A novel mechanism for induction of Tregs to control allergic disease

Authors

[1,2]Gorczynski RM,
[2]Hoffmann G

Affiliations

[1]Universityof Toronto, ON, Canada

[2]Network Immunology, Vancouver, BC, Canada,

Correspondence to:

Dr R. Gorczynski, 429 Drewry Avenue, Toronto, ON, M2R2K6, Canada

Email:
reg.gorczynski@utoronto.ca

Tel: 647-267-9739

Supported by: R Gorczynski and Network Immunology, Vancouver, BC

Abstract

In previous studies we reported immune regulation of colitis, transplanted tissue, and breast cancer in mice could be achieved by modifying immune networks using combinations of immune Ig and anti-idiotypic Ig. We have explored features of this regulation as it relates to altered allergic responses to ovalbumin, exploring attenuation of allergic reactivity in animals receiving combinations of either mouse or heterologous (human) Igs. Our data show a long-lasting suppression of allergic reactivity in mice (>14 weeks post cessation of treatment with Igs). This was seen both in mice receiving treatment from the time of allergen administration (suppression of induction of allergy), as well as in mice already sensitized to allergens before treatment began (suppression of pre-existing symptoms). In either case, attenuation of symptoms was abolished by infusion of T deleting antibodies during the time of Ig administration. Further confirming an importance for induction of ($CD4^+$) Tregs in suppression of allergic responses, we showed that after enrichment, these cells derived from pre-immunized mice treated with combinations of Igs suppressed OVA-induced Th2 responses in cells from fresh populations of immunized animals in vitro. We hypothesize that this treatment regimen opens up new avenues of approach to the treatment of chronic allergic disease in man and in other animals.

Keywords: Immunoregulation; idiotypic networks; allergic responses; IL-4; IgE

1. Introduction:

Allergic and atopic disorders are a significant global problem. Allergen specific immunity results in priming of IL-4, IL-5 and IL-13 cytokine producing Th2 type cells which augment IgE production by B cells along with eosinophil activation and recruitment and bronchial hyperreactivity [1-3]. Th17 cells and their cytokines, and Tregs, further regulate the allergic response [4-6].

While non allergen specific therapies are commonly used for treatment, including liberal use of anti-histamines, steroids and other anti-inflammatory reagents directed at the leukotriene cascade, specific subcutaneous Immunotherapy (SCIT: often referred to as desensitization therapy, [7,8]) is a more intriguing focused treatment with recognized efficacy and safety [9-11]. A limitation to SCIT, or more recent sublingual immunotherapy (SLIT) is that the allergen used must be carefully identified and purified for use in treatment. Moreover the mechanisms of action of allergen specific immunotherapy remain ill-understood, though it is thought that Treg development and suppression of production of Th2 type cytokines is critical [12,13], with newer therapies aimed at targeting Tregs directly [14]. A further issue with SLIT/SCIT is the concern that treatment must be given seasonally and or continuously to maintain efficacy [15].

Against this background we became interested in whether the application of immune network theory, as initially proposed by Jerne [16] might assist development of alternative strategies to regulate allergic responses. There is a broad literature concerning the importance of such networks in diverse fields, including the regulation of immune memory and more recently, as a novel strategy for development of anti-HIV immunity [17-22]. We recently reported on the use of a combined injection of polyclonal anti-idiotype antibodies, along with polyclonal immune antibodies, as a treatment which augmented immunoregulation in rodents in a number of models, including an inflammatory colitis model and metastatic breast cancer model; a transplant model; and a rodent allergy model induced in response to ovalbumin (OVA) [23]. The regulation seen was independent of the specific antigens used to prepare the polyclonal immune Ig. We hypothesized that in the last model at least, perturbation of regulatory T cell networks implicated in control of allergic immunity was an important mechanism of action [24]. In addition we predicted, based on shared germ-line V region genes across vertebrate species [16] that immunoregulation could be induced using treatment not just with immune Ig and anti-idiotype Ig from the same species (homologous), but across species (heterologous Ig treatment)-recent data has been presented supporting this claim, using human Igs in mice, along with additional evidence that T cells are implicated in regulation of suppression of Th2 activation in naïve mice by homologous anti-idiotype+immune Ig [25].

In the current study we have reviewed previous data and addressed several other important issues. Firstly, we confirm that attenuation of immunity to OVA occurs in mice receiving both homologous (mouse) and heterologous (human derived) immune Ig and anti-idiotype Ig (see also [26-31]). We show that after cessation of human Ig treatment, the attenuation of Th2 immunity, defined by IgE levels and antigen induced IL-4 and IL-13 production persists > 14 weeks. Finally, we confirm that desensitization in OVA-immune mice can be achieved using human reagents, and that $CD4^+$ enriched T cells from these treated animals can suppress production of Th2 cytokines from a new population of OVA-immune cells stimulated

in vitro. Using mAbs known to expand Treg [24,32,33], we show increased suppression of Th2 responses in immune mice treated with combined human Igs after infusion of these mAbs, with increased regulatory activity in $CD4^+$ cells isolated from those same mice.

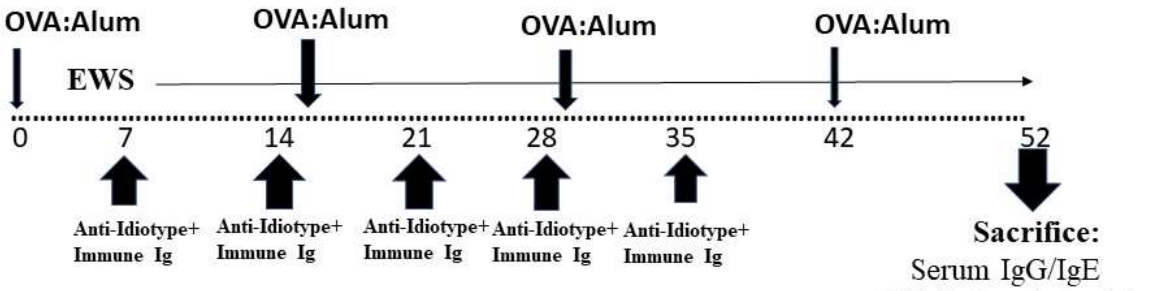
3. Materials and Methods

3.1Mice

All mouse experiments were conducted after ethics approval of a local animal care committee, in compliance with the Canadian Council on Animal Care (CCAC). Female BALB/c, C3H and C57BL/6 mice were purchased from Jax Labs, Bar Harbor Maine. All mice were housed 5 per cage and maintained at the Cedarlane animal facility. Mice were used at 8 weeks of age.

3.2Induction of IgE after Ovalbumin (OVA) Immunization

BALB/c mice were immunized to produce IgE against OVA in a protocol described elsewhere [23,25] and in Figure 1. Animals received 10μg OVA emulsified in alum in 0.2ml PBS at days 0, day 14, 28 and 42 with exposure from day 7 to egg white solution (EWS) in their drinking water. The treatment protocol to attenuate development of a Th2-type (IgE/IL-4/IL-13 dominated allergic response) involved weekly intramuscular (im) injections, beginning at day 7 post first OVA immunization, of 75μg of pooled polyclonal anti-idiotype Ig and 10μg of pooled polyclonal immune Ig (Figure 1).



