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Suppression of OVA-alum induced allergy by *Heligmosomoides polygyrus* products is MyD88-, TRIF-, Regulatory T- and B cell-independent, but is associated with reduced Innate Lymphoid Cell activation

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Abstract

The murine intestinal nematode *Heligmosomoides polygyrus* exerts multiple immunomodulatory effects in the host, including the suppression of allergic inflammation in mice sensitized to allergen presented with alum adjuvant. Similar suppression is attained by co-administration of *H. polygyrus* excretory/secretory products (HES) with the sensitizing dose of ovalbumin (OVA) in alum. We investigated the mechanism of suppression by HES in this model, and found it was maintained in MyD88xTRIF-deficient mice, implying no role for helminth- or host-derived TLR ligands, or IL-1 family cytokines that signal in a MyD88- or TRIF-dependent manner. We also found suppression was unchanged in μ MT mice, which lack B2 B cells, and that suppression was not abrogated when regulatory T cells were depleted in Foxp3.LuciDTR-4 mice. However, reduced IL-5 production was seen in the first 12 h after injection of OVA-alum when HES was co-administered, associated with reduced activation of IL-5⁺ and IL-13⁺ group 2 innate lymphoid cells. Thus, the suppressive effects of HES on alum-mediated OVA sensitization are reflected in the very earliest innate response to allergen exposure in vivo.

Introduction

Both epidemiological and experimental studies have established a consistent negative correlation between parasitic helminth infections and the incidence of allergic reactivity 1, 2, 3, 4. These observations have led both to a revised “hygiene hypothesis” - the proposition that in the absence of infections, the immune system is prone to hyperactivity 1, 5 - and to “helminth therapy”, the concept of using parasitic organisms as therapeutic agents against immune-mediated diseases 6, 7, 8. Furthermore, vaccination responses, dependent on effective Th2-mediated antibody production, have been shown to be less effective in

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parasite-endemic areas 9, 10. Hence it is widely thought that parasites release immunomodulatory products tailored to manipulate host immune responses, especially the type 2/Th2 pathway which generates both anti-helminth immunity and allergy 11, 12, 13, 14. In order to more completely define the molecular motifs and immune mechanisms by which helminths modulate type 2 responses, we and others have used the products of helminth parasites, administered into uninfected mice, to replicate the beneficial effects of infection 15, 16, 17, 18, 19, 20, 21.

The mouse intestinal nematode parasite, *Heligmosomoides polygyrus* exerts a broad range of immunomodulatory effects in the infected host, including suppression of allergy, autoimmunity and colitis 22. Many of these effects can be recapitulated by soluble *H. polygyrus* excretory/secretory products (HES), while in vitro HES is able to suppress dendritic cell maturation 23 and to induce regulatory T cells (Tregs) through the TGF- β R pathway 24. In the context of allergy, we recently showed that HES could replicate the suppressive effects of *H. polygyrus* infection, when administered at sensitization in asthma models driven by *Alternaria* fungal extract 21 or ovalbumin (OVA) co-precipitated onto alum adjuvant 17. In the *Alternaria* model of asthma, fungal allergens are introduced directly to the airways without recourse to exogenous adjuvant 25. In this setting, HES pre-emptively suppresses adaptive immune responses by suppressing the release of IL-33 immediately after administration of allergen into the lungs. This led to reduced group 2 innate lymphoid cell (ILC2) activation and reduced subsequent T cell-mediated inflammation in the lungs 21. In the OVA-alum model of asthma, mice are sensitized by intraperitoneal immunization of OVA with the type-2 promoting adjuvant alum 26, 27. In this model, a mechanism of suppression by HES has not yet been identified.

TLR ligands, such as bacterial endotoxin, when co-administered at sensitization with OVA-alum, can result in suppression of subsequent immune responses at challenge 28, 29. Although HES from our laboratory contained below-threshold levels of endotoxin contamination, helminth secretions are known to contain other TLR ligands 30, which potentially could act in a similar manner to suppress responses at sensitization. Furthermore, HES has recently been shown to induce the release of IL-1 β from macrophages, resulting in the suppression of pro-allergenic IL-25 and IL-33 responses, and a diminished Th2 response 31. Of note, for functional TLR and IL-1R responses, both of the signaling molecules MyD88 and TRIF are required, although alum-induced allergic responses are intact in mice lacking either or both of these adapter proteins 32, 33, 34.

