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Original Article

# Examination of intravenous immunoglobulin (IVIG) therapy and regulatory T cell-related biomarkers in hematopoietic stem cell transplantation

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## ABSTRACT

The conditioning chemotherapies for hematopoietic stem cell transplantation (HSCT), particularly the powerful conditioning regimen that includes total body irradiation can result in serious infectious diseases due to the associated hypoglobulinemia. To counteract this effect, intravenous immunoglobulin (IVIG) therapy may be performed. However, IVIG therapy can also drive regulatory T-cell (Treg) production *via* stimulation by a T cell epitope (Tregitope) for major histocompatibility complex (MHC) Class II. In the present study, we measured a subset of Treg-related biomarkers, i.e., interleukin (IL)-10, IL-12, transforming growth factor (TGF)β1, and soluble cytotoxic T lymphocyte-associated antigen 4 (sCTLA-4) in HSCT patients and examined the relationship between these biomarkers and IVIG. Significant elevations of the IL-10, TGFβ1, and sCTLA-4 levels were observed in the IVIG group compared with the non-IVIG group, whereas the IL-12 levels exhibited a decreasing trend in the IVIG group. These results suggest the possibility that IVIG could have a therapeutic effect for Treg-related complications such as graft-versus-host disease after HSCT.

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**KEYWORDS:** IVIG, hematopoietic stem cell transplantation, regulatory T-cell, sCTLA-4, GVHD.

## INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) often involves serious transplant-related complications including graft-versus-host disease (GVHD) [1]. Although the complex pathophysiology of acute GVHD is also affected by the conditioning regimen, cytokines, nitric oxide, and non-T effector cells, the cytolytic activity of donor T-cells is essential for its development [2]. The cytolytic activity of cytotoxic T-lymphocytes (CTLs) is primarily mediated through effectors such as the Fas/Fas ligand (FasL) system and perforin/granzyme pathway [3]. Some studies have indicated that the Fas/FasL system is involved in the pathogenesis of GVHD [2, 4]. Furthermore, endothelial damage perpetuated by $CD8^+$ CTLs has been linked to GVHD and described for both the skin and gut [5-8].

$CD4^+$ regulatory T cells (Tregs) play a critical role in the maintenance of peripheral tolerance, by suppressing the activation and proliferation of immune cells [9, 10]. They express cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and the transcription factor forkhead-box p3 (Foxp3) [9]. Tregs are essential in maintaining immune tolerance to self-antigens in secondary lymphoid organs and peripheral tissues, and they play an important role in controlling the inflammatory

response [11-14]. In addition, a recent work showed that the adoptive transfer of Tregs prevents GVHD [15]. Soluble CTLA-4 (sCTLA-4) can modulate and terminate immune responses [16]. Several reports have shown that sCTLA-4 levels are changed in patients who have certain autoimmune disorders [16, 17]. However, to our knowledge, sCTLA-4 levels with regard to GVHD after HSCT have not been previously investigated.

Intravenous immunoglobulin (IVIG) therapy can help people with weakened immune systems or other diseases fight off infections [18, 19]. Some of the diseases that IVIG can treat include autoimmune or intractable diseases [19]. Immunoglobulin is a highly diverse autologous molecule that can influence immunity in various physiological and diseased states. Its effect may be visible in terms of both the development and function of B and T lymphocytes [19]. Therefore, IVIG may affect GVHD [20].

We measured and compared the levels of interleukin (IL)-10, IL-12, transforming growth factor $\beta_1$ (TGF$\beta_1$), and sCTLA-4 in patients undergoing allogeneic HSCT with or without subsequent IVIG treatment. The aims of this study are to investigate the role of Tregs in the mechanisms underlying GVHD development and to examine the prophylactic use of IVIG for GVHD.

## MATERIALS AND METHODS

### Subjects

The subjects were 50 patients who underwent allogeneic HSCT between July 2011 and September 2017 at our institution (Table 1). The 26 male and 24 female allogeneic HSCT patients ranged in age from 23 to 67 years (median: 47 years). The applied conditioning regimen was total body irradiation for 36 subjects and non-total body irradiation for 14. The details of HSCT were as follows: bone marrow transplantation, 30; peripheral blood stem cell transplantation, 11; cord blood transplantation, 9. Written informed consent was obtained from all patients who were officially registered with Kansai Medical University prior to HSCT.

