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

Saghari, M.

SECTION I

KLH IMMUNE CHALLENGE MODEL BACKGROUND

CHAPTER II

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

Mahdi Saghari[1,2], Manon A. A. Jansen[1], Hendrika W. Grievink[1,3], Robert Rissmann[1,2,3], and Matthijs Moerland[1,2]

1. Centre for Human Drug Research (CHDR), Leiden, the Netherlands
2. Leiden University Medical Centre (LUMC), Leiden, the Netherlands
3. Leiden Academic Centre for Drug Research (LACDR), Leiden, the Netherlands

Frontiers in Drug Discovery, 2022;2. DOI: 10.3389/fddsv.2022.992087

Read our original article here

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.

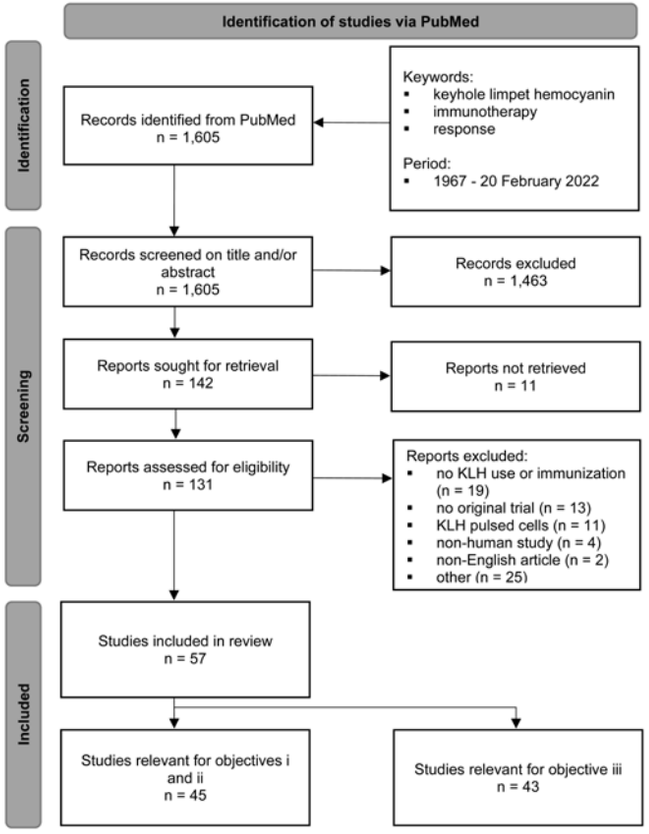


TABLE 1 Study design in relation to KLH challenge

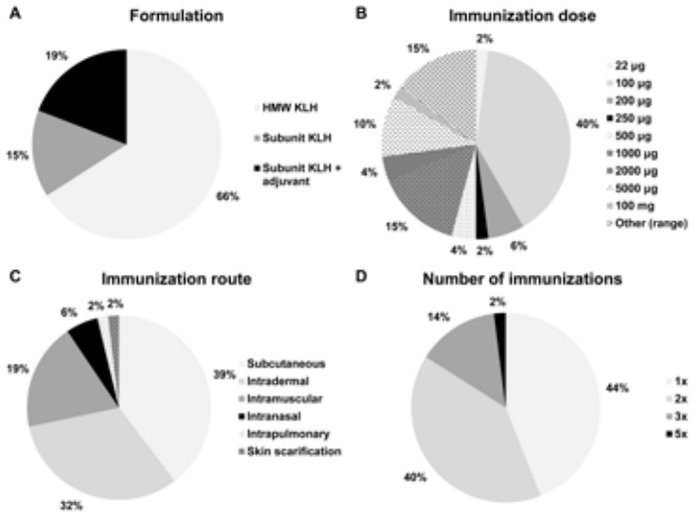
Author article, year	No. sub.	Formulation	Adjuvant	Immunization dose	Route	Frequency	Interval	Rechallenge skin dose**
Saghari M, et al. 2022[16]	10	Subunit KLH	ALUM	100 µg	I.M.	1×		1 µg
Yang J, et al. 2021[35]	16	Subunit KLH		1,000 µg	S.C.	1×		NA
Otterhaug T, et al. 2021[22]	12	Subunit KLH	Hiltonol	100 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.