Mouse Igs: *anti-idiotype:* C3H anti-anti-C3H Ig (C3H anti-BL/6 absorbed with BL/6 (X)

immune Ig: BL/6 anti-C3H

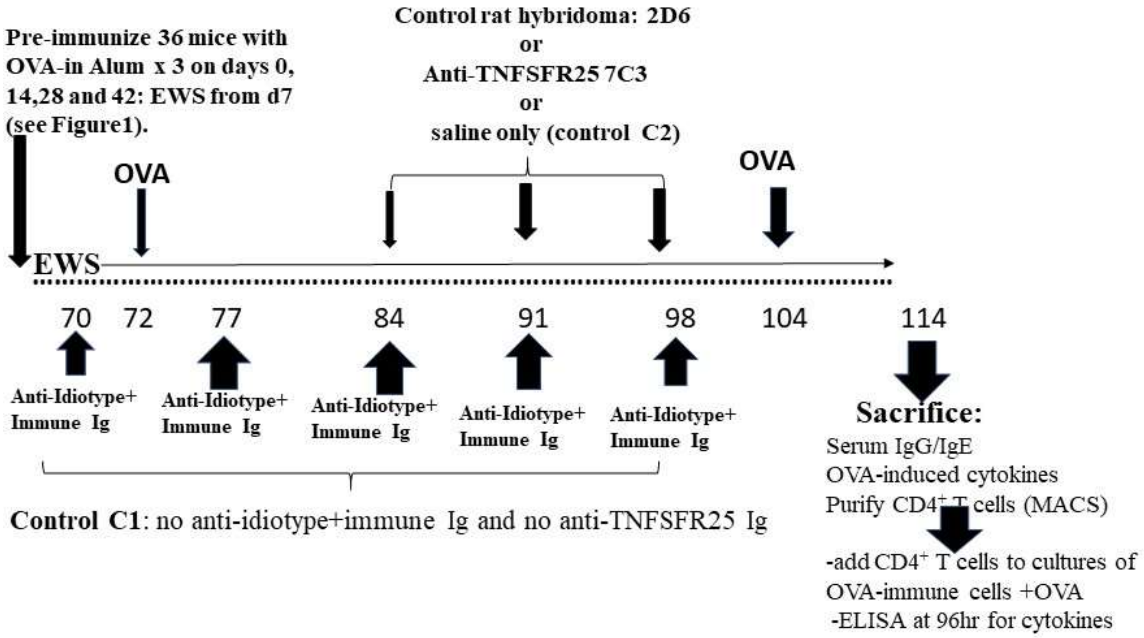
Human Igs: *anti-idiotype:* Commercial IVIG (Grifols: (24))

immune Ig: Commercial anti-Tet Ig (Grifols: (24))

Legend to Figure1: Schematic showing protocol for OVA immunization, and sequence of delivery of immune Ig and anti-idiotype Ig (given im in separate locations), to mice as a method to attenuate Th2-induced immunity (measured by suppression of OVA-specific IgE levels and OVA-induced cytokines). Both homologous (mouse) and heterologous (human) Igs were used. Data for such studies are shown in subsequent Figures (3&4).

This was of mouse [23] or human origin (the latter 75microgram of human IMIG or 10microgram human anti-Tet Ig (both from GRIFOLS, Canada [25]). All mice received a boost of OVA in alum 7 days following treatment with sacrifice 10d after the final immunization for analysis of serum OVA-specific IgE/IgG and production of OVA-induced IL-2/IL-4/IL-13 over 72hr in culture from stimulated splenocytes. When pre-

immune mice were treated to suppress Th2 immunity, a group of mice were immunized with OVA-Alum as above (days 0,14,28,42, with EWS from d7), rested for 4 weeks, and then began 5 serial injections with combined anti-idiotype and immune Igs from day 70 as shown in Figure 2.



Legend to Figure2: Schematic showing protocol for assessment of the ability of combined human anti-TetIg Ig and IMIG to attenuate Th2-induced immunity (measured by suppression of OVA-specific IgE levels and OVA-induced cytokines) in pre-immunized mice. Also shown is the timing of additional iv infusions of anti-TNFSFR25 mAb 7C3, or control antibody 2D6, or saline, in studies exploring the role of TNFSFR25$^+$ Tregs in suppressing Th2 immunity. CD4+Tregs were subsequently recovered from mice at the times shown using MACS columns,and used in vitro to suppress OVA-induced IL-4 cytokine induction from fresh OVA-immune cells (Figures 5&6).

Mice received further boosts with OVA in Alum on days 72 and 104, with sacrifice on day 114 to measure serum IgG/IgE and OVA-stimulated cytokines in vitro.

In studies examining possible mechanism(s) responsible for inhibition of Th2 immunity inn pre-immunized mice resulting from the Ig treatments described, mice also received infusions of either control rat monoclonal Ig or a mAb to TNFSFR25, known to expand Tregs implicated in regulation of immunity [24,32,33]-see Figures 2&5. Putative Tregs were subsequently harvested from pooled (6 donors/group) splenocytes of all treated mice and enriched for CD4$^+$ cells on MACS columns (Miltenyi Biotech). Enriched cells were stimulated for 48hr on anti-CD3-coated dishes with 2μg/ml anti-CD28 and 10ng/ml TGFβ [32], harvested and washed x3, and added to OVA-stimulated splenocytes prepared from a pool of 3 OVA-immune mice. 72hr culture supernatants were assayed for IL-2 and IL-4 (Figures 2&6).

3.3Measurement of serum IgE and serum IgG to OVA, and of antigen (or mitogen)-induced cytokine production by splenocytes in ELISA

Blood was obtained from mice by tail vein sampling (Figure 4) or at sacrifice by cardiac puncture, along with spleen. 5x10^6 spleen cells from individual animals were cultured in alpha MEM with 10% fetal calf serum (αF10)for 72hr with 0.1μg/ml

OVA and culture supernatants assayed by commercial ELISA kits for IL-2, IL-4 and IL-13 (BioLegend, CA, USA) production..

OVA-specific IgE or IgG was measured in all serum samples by ELISA using plates coated with 100ng/well of OVA and developed with HRP-anti-mouse IgE or HRP anti-IgG (ThermoFisher), followed by appropriate substrate.

