Intravenous IgG Reduces Pathogenic Autoantibodies, Serum IL-6 Levels, and Disease Severity in Experimental Bullous Pemphigoid Models

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Bullous pemphigoid (BP) is an autoimmune blistering disease characterized by autoantibodies to COL17. Currently, systemic corticosteroids are used as first-line treatments for BP; alternatively, intravenous administration of high-dose IgG (IVIG) has been shown to be effective for patients with steroid-resistant BP in clinical practice. However, the effect of IVIG on BP has not fully been investigated. To examine the effects and mechanisms of action of IVIG against BP, we performed IVIG experiments using two experimental BP mouse models. One is a passive-transfer BP model that reproduces subepidermal separation in neonatal mice by the passive transfer of IgGs against COL17, such as polyclonal or monoclonal mouse IgG or IgG from BP patients. The other is an active BP model that continuously develops a disease phenotype in adult mice. IVIG decreased pathogenic IgG and the disease scores in both models. Injected IVIG distributed throughout the dermis and the intercellular space of the lower epidermis. Notably, IVIG inhibited the increase of IL-6 in both models, possibly by suppressing the production of IL-6 by keratinocytes. These results suggest that the inhibitory effects of IVIG on BP are associated with the reduction of pathogenic IgG and the modulation of cytokine production.


Bullous pemphigoid (BP) is characterized by tense blisters with itchy urticarial plaques and erythema that develop on the entire body. It is induced by autoantibodies to COL17 (also called BP180), a hemidesmosomal transmembrane protein at the dermal–epidermal junction (DEJ). The juxta-membranous extracellular noncollagenous 16A (NC16A) domain is preferentially recognized by autoantibodies in the sera of BP patients (Zillikens et al., 1997). The pathogenicity of IgG from BP patients (BP-IgG) was directly proven by our previous studies using COL17-humanized (COL17m−/−.h+) mice, which lack murine COL17 but express human COL17 in the skin (Nishie et al., 2007). Previous studies using an IgG passive-transfer neonatal mouse model have demonstrated that pathogenic IgG binding to murine COL17 triggers immune responses that include complement activation (Liu et al., 1995), mast cell degranulation (Chen et al., 2001), and neutrophil infiltration (Liu et al., 1997). The infiltrating neutrophils are activated via Fcγ receptor III (Schulze et al., 2014; Zhao et al., 2006) and IV (Schulze et al., 2014) and release neutrophil elastase and matrix metalloproteinase-9, which are responsible for the dermal–epidermal separation (Liu et al., 2000). Meanwhile, we demonstrated that BP-IgG is able to induce dermal–epidermal separation in complement-deficient neonatal mice (Ujiie et al., 2014). Cultured human keratinocytes produce proinflammatory cytokines, such as IL-6 and IL-8, by stimulation with BP-IgG (Schmidt et al., 2000). Thus, several complement-independent pathomechanisms also play important roles in blister formation in BP.

Currently, in clinical practice, systemic corticosteroids are used as the first-line treatment for moderate to severe BP, but long-term corticosteroid treatment may cause many dose-related adverse effects. Intravenous administration of high-dose IgG (IVIG) has also been reported as a safe, beneficial therapy for BP (Amagai et al., 2017). In general, several mechanisms of anti-inflammatory effects for IVIG have already been proposed, such as (i) the inhibition of the recycling of pathogenic autoantibodies via neonatal Fc receptor saturation; (ii) the suppression or neutralization of pathogenic autoantibodies by anti-idiotypic antibody action; (iii) the non-specific blocking of the Fcγ receptor via the Fc portion derived from IgG preparations; and (iv) the modulation of cytokine production, such as decreases in TNF-α, IL-1β, IL-4, IL-6, IL-13, and IL-33, and an
increase in IL-10 (Gelfand, 2012; Tjon et al., 2015). DC-SIGN ligation by sialylated IgG Fc fragments in IVIG results in FcylRIB upregulation on monocytes and macrophages (Anthony et al., 2011). Other mechanisms of IVIG have also been reported, such as the inhibition of dendritic cells, B cells, T helper type 1 cells, and T helper type 17 cells, the stimulation of regulatory T cells (Tjon et al., 2015) and the IVIG-mediated death effects on human neutrophils (Schneider et al., 2017). However, the modes of action and the impact of IVIG on BP have not been fully investigated in vivo.

