Development of Immunotherapy for Equine Insect Bite Hypersensitivity

Sara Björk Stefánsdóttir

Thesis for the degree of Philosophiae Doctor

March 2024

School of Health Sciences

FACULTY OF MEDICINE

UNIVERSITY OF ICELAND

Development of Immunotherapy for Equine Insect Bite Hypersensitivity

Sara Björk Stefánsdóttir

Thesis for the degree of Philosophiae Doctor

Supervisors

Sigurbjörg Þorsteinsdóttir, Vilhjálmur Svansson

Doctoral committee

Arna Rúnarsdóttir, Eliane Marti, Jón Már Björnsson

March 2024

School of Health Sciences

FACULTY OF MEDICINE

UNIVERSITY OF ICELAND

Þróun ónæmismeðferðar gegn sumarexemi í hestum

Sara Björk Stefánsdóttir

Ritgerð til doktorsgráðu

Leiðbeinendur

Sigurbjörg Þorsteinsdóttir, Vilhjálmur Svansson

Doktorsnefnd

Arna Rúnarsdóttir, Eliane Marti, Jón Már Björnsson

Mars 2024

Heilbrigðisvísindasvið

LÆKNADEILD

HÁSKÓLI ÍSLANDS

ORCID: 0000-0001-9560-7574

Reykjavik, Iceland 2024

Ágrip

Sumarexem er húðofnæmi af gerð I í hestum sem orsakast af framleiðslu á IgE mótefnum gegn ofnæmisvökum upprunnum úr biti smámýs (*Culicoides spp.*). Sjúkdómsvaldurinn lifir ekki á Íslandi. Tíðni sjúkdómsins er mun hærri í útfluttum hestum samanborið við íslenska hesta sem eru fæddir á flugusvæðum. Sumarexem hefur mikil áhrif á velferð hrossa. Aðalofnæmisvakarnir sem valda exeminu hafa verið skilgreindir og rannsóknir á ónæmissvari sjúkdómsins hafa leitt í ljós að meingerð sjúkdómsins einkennist af ójafnvægi milli Th2 hjálparfrumna og T stýrifrumna. Sökum þessa opnast sá möguleiki að þróa ofnæmisvakasértæka ónæmismeðferð við sumarexemi. Við þróun á fyrirbyggjandi ónæmismeðferð höfum við áður sýnt fram á að bólusetning í kjálkabarðseitla með hreinsuðum ofnæmisvökum í ónæmisglæði framkallar týpu 1 (Th1)/T-stýrifrumu miðað ónæmissvar. Markmið verkefnisins var frekari þróun á fyrirbyggjandi ónæmismeðferð gegn sumarexemi. Einnig framleiðsla á endurröðuðum ofnæmisvökum í skordýrafrumum og byggi auk hreinsunar þeirra til notkunar í ónæmisprófum.

Nýskilgreindir aðalofnæmisvakar sem valda sumarexemi (Cul o 8, Cul o 9, Cul o 10 og Cul o 11) voru tjáðir í skordýrafrumum með Baculoveirukerfi (Bac). Endurröðuðu ofnæmisvakarnir voru hreinsaðir og notaðir í ónæmisprófum til að meta ónæmissvörun í kjölfar bólusetningar. Einnig var sýnt fram á notagildi þeirra í greiningaprófi fyrir sumarexem. Fjórir ofnæmisvakar (Cul o 2, Cul o 2P, Cul o 3 og Cul n 8) voru tjáðir í byggi í samstarfi við ORF Líftækni. Tveir þeirra (Cul o 2P og Cul o 3) voru hreinsaðir úr byggi.

Í ritgerðinni er gerð samanburðarrannsókn á sprautun í eitla og sprautun undir húð. Tólf heilbrigðum hestum á Íslandi var skipt í tvo hópa sem sprautaðir voru í kjálkabarðseitla eða undir húð, þrisvar sinnum með fjögurra vikna millibili með hreinsuðum *Culicoides* ofnæmisvökum (rCul o 2P, rCul o 3 og rCul n 4) í ónæmisglæði (aluminum hydroxide (alum) og monophosphoryl lipid A (MPLA)). Sex hross til viðbótar voru bólusett undir húð með hreinsuðum ofnæmisvökum og veirulíkum ögnum (VLP) til samanburðar við MPLA á stýringu ónæmissvars á Th1 braut. Allar þrjár bólusetningaraðferðirnar örvuðu myndun á ofnæmisvaka sértækum IgG1, IgG4/7 og IgG5 mótefnum án framleiðslu á IgE. Mótefnin hindruðu að hluta bindingu IgE úr sumarexemshestum við ofnæmisvakana.

Engin marktækur munur var á mótefnasvörun í kjölfar sprautunar í eitla samanborið við sprautun undir húð. Boðefnasvörun í kjölfar örvunar á hvítfrumum sýndi fram á marktæka aukningu á IFN-γ og IL-4 framleiðslu hjá eitla og undir húð bólusetningahópum og IL-10 í hestum bólusettum í eitla. Engin marktækur munur var á

boðefnasvörun milli hópanna, en framleiðsla á IFN-γ var aðeins hærri í hestum bólusettnum í eitla samanborið við hesta bólusetta undir húð og IL-4 framleiðsla var minni. Hægt er að álykta að sprautun undir húð geti verið nothæf við ónæmismeðferð gegn sumarexemi í hestum.

Bólusetning með alum/VLP örvaði lægri IgG4/7 og IgG5 mótefnasvörun samanborið við alum/MPLA, en IgG1 svörun var sambærileg. IgG4/7 svörun var marktækt lægri gegn Bac-rCul o 2P og Bac-rCul n 4 í hestum bólusettum með alum/VLP, einnig IgG5 svörun gegn Bac-rCul o 2P. Enn fremur var hindrunargeta á IgE bindingu við ofnæmisvakana minni í hestum bólusettum með alum/VLP. Heilt yfir var mótefnasvar minna með VLP sem ónæmisglæði samanborið við MPLA og hlutfall IgG5:IgG4/7 lægra. IgG5 hefur verið tengt við ofnæmi. Í ljósi þess að IgG5 svörun var lægri með VLP gæti það verið mögulegur kostur við ofnæmisvakasértæka ónæmismeðferð gegn sumarexemi í hestum, en frekari rannsókna er þörf.

Sermi úr fyrri rannsókn var notað til að bera saman mótefnasvörun í kjölfar bólusetningar með 10 µg og 20 µg af ofnæmisvaka. Marktæk aukning á ofnæmisvakasértækum IgG mótefnum varð bæði með 10 µg og 20 µg af ofnæmisvökum. Framleiðsla á IgG1 mótefnum var marktækt minni, IgG4/7 nokkuð minni og IgG5 sambærileg með 10 µg af ofnæmisvökum samanborið við 20 µg. Draga má þá ályktun að 20 µg af hverjum ofnæmisvaka í bólusetningu sé ákjósanlegur kostur við frekari þróun á ónæmismeðferð gegn sumarexemi í hestum.

Til að meta hvort bólusetning með hreinsuðum ofnæmisvökum í ónæmisglæði veiti vörn gegn sumarexemi var framkvæmd áskorunartilraun. Tuttugu og sjö heilbrigðir íslenskir hestar voru bólusettir í kjálkabarðseitla með níu hreinsuðum ofnæmisvökum (Cul o 1P, Cul o 2P, Cul o 3, Cul o 5, Cul o 7, Cul o 8, Cul o 9, Cul o 11 and Cul n 4) í alum/MPLA ónæmisglæðum. Bólusetningin var framkvæmd á Íslandi og í kjölfarið voru hrossin flutt út til Sviss. Hestarnir voru óvarðir gegn biti smámýs og þeim fylgt eftir í þrjú ár með reglulegri klínískri skoðun, blóðtöku og örvunarbólusetningu í upphafi annars og þriðja sumars. Á fyrsta ári höfðu 6 hestar (23,1%) fengið sumarexem, 13 hestar (50,0%) á öðru ári og 16 (61,5%) á því þriðja. Mótefnasvörun á fyrstu tveimur árunum var mæld gegn þremur ofnæmisvökum (Cul o 2P, Cul o 8 og Cul o 9). Sambærilegt mótefnasnið (miðgildi) var á IgG1, IgG4/7 og IgG5 gegn öllum þremur ofnæmisvökunum eftir bólusetningu og örvunarbólusetningu en mikill breytileiki var milli einstaklinga. Engin marktækur munur var á IgG mótefnasvari á neinum tímapunkti fyrstu tvö árin milli bólusettra hesta sem fengu sumarexem og þeirra sem voru heilbrigðir út tímabilið. Við lok annars árs var IgE mótefna framleiðsla gegn Bac-rCul o 8 og Bac-rCul o 9 ofnæmisvökum marktækt hærri í bólusettum hestum sem fengu sumarexem samanborið við þau sem voru heilbrigð út tímabilið. Bólusetningin veitti því ekki vörn gegn sumarexemi en frekari prófun á þeim sýnum sem safnað var gæti gefið innsýn í orsök þess að bóluefnið varði ekki hestana.

Lykilorð: Sumarexem, hestar, ofnæmisvakar, ónæmismeðferð, bygg

Abstract

Insect bite hypersensitivity (IBH) is an allergic dermatitis of horses caused by IgE-mediated reactions to allergens of biting midges (*Culicoides* spp). Iceland is free of the causative agents but the prevalence of IBH in exported Icelandic horses is much higher than in Icelandic horses born in *Culicoides* rich environment. IBH can severely impact the well-being of affected horses. Numerous *Culicoides* allergens have been identified and studies on the pathogenesis demonstrate an imbalance between T-helper 2 (Th2) and regulatory T (Treg) cell subsets. This knowledge opens the possibility to develop an allergen-specific immunotherapy (AIT) against IBH. First studies showed that intralymphatic (i.l) injection of purified *E. coli* produced recombinant (r-) allergens with adjuvants induce a T-helper 1(Th1)/Treg focused immune response and is thus a promising approach for preventive AIT. The aim of the project is further development of preventive immunotherapy and production of r-*Culicoides* allergens in insect cells and transgenic barley for application in immunoassays.

Newly identified major *Culicoides* allergens (Cul o 8, Cul o 9, Cul o 10 and Cul o 11) were expressed and produced in insect cells (Bac). The r-allergens were purified and used in immune assays to evaluate responses following vaccination. The purified r-allergens were also found promising for use in diagnostic tests, distinguishing between IBH-affected and healthy horses. Four *Culicoides* allergens (Cul o 2, Cul o 2P, Cul o 3 and Cul n 8) were expressed in transgenic barley in collaboration with ORF Genetics and two of them (Cul o 2P and Cul o 3) purified.

For the development of preventive immunization, i.l. injection of purified *E. coli* produced r-allergens mixed with adjuvants was compared to the more practical subcutaneous (s.c.) route. Twelve healthy horses living in Iceland were injected, i.l. or s.c., three times with four-week interval, using the purified r-*Culicoides* allergens (rCul o 2P, rCul o 3 and rCul n 4) in a mixture with aluminium hydroxide (alum) and monophosphoryl lipid A (MPLA) as adjuvants. Six additional healthy horses were vaccinated s.c. with the same r-*Culicoides* allergens in alum and virus-like particles (VLP) for comparison with MPLA adjuvant for induction of Th1 type immune responses. The three vaccination methods significantly induced allergen-specific IgG1, IgG4/7 and IgG5 subclass antibody responses without allergen-specific IgE production. Furthermore, the antibodies generated were able to block the binding of IgE from IBH-affected horses to the corresponding r-allergens. Interestingly, there was no significant difference in the antibody response between the i.l. and s.c. injection routes at any time point following immunisation. Upon re-stimulation of peripheral blood mononuclear cells (PBMC), there was a significant increase in IFN-γ and IL-4 production in both vaccination groups compared to pre-vaccination, while a significant increase in IL-10

was only observed in the i.l. group. Horses vaccinated i.l. produced slightly more IFN-γ and less IL-4 as compared to the horses injected s.c., but the difference did not reach significance. It can be concluded that the simpler s.c. injection could be an option in immunotherapy against IBH. Use of VLP instead of MPLA as immunomodulator induced weaker IgG4/7 and IgG5 while IgG1 was similar in both groups. The IgG4/7 response was significantly lower against Bac-rCul o 2P and Bac-rCul n 4 in the s.c./VLP vaccination group compared to s.c./MPLA, as well for IgG5 responses against Bac-rCul o 2P. Furthermore, the IgE blocking capacity of the allergen-specific IgG antibodies induced following vaccination with alum/VLP was weaker compared to alum/MPLA. In conclusion, addition of VLPs as immunomodulator instead of MPLA resulted in a weaker antibody response and lower IgG5:IgG4/7 ratio. IgG5 has been associated with allergy. In the light of the low IgG5 response, VLP might present a promising adjuvant system for AIT against IBH but further investigation is needed. Sera from a previous vaccination experiment was used for comparison of the antibody response following i.l. immunization with 10 or 20 µg r-allergen in alum/MPLA. Both induced a significant IgG antibody response following vaccination. The 10 µg induced significantly lower IgG1 antibody levels as compared to 20 µg, the IgG4/7 response was slightly lower and the IgG5 was similar. Based on these results, 20 µg of each r-allergen in vaccination seems preferable for AIT against IBH.

To test the efficacy of a preventive allergen immunotherapy, a challenge experiment was conducted. Twenty - seven healthy Icelandic horses were vaccinated i.l. with 20 µL each of nine *E. coli* produced r-*Culicoides* allergens (Cul o 1P, Cul o 2P, Cul o 3, Cul o 5, Cul o 7, Cul o 8, Cul o 9, Cul o 11 and Cul n 4) in alum/MPLA. The vaccination was done in Iceland and the horses subsequently exported to Switzerland. They were kept unprotected from insect bites to ensure exposure to *Culicoides* and followed for three years with regular clinical examination and blood sampling. One horse was withdrawn from the study and excluded from sample analysis. Six horses developed IBH (23.1%) in the first year. In the second year a total of 13 horses (50.0%) and in the third year 16 (61.5%) horses were affected with IBH. The antibody response during the first two years was analysed against three *Culicoides* allergens (Cul o 2P, Cul o 8 and Cul o 9). A comparable time course pattern between horses that remained healthy and those that developed IBH was observed for IgG1, IgG4/7 and IgG5 antibody levels against all three r-allergens following vaccination and booster vaccination. However, there was considerable individual variability among horses. The IgG subclass response showed no difference at any time-point between horses which developed IBH and horses that remained healthy. However, at the end of the second *Culicoides* season IBH-affected horses had significantly higher IgE levels against Bac-rCul o 8 and Bac-rCul o 9. In conclusion, the vaccine did not reduce the incident of IBH in exported horses based on reported prevalence. Further analysis of collected samples could provide insights on why the vaccine does not protect the horses against IBH.

Keywords: Insect bite hypersensitivity, horses, allergen, immunotherapy, barley

Acknowledgements

Now my doctoral studies have reached the end with the completion of this thesis. It has been a journey filled with many challenges but first and foremost been educational and rewarding. There are many people that have followed me through this journey with participation in the project and/or support while carrying it out, that I would like to thank.

The project was mainly carried out at the Institute for Experimental Pathology, University of Iceland, Keldur, also at Prof. Eliane Marti's lab at the Vetsuisse Faculty, University of Berne Switzerland and ORF Genetics, Reykjavík, Iceland. I am thankful to the administration of those institutions for allowing me to carry out my doctoral studies at their facilities.

With deep respect, I thank my supervisors Sigurbjörg Þorsteinsdóttir and Vilhjálmur Svansson for the opportunity to work on this project at Keldur, their patience, motivation and guidance throughout all the ups and downs of this journey. They have now supervised me through my B.Sc., M.Sc. and Ph.D. degrees and I have grown immensely as scientist under their supervision where they have provided guidance but also allowed me to take on challenges individually. I'm also grateful for their friendship, kindness and all the good times we have shared over the years.

I would also like to express my sincere gratitude to Eliane Marti, member of my doctoral committee, for her guidance, valuable input in the project and welcoming me to her lab in the University of Berne, Switzerland. I would also like to thank her for welcoming me to her home, for taking the time to show me many beautiful places in Switzerland and all the good times we have shared.

I am sincerely grateful to Arna Rúnarsdóttir and Jón Már Björnsson, members of my doctoral committee, for their support throughout the project and for showing me the wonders of transgenic barley as protein expression system and expanding my world of protein purification.

I would like to thank all members of the doctoral committee for their valuable input on the project and specially the thesis.

I give special thanks to Sigríður Jónsdóttir my great friend and role model for endless motivation, guidance and all her hard work and dedication to the IBH project. I am thankful for her housing me during my time in Switzerland and all the fun trips, laughs and meaningful conversations we have had through the years.

I thank my dear colleagues and co-workers at Keldur for their companionship, knowledge sharing and discussions during coffee breaks. Most of all I am grateful for my fellow IBH team members Lilja, Hólmfríður and Ragna Brá for our many laughs in and out of the lab and horse stables.

Moreover, I would like to thank the whole team at ORF Genetics for welcoming me to their lab with such kindness and in particular R&D Cool Kidz™ for all the fun times, laughter and jokes in the lab. Special thanks to Hildur Björg for guidance and assistance in the lab with screening of transgenic barley lines and Melkorka Mist for assistance with protein purification.

I thank all the great people and administration at Department of Immunology at Landspítali for allowing me to set-up FACS and Luminex methods for IBH at their lab and the use of the devices. Especially Stefanía and Siggeir for their warm welcome to the lab, guidance and their valuable input.

I would like to thank Bettina Wagner and her staff for welcoming me to her laboratory at Cornell University, USA.

Writing of the thesis was performed alongside work at ORF Genetics and Alvotech, Reykjavík Iceland and I would like to thank the administrators for giving me the understanding and flexibility needed during the writing. At ORF genetics, my special thanks go to Ingi and the whole production team. At Alvotech, I would like to thank Hörður, Head of Drug Product Development, my managers María and Haffi and the greatest girl power project team lead by Jóhanna for all their patience and support.

I am incredibly grateful for the everlasting love, constant support and endless encouragement of my family and friends throughout the years, especially my parents Þorbjörg Dóra and Stefán Þór. I am incredibly lucky to have large family with many siblings, to get to enjoy their company, share with them many laughs and get inspired by. I dedicate this work to amma Harpa which always had unwavering belief in me.

Finally, my sincere gratitude goes to the most important people in my life, my husband Jónas and our sons Breki Snær and Aron Darri. Throughout this journey they have brought me endless love, support, patience and encouragement. You light up my life and make it all worth it. To my sons, I want you to know you can achieve anything you set your mind and hard work to.

This work was funded by The Icelandic Research Fund (grant no. 184998-051 and 206528-051), The University of Iceland Research Fund, The Horse Breeders Association of Iceland, Stofnverndarsjóður íslenska hestakynsins, Náttúruverndasjóður Pálma Jónssonar, Morris Animal Foundation (grant no. D20EQ-032), Stiftung pro Pferd and Swiss National Science Foundation (grant no. 310030_208152).

Contents

List of Abbreviations

a.a.	Amino acids
AD	Atopic dermatitis
AIT	Allergen-specific immunotherapy
Alum	Aluminum hydroxide
AP	Alkaline phosphatase
APCs	Antigen-presenting cells
AR	Allergic rhinitis
ARC	Allergic rhinoconjunctivitis
ASIT	Allergen-specific immunotherapy
B cells	B lymphocytes
BSA	Bovine serum albumin
CCMV	Cowpea chlorotic mottle virus
CCDs	Cross-reactive carbohydrate determinants
CD	Cluster of differentiation
CH	Culicoides hypersensitivity
CTLs	Cytotoxic T lymphocytes
Cul n	*Culicoides nubeculosis*
Cul o	*Culicoides obsoletus*
Cul s	*Culicoides sonorensis*
CuMV	Cucumber mosaic virus
$CuMV_{TT}$	VLP derived from Cucumber mosaic virus covalently linked to tetanus toxin
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DNA	Deoxribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELA	Equine leukocyte antigen
ELISA	Enzyme-linked immunosorbent assay

elu	Elution
EPIT	Epicutaneous immunotherapy
ext	Extract
FACS	Fluorescence activated cell sorting
FceRI	High-affinity IgE receptor
FDA	U.S. Food and Drug Administration
FoxP3	Forkhead box 3
gp67 sec	Glycoprotein secretion signal originated from baculoviruses
GRAS	Generally recognized as safe
GWA	Genome-wide association
HBM	Honey-bee melittin
HBV	Hepatitis B Virus
High-five	Cell line from *Trichpulsia ni*
HPV	Human Papilloma Virus
h	Hour
HRP	Horseradish peroxidase
hya	Hyaluronidase
i.d.	Intradermal
i.l.	Intralymphatic
IBH	Insect bite hypersensitivity
IDIT	Intradermal immunotherapy
IDT	Intradermal test
IFN	Interferon
Ig	Immunoglobulin
IGHC	Ig heavy-chain constant
IL	Interleukin
ILCs	Innate lymphoid cells
ILIT	Intralymphatic immunotherapy
IMAC	Immobilized metal affinity chromatography
INIT	Intranasal immunotherapy
IPTG	Isopropyl β- d-1-thiogalactopyranoside
IUIS	International Union of Immunological Societies

kDA	Kilo Dalton
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MES	2-(N-morpholino) ethane sulfonic acid
MHC	Major histocompatibility complex
MPLA	Monophosphoryl lipid A
NK cells	Natural killer cells
o.n.	Over night
OBP	Odorant Binding Protein
OD	Optical density
OIT	Oral immunotherapy
pAb	Polyclonal antibody
PAMPs	Pathogen-associated molecular patterns
PAR2	Protease-activated receptor 2
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PHA	Phytohaemaglutinin
PPRs	Pattern recognition receptors
PTM	Post-translational modifications
qPCR	*Quantitative real-time polymerase chain reaction*
r-	Recombinant
RNA	Ribonucleic acid
RT	Room temperature
s.c.	Subcutaneous
SCIT	Subcutaneous immunotherapy
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sf-9	Cell line from *Spodoptera frugiperda*
SLIT	Sublingual immunotherapy
sLT	Sulfidoleukotriene
SNP	Single nucleotide polymorphism
SP	Sulphopropyl

SP1	SUMOstar Protease 1 cleavage site
T cells	T lymphocytes
T_1	Transgenic barley seed line generartion 1
T_2	Transgenic barley seed line generartion 2
T_3	Transgenic barley seed line generartion 3
Th1	Type 1 immune responses
Th2	Type 2 immune responses
Th3	Type 3 immune responses
TBS	Tris buffer saline
TEV	Tobacco etch virus
TFF	Tangential flow filtration
Tfh cells	Follicular T helper cells
TGF	Transforming growth factor
Th cells	T helper cells
TLRs	Toll-like receptors
Treg	T regulatory cell
TSLP	Thymic stromal lympopoietin
TT	Tetanus toxin
VLP	Virus-like particles
WB	Western blot
WBE	Whole body extract
WHO	World Health Organization
wo	Without

List of Figures

List of Tables

List of Original Papers

This thesis is based on the following original publications, which is referred to in the text as:

I. **Establishment of a protocol for preventive vaccination against equine insect bite hypersensitivity.**
Stefansdottir S., Jonsdottir S., Kristjansdottir H., Svansson V., Marti E., Torsteinsdottir S., *Veterinary Immunology and Immunopathology,* 2022, 253, 110502

Paper is reprinted with kind permission of the publishers.

Declaration of Contribution

Production and purification of r-allergens in different expression systems:

For application of r-allergens in immunotherapy and immunoassays evaluating immune response, the r-allergens were expressed in three different expression systems and purified. I expressed three IBH r-allergens in *E. coli* and purified one successfully for use in vaccination. The other eight *E. coli* r-allergens used in vaccinations were expressed and purified by Dr. Sigríður Jónsdóttir. Four newly discovered major *Culicoides* allergens were expressed in insect cells, all the work was done by me with assistance from Dr. Sigurbjörg Þorsteinsdóttir with cell cultures. These four r-allergens and eight r-allergens expressed in insect cells prior to this study were purified for use in immunoassays for evaluation of immune response following immunotherapy. Purification of Bac-r-allergens was carried out by me and students in the lab, Hólmfríður Kristjánsdóttir and Ragna Brá Guðnadóttir.

Expression of r-allergens in barley was done in collaboration with ORF Genetics (Kópavogur, Iceland), molecular cloning and transformation of barley done by Dr. Jón Már Björnsson and Hildur Björg Birnisdóttir. Screening of three generations of r-barley seed lines was performed by me with assistance from Hildur Björg Birnisdóttir. Purification of the r-allergens from barley was performed by me at ORF Genetics under the guidance of Dr. Arna Rúnarsdóttir.

Sulfidoleukotriene release test was performed by Prof. Dr. Eliane Marti and Jelena Mirkovitch at Vetsuisse Faculty, University of Berne, Switzerland using r-allergens I expressed and purified. Data analysis and presentation was carried out by me.

Experimental vaccination comparing injection routes (Paper):

This paper describes the vaccination of 12 horses i.l. or s.c. with purified r-allergens in alum/MPLA adjuvant. I took part in all steps of the experiment. I assisted with vaccination and blood sampling performed by Dr. Vilhjálmur Svansson. Immunoassays were done by me. Immunoblot analysis and *in vitro* stimulation was carried out at Keldur, University of Iceland, the antibody ELISA was carried out Vetsuisse Berne and the bead-based multiplex assay measuring cytokines in supernatant of *in vitro* re-stimulated PBMC at Prof. Dr. Bettina's Wagners lab at Cornell University, USA. FACS was carried out at Department of Immunology at Landspítali University Hospital with help from Associate Prof. Dr. Stefanía P. Bjarnarson. I collected the data and carried out data analysis.

Experimental vaccination comparing adjuvant effect:

This study describes the vaccination of 6 horses s.c. with purified r-allergens in alum/VLP adjuvant which antibody response was compared to 6 horses vaccinated s.c. with same r-allergens in alum/MPLA. I took part in all steps of the experiment. I assisted with vaccination and blood sampling performed by Dr. Vilhjálmur Svansson. Immunoassays were done by me. Immunoblot analysis and *in vitro* stimulation was carried out at Keldur, the antibody ELISA was carried out at Vetsuisse Berne and the bead-based multiplex assay measuring cytokines in supernatant of *in vitro* re-stimulated PBMC at Landspítali. I collected the data and carried out data analysis.

Comparison of different amounts of r-allergens in vaccination:

In this chapter the amounts of r-allergens in vaccination are compared in antigen ELISA using serum samples of horses from two different vaccination experiments. The ELISA was carried out by master student Elisa Bach using serum from vaccination experiments I participated in with r-allergen expressed and purified by me. Data analysis and presentation was performed by me.

Challenge experiment:

This study describes a challenge experiment where 27 horses were vaccinated i.l. with r-allergens in alum/MPLA adjuvant in Iceland followed by export to *Culicoides* infested areas in Switzerland and Germany. I took part in all steps of the experiment. I assisted with vaccination, blood sampling and clinical examination performed by Dr. Vilhjálmur Svansson, Prof. Dr. Eliane Marti, Dr. Charlotta Oddsdóttir and Dr. Anja Ziegler. I participated in sample collection and *in vitro* stimulation of PBMC carried out at Keldur and Vetsuisse Berne. Antibody ELISA was carried out at Keldur and data analysis was performed by me.

1 Introduction

1.1 The immune system in brief

The immune system is a biological defence mechanism which constitutes of different types of effector cells and molecules which cooperate in response against foreign substances and the damage they can cause. Immune responses are not only elicited by infectious agents but also by non-infectious foreign substances and damaged and malignant (tumor) cells. Based on the speed and specificity of the reaction, the immune system can be classified into the innate- and the adaptive immune system. The innate immune system responds immediately upon encounter of pathogens with inflammation, that is recruitment of phagocytes and other leukocytes which destroy microbes, block viral replication and eliminate virally infected cells. The first line of defence is the physical barrier provided by the skin and mucosal surfaces. Antimicrobial enzymes and peptides in blood plasma and mucus digest and lyse bacterial cell membranes while blood proteins which make up the complement system target pathogens for lysis and phagocytosis by effector cells of the innate immune system. Innate immune responses are elicited upon activation of pattern recognition receptors (PRRs) expressed by cells of the immune system. PRRs recognize molecular structures of microbial pathogens called pathogen-associated molecular patterns (PAMPs) and substances produced or released from damaged and dying cells called damage-associated molecular patterns (DAMPs) and include transmembrane receptors like Toll-like receptors (TLRs) and intracellular compartments receptors (Abbas et al., 2022a, 2022c; Murphy et al., 2022a, 2022b, 2022c).

Cells of the immune system originate from the same hematopoietic stem cells in the bone marrow that give rise to leukocytes of myeloid origin and lymphocytes of lymphoid origin. The various cell types of the immune system express cell surface molecules that are numbered according to the "cluster of differentiation" or CD numbers. Leukocytes of the innate immune system include macrophages, neutrophils, mast cells, basophils, eosinophils, dendritic cells (DCs) and innate lymphocytes that lack specific antigen receptors, which are innate lymphoid cells (ILCs) and natural killer (NK) cells. B and T lymphocytes (B cells and T cells) are the main effector cells of the adaptive immune system. Neutrophils are the most abundant leukocytes and are rapidly recruited to the site of inflammation where their main function is phagocytosis and microbial killing. Monocytes are recruited to the site of inflammation where they differentiate into macrophages. Macrophages recognize pathogens through a number of receptors they express and function through phagocytosis and killing of microbes and dead host cells. They also promote recruitment of leukocytes from blood by

secretion of cytokines and chemokines and initiate reparation of the damaged tissue. Mast cells, basophils and eosinophils release enzymes and toxic proteins upon activation, important in defences against parasites and play a major role in allergy. The main role of DCs is antigen presentation, they either take up particulate matter by phagocytosis or larger amount of extracellular fluid by micropinocytosis. Mature lymphocytes that have not been introduced to antigens are called naïve B- and T-cells which proliferate and differentiate upon antigen encounter into effector lymphocytes which engage in protective immune responses. Effector B cells are antibody-secreting plasma cells and effector T cells include cytokine-secreting $CD4^+$ T helper (Th) cells and cytotoxic T lymphocytes (CTLs) expressing $CD8^+$. Naïve T-cells are initially activated by DCs which are an important link between innate and adaptive immune responses. Activation of T cells leads to differentiation into specialized effector T cells with changes of surface molecular expression and production of cytokines (Figure 1). Cytokines produced by each T cell subset result in further development of the subset while inhibiting development of other subsets causing increased polarization of the response. Follicular helper T (Tfh) cells are essential in B cell differentiation, affinity maturation and production of antibodies. They are found in the B cell zone (follicles) of secondary lymphoid organs. ILCs have similar functions as $CD4^+$ and $CD8^+$ T cells but are not antigen-specific as they lack antigen-specific receptors. ILCs can be divided into three subsets ILC1, ILC2 and ILC3 which secrete the same cytokines as Th1, Th2 and Th17 cells respectively. NK cells have a cytotoxic function resembling CTLs (Abbas et al., 2022b; Murphy et al., 2022a).

Activation of adaptive immune responses is initiated by antigen uptake of antigen-presenting cells (APCs) including DCs, macrophages and B-cells. Following antigen uptake, APCs migrate to the draining lymph node while processing the antigen. In the lymph node, antigen peptides are presented on the surface of major histocompatibility complex (MHC) molecules and through cellular specification activate T cells. Antigens that are presented in MHC I expressed on all nucleated cells are recognized by $CD8^+$ T cells and those presented by MHC II expressed on APCs are recognized by $CD4^+$ T cells. Some activated T cells differentiate into memory T cells, long-living with rapid responses upon antigen re-encounter. When antigen has been eliminated, T-cell responses decline. B-cell or humoral immune responses result in production of secreted antibodies by B-cells engaging in antigen elimination. The activation of B-cells can be T-cell independent where antigens induce humoral responses without the involvement of Th cells. These antigens are usually of non-protein origins and include polysaccharides, membrane glycolipids and nucleic acids. T cell dependent activation of humoral responses is involved with the interaction of B- and T-cells. Cytokines secreted by the T cell subsets and the environment promotes activation of B cell class-switching and production of various immunoglobulin (Ig) isotypes followed by affinity maturation resulting in high affinity B cells. Activated B-cells differentiate into plasma cells and long-lived memory cells with rapid responses against antigens upon challenge.

Functions of antibodies include neutralization or blocking of microbe infectivity, opsonization for phagocytosis and activation of the complement system (Abbas et al., 2022d, 2022e; Murphy et al., 2022d, 2022e).

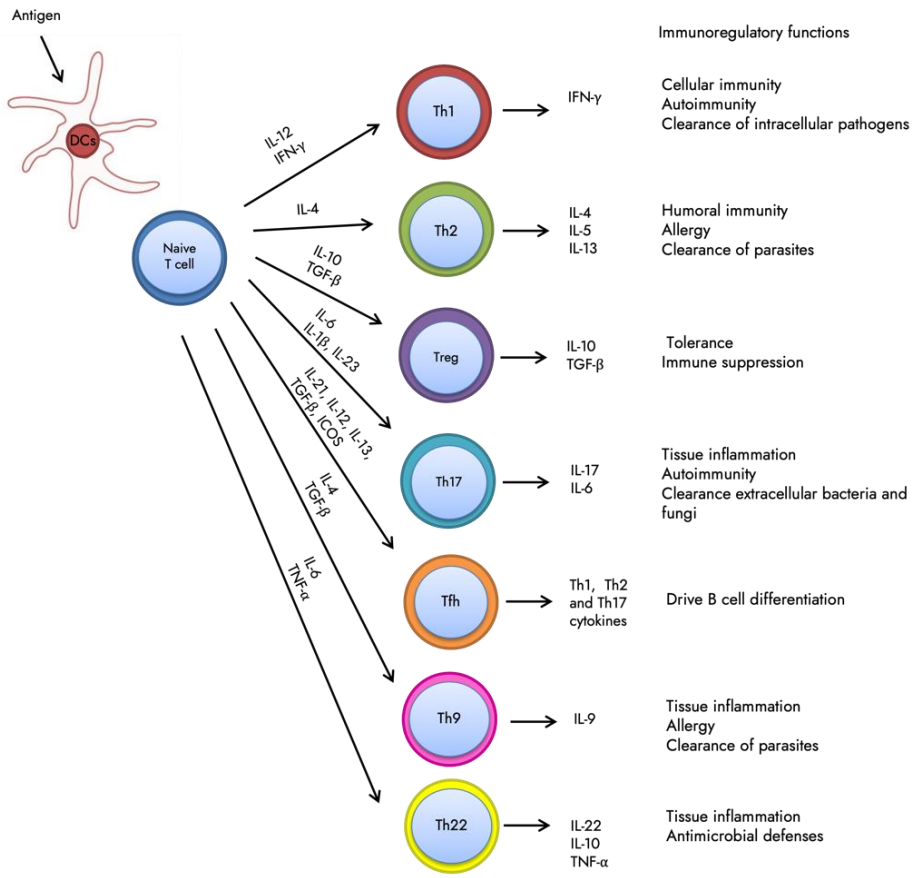


Figure 1. Differentation of naïve CD4+ T cells into effector cells. The antigen and environmental factors determine the signals of the DCs in the form of cytokines that induce the development of CD4+ T cells. Each effector T cell subset produces unique cytokines and has unique immunoregulatory function. The figure was modified in accordance with Crotty (2014); Soyer et al. (2013). The figure and figure text are reprinted from Stefansdottir, 2015.

Immune responses can be categorized into different immune models based on innate and adaptive mechanisms activated to eliminate distinct type of pathogens. Main effector cells in cytotoxic responses are NK cells and CTLs which target host cells infected with intracellular pathogens, stressed, or damaged host cells and tumor cells. Mechanisms of type 1 immune responses (Th1 responses) are characterized by ILC1, Th1 cells, opsonizing IgG antibody isotypes targeting intracellular pathogens, including bacteria, viruses and parasites. Mechanisms of type 2 immune responses (Th2 responses) are characterized by ILC2, Th2 cells, IgE, tissue mast cells, eosinophils and

basophils targeting multicellular parasites or helminths. Mechanisms of type 3 immune responses (Th3 responses) are characterized by ILC3, Th17 cells, opsonizing IgG isotypes and neutrophils targeting extracellular bacteria and fungi (Murphy et al., 2022f).

Adaptive immune responses are important in the fight against infections, but these responses are sometimes elicited by harmless antigens, causing disease. Hypersensitivity reactions are exaggerated immune and inflammatory responses triggered by interaction with foreign antigen, then termed allergen (Abbas et al., 2022f, 2022g; Murphy et al., 2022g). Hypersensitivity reactions were originally classified into four different types, Type I to IV based on sensitisation and effector phase (Gell & Coombs, 1968). Recent advances in disease characterization has led to extension into nine different classes; type I-III are antibody driven, type IVa-c cell-mediated, type V-VI tissue-driven mechanisms and type VII are direct responses to chemicals (Jutel et al., 2023), summarized in Table 1.

The most common immune disorder is type I hypersensitivity or IgE-mediated allergy. It is a major health problem affecting up to 30% of the human population worldwide and decreases quality of life (Valenta et al., 2018). The main allergic diseases are allergic rhinitis, allergic asthma, food allergy, atopic eczema or dermatitis and anaphylaxis (Palomares et al., 2017). The most severe allergic reaction is anaphylaxis which is potentially fatal systematic reaction which can be caused by allergens in food, insect venoms and drugs (Galli et al., 2008; Valenta et al., 2018). Type II hypersensitivity reactions or cytotoxic-mediated responses are mediated through IgG and IgM antibodies against cell surface and extracellular matrix proteins. Type II reactions may be subcategorized based on three different mechanisms of cellular destruction, functional loss, or tissue damage; Type IIa through opsonization and phagocytosis with complement system activation; Type IIb through neutrophilic inflammation mediated through the complement system and Fc receptor; Type IIc through antibody dependent cellular cytotoxicity. Immune cytopenia, Graves disease and chronic idiopathic urticaria are among type II hypersensitivity diseases (Dispenza, 2019; Joshi & Khan, 2021). Type III hypersensitivity reactions or immune complex-mediated reactions occur through the formation of antigen-antibody aggregates, called immune complexes, which can precipitate in various tissues leading to activation of the complement system. Common sites are small arteries, renal glomeruli and synovial capsules of joints. Activation of the complement system leads to recruitment of inflammatory cells causing tissue damage (Dispenza, 2019; Jutel et al., 2023). Serum sickness, drug induced lupus and vasculitis are among the most common type III allergic diseases (Dispenza, 2019). Type IV hypersensitivity or delayed reaction mediated through T cells and can be divided into four subtypes based on the T cells involved and immune mechanism. Type IVa hypersensitivity reaction is mediated by Th1 responses, activation of macrophages secreting cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α. Common diseases of type IVa are type I diabetes and contact dermatitis along with type

IVc hypersensitivity reaction. Type IVb reactions are mediated by Th2 responses with production of interleukin (IL)-4, IL-5 and IL-13 inducing eosinophilic inflammation and IgE production. Type IVb reactions are involved in persistent asthma and allergic rhinitis (Dispenza, 2019; Jutel et al., 2023).

Table 1. Overview of the hypersensitivity reaction classification by Jutel et al., 2023

Hypersensitivity reaction			**Effector cells**	**Example of diseases**
Inflammation – Immune system driven	Antibody-mediated	**Type I Immediate**	B cells: IgE Th2, ILC2 (IL-4, IL-5, IL-9, IL-13) Mast cells, basophils	AR, ARC, asthma, AD, acute urticaria, angioedema, food allergy, venom allergy, drug allergy
		Type II Cytotoxic	B cells: IgM, IgG Neutrophils, macrophages C-dependent cytotoxicity NK cells	Drug-induced cytopenia
		Type III Immune complexes	B cells: IgM, IgG Immune complexes Complement, basophils, mast cells, platelets Neutrophils, macrophages and monocytes	Acute phase of hypersensitivity pneumonitis, drug-induces vasculitis, serum sickness/ Arthus reaction
	Cell-mediated	**Type IVa Th1 responses**	Th1, ILC1, CTL1, NK (IFN-γ, TNF-α) Macrophages	Allergic contact dermatitis, acute phase of hypersensitivity pneumonitis, celiac disease, asthma, AR, ARC, chronic rhinosinusitis, AD, drug allergy
		Type IVb Th2 responses	Th2, ILC2, CTL2, NK-T (IL-4, IL-5, IL-9, IL-13, IL-31) Eosinophil, B cells, mast cells, basophils	Asthma, AR, chronic rhinosinusitis, AD, eosinophilic oesophagitis, food allergy, drug allergy
		Type IVc Th3 responses	Th17, ILC3, CTL17 (IL-17, IL-22, IL-23) Neutrophils	Neutrophilic asthma, AD, drug allergy
Tissue-driven mechanisms		**Type V Epithelial**	Epithelial barrier defect, leaky junctions, resident cells changes, immune modulation (alarmins: TSLP, IL-25, IL-33), epigenetic impact	Asthma, AR, ARC, chronic rhinosinusitis, AD, food protein-induced enterocolitis syndrome, eosinophilic oesophagitis, celiac disease
		Type VI Metabolic	Metabolic-induced immune dysregulation	Obesity & asthma, histamine-driven disorders
Direct response to chemicals		**Type VII**	Direct cellular and inflammatory response to chemical substances	Aspirin-exacerbated respiratory diseases, idiosyncratic reactions

Type IVc reactions are mediated by Th3 responses with production of IL-17 inducing neutrophilic inflammation. In Type V reactions or epithelial barrier defects there is impairment in the function of the epithelial barrier not derived from primary immune dysregulation. Type VI reactions are caused by dysregulation in immune responses induced by metabolic dysregulation like obesity. Type VII reactions are derived from direct cellular and inflammatory response to chemical substances and are for example seen in drug allergy (Jutel et al., 2023).

1.1.1 The skin immune system

The skin is a complex organ maintaining physiological functions and serving as barrier protecting the body from pathogen and environmental compound exposure. The skin consists of three layers, epidermis, dermis and hypodermis, each of which contains immune cells contributing to host defence and tissue homeostasis. The skin immune system, also called cutaneous immune system, consists of different specialized immune cells and molecules strategically positioned to detect and eliminate pathogens encountered via the skin. The epidermis is the outermost layer of the skin where the most dominant cell type is keratinocytes. Keratinocytes play important role in the immune defence of the body expressing range of PRRs identifying microbial elements (i.e. PAMPs) and tissue damage (i.e. DAMPs) such as TLRs, Rig-like receptors (RLRs), NOD-like receptors (NLRs) and inflammasomes. Cell surface endosomal PAMPs are recognized by TLRs and cytosolic PAMPs by RLRs. Engagement through PRRs leads to production of secondary mediators (i.e. cytokines, chemokines and growth factors) initiating immune responses. Keratinocytes produce IL-1β and TNF to initiate Th1 and Th3 responses upon infection by intracellular pathogens like bacteria and viruses with recruitment of neutrophils and activation of macrophages. Keratinocytes trigger Th2 responses upon encounter of extracellular pathogens, toxins and tissue damage via production of thymic stromal lymphopoietin (TSLP), IL-33 and IL-25. A unique population of APCs, the Langerhans cells, are present in the epidermis, screening the epidermal microenvironment. The immune cells found in the underlying dermis are several distinct DCs and T cell subsets, macrophages, mast cells, basophils and eosinophils. Dysregulation of the immunological mechanisms against infectious agents in the skin leads to inflammatory skin diseases (reviewed in Kobayashi et al., 2019). Skin diseases are among the most prevalent disorders. Immune responses contribute to pathogenesis of many skin diseases which are mediated by T-cells, humoral responses and in some the inflammation is unspecific and uncontrolled. Th-dependent diseases are characterized based on the type of T cells mediating the response, for example Th1-dominant in vitiligo, Th2-dominant in acute atopic dermatitis (AD), Th17/Th22-dominant in psoriasis and Treg-dominant in melanoma (Sabat et al., 2019).

1.1.1.1 Itch

Itch or pruritus is one of the most common symptoms reported in skin diseases and may be described as a sensation which triggers the urge to scratch the skin. Itch sensation is mostly transmitted by unmyelinated type C and thinly myelinated type Aδ nerve fibres in the skin, spinal cord and brain through interaction between the immune and neural systems. Two families of receptors are involved in itch sensation, G-protein-coupled receptors and transient receptor potential (TRP) channels. The pathogenesis of itch can be classified into histaminergic and nonhistaminergic pathways. Histaminergic itch is activated by histamine mainly secreted by mast cells, basophils and keratinocytes while nonhistaminergic itch is activated by other pruritogens. Effector cells releasing various pruritogens include mast cells, granulocytes, macrophages, lymphocytes, keratinocytes and neurons. The most studied pruritogenic cytokines are the Th2 cytokines, IL-4, IL-13 and IL-31. TSLP and IL-33 released by keratinocytes are also important in itch by promoting Th2 responses. Other cytokines have also been associated with itch as IL-17 and IL-23 in psoriasis pruritus and IL-2 and IL-31 in uraemic pruritus (reviewed in Sutaria et al., 2022).

1.1.2 The immune system of the horse

In general, the immune response of horses is mediated through the same cells, molecules and mechanisms as in other mammals (Felippe, 2016). Equine immunoglobulins (Ig) have been studied for many years as hyperimmune sera has been important in serum therapy and prophylaxis for various diseases in humans and animals. Equine Ig has also been used to support immunosuppression and regulate autoimmune diseases in humans (reviewed in Walther, Rusitzka et al. 2015). As in humans and mice there are five major immunoglobulin isotypes, IgM, IgD, IgA, IgE and IgG. The immunoglobulin isotype responsible for the primary immune response and activation of the complement system is IgM and the first isotype produced in the fetus (Schrenzel et al., 1997; Tallmadge et al., 2009). IgA has neutralizing activity and is essential in mucosal immunity (Lewis et al., 2010), it is a major immunoglobulin isotype in nasal secretions and saliva but minor in serum and colostrum (Schnabel et al., 2017). Presence of IgD has been confirmed in horses but its basic immune function remains unclear (Perkins & Wagner, 2015; Wagner et al., 2004). IgE is found soluble in equine serum and is associated with parasite immunity (Wagner, 2009b) and the parasitic burden is relatively high in horses (Hamza et al., 2010). Total concentration of soluble IgE in equine serum can be up to 1000-fold higher than in human serum (Wagner, 2009b). IgE has been shown to be responsible for allergic responses is in horses (Wagner et al., 2006c). The major immunoglobulin in horse sera and colostrum is IgG, contributing up to 75% of antibodies. Transfer of Igs to fetus is hindered by epitheliochorial placentation of the mare, therefore soon after birth the foal needs to absorb Igs, cytokines and maternal cells from the colostrum (reviewed in Perkins & Wagner, 2015). The function of IgG is variable such as antigen binding, complement

fixation and binding various cells as phagocytic cells, lymphocytes, platelets, mast cells and basophils (reviewed in Walther, Rusitzka et al. 2015).

Four IgG subclasses were found, IgGa, IgGb, IgGc and IgG(T) based on their allergenic differences and serological and electrophoretic properties (reviewed in Wagner 2006). However, with complete mapping and nucleotide sequencing of Ig heavy-chain constant (IGHC) gene region of the horse was shown that horses have seven IgG subclasses termed IgG1 – IgG7 (Wagner et al., 2004) and monoclonal antibodies have been produced against them all (Keggan et al., 2013). The old IgG subclasses have been linked to the new nomenclature as follows: IgGa is IgG1, IgGb is IgG4 and IgG7, IgGc is IgG6 and IgG(T) is IgG3 and IgG5 (Wagner, 2006a). The evolutional events leading to seven distinct equine IGHG genes may have been driven by the need to adapt to environmental conditions and pathogens (Wagner, 2006a). The seven IgG subclasses have different effector function. IgG1, IgG3, IgG4 and IgG7 are the equine immunoglobulins leading to activation of the complement pathway (Lewis et al., 2008) and the immunoglobulins important in protection against viral and bacterial infections (Goodman et al., 2012; Svansson et al., 2009). The genes encoding IgG4 and IgG7 share high homology of 96% which is unusual for IGHC genes of the horse indicating that these two genes duplicated recently during the evolution (Wagner et al., 2004). The most abundant antibody in horse serum is the IgG4/7, however rarely found in clinically healthy horses (Keggan et al., 2013). IgG4/7 is important for latent infections and secondary immune responses IgG1 is primarily produced in primary infections and is rather short lived (Goodman et al., 2012; Svansson et al., 2009; Wagner et al., 2006c). IgG3/5 is important against extracellular pathogens (Dowdall et al., 2002) and IgG5 has been linked to allergy in horses (Wagner et al., 2006c).

1.2 Type I hypersensitivity – IgE-mediated allergy

IgE-mediated allergy is associated with Th2 immune responses where main effector cells are Th2 cells, ILC2, B-cells, small fraction of IL-4 secreting NK cells, IL-4 secreting NK-T cells, basophils, eosinophils and mast cells. The mechanism of the disease (Figure 2) can be divided into two phases, (I) sensitisation and (II) effector phase. Allergens trigger the immune systems through different routes, for food allergens in the oral and gastrointestinal mucosa, aeroallergens in the nasal and airway mucosa and for contact allergens in the skin (Palomares et al., 2014). Impairment of epithelial barriers allows the entrance of allergens through the tissue where submucosal DCs take up the allergen. Stimulated epithelial cells contribute to Th2 polarization and sensitisation by secreting IL-25, IL-33, IL-31 and TSLP which act as alarmins activating DCs and ILC2. Following activation and allergen uptake, mature DCs migrate to the draining lymph node while processing allergen peptide and present through MHC class II molecules to naïve T-cells. During the sensitization phase there is polarization of naïve T cells towards Th2 cells resulting in production of Th2 cytokines such as IL-4 and IL-13. The Th2

cytokine environment induces the class-switching of naïve B-cells to ε immunoglobulin heavy chain and production of allergen-specific IgE. Mast cells and basophils are sensitized as IgE binds to the high-affinity IgE receptor (FcεRI) expressed on the surface of these cells. Other IL-4 producing cells such as basophils and NKT cells could also contribute to sensitization as an early source of IL-4. The immediate effector phase occurs within minutes of allergen re-encounter. Crosslinking of the IgE-FcεRI complexes by allergen activates mast cells and basophils leading to degranulation with release of vasoactive amines (mostly histamine), lipid mediators (prostaglandins and cysteinyl leukotrienes), chemokines and other cytokines causing acute inflammation. Late phase reaction occurs in the tissue 2-6 hours after allergen exposure and peaks after 6-9 hours. Memory Th2 cells and activated ILC2 in cooperation produce IL-4, IL-5, IL-9, IL-13 and IL-31, maintaining allergen-specific IgE levels and causing infiltration of eosinophils and inflammatory cells. The late phase response and chronicity of inflammation is result of activation, migration and prolonged life span of eosinophils including type IVb cell-mediated responses (Figure 3) (reviewed in Larché et al., 2006; Galli et al., 2008; Palomares et al., 2017; Akdis et al., 2020; Jutel et al., 2023).

The cytokines promoting Th2 responses have different roles in the induction and maintenance of allergic responses. TSLP has multiple functions and is involved in various diseases. In allergy the main responsibility of TSLP is stimulation of naïve T cells and DCs to induce Th2 responses and formation of memory Th2 cells. TSLP directly affects eosinophils, mast cells and macrophages by promoting release of Th2 cytokines and chemokines but the importance in basophil reactions remains unclear (Ebina-Shibuya & Leonard, 2023). IL-5 is the main cytokine promoting maturation and recruitment of eosinophils. Th2 and Th9 cells are the main source of IL-9, but it has also been shown to be produced by ILC2s and some populations of mast cells. IL-9 induces recruitment of eosinophils and mast cells and stimulates production of mucus by epithelial cells (Angkasekwinai & Dong, 2021). Both IL-4 and IL-13 have been shown to affect the epithelial barrier by decreasing expression of structural proteins, lipid composition and disrupting tight junctions. Recent studies have indicated that IL-13 might be more important mediator of Th2 responses in the skin and IL-4 responses centralized in the lymph nodes contributing to the generation and regulation of humoral responses (Bieber, 2020). IL-31 activates peripheral nerve endings leading to itch signal to the central nervous system and therefor acting as pruritic sensitizer or activator. IL-31 also contributes to allergic reaction by activation of mast cells, macrophages, basophils and eosinophils (Datsi et al., 2021).

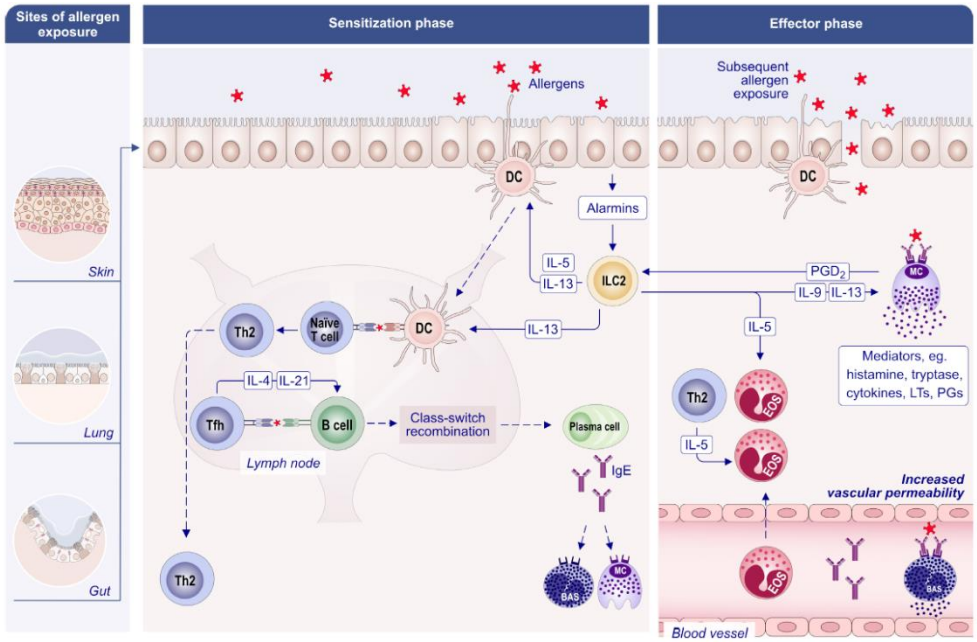


Figure 2. Mechanisms of type I hypersensitivity in allergic rhinitis (AR), allergic rhinoconjunctivitis (ARC), asthma, atopic dermatitis (AD), acute urticaria/angioedema, food, venom and drug allergy. The allergen is deposited on the epithelial cells, in the respiratory tract, gut or skin. The sensitization phase occurs after the first contact with the allergen, APCs, for example, DCs, present the antigen to the naïve Th cells. ILC2 are activated by cytokines released by epithelial cells (called alarmins), such as IL-25, IL-33 and TSLP. Upon activation, they produce large amounts of type 2 cytokines, including IL-5, IL-9 and IL-13, further supporting the Th2-cell response. Tfh help B cells to maturate and produce high-affinity sIgE. Mast cells (MC) and basophils (BAS) possess the high affinity receptor for the Fc fragment of sIgE (FcεRI) and are coated with sIgE, thus concluding the sensitization phase. The effector phase occurs upon subsequent exposure to the same allergen. The allergen crosslinks sIgE bound to MC and BAS, triggering degranulation. MCs are located in various tissues throughout the body, while BAS circulate in the blood. Preformed mediators inside MC and BAS, like histamine, induce symptoms upon release into the microenvironment, like vasodilation, bronchial muscle contraction and increased mucus secretion. Eosinophils play a significant role in the delayed allergic response and the persistence of inflammation, engaging mechanisms related to type IVb hypersensitivity. Therefore, the mutual interaction between type I and IVb-related processes is vital to both the sensitization and the chronic phase. Asthma, AR, ARC and AD endotypes can show Th2-type cytokine overexpression (IL-4, IL-5 and IL-13) and high serum sIgE levels. Food/venom/drug allergy can be induced directly by a trigger with a potentially life-threatening anaphylactic reaction. Acuteurticaria/angioedema can be induced by allergens (e.g. foods, medications, insect bites or stings). The figure and figure text are reprinted from Jutel et al. 2023 with kind permission from John Wiley and Sons.

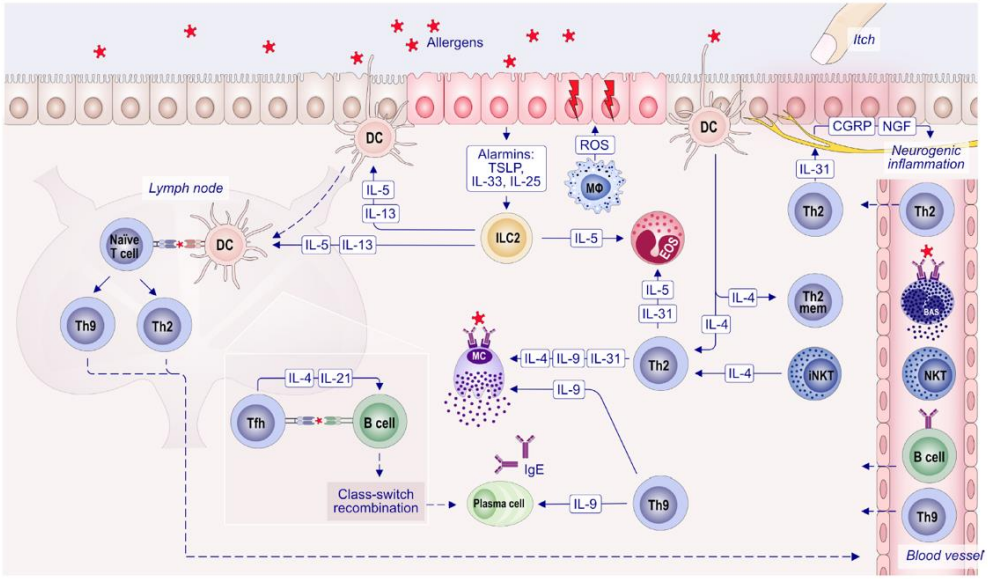


Figure 3. Mechanisms of type IVb hypersensitivity, including allergic rhinitis (AR), atopic dermatitis (AD), chronic rhinosinusitis with nasal polyposis and asthma (Th2 endotype), but also eosinophilic oesophagitis (EoE) and food allergy. In Type IVb hypersensitivity reactions, Th2 cells play a central role, driven by cytokines such as IL-4, IL-13, IL-5, IL-9 and IL-31. These cytokines stimulate B cells to class switch to IgE (IL-4 and IL-13) and mediate eosinophilia (IL-5), causing inflammation and tissue damage. IL-31 mainly produced by Th2 cells, activates IL-31 receptors on sensory neurons, which release calcitonin gene-related peptide (CGRP) and nerve growth factor (NGF) causing neurogenic inflammation and itch. Th9 cells, which differentiate with IL-4 and TGF-β, contribute significantly to this response,enhancing sIgE synthesis and promoting mast cell (MC) growth. The response is further complicated by the ILC2 cells, MC and alternatively activated macrophage (Mφ). ILC2, DC and Th2 cells, activated by IL-25, IL-33 or TSLP, cooperate, producing cytokines and affecting epithelial barriers. They facilitate eosinophil and basophil recruitment and modulate APC function, contributing to the chronicity of Type IVb reactions. (invariant) natural killer T cells (iNK-T) cells contribute to this response by producing IL-4 and IL-13, which induce alternative activation in Mφ and promote inflammation. Eosinophils migrate to inflammatory sites, activate various cytokines and chemokines and release cytotoxic granules contributing to tissue damage, cell death and chronic inflammation. At the final stage when IgE synthesis is triggered, type IVb and I overlap. T2-high asthma is characterized by eosinophilic airway infiltrates and Th2-dependent cytokine overexpression (IL-4, IL-5 and IL-13). In AD, the most common endotype is characterized by high serum IgE levels and a strong association with other allergic diseases such as asthma and AR. Both IgE-dependent and IgE-independent pathways characterize mixed food allergy. Atopic manifestations arising from IgE-independent factors include delayed food-allergy-associated AD (6–48 h post-exposure) caused by the Th2 cells (hypersensitivity type IVb) and eosinophilic gastrointestinal disorders, such as EoE. The oesophageal epithelium is the source of the IL-1 cytokine family members (IL-33, IL-36) and TSLP,involved in the balancing of pro- and anti-inflammatory responses. The figure and figure text are reprinted from Jutel et al. 2023 with kind permission from John Wiley and Sons.

Since allergy was first described in the nineteenth century, prevalence of allergic diseases has increased considerably and is reaching epidemic proportions. The prevalence is highest in industrialized countries, exceeding 50% of the population in Europe, Northern America and Australia (reviewed in Akdis, 2021). Onset of allergy is the result of complicated interactions between genetic (Holloway et al., 2010) and environmental factors with interplay of epigenetic mechanisms (Agache et al., 2020; Alashkar Alhamwe et al., 2020). Studies of gene - environment interactions and approaches with Mendelian randomization underline the importance of environmental factors in development of allergy (Celebi Sozener et al., 2022). To explain the steep increase in prevalence of allergy the hygiene hypothesis was proposed in 1989 by Strachan. The hypothesis suggests that early and regular exposure to harmless microorganisms is associated with protection against allergy. This protective effect has been compromised with excessive hygiene, sanitation and limited contact with microorganisms. The 'Old friends' hypothesis is an extension to the hygiene hypothesis proposing that mammals co-evolved with array of microbial species and parasites which needed to be tolerated. Loss of exposure to these organisms due to modern lifestyle and hygiene results in imbalanced immunoregulation and therefore allergic diseases. Another extension of the hygiene hypothesis is the biodiversity hypothesis stating that healthy microbiota is essential in maintaining tolerogenic immune status. It proposes that allergy is associated with reduction of biodiversity or dysbiosis which is imbalance in bacterial composition and changes in bacterial distribution and metabolic activities (Akdis, 2021; Pfefferle et al., 2021). The epithelium hypothesis focuses on the role of the epithelial barrier in immune regulation and development of allergy. Disruption of the epithelia barrier leads to increased permeability and loss in integrity, allowing the entry of allergens and microbial species. The epithelial barrier hypothesis suggests that increase in epithelial-disrupting substances associated with industrialization, urbanization and modern lifestyle correlates with increased onset of allergy (Akdis, 2021; Celebi Sozener et al., 2022).

1.2.1 Allergens

Allergens are ubiquitous, harmless environmental antigens causing allergic reactions in predisposed individuals (Pekar et al., 2018; Scheurer et al., 2015). According to the World Health Organization (WHO) and International Union of Immunological Societies (IUIS), an antigen is classified as an allergen when it causes IgE antibody response in at least five individuals (Breiteneder H, 2014; Traidl-Hoffmann et al., 2009). A protein is termed as major allergen when more than 50% of allergic patients show a positive reaction with the corresponding allergen-specific IgE in the given test system (Breiteneder H, 2014; Larsen & Lowenstein, 1996). A systematic nomenclature for allergens has been established and is overseen by the Allergen Nomenclature Sub-Committee of WHO and IUIS which maintains a database of approved allergen names (http://www.allergen.org). Allergens are named using the first three letters of the genus, one letter form the originating species followed by Arabic numeral usually

according to the order in which identified (Pomés et al., 2018). The allergens listed in the database are allergens reported to cause reaction in humans, but allergy is also known in other mammals. Allergens in veterinary medicine have been identified but are not included in the allergen database (Jensen-Jarolim et al., 2015; Mueller et al., 2016).

Great progress has been made in defining the intrinsic properties of allergens affecting allergenicity but the knowledge remains limited (Soh et al., 2023). Certain structural characteristics have been described along with other factors contributing to allergenicity such as the route and level of exposure (Scheurer et al., 2015). However, external and internal factors of patients leading to dysregulation of immune responses against allergens which would normally be harmless in healthy individuals are significant (Palomares et al., 2014). Most allergens are proteins or glycoproteins derived from animals, plants or fungi (Traidl-Hoffmann et al., 2009). Comparison of major protein allergens show that most of them are relatively small (5-100 kDa), water soluble, negatively charged and with high stability (Chapman et al., 2007; Scheurer et al., 2015). Allergenicity of allergens is affected by post-translational modifications (PTM) such as glycosylation or disulfide bonds which affect solubility, stability and size of proteins (Aalberse, 2000). However, there are no structural features which can be used to distinguish between allergenic and non-allergenic proteins (Aalberse, 2000; Chapman et al., 2007; Pekar et al., 2018). Route of allergen exposure has a strong effect on required characteristics of allergens. Allergens entering via the skin must show hydrophobic properties, inhalant allergens need to be soluble in the damp milieu of mucosal surfaces and be of particular size to get away from mucus binding properties and allergens entering via the gastrointestinal tract require proteolytic and hydrolytic resistance (reviewed in Pekar et al 2018).

By analysing specific physicochemical properties of food plant allergens, a certain pattern has been identified to affect allergenicity in allergens belonging to the same protein family although exceptions can be observed. These factors include heat stability, resistance to proteolysis and structural stability (Costa et al., 2022a). Glycosylation and phosphorylation are common PTM of food animal allergens that are important for allergenicity. Contradictorily, both structural stability and structural loss revealing hidden epitopes can have similar effects on allergenicity (Costa et al., 2022b). Both studies showed that there are important gaps in the literature and knowledge of physicochemical properties of allergens affecting allergenicity. The comparability of data is not sufficient and there is need for comparison of these properties both in allergens and non-allergens of the same family and even same type (Costa et al., 2022a; Costa et al., 2022b).

Protein allergens can be divided into two classes, whereas type I represent proteases able to promote Th2 responses and type II allergens are non-proteases capable of inducing Th2 responses after disrupting the epithelial barrier by various mechanisms. As an example of non-protease allergens, Der p 2 tiggers TLR4 signalling, even in the

absence of lipopolysaccharide (LPS) by simulating units of the TLR4 signalling complex. Pollen and food allergens have been shown to transport through the epithelial barrier by interaction with lipid rafts and induce Th2-promoting cytokines (Palomares et al., 2014). There are 1089 allergen entries on the WHO/IUIS allergen database, of which 57 are classified as proteases or peptidases. Allergens with proteolytic activity can overcome host tolerance by altering the integrity of the epithelial barrier and antiprotease defences thereby inducing pro-Th2 innate immune signalling pathways. Protease allergens act as allergic adjuvants for enzymatically inactive proteins or otherwise non allergenic proteins, contributing to making them allergenic. Therefore, protease allergens have been linked to development of allergic responses by being initiator allergens (Soh et al., 2023). In a study on IgE-mediated reaction to cockroaches, mice were sensitized and challenged with ovalbumin alone or in co-administration with active or inactive serine protease allergen (Per a 10) from *Periplaneta americana*. The exposure to ovalbumin together with active Per a 10 resulted in significant increase of infiltrated cells and production of ovalbumin-specific IgE and IgG1 as compared to exposure of ovalbumin alone or with inactive Per a 10 (Sudha et al., 2009). Various molecular mechanisms elicited by protease allergens have been recognized which trigger allergic responses. Proteolytic cleavage of fibrinogen or thrombin, proteins playing role in coagulation and blood clotting, induce allergic responses through TLR4 activation. Few serine proteases have been shown to directly cause protease-activated receptor 2 (PAR2) activation, subsequently activation of the *TSLP* gene and thereby Th2 reactions (reviewed in Soh et al., 2023). PAR2 activation has been shown to be important in the sensitization phase for various allergens (Asaduzzaman et al., 2015; Davidson et al., 2013; Gao, 2012). Recent study using a mouse model of atopic dermatitis shows PAR2 to mediate itch through transient receptor potential cation channel, subfamily V, member 3 (TRPV3) in keratinocytes (Zhao et al., 2020). Secretion of the alarmin IL-33 is induced by protease allergens but not limited by them. Protease-dependent IL-33 secretion is important for ILC2 recruitment and activation. Another mechanism of protease allergens is direct activation of nociceptive neurons with transient receptor potential vanilloid-type 1 $(TRPV1)^+$ neurons stimulating Th2-skewing DCs migration (Soh et al., 2023).

1.2.2 Insect allergy

Allergic reactions to biting and stinging insects are well known in both humans and animals (Arlian, 2002; Mueller et al., 2016). Biting insects inject venom using their mouthparts which has evolved for handling pray (Vetter & Visscher, 1998). Their saliva contains substances which work against host barriers such as haemostasis, inflammation and immunity for enabling blood-feeding (Ribeiro & Francischetti, 2003). Common biting insects are bedbugs, ants, chiggers, fleas, flies, mosquitoes and ticks (Lee et al., 2016). Stinging insects inject venom with their stingers which have evolved for defence and therefore cause immediate pain in the host (Vetter & Visscher, 1998). Common stinging insects introducing venom into host include ants, bees and wasps (Sahiner &

Durham, 2019). Insect bites and stings are common and most people show clinically mild local reactions but serious systematic reactions and potentially life-threatening anaphylactic reactions are known especially against Hymenoptera insect stings (Lee et al., 2016; Sahiner & Durham, 2019).

There are over 15.000 species of biting insects which evolved to feed on warm blood from vertebrate animals (Cantillo et al., 2014). In biting insects most allergens originate in the salivary glands and in a study where salivary gland ducts in mosquitoes were cut, they could still feed and produce eggs without causing reactions in humans (Hudson et al., 1960). However, mosquito allergens have also been shown to originate from their body and induce respiratory symptoms through inhalation (Bemanian et al., 2012). Mosquito salivary and body allergens have shown to have different biological activities (Peng & Simons, 1996). There are more than 3000 mosquito species distributed worldwide and mosquito allergy is highly prevalent and variable depending on geographical region. Climate change has affected the geographical localization of mosquito species where some have invaded areas where they were previously not found and others have been displaced affecting the accuracy of mosquito allergy prevalence (Cantillo & Puerta, 2021). According to the allergen database (http://www.allergen.org), allergens from four different mosquito species have been identified, most of them from *Aedes aegypti* (Yellow fever mosquito). Saliva and salivary gland extracts from 10 different mosquitos were compared using immunoblot with pooled sera from human allergic patients. Results showed these 10 species to have 3 to 16 salivary allergens with some species-specific allergens, but some allergens conserved between different species (Peng et al., 1998). The major allergens of mosquitoes are of apyrase, D7 protein and α-glucosidase protein families (Cantillo & Puerta, 2021). Mosquito allergens have been shown to cross-react with allergens from other biting insects and even stinging insects (Peng & Simons, 2004). Local and systematic reactions against bites of blackflies (*Simulium* spp.) in humans have been reported (Hempolchom et al., 2019; Sitarz et al., 2021; Sitarz et al., 2022). Nine allergens from *Simulium nigrogilvum* have been identified. Four allergens, antigen 5-related protein, salivary serine protease, erythema protein and hypothetical secreted protein, bound IgE in 100% of tested human sera and three allergens, salivary serine protease, salivary D7 secreted protein, hypothetical protein, bound IgE in over 50% tested sera (Hempolchom et al., 2019). Four allergens from *Simulium vittatum* relevant to horses have been isolated: antigen 5-like protein (Sim v 1), serine protease inhibitor (Sim v 2) and two α-amylases (Sim v 3 and Sim v 4). Horses suffering from insect bite hypersensitivity (IBH) had significantly higher IgE levels than controls against the four allergens and *S. vittatum* extract, where combined 92% IBH-affected horses reacted with one or more of the four r-Sim v allergens (Schaffartzik et al., 2009). However, in equine insect bite hypersensitivity (IBH) sensitization to *S. vittatum* has been shown to be secondary to sensitization to *Culicoides nubeculosus* (Torsteinsdottir et al., 2018). When comparing antigen 5-like protein from *C. nubeculosus* (Cul n 1) and *S. vittatum* (Sim v 1) there is about 48% sequencing homology. Full cross-reactivity has been demonstrated between these two allergens which share common IgE binding epitopes

(Schaffartzik et al., 2010) and over 50% of IBH-affected horses which react with *Culicoides* extract also react with *S. vittatum* extract (Baselgia et al., 2006). IBH and *Culicoides* allergens will be further discussed in chapters 1.3 and 1.3.8, respectively.

Hymenoptera insect stings are common with a prevalence of being stung during life ranging from 56.6 to 94.5% in adults. Members of the Hymenoptera insect group include bees and vespids (Sahiner & Durham, 2019). The venom of these insects is the major allergen source and contains both species-specific allergens and some homologous in different species. Therefore, patients with stinging insect allergy can be sensitive to multiple species of stinging insects due to cross-reactivity of exposure to venoms of multiple insects (King & Spangfort, 2000). The most probable cause of cross-reactivity are common epitopes of the homologous allergens present in venom, or the IgE antibodies could be directed against related N-linked carbohydrate structures of the allergens (i.e. cross-reactive carbohydrate determinants (CCDs)) (Brehler et al., 2013; Spillner et al., 2014). The nature of stinging and venom injection is different between species, bees inject 50-140 μg of venom and wasps about 3 μg but bees can only sting once and wasps have the capacity of stinging multiple times (Fitzgerald & Flood, 2006; Sahiner & Durham, 2019). Twelve allergens have been identified from honey bees (*Apis mellifera*) according to the allergen database (http://www.allergen.org) and the most prominent and high abundance allergens are phospholipase A_2, hyaluronidase and melittin (Hoffman, 2006; Spillner et al., 2014). According to the allergen database (http://www.allergen.org) five allergens have been identified from yellow jackets (*Vespula vulgaris*) venom where the most prominent allergens are phospholipase A_1, hyaluronidase and antigen 5 (Hoffman, 2006; Spillner et al., 2014). These two sets of prominent allergens in honey bees and yellow jackets are found throughout most Hymenoptera species with modifications (Spillner et al., 2014). Cross-reactivity between the hyaluronidase in bee venom (Api m 2) and vespid venom (Ves v 2) is due to homology but for majority of allergens it is caused by CCDs (Brehler et al., 2013).

1.3 Insect bite hypersensitivity

Insect bite hypersensitivity is a recurrent seasonal IgE-mediated allergy, type I hypersensitivity of horses. Reports of IBH go back to 1840 (Brostrom et al., 1987) in France and 1888 in Australia (Riek, 1953). The disease has been described worldwide under different names such as summer eczema, sweet itch, Queenlands itch, kasen and *Culicoides*- hypersensitivity (CH) (Baker & Quinn, 1978; Brostrom et al., 1987; Fadok & Greiner, 1990; Halldorsdottir & Larsen, 1991; Kurotaki et al., 1994; Riek, 1953). Early hypothesis of the probable causes of IBH led climate conditions, sunlight, grass allergy, nutritional deficiencies, bacteria, fungi, microfilariel and insect bites (Brostrom et al., 1987). In the 1980s it was confirmed that *Culicoides* spp. were the causative agent of IBH (Baker & Quinn, 1978; Fadok & Greiner, 1990; Mellor & McCraig, 1974).

1.3.1 Epidemiology

All horse breeds can be affected by IBH and it has been described worldwide where *Culicoides* midges are indigenous (reviewed in Schaffartzik et al., 2012). The prevalence varies greatly between regions from 3% in Great Britain (Anderson et al., 1996) to 37.7% in Germany (Littlewood, 1998) and 60% in Queensland Australia (Riek, 1953). Prevalence of IBH is affected by environmental factors that might affect the biting midges such as climate and habitat factors and study showed the prevalence of IBH in the Netherlands to vary from 0% to 71.4% depending on regions. A lower prevalence was found in areas based along the coast-line and with more rainfalls, many cold and few warm days per year. In areas with soils of clay and heather and woody vegetation there was increasing prevalence of IBH (van Grevenhof et al., 2007).

The causative *Culicoides* species are not indigenous in Iceland and therefore IBH is not found in the native horse population. However Icelandic horses exported to *Culicoides* infested areas are more often affected compared to other horse breeds. Two years after export more than 50% have developed this allergy if unprotected in heavily infested areas (Bjornsdottir et al., 2006). In Icelandic horses born in infested areas the prevalence is only 6 - 8% (Brostrom et al., 1987; Halldorsdottir & Larsen, 1991; Schaffartzik et al., 2012). The age of exposure to *Culicoides* has been shown to make a difference in the prevalence of IBH as weanlings exported from Iceland to Europe at age 7 to 10 months do not develop IBH more frequently than Icelandic horses born in *Culicoides* infested areas (Sommer-Locher et al., 2012). The high prevalence among Icelandic horses exported to *Culicoides* infested areas is probably due to lack of exposure to the midges in early life (Bjornsdottir et al., 2006).

