IVIG ameliorate inflammation in collagen induced arthritis- projection for IVIG therapy in rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disease that leads to joint destruction and disability. Despite a significant progress in administration of biological agents for RA patients, there is still a need for improved therapy. Intravenous-immunoglobulins (IVIG) , a pooled polyspecific immunoglobulin-G (IgG) extracted from 20,000 healthy subjects, showed beneficial therapeutic effect in patients with immune-deficiency, sepsis, and autoimmune diseases. The current study aim to investigate the beneficial effect of treatment with IVIG in established collagen induced arthritis in DBA/1j mice. Murine arthritis was induced in DBA/1j mice. The treatment with IVIG started when the disease was established. The clinical score was followed twice a week until day 48. The mice were bled for plasma, the paws were H&E stained. Cytokine profile in the plasma was analyzed by Luminex technology, titers of circulating anti-collagen antibodies in the plasma was tested by ELISA. Our results show that treatment with IVIG in murine, significantly rreduced the clinical arthritis score ($P<0.001$). Moreover, mode of action show that IVIG significantly reduced circulating level of inflammatory cytokines (IFN?, IL-1β, IL-17, IL-6, TNFα) ($P<0.001$), inhibit anti-collagen antibodies ($P < 0.001$) in the plasma of CIA mice. Importantly, histopathological examination revealed that IVIG treatment prevented the migration of inflammatory immune cells into the cartilage and synovium, reduced the extent of joint damage and preserved joint architecture. Our results proved for the first time the valuable anti-inflammatory treatment of IVIG in experimental RA. We propose IVIG therapy for a subgroup of patients with as rheumatological-related diseases.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory disease that leads to painful joint destruction and disability [1,2]. Traditional RA therapy includes disease-modifying anti-rheumatic drugs (DMARDs), such as first line therapy methotrexate, hydroxychloroquine, sulfasalazine, minocycline and leflunomide [3]. In recent years, emerging novel biologic agents targeting specific pathways proven to contribute to inflammatory pathways have also been included: anti-interleukine-1 (anakinra), anti-IL-6 (tocilizumab), tumor necrosis factor α (TNFα) blockers (infliximab, etanercept, adalimumab Enbrel), Janus-kinase inhibitors (tofacitinib, baricitinib) and anti-CD20 (rituximab) [4-7]. Despite the significant progress that has been achieved with the administration of biological therapies in changing the natural history of RA, such medications induce immune suppression, which is nonselective to the pathogenesis of the disease, resulting in higher rates of common and opportunistic infections. Thus, different strategies used to overcome these issues. For example, processing of antibodies under specific high dilution technology to produce drugs that change conformation state of the antigen and have specific target-modification activity [8,9], or produce

domain antibodies such as nanobodies recently approved by FDA for the first time [10]. One of another highly promising approach is the usage of Intravenous immunoglobulins (IVIG) as a therapy for RA.

IVIG is a blood product, predominantly IgG (>95%), isolated from 5,000-20,000 healthy donors. The first mentioned triumph of the use of IVIG therapy was in primary immunodeficiency diseases in the 1950s. IVIG has a good proven beneficial and safety profile, and is one of the first biological therapies which was introduced already in 1981 by Imbach I et al [11], for immune thrombocytopenic purpura (ITP). To date, the Food and Drug Administration (FDA) has approved the use of IVIG as a first line therapy in B-cell chronic lymphocytic leukemia, primary humoral immunodeficiency, ITP, Kawasaki syndrome and multifocal motor neuropathy [12]. Despite FDA off labeling, IVIG therapy has been expanded for diverse autoimmune diseases such as : specific subgroups of RA patients, juvenile chronic arthritis (JCA), Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy occurring in the context of rheumatic disease, systemic lupus erythematosis (SLE), idiopathic inflammatory myopathies, systemic sclerosis, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides, Still's disease and more [13-26]. The mode of action of IVIG encompass various mechanisms, attributed to the F(ab)2 or Fc portions of the molecule [27-31]. The Fab part of the IVIG is related to neutralization of inflammatory cytokines, anti-idiotypic activity, blocking of cellular receptors, antibody dependent cellular cytotoxicity (ADCC), and anaphylatoxin scavenging. Whereas the Fc related activities encompass regulation of FcγR, immunomodulation of the function of dendritic cells, blocking activating receptors, expansion of T regulatory cells and saturation of FcRn.