In this study, we used two experimental BP mouse models. One is a passive-transfer BP model that reproduces dermal–epidermal separation by the passive-transfer of IgG antibodies to COL17 into neonatal COL17-humanized mice (Nishie et al., 2007). The other is an active BP model that was generated by the adoptive transfer of human COL17-immunized spleen cells into adult immunodeficient COL17-humanized mice. This model continuously produces anti-human COL17 IgG in a CD4+ T-cell–dependent manner and reproduces the BP phenotype (Ujiie et al., 2010). We demonstrate that IVIG reduces pathogenic antibodies to COL17, including polyclonal mouse IgG, monoclonal mouse IgG, and BP-IgG, and suppresses BP phenotype in experimental models. We also demonstrate the distribution of administered IVIG in the skin. Furthermore, we show that IVIG modulates the production of cytokines and chemokines, and that, of these, IL-6 is suppressed by IVIG both in vivo and in vitro. These findings clarify the mechanisms of action of IVIG in BP.

RESULTS

IVIG reduced circulating polyclonal antibodies to COL17 and skin fragility in the passive-transfer BP model

To examine whether IVIG suppresses skin fragility that is simply induced by IgGs to COL17, we performed an IVIG experiment using an IgG passive-transfer BP model. First, antibodies to COL17 were isolated from the pooled sera of wild-type mice that had been immunized by grafting human COL17-expressing transgenic mice skin to their back (SG-IgG). We treated the neonatal mice with saline at 400 mg/kg/d (IVIG-400) or at 2,000 mg/kg/d (IVIG-2,000) at 2 hours prior to the injection of 150 µg/g of SG-IgG, and we examined them at 18 hours after injection (n = 15, n = 10, and n = 15, respectively). All of the saline-treated or IVIG-400–treated mice showed skin fragility and dermal–epidermal separation histologically (Figure 1a). Notably, 40% of the mice treated with IVIG-2,000 prior to SG-IgG were resistant to skin fragility (positive/total = 9/15). The intensities of IgG and C3 deposition at the DEJ of the skin were similar in all groups (Figure 1a). Inflammatory cell infiltration was not obvious in any of the mice. IVIG significantly decreased circulating antibodies to the NC16A domain of COL17 in a dose-dependent manner (Figure 1b). Titers of injected human IgG remained elevated for 48 hours after the IVIG-2,000 (Figure 1c). These findings show that passively transferred human IgGs persist for at least 2 days in mice, reduce circulating antibodies to the NC16A domain and mitigate skin fragility in the passive-transfer BP model.

IVIG reduces the binding of monoclonal antibodies against the NC16A domain of COL17 to the antigen

Next, we performed IgG passive-transfer experiments by using monoclonal mouse IgG1 to the NC16A domain of COL17 (TS39-3) (Ujiie et al., 2014) instead of SG-IgG, which consists of polyclonal antibodies to various epitopes on COL17. In this setting, we were able to directly evaluate the effect of IVIG on the BP model induced by an antibody to a single pathogenic epitope. Neonatal mice were treated with saline or IVIG-2,000 at 2 hours prior to the injection of 50 µg/g of TS39-3 and were examined at 24 hours after injection. All the saline-treated mice showed skin fragility (Figure 1d). Meanwhile, 86% of the mice treated with IVIG-2,000 prior to TS39-3 were resistant to skin fragility (positive/total = 1/7) (Figure 1d). Notably, the intensities of IgG deposition at the DEJ were significantly lower in the IVIG-2,000–treated mice than in the saline-treated mice (Figure 1e). Interestingly, human IgGs injected as IVIG distributed diffusely throughout the dermis, the subcutaneous tissues, and the intercellular space of the lower epidermis, but not at the DEJ (Figure 1d). IVIG reduced circulating TS39-3 as determined by ELISA (Figure 1f), although it failed to reduce the amount of total mouse IgG in the plasma (Supplementary Figure S1 online). Thus, IVIG eliminates the pathogenic antibodies and reduces the skin fragility in a passive-transfer BP model.

