Veterinary Immunology and Immunopathology 201 (2018) 32–37

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

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

Barley produced *Culicoides* allergens are suitable for monitoring the immune response of horses immunized with *E. coli* expressed allergens

Sigridur Jonsdottir[a,d,*], Sara Bjork Stefansdottir[a], Sæmundur Bjarni Kristinarson[a], Vilhjalmur Svansson[a], Jon Mar Bjornsson[b], Arna Runarsdottir[b], Bettina Wagner[c], Eliane Marti[d], Sigurbjorg Torsteinsdottir[a]

[a] Institute for Experimental Pathology, Biomedical Center, University of Iceland, Keldur, Keldnavegur 3, 112 Reykjavik, Iceland
[b] ORF Genetics Ltd., Vikurhvarfi 7, 203 Kopavogur, Iceland
[c] Departments of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, 14853, USA
[d] 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
Culicoides allergens
Barley
Antibodies
Cytokines
Horses

ABSTRACT

Insect bite hypersensitivity is an allergic dermatitis of horses caused by bites of *Culicoides* midges. Sufficient amount of pure, endotoxin-free allergens is a prerequisite for development and monitoring of preventive and therapeutic allergen immunotherapy.

Aims of the study were to compare the *Culicoides nubeculosus* (Cul n) allergens Cul n 3 and Cul n 4, produced in transgenic barley grains with the corresponding *E. coli* or insect cells expressed proteins for measuring antibody and cytokine responses.

Allergen-specific IgG responses were measured by ELISA in sera from twelve horses not exposed to *Culicoides*, before and after vaccination with *E. coli*-rCul n 3 and 4. Before vaccination no IgG binding to the barley and insect cell produced proteins was detected and a similar increase in specific IgG was observed after vaccination. While IgG levels to the *E.coli* expressed proteins were higher in the post-vaccination sera, some background binding was observed pre-vaccination. *In vitro* re-stimulation of PBMC was performed for measurements of cytokines. *E. coli* expressed proteins resulted in high background in PBMC from non-vaccinated controls. The barley and insect cell expressed proteins induced similar amount of IFN-γ and IL-4 in PBMC from vaccinated horses. Barley produced allergens are promising tools for use in immunoassays.

1. Introduction

Insect bite hypersensitivity (IBH) is an allergic dermatitis of horses caused by IgE-mediated reactions to *Culicoides,* blood feeding midges, which affects between 5 and 50% of horses, depending on the breed, origin and environment (reviewed in Schaffartzik et al., 2012). The causative allergens originating from the salivary glands of the midges have been isolated and produced as recombinant proteins in *E. coli* (Peeters et al., 2013; Schaffartzik et al., 2010; Schaffartzik et al., 2011; van der Meide et al., 2013). Allergen specific immunotherapy (AIT) based on the disease causing allergens is presently the only curative treatment against allergies in human (van de Veen et al., 2017) while prophylactic immunotherapy to prevent allergic sensitization is not yet practiced although gaining attention (Valenta et al., 2012). Following prophylactic or therapeutic treatment sufficient amount of purified allergens are important for monitoring the immune response. *E. coli* is the most straight forward production system. However, the formation of inactive insolvable inclusion bodies can be a problem (reviewed in Curin et al., 2017; Jonsdottir et al., 2015). Furthermore, endotoxin contamination can lead to high background cytokine production and *E.coli* expressed proteins are thus of limited use for monitoring the cellular immune response (Jonsdottir et al., 2016). Additionally, it is beneficial to use antigens produced in a differnet expression systems that those used for the immunization, as it allows to monitor the immune response specific for the relevant antigens and not to the contaminating proteins, which even at very low concentrations (purity of the r-allergens > 95%) could still induce some immune response. Some of the *Culicoides* allergens have been expressed in insect cells, a system resulting in recombinant proteins closest to the native ones (Langner et al., 2009). However, production of proteins in insect cells is laborious and can be difficult to scale up (reviewed in Curin et al., 2017). Alternatively, barley grains are an excellent production platform for

* Corresponding author at: Department of Clinical Research and Veterinary Public Health, Vetsuisse Faculty, University of Berne, Länggass-Strasse 124, 3012, Berne, Switzerland.
E-mail address: sigridur.jonsdottir@vetsuisse.unibe.ch (S. Jonsdottir).

https://doi.org/10.1016/j.vetimm.2018.05.005
Received 4 February 2018; Received in revised form 4 May 2018; Accepted 13 May 2018

endotoxin-free recombinant proteins and ideal for long term storage. Recombinant proteins have been retained in barley grains for over two years without loss of function (Horvath et al., 2000). In addition, cultivation costs are low in proportion to total production costs. An efficient protein purification method using histidine tagged recombinant proteins and an inexpensive affinity purification matrix have been developed. Barley has previously been used to successfully produce other functional proteins such as bioactive growth factors that are marketed for stem-cell based research worldwide (Erlendsson et al., 2010; Magnusdottir et al., 2013).