REFERENCES

1 Saghari M, Gal P, Ziagkos 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

2 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

3 Harris J., Markl J. Keyhole limpet hemocyanin (KLH): a biomedical review. Micron. 1999;30(6):597-623. doi:10.1016/S0968-4328(99)00036-0

4 Wimmers F, de Haas N, Scholzen A, et al. Monitoring of dynamic changes in Keyhole Limpet Hemocyanin (KLH)-specific B cells in KLH-vaccinated cancer patients. Sci Rep. 2017;7(1):43486. doi:10.1038/srep43486

5 Swanson MA, Schwartz RS. Immunosuppressive Therapy. N Engl J Med. 1967;277(4):163-170. doi:10.1056/NEJM196707272770401

6 Perabo FGE, Müller SC. Current and new strategies in immunotherapy for superficial bladder cancer. Urology. 2004;64(3):409-421. doi:10.1016/j.urology.2004.04.026

7 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

8 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

9 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

10 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

11 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

12 Lebrec H, Hock MB, Sundsmo JS, et al. T-cell-dependent antibody responses in the rat: Forms and sources of keyhole limpet hemocyanin matter. J Immunotoxicol. 2014;11(3):213-221. doi:10.3109/1547691X.2013.822948

13 Miller JS, Curtsinger J, Berthold M, et al. Diminished neo-antigen response to keyhole limpet hemocyanin (KLH) vaccines in patients after treatment with chemotherapy or hematopoietic cell transplantation. Clin Immunol. 2005;117(2):144-151. doi:10.1016/j.clim.2005.07.005

14 Geyer H, Wuhrer M, Resemann A, Geyer R. Identification and Characterization of Keyhole Limpet Hemocyanin N-Glycans Mediating Cross-reactivity with Schistosoma mansoni. J Biol Chem. 2005;280(49):40731-40748. doi:10.1074/jbc.M505985200

15 Edelman R. Vaccine Adjuvants. Clin Infect Dis. 1980;2(3):370-383. doi:10.1093/clinids/2.3.370

16 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

17 Poirier N, Blancho 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

18 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

19 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

20 Smith TP, Kennedy SL, Fleshner M. Influence of age and physical activity on the primary in vivo antibody and T cell-mediated responses in men. J Appl Physiol. 2004;97(2):491-498. doi:10.1152/japplphysiol.01404.2003

21 Smith A, Vollmer-Conna U, Bennett B, Wakefield D, Hickie I, Lloyd A. The relationship between distress and the development of a primary immune response to a novel antigen. Brain Behav Immun. 2004;18(1):65-75. doi:10.1016/S0889-1591(03)00107-7

22 Otterhaug T, Janetzki S, Welters MJP, et al. Photochemical Internalization Enhanced Vaccination Is Safe, and Gives Promising Cellular Immune Responses to an HPV Peptide-Based Vaccine in a Phase I Clinical Study in Healthy Volunteers. Front Immunol. 2021;11:3361. doi:10.3389/fimmu.2020.576756

23 Kantele A, Häkkinen MP, Zivny J, Elson CO, Mestecky J, Kantele JM. Humoral Immune Response to Keyhole Limpet Haemocyanin, the Protein Carrier in Cancer Vaccines. Clin Dev Immunol. 2011;2011:1-6. doi:10.1155/2011/614383

24 Moldoveanu Z, Oliver F, Mestecky J, Elson CO. Oral tolerance in humans: failure to suppress an existing immune response by oral antigen administration. Ann N Y Acad Sci. 2004;1029(1):299-309. doi:10.1196/annals.1309.051

25 Kraus TA, Toy L, Chan L, Childs J, Mayer L. Failure to induce oral tolerance to a soluble protein in patients with inflammatory bowel disease. Gastroenterology. 2004;126(7):1771-1778. doi:10.1053/j.gastro.2004.03.076

26 Lange CG, Lederman MM, Medvik K, et al. Nadir CD4+ T-cell count and numbers of CD28+ CD4+ T-cells predict functional responses to immunizations in chronic HIV-1 infection. AIDS. 2003;17(14):2015-2023. doi:10.1097/00002030-200309260-00002