IVIG suppresses the skin fragility induced by BP-IgG

To examine the effect of IVIG in a more clinically relevant condition, we examined the effect of IVIG on a passive-transfer BP model induced by BP-IgG. Neonatal mice were treated with saline or IVIG-2,000 at 2 hours prior to the injection of 750 µg/g of BP-IgG and were examined at 24 hours after injection (n = 7 and 8, respectively). All of the saline-treated mice showed skin fragility, whereas 75% of the mice treated with IVIG-2,000 prior to BP-IgG were resistant to skin fragility (positive/total = 2/8) (Figure 2a). The deposition of BP-IgG at the DEJ was difficult to evaluate because both the BP-IgG and the IVIG were human IgGs (Figure 2a). Notably, the intensities of C3 deposition at the DEJ were significantly diminished in the IVIG-2,000–treated mice (Figure 2a, 2b). IVIG reduced circulating BP-IgG as determined by ELISA (Figure 2c). Thus, IVIG reduced BP-IgG, suppressed C3 deposition, and prevented skin fragility in the passive-transfer BP model.

IVIG modulates inflammatory cytokines and chemokines in the passive-transfer BP model

To further examine the mechanism of action of IVIG, we measured plasma cytokine and chemokine levels at different time points in the SG-IgG passive-transfer BP model. Most of the examined cytokines and chemokines, including IFN-γ, TNF-α, IL-1β, IL-2, IL-6, IL-10, IL-13, IL-17, eotaxin, G-CSF, GM-CSF, MIP-1α, MIP-1β, and MCP-1, increased after SG-IgG injection, whereas IL-1γ, IL-12p40, and RANTES did not. IVIG-2,000 significantly decreased IL-6, IL-17, and MIP-1β at the early phase (Figure 3). Eotaxin also tended to be decreased at the early phase, and IFN-γ, MIP-1α, and MCP-1 tended to be decreased at the late phase by IVIG-2,000, although not significantly. Conversely, IL-10, an anti-inflammatory cytokine, was elevated in the IVIG-2,000–treated mice, although not significantly (Figure 3).
IVIG reduces disease severity and circulating autoantibodies in the active BP model

We next tried to examine the effect of IVIG on the active BP model that mimics the human BP phenotype better than the passive-transfer BP model does (Ujiie et al., 2010). Immunized spleen cells reacting with COL17 were intravenously transferred into Rag-2−/−/COL17-humanized mice at day 0. This model produces antibodies to the NC16A domain of COL17 in IVIG-treated and saline-treated mice 18 hours after the passive transfer of SG-IgG (normal mice, n = 15; saline + SG-IgG, n = 15, IVIG-400 + SG-IgG, n = 10; IVIG-2,000 + SG-IgG, n = 15). The titer of circulating human IgG at baseline and 6, 12, 18, and 24 hours after SG-IgG injection with or without IVIG-2,000, n = 6, respectively; at 48 hours, n = 3, respectively) (Figure 1). Linear deposition of mouse IgG (TS39-3) at the DEJ of the mice is indicated by arrows. (d) Representative clinical presentations and IgG deposition of the back skin 24 hours after the passive transfer of TS39-3 (50 μg/g), a monoclonal antibody to the NC16A domain. Skin detachment with gentle rubbing is indicated by an arrowhead. Linear deposition of mouse IgG (TS39-3) at the DEJ of the mice is indicated by arrows. Administered human IgG (IVIG) is visualized by anti-human IgG antibody. Scale bar = 200 μm. (e) Fluorescence intensity of direct immunofluorescence using the back skin of IVIG-treated and saline-treated mice 24 hours after the passive transfer of TS39-3. (f) The titer of IgG to the NC16A domain of COL17 in IVIG-treated and saline-treated mice 24 hours after the passive transfer of TS39-3. Results are shown as mean ± standard deviation. *P < 0.05, **P < 0.01 versus control. BP, bullous pemphigoid; COL17, type XVII collagen; DEJ, dermal-epidermal junction; IVIG, intravenous administration of high-dose IgG; NC16A, noncollagenous 16A domain; SG-IgG, IgG from skin-grafted mice.
Next, we examined the therapeutic effect of IVIG-2,000 by administering it for 5 consecutive days at two different timings: from days 1 to 5 (IVIG-2,000-1) or from day 6 to 10 (IVIG-2,000-2). In the early phase (2 to 4 weeks after transfer), the IVIG-2,000-2 tended to reduce the disease severity, whereas the saline or IVIG-2,000-1 did not, but in the late phase, there were no differences between the regimens (Figure 5a). Neither regimen reduced the titers of circulating autoantibodies (Figure 5b).

