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Short communication

A preventive immunization approach against insect bite hypersensitivity: Intralymphatic injection with recombinant allergens in Alum or Alum and monophosphoryl lipid A

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ABSTRACT

Insect bite hypersensitivity (IBH) is an IgE-mediated dermatitis of horses caused by bites of *Culicoides* insects, not indigenous to Iceland. Horses born in Iceland and exported to *Culicoides*-rich areas are frequently affected with IBH.

The aims of the study were to compare immunization with recombinant allergens using the adjuvant aluminum hydroxide (Alum) alone or combined with monophosphoryl lipid A (MPLA) for development of a preventive immunization against IBH.

Twelve healthy Icelandic horses were vaccinated intralymphatically three times with 10 μg each of four recombinant *Culicoides nubeculosus* allergens in Alum or in Alum/MPLA.

Injection with allergens in both Alum and Alum/MPLA resulted in significant increase in specific IgG subclasses and IgA against all r-allergens with no significant differences between the adjuvant groups. The induced antibodies from both groups could block binding of allergen specific IgE from IBH affected horses to a similar extent. No IgE-mediated reactions were induced. Allergen-stimulated PBMC from Alum/MPLA horses but not from Alum only horses produced significantly more IFNγ and IL-10 than PBMC from non-vaccinated control horses.

In conclusion, intralymphatic administration of small amounts of pure allergens in Alum/MPLA induces high IgG antibody levels and Th1/Treg immune response and is a promising approach for immunoprophylaxis and immunotherapy against IBH.

1. Introduction

Equine insect bite hypersensitivity (IBH), or summer eczema, is an allergic recurrent seasonal dermatitis of horses. It is caused by bites of insects mainly of the genus *Culicoides* (biting midges) (for review Schaffartzik et al., 2012) and characterized by papules and intense pruritus affecting the feeding sites of the midges (Bröstrom et al., 1987). IBH affects all horse breeds and is found almost worldwide with the exception of places where *Culicoides* species are not indigenous, as in Iceland (Illies, 1978). However, IBH is a severe problem in Icelandic horses exported from Iceland to the European continent. Over 50% of these horses develop the disease two years or more after importing into heavily *Culicoides* infested areas (Bjornsdottir et al., 2006), while Icelandic horses foaled in Europe develop the disease with a similar prevalence as most other horse breeds (Bröstrom et al., 1987). Presently, there is no satisfactory treatment of IBH (Schaffartzik et al., 2012), and thus preventive immunization may be an attractive option for horses exported from Iceland to countries where *Culicoides* are present. In humans, prophylactic immunotherapy to prevent allergic sensitization is not yet practiced but has been considered (Valenta et al., 2012). The allergen extracts mostly used in allergen immunotherapy are not suited as they risk inducing sensitization to other proteins in the mixture. For prophylactic treatment it is essential to use well-defined pure allergens and to treat before sensitization occurs (Valenta et al., 2012). IBH in Icelandic horses is thus an interesting model for development and study of prophylactic immunotherapy

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as Iceland is free of the causative agent and the horses are only sensitized after export. Furthermore, *Culicoides* allergens have been identified and characterized at the molecular level and are available as pure recombinant proteins (Mueller et al., 2016).

According to our studies on the immune response and pathogenesis of IBH, Icelandic horses exported from Iceland to Europe develop a stronger Th2 polarized immune response than their disease status matched counterparts born in Switzerland (Hamza et al., 2007). Further studies demonstrated an imbalance between the Th2/Treg immune response in IBH, using both *Culicoides* stimulated PBMC and skin biopsies (Hamza et al., 2008, 2013; Heimann et al., 2011). These findings indicate that a preventive immunization against IBH should aim at inducing an allergen-specific Treg and/or Th1 immune response.

Due to the high cost of production and purification of recombinant proteins and the risk of side effects when using a high dose of allergens (Klimek and Pfaar, 2013) it is of great importance to have an efficient route of injection and a vigorous adjuvant to be able to use small amounts of the allergens. In a previous study we could show that when using the Th1 adjuvant IC31® (Schellack et al., 2006) intralymphatic injection gave a slightly stronger immune response than intradermal injections (Jonsdottir et al., 2015). Based on these findings and on results from immunotherapy in humans (Senti et al., 2008), it was of interest to test further this injection route with other adjuvants.