Our goal was to investigate whether barley produced allergens could be used for evaluation of antibodies in serum and activation of cytokine production from PBMC following immunization with *E. coli* expressed allergens.

2. Materials and methods

2.1. Expression of Cul n 3 and Cul n 4

Cul n 3 and Cul n 4 were expressed in *E. coli* (*E. coli*-rCul n 3 and *E. coli*-rCul n 4), purified under denaturing conditions and refolded in H_2O in accordance with Schaffartzik et al., 2011. For long term storage the allergens were lyophilized after addition of 5% trehalose. Before immunization, the allergens were re-constituted in H_2O. The allergens were also expressed in insect cells with the Bac-to Bac® Baculovirus expression system (Invitrogen, Waltham, MA, USA) (Bac-rCul n 3 and Bac-rCul n 4) according to Jonsdottir et al., 2016, with the exception of Cul n 4 which was cloned with 6xHis-tag into the pFastBac1 vector (Life Technology, Carlsbad, CA, USA). The proteins were purified under native conditions and dialyzed against 2xPBS. For protein expression in barley grains, the cDNAs for Cul n 3 and Cul n 4 with N-terminal 6xHis-tag were codon-optimized according to barley codon usage (Genscript, George Town, Cayman Islands) and subsequently used to prepare an expression cassette under the control of a natural seed specific promoter. A natural 21 amino acid signal peptide sequence (D-hordein) was included N-terminally; this sequence is cleaved off in the cell resulting in soluble forms of the target proteins. Transgenic barley (*Hordeum vulgare* L. Cv Golden Promise) expressing Cul n 3 and Cul n 4 was generated by utilizing the *Agrobacterium tumefaciens* mediated transformation method. After milling of the grains and extraction, the proteins were purified by using an IMAC column (GE Healthcare Life Sciences, Pittsburgh, PA, USA) as the capture step and an IEX or a cation exchanger (GE Healthcare Life Sciences, Pittsburgh, PA, USA) for the subsequent polishing step. Buffer exchange was performed on the final products using 2xPBS. All proteins (1 μg) were analysed with coomassie blue staining and western blot with specific mouse polyclonal antibodies (made at the Institute for Experimental Pathology University of Iceland, Keldur) against *E. coli*-rCul n 3 and -rCul n 4 diluted 1:4000 (Jonsdottir et al., 2015; Schaffartzik et al., 2011).

2.2. Horses

Antibody measurements were performed in serum samples from twelve healthy Icelandic horses, one mare and eleven geldings (age 6–11, mean age 8), located in Iceland, *i.e.* not exposed to *Culicoides* bites. All horses were vaccinated intralymphatically on week 0, 4 and 8 with a combination of 10 μg each of *E. coli*-rCul n 3 and rCul n 4 in either 500 μg of aluminium hydroxide (alum) or 500 μg alum and 50 μg monophosphoryl lipid A (MPLA) in a total volume of 400 μL (Jonsdottir et al., 2016). Blood was taken before (week 0) and after the third vaccination (week 10) and sera collected for allergen specific total IgG and IgG subclasses determination by ELISA.

For measurement of cytokine production, three additional healthy Icelandic horses, one mare and two geldings (age 7, 10, 12), located in Iceland, were vaccinated as described above using the combination of alum and MPLA as adjuvant. At weeks 0, 6 and 10 heparinized blood was collected from the three vaccinated horses and from three non-vaccinated control horses, three geldings (age 9, 10, 11), located in Iceland, for isolation of peripheral blood mononuclear cells (PBMC). Blood was also collected for preparation of serum for antibody measurements. The experiment was carried out in accordance with a permit from the National Animal Research Committee of Iceland, no. 2016-01-03.

