IMMUNOGLOBULIN THERAPY IN THE 21ST CENTURY: THE DARK SIDE OF THE MOON

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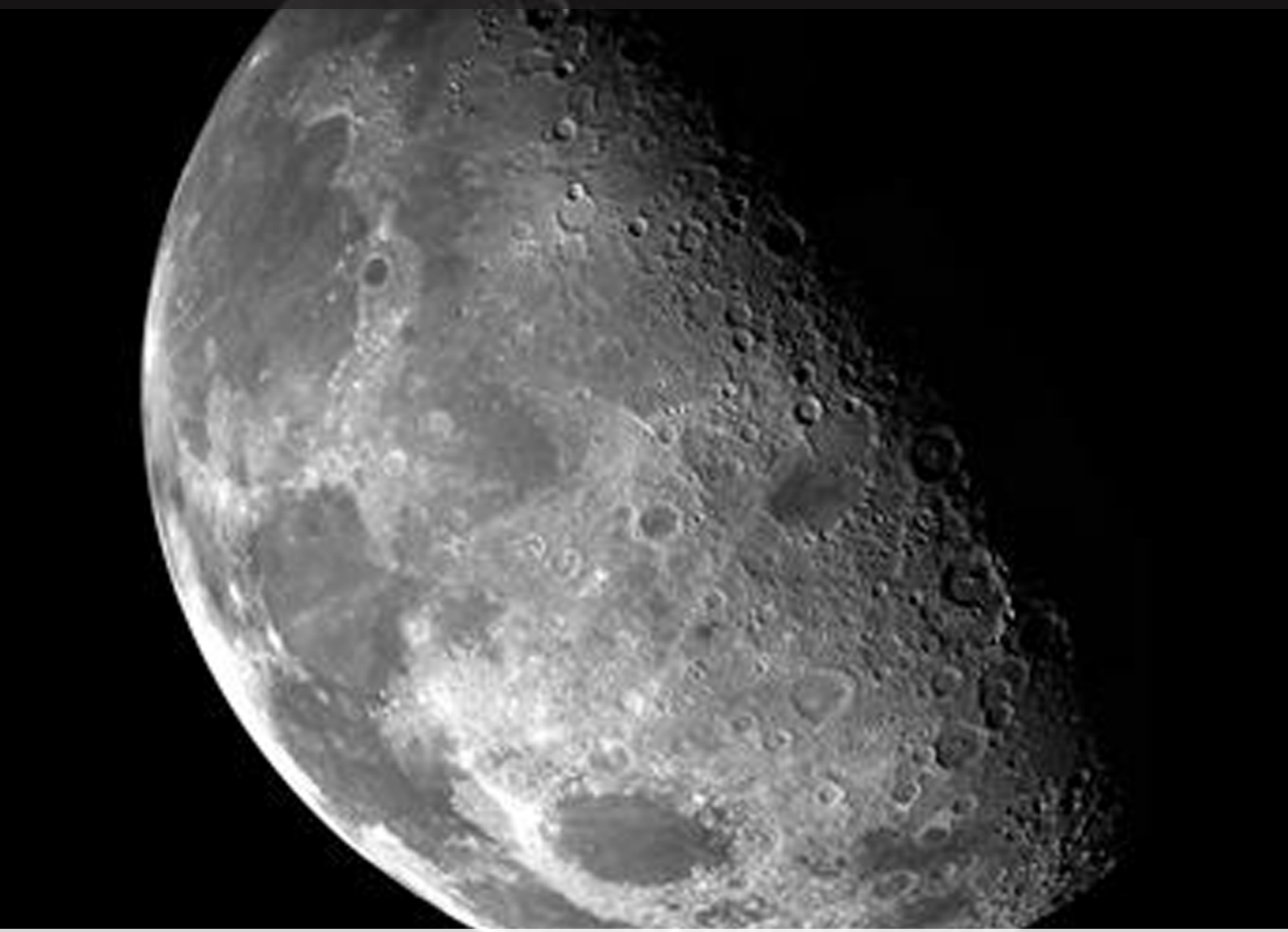

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IMMUNOGLOBULIN THERAPY IN THE 21ST CENTURY: THE DARK SIDE OF THE MOON

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In the early decades since the introduction in the early '80s of immunoglobulin therapy many studies tried to identify which clinical indications might benefit from the therapy, which treatment's schedules are effective and safe. It is universally accepted that immunoglobulin therapy is a life-saving treatment in patients with PID. The rise of new indications for further different clinical conditions resulted in a steady increase in demand for immunoglobulins. Currently the consumption of immunoglobulin for PID represents a small fraction of the market.