In *H. polygyrus* infection, Tregs expand in the draining mesenteric lymph nodes, and can, when transferred to naïve mice, suppress allergic airway disease 35, 36. Likewise, Tregs induced in vitro by HES or mammalian TGF- β can be transferred into uninfected mice, and also suppress allergic immune responses in their adoptive hosts 24. When we investigated the effects of HES in mice receiving OVA-alum injections, however, we found reduced rather than greater Treg numbers in the lung; moreover, administration of recombinant mammalian TGF- β with equivalent activity to the TGF- β mimic in HES could not replicate suppression 17. These data argued against, but did not exclude, a role for Treg induction in suppression of asthmatic responses by HES in the OVA-alum model.

Previous work by Wilson *et al* showed that infection with *H. polygyrus* also results in a regulatory B cell population able to suppress allergic airway disease 37. These B cells expressed follicular B2 B cell markers, with suppression independent of IL-10. Regulatory B cells induced by schistosomes have also been shown to suppress responses in models of asthma 38, 39, 40 suggesting that the ability of parasites to drive regulatory B cells may be a common mechanism of asthma suppression. The effect on B cells is further emphasized by the potent suppression of B cell antibody responses observed in mice given HES with OVA-alum 17, although the role of regulatory B cells had not yet been investigated in this model.

Here, we test the hypotheses that HES could be suppressing alum-driven allergic immune responses through TLR or IL-1-family members signaling through MyD88 or TRIF, or regulatory B cell (Breg) or Treg induction. Using MyD88xTRIF double-deficient mice, we found that suppression was not associated with TLR or IL-1 family members signaling through these adaptor proteins. Furthermore, using μ MT and Foxp3.LuciDTR-4 mice, we found that suppression was unaffected in the absence of B2 B cells or with depletion of regulatory T cells. Instead we found that HES co-administration was associated with reduced early (<12 h post-administration) production of type 2 cytokines by group 2 innate lymphoid cells at the site of administration.

Materials and Methods

Parasites and reagents

The life cycle of *H. polygyrus bakeri* was maintained, and HES products prepared, as described elsewhere 41. Class IV Ovalbumin was purchased from Sigma, Gillingham, Dorset, UK.

Mice

BALB/c, C57BL/6, MyD88xTRIF-double deficient 42, μ MT 43 and Foxp3.LuciDTR-4 44 mice were bred in-house at the University of Edinburgh and accommodated according to Home Office regulations.

OVA-alum model

Induction of airway allergic inflammation was performed as previously described 17, 35. Briefly, mice were sensitized with two intraperitoneal injections (d0 and d14) of 20 μ g OVA precipitated with alum. At days 28, 29 and 30 mice were challenged by intranasal instillation of 20 μ g OVA in PBS. Mice were culled at either day 31, or at early timepoints, as indicated.

Tissue preparation

At cull, bronchoalveolar cells were prepared by lavaging lungs with 500 μ l PBS containing 0.5% BSA (for cytokine measurement in cell-free supernatant), followed by another three lavages to collect remaining cells. Single-cell suspensions of lung tissue were prepared by digesting the right lobes of the lung in 2 U ml⁻¹ liberase TL (Roche, Burgess Hill, UK) and 80 U ml⁻¹ DNase (Life Technologies, Paisley, UK) at 37°C with agitation for 35 min. Digested lung tissue were macerated through 70 μ m cell strainers. Peritoneal lavage cells were collected in one wash with 1 ml RPMI medium (for cytokine measurement in cell-free

supernatant), followed by 2 washes of a further 5 ml RPMI medium to collect remaining cells.