Despite being a Th2 focusing adjuvant, Alum is the classical adjuvant used in immunotherapy for humans (Moingeon, 2012). However, when T-cell-mediated immunity is necessary additional components are needed (Brewer, 2006). Monophosphoryl lipid A (MPLA) is a detoxified derivative of lipopolysaccharide (LPS) that binds to Toll like receptor 4 (TLR4) and retains most of the adjuvant capacity of LPS (Evans et al., 2003; Qureshi et al., 1982). MPLA is used in vaccine formulations and has been registered for use in humans (Casella and Mitchell, 2008). Since MPLA promotes primarily a Th1 type of response (Puggioni et al., 2005), it is being applied as an adjuvant in immunotherapy and has been shown to enhance specific IgG1 and IgG4 antibodies, and reduce allergy symptoms (Drachenberg et al., 2001; Mothes et al., 2003). The mixture of aluminum hydroxide and MPLA (AS04) is being used successfully in viral vaccines (Garçon et al., 2011).

The aim of our study was to compare the immune response induced by intralymphatic immunization with pure recombinant allergens in Alum alone or a mixture of Alum and MPLA in the search for optimal conditions for prophylactic immunotherapy against equine IBH.

2. Materials and methods

2.1. Animals

Twelve healthy Icelandic horses, 7–10 years of age, were vaccinated. In addition three healthy horses were used as controls in the skin test and six horses as control in the *in vitro* stimulation of PBMC, age 5–13 years. All horses were living in Iceland. The experiment was performed in accordance with a permit from the National Animal Research Committee of Iceland (no. 0113–16).

2.2. Vaccination and sample collection

Twelve horses were vaccinated into the submandibular lymph nodes with four *Escherichia coli*-expressed recombinant *Culicoides nubeculosus* allergens (rCul n 3, rCul n 4, rCul n 8 and rCul n 10) (Schaffartzik et al., 2011). The horses were vaccinated three times (week 0, 4, 8) with 10 μg of each allergen. The horses were randomly divided into two groups. Six horses were vaccinated with the allergens in 500 μg aluminum hydroxide gel (Alhydrogel® 2%, Invivogen) and the other six with allergens in a mixture of Alum (500 μg) and 50 μg MPLA (Avantilipids). The total volume per vaccination was 400 μL. Blood was collected by jugular venipuncture at week 0, every other week for 16 weeks, and then monthly until week 32. Serum was stored at −20 °C until used. A differential count of leukocytes from EDTA blood was carried out (Jonsdottir et al., 2015).

2.3. Production of Cul n 3 in insect cells

Cul n 3 was expressed in insect cells because pilot experiments had shown with *E. coli* derived Cul n 3 the cytokine production by PBMC was extremely low or absent, probably because this *E. coli* expressed protein precipitated in medium. The *Cul n 3* gene was amplified from a λZAPII cDNA library, made from salivary glands of *C. nubeculosus* (Schaffartzik et al., 2011), with primers (Fw: 5′- ATGCATAATTTTGCAGGGAT-3′ and Re: 5′-CGCATATGTGGTCAAAGTAG-3′) designed based on the gene sequence of Cul n 3 (GenBank Accession No. HM145951). The protein was expressed in insect cells according to Bac-to-Bac® HBM-TOPO® Secreted Expression system. The Baculovirus was amplified in Sf-9 cells and the protein expressed in High five cells.

The Bac-rCul n 3 protein was purified under native conditions with HIS-Select™ HF Nickel Affinity Gel (Sigma) in conformity with the manufacturer's protocol and dialyzed against PBS. The protein was analyzed with Coomassie blue staining (Wong et al., 2000) and detected with Cul n 3 specific polyclonal antibodies diluted 1:4000 (Schaffartzik et al., 2011) in WB (Fig. 1A) (Jonsdottir et al., 2015).