**Table 1.** Patient and treatment characteristics.

| | Allogeneic HSCT |
|---|---|
| Sex | |
| Male/Female | 26 /24 |
| Median age (range) | 47 (23-67) |
| Patient diagnosis at transplantation | |
| Acute myeloblastic leukemia (AML) | 12 |
| Acute lymphoblastic leukemia (ALL) | 18 |
| Myelodysplastic syndrome (MDS) | 7 |
| Other | 13 |
| Conditioning regimen | |
| TBI-conditioning | 36 |
| CY/TBI | 12 |
| Flu/Bu/TBI | 6 |
| Flu/Mel/TBI | 3 |
| VP16/CY/TBI | 3 |
| Other | 12 |
| Non-TBI-conditioning | 14 |
| Flu/Bu | 6 |
| Bu/CY | 4 |
| Flu/Bu/ATG | 1 |
| Other | 3 |
| Donor source | |
| Bone marrow transplantation (BMT) | 30 |
| Peripheral blood stem cell transplantation (PBSCT) | 11 |
| Cords blood transplantation (CBT) | 9 |
| Prophylaxis for GVHD | |
| FK/sMTX | 24 |
| CyA/sMTX | 10 |
| FK/mPSL | 4 |
| FK/MMF | 4 |
| Other | 8 |

TBI: total body irradiation; CY: cyclophosphamide; Flu: fludarabine; Bu: busulfan; Mel: melphalan; ATG: anti-thymocyte globulin; GVHD: graft-versus-host disease; FK: tacrolimus; sMTX: short-term methotrexate; CyA: cyclosporine; mPSL: methylprednisolone; MMF: mycophenolate mofetil.

### IVIG therapy

The serum IgG was measured at 7, 14, and 21 days after HSCT. When the IgG concentration was less than 700 mg/dL, two vials (2.5 g x 2) per day of IVIG drug (Venoglobulin IH, JBPO, Japan) were administered over 2 days. If the IgG concentration remained less than 700 mg/dL at 2 weeks after IVIG administration the same volume of IVIG was given again, but the upper limit of the medication was twice the prescribed amount.

### Measurement of IL-10, IL-12, TGF$\beta_1$ and sCTLA-4

Blood samples from patients were collected into tubes containing sodium citrate or into tubes without any anticoagulant and the blood samples were allowed to clot at room temperature for a minimum of 1 h. The serum or citrated plasma was isolated by centrifugation for 20 min at 1,000 ×g at 4 °C. The serum was divided into aliquots and frozen at -30 °C until use. As a positive control, recombinant products as well as standard solutions provided with the commercial kits were used in each assay. Plasma concentrations of IL-10, IL-12, and TGF$\beta_1$ were measured using monoclonal antibody-based ELISA kits (Invitrogen Inc.; Camarillo, CA, USA). An ELISA kit from BioLegend, Inc. (San Diego, CA, USA) was used to measure sCTLA-4. The instructions warned that for measurement of TGF$\beta_1$, the activation of platelets should be avoided; so precautions to avoid platelet activation were taken. All kits were used according to the manufacturer's instructions.

### Statistical analysis

Data are expressed as the means ± SD. The statistical significance of differences between the groups was analyzed using chi-squared, Newman-Keuls, or Scheffe's tests. All statistical analyses were performed using StatFlex (ver. 6) software (Artech, Osaka, Japan). Values of $p < 0.05$ were considered statistically significant.

## RESULTS

Figure 1 shows the number of separate IVIG administrations over time. The time of IVIG administration ranged from day 8 to day 32 post HSCT and mean IVIG administration timing was 12.9 days after HSCT. Thirty-one patients were administered IVIG within 28 days after HSCT, 24 of them received IVIG administration within 14 days post HSCT. Three patients received an IVIG-regimen (repeated administration), 19 patients were not administered IVIG.

