

Letter to the Editor

[DOI: 10.1002/eji.201847774]

Intravenous immunoglobulin protects from experimental allergic bronchopulmonary aspergillosis via a sialylation-dependent mechanism

Aspergillus fumigatus has been implicated in causing life-threatening infections as well as severe asthma and allergic diseases. Allergic affliction like allergic bronchopulmonary aspergillosis (ABPA) is a disabling lung disease seen in atopic individuals and particularly in asthma and cystic fibrosis patients. ABPA is characterized by markedly elevated *Aspergillus*-specific IgE and total IgE levels, and eosinophilia. Both Th2 and Th17 cells are implicated in the pathogenesis of ABPA [1]. Corticosteroids are most commonly used to control ABPA but are associated with immunosuppression and resistance. Therefore, alternative immunotherapies that target inflammatory process without causing immunosuppression and toxicity are preferred.

Immunoglobulins from the pooled plasma of healthy donors are available in liquid formulations, suitable for i.v. or s.c. application (IVIG/SCIG) for the immunotherapy of diverse autoimmune and inflammatory diseases [2]. Experimental use of IVIG/SCIG in allergy and asthma has been described and protective

effects have been shown in experimental OVA-induced allergic airway inflammation (AAI) [3, 4]. However, IgG immunotherapy has never been explored in fungus-mediated allergic conditions. Based on several reports including ours showing that intravenous immunoglobulin (IVIG) reciprocally regulates Th17 cells that are one of the pathogenic cells in fungal allergic diseases, and Tregs that are immunoprotective [1, 5, 6], we hypothesized that IVIG therapy protects from experimental ABPA.

ABPA was induced in C57BL/6 (H-2^b) mice (Fig. 1A) [7] (See Supporting Information). Experiments were performed according to the Italian Approved Animal Welfare Assurance A-3143-01. Mice were sensitized by concomitant intraperitoneal (i.p.) and subcutaneous administration of *A. fumigatus* culture filtrate extract dissolved in incomplete Freund's adjuvant. Twice at weekly interval, the extract was instilled to these mice by intranasal route. At third week, bronchial colonization of *A. fumigatus* was induced (Fig. 1A). For the infection model, naïve mice were infected at third week for two consecutive days with resting conidia by intranasal route to induce bronchial colonization of *A. fumigatus* (Fig. 1B).

The protective role of IVIG (1g/kg, i.p. route) was evaluated both during the induction (prophylaxis) and the elicitation (therapy) phase of ABPA. For the prophylaxis, IgG was administered on day 0, 7 and 14 of induction of allergy. For the therapy, IgG was administered on day 21. The protective role of both native and desialylated IVIG was investigated. Desialylation of IVIG was confirmed by reverse phase HPLC (Fig. 1C). At day 28, mice were assessed for the various parameters of allergic airway inflammation.

IVIG-treated mice either under prophylaxis or therapeutic regime have shown minimal fungal burden in the lungs as assessed by CFU (Fig. 1D). In addition, eosinophils, one of the major players of allergic response to *A. fumigatus*, were mostly absent in the BAL fluid of IVIG-treated mice (Fig. 1E). Histopathology analysis of lungs confirmed the induction of extensive goblet cell hyperplasia in untreated allergic mice but not in IVIG group (Fig. 1F). Equimolar dose of human serum albumin, used as an irrelevant protein control for IVIG had no effect on allergic responses (Fig. 1D–F). The fungal growth and the occurrence of allergy were inversely related.

Studies in experimental model of autoimmune diseases have shown that α 2,6-sialic acid linkages at Asn297 of Fc-domain of IgG plays an important role in mediating the anti-inflammatory action of IVIG [8]. Sialylation of IgG was also reported to be critical for the protection against OVA-induced airway hyperresponsiveness (AHR) [4]. Therefore, we investigated if sialylation of IgG is key for the IVIG-mediated inhibition of hypersensitivity to *A. fumigatus*. We found that desialylated IVIG failed to inhibit inflammatory responses in the lungs and these mice had elevated fungal burden (Fig. 1D–F), thus confirming that sialylation of IgG is indispensable for rendering the protection against allergic response to *A. fumigatus*.

ABPA is characterized by a protracted Th2 and Th17 responses in the lungs, and experimental models have demonstrated that Tregs have a pivotal role in the protection [1, 7]. Analysis of CD4⁺ T-cell response in the thoracic lymph nodes by using isolated CD4⁺ T cells or in the lungs