2.3. ELISA

The ELISA plates, 96 well flat bottom (MaxiSorp, Thermo Fisher Scientific, Waltham, MA, USA), were coated with 0.2 μg/well of the allergens: *E. coli*-, Bac- or Barley-rCul n 3 or -rCul n 4 in coating buffer (carbonate-bicarbonate buffer, pH 9.5, Sigma-Aldrich, St. Louis, MO, USA). The plates were incubated for 2 h at 37 °C and then stored at −20 °C. In each step of the ELISA 100 μL were added to each well, except in the blocking step, where 200 μL were used. The sera and the antibodies were diluted in blocking buffer (PBS containing 500 mM NaCl and 5% Tween 20 and 5% dried milk powder). Before use the plates were thawed at 37 °C and washed with high salt ELISA wash buffer (PBS containing 500 mM NaCl and 0.05% Tween 20). The washing was done after each incubation step until addition of substrate. Non-specific binding sites were blocked with blocking buffer for 1 h at 37 °C. Serum from all the vaccinated horses, diluted 1:1600 was added in triplicate and incubated for 1 h at 37 °C. Then conjugate HRP-labelled Goat anti-horse IgG (Jackson ImmunoResearch, Ely, Cambridgeshire, UK) diluted 1:7000 was added and incubated for 1 h at 37 °C. The substrate, o-phenylenediamine dihydrochloride (OPD, Dako, Santa Clara, CA, USA) and peroxide, was added and incubated in the dark for 10 min at RT. The reaction was stopped with 75 μL/well of 4N H_2SO_4 and optical density (OD) measured at 490 nm. For measurement of IgG subclasses, horse sera were incubated at a dilution of 1:800 and detected with antibodies against IgG1 (IgGa), IgG4/7 (IgGb), IgG5 (part of IgG(T)), used at a concentration of 1 μg/ml (Keggan et al., 2013) and IgG (T) (IgG3/5) (0.5 μg/ml, Serotec, Hercules, CA, USA) followed by HRP-labelled anti-mouse IgG (Jackson ImmunoResearch) as above. Optimal serum dilutions for the ELISA were chosen based on the results from a titration pilot experiment (data not shown). All sera could be tested on a single plate for each allergen. Serum with a previously determined high titer was included on every plate in twofold dilutions 1:100–1:25,600 in duplicate and used as positive control. The intra-assay coefficient of variation was below 7%.

2.4. Stimulation of PBMC and determination of cytokines

PBMC from the 3 vaccinated horses and 3 non-vaccinated controls were isolated by Ficoll-Hypaque (Hamza et al., 2007; Jonsdottir et al., 2016) before vaccination and two weeks after the second (week 6), and third vaccination (week 10). PBMC were stimulated in duplicate for 24 and 48 h with *E. coli,* Bac- and Barley-rCul n 3 and rCul n 4, 2 μg/ml (previously determined with titration, data not shown) or the two from the same expression system mixed together at a concentration of 2 μg/ml each (Jonsdottir et al., 2016). Concanavalin A (Con A 10 μg/ml) was used as a positive control and cells in medium alone as a control for spontaneous cytokine release. IL-4, IFN-γ and IL-10 were measured in cell supernatants using a fluorescent bead-based Cytokine Multiplex assay (Wagner and Freer, 2009). Correction for spontaneous cytokine release was performed on the values for the allergen and Con A stimulations. The data for IL-4 and IL-10 are shown as pg/mL and for the IFN-γ as U/mL.

2.5. Statistical analysis

Statistical analyses were carried out using the software program NCSS 10 (NCSS Statistical Software, Kaysville, UT, USA). Descriptive statistics were run and Shapiro-Wilk W test showed that the data were

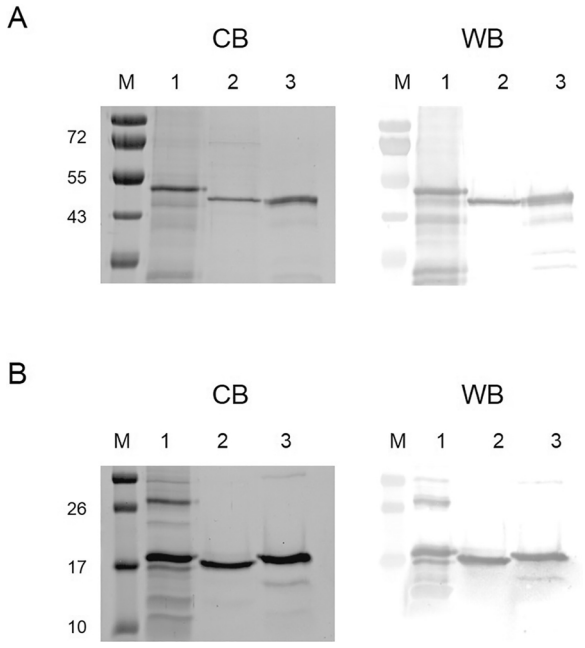

Fig. 1. Expression of rCul n 3 and rCul n 4 in *E. coli,* insect cells and barley. A) rCul n 3 (1 µg/lane) and B) rCul n 4 (1 µg/lane), M) Molecular weight marker, 1) *E. coli*-, 2) insect cell- 3) Barley-expressed allergens, Coomassie blue staining (CB) and western blot (WB). Primary antibody, mouse anti-*E. coli*-rCul n 3 and -Cul n 4 diluted 1:4000 (Schaffartzik et al., 2011) and secondary antibody AP-labelled anti-mouse diluted 1:5000 (Jackson ImmunoResearch).

not normally distributed, even after log-transformation of the data. Non-parametric tests were thus performed. Paired Wilcoxon signed-rank test was used for the comparison of IgG antibody values between weeks 0 and 10 within proteins. The Kruskal-Wallis multiple comparison Z value test was applied for comparison of the antibody response within time points between proteins. Significant difference between expression systems at week 0 or week 10 is indicated with the same superscript letter (a, b, c, d). The level of significance was set at $p \leq 0.05$ throughout the study.