2.4. Serological tests

2.4.1. ELISA

Sera from six unvaccinated horses living in Iceland and from five IBH-affected horses living in Switzerland were included on each plate, as negative and positive controls, respectively. Allergen-specific antibody levels were determined by ELISAs as described in Jonsdottir et al. (2015) with the following changes. Plates were coated with 2 μg/mL of the recombinant allergens rCul n 3, rCul n 4, rCul n 8, rCul n 10 and Bac-rCul n 3. Sera (weeks 0, 2, 6, 10, 16) were added at a dilution of 1:5 for IgE detection, 1:10 for IgA (weeks 0, 6, 10, 24, 32) and 1:200 for IgG subclasses (weeks 0, 2, 6, 10, 16, 24, 32). Specific monoclonal antibodies were used for IgG subclasses and IgE detection as described by Jonsdottir et al. (2015) and for IgA, a monoclonal equine IgA specific antibody in a 1:250 dilution (Serotec) was applied.

2.4.2. Competitive inhibition ELISA

Pools of sera from the Alum group and Alum/MPLA group, containing the same amount of serum from each of the six horses, were used to analyze the ability of sera from vaccinated horses to block the IgE-binding of serum from an IBH-affected horse to the recombinant allergens. Pools of preimmune sera (negative control) and postimmune sera (two weeks after the third vaccination) were used in the competitive inhibition ELISA performed as described by Jonsdottir et al. (2015). The percentage of inhibition for each dilution of the pre- and postimmune serum pools was calculated.

2.5. Stimulation of PBMC and determination of cytokines

PBMC were isolated from the horses by Ficoll-Hypaque in conformity with Hamza et al. (2007) three weeks (week 11) after the 3rd vaccination, and from six unvaccinated control horses. PBMC were stimulated for 24 h (for RNA isolation) or for 4 days (for analysis of the supernatant) with ConA (10 μg/mL) as a positive control