One of the frequent murine model used in preclinical studies to evaluate potential anti-rheumatic agents, is the collagen induced arthritis (CIA) model which imitate human RA. CIA-treated mice share several pathological features with RA including generation of autoantibodies, synovial inflammatory cell infiltration, synovial hyperplasia, cartilage destruction, and bone erosion [32].

MATERIALS AND METHODS

Mice and experimental design

Experimental arthritis was induced in 7-8 week old DBA/1J male mice (ENVIGO, Blackthorn,UK). The mice were maintained in a conventional animal housing facility at Sheba Medical Center and kept in individually ventilated cages. All experiments were approved and executed according to the protocols of the ethics committee of the Israeli Ministry of Health (no.1221/19) and fulfill "The ARRIVE guidelines 2.0" for animal research. The Collagen induced arthritis (CIA) model was performed as previously described by us [32-34]: Bovine type II collagen (Chondrex, Redmond, WA, USA) was emulsified 1:1 with mycobacterium tuberculosis H37RA in Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI, USA). DBA/1J males were subcutaneously injected into the base of the tail with 100 μg emulsion. A boost injection of bovine type II collagen in PBS, at the base of the tail, was given 21 days later. Intraperitoneal injections of IVIG (OMRIX ltd, Rehovot, Israel), 3mg/0.1 ml/mouse, started at score of 2-3 after the boost injection and repeated on a weekly base. PBS as vehicle (volume) and non-treated mice were used as controls, n = 10 per each group. The mice were sacrificed after 48 days.

Arthritis scoring The clinical scores and the hind paw widths of the mice were monitored daily over the course of each experiment. The development of arthritis was assessed daily, and the severity of arthritis was scored for each paw on a 3-point scale, in which 0 = normal appearance, 1 = localized edema/ erythema over one surface of the paw, 2 = edema/ erythema involving more than one surface of the paw, 3 = marked edema/erythema involving the whole paw. The scores of all four paws were added for a composite score, with a maximum score of 12 per mouse. Ankle thickness of the hind paws was measured in millimeters (mm) at the widest point (the malleoli) with the legs fully extended with digital calipers (Manostat, Herisau, Switzerland).

Histopathological assessment

The paws of the mice were obtained from the sacrificed mice and fixed in 4% formalin (Sigma-Aldrich St Louis, MO, USA), decalcified, cut, and stained with H&E. Samples were examined by light microscopy (×200

magnification). All histological evaluations performed by pathologists were double blinded.

Quantification of Cytokines with Luminex

At the end of the experiment, blood was collected into an EDTA containing Eppendorf tube and then centrifuged (2000 rpm 10 min) to obtain plasma samples.

Luminex technology (Mouse High Sensitivity T Cell Magnetic Bead Panel (MHSTCMAG-70K); Merck GmbH, Darm stadt, Germany) was used to measure IFNγ, IL-1β, IL-6, IL-17, and TNFα in the plasma. All quantifications were done according to the protocols provided by the manufacturer.

Anti-collagen type II antibodies determination

Circulating anti-collagen type II antibodies were determined by homemade enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated with collagen type II [10 μg/ml phosphate-buffered saline (PBS)]. Following overnight incubation at 4°C, the plates were blocked with 3% bovine serum albumin (BSA). Mouse plasma at dilution of 1:800 were added to the blocked ELISA plates and incubated for 2 h at room temperature ($n = 10$ for each treatment group). The binding was probed with goat anti-mouse IgG conjugated with alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), followed by the addition of appropriate substrate. The data were read by ELISA reader at optical density (OD) 405 nm and presented as OD at 405nm.

Statistical analysis

Statistical analysis was made using the repeated measures ANOVA procedure, while the weights data fitted all the required assumptions, arthritis score data did not meet the linear and sphericity assumption. Thus, increasing type 1 error for the model furthermore, violation for this assumptions is not known to bias the post hoc analysis (36). Therefore, we aggressively adjusted for multiple comparisons using the Bonferroni adjustment. For both models, time was set as the within subject effect while, the treatment group as the between subjects effect. The significance level was set at 5%. Statistical analysis and graph plotting were made with IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp. Cytokine and anti-collagen concentration were first assessed for distribution. Since, normal distribution was not met, non-parametric tests were chosen throughout the statistical analysis. Comparisons of the mean concentrations began with the Kruskal-Wallis H test and followed with the Dunnett's test for pair-wise comparisons. Post-hoc comparisons, including plotted comparisons, were adjusted for multiple comparisons following the Bonferroni procedure. Both plots and statistical analysis were conducted using R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/

RESULTS

IVIG treatment significantly abrogates the arthritis clinical score

CIA is a mouse model which imitates rheumatoid arthritis in genetically prone human RA patients, in which inflammation leads to the joint destruction. We investigated the effect of IVIG treatment in CIA mice and compared its efficacy with CIA mice treated with PBS (vehicle) or non-treated CIA mice.