3. Results and discussion

3.1. Expression of rCul n 3 and rCul n 4 in E. coli, insect cells and barley

The *Culicoides* allergens rCul n 3 and rCul n 4 were expressed in the three expression systems. The Cul n 3 proteins were detected as bands between 43 and 55 kDa. The estimated size is 45 kDa (Fig. 1A). The estimated size of Cul n 4 is 17 kDa and the proteins were detected as approximately 17 kDa bands (Fig. 1B). Staining of the western blot with specific polyclonal antibodies (Schaffartzik et al., 2011) made against *E. coli*-rCul n 3 and 4 confirmed that the bands seen in the coomassie blue staining were the recombinant proteins (Fig. 1B).

Barley, like other expression systems of recombinant proteins, can display a wide range of expression level depending on the target protein (Magnusdottir et al., 2013). The yield of our barley expressed proteins differed greatly: 0.43 mg/kg for rCul n 3 and 14 mg/kg for rCul n 4. Several methods are available to increase the yield of rCul n 3 from the barley grain, *e.g.* improve vector design and generation of double haploid plants (lines), which can result in higher expression levels (Magnusdottir et al., 2013).

3.2. Specific IgG response of vaccinated horses against the rCul n 3 and rCul n 4 expressed in E. coli, insect cells and barley

After vaccination significant increases were observed between pre- (week 0) and post- vaccination sera (week 10) in total IgG and IgG subclass levels against each protein, (Figs. 2 and 3). As we were interested in comparing the antibody response within time points between proteins, a Kruskal-Wallis multiple comparison Z value test was performed either between antibody levels at week 0 to compare the serological background reactivity or at week 10 (Figs. 2 and 3) to compare the specific IgG-binding to the recombinant proteins from different expression systems after vaccination.

The total IgG levels were significantly higher, both pre- and post-vaccination on *E. coli*, as compared to the Bac- and barley rCul n 3, whereas on rCul n 4 the difference was only significant for the pre-vaccination values. The *E. coli*-produced proteins gave the strongest responses after vaccination. This was to be expected since they were used as immunogens (Fig. 2). While the background observed on the *E.coli* expressed proteins might be problematic in other studies it did not affect our results because of the large difference between pre- and post-vaccination values.