Flow cytometry

Cells were surface stained with fluorescently labeled antibodies to CD3, CD4, CD8 α , CD11b, CD11c, CD49b, Gr1, ICOS, SiglecF and TcR β , or the relevant isotype controls. For intracellular Foxp3 staining, the eBioscience Foxp3 Fix/Perm kit (eBioscience, Hatfield, UK) was used. For intracellular cytokine staining, cells were stimulated for 4 h at 37°C with 500 ng ml⁻¹ Phorbol Myristate Acetate, 1 mg ml⁻¹ Ionomycin, and 10 mg ml⁻¹ Brefeldin A (Sigma). Cells were surface stained, then permeabilized with the BD Biosciences Fixation/Permeabilisation kit (BD Biosciences, Oxford, UK) before staining for IL-5 and IL-13 (eBioscience). Live/dead Fixable Blue or Aqua dyes (Invitrogen) were used to exclude dead cells. Samples were analyzed by flow cytometry using Becton-Dickinson FACSCanto or LSR-II flow cytometers (BD Biosciences). Cell types were characterized as: CD11c⁺SiglecF⁺ (alveolar macrophages), SiglecF⁺CD11c⁻ (eosinophils), Gr1^{hi}CD11b^{hi}CD11c⁻SiglecF⁻ (neutrophils) and CD4⁺CD11b⁻ (T helper cells). ILC2s were characterized as lineage-negative (CD3/CD4/CD5/CD8 α /CD11b/CD11c/CD19/CD49b/GR1) and ICOS⁺.

Soluble cytokine measurement

IL-33, RELM- α , TSLP and Ym1 were measured by ELISA, using DuoSet kits (R&D Systems) according to the manufacturer's instructions. IL-1 α , IL-1 β , IL-4, IL-5, IL-10, and IL-13 levels were detected in cell-free BAL of peritoneal lavage supernatant using BD cytometric bead array Flex-set kits (BD Biosciences), and were acquired on a BD FACSArray.

Statistics

All data were analyzed using Prism 6 (Graphpad Prism, La Jolla, CA). When comparing 3 or more groups at a single timepoint, data were analyzed by one-way ANOVA, with a Tukey's post test. When comparing 2 groups at multiple timepoints (Figure 3), data were analysed by t tests, with a Holm-Sidak correction for multiple comparisons. Unless otherwise indicated differences are not significant. *** = P<0.001, ** = P<0.01, * = P<0.05.

Results

OVA-alum induced airway allergy, and its suppression by HES, do not require MyD88 or TRIF signaling

MyD88, together with TRIF, are central adapter molecules which transmit signals from cell receptors for Toll-like ligands and IL-1 family cytokines 45. To evaluate if these pathways are involved in HES-driven suppression of the allergic response to OVA-alum sensitization, we made use of MyD88xTRIF double-deficient mice, which (except in rare cases 46, 47) cannot respond to ligation of TLR and IL-1 family receptors 48. However, as shown in Fig. 1 A, B, the absence of these signaling pathways did not greatly affect the allergic response itself to OVA-alum, consistent with published reports 32, 33, 34. Thus, eosinophilia (Fig. 1 A), type 2 cytokines, and the type 2 response markers RELM- α and Ym1 (Fig. 1 B) were unchanged in OVA-alum treated double-deficient mice compared to C57BL/6 wild-types.

The low level of neutrophilia generally seen in the lung after challenge of wild-type mice, however, was ablated in double-deficient mice (Fig. 1 A), indicating that lung neutrophil recruitment is dependent on TLR and/or IL-1 family cytokine signaling, as previously reported 49 50 51 52.

Significantly, the potent suppression of allergic responses by HES administration was also fully intact in double-deficient mice. Thus in both genotypes, HES markedly reduced eosinophilia, type 2 cytokine production and expression of RELM α and Ym1 in the lungs to the same degree (Fig. 1 A, B). Therefore HES suppression of allergic immune responses elicited by alum adjuvant does not depend upon signaling from host or parasite ligands through MyD88- or TRIF-dependent TLRs or IL-1 family receptors.

Airway eosinophilia, and HES suppression in the OVA-Alum model, is independent of B cell populations

We next utilized μ MT mice to investigate the role of B cells in OVA-alum induced airway allergy and in HES suppression, which lack conventional B2 B cells and cannot mount allergen-specific antibody responses 53. In these mice, we found that airway eosinophilia was unchanged compared to wild-type (Fig. 2), indicating that, as previously indicated 54, B cells are not critically required for the recall eosinophil response in this model of asthma. Importantly, it was found that HES administration at sensitization caused equivalent suppression of airway eosinophilia to that seen in wild-type mice, indicating that B cells are not required for HES suppression of airway eosinophilia and excluding an inhibitory mechanism involving regulatory B cells.

