Anti-inflammatory intravenous immunoglobulin (IVIg) suppresses homeostatic proliferation of B cells

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Abstract An intravenous injection of plasma-derived immunoglobulins is used for the treatment of severe infectious and autoimmune disorders. Despite of its clinical efficacy, precise mechanisms by which intravenous immunoglobulin (IVIg) suppresses proinflammatory immune response are still enigmatic. Here, we provide in vitro evidence that IVIg inhibits homeostatic proliferation of B cells accompanied by induction of their cell aggregation. The IVIg-driven suppression of B cell proliferation and induction of cell aggregation are both unaffected by treatment with a neutralizing antibody against low-affinity Fc receptors for IgG (CD16/FcγRIII and CD32/FcγRII), known cell surface ligands for IVIg. Our observations propose a new immunosuppressive action of IVIg, which directly acts on steady-state B cells to suppress their homeostatic expansion.

Keywords Aggregation · B cells · Homeostatic proliferation · Intravenous immunoglobulin (IVIg)

Introduction

Polyclonal plasma-derived immunoglobulins are used for the treatment of severe infectious diseases and autoimmune disorders (Barahona Afonso and Joao 2016; Dourmishev et al. 2016; Zuercher et al. 2016). The supplementation of polyclonal plasma-derived immunoglobulins via the intravenous route is used as intravenous immunoglobulin (IVIg) therapy. Human immunoglobulins for IVIg therapy are prepared from pooled serum immunoglobulins obtained from a minimum of 1000–100,000 healthy donors (Barahona Afonso and Joao 2016). The first report of IVIg treatment described its application to diphtheria and tetanus in 1890 (Von Behring 1890; Von Behring and Kitasato 1890), and since then, IVIg treatment has been used for autoimmune and proinflammatory diseases such as primary immunodeficiency, Kawasaki disease, and Guillain–Barre syndrome (Barahona Afonso and Joao 2016). To obtain positive clinical effect of IVIg, a large amount of immunoglobulins (0.1–2 g/kg body mass) is used for intravenous (IVIg) or subcutaneous (SCIg) administration to treat infectious and autoimmune disorders (Barahona Afonso and Joao 2016; Zuercher et al. 2016).

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Despite of the broad application of IVIg therapy on a wide array of proinflammatory disorders, the exact molecular mechanisms underlying the therapeutic effect remains controversial in humans as well as in mouse model studies (Ballow 2014; Dourmishev et al. 2016; Hartung 2008). Some of the suggested therapeutic mechanisms include: the inhibition of cytokine production from T cells and B cells (Amran et al. 1994; Andersson et al. 1996; Seite et al. 2011), the inhibition of antibody production from B cells (Sigman et al. 1998), the induction of B cell anergy against B cell receptor (BCR) crosslinking (Seite et al. 2014), the suppression of proinflammatory immune responses via inhibitory Fc receptor (FcγRIIb) on macrophages (Kaneko et al. 2006), the blockage of Fas-mediated cell death by direct binding to CD95/Fas (Viard et al. 1998), and the inactivation of complements (Basta et al. 1989; Mollnes et al. 1997). However, precise mechanisms underlying the IVIg-driven immunosuppression are not fully understood, and responsible molecules for IVIg therapy differ among reports and disorders (Ballow 2014; Issekutz et al. 2015; Nagelkerke and Kuijpers 2015).

Dysregulation of immune homeostasis can induce chronic inflammation and autoimmune disorders (Pedros et al. 2016; Yanes et al. 2017). T cells and B cells play important roles in the maintenance of immune homeostasis to prevent those proinflammatory conditions (Akdis et al. 2012; Ray and Dittel 2017). In particular, a breakdown of the homeostatic control of B cells in the steady-state leads to the induction of autoimmune diseases, and those hyper-activated B cells are involved in the deterioration of chronic inflammation (Kim and Won 2012; Wunsch et al. 2016). Therefore, a down-regulation of overactivated B cells and the maintenance of normal B cell homeostasis are important to prevent proinflammatory disorders.