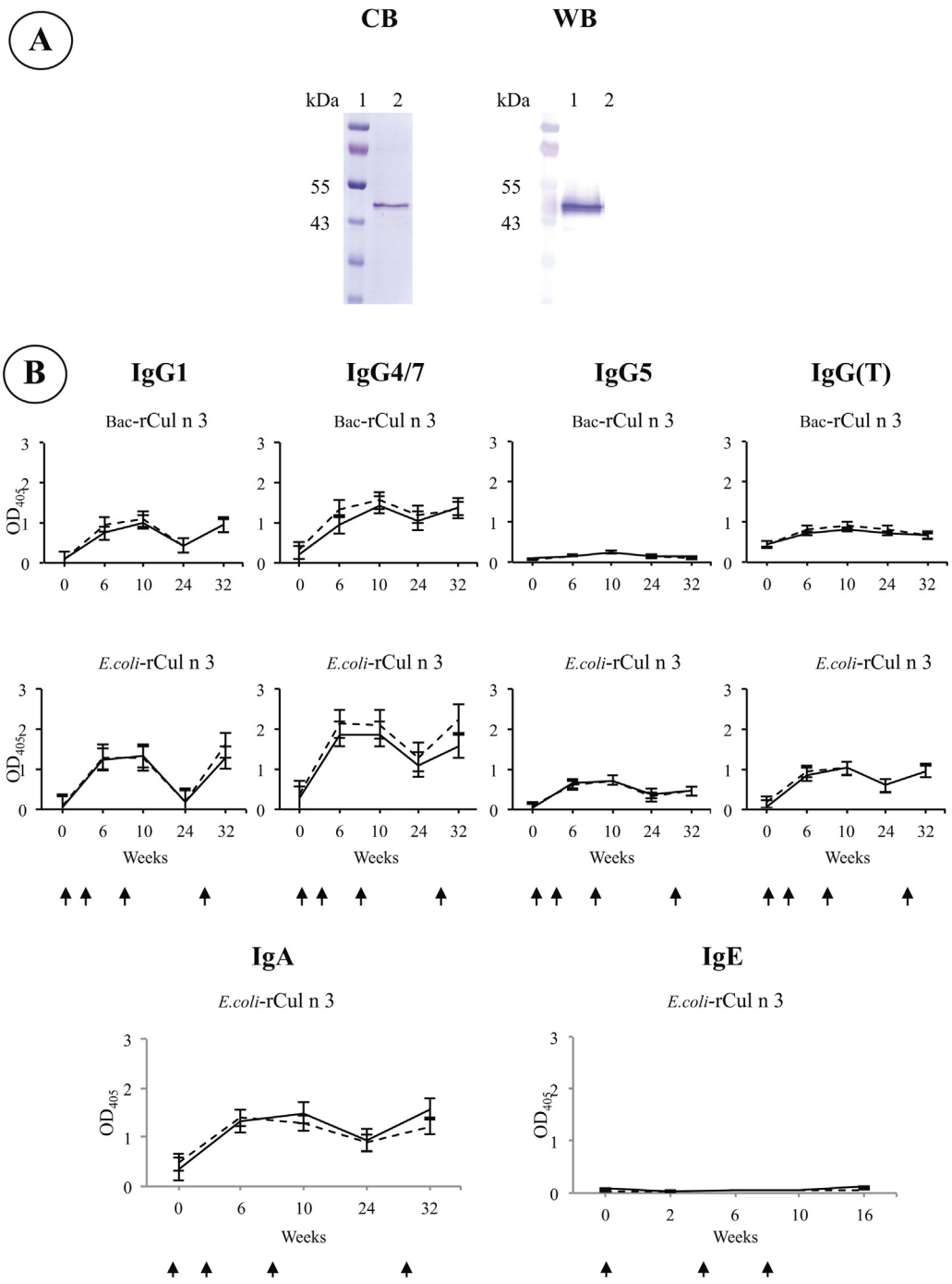

Fig. 1. (A) Purification and detection of Bac-rCul n 3 produced in High five insect cells. Coomassie blue staining (CB) and Western blot (WB) of purified Bac-rCul n 3 in PBS, detected with Cul n 3 specific polyclonal antibody (1:4000). (B) Cul n 3 specific antibody response. Time course of IgG subclass, IgA and IgE response against *E. coli*-rCul n 3 measured by ELISA and comparison of IgG subclass response against Bac-Cul n 3. Corrected OD mean values and standard error for the six horses in the two groups at different time points, Alum (——) and Alum/MPLA (- - - -). The first three arrows indicate the vaccination time points and the last the intradermal test.

and Bac-rCul n 3 (2 μg/mL) or cultured in medium alone. The stimulation was performed in accordance with Jonsdottir et al. (2015). After 24 h PBMC were harvested, lysed and homogenized in RA1 lysis buffer (Macherey–Nagel) and stored at −80 °C until used for total RNA isolation. After 4 days' incubation, cell supernatants were harvested and stored at −80 °C until cytokine measurement.

Determination of IL-4, IFNγ and IL-10 in cell supernatants was performed at the Department of Population Medicine and Diagnostic Sciences, Cornell University, with bead-based multiplex assays (Wagner and Freer, 2009). The values for the medium alone were subtracted and the results shown as mean and standard errors for each group as pg/mL for IL-4 and IL-10 and U/mL for IFNγ. Total RNA isolation, Reverse Transcription of RNA and Q RT-PCR for Cytokine mRNA expression was performed as described previously (Jonsdottir et al., 2015).

2.6. Skin test

Skin tests were performed according to Schaffartzik et al. (2011) at week 30. In short, horses were injected in the lateral neck with 100 μL of the allergens rCul n 3, 4, 8 and 10 diluted to 1 μg/mL and 10 μg/mL. Histamine 0.2 mg/mL was used as the