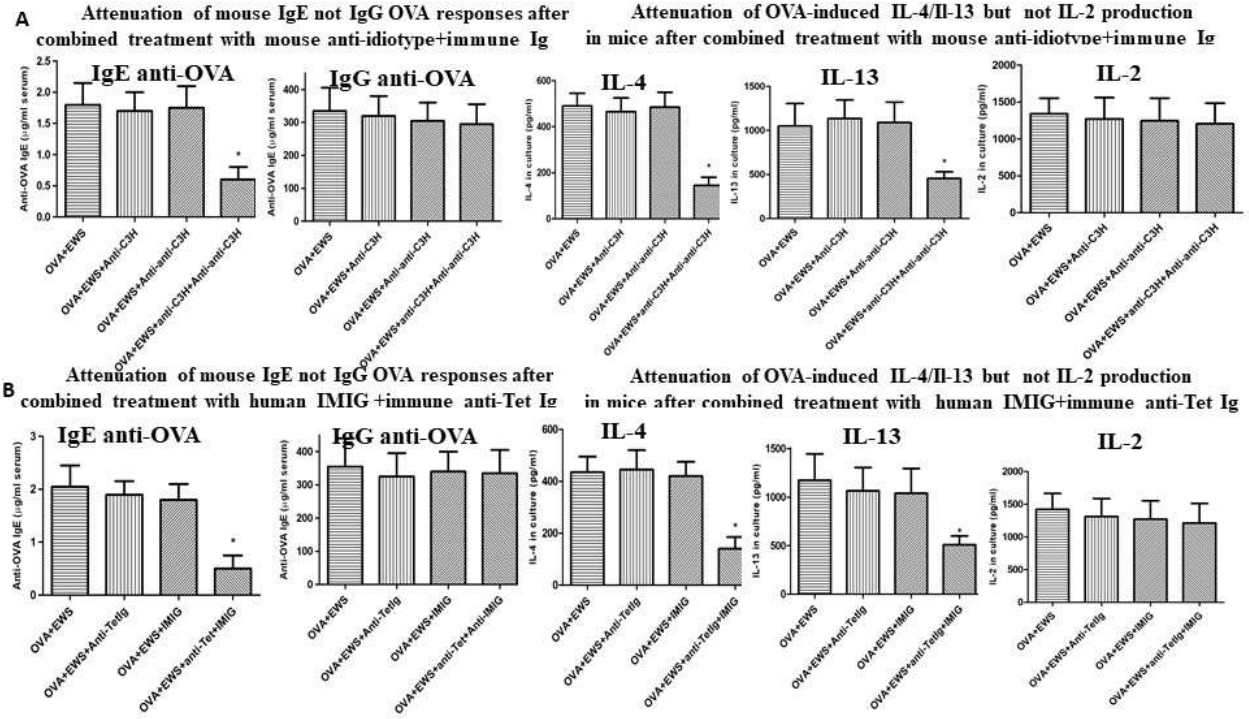
3.4Statistics

All experiments were performed at least twice, with a minimum of 16 mice in all groups. For studies with multiple groups, a multivariate analysis of variance (MANOVA) test was first applied to assess for any significant differences between groups, and subsequently, paired *t*-tests used to compare individual groups with the documented control.

4. Results

4.1 Combined mouse (human) immune Ig and anti-idiotype Ig, attenuate OVA-induced Th2 immunity

Data in Figure 3 confirms previous reports that BALB/c mice receiving a mixture of antibodies designed to stimulate idiotype: anti-idiotypic interactions [23,25] as in Figure 1 show attenuation of an OVA Th2-allergic type response, as assayed by suppression of serum IgE levels or antigen-induced IL-4/IL-13 production in vitro, with preservation of serum IgG levels and IL-2 production [25].

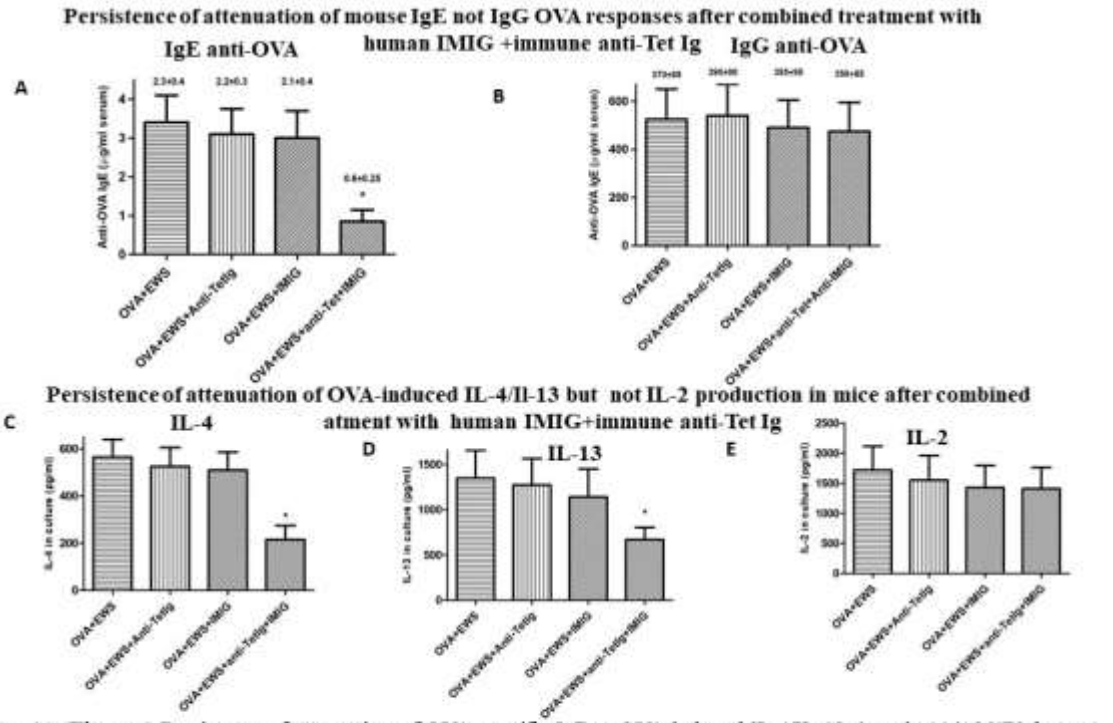


Legend to Figure3: Attenuation of OVA-specific IgE and OVA-induced IL-4/IL-13 but not IgG and OVA-induced IL-2 in groups of naïve BALB/c mice immunized with OVA in alum (see Figure1) and receiving combinations of mouse immune serum, with/without mouse anti-idiotype serum (panel A), or the human counterparts (panel B). All mice received egg white solution (EWS) in their drinking supply as in Figure1. Data show mean $\pm$ SD of IgE/IgG levels in serum, or 72hr-induced cytokines in splenocyte cultures in animals receiving a final boost of OVA in alum 7d after the final immunoglobulin treatment and sacrificed 10d later. The control group to the far left in each subpanel received OVA and EWS only.
*, $p<0.05$ compared with all other groups in each subpanel.

Our initial studies had used (as in the upper panels **A** of Figure 3) homologous mouse Igs (23,25) but we have subsequently shown, as is evident also in the lower panels **B** of this Figure, that human IMIG and anti-Tet Ig produce an equivalent attenuation of IgE and IL-4/IL-13 production to that seen with the murine counterparts [25]. This is consistent with the hypothesis that the immunoregulation seen occurs through perturbation of a T (and B) cell immune idiotypic network in the host animals, whose V-region domains are shared across vertebrate species [16]. We recently found also that human immune Ig (anti-Tet) and human anti-idiotype Ig, IMIG, also produce desensitization of skin allergic responses (to peanut butter) in dogs [34]. It is important to note that serum from mice receiving human Ig as in Figure 1produced no detectable anti-human Ig reactivity as reported elsewhere [25], as expected given the low doses of human Ig given, and the absence of adjuvant used.