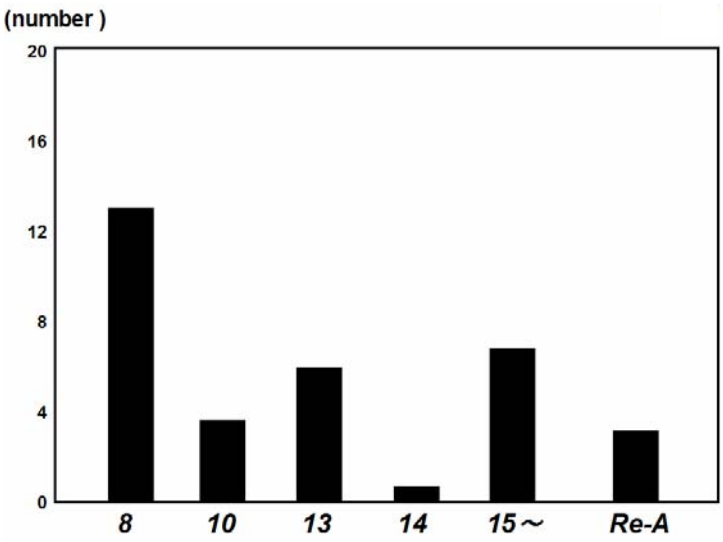


**Figure 1.** The number of separate IVIG administrations over time post HSCT. Re-A: re-administration.

**Table 2.** Changes in various factors with HSCT.

| Period before and after HSCT | TGFβ$_1$ (pg/ml) | sCTLA-4 (pg/ml) | IL-10 (pg/ml) | IL-12 (pg/ml) |
|---|---|---|---|---|
| Basal (before) | 1,462 ± 379 | 321 ± 138 | 1.2 ± 0.9 | 122 ± 25 |
| Day 0 | 1,285 ± 366 | 356 ± 128 | 1.3 ± 1.1 | 88 ± 21 |
| Day 4 | 1,087 ± 294$^{NS}$ | 352 ± 131 $^{NS}$ | 1.3 ± 1.2 $^{NS}$ | 82 ± 23 $^{NS}$ |
| Day 7 | 1,114 ± 313 $^{NS}$ | 313 ± 141 $^{NS}$ | 1.5 ± 1.4 $^{NS}$ | 69 ± 24 $^{NS}$ |
| Day 14 | 1,125 ± 359 $^{NS}$ | 337 ± 145 $^{NS}$ | 2.8 ± 1.6 $^{NS}$ | 72 ± 26 $^{NS}$ |
| Day 21 | 1,289 ± 384 $^{NS}$ | 429 ± 142 $^{NS}$ | 3.4 ± 2.1 $^{NS}$ | 75 ± 29 $^{NS}$ |
| Day 28 | 1,652 ± 486*$^{1}$ | 604 ± 158*$^{1}$ | 5.3 ± 2.6*$^{1}$ | 78 ± 31 $^{NS}$ |
| Day 35 | 2,213 ± 517*$^{2}$ | 785 ± 176*$^{2}$ | 8.8 ± 3.9*$^{2}$ | 82 ± 25 $^{NS}$ |

Data represent the means ± SD. TGFβ$_1$: transforming growth factor β$_1$; sCTLA-4: soluble cytotoxic T lymphocyte-associated antigen 4; IL-10: interleukin-10; IL-12: interleukin-12. The *p* values are for day 0 vs. day 4, 7, 14, 21, 28, and 35 post HSCT. NS: not significant. *$^{1}$: $p < 0.05$, *$^{2}$: $p < 0.01$.

Table 2 shows the changes in the levels of TGFβ$_1$, sCTLA-4, IL1-10, and IL12 following HSCT. The TGFβ$_1$, sCTLA-4, and IL-10 levels were all significantly increased following HSCT (day 28, $p < 0.05$; day 35, $p < 0.01$); in contrast, the level of IL12 was not changed.

We divided the patients into two groups based on whether they received IVIG (IVIG group) or not (no-IVIG group) after HSCT (Figure 2). Neither the IVIG group nor the no-IVIG group showed any significant changes in the four measured biomarkers from day 0 to day 21 post HSCT. However, the IVIG group showed significant increases in the plasma concentrations of TGFβ$_1$, sCTLA-4, and IL-10 relative to those in the no-IVIG group at days 28 and 35 post HSCT ($p < 0.05$ or $p < 0.01$; Figure 2). In addition, the IVIG group showed a significant decrease in the plasma concentrations of IL-12 relative to those in the no-IVIG group at days 21, 28, and 35 post HSCT ($p < 0.05$ or $p < 0.01$; Figure 2).