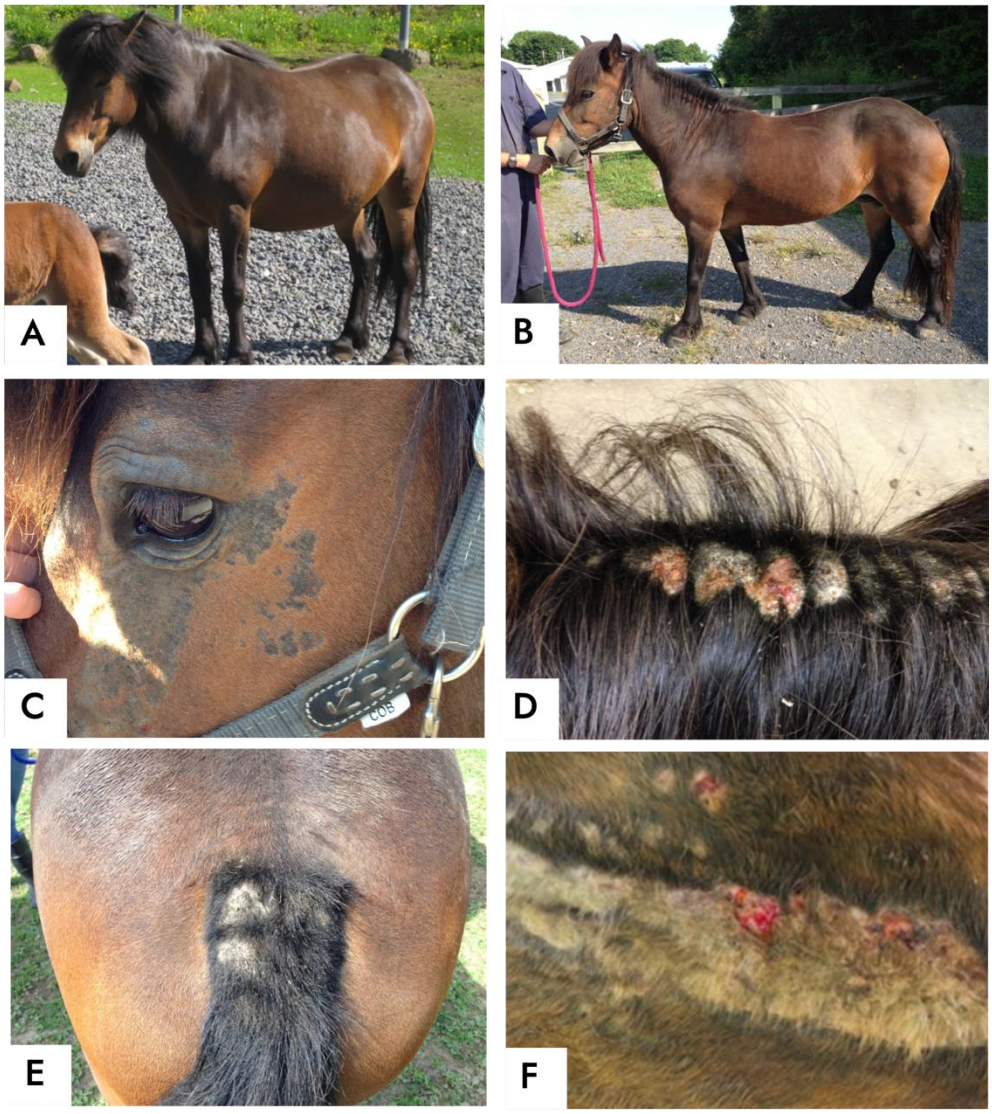


Figure 4. Clinical signs of Insect Bite Hypersensitivity. **A.** Mare in Iceland, before developing clinical signs of IBH. **B.** Same mare after developing clinical signs of IBH following exportation to *Culicoides* infested area. **C.** Face, **D.** Mane, **E.** Tale, **F.** Belly. Photo **A.** Sigurbjörg Þorsteinsdóttir, **B.-F.** Bettina Wagner.

1.3.2 Clinical signs

The clinical signs of IBH (Figure 4) are characterized by papules and severe pruritus that leads to localized hair loss, thickening of the skin (hyperkeratosis) and excoriation that can induce secondary infections (Baker & Quinn, 1978; Brostrom et al., 1987; Riek, 1953). Lesions caused by IBH are mostly localized at preferred feeding sites of the biting midges typically along the dorsal and ventral bodyline, the base of the mane, neck and tail, also the face, ears and withers (Brostrom et al., 1987; Schaffartzik et al., 2012; Townley et al., 1984). Clinical signs of IBH are seasonal and appear from spring to autumn when *Culicoides* are active and disappear in the absence of exposure during winter, however clinical signs can persist in the winter in severe chronic cases (Schaffartzik et al., 2012; Wilson et al., 2001).

1.3.3 Pathogenesis

1.3.3.1 B cell responses

IBH is type I, IgE-mediated hypersensitivity (Figure 5) caused by allergens of biting midges (*Culicoides* spp.). Some studies have shown delayed reactions, but it is not thought to be the major reaction in IBH (Zeller et al., 2009). Involvement of IgE in IBH has been confirmed with Prausnitz-Krüstner experiment by transferring IgE purified from serum of IBH-affected horse to the skin of healthy horse resulting in the induction of type I allergic reaction upon allergen challenge at the site of the IgE injection (Wagner et al., 2006c). Using western blot analysis, it has been shown that both healthy and allergic horses have serum IgG antibodies against *Culicoides* allergens but IgE antibodies against *Culicoides* allergens were only detected in allergic horses (Wilson et al., 2001). Furthermore, not only specific IgE antibody levels but also IgG3/5 and IgG1 against *Culicoides* allergens have been shown to be significantly higher in sera of IBH-affected horses compared to healthy horses (Hellberg et al., 2006). In skin lesions of IBH-affected horses, significantly more IgE-protein bearing cells and IgE-mRNA positive cell have been detected compared to skin biopsies of healthy horses (van der Haegen et al., 2001). Recently, IgE-secreting plasmablasts were identified and their proportion and secretion of IgE was shown to be significantly higher in IBH-affected horses with clinical allergy compared to healthy horses. The severity of the clinical signs of allergy and IgE concentrations correlated with the proportion of circulating IgE^+ plasmablasts (Simonin et al., 2022).

IgG5 antibodies have been shown to be associated with IBH (Wagner et al., 2006c). Studies have even shown that Cul o 1, Cul o 2 and Cul o 3 specific IgG3 and IgG5 antibodies are present in the circulation of IBH-affected horses before developing clinical signs (Raza et al., 2020; Ziegler et al., 2018). During the first season of *Culicoides* exposure in Icelandic horses exported to the Northeastern United States, Cul o 2 specific IgG3/5 antibody levels were significantly higher in horses who later

developed IBH compared to horses which did not develop the disease (Raza et al., 2020). Also, during and after the *Culicoides* season, IgG5 antibodies against Cul o 2 and IgG3/5 against Cul o 3 were significantly higher in IBH-affected compared to healthy horses (Raza et al., 2020). In a study on Icelandic born horses exported to Europe, IgG5 specific antibody levels against Cul o 1 and Cul o 3 before clinical signs of IBH were significantly higher in the horses who developed IBH compared to those that were healthy at end-point (Ziegler et al., 2018). These studies indicate that allergen specific IgG3 and IgG5 antibodies might be relevant as predictive biomarker of IBH in horses.

1.3.3.2 T cell responses

The T-cell response in the pathogenesis of IBH has been investigated both in the skin and the circulation showing there is imbalance between Th-cell subsets. Horses born in Iceland and exported to *Culicoides* infested areas as adults (1st generation) were compared to Icelandic horses born in *Culicoides* infested areas (2nd generation). During the *Culicoides* season there was a reduced number of IFN-γ mRNA and IFN-γ producing cells in 1st and 2nd generation IBH-affected horses compared to healthy controls and a significant increase in IL-4 mRNA levels and number of IL-4 producing cells. Healthy 2nd generation horses had increased levels of IL-10 (Hamza et al., 2007). IL-4 production of PBMC in 1st generation IBH-affected horses could be down-regulated using supernatant of PBMC from healthy 2nd generation horses. The same effects were obtained using IL-10 and TGF-β1 in combination but not as single cytokines. This demonstrates the role of IL-10 and TGF-β1 in IL-4 suppression of allergen-specific Th2 cells leading to reduction of IBH incidence (Hamza et al., 2008).

Horses in Iceland have higher burden of endoparasites compared to horses living in Switzerland and higher total IgE concentration. Furthermore, they showed low production of IL-4 and high production of IL-10 and TGF-β1 demonstrating a strong T cell regulation in parasitic infection opposite to what is seen in IBH (Hamza et al., 2010).

Equine FoxP3 is expressed by $CD4^+CD25^+$ T cells, population containing natural Treg cells and functional Treg cells (Hamza et al., 2011). Using flow cytometry, it has been shown that there is no difference in the proportion of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells in IBH-affected and healthy horses and importantly in the proportion of $CD4^+CD25^+FoxP3^+$ T cells. Stimulation of $CD4^+CD25^+$ T cells from healthy horses with *Culicoides* allergens induced more FoxP3 compared to IBH-affected horses, suggesting that allergen-specific-$FoxP3^+$ T cells contribute to regulation of allergen-specific immune responses in healthy horses. When adding rIL-4 to PBMC stimulated with *Culicoides* allergen of healthy horses there was a significant decrease in FoxP3 expressing $CD4^+CD25^+$ T cells, suggesting that lower FoxP3 expression in IBH-affected horses is caused by increased IL-4 production by PBMC (Hamza et al., 2012). When stimulating $CD4^+CD25^{high}$ T cells with *Culicoides* allergens, there was significantly lower ability to

suppress proliferation of $CD4^+CD25^-$ T cells in IBH-affected compared to healthy horses which was associated with a higher proportion of $IL\text{-}4^+$ cells and a lower proportion of $FoxP3^+IL\text{-}10^+$ cells (Hamza et al., 2013).

Imbalance of T cell subset has also been observed in the skin of IBH-affected compared to healthy horses. Following intradermal *Culicoides* allergen injection there was a significant increase in IL-4-mRNA expression in skin biopsies of IBH-affected horses, however in healthy horses there was a significant increase in IFN-γ-mRNA expression (Meulenbroeks et al., 2015). Immunohistochemical staining in skin of IBH-affected horses showed significantly higher numbers of $CD4^+$ T cells compared to healthy controls but the number of $FoxP3^+$ cells was not different between the groups. In lesional skin of IBH-affected horses the total number of T cells was significantly higher compared to non-lesional skin and furthermore, there was a higher number of $CD4^+$ cells. On mRNA level there was significant reduction of FoxP3-mRNA in lesional skin compared to non-lesional and healthy controls. There was increased expression of IL-13 but not of IL-4 and IL-5 in lesional skin of IBH-affected horses compared to non-lesional skin. In contrast there was decreased expression of IL-10 in lesional skin compared to non-lesional skin of IBH-affected horses. Also, expression of IL-10 in blood was significantly decreased in IBH-affected compared to healthy horses (Heimann et al., 2011). Seasonal changes in cytokine expression in the skin has been observed, where expression of IL-4, IL-13 and IFN-γ-mRNA was significantly increased during the season of *Culicoides* exposure compared to off season in both IBH-affected and healthy horses. This increase in cytokines was shown to be in correlation with upregulation of CD3-mRNA in the skin contributing to increased T cell infiltration during *Culicoides* season (Meulenbroeks et al., 2013).

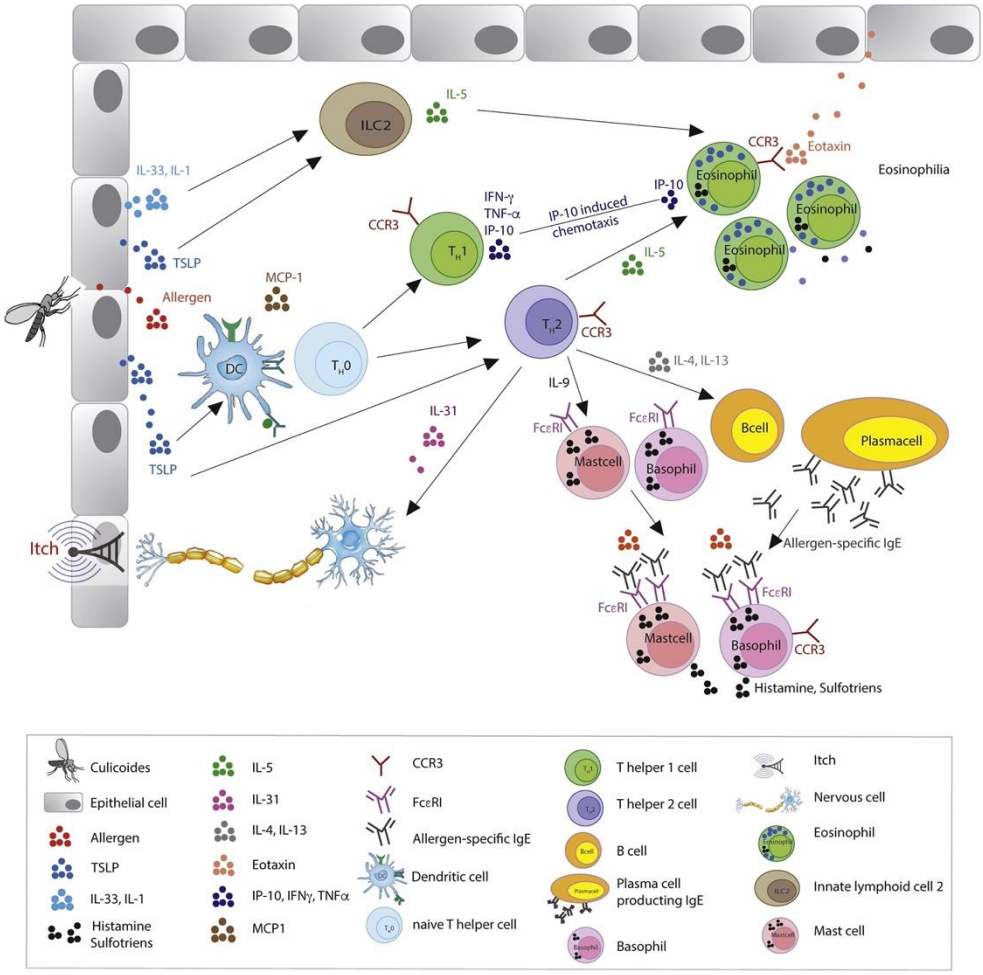


Figure 5. Molecular mechanisms of Culicoides hypersensitivity (CH). Multifactorial and complex interplay of molecular pathways in CH are dominated by Th2 cells, also include innate factors and show some Th1 characteristics. Main effector molecules and mechanisms are type-I mediated histamines, IL-5- derived eosinophilia and IL-31-induced allergic pruritus. The figure and figure text are reprinted from Fettelschoss-Gabriel et al. 2021 (https://creativecommons.org /licenses/by-nc-nd/4.0/).

1.3.3.3 Innate immunity responses

The main allergic inflammatory cell mediators in horses with immediate response upon allergen exposure are mast cells and basophils (Larson & Wagner, 2021b). Mast cells are long-lived cells found in the skin of IBH-affected horses (van der Haegen et al., 2001; Wagner et al., 2009c) and basophils are short-lived cells found in the circulation (Wagner et al., 2008b). Eosinophils have also been described in the lesional skin of IBH-affected horses (Benarafa et al., 2002; McKelvie et al., 1999). These cells have granules containing inflammatory mediators like histamine exocytosed through degranulation upon allergen encounter. They also produce and release leukotrienes and cytokines following IgE cross-linking (Langner et al., 2008; Marti et al., 1999; Wagner et al., 2008b).

It has been demonstrated that peripheral blood basophils play important role in IL-4 secretion in IBH-affected horses (Raza et al., 2021). Following stimulation of PBMC with *Culicoides* extract there was significant increase in IL-4$^+$ cells in IBH-affected compared to healthy horses but a similar proportion of IL-4$^+$ cells was seen in both groups following IgE crosslinking. These IL-4$^+$ cells were characterized as basophils. It was also shown that IBH-affected horses maintain higher proportion of basophils throughout the year than healthy horses. During the *Culicoides* season there was no significant difference in IL-4 production comparing IBH-affected and healthy horses, however during the months with no *Culicoides* exposure there was significantly higher secretion of IL-4 from PBMC of IBH-affected horses (Raza et al., 2021).

Equine IgE-binding monocytes have been characterized and shown to produce IL-10 and IL-8 following IgE cross-linking (Larson et al., 2020, 2021a). Similar levels of circulating IgE- binding monocytes are found with no difference in IL-10 production following IgE cross-linking between healthy and IBH-affected horses (Larson et al., 2020). However, significantly higher proportion of IL-8 producing IgE-binding monocytes following IgE cross-linking are found in IBH-affected horses compared to healthy promoting allergic inflammation (Larson et al., 2021a). Two mechanisms have been suggested to demonstrate IL-8 production in some but not all IgE-binding monocytes; (1) polarization of IgE-binding monocytes to produce either IL-10 and IL-8 depending on local environment of cytokines and chemokines or (2) there might be different subpopulation of IgE-binding monocytes in IBH-affected and healthy horses (Larson et al., 2021a).

Further investigation of the skin shows there is an impairment in the epithelial barrier and the immune signature of IBH-affected horses (Cvitas et al., 2020b). Lesional skin of IBH-affected horses is characterized by a downregulation of genes that are involved with tight junction formation, alterations in keratins and indication that both Th1 and Th2 responses are involved with specific upregulation of *IL13*. Non-lesional skin of IBH-affected horses was similar to the skin of healthy horses (Cvitas et al., 2020b). There is significantly more expression of thymic stromal lymphopoietin (TSLP) in lesional skin of IBH-affected horses compared to healthy horses. The TSLP expression in keratinocytes was significantly upregulated by TLR-1-8-L, strongest by TLR 1/2-L and TLR 3-L with further increase of TSLP expression in combination with Th2 cytokines. IL-6 expression was shown to be significantly increased by TLR-L 1-5 stimulation (Cvitas et al., 2020a). Keratinocytes contribute to innate immune responses and might thereby play role in IBH pathogenesis (Cvitas et al., 2022). Isolated keratinocytes from non-lesional skin of IBH-affected and healthy horses stimulated with *Culicoides* allergens alone showed no transcriptional changes but significant changes in gene expression were observed when stimulating additionally with Th2 promoting cytokines. Comparing the transcriptomes of keratinocytes of IBH-affected and healthy horses, they were similar but stimulation with *Culicoides* allergens along with Th2 promoting cytokines resulted in difference in 23 genes and seven genes with TLR-1/2-L stimulation alone (Cvitas et al., 2022).

1.3.4 Genetics

IBH is a multifunctional disease, both environmental and genetic factors play a role in the development and persistence of the disease (Steinman et al., 2003). The frequency of IBH is higher in some horse families than in others (Marti et al., 1992). The heritability varies between different horse breeds and on the liability scale it has shown to be 0.16 in Friesian horses (Schurink et al., 2011), between 0.08 and 0.24 in Dutch Shetland ponies (Schurink et al., 2009), between 0.36 and 0.63 in Old Kladruber horses (Citek et al., 2017) and between 0.27 and 0.33 in Swedish-born Icelandic horses (Eriksson et al., 2008). Genome-wide association (GWA) studies have revealed the polygenic properties of IBH and have proposed several candidate regions on different chromosomes to be linked with disease susceptibility (Schurink et al., 2013; Schurink et al., 2012; Shrestha et al., 2015; Velie et al., 2016). Both breed-specific and common genomic regions associated with IBH have been identified (Schurink et al., 2012). Serological studies on Icelandic horses showed IBH heritability to be associated with certain major histocompatibility complexes (MHC) i.e., equine leukocyte antigen (ELA) class II (Halldorsdottir & Larsen, 1991; Lazary et al., 1994; Marti et al., 1992). The association of ELA and susceptibility to IBH was confirmed using intra-MHC microsatellites and extended to another horse breed, Exmoor ponies (Andersson et al., 2012). ELA was also linked to IBH in GWA study on Shetland ponies and Icelandic horses (Schurink et al., 2012) and in Friesian horses using a single nucleotide polymorphism (SNP) array (Schurink et al., 2018). An inbreeding depression study in Old Kladruber horses showed that increase of *ELA class II* inbreeding was associated with higher prevalence of IBH (Vostry et al., 2021). However, other studies using SNP arrays on Icelandic horses (Shrestha et al., 2015), Exmoor ponies (Velie et al., 2016) and Dutch Shetland ponies (Schurink et al., 2013) showed no relations between ELA and susceptibility to IBH. Non-ELA genes which influence immunity and allergy have also been shown to be associated with IBH in Old Kladruber horse breed (Vychodilova et al., 2013). There is clearly evidence for genetic susceptibility and involvement of the immune system in pathogenesis of IBH but the specific genetic mechanisms are still unknown (Tallmadge et al., 2020).

1.3.5 Diagnosis

Currently IBH is mainly diagnosed by evaluating clinical signs and the history of recurrent symptoms. However, there is no validated clinical scoring system for the quantification of clinical signs (Miller et al., 2019). Various diagnostic tests have been developed using *Culicoides* whole body extract (WBE) like serological tests detecting allergen-specific IgE (Frey et al., 2008). However, commercially available tests have rather low sensitivity and specificity as the allergen content of the *Culicoides* WBE has not been standardized and there is considerable variation between batches (Frey et al., 2008; Morgan et al., 2007). Another factor decreasing the sensitivity is the high concentration of IgGs in serum with allergen-specific-IgGs competing for IgE binding

sites (Morgan et al., 2007). Performance of these tests needs to be improved using high affinity IgE reagents and pure recombinant allergens (Marti et al., 2008; Valenta et al., 1999; van der Meide et al., 2012; van der Meide et al., 2014). WBE has also been used in cellular and functional tests measuring histamine (Wagner et al., 2008b) or sulfidoleukotriene release (Baselgia et al., 2006). Allergen microarray for IBH has been set up using extracts from midges and purified allergens from various *Culicoides* species, expressed in different production systems which allows the investigation of the sensitization profiles of horses to *Culicoides* (Marti et al., 2015; Novotny et al., 2021).

1.3.6 Treatment

Currently, there is no specific and effective treatment for IBH other than avoidance of *Culicoides* midges. Horses are kept in stables around dawn and dusk when the midges are most active or dressed in special blankets with hoods (Figure 6) which enclose the body to keep symptoms mild or moderate (reviewed in Schaffartzik et al. 2012). Application of insect repellents, shampoos, leave-on topical therapies (Fettelschoss-Gabriel et al., 2021), essential oils spray (Cox et al., 2020a) and cremes with ingredients like omega 3/6 fatty acids, emollients and skin moisturizers (Huhmann & Mueller, 2019) have also been recommended for reducing clinical signs. Symptomatic treatment with corticosteroids is still the most efficient therapy for control of clinical signs of IBH. However, aggressive use of corticosteroids may cause severe side-effects such as cortisol suppression, hepatopathy, muscle wasting, altered bone metabolism, hyperglycemia, polyuria, polydipsia, laminitis and increased susceptibility to infection (Leclere, 2017; Yu, 2014). Use of the antihistamine cetirizine was not effective for treatment of IBH (Olsen et al., 2011) but it may have needed to be applied earlier in the disease process (Yu, 2014).

Figure 6. Horse wearing a blanket for protection against *Culicoides* bites. Photos: Sara Björk Stefánsdóttir.

Therapeutic vaccines with long-term reduction of clinical signs using virus-like particles (VLP) either targeting IL-5-induced eosinophilia or IL-31-induced allergic pruritus have been developed and tested (Fettelschoss-Gabriel et al., 2019; Fettelschoss-Gabriel et

al., 2018; Olomski et al., 2020). The vaccines consisted of either equine IL-5 or equine IL-31 covalently linked to a VLP derived from cucumber mosaic virus (CuMV). Following vaccination with eIL-5-VLP there was significant reduction of eosinophil levels in blood of treated horses compared to placebo treated horses. There was also significant reduction in clinical lesion scores compared to the prior season and placebo treated horses. Improvements of clinical scores the first year reaching 50% and 75% was seen in up to 69% and 31% of vaccinated horses, respectively. In the second treatment year, 50% and 75% improvements of clinical signs were reached in 88% and 57% of vaccinated horses respectively. In the first treatment year horses were injected twice resulting in short-lived antibody titers and therefore booster vaccination mid-season was needed. However, following three vaccinations, only one booster vaccination prior to IBH season resulted in antibody titer lasting the following IBH season (Fettelschoss-Gabriel et al., 2019). The IL-5-VLP vaccine was shown to induce strong reversible B-cell responses without induction of IL-5 specific T-cell responses, showing the vaccine to be a safe therapeutic treatment against IBH (Jonsdottir et al., 2020). IL-31-VLP vaccinated horses showed significant reduction in clinical signs compared to placebo treated horses (Olomski et al., 2020). Promising therapeutic antibody candidate for treatment against IBH, targeting equine IL-5 has been identified. Using phage display, eleven inhibiting antibody candidates were found and two of those further improved with *in vitro* affinity maturation and the final antibody selected showed a strong inhibition of IL-5 (Langreder et al., 2023).

1.3.7 Culicoides spp.

Culicoides are small biting midges belonging to Ceratopogonidae, of the order Diptera in the insecta class. There are over thousand species with distribution worldwide, except Antarctica, New Zealand (Mellor et al., 2000) and until recently Iceland. In 2015 *C. reconditus* (Icelandic: lúsmý) (Figure 7) was found in Iceland (Olafsson, 2015, 2019) but it is not known to bite horses, only humans.

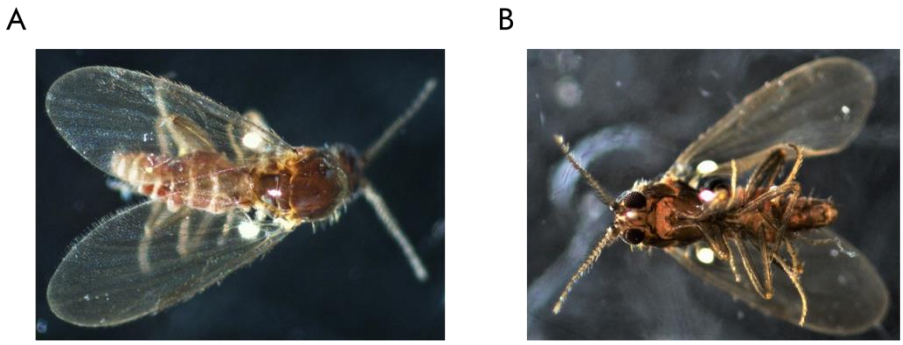


Figure 7. *Culicoides reconditus* caught in Iceland. **A.** dorsal side, **B.** ventral side. Photos: Karl Skírnisson.

Culicoides flies are small with wingspan less than 2 mm and weight about 0,5 µg. They require moist habitat and access to water for their different life cycles including egg,

larval, pupa and adult stages. The lifespan of adult flies is short, 20-30 days. The midges are haematophagous, but only the female flies suck blood which is important for egg production of some species like *C. nubeculosus*. Other species are autogenous like *C. impunctatus* and opportunistic feeding on mammals (Featherstone, 2010; Mordue & Mordue, 2003). Some species have host preferences (Ninio et al., 2011) and some are vectors transmitting pathogens such as bluetongue virus of ruminants and African horse sickness virus of equines (Mellor et al., 2000; Mordue & Mordue, 2003; Sick et al., 2019).

Numerous species have been reported to cause IBH in horses for example *C. obsoletus* (Anderson et al., 1991; van der Meide et al., 2012), *C. sonorensis* (Langner et al., 2009) and *C. nubeculosus* (Hellberg et al., 2009). The geographical distribution of *Culicoides* species varies and it differs between regions which species is the most abundant. *C. obsoletus* is the most abundant species in Switzerland (Cagienard et al., 2006; Casati et al., 2009), Ireland (Townley et al., 1984), England (Wilson et al., 2008), Germany (Vorsprach et al., 2009), Italy, Sweden (Möhlmann et al., 2018) and in the Netherlands (de Raat et al., 2008; Möhlmann et al., 2018; van der Rijt et al., 2008). IBH affected horses in The Netherlands had significantly higher IgE ELISA titers against whole body extract (WBE) from the most abundant species, *C. obsoletus*, than against WBE from *C. nubeculosus* and *C. sonorensis* (van der Meide et al., 2012). Nevertheless, horses with IBH can react to antigens from *Culicoides* species they have not been exposed to before in skin test showing IgE cross-reactivity between species (Anderson et al., 1993).

1.3.8 Culicoides allergens

The allergens causing IBH are originated in the salivary glands of *Culicoides* midges. When the midges bite the host, they secrete proteins and compounds which overcome the host defences, like haemostasis and immune responses and these proteins become allergens in some individuals (Ribeiro & Francischetti, 2003; Russell et al., 2009). Analysis of the salivary secretome using mass spectrometry analysis has been carried out in two *Culicoides* species (Lehiy & Drolet, 2014; Russell et al., 2009). Fifty-four novel protein sequences including possible allergens were revealed the in salivary secretome of *C. nubeculosus,* including the enzymes maltase, hyaluronidase and two serine proteases also some members of the D7 family and protease inhibitors with putative anticoagulant activity (Russell et al., 2009). From *C. sonorensis*, a total of 45 proteins have been identified including members of D7 family, Kunitz-like protease, maltase and trypsin (Lehiy & Drolet, 2014).

Some midge species are more accessible for research as they can be maintained in laboratory bred colonies, like *C. nubeculosus* and *C. sonorensis* (Boorman, 1974; Nunamaker & Lockwood, 2001) but other species like *C. obsoletus* have to be collected from the wild (Boorman, 1985). Allergens have been isolated from three different

species, *C. sonorensis* (Cul s), *C. nubeculosus* (Cul n) and *C. obsoletus* (Cul o) and expressed in *Escherichia coli*, some in insect cells, yeast and barley (Table 2). Some of the allergens share homology between *Culicoides* species (van der Meide et al., 2013). Some *Culicoides* allergens are proteins corresponding to allergens in humans for example Cul o 2 and Cul n 2 are both a hyaluronidase (hya), major allergen in honeybee and vespid venom allergy (Arlian, 2002; Hoffman, 2006; Schaffartzik et al., 2011; van der Meide et al., 2013). Cul s 1 is α-glucosidase (maltase) known in mosquito allergy (Cantillo & Puerta, 2021; Langner et al., 2009). Cul n 9, Cul o 2P, Cul o 3P and Cul o 6 are D7- related odorant binding proteins (OBP) also known in mosquitos (Cantillo & Puerta, 2021; Malafronte Rdos et al., 2003; Peeters et al., 2013; Schaffartzik et al., 2011; van der Meide et al., 2013). Cul n 1, Cul o 3 and Sim v 1, a major allergen in reactions to *S. vittatum* of IBH-affected horses, are antigen-5 like protein, with similarity to antigen 5, a major allergen in vespid venom allergy (Blank et al., 2020; Hoffman, 2006; Schaffartzik et al., 2010; van der Meide et al., 2013).

Table 2. List of isolated *Culicoides* allergens.

***Culicoides* species**	**r-allergen**	**GeneBank accesion nr.**	**a.a.**	**MW (kDa)**	**Protein family**	**Reference**
C. sonorensis	Cul s 1	n.s.	n.s.	68.6	Maltase (α-glucosidase) (AY603565)[a]	Langner et al (2009)
C. nubeculosus	Cul n 1	EU978899	209	25.4	PR1-like (antigen-5 like)	Schaffaritzik et al (2010)
	Cul n 2	HM145950	403	46.7	Hyaluronidase	Schaffaritzik et al (2011)
	Cul n 3	HM145951.1	391	44.6	DUF4803 superfamily	Schaffaritzik et al (2011)
	Cul n 4	HM145952	153	17.5	Unknown salivary protein	Schaffaritzik et al (2011)
	Cul n 5	HM145953	406	45.7	DUF4803 superfamily	Schaffaritzik et al (2011)
	Cul n 6	HM145954	147	16.9	Unknown salivary protein	Schaffaritzik et al (2011)
	Cul n 7	HM145955	186	20.9	Unknown salivary protein	Schaffaritzik et al (2011)
	Cul n 8	HM145956	603	68.7	Maltase (alpha amylase)	Schaffaritzik et al (2011)
	Cul n 9	HM145957	142	15.5	D7-related/OBP	Schaffaritzik et al (2011)
	Cul n 10	HM145958	420	47.8	DUF4803 superfamily	Schaffaritzik et al (2011)
	Cul n 11	HM145959	275	30.1	Serine protease/ trypsin	Schaffaritzik et al (2011)
C. obsoletus	Cul o 1	KC339671	602	66.8	Maltase (alpha amylase)	Van der Meide et al (2013)
	Cul o 1P	JX512273	205	23.3	Kunitz protease inhibitor	Peeters et al (2013)
	Cul o 2 (hya)	KC339672	388	44.8	Hyaluronidase	Van der Meide et al (2013)
	Cul o 2P	JX512274	151	17.5	D7-related/OBP	Peeters et al (2013)
	Cul o 3	KC339673	261	29.9	PR1-like (antigen-5 like)	Van der Meide et al (2013)
	Cul o 3P	JX512275	146	16.9	D7-related/OBP	Novotny et al (2021)
	Cul o 4	KC339674	273	27.1	Serine protease/ trypsin	Van der Meide et al (2013)
	Cul o 5	KC339675	181	20.1	Unknown salivary protein	Van der Meide et al (2013)
	Cul o 6	KC339676	148	15.2	D7-related/OBP	Van der Meide et al (2013)
	Cul o 7	KC339677	159	17.6	Unknown salivary protein	Van der Meide et al (2013)
	Cul o 8	MN123710	168	19.3	Kunitz protease inhibitor	Novotny et al (2021)
	Cul o 9	MN123712	157	17.6	WSC superfamily, carbohydrate-binding domain	Novotny et al (2021)
	Cul o 10	MN123711	635	72.9	DUF4803 superfamily	Novotny et al (2021)
	Cul o 11	MN123713	385	39.2	Apoliophorin III-like	Novotny et al (2021)
	Cul o 12	MN123714	309	35.8	Leucine-rich repeat	Novotny et al (2021)
	Cul o 13	MN123715	152	16.9	D7-related/OBP	Novotny et al (2021)
	Cul o 14	MN123716	275	30.8	Serine protease/ trypsin	Novotny et al (2021)
	Cul o 15	MN123717	553	62.1	Apyrase	Novotny et al (2021)

The first *Culicoides* allergen described and isolated originated from *C. sonorensis* (Cul s 1), a maltase which was expressed in insect cells. Eight IBH-affected horses reacted in skin test to the Cul s 1 and IgE from serum bound to the allergen in 7 of 8 IBH-affected horses (Langner et al., 2009).

Eleven Cul n allergens have been isolated (Cul n 1 – 11) and expressed in *E. coli* and some in insect cells and barley (Jonsdottir et al., 2018; Jonsdottir et al., 2016; Schaffartzik et al., 2011; Stefansdottir, 2015). The ability of the allergens to bind IgE from serum of IBH-affected horses located in Switzerland was shown with western blot analysis and ELISA showed a frequency of IgE sensitization ranging from 13% - 57% against the Cul n r-allergens (Schaffartzik et al., 2010; Schaffartzik et al., 2011).

Eighteen allergens originated in *C. obsoletus* have been isolated (Cul o 1 – 15, Cul o 1P – 3P) and expressed in *E. coli* and some of them also in insect cells and Pichia (Kristinarson, 2017; Novotny et al., 2021; Peeters et al., 2013; Stefansdottir, 2015; van der Meide et al., 2013). ELISA showed that IgE from serum of IBH-affected horses located in the Netherlands bound to the allergens Cul o 1 – Cul o 7 in the frequency of 38-67% (van der Meide et al., 2013). In another study, IgE from 45% and 40% of IBH-affected horses bound to Cul o 1P and Cul o 2P respectively (Peeters et al., 2013).

The major allergens of IBH have been identified with an allergen microarray using serum from a large panel of IBH-affected horses from Switzerland, Germany, Sweden, Ireland and United Kingdom together with healthy controls from the same countries (Novotny et al., 2021). Out of 27 *Culicoides* allergens tested for binding of IgE in serum from IBH-affected horses, the nine allergens Cul o 8, Cul o 1P, Cul o 2P, Cul o 10, Cul o 9, Cul o 11, Cul o 7, Cul o 5 and Cul o 3 were major allergens as IgE in sera from over 50% of the IBH-affected horses tested bound to these proteins. All nine major *Culicoides* allergens in IBH originate from *C. obsoletus* and seven of them bound IgE in sera from >70% of the IBH-affected horses. Out of the 27 r-allergens tested, 13 allergens were showen to be major in IBH-affected horses of the Icelandic horse breed (Figure 8), where >50% reacted to Cul o 8, Cul o 1P, Cul o 2P, Cul o 10, Cul o 9, Cul o 11, Cul o 7, Cul o 5, Cul o 3, Cul o 12, Cul n 5, Cul o 13 and Cul n 4. Comparison of IBH-affected horses of the Icelandic breed and other breeds showed similar sensitization to the top five r-allergens Cul o 8, Cul o 1P, Cul o 2P, Cul o 9 and Cul o 10 (Figure 8). However, positive IgE results were significantly higher for other allergens tested compared to other horse breeds indicating that imported Icelandic horses are sensitised to more *Culicoides* r-allergens than other horse breeds born in *Culicoides* infested areas. (Novotny et al., 2021).

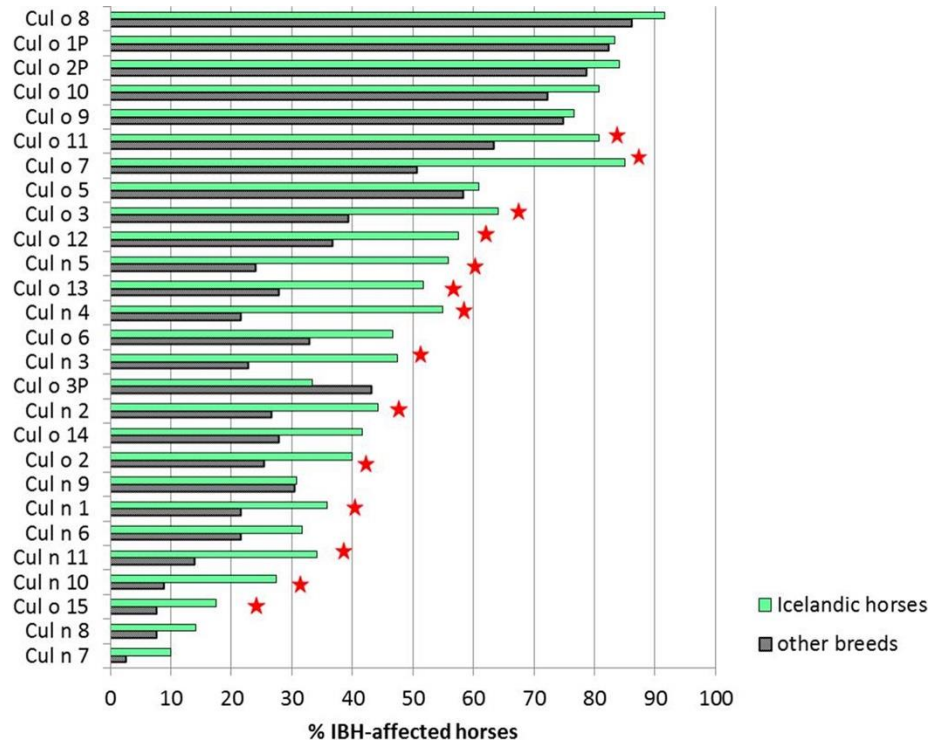


Figure 8. Comparison of IgE levels of IBH-affected horses to r-*Culicoides* allergens. Comparison of the IgE seropositivity (shown as % horses with IgE levels above cut off values) to the 27 recombinant Culicoides allergens between IBH-affected horses from the Icelandic breed (n = 120) versus horses belonging to other breeds (n = 79) within the horse group from Switzerland. Significant differences between groups in Fisher's exact test are shown with a star. The figure and figure text are reprinted from Novotny et al. 2020 (https://creativecommons.org/licenses/by-nc-nd/4.0/).

Analysis of the natural course of sensitization to *Culicoides* allergens, showed that in the first summer of clinical signs, IBH horses were sensitized to a median of eleven allergens demonstrating IgE-reactivity to be due to co-sensitization rather than cross-reactivity between *Culicoides* allergens (Birras et al., 2021). The major primary sensitizing allergens have been identified as Cul o 8, Cul o 11, Cul o 2P, Cul o 7, Cul o 1P, Cul o 13 and Cul o 10, binding IgE in over 50% of sera the first year of clinical signs of IBH (Figure 9). Furthermore, Cul o 3 and Cul o 9 were also identified as important primary sensitizing *Culicoides* allergens binding IgE in nearly 50% of the sera (Figure 9). For horses exported from Iceland to *Culicoides* infested areas the allergen Cul o 13 was showen to be of high importance with >50% IgE binding to the allergen in sera. Also, IgE levels against Cul o 13 of IBH-affected horses exported from Iceland were significantly higher compared to horses born in *Culicoides* infested areas (Birras et al., 2021).

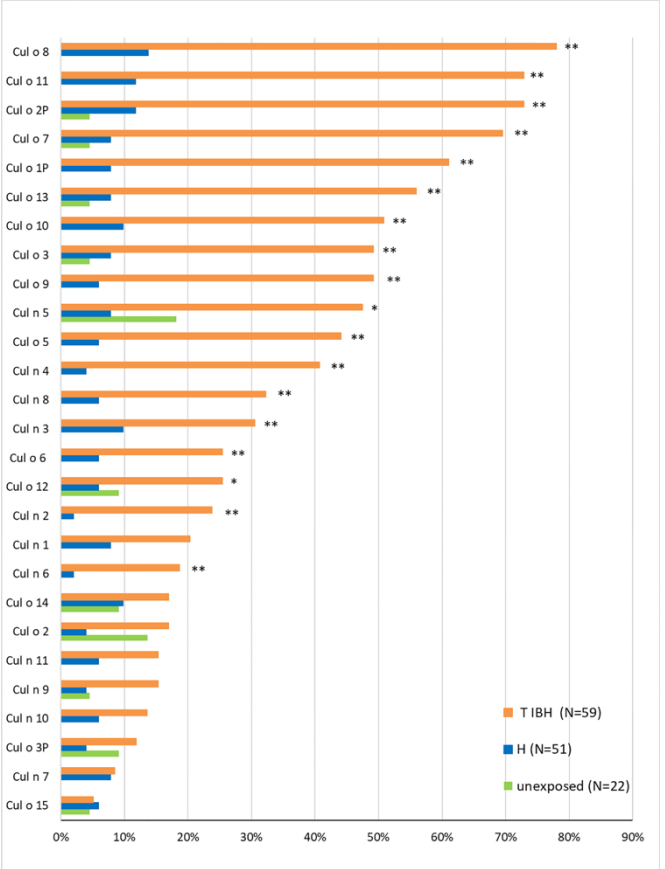


Figure 9. IgE sero-positivity to 27 Culicoides recombinant (r-) allergens. Percentage of horses with IgE levels above the cut-off values in horses exported from Iceland to Switzerland that developed insect bite hypersensitivity (IBH; n = 59) or remained healthy (H; n = 51) and in horses living in Iceland (unexposed; n = 22). Serum samples were taken the summer of clinical onset of IBH (T_{IBH}) and at the corresponding time in the H group. The allergens are listed in decreasing order from those binding serum IgE in the highest number of horses at time of clinical onset of IBH (T_{IBH}). P values were calculated with the Fisher's exact test and Bonferroni correction done for multiple comparisons. **IBH significantly different from H and unexposed (cP <0.05). *IBH significantly different from H (cP<0.05). No significant differences between H and unexposed for any r-allergen. The figure and figure text are reprinted from Birras et al 2021 (https://creativecommons.org/licenses/by/4.0/).

1.4 Allergen specific immunotherapy

Allergen-specific immunotherapy (AIT) is the only specific causative treatment of allergic diseases by making alteration of immune response to allergen resulting in desensitization and/or tolerance (Jutel et al., 2016; Jutel et al., 2015). First reports about AIT go back to 1911 when patients suffering from hay fever showed decreased sensitivity to pollen following subcutaneous injection (s.c.) with pollen extract (Noon, 1911). However, only recently efficacy of AIT was scientifically demonstrated with double-blind placebo-controlled clinical trials in allergic human patients (Roberts et al.,

2018; Sturm et al., 2018). Still there are no specific biomarkers generally accepted for the outcome of AIT (Shamji et al., 2017). The benefits of AIT for patients are reduction of clinical symptoms of allergy, prevention of new sensitization, induction of long-term tolerance and decrease in medication leading to improvement in quality of life (Alvaro-Lozano et al., 2020). Also, there is strong evidence that AIT is cost-effective compared to symptomatic treatment (Cox et al., 2020b).

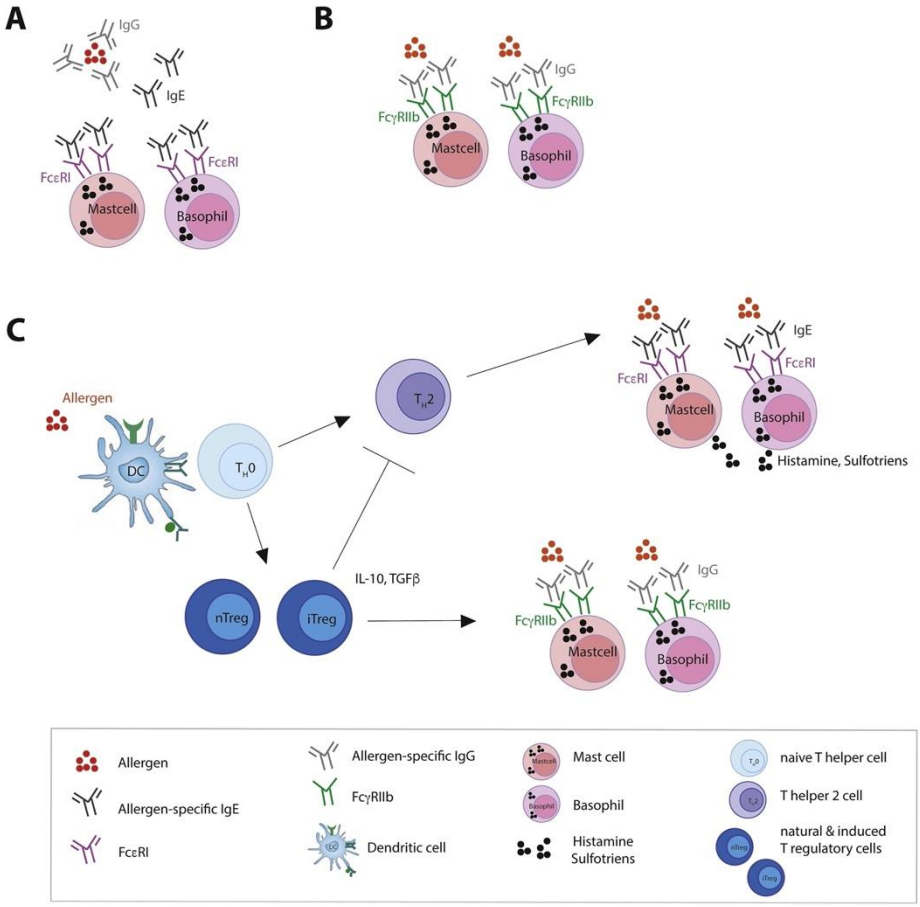


Figure 10. Mechanism of action allergen-specific immunotherapy (ASIT). ASIT-mediated induction of allergen-specific IgG antibodies, which **(A)** compete with allergen-specific IgE for binding sites thereby limiting IgE-mediated type I response and **(B)** bind to inhibitory FcγRIIb, stopping mast cell and basophil degranulation. **(C)** ASIT-mediated Treg induction that dampen Th2 mediated downstream responses. The figure and figure text are reprinted from Fettelschoss-Gabriel et al. 2021 (https://creativecommons.org/licenses/by-nc-nd/4.0/).

Imortant for a successful AIT (Figure 10) is the induction of allergen-specific IgG antibodies that block the binding of allergen-specific IgE antibodies to the allergens by competition of allergen binding site (Dorofeeva et al., 2020). AIT aims to shift Th2 responses towards Th1/Treg responses with generation of allergen-specific $CD4^+$ Treg and/or Th1 cells and induction of allergen-specific Breg cells. Treg cells produce

cytokines such as IL-10, TGF-β and IL-35 which promote immune regulation of allergic responses with suppression of Th2 responses. Suppression of Th2 cells leads to decreased production of Th2 cytokines like IL-4, IL-5, IL-9 and IL-13 and subsequently reduction of infiltrating inflammatory cells which include mast cells, eosinophils and basophils. The key cytokine of Breg is IL-10 promoting immune suppression with regulating development, proliferation and maintenance of $CD4^+$ T cells. During antigen uptake in suppressive cytokine milieu, immature DCs convert into tolerogenic DCs which promote to further immune suppression with expression of suppressive molecules and production of TGF-β and IL-10 (reviewed in Kucuksezer et al. 2020). There is a strong interest in prophylactic immunotherapy in humans to prevent the development of allergic symptoms. This is not yet practiced but some pilot studies have been performed (Campana et al., 2019; Tulaeva et al., 2020).

1.4.1 The route of administration

Different routes for administration of allergen vaccines in AIT have been tested and some are in use. The allergen application primarily used is subcutaneous immunotherapy (SCIT). It has been used for over hundred years and is the most documented route with the best establishment of underlying mechanisms (Dorofeeva et al., 2020; Noon, 1953). SCIT is applicable for multiple allergens and controlled trials on the efficacy have already been carried out for treatment of allergic rhinitis. It has been shown be safe and well-controlled in most cases, but severe systematic reactions can occur (Field & Blaiss, 2020). SCIT is currently used in humans for treatment against allergic asthma and allergic rhinitis. The treatment consists of multiple and frequent injections of vaccine, starting with initial exposure to a diluted concentration of allergen, then escalation phase with dosage increase and finally a maintenance phase which is continued for 3 to 5 years (Roche & Wise, 2014).

Sublingual immunotherapy (SLIT) is a non-invasive route where the allergen is administered under the tongue with formulation as drops or fast-dissolving tablets (Roberts et al., 2018). In the last 30 years, SLIT has been demonstrated to improve safety of AIT and shown to be effective in many clinical trials. The advantage of SLIT is that it can be self-managed and self-administrated by patients (Passalacqua et al., 2020). The drawback of SLIT is the need for high dose of allergens and long duration of the treatment (Moingeon et al., 2017). In meta-analysis of AIT against allergic rhinitis no significant difference was observed in efficacy comparing SCIT and SLIT but SLIT showed to have more local side effects but less systematic (Field & Blaiss, 2020). However, meta-analysis comparing efficacy of the two methods against house-dust mite allergy indicate that SCIT show a higher efficiency in symptoms control (Kim et al., 2021)

Intralymphatic immunotherapy (ILIT) is the application of allergen vaccine directly into the lymph node. The advantages of ILIT over SCIT and SLIT is a shorter duration of

treatment and low allergen dose for application (Figure 11). This novel technique is promising but more clinical trials are needed (Senti et al., 2019). In a study comparing ILIT and SCIT against grass pollen, patients either received 54 s.c. injections of pollen extract over 3 years or 3 i.l. injections over 2-month period. The results showed that ILIT caused fewer adverse events compared to SCIT, faster recovery and was less painful. Tolerance induced was long-lasting and comparable to what obtained with SCIT (Senti et al., 2008).

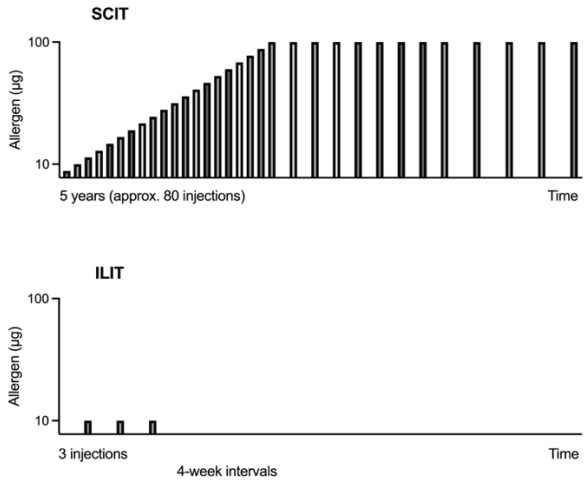


Figure 11. Schematic overview comparing SCIT and ILIT injection protocol. Modified in accordance to Senti et al. (2019).

Other application routes currently in development are oral immunotherapy (OIT), epicutaneous (EPIT), intradermal (IDIT) and intranasal (INIT). OIT has only shown to be effective for some food allergens but not for respiratory allergens or allergens which are easily digested in the gastrointestinal tract. EPIT is a novel technique with administration of allergens with repeated applications to the skin targeting skin antigen-presenting cells. EPIT has been shown to be safe and tolerable administration route, however efficacy has yet to be determined but results show that it its promising especially against food allergies (Esposito et al., 2018; Kim & Burks, 2020). The efficacy of IDIT and INIT remains unclear and further studies are needed (Atipas et al., 2022; Sadeghi et al., 2022).

1.4.2 Adjuvants

An adjuvant is a substance which modifies and enhances the immune response to antigens. Adjuvants enhance the efficacy of AIT when co-administered with the causative allergens (Figure 12) and reduce the number of doses by increasing immunogenicity of the allergens (Chesne et al., 2016). The ideal adjuvant should be stable, sustainable, biodegradable, nontoxic, cost-effective and promote appropriate immune responses (Aguilar & Rodríguez, 2007).

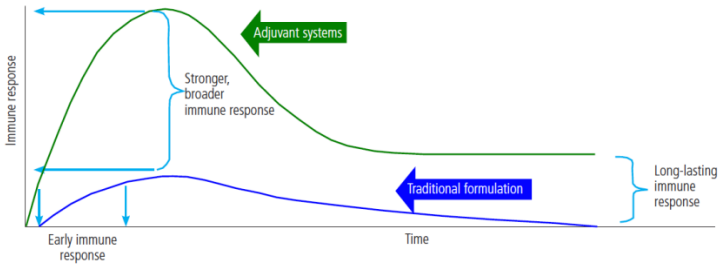


Figure 12. Advantages of adjuvant-formulated vaccines. The figure and figure text are reprinted from Zubelida et al. (2019) with permission from Journal of Investigational Allergology and Clinical Immunology ESMON Publicidad.

Adjuvants may generally be classified into three groups, delivery systems, immunomodulatory molecules and combination system composed of the prior two groups. Delivery systems consist of non-immunomodulatory compounds presenting the antigen to the immune system whereas direct activation of innate immune receptors is provided by immunomodulatory molecules (Reed et al., 2013).

Delivery systems have been considered as first-generation adjuvants. Their general mechanisms of action are similar but they vary considerably in structure and composition (O'Hagan & Fox, 2015). These systems generate depot effect with slow release of allergen, increasing the time of exposure of allergen to the immune system and prolonged presence at injection site contributing to immune tolerance (Klimek et al., 2017). Currently there are three first-generation adjuvants commercially available and used in AIT, aluminium hydroxide (alum), calcium phosphate and microcrystalline tyrosine (MCT).

The most frequently used adjuvant in immunotherapy and vaccines is alum. The mechanism of action remains unclear but when used in AIT it has shown to intensify allergen immunogenicity, tolerability and increase IgG and IgE antibody titers. Three mechanisms of action have been suggested: (a) the depot effect in which there is slow release of antigen enhancing antibody production, (b) induction of inflammation with recruitment and activation of APCs capturing the antigen, (c) conversion of soluble antigen into particulate form which gets phagocytosed by APCs such as macrophages, DCs and B cells (reviewed in Zubeldia et al. 2019). Th2 immune responses are especially enhanced by alum. Alum-formulated antigen causes inflammation at injection site, with recruitment of innate immune cells as muscle cells immediately release chemokines and cytokines upon injection. Furthermore, the damaged tissue releases uric acid an endogenous danger signal. Monocytes react to the alum and uric acid through inflammasomes (NALP3) and while migrating to the draining lymph nodes the antigen is processed for MHCI and MHCII molecules. In the lymph nodes, DCs select antigen-specific T cells which differentiate into Th effector cells shown to be Th2 biased in mouse model (Kool et al., 2008).

Immunomodulatory molecules alter the immune response against antigen with activation of innate immune receptors on APCs. These so-called second-generation adjuvants are synthetic bacterial derivatives which interact with TLRs on immune cells. The only second-generation adjuvant on the market and the only one used in AIT is monophosphoryl lipid A (MPLA) (reviewed in Zubeldia et al. 2019). MPLA is a detoxified LPS derivative, inducing Th1 cytokine production by binding to TLR-2 and 4 in human $CD4^+$ T cells (Komai-Koma et al., 2021). MPLA is used in vaccines against infectious diseases (Mitchell & Casella, 2017) and successfully in immunotherapy against allergic rhinitis to grass pollen in human patients (Mothes et al., 2003; Rosewich et al., 2010; Zielen et al., 2018).

Other adjuvants currently under research include immunostimulatory sequences (ISSs), nanoparticles (liposomes, virus-like particles (VLP) and biodegradable polymers) and phosphatidylserine derivatives). Nanoparticles are a novel adjuvant/vaccine delivery system with the potential of greatly enhancing AIT with strong immunogenic effect and low allergenic potency (reviewed in Zubeldia et al. 2019). Virus-like particles (VLP) are vaccine delivery systems that are assembled by one or more recombinantly expressed viral structural proteins which are overexpressed and spontaneously self-assembled into capsid particles. These structures lack a viral genome and therefore have no replicative capacity. The symmetry in their structure is similar to native viruses and they can therefore induce immune responses. Due to appropriate size, they drain freely into adjacent lymph nodes (Bachmann & Jennings, 2010; Chackerian, 2007). Due to size and surface structure, VLPs are recognized by the innate immune system. They are designed to target B-cells, induce antibody response and are also effective in priming of $CD8^+$ T cells (Mohsen et al., 2018). Recent study has shown that the size and structure of VLP can affect drainage dynamics and antibody response. Interestingly the same VLP monomer formed nano-sized icosahedra or rods in the micrometre size depending on whether tetanus toxin (TT) epitope was incorporated at the N-terminus or C-terminus of VLP derived from Cowpea chlorotic mottle virus (CCMV). Results showed the round-shaped $CCMV_{TT}$-VLPs to be more efficient in draining to the secondary lymphoid organs and inducing more than 100- fold IgG and IgA antibody response compared to the rod-shaped $CCMV_{TT}$-VLPs (Zinkhan et al., 2021). Several vaccines with VLPs are in use in humans e.g. against Human Papilloma Virus (HPV) and Hepatitis B Virus (HBV) (Mohsen et al., 2017).

1.4.3 Allergen preparation

The first AIT studies used allergen extract as basis for the vaccine and currently the only AIT forms on the market are allergen-extract based (Akinfenwa et al., 2021b). However, the disadvantage of using extracts is that the mixture is composed of both allergenic and nonallergenic substances where the content is variable between batches. Important allergens might be poorly immunogenic, present in too low concentration and sometimes absent from the extract (Curin et al., 2017). Hence, availability of well-

defined and pure allergens targeting a specific immunological mechanism is important for the development of efficient and standardized AIT (Valenta et al., 2016; Zhernov et al., 2019). With sequencing of the most relevant allergens the basis for next generation form of AIT has been made allowing for novel molecular strategies including recombinant native-like allergens, nucleic acid-based vaccines, T cell peptides, recombinant hypoallergens and peptide carrier-based B cell epitopes (Curin et al., 2018).

1.4.4 Production of recombinant proteins

Modern biotechnology and genetic engineering have enabled the production of recombinant proteins (r-proteins) for use in biomedical research, therapeutic treatments, vaccines and diagnosis. r-Proteins can be produced in prokaryotic or eukaryotic expression host systems. The production systems used are genetically modified bacteria, mammalian cells, yeast, insect cells, transgenic animals and transgenic plants (Tripathi & Shrivastava, 2019). When choosing an optimal expression system, different factors need to be considered depending on the target protein such as PTM including protein glycosylation. No or incorrect PTM can have significant effect on the target protein by changing its properties such as biological activity, immunogenicity and pharmacokinetics. Prokaryotic expression systems are therefore mostly used for production of relatively simple r-proteins and eukaryotic systems for more complex r-proteins (Rozov & Deineko, 2019).

1.4.4.1 E.coli

The most widely used expression host for r-proteins is bacteria, especially *E. coli* with advantages of rapid growth, good productivity and cost-effectiveness. The system has well-known genetics and is easily genetically modified. However, the system has some disadvantages such as lack of PTM, contamination of endotoxins, codon bias and formation of inclusion bodies. Also, proteins with many disulfide bonds are difficult to express. Proteins produced as inclusion bodies are difficult to purify as they are inactive, aggregated and insoluble (Demain & Vaishnav, 2009). Some of these disadvantages can be managed with some techniques such as addition of a tag to enhance protein solubility, co-expression of molecular or chemical chaperones to avoid inclusion bodies or allowing certain PTMs (Tripathi & Shrivastava, 2019). Endotoxins can be minimized or eliminated by certain purification processes (Choi et al., 2014). Codon optimization has in some cases been shown to increase r-protein expression by many folds (Rosano et al., 2019), other strategies for increasing protein expression include change in expression vector or host strain and cultivation parameters such as temperature and incubation time (Gopal & Kumar, 2013).

1.4.4.2 Insect cells

For production of r-proteins in insect cells, the baculovirus expression vector system (Figure 13) is the most commonly used. The insect cells are grown to a certain density and then infected with recombinant baculovirus (r-baculovirus) containing the gene of interest (Irons et al., 2018). Insect cells offer many advantages as a protein expression system such as complex eukaryotic PTMs and proper protein folding making the system quite suitable for expression of soluble proteins of mammalian origin (Altmann et al., 1999) and even more so for proteins of insect origin. The system has high expression levels and can easily be scaled up with no limit on protein size and multiple genes can be expressed simultaneously (Demain & Vaishnav, 2009). The glycosylation pattern is comparable to mammalian expression systems, but cannot carry out N-glycosylation (Altmann et al., 1999) which can be overcome by co-expression of enzymes for synthesis of N-glycans (Palmberger et al., 2012). Other disadvantages include inefficient secretion of r-proteins, which can be overcome by adding secretion signal sequences originating from insects for example honeybee melittin sequence. Low expression levels can be increased with optimisation of expression time and infection level (Demain & Vaishnav, 2009). The small ubiquitin-related modifier (SUMO) tag was developed to be used in the baculovirus expression system, shown to enhance expression levels and ease the expression of proteins proven to be difficult and for those who need native N-terminal residues for function (Liu et al., 2008).

Baculoviruses have a rod-shaped nucleocapsid enclosing a double-stranded circular DNA genome. They are naturally pathogenic,arthropod-specific and known to infect more than 600 host species. As they are host-specific they are safe for the environment and human health. The most widely used virus is derived from *Autographa californica* multiple capsid nucleopolyhedrovirus (AcMNPV) and the cell lines used are usually Sf-9 and High-Five™. The baculovirus replication cycle results in two structural forms of the virus, budded virus and occlusion-derived virus and includes expression of genes in four phases: immediate-early, delayed-early, late and very late. The early and late phases are associated with production of budded virus leading to spreading of the infection. The very late lead to formation of occlusion-derived virus in the nucleus of the host cell. The occlusion-derived virus becomes occulated in occlusion bodies made from polyhederin protein which is expressed in high levels under a strong promoter (reviewed in Chambers, Aksular et al. 2018). The very late genes encoding for polyhederin can be excluded from the virus genome without affecting the production of infectious virus particles. In the baculovirus expression system the polyhederin gene is replaced with the cloned gene of interest, under the strong polyhederin promoter allowing for the expression of r-protein (Possee, 1997).

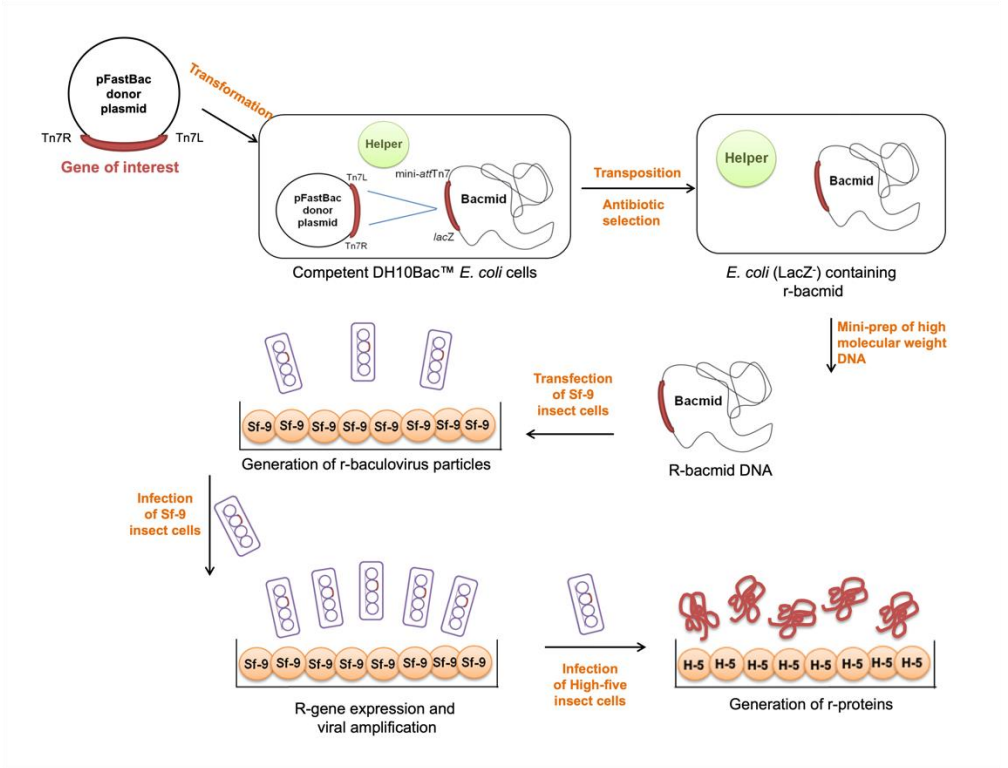


Figure 13. Schematic overview of the Bac-to-Bac expression system. The gene of interest is cloned into pFastBac vector followed by transformation of DH10Bac *E. coli* cells. With antibiotic selection, colonies containing the gene of interest are cultured and recombinant bacmid isolated. Sf-9 insect cells are transfected with the isolated r-bacmids and baculovirus produced. For amplification of the viral stock, fresh Sf-9 insect cells are infected with the r-baculoviruses produced. For generation of r-proteins, fresh High-five insect cells are infected with amplified r-baculoviruses. The figure was modified in accordance with Invitrogen instruction manual.

The baculovirus expression system has been used for production of a number recombinant allergens. The bioactivities of these recombinant allergens are similar to the native proteins and they have IgE-binding capacity (Schmidt & Hoffman, 2002). Honeybee venom hyaluronidase allergen was produced in insect cells and *E. coli* for comparison of biological activity to natural hyaluronidase. The result showed the hyaluronidase produced in insect cells to have enzymatic activity equal to natural hyaluronidase, whereas the *E. coli* produced hyaluronidase had only 20-30 % activity compared to natural hyaluronidase (Soldatova et al., 1998).

1.4.4.3 Transgenic barley

The use of plants to produce recombinant proteins is referred to as molecular farming (Fischer & Buyel, 2020). Production in transgenic plants has many advantages such as low costs, enabling of PTMs, easy scale-up and safety (i.e. low risk of endotoxins and animal pathogens contamination) (Tripathi & Shrivastava, 2019). The structures of

oligosaccharide attached to glycoproteins in plants and mammals differ as the Golgi apparatus which processes *N*-glycan and *O*-glycan synthesises is kingdom specific. But with intensive glycoengineering, plant glycosylation can be humanized (Gomord et al., 2010; Strasser, 2016). A major advantage of this system is the expression of recombinant protein in the desired plant compartment, both whole terrestrial plants are used and plant-based systems as plant cell and tissue cultures. For whole terrestrial plants there are two main platforms for expression of recombinant proteins, leafy crops such as tobacco and cereal crop such as maize, rice and barley (Fischer & Buyel, 2020). The main disadvantages of plant expression systems are low yield of target recombinant protein compared with bacterial systems and longer gene to protein timeframe because of slower growth. Purification of whole plants requires disruption of plant tissue for recovering r-protein whereas in animal cells and microbes the r-protein is generally secreted into the medium. R-protein expression may be increased with optimization of genetic construction and conditions of plant cell cultivation (Fischer & Buyel, 2020; Rozov & Deineko, 2019). Protein purification from plants requires laborious steps, harvest of plant material, extraction of total proteins and purification of target r-protein. However, r-proteins produced in edible plant parts may only need partial purification steps or none at all (Moustafa et al., 2016). Many plants have edible leaves, seeds and fruits and have been used in pharmaceutical products and vaccines via oral administration (Fischer & Buyel, 2020).

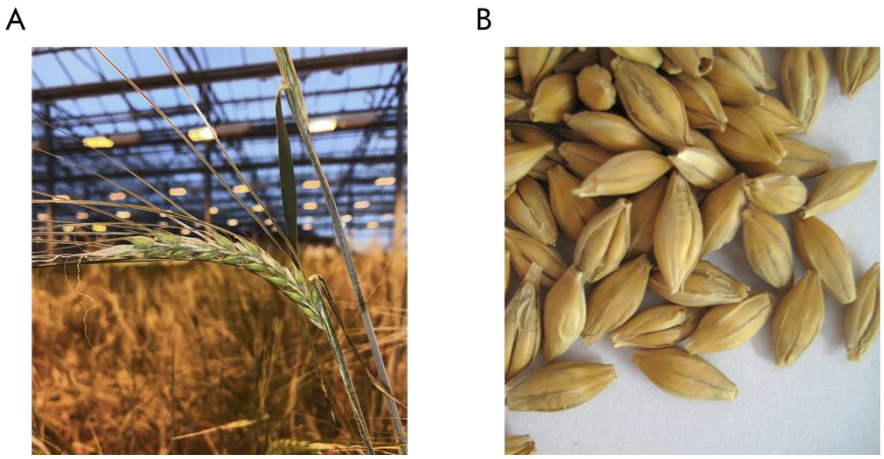


Figure 14. Barley (*Hordeum vulgare*). **A.** Transgenic barley in the greenhouse of ORF Genetics. **B.** Harvested seeds of transgenic barley. Photos: Sara Björk Stefánsdóttir.

Barley (*Hordeum vulgare*) (Figure 14) is cultivated worldwide and one of the most important cereal crops, mostly for animal feed but also as food, for malting and brewing (Ritala et al., 2008). Extended knowledge of barley cultivation has accumulated for over 10.000 years and optimal conditions for cultivation are well established. Barley is sturdy and can be grown in agriculturally challenging areas (Magnusdottir et al., 2013). Barley is an attractive system for r-protein production as it is

a strong protein producer, storing a large amount of proteins or between 8% and 30% of total seed mass (Jaeger et al., 2021; Ritala et al., 2008). The barley seed is a natural storage environment for long-term preservation of proteins and nutrition for the embryo. Therefore the barley seed is ideal as storage for r-proteins for 5-10 years (Ritala et al., 2008) with the possibility of harvesting a stockpile of unprocessed material and separating protein production from protein purification (Magnusdottir et al., 2013). There is low risk of gene transfer as barley is a self-pollinated crop with no outcrossing (Ritala et al., 2008). Barley seeds are endotoxin-free, low in toxic compounds and do not harbour viruses or pathogens known to infect mammals (Magnusdottir et al., 2013). Furthermore, barley is approved as a GRAS (generally recognized as safe) organism for human consumption by the U.S. Food and Drug Administration (FDA). The major disadvantage of barley as an r-protein production system is long gene-to-protein timeframe. It usually takes 6-7 months for generating new transgenic barley lines and two more generations are required to achieve sufficient amount of barley seeds for extraction and purification of target r-protein (Magnusdottir et al., 2013). ORF Genetics (Kopavogur, Iceland) has engineered barley for production of endotoxin-free human and animal derived growth-factors and cytokines (ORF Genetics 2023).

1.5 Development of immunotherapy against IBH

Attempts have been made treating IBH-affected horses with AIT using *Culicoides* WBE with inconclusive results (Anderson et al., 1996; Barbet et al., 1990; Ginel et al., 2014). In one trial significant improvement was reported in clinical signs but no control group was included in the study (Anderson et al., 1996). In the other two trials clinical signs improved post vaccination but the difference did not reach significance when compared to control group (Barbet et al., 1990; Ginel et al., 2014). The drawback of using WBE is how difficult it is to standardize and the mixture is composed of both allergenic and nonallergenic substances. Also, the content of WBE may vary between batches and some important allergens might be absent (Curin et al., 2017). These studies show that it is important to have well-defined allergens ideally suiting the sensitization profile of the IBH-affected horse being treated (Jonsdottir et al., 2019; Novotny et al., 2021).

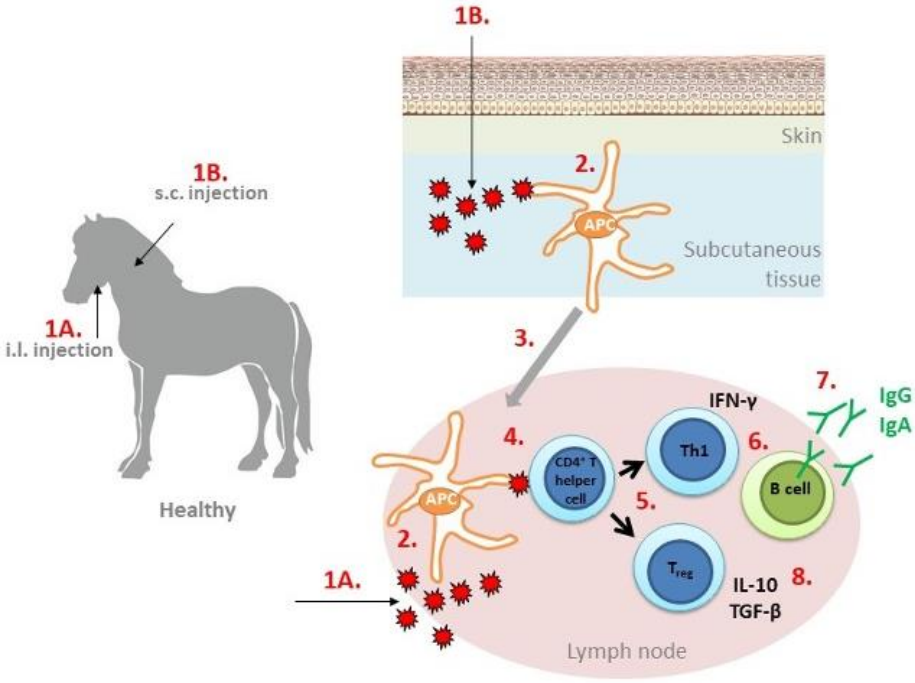


Figure 15. Simplified scheme of preventive allergen-specific immunotherapy. Immune reactions that have been demonstrated in horses are indicated as bold in the fig. legend. **(1)** The vaccine consisting of recombinant Culicoides allergens in adjuvants is injected directly into the submandibular lymph node **(A)** or subcutaneously **(B)** or intradermal. The advantage of the intralymphatic injections being that the allergens are delivered directly at the site of the immune response. **(2)** Antigen-presenting cells (APCs) take up the allergens **(3)** and bring them to the draining lymph node (only B). **(4)** In the lymph node, APCs present allergen peptides on MHC class II to naïve CD4+ T helper cells. **(5)** Subsequently, naïve CD4+ T helper cells differentiate into T helper type 1 (Th1) cells and/or T regulatory cells (Tregs). **(6)** The Th1 cells produce **IFN-γ** and instruct the B cells to undergo class switching, **(7)** and start producing **IgG** and **IgA** antibodies. **(8)** Additionally, Treg cells produce regulatory cytokines **IL-10** and TGFβ. The figure and figure text are reprinted from Jonsdottir et al. 2019 (https://www.creativecommons.org /licenses/by/4.0/).

Currently, research aims to improve AIT by using purified recombinant allergens instead of WBE. In development of prophylactic immunotherapy against IBH, two experiments have been carried out in healthy Icelandic horses using purified recombinant *Culicoides* allergens. In the first study, the purified r-allergens either with or without Th1 focusing adjuvant, IC31®, where injected three times with four-week interval either intradermally (i.d.) or intralymphatically (i.l.) (Figure 15). The result showed significant increase in allergen-specific IgG subclasses, after vaccination with the r-allergens. Injection of r-allergens with IC31® resulted in significantly higher level of allergen specific IgG1, IgG1/3 and IgG4/7 antibodies compared to injection with r-allergens alone. The specific IgG antibodies induced were able to partly block the binding of serum IgE from IBH-affected horses to the r-allergens. Comparing i.l. and i.d. injection routes, the difference did not reach significance but i.l. injection was

slightly more efficient than i.d. injection (Jonsdottir et al., 2015). In the second study the commonly used adjuvant alum alone was compared to mixture of alum and monophosphoryl lipid A (MPLA) as the adjuvant IC31® was no longer available for use in horses. The horses were injected i.l. (Figure 16) with purified r-allergens either with alum alone or mixture of alum/MPLA three times with four-week interval. The immunizations significantly induced allergen-specific IgG subclasses which were able to block the binding of serum IgE from IBH-affected horses to the allergens. There was no significant difference between the vaccination groups in induction of IgG antibodies. Compared to unvaccinated control horses, r-allergen stimulated PBMC from horses vaccinated with alum/MPLA produced significantly more IFNγ and IL-10 but not horses vaccinated with alum alone. These results indicate that vaccination i.l. with purified r-allergens in alum/MPLA induces Th1/Treg immune response (Jonsdottir et al., 2016).