Both IVIG and PBS treatments started when the baseline arthritic score was almost 3. We observed a significantly lower marginal arthritis score in IVIG-treated mice as compared to marginal arthritis score of PBS-treated mice or non-treated mice ($P < 0.001$) as illustrated in Fig. 1. Repeated measures ANOVA analysis revealed a significant difference between the treatment groups mean arthritis score over time F=30.224, P <0.0001 (lower-bound adjusted). Marginal arthritis score of the different studied groups was as follow: The IVIG group had the lowest arthritis score marginal mean of 4.128 95% CI (3.995, 4.262), in comparison to 5.78 95% CI (5.56, 6.01) for non-treated CIA mice, and 5.62 95%CL (5.45, 5.77) for PBS treated CIA mice P <0.0001 (Bonferroni adjusted). The significantly lower arthritis score lasted until the mice were sacrificed at day 48 (Fig.1). No significant change in mice body weight was documented overtime in the three studied groups, P >0.05 (data not shown).

Analyzing the clinical score at day 27 after the boost administration (day 48 from day 0 of diseases induction), showed score of 4.48+-0.98 in CIA mice treated with IVIG, P <0.001, whereas a score of 8.09 +-2.13 and 7.51+-1.05 for PBS or non-treated CIA mice was observed, respectively (Fig.2).

H&E staining of the joints tissue sections from the IVIG-treated mice as described in Fig.3A, demonstrates a significantly less synovial hyperplasia, no inflammatory process or neutrophil infiltration, normal cartilage layer and muscle structure, typical bone organization and uninflamed fat tissue, as in healthy non injected mice (Fig.3B). Whereas in CIA mice treated with PBS (Fig.3C) and in non-treated CIA mice (Fig.3D), a massive infiltration of neutrophils can be observed as well as bone and joint destruction.

IVIG treatment significantly reduced the production of anti-collagen antibodies in CIA mice

IVIG is known to consist neutralizing antibodies targeting the Fab portion of pathogenic autoantibodies, a phenomenon previously described as an anti-idiotypic activities. Therefore, we analyzed the effect of IVIG in amelioration of anti-collagen antibodies in the plasma of CIA mice +-treatment (Fig.4).

As illustrated in Fig.4, ELISA examination of antibodies against collagen in IVIG-CIA group showed OD of 1.15+-0.28 at 405nm, as compared to 1.56+-0.31 OD and 1.76 +-0.7 OD in PBS or untreated CIA mice, respectively, P <0.001.

IVIG treatment, regulated circulating cytokines levels in CIA mice

A direct effect of the cytokines on the destruction of the joint or another mechanism beside recruitment of inflammatory cells has been shown [1,2,37]. We evaluated the circulating inflammatory cytokines (IFNγ, IL-1β, IL-17, IL-6, TNFα) levels in the CIA mice plasma upon treatment with IVIG, vehicle or non-treatment, Fig,5A-E. The inflammatory cytokines level in IVIG-treated CIA mice were significantly lower in comparison to the control PBS or non-treated mice for all of the tested cytokines , P <0.001. For example: IL-6 levels in the plasma of CIA mice treated with IVIG were found to be 896±168pg/ml in comparison to 13,999±917 pg/ml and 13,481±869 pg/ml, for non-treated CIA mice and PBS treated CIA mice, respectively,P <0.001. The IL-17 levels in the plasma of IVIG treated CIA mice were 1,173±497 pg/ml, whereas in the non-treated or PBS treated CIA mice the levels were 10,318±1,521 pg/ml and 11,487±371 pg/ml respectively, P <0.001. The TNFα levels in the CIA mice treated with IVIG were 2,164.28±652 pg/ml compared to 11,099±4176 pg/ml and 13,969±3,044 pg/ml in the non-treated CIA mice or PBS treated CIA mice, respectively, P <0.001.