4.2 Attenuation of IgE production, and of OVA induce IL-4 release, persists >14 weeks post Ig treatment with anti-idiotype and immune Ig:

To assess the persistence of the suppression of IgE and IL-4 production in animals receiving the 5-week Ig treatment with IMIG and anti-Tet Ig given in Figures 1&3, the same groups of mice received the boost with OVA in alum on d42, as shown in Figure1, with only a test blood sample obtained from the tail (50μl) 10d later (d52). OVA-specific IgE and IgG were measured by ELISA (see figures in parentheses above histograms in panels A/B of Figure 4).



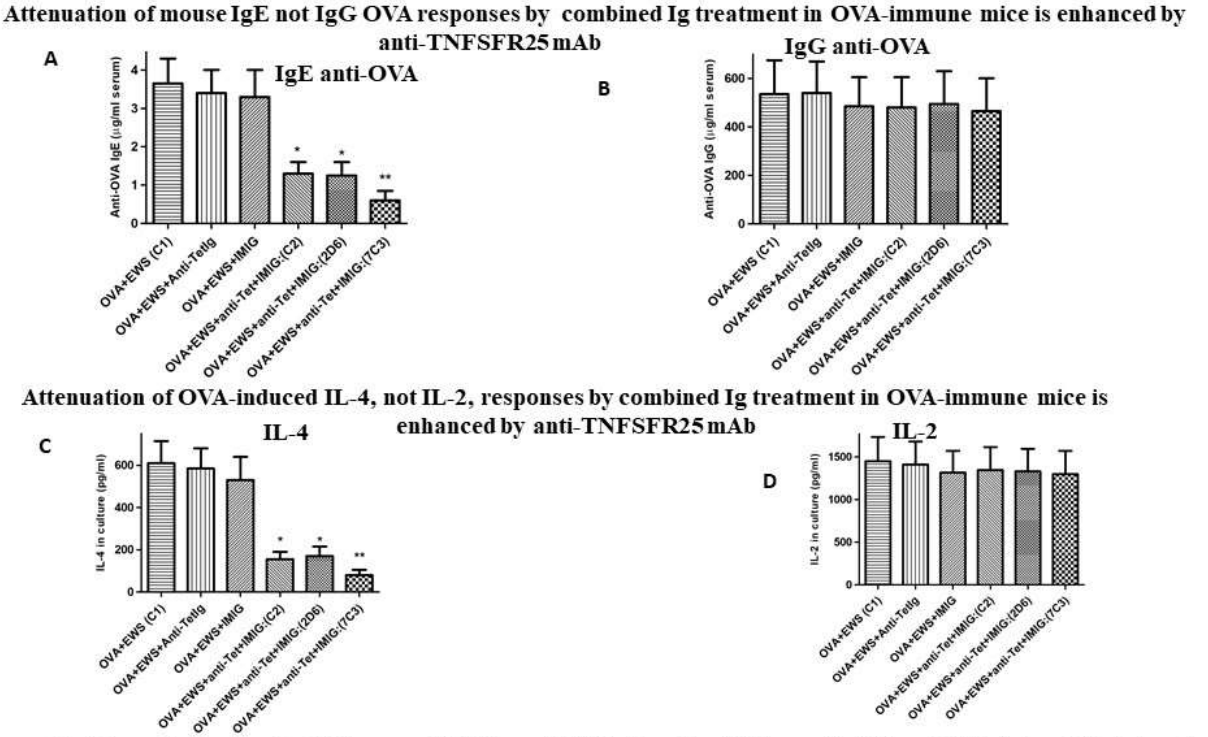
Legend to Figure 4: Persistence of attenuation of OVA-specific IgE or OVA-induced IL-4/IL-13 (panels 4A/4C/4D) but not IgG or OVA-induced IL-2 (panels 4B/4E) in mice treated as in Figures 1&3 with human immune Ig and IMIG, and rested for 10 weeks (but remaining on EWS in the drinking supply) before receiving a final boost of OVA in alum and sacrificed 10d later (day 132)-this represents 14 weeks post the last combined IG treatment. IgE and IgG levels were measured from a tail vein sample at d52 (shown above the histogram bars representing levels at 132d in serum). *, $p<0.05$ compared with all other groups in the same subpanels.

Animals remained exposed to EWS in the drinking water for a further 10 weeks (to d122), but without further Ig treatments. They then received a final boost of OVA in alum (with sacrifice and harvest of splenocytes and serum 10d later on d132, with serum OVA-specific IgE and IgG measured (Figure 4A/B) along with OVA-induced IL-4, IL-13 and IL-2 from splenocytes stimulated for 72hrs in vitro (Figure 4C/D/E).

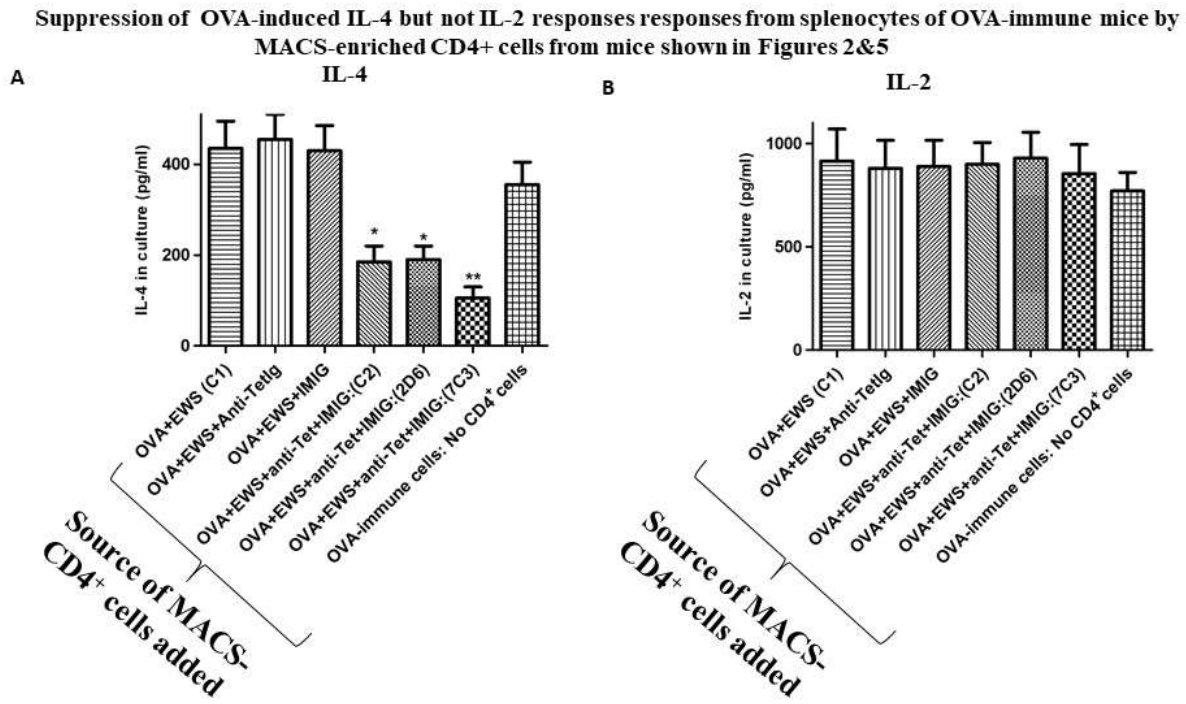
These data confirm that from an earlier study performed in mice receiving homologous (mouse) Igs [25]. The attenuation of OVA-specific IgE, but not IgG, already evident immediately after completion of combined Ig treatment (seen numbers in parentheses above histograms in Figures 4A/B) persisted in the absence of further Ig treatments when animals were reimmunized and re-tested some 14 weeks post the last Ig injection (panels 4A/B). The same conclusion was reached following analysis of OVA-induced IL-4 and IL-13 production in vitro (compare panels 4C/D with data in the lower panels of Figure3), with again no attenuation of IL-2 production following Ig treatment (Figure 4E).