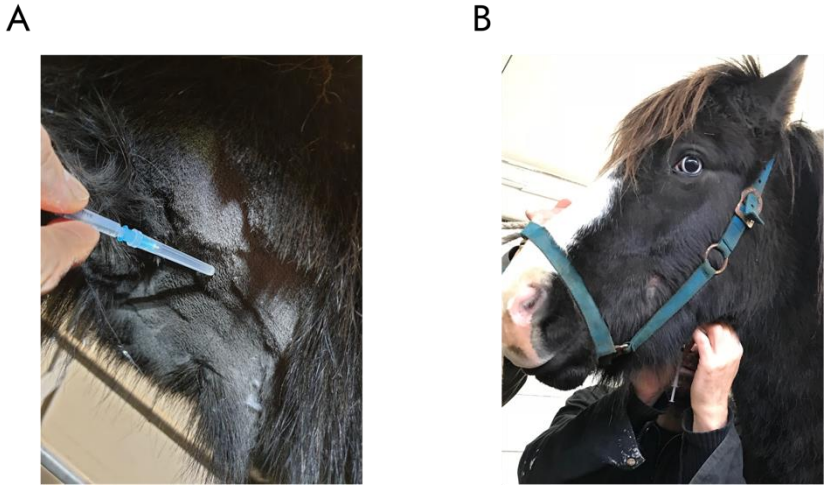


Figure 16. Intralymphatic vaccination. **A.** Submandibular lymph node. **B.** Injection into the lymph node. Photo: **A.** Sigríður Jónsdóttir, **B.** Sara Björk Stefánsdóttir.

Recombinant barley was used to develop a method for curative treatment of horses via the mucosa of the mouth (Jonsdottir et al., 2017). In a pilot study, four healthy Icelandic horses were treated with transgenic barley porridge and three with control barley 7 times over a 20-week period using a device developed to treat horses orally. The barley used for the porridge was transgenic barley expressing *C. nubeculosus* allergen, Cul n 2. Horses treated with transgenic barley induced Cul n 2 specific IgG1 and IgG 4/7 antibodies in blood and saliva. The IgG1 response in serum and saliva was significantly higher in horses treated with transgenic barley compared to horses treated with control barley. The same applied to IgG1 antibody response in saliva. The antibodies induced were able to partly inhibit IgE binding to Cul n 2 as well to the corresponding allergen from *C. obsoletus,* Cul o 2 (Jonsdottir et al., 2017).

2 Aims

Insect bite hypersensitivity (IBH) is a seasonal recurring IgE-mediated allergy in horses caused by biting midges of the genus *Culicoides*. IBH has been reported worldwide and affects all horse breeds. The prevalence has been reported particularly high in adult Icelandic born horses exported to *Culicoides* infested areas (>50%). Currently no long-term treatment is available except for symptomatic treatment and avoidance of the midges. IBH is a serious animal welfare issue as it greatly affects the quality of life of affected horses.

Studies of IBH at the Institute for Experimental Pathology, University of Iceland at Keldur in collaboration with the Vetsuisse Faculty, University of Berne, Switzerland have been ongoing since the year 2000 with the final aim of developing immunotherapy against equine IBH. Numerous allergens have been identified and studies of the pathogenesis demonstrate an imbalance within T-cell subsets. This opens the possibility to develop an allergen-specific immunotherapy against IBH by shifting the immune response towards Th1 and inducing Treg cells.

The aim of the project was to develop allergen specific immunotherapy against IBH with two approaches.

I) Development of preventive immunotherapy by vaccination before sensitization with purified IBH-allergens in adjuvants.

- Expression and purification of newly identified major *Culicoides* allergens in insect cells.
- Comparison of subcutaneous and intralymphatic injection for immunotherapy using purified recombinant allergens with Th1 focusing adjuvant Monophosphoryl lipid A (MPLA) and aluminum hydroxide (alum).
- Comparison of the adjuvant effect of virus-like particles (VLP) in alum and the MPLA/alum mixture in subcutaneous vaccination with purified r-allergens.
- Analysis of the antibody response of horses vaccinated in Iceland and challenged by exportation to *Culicoides* infested areas in Switzerland.

II) Curative treatment by treating IBH-affected horses via the oral mucosa of the mouth with transgenic barley expressing IBH-allergens.

- Expression and production of r-allergens in barley for development of immunotherapy via the oral mucosa with transgenic barley flour mixture.
- Purification of the barley expressed r-allergens for improvement of diagnostic tests.

3 Materials and Methods

3.1 Allergens

3.1.1 Cloning of allergen genes

3.1.1.1 Origin of genes

The allergen genes were codon optimized and synthesized by GenScript (George Town, Cayman Island) for expression in the appropriate expression system; *Cul o 1P, Cul o 2P, Cul o 3 and Cul n 4* as N-terminal fusion to a hexa-histidine tag ($(His)_6$) in the high expression vector pET-17b with a 3-amino-acid C-terminal linker (GGC) for *E. coli* production (appendix I). *Cul o 8, Cul o 9, Cul o 10, Cul o 11 and Cul o 12* on the pFastBac™1 vector) or expression in insect cells (appendix II). *Cul o 2 (hya), Cul o 2P, Cul o 3, Cul o 5 and Cul n 8* on expression cassette under the control of a natural seed specific promoter for expression in barley (appendix III) with natural signal sequence (D-hordein) and $(His)_6$ tag on the N-terminus (Jonsdottir et al., 2018).

3.1.1.2 Primer design

Primers for amplifying the entire genes and gene parts were designed from the codon optimized gene sequence and those for cloning with appropriate cleavage sites. All primers were purchased from TAG Copenhagen (Frederiksberg, Denmark) and are listed in appendix IV.

3.1.1.3 Polymerase chain reaction (PCR) and DNA electrophoresis

PCR reactions were done with Phusion® Hot Start Flex DNA polymerase (New England Biolabs® Inc., Ipswich, MA, USA) for amplification of the allergen genes for cloning and *Taq* DNA polymerase (New England Biolabs® Inc.) for testing bacterial cultures and minipreps after transformation according to manufacturer's protocol. The PCRs were performed in Veriti 96 Well Thermal Cycler from Applied Biosystems (Thermo Fischer Scientific, Waltham, MA, USA). The PCR products were run on 1% agarose gel. The Agarose low EEO (Agarose Standard) (PanReac AppliChem ITW Reagents, Darmstadt, Germany) powder was added to 0.5% Tris borate – Ethylenediaminetetraacetic acid (EDTA) (45 mM Tris borate, 0.1 mM EDTA) and melted. Before solidification 1-4 drops of ethidium bromide (1.33 mg/mL, Merck, Darmstadt, Germany) added to the melted agar depending on the size of the gel. Restriction buffer (10x RSB; 50% glycerol, 15 mM EDTA, 0.25% bromophenol blue) was added to each sample of the PCR product before loading to the agarose gel. PCR products were

visualized under UV light in InGenius (SynGene International Ltd, Bangalore, India) and photographed using the GeneSnap program (SynGene International Ltd). The size of the products was estimated by comparison with a 2-log ladder (New England Biolabs® Inc.).

3.1.1.4 Purification of amplicons and DNA quantification

Before cloning, amplified allergen genes were purified with gel extraction using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific), according to manufacturer's protocol. The concentration of nucleic acid in the DNA samples was measured in NanoDrop®ND-1000 Spectrometer (NanoDrop Technologies Inc., Wilmington, DE, USA) according to manufacturer's user manual.

3.1.1.5 DNA constructs

The allergen genes were cloned into different expression vectors either with or without the native secretion signal sequence of the allergen. The secretion signal sequence of the allergens was determined by the prediction program SignalP 5.0 Server (Nielsen et al., 2019). The vector used for recombinant allergen (r-allergen) expression in *E. coli* was pET-42b(+) (Merck) containing the kanR gene coding for kanamycin antibiotic resistance.

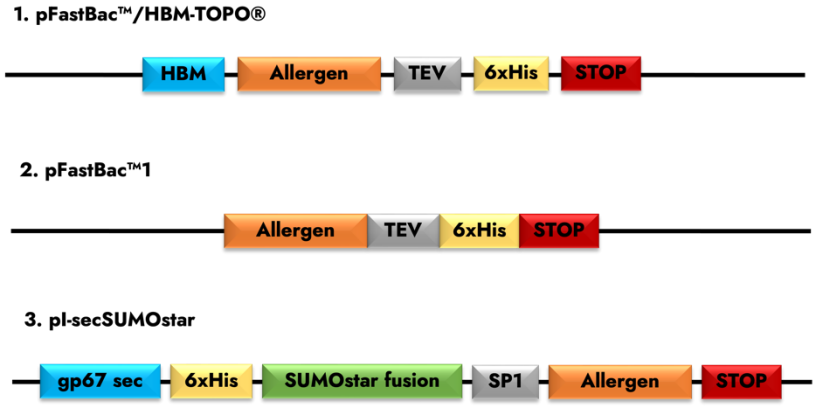


Figure 17. Schematic picture of the vectors used in the study. **1.** pFastBac™/HBM-TOPO® vector contains Honey-bee melittin secretion signal at the N-terminus of the allergen, TEV (tobacco etch virus) protease cleavage site and 6xHis-tag at the C-terminus. **2.** From pFastBac™/HBM-TOPO® vector the TEV protease cleavage site and 6xHis-tag is cloned with the allergen gene into the pFastBac™1 vector. **3.** pI-secSUMOstar vector contains glycoprotein secretion signal (gp67 sec) originated from baculoviruses, 6xHis-tag, a SUMOstar fusion protein and SUMOstar Protease 1 cleavage site (SP1) at the N-terminus. of the allergen.

The vectors used for r-allergen expression in insect cells were pFastBac™/HBM-TOPO (Invitrogen, Thermo Fischer Scientific), pFastBac™1 (Invitrogen, Thermo Fischer Scientific) and pI-secSUMOstar (LifeSensors, Malwern, PA, USA) (Figure 17), all three containing the ampR gene coding for ampicillin antibiotics resistance. The pFastBac™/HBM-TOPO vector contains Honey-bee melittin (HBM) secretion signal at

the N-terminus of the allergen cloning site, TEV protease cleavage site and $(His)_6$ tag at the C-terminus. From pFastBac™/HBM-TOPO vector the TEV protease cleavage site and $(His)_6$ tag is cloned with the allergen gene into the pFastBac™1 vector. pI-secSUMOstar vector contains glycoprotein secretion signal (gp67 sec) originated from baculoviruses, $(His)_6$ tag and a SUMOstar fusion protein at the N-terminus of the allergen.

3.1.1.6 TOPO cloning

The genes were TOPO cloned into pFastBac™/HBM-TOPO vector using Bac-to-Bac TOPO Cloning Kit (Thermo Fischer Scientific) according to manufacturer's protocol.

3.1.1.7 Restriction enzyme digestion and ligation

Restriction sites were incorporated at the 5'- end of the forward and reverse primers to facilitate cloning of the coding sequences into pFastBac™1 vector or pI-secSUMOstar vector. The genes were amplified with primers containing *Bam*HI and *Hind*III restriction sites for cloning into pFastBac™1 vector. The genes and vectors were digested with endonucleases *Bam*HI (New England Biolabs® Inc.) and *Hind*III (New England Biolabs® Inc.) according to manufacturer's protocol. For cloning into pI-secSUMOstar vector the genes were amplified with primers containing *Bsm*BI and *Xba*I restriction sites and the genes and vectors digested with endonucleases *Bsm*BI (New England Biolabs® Inc.) and *Xba*I (New England Biolabs® Inc.) according to manufacturer's protocol. After restriction digestion the products were run on agarose gel, excised, extracted from gel and concentration of nuclease acid measured. The genes were ligated into the vectors with T4 DNA ligase (Thermo Fischer Scientific) according to manufacturer's protocol, except the ratio between gene and vector was 3:1 and 6:1 and the ligation reaction was caried out at 16°C for 24-48 hrs.

3.1.1.8 Production of DH5α chemically competent E.coli cells

E. coli strain DH5α (Thermo Fischer Scientific) from stock was plated on LB agar (1% N-Z amine, 0.5% yeast extract, 1% NaCl, 1.5% bacto agar) and incubated o.n. at 37°C. Colonies were picked and cultured in SOB medium (2% N-Z amine, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl) on a shaker at 16°C until the OD_{580} was between 0.45 - 0.60, put on ice for 10 min, centrifuged for 15 min at 2000 x g at 4°C. The supernatant was discarded and the pellet dissolved with cold HTB buffer (10 mM HEPES, 15 mM $CaCl_2$, 250 mM KCl, 55mM $MnCl_2$, pH 6.7; 3.2 mL per 10 mL of SOB medium) and kept on ice for 10 min, centrifuged for 15 min at 2000 x g at 4°C. The pellet was resuspended in HTB buffer (0.8 mL per 10 mL SOB medium) and finally DMSO (dimethyl sulfoxide, 60 µL per 10 mL SOB) added. The competent cells were dispensed in 100 µL aliquots, quickly frozen in liquid nitrogen and stored at -80°C.

3.1.1.9 Transformation of DH5α chemically competent cells

The DH5α chemically competent *E. coli* cells, kept at -80°C, were put directly on ice. Six µL of the vector or ligation mix were added to the cells, they kept on ice for 30 min, heat-shocked at 42°C for 30 sec and cooled on ice for 2 min. Next 250 µL SOC medium (2% N-Z amine, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose) was added to each vial and incubated at 37°C for 1 hr while being agitated. The transformation mix was spread on preheated LB agar (1% N-Z amine, 0.5% yeast extract, 1% NaCl, 1.5% bacto agar) plates containing appropriate antibiotics (Ampicillin (100 µg/mL) or Kanamycin (50 µg/mL)) and cultured for 16-18 hrs at 37°C.

3.1.1.10 Plasmid purification

Following transformation of chemically competent cells, colonies were cultured in 2.5 mL of LB medium (1% N-Z amine, 0.5% yeast extract, 1% NaCl) with appropriate antibiotics for 16-18 hrs at 37°C while being agitated. The cultures were tested in PCR and plasmid isolated from positive cultures using Nucleospin® Plasmid Kit (Macherey-Nagel, Düren, Germany) according to manufacturer's protocol.

3.1.1.11 Sequencing

For confirmation of successful cloning of allergen gene into vector the products were sequenced with sanger sequencing by deCODE genetics (Reykjavik, Iceland) or GeneWiz Inc. (Leipzig, Germany). Results were analysed in the Sequencer™ program (Gene Codes Corporation, Ann Arbor, MI, USA).

3.2 Expression, production and purification of r-allergens

3.2.1 E. coli

3.2.1.1 Expression and production of r-allergens in E. coli

BL21 (DE3) competent *E. coli* cells (New England Biolabs® Inc.) were transformed with the PET-42b vectors containing the *Culicoides* genes according to manufacturer's protocol. Positive colonies were cultured in LB medium (1% N-Z amine, 0.5% yeast extract, 1% NaCl) overnight and then stored in 25% glycerol at -80°C for further production. For production, pre-culture was made by adding drops of glycerol stock in 20 mL of 2xYT medium (1.6% N-Z amine, 1% yeast extract, 0.5% NaCl) with 50 µg/mL kanamycin and cultured o.n. at 37°C while being agitated. The pre-culture was added to 1 L 2xYT medium (50 µg/mL kanamycin), cultured until OD_{580} reached 0.6, induced with 1 mM Isopropyl β- d-1-thiogalactopyranoside (IPTG) and incubated for 4 h at 37°C while being agitated. The culture was pelleted by centrifuging at 8000 x g for 8 min. The pellet was washed in five steps with three different wash buffers: 1. 100 mM tris pH 8, 2. 100 mM tris, 5 mM $CaCl_2xH_2O$ pH 8, 3. 100 mM tris, 10 mM EDTA pH 8, 4. 100

mM Tris pH 8, 5. 100 mM Tris pH8. Finally, centrifuged at 2500 x g for 30 min at 4°C and the pellet frozen in liquid nitrogen and stored at -80°C.

3.2.1.2 Purification of r-allergens expressed in E. coli

The cell pellet was lyzed with NZY buffer (NZYtech, Lisbon, Portugal) with Protease inhibitor cocktail (PIC) (Sigma – Aldrich, Merck) and incubated at RT under rotation for 30 min. After centrifugation at 16000 x g for 10 min at 4°C the pellet was washed four times in four different wash buffers: 1. 50 mM tris, 50 mM NaCl, 1% triton X114, 1 mM EDTA, pH 8, 2. 50 mM tris, 500 mM NaCl, 1% triton X114, 1 mM EDTA, pH 8, 3. 50 mM Tris, 500 mM NaCl, 0,5% triton X114, pH 8, 4. 50 mM tris, 500 mM NaCl, pH 8. The pellet was resuspended in 50 mM tris, 500 mM NaCl, 8 M urea, pH 8 and incubated under rotation at RT overnight and stored at 4°C. To remove insoluble material the lysate was centrifuged at 16000 x g for 10 min at RT. For r-allergen purification the lysate was incubated with HIS-Select™ HF Nickel Affinity Gel (Sigma – Aldrich, Merck) for 1 h at RT on a vertical rotator followed by centrifugation at 2500 x g for 5 min and the supernatant removed. The affinity gel was washed twice with 6M urea, 20 mM tris, 500 mM NaCl, pH 8 then the r-allergen was eluted and refolded in 20 mM tris, 500 mM NaCl, 400 mM L-arginine-HCl, 5 mM b-cyclodextrin, 10 mM glycerol, 340 mM imidazole, pH 8. The purified r-allergen was dialyzed in 20 mM tris, 500 mM NaCl, 400 mM L-arginine-HCl, 10 mM glycerol, pH 8.

3.2.2 Insect cells

3.2.2.1 Expression of r-allergens in Sf-9 insect cells

The allergens Cul o 8, Cul o 9, Cul o 10 and Cul o 11 were expressed in insect cells using the Bac-to-Bac™ baculovirus expression system (Thermo Fischer Scientific) according to manufacturer's protocol, in short: Plasmids were transformed in DH10Bac *E. coli* cells. Followed by transposition and selection of recombinant bacmids (r-bacmid) with antibiotic selection and isolation of r-bacmids. Sf-9 cells (ATCC®, Manassas, VA, USA) were transfected with the r-bacmids. Supernatant containing the infectious recombinant baculoviruses (r-baculovirus) was used to infect a fresh confluent layer of Sf-9 cells for virus amplification and cloning of the virus with limiting dilution. The cells were cultured in complement Sf-900™II Serum Free Medium (Gibco® by Life Technologies™, Thermo Fischer Scientific), supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 1% fetal bovine serum (Gibco® by Life Technologies™, Thermo Fischer Scientific), in closed culture at 27°C.

3.2.2.2 Production of r-allergens in High-five insect cells

The recombinant allergens were produced in High five cells (ATCC®). High five cells (1.0-1.3 x 10^6 cells/mL) in 96 mL of Sf-900™II medium supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin were infected with 4 mL of viral stock (passage 3)

and incubated at 15°C at 100 RPM in an orbital shaker incubator for 6-10 days or until sufficient cytopathy signs. The cultures were pelleted at 515 x g for 12 min, the pellet snap frozen in liquid nitrogen and stored at -80°C.

3.2.2.3 Purification of r-allergens expressed in insect cells under native conditions

The $(His)_6$ tagged r-allergens were purified using HIS-Select® HF Nickel Affinity Gel (Sigma – Aldrich, Merck) according to manufacturer's protocol, in short: The cell pellet ($100x10^6$ cells) was lysed in 8 mL lysis buffer (50 mM $NaH_2PO_4xH_2O$, 150 mM NaCl, 1% IgePal CA-630, pH 8) with 80 µL Protease Inhibitor Cocktail (PIC) (Sigma – Aldrich, Merck) and sonicated. The lysed cells were pelleted by centrifugation at 23428 x g at 4°C for 15 min and the supernatant incubated with the nickel affinity gel for 2 h at 4°C in vertical rotation. After incubation the gel was centrifuged at 1250 x g for 5 min at 4°C and the supernatant collected. The gel was washed two times in wash buffer (50 mM $NaH_2PO_4xH_2O$, 300 mM NaCl, pH 8) and two times in wash buffer with 10 mM imidazole. The r-allergen was then eluted two times from the gel in elution buffer 1 (50 mM $NaH_2PO_4xH_2O$, 300 mM NaCl, 250 mM imidazole, pH 8) in equal volume to the gel, then two times in elution buffer 2 (same as elution buffer 1 but at pH 6.5) and separated from the gel on Poly-Prep® Chromatography Columns (BioRad, Hercules, CA, USA). Elution fractions were stored in 4°C. The purified r-allergens were dialyzed in 2x phosphate buffered saline (PBS, 171 mM NaCl, 3.3 mM KCl, 1.8 mM KH_2PO_4, 10 mM $Na_2PO_4xH_2O$, pH 7.4) using Slide-A-Lyzer® Dialysis Cassette G2 (Thermo Fischer Scientific) with 7 to 20 MWCO depending on the size of the purified r-allergen.

3.2.2.4 Purification of r-allergens expressed in insect cells under denaturing conditions

r-Allergen purification under denaturing conditions was done using HIS-Select® HF Nickel Affinity Gel (Sigma – Aldrich, Merck) and in general carried out the same as for native purification but with different buffers. The cell pellet was lysed in lysis buffer (6M guanidinium-HCl, 100 mM $NaH_2PO_4xH_2O$, pH 8) and the lysed cell pellet sonicated and centrifuged. The supernatant was incubated with nickel affinity gel for 1 h at RT, centrifuged and the supernatant discarded. The nickel affinity gel wash washed two times in wash buffer (6 M urea, 100 mM $NaH_2PO_4xH_2O$, 10 mM tris-base, 100 mM NH_4Cl, pH 8). Then the r-proteins were eluted and refolded elution buffer (20 mM Tris, 500 mM NaCl, 400 mM L-arginine-HCl, 5 mM b-cyclodextrin, 10 mM glycerol, 340 mM Imidazole, pH 8) and subsequently dialysed in 2xPBS.

3.2.2.5 Deglycosylation of L-linked glycans

Deglycosylation was carried out using PNGase F (N-Glycosidase F) kit (New England Biolabs® Inc.) by cleavage of N-linked glycans of purified r-allergens according to manufacturer's protocol.

3.2.3 Barley

In collaboration with ORF Genetics Cul o 2P, Cul o 2 (hya), Cul o 3 and Cul o 5 originated from *Culicoides obsoletus* and Cul n 8 from *Culicoides nubeculosus* were expressed and produced in barley with $(His)_6$ tag using the Orfeus technique (Erlendsson et al., 2010; Magnusdottir et al., 2013).

3.2.3.1 Transformation of barley

The production of transgenic barley was performed at ORF Genetics according to Erlendsson et al., (2010), in brief: immature barley embryos were isolated from seeds of donor barley plants (*Hordeum vulgare* L. Cv Golden Promise). The embryos were inoculated on callus-inducing medium (CIM) with *Agrobacterium tumefaciens* having been transformed with plasmid containing the expression cassette of interest. In three days, the embryos were transferred to selective CIM containing hygromycin and timentin for 4-6 weeks and then selective shoot induction medium for 5-7 weeks followed by transfer of viable plants to selective root induction media. Plants with strong root systems were further cultivated in soil mixed with pumice.

3.2.3.2 Screening of barley seeds

Three generations of barley seeds, T_1, T_2 and T_3, are required for production and purification of r-allergens (Figure 18). Analysis of r-allergen expression of seeds from each generation was done to select the barley lines to be propagated. For analysis of T_1 lines, four seeds are harvested from the T_0 primary transformant and for analysis of T_2 lines, 5g of seeds are harvested from T_1 plants. Screening of T_1 seeds was done with WB and ELISA on the extract but for screening of T_2 seeds extract and eluate from mini-scale purification was analysed in WB and ELISA.

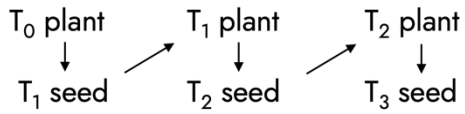


Figure 18. Diagram showing the barley generations required for production and purification of r-proteins.

Extraction: The seeds were milled and mixed in extraction buffer (50 mM $KiPO_4$, 500 mM NaCl, pH 7.4) for 1 h at RT for extraction of the r-allergen. The extracts were centrifuged at 3571 x g for 15 min at RT and the supernatant analysed in WB and ELISA.

Mini-scale purification: Immobilized metal-ion affinity chromatography (IMAC) magnetic beads Co SepFast MAG (Cat. 190103-50-mL, Biotoolomic, Consett, UK) were added to the extract and incubated for 1 h at RT on vertical rotation. Then a magnetic field was applied to attract IMAC magnetic beads and the supernatant removed. The magnetic beads were washed twice (50 mM $KiPO_4$, 500 mM NaCl, 50 mM imidazole,

pH 7.4), followed by incubation for 30 min in elution buffer (50 mM $KiPO_4$, 500 mM NaCl, 500 mM imidazole, pH 7.4) for elution of r-allergens and thereafter the eluate was analysed in WB and ELISA.

ELISA: Nunc-Immuno™ 96 well Microwell™ (Merck) plates were coated with 50 µL/well undiluted extract of barley seeds and incubated at 37°C for 1 h. The plates were washed with wash buffer (1xPBS_{ORF} (137mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4), 1.5 mM KH_2PO_4, pH 7.2), 0.005% Tween20) using Wellwash™ Microplate Washer (Thermo Fisher Scientific) between incubation steps. Blocking of unspecific binding was done with 100 µL/well blocking buffer (1xPBS, 3% bovine serum albumin (BSA, Reagent diluent concentrate 2 (10x), Cat. DY995, R&D Systems, Minneapolis, MN, USA) at 37°C for 1 h. Following, the plates were incubated at 37°C for 1 h in primary antibody, 50 µL/well Anti-His (Cat. Ab9108, Abcam, Cambridge, UK) diluted 1:3000 in dilution buffer (1xPBS, 1% BSA). Then incubated at 37°C for 1 h in secondary antibody, 50 µL/well goat anti-rabbit (Cat. P0448, Dako Agilent, Santa Clara, CA, USA) diluted 1:3000 in dilution buffer. The plates were developed with 100 µL/well TMB solution (Dilute substrate reagent Cat. DY999, R&D Systems) at RT for 30 sec. – 5 min. Once blue colour developed then the reaction was stopped using 100 µL/well 0.2 M sulfuric acid and OD measured at 450 nm using Multiskan™ FC Microplate Photometer (Thermo Ficher Scientific). The OD_{450} values of the barley seed lines screened were compared to OD_{450} values of positive control, transgenic barley expressing epidermal growth factor (EGF), which determined the cut-off value for positive and negative lines.

3.2.3.3 Optimization of barley r-allergen extraction

Extraction conditions of the r-allergens were tested with extraction buffers in a range of different pH (listed below) for selection of which gave highest yield of soluble r-allergen.

1. 50 mM Citrate, 500 mM NaCl, pH 4.0

2. 50 mM Acetate, 500 mM NaCl, pH 5.0

3. 50 mM MES, 500 mM NaCl, pH 6.0

4. 50 mM MES, 500 mM NaCl, pH 6.5

5. 50 mM $KiPO_4$, 500 mM NaCl, pH 7.0

6. 50 mM $KiPO_4$, 500 mM NaCl, pH 7.5

7. 50 mM Tris, 500 mM NaCl, pH 8.0

8. 50 mM Bicine, 500 mM NaCl, pH 8.8

3.2.3.4 Purification of barley r-allergen

R-allergens were purified using the ÄKTA avant chromatography system (Cytiva, Marlborough, MA, USA). Barley seeds expressing r-allergen, were milled and extracted in extraction buffer. The crude extract was clarified by centrifuging at 7450 x g for 10 min. Further clarification was achieved through tangential flow filtration (TFF) using diafiltration and ultrafiltration. First the supernatant was diafiltrated through 750 kDa hollow fiber membrane (Cytiva) and then the permeate was ultra-filtrated with 3 kDa hollow fiber membrane (Cytiva). The concentrated sample was loaded onto a HiTrap IMAC Sepharose FF affinity chromatography column (Cytiva) with binding conditions of 10 or 30 mM imidazole, washed with 20/40 and 40/80 mM imidazole, eluted with 500 mM imidazole and the peak representing r-allergen of interest collected. The protein eluate was diluted 10x in 50 mM MES (2-(N-morpholino) ethane sulfonic acid), pH 6.5 buffer and loaded on HiTrap sulphopropyl (SP) HP cation exchange chromatography column (Cytiva) and eluted with 700 mM and 1M NaCl. Finally, the r-allergen was dialyzed in 2xPBS using Slide-A-Lyzer® Dialysis Cassette G2 (Thermo Fisher Scientific) with 7 to 20 MWCO depending on the size of the purified r-allergen.

3.2.4 Protein analysis

3.2.4.1 SDS-PAGE

Proteins were separated by Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions based on their electrophoretic mobility in Mini-protean II system (Bio-Rad). Denaturing buffer (2xSample buffer, 0.5% 2-mercaptoethanol, 20% glycine, 2% SDS, 0.1% bromophenol blue, 130 mM tris) was added to the protein samples, then boiled for 5 min at 100°C, spun for 2 min and run on 12% or 14% acrylamide gels (Separating gel: 375 mM tris pH 8.8, 12-14% acrylamide, 0.01% SDS, 0.01% Ammonium Persulfate, 0.002% Tetramethylethylenediamine) at 200V in SDS-PAGE running buffer (25 mM tris, 192 mM glycine, 0.1% SDS). PageRuler™ Prestained Molecular Mass Marker (Cat. 26616, Thermo Fischer Scientific) was used to estimate the size of the r-allergens. The proteins were visualized using western blotting or protein staining.

3.2.4.2 Western blotting

The proteins were transferred to a PVDF membrane following SDS-PAGE by wet transfer in the Miniprotean II system (Bio-Rad) at 100V for 1 h in a transfer buffer (25 mM tris, 192 mM glycine, 20% methanol). The membranes were blocked for 30 min at RT in TBS-T (tris buffered saline containing 0.1% Tween 20) with extra 2% Tween 20. After washing, the membranes were incubated o.n. at 4°C with primary antibody: allergen specific polyclonal antibodies (pAb) or monoclonal antibodies (mAb) (Table 3) (Gudnadottir, 2021; Jonsdottir, 2011) or mouse-anti-Histidine Tag (1.0 mg/mL, Cat. MCA1396 BioRad) diluted 1:1000. Then after washing, incubated for 1 h at RT with the

secondary antibody: Alkaline Phosphatase (AP) conjugated AffiniPure goat-anti-mouse IgG (H+L) (0.6 mg/mL, Cat. 115-055-071, Jackson ImmunoResearch Labs, West Grove, PA, USA) with 1:5000 dilution. After washing, the membranes were developed using BCIP/NBT stock solution (Cat. 11681451001, Roche, Basel, Switzerland) diluted 1:50 in AP buffer (100 mM tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 0.005% Tween 20, pH 9.5).

Table 3. Dilutions of allergen specific antibodies used in the study. The antibodies were raised against purified *E. coli* expressed r-allergens.

Antibody		Origin	Dilution
α-Cul o 1P	pAb	Mouse ascites (Keldur)	1:2000
α-Cul o 2P	pAb	Mouse ascites (Keldur)	1:2000
α-Cul o 5	pAb	Mouse ascites (Artic LAS)	1:2000
α-Cul n 1 (Cul o 3)	pAb	Mouse ascites (Keldur)	1:20000
α-hya (e16 (Cul n 2))	mAb	Hybridoma supernatant	1:10
α-Cul n 4	pAb	Mouse ascites (Keldur)	1:4000
α-Cul n 8 (g13)	pAb	Mouse ascites (Keldur)	1:2000

Protein staining

SDS-PAGE gels were stained with Coomassie blue staining according to Wong et al. or with GelCode™ Blue Safe Protein stain (Thermo Fisher Scientific) according to manufacturer's protocol. After the staining the gels were dried between two Gel drying frames (Merck) at RT o.n. in storage solution (25% methanol, 7% acetic acid, 3% glycerol).

3.2.4.3 Bradford protein assay

The protein concentration of samples was measured using Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific) at 595 nm in a micro-plate spectrometer (Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific) according to manufacturer's protocol.

3.3 Experimental horses

Horses used for vaccination and challenge experiments were healthy Icelandic horses located in Iceland. All horses were maintained according to the Icelandic care guidelines for experimental animals. The experiments were accepted by the National Animal Research Committee of Iceland (no. 2018-05-04 and no. 2019-01-12). The horses used in the challenge experiment were exported to Switzerland following vaccination and placed in 13 different farms (Table 4). Before vaccination, the six horses in the s.c., alum/VLP group were used as control horses in immunoassays for experimental vaccination comparing route of injection. The six control horses listed in Table 4 were used for experimental vaccination comparing adjuvant effect. The control horses for the challenge experiment were exported at the same time as the vaccinated

horses to Switzerland but kept at different farms with different maintenance as they were protected from *Culicoides* bites either with a blanket or by stabling during dusk and dawn.

Table 4. Information about the healthy Icelandic horses used in the experimental vaccinations.

Experiment	Adjuvant	Route of administration	Mean age (range)	Male/Female
Comparison of injection route and adjuvant effect	alum/MPLA	i.l.	5.8 (4 - 8)	4/2
	alum/MPLA	s.c.	7.2 (6 - 9)	5/1
	alum/VLP	s.c.	7.3 (6 - 10)	6/0
	controls	N.A.	7.8 (4 - 10)	6/0
Challenge experiment	alum/MPLA	i.l.	9.5 (6 - 13)	15/12
	controls	N.A.	7.9 (6 – 14)	13/7

3.4 Vaccinations

3.4.1 Intralymphatic and subcutaneous vaccination with r-allergens in mixture with alum/MPLA or alum/VLP

Eighteen horses were divided into three groups. All horses received three *E.coli* expressed and purified r-allergens (rCul o 2P, rCul o 3 and rCul n 4), 20 µg of each r-allergen per injection, three times with four week interval (Figure 19). rCul o 2P was produced and purified according to Peeters et al. 2013 and eluted in 20 mM tris, 500 mM NaCl, pH 8. rCul o 3 was purchased from GenScript® and eluted in PBS, 10% glycerol, 1 M L-arginine-HCl, 2 mM DTT, pH 7.4. rCul n 4 was produced and purified as described in chapter 3.2.1. and eluted in 20 mM tris, 500 mM NaCl, 400 mM L-arginine-HCl, 10 mM glycerol, pH 8. Six horses were vaccinated i.l. with the r-allergens in mixture with 500 µg aluminum hydroxide gel (Alhydrogel® 2%, Invivogen, San Diego, CA, USA) (alum) and 50 µg MPLA (MPLA-SM VacciGrade™, InvivoGen) (alum/MPLA), six horses s.c. in the lateral neck using the same combination of allergens and adjuvants. The last group of six horses were vaccinated at another time point, s.c. with the same allergens in mixture of 500 µg alum and 300 µg of VLP derived from cucumber mosaic virus CuMV. Body temperature and site reactions were recorded before and after each vaccination except only for third vaccination in the i.l. and s.c. vaccination with alum/MPLA. Blood was collected before vaccination and every other week for 20 weeks and serum harvested for analysis of the antibody response. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized

blood before vaccination and two weeks after second and third vaccination for *in* vitro stimulation and analysis of the cytokine response following vaccination. Skin test was performed at week 18 (3.5.8.) (Figure 19).

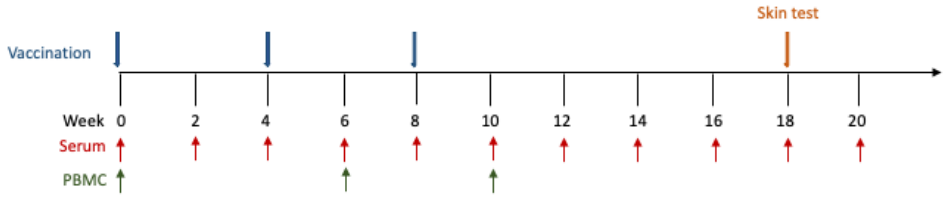


Figure 19. Diagram showing vaccination and blood sampling time-points of experimental vaccination comparing route of injection (i.l. and s.c.) and adjuvant effects (MPLA and VLP). Weeks are shown in black on the time-line. **Blue** arrows: vaccination time-points, **orange** arrows: time of skin test, **red** arrows: serum sampling time-points, **green** arrows: heparinized blood sampling for isolation of PBMC.

3.4.2 Challenge experiment

Twenty seven horses were vaccinated i.l. with nine *E .coli* expressed and purified r-allergens (rCul o 1P, rCul o 2P, rCul o 3, rCul o 5, rCul o 7, rCul o 8, rCul o 9, rCul o 11, rCul n 4), 20 µg of each per injection in mixture with alum/MPLA, three times with four week interval (Figure 20). Body temperature, site reactions and blood count were recorded before and after each vaccination. Blood was collected before vaccination and every other week for 12 weeks and serum harvested for analysis of antibody response. More samples were collected which are not relevant for this project; Heparinized blood was collected before vaccination and two weeks after third vaccination, PBMC isolated for *in vitro* re-stimulation. Three different set-ups of PBMC re-stimulation were performed to be used in three different methods for analysis of the cytokine response (i.e. RNA single cell sequencing, qPCR and bead-based multiplex assay). Before vaccination was carried out, two skin biopsies from groin were collected, whole skin for RNA sequencing and isolated epidermis. Following third vaccination and before export, skin and rectal swabs were collected for analysis of microbiota.

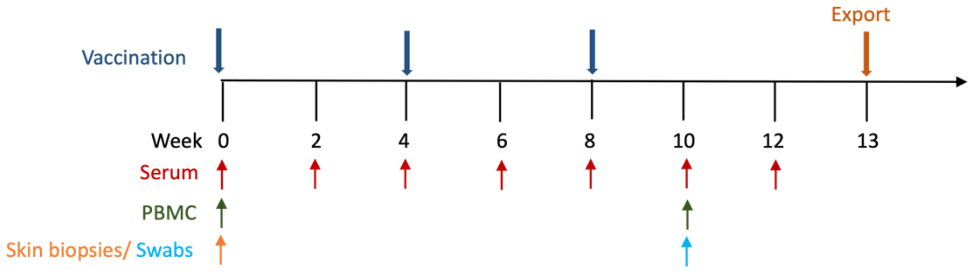


Figure 20. Diagram showing vaccination and sampling time-points of challenge experiment. Weeks are shown in black on the time-line. **Blue** arrows: vaccination time-points, **orange** arrows: time of export, **red** arrows: serum sampling time-points, **green** arrows: heparinized blood sampling for isolation of PBMC, **bright orange** arrow: time of skin biopsy sampling, **light blue** arrow: rectal and skin swabs collected.

In week 13 the horses were exported to Switzerland where they were placed in 13 different farms with known presence of *Culicoides*. The vaccinated horses were followed for three years with monthly blood collection for serum and clinical examination during the *Culicoides* season (May – Sept). The horses received booster vaccination before the *Culicoides* season the second and third year and heparinized blood was collected before and after the booster and after the *Culicoides* season for isolation of PBMC (Figure 21).

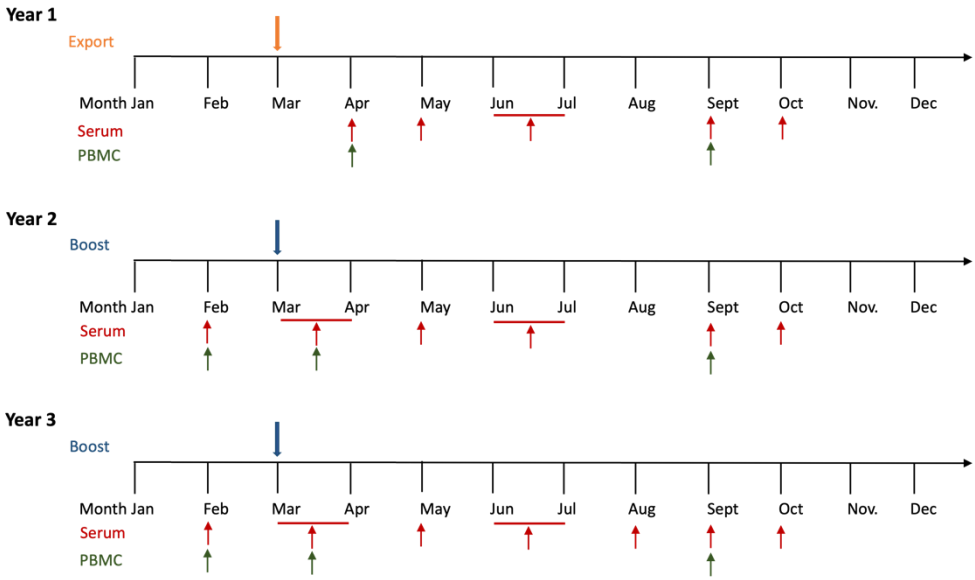


Figure 21. Diagram showing blood sampling time-points of challenge experiment the first two years. Weeks are shown in black on the time-line. **Blue** arrows: booster vaccination time-points, **orange** arrows: time of export from Iceland to Switzerland **red** arrows: serum sampling time-points, **green** arrows: heparinized blood sampling for isolation of PBMC.

3.5 Immunological assays

3.5.1 Antibody ELISA

Allergen specific antibodies were detected in serum of horses using three different ELISA protocols. For analysis of ELISA results the OD values were corrected by subtracting the blank and to correct for variations between plates a correction factor was calculated for each plate set (i.e. plates for the same allergens and antibody class) using the average OD values of positive control samples. Further data analysis was performed using corrected OD values and the data presented as corrected OD values (Peeters et al., 2013).

3.5.1.1 ELISA protocol 1 – total IgG:

Nunc-Immuno™ 96 well Microwell™ plates (Merck) were coated with 0.2 µg/well (100 µL/well) of r-allergen in Carbonate-Bicarbonate Buffer, pH 9.6 (Sigma – Aldrich, Merck), incubated for 2 h at 37°C and frozen at -20 °C. Before use, the plates were thawed at 37°C. Plates were washed in high-salt wash buffer (0.5 M NaCl, 0.05% Tween 20 in PBS) using Wellwash™ Microplate Washer (Thermo Fisher Scientific) between incubation steps. Blocking of unspecific binding was done with 200 µL/well blocking buffer (5% Tween 20, 5% skimmed milk) for 1 h at RT. For detection of total allergen-specific IgG in horse sera, serum samples were added to the plates in serial dilutions from 1:200 to 1:800 (100 µL/well) and incubated for 1 h at 37°C. Then incubated for 1 h at 37°C in primary antibody Horseradish Peroxidase (HRP) conjugated AffiniPure goat-anti-horse IgG (H+L) (0.8 mg/mL, Cat. 108-035-003, Jackson ImmunoResearch Labs) in dilution 1:7000 (100 µL/well). Dilutions of samples and conjugates were made in blocking buffer. The plates were developed with 200 µL/well SIGMA*FAST*™ OPD peroxidase substrate (Cat. P9187, Sigma – Aldrich, Merck) for 15 min at RT. The reaction stopped with 50 µL/well sulfuric acid (3N H_2SO_4) and the absorbance (optical density (OD)) was measured at 492 nm using Multiskan™ FC Microplate Photometer (Thermo Ficher Scientific).

3.5.1.2 ELISA protocol 2 – IgE and IgG subclasses (Vetsuisse, Berne, Switzerland):

ELISA protocol 2 was used for evaluation of immune response following experimental vaccinations comparing route of injection (section 4.2.5) and adjuvant effect (section 4.3.4). The plates were coated with 0.2 µg/well of r-allergen in BupH Carbonate – Bicarbonate Buffer packs (Thermo Fisher Scientific) (100 µL/well) and incubated for 2 h at 37°C. Plates were washed manually in wash buffer (150 mM NaCl, 0.005% Tween 20) between incubation steps. Blocking of unspecific binding was done with 200 µL/well blocking buffer (PBS, 5% Tween 20, 5% skimmed milk) for 1 h at 37°C. For detection of IgG subclasses, the serum samples were added to the plates in dilution 1:800 and 1:10 for IgE. The serum dilutions were determined by serum titrations in a

pilot experiment (appendix XIII). Dilutions of samples and conjugates was made in blocking buffer. Plates were incubated for 2 h at RT in primary antibody specific for the IgG subclasses IgG1, IgG4/7, IgG5 (2mg/mL) (Goodman et al., 2012) diluted 1:2000 (100 µL/well) in blocking buffer or IgE (1.81 mg/mL, clone 3H10) (Wilson et al., 2006) at dilution 1:1500 (100 µL/well) or goat-anti-horse IgA (BioRad) at dilution 1:250 (100 µL/well). They were then incubated in secondary antibody AP conjugated goat-anti-mouse IgG (Fcγ) (0.6 mg/mL, Cat. 115-055-071, Jackson ImmunoResearch Labs) diluted 1:2000 (100 µL/well) for 1 ½ h at RT. The plates were developed with 200 µL/well phosphatase substrate (1.5 mg/mL, Cat. P4744, Sigma – Aldrich, Merck) in 10% diethanolamine (Fluka, Honeywell, Charlotte, NC, USA) pH 9.8 and the OD at 405 nm measured after 15, 20, 30 min and 1 h.

3.5.1.3 ELISA protocol 3 – IgG subclasses (Keldur, Iceland):

ELISA protocol 3 was used in for evaluation of immune response of horse vaccinated in Iceland and challenge by export to Switzerland with known presence of *Culicoides* (section 4.5.5). In principle, the protocol uses same set-up as ELISA protocol 2 with few changes in buffers and reagents. The plates were coated with 0.2 µg/well (100 µL/well) of r-allergen in Carbonate-Bicarbonate Buffer, pH 9.6 (Merck) and washing done with high-salt wash buffer (0.5 M NaCl, 0.05% Tween 20 in PBS) using Wellwash™ Microplate Washer (Thermo Fisher Scientific). The secondary antibody used was HRP conjugated goat-anti-mouse IgG (Fcγ) (0.8 mg/mL, Cat. 115-035-071, Jackson ImmunoResearch Labs) diluted 1:2000 (100 µL/well). The plates were developed with 200 µL/well SIGMA*FAST*™ OPD peroxidase substrate (Cat. P9187, Sigma – Aldrich, Merck) for 15 min at RT, the reaction stopped with 50 µL/well sulfuric acid (3N H_2SO_4) and the OD was measured at 492 nm.

3.5.2 Blocking ELISA

The IgE blocking capacity of the serum antibodies induced by immunotherapy were determined as follows: The ELISA was carried out as the IgE antibody ELISA (3.5.1.2) except that serial dilutions of pooled sera from treated horses (before and after immunization) were added to the plate and incubated for 1 h at 37°C, prior to the addition of serum from an IBH affected horse with high IgE to the corresponding r-allergen (diluted 1:10 or 1:16 depending on r-allergen tested). The percentage of blocking was calculated using the following equation.

$$\%\ \text{blocking} = 100 - \left(\frac{\text{OD of IBH serum after blocking with serum from treated horse}}{\text{OD of IBH serum}} \times 100\right)$$

3.5.3 Immunoblot analysis

The immunoblot analysis was used for detection of allergen specific antibodies, total IgG or IgG1, in serum. In general, the procedure is same as for SDS-PAGE and Western blotting (3.2.4.1 and 3.2.4.2) but after blocking, the PVDF membranes were cut into strips and used directly or dried and stored at RT in dark before use. Membranes were incubated o.n. at 4°C with horse sera diluted 1:2000 followed by incubation for 1 h at RT in secondary antibody AP conjugated goat-anti-horse IgG (H+L) (Jackson ImmunoResearch Labs1:2000 and development with BCIP/NBT (Merck).

3.5.4 Isolation and re-stimulation of PBMC *in vitro*

PBMC were isolated and re-stimulated for analysis of cytokine response following vaccination. Blood was collected by jugular puncture into vacutainer tubes (Vacuette, Greiner, Kremsmünster, Austria) containing lithium heparin. The blood was mixed and then erythrocytes allowed to sediment for 30 min at RT. The leucocyte-rich-plasma layer is harvested and laid on Histopaque®-1077 (1.077 g/mL, Cat.10771 Sigma – Aldrich, Merck) and centrifuged at 450 x g for 20 min at RT without brake. The interphase band with the PBMC harvested and washed to get rid of platelets with PBS supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin and centrifuged at 290 x g for 20 min with brake at 7; the wash was repeated twice and centrifuged at 200 x g for 10 min with brake on. After the wash the cells were resuspended in RPMI 1640 Medium GlutaMAX™ (Gibco® by Life Technologies™, Thermo Fisher Scientific) supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 10% inactivated horse sera (Keldur) and 50 µM 2-mercaptoethanol, the cells counted and seeded in cell culture plates. PBMC were re-stimulated for analysis of expression and production of cytokines (IFN-γ, IL-10 and IL-4) following vaccination. The stimulants used were a mixture of r-allergens used for vaccination (2 µg/mL each) mixture of r-*Culicoides* allergens not included in the vaccine, medium alone was used as negative control and Phytohaemagglutinin (Sigma – Aldrich, Merck) (PHA 1 mg/mL) as positive control. The PBMC were incubated with the stimulants at 37°C and 5% CO_2 for 18-48 h depending on the method used for analysis.

For cytokine measurement at mRNA level, $3x10^6$ cells/well (1.5 mL/well) for each stimulant were re- stimulated in a 24-well plate for 24 h. The PBMC were harvested by centrifuging the plate at 600 x g for three minutes without brake, supernatant discarded and PBMC lysed in RNA easy Plus lysis buffer (Quiagen, Hilden, Germany) and kept at 4°C until isolation of RNA.

For analysis of intracellular cytokines, $5x10^6$ cells/well (1.5 mL/well) for each stimulant were re-stimulated in 24-well plate for 18 h at 37°C. After 14 h incubation, ™Brefeldin A (Cat. B7651, Sigma – Aldrich, Merck) was added to the cells for inhibition of intracellular protein transport. The cells were harvested and immediately stained for flow cytometry.

For measurement of secreted cytokines in the supernatant of re-stimulated PBMC, $0.5x10^6$ cells/well (200 µL/well) for each stimulant in duplicate were re-stimulated in 96-well plate for 48 h at 37°C. The supernatant was harvested and kept at -80°C until measurement with bead-based multiplex assay.

3.5.5 Isolation of RNA and quantitative real time PCR (qPCR)

RNA was isolated with RNasy 96 kin in Quiacube HT (Quiagen) according to manufacturer's protocol. The isolated RNA was stored at -80°C until measurement of mRNA with qPCR. Isolated RNA was tested for the expression of IFN-γ, IL-10 and IL-4 cytokines with 18S as reference according to Heimann et al. (2011). The primers and probes for IFN-γ and IL-10 were obtained from Lanz et al. (2013) and for IL-4 from Svansson et al. (2009). The reactions were performed with AgPath-ID One-Step RT-PCR Kit master mix (Life Technologies, Thermo Fischer Scientific) by StepOnePlus™ RealTime PCR System (Applied BioSystem, Thermo Fischer Scientific).

3.5.6 Bead-based multiplex assay

Secreted cytokines, IFN-γ, IL-10 and IL-4 were measured in the supernatant of re-stimulated PBMC with bead-based multiplex capture sandwich immunoassay (Luminex) according to Wagnes & Freer (2009).

3.5.6.1 Coupling of antibodies

MagPlex-C Microspheres (Luminex, Austin, TX, USA) were coupled with monoclonal Ab; bead 33 coupled with mouse-anti-equine IL-10 mAb (clone 492-1) (Wagner et al., 2008a), bead 35 coupled with mouse-anti-equine IL-4 mAb (clone 25) (Wagner et al., 2012), beads 38 to mouse-anti-bovine IFN-γ mAb (clone CC302) (BioRad). The coupling in brief: 5.0 x 10^6 microspheres were washed with 100 µL dH_2O, resuspended in 80 µL 0.1 M Sodium Phosphate (monobasic) pH 6.2. For activation, 10 µL of 50 mg/mL Sulfo-NHS (N-hydroxysulfosuccinimide) diluted in activation buffer (0.1 M NaH_2PO_4, pH 6.2) were added and 10 µL of 50 mg/mL EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide) to the microspheres and incubated for 20 min at RT. By using magnetic separator, the supernatant is discarded and the microspheres washed two times in 250 µL of 50 mM MES, pH 5.0 and then resuspended in 100 µL of 50 mM MES pH 5.0. 100 µg of antibody was diluted in 400 µL 50 mM MES is added to the activated microspheres and coupling performed for 3h with rotation. The coupled beads are blocked by incubation in 500 µL 1% bovine serum albumin (BSA) in PBS for 30 min at RT by rotation, then washed 2x with 1 mL PBS-TB (0.1% BSA, 0.02% Tween 20 in PBS) and finally resuspended in 1 mL PBS-TB, counted and stored at 4°C in the dark.

3.5.6.2 Capture Sandwich Immunoassay

The assay in brief: the coupled beads were diluted to 50 microspheres of each set per µL in assay buffer (1% BSA in PBS, Sigma – Aldrich, Merck) and 50 µL of mixture dispensed in appropriate wells. 50 µL of samples and standards were added to respective wells and incubated for 30 min at RT on shaker in the dark. Supernatants of equine recombinant cytokines/IgG fusion proteins were used as standards for quantification of the individual cytokines (Wagner & Freer, 2009a). The plates were washed between incubation steps in assay buffer. Primary antibodies were added in appropriate dilution, biotinylated mouse-anti-equine IL-4 mAb (clone 13G7, 2 mg/mL) (Wagner et al., 2006b) at dilution 1:800, biotinylated mouse-anti-equine IL-10 mAb (clone 492-2, 2 mg/mL) (Wagner et al., 2008a) at 1:100 and biotinylated mouse-anti-equine IFN-γ mAb (clone 36, 2mg/mL) (Wagner et al., 2015) at 1:500 and incubated for 30 min at RT in dark on shaker. R-phycoerythrin conjugate streptavidin (1 mg/mL, Cat. S866, Thermo Fisher Scientific) was added at dilution 1:100 and incubated for 30 min at RT in dark on shaker and finally the microspheres resuspended in 100 µL PBN and incubated for 10-15 min before measurement in Bio-Plex®200 Systems (BioRad). The measurements of IFN-γ and IL-4 were performed in one plate and IL-10 on another plate because of unspecific background measurement of IL-10 when in mixture.

3.5.7 Fluorescence activated cell sorting (FACS)

Isolated and re-stimulated PBMC were stained and analysed for expression of CD4, CD8, IFN-γ, IL-10 and IL-4 using SH800s Cell Sorter (Sony Biotechnology). Dilutions of conjugated antibodies was decided with compensation experiments (data not shown). The PBMC were washed with cold PBS, then dissolved in 210 µL cold PBS and placed on 96-well plate. The plate was centrifuged at 600 x g for 4 min at 4°C, the supernatant discarded and the plate placed on ice. Mouse anti-equine CD4 mAb (clone CVS4, 0.1 mg/mL) FITC conjugated (Cat. MCA1078F, BioRad) diluted 1:50 and mouse-anti-equine CD8 mAb (clone HT14A, 1.0 mg/mL) (Cat.HRS2007, Monoclonal antibody center, Washington State University) conjugated with Pacific Blue™ Antibody Labeling Kit (Cat. P30013, Thermo Fisher Scientific) diluted 1:67 was added to the PBMC for surface staining and incubated for 15 min. The cells were washed with 200 µL cold PBS. The PBMC were fixed and permeabilizated using 100 µL of BD fix solution from Fixation/Permeabilization Solution Kit (Cat. 554714, BD Biosciences, Franklin Lakes, NJ, USA) and incubated for 30 min at 4°C, then washed with 200 µL BD wash (BD Biosciences). For intracellular staining, mouse-anti-equine IFN-γ mAb (clone 38-1, 2 mg/mL) A647 directly labelled (Wagner et al., 2015) diluted 1:700, mouse-anti-equine IL-10 mAb (clone 165-2, 2 mg/mL) (Wagner et al., 2008a) conjugated with Qdot 800 (Cat. S10455 , SiteClick™ Antibody Labelling Kit, Thermo Fisher Scientific) diluted 1:10 and mouse-anti-equine IL-4 mAb (clone 13G7, 2 mg/mL) (Wagner et al., 2006b) conjugated with Zenon R-Phycoerythrin (R-PE) (Cat. Z25055, Zenon ™ Mouse IgG_1 Labeling Kit, Thermo Fisher Scientific) diluted 1:20 in BD wash were added to the

PBMC and incubated for 30 min at 4°C. Then the PBMC were washed in BD wash and finally dissolved in BD wash and transferred to tubes for FACS. Results were analysed in Kaluza® analysis software (version 2.1 from Beckman Coulter).

3.5.8 Intradermal test (IDT)

Following vaccination, IDT was performed to analyse whether vaccinated horses were sensitized to the r-allergens used in the vaccine. The horses were injected i.d. in the lateral neck with 0.1 mL of each of the r-allergens tested diluted 10 µg/mL (1 µg) and 1 µg/mL (0.1 µg). Allergen dilution buffers alone and PBS were used as negative controls and histamine, 0.2 mg/mL (20 µg) as positive control. The response was measured in diameters of the wheal reaction at injection site 30 min, 1 h, 4 h, 24 h and 48 h post injection. Negative controls were used as base line for interpretation of positive reaction against the r-allergens.

3.5.9 Sulfidoleukotriene release test

A sulfidoeukotriene (sLT) release test was used to analyse whether IBH-affected horses were sensitized to the allergens expressed in insect cells (or whether the allergens expressed in insect cells were functionally relevant for IBH). Peripheral blood leukocytes of IBH-affected and control horses were incubated with Bac-r-allergens at 37°C for 40 min and the supernatant harvested. Released sLTs in the supernatants were determined using CAST 2000-ELISA according to the manufacturer's instructions (Bühlmann Laboratories AG, Schönenbuch, Switzerland) (Baselgia et al., 2006).

3.6 Statistical analysis

Statistical analysis and plotting of graphs were carried out in GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA). Non-parametric tests were used for analysis of the data as Shapiro Wilk test showed it did not follow normal distribution. For analysis of IgG subclass antibody response, Friedman's test was used for comparison of time points within each of the vaccination groups. Mann-Whitney U test was used for comparison of vaccination groups, each time point tested in the vaccination was examined for statistical difference between groups and Bonferroni correction used to correct for multiple comparisons. Cytokine response analysis was done using Wilcoxon test for comparisons of time points within each vaccination group and using Kruskal-Wallis test for comparisons of vaccination groups and control horses. For all tests $p \leq 0.05$ was considered the significance threshold.

4 Results

4.1 Production and purification of r-allergens in different expression systems

4.1.1 E.coli

4.1.1.1 Expression of r-allergens in E. coli

The codon optimized allergen genes (appendix I) *Cul o 1P*, *Cul o 2P*, *Cul o 3* and *Cul n 4* were cloned in PET-42b expression vector (GenScript®) with 3-amino-acid C-terminus linker (GGC). Following, BL21 (DE3) competent *E. coli* cells were transformed with the vectors containing an allergen gene.. To ensure the right reading frame of the allergen genes the vectors containing *Cul o 1P* and *Cul n 4* were sequenced (data not shown). For production of recombinant proteins, the cells were cultured in rich growth, 2xYT medium and induced with IPTG.

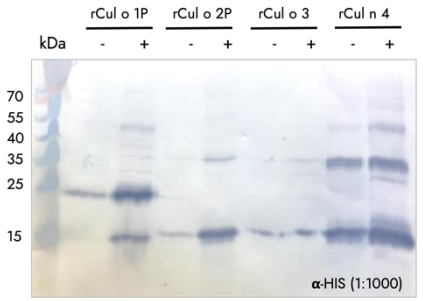


Figure 22. Protein expression in *E.* coli. WB showing expression of rCul o 1P (24.3 kDa), rCul o 2P (18.5 kDa), rCul o 3 (30.9 kDa) and rCul n 4 (18.4 kDa) containing 3-amino-acid linker (GGC) in BL21 (DE3) *E.coli* cell culture before (–) and following IPTG induction (+).

Following IPTG induction of the *E. coli* cultures there was an increase in expression of three of the r-allergens, rCul o 1P (GGC), rCul o 2P (GGC) and rCul n 4 (GGC) (Figure 22). There was no visible expression of rCul o 3 (GGC).

4.1.1.2 Purification of r-allergens from E. coli

All three r-allergens, rCul o 1P (GGC), rCul o 2P (GGC) and rCul n 4 (GGC). were purified from BL21 (DE3) *E.coli* cells using HIS-select nickel affinity gel under denaturing conditions with refolding in the elution steps. The expression levels of rCul o 1P (GGC) and rCul o 2P (GGC) were low based on WB analysis (Figure 22) and therefore the purification resulted in low amount of purified r-protein (appendix IX). The

r-proteins needed to be dialysed to eliminate imidazole as they were intended for vaccination. This resulted in complete loss of both rCul o 1P (GGC) and rCul o 2P (GGC) (data not shown).

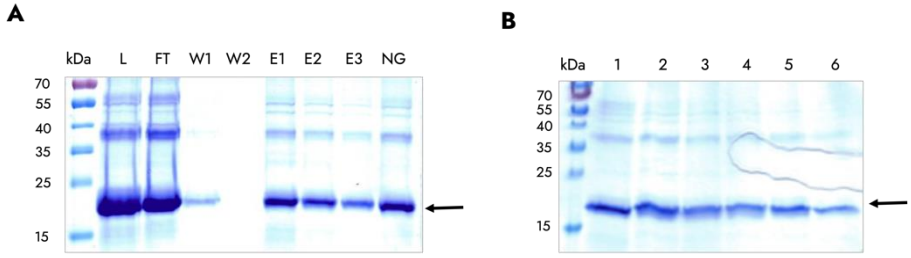


Figure 23. rCul n 4 (GGC) protein purification from *E. coli* cells. Protein stained gel showing **A.** Purification of rCul n 4 (GGC) from BL21 (DE3) *E.coli* cells using HIS-select nickel affinity gel. **L:** lysed cell pellet; **FT:** Flowthrough; **W 1-2:** Wash 1-2; **E 1-3:** Elution 1-3; **NG:** Nickel gel following purification. **B.** Dialysis of purified rCul n 4 (GGC); **1.** rCul n 4 (GGC) eluted in 20 mM Tris, 500 mM NaCl, 400 mM L-arginine, 5 mM b-cyclodextrin, 10 mM glycerol, 340 mM imidazole pH 8; **2.** rCul n 4 (GGC) following dialysis eliminating imizazole; **3.** After elimination of cyclodextrin; **4.** After elimination of glycerol, **5.** After dialysa reducing L-arginine to 200 mM; **6.** After elimination of L-arginine. The arrows indicate rCul n 4 (GGC).

The highest protein expression levels in *E. coli* were observed for rCul n 4 (Figure 22), resulting in higher amount of purified r-protein following purification (Figure 23A) compared to rCul o 1P and rCul o 2P. Following purification, the rCul n 4 was eluted in 20 mM Tris, 500 mM NaCl, 400 mM L-arginine, 5 mM b-cyclodextrin, 10 mM glycerol, 340 mM Imidazole pH 8. Stepwise dialysis was performed to determine which chemicals could be eliminated without great loss of the purified r-protein. There was not much loss of the purified rCul n 4 following elimination of imidazole based on band intensity on the protein-stained gel (Figure 23B). However, elimination of the aggregation suppressors as cyclodextrin and L-arginine and the stabilizing agent glycerol resulted in slight loss of the purified rCul n 4 (Figure 23B). It was decided that before use in vaccination the purified rCul n 4 would be dialyzed to eliminate imidazole and cyclodextrin, but glycerol and L-arginine would be left in the formulation.

4.1.2 Insect cells

4.1.2.1 Expression of r-allergens in insect cells

The allergen genes, *Cul o 8, Cul o 9*, *Cul o 10*, *Cul o 11 and Cul o 12* were codon optimized by GenScript® for expression in insect cells (appendix II). The genes were cloned into pFastBac-HBM-TOPO, pFastBac1 with $(His)_6$ tag and STOP signal from the pFastBac-HBM-TOPO vector and pI-secSUMOstar vector (appendix V). Successful cloning of the genes into different vectors is shown in Table 5.

Table 5. List of vectors the allergen genes were cloned into.

Allergen gene	Size of insert (bp)		Vector
Cul o 8	full length	504	pFastBac-HBM-TOPO pFastBac-1
	wo secretion signal sequence	447	pI-secSUMOstar
Cul o 9	full length	471	pFastBac-HBM-TOPO pFastBac-1
	wo secretion signal sequence	408	pI-secSUMOstar
Cul o 10	full length	1905	pFastBac-HBM-TOPO pFastBac-1
Cul o 10a	first 217 a.a. following secretion signal sequence	651	pFastBac-HBM-TOPO pFastBac-1 pI-secSUMOstar
Cul o 10b	397 a.a. following *Cul o 10 a*	1188	pFastBac-1 pI-secSUMOstar
Cul o 11	Full length	1155	pFastBac-HBM-TOPO pFastBac-1
	wo secretion signal sequence	1095	pI-secSUMOstar
Cul o 12	full length	927	pFastBac-HBM-TOPO pFastBac-1

Following, DH5α *E. coli* cells were transformed. Vectors containing the gene of interest were purified from positive cultures and sequenced to confirm the right reading frame of the genes (appendix VII). DH10Bac *E. coli* cells were transformed with the recombinant constructs to produce r-bacmids. They were then isolated and analysed in PCR with gene and bacmid specific primers to verify the presence of the gene in the isolated r-bacmids (data not shown).

The isolated r-bacmids containing the gene of interest were used to transfect Sf-9 cells for production of r-baculoviruses (appendix VIII). To generate high-titer homogenous viral stock used for production of r-protein, the r-baculoviruses were cloned and amplified with 3 passages (appendix VIII). For production of r-allergens, High-five insect cells were infected with cloned r-baculovirus. Successful transfections of r-allergen constructs and clones are listed in Table 6.

Table 6. List of r-allergen constructs successfully expressed in insect cells.

Allergen		Construct
Cul o 8	full length	Bac-1-rCul o 8
Cul o 9	without secretion signal sequence	Bac-SUMO-rCul o 9
Cul o 10	full length	Bac-1-Cul o 10
Cul o 10a	partial protein	Bac-SUMO-rCul o 10a
		Bac-HBM-rCul o 10a
Cul o 10b	partial protein	Bac-SUMO-rCul o 10b
		Bac-1-rCul o 10b
Cul o 11	full length	Bac-1-rCul o 11

4.1.2.2 Purification of r-allergens from insect cells

The newly expressed r-allergens Bac-1-rCul o 8, Bac-SUMO-rCul o 9 and Bac-1-rCul o 11 were successfully purified under native conditions using HIS-select® HF nickel affinity gel (Figure 24).

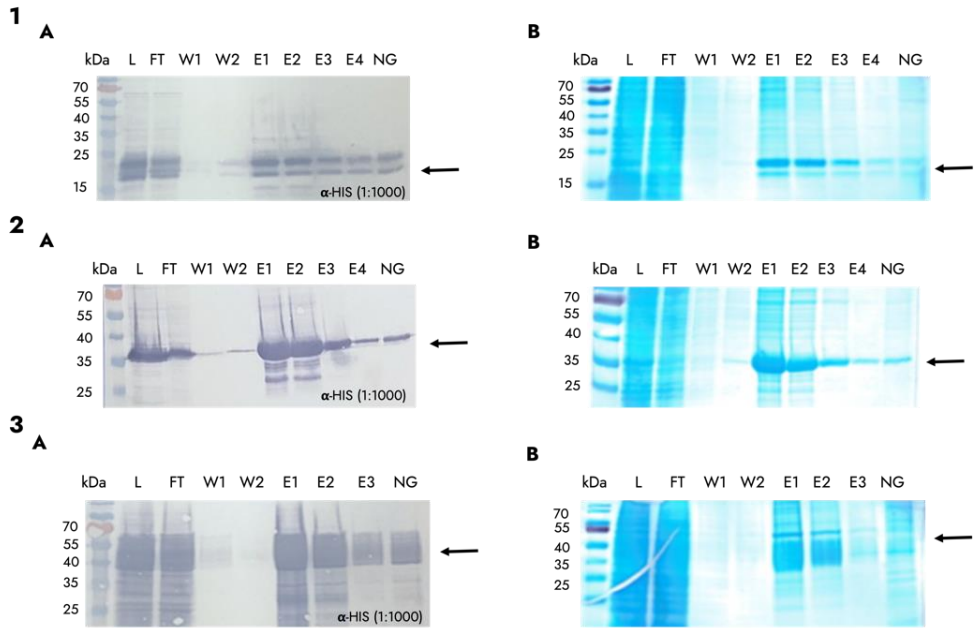


Figure 24. Purification of r-Bac allergens under native conditions. **1.** Bac-1-rCul o 8 (21.2 kDa), **2.** Bac-SUMO-rCul o 9 (34.0 kDa), **3.** Bac-1-rCul o 11 (41.1 kDa). **A.** WB; **B.** Protein staining; **L:** lysed cell pellet; **FT:** Flowthrough; **W 1-2:** Wash 1-2; **E 1-4:** Elution 1-4; **NG:** Nickel gel following purification. The arrows indicate the representative r-proteins.

Bac-1-rCul o 8 was seen as double band between the 15 kDa and 25 kDa marker on the ladder (Figure 24-1), based on calculation of the protein size it is 21.2 kDa with its secretion signal sequence but 19.2 kDa without. Following N-linked deglycosylation of

Bac-1-rCul o 8 the upper band disappears and the lower band is left (Gudnadottir, 2021). Bac-SUMO-rCul o 9 is 34.0 with the gp67 secretion signal of the pI-secSUMOstar construct but 29.6 kDa without. The expressed and purified Bac-SUMO-rCul o 9 was seen as single band close to the 35 kDa marker (Figure 24-2). Bac-1-rCul o 11 was seen as broad and fuzzy band covering the gap between the 40 kDa and 55 kDa (Figure 24-3), indicating that the r-protein is heavily glycosylated. N-linked deglycosylation resulted in narrow and sharp band by the 40 kDa marker (Figure 25). Bac-1-rCul o 11 is calculated to be 41.1 kDa with its secretion signal sequence but 39.0 kDa without.

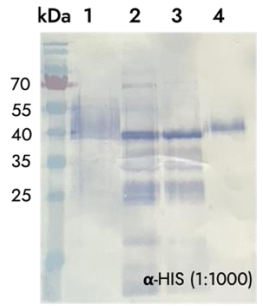


Figure 25. N-linked glycan deglycosylation of Bac-rCul o 11 with PNGase F. WB showing deglycosylation of Bac-rCul o 11; **1.** Bac-rCul o 11 untreated, **2.** Bac-rCul o 11 deglycosylation with non-denaturing reaction conditions, **3.** Bac-rCul o 11 deglycosylation with denaturing reaction conditions, **4.** E.coli-rCul o 11.

Bac-1-rCul o 10 full size (74.7 kDa) could neither be purified under native (Figure 26) nor denaturing conditions (data not shown) with HIS-select® HF nickel gel. The allergen was therefore expressed in two parts (Table 6) were Cul o 10a represents the first 217 a.a. (25.3 kDa) following the secretion signal sequence and has no homology with other insect proteins. Cul o 10b represents the final 397 a.a. (45.4 kDa) with homology to Cul n 3. Bac-SUMO-rCul o 10a and Bac-SUMO-rCul o 10b were successfully purified under native conditions. Bac-SUMO-Cul o 10a predicted to be 42.5 kDa with secretion signal sequence and 38.1 kDa without in the pI-secSUMOstar construct, was seen as double band right above the 40 kDa marker. Bac-SUMO-rCul o 10b was seen as double band, with the upper band fuzzy between the 55 kDa and 70 kDa. The predicted size of Bac-SUMO-rCul o 10b is 62.2 kDa with the secretion signal sequence but 58.2 kDa without.

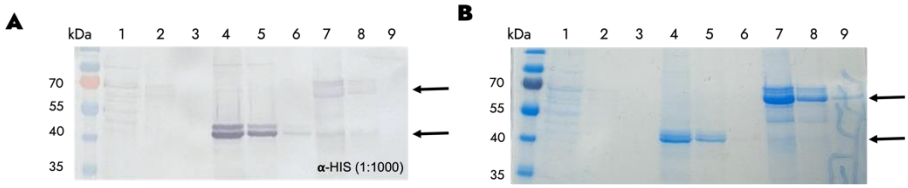


Figure 26. Purification of Bac-rCul o 10 from High-five insect cells. **A.** WB; **B.** Protein staining; **1.** Bac-1-rCul o 10 elution 1, **2.** elution 2, **3.** elution 3, **4.** Bac-SUMO-rCul o 10a elution 1, **5.** elution 2, **6.** elution 3, **7.** Bac-SUMO-rCul o 10b elution 1, **8.** elution 2, **9.** elution 3. The arrows indicate the representative r-proteins.

Successful purification of Bac-r-allergens (Figure 78, appendix X) used in this study is listed in Table 7, done under native conditions except for Bac-SUMO-rCul o 1P and Bac-HBM-rCul o 1P under denaturing conditions with refolding in the elution steps. Following protein purification, buffer exchange in 2xPBS was done for all Bac-r-allergens.

Table 7. Bac-r-allergens successfully purified using HIS-select® HF nickel affinity gel.

Allergen	Bac-construct	Codon optimized[1]	Vector	Allergen secretion signal squence[2]	Size with or without construct secretion signal sequence	
					with	without
Cul o 1P	Bac-HBM-Cul o 1P	No	pFastBac-HBM TOPO	Without	26.1	23.3
	Bac-SUMO-rCul o 1P	No	pI-secSUMOstar	Without	38.5	34.1
Cul o 2P	Bac-HBM-rCul o 2P	No	pFastBac-HBM TOPO	Without	22.3	17.5
Cul o 3	Bac-SUMO-rCul o 3	No	pI-secSUMOstar	Without	45.2	40.8
Cul o 5	Bac-HBM-rCul o 5	Yes	pFastBac-HBM TOPO	Without	22.7	19.9
Cul o 7	Bac-1-rCul o 7	Yes	pFastBac 1	With	19.5	16.9
Cul o 8	Bac-1-rCul o 8	Yes	pFastBac 1	With	21.2	19.2
Cul o 9	Bac-SUMO-rCul o 9	Yes	pI-secSUMOstar	Without	34.0	29.6
Cul o 10	Bac-SUMO-rCul o 10a	Yes	pI-secSUMOstar	Without	42.5	38.1
	Bac-SUMO-rCul o 10b		pI-secSUMOstar	Not relevant	62.2	58.2
Cul o 11	Bac-1-rCul o 11	Yes	pFastBac 1	With	41.1	39.0
Cul n 3	Bac-rCul n 3	No	pFastBac-HBM TOPO	With	49.3	46.5
Cul n 4	Bac-1-rCul n 4	No	pFastBac 1	With	19.3	16.9
Cul n 8	Bac-rCul n 8	No	pFastBac HT B	With	68.6	66.5

[1]Gene codon optimized for insect cells.

[2]Expressed with or without predicted allergen secretion signal sequence.

4.1.2.3 Sulfidoleukotriene (sLT) release test

Four of the r-allergens expressed in insect cells were used in sLT release test to test the relevance of the allergens in IBH-affected horses. Isolated peripheral blood leukocytes from healthy and IBH-affected horses were re-stimulated with Bac-1-Cul o 1P, Bac-SUMO-Cul o 9, Bac-SUMO-Cul o 10b or Bac-1-rCul o 11 and sLT release measured using CAST ELISA. The measured sLT release was significantly higher in IBH-affected horses compared to healthy once following stimulation of peripheral blood leukocytes with all four insect cell expressed r-allergens (Figure 27).