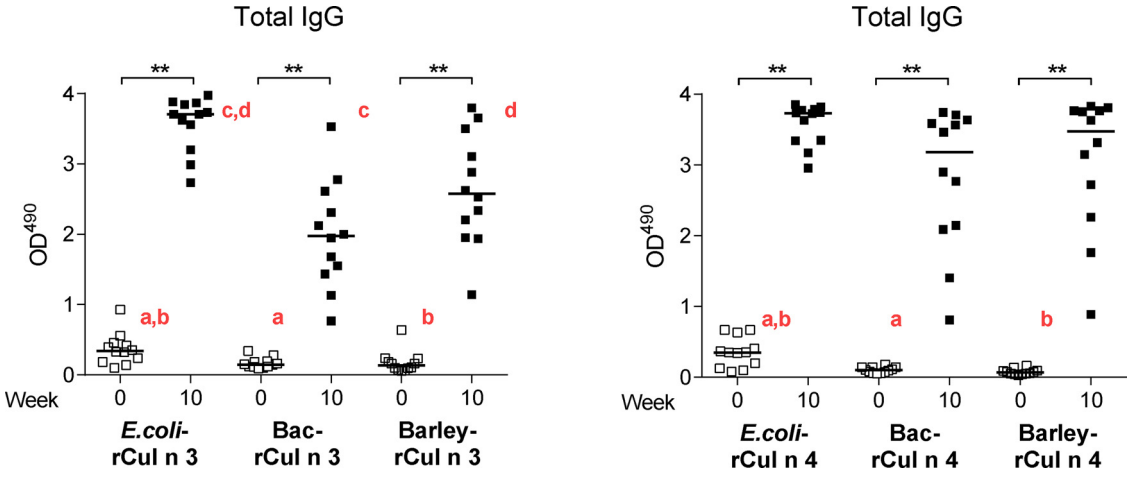

Fig. 2. Total IgG response of vaccinated horses against rCul n 3 and rCul n 4 expressed in *E. coli*, insect cells and barley.
Total serum IgG was measured by ELISA using *E. coli*-, Bac- (insect cells) and Barley-rCul n 3 and -rCul n 4. The results are shown as OD value for each horse with the median for the groups, before (week 0 □) and after the third vaccination (week 10 ■). Comparison of IgG levels between weeks 0 and 10 within proteins was performed using the paired Wilcoxon signed-rank test. Asterisks indicate significant differences between week 0 and week 10 within the proteins (*$p \leq 0.05$, **$p \leq 0.001$). Comparison of the antibody response within time points between proteins was performed with the Kruskal-Wallis multiple comparison Z value test. A significant difference between expression systems at week 0 or week 10 is indicated with the same superscript letter (a, b, c, d) $p \leq 0.05$.