DISCUSSION

RA is a systemic autoimmune inflammatory disease that leads to painful joint destruction and disability [11]. IVIG with good proven beneficial and safety profile, is one of the first biological therapies which was introduced already in 1981 by Imbach I et al, for immune thrombocytopenic purpura (ITP) [11] . Since then, IVIG was employed successfully in wide range of conditions such as immune deficiency, sepsis, autoimmunity (e.g specific subgroups of RA patients, juvenile chronic arthritis (JCA), Still's disease, Guillain-Barré syndrome and chronic inflammatory demyelinating polyneuropathy occurring in the context of rheumatic disease, as well as in SLE, idiopathic inflammatory myopathies, systemic sclerosis, and ANCA-associated vasculitides, Still's disease and more) [13-24].

We show herein a beneficial effect of IVIG in the treatment in established murine CIA. IVIG inhibited the arthritis clinical score, inflammatory condition, exemplified by reduced the levels of circulating inflammatory cytokines. IVIG prevent infiltration of immune cells by histopathological examination inhibit joints destruction, as compared with the untreated or PBS subjected CIA mice. The lower clinical score was accompanied by reduced levels of anti-collagen antibodies (P <0.001). Likewise, the diminished levels of circulating inflammatory cytokines (IFNγ, IL-1β, IL-6, IL-17, TNFα) were recorded, P <0.001. Our data support and strength our previous observation that prophylactic treatment with IVIG inhibited the severity of disease inflammation in murine CIA. In that study, IVIG affinity purified on curillin peptides (ACPA-specific IVIG) and more significantly citrullinated-peptides specific IVIG was found to be 200 times more efficient in the reduction of CIA inflammation activity, inhibiting the inflammatory cytokines production *ex-vivo* by spleen

cells and enhanced expansion of spleen T regulatory cells as compared to regular IVIG treatment [35]. Moreover , the immunomodulatory makeup of IVIG in this study was attributed to its anti-idiotypic activity *via* the Fab portion of the molecules, although we can't exclude the Fc contribution *via* elevation of T regulatory cells number.

Lee SY et al, using a prophylactic protocol for the IVIG treatment in CIA mice, showed that IVIG inhibited the development of disease score in CIA mice, reduce the number of Th17 cells in the spleen, expansion of spleen T-regulatory cells and inflammatory cytokines in the joints [38] . Similarly, IVIG up-regulated IL-10 and Fcγ receptor IIB expression by spleen cells (38). It is worth mentioning that in our current study, IVIG was found to abrogate the course of disease, even when the treatment started when the mice have already an arthritis score of 2-3, as compared to the prophylactic treatment of (name of group).. Passive transfer of sera from RA patients to K/BxN mice is an additional murine model of arthritis [39]. IVIG given in a prophylactic protocol or recombinant IgG1 Fc hexamer (Fc-μTP-L309C), resulted in attenuation of arthritis score in the artherogenic K/BXN mice [40].

IVIG mode of action encompass numerous biological functions related in part to the $F(ab)_2$ of the molecule and/or to the Fc activities. The anti-inflammatory activity of IVIG is based on the following activities: **a)** Neutralization of inflammatory cytokines since IVIG targets at least 13 different cytokines or cytokine-inhibitors [41-45]. Due to the fact that IVIG has anti-inflammatory cytokine activities, it was shown that IVIG is an efficient therapy in the cytokine storm in cases of catastrophic antiphospholipid syndrome, severe influenza, and also currently in COVID-19 [43-48]. IVIG bind specifically the spike protein on RS-Cov-2 as well as the receptor binding protein (personal data) ;**b)** IVIg impairs the generation of human monocyte-derived anti-inflammatory macrophages by inducing JNK activation and activin , limiting the production of inflammatory macrophage differentiation by inhibiting GM-CSF-driven STAT5 activation *in-vitro* [49].**c)** IVIG can function through the Fc portion thus contributing to the expansion of T regulatory cells , Fc receptor blocking including the sialic acid content of the Fc portion of the antibodies and the interaction with ITAM-bearing Fc receptors [50,51]. Of note, and as opposed to some common assumptions in the medical scientific community, IVIG was found to have less side effects as compared to current biological treatments which might cause severe side effect and loss of efficacy in the long-term. Moreover, IVIG might be even cheaper than current expansive biological agents, therefore it might change the financial burden of biological treatment in RA and other inflammatory/autoimmune diseases.