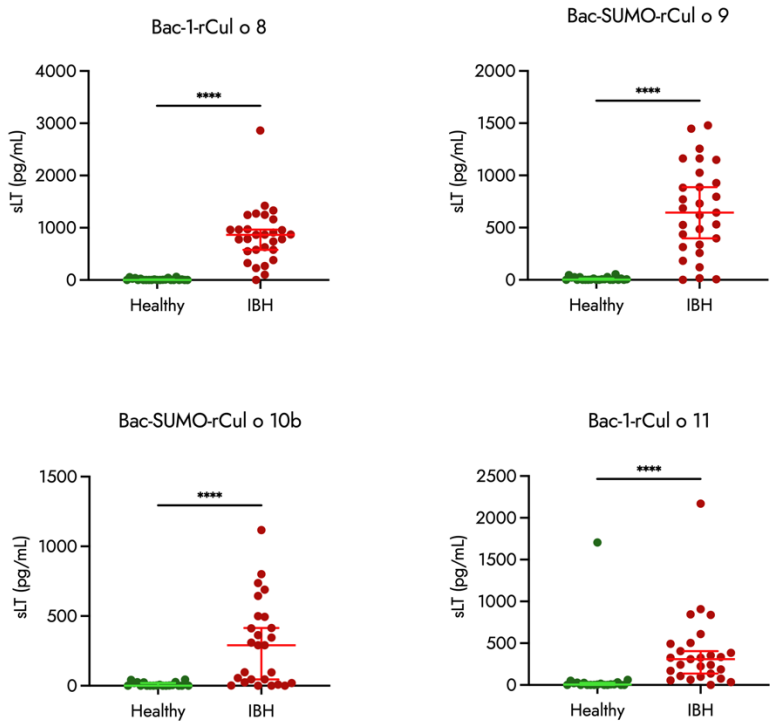


Figure 27. Sulfidoleukotriene (sLT) production after stimulation of peripheral blood leukocytes with *Culicoides* r-allergens expressed in insect cells. Peripheral blood leukocytes from healthy and IBH-affected horses were re-stimulated with the four Bac- r-allergens separately, Bac-1-rCul o 8, Bac-SUMO-rCul o 9, Bac-SUMO-rCul o 10b or Bac-1-rCul o 11 and sLT release measured using CAST ELISA. Comparison of sLT release between healthy and IBH-affected horses was done using Mann-Whitney U test. The graphs show individual values of sLT release, median and 95% confidence interval. An asterisk (*) with line indicates statistical difference of sLT release between healthy and IBH-affected horses.

4.1.3 Barley

4.1.3.1 Expression of r-allergens in barley and screening of seed lines

Five r-allergens were expressed in barley in three generations of each seed line (Table 8). T_1 generation was screened with extractions analysed in ELISA and WB (appendix XI) but T_2 (appendix XII) and T_3 generations with extraction and mini-scale purification analysed in WB (Figure 28). Two positive T_2 seed lines were sowed for T_3 generation for all r-allergens except for barley-rCul o 3 where four seed lines were sowed (Table 8).

Table 8. Summary of screened and positive r-barley seed lines

r-allergen	Size (kDa)	T1 seed lines				T2 seed lines			T3 seed lines		
		Nr[1]	Pos[2]	Antibody	Sowed for T2	Pos[2]	Antibody	Sowed for T3	Pos[2]	Antibody	Total[3] (kg)
Barley-rCul o 2 (hya)	43.2	38	27	α-Cul n 2	7	7	α-Cul n 2	2	2	α-Cul n 2	10.2
Barley-rCul o 2P	16.1	44	31	α-Cul o 2P	10	10	α-Cul o 2P	2	2	α-Cul o 2P	17.2
Barley-rCul o 3	28.8	79	26	α-His	11	3	α-His	4	2	α-Cul n 1	7.7
Barley-rCul o 5	18.7	147	83	α-His	11	11	α-His	2	0	α-Cul o 5	N/A
Barley-rCul n 8	67.5	123	37	α-His	11	2	α-His	2	1	α-Cul n 8	2.5

[1]Number of seed lines

[2]Number of positive seed lines

[3]Total weight of positive seeds (kg)

Four allergens were successfully expressed in T_3 generation seed lines of r-barley. Both T_3 seed lines were positive for barley-rCul o 2 giving final harvest of 10.2 kg of positive seeds (Table 8). Barley-rCul o 2 was seen as a double band around the 40 kDa marker in extraction but following mini-scale purification as a single band (Figure 28), predicted size was 43.2 kDa. Total harvest of barley-rCul o 2P was 17.2 kg from two positive T_3 seed lines, seen as single band by the 15 kDa marker (Figure 28) matching the predicted size of 16.1 kDa. Two of four barley-rCul o 3 T_3 seed lines tested positive resulting in total 7.7 kg of positive seeds. The predicted size of barley-rCul o 3 was 28.8 kDa and the r-allergen was seen as a single band between the 25 kDa and 35 kDa (Figure 28). One T_3 seed line of barley-rCul n 8 was positive, giving a total of 2.5 kg of positive seeds. Barley-rCul n 8 predicted size was 67.5 kDa and was seen as single band by the 70 kDa marker (Figure 28). Barley-rCul o 5 showed positive seed lines in the T_1 and T_2 generations, but both T_3 seed lines tested negative (Table 8).

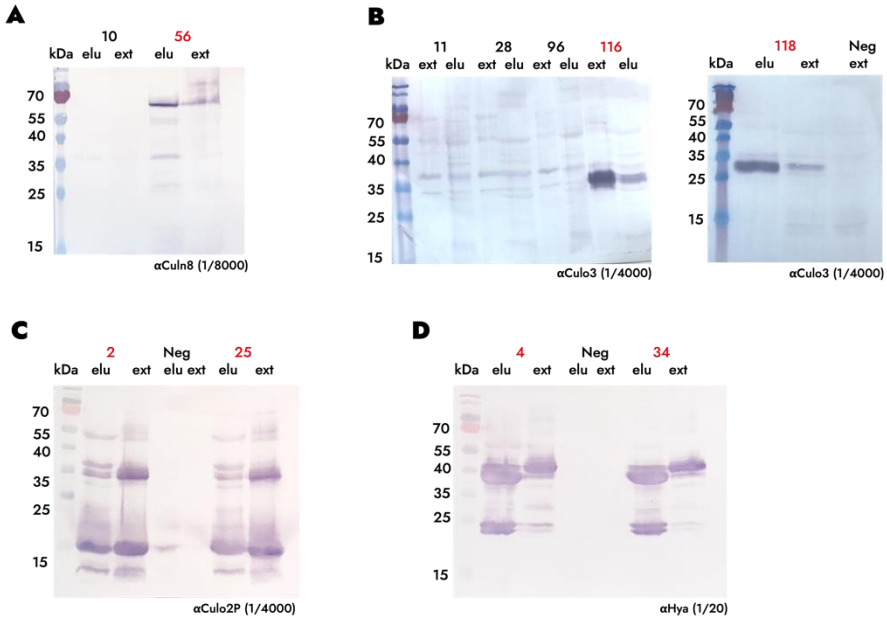


Figure 28. T_3 screening of recombinant barley. WB showing screening with extraction (ext) and elution from miniscale (elu) purification of T_3 r-barley seeds; numer represent the number of seed line (i.e. 10 represents seed line #10), Neg represents negative control, untransgenic barley. Red line numbers represesent positive seed lines. **A.** Barley-rCul n 8, **B.** Barley-rCul o 3, **C.** Barley-rCul o 2P, **D.** Barley-rCul o 2 (hya).

4.1.3.2 Purification of r-allergens from barley

An attempt was made to purify barley-rCul o 2P, barley-rCul o 3 and barley-rCul n 8. Results are shown for barley-rCul o 2P and barley-rCul o 3, barley-rCul n 8 could not be successfully purified.

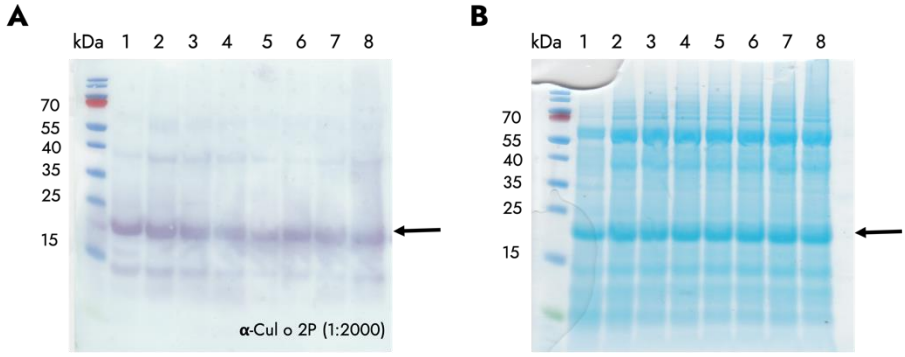


Figure 29. Extraction of barley-rCul o 2P (16.1 kDa). **A.** WB; **B.** Protein staining; **1.** 50 mM citrate, 500 mM NaCl, pH 4,0, **2.** 50 mM acetate, 500 mM NaCl, pH 5.0, **3.** 50 mM MES, 500 mM NaCl, pH 6.0, **4.** 50 mM MES, 500 mM NaCl, pH 6.5, **5.** 50 mM $KiPO_4$, 500 mM NaCl, pH 7.0, **6.** 50 mM $KiPO_4$, 500 mM NaCl, pH 7.5, **7.** 50 mM tris, 500 mM NaC, pH 8.0, **8.** 50 mM bicine, 500 mM NaCl, pH 8.8. The arrows indicate the representative r-allergen.

Before purification of the r-barley-allergens, different extraction conditions were tested to see what pH would give the best yield of soluble r-allergen (Figure 29).

Based on visual assessment of the band intensity of barley-rCul o 2P on the WB in Figure 29A it was decided to do the extraction using buffer 50 mM MES, 500 mM NaCl, pH 6.0. The extraction buffer chosen for barley-rCul o 3 was 50 mM tris, 500 mM NaCl, pH 8.0 (Figure 30) and for barley-rCul n 8 was 50 mM $KiPO_4$, 500 mM NaCl, pH 7.5 (data not shown).

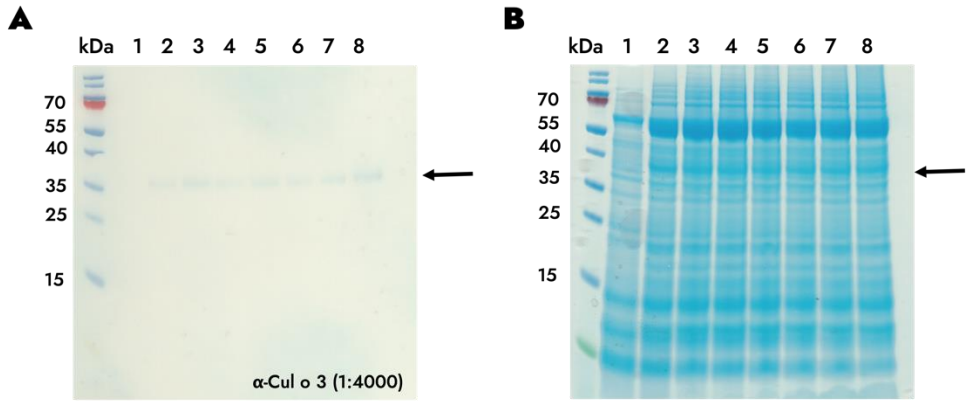


Figure 30. Extraction of barley-rCul o 3 (28.8 kDa). **A.** WB; **B.** Protein staining; **1.** 50 mM citrate, 500 mM NaCl, pH 4,0, **2.** 50 mM acetate, 500 mM NaCl, pH 5.0, **3.** 50 mM MES, 500 mM NaCl, pH 6.0, **4.** 50 mM MES, 500 mM NaCl, pH 6.5, **5.** 50 mM $KiPO_4$, 500 mM NaCl, pH 7.0, **6.** 50 mM $KiPO_4$, 500 mM NaCl, pH 7.5, **7.** 50 mM tris, 500 mM NaC, pH 8.0, **8.** 50 mM bicine, 500 mM NaCl, pH 8.8. The arrows indicate the representative r-allergen.

For purification, 300 g of transgenic barley expressing Cul o 2P were milled and extracted in 50 mM MES, 500 mM NaCl, pH 6.0. Following, the extract was clarified by centrifuging then TFF by using hollow fibre for diafiltration and ultrafiltration, before IMAC. Initially the binding condition of 30 mM imidazole was tested followed by 40 mM and 80 mM imidazole wash and finally elution with 500 mM imidazole in 50 mM $KiPO_4$, 500 mM NaCl, pH 7.4.

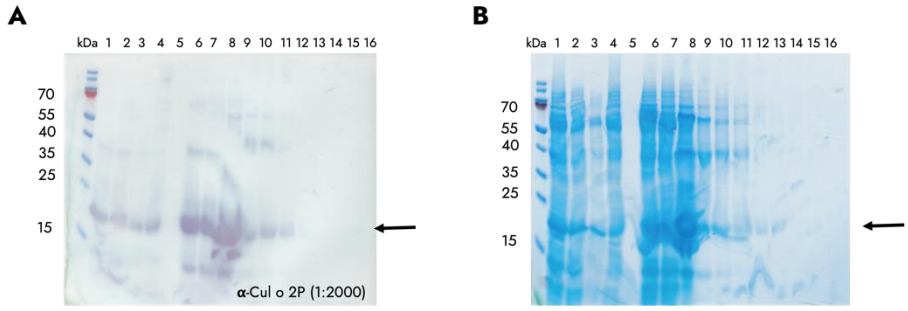


Figure 31. Barley-rCul o 2P IMAC affinity chromatograph purification with 30 mM imidazole binding conditions. **A.** WB, **B.** Protein staining; **1.** Extract, **2.** 2x diafiltrate, **3.** 3x washed diafiltrate, **4.** Retentate, **5.** Permeate, **6.** IMAC load, **7.** Flow through, **8.** 40 mM imidazole wash, **9.** 80 mM imidazole wash, **10.-16.** 500 mM imidazole elute. The arrows indicate the representative r-allergen.

Purification of barley-rCul o 2P with binding condition at 30 mM imidazole during IMAC resulted in poor binding capacity, as much of the target r-protein was released with the flow through and the remaining bound protein was released during the 40 mM and 80 mM imidazole wash. Therefore, only a portion of the target r-protein was in the elute (Figure 31). The flow through, 40 mM wash and 80 mM wash were pooled and the imidazole diluted to 10 mM imidazole before re-loading on IMAC and run with 20 mM and 30 mM imidazole wash and 500 mM imidazole elution.

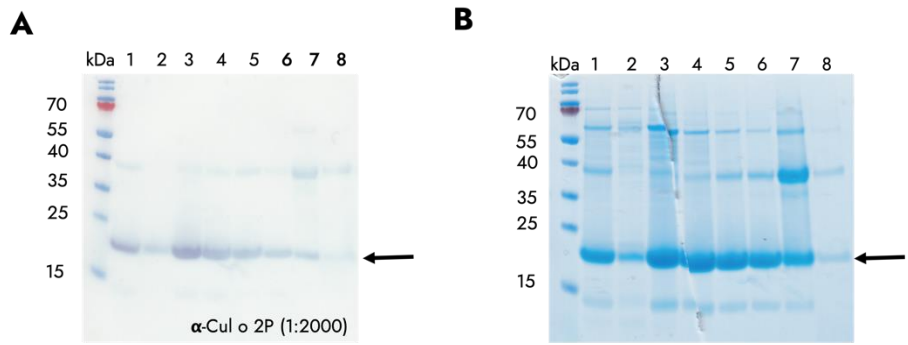


Figure 32. Barley-rCul o 2P IMAC affinity chromatograph purification with 10 mM imidazole binding conditions. **A.** WB, **B.** Protein staining; **1.** Load, **2.** Flow through, **3.-4.** 20 mM imidazole wash, **5-6.** 30 mM imidazole wash, **7.-8.** 500 mM imidazole elute. The arrows indicate the representative r-allergen.

With 10 mM imidazole binding conditions there was still some barley-rCul o 2P found in the flow through (Figure 32). The barley-rCul o 2P bound to the IMAC column eluted in both wash steps and the elution step. Both wash steps, the 2nd eluate and eluate from the first IMAC purification were pooled and diluted 10x in 50 mM MES, pH 6.5 before applying to SP cation exchange column.

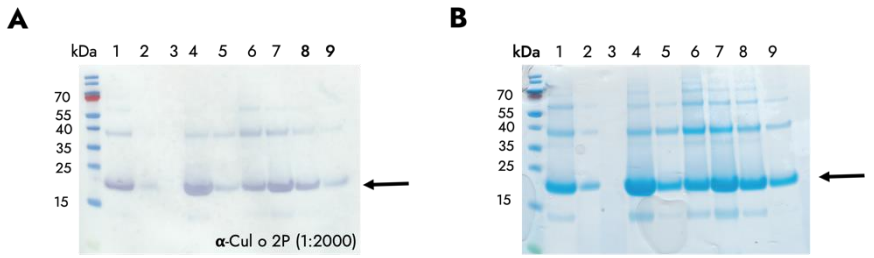


Figure 33. Barley-rCul o 2P SP cationic exchange chromatograph purification. **A.** WB, **B.** Protein staining; **1.** IMAC pool, **2.** Load, **3.** Flow through**, 4.-5.** 700 mM NaCl elute, **6.-9.** 1 M NaCl elute. The arrows indicate the representative r-proteins.

In the SP cation exchange chromatography purification, the barley-rCul o 2P bound to the column and was not found in the flow through (Figure 33). Barley-rCul o 2P was found in both 700 mM and 1 M NaCl eluates (Figure 33). The eluates were pooled and buffer exchanged in 2xPBS (data not shown).

For purification of barley-rCul o 3, 300 g of transgenic barley were milled and extracted in 50 mM Tris, 500 mM NaCl, pH 8.0. Following, the extract was clarified as done for barley-rCul o 2P and loaded onto IMAC using the same initial conditions; binding with 30 mM imidazole, wash with 40 mM and 80 mM imidazole wash and elution with 500 mM imidazole in 50 mM $KiPO_4$, 500 mM NaCl, pH 7.4. IMAC purification resulted in main peak in the 500 mM imidazole elution step representing the r-allergen (Figure 34). But, barley-rCul o 3 was also found in the flow through. The IMAC eluates containing barley-rCul o 3, were pooled and diluted 10x in 50 mM MES, pH 6.5 before applying to SP cation exchange column.

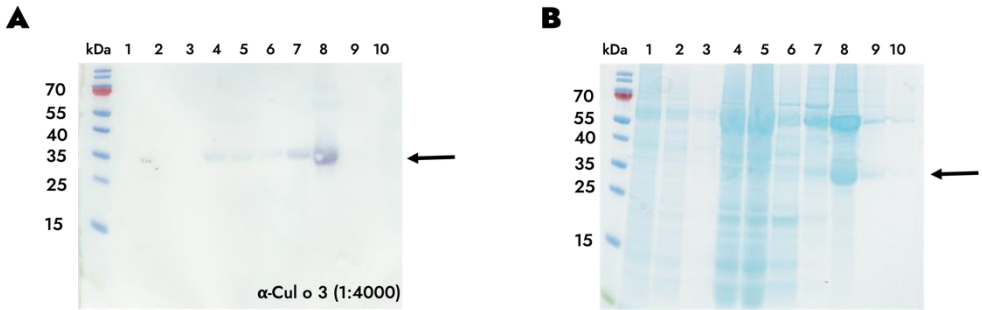


Figure 34. Barley-rCul o 3 IMAC affinity chromatograph purification with 30 mM imidazole binding conditions. **A.** WB, **B.** Protein staining; **1.** Retentate, **2.** 3x washed diafiltrate, **3.** Permeate, **4.** IMAC load, **5.**Flow through, **6.** 40 mM imidazole wash, **7.-9.** 500 mM imidazole elute, **10.** 80 mM imidazole wash. The arrows indicate the representative r-allergen.

The barley-rCul o 3 bound to the column and was not found in the flow through in the SP cation exchange chromatography purification. The protein eluted in 1 M NaCl (Figure 35).

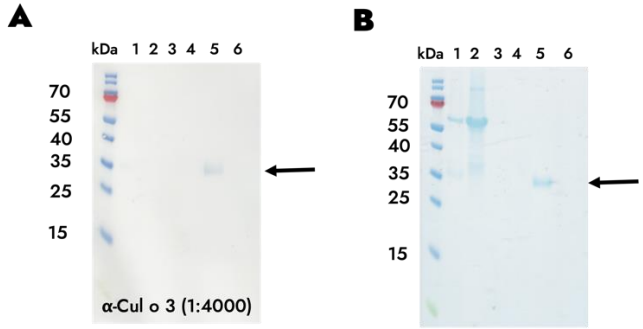


Figure 35. Barley-rCul o 3 SP cationic exchange chromatograph purification. **A.** WB, **B.** Protein staining; **1.** Load, **2.** 300 mM NaCl elute, **3.** 500 mM NaCl elute**, 4.** 700 mM NaCl elute, **5.** 1 M NaCl elute. **6.** Flow through. The arrows indicate the representative r-proteins.

4.2 Experimental vaccination comparing injection route (Paper I)

Paper I is a short communication, this section contains additional and more detailed results.

4.2.1 Allergens

Three *Culicoides* r-allergens expressed in *E. coli* and purified (rCul o 2P, rCul o 3 and rCul n 4) were used for experimental vaccination comparing i.l. and s.c. injection (Figure 36A). For immunoassays three corresponding r-allergens produced in insect cells and purified were used (rBac-Cul o 2P, rBac- Cul o 3 and rBac-Cul n 4) and three *Culicoides* r-allergens not used in the vaccination (rBac-Cul o 7, rBac-Cul n 3 and rBac-Cul n 8) as negative controls (Figure 36B).

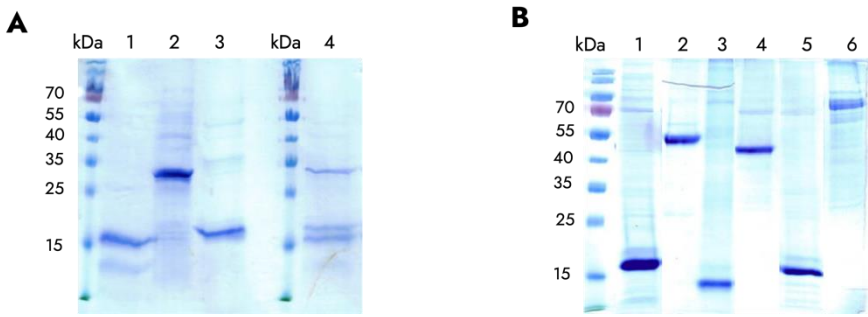


Figure 36. r-Allergens used in experimental vaccination comparing injection route and adjuvant effect. Protein stained gel showing **A. *E.coli* produced r-allergens; 1.** purified rCul o 2P from Bern, **2.** purified rCul o 3 from GeneScript, **3.** purified rCul n 4, **4.** vaccination mixture. **B. Insect cells produced r-allergens; 1.** Bac-rCul o 2P; **2.** Bac-rCul o 3; **3.** Bac-rCul o 7; **4.** Bac-rCul n 3; **5.** Bac-rCul n 4; **6.** Bac-rCul n 8.

4.2.2 Clinical examination

Following vaccination there was no rise in body temperature observed or pain at injection site on palpation showing the vaccination was well tolerated (Figure 37.). Following second vaccination a mild swelling at injection site was observed in some of the horses (data not shown). Leukocyte count was performed following the third vaccination and was within normal range (data not shown).

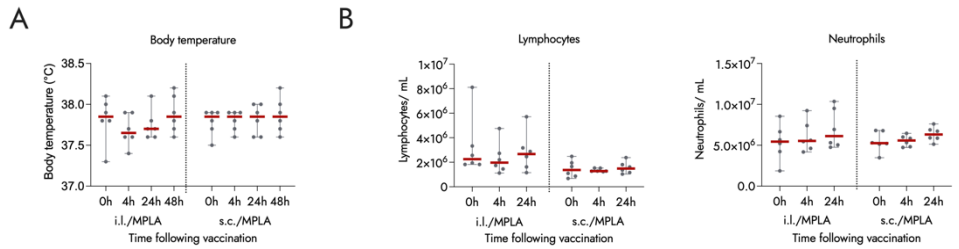


Figure 37. Body temperature and leukocyte count following third vaccination comparing injection sites. **A.** Body temperature measurement and **B.** leukocyte count before vaccination (0h), 4h, 24 and 48h following vaccination. Comparison between timepoints within each vaccination was performed using Friedman multiple comparisons test. The graphs show individual values for the horses, median and 95% confidence interval. An asterisk (*) with line indicates statistical difference between timepoints within vaccinations.

4.2.3 Intradermal test

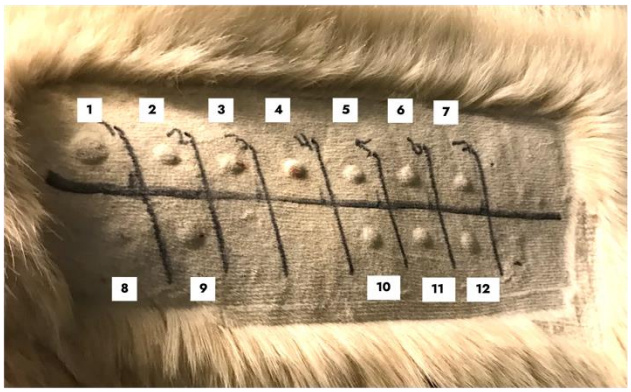


Figure 38. IDT following vaccination. **1.** Histidine (20 µg) positive control, **2.** rCul n 4 buffer, **3.** rCul o 2P buffer, **4.** rCul o 3 buffer, **5.** rCul n 4 (0.1 µg), **6.** rCul o 2P (0.1 µg), **7.** rCul o 3 (0.1 µg), **8.** PBS, **9.** 2xPBS, **10.** rCul n 4 (1 µg), **11.** rCul o 2P (1 µg), **12.** rCul o 3 (1 µg).

The vaccinated horses were tested *in vivo* by IDT to determine the ability of the r-allergens and their formulation buffers to induce type I hypersensitivity reactions. All but one horse reacted to the histamine control. Reactions to r-allergens did not differ from the negative buffer controls (data not shown), IDT reactions for one horse are shown in Figure 38.

4.2.4 Total IgG response following vaccination

Total IgG response was analysed with immunoblot before vaccination and two weeks following third vaccination. Sera were tested on the vaccination mixture which included the *E. coli* r-allergens rCul o 2P, rCul o 3 and rCul n 4.

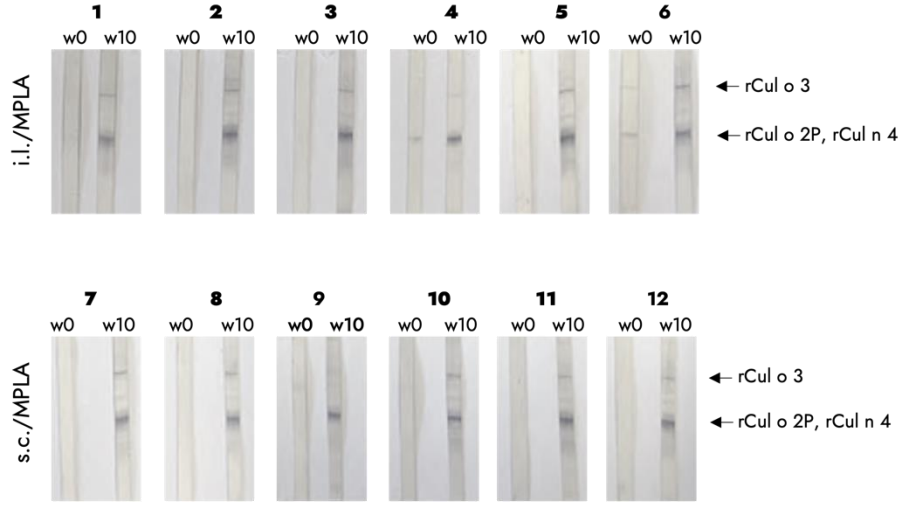


Figure 39. Binding of IgG serum antibodies from i.l./MPLA and s.c./MPLA vaccinated horses to vaccine allergens. WB strips with the vaccine containing rCul o 2P, rCul o 3 and rCul n 4 incubated with serum from i.l./MPLA (1 – 6) and s.c./MPLA (7 – 12) vaccinated horses pre- (w0) and post- (w10) vaccination. The allergens rCul o 2 and rCul n 4 are similar in size and therefore one band on strips represents both allergens.

All i.l./MPLA and s.c./MPLA vaccination horses showed stronger total IgG response against the r-allergens following vaccination (w10) compared to pre-vaccination (w0) (Figure 39). For all vaccination horses the lower band representing two r-allergens (rCul o 2P and Cul n 4) was stronger than the upper band representing one r-allergen (rCul o 3) (Figure 39). Visually the intensity of the protein bands on the strips were similar for both vaccination groups.

4.2.5 Allergen-specific antibody response

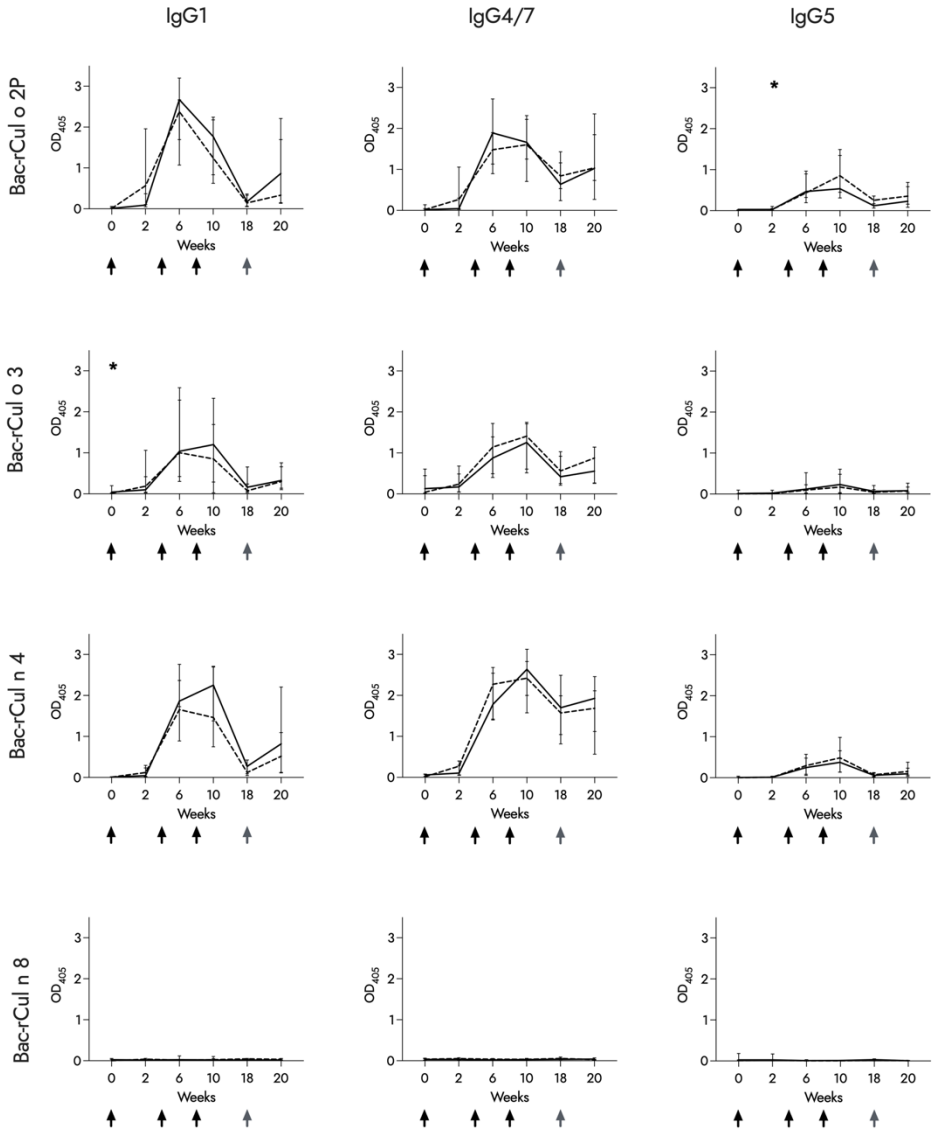


Figure 40. IgG subclass response of i.l./MPLA and s.c./MPLA vaccinated horses. Time course of allergen-specific IgG subclass response of i.l./MPLA (—) and s.c./MPLA (- - -) vaccinated horses, measured by ELISA against Bac-rCul o 2P, Bac-rCul o 3, Bac-rCul n 4 and Bac-rCul n 8 (not included in the vaccine). The graphs show corrected OD_{405} median values with 95% confidence interval for the six horses in each vaccination group at different time points. Black arrows indicate vaccinations and grey arrow intradermal test. Comparisons between vaccination groups were performed using Mann-Whitney U test with Bonferroni corrections. An asterisk (*) indicates statistical difference between vaccination groups at a given time-point (Stefansdottir et al., 2022).

Sera from i.l./MPLA and s.c./MPLA vaccinated horses were tested for IgG subclass response by ELISA against four Bac-r-allergens before, two weeks after each of the vaccinations, before and after IDT. Similar IgG subclass response was observed between the i.l./MPLA and s.c./MPLA vaccination groups against the three rBac-allergens used in the vaccination and no statistical difference (Figure 40). In the i.l./MPLA vaccination group specific IgG1 levels against Bac-rCul n 4 and Bac-rCul o 3 were highest two weeks after third vaccination (week 10) but against Bac-rCul o 2P two weeks after second vaccination (week 6) (Figure 40). The same pattern was seen for the IgG4/7 antibody response. In the s.c./MPLA vaccination group IgG1 levels against the vaccine Bac-r-allergens were highest two weeks after second vaccination (week 6) and IgG4/7 highest two weeks after third vaccination (week 10). The specific IgG5 antibody response was highest at week 10 in both groups against the vaccine Bac-r-allergens. No response at any time-point for either group was observed against rBac-Cul n 8, not used in the vaccine (Figure 40).

After the second and third vaccination there was significant increase in IgG1, IgG4/7 and IgG5 against all three rBac-allergens used in the vaccination within both vaccination groups, i.l./MPLA and s.c./MPLA (Figure 41).

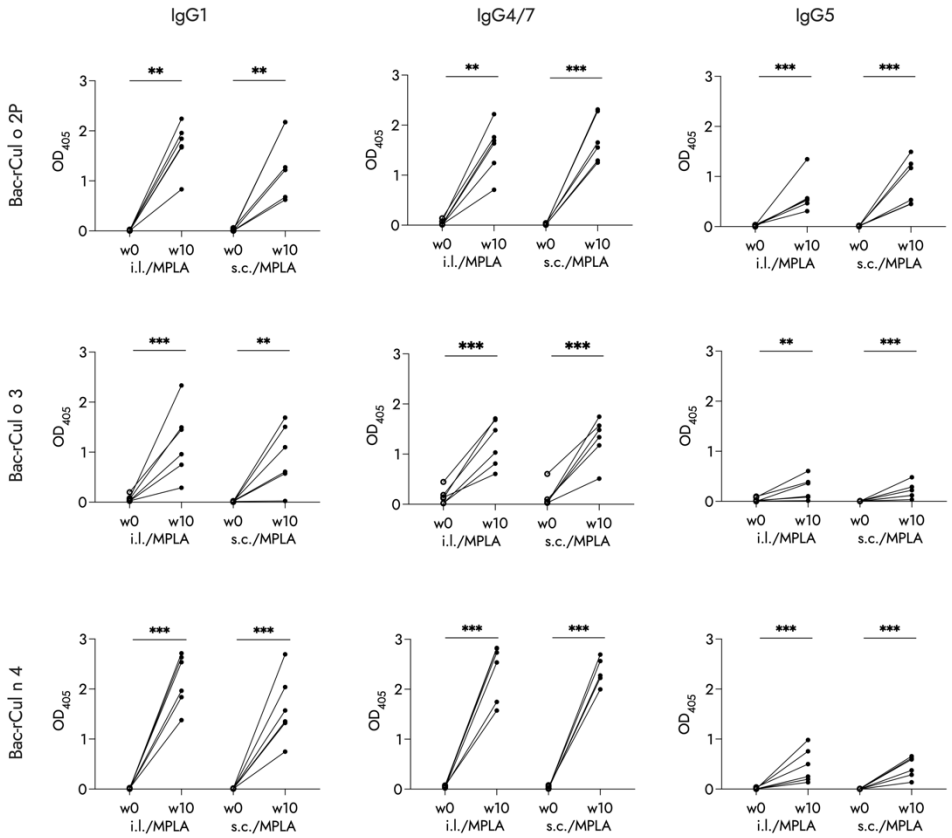


Figure 41. IgG subclass response pre- and post- vaccination of i.l./MPLA and s.c./MPLA vaccinated horses. IgG subclass response measured by ELISA of i.l./MPLA and s.c./MPLA vaccinated horses pre- and post-vaccination (week 0 and 10) against rBac-Cul o 2P, Bac-rCul o 3 and Bac-rCul n n 4. The graphs show individual correct OD_{405} values of the six horses in each group. Comparisons between the time-points within the horse groups were performed using Friedman test. An asterisk (*) with line indicates statistical difference (Stefansdottir et al., 2022).

Allergen-specific IgE and IgA was measured in sera against three Bac-r-allergens used in the vaccination (Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4) at same time-points as for IgG subclasses. No IgE was detected at any time-point in neither vaccination group (Figure 42).

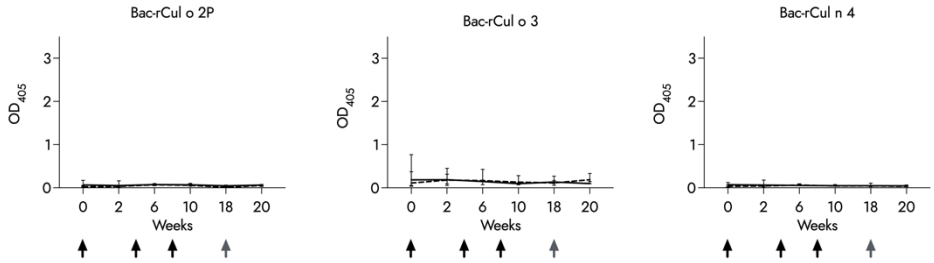


Figure 42. No IgE antibody response was detected in the i.l./MPLA or s.c./MPLA vaccinated horses. Time course of allergen-specific IgE subclass response of i.l./MPLA (—) and s.c./MPLA (- - -) vaccinated horses, measured by ELISA against Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4. The graphs show corrected OD_{405} median values with 95% confidence interval for the six horses in each vaccination group at different time points. Black arrows indicate vaccinations and grey arrow intradermal test. Comparisons between vaccination groups were performed using Mann-Whitney U test with Bonferroni corrections.

Both vaccination groups showed similar patterns in IgA response against the three Bac-r-allergens used in the vaccination, but there was great individual variability within both groups (Figure 43). Against Bac-rCul o 2P the IgA response gradually increased and was highest at week 6 for s.c./MPLA vaccination but highest at week 10 for i.l./MPLA. Two weeks following IDT or at week 20 the IgA response increased again. Against Bac-Cul n 4 both groups had highest IgA response two weeks after second vaccination at week 6 and slight increase following IDT or at week 20. Both groups had detectable IgA to Bac-rCul o 3 before vaccination. Following week 6 there was slight increase in IgA response for i.l./MPLA vaccination group, which decreased following week 18. For s.c./MPLA vaccination group there was slight increase in the IgA response at week 2 but following there was gradual decrease throughout week 20 (Figure 43).

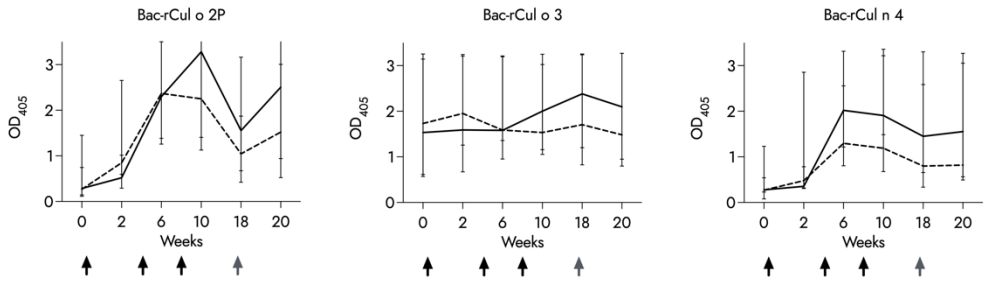


Figure 43. IgA antibody response of i.l./MPLA and s.c./MPLA vaccinated horses. Time course of allergen-specific IgA subclass response of i.l./MPLA (—) and s.c./MPLA (- - -) vaccinated horses, measured by ELISA against Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4. The graphs show corrected OD_{405} median values with 95% confidence interval for the six horses in each vaccination group at different time points. Black arrows indicate vaccinations and grey arrow intradermal test. Comparisons between vaccination groups were performed using Mann-Whitney test U with Bonferroni corrections.

4.2.6 Blocking capacity of sera from vaccinated horses

Blocking capacity of allergen-specific IgG in sera following vaccination against IgE-binding to allergen of IBH-affected horse was tested in ELISA. Serum pools from both vaccination groups were tested in different dilutions and percentage of IgE blocking calculated for each dilution.

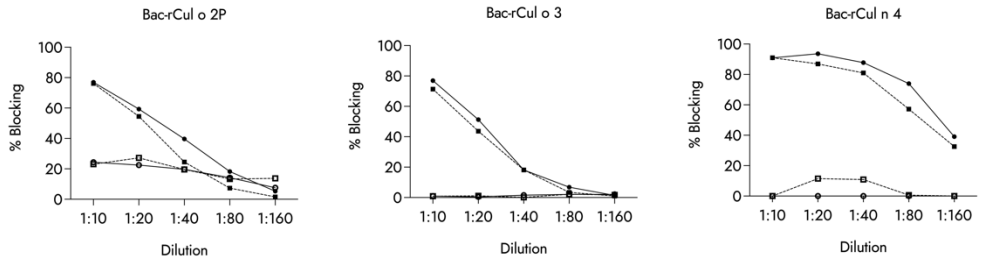


Figure 44. Blocking of IgE binding to r-allergens. Blocking capacity of sera from i.l. and s.c. vaccinated horses of IgE binding from serum of IBH-affected horse to Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4. The graphs show mean % blocking of sera pools, i.l./MPLA post-vaccination (–●–), i.l./MPLA pre-vaccination (–○–), s.c./MPLA post-vaccination (-■–), s.c./MPLA pre- vaccination (-□–) diluted 1:10 – 1:160. The serum was added to ELISA plate coated with r-allergen prior to adding serum from IBH-affected horse at dilution 1:10 (Stefansdottir et al., 2022).

The IgE blocking was similar for both groups, i.l./MPLA and s.c./MPLA (Figure 44). The most effective IgE blocking in both vaccination groups in post-immune sera was against Bac-rCul n 4 reaching 90% at dilution 1:10 and over 80% at dilutions 1:20 and 1:40. Blocking capacity of post-immune sera against Bac-rCul o 2 and Bac-rCul o 3 was close to 80% at dilution 1:10, decreasing gradually in the following dilutions. There was no or only marginal IgE blocking in pre-immune sera against Bac-rCul o 3 and Bac-rCul n 4, but around 20% blocking against Bac-rCul o 2P at dilution 1:10 and 1:20 and decreasing in the following dilution.

4.2.7 Cytokine response following *in vitro* stimulation of PBMC

4.2.7.1 Cytokine expression at mRNA level following vaccination

To analyse cytokine expression response following vaccination, PBMC were re-stimulated *in vitro* two weeks after third vaccination with pool of the three Bac-r-allergens used in the vaccination (Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4). The cells were stimulated for 24 h for RNA isolation and IFN-γ, IL-10 and IL-4 mRNA expression measured with qPCR.

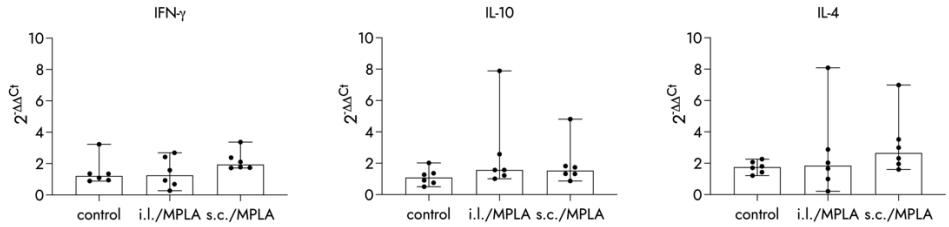


Figure 45. Cytokine expression following i.l./MPLA and s.c./MPLA vaccination. Cytokine expression after *in vitro* re-stimulation of PBMC from vaccinated horses with mixture of Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4. The cytokine expression was measured by qPCR. Comparison of i.l./MPLA, s.c./MPLA and control horses was performed using Kruskal-Wallis multiple comparisons test. The graphs show individual $2^{-\Delta\Delta Ct}$ values for the horses, median and 95% confidence interval for the groups.

Comparing mRNA cytokine expression there was no statistical difference observed between the two vaccination groups i.l./MPLA and s.c./MPLA or control horses for any of the three cytokines analysed, IFN-γ, IL-10 and IL-4 (Figure 45).

4.2.7.2 Analysis of the intracellular expression of cytokines following vaccination

Two weeks following third vaccination, PBMC were re-stimulated *in vitro* with the three Bac-r-allergens used in the vaccination and cell populations analysed using FACS flow cytometry and compared to healthy controls (Appendix XIV). Comparing the frequency of IFN-γ, IL-10 and IL-4 producing lymphocytes there was no significant difference observed between the vaccination groups and healthy controls (Figure 46).

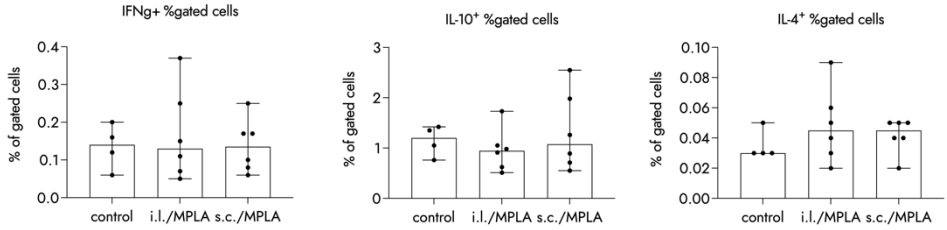


Figure 46. Frequency of IFN-γ, IL-10 and IL-4 producing cells of vaccinated horses and healthy controls. Frequency of IFN-γ, IL-10 and IL-4 producing lymphocytes measured with FACS flow cytometry following *in vitro* re-stimulation of PBMC with pool of Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4 two weeks after third vaccination (week 10). Comparison of i.l./MPLA, s.c./MPLA and control horses was performed using Kruskal-Wallis multiple comparisons test. The graphs show individual IFN-γ, IL-10 or IL-4% of lymphocytes for the horses, median and 95% confidence interval for the groups. The graphs show individual values for the horses, median and 95% confidence interval for the groups.

When analyzing frequenzy of IFN-γ, IL-10 and IL-4 producing $CD4^+CD8^-$, no significant difference was observed between i.l./MPLA-, s.c./MPLA vaccinated horses and heathly controls (Figure 47). The highest frequency of $CD4^+CD8^-$ seen were IL-10 producing

cells with no IFNγ (Figure 47B) and no IL-4 (Figure 47G) production with medium around 1% of gated cells for vaccinated horses and healthy controls. The lowest frequency of $CD4^+CD8^-$ cells observed were those producing two cytokines with frequency equal to or under 0.01% of gated cells for all horses except for one vaccinated s.c./MPLA (Figure 47C, F and I). Frequency of IFNγ producing $CD4^+CD8^-$ cells was around 0.2% of gated cells (Figure 47A and E) while IL-4 producing $CD4^+CD8^-$ cells were under 0.1% of gated cells (Figure 47D and H).

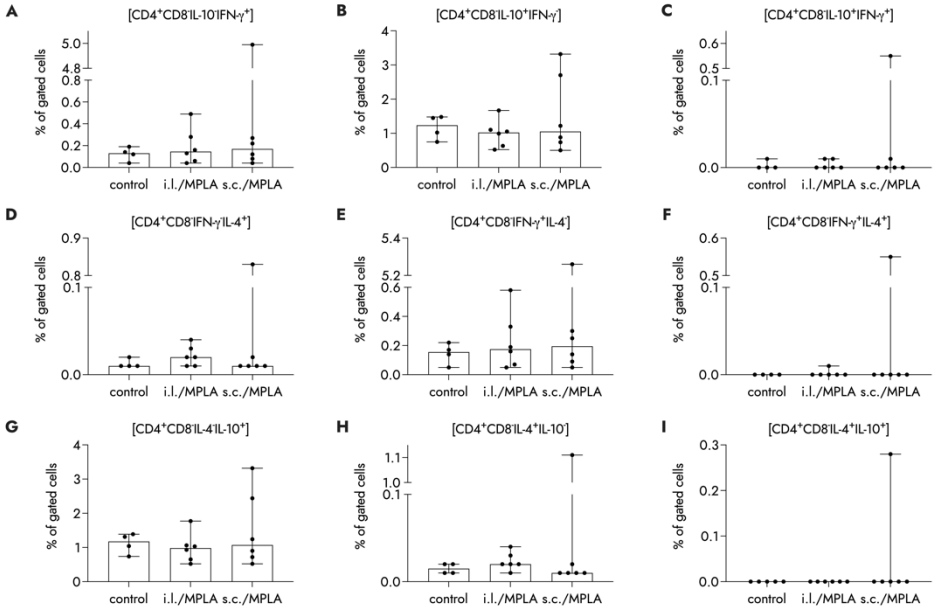


Figure 47. Frequency of IFN-γ, IL-10 and IL-4 producing $CD4^+CD8^-$ cells of horses following i.l./MPLA or s.c./MPLA vaccination. Frequency of IFN-γ, IL-10 and IL-4 producing cells measured with FACS flow cytometry following *in vitro* re-stimulation of PBMC with pool of Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4 two weeks after third vaccination (week 10). Comparison of i.l./MPLA, s.c./MPLA and control horses was performed using Kruskal-Wallis multiple comparisons test. The graphs show individual IFN-γ, IL-10 or IL-4% of gated cells for the horses, median and 95% confidence interval for the groups. The graphs show individual values for the horses, median and 95% confidence interval for the groups. (+) in superscript represents production of the respective cytokine and (–) in superscript represents no expression.

4.2.7.3 Secreted cytokine expression following vaccination

Secreted cytokines were analysed in supernatant of *in vitro* re-stimulated PBMC with the pool of the three Bac-r-allergens used in the vaccination (Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4) and three Bac-r-allergens not used in the vaccine (Bac-rCul o 7, Bac-rCul n 3 and Bac-rCul n 8). The cells were incubated for 96 h, supernatant harvested and IFN-γ, IL-10 and IL-4 measured with Luminex, bead-based multiplex assay.

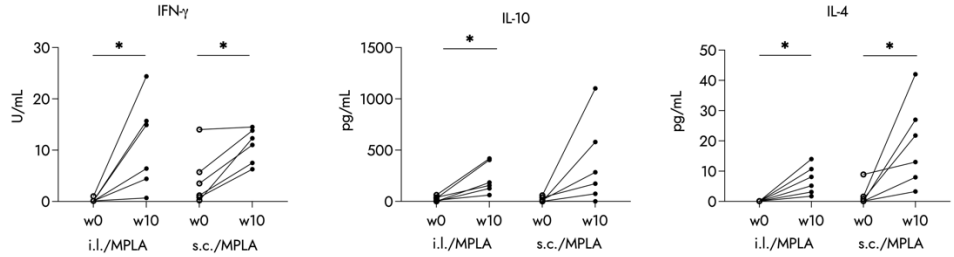


Figure 48. Comparison of cytokine secretion pre- and post i.l./MPLA and s.c./MPLA vaccination. Cytokine secretion after *in vitro* re-stimulation of PBMC with mixture of Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4. The cytokine secretion was measured in supernatant of *in* vitro re-stimulated PBMC by bead-based multiplex assay. Comparison of cytokine secretion pre- and post-vaccination of i.l./MPLA and s.c./MPLA vaccinated horses. Comparisons were performed using Wilcoxon test. An asterisk (*) with line indicates statistical difference between pre- and post-vaccination within horse groups (Stefansdottir et al., 2022).

Following vaccination there was significant increase in secreted IFN-γ, IL-10 and IL-4 cytokine levels within both vaccination groups i.l./MPLA and s.c./MPLA, except for IL-10 in the s.c./MPLA group (Figure 48).

In vitro re-stimulation following vaccination (week 10) with Bac-r-allergens used in the vaccine resulted in significantly higher levels of all three cytokines, IFN-γ, IL-10 and IL-4 compared to *in vitro* re-stimulation with *Culicoides* Bac-r-allergens not used in the vaccine for both vaccination groups, except for IL-10 secretion in the s.c./MPLA group (Figure 49).

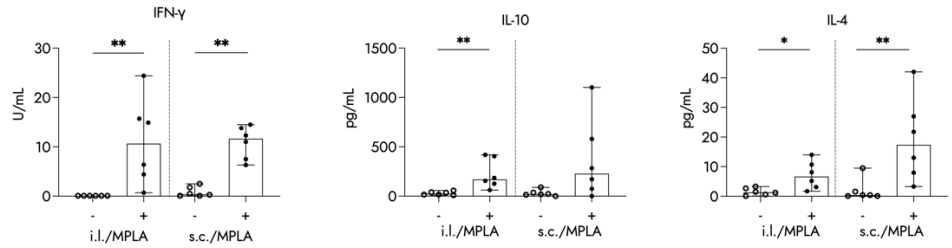


Figure 49. Comparison of cytokine secretion after *in vitro* re-stimulation with r-allergens included in vaccine (+) and *Culicoides* allergens not included (-). Comparison of *in vitro* re-stimulation of PBMC post-vaccination from i.l./MPLA and s.c./MPLA vaccinated horses using r-allergens included in the vaccine (Bac-Cul o 2P, Bac-rCul o 3, Bac-rCul n 4) and *Culicoides* r-allergens not included in the vaccine (Bac-rCul o 7, Bac-rCul n 3 and Bac-rCul n 8). The graps show individual values for the horses and median and 95% confidence interval for the groups. Comparison between r-allergens used for *in vitro* re-stimulation were performed using Mann-Whitney test U, asterisk (*) with line indicates statistical difference (Stefansdottir et al., 2022).

Comparing the two vaccination groups i.l./MPLA and s.c./MPLA there was no significant difference for any of the three cytokines measured (Figure 50). Compared to healthy controls there was no significant difference observed for IFN-γ levels, two control horses had relatively high IFN-γ levels. Both vaccination groups had significantly

higher IL-10 levels following vaccination compared to controls. The s.c./MPLA group showed significantly higher IL-4 levels compared to controls.

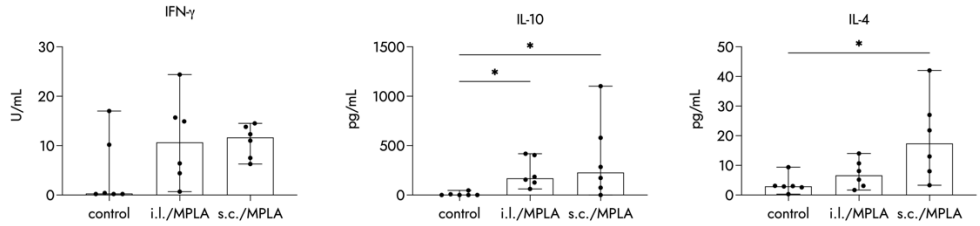


Figure 50. Cytokine secretion following i.l./MPLA and s.c./MPLA vaccination. The cytokine secretion was measured by bead-based multiplex assay. Comparison of i.l./MPLA, s.c./MPLA and control horses was performed using Kruskal-Wallis multiple comparisons test. The graphs show individual values for the horses and median and 95% confidence interval for the groups. An asterisk (*) with line indicates statistical difference between the groups of horses (Stefansdottir et al., 2022).

4.3 Experimental vaccination comparing adjuvant effect

For comparison of adjuvant effect of MPLA and VLP, 6 horses were vaccinated identically to s.c./MPLA vaccinated horses in experimental vaccination comparing injection routes (section 4.2.) but with VLP instead of MPLA. Sera from s.c./MPLA vaccinated horses that was used to compare the IgG subclass response and blocking of IgE binding in ELISA with sera from the horses vaccinated with VLP (s.c./VLP).

4.3.1 Allergens

The same r-allergens as used in experimental vaccination comparing injection routes (Figure 36 in section 4.2.1) were used for injection and immunoassays following vaccination.

4.3.2 Clinical examination and IDT

Rise in temperature was observed following first vaccination but was within normal range, but no rise following second or third vaccination (Figure 51) or pain at injection site on palpation was observed by clinical examination following vaccination, indicating that the vaccination was well tolerated (data not shown). IDT was performed following vaccination to check if the allergens induced type I hypersensitivity by the r-allergens. All horses reacted to the histamine control but reaction to r-allergens were not observed (data not shown).

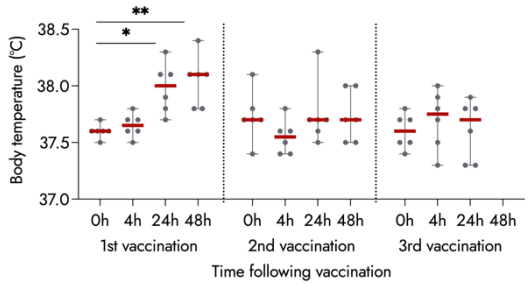


Figure 51. Body temperature following s.c. vaccination with allergens in alum/VLP. Body temperature measurement before vaccination (0h), 4h, 24 and 48h following vaccination. Comparison between timepoints within each vaccination was performed using Friedman multiple comparisons test. The graphs show individual values for the horses and median and 95% confidence interval for the body temperature measured within timepoints. An asterisk (*) with line indicates statistical difference between the body temperature measurements between timepoints within vaccinations.

4.3.3 Total IgG response following vaccination

Before and two weeks following vaccination, total IgG antibody response in serum was analysed using immunoblot against the vaccination mixture as in 4.2.4.

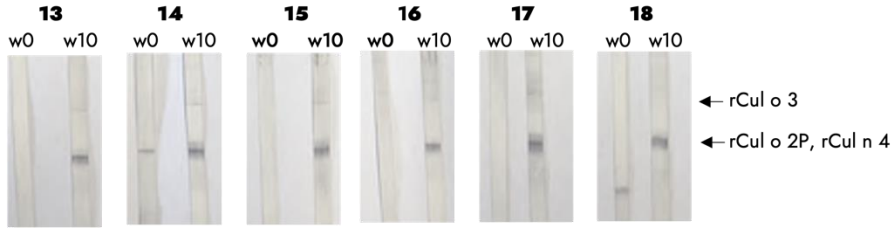


Figure 52. Binding of IgG serum antibodies from s.c./VLP vaccinated horses to vaccine allergens. WB strips with the vaccine containing rCul o 2P, rCul o 3 and rCul n 4 incubated with serum from s.c./VLP (13 – 18) vaccinated horses pre- (w0) and post- (w10) vaccination. The allergens rCul o 2 and rCul n 4 are similar in size and therefore one band on strips represents both allergens.

The total IgG response against the r-allergens following vaccination (w10) was stronger in all s.c./VLP vaccination horses compared to pre-vaccination (w0) (Figure 52). As in section 4.2.4, two r-allergens, rCul o 2P and rCul n 4 represent one band which shows higher intensity compared to the rCul o 3 band.

4.3.4 Allergen-specific antibody response

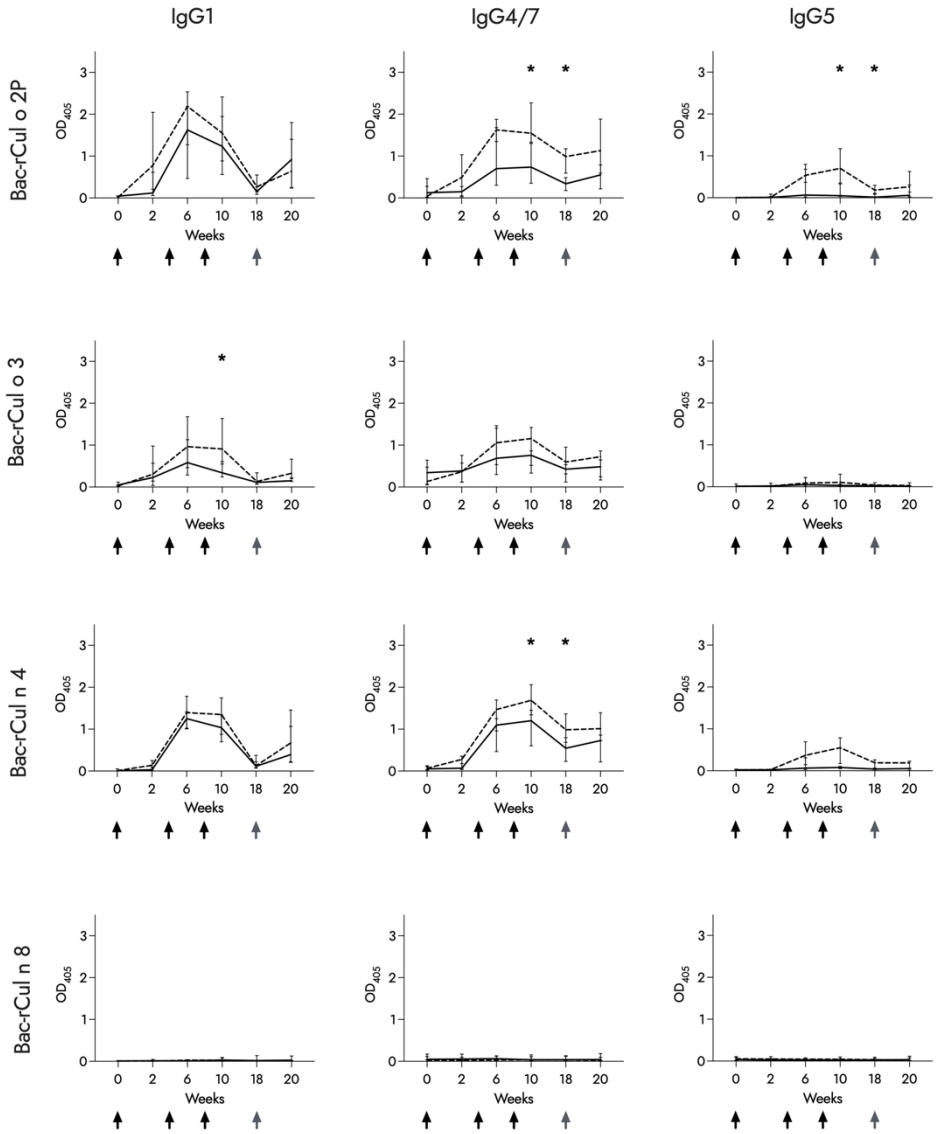


Figure 53. IgG subclass response of s.c./VLP and s.c./MPLA vaccinated horses. Time course of allergen-specific IgG subclass response of s.c./VLP (—) and s.c./MPLA (- - -) vaccinated horses. Measured by ELISA against Bac-rCul o 2P, Bac-rCul o 3, Bac-rCul n 4 and Bac-rCul n 8 (not included in the vaccine). The graphs show corrected OD_{405} median values with 95% confidence interval for the six horses in each vaccination group at different time points. Black arrows indicate vaccinations and grey arrow intradermal test. Comparisons between vaccination groups were performed using Mann-Whitney U test with Bonferroni corrections. An asterisk (*) indicates statistical difference between vaccination groups at a given time-point.

The IgG subclass response of the s.c./MPLA horses had the same pattern as before (4.2.5) but the corrected OD values were in general lower (Figure 53). The specific IgG antibody response was lower in the s.c./VLP vaccination group compared to the

s.c./MPLA. The difference reaches significance at week 10 for IgG1 against Bac-rCul o 3, for IgG4/7 against Bac-r-Cul n 4, for IgG4/7 and IgG5 against Bac-rCul o 2P. Significant difference was also observed between the groups at week 18 for IgG4/7 against rBac-Cul o 2P and rBac-Cul n 4 and IgG5 against Bac-rCul o 2 and at week 20 for IgG1 against Bac-rCul o 3 (Figure 53).

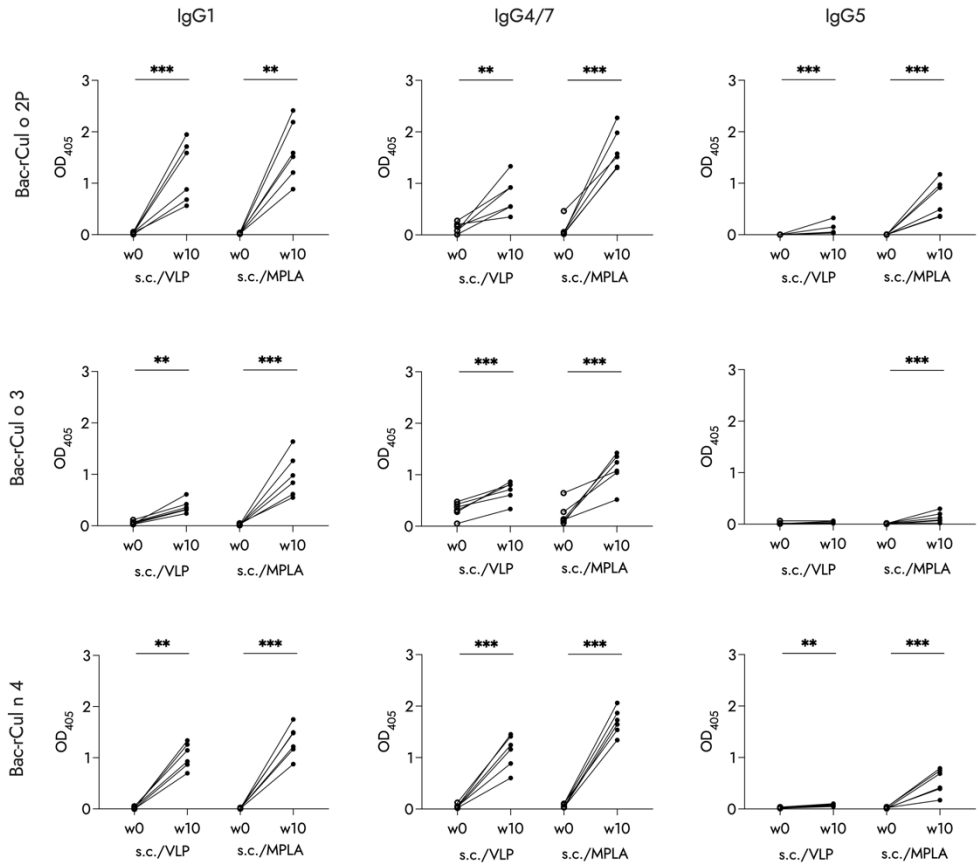


Figure 54. IgG subclass resonse pre- and post- vaccination of s.c./VLP and s.c./MPLA vaccinated horses. IgG subclass response measured by ELISA of s.c./VLP and s.c./MPLA vaccinated horses pre- and post-vaccination (week 0 and 10) against rBac-Cul o 2P, Bac-rCul o 3 and Bac-rCul n 4. The graphs show individual correct OD_{405} values of the six horses in each group. Comparisons between the time-points within the horse groups were performed using Friedman test. An asterisk (*) with line indicates statistical difference.

There was a significant increase in IgG subclass response two weeks after the second (data not shown) and third vaccination within both vaccination groups against the three r-allergens used in the immunization (Figure 54). The exception was the IgG5 response to rBac-Cul o 3 in the s.c./VLP group where the difference did not reach significance. Within the s.c./VLP vaccination group specific IgG1 subclass antibody levels were highest at week 6 for all three r-allergens and IgG4/7 levels highest at week 10 (Figure 53). The IDT at week 18 boosted the specific antibody response against all r-allergens.

No IgG subclass response at any time-point for either group was observed against rBac-Cul n 8, not used in the vaccine (Figure 53).

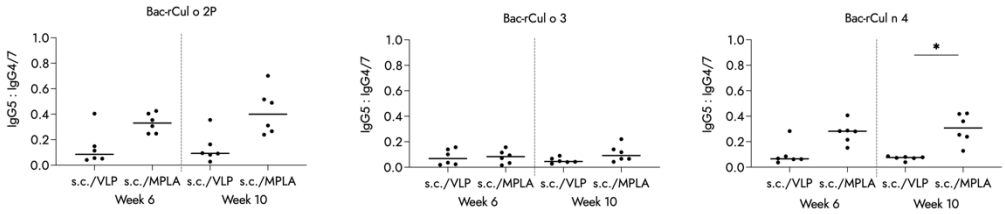


Figure 55. IgG5 and IgG4/7 ratio following s.c./VLP and s.c./MPLA vaccination. Comparison of ratio between IgG5 and IgG4/7 response of horses following vaccination with s.c./MPLA and s.c./VLP at week 6 and 10. Comparisons were performed using Mann-Whitney U test with Bonferroni corrections. An asterisk (*) indicates statistical difference between the groups of horses at a given time-point.

The ratio of IgG5:IgG4/7 antibody levels at week 6 and 10 was compared to analyse whether proportionally less IgG5 than IgG4/7 was induced by alum/VLP compared to alum/MPLA or whether there was just an overall lower antibody response in the alum/VLP group. The IgG5 was indeed proportionally lower in the s.c./VLP group compared to the s.c./MPLA group (Figure 55) for Bac-rCul o 2P and Bac-rCul n 4 at both week 6 and 10. This difference reached significance at week 10 against Bac-rCul n 4. The ratio was similar against Bac-rCul o 3 both at week 6 and week 10 (Figure 55).

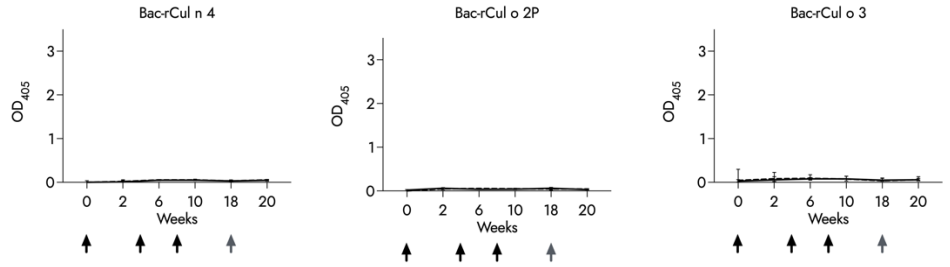


Figure 56. No IgE antibody response was detected in the s.c./VLP or s.c./MPLA vaccinated horses. Time course of allergen-specific IgE subclass response of s.c./VLP (—) and s.c./MPLA (- - -) vaccinated horses, measured by ELISA against Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4. The graphs show corrected OD_{405} median values with 95% confidence interval for the six horses in each vaccination group at different time points. Black arrows indicate vaccinations and grey arrow intradermal test. Comparisons between vaccination groups were performed using Mann-Whitney U test with Bonferroni corrections.

As for experimental vaccination comparing the injection route, allergen-specific IgE and IgA antibodies were measured in serum at the same time-points as for IgG subclasses. No IgE antibody response was detected at any time-point for either vaccination group (Figure 56).

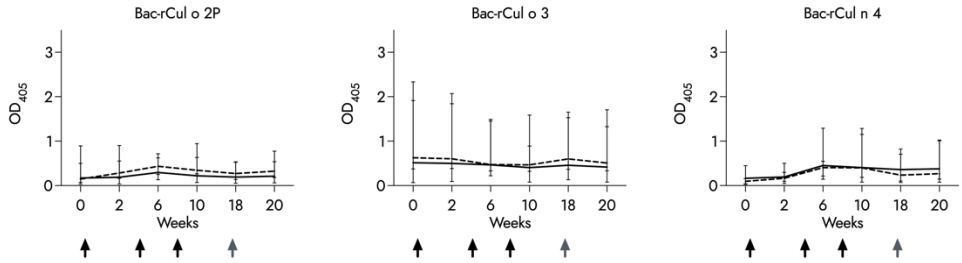


Figure 57. IgA antibody response of s.c./VLP and s.c./MPLA vaccinated horses. Time course of allergen-specific IgE subclass response of s.c./VLP (—) and s.c./MPLA (- - -) vaccinated horses, measured by ELISA against Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4. The graphs show corrected OD_{405} median values with 95% confidence interval for the six horses in each vaccination group at different time points. Black arrows indicate vaccinations and grey arrow intradermal test. Comparisons between vaccination groups were performed using Mann-Whitney U test with Bonferroni corrections.

A similar pattern with a very weak IgA response was seen in the s.c./MPLA group as in Figure 43 (4.2.5). The s.c./VLP group showed similar IgA response. Against Bac-rCul o 2P both groups had highest IgA response against Bac-rCul o 2P at week 6 and against rBac-Cul n 4 the response was similar between week 6 and 10. For Bac-rCul o 3, both groups already had rather high IgA levels before vaccination with no increase following vaccination.

4.3.5 Blocking capacity of sera from vaccinated horses

Testing of the blocking capacity of the serum pool from the s.c./MPLA group gave similar results as obtained before (4.2.6) and was in general somewhat more effective than the serum pool from the s.c./VLP group for all allergens (Figure 58).

Against Bac-rCul n 4 the blocking capacity of s.c./VLP serum from post-immunization was over 90% at the 1:10 dilution and over 80% at 1:20 dilution and thus similar to the s.c./MPLA group, but in the 1:40 dilution the blocking of s.c./MPLA serum was still over 80% but only 60% in s.c./VLP (Figure 58). The blocking capacity of IgE binding to Bac-rCul o 2P and Bac-rCul o 3 was lower in the s.c./VLP in all dilutions. At dilution 1:10 the blocking capacity to Bac-rCul o 2P was slightly over 70% but close to 90% for s.c./MPLA and to Bac-rCul o 3 around 65% for s.c./VLP but 85% for s.c./MPLA. Pre-immune sera showed no or marginal blocking against Bac-rCul o 2P and Bac-rCul n 4. The blocking against Bac-rCul o 3 was around 20 % at dilution 1:10 for both vaccination groups.

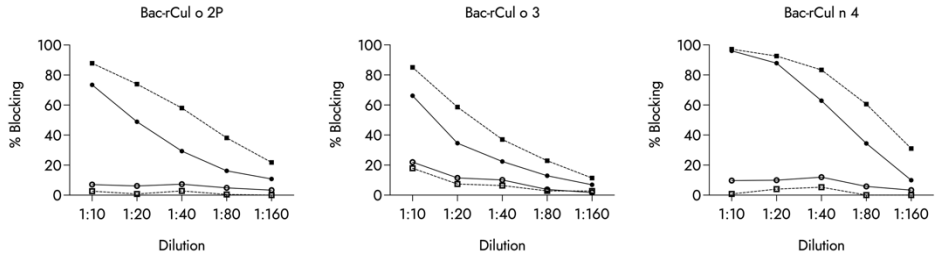


Figure 58. Blocking of IgE binding to r-allergens. Blocking capacity of sera from s.c./VLP and s.c./MPLA vaccinated horses of IgE binding from serum of IBH-affected horse to Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4. The graphs show mean % blocking of sera pools, s.c./VLP post-vaccination (–●–), s.c./VLP pre-vaccination (–○–), s.c./MPLA post-vaccination (- -■- -), s.c./MPLA pre- vaccination (- -□- -) diluted 1:10 – 1:160. The serum was added to ELISA plate coated with r-allergen prior to adding serum from IBH-affected horse at dilution 1:10.

4.3.6 Cytokine response after *in vitro* stimulation of PBMC

4.3.6.1 Cytokine expression at mRNA level following vaccination

Cytokine expression was analysed at mRNA level in the s.c./VLP vaccination group before and two weeks following third vaccination by *in vitro* re-stimulation of PBMC with pool of the three Bac-r-allergens used in the vaccination (Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4). After 24 h stimulation the cells were harvested, RNA isolated and IFN-γ, IL-10 and IL-4 mRNA expression measured with qPCR.

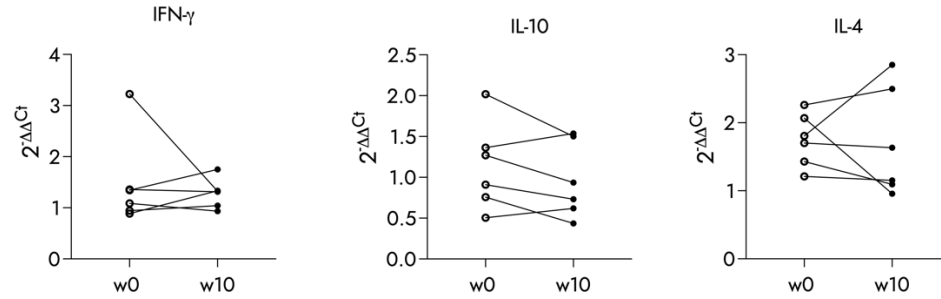


Figure 59. Cytokine expression following s.c./VLP vaccination. Cytokine expression after *in vitro* re-stimulation of PBMC pre- (w0) and post-vaccination (w10) with mixture of Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4. The cytokine expression was measured by qPCR. Comparison of pre- and post-vaccination was performed using Wilcoxon test. The graphs show individual $2^{-\Delta\Delta Ct}$ values for the horses.

No significant difference was seen in IFN-γ, IL-10 and IL-4 mRNA expression before and after vaccination (Figure 59). For all three measured cytokine mRNA expression, there was increase in two horses but the other four showed no difference or decrease.