27 Kantele A, Zivny J, Häkkinen M, Elson CO, Mestecky J. Differential homing commitments of antigen-specific T cells after oral or parenteral immunization in humans. J Immunol. 1999;162(9):5173-5177. doi:10.4049/jimmunol.162.9.5173

28 Waldo FB, van den Wall Bake AWL, Mestecky J, Husby S. Suppression of the Immune Response by Nasal Immunization. Clin Immunol Immunopathol. 1994;72(1):30-34. doi:10.1006/clin.1994.1103

29 Husby S, Mestecky J, Moldoveanu Z, Holland S, Elson CO. Oral tolerance in humans. T cell but not B cell tolerance after antigen feeding. J Immunol. 1994;152(9):4663-4670. doi:10.4049/jimmunol.152.9.4663

30 Snyder BK, Roghmann KJ, Sigal LH. Stress and psychosocial factors: Effects on primary cellular immune response. J Behav Med. 1993;16(2):143-161. doi:10.1007/BF00844890

31 Ochs HD, Junker AK, Collier AC, Virant FS, Handsfield HH, Wedgwood RJ. Abnormal antibody responses in patients with persistent generalized lymphadenopathy. J Clin Immunol. 1988;8(1):57-63. doi:10.1007/BF00915157

32 Birdsall HH, Rossen RD. Characterization of anti-Fab' antibodies in human sera: identification of soluble immune complexes that contain hidden anti-KLH and blocking anti-immunoglobulins following immunization with keyhole limpet haemocyanin. Clin Exp Immunol. 1983;53(2):497-504. http://www.ncbi.nlm.nih.gov/pubmed/6603934

33 Paty JG, Sienknecht CW, Townes AS, Hanissian AS, Miller JB, Masi AT. Impaired cell-mediated immunity in systemic lupus erythematosus (SLE). Am J Med. 1975;59(6):769-779. doi:10.1016/0002-9343(75)90462-3

34 Belson A, Schmidt T, Fernando D, et al. Characterisation of the clinical and activated T cell response to repeat delayed-type hypersensitivity skin challenges in human subjects, with KLH and PPD, as a potential model to test T cell-targeted therapies. Inflamm Res. 2016;65(5):389-404. doi:10.1007/s00011-016-0923-4

35 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

36 Boelens PG, Fonk JCM, Houdijk APJ, et al. Primary immune response to keyhole limpet haemocyanin following trauma in relation to low plasma glutamine. Clin Exp Immunol. 2004;136(2):356-364. doi:10.1111/j.1365-2249.2004.02447.x

37 Rentenaar RJ, Van Diepen FNJ, Meijer RT, et al. Immune responsiveness in renal transplant recipients: Mycophenolic acid severely depresses humoral immunity in vivo. Kidney Int. 2002;62(1):319-328. doi:10.1046/j.1523-1755.2002.00425.x

38 Schuyler M, Lyons CR, Masten B, Bice D. Immunoglobulin response to intrapulmonary immunization of asthmatics. Immunology. 1997;91(2):167-175. doi:10.1046/j.1365-2567.1997.00234.x

39 Kondratenko I, Amlot PL, Webster AD, Farrant J. Lack of specific antibody response in common variable immunodeficiency (CVID) associated with failure in production of antigen-specific memory T cells. MRC Immunodeficiency Group. Clin Exp Immunol. 1997;108(1):9-13. doi:10.1046/j.1365-2249.1997.d01-993.x

40 Falconer AE, Friedmann PS, Bird P, Calvert JE. Abnormal immunoglobulin G subclass production in response to keyhole limpet haemocyanin in atopic patients. Clin Exp Immunol. 1992;89(3):495-499. doi:10.1111/j.1365-2249.1992.tb06987.x

41 Ward MM, Hall RP, Pisetsky DS. Serum interleukin-2 receptor responses to immunization. Clin Immunol Immunopathol. 1990;57(1):120-124. doi:10.1016/0090-1229(90)90027-N