In the recent past we have been observing:

1) An increase in the demand for plasma and in the consequent need to increase the number of donors;
2) Changes in methods to improve IgG recovery and to increase productivity as a response to growing clinical demand;
3) Introduction of immunoglobulin treatments with higher concentration;
4) Changes in the timing of administration with an increase in the rate of infusion;
5) Introduction of immunoglobulin treatment administered subcutaneously mainly confined initially to patients with PID and later extended to other clinical indications which often require higher volumes of infusion.

Doctors following patients with PID were initially alarmed only to a possible risk of shortage. More relevant and less discussed appear the possible consequences of:

1) the risk of an improper transfer of information on treatments from a clinical indication to another. In particular, the idea of a mere replacement function in patients with PID might possibly be borrowed from the model of other clinical conditions requiring a replacement such as haemophilia. In PID, immunoglobulin treatment instead is obviously replacing a missing feature. However, other immune alterations are responsible for the large number of PID-associated diseases including inflammatory manifestations and tumors, common causes of morbidity and mortality. The immunomodulatory effects of immunoglobulin administered at replacement dosages on multiple cells and immune system functions are still largely to be checked in in vitro studies and in vivo.

2) the changes in the immunoglobulin production and schedules of administration. These should have been assessed in studies of drug surveillance, necessary in order to evaluate on large numbers of what it is initially reported on patients enrolled in the pivotal clinical trials, usually in the absence of most of the main disease-associated clinical conditions affecting pharmacokinetics, efficacy and tolerability. Severe side effects are now more frequently reported. This requires surveillance studies in order to verify the tolerability. Nowadays, personalized health research presents methodologic challenges, since emphasis is placed on the individual response rather than on the population. Even within a universally accepted indication, such as in PID, the identification of prognostic markers should guide the therapeutic intervention.

3) the risk of a decrease in the surveillance and monitoring of PID-associated clinical conditions. In fact, self- administration of immunoglobulins administered subcutaneously increased the independence of a number of patients. On the other hand, it led to the reduction in the number of contacts between specialized centers and patients who often require a close monitoring of disease-associated conditions.

A wide debate between experts is necessary to afford the new challenge on immunoglobulin usage.

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EDITORIAL
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Editorial: Immunoglobulin therapy in the 21st century – the dark side of the moon

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Keywords: immunoglobulin G, immunedeficiency, dosage, safety, individualized medicine

Since its widespread introduction in the early 1980s, immunoglobulin therapy (IG) has been extensively investigated to understand its mechanism of action and clinical benefits. Research has also continued to improve its production. The survival benefits of IG for patients with primary immunodeficiency (PID) are accepted (1). The basic principle of replacing the missing protein has led to proposals for progressive increases in dosage, delivered intravenously (2), or subcutaneously (3). It is suggested that continuously increasing IG trough levels decreases pulmonary infections and damage. In contrast, other studies on large patient cohorts found no correlation between IG trough level and the incidence of pneumonia and serious infections when trough levels were raised above 400 mg/dl (4, 5). Dosage and other aspects of the therapeutic regimen remain open questions even in the mainstream indication of substitution therapy in PID. These issues have influenced the development of product modifications such as highly concentrated solutions and fast infusion rates. They have also contributed to the increased usage of 16–20% IG infused subcutaneously.

Simple replacement of the antibody defect in PID is now known to be an incomplete explanation of the mechanism of IG. A range of immunomodulatory and anti-inflammatory mechanisms are involved (6). These mechanisms are important in the role of IG in autoimmune disorders, particularly neuropathies including chronic inflammatory demyelinating polyneuropathy (CIDP), Guillain-Barré syndrome (GBS), and multifocal motor neuropathy (MMN). These indications represent the largest area of IG use in the established economies. They contribute greatly to the steady increase in demand for immunoglobulins experienced in the past 20 years, despite uncertainty in mechanisms of action. The increase in adverse events, such as thrombogenicity (7) and haemolysis (8), experienced in recent years makes a better understanding of mechanism and dosage even more important. The substantial increase in the usage of expensive IG products has also influenced developments in formulation and infusion practices. Faster infusion of more concentrated solutions will decrease hospital stay and costs. The subcutaneous route is supposedly easier and more convenient to deliver in home therapy settings (9), also potentially decreasing hospital stay. Although approved by regulatory agencies, these developments have yet to be validated through the long period of clinical practice experienced with the previous range of IG products.