## DISCUSSION

Although the therapeutic performance of allogeneic HSCT has made remarkable progress, GVHD remains the most important complication of HSCT [21]. GVHD is caused by alloreactive T cells that recognize alloantigens initially presented by host/donor antigen-presenting cells (APCs) [22, 23]. Although GVHD can sometimes be constrained by using rigorously T cell-depleted donor grafts or pharmacologic agents, such treatments predispose patients to relapses, in malignancy and opportunistic infections [24]. Treg-based cell therapies are currently being employed in clinical trials to assess their ability to prevent GVHD post HSCT [25]. However, as Tregs suppress the function of conventional T cells and other immune cells, Treg-based cell therapies for the treatment of GVHD are under careful evaluation. In the present study, we evaluated the changes in TGFβ$_1$, sCTLA-4, IL-10, and IL-12 levels after HSCT. The results revealed significant increases in the TGFβ$_1$, sCTLA-4, and IL-10 levels following HSCT. Furthermore, these increases were significantly higher in patients who received IVIG therapy. Together, our results suggest that IVIG causes the elevation of Treg-related biomarkers such as TGFβ$_1$, sCTLA-4, and IL-10.

IVIG is a purified concentrated human immunoglobulin solution composed primarily of IgG, obtained by fractionating blood plasma from a pool of healthy donors [19]. Due to its immunomodulatory and anti-inflammatory effects, IVIG is used as a therapeutic modality for a variety of immune disorders [19]. Although several previous clinical studies failed to clearly

