of a Novel Anti-CD28 Domain Antibody Antagonist in Healthy Subjects. *J Clin Pharmacol*. 2017;57(2):161-172. doi:10.1002/jcph.791
- 57 Abrams JR, Lebowitz MG, Guzzo CA, et al. CTLA4Ig-mediated blockade of T-cell costimulation in patients with psoriasis vulgaris. *J Clin Invest*. 1999;103(9):1243-1252. doi:10.1172/JCI5857
- 58 Yang J, Lickliter JD, Hillson JL, et al. First-in-human study of the safety, tolerability, pharmacokinetics, and pharmacodynamics of ALPN-101, a dual CD28/ICOS antagonist, in healthy adult subjects. *Clin Transl Sci*. 2021;14(4):1314-1326. doi:10.1111/cts.12983
- 59 Sullivan BA, Tsuji W, Kivitz A, et al. Inducible T-cell co-stimulator ligand (ICOSL) blockade leads to selective inhibition of anti-KLH IgG responses in subjects with systemic lupus erythematosus. *Lupus Sci Med*. 2016;3(1):e000146. doi:10.1136/lupus-2016-000146
- 60 Espié P, He Y, Koo P, et al. First-in-human clinical trial to assess pharmacokinetics, pharmacodynamics, safety, and tolerability of iscalimab, an anti-CD40 monoclonal antibody. *Am J Transplant*. 2020;20(2):463-473. doi:10.1111/ajt.15661
- 61 Jain A, Kovacs JA, Nelson DL, et al. Partial immune reconstitution of X-linked hyper IgM syndrome with recombinant CD40 ligand. *Blood*. 2011;118(14):3811-3817. doi:10.1182/blood-2011-04-351254
- 62 Karnell JL, Albulescu M, Drabic S, et al. A CD40L-targeting protein reduces autoantibodies and improves disease activity in patients with autoimmunity. *Sci Transl Med*. 2019;11(489). doi:10.1126/scitranslmed.aar6584
- 63 Cerny T, Borisch B, Introna M, Johnson P, Rose AL. Mechanism of action of rituximab. *Anticancer Drugs*. 2002;13:S3-S10. doi:10.1097/00001813-200211002-00002
- 64 Bingham CO, Looney RJ, Deodhar A, et al. Immunization responses in rheumatoid arthritis patients treated with rituximab: Results from a controlled clinical trial. *Arthritis Rheum*. 2010;62(1):64-74. doi:10.1002/art.25034
- 65 Pournajaf S, Dargahi L, Javan M, Pourgholami MH. Molecular Pharmacology and Novel Potential Therapeutic Applications of Fingolimod. *Front Pharmacol*. 2022;13. doi:10.3389/fphar.2022.807639
- 66 Boulton C, Meiser K, David OJ, Schmouder R. Pharmacodynamic Effects of Steady-State Fingolimod on Antibody Response in Healthy Volunteers: A 4-Week, Randomized, Placebo-Controlled, Parallel-Group, Multiple-Dose Study. *J Clin Pharmacol*. 2012;52(12):1879-1890. doi:10.1177/0091270011427908
- 67 Issekutz TB. Effects of anti-inflammatory agents on lymphocyte migration stimulated by the interferons, tumor necrosis factor and cutaneous inflammation. *Int J Immunopharmacol*. 1989;11(7):725-732. doi:10.1016/0192-0561(89)90126-4
- 68 Holsapple MP, Page DG, Bick PH, Shopp GM. Characterization of the delayed hypersensitivity response to a protein antigen in the mouse—I. Kinetics of reactivity and sensitivity to classical immunosuppressants. *Int J Immunopharmacol*. 1984;6(5):399-405. doi:10.1016/0192-0561(84)90077-8
- 69 Exon JH, Bussiere JL, Mather GG. Immunotoxicity testing in the rat: An improved multiple assay model. *Int J Immunopharmacol*. 1990;12(6):699-701. doi:10.1016/0192-0561(90)90108-Y
- 70 Devlin H. James P Allison and Tasuku Honjo win Nobel prize for medicine. *The Guardian*. Published 2018. Accessed March 24, 2024. <https://www.theguardian.com/science/2018/oct/01/james-p-allison-and-tasuku-honjo-win-nobel-prize-for-medicine>
- 71 Poon E, Mullins S, Watkins A, et al. The MEK inhibitor selumetinib complements CTLA-4 blockade by reprogramming the tumor immune microenvironment. *J Immunother Cancer*. 2017;5(1):63. doi:10.1186/s40425-017-0268-8