42 Ashorn RG, Vandenbark AA, Acott KM, Krohn KJ. Dialysable leukocyte extracts (transfer factor) augment nonspecifically keyhole limpet haemocyanin and horseshoe crab haemocyanin skin reactivity in unimmunized human recipients. Scand J Immunol. 1986;23(2):161-167. doi:10.1111/j.1365-3083.1986.tb01954.x

43 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

44 Ford D, Burger D. Precursor frequency of antigen-specific T cells: Effects of sensitization in vivo and in vitro. Cell Immunol. 1983;79(2):334-344. doi:10.1016/0008-8749(83)90075-8

45 Brunner CM, Horwitz DA, Shann MK, Sturgill BA, Davis JS. Clinical and immunologic studies in identical twins discordant for systemic lupus erythematosus. Am J Med. 1973;55(2):249-254. doi:10.1016/0002-9343(73)90176-9

46 Giesecke C, Meyer T, Durek P, et al. Simultaneous Presence of Non- and Highly Mutated Keyhole Limpet Hemocyanin (KLH)-Specific Plasmablasts Early after Primary KLH Immunization Suggests Cross-Reactive Memory B Cell Activation. J Immunol. 2018;200(12):3981-3992. doi:10.4049/jimmunol.1701728

47 Ferbas J, Belouski SS, Horner M, et al. A novel assay to measure B cell responses to keyhole limpet haemocyanin vaccination in healthy volunteers and subjects with systemic lupus erythematosus. Br J Clin Pharmacol. 2013;76(2):188-202. doi:10.1111/bcp.12172

48 Milgrom H, Kesler K, Byron M, Harbeck R, Holliday R, Leung DYM. Response to Cutaneous Immunization with Low-Molecular-Weight Subunit Keyhole Limpet Hemocyanin. Int Arch Allergy Immunol. 2012;157(3):269-274. doi:10.1159/000328784

49 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

50 Valdez H, Smith KY, Landay A, et al. Response to immunization with recall and neoantigens after prolonged administration of an HIV-1 protease inhibitor-containing regimen. AIDS. 2000;14(1):11-21. doi:10.1097/00002030-200001070-00002

51 Bird P, Calvert JE, Amlot PL. Distinctive development of IgG4 subclass antibodies in the primary and secondary responses to keyhole limpet haemocyanin in man. Immunology. 1990;69(3):355-360. http://www.ncbi.nlm.nih.gov/pubmed/2312160

52 Volkman DJ, Lane HC, Fauci AS. Antigen-induced in vitro antibody production in humans: a model for B cell activation and immunoregulation. Proc Natl Acad Sci. 1981;78(4):2528-2531. doi:10.1073/pnas.78.4.2528

53 Powell AE, Sloss AM, Smith RN. Leukocyte-adherence inhibition: a specific assay of cell-mediated immunity dependent on lymphokine-mediated collaboration between T lymphocytes. J Immunol. 1978;120(6):1957-1966. doi:10.4049/jimmunol.120.6.1957

54 Curtis JE, Hersh EM. The human secondary immune response to Keyhole limpet haemocyanin. Clin Exp Immunol. 1972;10(1):171-177. http://www.ncbi.nlm.nih.gov/pubmed/5017367

55 Salvaggio J, Castro-Murillo E, Kundur V. Immunologic response of atopic and normal individuals to keyhole limpet hemocyanin. J Allergy. 1969;44(6):344-354. doi:10.1016/0021-8707(69)90026-4

56 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

57 Diaz-Sanchez D, Garcia MP, Wang M, Jyrala M, Saxon A. Nasal challenge with diesel exhaust particles can induce sensitization to a neoallergen in the human mucosa. J Allergy Clin Immunol. 1999;104(6):1183-1188. doi:10.1016/S0091-6749(99)70011-4

58 de Fijter JW, Eijgenraam JW, Braam CA, et al. Deficient IgA1 immune response to nasal cholera toxin subunit B in primary IgA nephropathy. Kidney Int. 1996;50(3):952-961. doi:10.1038/ki.1996.396