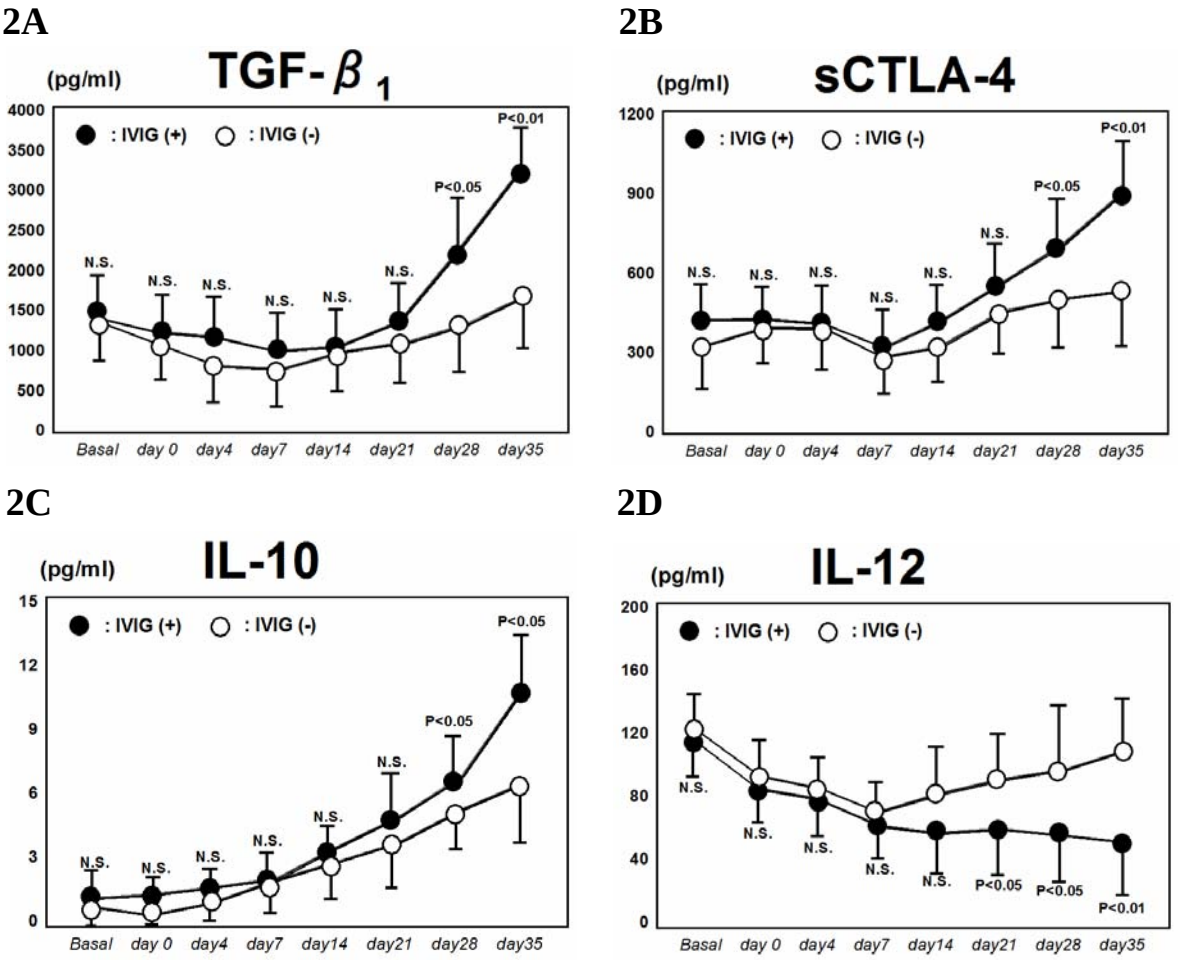


**Figure 2(A-D).** Changes in TGF$\beta_1$, sCTLA-4, IL-10, and IL-12 levels in patients who were and were not administered IVIG therapy post HSCT. Values are presented as means ± SD. The *p*-values shown for various biomarker levels in patients with IVIG are for the comparisons with the corresponding levels in patients who did not receive IVIG treatment (Basal, day 0, day 4, day 7, day 14, day 21, day 28, and day 35 post HSCT). N.S.: not significant.

demonstrate the therapeutic efficacy of IVIG in the prevention of GVHD, there is no doubt about the fact that IVIG is effective for treating GVHD [26]. However, Gregoire-Gauthier *et al*. [20] showed, in a xeno-GVHD model, that IVIG therapy did not modify the percentage or absolute numbers of human T lymphocytes, suggesting that IVIG may contribute to the prevention of GVHD *via* an immunomodulating effect. The relationship between IVIG and Tregs in the therapeutic effect of IVIG against GVHD after HSCT remains unclear.

Fc receptors, particularly the Fcγ receptor IIb, play an important role in the anti-inflammatory and immunomodulatory effect of IVIG [27]. Therefore, Fc receptors are likely to be involved the mechanism by which IVIG and Tregs interact to impart the therapeutic effect of IVIG for GVHD after HSCT. However, $CD4^+$ T lymphocyte lack Fc receptors, so IVIG probably does not have a direct Treg-increasing effect. Thus, we propose that IVIG acts on APCs possessing Fc receptors, such as macrophages, dendritic cells, and B cells, rather than directly on Tregs, and that these cells subsequently interact with T cells.

The existence of Tregitopes is currently an active topic of research. Tregitope peptides were first discovered because they contain *in silico* signatures that exhibit high-affinity binding to

multiple human class II major histocompatibility complexes (class II MHC; HLA-DR) [28]. Like IVIG, Tregitopes are capable of engaging Tregs. The discovery of Tregitopes in IgG may contribute to an improved understanding of the mechanism of action of IVIG therapy and lead to the application of these powerful immunomodulators to improving transplantation success [29, 30]. The observed changes in TGF$\beta_1$, sCTLA-4, and IL-10 levels in subjects who received IVIG post HSCT may have been caused by the action of Tregitopes. Further work is needed to study the potential GVHD-preventing effect of Tregitopes.

## CONCLUSION

Our results demonstrate that TGF$\beta_1$, sCTLA-4, and IL-10 levels were increased after allogeneic HSCT. Additionally, we found that the elevation of TGF$\beta_1$, sCTLA-4, and IL-10 levels after allogeneic HSCT is further increased by IVIG therapy. Nevertheless, our study has some limitations: this was not a randomized study, and we were unable to determine the relationship between the effects of IVIG therapy and GVHD occurrence. In addition, we did not uncover the mechanism by which IVIG therapy increases the TGF$\beta_1$, sCTLA-4, and IL-10 levels after HSCT. Further confirmation of these observations in prospective studies is necessary.

## CONFLICT OF INTEREST STATEMENT

The authors do not have any conflicts of interest to report for this work.

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