Many studies demonstrated impact of IVIg's immunosuppressive effects on in vitro activated B cells (e.g. B cells stimulated via B cell receptor (BCR), toll-like receptor (TLR), or by mitogen) or in vivo activated B cells isolated from patients with inflammation or animals of disease models (Mitrevski et al. 2015; Rigal et al. 1994; Tha-In et al. 2008). However, as far as we know, there is no report that described anti-proliferative activity of IVIg onto naive steady-state B cells. In this study, we show that IVIg acts directly on steady-state B cells to suppress their

homeostatic proliferation upon triggering of B cell-specific aggregation. Present results provide new insight into the anti-inflammatory mechanisms of IVIg therapy.

Materials and methods

Experimental animals and ethical statement

Five-week-old female BALB/c mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan) and kept under specific pathogen-free conditions. All animal experiments were carried out using protocols approved by the Committee on Animal Experimentation of Hiroshima University, Japan.

Reagents

Pooled human immunoglobulin prepared for IVIg (Sanglopor® I.V. Infusion) was purchased from CSL Behring (King of Prussia, PA, USA). Purchased IVIg was reconstituted with endotoxin-free saline (Otsuka Pharmaceutical Factory, Tokushima, Japan) as 200 mg/ml aliquots and kept at -20°C until use. All chemicals used were of biochemical grade or cell culture grade, and were purchased from Wako Pure Chemical Industries (Osaka, Japan) unless otherwise indicated.

Isolation of B and T cells from splenocytes and cell labeling with fluorescent dye

CD19-positive (CD19^{+}) B cells and CD3-positive (CD3^{+}) T cells were isolated using anti-CD19 antibody-conjugated magnetic beads and the Pan T cell Isolation Kit II (Miltenyi Biotec, Bergisch-Gladbach, Germany) following the manufacturer's instructions. Three millions of isolated CD19^{+} B cells were stained with 400-fold-diluted Orange Fluorescent Cytoplasmic Membrane Staining Kit (Takara Bio, Shiga, Japan) for 2 min, and 5×10^6 isolated CD3^{+} T cells were stained with $1 \mu\text{M}$ 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Affymetrix Japan, Tokyo) for 10 min. Splenocytes were then reconstituted with CFSE-labeled CD3^{+} T cells, orange fluorescence-labeled B cells, and non-labeled non-T/B cells.

Cell culture conditions and microscopic analysis

For the culture of whole splenocytes, 3×10^5 splenocytes prepared from a naive female BALB/c mouse were cultured with 10 mg/ml IVIg or human albumin in 200 μ l of culture medium; RPMI medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich), 50 μ M 2-mercaptoethanol (Nacalai Tesque, Kyoto, Japan), 100 units/ml penicillin (Thermo Fisher Scientific, Waltham, MA, USA) and 100 μ g/ml streptomycin (Thermo Fisher Scientific) for 2 days in 96-well flat-bottom culture plates (Corning, Corning, NY, USA) for a proliferation assay, or a triple-well glass-base dish (Asahi Glass, Tokyo) for microscopic observation.

CD19⁺ B cells or CD3⁺ T cells were depleted from murine splenocytes using anti-CD19 antibody-conjugated magnetic beads or the Pan T cell Isolation Kit II (Miltenyi Biotec), respectively, according to the manufacturer's instructions. Whole splenocytes, isolated CD19⁺ B cells, isolated CD3⁺ T cells, splenocytes depleted with CD19⁺ B cells or CD3⁺ T cells were cultured as 3×10^5 cells in 200 μ l of culture medium for 2 days with 10 mg/ml IVIg, 10 mg/ml human albumin, 50 μ g/ml mitomycin C, or saline as a vehicle control. The aggregation of fluorescent dye-labeled cells was analyzed by fluorescence microscopy (BZ-9000, Keyence, Osaka, Japan) at 48 h after cell cultivation.