59 Aydin S. A short history, principles, and types of ELISA, and our

laboratory experience with peptide/protein analyses using ELISA. Peptides. 2015;72:4-15. doi:10.1016/j.peptides.2015.04.012
60 Thiha A, Ibrahim F. A Colorimetric Enzyme-Linked Immunosorbent Assay (ELISA) Detection Platform for a Point-of-Care Dengue Detection System on a Lab-on-Compact-Disc. Sensors. 2015;15(5):11431-11441. doi:10.3390/s150511431
61 Morgan E, Varro R, Sepulveda H, et al. Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. Clin Immunol. 2004;110(3):252-266. doi:10.1016/j.clim.2003.11.017
62 Phetsouphanh C, Zaunders J, Kelleher A. Detecting Antigen-Specific T Cell Responses: From Bulk Populations to Single Cells. Int J Mol Sci. 2015;16(8):18878-18893. doi:10.3390/ijms160818878
63 Ji N, Forsthuber TG. ELISPOT Techniques. In: Methods in Molecular Biology. Vol 1304. Humana Press, New York, NY; 2014:63-71. doi:10.1007/7651_2014_111
64 Vignali DAA. Multiplexed particle-based flow cytometric assays. J Immunol Methods. 2000;243(1-2):243-255. doi:10.1016/S0022-1759(00)00238-6
65 Koppi TA, Maluish AE, Halliday WJ. The cellular mechanism of leukocyte adherence inhibition. J Immunol. 1979;123(5):2255-2260. doi:10.4049/jimmunol.123.5.2255
66 Lange CG, Xu Z, Patterson BK, et al. Proliferation responses to HIVp24 during antiretroviral therapy do not reflect improved immune phenotype or function. AIDS. 2004;18(4):605-613. doi:10.1097/00002030-200403050-00004
67 Sokal JE. Measurement of Delayed Skin-Test Responses. N Engl J Med. 1975;293(10):501-502. doi:10.1056/NEJM197509042931013
68 Pouchot J. Reliability of Tuberculin Skin Test Measurement. Ann Intern Med. 1997;126(3):210. doi:10.7326/0003-4819-126-3-199702010-00005
69 Deegan AJ, Wang RK. Microvascular imaging of the skin. Phys Med Biol. 2019;64(7):07TR01. doi:10.1088/1361-6560/ab03f1
70 Linming F, Wei H, Anqi L, et al. Comparison of two skin imaging analysis instruments: The VISIA ® from Canfield vs the ANTERA 3D ® CS from Miravex. Ski Res Technol. 2018;24(1):3-8. doi:10.1111/srt.12381
71 Ly BCK, Dyer EB, Feig JL, Chien AL, Del Bino S. Research Techniques Made Simple: Cutaneous Colorimetry: A Reliable Technique for Objective Skin Color Measurement. J Invest Dermatol. 2020;140(1):3-12.e1. doi:10.1016/j.jid.2019.11.003
72 Kimura M, Comstock GW, Mori T. Comparison of erythema and induration as results of tuberculin tests. Int J Tuberc Lung Dis. 2005;9(8):853-857. http://www.ncbi.nlm.nih.gov/pubmed/16104630
73 Platts-Mills TAE, Vaughan JW, Blumenthal K, Woodfolk JA, Sporik RB. Decreased prevalence of asthma among children with high exposure to cat allergen: relevance of the modified Th2 response. Mediators Inflamm. 2001;10(6):288-291. doi:10.1080/09629350152700902
74 Poulsen LK, Hummelshoj L. Triggers of IgE class switching and allergy development. Ann Med. 2007;39(6):440-456. doi:10.1080/07853890701449354
75 Vercelli D. One cytokine, two isotypes: a trojan horse, pandora's box, and an evolving paradigm. Am J Respir Crit Care Med. 2000;162(3 Pt 2):S86-90. doi:10.1164/ajrccm.162.supplement_2.ras-6
76 Spencer LA, Weller PF. Eosinophils and Th2 immunity: contemporary insights. Immunol Cell Biol. 2010;88(3):250-256. doi:10.1038/icb.2009.115
77 Dannenberg AM. Delayed-type hypersensitivity and cell-mediated immunity in the pathogenesis of tuberculosis. Immunol Today. 1991;12(7):228-233. doi:10.1016/0167-5699(91)90035-R