4.3.6.2 Secreted cytokine expression following vaccination

The cytokine production of the s.c./VLP horses was measured in supernatant of PBMC upon re-stimulation with the allergens (Figure 60). Following stimulation with the three r-allergens used in the vaccination there was no significant increase in any of the three cytokines after vaccination compared to pre-vaccination. Two of the horses showed very high IFN-γ levels before vaccination and there was no further increase following vaccination, but in the other horses IFN-γ production increased after vaccination. In four out of the six horses there was an increase in the IL-10 response and in four horses IL-4 production was lower at week 10 compared to week 0 (Figure 60A).

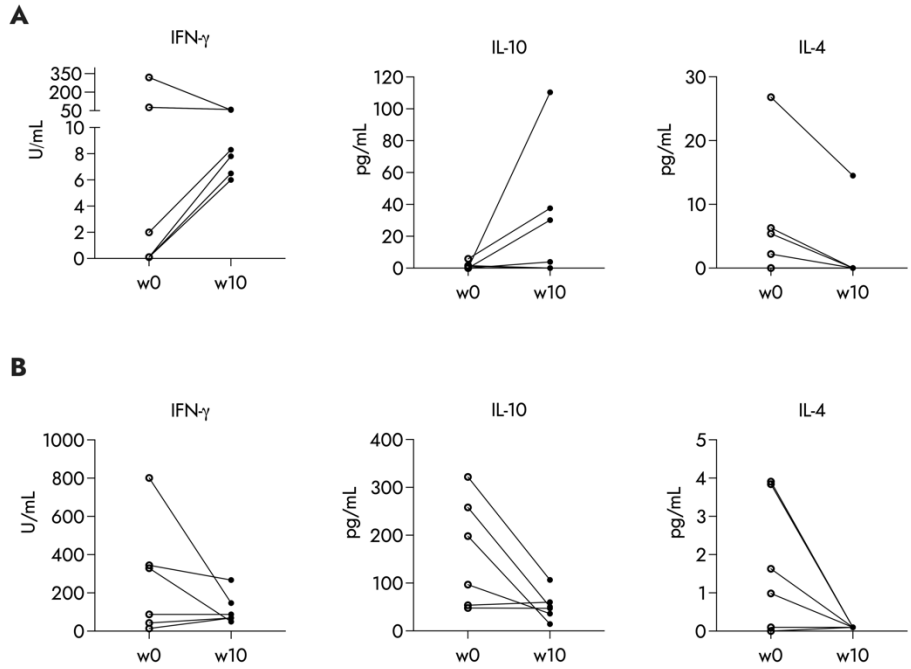


Figure 60. Comparison of cytokine secretion pre- and post s.c./VLP vaccination. The cytokine secretion was measured in supernatant of *in vitro* re-stimulated PBMC by bead-based multiplex assay. Comparison of cytokine secretion pre- (w0) and post-vaccination (w10) of s.c./VLP were performed using Wilcoxon test. An asterisk (*) with line indicates statistical difference between pre- and post-vaccination within horse groups. **A.** Cytokine secretion after *in vitro* re-stimulation of PBMC with mixture of Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4. **B.** Cytokine secretion after *in vitro* re-stimulation of PBMC with CuMV-TT.

Following *in vitro* stimulation of PBMC with CuMV-TT (VLP derived from Cucumber mosaic virus covalently linked to tetanus toxin) there was no significant difference before and two weeks following vaccination (Figure 60B). CuMV-TT induced strong IFN-γ and IL-10 response already before vaccination (w0). Following vaccination there was increase in IFN-γ levels in three horses but decrease in the other three horses. All but two horses showed decrease in IL-10 response and there was decrease in IL-4 levels for all six horses.

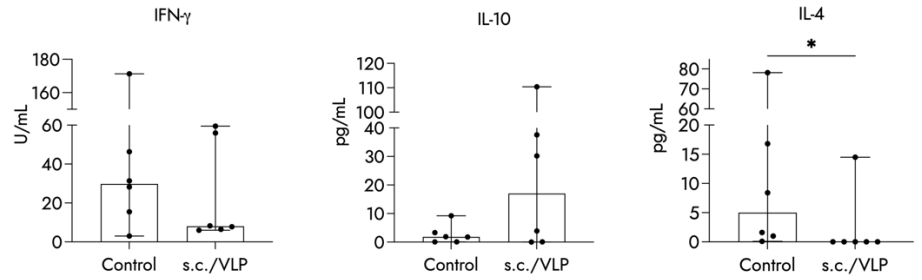


Figure 61. Cytokine secretion following s.c./VLP vaccination. The cytokine secretion was measured by bead-based multiplex assay. Comparison of s.c./VLP and control horses was performed using Mann Whitney U test. The graphs show individual values for the horses and median and 95% confidence interval for the groups. An asterisk (*) with line indicates statistical difference between the groups of horses.

Compared to non-vaccinated controls at week 10 there was no significant difference in IFN-γ and IL-10 levels against the r-allergens used in the vaccination, but IL-4 levels were significantly higher in control horses (Figure 61).

4.4 Comparison of different amounts of r-allergens in vaccination

In a former study, horses were vaccinated i.l. using alum/MPLA as adjuvant, with four r-allergens (rCul n 3, rCul n 4, rCul n 8, rCul n 10) using 10 µg per allergen (Jonsdottir et al., 2016). To test whether the IgG response was different when using 20 µg instead of 10 µg r-allergen, the IgG subclass responses were determined by ELISA using rCul n 4, the allergen that was common to both study groups. Both vaccinations with 10 µg and 20 µg resulted in increase in IgG subclass response which reaches significant difference both two weeks after second (week 6) (data not shown) and third vaccination (week 10) (Figure 62).

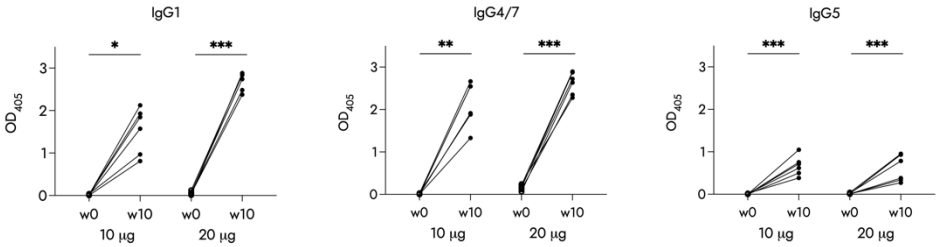


Figure 62. IgG subclass resonse pre- and post- vaccination of horses vaccinated with 10 µg and 20 µg r-allergens. IgG subclass response against Bac-rCul n 4, before and after immunization (week 0 and 10) of i.l./MPLA/10 µg and i.l./MPLA/20 µg vaccinated horses measured by ELISA. Individual corrected OD_{405} values for the six horses in each group. Comparisons were performed using Friedman test. An asterisk (*) with line indicates statistical difference between the time-points within the horse groups.

Two weeks after the third vaccination (week 10) and at week 16 the IgG1 levels in the i.l./MPLA/10 µg group was significantly lower than in the i.l./MPLA/20 µg horses (Figure 63). However, the IgG4/7 was only slightly but not significantly lower at those

time points and no difference was observed for the IgG5. Interestingly though, the response was more uniform when using the dose of 20 µg, i.e. antibody levels at week 10 were very similar between the horses (Figure 62).

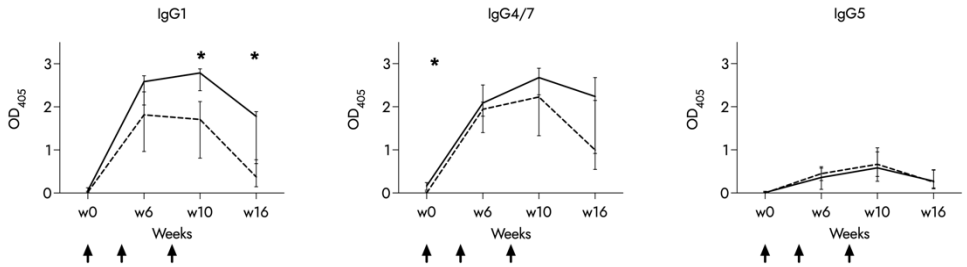


Figure 63. IgG subclass response of horses vaccinated with 10 µg and 20 µg r-allergens. Time course of IgG subclass response against Bac-rCul n 4 measured by ELISA. Corrected OD_{405} median values and standard error for the six horses in each group at different time points. Horses vaccinated i.l./MPLA/10 µg (- - -) and i.l./MPLA/20 µg (—). Black arrows indicate vaccinations. Comparisons were performed using Mann-Whitney test with Bonferroni corrections. An asterisk (*) with line indicates statistical difference between the groups of horses.

4.5 Challenge experiment

In a challenge experiment, 27 healthy horses in Iceland were vaccinated and exported to *Culicoides* infested areas in Switzerland. Following export, one horse was withdrawn from the experiment and is therefore not included in the analysis.

4.5.1 Allergens

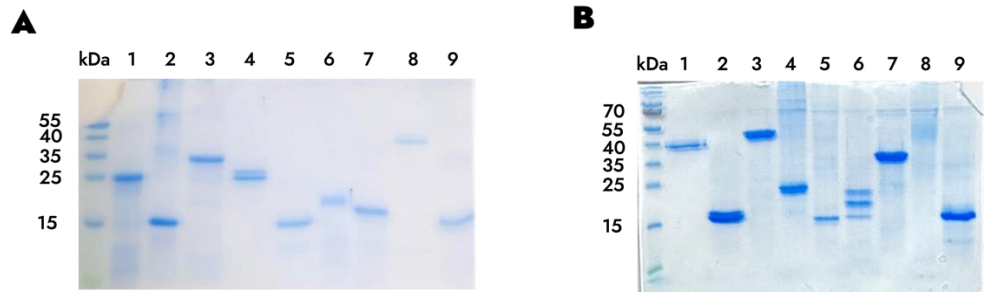


Figure 64. R-allergens purified from *E. coli* and insect cells used in the challenge experiment. **A.** r-allergens purifed from *E. coli* used for vaccinating horses before challenge, **B.** r-allergens purified from insect cells used in immunoassays evaluating immune response following vaccination. **1.** rCul o 1P; **2.** rCul o 2P; **3.** rCul o 3; **4.** rCul o 5; **5.** rCul o 7; **6.** rCul o 8; **7.** rCul o 9; **8.** rCul o 11; **9.** rCul n 4.

Nine *Culicoides* r-allergens expressed in *E.coli* and purified (Figure 64A) (rCul o 1P, rCul o 2P, rCul o 3, rCul o 5, rCul o 7, rCul o 8, rCul o 9, rCul o 11 and rCul n 4) were used to vaccinate 27 horses in Iceland before they were challenged by exporting them to *Culicoides* infested areas in Switzerland and Germany. For immunoassays nine corresponding r-allergens were produced in insect cells and purified (Bac-rCul o 1P,

Bac-rCul o 2P, Bac-rCul o 3, Bac-rCul o 5, Bac-rCul o 7, Bac-rCul o 8, Bac-rCul o 9, Bac-rCul o 11 and Bac-rCul n 4) (Figure 64B).

4.5.2 Clinical examination following vaccination

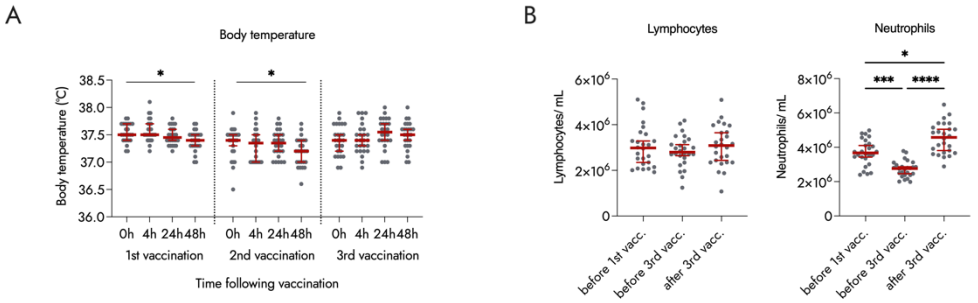


Figure 65. Body temperature and leukocyte count before and after vaccination in challenge experiment. **A.** Body temperature before, 4h, 24h and 48h following the three vaccinations. **B.** Leucocyte count before first and third and following third vaccination. Comparison between timepoints was performed using Friedman multiple comparisons test. The graphs show individual values, median and 95% confidence interval. An asterisk (*) with line indicates statistical difference between timepoints; **A.** shows comparison of timepoints within each vaccination.

Clinical examination following vaccination showed no rise in body temperature (Figure 65A) nor pain at injection site on palpation indicating the vaccination was well tolerated (data not shown). Leukocyte count was performed following vaccination (Figure 65B) statistical difference was observed before and after vaccination in neutrophil count, however within normal range.

4.5.3 Total IgG response following vaccination

Following third vaccination and before export, total IgG response of the 26 challenge horses was tested against Bac-rCul o 1P, Bac-rCul o 8 and Bac-rCul n 4 (Figure 66) for preliminary results on whether the vaccination resulted in generation of allergen-specific IgG antibodies.

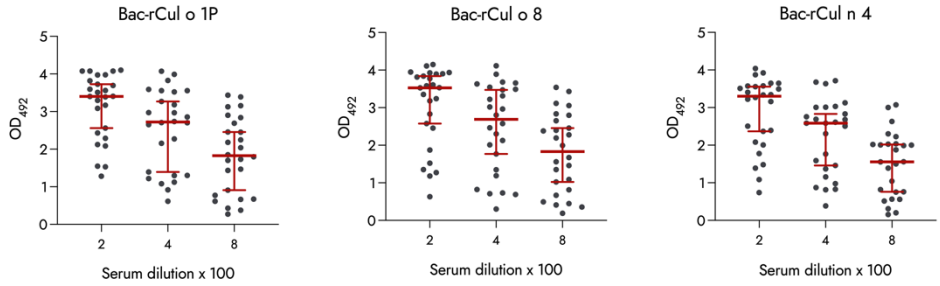


Figure 66. Total IgG response at week 10 of vaccinated horses in challenge experiment. Total IgG response against Bac-rCul o 1, Bac-rCul o 8 and Bac-rCul n 4 tested in different serum dilution (1:200 – 1:800). The graphs show individual OD_{492} values, median and 95% confidence interval.

The total IgG antibody response of the 26 vaccinated horses was similar against the three r-allergens, Bac-rCul o 1P, Bar-rCul o 8 and Bac-rCul n 4. In serum dilution 1:200 the median OD_{492} value was over 3, decreased under 3 in serum dilution 1:400 and was under 2 in serum dilution 1:800. IgG responses of the horses ranged from around 1 in OD_{492} value of low responders to around 4 OD_{492} value high responders at serum dilution 1:200 (Figure 66).

4.5.4 Clinical examination three years following export

Following vaccination in the challenge experiment, 27 horses were exported to Switzerland where they were kept unprotected from *Culicoides* midges, that is not covered in blanket or housed during dusk and dawn. During midge season clinical examination and blood collection was carried out monthly. The IBH status was confirmed with sLT release test (data not shown).

Table 9. IBH status of vaccinated horses three years following export from Iceland.

	Healthy horses	**IBH – affected horses**	**% IBH affected horses**
1st year	20	6	23.1
2nd year	13	13	50.0
3rd year	10	16	61.5

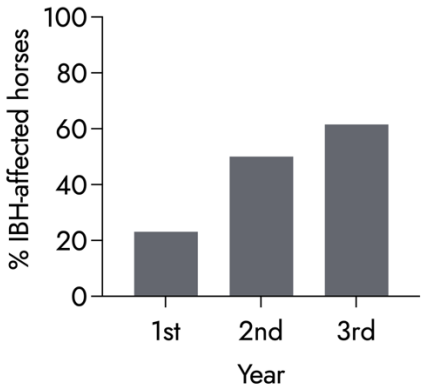


Figure 67. IBH status of vaccinated horses three years following export. The graph shows percentage of IBH-affected horses each year for three years following export to *Culicoides* infested areas in Switzerland ang Germany.

Following the first year after export from Iceland, 6 horses developed IBH or 23.1%, additionally 7 horses after the second year or total 50% and then 3 more resulting in 61.5% IBH - affected horses in the end of the third year (Table 9 and Figure 67).

4.5.5 Allergen-specific IgG subclass and IgE response following vaccination and the first two years after export

Sera from the challenge horses were tested for IgG subclass and IgE response by ELISA against three Bac-r-allergens before, two weeks after vaccination (week 10) and monthly during the *Culicoides* season (April – September) over the first two years following vaccination. A similar time course pattern of IgG subclass responses was observed against the three Bac-r-allergens, Bac-rCul o 2P, Bac-rCul o 8 and Bac-rCul o 9 (Figure 68).

In general, based on median corrected OD values, there was an increase in IgG1 levels two weeks following the third vaccination (week 10) and this was the highest response observed during the time course. The IgG1 response decreased over time during the first year following export. Before the *Culicoides* season of the second year following export, the horses received a booster vaccination. There was an increase in IgG1 following the booster. As in the first year, the IgG1 responses decreased and were low at the end of the *Culicoides* season of the second year. The IgG1 increase following vaccination and booster vaccination was significant against all three Bac-r-allergens (Figure 69).

A similar pattern was seen for IgG4/7 responses against the three allergens, the highest median OD value were reached at week 10 following vaccination, but the decrease in IgG4/7 antibody levels following vaccination and booster vaccination was not as steep as compared to the IgG1 (Figure 68). There was a significant increase in

IgG4/7 response following vaccination and booster vaccination against all three Bac-r-allergens except for Bac-rCul o 9 following the booster vaccination (Figure 69).

The IgG5 antibody response was low against Bac-rCul o 2P and there was a slight elevation throughout the *Culicoides* seasons, highest in September both in the first- and second year following export. Against Bac-rCul o 8 the response increased significantly following vaccination and booster vaccination (Figure 69) and levels remained similar during the *Culicoides* season both years (Figure 68). A significant increase in IgG5 response against Bac-rCul o 9 was observed following vaccination (Figure 69). There was a slight decrease in IgG5 levels following export which then remained similar during the *Culicoides* season of the first year. There was an elevation in IgG5 levels following booster vaccination, but the difference did not reach significance and the response increased throughout the *Culicoides* season during the second year (Figure 68).

IgE responses were barely detectable against Bac-rCul o 2P. In September of the first year, two horses showed slight elevation in IgE levels compared to the other horses but no increased IgE levels were detected during the second year (Figure 68). One horse showed elevated IgE (OD_{492} >0.5) to Bac-rCul o 9 in September of the first year and both in June and September of the second year. Additionally, one horse showed elevated IgE levels against Bac-rCul o 9 in the middle of the *Culicoides* season (June) of second year and three horses at the end of the season (September). In total five IBH-affected horses showed elevated IgE levels against Bac-rCul o 9 over the two years following export. IgE responses against Bac-rCul o 8 were stronger based on OD_{492} values when compared to Bac-rCul o 2P and Bac-rCul o 9 (Figure 68). In the first year, elevated IgE against Bac-rCul o 8 was observed in one horse at the beginning of *Culicoides* season (April) and in two horses in the middle of the season (June), as well as a slight elevation in one horse. In September, one of these horses still had elevated IgE responses to Bac-rCul o 8. Furthermore, an increase of IgE levels to this allergen was detected in four horses that had been low on Cul o 8 specific IgE so far. Before and following booster, four horses had slightly higher IgE levels to this allergen compared to the other horses. In the middle of the second summer (June), six horses showed elevated IgE levels and in three of them the OD_{492} value exceeded 0.5. In September, nine horses had elevated IgE against Bac-rCul o 8 of which seven had values above 0.5 OD_{492} (Figure 68). Over the two years following export a total of eleven IBH-affected horses showed elevated IgE levels against Bac-rCul o 8. One of the IBH-affected horses showed IgE reaction against all three Bac-r-allergens tested (Bac-rCul o 2P, Bac-rCul o 8 and Bac-rCul o 9) and three horses against two of them.

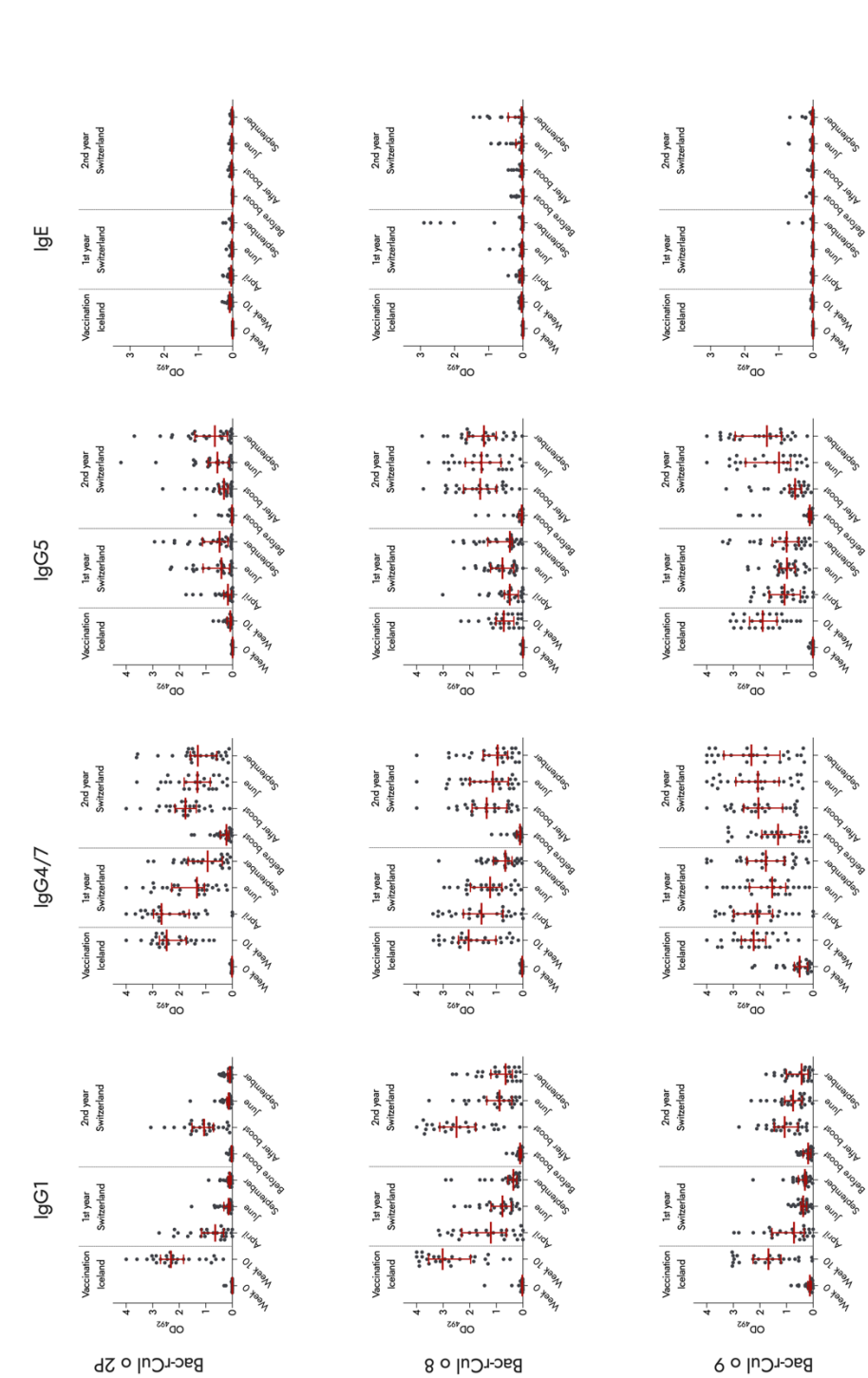


Figure 68. IgG subclass and IgE response before and following vaccination of challenge horses (n = 26). Time course of allergen-specific IgG subclass and IgE response measured by ELISA against Bac-rCul o 2P, Bac-rCul o 8 and Bac-rCulo 9. The graphs show corrected OD_{492} values, median and 95% confidence interval.

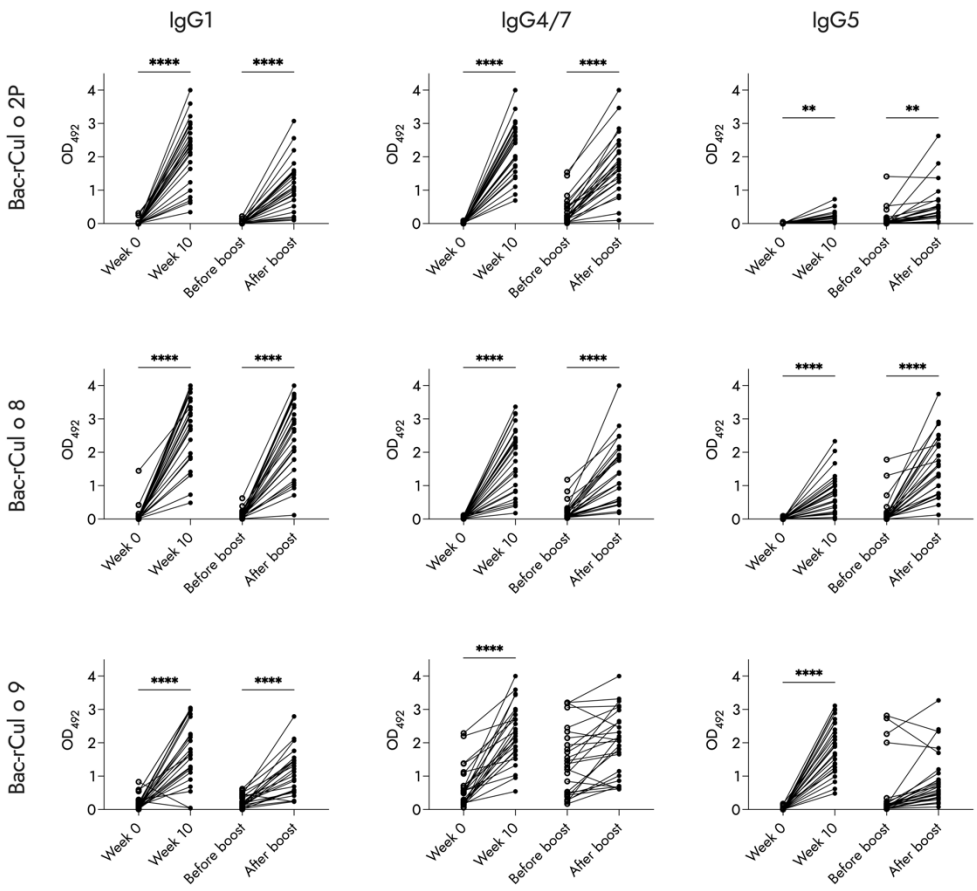


Figure 69. IgG subclass resonse pre- and post- vaccination and booster vaccination of challenge horses. IgG subclass response measured by ELISA of horses pre- and post- vaccination (week 0 and 10) and booser vaccination following first year after export from Iceland against rBac-Cul o 2P, Bac-rCul o 8 and Bac-rCul o 9. The graphs show individual correct OD_{492} values of the 26 horses. Comparisons between the time-points within the horse groups were performed using Friedman test. An asterisk (*) with line indicates statistical difference.

4.5.6 Comparison of allergen-specific IgG subclass and IgE response of IBH-affected and healthy horses following vaccination

IgG subclass and IgE response of the challenge horses was further analysed based on IBH status in the end of second- and third year following vaccination by comparing healthy and IBH-affected horses. No significant difference was seen for any IgG subclass response at any time-point tested between the horses grouped according to their clinical diagnosis at the end of the second (Figure 95, appendix XV) or third year (Figure 70). When the IBH status at the end of the second study year was used, there was a significant difference between IBH and healthy horses for IgE levels against Bac-rCul o 8 and Bac-rCul o 9 in September of the second year (Figure 96, appendix XV).

If based on the IBH status at the end of the third-year, differences between the groups reached significance for IgE against rBac-Cul o 8 in June and September of the second study year and against Bac-rCul o 9 in September of the same year (Figure 71).

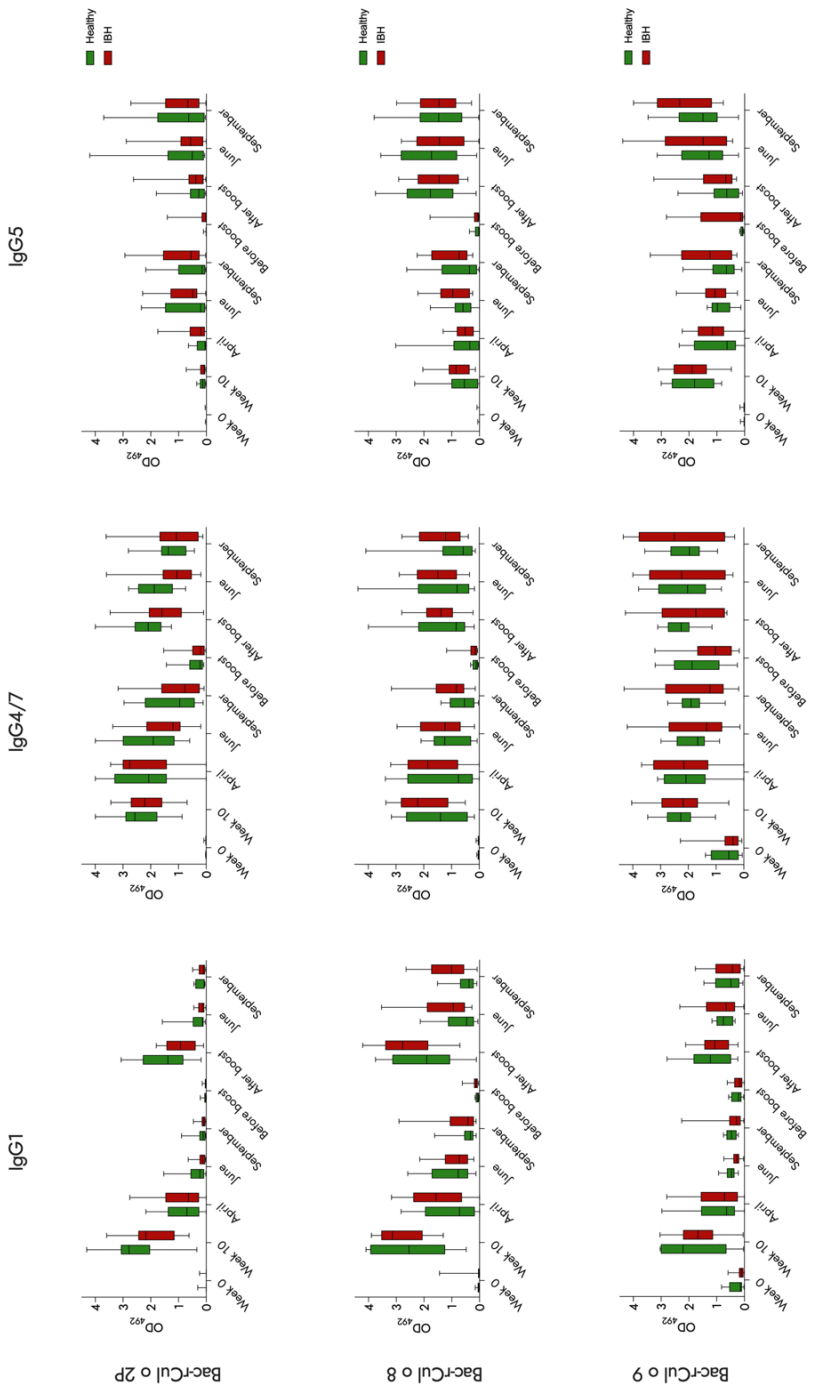


Figure 70. IgG subclass response of vaccinated horses in challenge experiment based on IBH status three years after export. Allergen-specific IgG subclass response of vaccinated horses measured by ELISA against Bac-rCul o 2P, Bac-rCul o 8 and Bac-rCul 0 9. The graphs show corrected OD_{492} median values in box and whisker plot for the twenty six horses at different time points divided in healthy (green) and IBH-affected horses (red). Comparisons between vaccination groups were performed using Mann-Whitney U test with Bonferroni corrections.

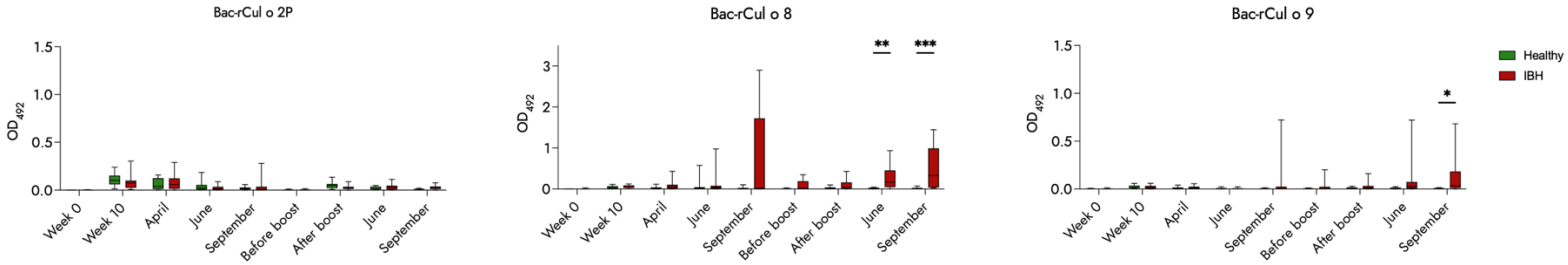


Figure 71. IgE response of vaccinated horses in challenge experiment based on IBH status three years after export. Allergen-specific IgE response of vaccinated horses measured by ELISA against Bac-rCul o 2P, Bac-rCul o 8 and Bac-rCul 0 9. The graphs show corrected OD_{492} median values in box and whisker plot for the twenty six horses at different time points divided in healthy (green, n= 10) and IBH-affected horses (red, n= 16). Comparisons between outcome groups were performed using Mann-Whitney U test with Bonferroni corrections. An asterisk (*) indicates statistical difference between vaccination groups at a given time-point.

5 Discussion

5.1 The Icelandic horse and IBH

The only horse breed in Iceland is the native Icelandic horse, pure bred since it was brought to the country during the early Viking settlement, in the 9th and 10th centuries. Importation of horses has been prohibited by law since 1882 and there is no historical documentation for import of breeding animals after the settlement period. Due to the long period of isolation, Icelandic horses are immunologically naïve to various agents known to infect horses in other countries. The Icelandic horse was exported to Europe in the mid 19th century to serve as a working horse, a practice that decreased when machines replaced horses in the workplace (Bjornsdottir et al., 2006). They are unique for their temperance and diligence and are excellent riding horses with five gaits. The Icelandic horse has become a popular riding horse especially in Scandinavia and Western Europe and the export has increased greatly again since the mid 20th century. Around 1400 horses are annually exported and 2021 was a record year with 3341 exported horses (Hreinsdottir, 2021; Möller, 2009). With increased export, high prevalence of IBH was noted in horses born in Iceland and exported to *Culicoides* infested areas, becoming a major problem for the Icelandic horse export industry and animal welfare issue with reported prevalence of over 50% if not protected from insect bites (Bjornsdottir et al., 2006; Torsteinsdottir et al., 2018). IBH-affected horses suffer from severe itch resulting in hair loss and skin damage which can lead to secondary infections greatly compromising the welfare of these horses. The causative *Culicoides* species are not indigenous in Iceland and therefore the disease does not occur domestically (Schaffartzik et al., 2012). The black fly (*Simulium vittatum*) is known to bite horses in Iceland but not resulting in sensitization. It has been shown that sensitization to *S. vittatum* in IBH-affected horses is secondary to *Culicoides* due to cross-reactivity (Schaffartzik et al., 2010; Torsteinsdottir et al., 2018).

Studies of IBH have been ongoing since the year 2000 at the Institute for Experimental Pathology at Keldur in collaboration with the Vetsuisse Faculty, University of Berne, Switzerland, with the final aim of developing immunotherapy against equine IBH. Initially, the research aimed at identifying and characterizing the causative allergens of IBH and analysing the immune response leading to IBH in horses. Presently, numerous *Culicoides* allergens have been identified and studies of the pathogenesis demonstrate an imbalance within T-cell subsets. This has opened the possibility to develop an allergen-specific immunotherapy against IBH. In the current thesis the focus is on two approaches: I) development of preventive allergen-specific immunotherapy by vaccination before sensitization with purified IBH-allergens in adjuvants, II) curative

treatment by treating IBH-affected horses via the oral mucosa of the mouth with transgenic barley expressing IBH-allergens. It was shown that adjuvant was essential for obtaining preferable allergen-specific antibody response and i.l. injection gave stronger response compared to i.d. injection (Jonsdottir et al., 2015). The first experimental vaccination used the adjuvant IC31, but as collaboration with the company providing the adjuvant discontinued, commercially available adjuvants were tested, a mixture of alum/MPLA resulted in a Th1/Treg focused immune response (Jonsdottir et al., 2016). The aims of the current study were to further investigate the route of injection and adjuvant effect before a challenge experiment would be carried out where vaccinated horses would be exposed to *Culicoides* midges. The second approach is done in collaboration with ORF Genetics by expression of r-allergens in transgenic barley. In a prior study, healthy horses were treated with transgenic barley expressing Cul n 2. This resulted in induction of allergen specific IgG in blood and saliva able to partly block IgE binding to the corresponding allergen (Jonsdottir et al., 2017). The specific aim of the current study was to extend the study and treat IBH-affected horses with transgenic barley based on sensitization profile in collaboration with Dr. Bettina Wagner, Cornell University, USA. At Cornell University, at that time, experimental Icelandic horses with IBH were available and some preliminary testing of allergen sensitization had been done. However, the treatment could not be performed due to border restrictions of USA during the Covid-19 pandemic. Since this was impossible and in view of the microarray results obtained on horses in Europe, it was important to express alternative allergens in barley.

Preventive vaccination is well established against various infectious agents. However, vaccination to prevent allergic sensitization is not yet practiced in humans, although being considered with great interest as allergic diseases affect almost 30% of the human population in industrialized countries (Shamji et al., 2021). An important factor in preventive immunotherapy against allergy is that the administration of the vaccine and formation of allergen-specific IgG is carried out before sensitization (Akinfenwa et al., 2021a). Iceland is free of the causative IBH agent and horses are only sensitized after export, providing a good opportunity for development and study of preventive immunotherapy against allergy. Horses present a natural disease model for studying immune mechanisms (Larson & Wagner, 2021b). They share many diseases with humans and unlike mice, provide a heterogeneous large animal model with naturally occurring inflammatory disorders, filling in gaps of information that murine models eventually cannot provide. The experimental settings and environment of horse studies can be controlled eliminating confounders of human clinical studies. Additionally, blood can be collected routinely in large volumes (reviewed in Horovhov, 2015 and Larson & Wagner, 2021). However, horses are large, expensive animals and maintenance is relatively costly. As they are not frequently used, reagents for evaluating immune responses have not been generated to the same extend as for mice and man (Wagner, 2023). IBH is caused by IgE-mediated reactions and is thus a good model for

allergy. Before a preventive immunotherapy study for IBH can be carried out, a suitable immunization protocol had to be established and the major allergens for IBH in horses exported from Iceland had to be identified.

5.2 Allergen preparation for immunotherapy against IBH

AIT is the only curative treatment of allergy practised. Pure, well-defined allergens displaying the relevant epitopes should preferably be used in AIT. Crude extracts vary in allergen composition with different immunogenicity profile and often miss major allergens (Huang et al., 2023). The advantage of recombinant native-like allergens is that they are well-defined and can be produced in a cost-effective and reproducible manner (Zhernov et al., 2019). B-cells and antibodies recognize the native form of allergens (Heesters et al., 2016). T-cells recognize processed allergen peptides bound to MHC molecules on the surface of APC. Some peptides may contain PTM affecting antigen recognition (Petersen et al., 2009). Native-like r-allergens retain IgE and T-cell epitopes of corresponding natural allergens increasing the risk of side effects and sensitization (Dorofeeva et al., 2020). Major factors driving allergy sensitization are inherent susceptibility of patient, exposure to allergens in terms of conditions, duration and route of exposure as well as co-factors and physicochemical properties of allergens. Characteristics of allergens important for allergenicity are structural features, enzymatic activity, PTMs (in particular glycosylation) and physicochemical properties such as stability (Krutz et al., 2020).

Numerous *Culicoides* allergens have been isolated and expressed as r-allergens and recently some major allergens causing IBH were identified (Novotny et al., 2021). This is essential for deciding which allergens are important for AIT. These allergens belong to protein families of Kunitz protease inhibitors, D7-related proteins, WSC superfamily, Apolipophorin III like, Antigen-5 like and proteins with unknown function (Novotny et al., 2021). Some were shown to be important in enabling blood feeding as Kunitz-type inhibitors in Ticks (Jmel et al., 2023) and D7-related proteins in Nematocera (Alvarenga & Andersen, 2022).

5.2.1 Expression and purification of r-allergens in *E. coli* and insect cells

The choice of the appropriate protein expression system depends on specific characteristics of the protein of interest, application of use and desired properties of the r-protein. In our studies the r-allergens used for vaccination are produced in *E. coli* and purified under denaturing conditions and therefore, lacking the native structure and some physicochemical properties of *Culicoides* allergens. For example, both in terms of PTMs and protein folding, thus reducing the risk of sensitization and allergic side-effects. Unpublished studies show that some *E coli* expressed *Culicoides* allergens were not able to induce sLT release while insect cell expressed allergens did (E. Marti,

personal communication). Despite that, the vaccinated horses in our experiments develop an immune response against native-like r-allergens produced in insect cells.

For vaccination using VLP as adjuvant system, it is beneficial to couple the purified r-allergens to the VLP. Therefore, the r-allergens intended for use in vaccination with VLP were expressed in *E.coli* with amino acid linker, GGC, for coupling. Overexpressed *E.coli* produced r-allergens often result in formation of inclusion bodies which need to be solubilized with denaturation when purified and refolded to recover purified r-allergen (Singhvi et al., 2020). Also, elimination of endotoxins such as LPS can be tedious in purification of r-proteins produced in *E. coli*. Purification protocols have been established to increase protein purity and reduce endotoxin contamination using chelating agents for cell membrane washing and reduction of endotoxins and non-ionic surfactant treatment for isolation of inclusion bodies (Choi et al., 2014). These methods were applied as pre-steps for purification under denaturing conditions of *E.coli* produced r-allergens successfully expressed in this study (i.e. *E. coli*-rCul o 1P rCul o 2P and rCul n 4 with GGC amino acid linker). The purified r-allergens were refolded using compounds shown to facilitate refolding by stabilizing effects and inhibition of aggregation. These allergens had been successfully expressed in *E. coli* and purified previously. Due to low expression of the GGC linked allergens *E. coli*-rCul o 1P and -rCul o 2P, the purification resulted only in a sufficient yield of *E. coli*-rCul n 4. Further optimization of *E. coli* expression of *E. coli*-rCul o 1P, rCul o 2P and rCul o 3 containing GGC is needed to obtain purified r-allergens. Endotoxin levels were not measured in the purified r-allergen (*E. coli*-rCul n 4 (GGC)) and therefore reduction of endotoxins was not confirmed.

All horses have immune responses against *E. coli* proteins and LPS is a mitogen which unspecifically stimulates lymphocytes. LPS stimulates Th cells through TLR4 and is known to induce Th1 responses (McAleer & Vella, 2008). The r-allergens purified from *E. coli* risk having traces of impurities and have proven to be difficult in cellular immunoassays i.e. *in vitro* re-stimulation of PBMC, giving too much background and they do not work in sLT release test probably because they cannot crosslink IgE due to incorrect protein folding (Jonsdottir et al., 2015; Jonsdottir et al., 2021). As *Culicoides* allergens originate in the salivary gland of an insect, the Bac-to-Bac insect cell expression system should be able to provide native-like PTMs and structural properties of r-allergens. *E. coli* as expression system provides well- characterized system with fast growth, simple genetic manipulation and short production cycle while insect cells are more complex expression system with higher cost, especially regarding cell medium, longer production cycle as compared to *E. coli* (Tripathi & Shrivastava, 2019). Unlike *E. coli*, insect cells carry chaperones providing proper protein folding and metabolic pathways essential for complex PTMs making insect cells more suitable for expression of complex proteins that require proper folding and PTMs (Rivera-de-Torre et al., 2021). The choice of expression system should therefore be assessed case-by-case with required properties of r-proteins in mind, for example activity. In comparative study,

recombinant honeybee chymotrypsin-like protease was expressed and produced in both *E. coli* and insect cells. Both enzymes were shown to be of comparable molecular size and to have comparable specificity. However, the enzyme produced in insect cells was shown to have higher specific activity as compared to the enzyme produced in *E. coli*. (Matsuoka et al., 2017).

In this study four newly identified major allergens were expressed in insect cells with $(His)_6$- tag using Bac-to-Bac expression with two different expression vectors (i.e. pFastBac1 and pI-secSUMOstar) commonly used for optimizing expression and purification. The cell lines used were Sf9 (Spodoptera frugiperda) for production of recombinant baculoviruses and High-five (Trichoplusia ni) for production of r-allergen. Different host cell lines have been shown to affect expression level, production yield and glycosylation pattern and therefore screening of cell lines has been recommended for different r-proteins (Rivera-de-Torre et al., 2021). Eight r-allergens (Bac-rCul o 1P, -Cul o 2P, -rCul o 3, -rCul o 5, -rCul o 7, rCul n 3, -rCul n 4, -rCul n 8) expressed in insect cells prior to this study were as well produced and purified for application in immunoassays following vaccination experiments. The same purification method using nickel affinity gel binding the $(His)_6$-tag of the target and elution with imidazole was used for the insect cell expressed r-allergens. All allergens were purified under native conditions except for Bac-rCul o 1P which required purification by denaturation followed by refolding using the same refolding facilitating compounds as the purified *E. coli*–rCul n 4. There is still room for improvements in the purification and quantification strategy for obtaining better defined purified r-allergens. The structure and properties of the allergens are variable and the same purification method might not be optimal for all of them. With further characterization of the physical properties of the r-allergens, purification strategy might be optimized for increased yield, purity and stability of the r-allergens.

Good sources of purified r-allergens preferably with tertiary structure and PTM close to native proteins, are essential for effective diagnostic test and for analysing the sensitization profile of IBH-affected horses as guidance for immunotherapy. Currently, diagnostic tests are performed using crude WBE giving low specificity and/or sensitivity for diagnosis of IBH also lacking standardization (Schaffartzik et al., 2012). In terms of WBE, *C. obsoletus* extract has proven to be superior for diagnosis compared to *C. nubeculosus* extract (van der Meide et al., 2012) concurring with that *C. obsoletus* has been reported the most abundant species in Europe (Casati et al., 2009; Möhlmann et al., 2018; Townley et al., 1984; Vorsprach et al., 2009; Wilson et al., 2008). However, *C. obsoletus* cannot be bred in the laboratory like *C. nubeculosus* and must be collected in the wild which can be a difficult task. For that reason, it would be beneficial to produce and purify r-Cul o allergens for application in diagnostic tests. The four Bac-r-allergens, Bac-1-rCul o 8, Bac-SUMO-rCul o 9, Bac-SUMO-rCul o 10b and Bac-1-rCul o 11 were used in sLT release assay with good results: the sLT release was significantly higher in IBH-affected compared to healthy horses following re-stimulation

of peripheral blood leukocytes. Combination of severeal *Culicoides* Bac-r-allergens allowed to identify all IBH-affected horses, with only one false positive reaction (unpublished results). These results show that Bac-r-allergens are promising for use in diagnostic tests for IBH.

5.2.2 Expression and purification of barley-r-allergens

Barley is an excellent endotoxin-free expression system for r-proteins providing PTMs, stable long term storage of r-proteins in seeds (Magnusdottir et al., 2013). Development of curative immunotherapy via the mucosa of the mouth using recombinant barley expressing causative allergen of IBH, barley-rCul n 2, has shown promising results on healthy horses. It showed induction of allergen-specific IgG in blood and saliva that was able to inhibit binding of serum IgE from IBH-affected horse to rCul n 2 and rCul o 2 (hya) (Jonsdottir et al., 2017). An advantage of this method is that protein purification is not needed as the barley seeds are used directly. This method of using transgenic barley expressing r-allergens in desensitization of allergy could be applied as a novel approach in humans by feeding of transgenic barley expressing relevant allergen for example in the form of porridge. OIT in children with peanut allergy has shown to be promising by effectively inducing desensitization (Jones et al., 2022).

For expansion of using transgenic barley in curative treatment for IBH more allergens need to be expressed in barley, especially those identified as major and relevant for desensitisation of IBH-affected horses. Four r-allergens were successfully expressed in barley in collaboration with ORF Genetics, barley-rCul o 2P, barley-rCul o 2, barley-rCul o 3 and barley-rCul n 8, an attempt was also made for expressing barley-rCul o 5 but unfortunately it has not been successful so far, probably due to misinterpretation of T_1 and T_2 seed lines. Three generations of transgenic barley are required for generating sufficient amount for extraction and purification of r-protein leading to long gene-to-protein timeframe (Magnusdottir et al., 2013) compared to *E. coli* and insect cells. Barley seeds contain an abundance of proteins (Jaeger et al., 2021) and production in a plant system often results in low yield of r-proteins as compared to bacterial systems (Fischer & Buyel, 2020; Rozov & Deineko, 2019). Therefore, it is important to have specific and sensitive methods as WB and preferably target specific antibodies for screening the transgenic barley generations. For screening by WB, barley-rCul o 3, barley-rCul o 5 and barley-rCul n 8, α-his tag antibodies were used. This resulted in unspecific binding of irrelevant barley proteins making the interpretation of the results difficult. T3 generation barley lines were re-tested with allergen-specific antibodies. Screening of barley-rCul o 2P and barley-rCul o 2 was made with allergen-specific antibodies already starting from first generation seed lines and all seed lines sowed were positive in the following generations. This shows the importance of using protein-specific antibodies for screening of transgenic barley lines to eliminate mistakes in analysing results due to background binding of irrelevant proteins. Purified r-allergens from transgenic barley have been shown to be comparable to *E. coli* and insect cell

produced r-allergens in serological assays for evaluating immune response following immunotherapy against IBH (Jonsdottir et al., 2018) and diagnosis of IBH based on serum IgE antibody titer (Jonsdottir et al., 2021). In addition, barley produced r-allergens showed good performance in re-stimulation of PBMCs for analysis of cytokine production and were comparable with insect cell produced r-allergens (Jonsdottir et al., 2018). Barley-rCul o 2P and barley-Cul o 3 were successfully purified and their performance in immunoassays is under investigation. Purified barley-rCul allergens could be valuable for application in diagnostic tests. As the barley seed presents an excellent storage medium for protein allergens, they could be produced in mass and the barley-rCul allergens purified as needed.

5.3 Immunotherapy against equine IBH

5.3.1 Comparison of injection sites

Lymph nodes are highly organized and strategically positioned secondary lymphoid organs engaging in immune responses against pathogens by ensuring encounter of B- and T-cells with APCs. They are composed of subcapsular area, medulla, paracortex and cortex in which B- and T-cells home to separate areas. Pathogens are filtered from the lymph in the antigen-sampling zone located in the subcapsular zone and medulla of lymph nodes, dense in APCs which deliver antigens to the B- and T-cells zones. The B-cell zone also referred to as B-follicle is positioned in the cortex and contains B-cells while the T-cell zone containing T-cells is located in the paracortex (Junt et al., 2008; Willard-Mack, 2006). The lymph node is dense of B- and T-cells and by direct intralymphatic administration there is increased probability of antigen and specific lymphocyte encounter as compared to peripheral administration (Senti et al., 2019).

The anatomy of the skin presents three layers with the epidermis at the surface, the next layer is the dermis and the third layer is hypodermis or subcutaneous connective tissue. Antigens administered subcutaneously in the hypodermis elicit uptake, processing and maturation of DCs and Langerhans DCs located in the dermis and epidermis, respectively which migrate into the draining lymph node and secondary lymphoid tissue. Also, secondary antigen presentation takes place in lymph nodes following distribution of antigen to the draining lymph node (reviewed in Fathallah et al., 2013).

In a pilot study in healthy Icelandic horses using alum/MPLA, we have shown that i.l. injection gave the desirable immune response (Jonsdottir et al., 2016). However, injecting into the submandibular lymph nodes can be challenging, they can be difficult to locate and vary in size and accessibility between horses. We therefore wanted to see if it would be sufficient to use the more convenient s.c. route applying the same dosage and frequency of injection. Thus, the major aim of the study was a direct comparison of i.l. and s.c. injection.

In this study we used three allergens, two originating from the salivary glands of *C. obsoletus*; Cul o 2P (Peeters et al., 2013) and Cul o 3 (van der Meide et al., 2013) and one from *C. nubeculosus*; Cul n 4 (Schaffartzik et al., 2011). All three allergens are major allergens for horses of the Icelandic breed where over 80% of IBH-affected horses had IgE to Cul o 2P, over 60% to Cul o 3 and over 50% to Cul n 4 (Novotny et al., 2021).

The specific antibody response induced following i.l. and s.c. vaccination was similar for all IgG subclasses and the IgE blocking capacity did not differ between the groups. No specific antibody response was observed against Bac-rCul n 8, an irrelevant allergen not included in the vaccine. That implies that the induced antibody responses were allergen specific. IgE blocking capacity of allergen-specific IgG antibodies induced following vaccination is an important factor in successful AIT (López et al., 2022). In humans IgG4 antibodies have been shown to mediate protection against allergy symptoms by blocking of IgE binding to allergen(Rispens & Huijbers, 2023). In horses, linkage between Th1 responses and production of IgG4/7 has been postulated and research suggested IgG4 in horses to represent protective immunity (reviewed in Wagner et al., 2005 and Perkins & Wagner, 2015). We have not investigated which of the IgG subclass antibodies are responsible for blocking the binding of IgE to allergens, presenting protective immune responses against allergic symptoms. Generation of allergen tolerance is also important in AIT with increase in suppressive mediators such as IL-10 (Celebi Sözener et al., 2020). A significant increase in secreted IFN-γ and IL-4 was observed following i.l. and s.c. vaccination and in IL-10 following i.l. vaccination at week 10 post-vaccination compared to pre-vaccination measured with bead-based multiplex assay. Furthermore, the expression of IL-10 was significantly higher in both vaccination groups compared to control horses. However, there was not a significant difference between the two vaccination groups regarding any of the cytokines. This might be due to the small number of horses in each group and the high individual variation. These results indicate that i.l. injection may be replaced with the more convenient s.c. injection in AIT against equine IBH.

5.3.2 Comparison of adjuvant effect

VLP have been shown to be strong inducers of Th1 responses (Alam et al., 2021). VLP have been used effectively in development of long-term treatment of IBH by targeting equine IL-5 and IL-31 (Fettelschoss-Gabriel et al., 2019; Olomski et al., 2020). Therefore, we wanted to investigate whether VLP could be applied instead of MPLA as adjuvant. The three allergens used in the study had been expressed and purified as r-proteins from *E. coli* and insect cells (Jonsdottir et al., 2018; Novotny et al., 2021; Peeters et al., 2013; Schaffartzik et al., 2011). Attempts were made to express these allergens again with 3-amino-acid linker (GGC) at C-terminus in *E. coli* for coupling with the VLP-CuMV$_{TT}$. However, the proteins could not be successfully coupled with the VLP as the proteins had to be soluble in low salt concentration which was not feasible.

Therefore, the VLP were mixed in solution with the allergens and alum. This is not an optimal procedure as studies indicate that allergen fused with VLP induce more effective allergen-specific immune response than a combination of them (Sani et al., 2021). Novel VLP platforms consist of r-allergens genetically fused with VLP subunits or co-expression of r-allergen and VLP subunits (Ogrina et al., 2023). This technique has shown promising results in treatment of peanut allergy in murine model where the major peanut allergen Ara h 2 was genetically fused with $CuMV_{TT}$ forming mosaic VLPs (Sobczak et al., 2023).

In general, the vaccine containing VLP induced a lower IgG subclass response and a weaker IgE blocking capacity. Interestingly, IgG5 levels to Bac-rCul o 3 and Bac-rCul n 4 were slightly lower in the VLP compared to the s.c./MPLA group and the differences reached significance for Bac-rCul o 2P. As IgG5 has been linked to allergic responses (Wagner, 2006a) whereas IgG4/7 should be protective, we calculated the IgG5:IgG4/7 ratio. It was significantly lower for s.c./VLP at week 10 against Bac-rCul n 4 compared to s.c./MPLA and slightly lower for both week 6 and 10 for Bac-rCul o 2P and Bac-rCul n 4. This suggests that VLP might be a favourable immunomodulator for Th1/Treg focusing. Disappointingly we cannot draw any conclusion from the cytokine results obtained from the s.c./VLP as re-stimulation with the r-allergens induced cytokines in several horses already at week 0 while others did not respond at week 10. Nevertheless, this pilot study indicates that it would be worth trying other methods to couple the allergens to VLPs or by genetically engineered fusion of allergen and VLP (Sani et al., 2021).

5.3.3 Comparison of different amounts of r-allergen in vaccination

Horses were vaccinated i.l. using alum/MPLA using 10 µg per allergen with four r-allergens including *E. coli*-rCul n 4 prior to the study (Jonsdottir et al., 2016). The vaccination study comparing injection sites followed the same vaccination protocol except using 20 µg per allergen with three r-allergens including *E. coli*-rCul n 4. This provided opportunity of comparing different amounts of r-allergens used in vaccination. The antibody response against Bac-rCul n 4 was evaluated in the same ELISA using serum obtained from both vaccination studies. The IgG1 response was significantly higher at week 10 following vaccination with 20 µg per r-allergen than 10 µg and the IgG4/7 response was slightly higher, while importantly, the IgG5 response was similar. Interestingly, there was less variation at week 10 between horses vaccinated with 20 µg r-allergen i.e., suggesting that low responders have a stronger response with 20 than with 10 µg. For this reason, 20 µg per r-allergen were used in the vaccination protocol of the challenge experiment.

5.3.4 Challenge experiment

In the development of preventive immunotherapy against IBH, we have shown that vaccination of healthy Icelandic horses using purified r-allergens in adjuvant leads to

significant increase in allergen-specific IgG1 and IgG4/7 antibodies with the capacity of blocking allergen-specific IgE, without induction of IgE (Jonsdottir et al., 2015; Jonsdottir et al., 2016; Stefansdottir et al., 2022). To investigate the efficacy of the immunotherapy a challenge experiment had to be conducted with exposure of vaccinated horses to the bites of *Culicoides* midges. For that, healthy horses were vaccinated in Iceland and subsequently exported to *Culicoides* infested areas before the *Culicoides* season (April – September). The vaccination protocol was based on prior vaccination studies and the identification of the major allergens of IBH (Novotny et al., 2021).

For the challenge experiment, 27 healthy Icelandic horses were vaccinated i.l. with nine purified r-allergens, 20 µg each allergen per injection, in alum/MPLA adjuvant, three times with four-week interval. Following vaccination, the horses were exported to *Culicoides* infested areas, 27 horses to 13 different places or horse stables in Switzerland. For the challenge experiment it is important that the horses are exposed to bites of *Culicoides* midges, meaning they could not be dressed in blankets or housed during dusk and dawn when the midges are most active as usually is done to protect horses against *Culicoides* bites. Clinical examination of the vaccinated horses was carried out monthly during the *Culicoides* season (April – September) and blood samples taken over three years, the reported time-line in which IBH following export occurs (Sommer-Locher et al., 2012). One vaccinated horse was withdrawn from the experiment because of severe urticaria with need of corticosteroid treatment and thus not included in data analysis.

The nine allergens used in the vaccination were *E. coli*- rCul o 1P, rCul o 2P (Peeters et al., 2013), rCul o 3, rCul o 5, rCul o 7 (van der Meide et al., 2013), rCul o 8, rCul o 9, rCul o 11 (Novotny et al., 2021) and rCul n 4 (Schaffartzik et al., 2011), all major allergens in IBH for the Icelandic horse breed, with six of them having >70% IgE binding in sera of IBH-affected horses (Novotny et al., 2021). Seven of these allergens have been shown to be important in sensitization of IBH in Icelandic horses with IgE binding in sera >50% of IBH-affected horses the year of sensitization. These allergens are Cul o 8, Cul o 11, Cul o 2P, Cul o 7, Cul o 1P, Cul o 13 and Cul o 10. Additionally, Cul o 3 and Cul o 9 were showen to be important with IgE binding in sera of nearly 50% IBH-affected horses at first clinical signs of IBH. Horses are sensitized against multiple allergens simultaneously, with average of 11 allergens whereof 9 are major allergens (Birras et al., 2021). As horses are sensitized to multiple allergens at the same time, one or two allergens would probably not be sufficient for preventive AIT, it would need to be performed with multiple allergens. Nine of the most important r-*Culicoides* allergens were therefore chosen for the vaccine, based on Novotny et al., 2021. Cul o 10 could not be successfully produced and purified in *E. coli* prior to the study and was therefore not included. Analysis of the antibody response against the vaccine allergens is ongoing. For this work, the antibody response was tested against three r-allergens (Bac-rCul o 2P, rBac-rCul o 8 and Bac-rCul o 9). The vaccination resulted in significant

increase in IgG1, IgG4/7 and IgG5 antibody levels against the three r-allergens. There was considerable individual variation in the antibody response. There were high and low responders ranging from <1 to >3 OD_{492} for IgG1 and IgG4/7. The vaccinated horses were exported to Switzerland in March, five weeks (i.e. week 13) following the third vaccination. The *Culicoides* season in Switzerland is approximately from April to September and the midges are most active during dusk and dawn (Mordue & Mordue, 2003). It is usual to protect Icelandic imported horses from the *Culicoides* midges by housing them during dusk and dawn or dressing them in blankets, however the vaccinated challenge horses were kept unprotected. The control horses in the experiment were privately owned horses, exported from Iceland to Switzerland at the same time as the vaccinated horses. They were almost all protected from *Culicoides* to minimize the risk of IBH by their owners. Therefore, the controls were not completely comparable to the vaccinated horses. Following the first summer, 6 vaccinated horses or 23.1% showed clinical symptoms of IBH and the diagnosis was confirmed with the sLT release test. IBH incidences increased to a total 13 affected horses or 50% following the second summer and to a total of 16 affected horses or 61.1% following the third summer. The prevalence of IBH among horses exported from Iceland has been reported to be 26% - 72% in heavily *Culicoides* infested areas (reviewed in Eriksson et al., 2008) but preventive measures against IBH might influence the reported prevalence. Our results confirm the high prevalence of IBH among exported Icelandic horses. Preferably unvaccinated control horses should have been exported from Iceland simultaneously and placed in same stables as the vaccinated horses with no use of preventive measures against IBH. That could not be executed due to high costs. Also, the horse stables would only take 1-2 horses each and the horses were therefore distributed over a large area making sampling laborious.

The antibody response during the first two years was compared based on the IBH status of the vaccinated challenge horses (i.e. healthy or IBH-affected) at the end of the third year. There was no significant difference in IgG1, IgG4/7 nor IgG5 between the two groups but IgE responses against Bac-rCul o 8 and Bac-rCul o 9 were significantly higher in IBH-affected horses. Some studies have indicated that elevated IgG5 responses have predictive value of IBH. IBH-affected horses had significantly higher IgG5 before they developed clinical signs as compared to healthy horses (Raza et al., 2020; Ziegler et al., 2018). The results from the challenge experiment did not support those findings, or it might be due to the effect of the vaccination. There was no significant difference in IgG5 responses between IBH-affected and healthy vaccinated horses against any of the three r-allergens, neither before nor after sensitization. It is possible that this might be an effect of the vaccination.

The vaccine did not provide protection and the data analysed so far does not offer any indication regarding the reason for this outcome. However, the sensitization profile of the IBH-affected horses is still being investigated and a large part of the samples have still to be analysed, in particular also the analysis of antibody responses against the six

other r-allergens (Cul o 1P, Cul o 3, Cul o 5, Cul o 7, Cul o 11 and Cul n 4) included in the vaccine. PBMC were isolated and re-stimulated before and after the vaccination, the booster vaccinations and at the end of the *Culicoides* season each year for analysing cytokine responses. Antibody and cytokine responses of the vaccinated horses will be compared the control horses. Furthermore, skin biopsies before vaccination and at the end of three years were taken with the aim of analysing immunological changes in the skin. Hopefully the analysis of all samples will reveal if the vaccine did somehow modify the immune response against the allergens and maybe suggest what the next steps should be in the development of preventive immunization against equine IBH.

6 Conclusions

Four newly identified major IBH-allergens (Cul o 8, Cul o 9, Cul o 10 and Cul o 11) were expressed in insect cells and purified. Their application in immunoassays showed good performance both for analysis of immune responses following vaccination against IBH and for distinguishing between IBH-affected and healthy horses. The results show that pure r-allergens expressed in insect cells may improve *in vitro* diagnostics of IBH. In the future these allergens could be used to identify the sensitisation profiles of IBH-affected horses in order to develop patient tailored allergen immunotherapy for IBH using r-*Culicoides* allergens. Four out of five r-allergens were expressed in transgenic barley, for screening of transgenic barley lines it is important to use allergen-specific antibodies. Two barley-r-allergen were purified. Transgenic barley could present a good option for production of r-allergens in large quantities for commercial IBH diagnostic tests. For that, more major allergens need to be expressed in barley, purified and tested.

The two experimental vaccination studies of healthy horses with no exposure to *Culicoides* allergens gave promising results. Both i.l. and s.c. injection, with r-allergens in alum/MPLA or alum/VLP were well-tolerated and induced production of the important IgG subclasses, IgG1 and IgG4/7, without induction of IgE antibodies. The antibodies were able to block IgE-binding to the corresponding r-allergens used in the vaccine. As there was no major significant difference observed between i.l. and s.c. vaccination it can be concluded that the i.l. injection could be replaced with the s.c. injection route. Using VLP along with alum in a mixture with r-allergens induced a lower antibody response compared to alum/MPLA and most interestingly, lower IgG5 responses. Hence it would be interesting to test the Th1 focusing capacity of VLP coupled to the r-allergens or of r-allergens co-expressed with VLP.

Comparison of vaccination with two different doses of r-allergens showed less individual variation in IgG1 and IgG4/7 levels following vaccination with 20 μg compared to 10 μg r-*Culicoides* allergen, while the IgG5 response did not increase and no IgE was induced. We therefore conclude that the use of 20 μg dosage of each allergen per vaccination is preferable for further development of immunotherapy in horses.

Exposure to *Culicoides* following preventive i.l. vaccination with nine *Culicoides* r-allergens in alum/MPLA did not result in effective protection as incidence of IBH following the vaccination was not reduced compared to reported prevalence of the disease. Optimally unvaccinated horses should have been exported from Iceland together with the vaccinated horses and maintained at the same locations without use of

preventive measures against IBH. During the first two years, no significant differences were observed between vaccinated horses that remained healthy and those that developed IBH regarding IgG1, IgG4/7 and IgG5 antibody responses against the vaccine allergens, Bac-rCul o 2P, Bac-rCul o 8 and Bac-rCul o 9. At the end of the second *Culicoides* season, IgE antibody levels against Bac-rCul o 8 and Bac-rCul o 9 were significantly higher in IBH-affected horses compared to horses remaining healthy. The analysis of data collected from the challenge experience is still ongoing. Once completed, it may potentially provide some indications why the vaccine failed to provide protection against IBH.

References

Aalberse, R. C., 2000. Structural biology of allergens. Journal of Allergy and Clinical immunology 106, 228-238.

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2022a). Properties and Overview of Immune Responses. In A. K. Abbas, A. H. Lichtman, & S. Pillai (Eds.), *Cellular and Molecular Immunology* (Tenth ed., pp. 1-12). United States of America: Jeremy Bowes.

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2022b). Cells and Tissues of the Immune System. In A. K. Abbas, A. H. Lichtman, & S. Pillai (Eds.), *Cellular and Molecular Immunology* (Tenth ed., pp. 13-42). United States of America: Jeremy Bowes.

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2022c). Innate Immunity. In A. K. Abbas, A. H. Lichtman, & S. Pillai (Eds.), *Cellular and Molecular Immunology* (Tenth ed., pp. 63-102). United States of America: Jeremy Bowes.

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2022d). Antibodies and Antigens. In A. K. Abbas, A. H. Lichtman, & S. Pillai (Eds.), *Cellular and Molecular Immunology* (Tenth ed., pp. 103-122). United States of America: Jeremy Bowes.

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2022e). Antigen Presentation to T Lymphocytes and Function of Major Histocompatibility Complex Molecules. In A. K. Abbas, A. H. Lichtman, & S. Pillai (Eds.), *Cellular and Molecular Immunology* (Tenth ed., pp. 123-150). United States of America: Jeremy Bowes.

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2022f). Hypersensitivity Disorders. In A. K. Abbas, A. H. Lichtman, & S. Pillai (Eds.), *Cellular and Molecular Immunology* (Tenth ed., pp. 437-458). United States of America: Jeremy Bowes.

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2022g). Allergy. In A. K. Abbas, A. H. Lichtman, & S. Pillai (Eds.), *Cellular and Molecular Immunology* (Tenth ed., pp. 459-480). United States of America: Jeremy Bowes.

Agache, I., Cojanu, C., Laculiceanu, A., & Rogozea, L., 2020. Genetics and epigenetics of allergy. Current Opinion in Allergy and Clinical Immunology. 20, 223-232.

Aguilar, J. C., & Rodríguez, E. G., 2007. Vaccine adjuvants revisited. Vaccine. 25, 3752-3762.

Akdis, C. A., 2021. Does the epithelial barrier hypothesis explain the increase in allergy, autoimmunity and other chronic conditions? Nature Reviews. Immunology. 21, 739-751.

Akdis, C. A., Arkwright, P. D., Brüggen, M. C., Busse, W., Gadina, M., Guttman-Yassky, E., Kabashima, K., Mitamura, Y., Vian, L., Wu, J., & Palomares, O., 2020. Type 2 immunity in the skin and lungs. Allergy. 75, 1582-1605.

Akinfenwa, O., Huang, H. J., Linhart, B., Focke-Tejkl, M., Vrtala, S., Poroshina, A., Nikonova, A., Khaitov, M., Campion, N. J., Eckl-Dorna, J., Niederberger-Leppin, V., Kratzer, B., Tauber, P. A., Pickl, W. F., Kundi, M., Campana, R., & Valenta, R., 2021a. Preventive Administration of Non-Allergenic Bet v 1 Peptides Reduces Allergic Sensitization to Major Birch Pollen Allergen, Bet v 1. Frontiers in Immunology. 12, 744544.

Akinfenwa, O., Rodríguez-Domínguez, A., Vrtala, S., Valenta, R., & Campana, R., 2021b. Novel vaccines for allergen-specific immunotherapy. Current Opinion in Allergy and Clinical Immunology. 21, 86-99.

Alam, M. M., Jarvis, C. M., Hincapie, R., McKay, C. S., Schimer, J., Sanhueza, C. A., Xu, K., Diehl, R. C., Finn, M. G., & Kiessling, L. L., 2021. Glycan-Modified Virus-like Particles Evoke T Helper Type 1-like Immune Responses. ACS Nano. 15, 309-321.

Alashkar Alhamwe, B., Alhamdan, F., Ruhl, A., Potaczek, D. P., & Renz, H., 2020. The role of epigenetics in allergy and asthma development. Current Opinion in Allergy and Clinical Immunology. 20, 48-55.

Altmann, F., Staudacher, E., Wilson, I. B., & März, L., 1999. Insect cells as hosts for the expression of recombinant glycoproteins. Glycoconjugate Journal. 16, 109-123.

Alvarenga, P. H., & Andersen, J. F., 2022. An Overview of D7 Protein Structure and Physiological Roles in Blood-Feeding Nematocera. Biology (Basel). 12.

Alvaro-Lozano, M., Akdis, C. A., Akdis, M., Alviani, C., Angier, E., Arasi, S., Arzt-Gradwohl, L., Barber, D., Bazire, R., Cavkaytar, O., Comberiati, P., Dramburg, S., Durham, S. R., Eifan, A. O., Forchert, L., Halken, S., Kirtland, M., Kucuksezer, U. C., Layhadi, J. A., Matricardi, P. M., Muraro, A., Ozdemir, C., Pajno, G. B., Pfaar, O., Potapova, E., Riggioni, C., Roberts, G., Rodríguez Del Río, P., Shamji, M. H., Sturm, G. J., & Vazquez-Ortiz, M., 2020. EAACI Allergen Immunotherapy User's Guide. Pediatric Allergy and Immunology. 31 Suppl 25, 1-101.

Anderson, G. S., Belton, P., Jahren, E., Lange, H., & Kleider, N., 1996. Immunotherapy trial for horses in British Columbia with Culicoides (Diptera:Ceratopogonidae) hypersensitivity. Journal of Medical Entomology. 33, 458-466.

Anderson, G. S., Belton, P., & Kleider, N., 1991. *Culicoides obsoletus* (Diptera: *Ceratopogonidae*) as a causal agent of *Culicoides* hypersensitivity (sweet itch) in British Columbia. J Med Entomol. 28, 685-693.

Anderson, G. S., Belton, P., & Kleider, N., 1993. Hypersensitivity of horses in British Columbia to extracts of native and exotic species of Culicoides (Diptera: Ceratopogonidae). J Med Entomol. 30, 657-663.

Andersson, L. S., Swinburne, J. E., Meadows, J. R., Broström, H., Eriksson, S., Fikse, W. F., Frey, R., Sundquist, M., Tseng, C. T., Mikko, S., & Lindgren, G., 2012. The same ELA class II risk factors confer equine insect bite hypersensitivity in two distinct populations. Immunogenetics. 64, 201-208.

Angkasekwinai, P., & Dong, C., 2021. IL-9-producing T cells: potential players in allergy and cancer. Nature Reviews. Immunology. 21, 37-48.

Arlian, L. G., 2002. Arthropod allergens and human health. Annual Review of Entomology. 47, 395-433.

Asaduzzaman, M., Nadeem, A., Arizmendi, N., Davidson, C., Nichols, H. L., Abel, M., Ionescu, L. I., Puttagunta, L., Thebaud, B., Gordon, J., DeFea, K., Hollenberg, M. D., & Vliagoftis, H., 2015. Functional inhibition of PAR2 alleviates allergen-induced airway hyperresponsiveness and inflammation. Clinical & Experimental Allergy. 45, 1844-1855.

Atipas, K., Kanjanawasee, D., & Tantilipikorn, P., 2022. Intradermal Allergen Immunotherapy for Allergic Rhinitis: Current Evidence. Journal of Personalized Medicine. 12, 1341.

Bachmann, M. F., & Jennings, G. T., 2010. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. Nature Reviews. Immunology. 10, 787-796.

Baker, K. P., & Quinn, P. J., 1978. A report on clinical aspects and histopathology of sweet itch. Equine Veterinary Journal. 10, 243-248.

Barbet, J. L., Bevier, D., & Greiner, E. C., 1990. Specific immunotherapy in the treatment of *Culicoides* hypersensitive horses: a double-blind study. Equine Veterinary Journal. 22, 232-235.

Baselgia, S., Doherr, M. G., Mellor, P., Torsteinsdottir, S., Jermann, T., Zurbriggen, A., Jungi, T., & Marti, E., 2006. Evaluation of an *in vitro* sulphidoleukotriene release test for diagnosis of insect bite hypersensitivity in horses. Equine Veterinary Journal. 38, 40-46.

Bemanian, M. H., Alizadeh Korkinejad, N., Shirkhoda, S., Nabavi, M., & Pourpak, Z., 2012. Assessment of sensitization to insect aeroallergens among patients with allergic rhinitis in Yazd City, Iran. Iranian Journal of Allergy, Asthma and Immunology. 11, 253-258.

Benarafa, C., Collins, M. E., Hamblin, A. S., & Cunningham, F. M., 2002. Role of the chemokine eotaxin in the pathogenesis of equine sweet itch. Veterinary Record. 151, 691-693.

Bieber, T., 2020. Interleukin-13: Targeting an underestimated cytokine in atopic dermatitis. Allergy. 75, 54-62.

Birras, J., White, S. J., Jonsdottir, S., Novotny, E. N., Ziegler, A., Wilson, A. D., Frey, R., Torsteinsdottir, S., Alcocer, M., & Marti, E., 2021. First clinical expression of equine insect bite hypersensitivity is associated with co-sensitization to multiple Culicoides allergens. PLoS One. 16, e0257819.

Bjornsdottir, S., Sigvaldadottir, J., Brostrom, H., Langvad, B., & Sigurdsson, A., 2006. Summer eczema in exported Icelandic horses: influence of environmental and genetic factors. Acta Veterinaria Scandinavica. 48, 3.

Blank, S., Bazon, M. L., Grosch, J., Schmidt-Weber, C. B., Brochetto-Braga, M. R., Bilò, M. B., & Jakob, T., 2020. Antigen 5 Allergens of Hymenoptera Venoms and Their Role in Diagnosis and Therapy of Venom Allergy. Current Allergy and Asthma Reports. 20, 58.