4.3 Importance of $CD4^+$ Tregs in desensitization of OVA-immune animals by combined treatment with human anti-Tet IG and IMIG:

In a previous study we reported that the attenuation of Th2 immunity seen in mice receiving combinations of immune Ig and anti-idiotype Ig was abolished by infusion of anti-$CD4^+$ and anti-$CD8^+$ antibodies into treated mice [25]. In naïve mice receiving combinations of mouse immune Ig and anti-idiotype Ig, further evidence implicating an important role for Tregs in suppression of immunity was obtained with the observation that suppression was enhanced if mice received, in concert with the combined mouse Ig treatment, infusions of monoclonal antibodies to TNFSFR25 [25]. Such treatment has been shown elsewhere to expand populations of $CD4^+$ human and murine Tregs [25,32,33]. We have expanded upon these data by characterizing $CD4^+$ cells isolated by MACS columns from, in this case, pre-immunized mice treated in vivo with human IMIG and anti-TetIg along with anti-TNFSFR25 antibody. We explored the ability of these enriched $CD4^+$ cells obtained from mice with/without treatment with anti-TNFSFR25, to suppress IL-4 production in vitro from a separate preparation of OVA-immune splenocytes. The protocol used for this study is shown in Figure 2, with subsequent data presented in Figures 5&6.



Legend to Figure 5: *T*reatment with human anti-TetIg and IMIG attenuates OVA-specific IgE and OVA-induced IL-4 (panels 5A/5C) but not IgG or OVA-induced IL-2 production (panels 5B/5D) in OVA-immune mice, with further suppression after added infusion of anti-TNFSFR25 mAb. 36 mice were immunized with OVA in alum as in Figure2. 6mice/group received human anti-TetIg and/or IMIG as in Figure 2-a control group (C1) received no human Igs. Subgroups of mice receiving both the anti-TetIg and IMIG also received 3 iv infusions of saline only (C2), an inactive anti-TNFSFR25 (2D6), or a high binding anti-TNFSFR25 mAb (7C3) at the times shown in Figure2. OVA was given 6d after the last human Ig treatment with sacrifice 10d later.. *, $p<0.05$ compared with Control C1. **, $p<0.05$ compared with Control C2.



Legend to Figure 6: Suppression of 72hr OVA-induced IL-4, but not IL-2, production from OVA-immune cells in vitro using MACS-enriched $CD4^+$ cells from mice shown in Figures 2 and 5. $4x10^6$ immune splenocytes pooled from 3 OVA-immune mice (4x immunization with OVA in alum over 8 weeks) were stimulated in triplicate alone (far left in Figures 6A/B) or after addition of $1x10^6$ MACS-enriched $CD4^+$ cells from pooled mice of the 6 groups shown in Figures 2&5. Data show mean $\pm$ SD of cytokines in culture supernatants.
*, $p<0.05$ compared with OVA immune cells (no $CD4^+$ cells added); ** $p<0.05$ compared with control C2.

Data in Figure 5 confirm that the combined treatment with anti-TetIg and IMIG did indeed suppress mouse Th2 responses in pre-immunized mice as defined by attenuation of OVA-IgE serum antibody (Fig5A), or OVA-induced IL-4 in vitro (Fig 5C)-compare C2 with C1 in both panels. No suppression of OVA-IgG or OVA-induced IL-2 responses was seen (Figures 5B/D). Importantly, additional infusion of a high binding anti-TNFSFR25mAb (7C3), but not saline (C2) or a non-binding anti-TNFSFR25 mAb (2D6), further augmented the suppression of Th2 immunity seen (** in panels of Figure 5A/C vs * in the same panels).

When we compared the ability of MACS-enriched $CD4^+$ cells prepared from pooled splenocytes of the 6 groups shown in Figure 5 for their ability to inhibit IL-4 production from a separate population of OVA-immune cells, the data of Figure 6 were obtained. Clearly $CD4^+$ cells from mice receiving both anti-TetIg and IMIG suppressed IL-4, but not IL-2 production from immune cells in vitro. This effect was most pronounced (2-fold greater inhibition) using $CD4^+$ cells obtained from mice receiving in addition 7C3 mAb (** in Figure 6), compared with saline (C2) or 2D6 mAb (* in Figure 6). These data are consistent with our findings reported earlier investigating suppression of Th2 immunity in naïve mice [25].

5. Discussion:

Most newer immunotherapies designed for treatment of allergic diseases are directed at decreasing both IgE levels, and production of those cytokines (e.g. IL-4,

IL-5, IL-31) implicated in regulating IgE producing B cells and other cells (eosinophils/basophils) associated with allergic disease [35-38]. These methodologies add to the previously discussed SCIT (SLIT) therapies which in general depend upon identification and purification of unique allergens causing pathological responses [8-11; 14, 15]. As a radical alternative to such treatment we have proposed a novel therapy aimed at manipulating self-reactive immune regulatory networks through deliberate perturbation of idiotype: anti-idiotype interactions [23,25] There is already a body of literature supporting the rationale for such therapy, using human IVIG, in a number of autoimmune scenarios, including allergic diseases [26-31]-reviewed in [31] .

IVIG has been used in a variety of clinical conditions, initially primarily related to immunodeficiency [39], but subsequently in a number of off-label scenarios where it has shown proven efficacy [40-46]. The role of natural antibodies (whose production is presumed to be driven by self-antigens) in the IVIG preparations in such protection, has been recently reviewed [47]. Interestingly, primary immunodeficiency in adults, particularly IgG3 subclass deficiency, has a known correlation with asthma, and recent data suggests consideration of IVIG replacement therapy for this group, though no known mechanism was suggested for the effects seen [48]. In a recent study which explored possible mechanism(s) of action of IVIG in inflammatory demyelinating polyneuropathy syndromes [49], the observation that some effects of IVIG were rapidly reversible led to the suggestion that there may be a competition between IVIG and the endogenous autoantibodies which affect nerve conduction. While not investigated further in the publication cited [49], such competition may involve anti-idiotypic [26-31] neutralization of (auto)antibody, as previously suggested, but may also involve a more indirect immunomodulatory effect (through induction of Tregs/Bregs) as we imply in our studies above. The notion that a key component of the IVIG preparation may indeed be the anti-idiotypic moiety [26-31] was supported by a study by investigators who used affinity purified anti-idiotype preparations which they showed were more efficacious in immunoregulation in various experimental models of autoimmune disease [50].