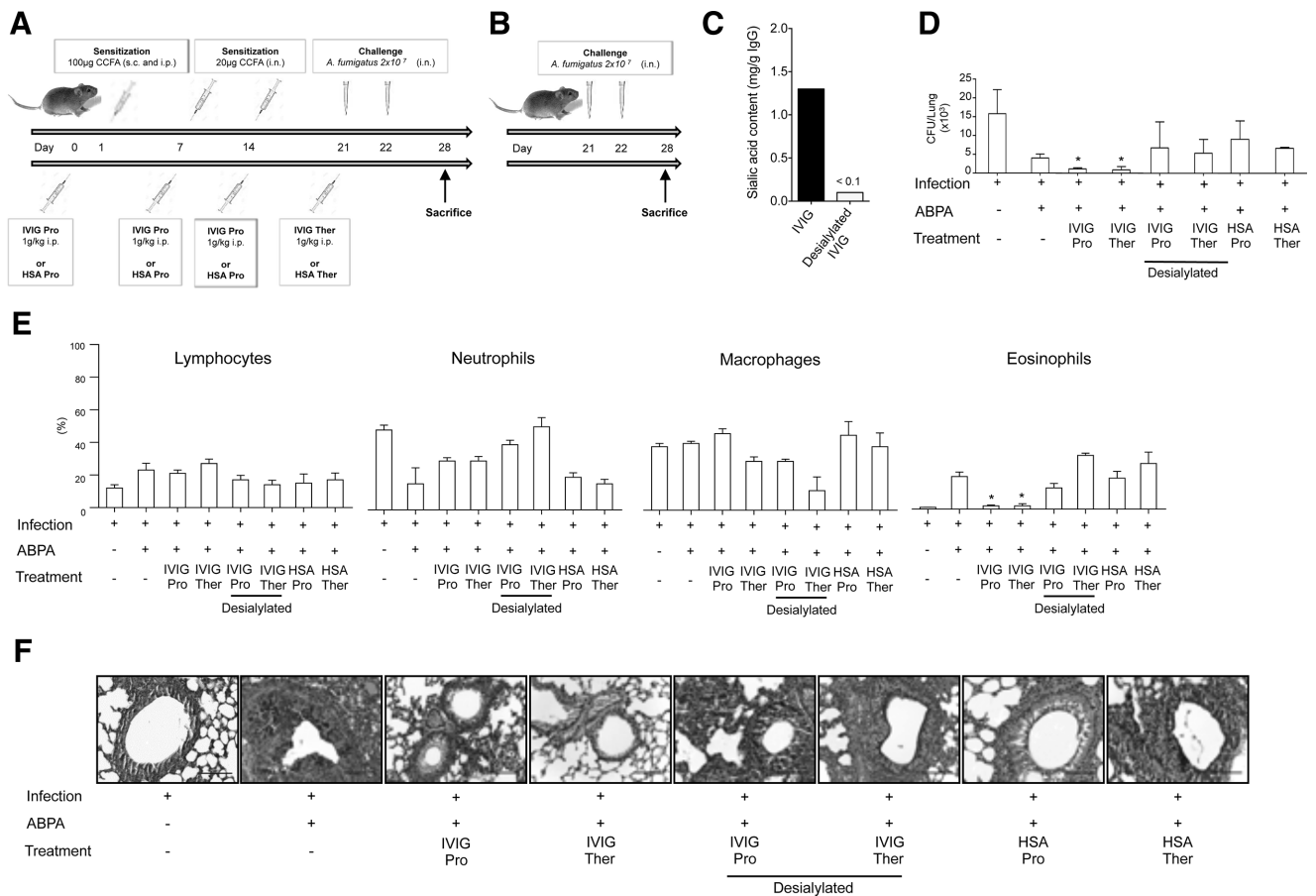


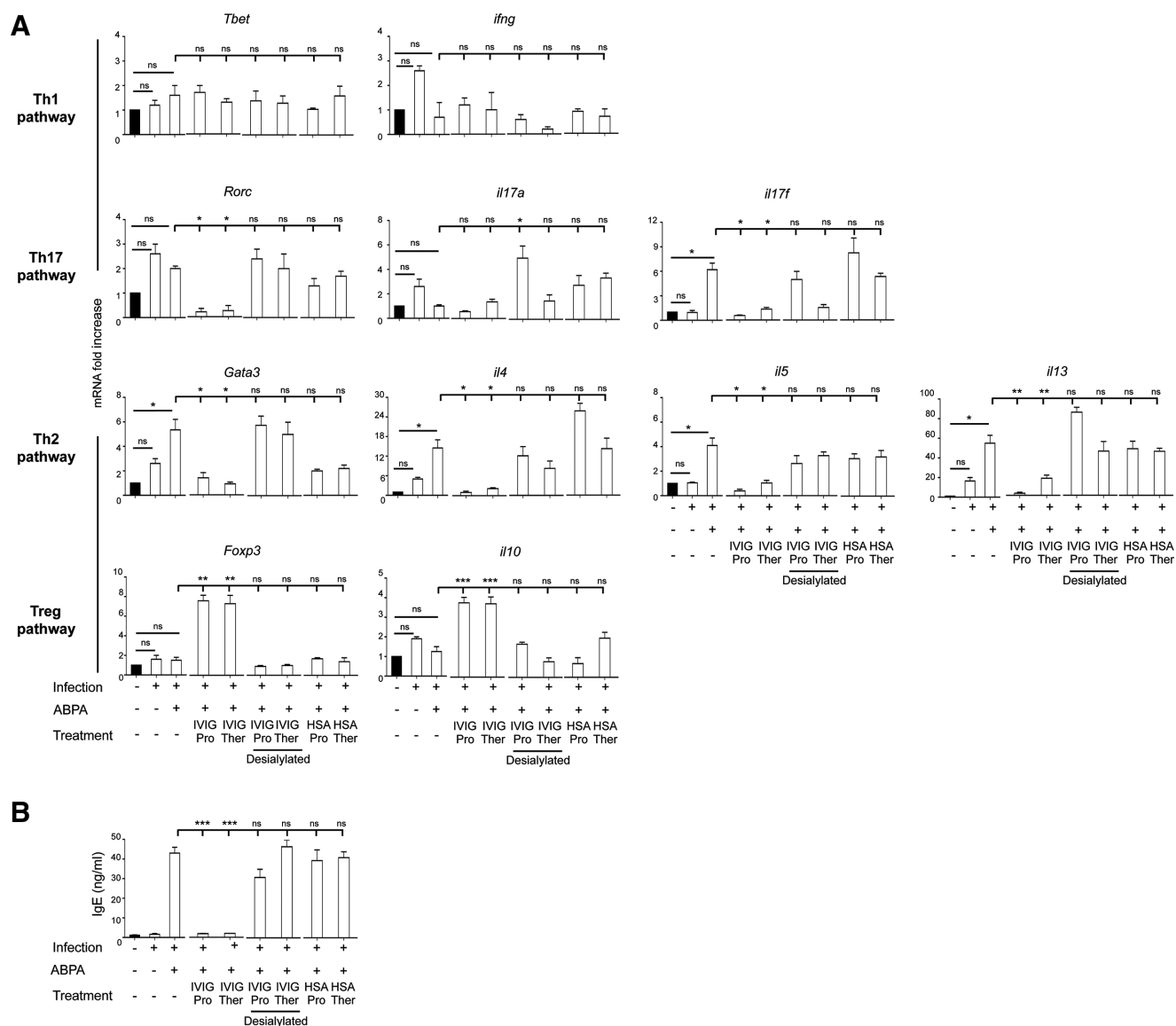
Figure 1. IVIG protects from experimental ABPA in a sialylation-dependent mode. (A) Schematic outline of experimental ABPA. For the sensitization, C57BL/6 mice received intraperitoneal (i.p.) and subcutaneous (s.c.) administration of crude culture filtrate antigens (CCFA) dissolved in incomplete Freund's adjuvant followed by intranasal (i.n.) instillation of CCFA. At third week, bronchial colonization of *A. fumigatus* was induced. For the prophylaxis treatment, IVIG (IVIG Pro) or HSA (HSA Pro) were administered in concomitance with CCFA sensitization. For the therapeutic regime, IgG were administered during elicitation phase of allergy (IVIG Ther). (B) Schematic outline of fungal infection model. (C) Sialic acid content of IgG as analyzed by reverse phase HPLC. (D) Lung colonization of *A. fumigatus*. (E) The abundance of immune cells in BAL fluid as analyzed by May-Grünwald Giemsa staining of cytospin preparations. (D–E) Data represent the mean $\pm$ SD of six mice per group and are representative of three independent experiments. * $p < 0.05$ Treated versus untreated allergic mice, one-way ANOVA (Bonferroni's post-hoc test). (F) Goblet cells in the lung sections ($\times 40$, scale bars = 200 μ m). Representative from at least three independent experiments. HSA: human serum albumin.

by quantitative RT-PCR assessment of either lineage-specific transcription factors (*Tbet*, *Rorc*, *Gata3*, *Foxp3*) or cytokines (*ifng*, *il17a*, *il17f*, *il4*, *il5*, *il13*, *il10*) revealed that hypersensitive response to *A. fumigatus* is characterized by predominant Th2-signature cytokines and *il17f* (Fig. 2A). However, Th1 and Treg responses were not altered in ABPA mice as compared to infected or control mice. IVIG significantly attenuated Th2-transcription factor *Gata3* and cytokines *il4*, *il5*, and *il13* gene expression. In addition, Th17 response, and *il17f* in particular, was also diminished in IVIG-treated mice (Fig. 2A). Interestingly, attenuation of Th2 and Th17 response by IVIG was associated