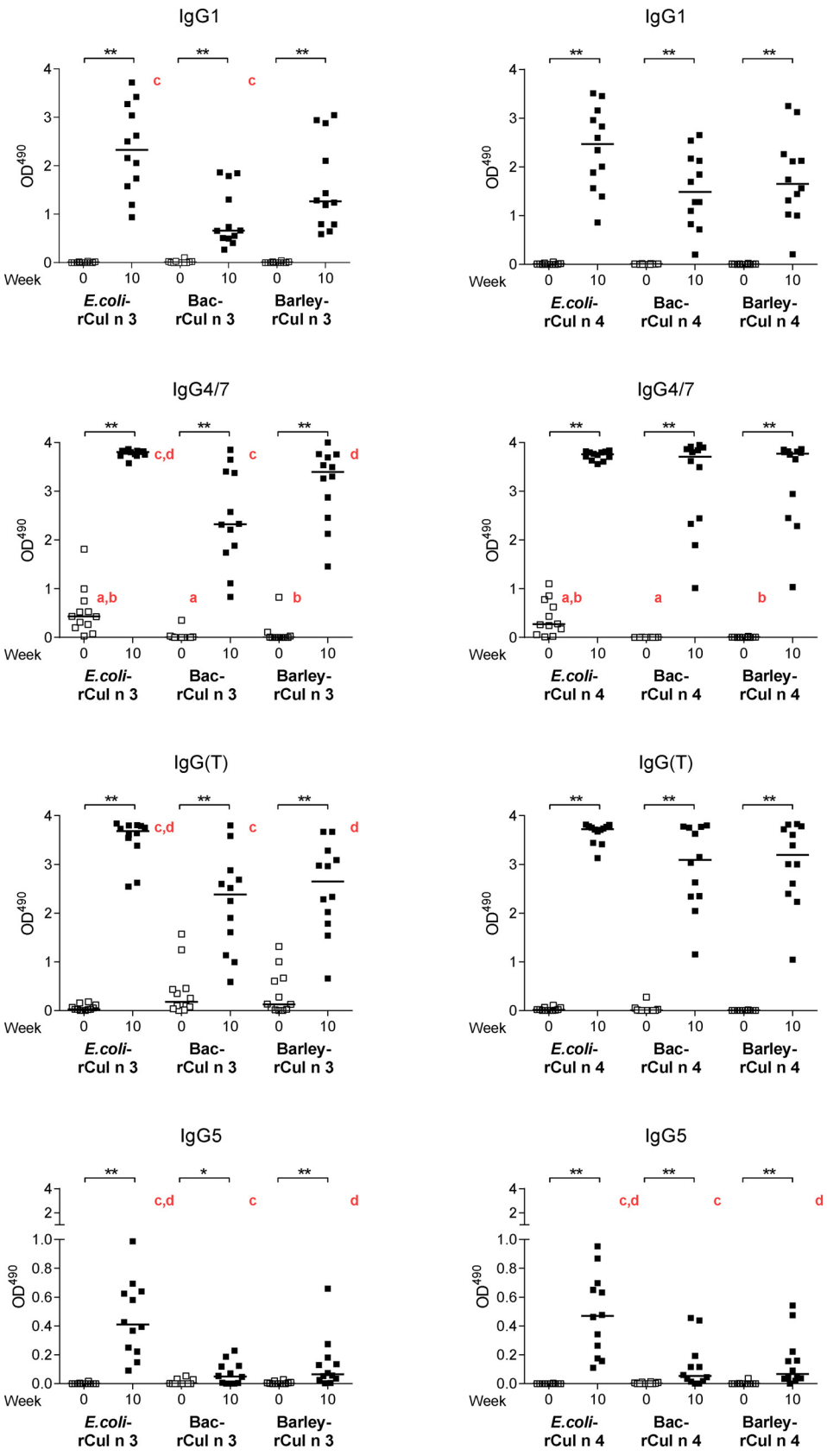

Fig. 3. IgG isotype responses of vaccinated horses against rCul n 3 and rCul n 4 expressed in *E. coli*, insect cells and barley.
Serum IgG isotypes were measured by ELISA against *E. coli*-, Bac- (insect cells) and Barley-rCul n 3 and -rCul n 4 and are shown as OD value for each horse, with the median for the groups, before (week 0 □) and after the third vaccination (week 10 ■). Comparison of IgG subclass levels between week 0 and 10 within proteins was performed using the paired Wilcoxon signed-rank test. Asterisks indicate significant differences between week 0 and week 10 within the proteins (*$p \leq 0.05$, **$p \leq 0.001$). Comparison of the antibody response within time points between proteins was performed with the Kruskal-Wallis multiple comparison Z value test. A significant difference between expression systems at week 0 or week 10 is indicated with the same superscript letter (a, b, c, d) $p \leq 0.05$.

Specific IgG1 levels were not detected before vaccination for any of the proteins, whereas the vaccination induced a strong reaction against all of them. The Cul n 3 specific response was significantly stronger on the protein produced in *E. coli* than against Bac-rCul n 3. The others Cul n 3 responses did not differ (Fig. 3). Pre-vaccination IgG4/7 levels on both rCul n 3 and rCul n 4 produced in *E. coli* were significantly higher than on the proteins from insect cells and barley, suggesting that IgG4/7 antibodies may bind to *E. coli* contamination in the *E. coli* expressed