Figures 1&3 confirm that the use of combine immune Ig and anti-idiotype Ig (from human IVIG injection) strategy is effective in suppressing Th2 immunity to OVA in mice. Moreover, Figure 3 confirms that unlike SCIT/SLIT, long-term suppression of immunity persists following cessation of treatment with Ig therapy (>14 weeks in the mice shown). In more recent extensions of these earlier studies we proposed that the mechanism(s) involved in regulating immunity by this approach involved generation and expansion of both T and B cell regulatory cell immune networks in animals receiving combined immune Ig and anti-idiotype Ig [20-23, 25]. Given that vertebrates share common ancestral V region genes [16], we predicted that both homologous and heterologous combinations of immune Ig and anti-idiotype Ig would produce similar re-setting of immune networks in appropriately treated animals, and hence our interest in the use of human IVIG products (delivered as im injections), particularly given the evidence that such products are a rich source of polyclonal anti-idiotypic Igs [26-31]. Data in Figure 3 and elsewhere [25] confirm that attenuation of Th2 immunity to OVA in BALB/c mice was indeed seen following treatment with commercial immune Ig (Anti-Tet) and anti-idiotype Ig of human origin (using commercial IVIG as a source of anti-idiotype [25-31].

Other studies have shown an attenuation of IgE and IL-4 levels in mice receiving both immune and anti-idiotype Igs over a broad dose range, and even following subcutaneous injection (rather than im as reported above) at weekly intervals [25]. It is also important to acknowledge that we have shown unequivocally elsewhere [25] and above (Figures 5,6) that attenuation of Th2 immunity was seen using both a preventative strategy (injections into naïve animals newly exposed to allergen [23,25,50]), and as a therapeutic strategy in allergen-sensitized individuals, already with high levels of IgE and IL-4/IL-13 production capacity (Figures 5&6). Returning to the mechanism(s) involved in regulation induced by the combined immune Ig and anti-idiotype Ig, we reported earlier that depletion of both $CD4^+$ and $CD8^+$ T cells in treated mice caused some loss of immunoregulation [25], consistent with data elsewhere suggesting a role for T cells in allergic diseases [12,13, 24]. We have now added to these data with the studies shown in Figure 5&6. Using mAbs which expand TNFRSF25 Tregs [24,32,33], reported by others to be implicated in T cell regulation of allergic responses [24]) and using immune mice treated with combinations of human anti-Tet Ig and IMIG, we found increased suppression of Th2 immunity in TNFSFR25-treated mice (Figure 5) and increased activity in CD4+ enriched suppressor T cells from such mice (Figure 6). It is important to note that we have reported elsewhere that the TNFSFR25 mAb used in Figure 6 did not in induce Tregs directly [32].

There are a number of studies from others which have also addressed the issue of Treg involvement, including $TNFR^+$ Tregs in regulation of allergic-type responses following allergen specific immunotherapy in animals and humans. Oral administration of nanoparticles containing a food allergen peptide with a TLR7 ligand adjuvant led to development of tolerogenic intestinal DCs which in turn could promote the differentiation of Tregs, enabling protection of mice from food allergic responses [51]. More recently, in a murine model of allergic rhinitis in response to prolonged intranasal OVA administration, both $TGF\beta1^+B$ regs and $CD4^+$Tregs were shown to cooperate in regulating inflammatory responses in the nasal mucosa [52]. This in turn is consistent with more recent data analyzing the role for Bregs and Tregs in a human allergen immunotherapy trial [53]. In terms of a role for $TNFR^+$ Tregs in regulation of allergic responses, it is worthy of note that 4-1BB (CD137), also a member of the TNFR family, is expressed on Tregs and enhanced expression has been shown during allergen-specific immunotherapy in mite-sensitive allergic rhinitis patients [54]. In addition, there is a body of data implicating a role for another TNFR family member, glucocorticoid-induced tumour necrosis factor receptor-related gene (GITR) known to be expressed on $CD4^+CD25^+$Tregs, and its ligand (GITRL), in the regulation of atopic dermatitis [55] and in murine asthma [56]. More recently other workers have identified an important role for Tregs in regulation of food allergies through attenuation of IL-4 responses (see also above data) [57].

Although the study by Zissler et al [53] implicates Bregs in regulation of allergen-induced immunity, we must acknowledge that we have not explored the possibility that cells other than Tregs, including Bregs, play a role in the regulation induced by anti-idiotype+ immune Ig treatment. Bregs are known to cause immunosuppression [58-60] possibly through increasing the number of Treg cells[61]. Other studies in allergy investigating a role for B regs [62,63], suggest an important role for a Th2 cytokine

mediated increase in Breg IL-10 production as an alternative mechanism of action [64].

6. Conclusion:

In summary, we have reviewed above the evidence that therapy using a combination of immune Ig and anti-idiotype Ig, of either homologous or heterologous origin, can induce long-lasting suppression of Th2 allergic responses in both naïve and pre-sensitized animals. WE have shown that this suppression occurs independent of any relation between the immune Igs/anti-idiotype Igs and the allergen, obviating any need for biological characterization definition of the allergen prior to use. Our data show that the effect of treatment persists for prolonged times after completion of therapy, despite (or perhaps because of) ongoing exposure to allergen. In previous reports [25] we have also shown that immunoregulation occurs following delivery of Igs by any of multiple routes, intravenous, intramuscular or subcutaneous. Given that we have successfully used this same therapy in both small (mice) and large (dogs [34]) animals, we propose that this treatment offers now hope for the clinical management of allergic disease.

Declarations:

Ethics approval and consent to participate: All moue studies were performed after approval by a local animal care committee, as documented in Materials and Methods.

Consent for publication: Both authors have read the manuscript, are fully conversant with the data below, and agree to publication in its present form.

Availability of data and material: Data and materials (where available) included in this study will be made freely available.

Competing interests: GW Hoffman and RM Gorczynski declare purchase of shares in NI Inc. GW Hoffman is a member of the Research Advisory Board of NI Inc. Neither of the authors is a paid employee of NI Inc., and no-one received remuneration for work performed in this study.

Funding: All research costs, supplies, reagents, animals and laboratory overheads were financed by R Gorczynski and Network Immunology Inc (NI Inc., BC, Canada).

Authors' contributions: RMG designed and carried out these experiments and drafted the initial and final version of the manuscript. GWH assisted in experimental design and data analysis.

Acknowledgements: The authors and NI acknowledge the additional assistance of Tahir Maqbool, Moyo Williams and Asmahan Haddad at Cedarlane Labs for the ELISA studies reported and Christopher Gorczynski (AccessiFly) for data analysis; and the management at Cedarlane (Eddie Johnson and John Course) for their support throughout the pursuit of this research.

7. References:

1.Wheatley LM, Togias A Clinical Practice. Allergic rhinitis. The New England Journal of Medicine. 2015. 372 (5): 456–63.

2. Thomsen SF Atopic dermatitis: natural history, diagnosis, and treatment. "ISRN Allergy. 2014: 354250.

3. Pawankar R. Allergic diseases and asthma: a global public health concern and a call to action. World *Allergy Organization Journal*. 2014: 7:55: https://doi.org/10.1186/1939-4551-7-12

4. Nauta AJ. Mechanisms of allergy and asthma. Eur J Pharmacol 2008; 585:354-60.

5. Ciprandi G, Filaci G, Fenoglio D. Th17 cells and allergic rhinitis: Is there clinical relevance? Otolaryngol Head Neck Surg 2010; 143: 604-5.