Airway allergy is exacerbated by Treg depletion after sensitization, but HES suppression is unaffected

To assess the involvement of Foxp3⁺ Tregs in HES suppression of alum-induced allergy, we took advantage of a genetic model for Treg depletion through the Foxp3-dependent expression of the diphtheria toxin receptor in transgenic mice 44. We chose to deplete the Tregs after sensitization but prior to challenge, thereby removing pre-existing Tregs but allowing natural reconstitution of the regulatory compartment by the time of challenge. We chose this depletion regime as we hypothesized that if HES-induced OVA-specific Tregs were involved in the suppression of Th2 responses at challenge, these would be depleted between sensitization and challenge. Then, natural self-antigen specific Tregs (but not HES-induced OVA-specific Tregs) would be allowed to repopulate the host, resulting in a normalised immune system, and, we hypothesized, giving near-normal responses at challenge.

Accordingly, we depleted Foxp3⁺ cells by diphtheria toxin administration to Foxp3.LuciDTR-4 or wild-type mice, commencing 14 days after the second sensitization (Fig. 3 A). The Foxp3 transgene also drives GFP expression, allowing Treg numbers to be monitored during and after depletion. We found, as shown previously 44, that 5 daily injections of diphtheria toxin resulted in almost total depletion of GFP⁺ Tregs (data not shown), but left a small residual population of CD4⁺Foxp3⁺GFP⁻ Tregs (Fig. 3 B). Mice were then left untreated for 16 days to allow levels of Foxp3⁺ Tregs to recover 44, by which

time numbers in fact overshot the original levels (Fig. 3 B), as seen in other Foxp3-DTR studies 55, 56. Reflecting the “rebound” of Tregs in the post-depletion Foxp3.LuciDTR-4 mice, higher levels of CD4⁺Foxp3⁺ Treg proportions and numbers were recovered from the lungs in the transgenic mice (Fig. 3 C, D).

Following restoration of Tregs in the treated mice, all groups were then subjected to allergen provocation in the airways, with soluble OVA alone; no HES was given to mice at this challenge stage. As shown in Fig. 3 E, in both wild-type and Foxp3.LuciDTR-4 mice, the development of lung eosinophilia in response to OVA challenge was profoundly suppressed in animals exposed to HES during the sensitization phase. These results, although not conclusive, support our proposition that HES induction of Tregs is not crucial for suppression of alum-induced airway allergy.

It was interesting to note that higher levels of BAL eosinophils were seen in Foxp3.LuciDTR-4 mice compared to wild-types, implying that Treg depletion resulted in increased OVA-specific effector cell responses, even after reconstitution of the Treg population. One possible explanation is that removal of Tregs allows stronger T cell memory to be maintained, as it is noticeable that the magnitude of response in wildtype mice markedly declines over the longer 52-day protocol used in this experiment compared to the shorter 31-day protocol used in Fig. 1 (compare axes of Figs. 1 A and 3 E). This scenario would also account for the observation in a 34-day study in which depletion of Tregs prior to challenge in the OVA-Alum model did not affect airway allergy 57.

HES co-administration suppresses early innate cytokine responses to Alum

As neither Bregs and Tregs are required for the suppression of alum-induced immune responses by HES, we hypothesized that HES may interfere in a MyD88/TRIF-independent manner with the early innate response to alum administration, resulting in deficient TH2 induction and reduced allergic responses at recall. To test this, we assayed soluble cytokines released at the site of intraperitoneal injection in the first 24 hours following administration. Early innate “alarmin” cytokines were induced within the first 12 h after alum administration: IL-1 α , IL-1 β and IL-33 peaked at 2 h after OVA-alum injection, regardless of the presence of HES (Fig. 4 A-C). Levels of TSLP peaked later (4 h post-administration), and were maintained at increased levels to 12 h post-administration, showing a transient reduction at 6 h (which did not quite reach statistical significance: $p=0.066$) consequent on HES co-administration (Fig. 4 D). Levels of IL-25 were also measured, however were very low and did not exceed PBS control levels at any timepoint after OVA-alum administration, regardless of the presence of HES (data not shown).