with reciprocal enhancement of Treg response and significantly increased *il10* (Fig. 2A). The inhibitory effect of IVIG on Th2 allergic response was also reflected in significantly decreased IgE levels in these mice (Fig. 2B). Again, desialylated IVIG lacked the ability to suppress Th2, Th17, and IgE responses (Fig. 2A, B). Further work is necessary to corroborate these data with intra cellular staining of T-cell subsets and cytokine analysis in the lungs at protein level.

It was reported that IVIG exerts anti-inflammatory actions through interaction of $\alpha(2,6)$ sialylated linkages at Asp-297 of Fc fragment with type II Fc receptors, such as DC-SIGN (or SIGN-R1) or

CD23 expressed on splenic myeloid regulatory cells [8]. This interaction induces IL-33 that acts on basophils for the production of IL-4. The action of IL-4 on effector macrophages enhances inhibitory Fc γ RIIB and renders them tolerogenic for the activation by immune complexes. However, this concept has met with controversies as other reports have demonstrated sialylation- and Fc γ RIIB-independent protective effects of IVIG (discussed in [5]). F(ab')₂ fragments of IVIG exerted protection similar to intact IVIG and reciprocally regulated Tregs and Th17 cells in EAE [6]. IVIG could protect from AAI by Fc γ RIIIA-dependent inhibitory effect on iNKT cells [3]. Also, Fc γ RIIB on dendritic cells



was dispensable for mediating protection against OVA-induced AHR [4], thus pointing out that IVIG might protect from experimental ABPA and modulate inflammatory responses independent of Fc γ RIIB. Further exploration is essential to dissect the role of Fc γ Rs and the contribution of F(ab')₂ versus Fc-fragments in IVIG-mediated protection against experimental ABPA.