Proliferation assay

Homeostatic cell proliferation was estimated by BrdU incorporation during the last 18 h of the 2-days culture. BrdU incorporation was determined by Cell Proliferation Enzyme-linked Immunosorbent Assay (ELISA) BrdU (colorimetric) Kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions.

Blocking experiments by anti-Fc γ receptors (CD16/CD32) and anti-integrin β 2 (CD18) antibodies

Naive splenocytes were incubated with 16 μ g/ml anti-CD16/CD32 antibody (eBioscienceTM, Thermo Fisher Scientific), 16 μ g/ml rat IgG2a isotype control antibody (eBioscienceTM, Thermo Fisher Scientific),

20 μ g/ml anti-CD18 antibody (BioLegend, San Diego, CA, USA), or 20 μ g/ml rat IgG2a isotype control antibody (BioLegend) for 15 min at 4 °C. After the incubation, 20 mg/ml IVIg, 20 mg/ml human albumin, 100 μ g/ml mitomycin C or saline was added and cells were cultured for 2 days, final concentration for each agent was 8 μ g/ml anti-CD16/CD32 antibody and their isotype control antibody (rat IgG2a), 10 μ g/ml anti-CD18 antibody and its isotype control antibody (rat IgG2a), 10 mg/ml IVIg and human albumin, and 50 μ g/ml mitomycin C.

Statistical analysis

Data are represented as mean $\pm$ SD. Representative data are from at least three independent experiments. A non-repeated analysis of variance (ANOVA) was applied to the comparisons of data among four groups; when a difference was significant, a post hoc analysis using the Tukey's test was performed. Statistical significance was defined as $p < 0.05$.

Results and discussion

IVIg suppresses homeostatic proliferation of B cells

We first tested the effect of IVIg on leukocyte cell proliferation under a homeostatic steady-state condition with no exogenous stimulation. Firstly, we observed that naive whole splenocytes showed a homeostatic proliferative response (shown by a purple bar in Fig. 1). This leukocyte cell proliferation was completely abolished by B cell depletion but not by T cell depletion (see corresponding purple bars). In addition, purified B cells, but not purified T cells, also showed a homeostatic proliferative response comparable to that seen in whole splenocytes. These results correctively indicate that B cells are dominant immune cells that show homeostatic proliferation without any exogenous stimulation.

We found that supplementation with IVIg significantly suppressed this B cell-intrinsic homeostatic proliferation (shown by red bars in Fig. 1) in whole splenocytes, T cell-depleted splenocytes, and purified B cells as compared with those stimulated with albumin (negative control protein) or saline (vehicle control).

Fig. 1 IVIg suppresses homeostatic proliferation of B cells. Whole splenocytes, B cell- or T cell-depleted splenocytes, and purified B cells or T cells were cultured with IVIg, albumin, saline, or mitomycin C for 48 h. Mitomycin C treatment was used as a negative proliferative control. The cultured cells were pulsed with BrdU for the last 18 h of the culture. The amount of incorporated BrdU was determined by a colorimetric ELISA (A_{450}). * $p < 0.05$ as estimated by post hoc Tukey's test compared among saline, human albumin, IVIg, and mitomycin C. Data are presented as mean $\pm$ SD ($n = 3$)

IVIg-driven suppression of B cell homeostatic proliferation is accompanied by induction of B cell-specific aggregation

We next analyzed morphological changes in immune cells incubated with IVIg by microscopic inspection. We found that IVIg induced splenocyte aggregation after a 48 h culture as compared with those cultured with albumin as a nonfunctional protein control or saline as a vehicle control (Fig. 2a). We also observed that the IVIg-induced splenocyte aggregation was seen from 3 h after the start of culture (data not shown).