Type 2 immune response cytokines IL-4, IL-5 and IL-13 were also measured in peritoneal lavage fluids. While IL-4 was not detectable in any samples, IL-5 was potently induced, peaking at 6 h post-OVA-alum administration and remaining at high levels to 12 h post-administration. A striking effect of HES co-administration was to reduce levels of IL-5 to near baseline throughout the time course assayed (Fig. 4 E). A similar, but much more limited, effect was observed with IL-13, however this was low and variable between experiments (data not shown). Levels of the TH1 and TH17 cytokines IFN- γ and IL-17A were also measured, but were consistently below the level of detection (data not shown).

Finally, levels of the regulatory cytokine IL-10 peaked at 1 h post OVA-alum administration, and although HES tended to suppress this response, this also did not reach significance (Fig. 4 F).

Alum administration results in local ILC2 activation, which is suppressed by HES

IL-5 is produced in large quantities by Th2 cells and ILC2s. As release of this cytokine peaked as early as 6-12 h post administration, it is implausible to attribute its production to OVA-specific TH2 cells, which would not yet have differentiated. Indeed, no T cell IL-5 expression could be seen at 12 h post-administration (data not shown). Therefore, we assessed ILC2 activation and cytokine production in the peritoneal cavity, using a flow cytometric gating strategy that analysed CD45⁺ICOS⁺ hematopoietic cells which were negative for a cocktail of markers for other cell lineages (Fig. 5 A).

In naïve mice, nearly 40% of ILC2s expressed detectable intracellular IL-5, in keeping with reports of constitutive IL-5 production by this cell type 58. In alum-immunized allergic mice, this proportion increased significantly, but when sensitized in the presence of HES, levels were reduced to those of naïve mice (Fig. 5 B, C). Similarly, IL-13 expression by ILC2s increased from a much lower baseline in OVA-alum-immunized mice, and increase which was entirely abolished in animals that had also received HES (Fig. 5 B, D). Hence, with respect to the two canonical markers of ILC2 activation, IL-5 and IL-13, both were completely reduced to the levels of unimmunized mice in the presence of HES.

Discussion

The products of helminth parasites have extraordinary capability to suppress a broad spectrum of immune responses to model immunogens, allergens, autoantigens and vaccines 4, 5, 12, 59, 60. We previously showed that *H. polygyrus* HES strongly inhibits pathology in the OVA-alum model of asthma, in which OVA-specific Th2 responses are induced by systemic injection of OVA protein adsorbed to an alum adjuvant 17. Although widely-used, the OVA-alum model is not the most physiologically relevant pathway of airway allergy induction 26. However, as alum remains the key adjuvant in most human vaccines, the modulation of responses following alum immunization is of both fundamental and practical importance. Moreover, helminth infections are associated with the suppression of both vaccine-induced and allergen-induced immune responses 61, leading us to investigate the mechanisms of suppression of HES against alum-induced immune responses.

We found that HES suppression of alum-induced type 2 responses was independent of MyD88- or TRIF-dependent TLR or IL-1-family cytokine signaling, B2 B cells or Foxp3⁺ regulatory T cells, as inhibition was maintained in MyD88xTRIF-deficient mice (which cannot signal through TLRs or IL-1 family receptors), μ MT mice (which lack B2 B cells), or in Foxp3⁺ Treg depletion (in Foxp3.LuciDTR-4 mice) respectively. It is interesting to note that eosinophilia in MyD88xTRIF double-deficient mice was unchanged in OVA-alum controls compared to wild-types, as has been shown previously with MyD88-deficient mice 28. This implies that MyD88- and TRIF-dependent TLR signaling, IL-1 and IL-33 are also not involved in induction of the Th2 response to alum, even though each of these players have a non-redundant role in the initiation of allergic responses through sensitization in the

airways 32, 62. **Some studies 46, 47 have shown evidence of MyD88- and TRIF-independent TLR and IL-1R signaling, therefore the possibility remains for a role for these TLR or IL-1 family members signaling through unconventional signaling pathways.**

The role of IL-1 β and the inflammasome in the adjuvant properties of alum is controversial: early reports suggested that activation of the NLRP3-dependent inflammasome (and IL-1 β release) was crucial for alum adjuvanticity 34, 63, but these findings were later challenged by reports showing that mice which lacked essential components of the inflammasome pathway produced normal Th2 responses to alum 64, 65, 66, 67, 68. Our results agree with a lack of a role for the inflammasome for Th2 induction by alum, as although inflammasome activation can take place in MyD88xTRIF-deficient mice, release of IL-1 β (or IL-18) is unlikely to produce a response.