Although sialylation of IgG is critical for the protection against ABPA, it is not clear whether sialylation of Fc- or Fab-domain mediates this effect. Fab region

is glycosylated in 15–20% of IgG and $\alpha(2,6)$ sialylated linkages are represented in nearly 70% of those IgG [9]. SIGN-R1 was initially reported as a receptor for sialylated Fc-domain, but subsequent report later confirms that SIGNR-1 has a partial role in mediating anti-inflammatory effects of IVIG [10]. Hence other lectin receptors could also mediate protective effects of IVIG. In fact, in OVA-induced AHR model, interaction of sialylated IgG with dendritic cell immunoreceptor (DCIR) on pulmonary CD11c⁺ dendritic cells but

not with SIGN-R1⁺ macrophages mediates protection [4]. Thus, the target receptors for sialic acid residues of IgG might diverge depending on the pathology and/or target organ. Future investigations should aim at exploring the mode of action of sialylated IgG in ABPA, in particular the requirement of DCIR and DC-SIGN receptors and the cell types that are targeted by IVIG.

To conclude, our data show that IVIG protects from experimental ABPA by sialylation-dependent mechanism. As safer therapy for ABPA is not yet available, IVIG

immunotherapy could be explored in ABPA patients.

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Conflicts of interests: Fabian Käsermann is an employee of CSL Behring, Switzerland.

land. Other authors have no conflicts of interests to declare.

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Abbreviations: AAI: allergic airway inflammation · ABPA: allergic bronchopulmonary aspergillosis · AHR: airway hyperresponsive-

ness · DCIR: dendritic cell immunoreceptor · DC-SIGN: dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin · IVIG: intravenous immunoglobulin · SIGN-R1: specific intercellular adhesion molecule-3-grabbing non-integrin-related 1

Keywords: ABPA · *Aspergillus fumigatus* · IL-17F · IVIG · Tregs

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The detailed *Materials and methods* for Technical comments are available online in the Supporting information

Intravenous immunoglobulin protects from experimental allergic bronchopulmonary aspergillosis via a sialylation-dependent mechanism

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	First Editorial decision:	26-Jul-2018
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	Accepted:	27-Sep-2018

Handling Executive Committee member: Prof. Annette Oxenius

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision - 26-Jul-2018

Dear Dr. Bayry,

Manuscript ID eji.201847774 entitled "Sialylation-dependent protection from experimental allergic bronchopulmonary aspergillosis by intravenous immunoglobulin" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter.

We have asked the Referees to assess your manuscript as a Letter to the Editor because we felt that without mechanistic insights your study may be a better fit for this article type. You will note that Referee 1 still prefers to keep a Short Communication format, however, this referee requested that mechanistic data should be provided. With this in mind the Executive Committee is proposing the following:

- 1) you can opt to perform additional mechanistic experiments and work on the revisions as a Short Communication; please note that if you decide for this option some of the mechanistic experiments have to be added for example the Treg cell depletion experiment is preferred. It would be nice to show

possibly involved Ig receptors suggested by the referee but this is not a strict requirement of the Executive Committee

2) we also offer you to convert your manuscript to a Letter to the Editor, and use the comments of referees as a guideline for revisions (without performing major experiments).

We do encourage you to choose option 1, however, we understand that this may not be possible and therefore option 2 would also be acceptable.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. Should you disagree with any of the referees' concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

You should also pay close attention to the editorial comments included below. **In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Please clarify if the data shown are combined from multiple experiments or rather representative. Failure to do this will result in delays in the re-review process.**

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Nadja Bakocevic

On behalf of Prof. Annette Oxenius

Dr. Nadja Bakocevic
Editorial Office
European Journal of Immunology

Reviewer: 1

Comments to the Author

The manuscript by Bozza et al. investigates the effect of intravenous immunoglobulin (IVIG) administration as a strategy to combat allergic bronchopulmonary aspergillosis (ABPA) in an experimental setup in mice. IVIG has been shown before to protect from various autoimmune and inflammatory diseases, whereby the mechanism of action remains unclear and controversial. More recently, IVIG was also proposed to be beneficial in case of allergic airway inflammation in an experimental OVA-induced model. Here, the authors evaluate the efficacy of IVIG against ABPA in mice. They observe a reduction in allergy and in the fungal load upon intravenous administration of IgG, but not of desialylated IgG, both in a prophylactic and a therapeutic setting. They further correlate this phenotype with a shift from a type 2 / type 17-dominated cytokine response towards a regulatory response, as assessed by quantifying cytokine transcripts and serum IgE levels. Although the statements made by the authors are plausible based on the presented results, the study would benefit from a couple of additional experiments to substantiate the conclusions and increase the relevance.