Boorman, J., 1974. The maintenance of laboratory colonies of Culicoides variipennis (Coq.), C. nubeculosus (Mg.) and C. riethi Kieff. (Diptera, Ceratopogonidae). Bulletin of Entomological Research. 64, 371-377.

Boorman, J., 1985. Rearing *Culicoides obsoletus* (Diptera, *Ceratopogonidae*) on agar cultures of nematodes. Progress in Clinical and Biological Research. 178, 229-231.

Brehler, R., Grundmann, S., & Stöcker, B., 2013. Cross-reacting carbohydrate determinants and hymenoptera venom allergy. Curr Opin Allergy Clin Immunol. 13, 360-364.

Breiteneder H, C. M. (2014). Allergen Nomenclature. In L. D. K. Lockey R. F. (Ed.), *Allergens and Allergen Immunotherapy: Subcutaneous, sublingual and oral* (5th ed.). Florida, USA: CRC Press.

Brostrom, H., Larsson, A., & Troedsson, M., 1987. Allergic dermatitis (sweet itch) of Icelandic horses in Sweden: an epidemiological study. Equine Veterinary Journal. 19, 229-236.

Cagienard, A., Griot, C., Mellor, P. S., Denison, E., & Stark, K. D., 2006. Bluetongue vector species of Culicoides in Switzerland. Medical and Veterinary Entomology. 20, 239-247.

Campana, R., Marth, K., Zieglmayer, P., Weber, M., Lupinek, C., Zhernov, Y., Elisyutina, O., Khaitov, M., Rigler, E., Westritschnig, K., Berger, U., Wolkersdorfer, M., Horak, F., Jr., Horak, F., & Valenta, R., 2019. Vaccination of nonallergic individuals with recombinant hypoallergenic fragments of birch pollen allergen Bet v 1: Safety, effects, and mechanisms. Journal of Allergy and Clinical Immunology. 143, 1258-1261.

Cantillo, J. F., Fernandez-Caldas, E., & Puerta, L., 2014. Immunological aspects of the immune response induced by mosquito allergens. International Archives of Allergy and Immunology. 165, 271-282.

Cantillo, J. F., & Puerta, L., 2021. Mosquitoes: Important Sources of Allergens in the Tropics. Frontiers in Allergy. 2, 690406.

Casati, S., Racloz, V., Delecolle, J. C., Kuhn, M., Mathis, A., Griot, C., Stark, K. D., & Vanzetti, T., 2009. An investigation on the Culicoides species composition at seven sites in southern Switzerland. Medical and Veterinary Entomology. 23, 93-98.

Celebi Sözener, Z., Mungan, D., Cevhertas, L., Ogulur, I., Akdis, M., & Akdis, C., 2020. Tolerance mechanisms in allergen immunotherapy. Current Opinion in Allergy and Clinical Immunology. 20, 591-601.

Celebi Sozener, Z., Ozdel Ozturk, B., Cerci, P., Turk, M., Gorgulu Akin, B., Akdis, M., Altiner, S., Ozbey, U., Ogulur, I., Mitamura, Y., Yilmaz, I., Nadeau, K., Ozdemir, C., Mungan, D., & Akdis, C. A., 2022. Epithelial barrier hypothesis: Effect of the external exposome on the microbiome and epithelial barriers in allergic disease. Allergy. 77, 1418-1449.

Chackerian, B., 2007. Virus-like particles: flexible platforms for vaccine development. Expert Review of Vaccines. 6, 381-390.

Chambers, A. C., Aksular, M., Graves, L. P., Irons, S. L., Possee, R. D., & King, L. A., 2018. Overview of the Baculovirus Expression System. Current Protocols in Protein Science. 91, 5.4.1-5.4.6.

Chapman, M. D., Pomes, A., Breiteneder, H., & Ferreira, F., 2007. Nomenclature and structural biology of allergens. Journal of Allergy and Clinical Immunology. 119, 414-420.

Chesne, J., Schmidt-Weber, C. B., & Esser von-Bieren, J., 2016. The Use of Adjuvants for Enhancing Allergen Immunotherapy Efficacy. Immunology and Allergy Clinics of North America. 36, 125-145.

Choi, B. H., Cheong, H., Jo, Y. K., Bahn, S. Y., Seo, J. H., & Cha, H. J., 2014. Highly purified mussel adhesive protein to secure biosafety for in vivo applications. Microbial Cell Factories. 13, 52.

Citek, J., Vostry, L., Vostra-Vydrova, H., Brzakova, M., & Prantlova, V., 2017. Genetic parameters of insect bite hypersensitivity in the Old Grey Kladruber horse1. Journal of Animal Science. 95, 53-58.

Costa, J., Bavaro, S. L., Benedé, S., Diaz-Perales, A., Bueno-Diaz, C., Gelencser, E., Klueber, J., Larré, C., Lozano-Ojalvo, D., Lupi, R., Mafra, I., Mazzucchelli, G., Molina, E., Monaci, L., Martín-Pedraza, L., Piras, C., Rodrigues, P. M., Roncada, P., Schrama, D., Cirkovic-Velickovic, T., Verhoeckx, K., Villa, C., Kuehn, A., Hoffmann-Sommergruber, K., & Holzhauser, T., 2022a. Are Physicochemical Properties Shaping the Allergenic Potency of Plant Allergens? Clinical Reviews in Allergy & Immunology. 62, 37-63.

Costa, J., Villa, C., Verhoeckx, K., Cirkovic-Velickovic, T., Schrama, D., Roncada, P., Rodrigues, P. M., Piras, C., Martín-Pedraza, L., Monaci, L., Molina, E., Mazzucchelli, G., Mafra, I., Lupi, R., Lozano-Ojalvo, D., Larré, C., Klueber, J., Gelencser, E., Bueno-Diaz, C., Diaz-Perales, A., Benedé, S., Bavaro, S. L., Kuehn, A., Hoffmann-Sommergruber, K., & Holzhauser, T., 2022b. Are Physicochemical Properties Shaping the Allergenic Potency of Animal Allergens? Clinical Reviews in Allergy & Immunology. 62, 1-36.

Cox, A., Wood, K., Coleman, G., Stewart, A. J., Bertin, F. R., Owen, H., Suen, W. W., & Medina-Torres, C. E., 2020a. Essential oil spray reduces clinical signs of insect bite hypersensitivity in horses. Australian Veterinary Journal. 98, 411-416.

Cox, L. S., Murphey, A., & Hankin, C., 2020b. The Cost-Effectiveness of Allergen Immunotherapy Compared with Pharmacotherapy for Treatment of Allergic Rhinitis and Asthma. Immunology and Allergy Clinics of North America. 40, 69-85.

Curin, M., Garib, V., & Valenta, R., 2017. Single recombinant and purified major allergens and peptides: How they are made and how they change allergy diagnosis and treatment. Annals of Allergy, Asthma & Immunology. 119, 201-209.

Curin, M., Khaitov, M., Karaulov, A., Namazova-Baranova, L., Campana, R., Garib, V., & Valenta, R., 2018. Next-Generation of Allergen-Specific Immunotherapies: Molecular Approaches. Current Allergy and Asthma Reports. 18, 39.

Cvitas, I., Galichet, A., Ling, S. C., Müller, E. J., & Marti, E., 2020a. Toll-like receptor-ligand induced thymic stromal lymphopoietin expression in primary equine keratinocytes. Veterinary Dermatology. 31, 154-162.

Cvitas, I., Oberhaensli, S., Leeb, T., & Marti, E., 2022. Equine keratinocytes in the pathogenesis of insect bite hypersensitivity: Just another brick in the wall? PLoS One. 17, e0266263.

Cvitas, I., Oberhansli, S., Leeb, T., Dettwiler, M., Muller, E., Bruggman, R., & Marti, E. I., 2020b. Investigating the epithelial barrier and immune signatures in the pathogenesis of equine insect bite hypersensitivity. PLoS One. 15, e0232189.

Datsi, A., Steinhoff, M., Ahmad, F., Alam, M., & Buddenkotte, J., 2021. Interleukin-31: The "itchy" cytokine in inflammation and therapy. Allergy. 76, 2982-2997.

Davidson, C. E., Asaduzzaman, M., Arizmendi, N. G., Polley, D., Wu, Y., Gordon, J. R., Hollenberg, M. D., Cameron, L., & Vliagoftis, H., 2013. Proteinase-activated receptor-2 activation participates in allergic sensitization to house dust mite allergens in a murine model. Clinical & Experimental Allergy. 43, 1274-1285.

de Raat, I. J., van den Boom, R., van Poppel, M., & van Oldruitenborgh-Oosterbaan, M. M., 2008. The effect of a topical insecticide containing permethrin on the number of *Culicoides* midges caught near horses with and without insect bite hypersensitivity in the Netherlands. Tijdschrift voor Diergeneeskunde. 133, 838-842.

Demain, A. L., & Vaishnav, P., 2009. Production of recombinant proteins by microbes and higher organisms. Biotechnology Advances. 27, 297-306.

Dispenza, M. C., 2019. Classification of hypersensitivity reactions. Allergy & Asthma Proceedings. 40, 470-473.

Dorofeeva, Y., Shilovskiy, I., Tulaeva, I., Focke-Tejkl, M., Flicker, S., Kudlay, D., Khaitov, M., Karsonova, A., Riabova, K., Karaulov, A., Khanferyan, R., Pickl, W. F., Wekerle, T., & Valenta, R., 2020. Past, presence, and future of allergen immunotherapy vaccines. Allergy.

Dowdall, S. M., Matthews, J. B., Mair, T., Murphy, D., Love, S., & Proudman, C. J., 2002. Antigen-specific IgG(T) responses in natural and experimental cyathostominae infection in horses. Veterinary Parasitology. 106, 225-242.

Ebina-Shibuya, R., & Leonard, W. J., 2023. Role of thymic stromal lymphopoietin in allergy and beyond. Nature Reviews. Immunology. 23, 24-37.

Eriksson, S., Grandinson, K., Fikse, W. F., Lindberg, L., Mikko, S., Broström, H., Frey, R., Sundquist, M., & Lindgren, G., 2008. Genetic analysis of insect bite hypersensitivity (summer eczema) in Icelandic horses. Animal. 2, 360-365.

Erlendsson, L. S., Muench, M. O., Hellman, U., Hrafnkelsdóttir, S. M., Jonsson, A., Balmer, Y., Mäntylä, E., & Orvar, B. L., 2010. Barley as a green factory for the production of functional Flt3 ligand. Biotechnology Journal. 5, 163-171.

Esposito, S., Isidori, C., Pacitto, A., Salvatori, C., Sensi, L., Frati, F., Di Cara, G., & Marcucci, F., 2018. Epicutaneous immunotherapy in rhino-conjunctivitis and food allergies: a review of the literature. Journal of Translational Medicine. 16, 329.

Fadok, V. A., & Greiner, E. C., 1990. Equine insect hypersensitivity: skin test and biopsy results correlated with clinical data. Equine Veterinary Journal. 22, 236-240.

Fathallah, A. M., Bankert, R. B., & Balu-Iyer, S. V., 2013. Immunogenicity of subcutaneously administered therapeutic proteins–a mechanistic perspective. The AAPS Journal. 15, 897-900.

Featherstone, A. W. (2010). Highland biting midge.

Felippe, M. J. (2016). The immune system. In M. J. Felippe (Ed.), *Equine Clinical Immunology* (pp. 1-10). Ames, Iowa, USA: Wiley-Blackwell.

Fettelschoss-Gabriel, A., Birkmann, K., Pantelyushin, S., & Kündig, T. M., 2021. Molecular mechanisms and treatment modalities in equine Culicoides hypersensitivity. The Veterinary Journal. 276, 105741.

Fettelschoss-Gabriel, A., Fettelschoss, V., Olomski, F., Birkmann, K., Thoms, F., Buhler, M., Kummer, M., Zeltins, A., Kundig, T. M., & Bachmann, M. F., 2019. Active vaccination against interleukin-5 as long-term treatment for insect-bite hypersensitivity in horses. Allergy. 74, 572-582.

Fettelschoss-Gabriel, A., Fettelschoss, V., Thoms, F., Giese, C., Daniel, M., Olomski, F., Kamarachev, J., Birkmann, K., Buhler, M., Kummer, M., Zeltins, A., Marti, E., Kundig, T. M., & Bachmann, M. F., 2018. Treating insect-bite hypersensitivity in horses with active vaccination against IL-5. Journal of Allergy and Clinical Immunology. 142, 1194-1205 e1193.

Field, K., & Blaiss, M. S., 2020. Sublingual Versus Subcutaneous Immunotherapy for Allergic Rhinitis: What Are the Important Therapeutic and Real-World Considerations? Current Allergy and Asthma Reports. 20, 45.

Fischer, R., & Buyel, J. F., 2020. Molecular farming – The slope of enlightenment. Biotechnology Advances. 40, 107519.

Fitzgerald, K. T., & Flood, A. A., 2006. Hymenoptera stings. Clinical Techniques in Small Animal Practice. 21, 194-204.

Frey, R., Bergvall, K., & Egenvall, A., 2008. Allergen-specific IgE in Icelandic horses with insect bite hypersensitivity and healthy controls, assessed by FcepsilonR1alpha-based serology. Veterinary Immunology and Immunopathology. 126, 102-109.

Galli, S. J., Tsai, M., & Piliponsky, A. M., 2008. The development of allergic inflammation. Nature. 454, 445-454.

Gao, P., 2012. Sensitization to cockroach allergen: immune regulation and genetic determinants. Clinical and Developmental Immunology. 2012, 563760.

Gell, P. G. H., & Coombs, R. R. A. (1968). *Clinical Aspects of Immunology*: Blackwell Scientific.

Genetics, O. (2023). We Design Plants to Bring Quality to Life.

Ginel, P. J., Hernandez, E., Lucena, R., Blanco, B., Novales, M., & Mozos, E., 2014. Allergen-specific immunotherapy in horses with insect bite hypersensitivity: a double-blind, randomized, placebo-controlled study. Veterinary Dermatology. 25, 29-e10.

Gomord, V., Fitchette, A.-C., Menu-Bouaouiche, L., Saint-Jore-Dupas, C., Plasson, C., Michaud, D., & Faye, L., 2010. Plant-specific glycosylation patterns in the context of therapeutic protein production. Plant Biotechnology Journal. 8, 564-587.

Goodman, L. B., Wimer, C., Dubovi, E. J., Gold, C., & Wagner, B., 2012. Immunological correlates of vaccination and infection for equine herpesvirus 1. Clinical and Vaccine Immunology. 19, 235-241.

Gopal, G. J., & Kumar, A., 2013. Strategies for the production of recombinant protein in Escherichia coli. The Protein Journal. 32, 419-425.

Gudnadottir, R. B. (2021). *Immune response following vaccination against equine insect bite hypersensitivity*. (Master's thesis), University of Iceland, Reykjavík. Retrieved from http://hdl.handle.net/1946/38765

Halldorsdottir, S., & Larsen, H. J., 1991. An epidemiological study of summer eczema in Icelandic horses in Norway. Equine Veterinary Journal. 23, 296-299.

Hamza, E., Akdis, C. A., Wagner, B., Steinbach, F., & Marti, E., 2013. In vitro induction of functional allergen-specific $CD4^+$ $CD25^{high}$ Treg cells in horses affected with insect bite hypersensitivity. Clinical & Experimental Allergy. 43, 889-901.

Hamza, E., Doherr, M. G., Bertoni, G., Jungi, T. W., & Marti, E., 2007. Modulation of allergy incidence in icelandic horses is associated with a change in IL-4-producing T cells. International Archives of Allergy and Immunology. 144, 325-337.

Hamza, E., Gerber, V., Steinbach, F., & Marti, E., 2011. Equine CD4(+) CD25(high) T cells exhibit regulatory activity by close contact and cytokine-dependent mechanisms in vitro. Immunology. 134, 292-304.

Hamza, E., Steinbach, F., & Marti, E., 2012. $CD4^+CD25^+$ T cells expressing FoxP3 in Icelandic horses affected with insect bite hypersensitivity. Veterinary Immunology and Immunopathology. 148, 139-144.

Hamza, E., Torsteinsdottir, S., Eydal, M., Frey, C. F., Mirkovitch, J., Brcic, M., Wagner, B., Wilson, A. D., Jungi, T. W., & Marti, E., 2010. Increased IL-4 and decreased regulatory cytokine production following relocation of Icelandic horses from a high to low endoparasite environment. Veterinary Immunology and Immunopathology. 133, 40-50.

Hamza, E., Wagner, B., Jungi, T. W., Mirkovitch, J., & Marti, E., 2008. Reduced incidence of insect-bite hypersensitivity in Icelandic horses is associated with a down-regulation of interleukin-4 by interleukin-10 and transforming growth factor-beta1. Veterinary Immunology and Immunopathology. 122, 65-75.

Heesters, B. A., van der Poel, C. E., Das, A., & Carroll, M. C., 2016. Antigen Presentation to B Cells. Trends Immunol. 37, 844-854.

Heimann, M., Janda, J., Sigurdardottir, O. G., Svansson, V., Klukowska, J., von Tscharner, C., Doherr, M., Brostrom, H., Andersson, L. S., Einarsson, S., Marti, E., & Torsteinsdottir, S., 2011. Skin-infiltrating T cells and cytokine expression in Icelandic horses affected with insect bite hypersensitivity: a possible role for regulatory T cells. Veterinary Immunology and Immunopathology. 140, 63-74.

Hellberg, W., Mellor, P. S., Torsteinsdottir, S., & Marti, E., 2009. Insect bite hypersensitivity in the horse: comparison of IgE-binding proteins in salivary gland extracts from Simulium vittatum and Culicoides nubeculosus. Veterinary Immunology and Immunopathology. 132, 62-67.

Hellberg, W., Wilson, A. D., Mellor, P., Doherr, M. G., Torsteinsdottir, S., Zurbriggen, A., Jungi, T., & Marti, E., 2006. Equine insect bite hypersensitivity: immunoblot analysis of IgE and IgG subclass responses to Culicoides nubeculosus salivary gland extract. Veterinary Immunology and Immunopathology. 113, 99-112.

Hempolchom, C., Sookrung, N., Srisuka, W., Reamtong, O., Sakolvaree, Y., Chaicumpa, W., Dedkhad, W., Jariyapan, N., Takaoka, H., & Saeung, A., 2019. Characterization of IgE-binding proteins in the salivary glands of Simulium nigrogilvum (Diptera: Simuliidae). Parasitology Research. 118, 2353-2359.

Hoffman, D. R., 2006. Hymenoptera venom allergens. Clinical Reviews in Allergy & Immunology. 30, 109-128.

Holloway, J. W., Yang, I. A., & Holgate, S. T., 2010. Genetics of allergic disease. Journal of Allergy and Clinical Immunology. 125, S81-94.

Hreinsdottir, H. (2021). Metár í útflutningi hrossa árið 2021. Retrieved from https://www.rml.is/is/starfsemi/frettir/metar-i-utflutningi-hrossa-arid-2021

Huang, H.-J., Sarzsinszky, E., & Vrtala, S., 2023. House dust mite allergy: The importance of house dust mite allergens for diagnosis and immunotherapy. Molecular Immunology. 158, 54-67.

Hudson, A., Bowman, L., & Orr, C. W., 1960. Effects of absence of saliva on blood feeding by mosquitoes. Science. 131, 1730-1731.

Huhmann, R., & Mueller, R. S., 2019. A cream containing omega-3-fatty acids, humectants and emollients as an aid in the treatment of equine Culicoides hypersensitivity. Veterinary Dermatology. 30, 155-e146.

Irons, S. L., Chambers, A. C., Lissina, O., King, L. A., & Possee, R. D., 2018. Protein Production Using the Baculovirus Expression System. Current Protocols in Protein Science. 91, 5.5.1-5.5.22.

Jaeger, A., Zannini, E., Sahin, A. W., & Arendt, E. K., 2021. Barley Protein Properties, Extraction and Applications, with a Focus on Brewers' Spent Grain Protein. Foods. 10.

Jensen-Jarolim, E., Einhorn, L., Herrmann, I., Thalhammer, J. G., & Panakova, L., 2015. Pollen Allergies in Humans and their Dogs, Cats and Horses: Differences and Similarities. Clinical and Translational Allergy. 5, 15.

Jmel, M. A., Voet, H., Araújo, R. N., Tirloni, L., Sá-Nunes, A., & Kotsyfakis, M., 2023. Tick Salivary Kunitz-Type Inhibitors: Targeting Host Hemostasis and Immunity to Mediate Successful Blood Feeding. International Journal of Molecular Sciences. 24.

Jones, S. M., Kim, E. H., Nadeau, K. C., Nowak-Wegrzyn, A., Wood, R. A., Sampson, H. A., Scurlock, A. M., Chinthrajah, S., Wang, J., Pesek, R. D., Sindher, S. B., Kulis, M., Johnson, J., Spain, K., Babineau, D. C., Chin, H., Laurienzo-Panza, J., Yan, R., Larson, D., Qin, T., Whitehouse, D., Sever, M. L., Sanda, S., Plaut, M., Wheatley, L. M., & Burks, A. W., 2022. Efficacy and safety of oral immunotherapy in children aged 1-3 years with peanut allergy (the Immune Tolerance Network IMPACT trial): a randomised placebo-controlled study. Lancet. 399, 359-371.

Jonsdottir, S. (2011). *Insect Bite Hypersensitivity in Horses; Production of Allergens in Insect Cells and Barley*. (Master's thesis), University of Iceland, Reykjavík. Retrieved from http://hdl.handle.net/1946/16951

Jonsdottir, S., Cvitas, I., Svansson, V., Fettelschloss-Gabriel, A., Torsteinsdottir, S., & Marti, E., 2019. New Strategies for Prevention and Treatment of Insect Bite Hypersensitivity in Horses. Current Dermatology Reports. 8, 303-312.

Jonsdottir, S., Fettelschoss, V., Olomski, F., Talker, S. C., Mirkovitch, J., Rhiner, T., Birkmann, K., Thoms, F., Wagner, B., Bachmann, M. F., Kündig, T. M., Marti, E., & Fettelschoss-Gabriel, A., 2020. Safety Profile of a Virus-Like Particle-Based Vaccine Targeting Self-Protein Interleukin-5 in Horses. Vaccines (Basel). 8.

Jonsdottir, S., Hamza, E., Janda, J., Rhyner, C., Meinke, A., Marti, E., Svansson, V., & Torsteinsdottir, S., 2015. Developing a preventive immunization approach against insect bite hypersensitivity using recombinant allergens: A pilot study. Veterinary Immunology and Immunopathology. 166, 8-21.

Jonsdottir, S., Stefansdottir, S. B., Kristinarson, S. B., Svansson, V., Bjornsson, J. M., Runarsdottir, A., Wagner, B., Marti, E., & Torsteinsdottir, S., 2018. Barley produced *Culicoides* allergens are suitable for monitoring the immune response of horses immunized with *E. coli* expressed allergens. Veterinary Immunology and Immunopathology. 201, 32-37.

Jonsdottir, S., Svansson, V., Stefansdottir, S. B., Mantyla, E., Marti, E., & Torsteinsdottir, S., 2017. Oral administration of transgenic barley expressing a Culicoides allergen induces specific antibody response. Equine Veterinary Journal. 49, 512-518.

Jonsdottir, S., Svansson, V., Stefansdottir, S. B., Schupbach, G., Rhyner, C., Marti, E., & Torsteinsdottir, S., 2016. A preventive immunization approach against insect bite hypersensitivity: Intralymphatic injection with recombinant allergens in Alum or Alum and monophosphoryl lipid A. Veterinary Immunology and Immunopathology. 172, 14-20.

Jonsdottir, S., Torsteinsdottir, S., Svansson, V., Gudbrandsson, J., Stefansdottir, S. B., Mar Bjornsson, J., Runarsdottir, A., & Marti, E., 2021. Comparison of recombinant Culicoides allergens produced in different expression systems for IgE serology of insect bite hypersensitivity in horses of different origins. Veterinary Immunology and Immunopathology. 238, 110289.

Joshi, S. R., & Khan, D. A., 2021. Non-IgE-Mediated Drug Hypersensitivity Reactions. Current Allergy and Asthma Reports. 21, 41.

Junt, T., Scandella, E., & Ludewig, B., 2008. Form follows function: lymphoid tissue microarchitecture in antimicrobial immune defence. Nature Reviews Immunology. 8, 764-775.

Jutel, M., Agache, I., Bonini, S., Burks, A. W., Calderon, M., Canonica, W., Cox, L., Demoly, P., Frew, A. J., O'Hehir, R., Kleine-Tebbe, J., Muraro, A., Lack, G., Larenas, D., Levin, M., Martin, B. L., Nelson, H., Pawankar, R., Pfaar, O., van Ree, R., Sampson, H., Sublett, J. L., Sugita, K., Du Toit, G., Werfel, T., Gerth van Wijk, R., Zhang, L., Akdis, M., & Akdis, C. A., 2016. International Consensus on Allergen Immunotherapy II: Mechanisms, standardization, and pharmacoeconomics. Journal of Allergy and Clinical Immunology. 137, 358-368.

Jutel, M., Agache, I., Bonini, S., Burks, A. W., Calderon, M., Canonica, W., Cox, L., Demoly, P., Frew, A. J., O'Hehir, R., Kleine-Tebbe, J., Muraro, A., Lack, G., Larenas, D., Levin, M., Nelson, H., Pawankar, R., Pfaar, O., van Ree, R., Sampson, H., Santos, A. F., Du Toit, G., Werfel, T., Gerth van Wijk, R., Zhang, L., & Akdis, C. A., 2015. International consensus on allergy immunotherapy. Journal of Allergy and Clinical Immunology. 136, 556-568.

Jutel, M., Agache, I., Zemelka-Wiacek, M., Akdis, M., Chivato, T., Del Giacco, S., Gajdanowicz, P., Gracia, I. E., Klimek, L., Lauerma, A., Ollert, M., O'Mahony, L., Schwarze, J., Shamji, M. H., Skypala, I., Palomares, O., Pfaar, O., Torres, M. J., Bernstein, J. A., Cruz, A. A., Durham, S. R., Galli, S. J., Gómez, R. M., Guttman-Yassky, E., Haahtela, T., Holgate, S. T., Izuhara, K., Kabashima, K., Larenas-Linnemann, D. E., von Mutius, E., Nadeau, K. C., Pawankar, R., Platts-Mills, T. A. E., Sicherer, S. H., Park, H. S., Vieths, S., Wong, G., Zhang, L., Bilò, M. B., & Akdis, C. A., 2023. Nomenclature of allergic diseases and hypersensitivity reactions: Adapted to modern needs: An EAACI position paper. Allergy.

Keggan, A., Freer, H., Rollins, A., & Wagner, B., 2013. Production of seven monoclonal equine immunoglobulins isotyped by multiplex analysis. Veterinary Immunology and Immunopathology. 153, 187-193.

Kim, E. H., & Burks, A. W., 2020. Food allergy immunotherapy: Oral immunotherapy and epicutaneous immunotherapy. Allergy. 75, 1337-1346.

Kim, J. Y., Jang, M. J., Kim, D. Y., Park, S. W., & Han, D. H., 2021. Efficacy of Subcutaneous and Sublingual Immunotherapy for House Dust Mite Allergy: A Network Meta-Analysis-Based Comparison. Journal of Allergy and Clinical Immunology. 9, 4450-4458.e4456.

King, T. P., & Spangfort, M. D., 2000. Structure and biology of stinging insect venom allergens. International Archives of Allergy and Immunology. 123, 99-106.

Klimek, L., Schmidt-Weber, C. B., Kramer, M. F., Skinner, M. A., & Heath, M. D., 2017. Clinical use of adjuvants in allergen-immunotherapy. Expert Review of Clinical Immunology. 13, 599-610.

Komai-Koma, M., Ji, Y., Cao, H., Liu, Z., McSharry, C., & Xu, D., 2021. Monophosphoryl lipid A directly regulates Th1 cytokine production in human CD4(+) T-cells through Toll-like receptor 2 and 4. Immunobiology. 226, 152132.

Kool, M., Soullié, T., van Nimwegen, M., Willart, M. A., Muskens, F., Jung, S., Hoogsteden, H. C., Hammad, H., & Lambrecht, B. N., 2008. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. Journal of Experimental Medicine. 205, 869-882.

Kristinarson, S. B. (2017). *Expression and purification of the causative allergens of insect bite hypersensitivity in horses and comparison of different expression systems.* (Master's thesis), University of Iceland, Reykjavík. Retrieved from http://hdl.handle.net/1946/27981

Kristjansdottir, H. (2019). *Samanburður á mótefnasvari hesta eftir bólusetningu í eitla og undir húð*. (Bachelor's thesis), University of Iceland, Reykjavík. Retrieved from http://hdl.handle.net/1946/33261

Krutz, N. L., Kimber, I., Maurer-Stroh, S., & Gerberick, G. F., 2020. Determination of the relative allergenic potency of proteins: hurdles and opportunities. Critical Reviews in Toxicology. 50, 521-530.

Kucuksezer, U. C., Ozdemir, C., Cevhertas, L., Ogulur, I., Akdis, M., & Akdis, C. A., 2020. Mechanisms of allergen-specific immunotherapy and allergen tolerance. Allergology International. 69, 549-560.

Kurotaki, T., Narayama, K., Oyamada, T., Yoshikawa, H., & Yoshikawa, T., 1994. Immunopathological study on equine insect hypersensitivity ("kasen") in Japan. Journal of Comparative Pathology. 110, 145-152.

Langner, K. F., Darpel, K. E., Drolet, B. S., Fischer, A., Hampel, S., Heselhaus, J. E., Mellor, P. S., Mertens, P. P., & Leibold, W., 2008. Comparison of cellular and humoral immunoassays for the assessment of summer eczema in horses. Vet Immunol Immunopathol. 122, 126-137.

Langner, K. F., Jarvis, D. L., Nimtz, M., Heselhaus, J. E., McHolland, L. E., Leibold, W., & Drolet, B. S., 2009. Identification, expression and characterisation of a major salivary allergen (Cul s 1) of the biting midge *Culicoides sonorensis* relevant for summer eczema in horses. International Journal for Parasitology. 39, 243-250.

Langreder, N., Schäckermann, D., Meier, D., Becker, M., Schubert, M., Dübel, S., Reinard, T., Figge-Wegener, S., Roßbach, K., Bäumer, W., Ladel, S., & Hust, M., 2023. Development of an inhibiting antibody against equine interleukin 5 to treat insect bite hypersensitivity of horses. Scientific Reports. 13, 4029.

Lanz, S., Gerber, V., Marti, E., Rettmer, H., Klukowska-Rötzler, J., Gottstein, B., Matthews, J. B., Pirie, S., & Hamza, E., 2013. Effect of hay dust extract and cyathostomin antigen stimulation on cytokine expression by PBMC in horses with recurrent airway obstruction. Veterinary Immunology and Immunopathology. 155, 229-237.

Larsen, J. N., & Lowenstein, H., 1996. Allergen nomenclature. Journal of Allergy and Clinical Immunology. 97, 577-578.

Larson, E. M., Babasyan, S., & Wagner, B., 2020. Phenotype and function of IgE-binding monocytes in equine Culicoides hypersensitivity. PLoS One. 15, e0233537.

Larson, E. M., Babasyan, S., & Wagner, B., 2021a. IgE-Binding Monocytes Have an Enhanced Ability to Produce IL-8 (CXCL8) in Animals with Naturally Occurring Allergy. The Journal of Immunology. 206, 2312-2321.

Larson, E. M., & Wagner, B., 2021b. Viral infection and allergy - What equine immune responses can tell us about disease severity and protection. Molecular Immunology. 135, 329-341.

Lazary, S., Marti, E., Szalai, G., Gaillard, C., & Gerber, H., 1994. Studies on the frequency and associations of equine leucocyte antigens in sarcoid and summer dermatitis. Animal Genetics. 25 Suppl 1, 75-80.

Leclere, M., 2017. Corticosteroids and Immune Suppressive Therapies in Horses. Veterinary Clinics of North America: Equine Practice. 33, 17-27.

Lee, H., Halverson, S., & Mackey, R., 2016. Insect Allergy. Primary Care: Clinics in Office Practice. 43, 417-431.

Lehiy, C. J., & Drolet, B. S., 2014. The salivary secretome of the biting midge, *Culicoides sonorensis*. PeerJ. 2, e426.

Lewis, M. J., Wagner, B., Irvine, R. M., & Woof, J. M., 2010. IgA in the horse: cloning of equine polymeric Ig receptor and J chain and characterization of recombinant forms of equine IgA. Mucosal Immunology. 3, 610-621.

Lewis, M. J., Wagner, B., & Woof, J. M., 2008. The different effector function capabilities of the seven equine IgG subclasses have implications for vaccine strategies. Molecular Immunology. 45, 818-827.

Littlewood, J. D., 1998. Incidence of recurrent seasonal pruritus ('sweet itch') in British and German shire horses. The Veterinary Record. 142, 66-67.

Liu, L., Spurrier, J., Butt, T. R., & Strickler, J. E., 2008. Enhanced protein expression in the baculovirus/insect cell system using engineered SUMO fusions. Protein Expression and Purification. 62, 21-28.

López, J. F., Bel Imam, M., Satitsuksanoa, P., Lems, S., Yang, M., Hwang, Y. K., Losol, P., Choi, J. P., Kim, S. H., Chang, Y. S., Akdis, M., Akdis, C. A., & van de Veen, W., 2022. Mechanisms and biomarkers of successful allergen-specific immunotherapy. Asia Pac Allergy. 12, e45.

Magnusdottir, A., Vidarsson, H., Bjornsson, J. M., & Orvar, B. L., 2013. Barley grains for the production of endotoxin-free growth factors. Trends in Biotechnology. 31, 572-580.

Malafronte Rdos, S., Calvo, E., James, A. A., & Marinotti, O., 2003. The major salivary gland antigens of *Culex quinquefasciatus* are D7-related proteins. Insect Biochemistry and Molecular Biology. 33, 63-71.

Marti, E., Gerber, H., & Lazary, S., 1992. On the genetic basis of equine allergic diseases: II. Insect bite dermal hypersensitivity. Equine Veterinary Journal. 24, 113-117.

Marti, E., Gerber, V., Wilson, A. D., Lavoie, J. P., Horohov, D., Crameri, R., Lunn, D. P., Antczak, D., Bjornsdottir, S., Bjornsdottir, T. S., Cunningham, F., Derer, M., Frey, R., Hamza, E., Horin, P., Heimann, M., Kolm-Stark, G., Olafsdottir, G., Ramery, E., Russell, C., Schaffartzik, A., Svansson, V., Torsteinsdottir, S., & Wagner, B., 2008. Report of the 3rd Havemeyer workshop on allergic diseases of the Horse, Holar, Iceland, June 2007. Veterinary Immunology and Immunopathology. 126, 351-361.

Marti, E., Urwyler, A., Neuenschwander, M., Eicher, R., Meier, D., de Weck, A. L., Gerber, H., Lazary, S., & Dahinden, C. A., 1999. Sulfidoleukotriene generation from peripheral blood leukocytes of horses affected with insect bite dermal hypersensitivity. Veterinary Immunology and Immunopathology. 71, 307-320.

Marti, E., Wang, X., Jambari, N. N., Rhyner, C., Olzhausen, J., Pérez-Barea, J. J., Figueredo, G. P., & Alcocer, M. J., 2015. Novel in vitro diagnosis of equine allergies using a protein array and mathematical modelling approach: a proof of concept using insect bite hypersensitivity. Veterinary Immunology and Immunopathology. 167, 171-177.

Matsuoka, T., Kawashima, T., Nakamura, T., & Yabe, T., 2017. Characterization and comparison of recombinant honeybee chymotrypsin-like protease (HCLPase) expressed in Escherichia coli and insect cells. Biosci Biotechnol Biochem. 81, 1401-1404.

McAleer, J. P., & Vella, A. T., 2008. Understanding how lipopolysaccharide impacts CD4 T-cell immunity. Crit Rev Immunol. 28, 281-299.

McKelvie, J., Foster, A. P., Cunningham, F. M., & Hamblin, A. S., 1999. Characterisation of lymphocyte subpopulations in the skin and circulation of horses with sweet itch (Culicoides hypersensitivity). Equine Veterinary Journal. 31, 466-472.

Mellor, P. S., Boorman, J., & Baylis, M., 2000. Culicoides biting midges: their role as arbovirus vectors. Annual Review of Entomology. 45, 307-340.

Mellor, P. S., & McCraig, J., 1974. The probable cause of "sweet itch" in England. The Veterinary Record. 95, 411-415.

Meulenbroeks, C., van der Lugt, J. J., van der Meide, N. M., Willemse, T., Rutten, V. P., & Zaiss, D. M., 2015. Allergen-Specific Cytokine Polarization Protects Shetland Ponies against Culicoides obsoletus-Induced Insect Bite Hypersensitivity. PLoS One. 10, e0122090.

Meulenbroeks, C., van der Meide, N. M. A., Zaiss, D. M. W., Sloet van Oldruitenborgh-Oosterbaan, M. M., van der Lugt, J. J., Smak, J., Rutten, V. P. M. G., & Willemse, T., 2013. Seasonal differences in cytokine expression in the skin of Shetland ponies suffering from insect bite hypersensitivity. Veterinary Immunology and Immunopathology. 151, 147-156.

Miller, J. E., Mann, S., Fettelschoss-Gabriel, A., & Wagner, B., 2019. Comparison of three clinical scoring systems for Culicoides hypersensitivity in a herd of Icelandic horses. Veterinary Dermatology. 30, 536-e163.

Mitchell, T. C., & Casella, C. R., 2017. No pain no gain? Adjuvant effects of alum and monophosphoryl lipid A in pertussis and HPV vaccines. Current Opinion in Immunology. 47, 17-25.

Möhlmann, T. W. R., Wennergren, U., Tälle, M., Favia, G., Damiani, C., Bracchetti, L., Takken, W., & Koenraadt, C. J. M., 2018. Community analysis of the abundance and diversity of biting midge species (Diptera: Ceratopogonidae) in three European countries at different latitudes. Parasites & Vectors. 11, 217.

Mohsen, M. O., Gomes, A. C., Vogel, M., & Bachmann, M. F., 2018. Interaction of Viral Capsid-Derived Virus-Like Particles (VLPs) with the Innate Immune System. Vaccines. 6, 37.

Mohsen, M. O., Zha, L., Cabral-Miranda, G., & Bachmann, M. F., 2017. Major findings and recent advances in virus–like particle (VLP)-based vaccines. Seminars in Immunology. 34, 123-132.

Moingeon, P., Lombardi, V., Baron-Bodo, V., & Mascarell, L., 2017. Enhancing Allergen-Presentation Platforms for Sublingual Immunotherapy. Journal of Allergy and Clinical Immunology. 5, 23-31.

Möller, Á., Hilmarsdóttir, F., Gústafsdóttir, H., Hugason, K., Guðmundsdóttir, M., Eiríksson, P.J., Sveinbjörnsson, S. (2009). *Markaðssetning íslenska hestsins erlendis*. Retrieved from Reykjavík:

Mordue, J., & Mordue, B., 2003. Biting midge chemical ecology. Biologist. 50, 159-162.

Morgan, E. E., Miller, W. H., & Wagner, B., 2007. A comparison of intradermal testing and detection of allergen-specific immunoglobulin E in serum by enzyme-linked immunosorbent assay in horses affected with skin hypersensitivity. Veterinary Immunology and Immunopathology. 120, 160-167.

Mothes, N., Heinzkill, M., Drachenberg, K. J., Sperr, W. R., Krauth, M. T., Majlesi, Y., Semper, H., Valent, P., Niederberger, V., Kraft, D., & Valenta, R., 2003. Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. Clinical & Experimental Allergy. 33, 1198-1208.

Moustafa, K., Makhzoum, A., & Trémouillaux-Guiller, J., 2016. Molecular farming on rescue of pharma industry for next generations. Critical Reviews in Biotechnology. 36, 840-850.

Mueller, R. S., Janda, J., Jensen-Jarolim, E., Rhyner, C., & Marti, E., 2016. Allergens in veterinary medicine. Allergy. 71, 27-35.

Murphy, K., Weaver, C., & Berg, L. (2022a). Basic Concepts in Immunology. In K. Murphy, C. Weaver, & L. Berg (Eds.), *Janeway´s Immunobiology* (10th ed., pp. 1-36). London and New York: W. W, Norton & Company.

Murphy, K., Weaver, C., & Berg, L. (2022b). Innate Immunnity: First Lines of Defense. In K. Murphy & C. Weaver (Eds.), *Janeway´s Immunobiology* (10th ed., pp. 37-78). London and New York: W. W. Norton & Company.

Murphy, K., Weaver, C., & Berg, L. (2022c). Cellular Mechanisms of Innate Immunity. In K. Murphy, C. Weaver, & L. Berg (Eds.), *Janeway´s Immunobiology* (10th ed., pp. 79-140). London and New York: W. W. Norton & Company.

Murphy, K., Weaver, C., & Berg, L. (2022d). Antigen Recognintion by B-cell and T-cell Receptors. In K. Murphy, C. Weaver, & L. Berg (Eds.), *Janeway´s Immunobiology* (10th ed., pp. 141-178). London and New York: W. W. Norton & Company.

Murphy, K., Weaver, C., & Berg, L. (2022e). T Cell-Mediated Immunity. In K. Murphy, C. Weaver, & L. Berg (Eds.), *Janeway´s Immunobiology* (10th ed., pp. 347-404). London and New York: W. W. Norton & Company.

Murphy, K., Weaver, C., & Berg, L. (2022f). Integrated Dynamics of Innate and Adaptive Immunity. In K. Murphy, C. Weaver, & L. Berg (Eds.), *Janeway´s Immunobiology* (10th ed., pp. 453-503). London and New York: W. W. Norton & Company.

Murphy, K., Weaver, C., & Berg, L. (2022g). Allergy and Allergic Diseases. In K. Murphy, C. Weaver, & L. Berg (Eds.), *Janeway´s Immunobiology* (10th ed., pp. 663-698). London and New York: W. W. Norton & Company.

Nielsen, H., Tsirigos, K. D., Brunak, S., & von Heijne, G., 2019. A Brief History of Protein Sorting Prediction. The Protein Journal. 38, 200-216.

Ninio, C., Augot, D., Delecolle, J. C., Dufour, B., & Depaquit, J., 2011. Contribution to the knowledge of Culicoides (Diptera: Ceratopogonidae) host preferences in France. Parasitology Research. 108, 657-663.

Noon, L., 1911. Prophylactic Inoculation against Hay Fever. The Lancet. 177, 1572-1573.

Noon, L., 1953. Prophylactic inoculation against hay fever. International Archives of Allergy and Immunology. 4, 285-288.

Novotny, E. N., White, S. J., Wilson, A. D., Stefánsdóttir, S. B., Tijhaar, E., Jonsdóttir, S., Frey, R., Reiche, D., Rose, H., Rhyner, C., Schüpbach-Regula, G., Torsteinsdóttir, S., Alcocer, M., & Marti, E., 2021. Component-resolved microarray analysis of IgE sensitization profiles to Culicoides recombinant allergens in horses with insect bite hypersensitivity. Allergy. 76, 1147-1157.

Nunamaker, R. A., & Lockwood, J. A., 2001. Cryopreservation of embryos of *Culicoides sonorensis* (Diptera: *Ceratopogonidae*). Journal of Medical Entomology. 38, 55-58.

O'Hagan, D. T., & Fox, C. B., 2015. New generation adjuvants–from empiricism to rational design. Vaccine. 33 Suppl 2, B14-20.

Ogrina, A., Balke, I., Kalnciema, I., Skrastina, D., Jansons, J., Bachmann, M. F., & Zeltins, A., 2023. Bacterial expression systems based on Tymovirus-like particles for the presentation of vaccine antigens. Front Microbiol. 14, 1154990.

Olafsson, E. (2015). Lúsmý herjar á íbúa sumarhúsa. Retrieved from https://www.ni.is/is/frettir/2015/06/lusmy-herjar-ibua-sumarhusa

Olafsson, E. (2019). Lúsmý nýtur góðs af góðviðrinu. Retrieved from https://www.ni.is/is/frettir/2019/06/lusmy-nytur-gods-af-godvidrinu

Olomski, F., Fettelschoss, V., Jonsdottir, S., Birkmann, K., Thoms, F., Marti, E., Bachmann, M. F., Kundig, T. M., & Fettelschoss-Gabriel, A., 2020. Interleukin 31 in insect bite hypersensitivity-Alleviating clinical symptoms by active vaccination against itch. Allergy. 75, 862-871.

Olsen, L., Bondesson, U., Brostrom, H., Olsson, U., Mazogi, B., Sundqvist, M., Tjalve, H., & Ingvast-Larsson, C., 2011. Pharmacokinetics and effects of cetirizine in horses with insect bite hypersensitivity. The Veterinary Journal. 187, 347-351.

Palmberger, D., Wilson, I. B., Berger, I., Grabherr, R., & Rendic, D., 2012. SweetBac: a new approach for the production of mammalianised glycoproteins in insect cells. PLoS One. 7, e34226.

Palomares, O., Akdis, M., Martín-Fontecha, M., & Akdis, C. A., 2017. Mechanisms of immune regulation in allergic diseases: the role of regulatory T and B cells. Immunological Reviews. 278, 219-236.

Palomares, O., Crameri, R., & Rhyner, C., 2014. The contribution of biotechnology toward progress in diagnosis, management, and treatment of allergic diseases. Allergy. 69, 1588-1601.

Passalacqua, G., Bagnasco, D., & Canonica, G. W., 2020. 30 years of sublingual immunotherapy. Allergy. 75, 1107-1120.

Peeters, L. M., Janssens, S., Goddeeris, B. M., De Keyser, K., Wilson, A. D., Kaufmann, C., Schaffartzik, A., Marti, E., & Buys, N., 2013. Evaluation of an IgE ELISA with *Culicoides* spp. extracts and recombinant salivary antigens for diagnosis of insect bite hypersensitivity in Warmblood horses. The Veterinary Journal. 198, 141-147.

Pekar, J., Ret, D., & Untersmayr, E., 2018. Stability of allergens. Molecular Immunology. 100, 14-20.

Peng, Z., Li, H., & Simons, F. E., 1998. Immunoblot analysis of salivary allergens in 10 mosquito species with worldwide distribution and the human IgE responses to these allergens. Journal of Allergy and Clinical Immunology. 101, 498-505.

Peng, Z., & Simons, F. E., 1996. Comparison of proteins, IgE, and IgG binding antigens, and skin reactivity in commercial and laboratory-made mosquito extracts. Annals of Allergy, Asthma & Immunology. 77, 371-376.

Peng, Z., & Simons, F. E., 2004. Mosquito allergy: immune mechanisms and recombinant salivary allergens. International Archives of Allergy and Immunology. 133, 198-209.

Perkins, G. A., & Wagner, B., 2015. The development of equine immunity: Current knowledge on immunology in the young horse. Equine Veterinary Journal. 47, 267-274.

Petersen, J., Purcell, A. W., & Rossjohn, J., 2009. Post-translationally modified T cell epitopes: immune recognition and immunotherapy. J Mol Med (Berl). 87, 1045-1051.

Pfefferle, P. I., Keber, C. U., Cohen, R. M., & Garn, H., 2021. The Hygiene Hypothesis - Learning From but Not Living in the Past. Frontiers in Immunology. 12, 635935.

Pomés, A., Davies, J. M., Gadermaier, G., Hilger, C., Holzhauser, T., Lidholm, J., Lopata, A. L., Mueller, G. A., Nandy, A., Radauer, C., Chan, S. K., Jappe, U., Kleine-Tebbe, J., Thomas, W. R., Chapman, M. D., van Hage, M., van Ree, R., Vieths, S., Raulf, M., & Goodman, R. E., 2018. WHO/IUIS Allergen Nomenclature: Providing a common language. Molecular Immunology. 100, 3-13.

Possee, R. D., 1997. Baculoviruses as expression vectors. Current Opinion in Biotechnology. 8, 569-572.

Raza, F., Babasyan, S., Larson, E. M., Freer, H. S., Schnabel, C. L., & Wagner, B., 2021. Peripheral blood basophils are the main source for early interleukin-4 secretion upon in vitro stimulation with Culicoides allergen in allergic horses. PLoS One. 16, e0252243.

Raza, F., Ivanek, R., Freer, H., Reiche, D., Rose, H., Torsteinsdóttir, S., Svansson, V., Björnsdóttir, S., & Wagner, B., 2020. Cul o 2 specific IgG3/5 antibodies predicted Culicoides hypersensitivity in a group imported Icelandic horses. BMC Veterinary Research. 16, 283.

Reed, S. G., Orr, M. T., & Fox, C. B., 2013. Key roles of adjuvants in modern vaccines. Nature Medicine. 19, 1597-1608.

Ribeiro, J. M., & Francischetti, I. M., 2003. Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. Annual Review of Entomology. 48, 73-88.

Riek, R. F., 1953. Studies on allergic dermatitis ("Queensland itch") of the horse. I. - Description, Distribution, Symptoms and Pathology. Australian Veterinary Journal. 29, 177-184.

Rispens, T., & Huijbers, M. G., 2023. The unique properties of IgG4 and its roles in health and disease. Nat Rev Immunol. 23, 763-778.

Ritala, A., Wahlström, E. H., Holkeri, H., Hafren, A., Mäkeläinen, K., Baez, J., Mäkinen, K., & Nuutila, A. M., 2008. Production of a recombinant industrial protein using barley cell cultures. Protein Expression and Purification. 59, 274-281.

Rivera-de-Torre, E., Rimbault, C., Jenkins, T. P., Sørensen, C. V., Damsbo, A., Saez, N. J., Duhoo, Y., Hackney, C. M., Ellgaard, L., & Laustsen, A. H., 2021. Strategies for Heterologous Expression, Synthesis, and Purification of Animal Venom Toxins. Frontiers in Bioengineering and Biotechnology. 9, 811905.

Roberts, G., Pfaar, O., Akdis, C. A., Ansotegui, I. J., Durham, S. R., Gerth van Wijk, R., Halken, S., Larenas-Linnemann, D., Pawankar, R., Pitsios, C., Sheikh, A., Worm, M., Arasi, S., Calderon, M. A., Cingi, C., Dhami, S., Fauquert, J. L., Hamelmann, E., Hellings, P., Jacobsen, L., Knol, E. F., Lin, S. Y., Maggina, P., Mösges, R., Oude Elberink, J. N. G., Pajno, G. B., Pastorello, E. A., Penagos, M., Rotiroti, G., Schmidt-Weber, C. B., Timmermans, F., Tsilochristou, O., Varga, E.-M., Wilkinson, J. N., Williams, A., Zhang, L., Agache, I., Angier, E., Fernandez-Rivas, M., Jutel, M., Lau, S., van Ree, R., Ryan, D., Sturm, G. J., & Muraro, A., 2018. EAACI Guidelines on Allergen Immunotherapy: Allergic rhinoconjunctivitis. Allergy. 73, 765-798.

Roche, A. M., & Wise, S. K., 2014. Subcutaneous immunotherapy. International Forum of Allergy & Rhinology. 4 Suppl 2, S51-54.

Rosano, G. L., Morales, E. S., & Ceccarelli, E. A., 2019. New tools for recombinant protein production in Escherichia coli: A 5-year update. Protein Science. 28, 1412-1422.

Rosewich, M., Schulze, J., Eickmeier, O., Telles, T., Rose, M. A., Schubert, R., & Zielen, S., 2010. Tolerance induction after specific immunotherapy with pollen allergoids adjuvanted by monophosphoryl lipid A in children. Clinical and Experimental Immunology. 160, 403-410.

Rozov, S. M., & Deineko, E. V., 2019. Strategies for Optimizing Recombinant Protein Synthesis in Plant Cells: Classical Approaches and New Directions. Molecular Biology. 53, 157-175.

Russell, C. L., Heesom, K. J., Arthur, C. J., Helps, C. R., Mellor, P. S., Day, M. J., Torsteinsdottir, S., Bjornsdottir, T. S., & Wilson, A. D., 2009. Identification and isolation of cDNA clones encoding the abundant secreted proteins in the saliva proteome of *Culicoides nubeculosus*. Insect Molecular Biology. 18, 383-393.

Sabat, R., Wolk, K., Loyal, L., Döcke, W. D., & Ghoreschi, K., 2019. T cell pathology in skin inflammation. Seminars in Immunopathology. 41, 359-377.

Sadeghi, M., Asadirad, A., Koushki, K., Keshavarz Shahbaz, S., & Dehnavi, S., 2022. Recent advances in improving intranasal allergen-specific immunotherapy; focus on delivery systems and adjuvants. International Immunopharmacology. 113, 109327.

Sahiner, U. M., & Durham, S. R., 2019. Hymenoptera Venom Allergy: How Does Venom Immunotherapy Prevent Anaphylaxis From Bee and Wasp Stings? Frontiers in Immunology. 10, 1959.

Sani, M. Z., Bargahi, A., Momenzadeh, N., Dehghani, P., Moghadam, M. V., Maleki, S. J., Nabipour, I., Shirkani, A., Akhtari, J., Hesamizadeh, K., Heidari, S., Omrani, F., Akbarzadeh, S., & Mohammadi, M., 2021. Genetically engineered fusion of allergen and viral-like particle induces a more effective allergen-specific immune response than a combination of them. Applied Microbiology and Biotechnology. 105, 77-91.

Schaffartzik, A., Hamza, E., Janda, J., Crameri, R., Marti, E., & Rhyner, C., 2012. Equine insect bite hypersensitivity: what do we know? Veterinary Immunology and Immunopathology. 147, 113-126.

Schaffartzik, A., Marti, E., Crameri, R., & Rhyner, C., 2010. Cloning, production and characterization of antigen 5 like proteins from *Simulium vittatum* and *Culicoides nubeculosus*, the first cross-reactive allergen associated with equine insect bite hypersensitivity. Veterinary Immunology and Immunopathology. 137, 76-83.

Schaffartzik, A., Marti, E., Torsteinsdottir, S., Mellor, P. S., Crameri, R., & Rhyner, C., 2011. Selective cloning, characterization, and production of the *Culicoides nubeculosus* salivary gland allergen repertoire associated with equine insect bite hypersensitivity. Veterinary Immunology and Immunopathology. 139, 200-209.

Schaffartzik, A., Weichel, M., Crameri, R., Bjornsdottir, T. S., Prisi, C., Rhyner, C., Torsteinsdottir, S., & Marti, E., 2009. Cloning of IgE-binding proteins from *Simulium vittatum* and their potential significance as allergens for equine insect bite hypersensitivity. Veterinary Immunology and Immunopathology. 132, 68-77.

Scheurer, S., Toda, M., & Vieths, S., 2015. What makes an allergen? Clinical & Experimental Allergy. 45, 1150-1161.

Schmidt, M., & Hoffman, D. R., 2002. Expression systems for production of recombinant allergens. International Archives of Allergy and Immunology. 128, 264-270.

Schnabel, C. L., Babasyan, S., Freer, H., & Wagner, B., 2017. Quantification of equine immunoglobulin A in serum and secretions by a fluorescent bead-based assay. Veterinary Immunology and Immunopathology. 188, 12-20.

Schoos, A.-M. M., Bullens, D., Chawes, B. L., Costa, J., De Vlieger, L., DunnGalvin, A., Epstein, M. M., Garssen, J., Hilger, C., Knipping, K., Kuehn, A., Mijakoski, D., Munblit, D., Nekliudov, N. A., Ozdemir, C., Patient, K., Peroni, D., Stoleski, S., Stylianou, E., Tukalj, M., Verhoeckx, K., Zidarn, M., & van de Veen, W., 2020. Immunological Outcomes of Allergen-Specific Immunotherapy in Food Allergy. Frontiers in Immunology. 11.

Schrenzel, M. D., King, D. P., McKnight, M. L., & Ferrick, D. A., 1997. Characterization of horse (Equus caballus) immunoglobulin mu chain-encoding genes. Immunogenetics. 45, 386-393.

Schurink, A., da Silva, V. H., Velie, B. D., Dibbits, B. W., Crooijmans, R., François, L., Janssens, S., Stinckens, A., Blott, S., Buys, N., Lindgren, G., & Ducro, B. J., 2018. Copy number variations in Friesian horses and genetic risk factors for insect bite hypersensitivity. BMC Medical Genetics. 19, 49.

Schurink, A., Ducro, B. J., Bastiaansen, J. W., Frankena, K., & van Arendonk, J. A., 2013. Genome-wide association study of insect bite hypersensitivity in Dutch Shetland pony mares. Animal Genetics. 44, 44-52.

Schurink, A., Ducro, B. J., Heuven, H. C., & van Arendonk, J. A., 2011. Genetic parameters of insect bite hypersensitivity in Dutch Friesian broodmares. Journal of Animal Science. 89, 1286-1293.

Schurink, A., van Grevenhof, E. M., Ducro, B. J., & van Arendonk, J. A., 2009. Heritability and repeatability of insect bite hypersensitivity in Dutch Shetland breeding mares. Journal of Animal Science. 87, 484-490.

Schurink, A., Wolc, A., Ducro, B. J., Frankena, K., Garrick, D. J., Dekkers, J. C., & van Arendonk, J. A., 2012. Genome-wide association study of insect bite hypersensitivity in two horse populations in the Netherlands. Genetics Selection Evolution. 44, 31.

Senti, G., Freiburghaus, A. U., Larenas-Linnemann, D., Hoffmann, H. J., Patterson, A. M., Klimek, L., Di Bona, D., Pfaar, O., Ahlbeck, L., Akdis, M., Weinfeld, D., Contreras-Verduzco, F. A., Pedroza-Melendez, A., Skaarup, S. H., Lee, S. M., Cardell, L. O., Schmid, J. M., Westin, U., Dollner, R., & Kundig, T. M., 2019. Intralymphatic Immunotherapy: Update and Unmet Needs. International Archives of Allergy and Immunology. 178, 141-149.

Senti, G., Prinz Vavricka, B. M., Erdmann, I., Diaz, M. I., Markus, R., McCormack, S. J., Simard, J. J., Wuthrich, B., Crameri, R., Graf, N., Johansen, P., & Kundig, T. M., 2008. Intralymphatic allergen administration renders specific immunotherapy faster and safer: a randomized controlled trial. Proceedings of the National Academy of Sciences. 105, 17908-17912.

Shamji, M. H., Kappen, J. H., Akdis, M., Jensen-Jarolim, E., Knol, E. F., Kleine-Tebbe, J., Bohle, B., Chaker, A. M., Till, S. J., Valenta, R., Poulsen, L. K., Calderon, M. A., Demoly, P., Pfaar, O., Jacobsen, L., Durham, S. R., & Schmidt-Weber, C. B., 2017. Biomarkers for monitoring clinical efficacy of allergen immunotherapy for allergic rhinoconjunctivitis and allergic asthma: an EAACI Position Paper. Allergy. 72, 1156-1173.

Shamji, M. H., Valenta, R., Jardetzky, T., Verhasselt, V., Durham, S. R., Würtzen, P. A., & van Neerven, R. J. J., 2021. The role of allergen-specific IgE, IgG and IgA in allergic disease. Allergy. 76, 3627-3641.

Shrestha, M., Eriksson, S., Schurink, A., Andersson, L. S., Sundquist, M., Frey, R., Broström, H., Bergström, T., Ducro, B., & Lindgren, G., 2015. Genome-Wide Association Study of Insect Bite Hypersensitivity in Swedish-Born Icelandic Horses. Journal of Heredity. 106, 366-374.

Sick, F., Beer, M., Kampen, H., & Wernike, K., 2019. Culicoides Biting Midges-Underestimated Vectors for Arboviruses of Public Health and Veterinary Importance. Viruses. 11.

Simonin, E. M., Babasyan, S., & Wagner, B., 2022. Peripheral CD23(hi)/IgE(+) Plasmablasts Secrete IgE and Correlate with Allergic Disease Severity. The Journal of Immunology. 209, 665-674.

Singhvi, P., Saneja, A., Srichandan, S., & Panda, A. K., 2020. Bacterial Inclusion Bodies: A Treasure Trove of Bioactive Proteins. Trends in Biotechnology. 38, 474-486.

Sitarz, M., Buczek, A., & Buczek, W., 2021. Skin Lesions and Systemic Reactions in Humans Infested by Blackflies (Diptera: Simullidae) in Recreational Areas in Southeastern Poland. Journal of Clinical Medicine. 10.

Sitarz, M., Buczek, A. M., Buczek, W., Buczek, A., & Bartosik, K., 2022. Risk of Attacks by Blackflies (Diptera: Simuliidae) and Occurrence of Severe Skin Symptoms in Bitten Patients along the Eastern Border of the European Union. International Journal of Environmental Research and Public Health. 19.

Sobczak, J. M., Krenger, P. S., Storni, F., Mohsen, M. O., Balke, I., Reseviča, G., Heath, M. D., Carreno Velazquez, T. L., Kramer, M. F., Scott, C. J. W., Skinner, M. A., Zeltiņš, A., Kündig, T. M., Vogel, M., & Bachmann, M. F., 2023. The next generation virus-like particle platform for the treatment of peanut allergy. Allergy. 78, 1980-1996.

Soh, W. T., Zhang, J., Hollenberg, M. D., Vliagoftis, H., Rothenberg, M. E., Sokol, C. L., Robinson, C., & Jacquet, A., 2023. Protease allergens as initiators-regulators of allergic inflammation. Allergy.

Soldatova, L. N., Crameri, R., Gmachl, M., Kemeny, D. M., Schmidt, M., Weber, M., & Mueller, U. R., 1998. Superior biologic activity of the recombinant bee venom allergen hyaluronidase expressed in baculovirus-infected insect cells as compared with *Escherichia coli*. Journal of Allergy and Clinical Immunology. 101, 691-698.

Sommer-Locher, B., Endriss, V., & Fromm, E., 2012. Various Circumstances Regarding Initial Allergen Exposure and Their Influence on Development of Insect Bite Hypersensitivity in Horses. Journal of Equine Veterinary Science. 32, 158-163.

Spillner, E., Blank, S., & Jakob, T., 2014. Hymenoptera allergens: from venom to "venome". Frontiers in Immunology. 5, 77.

Stefansdottir, S. B. (2015). *Immunotherapy for Insect Bite Hypersensitivity in horses; Development of methods for expressing and purifying recombinant allergens in insect cells and their application for evaluating immunotherapy.* (Master's thesis), University of Iceland, Reykjavík. Retrieved from http://hdl.handle.net/1946/21603

Stefansdottir, S. B., Jonsdottir, S., Kristjansdottir, H., Svansson, V., Marti, E., & Torsteinsdottir, S., 2022. Establishment of a protocol for preventive vaccination against equine insect bite hypersensitivity. Veterinary Immunology and Immunopathology. 253, 110502.

Steinman, A., Peer, G., & Klement, E., 2003. Epidemiological study of Culicoides hypersensitivity in horses in Israel. Veterinary Record. 152, 748-751.

Strasser, R., 2016. Plant protein glycosylation. Glycobiology. 26, 926-939.

Sturm, G. J., Varga, E. M., Roberts, G., Mosbech, H., Bilò, M. B., Akdis, C. A., Antolín-Amérigo, D., Cichocka-Jarosz, E., Gawlik, R., Jakob, T., Kosnik, M., Lange, J., Mingomataj, E., Mitsias, D. I., Ollert, M., Oude Elberink, J. N. G., Pfaar, O., Pitsios, C., Pravettoni, V., Ruëff, F., Sin, B. A., Agache, I., Angier, E., Arasi, S., Calderón, M. A., Fernandez-Rivas, M., Halken, S., Jutel, M., Lau, S., Pajno, G. B., van Ree, R., Ryan, D., Spranger, O., van Wijk, R. G., Dhami, S., Zaman, H., Sheikh, A., & Muraro, A., 2018. EAACI guidelines on allergen immunotherapy: Hymenoptera venom allergy. Allergy. 73, 744-764.

Sudha, V. T., Arora, N., & Singh, B. P., 2009. Serine protease activity of Per a 10 augments allergen-induced airway inflammation in a mouse model. European Journal of Clinical Investigation. 39, 507-516.

Sutaria, N., Adawi, W., Goldberg, R., Roh, Y. S., Choi, J., & Kwatra, S. G., 2022. Itch: Pathogenesis and treatment. J Am Acad Dermatol. 86, 17-34.

Svansson, V., Roelse, M., Olafsdottir, G., Thorsteinsdottir, L., Torfason, E. G., & Torsteinsdottir, S., 2009. Immune response against equine gammaherpesvirus in Icelandic horses. Veterinary Microbiology. 137, 363-368.

Tallmadge, R. L., Antczak, D. F., & Felippe, M. J. B., 2020. Genetics of Immune Disease in the Horse. Veterinary Clinics of North America: Equine Practice. 36, 273-288.

Tallmadge, R. L., McLaughlin, K., Secor, E., Ruano, D., Matychak, M. B., & Flaminio, M. J., 2009. Expression of essential B cell genes and immunoglobulin isotypes suggests active development and gene recombination during equine gestation. Developmental & Comparative Immunology. 33, 1027-1038.

Torsteinsdottir, S., Scheidegger, S., Baselgia, S., Jonsdottir, S., Svansson, V., Bjornsdottir, S., & Marti, E., 2018. A prospective study on insect bite hypersensitivity in horses exported from Iceland into Switzerland. Acta Veterinaria Scandinavica. 60, 69.

Townley, P., Baker, K. P., & Quinn, P. J., 1984. Preferential landing and engorging sites of *Culicoides* species landing on a horse in Ireland. Equine Veterinary Journal. 16, 117-120.

Traidl-Hoffmann, C., Jakob, T., & Behrendt, H., 2009. Determinants of allergenicity. Journal of Allergy and Clinical Immunology. 123, 558-566.

Tripathi, N. K., & Shrivastava, A., 2019. Recent Developments in Bioprocessing of Recombinant Proteins: Expression Hosts and Process Development. Frontiers in Bioengineering and Biotechnology. 7, 420-420.

Tulaeva, I., Kratzer, B., Campana, R., Curin, M., van Hage, M., Karsonova, A., Riabova, K., Karaulov, A., Khaitov, M., Pickl, W. F., & Valenta, R., 2020. Preventive Allergen-Specific Vaccination Against Allergy: Mission Possible? Frontiers in Immunology. 11, 1368.

Valenta, R., Campana, R., Focke-Tejkl, M., & Niederberger, V., 2016. Vaccine development for allergen-specific immunotherapy based on recombinant allergens and synthetic allergen peptides: Lessons from the past and novel mechanisms of action for the future. Journal of Allergy and Clinical Immunology. 137, 351-357.

Valenta, R., Karaulov, A., Niederberger, V., Gattinger, P., van Hage, M., Flicker, S., Linhart, B., Campana, R., Focke-Tejkl, M., Curin, M., Eckl-Dorna, J., Lupinek, C., Resch-Marat, Y., Vrtala, S., Mittermann, I., Garib, V., Khaitov, M., Valent, P., & Pickl, W. F., 2018. Molecular Aspects of Allergens and Allergy. Advances in Immunology. 138, 195-256.

Valenta, R., Lidholm, J., Niederberger, V., Hayek, B., Kraft, D., & Grönlund, H., 1999. The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). Clinical & Experimental Allergy. 29, 896-904.

van der Haegen, A., Griot-Wenk, M., Welle, M., Busato, A., von Tscharner, C., Zurbriggen, A., & Marti, E., 2001. Immunoglobulin-E-bearing cells in skin biopsies of horses with insect bite hypersensitivity. Equine Veterinary Journal. 33, 699-706.

van der Meide, N. M., Meulenbroeks, C., van Altena, C., Schurink, A., Ducro, B. J., Wagner, B., Leibold, W., Rohwer, J., Jacobs, F., van Oldruitenborgh-Oosterbaan, M. M., Savelkoul, H. F., & Tijhaar, E., 2012. *Culicoides obsoletus* extract relevant for diagnostics of insect bite hypersensitivity in horses. Veterinary Immunology and Immunopathology. 149, 245-254.

van der Meide, N. M., Roders, N., Sloet van Oldruitenborgh-Oosterbaan, M. M., Schaap, P. J., van Oers, M. M., Leibold, W., Savelkoul, H. F., & Tijhaar, E., 2013. Cloning and expression of candidate allergens from *Culicoides obsoletus* for diagnosis of insect bite hypersensitivity in horses. Veterinary Immunology and Immunopathology. 153, 227-239.

van der Meide, N. M., Savelkoul, H. F., Meulenbroeks, C., Ducro, B. J., & Tijhaar, E., 2014. Evaluation of a diagnostic ELISA for insect bite hypersensitivity in horses using recombinant Obsoletus complex allergens. The Veterinary Journal. 200, 31-37.

van der Rijt, R., van den Boom, R., Jongema, Y., & van Oldruitenborgh-Oosterbaan, M. M., 2008. *Culicoides* species attracted to horses with and without insect hypersensitivity. The Veterinary Journal. 178, 91-97.

van Grevenhof, E. M., Ducro, B., Heuven, H. C., & Bijma, P., 2007. Identification of environmental factors affecting the prevalence of insect bite hypersensitivity in Shetland ponies and Friesian horses in The Netherlands. Equine Veterinary Journal. 39, 69-73.

Velie, B. D., Shrestha, M., François, L., Schurink, A., Tesfayonas, Y. G., Stinckens, A., Blott, S., Ducro, B. J., Mikko, S., Thomas, R., Swinburne, J. E., Sundqvist, M., Eriksson, S., Buys, N., & Lindgren, G., 2016. Using an Inbred Horse Breed in a High Density Genome-Wide Scan for Genetic Risk Factors of Insect Bite Hypersensitivity (IBH). PLoS One. 11, e0152966.

Vetter, R. S., & Visscher, P. K., 1998. Bites and stings of medically important venomous arthropods. International Journal of Dermatology. 37, 481-496.

Vorsprach, B., Meiser, C. K., Werner, D., Balczun, C., & Schaub, G. A., 2009. Monitoring of ceratopogonidae in southwest Germany. Parasitology Research. 105, 337-344.

Vostry, L., Vostra-Vydrova, H., Citek, J., Gorjanc, G., & Curik, I., 2021. Association of inbreeding and regional equine leucocyte antigen homozygosity with the prevalence of insect bite hypersensitivity in Old Kladruber horse. Animal Genetics. 52, 422-430.

Vychodilova, L., Matiasovic, J., Bobrova, O., Futas, J., Klumplerova, M., Stejskalova, K., Cvanova, M., Janova, E., Osickova, J., Vyskocil, M., Sedlinska, M., Dusek, L., Marti, E., & Horin, P., 2013. Immunogenomic analysis of insect bite hypersensitivity in a model horse population. Veterinary Immunology and Immunopathology. 152, 260-268.

Wagner, B., 2006a. Immunoglobulins and immunoglobulin genes of the horse. Developmental & Comparative Immunology. 30, 155-164.

Wagner, B., 2009b. IgE in horses: occurrence in health and disease. Veterinary Immunology and Immunopathology. 132, 21-30.

Wagner, B. (2023). Equine Reagents.

Wagner, B., Childs, B. A., & Erb, H. N., 2008b. A histamine release assay to identify sensitization to Culicoides allergens in horses with skin hypersensitivity. Veterinary Immunology and Immunopathology. 126, 302-308.

Wagner, B., & Freer, H., 2009a. Development of a bead-based multiplex assay for simultaneous quantification of cytokines in horses. Veterinary Immunology and Immunopathology. 127, 242-248.

Wagner, B., Goodman, L. B., Babasyan, S., Freer, H., Torsteinsdóttir, S., Svansson, V., Björnsdóttir, S., & Perkins, G. A., 2015. Antibody and cellular immune responses of naïve mares to repeated vaccination with an inactivated equine herpesvirus vaccine. Vaccine. 33, 5588-5597.

Wagner, B., Hillegas, J. M., & Antczak, D. F., 2006b. A monoclonal antibody to equine interleukin-4. Veterinary Immunology and Immunopathology. 110, 363-367.

Wagner, B., Hillegas, J. M., & Babasyan, S., 2012. Monoclonal antibodies to equine CD23 identify the low-affinity receptor for IgE on subpopulations of IgM+ and IgG1+ B-cells in horses. Veterinary Immunology and Immunopathology. 146, 125-134.

Wagner, B., Hillegas, J. M., Brinker, D. R., Horohov, D. W., & Antczak, D. F., 2008a. Characterization of monoclonal antibodies to equine interleukin-10 and detection of T regulatory 1 cells in horses. Veterinary Immunology and Immunopathology. 122, 57-64.

Wagner, B., Miller, D. C., Lear, T. L., & Antczak, D. F., 2004. The complete map of the Ig heavy chain constant gene region reveals evidence for seven IgG isotypes and for IgD in the horse. The Journal of Immunology. 173, 3230-3242.

Wagner, B., Miller, W. H., Jr., Erb, H. N., Lunn, D. P., & Antczak, D. F., 2009c. Sensitization of skin mast cells with IgE antibodies to *Culicoides* allergens occurs frequently in clinically healthy horses. Veterinary Immunology and Immunopathology. 132, 53-61.

Wagner, B., Miller, W. H., Morgan, E. E., Hillegas, J. M., Erb, H. N., Leibold, W., & Antczak, D. F., 2006c. IgE and IgG antibodies in skin allergy of the horse. Veterinary Research. 37, 813-825.

Walther, S., Rusitzka, T. V., Diesterbeck, U. S., & Czerny, C.-P., 2015. Equine immunoglobulins and organization of immunoglobulin genes. Developmental & Comparative Immunology. 53, 303-319.

Willard-Mack, C. L., 2006. Normal structure, function, and histology of lymph nodes. Toxicologic Pathology. 34, 409-424.

Wilson, A. D., Harwood, L., Torsteinsdottir, S., & Marti, E., 2006. Production of monoclonal antibodies specific for native equine IgE and their application to monitor total serum IgE responses in Icelandic and non-Icelandic horses with insect bite dermal hypersensitivity. Veterinary Immunology and Immunopathology. 112, 156-170.

Wilson, A. D., Harwood, L. J., Bjornsdottir, S., Marti, E., & Day, M. J., 2001. Detection of IgG and IgE serum antibodies to *Culicoides* salivary gland antigens in horses with insect dermal hypersensitivity (sweet itch). Equine Veterinary Journal. 33, 707-713.

Wilson, A. D., Heesom, K. J., Mawby, W. J., Mellor, P. S., & Russell, C. L., 2008. Identification of abundant proteins and potential allergens in Culicoides nubeculosus salivary glands. Veterinary Immunology and Immunopathology. 122, 94-103.

Yu, A. (2014). Culicoides hypersensitivity: therapy. In C. F. Noli, A.; Rosenkrantz, W. (Ed.), *Veterinary Allergy*: John Wiley & Sons Ltd.

Zeller, S., Rhyner, C., Meyer, N., Schmid-Grendelmeier, P., Akdis, C. A., & Crameri, R., 2009. Exploring the repertoire of IgE-binding self-antigens associated with atopic eczema. Journal of Allergy and Clinical Immunology. 124, 278-285, 285 e271-277.

Zhao, J., Munanairi, A., Liu, X. Y., Zhang, J., Hu, L., Hu, M., Bu, D., Liu, L., Xie, Z., Kim, B. S., Yang, Y., & Chen, Z. F., 2020. PAR2 Mediates Itch via TRPV3 Signaling in Keratinocytes. Journal of Investigative Dermatology. 140, 1524-1532.

Zhernov, Y., Curin, M., Khaitov, M., Karaulov, A., & Valenta, R., 2019. Recombinant allergens for immunotherapy: state of the art. Current Opinion in Allergy and Clinical Immunology. 19, 402-414.

Ziegler, A., Hamza, E., Jonsdottir, S., Rhyner, C., Wagner, B., Schüpbach, G., Svansson, V., Torsteinsdottir, S., & Marti, E., 2018. Longitudinal analysis of allergen-specific IgE and IgG subclasses as potential predictors of insect bite hypersensitivity following first exposure to Culicoides in Icelandic horses. Veterinary Dermatology. 29, 51-e22.

Zielen, S., Gabrielpillai, J., Herrmann, E., Schulze, J., Schubert, R., & Rosewich, M., 2018. Long-term effect of monophosphoryl lipid A adjuvanted specific immunotherapy in patients with grass pollen allergy. Immunotherapy. 10, 529-536.

Zinkhan, S., Ogrina, A., Balke, I., Reseviča, G., Zeltins, A., de Brot, S., Lipp, C., Chang, X., Zha, L., Vogel, M., Bachmann, M. F., & Mohsen, M. O., 2021. The impact of size on particle drainage dynamics and antibody response. Journal of Controlled Release. 331, 296-308.

Zubeldia, J. M., Ferrer, M., Dávila, I., & Justicia, J. L., 2019. Adjuvants in Allergen-Specific Immunotherapy: Modulating and Enhancing the Immune Response. JIACI Journal of Investigational Allergology and Clinical Immunology. 29, 103-111.