Furthermore, we found that in our hands eosinophilia in μ MT mice was equivalent to wild-type controls with OVA-alum. A previous study using chronic repeated exposure to OVA aerosol resulted in a modest reduction in eosinophilia in μ MT mice compared to wild-type mice 54, and this disparity could reflect the role of antibody and B cells in more chronic exposure to allergen.

Finally, we found that depletion of Tregs between sensitization and challenge resulted in a far larger eosinophilic response in the lungs at challenge. Depletion of Tregs just prior to challenge does not result in any change in the magnitude of the immune response 57, indicating that the differences seen here are likely linked to the memory immune response. This may indicate that Tregs are involved in the waning of the memory immune response prior to allergen challenge, and potentially in tolerance induction to allergen.

We also assessed cytokine responses in the first few hours after injection of OVA-alum. In keeping with other reports, we found that alum induced a range of inflammatory cytokines at the site of injection within the first 12 h post-administration, including potent induction of IL-5 64. When HES was co-administered with OVA-alum, we found potent suppression of IL-5 release. We assessed ILC2 activation and found that ILC2-derived IL-5 and IL-13 were both induced by alum, and that this response was ablated by HES co-administration.

ILC2s have been implicated in the induction of TH2 responses, either through stimulation of dendritic cells by production of IL-13 69, or as they can express MHC class II, and present peptide antigens 70, 71. Conversely, T cell derived IL-2 can activate ILC2s and induce proliferation, so TH2 and ILC2 cells clearly cooperate in allergic responses 70, 71. Thus it may be that the ILC2 activation seen here could be crucial to TH2 induction after alum administration.

Recently, it was implied that ILC2s are dispensable for the induction of allergy in the OVA-alum model of asthma 72. Gold *et al* produced mice selectively deficient in ILCs by transfer of ROR α -deficient bone marrow to lethally irradiated wild-type recipients, and found no difference in allergic responses after OVA-alum injection. However, ILCs are known to be partially radioresistant 73, and after irradiation in this protocol a significant residual population of wild-type ILCs could be detected remaining in the lung 72, while ILCs

remaining in the peritoneal cavity were not assessed. Therefore, it could be that these residual ILCs are sufficient to induce a TH2 response to alum. More definitive analysis of this question may in future be possible with immunocompetent mice in which ILC2 can be specifically depleted, such as strains expressing an inducible ICOS-diphtheria toxin receptor (iCOS-T) mice 74.

Suppression of allergy and vaccine responses by helminth parasites may be the result of similar pathways: they both result from parasites suppressing host TH2 responses, which allow the parasite to survive in their host. Using models of asthma which depend on allergic responses dependent on allergen exposure in the airways 21 or systemic alum adjuvant injection 17, we have shown that the products of *H. polygyrus* can suppress allergic immune responses. In airway allergen sensitization, in which reactivity is known to be MyD88-dependent, HES suppresses through abrogation of IL-33 release, resulting in decreased ILC2 responses. Remarkably, even though alum-driven allergy is IL-33-independent, we show here that an ILC2 response is initiated, which is very effectively suppressed by HES within 12 h of alum injection.

In this study we have not identified the mechanism by which HES suppresses ILC2 responses. Our previous work showed that HES was incapable of suppressing ILC2 responses to exogenous IL-33 administration 21, indicating that the suppressive effects of HES are not ILC2-intrinsic, but rather act on the upstream cytokines which activate ILC2s, such as IL-33, IL25 and TSLP. Of note, HES coadministration tended to reduce TSLP release at 6 h post-administration. The TSLP pathway has both been shown to activate ILC2s 75, and to be essential for the allergenicity of alum 76, therefore suppression of TSLP, as well as IL-33, could be a novel feature of parasitic suppression of pro-allergic cytokines.