The increased demand for IG has also seen the rapid development of new manufacturing methods replacing the traditional Cohn fractionation system (10). This system has demonstrated decades of safety and efficacy and caution is warranted as new methods are introduced into production and clinical use.

This Research Topic of Frontiers in Immunology has been assembled by an editorial team which have experienced sufficient "dark forebodings" (11) regarding the uncertainties outlined above. They have called upon a group of international experts to assess some of these issues from their perspective. The mechanism of IG on the immune system is explored by Nagelkerke and Kuijpers (12) who describe the different Fcγ receptors variants on immune cells and the direct IG effects at the level of the activating Fcγ receptors, including the more recently described FcgRIIc.

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Mitrevski et al. (13) assess analogous mechanisms in the action of IG in PID, showing that IG at replacement dosages could prime B cells to an anergic, apoptotic state through the generation of an increase in $CD21^{low}$ B cells. Matucci et al. (14) discuss the role of benefits additional to the direct substitution of deficient IG, such as the immunomodulatory and anti-inflammatory effects of IG preparations, while Paquin-Proulx and Sandberg (15) discuss the role of immune activation in the pathology of commonest PID – common variable immunodeficiency (CVID) – and its alleviation by IG therapies.

Taken together, this body of work mitigates our "dark forebodings" regarding the lack of clarity on the mechanism of action of IG. More work is also needed to optimize therapeutic practice. Kerr et al. (16) note the desirability of progressing beyond simple, mandated, weight-based dosages in PID, and the need to approach more individualized therapeutic regimens for different PID patients. Their approach is augmented by the review of Wolf et al. (17) demonstrating how the identification of impaired IG formation may differentiate patients requiring IG from those who do not. Patient data collected through long term monitoring led Lucas et al. (18) to conclude that "The goal of replacement therapy should be to improve clinical outcome and not to reach a particular IgG trough level" supporting previous work tailoring optimal IG prophylaxis regimens to clinical and immunological markers (19). Studies such as those cited should influence treatment protocols and a negate a "one size fits all" therapeutic approach as reflected in many current guidelines. Kerr et al. (16) also note how little evidence underpins the dosage regimens used in autoimmune indications. It is to be wondered that empirically driven doses developed for the treatment of idiopathic thrombocytopenic purpura have been extended into the treatment of the various autoimmune neuropathies. The importance of individualizing treatment is also discussed by Compagno et al. (20) in their review of the use of IG in the various acquired hypogammaglobulinemic diseases. We are aware of large discrepancies between and within countries in this area, leading to the need for better evidence based on individualized treatment. Overall, these particular contributions support the need to improve outcomes in PID as shown by the study of Tabolli et al. (21) who conclude that Health-Related Quality of Life in PID patients is not related to IG therapy but to more sophisticated personal and clinical preferences.

The final component of this Research Topic of Frontiers in Immunology examines the manufacture of IG therapies and its effect on the efficacy and safety of the products. Goldacker et al. (22) show that, despite the mandatory large plasma donation pool size for IG products, the content of specific, therapeutically important antibodies is still subject to geographical influence. This is very important in the context of continuing to ensure that IG therapies are relevant in the protection of PID patients in different countries. We suggest that this requires the attention of regulatory authorities. Finally, Farrugia and Quinti (23) and Späth et al. (24) discuss the history of the manufacture of IG therapies. They suggest ways whereby changes introduced by companies to optimize yield and minimize hospital related costs might alter the repertoire of specificities and the efficacy of IG. These changes may also have played a role in recently observed surges in adverse events. These observations suggest that a more cautionary approach in the usage IG therapies is warranted.

Overall, the therapeutics of diseases caused by a deficiency or an inappropriate type of antibodies continues to be an exciting and fruitful area of clinical research, and well suited to contribute to the ongoing evolution in medicine toward more individualized and patient centric paradigms. We hope that this Research Topic of Frontiers in Immunology contributes to this debate. Such a wide debate between experts is necessary to enable the new challenges in immunoglobulin usage.