Specific comments:

1. In Figure 2, the direct analysis of T cell subsets by intracellular antibody staining and flow cytometry is needed to provide direct evidence for the contribution of different CD4⁺ T cell subsets and Treg cells, as it is stated in the abstract, the results section and the conclusions.
2. Linked to point 2: Why did the authors analyze cytokine transcripts in the lung and transcription factors in the thoracic lymph nodes. In case of the latter, were CD4⁺ T cells purified from thoracic lymph nodes as the legend suggests? The description of the methods used is insufficient.
3. In Line 105ff, it is unclear which comparison the authors are referring to. They should indicate the statistical significance for differences between infection and uninfected control groups and between infection+ABPA and control groups.
4. The host-beneficial effect of IVIG appears to be sialylation-dependent. The relevance for the role of sialylation in IVIG has been controversially discussed in the literature. To underpin their statements, the authors should add more mechanistic data on the mode of action of sialylated IgG. In other experimental systems, diverse receptors have been implicated. Are e.g. Fc receptors, DC-SIGN/SIGNR1 or DCIR involved here?
5. Linked to point 4: The authors seem to claim that Tregs are responsible for the observed phenotype. Can they provide experimental evidence for the protective effect of IVIG in their system, e.g. by depletion of Tregs, which presumably would reduce the efficacy of IgG administration.

Minor comments:

1. A schematic outline of the experimental setup would be helpful for a better overview and to explain what is meant with "infection" and "ABPA" in the figures.
2. Figure 1B: showing the quantification of one cell type in the different experimental groups per panel would be more logical and allow direct comparison of the different groups, as in Fig. 1A and in Fig. 2. The images below the bar graphs are not helpful. More representative images with more cells per image should be shown for each experimental condition. The legends are incomplete, they do not mention the BAL smears and how the quantification was made.
3. Figure 1C: a higher magnification of the PAS-stained histology sections should be shown and the goblet cells should be quantified using several fields per sections and sections from different animals.
4. Line 67: the sentence "reciprocally regulates Th17 cells" should probably be completed "reciprocally regulates Th17 cells and Tregs"
5. Lines 88-90 mentions sialylation at Asn297 to be important for the anti-inflammatory effects of IVIG, while lines 115ff refer to sialylation at Asp289. This is confusing and should be corrected/clarified.
6. The efficacy of desialylation by recombinant neuraminidase should be shown.
7. In the abstract, IVIG should be defined

Reviewer: 2

Comments to the Author

The manuscript by Bozza and colleagues addresses a very interesting question. The authors show that IVIG protects mice from experimental allergic bronchopulmonary aspergillosis in a sialylation dependent manner. This finding is clearly of major importance for the field and I have only a few concerns.

Specific points to be considered:

The manuscript would be strengthened by showing if the IVIG Fc-fragment is sufficient to protect from allergic aspergillosis. If this is technically not feasible the authors may discuss this.

Some references are not cited in a correct way. For example Ref 22 does not show that type II FcRs are involved in IVIg activity. This study should be cited later in the text when discussing IVIG activity in human settings. The authors state in this section that especially in human settings IVIG activity was not dependent on sialic acid residues, which is only true in part. Ref 22 uses humanized mice to show that in sialylation is indeed required for IVIG activity in the context of a human immune system in vivo.

Ref 19 may be used to underscore the statement of the authors that SignR1 may not be involved in IVIG

activity under therapeutic conditions. The study clearly shows that therapeutic IVIG application in mice does only partially (to a minor extend) rely on SignR1 especially in inflammatory model systems.

First Revision – authors’ response 29-Aug-2018

Dear Prof. Oxenius,

Thank you very much for the e-mail dated 26th July 2018 with a decision on our manuscript eji.201847774. As the current funding for the project is already over, we would like to accept your offer to convert our manuscript to a ‘Letter to the Editor’. We used the comments of referees as a guideline for revisions without performing major experiments.

Please find enclosed revised manuscript entitled “Sialylation-dependent protection from experimental allergic bronchopulmonary aspergillosis by intravenous immunoglobulin” by Bozza et al., for the consideration in ‘European Journal of Immunology’ as ‘Letter to the Editor’.

We have revised the manuscript taking into consideration of reviewers’ comments. We have included additional figures that include experimental outlines (Fig 1A and B) and sialylation content following desialylation of IgG (Fig 1C). In addition, the presentation of graphs (Fig 1E) and denotation of statistical significance (Fig 2 A and B) have been modified. The revised discussion includes all the points raised by both the reviewers.

The instructions from the Journal staff have been followed for preparing the revised manuscript. We hope that our manuscript is now suitable for the publication in ‘European Journal of Immunology’

With best wishes,

Luigina ROMANI and Jagadeesh BAYRY

Reviewer: 1

Comments to the Author

The manuscript by Bozza et al. investigates the effect of intravenous immunoglobulin (IVIG)

administration as a strategy to combat allergic bronchopulmonary aspergillosis (ABPA) in an experimental setup in mice. IVIG has been shown before to protect from various autoimmune and inflammatory diseases, whereby the mechanism of action remains unclear and controversial. More recently, IVIG was also proposed to be beneficial in case of allergic airway inflammation in an experimental OVA-induced model. Here, the authors evaluate the efficacy of IVIG against ABPA in mice. They observe a reduction in allergy and in the fungal load upon intravenous administration of IgG, but not of desialylated IgG, both in a prophylactic and a therapeutic setting. They further correlate this phenotype with a shift from a type 2 / type 17-dominated cytokine response towards a regulatory response, as assessed by quantifying cytokine transcripts and serum IgE levels. Although the statements made by the authors are plausible based on the presented results, the study would benefit from a couple of additional experiments to substantiate the conclusions and increase the relevance.