In conclusion, we have shown that the products of *H. polygyrus* are capable of suppressing alum-induced type 2 immune responses, by a mechanism independent of MyD88, TRIF, B cells or regulatory T cells, but associated with suppressed ILC2 responses. Further knowledge of the induction of ILC2s in the effective vaccination response to alum, how this is abrogated by parasite products, and how these effects could be avoided will be important future advances in the quest for more rational and effective design of vaccines in parasite endemic areas.

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Highlights

- *H. polygyrus* ES (HES) suppresses alum-induced type 2 responses in MyD88xTRIF doubly-deficient mice;
- HES does not require the presence of B cells to block alum-induced type 2 responses;
- Regulatory T cell depletion does not reverse suppression of alum-induced type 2 responses by HES;
- HES-administered mice show significant inhibition of Type 2 innate lymphoid cell activation.

Figure 1.

OVA-Alum $\pm$ HES was injected intraperitoneally into C57BL/6 or MyD88xTRIF double-deficient mice, and then challenged with intranasal OVA. Numbers of BAL cell subtypes (A) and BAL cytokines (B) were measured. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$, comparing OVA-alum to OVA-alum+HES groups within a mouse strain. $\phi\phi\phi$ = $p < 0.001$, comparing OVA-alum groups between wild-type and transgenic strains. Data are representative of 2 repeat experiments, 4-5 mice per group. Unless otherwise indicated, differences are not significant.

Figure 2.

OVA-Alum $\pm$ HES was injected intraperitoneally into C57BL/6 or μ MT mice, and then challenged with intranasal OVA. Numbers of BAL eosinophils were measured. Data are representative of 2 repeat experiments, 4-5 mice per group. * = $p < 0.05$, unless otherwise indicated, differences are not significant.

Figure 3.

The OVA-alum model was carried out, with depletion of Foxp3-DTR Tregs as shown in (A). Foxp3 expression in blood CD4⁺ T cells was assessed at day 33 (post-depletion) and 49 (pre-challenge), representative FACS plots shown in (B). Foxp3⁺ proportions of lung CD4⁺ T cells (C), lung CD4⁺Foxp3⁺ Treg numbers (D) and BAL eosinophils (E), were measured at cull. (DTR= Foxp3-Luciferase). Results are pooled from 2 repeat experiments, to give a total of 7-9 mice per group, error bars are standard error of mean. *** = P<0.001, ** = P<0.01, unless otherwise indicated, differences are not significant.

Figure 4.

PBS, OVA-alum or OVA-alum+HES were injected intraperitoneally as indicated, and levels of IL-1α (A), IL-1β (B), IL-33 (C), TSLP (D), IL-5 (E), and IL-10 (F) were measured in peritoneal lavage supernatants over a timecourse post-administration. An outlier for IL-33 levels at 12 h in the OVA-alum group is shown separately in C. Results are pooled from 2 repeat experiments, to give a total of 6 mice per group, per timepoint. Error bars are standard error of mean. *** = $P < 0.001$, ** = $P < 0.01$, comparing OVA-alum and OVA-alum+HES groups. Unless otherwise indicated, differences are not significant.

Figure 5.

Mice were injected with PBS, OVA-alum and HES as indicated, and 12 h later peritoneal lavage cells taken, stimulated with PMA, Ionomycin and BrefeldinA for 4 h at 37°C and intracellular cytokine staining in ICOS⁺CD45⁺lineage⁻ type 2 innate lymphoid cells (ILC2) identified by flow cytometry. Representative gating strategy for live, singlet, CD45⁺, ICOS⁺lineage⁻ (CD3⁻/CD5⁻/CD8 α ⁻/CD11c⁻/CD19⁻/CD49b⁻/GR1⁻), CD4⁻CD11b⁻ cells shown in (A), and representative IL-5 and IL-13 staining shown in (B). IL-5- (C) and IL-13- (D) positive

proportions of ILC2s were assessed in all mice. Results pooled from 2 replicate experiments, giving a total of 6 mice per group. **** = $p < 0.0001$, *** = $p < 0.001$.