Original Publications

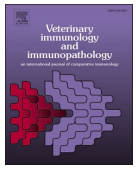
Paper I

Veterinary Immunology and Immunopathology 253 (2022) 110502

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm



Establishment of a protocol for preventive vaccination against equine insect bite hypersensitivity

Sara Bjork Stefansdottir [a,*], Sigridur Jonsdottir [a,b], Holmfridur Kristjansdottir [a], Vilhjalmur Svansson [a], Eliane Marti [b], Sigurbjorg Torsteinsdottir [a]

[a] Institute for Experimental Pathology, Biomedical Center, University of Iceland, Keldur, Keldnavegur 3, 112 Reykjavik, Iceland
[b] Department of Clinical Research and Veterinary Public Health, Vetsuisse Faculty, University of Berne, Länggass-Strasse 124, 3012 Berne, Switzerland

ARTICLE INFO

Keywords:
Insect bite hypersensitivity
Preventive allergen immunotherapy
Intralymphatic injection
Subcutaneous injection
Aluminum hydroxide
Monophosphoryl lipid A

ABSTRACT

Insect bite hypersensitivity (IBH) is a seasonal dermatitis of horses caused by IgE-mediated reactions to bites of *Culicoides* midges characterized by an imbalance of T-cell subsets. Iceland is free of the causative species but the prevalence of IBH in exported Icelandic horses is especially high. We have shown that intralymphatic (i.l.) vaccination with r-*Culicoides* allergens in Aluminum hydroxide (alum) and monophosphoryl lipid A (MPLA) adjuvants induces a desired Th1/regulatory T-cell response. The aim of this study was to compare i.l. to subcutaneous (s.c.) injection. Twelve healthy Icelandic horses were injected, i.l. or s.c., three times with four-week interval, using purified r-*Culicoides* allergens in alum/MPLA. Serum antibody levels and cytokine profile following *in vitro* re-stimulation of PBMC were analysed. Comparable allergen-specific IgG antibodies were induced following both routes of vaccinations. The antibodies showed similar capacity to block binding of IgE from IBH-affected horse to the allergens. Upon re-stimulation of PBMC, IL-10 was induced. Horses vaccinated i.l. produced more IFN-γ and less IL-4 as compared to the horses injected s.c., but the difference did not reach significance. It can be concluded that applying the simpler s.c. injection instead of i.l. to obtain a suitable immune response could be option in IBH immunotherapy.

1. Introduction

Equine insect bite hypersensitivity (IBH) or summer eczema is an allergic dermatitis in horses caused by bites of *Culicoides* spp. with the production of IgE antibodies against allergens derived from the salivary glands of the midges (Fadok & Greiner, 1990; Hellberg et al., 2006; Wilson et al., 2001). The clinical signs are eczema and intense pruritus resulting in broken hair and excoriation due to scratching which can contribute to secondary bacterial infections (Baker & Quinn, 1978; Brostrom et al., 1987; Riek, 1953).

IBH can affect all horse breeds with a prevalence most often < 10%. With the exception of Iceland and New Zealand, IBH has been described worldwide (reviewed in Schaffartzik et al., 2012). However, the prevalence is high in horses foaled in Iceland and exported as adults to *Culicoides* infested areas, where up to 50% of horses may develop the disease after two years or more (Bjornsdottir et al., 2006; Torsteinsdottir et al., 2018). The prevalence of IBH in offspring of Icelandic horses foaled in *Culicoides* infested areas or imported as weanlings is not higher than in most other breeds (Sommer-Locher et al., 2012).

Studies on the immune response in IBH both in the skin and using PBMC suggest that there is an imbalance between Th2 and regulatory-T (Treg) cells. Furthermore, horses born in Iceland and exported to Europe develop a stronger Th2 response than horses born in Europe (Hamza et al., 2007).

Presently there is no effective treatment for IBH available other than avoidance of *Culicoides* midges or symptomatic therapy (Schaffartzik et al., 2012). Vaccination using virus-like particles (VLP) linked to equine IL-5 is being developed and has been shown to reduce blood eosinophil levels, leading to reduction of clinical symptoms (Fettelschoss-Gabriel et al., 2019).

Allergen immunotherapy (AIT) is the only specific treatment of IgE associated allergies. It is based on administration of the causative allergens, aiming to restore immune tolerance resulting in reduction of symptoms and clinical improvement (Dorofeeva et al., 2020). In

Abbreviations: AIT, Allergen immunotherapy; Alum, Aluminum hydroxide; *E.coli*,, *Escherichia coli*; His, Histidine; i.l., Intralymphatic; IBH, insect bite hypersensitivity; IDT, Intradermal test; MPLA, Monophosphoryl lipid A; Treg, Regulatory T cells; WBE, Whole body extract.

* Corresponding author.
E-mail address: sbs27@hi.is (S.B. Stefansdottir).

https://doi.org/10.1016/j.vetimm.2022.110502
Received 18 June 2022; Received in revised form 3 October 2022; Accepted 10 October 2022
Available online 15 October 2022

effective AIT there is a switch from Th2 towards Th1/Treg immune response and induction of allergen-specific Tregs with production of regulatory cytokines. Also increase in allergen-specific B regulatory cells inducing IgG antibodies able to inhibit the binding of allergen-specific IgE antibodies. Followed by decrease in Th2 cytokines and suppression of allergic inflammation (Globinska et al., 2018).

Attempts have been made to treat IBH with AIT using whole body extract (WBE) with inconclusive results (Anderson et al., 1996; Barbet et al., 1990; Ginel et al., 2014). WBE is a mixture of both allergenic and nonallergenic components, the content is variable between batches and important allergens often present in too low concentration and even absent from the extract (Curin et al., 2017). Availability of pure and well-defined allergens is thus important for the development of standardized and efficient AIT (Valenta et al., 2016; Zhernov et al., 2019).

For development of both preventive and therapeutic immunotherapy against IBH it is important to consider allergen source, type of adjuvant and the route of administration (Jonsdottir et al., 2019).

Major allergens of IBH have been identified with a large panel of IBH-affected horses and healthy controls from various areas in Europe. Nine of the 27 tested r-allergens from *Culicoides* bound IgE in sera from > 50% of IBH-affected horses (Birras et al., 2021; Novotny et al., 2021).

Subcutaneous injection is one of the most frequently used allergen administration route in AIT (Field & Blaiss, 2020). Currently s.c. immunotherapy is used in humans for treatment against allergic rhinitis and allergic asthma performed with frequent injections over a long period of time (Roche & Wise, 2014). Intralymphatic immunotherapy, where lower doses of allergens are needed and shorter treatment time compared to other administration routes is in clinical development (Senti et al., 2019).

Aluminum hydroxide (alum) especially enhances Th2 immune responses (He et al., 2015). Monophosphoryl lipid A (MPLA), a detoxified LPS derivative has been shown to induce Th1 cytokine production and is used successfully in immunotherapy against allergic rhinitis to grass pollen in human patients (Mothes et al., 2003; Rosewich et al., 2010; Zielen et al., 2018). In a previous study we have shown that i.l. vaccination with r-allergens in alum alone or in mixture with MPLA significantly induced IgG subclass antibodies with strong blocking capacity (Jonsdottir et al., 2016). Furthermore, a significant increase in production of IFN-γ and IL-10 upon allergen-specific re-stimulation of PBMC was observed from horses vaccinated with r-allergens in alum/MPLA but not from horses vaccinated with the same r-allergens in alum alone compared to unvaccinated controls.

The i.l. injection route has been tried with promising results in experimental preventive vaccinations against IBH in horses (Jonsdottir et al., 2015, 2016). However, injection into the submandibular lymph nodes of horses can be laborious and requires experienced vaccinators. Therefore, we wanted to compare the s.c. and i.l. routes using the same vaccination protocol as previously (Jonsdottir et al., 2016).

2. Materials and methods

2.1. Animals

Eighteen healthy Icelandic horses (age: 4–10 years) living in Iceland and thus free of IBH, were included in the study. Twelve of the horses were used in the experimental vaccination study and six unvaccinated horses included as non-vaccinated controls (Table 1). The horses were maintained according to the Icelandic care guidelines for experimental animals. Experiment permit National Animal Research Committee of Iceland no. 2018–05–04.

Table 1
Information on the horses used in the study and their distribution in three different vaccination groups.

Horse group	Mean age (range)	Male/Female
i.l.	5.8 (4–8)	4/2
s.c.	7.2 (6–9)	5/1
Controls	7.3 (6–10)	6/0

2.2. Vaccination and sample collection

Twelve horses were vaccinated with three r-*Culicoides* allergens (Cul o 2P, Cul o 3 and Cul n 4) (Peeters et al., 2013; Schaffartzik et al., 2011; van der Meide et al., 2013) produced in *E. coli* and purified. They were vaccinated three times (week 0, 4, 8) with 20 μg of each allergen in mixture with 500 μg aluminum-hydroxide-gel (Alhydrogel® 2%, Invitrogen) and 50 μg MPLA (MPLA-SM VacciGrade™, InvivoGen) (alum/MPLA), totally 0.4 mL per vaccination. Six horses were vaccinated into the submandibular lymph nodes (i.l.) and six s.c. in the lateral neck. Blood was collected by jugular venipuncture before vaccination and every other week for 20 weeks. Serum was stored at − 20 °C. Before vaccination and two weeks after the second and third vaccination heparinized blood was collected for isolation of PBMC. A differential count of leukocytes from EDTA blood was carried out before and after the third vaccination. Body temperature and site reactions were recorded before and after each vaccination (Jonsdottir et al., 2015).

2.3. Protein production and purification

2.3.1. Proteins used for vaccination

The allergens used in the vaccination were produced in *E. coli*. Cul o 2P was produced and purified according to Peeters et al. 2013. Cul o 3 was purchased from GenScript® refolded in PBS, 10% glycerol, 1 M arginine, 2 mM DTT. Cul n 4 was codon optimized for *E. coli* and cloned as N-terminal fusion to a hexa-histidine tag ($(His)_6$) into the high expression vector pET-42b(+) (GenScript®). Transformation and protein production was performed as described by Schaffartzik et al. 2011. All proteins went through a LPS removal step during the purification. The culture was harvested and washed according to Novotny et al. 2021 except the pellet was resuspended in 50 mM Tris, 500 mM NaCl, 8 M urea pH 8. For protein purification the lysate was applied to HIS-Select™ HF Nickel Affinity Gel (Sigma Aldrich) according to manufacturer's protocol. The gel was washed with 6 M urea, 20 mM Tris, 500 mM NaCl pH 8, the protein eluted and refolded in 20 mM Tris, 500 mM NaCl, 400 mM Arginine-HCl, 5 mM b-cyclodextrin, 10 mM glycerol, 340 mM Imidazole pH 8. Eluate was dialyzed to eliminate b-cyclodextrin and imidazole.

2.3.2. Proteins used for analyzing the immune response

The allergens corresponding to those used in the vaccination and three *Culicoides* proteins not used for vaccination were produced in insect cells (Bac-to-Bac® Baculovirus expression system Invitrogen). Cul n 4 (Bac-rCul n 4) and Cul o 7 (Bac-rCul o 7) as described in Jonsdottir et al., 2018. Cul o 2P, Cul o 3, Cul n 3 and Cul n 8 (Bac-rCul o 2P, Bac-rCul o 3, Bac-rCul n 3, Bac-rCul n 8) according to Jonsdottir et al. 2016 with exception of Cul o 3 cloned into pI-secSUMOstar vector (Life-Sensors) and Cul n 8 cloned into pFastBacHT B vector (Invitrogen). The proteins were purified under native conditions as described in Jonsdottir et al., 2016 and dialyzed in 2xPBS.

2.4. Serological tests

2.4.1. ELISA

Allergen-specific IgG subclasses and IgE antibody levels were measured by ELISA in sera from the vaccinated horses (week 0, 2, 6, 10, 18 and 20). Sera from unvaccinated control horses were included as negative controls. Sera from two IBH-affected horses living in Switzerland with high IgE levels to the r-allergens were included as positive control. The ELISA was performed as described by Jonsdottir et al., 2015. Briefly, the plates were coated with 2 μg/mL of rBac-Cul o 2P, rBac-Cul o 3 and rBac-Cul n 4. For detection of IgG subclasses (IgG1,

IgG4/7 IgG5), the serum was diluted 1:800 and 1:10 for IgE. The serum dilution used was determined in a pilot experiment by titration (data not shown). IgG subclasses and IgE were detected using the corresponding mouse monoclonal antibodies, followed by alkaline-phosphatase conjugated goat-anti mouse IgG polyclonal antibodies, and developed with phosphatase substrate in 10% diethanolamine.

2.4.2. Blocking ELISA assay

The IgE blocking capacity of pools of serum (week 0 and 10) from the vaccinated groups, was tested in a blocking ELISA performed as described by Jonsdottir et al., 2015 against Bac-rCul o 2P, Bac-rCul o 3, Bac-rCul n 4 and Bac-rCul n 8.

2.5. Determination of cytokines

PBMC were re-stimulated *in vitro* before vaccination (week 0) and two weeks after second (week 6) and third (week 10) vaccination as well as from unvaccinated control horses. PBMC were isolated according to Hamza et al., 2007 and Jonsdottir et al., 2016. They were re-stimulated in medium alone as negative control, a mixture of the three allergens used for vaccination (Bac-rCul o 2P, Bac-rCul o 3, Bac-rCul n 4, each 2 μg/mL), a mixture of three *Culicoides* allergens not included in the vaccine (Bac-rCul o 7, Bac-rCul n 3, Bac-rCul n 8) (Schaffartzik et al., 2011; van der Meide et al., 2013) and Phytohaemagglutinin (L1665 Sigma) (PHA 1 μg/mL) as positive control. 0.5×10^6 cells/well were stimulated for 48 h in 96-well plates at 37 °C and 5% CO_2. Supernatants were harvested and stored at − 80 °C. Levels of IL-4, IFN-γ and IL-10 in cell supernatant were determined with bead-based multiplex assays performed at Cornell University as described by Wagner and Freer, 2009. Correction for spontaneous cytokine release was made by subtracting values from the medium alone.

2.6. Intradermal test

Intradermal test (IDT) was performed at week 18 with the allergens rCul o 2P, rCul o 3 and rCul n 4 produced in *E. coli* according to Jonsdottir et al., 2016.

2.7. Statistical analysis

Statistical analysis was carried out in Prism 9.0 (GraphPad). The distribution of the data was tested with the Shapiro Wilk test showing it did not follow normal distribution. Therefor non-parametric tests were used for analysis of the data. For analysis of IgG subclass response Friedman's test was used for comparison of time points within each of the vaccination groups. Each time point was examined for statistical difference between groups using Mann-Whitney U test and the Bonferroni correction used to correct for multiple comparison. For analysis of cytokine response comparison of time points, week 0 and week 10, within vaccination groups the Wilcoxon test was used. Comparison of stimulation of PBMC with *Culicoides* allergens used in the vaccine and not used was done with Mann-Whitney test within vaccination groups. For comparison of vaccination groups and control horses at week 10 the Kruskal-Wallis test was used. For all tests, $p \leq 0.05$ was considered significant difference.

3. Results and discussion

3.1. Clinical examination

The vaccinations were well tolerated. No rise in body temperature or pain on palpation was observed, but a mild swelling in some of the horses after the second vaccination. Leukocyte count following the third vaccination was within normal range (data not shown). IDT was performed to test if the r-allergens would induce type I hypersensitivity reactions following the vaccinations. All but one horse reacted to the histamine control but response to the r-allergens was not observed (data not shown). This confirms that the vaccination with these allergens seems to be safe, as already observed by Jonsdottir et al., 2016.

3.2. Specific antibody response of vaccinated horses

Horses have seven IgG subclasses with different functions (Wagner et al., 2004). The most abundant antibody in horse serum is IgG4/7, important for latent infections and secondary immune responses, while IgG1 is rather short lived and predominantly produced in primary infections (Goodman et al., 2012; Svansson et al., 2009; Wagner et al., 2006). In IBH IgG5 has been linked to allergy (Wagner, 2006) and there is some evidence that allergen-specific IgG5 may be used as a predictor for IBH (Ziegler et al., 2018). Thus, we assume that a preventive vaccine against allergy in horses should preferably induce no or only low IgG5. Following vaccination, no significant difference between the i.l. and s.c. vaccination groups in the IgG subclass response was observed (Fig. 1). Within both groups there was a significant increase in IgG1, IgG4/7 and IgG5 two weeks after the second (data not shown) and third vaccination against the three r-allergens Bac-rCul o 2P, Bac-rCul o 3, Bac-rCul n 4 (Supplementary Fig. 1). No response was observed against irrelevant allergen Bac-Cul n 8 (data not shown). The IDT at week 18 boosted the specific antibody response against the vaccine Bac-r-allergens as seen in Jonsdottir et al., 2016. No IgE production was detected at any time point and the horses were negative in a skin test carried out at week 18 (data not shown). The IgG subclasses induced were mainly IgG1 and IgG4/7 accompanied by a low IgG5 and no IgE confirming our previous studies (Jonsdottir et al., 2016). Together with the negative skin test results these data indicate that the vaccine did not induce sensitization to the allergens.

3.3. Blocking of allergen specific IgE binding by sera from vaccinated horses

An important factor in successful AIT is induction of allergen-specific IgG antibodies capable of blocking IgE from binding to allergen (Dorofeeva et al., 2020). The blocking capacity of allergen-specific IgG antibodies induced by the vaccination was tested by using pools of the pre- and post-immune sera (two weeks after third vaccination) in blocking ELISA. Serum from the two groups i.l. and s.c. showed similar blocking of IgE (Fig. 2). Blocking in the post-immune sera was most efficient against Bac-rCul n 4, reaching over 90% at the 1:10 dilution and still over 80% at the 1:40 dilution in both groups. The blocking capacity of the post-immune sera to Bac-rCul o 2P and Bac-rCul o 3 was lower, close to 80% at the 1:10 dilution and around 20% in the 1:40 dilution. Pre-immune sera showed no or only marginal blocking of IgE-binding against Bac-rCul n 4 and Bac-rCul o 3 but blocking against Bac-rCul o 2P was around 20% at the 1:10 and 1:20 dilutions, then dropping in the following dilutions. Our data thus indicates that the blocking capacity of the sera is rather depending on the allergen than on the immunization route. Horses in the continental Europe are more often exposed to *Culicoides obsoletus* than to *Culicoides nubeculosus* (van der Rijt et al., 2008) and have thus probably higher IgE or possibly IgE with higher affinity to Cul o than to Cul n allergens. Consequently, a higher blocking capacity of the IgG might be required to block IgE binding to Cul o compared to Cul n allergens. This might be a reason for lower blocking capacity of our postimmune sera for Cul o 2P and Cul o 3 compared to Cul n 4 (Birras et al., 2021; Novotny et al., 2021).

3.4. Cytokine production upon re-stimulation of PBMC

To characterize further the type of immune response induced by the two injection routes the cytokine response was analyzed by re-stimulation of PBMC with a mixture of Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4 and levels of IFN-γ, IL-10 and IL-4 analyzed in the supernatant. Important factor of AIT is generation of allergen tolerance

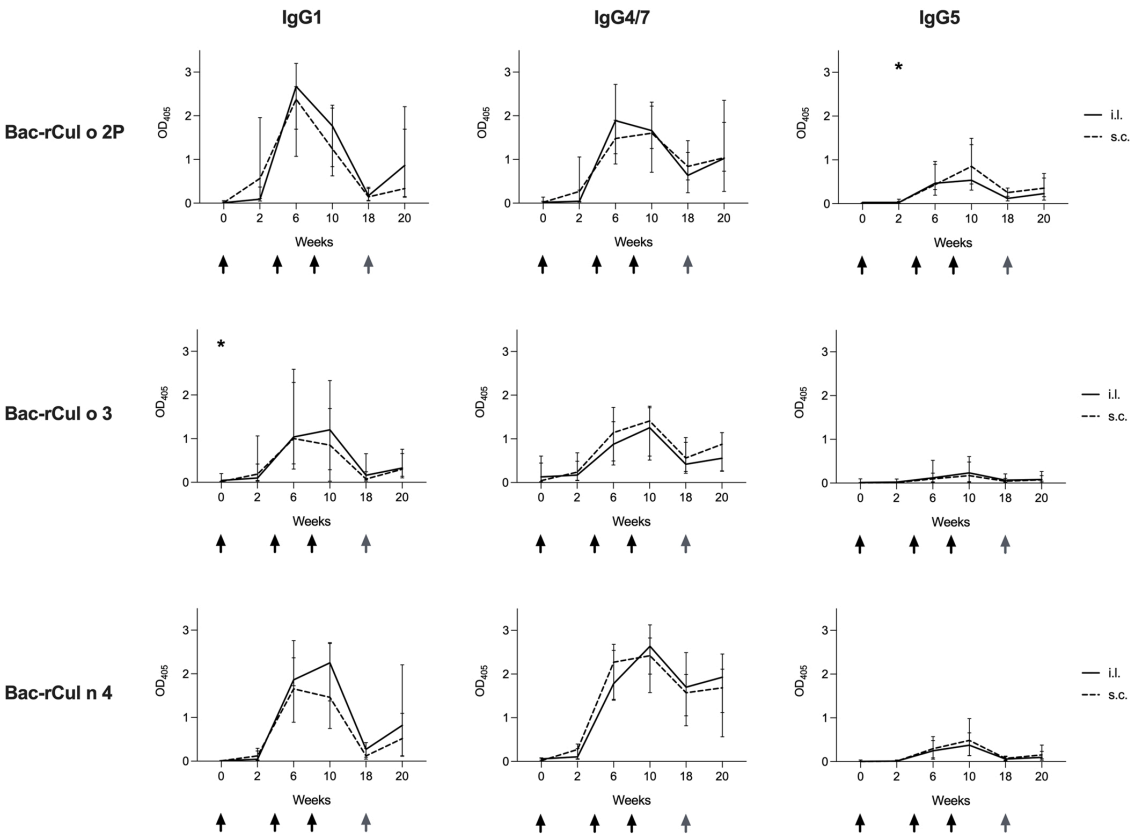


Fig. 1. Time course of IgG subclass response against Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4 of i.l./MPLA and s.c./MPLA vaccinated horses measured by ELISA. Corrected OD median values for the six horses in each group at different time points. The bars indicate 95% confidence interval. Comparisons were performed using Mann-Whitney test with Bonferroni corrections. An asterisk (*) indicates statistical difference between the groups of horses at a given time-point. Black arrows indicate vaccinations and gray arrow intradermal test.

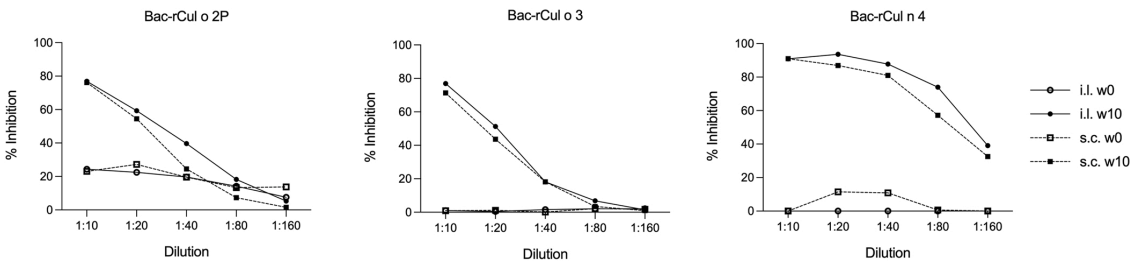


Fig. 2. Inhibition of IgE binding from serum of IBH-affected horse to Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4 with pool of sera from i.l./MPLA and s.c./MPLA vaccinated horses. Mean % inhibition by sera from horses in group i.l./MPLA post-vaccination, i.l./MPLA pre-vaccination, s.c./MPLA post-vaccination, s.c./MPLA pre- vaccination diluted 1:10 – 1:160. Serum added to ELISA plate coated with allergen prior to adding serum at dilution 1:10 from IBH-affected horse.

where IL-10 is a critical suppressive mediator (Celebi Sözener et al., 2020). The aim of preventive immunotherapy against IBH is to induce Th1/Treg immune response with production of IFN-γ and IL-10 (Jonsdottir et al., 2016). Cytokine levels following vaccination increased significantly in both vaccination groups, for all three cytokines except for IL-10 in the s.c. group probably because of one non-responding horse in this group (Fig. 3A). Stimulation with the vaccine Bac-r-allergens resulted in significantly higher cytokine levels compared to stimulation with *Culicoides* allergens not included in the vaccine except in the s.c. group for IL-10 (Supplementary Fig. 2). Comparing the i.l. and s.c. vaccination groups there was no significant difference for any of the measured cytokines (Fig. 3B). Compared to non-vaccinated controls, significantly higher IL-10 levels were detected in both groups and IL-4 levels were significantly higher in the s.c. vaccination group. No significant difference was observed for IFN-γ secretion, as two control horses showed high levels of IFN-γ (> 10 U/mL) (Fig. 3B). These

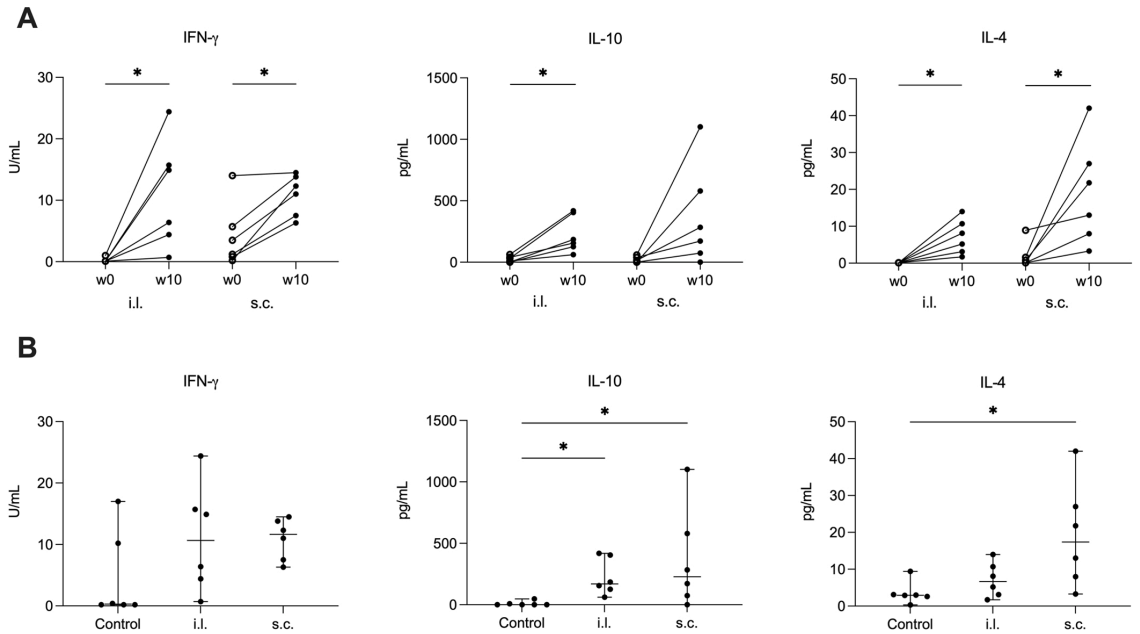


Fig. 3. Cytokine secretion after *in vitro* re-stimulation of PBMC from the vaccinated horses with a mixture of Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4. The cytokines were measured in supernatants using bead-based multiplex assay. A. Comparison of cytokine secretion pre- and post-vaccination of i.l./MPLA and s.c./MPLA vaccinated horses. Comparisons were performed using Wilcoxon test. An asterisk (*) with line indicates statistical difference between pre- and post-vaccination within horse groups. B. Comparison of cytokine secretion of i.l./MPLA and s.c./MPLA post-vaccination and control horses. Comparisons were performed using Kruskal-Wallis multiple comparisons test. An asterisk (*) with line indicates statistical difference between the groups of horses.

findings indicate that i.l. vaccination induces the preferable Treg cytokine profile with production of IL-10. The same applies to s.c. vaccination, even though the increase was not significant following vaccination, compared to control horses the difference was significant. Interpretation of the cytokine response is somewhat difficult because of high inter-individual variability, but both i.l. and s.c. resulted in significant Treg response accompanied by a less consistent, mixed Th1, Th2 response, with slightly higher IL-4 production in the s.c. group. However, no significant differences between the s.c. and i.l. groups were found for any cytokine, indicating that there are no major differences in the cytokine response following s.c. and i.l. vaccination.

4. Conclusion

Vaccination of healthy horses with no previous exposure to the allergens, both i.l. and s.c., with r-allergens in alum/MPLA was well-tolerated and induced immune responses without production of IgE antibodies. The antibodies produced following vaccination could block IgE-binding to the r-allergens. There was not a significant difference between the two vaccination groups regarding the IgG antibody and cytokine response, indicating that i.l. injection may be replaced with the more convenient s.c. injection in AIT against equine IBH.

Conflict of interest

No conflict of interest.

Acknowledgments

This work was supported by Icelandic Research Fund Grants nos. 184998-051 and 206528-051.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetimm.2022.110502.

References

Anderson, G.S., Belton, P., Jahren, E., Lange, H., Kleider, N., 1996. Immunotherapy trial for horses in British Columbia with Culicoides (Diptera:Ceratopogonidae) hypersensitivity. J. Med Entomol. 33, 458–466.
Baker, K.P., Quinn, P.J., 1978. A report on clinical aspects and histopathology of sweet itch. Equine Vet. J. 10, 243–248.
Barbet, J.L., Bevier, D., Greiner, E.C., 1990. Specific immunotherapy in the treatment of *Culicoides* hypersensitive horses: a double-blind study. Equine Vet. J. 22, 232–235.
Birras, J., White, S.J., Jonsdottir, S., Novotny, E.N., Ziegler, A., Wilson, A.D., Frey, R., Torsteinsdottir, S., Alcocer, M., Marti, E., 2021. First clinical expression of equine insect bite hypersensitivity is associated with co-sensitization to multiple Culicoides allergens. PLoS One 16, e0257819.
Bjornsdottir, S., Sigvaldadottir, J., Brostrom, H., Langvad, B., Sigurdsson, A., 2006. Summer eczema in exported Icelandic horses: influence of environmental and genetic factors. Acta Vet. Scand. 48, 3.
Brostrom, H., Larsson, A., Troedsson, M., 1987. Allergic dermatitis (sweet itch) of Icelandic horses in Sweden: an epidemiological study. Equine Vet. J. 19, 229–236.
Celebi Sözener, Z., Mungan, D., Cevhertas, L., Ogulur, I., Akdis, M., Akdis, C., 2020. Tolerance mechanisms in allergen immunotherapy. Curr. Opin. Allergy Clin. Immunol. 20, 591–601.
Curin, M., Garib, V., Valenta, R., 2017. Single recombinant and purified major allergens and peptides: How they are made and how they change allergy diagnosis and treatment. Ann. Allergy Asthma Immunol. 119, 201–209.
Dorofeeva, Y., Shilovskiy, I., Tulaeva, I., Focke-Tejkl, M., Flicker, S., Kudlay, D., Khaitov, M., Karsonova, A., Riabova, K., Karaulov, A., Khanferyan, R., Pickl, W.F., Wekerle, T., Valenta, R., 2020. Past, presence, and future of allergen immunotherapy vaccines. Allergy.
Fadok, V.A., Greiner, E.C., 1990. Equine insect hypersensitivity: skin test and biopsy results correlated with clinical data. Equine Vet. J. 22, 236–240.
Fettelschoss-Gabriel, A., Fettelschoss, V., Olomski, F., Birkmann, K., Thoms, F., Buhler, M., Kummer, M., Zeltins, A., Kundig, T.M., Bachmann, M.F., 2019. Active vaccination against interleukin-5 as long-term treatment for insect-bite hypersensitivity in horses. Allergy 74, 572–582.

Field, K., Blaiss, M.S., 2020. Sublingual versus subcutaneous immunotherapy for allergic rhinitis: what are the important therapeutic and real-world considerations? Curr. Allergy Asthma Rep. 20, 45.
Ginel, P.J., Hernandez, E., Lucena, R., Blanco, B., Novales, M., Mozos, E., 2014. Allergen-specific immunotherapy in horses with insect bite hypersensitivity: a double-blind, randomized, placebo-controlled study. Vet. Dermatol. 25, 29–e10.
Globinska, A., Boonpiyathad, T., Satitsuksanoa, P., Kleuskens, M., van de Veen, W., Sokolowska, M., Akdis, M., 2018. Mechanisms of allergen-specific immunotherapy: Diverse mechanisms of immune tolerance to allergens. Ann. Allergy Asthma Immunol. 121, 306–312.
Goodman, L.B., Wimer, C., Dubovi, E.J., Gold, C., Wagner, B., 2012. Immunological correlates of vaccination and infection for equine herpesvirus 1. Clin. Vaccine Immunol. 19, 235–241.
Hamza, E., Doherr, M.G., Bertoni, G., Jungi, T.W., Marti, E., 2007. Modulation of allergy incidence in icelandic horses is associated with a change in IL-4-producing T cells. Int. Arch. Allergy Immunol. 144, 325–337.
He, P., Zou, Y., Hu, Z., 2015. Advances in aluminum hydroxide-based adjuvant research and its mechanism. Hum. Vaccine Immunother. 11, 477–488.
Hellberg, W., Wilson, A.D., Mellor, P., Doherr, M.G., Torsteinsdottir, S., Zurbriggen, A., Jungi, T., Marti, E., 2006. Equine insect bite hypersensitivity: immunoblot analysis of IgE and IgG subclass responses to Culicoides nubeculosus salivary gland extract. Vet. Immunol. Immunopathol. 113, 99–112.
Jonsdottir, S., Hamza, E., Janda, J., Rhyner, C., Meinke, A., Marti, E., Svansson, V., Torsteinsdottir, S., 2015. Developing a preventive immunization approach against insect bite hypersensitivity using recombinant allergens: a pilot study. Vet. Immunol. Immunopathol. 166, 8–21.
Jonsdottir, S., Svansson, V., Stefansdottir, S.B., Schupbach, G., Rhyner, C., Marti, E., Torsteinsdottir, S., 2016. A preventive immunization approach against insect bite hypersensitivity: Intralymphatic injection with recombinant allergens in Alum or Alum and monophosphoryl lipid A. Vet. Immunol. Immunopathol. 172, 14–20.
Jonsdottir, S., Cvitas, I., Svansson, V., Fettelschloss-Gabriel, A., Torsteinsdottir, S., Marti, E., 2019. New strategies for prevention and treatment of insect bite hypersensitivity in horses. Curr. Dermatol. Rep. 8, 303–312.
Mothes, N., Heinzkill, M., Drachenberg, K.J., Sperr, W.R., Krauth, M.T., Majlesi, Y., Semper, H., Valent, P., Niederberger, V., Kraft, D., Valenta, R., 2003. Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. Clin. Exp. Allergy 33, 1198–1208.
Novotny, E.N., White, S.J., Wilson, A.D., Stefánsdóttir, S.B., Tijhaar, E., Jonsdóttir, S., Frey, R., Reiche, D., Rose, H., Rhyner, C., Schüpbach-Regula, G., Torsteinsdóttir, S., Alcocer, M., Marti, E., 2021. Component-resolved microarray analysis of IgE sensitization profiles to Culicoides recombinant allergens in horses with insect bite hypersensitivity. Allergy 76, 1147–1157.
Peeters, L.M., Janssens, S., Goddeeris, B.M., De Keyser, K., Wilson, A.D., Kaufmann, C., Schaffartzik, A., Marti, E., Buys, N., 2013. Evaluation of an IgE ELISA with Culicoides spp. extracts and recombinant salivary antigens for diagnosis of insect bite hypersensitivity in Warmblood horses. Vet. J. 198, 141–147.
Riek, R.F., 1953. Studies on allergic dermatitis ("Queensland itch") of the horse. I. - description, distribution, symptoms and pathology. Aust. Vet. J. 29, 177–184.
Roche, A.M., Wise, S.K., 2014. Subcutaneous immunotherapy. Int. Forum Allergy Rhinol. 4 (Suppl. 2), S51–S54.
Rosewich, M., Schulze, J., Eickmeier, O., Telles, T., Rose, M.A., Schubert, R., Zielen, S., 2010. Tolerance induction after specific immunotherapy with pollen allergoids adjuvanted by monophosphoryl lipid A in children. Clin. Exp. Immunol. 160, 403–410.
Schaffartzik, A., Marti, E., Torsteinsdottir, S., Mellor, P.S., Crameri, R., Rhyner, C., 2011. Selective cloning, characterization, and production of the *Culicoides nubeculosus* salivary gland allergen repertoire associated with equine insect bite hypersensitivity. Vet. Immunol. Immunopathol. 139, 200–209.
Schaffartzik, A., Hamza, E., Janda, J., Crameri, R., Marti, E., Rhyner, C., 2012. Equine insect bite hypersensitivity: what do we know? Vet. Immunol. Immunopathol. 147, 113–126.
Senti, G., Freiburghaus, A.U., Larenas-Linnemann, D., Hoffmann, H.J., Patterson, A.M., Klimek, L., Di Bona, D., Pfaar, O., Ahlbeck, L., Akdis, M., Weinfeld, D., Contreras-Verduzco, F.A., Pedroza-Melendez, A., Skaarup, S.H., Lee, S.M., Cardell, L.O., Schmid, J.M., Westin, U., Dollner, R., Kundig, T.M., 2019. Intralymphatic immunotherapy: update and unmet needs. Int. Arch. Allergy Immunol. 178, 141–149.
Sommer-Locher, B., Endriss, V., Fromm, E., 2012. Various circumstances regarding initial allergen exposure and their influence on development of insect bite hypersensitivity in horses. J. Equine Vet. Sci. 32, 158–163.
Svansson, V., Roelse, M., Olafsdottir, G., Thorsteinsdottir, L., Torfason, E.G., Torsteinsdottir, S., 2009. Immune response against equine gammaherpesvirus in Icelandic horses. Vet. Microbiol. 137, 363–368.
Torsteinsdottir, S., Scheidegger, S., Baselgia, S., Jonsdottir, S., Svansson, V., Bjornsdottir, S., Marti, E., 2018. A prospective study on insect bite hypersensitivity in horses exported from Iceland into Switzerland. Acta Vet. Scand. 60, 69.
Valenta, R., Campana, R., Focke-Tejkl, M., Niederberger, V., 2016. Vaccine development for allergen-specific immunotherapy based on recombinant allergens and synthetic allergen peptides: lessons from the past and novel mechanisms of action for the future. J. Allergy Clin. Immunol. 137, 351–357.
van der Meide, N.M., Roders, N., Sloet van Oldruitenborgh-Oosterbaan, M.M., Schaap, P. J., van Oers, M.M., Leibold, W., Savelkoul, H.F., Tijhaar, E., 2013. Cloning and expression of candidate allergens from Culicoides obsoletus for diagnosis of insect bite hypersensitivity in horses. Vet. Immunol. Immunopathol. 153, 227–239.
van der Rijt, R., van den Boom, R., Jongema, Y., van Oldruitenborgh-Oosterbaan, M.M., 2008. *Culicoides* species attracted to horses with and without insect hypersensitivity. Vet. J. 178, 91–97.
Wagner, B., 2006. Immunoglobulins and immunoglobulin genes of the horse. Dev. Comp. Immunol. 30, 155–164.
Wagner, B., Freer, H., 2009. Development of a bead-based multiplex assay for simultaneous quantification of cytokines in horses. Vet. Immunol. Immunopathol. 127, 242–248.
Wagner, B., Miller, D.C., Lear, T.L., Antczak, D.F., 2004. The complete map of the Ig heavy chain constant gene region reveals evidence for seven IgG isotypes and for IgD in the horse. J. Immunol. 173, 3230–3242.
Wagner, B., Miller, W.H., Morgan, E.E., Hillegas, J.M., Erb, H.N., Leibold, W., Antczak, D. F., 2006. IgE and IgG antibodies in skin allergy of the horse. Vet. Res. 37, 813–825.
Wilson, A.D., Harwood, L.J., Bjornsdottir, S., Marti, E., Day, M.J., 2001. Detection of IgG and IgE serum antibodies to *Culicoides* salivary gland antigens in horses with insect dermal hypersensitivity (sweet itch). Equine Vet. J. 33, 707–713.
Zhernov, Y., Curin, M., Khaitov, M., Karaulov, A., Valenta, R., 2019. Recombinant allergens for immunotherapy: state of the art. Curr. Opin. Allergy Clin. Immunol. 19, 402–414.
Ziegler, A., Hamza, E., Jonsdottir, S., Rhyner, C., Wagner, B., Schüpbach, G., Svansson, V., Torsteinsdottir, S., Marti, E., 2018. Longitudinal analysis of allergen-specific IgE and IgG subclasses as potential predictors of insect bite hypersensitivity following first exposure to Culicoides in Icelandic horses. Vet. Dermatol. 29, 51–e22.
Zielen, S., Gabrielpillai, J., Herrmann, E., Schulze, J., Schubert, R., Rosewich, M., 2018. Long-term effect of monophosphoryl lipid A adjuvanted specific immunotherapy in patients with grass pollen allergy. Immunotherapy 10, 529–536.

Appendix I

Nucleotide and amino acid sequences for codon optimized genes with $(His)_6$-tag and GGC link for VLP used for expression in *E. coli*.

Start and stop codons are in **bold** and the secretion signal sequence of the proteins is marked as green.

Cul o 1P (JX512273)

645 bp

ATGCGCTTCGCAACCATTTTTCTGTTGTCGGCATCAATCATCTTGCTGAGCACTGGGGAGG
CGTTGGCGAAGAAAAAGAAAAAAATCGACAAATCGATGCCTCCTGAATGCTTGCCCTTACC
GCCTAAAAAACGCGCGTCAGAGTGCACGAACCAGTCGGGGTTCAAATATTACCCGAAAACA
AATCAGTGCGGGCCTGTGCGTAACCAACTTTGTCAAGGACACGGAGGTTTCACTACGTTAG
ACGAATGCGTTTATAAATGCTACGATTACCGTAAAATGTCAACGACCAAAAATGTAGATGG
GTGTAATAAGTCTATTAAAGAAGAGGAGATGACCGAGGCGATTCGTGTGGTATCTCGCGGA
GACTCCCCAGATCTGATTAAAGACAATCGTTGCCGTGAACCAAATGAGTTGAGTTTGAAGA
ACGGGTCAGCCCGCGTCAAACCGGCATACAAGTTCAATAAAGACACAAATGAATGTGTGGC
GATGATGGACAAAGTTTGTTTGGGACGCAATCGCTTCAAGACGAAGGAAGAATGTGTCCAC
GTTTGTAATTGGAACCTGTCCAGTGGGCGTCATCGCCACATCGTCCGTGAAAGCAACGCGA
AGCAAGGCGGTTGCCATCATCATCACCATCAC**TAA**

214 a.a.

M R F A T I F L L S A S I I L L S T G E A L A K K K K K I D K
S M P P E C L P L P P K K R A S E C T N Q S G F K Y Y P K T N
Q C G P V R N Q L C Q G H G G F T T L D E C V Y K C Y D Y R K
M S T T K N V D G C N K S I K E E E M T E A I R V V S R G D S
P D L I K D N R C R E P N E L S L K N G S A R V K P A Y K F N
K D T N E C V A M M D K V C L G R N R F K T K E E C V H V C N
W N L S S G R H R H I V R E S N A K Q G G C H H H H H H **Stop**

Cul o 2P (JX512274)

483 bp

ATGACCACCTTCAAAACCTTTATCGTGCTGCTGAGCATTGCGCTGAGCAGCCTGGTTGACG
CGGGTACCCCGGGTACCACCCGTTGCGAGAAGAAAAACAAGCTGAGCAAAGAAACCATCGA
TACCTACCACAGCTGGCGTATGCCGACCAGCTTCCGTACCAAGGCGGAGAGCTGCCACCTG
CACTGCGTGCTGGAAAAAATCGGTTGGATGAAGGGCCACAAAATTCTGGACAAGGAGATCA
ACAGCGATATTAAGGCGAGCAAAGAAAGCCCGGAAGCGCGTAGCAGCCACCTGCGTACCCT
GCTGTTCGAGGACTGCAACATTCGTGAAAACAACAAGAAAGACAAATGCAAGAAAGCGCGT
GATGTGTATAAGTGCCTGGTTGAGAAATTTGAACCGCGTAGCACCTTCCAGAAGGCGTTTG
AGCGTGCGGAAAAGCGTAGCGAAAAAGGTGGCTGCCACCACCACCACCACCAC**TAA**

160 a.a.

M T T F K T F I V L L S I A L S S L V D A G T P G T T R C E K
K N K L S K E T I D T Y H S W R M P T S F R T K A E S C H L H
C V L E K I G W M K G H K I L D K E I N S D I K A S K E S P E
A R S S H L R T L L F E D C N I R E N N K K D K C K K A R D V
Y K C L V E K F E P R S T F Q K A F E R A E K R S E K G G C H
H H H H H **Stop**

Cul o 3 (KC339673)

813 bp

ATGTTCCGTATCTGCCTGTTTACCGTGCTGTGCGTTAACTTCGTGGTTGCGACCGACTTTT
GCGATCGTAAGCTGTGCCGTCGTCAGATTGAGCCGAACGTGTACCAAAACATCCCGCACAT
TGGCTGCAACCATGATGGTCGTAACAGCCCGGCGTGCCCGAGCGATGCGAAAATCCTGCCG
ATGAGCACCAAGCGTAAAAACCTGATTCTGCGTGTGCACAACCGTCTGCGTAACAAAGTTG
CGCTGGGTCAGCTGCCGGGTTACCCGAAAGCGGTGCGTATGCCGATCCTGCGTTGGGACGA
TGAGCTGGCGTATCTGGCGGAACTGAACGTTAAGCAGTGCGAAATGAAACACGACCAATGC
CGTAACACCGATAAGTTCCGTTACGCGGGCCAAAACCTGGCGTATATCGGTGGCGGTAAAG
AGCCGAACGCGGTGCGTATTAAAACCCTGGTTCGTGCGTGGTTCGACGAATACAAAGATGC
GAACAGCAGCTTTATCGACAAGTATCGTAGCCACCCGAACGGCAAAGCGATTGGTCACTTC
ACCGCGATGGTGCAGGACCGTACCGATACCGTTGGCTGCGCGATTCTGCGTCACACCAAGA
ACACCTACTTCTTTCTGGCGTGCAACTATAGCTTTACCAACATGGTGAAGGATAAAGTTTA
TACCCGTGGTGCGAAGAGCTGCAGCAAATGCCGTACCGGCTGCAGCCCGGTGTACAAGGGT
CTGTGCAAACCGCACGAGTATGTTAACCCGGACCCGGATGAAGACCTGGATGGCGGTTGCC
ACCACCACCACCACCAC**TAA**

270 a.a.

M F R I C L F T V L C V N F V V A T D F C D R K L C R R Q I E
P N V Y Q N I P H I G C N H D G R N S P A C P S D A K I L P M
S T K R K N L I L R V H N R L R N K V A L G Q L P G Y P K A V
R M P I L R W D D E L A Y L A E L N V K Q C E M K H D Q C R N
T D K F R Y A G Q N L A Y I G G G K E P N A V R I K T L V R A
W F D E Y K D A N S S F I D K Y R S H P N G K A I G H F T A M
V Q D R T D T V G C A I L R H T K N T Y F F L A C N Y S F T N
M V K D K V Y T R G A K S C S K C R T G C S P V Y K G L C K P
H E Y V N P D P D E D L D G G C H H H H H H **Stop**

Cul n 4 (HM145952)

486 bp

ATGAAGTTTCCGACCTTCCTGATCTTAGCTTTTTTCCTGAGTCTTTACATCTCTTCCACTG
CCTCCCGTCGCAAACATTTTCGCCACTTAAAACGCATTGAAGCTGCAAATGATTGCCCTGC
GAAGAACAGCGGAACGTATCAGAAGGTGTGCAAACAGTTGCAAAAATACTACGTCCTGACT
CCCGACGACAAGTTAGGTTCTTACTTAAAAGGAGGATTGCAGGAGGCAGCAAATCGTGTAC
TTACACCAGTTTCCAAATCCGATAAGATCACATTCGATATCGTTCAAAACTGCCTGAAGAA
TTTCCAGGTGATGGTCAACAAACATAACAAAGAAGCACTGCGTAAATATCGTGAGTGCAAG
AAAGAGTGTTTTACAGAGGTAGGCAAGGAATTTTCATCCGCCTTAGACAAAACTGGTGTCC
AAATTGCAGAATGTCTTAATGAGTCGCTTGGTGGTTGTCATCATCATCACCATCAC**TAA**

161 a.a.

M K F P T F L I L A F F L S L Y I S S T A S R R K H F R H L K
R I E A A N D C P A K N S G T Y Q K V C K Q L Q K Y Y V L T P
D D K L G S Y L K G G L Q E A A N R V L T P V S K S D K I T F
D I V Q N C L K N F Q V M V N K H N K E A L R K Y R E C K K E
C F T E V G K E F S S A L D K T G V Q I A E C L N E S L G G C
H H H H H H **Stop**

Appendix II

Nucleotide and amino acid sequences for the codon optimized genes used in the Bac-to-Bac baculovirus expression system.

Start and stop codons are in **bold** and the secretion signal sequence of the proteins is marked as green.

Cul o 8 (MN123710)

507 bp

ATGAGCATCTTCATCATCACCTTCGGAATCTTCCTGGCTATCATCGGTGTGTCTTGCCAGC
GCTCCAGCTCTACCCTGTCATCCGTCTGCCGCGGTGGTTCCTCCCGCGGCACCTGCAACGC
TAACGTGAGCCGCTTCTACTACAACGAGCGTACTAACAACTGCCAGAAGTTCTCATGGTCC
GGCTGCGGAGGTAACGAGAACAACTTCGTGTACAAGGAAAGCTGCAAGTCTAGGTGCGTGC
AGAAGCCAAAGCAGAACCTGCGCGACCACCCTGAGCTGAAGAAGTGCTTCCTGAAGCCTGA
CGAAGGTATCGGCCGCGCTATGCACAAGAAGTACTACTACGACCGTGGCTCACGCCGTTGC
CAGGACTTCTACTACGGCGGAATGTACGGAAACGAGAACAGGTTCGACTCAATGGACGACT
GCTACGAAAAGTGCGCCTCCCGCATCAACCCTTACCTGAAGCTGGTCCCCAACAACAACAA
GATCAAGCAGCAGTCC**TAA**

168 aa

M S I F I I T F G I F L A I I G V S C Q R S S S T L S S V C R
G G S S R G T C N A N V S R F Y Y N E R T N N C Q K F S W S G
C G G N E N N F V Y K E S C K S R C V Q K P K Q N L R D H P E
L K K C F L K P D E G I G R A M H K K Y Y Y D R G S R R C Q D
F Y Y G G M Y G N E N R F D S M D D C Y E K C A S R I N P Y L
K L V P N N N K I K Q Q S **Stop**

Cul o 9 (MN123712)

474 bp

ATGAAGACCTTCACTCCTTTCCTGCTGCTGCTGGTCATCTACTTCGCTAACTTCTCCCTGG
CCAAGGAGATCTGCCTGGAAACCACCGGCAAGACCCGCGGCCAGCACGTGGGATGCTTCGA
GGACCACGAAAACCAGCGCATGTTCCGTGGCTTCCTGGCTCACTACAACCAGGCCAACTCT
ATCGAGGCTTGCGTGAACACTTGCCACTCAAGGCACTTCGTCTACGCTGGAGCCCAGAACG
GACACGGTTGCTACTGCGGTAACTCCCACCCTCTGGAGGACTACCACGCCAGAGTGAGCGA
CAAGAAGTGCGACAAGAAGTGCCCCGGTCACTCTACCGAAAACTGCGGTGGCTACGGTTTC
GTGTCAGTCCACGAGACTGGCATCGAACAGTTCCTGATCTCCGGCAAGGGAAAGAGGCTGG
AAGACTCCAACACCATCAGCGACATGAAGTGGAAGATCAGCGCT**TAA**

157 aa

M K T F T P F L L L L V I Y F A N F S L A K E I C L E T T G K
T R G Q H V G C F E D H E N Q R M F R G F L A H Y N Q A N S I
E A C V N T C H S R H F V Y A G A Q N G H G C Y C G N S H P L
E D Y H A R V S D K K C D K K C P G H S T E N C G G Y G F V S
V H E T G I E Q F L I S G K G K R L E D S N T I S D M K W K I
S A **Stop**

Cul o 10 (MN123711)

1908 bp

ATGAAGTTCTCCGGCTTCCTGGCTATCCTGCTGGTCACCGCCTTCCCACTGTGCGTGCACG

GATTCTCTCTGACCACTGTCGCTTCAGAGTTCTTCGACTTCGTGCTGTCCAAGGTCTTCGA

CGCCAGCTGGGACCCTCTGCAGGGTCTGTTCGTCAAGAAGGAAACCGCCTACGAAAAGATC

ACTAAGCAGCTGACCGAGGTCATCTCCAAGATCGACAAGCTGACTGAAGACTTCAAGAACT

CCATCAAGGACGTGAAGAACCACATCACTCTGGAGATCCGCACCTCCAAGATCCAGGACCT

GCAGGAGAAGATCGAATCTAAGTTCAAGTCAACCATGGAGTTCGTCAACTCCATCCACAGC

CTGGAGAACACCACTATCCAGGAAATCGTGATGGACCTGACTCAGGGTCCTGACAGCATCG

AGAACCTGATCACCCAGTTCCACGTGAAGGTCTTCGGCTCTGACACTATCTACCTGGAAAA

CGTCATCCAGGCTATCCGTGCCAACAAGCACTACTTCTCTACCAACACTGGAACCTCAGAG

GAAGTGGTCCTGTACAAGTTCTTCCTGACCATGCTGTCAGTGCAGTACAAGGCTATGATCC

TGCAGAAGATCAACTGCTACCTGAAGTTCAGGCTGTCCATGGGTATCTTCCAGACTGAGAT

CGCCATCTACGAAAAGCGCTTCAAGAACCGTGTCCAGGAGAGC

ATGGAACGCATGCGTAACGCTTTCCTGCTGTCTAACCGCGACCTGTACAAGCTGGACGCTA

AGAAGTACGAGCGCGGTGTCACTTTCGCCGAAGTGACCCGTTTCAACCAGATCTACTTCAC

CTCTGAGTCAAACTTCGAATCTTGCTGGTCTGTGTGCGAGGACTACGCCAAGCCACGCCAC

AACTTCGAGGAACGTTACAGCTACCCTCAGCAGCGCCAGTGCAAGGGAACTATCTCCAAGT

GCCGTAGCGAGCACATCAACGACTACCACGTGTGGGTCACCACTGGTAACGAAGACAAGCG

CTACCAGTACGTCGCTCGCCGTCTGTCTTACGACAACTTCAGGCCCGGATACAGATACGGT

CAGATGTCCCACGTCACCAACAACAGCAAGATCGTGGACTTCTCTGGCTGGACTAGGGGAT

TCGGTCACTGCGACGTGTGCAGATGCCAGTGCGACGAGCCCGACGACAAGGAATCCGTGAG

GAGCTTCGTCCTGACTCCACTGAGAAGCGACACCGACAAGAACATGGTCATCACCGGTGTC

CAGCTGGCTGTGAAGGGCAAGTCTTTCGTCCTGAAGATCCACCAGGCCCCACTGATGCCTC

TGGGCAAGGTGAACTCCAGCGTGTCAGTCAAGATCTCTAACCCTTCACCCAAGGACAAGAT

CTTCAAGCTGGAGCACAACAAGAGGAGAGTGTACATCGGCACCGCTAAGCTGGACTCCATG

AAGTTCCCAAACTACGTCGCTACTGGAGTGAAGTTCGCCGAGAAGAACGGTAACCTGGCTC

TGGAAGTCAGGTTCACTAAGTTCAACTACACCGCCGGCCTGCTGTACAAGGCTGACGACAA

GGCCAACACTATCACCCGCTTCAACAACCTGAGCAAGAACGTGATCCAGCGTGGCAAGTCC

GGCAACAGCCCTCTGCAGTACAGGGACAACGCTCCCTCTGCCGAAACCAAGAGAGGCCTGA

TCGAGTTCGGACCTTCAAACCTGCACGACGACGTGGGACAGTCCACCATCCCCTTCCTGGA

CCTGCAGGAGGTCCGTTCAACTGTGACCGCTCTGACTGGCATCGGACTGCAGTTCAGGGGC

AGAGAAAAGTCCGGTGGCTTCATCGCCCCCACTCTGGTCACCTACCCAATGTTCATCGGAA

ACGAGGTGCAGGAAACTAAGGCTCTGCTGCAG**TAA**

635 aa

M K F S G F L A I L L V T A F P L C V H G F S L T T V A S E F
F D F V L S K V F D A S W D P L Q G L F V K K E T A Y E K I T
K Q L T E V I S K I D K L T E D F K N S I K D V K N H I T L E
I R T S K I Q D L Q E K I E S K F K S T M E F V N S I H S L E
N T T I Q E I V M D L T Q G P D S I E N L I T Q F H V K V F G
S D T I Y L E N V I Q A I R A N K H Y F S T N T G T S E E V V
L Y K F F L T M L S V Q Y K A M I L Q K I N C Y L K F R L S M
G I F Q T E I A I Y E K R F K N R V Q E S M E R M R N A F L L
S N R D L Y K L D A K K Y E R G V T F A E V T R F N Q I Y F T
S E S N F E S C W S V C E D Y A K P R H N F E E R Y S Y P Q Q
R Q C K G T I S K C R S E H I N D Y H V W V T T G N E D K R Y
Q Y V A R R L S Y D N F R P G Y R Y G Q M S H V T N N S K I V
D F S G W T R G F G H C D V C R C Q C D E P D D K E S V R S F
V L T P L R S D T D K N M V I T G V Q L A V K G K S F V L K I
H Q A P L M P L G K V N S S V S V K I S N P S P K D K I F K L
E H N K R R V Y I G T A K L D S M K F P N Y V A T G V K F A E
K N G N L A L E V R F T K F N Y T A G L L Y K A D D K A N T I
T R F N N L S K N V I Q R G K S G N S P L Q Y R D N A P S A E
T K R G L I E F G P S N L H D D V G Q S T I P F L D L Q E V R
S T V T A L T G I G L Q F R G R E K S G G F I A P T L V T Y P
M F I G N E V Q E T K A L L Q **Stop**

Cul o 11 (MN123713)

1158 bp

ATGAAGAACCACGTGTACTGCATCGGTCTGCTGCTGGTGGGTGTGGGACTGGCTAACGGTC

TGGGCTTCGGACTGCCATCCCTGCCTAAGCTGCCTAACCCCCTGAACCCTAAGGCCAGCTA

CAACAGCCCCTCTCTGCCAGGTGGCCCTTCATTCTCCGTGAACGTCTCAGGAAACGCTGGT

TCCGTGGCCGAGGGTCTGATGCAGCCCAAGCTGCCTCAGTTCCCTGGCCTGCCACAGCTGG

GAGGTAACAGCGGCTCTAACGGAAACTCTGGTTCCAGCTGCGGTCTGCCACAGCTGCCTGG

CGTCCCCTCATTCCCAGGAGCTCCTAAGCTGCCCGGTTTCCCATCACTGCCAGGAATGCCC

TCCCTGCCAGGATTCCCTGGTTCCAACTCCTCCTCCAACTCCCTGTCCAACAGCTCTTCAT

TCGGTAACCTGCCCGGCGTCCCAAACTTCTTCGACCCCAACGCTCTGTCAAACCTGATGCC

AAACGCTTCCAACTTCGCCAACCCTAACAGCACTGTGGGAAACGCCCTGGACGGACTGCAG

AGCGCTGTCAAGAACGCCAACAACAGCGCTAACCAGGCCCTGTCTAACTTCCAGGACTTCA

GCAACAACCTGTCTGAGCAGATCAAGAACGCTTCCGACGCTGCCCAGTCTAGGGTGAAGGA

ACAGCTGCAGGGACTGTCCAGCGGTGTGAGATCTTGCGTCGAGGAAAACGGTAACCCTGGA

CAGGCTGGAGTCCAGGCTGCTCAGCAGTCAGCCATGCAGTGCGTGCAGAAGAAGGTCGAGG

AAGCTGTGTCAATCGTCTCTAACACTAGGTCTGACGTGCAGGCTGCTCAGCTGGGACTGGA

CTACGTCCGCGGTAACCTGAGCAACTGCCGTGTGAACGTCAGCATGTCTCTGGCCGAGCTG

CCAACTTACGACTCTTCATCCCCTTCTTGCGTGGCTGCCGCTCTGTTCTCAATCCAGCCTG

AAACCATCCTGCTGCCCATCAACCTGGCTACAAACGGTGCTCAAGCTGTGGCTCTGGTCCA

GGGACTGCAGGCTTACGCCATGAAGCGCGTGGCCAACATGATGGGAAAGGTCGCTAAGGCC

TCTCTGGAAAACACCCTGGCTATCGGCAAGGGCACTCCAAACAGGGCCATCAAGGGC**TAA**

385 aa

M K N H V Y C I G L L L V G V G L A N G L G F G L P S L P K L
P N P L N P K A S Y N S P S L P G G P S F S V N V S G N A G S
V A E G L M Q P K L P Q F P G L P Q L G G N S G S N G N S G S
S C G L P Q L P G V P S F P G A P K L P G F P S L P G M P S L
P G F P G S N S S S N S L S N S S S F G N L P G V P N F F D P
N A L S N L M P N A S N F A N P N S T V G N A L D G L Q S A V
K N A N N S A N Q A L S N F Q D F S N N L S E Q I K N A S D A
A Q S R V K E Q L Q G L S S G V R S C V E E N G N P G Q A G V
Q A A Q Q S A M Q C V Q K K V E E A V S I V S N T R S D V Q A
A Q L G L D Y V R G N L S N C R V N V S M S L A E L P T Y D S
S S P S C V A A A L F S I Q P E T I L L P I N L A T N G A Q A
V A L V Q G L Q A Y A M K R V A N M M G K V A K A S L E N T L
A I G K G T P N R A I K G **Stop**

Cul o 12 (MN123714)

930 bp

ATGTCCCTGAAGTTCGTGCAGACTCTGCTGATCCTGGCCCTGACCAACACTAGCCGCGCTC
AGCGTCCCTGGGAAAAGAACATCACTTCCGTCATCTGCAGCGAAACCATCGACTTCTCCAA
CCACAACGTGATCCACGTCGCCAAGGACGCTTTCCAGTCCTGCCTGAGCGTGAAGAAGATC
ATCCTGGACAACAACTACCTGAAGACCCTGCACGTGGACACTTTCAGGGGTCTGACCGAGC
TGGAATGGCTGTCTATCGACCGCAACCGTCTGGAAACTCTGCCTGAGGAAATCTTCAAGGA
CCTGACCAACCTGAAGTACCTGAACCTGTGGAACAACAAGCTGACTTCACTGGAACCTCTG
GTGTTCCGTGGCCTGCAGAAGCTGGAGAAGCTGACCATCGGAGTCAACGAGCTGAACGAGT
TCCCCGAGGGTCTGCTGAAGGACCTGTCCAGCCTGAGGACTTTCTACGCCAACTCCAACAA
CATCCAGAGACTGAGCGACACCACTTTCTCTGGACTGTCACAGCTGAAGGAAATCGGTATG
GAGGGTAACGGCATCGTGTACCTGAGCGCTGACCTGTTCATCGGTCTGGACTCTCTGAAGT
CTATCTCATTCTGGTCAAACGAAATCATGAACGTGGACGCTGCTGCTCTGGTGCGCCACGT
CCCAGGCCTGTGGGAGTTCTACCTGGTGAACTTCAGGATGAGATGCGACCGCTTCCGTAAG
GTCCAGAACATCTTCCGTGAAGCCAACGTGGAGGTGGACATCCTGATCCCTGAAGCTCGCC
GTAGGAGAGACACCGAGCACGGCCTGATCTGCCTGGAAAACGACCGCTTCGAGAAGATGTT
CAGGGAAAGAGTGGAGTCTGGACTGATCTCACAGGAGGAAGCCGACAAGATCATCGCTCTG
AACGACGAGGTC**TAA**AAGCTT

309 aa

M S L K F V Q T L L I L A L T N T S R A Q R P W E K N I T S V
I C S E T I D F S N H N V I H V A K D A F Q S C L S V K K I I
L D N N Y L K T L H V D T F R G L T E L E W L S I D R N R L E
T L P E E I F K D L T N L K Y L N L W N N K L T S L E P L V F
R G L Q K L E K L T I G V N E L N E F P E G L L K D L S S L R
T F Y A N S N N I Q R L S D T T F S G L S Q L K E I G M E G N
G I V Y L S A D L F I G L D S L K S I S F W S N E I M N V D A
A A L V R H V P G L W E F Y L V N F R M R C D R F R K V Q N I
F R E A N V E V D I L I P E A R R R R D T E H G L I C L E N D
R F E K M F R E R V E S G L I S Q E E A D K I I A L N D E V
Stop

Appendix III

Nucleotide and amino acid sequences for the codon optimized genes used for expression in transgenic barley.

The protein genes are codon optimized without the secretion signal sequence.

Cul o 2P (JX512274.1)

390 bp

GGCACCCCAGGCACCACCAGGTGCGAGAAGAAGAACAAGCTCTCCAAGGAGACCATCGACA
CCTACCACTCCTGGAGGATGCCCACCAGCTTCCGCACCAAGGCCGAGAGCTGCCACCTCCA
CTGCGTGCTGGAGAAGATCGGCTGGATGAAGGGCCACAAGATCCTCGACAAGGAGATCAAC
TCCGACATCAAGGCCTCCAAGGAGTCCCCAGAGGCTAGGTCCAGCCACCTGCGCACCCTCC
TGTTCGAGGACTGCAACATCAGGGAGAACAACAAGAAGGACAAGTGCAAGAAGGCCCGCGA
CGTCTACAAGTGCCTGGTCGAGAAGTTCGAGCCGCGCTCCACCTTCCAGAAGGCCTTCGAG
AGGGCCGAGAAGCGCAGCGAGAAG

130 aa

G T P G T T R C E K K N K L S K E T I D T Y H S W R M P T S F
R T K A E S C H L H C V L E K I G W M K G H K I L D K E I N S
D I K A S K E S P E A R S S H L R T L L F E D C N I R E N N K
K D K C K K A R D V Y K C L V E K F E P R S T F Q K A F E R A
E K R S E K

Cul o 2 (hya) (KC339672.1)

1095 bp

CAGCACCTCATCCAGGCCGAGCTGCCAGACAGCCCTCTCAACATCGTCAAGGAGTTCGACG
ACGACGGCCGCAACAACAACAACCAGAACGACTTCAACTTCTACTGGAACATCCCATCCTT
CATGTGCGCCCAGCACAACATCACCTTCACCGACATGACCTCCAGCTACAACATCGTCCAG
AACAAGGACGACAAGTGGAGGGGCGACAAGATCGTGATCCTCTACGACCCAGGCAAGTTCC
CAGCTCTCCTGGAGCATCAGGGCCAGCTCTACCGCAGGAACGGCGGCGTCCCACAAGAGGG
CAACCTGCAGGAGCACATCGACATCCTCGCCGAGCACATCAACAAGCTGATCCCTGACACC
CAGTTCTCCGGCATCGGCGTGATCGACTTCGAGAGCTGGCGCCCGATCTTCAGGCAGAACT
CCGGCGTGCTCCAGCCCTACAAGGACCTCAGCTACAAGCTGGTCCACCGCGACCACAAGCT
CTGGAACAGGAAGCGCGTGGAGATCGAGGCCGCCCGCCTGTTCGAGGCTGCTGGCAGGACC
TTCGTGGAGGAGACGATCAACGTCGCCAAGATCCTCAGGCCAAAGGCCAAGTGGGGCTACT
ACGGCTTCCCTTACTGCTTCAACATGAACGGCGGCGCCAACATGAACGAGGACTGCCCGGC
CAACGTGAAGGAAGAGAACGACCAGATCAAGTGGCTGTGGGACATCGTGGACGTGGTCCTG
CCCAGCGTCTACCTCAACAACAAGATCACCTCCGCTCAGCGCGTGCAGTTCGTCAGGGGCA
GGATGAGGGAGGGCTACAGGGTCGCCAAGATGTCCAAGAAGAGCCCAAAGCCGCCAGTGCT
GGCTTACCTGCGCTACGTCTACACCGACACCCTCAAGTTCCTGTCCAACGAGGACCTCAAG
CAGGCCATCAAGGTGTCCAAGGAGCAGAAGAGCAAGGGCATGATCTTCTGGGGCTCCAGCT
ACGACGTCAAGACCAAGGAGCAGTGCATCGACTTCAGGAAGTATGTGGACAACAACCTGGG
CCCTATCGTCCTGCTCGCTAACAATAAGTCCCCCAAGGTGCTGACCCCAAATCTCGCT

365 aa

Q H L I Q A E L P D S P L N I V K E F D D D G R N N N N Q N D
F N F Y W N I P S F M C A Q H N I T F T D M T S S Y N I V Q N
K D D K W R G D K I V I L Y D P G K F P A L L E H Q G Q L Y R
R N G G V P Q E G N L Q E H I D I L A E H I N K L I P D T Q F
S G I G V I D F E S W R P I F R Q N S G V L Q P Y K D L S Y K
L V H R D H K L W N R K R V E I E A A R L F E A A G R T F V E
E T I N V A K I L R P K A K W G Y Y G F P Y C F N M N G G A N
M N E D C P A N V K E E N D Q I K W L W D I V D V V L P S V Y
L N N K I T S A Q R V Q F V R G R M R E GY R V A K M S K K S
P K P P V L A Y L R Y V Y T D T L K F L S N E D L K Q A I K V
S K E Q K S K G M I F W G S S Y D V K T K E Q C I D F R K Y V
D N N L G P I V L L A N N K S P K V L T P N L A

Cul o 3 (KC339673.1)

732 bp

ACCGACTTCTGCGACAGGAAGCTGTGCAGGCGCCAGATCGAGCCAAACGTGTACCAGAACA
TCCCTCACATCGGCTGCAACCATGACGGCCGCAACTCCCCAGCTTGCCCTAGCGACGCCAA
GATCCTGCCGATGTCCACCAAGAGGAAGAACCTCATCCTGCGCGTGCACAACCGGCTGAGG
AACAAGGTGGCTCTGGGCCAGCTGCCAGGCTACCCTAAGGCCGTGAGGATGCCAATCCTGA
GGTGGGACGACGAGCTGGCTTACCTGGCTGAGCTCAACGTCAAGCAGTGCGAGATGAAGCA
CGACCAGTGCAGGAACACCGACAAGTTCCGCTACGCTGGCCAGAACCTGGCTTACATCGGC
GGCGGCAAGGAGCCAAACGCCGTGAGGATCAAGACCCTCGTCCGCGCCTGGTTCGACGAGT
ACAAGGACGCCAACTCCAGCTTCATCGACAAGTACAGGAGCCACCCTAACGGCAAGGCCAT
CGGCCACTTCACCGCGATGGTGCAGGACAGGACCGACACCGTGGGCTGCGCTATCCTGAGG
CACACCAAGAACACCTACTTCTTCCTCGCCTGCAACTACTCCTTCACCAACATGGTGAAGG
ACAAGGTCTACACCAGGGGCGCCAAGTCCTGCAGCAAGTGCAGGACCGGCTGCAGCCCAGT
CTACAAGGGCCTGTGCAAGCCCCACGAGTATGTGAACCCGGACCCCGACGAGGACCTCGAC

244 aa

T D F C D R K L C R R Q I E P N V Y Q N I P H I G C N H D G
R. N S P A C P S D A K I L P M S T K R K N L I L R V H N R L
R N K V A L G Q L P G Y P K A V R M P I L R W D D E L A Y L A
E L N V K Q C E M K H D Q C R N T D K F R Y A G Q N L A Y I G
G G K E P N A V R I K T L V R A W F D E Y K D A N SS F I D K
Y R S H P N G K A I G H F T A M V Q D R T D T V G C A I L R H
T K N T Y F F L A C N Y S F T N M V K D K V Y T R G A K S C S
K C R T G C S P V Y K G L C K P H E Y V N P D P D E D L D

Cul o 5 (KC339675.1)

483 bp

TTCGACCTCTCCGACGCTCTGCCAGGCCACATCACCGACGACATGACCACCACCGAGAAGC
CAACCAACGTGGCTGCTGCTACCGCTGACTTCTCCGACGACGACCTCCTGGCCGTCATCAA
CGAGTCCAAGAAGAAGGTGAAGTCCAGCGACGTCAAGCCGACCAACAAGCTCCTGACCAAC
GTGAAGAACAAGCTGGAGTCCGTGGACTTCAAGAAGATCGACCAGAAGGTGGCCAGCCTCC
TGATCCCAATCTACAAGAAGGCCTCCGAGGCCATCCTCAACTGGATCAGCATGACCGGCGC
TGCTATCGAGACCAGCCCATGCTTCGAGAAGATCGACAGGATCTTCACCAAGATCCTGCAG
GACACCTCCAAGTACTTCACCCCTGCCAGGGCCAACATCGCCCAGAAGAGCTACGTCGAGA
AGCTCACCAAGGCCCTGTCCACCATCAGGCAGTGCATCATCAGCAAGATCAAGAAG

161 aa

F D L S D A L P G H I T D D M T T T E K P T N V A A A T A D F
S D D D L L A V I N E S K K K V K S S D V K P T N K L L T N V
K N K L E S V D F K K I D Q K V A S L L I P I Y K K A S E A I
L N W I S M T G A A I E T S P C F E K I D R I F T K I L Q D T
S K Y F T P A R A N I A Q K S Y V E K L T K A L S T I R Q C I
I S K I K K

Cul n 8 (HM145956.1)

1749 bp

GCTCCAGAGGGCGCCAGGGAGAAGGACTGGTGGGAGACCGGCAACTTCTACCAGGTCTACC
CCCGCTCCTTCATGGACAGCGACGGCGACGGCGTGGGCGACCTCAAGGGCATCACCGCCAA
AGTGGGCTACCTGAAGGAGATCGGCATGGACGGCGTGTGGCTCTCCCCAATCTTCGACAGC
CCTATGGCCGACTTCGGCTACGACATCAGCAACTTCACCAAGGTCTTCCCGCAGTTCGGCG
ACCTCTCCAGCATCGACGAGCTGGTGGCCGAATGCAACAAGAAGGACATCAAGCTCATCCT
GGACTTCGTGCCCAACCACACCTCCGACCAGTGCGAGTGGTTCAAGAAGAGCATCAAGAGG
GACCCAGAGTACGACAACTACTACATCTGGCACCCTGGCAAGCCAAACCCAGACGGTGGCA
GGAACCTCCCGCCCACCAACTGGGTCTCCGCCTTCAGGTCCAGCGCTTGGGAGTGGAACGA
GGAGCGCGGCGAGTACTACCTCCACCAGTTCCTGGCCGAGCAGCCAGACCTGAATTACAGG
AACCCTGAGGTGGTCGAGACCATGAAGAACGTGCTCAGGTTCTGGCTGAGCAAGGGCATCA
ACGGCTTCCGCATCGACGCTGTGCCATACCTCTTCGAGGTGGCTCCAGACGCTAACGGCAA
CTGCCCAGACGAGATCGAGACCAACGCCTGCGACGACCCACTCTCCCAGTGCTACCTGTAC
CACGACTACACCCAGAACAGGCCTGAGACCTTCGAGATGGTCACCGAGTGGAGGGCCACCC
TGGAGGACTACAAGAACAAGAACGGCGGCCCAACCAGGGTGCTCATGGTGGAGGCCTACGC
CCCGCTCACCAAGGTCATCCAGATCTACGGCCAGAACGGCCAGCTCAACGGCGCTCAGATC
CCATTCAACTTCGAGATCCTCAACTTCCTGGGCGCCACCTCCAACGCCCGCAACTTCAAGG
ACATCATCGACGAGTACCTGAGCACCATCCCAGAGGGCGCTACCCCCAACTGGGTCCAGGG
CAACCACGACCAGCACAGGTCCGCCAGCCGCCTCGGCACCCAGAAGGCTGACGCCGTGAAC
ATGCTGCTGCAGGTGCTCCCTGGCGCTGCTGTGACCTACTACGGCGAGGAGCTCGCGATGG
AGGACGTCTTCATCCCTTGGTCCAGGACCGTGGACCCGCAGGCCTGCACCACCAACCCCAA
CATCTTCCATGCTAAGAGCAGGGACCCGGCTAGGACCCCCATGATCTGGAACACCCAGAGG
AACGCCGGCTTCTCCAACGCCAACTACACCTGGCTCCCAACCGGCCCTGACTACCGCAAGA
ACAACGTGGACGTCCAGAGGTCCCAGAGGGGCAGCCACCTCAACATCTTCAAGAAGCTGAC
CCAGTTCAGGAAGCAGGACATCCTGAAGTACGGCACCTACGACAGCTACCTCGCCAACGAC
GACGTGCTGGTCATCAAGAGGGAGATCAAGAACAACCGCACCCTCATCGCCGTCCTCAACC
TGGGCTTCACCGAGCAGGTGGTCAACCTCAACCTGAACGAGAGGGACTGGCAGGTGCCAGA
GCGCATGGAGGTGACCACCGCTTCCGTCAACGCTGGCATGTTCGAGCGCCAGCCTATCGTC
ACCAGCGAGGTCTACGTGGCTGCTGGCGTGGGCGTGGTCCTGGACTACCAAGAGGGCAGGC
AGGTGCCGGCCCCGAGGGGCGACGACCCGGGCCTCTACGAG

583 aa

A P E G A R E K D W W E T G N F Y Q V Y P R S F M D S D G D G
V G D L K G I T A K V G Y L K E I G M D G V W L S P I F D S P
M A D F G Y D I S N F T K V F P Q F G D L S S I D E L V A E C
N K K D I K L I L D F V P N H T S D Q C E W F K K S I K R D P
E Y D N Y Y I W H P G K P N P D G G R N L P P T N W V S A F R
S S A W E W N E E R G E Y Y L H Q F L A E Q P D L N Y R N P E
V V E T M K N V L R F W L S K G I N G F R I D A V P Y L F E V
A P D A N G N C P D E I E T N A C D D P L S Q C Y L Y H D Y T
Q N R P E T F E M V T E W R A T L E D Y K N K N G G P T R V L
M V E A Y A P L T K V I Q I Y G Q N G Q L N G A Q I P F N F E
I L N F L G A T S N A R N F K D I I D E Y L S T I P E G A T P
N W V Q G N H D Q H R S A S R L G T Q K A D A V N M L L Q V L
P G A A V T Y Y G E E L A M E D V F I P W S R T V D P Q A C T
T N P N I F H A K S R D P A R T P M I W N T Q R N A G F S N A
N Y T W L P T G P D Y R K N N V D V Q R S Q R G S H L N I F K
K L T Q F R K Q D I L K Y G T Y D S Y L A N D D V L V I K R E
I K N N R T L I A V L N L G F T E Q V V N L N L N E R D W Q V
P E R M E V T T A S V N A G M F E R Q P I V T S E V Y V A A G
V G V V L D Y Q E G R Q V P A P R G D D P G L Y E

Appendix IV

List of primers used in this study.