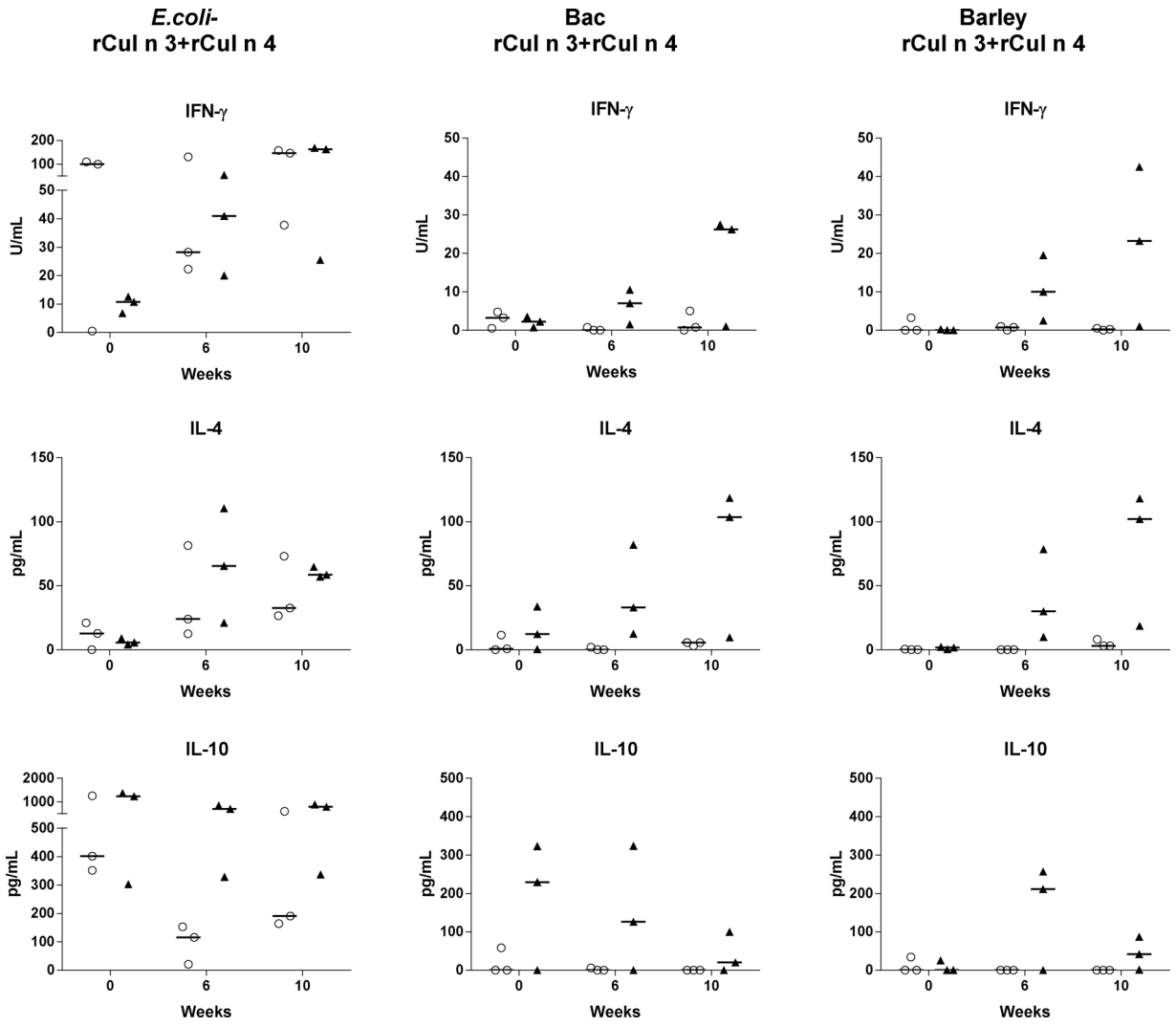

Fig. 4. Detection of IFN-γ, IL-4, and IL-10 following *in vitro* re-stimulation of PBMC from vaccinated horses and unvaccinated controls.
PBMC were re-stimulated with mixture of rCul n 3 and rCul n 4 expressed in *E. coli*, insect cells or barley. Cytokines production was measured in supernatants using a fluorescent bead-based multiplex assay. The results after 48 h stimulation of PBMC taken before (week 0), after the second (week 6) and after the third vaccination (week 10), are shown as U/mL for IFN-γ and pg/mL for IL-4 and IL-10 for each horse and the median for the three horses. The value for each horse is shown, non-vaccinated controls (○) and vaccinated (▲) with the median of the three horses.

proteins. There was a strong IgG4/7 increase after vaccination and, as for total IgG, significantly higher responses on *E. coli*-rCul n 3 and *E. coli*-rCul n 4 as compared to the corresponding proteins produced in the other two systems (Fig. 3). Again, this was expected as the *E. coli* proteins were used for immunization. Regarding IgG(T) there was no background on *E. coli* proteins whereas few horses had IgG(T) binding to rCul n 3 produced in insect cells and in barley before vaccinations. As before, the strongest response was found for the proteins produced in *E. coli*, both for rCul n 3 and rCul n 4 (Fig. 3). IgG5 serum levels were lower than the other subclasses. However, there was a significant difference before and after vaccination against all proteins and the post-vaccination values for the *E. coli*-produced proteins were significantly higher than for Bac- and barley-rCul n 3 and 4 (Fig. 3). Overall the barley produced proteins gave very similar results to those obtained with the insect cell expressed proteins for monitoring the IgG response of vaccinated horses.

3.3. Cytokine production following in vitro re-stimulation of PBMC from vaccinated and non-vaccinated horses

At the time of vaccination of the 12 horses the only purified allergens available were the *E. coli* produced rCul n 3 and 4 and Bac-rCul n 3. In a pilot vaccination study (Jonsdottir et al., 2015) a high background stimulation was observed when PBMC were stimulated with *E. coli* produced allergens. For that reason only insect cell expressed rCul n 3 was used to analyse the cytokine response of the twelve horses (Jonsdottir et al., 2016). Thus, for the comparison of the cytokine response following stimulation with rCul n 3 and rCul n 4 produced in different expression systems, three additional horses were vaccinated and *in vitro* re-stimulation of PBMC and determination of cytokines performed. PBMC from three non-vaccinated horses were stimulated at the same time points as controls. The IgG antibody response, to all proteins, of the three horses was comparable to the twelve horses analysed before (data not shown).

To determine the optimal stimulation time, PBMC were stimulated for 24 and 48 h. The 24 h stimulation with the allergens resulted in low or no cytokine production. These data are thus not shown. Furthermore, pooling of rCul n 3 and rCul n 4 from each expression system induced a higher cytokine production compared to single proteins (data not shown).

Fig. 4 shows the results after the 48 h stimulation with the pooled allergens. Compared to the proteins expressed in the other expression

systems, the *E. coli*-produced proteins induced high levels of IFN-γ and IL-10, but there was no difference between the vaccinated horses and the controls at any time point. We encountered this problem previously when using the *E. coli* produced proteins for stimulation of PBMC (Jonsdottir et al., 2015). It is assumed to result from LPS contamination in proteins expressed in *E. coli*. IL-4 production was similar between the proteins from the three expression systems. While IL-4 remained very low over time in the non-vaccinated horses following stimulation with the insect cell- and barley produced proteins, surprisingly a low increase was observed after stimulation with the *E.coli* produced proteins.