IVIG decreases IL-6 and increases IL-10 in the active BP model

We also measured cytokine levels in the active BP model at day 11. We focused on the four cytokines that were altered by IVIG in the passive-transfer BP model. IVIG-2,000 significantly decreased IL-6 and increased IL-10 in the active BP model, whereas IL-17 and MIP-1β showed no changes (Figure 6a). The changes could be associated with the reduction of disease severity by IVIG-2,000 in the active BP model.

IVIG decreases IL-6 release from cultured keratinocytes

IVIG reduced serum IL-6 levels in both the passive-transfer BP model and the active BP model. IL-6 is known to be produced by keratinocytes in response to stimulation with antibodies to COL17 (Schmidt et al., 2000). In addition, we found that administered IVIG distributes in the intercellular space of the lower epidermis (Figure 6b). Therefore, we examined the effect of IVIG on IL-6 production by keratinocytes. HaCaT cells, which are immortalized human keratinocytes, were stimulated with BP-IgG in the presence or absence of IVIG. The release of IL-6 from the HaCaT cells was significantly reduced by IVIG (Figure 6c), suggesting that IVIG may directly act on keratinocytes and inhibit the production of IL-6.

**DISCUSSION**

IVIG suppressed disease severity in both the passive-transfer BP model and the active BP model with the reduction of circulating antibodies to the NC16A domain of COL17. First, we examined the effect of IVIG on a neonatal passive-transfer BP model in which the disease is induced by SG-IgG, polyclonal antibodies to COL17. IVIG significantly reduced circulating antibodies to the NC16A domain and partially suppressed skin fragility in a dose-dependent manner. However, direct immunofluorescence failed to detect the reduction of the deposition of SG-IgG at the DEJ. We previously reported that antibodies to the NC16A domain but not to other domains on COL17 are relevant to the skin fragility in the BP model (Ujiie et al., 2012). Therefore, we speculated that the amount of SG-IgG binding to the NC16A domain was decreased by IVIG, but that the reduction was masked by the deposition of a large amount of antibodies to other domains on COL17. To address this issue, we next performed a passive-transfer experiment by using TS39-3, a pathogenic monoclonal antibody to the NC16A domain. As expected, IVIG reduced the circulating as well as the skin-binding TS39-3 antibody and suppressed skin fragility. However, we were unable to evaluate the C3 deposition in this model because TS39-3 is a mouse IgG1 antibody that has no complement activation ability. We next performed IVIG experiments using a BP-IgG passive-transfer model. Notably, IVIG significantly diminished C3 deposition at the DEJ induced by BP-IgG. Taken together, these findings suggest that IVIG reduces circulating antibodies to COL17 and diminishes the deposition of IgG and C3 at the DEJ, although the results depend on experimental system.
How does IVIG reduce the pathogenic antibodies in vivo? Previous studies have demonstrated that the IVIG saturates the neonatal Fc receptor, a protective receptor that prevents IgG degradation by lysosomes and returns intact IgG to circulation, and enhances the clearance of the free pathogenic autoantibody from circulation (Gelfand, 2012; Li et al., 2005). Human IgGs are able to bind to murine neonatal Fc receptor (Ober et al., 2001). The reduction of the pathogenic antibody by IVIG in our model might be explained by this mechanism.