6 Rivas MN, Chatila TA. Regulatory T cells in Allergic Diseases. J Allergy Clin Immunol 2016; 138:639-652.

7. Passalacqua G, Durham SR. Global Allergy and Asthma European Network. Allergic rhinitis and its impact on asthma update: allergen immunotherapy. J Allergy Clin Immunol 2007; 119(4):881-91.

8. Akdis CA, Akdis M. Mechanisms of allergen-specific immunotherapy and immune tolerance to allergens World Allergy Organ J. 2015; 8: 1-12.

9. Mohapatra SS, Qazi M, Hellermann G. Immunotherapy for allergies and asthma: present and future. Curr Opin Pharmacol 2010; 10(3):276-88.

10. Rolland JM, Gardner LM, O'Hehir RE. Functional regulatory T cells and allergen immunotherapy. Curr Opin Allergy Clin Immunol 2010; 10:559-66.

11. Canonica GW, Baena-Cagnani CE, Bousquet J, Bousquet PJ, Lockey RF, Malling HJ, et al. Recommendations for standardization of clinical trials with Allergen Specific Immunotherapy for respiratory allergy. A statement of a World Allergy Organization (WAO) taskforce. Allergy 2007; 62(3):317-24.

12. Bacher P, Scheffold A. The effect of regulatory T cells on tolerance to airborne allergens and allergen immunotherapy. J Allergy Clin Immunol 2018; 142:1697-1709

13.Palomares O. The role of regulatory T cells in IgE-mediated Food allergy. J Investig Allergol Clin Immunol. 2013; 23:371-382.

14. Calzada D, Baos S, Cremades L, Cardaba B. New treatments for allergy: Advances in peptide immunotherapy. Curr Med Chem 2018; 25:2215-2232

15. Simms E, Syed I, Rudulier C, Larche M. Nelson HS. Peptide immunotherapy; short but long lasting? Curr Treatment Options in Allergy 2015; 2:64-71.

16. Jerne, N. K "Towards a network theory of the immune system". *Annales d'immunologie*. 1974; 125C (1–2): 373–389. PMID 4142565.

17. Hoffmannn GW. Immune Network Theory. 2008 www.physics.ubc.ca/~Hoffmannn/ni.html.

18. Seledtsov VI, Seledtsova GV. A possible role for idiotype:anti-idiotype B-T cell interactions in maintaining immune memory. Front Immunol. 2017. https://doi.org/10.3389/fimmu.2017.00409

19. Hoffmann GW, Muller S, Kohler H. Towards an HIV vaccine based on immune

network theory Curr Trends Immunology 2012; 13: 69-79

20. Hoffmann GW, Otto T, Grant M, Muller S, Kohler H. J Infectious Diseases & Preventive Medicine 2019: 7: Iss.2 No:190: 1-5

21. Dosenovic P, Pettersson A-K, Wall A, Thientosapol ES, Feng J, Weidle C, Bhullar K et al. Anti-idiotypic antibodies elicit anti-HIV-1-specific B cell responses. J Exp Med 2019; 216:2316-2330

22. Bancroft T, DeBuysscher BL, Weidle C, Schwartz A, Wall A, Gray M, Feng J et al. Detection and activation of HIV broadly neutralizing antibody precursor B cells using anti-idiotypes. J Exp Med. 2019; 216: 2331-2347

23. Gorczynski RM and Hoffmannn GW. Towards a new kind of vaccine: a logical extension of the symmetrical immune network theory Interact J Med Res. 2017: PMID: 28879488. DOI: 10 2196/ijmr.7612

24. Schreiber TH, Wolf D, Tsai MS, Chirinos J, Deyev V V., Gonzalez L, et al. Therapeutic Treg expansion in mice by TNFRSF25 prevents allergic lung inflammation. J Clin Invest. 2010;120:3629–40

25. Gorczynski RM, Maqbool T, Hoffmann GW. Mechanism(s) of prolonged attenuation of allergic responses after modulation of idiotypic regulatory network. Allergy, Asthma & Clin Immunol. 2019: in press

26. Schaub A, von Gunten S, Vogel M, Wymann S, Rüegsegger M, Stadler BM, Spycher M, Simon HU, Miescher S. Dimeric IVIG contains natural anti-Siglec-9 autoantibodies and their anti-idiotypes. Allergy. 2011;66:1030-7.

27. Konova E, Atanasova M, Stoykov S, Velkova A, Shoenfeld Y. Idiotypic and anti-idiotypic elastin autoantibodies: Implications for IVIg and pregnancy loss. J Autoimmun. 2007;28:46-54.

28. Wu R, Shoenfeld Y, Sherer Y, Patnaik M, Matsuura E, Gilburd B, Koike T, Peter JB.
Anti-idiotypes to oxidized LDL antibodies in intravenous immunoglobulin preparations--possible immunomodulation of atherosclerosis.
Autoimmunity. 2003;36:91-7.

29. DeKeyser F, DeKeyser H, Kazatchkine MD, Rossi F, Dang H, Talal N. Pooled human immunoglobulins contain anti-idiotypes with reactivity against the SLE-associated 4B4 cross-reactive idiotype.Clin Exp Rheumatol. 1996;14:587-91.

30. Caccavo D, Vaccaro F, Ferri GM, Amoroso A, Bonomo L. Anti-idiotypes against antiphospholipid antibodies are present in normal polyspecific immunoglobulins for therapeutic use.J Autoimmun. 1994;7:537-48.

31. Dietrich G, Kaveri SV, Kazatchkine MD. Modulation of autoimmunity by intravenous immune globulin through interaction with the function of the immune/idiotypic network.Clin Immunol Immunopathol. 1992 Jan;62(1 Pt 2):S73-81. Review.

32. Gorczynski RM, Sadozai H, Zhu F, Khatri I. Effect of infusion of monoclonal antibodies to tumour necrosis factor-receptor super family 25 on graft rejection in allo-immune mice receiving autologous marrow transplantation. Immunology. 2017; 150:418-431.

33. Taraban VY, Ferdinand JR, Al-Shamkhani A. Expression of TNFRSF25 on conventional T cells and Tregs. J Clin Invest 2011; 121:463-4; author reply 5.

34. Gorczynski RM, Maqbool T, Hoffmann GW. Combined IMIG and Immune Ig attenuates allergic responses in Beagle dogs: submitted: 2019