Conversely to the *E. coli* expressed allergens, PBMC stimulation with allergens expressed in insect cells or barley lead to no or very low IFN-γ and IL-4 production at week 0, both in the vaccination and control groups. However, at week 6 (*i.e.* 2 weeks after the 2nd vaccination) an increased production of these cytokines could be detected in two of the three vaccinated horses but not in the controls. This IL-4 production further increased at week 10, *i.e.* two weeks after the 3rd vaccination. In one vaccinated horse IFN-γ values after stimulation with the insect cells and barley produced allergens were below the lower limit of detection of the assay and were very low for IL-4. This horse also reacted poorly after Con A stimulation, used as a positive control, especially with IFN-γ (Supplementary Fig. 1). In this horse, no IL-10 production was detected following PBMC stimulation with insect cell or barley expressed allergens. In the other two vaccinated horses, a clear increase in IL-10 production was observed between week 0 and week 6 following stimulation with the barley expressed allergens. Interestingly these values decreased at week 10. Surprisingly, after stimulation with the insect cell produced allergens the highest IL-10 concentration were seen at week 0. We cannot explain this background; the three control horses were negative and we have successfully used insect cell produced rCul n 3 to stimulate PBMC for IL-10 production (Jonsdottir et al., 2016). Again, stimulation with the *E.coli* expressed allergens resulted in high background IL-10 production at week 0.

As shown in Fig. 4, the horses showed considerable individual variation in cytokine production after re-stimulation of their PBMC with the allergens and, retrospectively, a larger number of horses would have been preferred for this experimental vaccination.

4. Conclusion

In conclusion, this study shows that both insect cell and barley expressed allergens are preferred for PBMC re-stimulation and to evaluate the cellular immune response to recombinant allergens compared to *E. coli* produced proteins. While the *E. coli* expressed allergens could be applied in the serological assays, it is of advantage to have the proteins available that can also be used for re-stimulation of PBMC and are free of endotoxins. The study also showed that barley expressed allergens perform similar to insect cell in the assays used. In addition, allergen amount in barley can easily be scaled up. They could be valuable tools to monitor the immune response of allergen immunized horses. Future studies are needed to evaluate their use for characterizing the immune response of IBH-affected horses.

Conflicts of interest

No conflict of interest.

Acknowledgments

This work was supported by Icelandic Research Fund grant no. 141071-051, the Eimskip University Fund, the Agricultural Productivity Fund of Iceland, and Swiss National Science Foundation grant no. 310030-160196/1. We are grateful to Sigurdur H. Helgason, Hilmar Össurarson and Elvar H. Rikardsson for all their assistance with the horses.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetimm.2018.05.005.

References

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 (3), 201–209.

Erlendsson, L.S., Muench, M.O., Hellman, U., Hrafnkelsdóttir, S.M., Jonsson, A., Balmer, Y., Mäntylä, E., Örvar, B.L., 2010. Barley as a green factory for the production of functional Flt3 ligand. Biotechnol. J. 5 (2), 163–171.

Hamza, E., Doherr, M.G., Bertoni, G., Jungi, T., 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 (4), 325–337.

Horvath, H., Huang, J., Wong, O., Kohl, E., Okita, T., Kannangara, C.G., von Wettstein, D., 2000. The production of recombinant proteins in transgenic barley grains. Proc. Natl. Acad. Sci. U. S. A. 97 (4), 1914–1919.

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 (1–2), 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.

Keggan, A., Freer, H., Rollins, A., Wagner, B., 2013. Production of seven monoclonal equine immunoglobulins isotyped by multiplex analysis. Vet. Immunol. Immunopathol. 153 (3–4), 187–193.

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. Int. J. Parasitol. 39 (2), 243–250.

Magnusdottir, A., Vidarsson, H., Bjornsson, J.M., Orvar, B.L., 2013. Barley grains for the production of endotoxin-free growth factors. Trends Biotechnol. 31 (10), 572–580.

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 (1), 141–147.

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. Vet. Immunol. Immnopathol. 137 (1–2), 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. Vet. Immunol. Immunopathol. 139 (2–4), 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 (3–4), 113–126.

Valenta, R., Campana, R., Marth, K., van Hage, M., 2012. Allergen-specific immunotherapy: from therapeutic vaccines to prophylactic approaches. J. Intern. Med. 272 (2), 144–157.

Wagner, B., Freer, H., 2009. Development of a bead-based multiplex assay for simultaneous quantification of cytokines in horses. Vet. Immunol. Immunopathol. 127 (3–4), 242–248.

van de Veen, W., Wirz, O.F., Globinska, A., Akdis, M., 2017. Novel mechanisms in immune tolerance to allergens during natural allergen exposure and allergen-specific immunotherapy. Curr. Opin. Immunol. 48, 74–81.

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 (3–4), 227–239.