Cul o 8	**Nucleotide sequence**
Co145_153-172_Fw	5′- CGAGCGTACTAACAACTGCC -3′
Co145_375-394_Re	5′- TTCCGCCGTAGTAGAAGTCC -3′
Co145_1-20_Fw	5′- ATGAGCATCTTCATCATCAC -3′
Co145_484-504_Re	5′- GGACTGCTGCTTGATCTTGT -3′
Co145_BamHI_Fw	5′- GCGGATCCGC ATGAGCATCTTCATCATCAC -3′
Co145_SUMO_Fw	5′- CGCGTCTCGAGGTCAGCGCTCCAGCTCTACC-3′
Co145_SUMO_Re	5′- CGTCTAGATTAGGACTGCTGCTTGAT-3′
Co145_306-326_Fw	5′- CTGTTCTCAATCCAGCCTGA –3′
Co145_210-227_Re	5′- GCAGCTTTCCTTGTACACG-5′
Co145_58-77_Fw	5′- CAGCGCTCCAGCTCTACCCT -3′

Cul o 9	**Nucleotide sequence**
Co120_201-220_Fw	5′- CGTGAACACTTGCCACTCAA -3′
Co120_419-438_Re	5′- GTCTTCCAGCCTCTTTCCCT -3′
Co120_1-21_Fw	5′- ATGAAGACCTTCACTCCTTTC -3′
Co120_451-471_Re	5′- AGCGCTGATCTTCCACTTCA -3′
Co120_BamHI_Fw	5′- GCGGATCCGCATGAAGACCTTCACTCCTTTC -3′
Co120_SUMO_Fw	5′- CGCGTCTCGAGGTAAGGAGATCTGCCTGGAA-3′
Co120_SUMO_Re	5′- CGTCTAGATTAAGCGCTGATCTTCCA-3′
Co120_215-235_Re	5′- CTCCAGCGTAGACGAAGTGCC -3′
Co120_275-294_Fw	5′- CTCTGGAGGACTACCACGCC -3′
Co120_64-81_Fw	5′- AAGGAGATCTGCCTGGAAAC -3′

Cul o 10	**Nucleotide sequence**
Co180_1345-1364_Fw	5′- TCCAGCGTGTCAGTCAAGAT -3′
Co180_1501-1520_Re	5′- ACTTCCAGAGCCAGGTTACC -3′
Co180_1-20_Fw	5′- ATGAAGTTCTCCGGCTTCCT -3′
Co180_1885-1905_Re	5′- CTGCAGCAGAGCCTTAGTTT -3′
Co180_BamHI_Fw	5′- GCGGATCCGCATGAAGTTCTCCGGCTTCCT -3′
Co180_SUMO_Fw	5′- CGCGTCTCGAGGTTTCTCTCTGACCACTGTC-3′
Co180_SUMO_Re	5′- CGTCTAGATTACTGCAGCAGAGCCTT-3′
Co180_215-234_Re	5′- GTCTTCAGTCAGCTTGTCGA -3′

Cul o 10	Nucleotide sequence
Co180_1710-1729_Fw	5′- CCTGCACGACGACGTGGGAC -3′
Co180_63-73_Fw	5′- TTCTCTCTGACCACTGTCGC -3′
Co180_1_BamHI_FW	5′- GCGGATCCGC TTCTCTCTGACCACTGTCGC -3′
Co180_2_BamHI_Fw	5′- GCGGATCCGCATGGAACGCATGCGTAACGC -3′
Co180_1_SUMO_Re	5′- CGTCTAGATTAGCTCTCCTGGACACG-3′
Co180_2_SUMO_Fw	5′- CGCGTCTCGAGGTATGGAACGCATGCGTAAC-3′
Culo180b_715-734_Fw	5′- ATGGAACGCATGCGTAACGC -3′
Co180a_545-564_FW	5′- CAGAGGAAGTGGTCCTGTAC -3′
Co180b_714-735_Re	5′- GCGTAGTCCTCGCACACAGAC -3′
Co180b_1026-1046_Fw	5′- GTACGTCGCTCGCCGTCTGTC -3′

Cul o 11	Nucleotide sequence
Co167_795-814_Fw	5′- CGAGGAAGCTGTGTCAATCG -3′
Co167_956-975_Re	5′- GATTGAGAACAGAGCGGCAG – 3′
Co167_1-21_Fw	5′- ATGAAGAACCACGTGTACTG -3′
Co167_1135-1155_Re	5′- GCCCTTGATGGCCCTGTTTG -3′
Co167_BamHI_Fw	5′- GCGGATCCGCATGAAGAACCACGTGTACTG -3′
Co167_SUMO_Fw	5′- CGCGTCTCGAGGTCTGGGCTTCGGACTGCCA -3′
Co167_SUMO_Re	5′- CGTCTAGATTAGCCCTTGATGGCCCT -3′
Co167_212-232_Re	5′- GGCCAGGGAACTGAGGCAGC -3′
Co167_958-977_Fw	5′- CTGTTCTCAATCCAGCCTGA -3′
Co167_61-80_Fw	5′- CTGGGCTTCGGACTGCCATC -3′

Cul o 12	Nucleotide sequence
Co15_506-525_Fw	5′- TGAGCGACACCACTTTCTCT –′3
Co15_684-403_Re	5′- CCAGGTAGAACTCCCACAGG -3′
Co15_1-21_Fw	5′- ATGTCCCTGAAGTTCGTGCAG -3′
Co15_907-927_Re	5′- GACCTCGTCGTTCAGAGCGA -3′
Co15_BamHI_Fw	5′- GCGGATCCGCATGTCCCTGAAGTTCGTGCAG -3′
Co15_SUMO_Fw	5′- CGCGTCTCGAGGTCAGCGTCCCTGGGAAAAG-3′
Co15_SUMO_Re	5′- CGTCTAGATTAGACCTCGTCGTTCAG-3′
Co15_211-230_Re	5′- CTCAGGCAGGACTGGAAAGC -3′
Co15_732-751_Fw	5′- GGTCCAGAACATCTTCCGTG -3′

Appendix V

Amplification of allergen genes for cloning into pFastBac-1 and pI-secSUMOstar vectors.

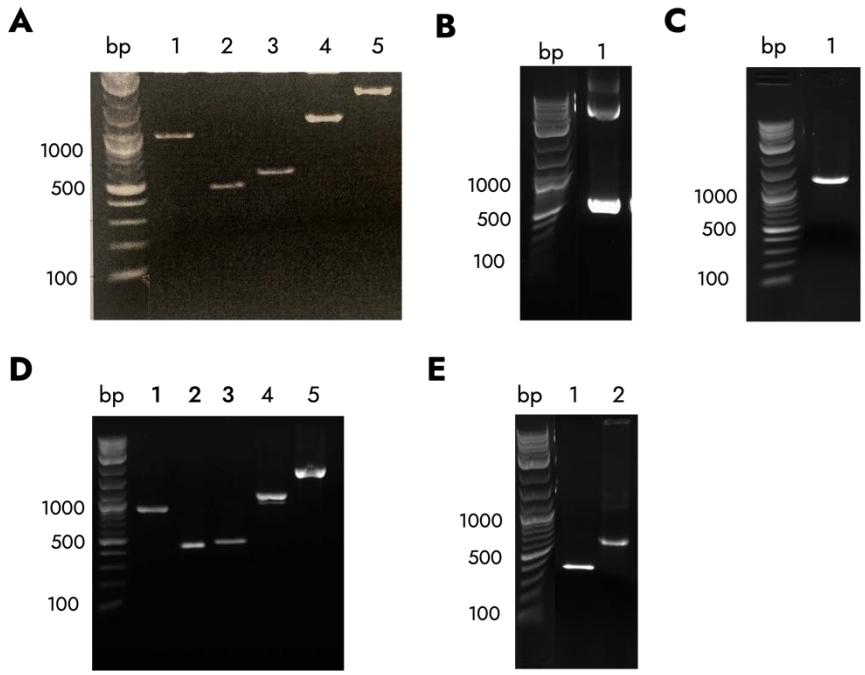


Figure 72. Amplification of allergen genes for cloning into. **A.** pFastBac-1 vector, 1. *Cul o 12*, 2. *Cul o 9*, 3. *Cul o 8*, 4. *Cul o 11*, 5. *Cul o 10*. **B.** pFastBac-1 vector, 1. *Cul o 10a*. **C.** pFastBac-1 vector, 1. *Cul o 10b*. **D.** pI-secSUMOstar vector, 1. *Cul o 12*, 2. *Cul o 9*, 3. *Cul o 8*, 4. *Cul o 11*, 5. *Cul o 10*. ***E.*** pI-secSUMOstar, 1. *Cul o 10a*, 2. *Cul o 10b*.

Appendix VI

Sequencing results of allergen genes for expression in *E. coli*

PET-42b - Cul n 4 (GGC):

ATGAAGTTTCCGACCTTCCTGATCTTAGCTTTTTTCCTGAGTCTTTACATCTCTTCCACTG
CCTCCCGTCGCAAACATTTTCGCCACTTAAAACGCATTGAAGCTGCAAATGATTGCCCTGC
GAAGAACAGCGGAACGTATCAGAAGGTGTGCAAACAGTTGCAAAAATACTACGTCCTGACT
CCCGACGACAAGTTAGGTTCTTACTTAAAAGGAGGATTGCAGGAGGCAGCAAATCGTGTAC
TTACACCAGTTTCCAAATCCGATAAGATCACATTCGATATCGTTCAAAACTGCCTGAAGAA
TTTCCAGGTGATGGTCAACAAACATAACAAAGAAGCACTGCGTAAATATCGTGAGTGCAAG
AAAGAGTGTTTTACAGAGGTAGGCAAGGAATTTTCATCCGCCTTAGACAAAACTGGTGTCC
AAATTGCAGAATGTCTTAATGAGTCGCTT

PET-42b - Cul o 1P (GGC):

ATGCGCTTCGCAACCATTTTTCTGTTGTCGGCATCAATCATCTTGCTGAGCACTGGGGAGG
CGTTGGCGAAGAAAAAGAAAAAAATCGACAAATCGATGCCTCCTGAATGCTTGCCCTTACC
GCCTAAAAAACGCGCGTCAGAGTGCACGAACCAGTCGGGGTTCAAATATTACCCGAAAACA
AATCAGTGCGGGCCTGTGAGTAACCAACTTTGTCAAGGACACGGAGGTTTCACTACGTTAG
ACGAATGCGTTTATAAATGCTACGATTACCGTAAAATGTCAACGACCAAAAATGTAGATGG
GTGTAATAAGTCTATTAAAGAAGAGGAGATGACCGAGGCGATTCGTGTGGTATCTCGCGGA
GACTCCCCAGATCTGATTAAAGACAATCGTTGCCGTGAACCAAATGAGTTGAGTTTGAAGA
ACGGGTCAGCCCGCGTCAAACCGGCATACAAGTTCAATAAAGACACAAATGAATGTGTGGC
GATGATGGACAAAGTTTGTTTGGGACGCAATCGCTTCAAGACGAAGGAAGAATGTGTCCAC
GTTTGTAATTGGAACCTGTCCAGTGGGCGTCATCGCCACATCGTCCGTGAAAGCAACGCGA
AGCAA

Appendix VII

Sequencing results of allergen genes for expression in insect cells

pFastBac1 Cul o 8:

ATGAGCATCTTCATCATCACCTTCGGAATCTTCCTGGCTATCATCGGTGTGTCTTGCCAGC
GCTCCAGCTCTACCCTGTCATCCGTCTGCCGCGGTGGTTCCTCCCGCGGCACCTGCAACGC
TAACGTGAGCCGCTTCTACTACAACGAGCGTACTAACAACTGCCAGAAGTTCTCATGGTCC
GGCTGCGGAGGTAACGAGAACAACTTCGTGTACAAGGAAAGCTGCAAGTCTAGGTGCGTGC
AGAAGCCAAAGCAGAACCTGCGCGACCACCCTGAGCTGAAGAAGTGCTTCCTGAAGCCTGA
CGAAGGTATCGGCCGCGCTATGCACAAGAAGTACTACTACGACCGTGGCTCACGCCGTTGC
CAGGACTTCTACTACGGCGGAATGTACGGAAACGAGAACAGGTTCGACTCAATGGACGACT
GCTACGAAAAGTGCGCCTCCCGCATCAACCCTTACCTGAAGCTGGTCCCCAACAACAACAA
GATCAAGCAGCAGTCCAAGGGCGAAAACTTGTACTTTCAAGGCCATCACCATCACCATCAC
TAG

pI-secSUMOstar Cul o 8:

CAGCGCTCCAGCTCTACCCTGTCATCCGTCTGCCGCGGTGGTTCCTCCCGCGGCACCTGCA
ACGCTAACGTGAGCCGCTTCTACTACAACGAGCGTACTAACAACTGCCAGAAGTTCTCATG
GTCCGGCTGCGGAGGTAACGAGAACAACTTCGTGTACAAGGAAAGCTGCAAGTCTAGGTGC
GTGCAGAAGCCAAAGCAGAACCTGCGCGACCACCCTGAGCTGAAGAAGTGCTTCCTGAAGC
CTGACGAAGGTATCGGCCGCGCTATGCACAAGAAGTACTACTACGACCGTGGCTCACGCCG
TTGCCAGGACTTCTACTACGGCGGAATGTACGGAAACGAGAACAGGTTCGACTCAATGGAC
GACTGCTACGAAAAGTGCGCCTCCCGCATCAACCCTTACCTGAAGCTGGTCCCCAACAACA
ACAAGATCAAGCAGCAGTCCTAA

pFastBac-HBM-TOPO Cul o 10a:

TTCTCTCTGACCACTGTCGCTTCAGAGTTCTTCGACTTCGTGCTGTCCAAGGTCTTCGACG
CCAGCTGGGACCCTCTGCAGGGTCTGTTCGTCAAGAAGGAAACCGCCTACGAAAAGATCAC
TAAGCAGCTGACCGAGGTCATCTCCAAGATCGACAAGCTGACTGAAGACTTCAAGAACTCC
ATCAAGGACGTGAAGAACCACATCACTCTGGAGATCCGCACCTCCAAGATCCAGGACCTGC
AGGAGAAGATCGAATCTAAGTTCAAGTCAACCATGGAGTTCGTCAACTCCATCCACAGCCT
GGAGAACACCACTATCCAGGAAATCGTGATGGACCTGACTCAGGGTCCTGACAGCATCGAG
AACCTGATCACCCAGTTCCACGTGAAGGTCTTCGGCTCTGACACTATCTACCTGGAAAACG
TCATCCAGGCTATCCGTGCCAACAAGCACTACTTCTCTACCAACACTGGAACCTCAGAGGA
AGTGGTCCTGTACAAGTTCTTCCTGACCATGCTGTCAGTGCAGTACAAGGCTATGATCCTG
CAGAAGATCAACTGCTACCTGAAGTTCAGGCTGTCCATGGGTATCTTCCAGACTGAGATCG
CCATCTACGAAAAGCGCTTCAAGAACCGTGTCCAGGAGAGC

pI-secSUMOstar Cul o 10a:

TTCTCTCTGACCACTGTCGCTTCAGAGTTCTTCGACTTCGTGCTGTCCAAGGTCTTCGACG
CCAGCTGGGACCCTCTGCAGGGTCTGTTCGTCAAGAAGGAAACCGCCTACGAAAAGATCAC
TAAGCAGCTGACCGAGGTCATCTCCAAGATCGACAAGCTGACTGAAGACTTCAAGAACTCC
ATCAAGGACGTGAAGAACCACATCACTCTGGAGATCCGCACCTCCAAGATCCAGGACCTGC
AGGAGAAGATCGAATCTAAGTTCAAGTCAACCATGGAGTTCGTCAACTCCATCCACAGCCT
GGAGAACACCACTATCCAGGAAATCGTGATGGACCTGACTCAGGGTCCTGACAGCATCGAG
AACCTGATCACCCAGTTCCACGTGAAGGTCTTCGGCTCTGACACTATCTACCTGGAAAACG
TCATCCAGGCTATCCGTGCCAACAAGCACTACTTCTCTACCAACACTGGAACCTCAGAGGA
AGTGGTCCTGTACAAGTTCTTCCTGACCATGCTGTCAGTGCAGTACAAGGCTATGATCCTG
CAGAAGATCAACTGCTACCTGAAGTTCAGGCTGTCCATGGGTATCTTCCAGACTGAGATCG
CCATCTACGAAAAGCGCTTCAAGAACCGTGTCCAGGAGAGCTAA

pFastBac1 Cul o 10b:

ATGGAACGCATGCGTAACGCTTTCCTGCTGTCTAACCGCGACCTGTACAAGCTGGACGCTA
AGAAGTACGAGCGCGGTGTCACTTTCGCCGAAGTGACCCGTTTCAACCAGATCTACTTCAC
CTCTGAGTCAAACTTCGAATCTTGCTGGTCTGTGTGCGAGGACTACGCCAAGCCACGCCAC
AACTTCGAGGAACGTTACAGCTACCCTCAGCAGCGCCAGTGCAAGGGAACTATCTCCAAGT
GCCGTAGCGAGCACATCAACGACTACCACGTGTGGGTCACCACTGGTAACGAAGACAAGCG
CTACCAGTACGTCGCTCGCCGTCTGTCTTACGACAACTTCAGGCCCGGATACAGATACGGT
CAGATGTCCCACGTCACCAACAACAGCAAGATCGTGGACTTCTCTGGCTGGACTAGGGGAT
TCGGTCACTGCGACGTGTGCAGATGCCAGTGCGACGAGCCCGACGACAAGGAATCCGTGAG
GAGCTTCGTCCTGACTCCACTGAGAAGCGACACCGACAAGAACATGGTCATCACCGGTGTC
CAGCTGGCTGTGAAGGGCAAGTCTTTCGTCCTGAAGATCCACCAGGCCCCACTGATGCCTC
TGGGCAAGGTGAACTCCAGCGTGTCAGTCAAGATCTCTAACCCTTCACCCAAGGACAAGAT
CTTCAAGCTGGAGCACAACAAGAGGAGAGTGTACATCGGCACCGCTAAGCTGGACTCCATG
AAGTTCCCAAACTACGTCGCTACTGGAGTGAAGTTCGCCGAGAAGAACGGTAACCTGGCTC
TGGAAGTCAGGTTCACTAAGTTCAACTACACCGCCGGCCTGCTGTACAAGGCTGACGACAA
GGCCAACACTATCACCCGCTTCAACAACCTGAGCAAGAACGTGATCCAGCGTGGCAAGTCC
GGCAACAGCCCTCTGCAGTACAGGGACAACGCTCCCTCTGCCGAAACCAAGAGAGGCCTGA
TCGAGTTCGGACCTTCAAACCTGCACGACGACGTGGGACAGTCCACCATCCCCTTCCTGGA
CCTGCAGGAGGTCCGTTCAACTGTGACCGCTCTGACTGGCATCGGACTGCAGTTCAGGGGC
AGAGAAAAGTCCGGTGGCTTCATCGCCCCCACTCTGGTCACCTACCCAATGTTCATCGGAA
ACGAGGTGCAGGAAACTAAGGCTCTGCTGCAG

pI-secSUMOstar Cul o 10b:

ATGGAACGCATGCGTAACGCTTTCCTGCTGTCTAACCGCGACCTGTACAAGCTGGACGCTA
AGAAGTACGAGCGCGGTGTCACTTTCGCCGAAGTGACCCGTTTCAACCAGATCTACTTCAC
CTCTGAGTCAAACTTCGAATCTTGCTGGTCTGTGTGCGAGGACTACGCCAAGCCACGCCAC
AACTTCGAGGAACGTTACAGCTACCCTCAGCAGCGCCAGTGCAAGGGAACTATCTCCAAGT
GCCGTAGCGAGCACATCAACGACTACCACGTGTGGGTCACCACTGGTAACGAAGACAAGCG
CTACCAGTACGTCGCTCGCCGTCTGTCTTACGACAACTTCAGGCCCGGATACAGATACGGT
CAGATGTCCCACGTCACCAACAACAGCAAGATCGTGGACTTCTCTGGCTGGACTAGGGGAT
TCGGTCACTGCGACGTGTGCAGATGCCAGTGCGACGAGCCCGACGACAAGGAATCCGTGAG
GAGCTTCGTCCTGACTCCACTGAGAAGCGACACCGACAAGAACATGGTCATCACCGGTGTC
CAGCTGGCTGTGAAGGGCAAGTCTTTCGTCCTGAAGATCCACCAGGCCCCACTGATGCCTC
TGGGCAAGGTGAACTCCAGCGTGTCAGTCAAGATCTCTAACCCTTCACCCAAGGACAAGAT
CTTCAAGCTGGAGCACAACAAGAGGAGAGTGTACATCGGCACCGCTAAGCTGGACTCCATG
AAGTTCCCAAACTACGTCGCTACTGGAGTGAAGTTCGCCGAGAAGAACGGTAACCTGGCTC
TGGAAGTCAGGTTCACTAAGTTCAACTACACCGCCGGCCTGCTGTACAAGGCTGACGACAA
GGCCAACACTATCACCCGCTTCAACAACCTGAGCAAGAACGTGATCCAGCGTGGCAAGTCC
GGCAACAGCCCTCTGCAGTACAGGGACAACGCTCCCTCTGCCGAAACCAAGAGAGGCCTGA
TCGAGTTCGGACCTTCAAACCTGCACGACGACGTGGGACAGTCCACCATCCCCTTCCTGGA
CCTGCAGGAGGTCCGTTCAACTGTGACCGCTCTGACTGGCATCGGACTGCAGTTCAGGGGC
AGAGAAAAGTCCGGTGGCTTCATCGCCCCCACTCTGGTCACCTACCCAATGTTCATCGGAA
ACGAGGTGCAGGAAACTAAGGCTCTGCTGCAGTAA

pFastBac1 Cul o 11:

ATGAAGAACCACGTGTACTGCATCGGTCTGCTGCTGGTGGGTGTGGGACTGGCTAACGGTC
TGGGCTTCGGACTGCCATCCCTGCCTAAGCTGCCTAACCCCCTGAACCCTAAGGCCAGCTA
CAACAGCCCCTCTCTGCCAGGTGGCCCTTCATTCTCCGTGAACGTCTCAGGAAACGCTGGT
TCCGTGGCCGAGGGTCTGATGCAGCCCAAGCTGCCTCAGTTCCCTGGCCTGCCACAGCTGG
GAGGTAACAGCGGCTCTAACGGAAACTCTGGTTCCAGCTGCGGTCTGCCACAGCTGCCTGG
CGTCCCCTCATTCCCAGGAGCTCCTAAGCTGCCCGGTTTCCCATCACTGCCAGGAATGCCC
TCCCTGCCAGGATTCCCTGGTTCCAACTCCTCCTCCAACTCCCTGTCCAACAGCTCTTCAT
TCGGTAACCTGCCCGGCGTCCCAAACTTCTTCGACCCCAACGCTCTGTCAAACCTGATGCC
AAACGCTTCCAACTTCGCCAACCCTAACAGCACTGTGGGAAACGCCCTGGACGGACTGCAG
AGCGCTGTCAAGAACGCCAACAACAGCGCTAACCAGGCCCTGTCTAACTTCCAGGACTTCA
GCAACAACCTGTCTGAGCAGATCAAGAACGCTTCCGACGCTGCCCAGTCTAGGGTGAAGGA
ACAGCTGCAGGGACTGTCCAGCGGTGTGAGATCTTGCGTCGAGGAAAACGGTAACCCTGGA
CAGGCTGGAGTCCAGGCTGCTCAGCAGTCAGCCATGCAGTGCGTGCAGAAGAAGGTCGAGG
AAGCTGTGTCAATCGTCTCTAACACTAGGTCTGACGTGCAGGCTGCTCAGCTGGGACTGGA
CTACGTCCGCGGTAACCTGAGCAACTGCCGTGTGAACGTCAGCATGTCTCTGGCCGAGCTG
CCAACTTACGACTCTTCATCCCCTTCTTGCGTGGCTGCCGCTCTGTTCTCAATCCAGCCTG
AAACCATCCTGCTGCCCATCAACCTGGCTACAAACGGTGCTCAAGCTGTGGCTCTGGTCCA
GGGACTGCAGGCTTACGCCATGAAGCGCGTGGCCAACATGATGGGAAAGGTCGCTAAGGCC
TCTCTGGAAAACACCCTGGCTATCGGCAAGGGCACTCCAAACAGGGCCATCAAGGGCAAGG
GCGAAAACTTGTACTTTCAAGGCCATCACCATCACCATCACTAG

pI-secSUMOstar Cul o 11:

CTGGGCTTCGGACTGCCATCCCTGCCTAAGCTGCCTAACCCCCTGAACCCTAAGGCCAGCT
ACAACAGCCCCTCTCTGCCAGGTGGCCCTTCATTCTCCGTGAACGTCTCAGGAAACGCTGG
TTCCGTGGCCGAGGGTCTGATGCAGCCCAAGCTGCCTCAGTTCCCTGGCCTGCCACAGCTG
GGAGGTAACAGCGGCTCTAACGGAAACTCTGGTTCCAGCTGCGGTCTGCCACAGCTGCCTG
GCGTCCCCTCATTCCCAGGAGCTCCTAAGCTGCCCGGTTTCCCATCACTGCCAGGAATGCC
CTCCCTGCCAGGATTCCCTGGTTCCAACTCCTCCTCCAACTCCCTGTCCAACAGCTCTTCA
TTCGGTAACCTGCCCGGCGTCCCAAACTTCTTCGACCCCAACGCTCTGTCAAACCTGATGC
CAAACGCTTCCAACTTCGCCAACCCTAACAGCACTGTGGGAAACGCCCTGGACGGACTGCA
GAGCGCTGTCAAGAACGCCAACAACAGCGCTAACCAGGCCCTGTCTAACTTCCAGGACTTC
AGCAACAACCTGTCTGAGCAGATCAAGAACGCTTCCGACGCTGCCCAGTCTAGGGTGAAGG
AACAGCTGCAGGGACTGTCCAGCGGTGTGAGATCTTGCGTCGAGGAAAACGGTAACCCTGG
ACAGGCTGGAGTCCAGGCTGCTCAGCAGTCAGCCATGCAGTGCGTGCAGAAGAAGGTCGAG
GAAGCTGTGTCAATCGTCTCTAACACTAGGTCTGACGTGCAGGCTGCTCAGCTGGGACTGG
ACTACGTCCGCGGTAACCTGAGCAACTGCCGTGTGAACGTCAGCATGTCTCTGGCCGAGCT
GCCAACTTACGACTCTTCATCCCCTTCTTGCGTGGCTGCCGCTCTGTTCTCAATCCAGCCT
GAAACCATCCTGCTGCCCATCAACCTGGCTACAAACGGTGCTCAAGCTGTGGCTCTGGTCC
AGGGACTGCAGGCTTACGCCATGAAGCGCGTGGCCAACATGATGGGAAAGGTCGCTAAGGC
CTCTCTGGAAAACACCCTGGCTATCGGCAAGGGCACTCCAAACAGGGCCATCAAGGGCTAA

Appendix VIII

Expression of r-allergens in Sf-9 insect cells and cloning of r-baculoviruses.

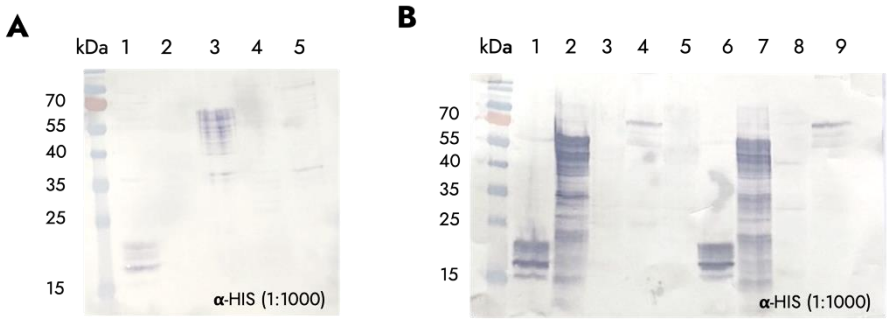


Figure 73. Transfection, 1P and 2P of Bac-1-Cul o 8, Bac-1-Cul o 11 and Bac-1-rCul o 10. WB showing **A.** Transfection, 1. Bac-1-rCul o 8, 2. cellfectin control, 3. Bac-1-rCul o 11, 4. cell control, 5. Bac-1-rCul o 10. **B.** 1 passage and 2 passage, 1. Bac-1-rCul o 8 1P, 2. Bac-1-rCul o 11 1P, 3. cellfectin control, 4. Bac-1-rCul o 10 1P, 5. cell control, 6. Bac-1-rCul o 8 2P, 7. Bac-1-rCul o 11 2P, 8. cell control, 9. Bac-1-rCul o 10 2P.

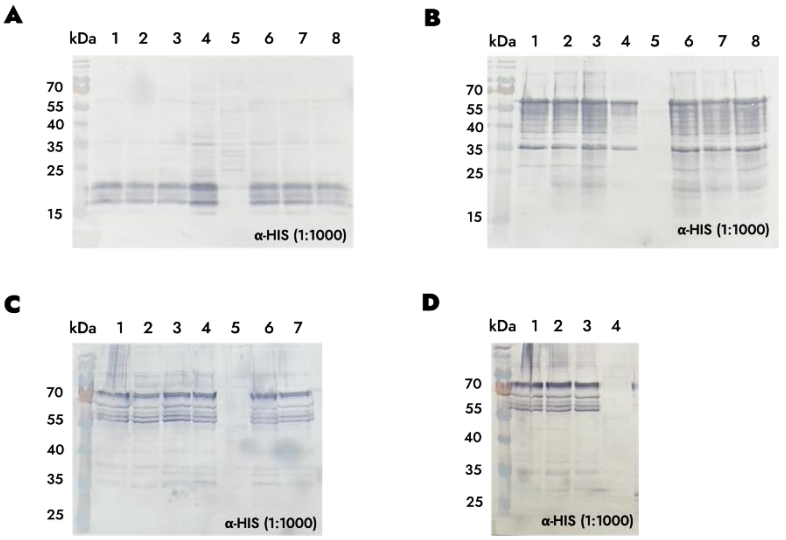


Figure 74 Cloning of Bac-1-rCul o 8, Bac-1-Cul o 11 and Bac-1-rCul o 10 r-baculoviruses. WB showing **A.** Bac-1-rCul o 8; 1. clone 1, 2. clone 2, 3. clone 3, 4. clone 4, 5. Cell control, 6. clone 5, 7. clone 6, 8. clone 7. **B.** Bac-1-Cul o 11; 1. clone 1, 2. clone 2, 3. clone 3, 4. clone 4, 5. cell control, 6. clone 5, 7. clone 6, 8. clone 7. **C.** Bac-1-rCul o 10; 1. clone 1, 2. clone 2, 3. clone 3, 4. clone 4, 5. Cell control, 6. clone 5, 7. clone 6. **D.** Bac-1-rCul o 10; 1. clone 7, 2. clone 8, 3. clone 9, 4. cell control.

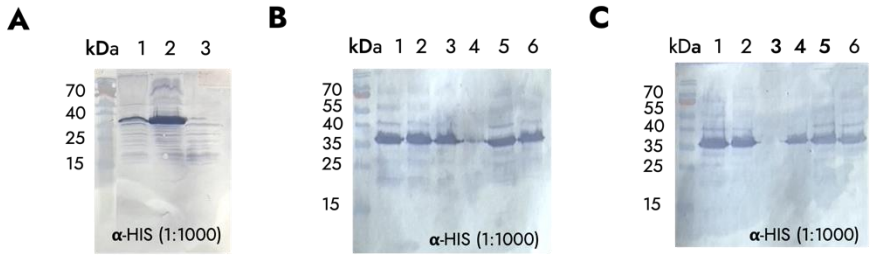


Figure 75. Transfection and cloning of Bac-SUMO-rCul o 9 r-baculoviruses. WB showing **A.** 1. Bac-SUMO-rCul o 9 transfection, 2. Bac-SUMO-rCul o 9 1P, 3. Cellfectin control 1P. **B.** 1. rBac-SUMO-rCul o 9 clone 1, 2. clone 2, 3. clone 3, 4. negative control, 5. clone 4, 6. clone 5. **C.** 1. clone 6, 2. clone 7, 3. Negative control, 4. clone 8, 5. clone 9, 6. clone 10.

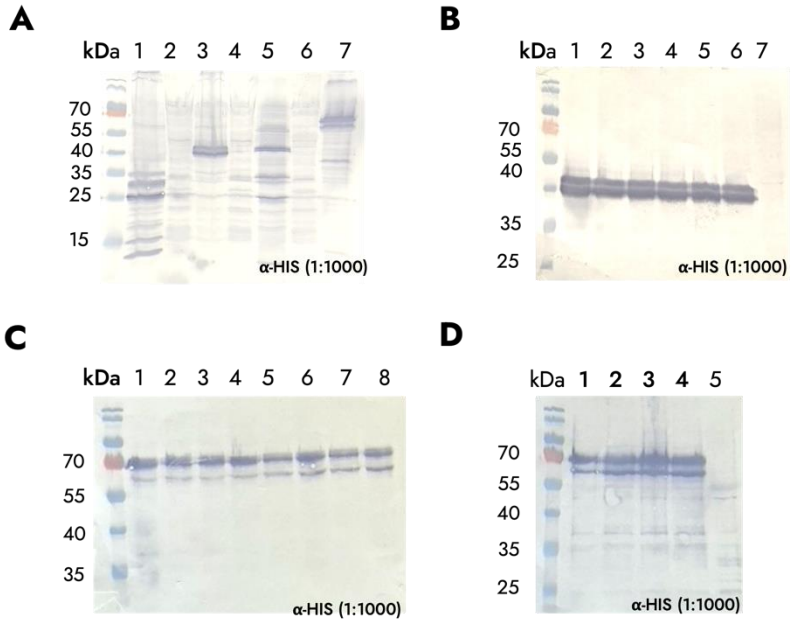


Figure 76. Transfection and clones of Bac-SUMO-rCul o 10a and Bac-SUMO-rCul o 10b. WB showing **A.** 1. Bac-HBM-rCul o 10a, 2. cellfectin control, 3. Bac-SUMO-rCul o 10a, 4. cell control, 5. Bac-1-rCul o 10b, 6. cell control, 7. Bac-SUMO-rCul o 10b. **B.** Bac-SUMO-rCul o 10a clones, 1. clone 1, 2. clone 2, 3. clone 3, 4. clone 4, 5. clone 5, 6. clone 6, 7. cell control. **C.** Bac-SUMO-rCul o 10b clones, 1. clone 1, 2. clone 2, 3. clone 3, 4. clone 4, 5. clone 5, 6. clone 6, 7. clone 7, 8. clone 8, 9. cell control. **D.** Bac-SUMO-rCul o 10b clones, 1. clone 9, 2. clone 10, 3. clone 11, 4. clone 12, 5. cell control.

Appendix IX

Purification of r-allergens from *E. coli* using HIS-select® HF nickel affinity gel.

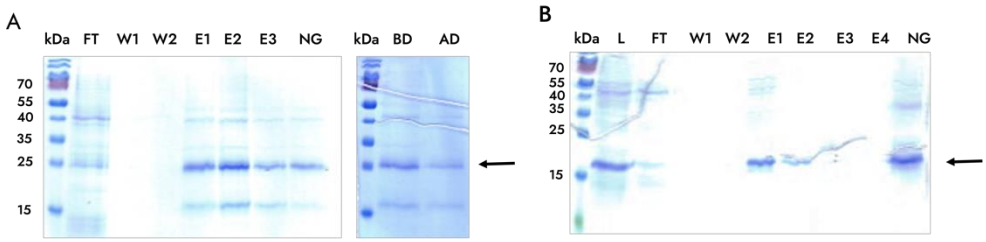


Figure 77. rCul o 1P (GGC) and rCul o 2P (GGC) protein purification from BL21 (DE3) *E.coli* cells. **A.** rCul o 1P (GGC); **B.** rCul o 2P (GGC); **L:** lysed cell pellet; **FT:** Flowthrough; **W 1-2:** Wash 1-2; **E 1-4:** Elution 1-4; **NG:** Nickel gel following purification, **BD:** before dialysis, **AD:** after dialysis in 2xPBS. The arrows indicate the representative proteins.

Appendix X

Purification of r-allergens from insect cells using HIS-select® HF nickel affinity gel.

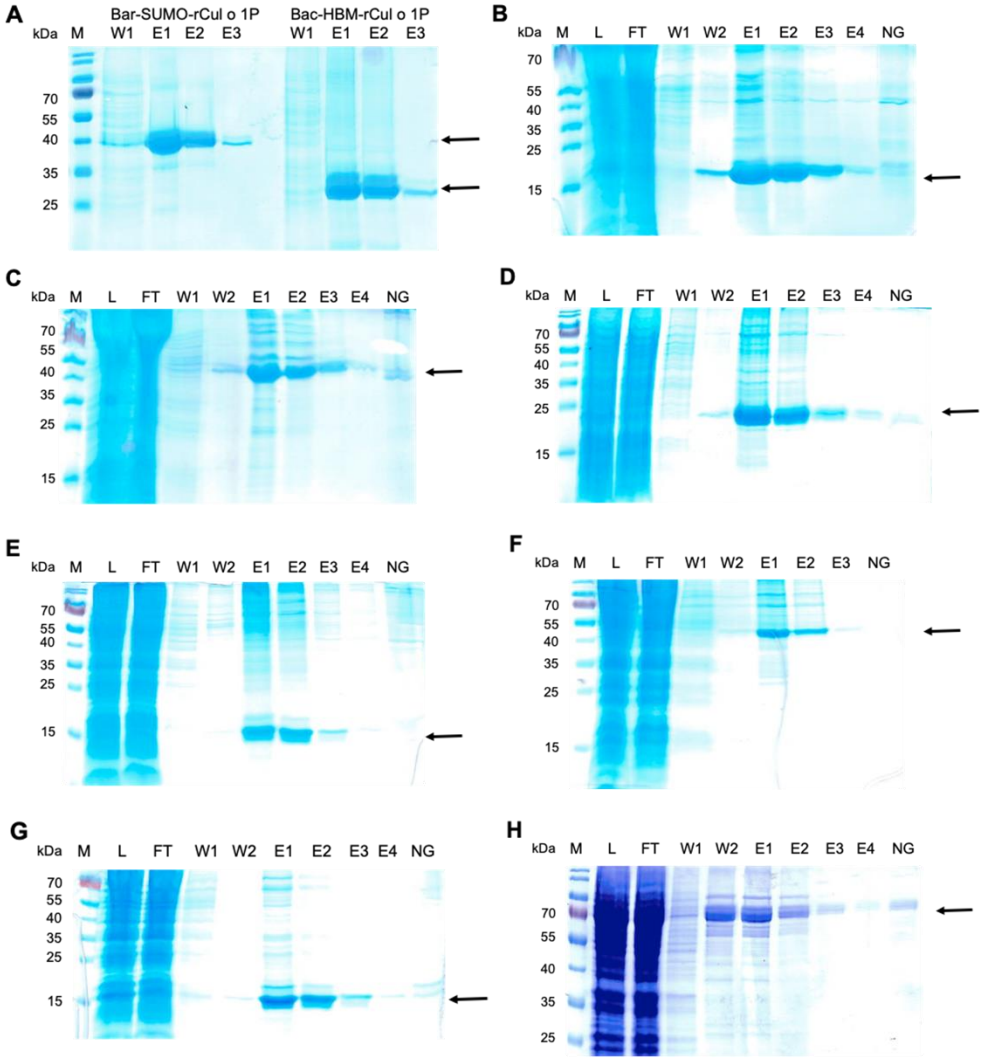


Figure 78. Bac-r-allergens protein purification using HIS-select® HF nickel affinity gel. **A.** Bac-SUMO-rCul o 1P and Bac-HBM-Cul o 1P, **B.** Bac-HBM-rCul o 2P, **C.** Bac-SUMO-rCul o 3, **D.** Bac-HBM-rCul o 5, **E.** Bac-1-rCul o 7, **F.** Bac-rCul n 3, **G.** Bac-1-rCul n 4, **H.** Bac-rCul n 8. **L:** lysed cell pellet; **FT:** Flowthrough; **W 1-2:** Wash 1-2; **E 1-4:** Elution 1-4; **NG:** Nickel gel following purification. The arrows indicate the representative r-proteins.

Appendix XI

T_1 screening of barley-r-allergen seed lines.

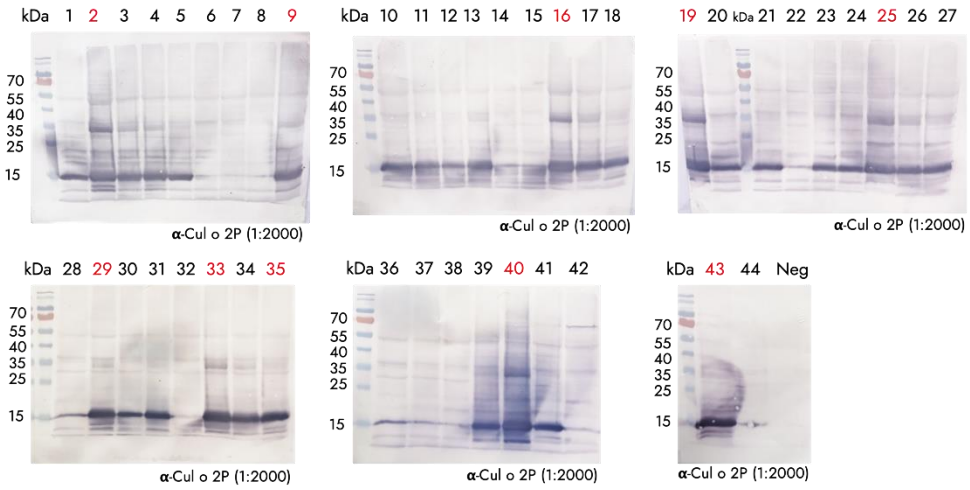


Figure 79. T_1 screening of barley-rCul o 2P. WB showing screening with extraction of T_1 barley-rCul o 2P seeds; numer represent the number of seed line (i.e. 1 seed line #1), Neg represents negative control, untransgenic barley. Red numbers represesent seed lines sowed for T_2 generation.

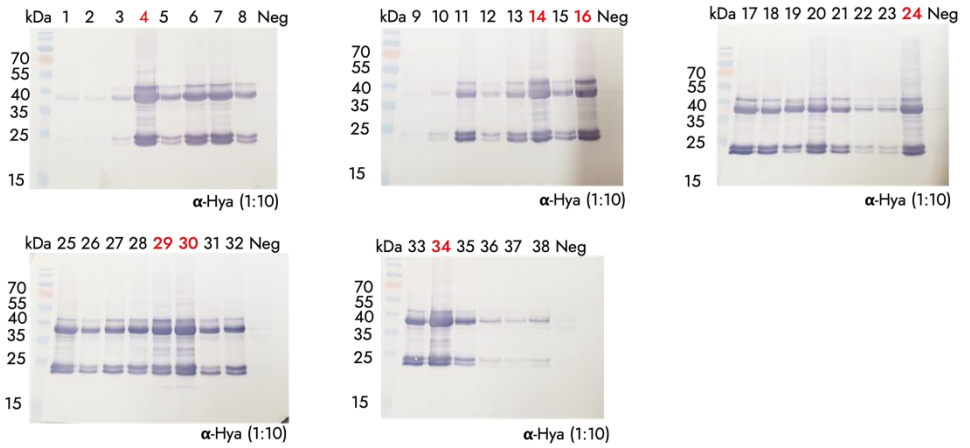


Figure 80. T_1 screening of barley-rCul o 2 (hya). WB showing screening with extraction of T_1 barley-rCul o 2 (hya) seeds; numer represent the number of seed line (i.e. 1 seed line #1), Neg represents negative control, untransgenic barley. Red numbers represesent seed lines sowed for T_2 generation.

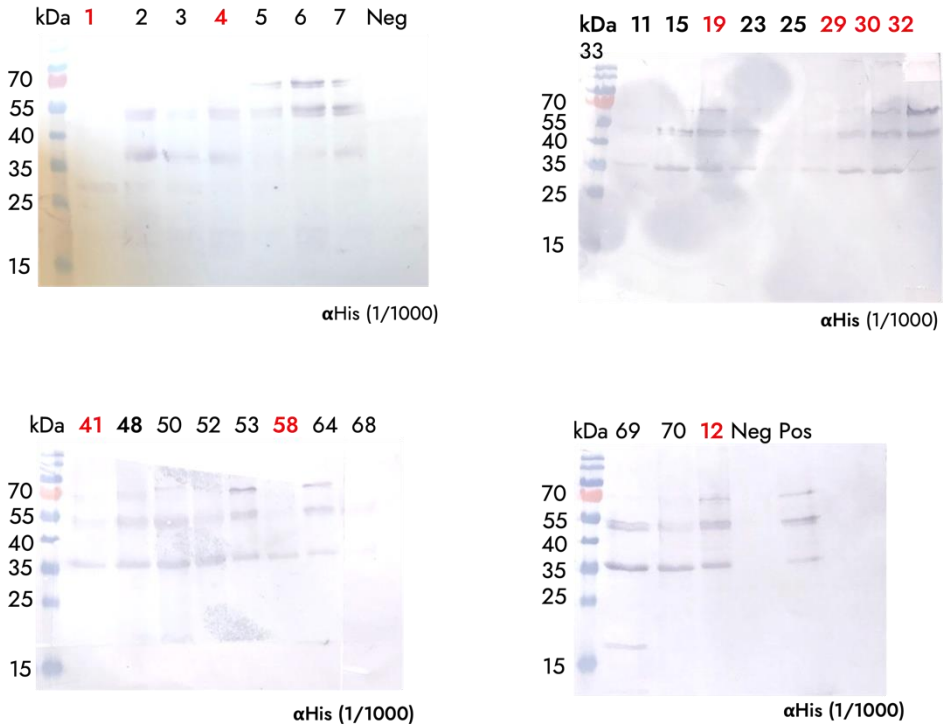


Figure 81. T_1 screening of barley-rCul o 3. WB with α-His showing screening with extraction of T_1 barley-rCul o 3 seeds; numer represent the number of seed line (i.e. 1 seed line #1), Neg represents negative control, untransgenic barley. Red numbers represesent seed lines sowed for T_2 generation.

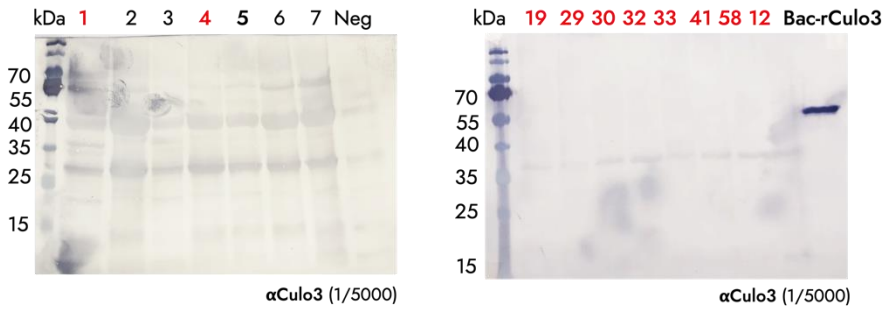


Figure 82. T_1 screening of barley-rCul o 3. WB with α-Culo3 showing screening with extraction of T_1 barley-rCul o 3 seeds; numer represent the number of seed line (i.e. 1 seed line #1), Neg represents negative control, untransgenic barley. Red numbers represesent seed lines sowed for T_2 generation.

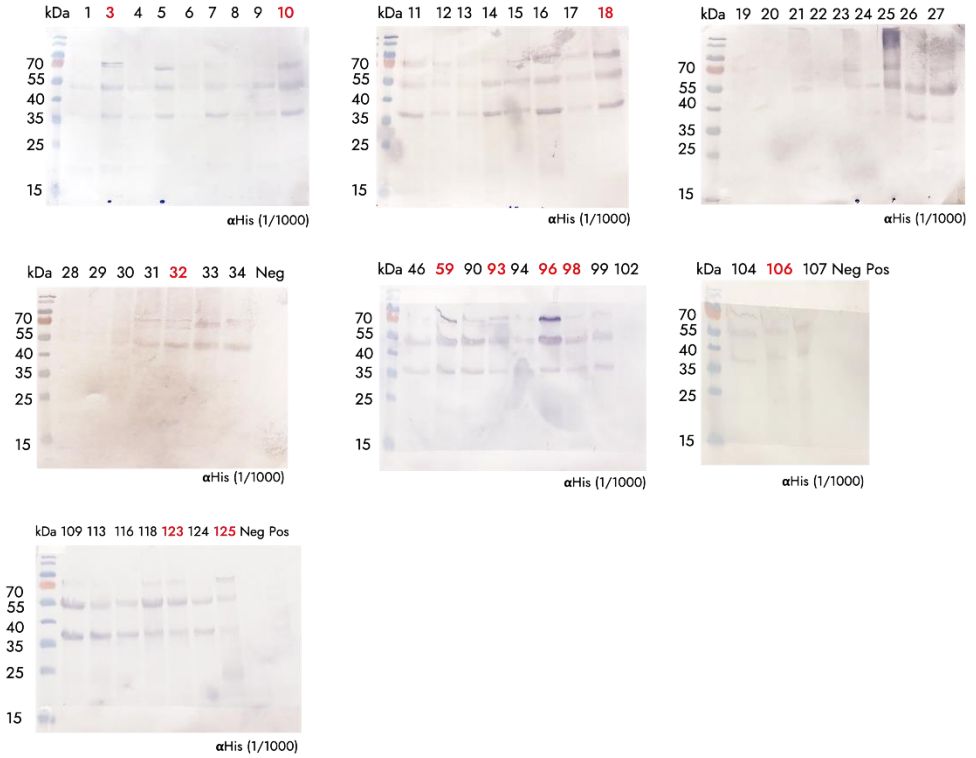


Figure 83. T_1 screening of barley-rCul n 8. WB with α-His showing screening with extraction of T_1 barley-rCul n 8 seeds; numer represent the number of seed line (i.e. 1 seed line #1), Neg represents negative control, untransgenic barley. Red numbers represesent seed lines sowed for T_2 generation.

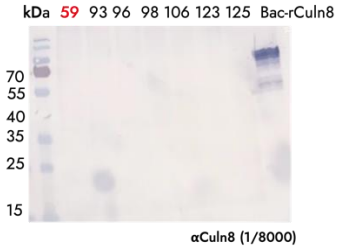


Figure 84. T_1 screening of barley-rCul n 8. WB with α-Cul n 8 showing screening with extraction of T_1 barley-rCul n 8 seeds; numer represent the number of seed line (i.e. 59 seed line #59). Red numbers represesent seed lines sowed for T_2 generation.

Appendix XII

T_2 screening of barley-r-allergen seed lines.

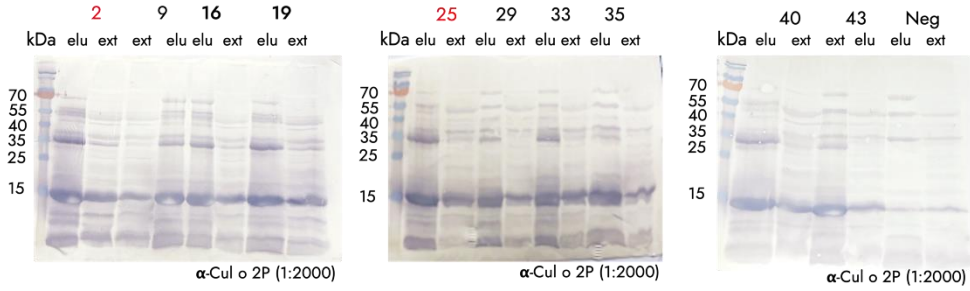


Figure 85. T_2 screening of barley-rCul o 2P. WB showing screening with extraction (ext) and elution from miniscale (elu) purification of T_2 barley-rCul o 2P seeds; numer represent the number of seed line (i.e. 2 seed line #2), Neg represents negative control, untransgenic barley. Red numbers represesent seed lines sowed for T_3 generation.

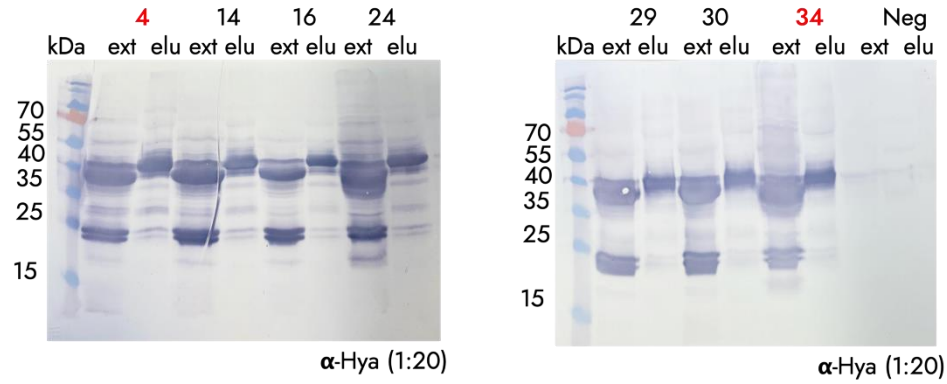


Figure 86. T_2 screening of barley-rCul o 2 (hya). WB showing screening with extraction (ext) and elution from miniscale (elu) purification of T_2 barley-rCul o 2 (hya) seeds; numer represent the number of seed line (i.e. 4 seed line #4), Neg represents negative control, untransgenic barley. Red numbers represesent seed lines sowed for T_3 generation.

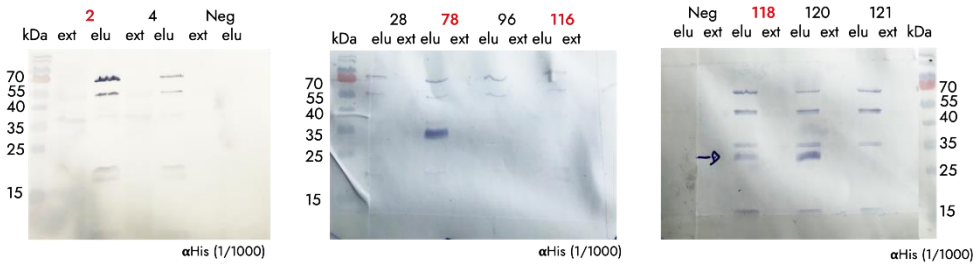


Figure 87. T_2 screening of barley-rCul o 3. WB with α-His showing screening with extraction (ext) and elution from miniscale (elu) purification of T_2 barley-rCul o 3 seeds; numer represent the number of seed line (i.e. 2 seed line #2), Neg represents negative control, untransgenic barley. Red numbers represesent seed lines sowed for T_3 generation.

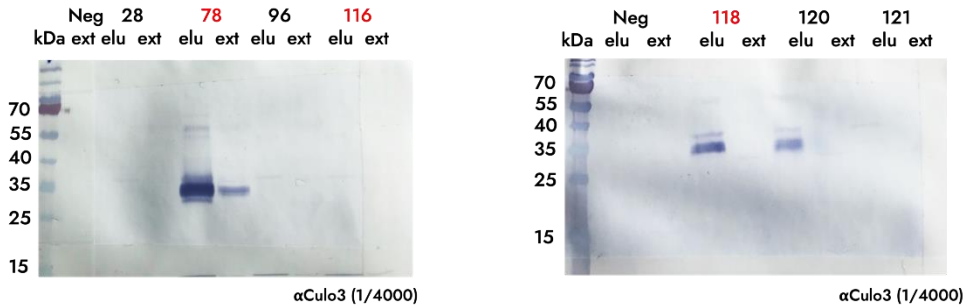


Figure 88. T_2 screening of barley-rCul o 3. WB with α-Cul o 3 showing screening with extraction (ext) and elution from miniscale (elu) purification of T_2 barley-rCul o 3 seeds; numer represent the number of seed line (i.e. 28 seed line #28), Neg represents negative control, untransgenic barley. Red numbers represesent seed lines sowed for T_3 generation.

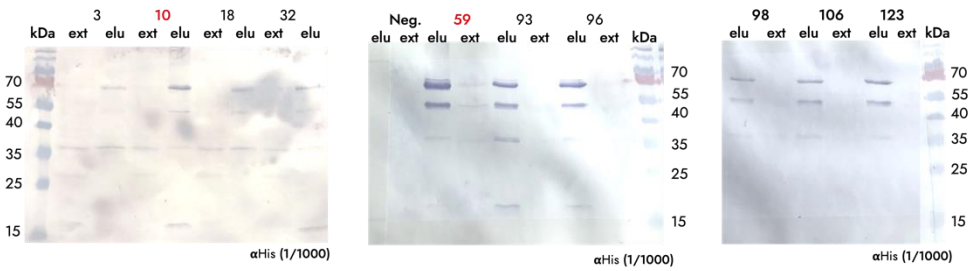


Figure 89. T_2 screening of barley-rCul n 8. WB with α-His showing screening with extraction (ext) and elution from miniscale (elu) purification of T_2 barley-rCul o 8 seeds; numer represent the number of seed line (i.e. 3 seed line #3), Neg represents negative control, untransgenic barley. Red numbers represesent seed lines sowed for T_3 generation.

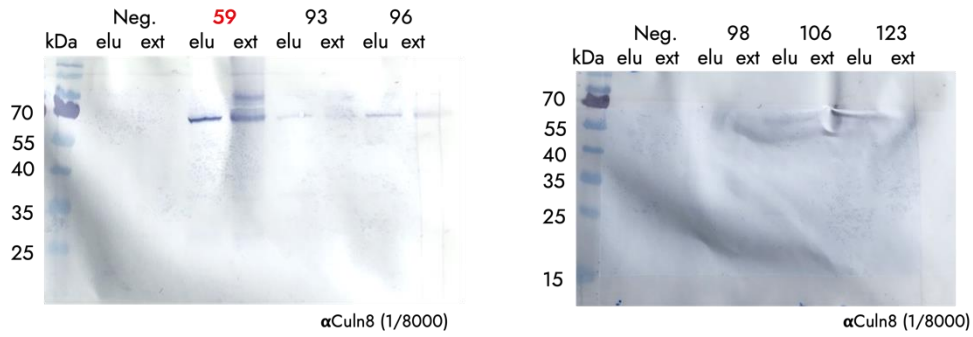


Figure 90. T_2 screening of barley-rCul n 8. WB with α-Cul n 8 showing screening with extraction (ext) and elution from miniscale (elu) purification of T_2 barley-rCul n 8 seeds; numer represent the number of seed line (i.e. 59 seed line #59), Neg represents negative control, untransgenic barley. Red numbers represesent seed lines sowed for T_3 generation.

Appendix XIII

For determination of the optimal serum dilution for ELISA runs to compare IgG subclass response against r-allergens used in vaccination (Bac-rCul o 2P, Bac-rCul o 3 and Bac-rCul n 4) experiment comparing route of injection (i.l./MPLA and s.c./MPLA, section 4.2) the IgG subclass response against the r-allergens was tested in ELISA using different dilution of serum (1:50 – 1:6400) (Figure 91). Prior, different serum dilutions had been tested in total IgG ELISA against Bac-rCul n 4 (Kristjansdottir, 2019). Based on those results, two horses, one from each vaccination group, with medium IgG response with consideration of IgG response of the group, were used for serum titration for IgG subclass ELISA. 1:800 dilution of serum was decided to be optimal for ELISA comparison of IgG subclass response as based on the titration curve IgG1 response was at median value in 1:800 dilution, the IgG4/7 response was starting to decrease in 1:800 dilution and the IgG5 response was in good detectable range (Figure 91).

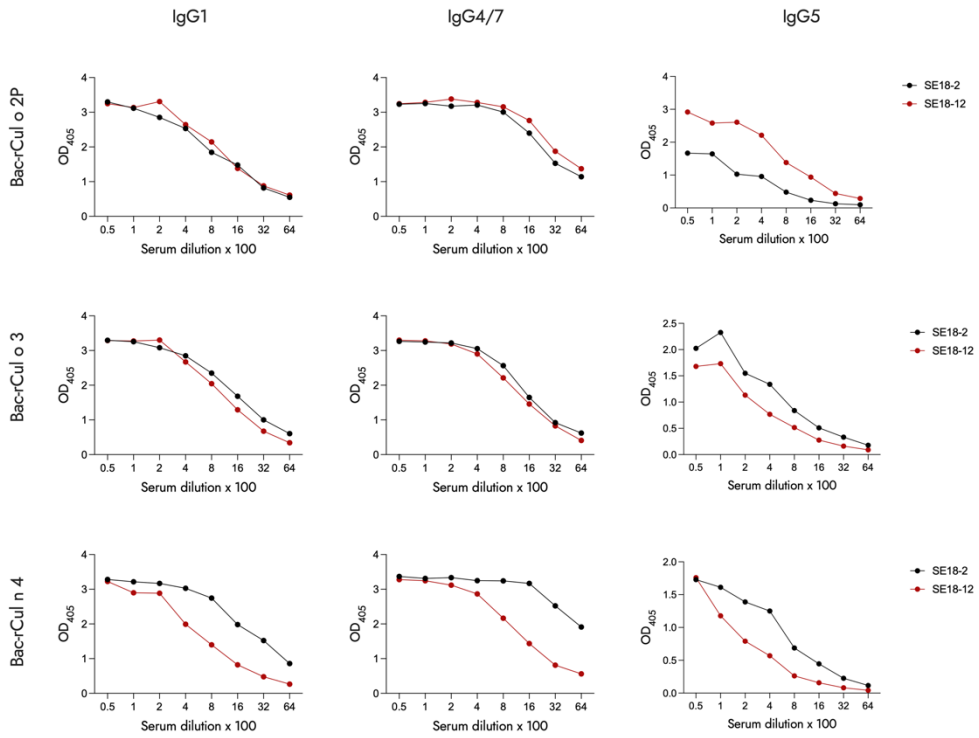


Figure 91. Testing of serum titration of i.l./MPLA and s.c./MPLA vaccination horses in IgG subclass ELISA against r-allergens. Allergen-specific IgG subclasses measured in ELISA against rBac-rCul o 2P, Bac-rCul o 3, Bac-rCul n 4 with different dilutions (1:50 – 1:6400) of serum from week 10 of two vaccinated horses, i.l./MPLA (SE18-2) and s.c./MPLA (SE18-12) to determine optimal serum dilution for ELISAs comparing the two vaccination groups.

Total IgG response of 26 challenge horses and IgG subclass response in 6 challenge horses (Gudnadottir, 2021) were tested in serum dilution against rBac- Cul o 8 (Figure 66). Based on these results and serum dilution of six horses tested in IgG4/7 subclass ELISA against Bac-rCul o 9 (Figure 92), the serum dilution of 1:800 was chosen to perform IgG subclass ELISA of challenge horses against Bac-rCul o 2P, Bac-rCul o 8 and Bac-rCul o 9.

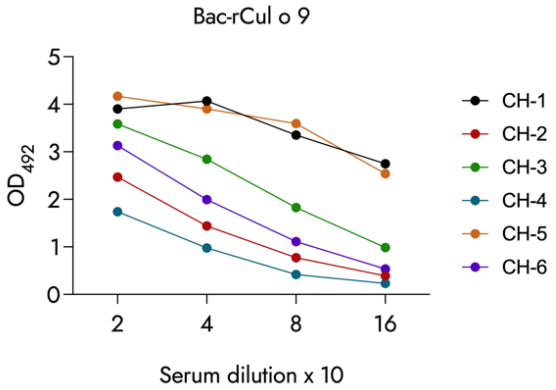


Figure 92. Testing of serum titration in IgG4/7 subclass ELISA against Bac-rCul o 9. Allergen-specific IgG4/7 subclasses measured in ELISA against rBac-rCul o 9 with different dilutions (1:200 – 1:1600) of serum from week 10 of six vaccinated horses in challenge (CH-1 – CH-6) experiment to determine optimal serum dilution for ELISA.

For setting up and optimizing IgE ELISA for challenge experiment, four horses shown to have IgE response against Bac-rCul o 8 were tested with serum titration (1:10 – 1:640) with three different dilutions of IgE antibody (1:2500, 1:4000, 1:5000) (Figure 93). Based on the results, serum dilution of 1:100 was decided to be used as the IgE of highest responder was decreasing between 1:80 and 1:160 dilution and the low responder still detectable. Antibody dilution of 1:4000 was decided as the performance was comparable to 1:2500 dilution (Figure 93).

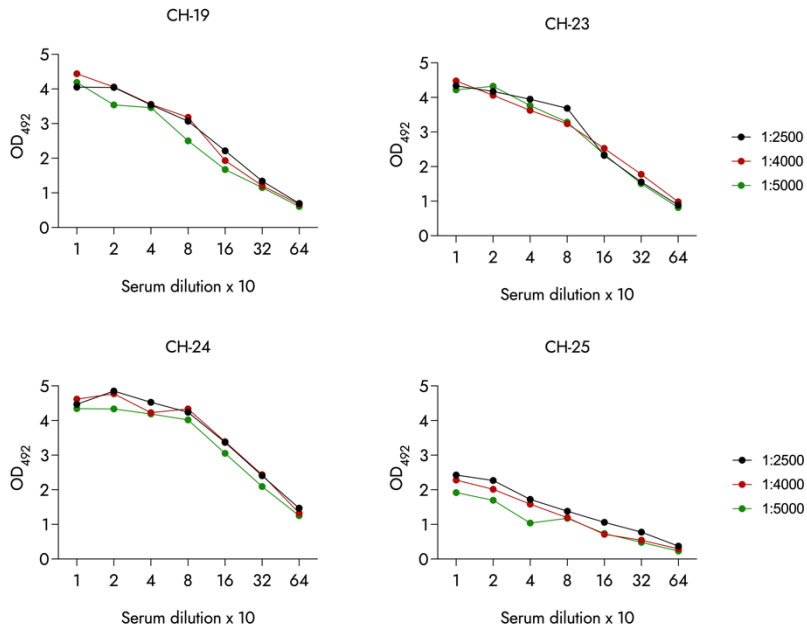


Figure 93. Testing of serum titration and IgE antibody dilution in ELISA against Bac-rCul o 8. Allergen-specific IgE measured in ELISA against rBac-rCul o 8 with different dilutions (1:10 – 1:640) of serum from September of first year following export of four vaccinated horses (CH-19, 23, 24, 25) in challenge experiment tested with three different dilution of IgE antibody (1:2500, 1:4000 and 1:5000) to determine optimal serum dilution and antibody dilution for IgE ELISA.

Appendix XIV

Example of gating strategy for FACS analysis

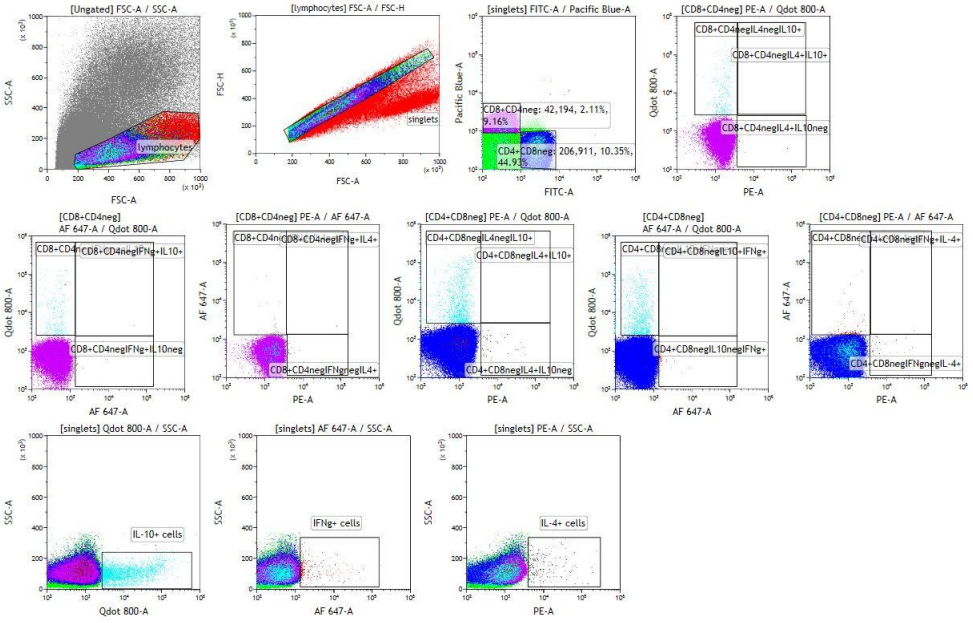


Figure 94. Example of gating strategy for FACS.

Appendix XV

Comparison of IgG subclass and IgE antibody response following vaccination and two years after export from Iceland to Switzerland on challenge horses based on IBH-status in the end of second summer following export.

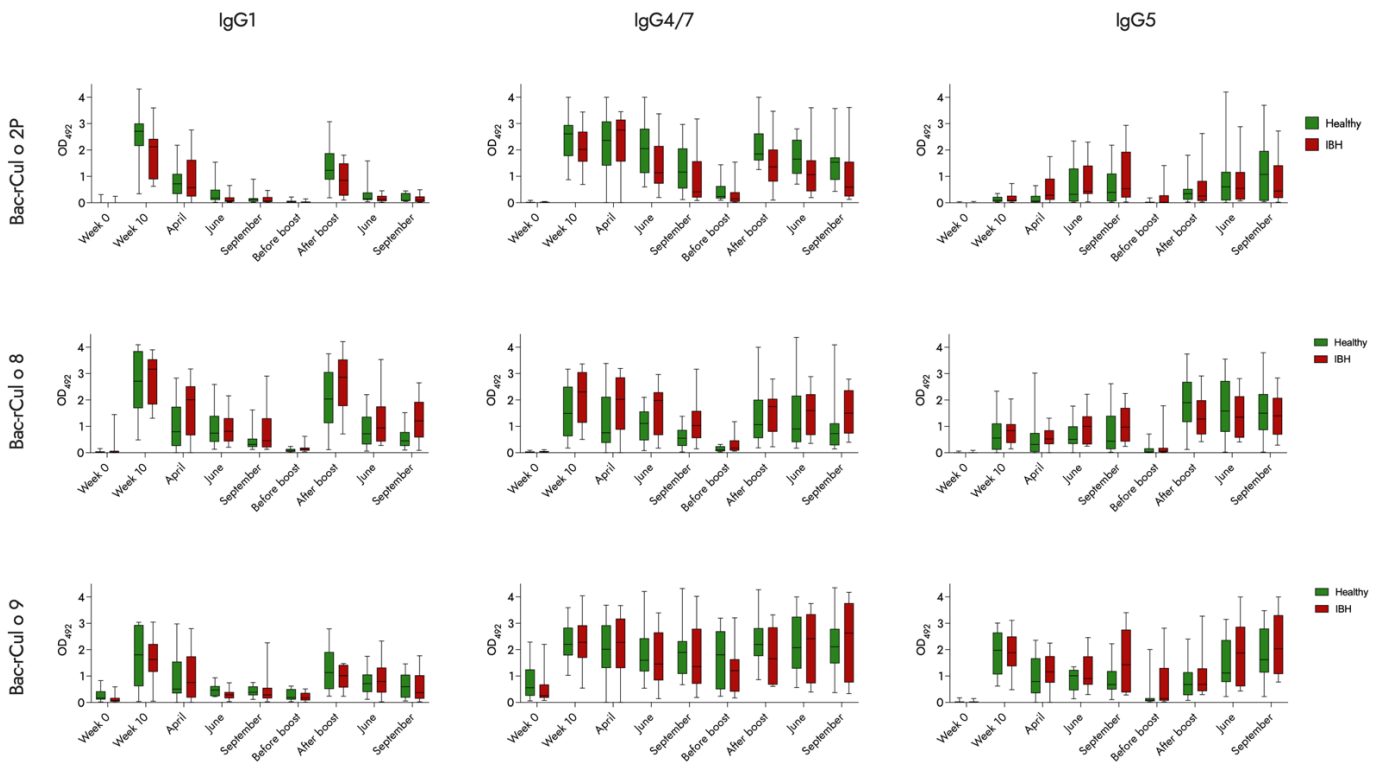


Figure 95. IgG subclass response of vaccinated horses in challenge experiment based on IBH-disease status two years after export. Allergen-specific IgG subclass response of vaccinated horses measured by ELISA against Bac-rCul o 2P, Bac-rCul o 8 and Bac-rCul 0 9. The graphs show corrected OD_{492} median values in box and whisker plot for the twenty six horses at different time points divided in healthy (green) and IBH-affected horses (red). Comparisons between vaccination groups were performed using Mann-Whitney U test with Bonferroni corrections.

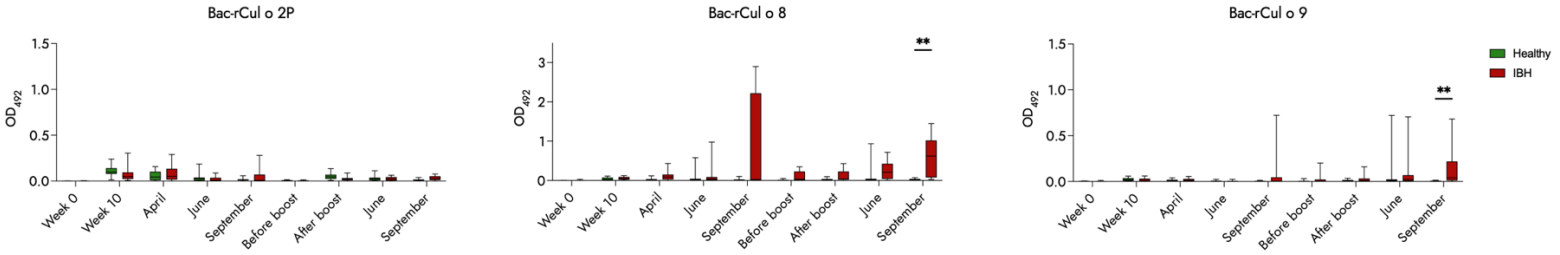


Figure 96. IgE response of vaccinated horses in challenge experiment based on IBH-disease status two years after export. Allergen-specific IgE response of vaccinated horses measured by ELISA against Bac-rCul o 2P, Bac-rCul o 8 and Bac-rCul 0 9. The graphs show corrected OD_{492} median values in box and whisker plot for the twenty six horses at different time points divided in healthy (green, n=13) and IBH-affected horses (red, n=13). Comparisons between outcome groups were performed using Mann-Whitney U test with Bonferroni corrections. An asterisk (*) indicates statistical difference between vaccination groups at a given time-point.