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Longitudinal study on health-related quality of life in a cohort of 96 patients with common variable immune deficiencies

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Health-related quality of life (HRQoL) in common variable immunodeficiency diseases (CVID) was evaluated by different tools, which were mainly used to compare different schedules of immunoglobulins administration in cross-sectional or short-term longitudinal studies. We assessed the HRQoL and psychological status of CVID patients in a longitudinal study over a 6-year period by a generic, non-disease-specific instrument (SF-36), and by a General Health Questionnaire (GHQ-12) for the risk of depression/anxiety. At baseline, 96 patients were enrolled. After 1 year, a second assessment was performed on 92 patients and, after 6 years, a third assessment was performed on 66 patients. Eighteen patients died during the study time. HRQoL was low, with mental health scales less affected than physical scales. A decline in the score on SF-36 scales was observed between the first and the third assessment for the Physical Functioning, Body Pain, General Health, Social Functioning, and Role-Emotional scales. The General Health scale showed a lower score in these patients, when compared to patients with other chronic diseases. Approximately one-third of the patients were at risk of anxiety/depression at all observation times, a percentage that reached two thirds of the patients, considering only the group of females. Over the 6 years of the study, the health condition of 11/66 patients worsened, passing from "GHQ-negative" to "GHQ-positive"; their score on SF-36 scales also decreased. A decrement of one point in each of the Physical Functioning, Vitality, Social Functioning, and Mental Health SF-36 scales increased the risk of developing anxiety/depression from three to five percent. A negative variation of the Physical Functioning score increased the risk of psychological distress. In a survival analysis with dichotomized variables, Physical Functioning scores <50 were associated with a relative risk (RR) of 4.4, whereas Social Functioning scores <37.5 were associated with a RR of 10.0. In our study, it was the clinical condition, as opposed to the different treatment strategies with immunoglobulins, which had a major role on the deterioration of HRQoL. Moreover, in a quality-of-life evaluation, disorders such as anxiety/depression should be assessed, as they yet often go unrecognized. Our results might be helpful in the interpretation of currently available data on quality of life in CVID patients.

Keywords: common variable immune deficiencies, health-related quality of life, SF-36, GHQ-12, immunoglobulins

INTRODUCTION

The health-related quality of life (HRQoL) is a multidimensional concept that encompasses measurements of physical, psychological, and social well-being and assesses the individual's perception of the impact of illness on his/her life (1).

Common variable immunodeficiency diseases (CVIDs) represent a heterogeneous group of rare chronic disorders of the immune system (2). The prognosis can vary from benign to very complex conditions (3). There is substantial evidence that the standard replacement treatment with immunoglobulins prolongs survival, reduces morbidity, and exerts a positive effect on the patients' HRQoL (4). Until now, different tools to evaluate HRQoL in CVID were used mainly to assess the patients' outcome and satisfaction related to different treatment choices (i.e., intravenous-IVIG vs. subcutaneous-SCIG immunoglobulin routes of administration) (5, 6). However, HRQoL in CVID should be assessed in the frame of the wide spectrum of the severity of the disease, taking into consideration the long life course of the disease.

There are several critical reasons to evaluate the available data on HRQoL in CVID. The absence of a disease-specific questionnaire is a major limitation. Only observational or short-term longitudinal studies on small cohorts were performed. Differences in HRQoL were mainly evaluated to compare different treatment regimens and routes of immunoglobulins administration.

With such limitations in evaluating quality of life in CVID, both ourselves and others (7–9) have used generic, non-disease-specific instruments, such as the Health Status Questionnaire (Medical Outcome Study 36-Items Short Form, SF-36) and the General Health Questionnaire (GHQ-12 Items) for the psychological assessment. All the studies agreed that CVID patients have a poor quality of life, especially in the physical domain, suffer a lot, and are at risk of psychological distress.

Because of the long lifetime of the disease, it is possible to speculate that quality of life in a population affected by a clinical and immunological heterogeneous disease may vary depending on age, treatment, clinical conditions, associated diseases, personal attitude, etc. Thus, it is evident that studies about the HRQoL outcome in CVID should be extended from a simple assessment to multimodal and longitudinal assessments and should include the patients' reported outcome measures.

The aim of this study was to assess the HRQoL and psychological status of patients with CVID over a 6-year period, using SF-36 and GHQ-12 questionnaires. Moreover, we investigated whether the psychological problems of patients, such as risk of depression/anxiety, could be associated with their health status.