Conclusion

In summary, in the current study we demonstrate for the first time an amelioration of CIA in mice with **established arthritis** using a treatment protocol. We show herein a powerful anti-inflammatory potential of IVIG in attenuating the pathogenesis in CIA in mice .The beneficial effect was presented by reducing the clinical score, reducing the titers of anti-collagen type II antibodies and declining the concentrations of circulating cytokine . Our study may pave the way for clinicians to reconsider the use of IVIG, as a safe and beneficial optional treatment in a subgroup of patients with rheumatoid arthritis.

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Author contribution

All authors were involved in drafting the article and all authors approved the current version of the manuscript. Study design: Blank, Tarasov, Petrova, Aital ; Performing the experiments: Halpert, Blank, Katz, Shovman; Pathology analysis: Volkov, Barshack; Statistical analysis: Katz. Manuscript writing: Ganina, Petrova, Tarasov, Blank, Halpert, Tocut, Amital.

Disclosure

Halpert, Katz, Shovman, Tocut, Volkov, Barshack, Blank, and Amital, declare that they have no conflict of interest.

Tarasov. Ganina, and Petrova are employees of OOO "NPF "MATERIA MEDICA HOLDING".

OOO "NPF "MATERIA MEDICA HOLDING" sponsored the study and took part in the design of the experiments and the manuscript writing.

OOO "NPF "MATERIA MEDICA HOLDING" does not manufacture IVIG or have any other commercial interest in the results of the experiment

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LEGEND TO FIGURES

Fig.1: IVIG effect on arthritis score in CIA mice

The data are presented as arthritis score over time in DBA/j CIA mice, upon treatment with IVIG, PBS or non-treated mice. N=10 per each studied group of mice, P <0.001.

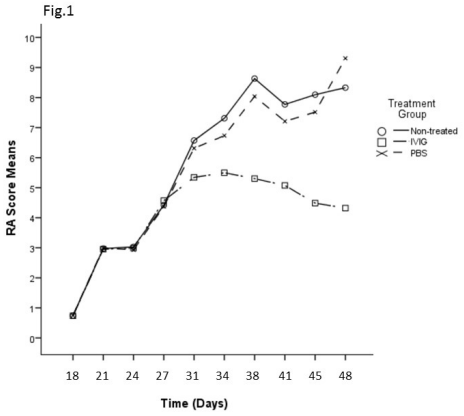

Fig.2: IVIG effect on arthritis score at the last point of the experiment

This arthritis score was evaluated at day 48 (27 days since the beginning of treatment with IVIG). Kruskal-Wallis, $\chi^2(2) = 38.65$, P <0.001.

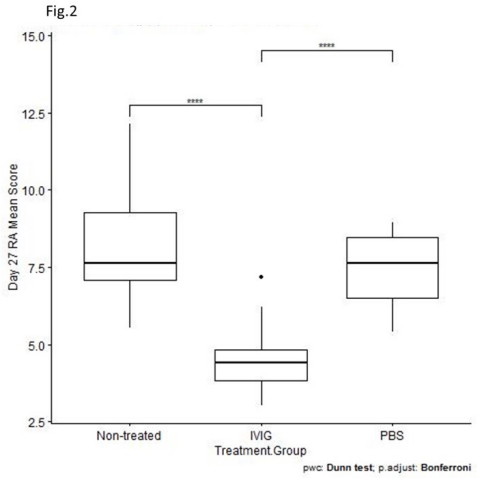

Fig.3: Histological analysis

H&E staining of representative arthritic paws, from each study group of CIA mice. Magnification presented × 200.A) CIA mouse treated with IVIG; B) healthy mouse without CIA; C) CIA mouse treated with PBS; D) non-treated CIA mouse.