35.Furue M, Yamamura K, Kido-Nakahara M, Nakahara T, Fukui Y. Emerging role of

interleukin-31 and interleukin-31 receptor in pruritus in atopic dermatitis. Allergy 2018; 73:29-36.
36. Weinstein SF, Katial R, Jayawardena S, Pirozzi G, Staudinger H, Eckert L, Joish VN, Amin N, Maroni J, Rowe P, Graham NMH, Teper A. Efficacy and safety of dupilumab in perennial allergic rhinitis and comorbid asthma. J Allergy Clin Immunol. 2018; 142:171-177.
37. Iftikhar IH, Schimmel M, Bender W, Swenson C, Amrol D. Comparative Efficacy of Anti IL-4, IL-5 and IL-13 Drugs for Treatment of Eosinophilic Asthma: A Network Meta-analysis. Lung. 2018; 196:517-530.
38. Ohta K, Nagase H, Suzukawa M, Ohta S. Antibody therapy for the management of severe asthma with eosinophilic inflammation. Int Immunol. 2017; 29:337-343.
39. Wasserman RL. Personalized Therapy: Immunoglobulin Replacement for Antibody Deficiency. Immunol Allergy Clin North Am. 2019;39:95-111.
40. Restrepo-Jiménez P, Rodríguez Y, González P, Chang C, Gershwin ME, Anaya JM.
The immunotherapy of Guillain-Barré syndrome.Expert Opin Biol Ther. 2018;18:619-631.
41. Wasserman RL. Gammaplex® 5 and 10% in the treatment of primary immunodeficiency and chronic immune thrombocytopenic purpura.
Immunotherapy. 2017;9:1071-1088.
42. Pascall B, Thakker A, Foo Y, Thakker P. Immunoglobulin for Kawasaki disease: a 3-year retrospective audit. BMJ Paediatr Open. 2019;3:e000451. doi: 10.1136/bmjpo-2019-000451.
43.Wawrzycki B, Krasowska D, Pietrzak A, Szumilo J, Blazowski L, Pietraszek-Mamcarz J, Lotti T.Infantile bullous pemphigoid successfully treated with combined pulse corticosteroids and high-dose IVIG. Dermatol Ther. 2018 Sep;31(5):e12635. doi: 10.1111/dth.12635. Epub 2018 Sep 14.
44. Marcus N, Smuel K, Almog M, Prais D, Straussberg R, Landau D, Scheuerman O.
Successful Intravenous Immunoglobulin Treatment in Pediatric Severe DRESS Syndrome.J Allergy Clin Immunol Pract. 2018;6:1238-1242.
45. Antoon JW, Goldman JL, Shah SS, Lee B.
A Retrospective Cohort Study of the Management and Outcomes of Children Hospitalized with Stevens-Johnson Syndrome or Toxic Epidermal Necrolysis.
J Allergy Clin Immunol Pract. 2019;7:244-250
46. Forbat E, Ali FR, Al-Niaimi F. Intravenous immunoglobulins in dermatology. Part 2: clinical indications and outcomes. Clin Exp Dermatol. 2018;43:659-666.
47. Maddur MS, Lacroix-Desmazes S, Dimitrov JD, Kazatchkine MD, Bayry J, Kaveri SV.
Natural Antibodies: from First-Line Defense Against Pathogens to Perpetual Immune Homeostasis. Clin Rev Allergy Immunol. 2019 Jun 3. doi: 10.1007/s12016-019-08746-9.
48. Lee SH, Ban GY, Kim SC, Chung CG, Lee HY, Lee JH, Park HS.
Association between primary immunodeficiency and asthma exacerbation in adult asthmatics. Korean J Intern Med. 2019 Jun 14. doi: 10.3904/kjim.2018.413.
49. Berger M, McCallus DE, Lin CS.Rapid and reversible responses to IVIG in autoimmune neuromuscular diseases suggest mechanisms of action involving competition with functionally important autoantibodies.J Peripher Nerv Syst. 2013;18:275-96.
50. Blank M, Bashi T, Shoenfeld Y.Idiotype-
specific intravenous immunoglobulin (IVIG) for therapy of autoimmune diseases.Methods Mol Biol. 2014;1060:353-61.
51.Hong J, Xiao X,Gao Q, Li S, Jiang B, Sun X, Ran P, Yang P.Co-delivery of

allergen epitope fragments and R848 inhibits food allergy by inducing tolerogenic dendritic cells and regulatory T cells. Int J Nanomedicine. 2019;14:7053-7064.

52. Wang M, Gu Z, Yang J, Zhao H, Cao Z.Changes among TGF-β1^{+} Breg cells and helper T cell subsets in a murine model of allergic rhinitis with prolonged OVA challenge. Int Immunopharmacol. 2019; 69:347-357.

53. Zissler UM, Jakwerth CA, Guerth FM, Pechtold L, Aguilar-Pimentel JA, Dietz K, Suttner K, Piontek G, Haller B, Hajdu Z, Schiemann M, Schmidt-Weber CB, Chaker AM.Early IL-10 producing B-cells and coinciding Th/Tr17 shifts during three year grass-pollen AIT. EBioMedicine. 2018;36:475-488.

54. Tsai YG, Yang KD, Wen YS, Hung CH, Chien JW, Lin CY.Allergen-specific immunotherapy enhances CD8^{+} CD25^{+} CD137^{+} regulatory T cells and decreases nasal nitric oxide. Pediatr Allergy Immunol. 2019;30:531-539.

55. Baumgartner-Nielsen J, Vestergaard C, Thestrup-Pedersen K, Deleuran M, Deleuran B.Glucocorticoid-induced tumour necrosis factor receptor (GITR) and its ligand (GITRL) in atopic dermatitis. Acta Derm Venereol. 2006;86:393-8.

56. Zhang M, Wan J, Xu Y, Zhang D, Peng J, Qi C, Guo Q, Xia S, Su Z, Wang S, Xu H. Simultaneously increased expression of glucocorticoid-induced tumor necrosis factor receptor and its ligand contributes to increased interleukin-5/13-producing group 2 innate lymphocytes in murine asthma. Mol Med Rep. 2017;15:4291-4299.

57. Nakajima-Adachi H, Shibahara K, Fujimura Y, Takeyama J, Hiraide E, Kikuchi A, Murakami H, Hosono A, Nochi T, Wakatsuki Y, Shimojo N, Kaminogawa S, Sato R, Kiyono H, Hachimura S. Critical role of intestinal interleukin-4 modulating regulatory T cells for desensitization, tolerance, and inflammation of food allergy. PLoS One. 2017 ;12(2): e0172795. doi: 10.1371/journal.pone.0172795.

58. Gorczynski RM, Farrokhi K, Sadozai H, Zhu F, and Khatri I. Importance of B cells to development of Tregs and prolongation of tissue allograft survival in recipients receiving autologous bone marrow transplantation. Immunology. 2018; 154:465-475

59. Wortel C, Heidt S. Regulatory B cells: Phenotype, function and role in transplantation. Transpl Immunol. 2017;41:1-9.

60. Goode I, Xu H, Ildstad ST. Regulatory B cells: The new "It" cell. Vol. 46, Transplantation Proceedings. 2014. p. 3–8.

61. Lee KM, Stott RT, Zhao G, Soohoo J, Xiong W, Lian MM, et al. TGF-β-producing regulatory B cells induce regulatory T cells and promote transplantation tolerance. Eur J Immunol. 2014;44(6):1728–36.

62. van de Veen W. The role of regulatory B cells in allergen immunotherapy. Curr Opin Allergy Clin Immunol. 2017;17:447-452.

63. Satitsuksanoa P, Jansen K, Głobińska A, van de Veen W, Akdis M Regulatory Immune Mechanisms in Tolerance to Food Allergy Front. Immunol., 2018; https://doi.org/10.3389/fimmu.2018.02939

64. Taitano SH, van der Vlugt LEPM, Shea MM, Jennifer Yang J, Nicholas W. Lukacs and Steven K. Lundy Differential Influence on Regulatory B Cells by T_H2 Cytokines Affects Protection in Allergic Airway Disease J Immunol; 2018; 201:1865-1874.