We next sought to determine responsible cell types for the IVIg-induced leukocyte aggregation. To this end, we first analyzed the involvement of B cells and T cells in the IVIg-mediated cell aggregation. We depleted T cells or B cells from splenocytes, and cultured those cells with IVIg for 48 h. We found that IVIg induced cell aggregation in whole splenocytes as well as in T cell-depleted splenocytes, but not in B cell-depleted splenocytes (Fig. 2b). In addition, IVIg also induced cell aggregation in purified B cells but not in purified T cells (Fig. 2b). These data indicate that B cells are responsible for the IVIg-driven leukocyte aggregation.

To further confirm whether these aggregated cells were B cells, we conducted cell type-specific fluorescent labeling (Fig. 2c). Purified B cells or T cells were

stained with orange or green fluorescent dye, and mixed with non-labeled other leukocytes to reconstitute whole splenocytes. The reconstituted splenocytes containing orange dye-labeled B cells and green dye-labeled T cells were incubated with IVIg, albumin, or saline for 48 h. We found that IVIg specifically induced formation of B cell clusters (stained in orange) in the reconstituted splenocytes, and that the B cell clusters are mainly comprised of only B cells (Fig. 2c). These results further confirm that B cells are responsible target cells for the IVIg-induced cell aggregation.

Blocking of $Fc\gamma$ receptors (CD16/ $Fc\gamma$ RIII and CD32/ $Fc\gamma$ RII) or integrin β 2 (CD18) cannot abrogate the IVIg-driven B cell aggregation and suppression of homeostatic proliferation

To explore target molecules responsible for the IVIg-induced B cell aggregation and inhibition of homeostatic proliferation, we tested the involvement of $Fc\gamma$ receptors for IgG (CD16/ $Fc\gamma$ RIII and CD32/ $Fc\gamma$ RII). In a human in vitro study, IVIg induces CD32-mediated platelet aggregation (Pollreisz et al. 2008). CD32 is a low-affinity $Fc\gamma$ receptor, and it is also expressed on the surface of conventional B cells. Moreover, $Fc\gamma$ receptors are also considered to be one of the key target molecules through which IVIg fulfills its immunosuppressive action (Kaneko et al. 2006; Mitrevski et al. 2015). We thus tested the involvement of $Fc\gamma$ receptors in the IVIg-induced B cell aggregation by using anti-CD16 ($Fc\gamma$ RIII)/CD32 ($Fc\gamma$ RII) blocking antibody. We stimulated reconstituted splenocytes (containing orange fluorescence-labeled B cells and green fluorescence-labeled T cells) with IVIg upon neutralization with anti-CD16/CD32 antibody or rat IgG2a antibody as an isotype control. As shown in Fig. 3a, B cell aggregation induced by IVIg (stained as orange clusters) was still intact even when supplemented with anti-CD16/CD32 antibody. We also tested the involvement of integrin β 2 (CD18) in the B cell aggregation induced by IVIg, because CD18 is another important molecule for the B cell aggregation (Bjorck et al. 1992). We found that the IVIg-mediated B cell aggregation was unaffected upon treatment with anti-CD18 antibody (Fig. 3a). These results suggest that $Fc\gamma$ receptors and integrin β 2 are dispensable for the IVIg-driven B cell aggregation. We further confirmed that treatment with anti-CD16/

Fig. 2 IVIg induces B cell aggregation. **a** IVIg induces leukocyte aggregation. Naive murine splenocytes were incubated with 10 mg/ml IVIg, human albumin, or saline for 48 h. Representative images of cultured cells are shown for each stimulant. All scale bars represent 100 μ m. **b** IVIg induces B cell aggregation. Whole naive splenocytes, splenocytes depleted with T cells or B cells, and purified B cells or T cells were cultured with IVIg for 48 h. All scale bars represent 100 μ m. **c** IVIg induces B cell aggregation in reconstituted splenocytes. Purified B cells or T cells were labeled with orange or green fluorescent dye, respectively. Whole splenocytes were then reconstituted by mixing those fluorescence-labeled B and T cells with unstained non-B/T cells. The reconstituted splenocytes were cultured with IVIg, albumin, or saline for 48 h, and analyzed by fluorescence microscopy. All scale bars represent 100 μ m

CD32 or anti-CD18 antibody did not abrogate the IVIg-driven suppression of homeostatic proliferation in naive leukocytes (Fig. 3b), suggesting that those cell surface molecules are also dispensable for the inhibition of homeostatic proliferation by IVIg.