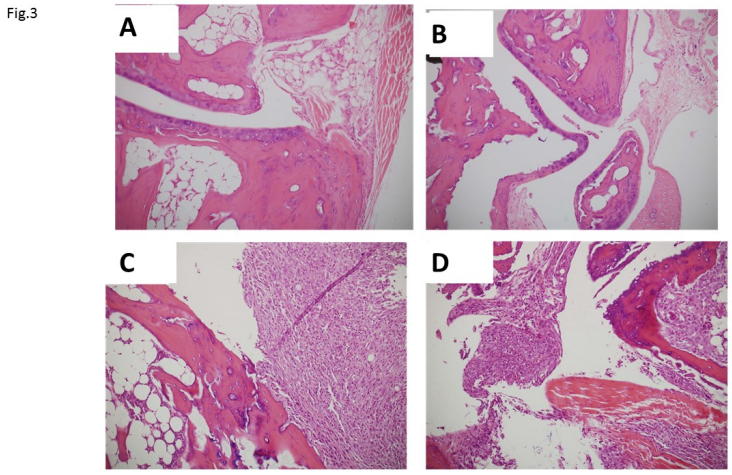

Fig.4: Circulating anti-collagen antibodies

Mouse anti-collagen type II circulating antibodies, were tested in the plasma of the three groups of mice at dilution of 1:800. Data are presented at OD at 405nm. Kruskal-Wallis, $\chi^2(2)=30.7$, $p<0.001$.

Fig.4

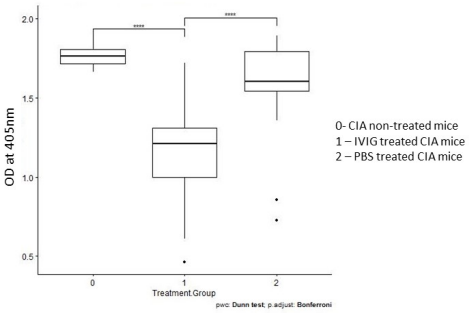

Fig.5: Levels of circulating cytokines in CIA mice upon treatment with IVIG

We analyzed the concentrations of the inflammatory cytokines IFNγ, IL-1β, IL-17, IL-6, TNFα, in the plasma of CIA mice ± treatment with IVIG, PBS or on-treated.

The data are presented as concentration in pg/ml. n = 10 per group. A) IFNγ Kruskal-Wallis, $\chi^2(2) = 42.76$; B) IL-1β Kruskal-Wallis, $\chi^2(2)=42.77$; C) IL-6 Kruskal-Wallis, $\chi^2(2)=43.22$; D) IL-17 Kruskal-Wallis, $\chi^2(2)=42.42$; E) TNFα Kruskal-Wallis, $\chi^2(2)=38.6$. $p<0.001$ for all studied groups of mice.

Fig.5A

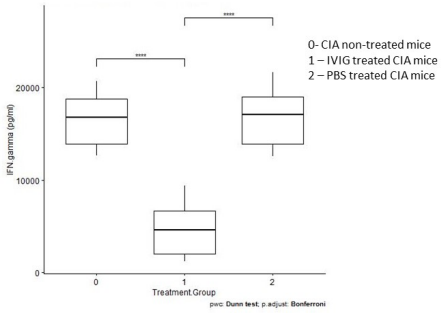

Fig.5B

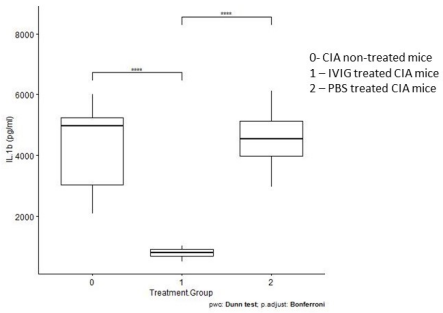

Fig.5C

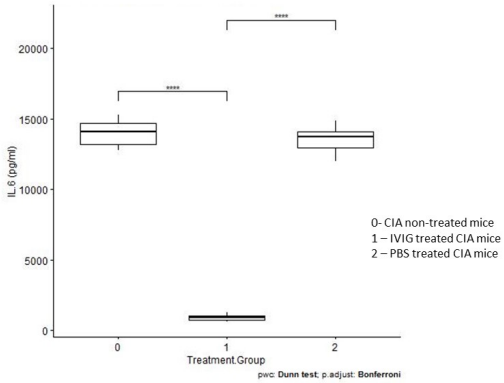

Fig.5E

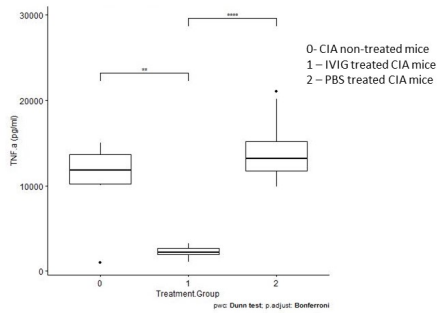

Fig.5D