Specific comments:

1. In Figure 2, the direct analysis of T cell subsets by intracellular antibody staining and flow cytometry is needed to provide direct evidence for the contribution of different CD4⁺ T cell subsets and Treg cells, as it is stated in the abstract, the results section and the conclusions.

Response) We thank the reviewer for the suggestion. As Tbet, RORc, Gata3 and FoxP3 are well known and T cell subset specific transcription factors of Th1, Th17, Th2 and Treg cells respectively, we opted for transcript analysis by using isolated CD4⁺ T cells from the thoracic lymph nodes. In line with suggestions from Editorial Board, we have decided to revise the article as 'Letter to Editor' and hence accepting the comment of reviewer, we have included this point as a limitation of our study. (lines 120-122)

2. Linked to point 2: Why did the authors analyze cytokine transcripts in the lung and transcription factors in the thoracic lymph nodes. In case of the latter, were CD4⁺ T cells purified from thoracic lymph nodes as the legend suggests? The description of the methods used is insufficient.

Response) Our previous data showed overlapping cytokine signatures (protein) in the lungs and transcription factor-based T cell subset analysis in the thoracic lymph nodes (Bozza et al J Immunol 2009). As we could not perform analyses of both cytokines and transcription factors from the available material, in order to judiciously use the precious material from the tissues (both for molecular studies

and histopathology), we opted for cytokine transcripts in the lung and transcription factors in the thoracic lymph nodes and are validated by several other articles (Bozza et al. J Immunol 2014; Fontaine et al. PLoS Pathog 2011; Bonifazi et al Mucosal Immunol 2010).

Nevertheless, in line with suggestions from Editorial Board, we have decided to revise the article as 'Letter to Editor'. Therefore, acknowledging the reviewer's comment, we have indicated in the revised manuscript that transcript analyses of cytokines and T cell subset-specific transcription factor should be validated in the future by protein analysis. (lines 120-122)

As correctly pointed out by the reviewer, CD4+ T cells were purified from thoracic lymph nodes. We have now detailed the description of the methods used in supplement methods. (lines 108-109; lines 222-223 of Figure 2 legend; lines 49-54 in Supporting Supplement file)

3. In Line 105ff, it is unclear which comparison the authors are referring to. They should indicate the statistical significance for differences between infection and uninfected control groups and between infection+ABPA and control groups.

Response) We have now corrected the statement as "Th1 and Treg responses were not altered in ABPA mice as compared to infected or control mice" (lines 112-113).

In addition, we have now edited the Fig 2 to highlight the statistical significance between "infection and uninfected control groups" and "infection+ABPA and control groups".

4. The host-beneficial effect of IVIG appears to be sialylation-dependent. The relevance for the role of sialylation in IVIG has been controversially discussed in the literature. To underpin their statements, the authors should add more mechanistic data on the mode of action of sialylated IgG. In other experimental systems, diverse receptors have been implicated. Are e.g. Fc receptors, DC-SIGN/SIGNR1 or DCIR involved here?

Response) We thank the reviewer for the excellent suggestion. In line with suggestions from Editorial Board, we have decided to revise the article as 'Letter to Editor' and hence acknowledging the reviewer's concern we have included a short discussion on the role of Fc receptors, DC-SIGN/SIGNR1 or DCIR in

mediating protective effects of IVIG in experimental ABPA. (lines 124-152)

5. Linked to point 4: The authors seems to claim that Tregs are responsible for the observed phenotype. Can they provide experimental evidence for the protective effect of IVIG in their system, e.g. by depletion of Tregs, which presumably would reduce the efficacy of IgG administration.

Response) We again thank the reviewer for this excellent suggestion. But we would like to clarify that we have never claimed that Tregs are responsible for the observed phenotype. Recently, it has been demonstrated that in the absence of endogenous Tregs, IVIG failed to confer protection against OVA-induced airway inflammation and that adoptive transfer of IVIg-generated Tregs rendered protection against challenge (Massoud AH et al. J Immunol 2017). Also our previous data in experimental autoimmune models showed that inhibition of Tregs or their depletion leads to abrogation of IVIG-mediated protection (Ephrem et al. Blood 2008 and Trinath et al. 2013). Although we do not claim Tregs are responsible for the protection in our report, as presented in the manuscript, we believe that combination of reciprocal regulation of Th17 and Tregs, inhibition of Th2 cells and associated effects on the other inflammatory cells and cytokines mediate protection against ABPA.

Minor comments:

1. A schematic outline of the experimental setup would be helpful for a better overview and to explain what is meant with ‚infection‘ and ‚ABPA‘ in the figures.

Response) We have now included a schematic outline of the experimental setup in the revised manuscript (New Fig. 1A and B)

2. Figure 1B: showing the quantification of one cell type in the different experimental groups per panel would be more logical and allow direct comparison of the different groups, as in Fig. 1A and in Fig. 2. The images below the bar graphs are not helpful. More representative images with more cells per image should be shown for each experimental condition. The legends are incomplete, they do not mention the BAL smears and how the quantification was made.

Response) This comment concerns modified Fig. 1E of revised manuscript.