MATERIALS AND METHODS

STUDY DESIGN

For this observational, longitudinal, cohort study (**Figure 1**), performed in a day hospital setting, patients' participation was obtained after signing an informed consent. The study was conducted in the period 2008–2013. One hundred twelve CVID patients were informed and considered eligible for the study. At basal time (T0), 96 patients were enrolled and 16 patients refused to participate. After 1 year, at the second assessment (T1) 92 out of 96 patients were evaluated (between T0 and T1 two patients died and two patients refused to continue the study). After 6 years, 66 out of 92 patients were available for the third assessment (T2). Between T1 and T2, 12 patients refused to participate and 16 patients died in the 5-year period. At the end of the study, data for the 66 patients were considered, taking into account all three observations. At T0, T1, and T2, patients were asked to fill in questionnaires concerning their health status and the possible presence of risk for depression and/or anxiety. The severity of disease judged by the physician (Physician Global Assessment, PhGA) and by the patient (Patient's Global Assessment, PtGA) was recorded. The study protocol was approved by the Ethical Board of the Sapienza, University of Rome.

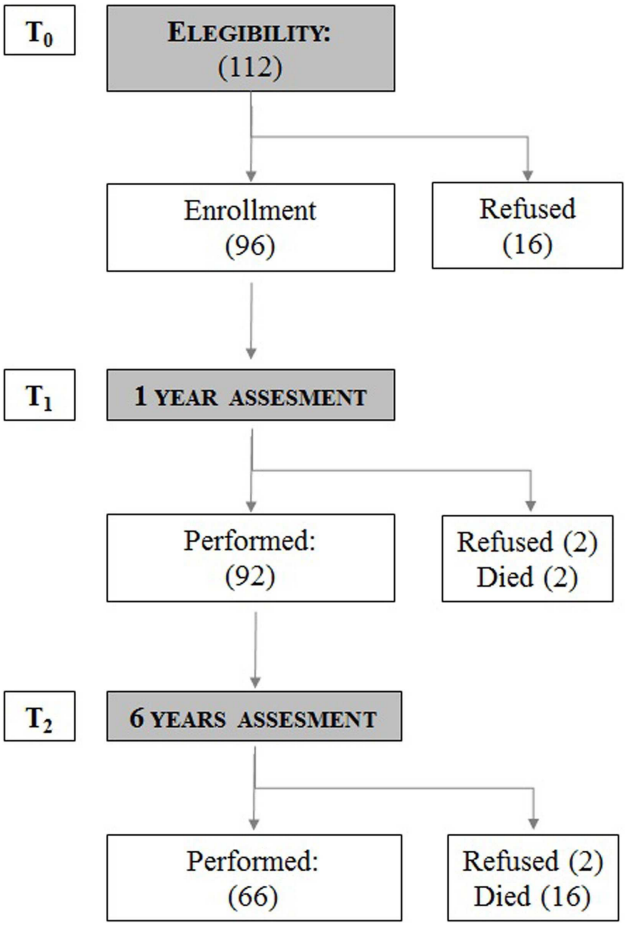

FIGURE 1 | Study flow chart. The number of patients analyzed at baseline (T0), after 1 year (T1), and after 6 years (T2). The number of patients who refused to continue the study and the number of patients who died during the study time is shown.

PATIENTS

We enrolled 112 patients with CVIDs attending our Reference Center for Primary Immune Deficiencies. Patients were diagnosed according to the ESID/PAGID criteria for CVIDs (10), based on IgG <500 mg/dL, IgA 2 SD below age-specific reference range, age onset >4 years, poor response to vaccines, and exclusion of other causes of hypogammaglobulinemia. No genetic causes of CVID were identified in this cohort. A detailed set of data was available, since all patients with a diagnosis of CVID have been regularly followed up in our center according to the Italian guidelines (www.aieop.org); their clinical and immunological data have been collected regularly in a national database, once a year. The dataset included age, date of CVID diagnosis, immunological data, including lymphocyte subsets and serum IgG, IgA, and IgM levels determined every 3 months, clinical manifestations, route, doses, and intervals of Ig replacement, and occurrence of adverse reactions. Route, dosage, and interval of Ig replacement were recorded once a month. At T0, 84 patients were on replacement therapy with IVIG and 12 with SCIG. Between T0 and T2, 6/66 patients shifted from IVIG to SCIG. High-resolution chest computerized tomography (HRCT) scans was performed once in every 4 years in all patients according to national guidelines. All patients have been on IVIG or SCIG replacement for at least 5 years.