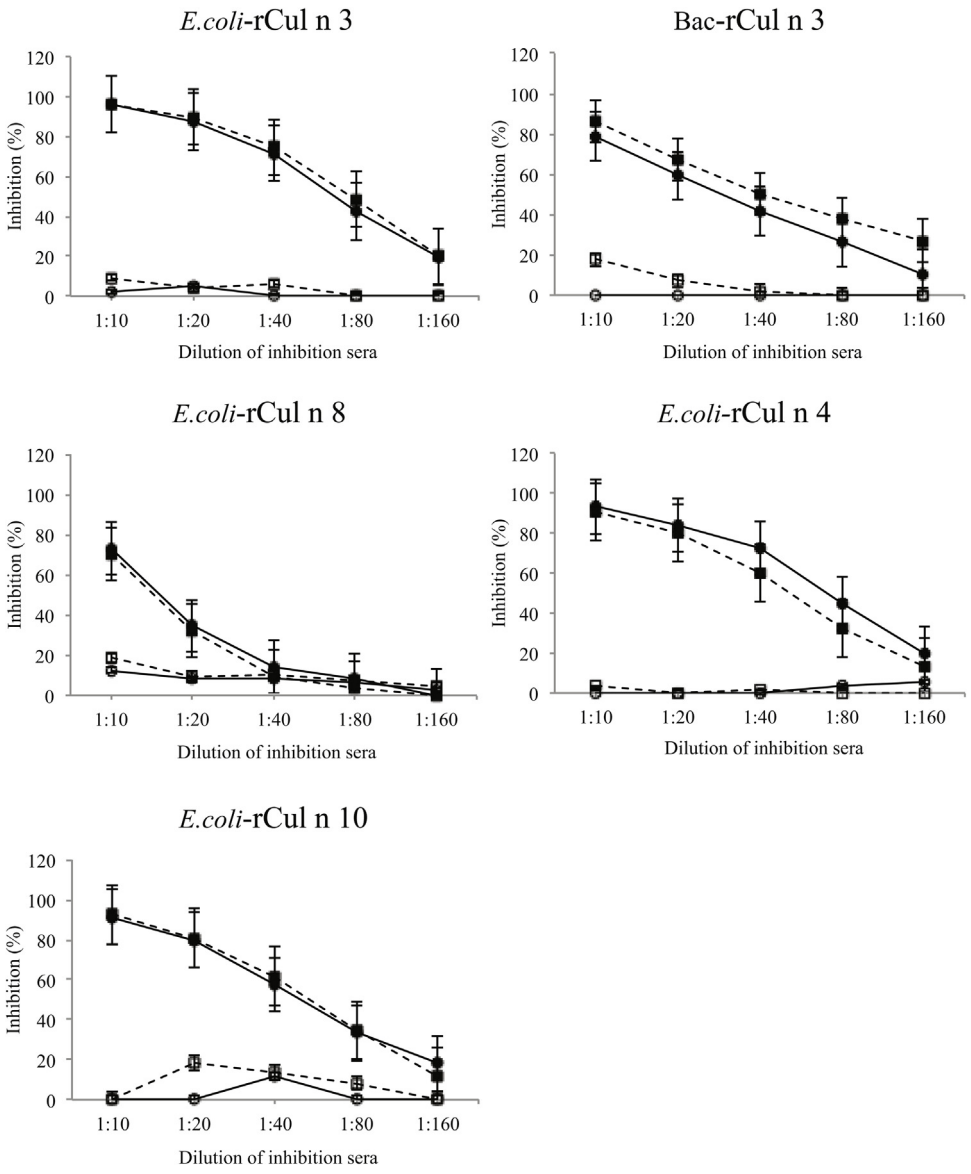

Fig. 2. Inhibition of IgE binding to the allergens with pool of sera from the two vaccination groups. Mean percent inhibition by pre and post vaccination sera from the two groups, diluted 1:10–1:160 applied to an ELISA plate coated with allergens, prior to adding serum from an IBH positive horse at a dilution 1:5. Preimmune sera Alum horses (—○—), preimmune sera Alum/MPLA horses (- -□- -), postimmune sera Alum horses (—●—), postimmune sera Alum/MPLA horses (- -■- -).

positive control. Diameters of the wheal reaction at injection site were measured 30 min post injection.

2.7. Statistical analyses

Descriptive statistics were run on the IgG subclasses, IgA and IgE ELISA results using the statistical software NCSS 8 (NCSS Statistical Software, 329 North 1000 East, Kaysville, Utah 84037, USA). Antibody levels were log transformed because they were not normally distributed. Allergen-specific logIgG subclasses and logIgA levels were then analyzed using Proc Mixed of SAS (SAS Inst. Inc., Cary, NC). Week of measurement was included as a repeated effect, with the individual horse as the subject. Separate models were run with levels of log IgG1, IgG1/3, IgG4/7, IgG5 and IgG(T) as outcome variable, respectively. The statistical model included effect of adjuvant (Alum or Alum/MPLA), recombinant allergen (rCul n 3, 4, 8, 10), and time (weeks after immunization: 0, 2, 6, 10, 16, 24, 32), and their interactions. Non-significant interactions were removed from the final model.