78 Vukmanovic-Stejic M, Reed JR, Lacy KE, Rustin MHA, Akbar AN. Mantoux Test as a model for a secondary immune response in humans. Immunol Lett. 2006;107(2):93-101. doi:10.1016/j.imlet.2006.08.002
79 Swaminathan A, Harrison SL, Ketheesan N, et al. Exposure to Solar UVR Suppresses Cell-Mediated Immunization Responses in Humans: The Australian Ultraviolet Radiation and Immunity Study. J Invest Dermatol. 2019;139(7):1545-1553.e6. doi:10.1016/j.jid.2018.12.025
80 Kaufman M, Pardo G, Rossman H, Sweetser MT, Forrestal F, Duda P. Natalizumab treatment shows no clinically meaningful effects on immunization responses in patients with relapsing-remitting multiple sclerosis. J Neurol Sci. 2014;341(1-2):22-27. doi:10.1016/j.jns.2014.03.035
81 Gallegos AM, Hoerger M, Talbot NL, et al. Toward Identifying the Effects of the Specific Components of Mindfulness-Based Stress Reduction on Biologic and Emotional Outcomes Among Older Adults. J Altern Complement Med. 2013;19(10):787-792. doi:10.1089/acm.2012.0028
82 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
83 Weide B, Pascolo S, Scheel B, et al. Direct Injection of Protamine-protected mRNA: Results of a Phase 1/2 Vaccination Trial in Metastatic Melanoma Patients. J Immunother. 2009;32(5):498-507. doi:10.1097/CJI.0b013e3181a00068
84 Smith A, Vollmer-Conna U, Geczy A, et al. Does genotype mask the relationship between psychological factors and immune function? Brain Behav Immun. 2005;19(2):147-152. doi:10.1016/j.bbi.2004.06.005
85 Smith AJ, Vollmer-Conna U, Bennett B, Hickie IB, Lloyd AR. Influences of Distress and Alcohol Consumption on the Development of a Delayed-Type Hypersensitivity Skin Test Response. Psychosom Med. 2004;66(4):614-619. doi:10.1097/01.psy.0000130962.28801.af
86 Abrams JR, Lebwohl 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
87 Grant RW, Mariani RA, Vieira VJ, et al. Cardiovascular exercise intervention improves the primary antibody response to keyhole limpet hemocyanin (KLH) in previously sedentary older adults. Brain Behav Immun. 2008;22(6):923-932. doi:10.1016/j.bbi.2008.01.006
88 Wishahi MM, Ismail IMH, Ruebben H, Otto T. Keyhole-Limpet Hemocyanin Immunotherapy in the Bilharzial Bladder: A New Treatment Modality? Phase II Trial: Superficial Bladder Cancer. J Urol. 1995;153(3S):926-928. doi:10.1016/S0022-5347(01)67605-5
89 Sidell N, Connor MJ, Chang B, Lowe NJ, Borok M. Effects of 13-Cis Retinoic Acid Therapy on Human Antibody Responses to Defined Protein Antigens. J Invest Dermatol. 1990;95(5):597-602. doi:10.1111/1523-1747.ep12505593
90 Berd D, Maguire HC, Mastrangelo MJ. Potentiation of human cell-mediated and humoral immunity by low-dose cyclophosphamide. Cancer Res. 1984;44(11):5439-5443. http://www.ncbi.nlm.nih.gov/pubmed/6488195
91 Parker DC. T Cell-Dependent B Cell Activation. Annu Rev Immunol. 1993;11(1):331-360. doi:10.1146/annurev.iy.11.040193.001555
92 Bain S, Schaefer-Jansen E, Delepine A, Pham N, Lauscher F. New, late-breaking data at EADV highlights emerging clinical profile of amlitelimab (formerly KY1005) in adults with inadequately controlled moderate-to-severe atopic dermatitis. Paris, France: Sanofi Press Release. Published 2021. Accessed May 25, 2022. https://www.sanofi.com/en/media-room/press-releases/2021/2021-09-30-14-30-00-2306183
93 White S. Sanofi buys Kymab for up to $1.5 billion to expand in immunotherapy. Reuters. Published 2021. Accessed May 25, 2022. https://www.reuters.com/article/us-sanofi-immunotherapy-idUSKBN29GoRT