In line with suggestion of the reviewer, we have now presented the graph showing the quantification of each cell type in the different experimental groups per panel (Fig 1E of revised manuscript). Also, we have decided to remove BAL smear to avoid overloading of Figure 1. The legends (lines 213-214) and Supplement supporting method sections (lines 40-45) are now modified to detail how the quantification of various immune cells was made.

3. Figure 1C: a higher magnification of the PAS-stained histology sections should be shown and the goblet cells should be quantified using several fields per sections and sections from different animals.

Response) This comment concerns modified Fig. 1F of revised manuscript.

We are presenting 40x magnification of the PAS-stained histology sections. As representative histology sections are self-explanatory, to avoid overloading of Figure, we have opted not to go with quantification of goblet cells.

4. Line 67: the sentence „...reciprocally regulates Th17 cells...” should probably be completed „...reciprocally regulates Th17 cells and Tregs...”

Response) We believe that reviewer has misjudged the sentence. In fact, the sentence is complete. It has two parts: first part on Th17 and its pathogenic role in ABPA, and second part Treg and its protective role.

5. Lines 88-90 mentions sialylation at Asn297 to be important for the anti-inflammatory effects of IVIG, while lines 115ff refer to salylation at Asp289. This is confusing and should be corrected/clarified.

Response) We regret oversight and it has been corrected now (line 125)

6. The efficacy of desialylation by recombinant neuraminidase should be shown.

Response) We have provided in Fig 1C the analysis of sialic acid content by reverse phase high performance liquid chromatography.

7. In the abstract, IVIG should be defined

Response) We have replaced IVIG with 'intravenous immunoglobulin'. However, due to space restrictions, we could not define further IVIG.

Reviewer: 2

Comments to the Author

The manuscript by Bozza and colleagues addresses a very interesting question. The authors show that IVIG protects mice from experimental allergic bronchopulmonary aspergillosis in a sialylation dependent manner. This finding is clearly of major importance for the field and I have only a few concerns.

Specific points to be considered:

1) The manuscript would be strengthened by showing if the IVIG Fc-fragment is sufficient to protect from allergic aspergillosis. If this is technically not feasible the authors may discuss this.

Response) We thank the reviewer for this excellent suggestion. Because of technical feasibility to obtain large amounts of Fc fragments, we have discussed the role of Fc to protect from allergic aspergillosis (lines 124-139)

2) Some references are not cited in a correct way. For example Ref 22 does not show that type II FcRs are involved in IVIg activity. This study should be cited later in the text when discussing IVIG activity in human settings. The authors state in this section that especially in human settings IVIG activity was not dependent on sialic acid residues, which is only true in part. Ref 22 uses humanized mice to show that in sialylation is indeed required for IVIG activity in the context of a human immune system in vivo.

Response) As 'Letter to Editor' manuscript could contain max 10 references, we have removed many relevant references including Ref 22. But we have discussed on sialylation of IgG and mechanisms of IVIG (lines 141-152).

3) Ref 19 may be used to underscore the statement of the authors that SignR1 may not be involved in IVIG activity under therapeutic conditions. The study clearly shows that therapeutic IVIG application in mice does only partially (to a minor extend) rely on SignR1 especially in inflammatory model systems.

Response) Thank you for the suggestion. We agree with the reviewer comment on Ref 19 that shows that therapeutic IVIG application in mice does only partially (to a minor extend) rely on SignR1 especially in inflammatory model systems. We have included these point in the discussion (lines 141-152).

Second Editorial Decision -13-Sep-2018

Dear Dr. Bayry,

It is a pleasure to provisionally accept your manuscript entitled "Sialylation-dependent protection from experimental allergic bronchopulmonary aspergillosis by intravenous immunoglobulin" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

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We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
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on behalf of
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**Intravenous immunoglobulin protects from experimental allergic
bronchopulmonary aspergillosis via a sialylation-dependent mechanism**

Supporting Information

Methodology

Animals

Female, 8- to 10-week-old inbred C57BL6 (*H-2^b*) mice were obtained from Charles River Breeding Laboratories (Calco, Italy). Experiments were performed according to the Italian Approved Animal Welfare Assurance A-3143-01.

Aspergillus fumigatus infection, allergy and treatments

Viable conidia from the *Aspergillus fumigatus* Af293 strain were obtained as described [1]. For allergic broncho-pulmonary aspergillosis (ABPA), C57BL6 mice were sensitized by concomitant intraperitoneal (i.p) and subcutaneous administration (100 µg) of *A. fumigatus* culture filtrate extract (cell culture filtrate antigens (CCFA) dissolved in incomplete Freund's adjuvant (Sigma-Aldrich, USA) [2]. Twice at weekly interval, 20 µg of the extract was instilled to these mice by intranasal route. At third week, bronchial colonization of *A. fumigatus* was induced by administering resting conidia (2×10^7) by intranasal route. IgG (IgPro10/IgPro20) (1g/kg, Privigen®/Hizentra®, CSL Behring, Switzerland) or equimolar dose of human serum albumin (HSA, Laboratoire Français du Fractionnement et des Biotechnologies, France) was administered in mice with ABPA during the induction phase (prophylaxis) or the elicitation (therapy) phase of allergy. For the prophylaxis, IgG was administered i.p. on day 0, day 7 and day 14 of induction of allergy. For the therapy, IgG was administered on day 21 by i.p. route. For infection, C57BL6 mice were anaesthetized (by inhalation of 3% isoflurane (Forane Abbot) in oxygen) and infected by the intranasal instillation of 2×10^7 resting conidia/20 µl saline.