As the cytokine values were not distributed normally, the non-parametric ANOVA Kruskal–Wallis Multiple-Comparison *Z*-Value Test was used to compare the differences on cytokine mRNA levels and in cytokines present in the supernatants in horses immunized with Alum, Alum/MPLA and non-immunized control horses. The Bonferroni correction was used to correct for multiple comparisons. Statistical significance was defined as $p < 0.05$.

3. Results and discussion

3.1. Clinical examination

The vaccinations in the submandibular lymph nodes were well tolerated. No rise in temperature or pain on palpation was observed, but mild local swelling occurred in a few cases. Leukocyte counts were within the normal range following vaccinations (data not shown). No difference was observed between vaccination with Alum or a mixture of Alum and MPLA.

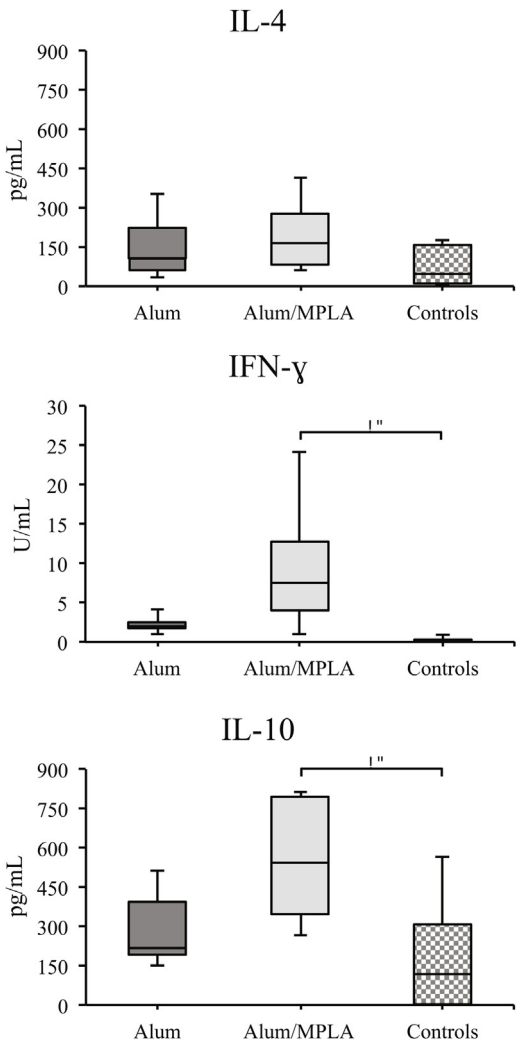

Fig. 3. Detection of IL-4, IL-10 and IFNγ after *in vitro* stimulation of PBMC from the Alum and Alum/MPLA vaccinated horses and six control horses. The cytokines were measured in supernatants using bead-based multiplex assays. Comparisons were performed using Kruskal–Wallis Multiple Comparison *Z* value Tests with Bonferroni corrections. An asterisk (*) with line indicates statistically significant differences between the groups of horses.

3.2. Specific antibody response of vaccinated horses

Immunization with 10 μg each of the four recombinant allergens led to a significant increase in all tested IgG subclasses as soon as two weeks after the first vaccination (Table 1), both in the Alum and Alum/MPLA groups, with further increase until week 10, *i.e.* 2 weeks after the 3rd vaccination (Fig. 1B). Antibody levels then decreased until week 24 but were still significantly higher than before vaccination (Table 1). Interestingly, the intradermal test performed at week 30 boosted the specific antibody responses (Fig. 1B).

Comparison of the two groups of horses vaccinated with Alum or Alum/MPLA, showed a similar IgG subclass response in both groups (Table 1 and exemplified for Cul n 3 in Fig. 1B).

Analysis of the data revealed significant differences in the IgG subclass levels between the different allergens. This confirms results from the previous study with the IC31® adjuvant (Jonsdottir et al., 2015).