A passive-transfer BP model using neonatal mice is a simple and easy method for assessing the efficacy of IVIG, but it has limitations: (i) IVIG was administered intraperitoneally and not intravenously due to technical difficulties; (ii) IVIG was administered in a single dose, and (iii) the observation period was short. To overcome these issues, we also utilized an active BP model. IVIG decreased circulating antibodies to the NC16A domain as well as disease severity in the active BP model in a dose-dependent manner. Furthermore, delayed short-term administration of IVIG temporally showed a reduction of disease severity, suggesting that IVIG may have not only a preventive effect but also a therapeutic effect on the active BP model.

To explore the other factors that contribute to diminished disease severity, we further examined plasma cytokine and chemokine levels. In the passive-transfer BP model, the administration of SG-IgG induced various cytokines and chemokines, especially at the early phase (6 hours after SG-IgG injection) and, interestingly, IVIG decreased IL-6, IL-17, and MIP-1β (CCL4). The skin fragility in a passive-transfer BP model is considered to be associated with the reduction of COL17 due to the binding of antibodies to the NC16A domain rather than due to the inflammatory process (Ujiie et al., 2014), whereas the disease severity in the active BP model is likely to be related to skin inflammation, because the model always scratches their skin and develop erythematous skin lesions (Ujiie et al., 2010). Therefore, we next examined several cytokines in the active model that were changed in the passive-transfer model. Interestingly, IL-6 was again decreased in IVIG-treated active BP model. IVIG was reported to suppress the release of IL-6 from blood cells of normal children and reduce serum IL-6 in children with Kawasaki disease (Gupta et al., 2001). IL-6, a proinflammatory cytokine, is well known as a key cytokine in BP because it is produced by keratinocytes in response to stimulation with antibodies to COL17 (Schmidt et al., 2000), and the serum levels correlate with disease activity (Inaoki and Takehara, 1998). Therefore, we assumed that IVIG may suppress IL-6 production by keratinocytes. As expected, IVIG reduced the release of IL-6 by cultured keratinocytes that were stimulated with BP-IgG, suggesting a novel mechanism of action of IVIG in BP.
Conversely, IL-10, an anti-inflammatory cytokine, was elevated by IVIG in the active BP model. Previous studies demonstrated that serum IL-10 levels (D’Auria et al., 1999) and the percentages of IL-10-producing T cells (Teraki et al., 2001) are significantly higher in BP patients after therapy with the resolution of the disease than before therapy. IVIG has been reported to induce IL-10 in various models of inflammatory disease (Kozicky et al., 2015; Ramakrishna et al., 2011). Although little is known about how IVIG affects the differentiation of IL-10-producing cells, our results suggest that IVIG may regulate the inflammatory responses of BP by promoting the increase of IL-10-producing cells.

In conclusion, IVIG decreases antibodies to the NC16A domain and modulates the production of cytokines and chemokines, resulting in the reduction of disease severity in experimental BP models. IVIG might act directly on keratinocytes to suppress the production of IL-6. This study provides significant evidence that IVIG is effective for BP through several mechanisms, although the details should be further investigated.
Figure 6. IVIG decreases IL-6 in the active BP model and from cultured keratinocytes. (a) Plasma IL-6, IL-17, IL-10, and MIP-1β levels were examined at day 11 after the adoptive transfer of immunized spleen cells in the active BP model (saline-treated, n = 4; IVIG-2,000–treated, n = 5). (b) Human IgG was visualized by FITC-conjugated anti-human IgG antibody in the skin of the IVIG-treated BP-IgG passive-transfer model. Positive staining is seen in the intercellular space of lower epidermis. Scale bar = 50 μm. (c) IL-6 level in the supernatant of cultured HaCaT cells with or without BP-IgG and IVIG. *P < 0.05. BP, bullous pemphigoid; IVIG, intravenous administration of high-dose IgG.