This is the first paper showing that IVIg induces B cell aggregation and suppresses its homeostatic proliferation under non-stimulatory conditions (Fig. 2). Functional significance of the IVIg-induced B cell aggregation on the anti-B cell proliferative effect of IVIg remains unclear. Proposed target molecules for IVIg are Fc receptors for IgG (Fc γ R/CD16 and CD32), surface IgM, C-type lectin SIGN-R1 (CD209b), Fas

(CD95), BAFF, APRIL, and so forth (Anthony et al. 2008; Le Pottier et al. 2007; Pollreis et al. 2008; Seite et al. 2014). However, most of those ligands are involved in cell proliferation and/or cell death, but not in cell aggregation. Although CD32 was a responsible ligand for aggregation of platelets by IVIg (Pollreis et al. 2008), blocking with anti-CD32 antibody had no impact on the IVIg-induced B cell aggregation as well as on the suppression of homeostatic proliferation (Fig. 3). Another candidate molecule for B cell aggregation was integrin β 2 (CD18) (Bjorck et al. 1992), but anti-CD18 antibody also could not abrogate IVIg-induced cell aggregation and its anti-

Fig. 3 $Fc\gamma$ receptors (CD16/CD32) and integrin $\beta 2$ (CD18) are dispensable for the IVIg-driven B cell aggregation and its anti-proliferative capacity. **a** Reconstituted splenocytes containing orange fluorescence-labeled B cells and green fluorescence-labeled T cells were cultured with IVIg upon pre-incubated with anti-CD16/CD32 antibody, anti-CD18 antibody, or corresponding isotype IgG2a antibody as a negative control. All scale bars represent 100 μ m. **b** Whole splenocytes pre-incubated with anti-

CD16/CD32, anti-CD18, or isotype IgG2a antibody were cultured with IVIg, albumin, saline, or mitomycin C for 48 h. The cultured cells were pulsed with BrdU for the last 18 h of the culture, and their BrdU incorporation was determined by a colorimetric ELISA (A_{450}). * $p < 0.05$ as estimated by post hoc Tukey's test compared among saline, human albumin, IVIg, and mitomycin C. Data are presented as mean $\pm$ SD ($n = 3$)

proliferative effect (Fig. 3). Identification of responsible target molecules is needed to elucidate molecular mechanisms underlying the IVIg-driven B cell aggregation and inhibition of homeostatic expansion.

Steady-state B cell plays a crucial role to prevent infection by producing polyspecific natural antibodies, which sometimes trigger crisis of autoimmune disorders (Choi and Baumgarth 2008; Haji-Ghassemi et al. 2017). IVIg may suppress activation of pathogen-activated B cells and steady-state B cells to reduce excess inflammation and to prevent overproduction of polyspecific natural antibodies by steady-state B cells, which may trigger crisis of autoimmune diseases.

It remains unclear what molecules are involved in anti-proliferative capacity and cell aggregation of steady-state B cell by IVIg. We are working onto elucidation of responsible mechanisms how IVIg induces anti-proliferative effects with B cell aggregation by omics analysis. Precise mechanisms and

clinical significance of anti-proliferative effects with cell aggregation in steady-state B cell should be clarified in the near future.

In summary, here we show that IVIg directly acts on unstimulated steady-state B cells to inhibit their homeostatic proliferation. This unique B cell regulatory function provides new mechanical insight into the immunosuppressive action of IVIg.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding this study.

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