The Bac-rCul n 3 antibody response showed a similar pattern as when the *E. coli*-Cul n 3 was used in the ELISA (Fig. 1B). As expected the measured antibody increase was usually lower on Bac-rCul n 3 than on *E. coli*-rCul n 3 that was used for the vaccination. These results confirm that the antibody response is mainly raised against the Cul n 3 protein and not against the *E. coli* contamination of the recombinant proteins used for immunization (purity of 95%).

As a protective role of IgA has been suggested in allergic diseases such as asthma (Gloudemans et al., 2013) and an increase in serum IgA is seen in allergen specific immunotherapy (Jutel et al., 2003), we also determined allergen-specific IgA levels in the sera. The horses in both groups had developed a significant IgA response against all four allergens (Table 1) already after the second immunization, as exemplified for rCul n3-specific IgA in Fig. 1B. The serum IgA levels were much lower than IgG as the sera could only be diluted 1:10 instead of 1:200 in the ELISA. No significant effect of MPLA could be observed on the IgA response compared to Alum alone.

Importantly immunization with the recombinant allergens did not induce sensitization as no IgE response was detected after the vaccinations, as exemplified for rCul n 3 in Fig. 1B. Nor did the horses respond to the *Culicoides* allergens in intradermal tests, irrelevant of the adjuvant used (data not shown).

3.3. Inhibition of allergen-specific IgE-binding by sera from the vaccinated horses

An important factor in immunotherapy is the induction of IgG antibodies that can block the binding of IgE to the relevant allergens (Flicker and Valenta, 2003). In the present study pools of the pre- and postimmune sera (two weeks after third vaccination) from the two groups of horses were tested in an inhibition ELISA. The inhibition of IgE-binding to rCul n 3, rCul n 4 and rCul n 10 was over 90% in the 1:10 serum dilution and still 60% or more in the 1:40 dilution. Inhibition of binding to rCul n 8 was lower, around 70% in the 1:10 serum dilution and the 1:40 dilution had no inhibitory activity. This was regardless of the use of MPLA (Fig. 2). In our previous vaccination study (Jonsdottir et al., 2015) with the adjuvant IC31®, the blocking activity of the 1:10 diluted sera was lower, only reaching 18 to 54%, depending on the allergen used. This result accords with higher antibody titers observed in the present study. However, we cannot distinguish whether this difference was due to the adjuvants or the allergens, as different recombinant *Culicoides* allergens were used. The inhibitory activity of the sera was also tested against Bac-rCul n 3 showing that the antibodies generated though immunization with *E. coli* expressed Cul n 3 were also able to efficiently inhibit IgE-binding to Bac-rCul n 3, although to a slightly lower extent (Fig. 2).

3.4. IL-10, IFNγ and IL-4 protein expression in PBMC stimulated in vitro with Bac-rCul n 3

To further characterize the type of immune response induced by these adjuvants, PBMC were stimulated *in vitro* with Bac-rCul n 3 and the cytokine production determined. In contrast to the antibody response, there was a significant difference between the adjuvant groups in the cytokine responses. Upon stimulation of PBMC with Bac-rCul n 3 the horses vaccinated with the allergens in Alum/MPLA produced significantly more IFNγ and IL-10 than the non-vaccinated controls, whereas this difference was not observed between the horses vaccinated with Alum and the control horses (Fig. 3). The same tendency was seen at the cytokine mRNA levels (data not shown). When the two vaccinated horse groups were compared without the controls the Alum/MPLA horses produced significantly more IFNγ and IL-10 than the Alum horses. These findings suggest that MPLA is important to obtain the preferable Th1/Treg cytokine profile and shows that in horses, like humans, a combination of Alum and MPLA (AS04) induces a higher level of IFNγ as compared to Alum alone (Didierlaurent et al., 2009).

Table 1
Mixed linear regression model of association between log-transformed levels of IgG subclasses and adjuvant and allergens with time (weeks after immunization) as repeated effect.