At day 28, the mice were evaluated for the allergic airway inflammation by i)

histopathological examination of lung tissues; ii) evaluation of bronchoalveolar lavage (BAL) fluid for the neutrophils, eosinophils and mononuclear cells; iii) evaluation of cytokine response in the lung tissues and CD4⁺ T cell subset (Th1, Th17, Th2 and Treg)-specific transcription factors in the purified CD4⁺ T cells of thoracic lymph nodes; and iv) determination of IgE in the sera of the mice.

Desialylation of IVIG

IVIG was desialylated by enzymatic digestion with recombinant neuraminidase (New England BioLabs, USA) as previously reported and desialylation was confirmed by reverse phase high performance liquid chromatography [3]. The content of sialylation in IVIG following neuraminidase treatment was >0.1 mg/g of IgG.

Histology and collection of bronchoalveolar lavage (BAL) fluid

Sections (3–4 µm) of paraffin-embedded tissues were stained with the periodic acid-Schiff (PAS) procedure. Bars indicate magnifications. Lungs were filled thoroughly with 1-ml aliquots of pyrogen-free saline through a 22-gauge bead-tipped feeding needle introduced into the trachea. BAL fluid was collected in a plastic tube on ice and centrifuged at 400 × g at 4°C for 5 min. For differential BAL fluid cell counts, cytopsin preparations were made and stained with May-Grünwald Giemsa reagents (Sigma-Aldrich, USA) before analysis. At least 200 cells/field/10 fields were counted, and the absolute number of each cell type was calculated.

ELISA and real-time RT-PCR

The total IgE in the serum samples was measured by ELISA as previously described [4]. Gene expression of cytokines mRNA was determined on total lung cells by real-time RT-PCR. Cells were lysed and total RNA was reverse transcribed with cDNA Synthesis Kit

(BioRad, Italy), according to the manufacturer's instructions. Transcription factors mRNA were determined in CD4⁺ T cells from thoracic lymph nodes. CD4⁺ T cells from the thoracic lymph nodes were separated by magnetic cell sorting with MicroBeads and MidiMacs (Miltenyi Biotec S.r.l, Italy). Real-time RT-PCR was performed using the Stratagene Mx3000P QPCR System and SYBR Green chemistry (Stratagene Cedar Creek, USA). Cells were lysed and total RNA was reverse transcribed with cDNA Synthesis Kit (BioRad, Italy), according to the manufacturer's instructions.

The sequences of gene-specific primers were as follows: *Foxp3*: forward, CAG CTG CCT ACA GTG CCC CTA G; *Foxp3*: reverse, CAT TTG CCA GCA GTG GGT AG; *Gata3*: forward, GAA GGC ATC CAG ACC CGA AAC; *Gata3*: reverse, ACC CAT GGC GGT GAC CAT GC; *ifn γ* : forward, TGA ACG CTA CAC ACT GCA TCT TGG; *ifn γ* : reverse, CGA CTC CTT TTC CGC TTC CTG AG; *Tbet*: forward, GGA CGA TCA TCT GGG TCA CAT TGT; *Tbet*: reverse, GCC AGG GAA CCG CTT ATA TG; *Rorc*: forward, AGC AGT GTA ATG TGG CCT AC; *Rorc*: reverse, GCA CTT CTG CAT GTA GAC TG; *il10*: forward, CCC TTT GCT ATG GTG TCC TT; *il10*: reverse, TGG TTT CTC TTC CCA AGA CC; *il4*: forward CGG CAT TTT GAA CGA GGT CAC AGG; *il4*: reverse, AGCACCTTGGAAGCCCTACAGACG; *il5*: forward, AAAGAGAAGTGTGGCGAGGA; *il5*: reverse, GCACAGTTTTGTGGGGTTTT; *il13*: forward, AGCTGAGCAACATCACACAA; *il13*: reverse, GAATCCAGGGCTACACAGAA; *il17a*: forward, GACTACCTCAACCGTTCCAC; *il17a*: reverse, CCTCCGCATTGACACAGC; *il17f*: forward, CCCTGGAGGATAACACTGTGAGAG; *il17f*: reverse, TTCCTGACCCTGGGCATTGATG; *Gapdh*: forward, CTGCCCAGAACATCATCCCT; *Gapdh*: reverse, ACTTGGCAGGTTTCTCCAGG.

Amplification efficiencies were validated and normalized against *Gapdh*. The thermal profile for SYBR Green real-time PCR was as follows: 95°C for 10 minutes, followed by 40 cycles

of denaturation for 30 s at 95°C and an annealing/extension step of 30 s at 72°C. Each data point was examined for integrity by analysis of the amplification plot.

Statistical analysis

Statistical significance was calculated by a one-way ANOVA (Bonferroni's post hoc test). Data were analyzed by GraphPad Prism 4.03 program (GraphPad software, USA). We considered all P values ≤ 0.05 as significant. The data shown in each figure represent the mean of three or more independent experiments. The *in vivo* groups consisted of six mice/group.

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