**MATERIALS AND METHODS**

**Generation of the passive-transfer BP model and IVIG treatment**

SG-IgG was passively transferred into neonatal COL17-humanized mice as described previously (Nishie et al., 2007; Ujiie et al., 2010). Different doses of IVIG (NIHON Pharmaceutical Co, Ltd, Japan) (400 mg/kg or 2,000 mg/kg) or saline was intraperitoneally administered to neonatal COL17-humanized mice, and 150 μg/g SG-IgG was intraperitoneally transferred at the same time or 2 hours later. At 18 hours after the injection of SG-IgG, the fragility of back skin was evaluated by up to four incidences of gentle rubbing. The evaluation was carried out by a blinded investigator. Ear or back skin was obtained after the evaluation of skin fragility and processed for light microscopy (hematoxylin and eosin) and for direct immunofluorescence using FITC-conjugated antibodies to mouse IgG and C3. In the hematoxylin and eosin sections, the histopathologic results were scored as follows: 0, negative; 1, minimal; 2, mild; 3, moderate; 4, marked. In some experiments, 50 μg/g TS39-3 or 750 μg/g BP-IgG was intraperitoneally transferred instead of SG-IgG and the mice were evaluated 24 hours after the injection. All animal procedures were conducted according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee.

**Generation of the active BP model**

Wild-type mice were immunized by human COL17-expressing Tg mouse skin grafts as described. Spleen cells were isolated and pooled from several immunized wild-type mice and intravenously transferred into Rag-2−/−/COL17-humanized mice through a tail vain in 500 μl phosphate-buffered saline (Ujiie et al., 2010). To determine the appropriate number of spleen cells, we compared the disease severity of mice at three different numbers of spleen cells (1.0, 2.0, and 4.0 × 10⁶ cells/mouse), and we found 1.0 × 10⁶ cells to be the best for inducing the grade of BP appropriate for this study.

**IVIG for the active BP model**

Spleen cells (1.0 × 10⁶) were transferred to Rag-2−/−/COL17-humanized mice at day 0. The mice were treated with IVIG from day 1 to day 21 (for 3 weeks) at different doses (400 mg/kg/d or 2,000 mg/kg/d) or saline. In some of the experiments, the mice were treated with 2,000 mg/kg/d of IVIG from day 1 to day 5, or from day 6 to day 10. Then, the mice were examined for general condition and for percentage of body surface area affected by cutaneous lesions (i.e., erythema, hair loss, blisters, erosions, and crusts) at days 1, 6, 9, 11, 14, 18, 21, 24, 28, 32, and 35. We also collected plasma to examine circulating anti-human COL17 IgG levels at days 0, 6, 9, 11, 18, 21, 28, and 35. The animals were then sacrificed at day 35, and skin sections were taken for histologic examination. Back, neck, or ear skin was processed for light microscopy (hematoxylin and eosin) and direct immunofluorescence using FITC-conjugated antibodies to IgG and C3. In the hematoxylin and eosin sections, the histopathologic results were scored as follows: 0, negative; 1, minimal; 2, mild; 3, moderate; 4, marked.

**Statistical analyses**

Data were expressed as mean ± standard deviation. Statistical analyses were performed using SAS, Release 9.3 (SAS Institute Inc, Cary, NC) and GraphPad Prism (GraphPad Software, La Jolla, CA). In the SG-IgG passive-transfer BP model, an unpaired t test was performed for plasma IgG titer. In the TS39-3 and BP-IgG passive-transfer BP models, the statistical differences in fluorescence intensity and plasma IgG titer were determined by unpaired t test. Comparisons of cytokine and chemokine levels in the passive-transfer model were made by using Dunnett’s multiple comparison test. For the active BP model, the Wilcoxon rank-sum test was performed for histopathologic examinations and ANOVA was performed for all other examinations. Multiplicity adjustment was performed by the Holm method. The unpaired t test was used for a comparison of cytokine levels in the active model. For comparison of IL-6 from HaCaT cells, Tukey’s multiple comparisons test was used. P < 0.05 was considered significant compared with the control.

**CONFLICT OF INTEREST**

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.01.005.

REFERENCES