	log IgG1			log IgG1/3			log IgG4/7			log IgG5			log IgG(T)			log IgA		
Effect	Estimate	SE	*p*-Value	Estimate	SE	*p*-Value	Estimate	SE	*p*-Value	Estimate	SE	*p*-Value	Estimate	SE	*p*-Value	Estimate	SE	*p*-Value
Adjuvant																		
Alum/MPLA[a]	0.23	0.05	**<.01**	1.27	0.21	**<.001**	0.01	0.02	.60	−0.04	0.05	.48	−0.06	0.03	<.05	−0.06	0.03	.08
Allergen																		
Cul n 3[b]	0.20	0.03	**<.0001**	1.75	0.21	**<.0001**	−0.27	0.07	**<.001**	1.12	0.19	**<.0001**	0.20	0.04	**<.0001**	0.22	0.05	**<.0001**
Cul n 4[b]	0.26	0.03	**<.0001**	1.70	0.21	**<.0001**	0.01	0.07	.87	0.27	0.19	.18	0.23	0.04	**<.0001**	0.06	0.05	.21
Cul n 10[b]	0.12	0.03	**<.001**	1.29	0.21	**<.0001**	−0.02	0.07	.75	0.57	0.19	**<.01**	0.19	0.04	**<.0001**	0.23	0.05	**<.0001**
Week																		
2[c]	1.25	0.05	**<.0001**	2.94	0.21	**<.0001**	0.42	0.07	**<.0001**	2.00	0.20	**<.0001**	1.04	0.05	**<.0001**	nd		
6[c]	1.46	0.05	**<.0001**	3.12	0.21	**<.0001**	0.54	0.07	**<.0001**	2.43	0.20	**<.0001**	1.32	0.05	**<.0001**	0.60	0.05	**<.0001**
10[c]	1.47	0.05	**<.0001**	3.13	0.21	**<.0001**	0.53	0.07	**<.0001**	2.49	0.20	**<.0001**	1.36	0.05	**<.0001**	0.64	0.05	**<.0001**
16[c]	0.95	0.05	**<.0001**	2.44	0.21	**<.0001**	0.47	0.07	**<.0001**	2.08	0.20	**<.0001**	1.25	0.05	**<.0001**	0.34	0.05	**<.0001**
24[c]	0.61	0.05	**<.0001**	2.18	0.21	**<.0001**	0.28	0.07	**<.0001**	1.67	0.20	**<.0001**	1.07	0.05	**<.0001**	nd		
32[c]	1.33	0.05	**<.0001**	2.95	0.21	**<.0001**	0.50	0.07	**<.0001**	2.01	0.20	**<.0001**	1.27	0.05	**<.0001**	0.55	0.05	**<.0001**
Interactions																		
Adjuvant × week									ns			ns			ns			ns
Alum/MPLA × 2[d]	−0.15	0.07	**<.05**	−1.23	0.29	**<.0001**												
Alum/MPLA × 6[d]	−0.20	0.07	**<.01**	−1.25	0.29	**<.0001**												
Alum/MPLA × 10[d]	−0.21	0.07	**<.01**	−1.23	0.29	**<.0001**												
Alum/MPLA × 16[d]	−0.25	0.07	**<.01**	−1.39	0.29	**<.0001**												
Alum/MPLA × 24[d]	−0.23	0.07	**<.01**	−1.40	0.29	**<.0001**												
Alum/MPLA × 32[d]	−0.07	0.07	.28	−1.10	0.29	**<.0001**												
Allergen × week			ns			**<.0001**			**<.05**			**<.05**			ns			ns
Adjuvant × allergen × week			ns			**<.05**			ns			ns			ns			ns

nd = not done.

[a] *vs* Alum: these significant differences for IgG1 and IgG1/3 and the resulting significant interaction between adjuvans × week, were due to the fact that mean IgG1 and IgG1/3 levels at week 0 in the Alum/MPLA group were slightly but consistently higher than in the Alum group (data not shown).

[b] *vs* Cul n 8.

[c] *vs* week 0.

[d] *vs* Alum × corresponding week.

Notably, in our study Th1/Treg biased immune response induced by MPLA could not be detected in the antibody response.

4. Conclusion

In conclusion, our study confirms that also in horses, intralymphatic immunization enables the use of a small amount of allergens and relatively few injections for procuring a potent response. Importantly, because of the induction of a Th1/Treg immune response, Alum/MPLA seems an interesting combination for preventive allergen immunization. For further development of immunoprophylaxis and therapy against equine IBH these results need to be confirmed by determining the cytokine response using additional allergens and in allergen immunotherapy trials with IBH affected horses.

Conflicts of interest

No conflict of interest.

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