QUESTIONNAIRES

We used validated tools: SF-36, GHQ-12, and PGA questionnaires.

SF-36

Despite the fact that it was designed as a generic health status indicator for use in population surveys and health policy evaluation studies, the SF-36 can also be used as an outcome measure (11, 12). The SF-36 includes 36 items in a Likert-type or forced-choice

format, intended for measuring the following eight dimensions: physical functioning (*PF*, limitations in performing physical activities such as bathing or dressing), role-physical (*RP*, limitations in work and other daily activities as a consequence of physical health), bodily pain (*BP*, how severe and limiting pain is), general health (*GH*, how general personal health is perceived by the patient), vitality (*VT*, feeling tired and worn out vs. feeling energetic), social functioning (*SF*, interference with regular social activities because of physical or emotional problems), role-emotional (*RE*, limitations in work and other daily activities as a consequence of emotional problems), and mental health (*MH*, feeling nervous and depressed vs. peaceful, happy, and calm). Scores for each domain ranged from 0 to 100, with higher scores indicating better health. Two additional summary measures, the physical component summary (PCS) and mental component scores (MCS), cross-culturally validated in the framework of the International Quality of Life Assessment project for the Italian version of the SF-36, were also obtained.

GHQ-12

The GHQ-12 is a self-administered, 12-item questionnaire, designed to measure psychological distress and to detect current non-psychotic, psychiatric disorders, such as depression and anxiety (13, 14). Answers are given on a 4-point scale; for instance, the item "in the last weeks, did you feel under strain?" allows for the following answers: "no," "not more than usual," "more than usual," and "much more than usual." When scored with the binary method (0–0–1–1), the GHQ-12 can be used as a screening tool to detect minor non-psychotic, psychiatric disorders, yielding final scores that range from 0 to 12. Operationally, patients scoring 4 or more were considered as "GHQ-positive" (GHQ+).

PGA

For each patient, an overall clinical severity evaluation of the disease was given by the provider and by the patient him/herself. The PhGA and the PtGA consisted of the following questions respectively: "In your opinion, compared to other patients with the same condition, how severe is the disease of patient X?" and "In your experience, how severe is your disease?" Answers were given on a 5-point scale: "very mild," "mild," "moderate," "severe," and "very severe." For the purpose of statistical analysis, "very mild"/"mild" were considered as low severity and "severe"/"very severe" as high severity, and were grouped. The same physician at T0, T1, and T2 recorded her evaluation at the end of the visit. Patients recorded his/her evaluation after the completion of the questionnaires.

STATISTICS

In the first part, for descriptive analyses and comparisons among groups, we used *t*-test for independent samples and ANOVA for the comparison of mean values, due to the samples' size. Chi-squared test was also used for the comparison of percentages. For the 66 patients present in each observation, paired tests were used. In the second part, we performed a logistic regression analysis to assess the independent role of SF-36 scales on GHQ-12 deterioration over time. In the last part, in order to investigate factors predicting mortality, survival analysis was performed, both through Kaplan–Meier curves and Cox regression analysis. All analyses were performed using the Stata version 11 (Stata Corp, College Station, TX, USA).

RESULTS

The study design flow chart on CVID patients enrolled in the cohort study is shown in **Figure 1**. One hundred twelve patients were enrolled; 96 patients accepted to participate in the study [M/F: 50/46; mean age: 48.2 ± 17.0 years old (range 14–85); mean time of disease since diagnosis: 10.7 years (range 5–36)]; 92 patients [M/F: 47/45; mean age: 49 ± 4.9 years old (range 15–86)] completed the second assessment. Sixty-six patients [M/F: 32/34; mean age: 50 ± 5.7 years old (range 20–76)] completed the T0, T1, and T2 assessments. Thirty patients refused to participate (16 at T0, 2 at T1, and 12 at T2). Eighteen patients [M/F: 9/9; mean age: 62.9 ± 14.7 years old (range 39–88)] died in the 6-year period. Causes of death were: gastrointestinal cancer (5 patients), lymphoproliferative diseases (5 patients), chronic lung disease (CLD) (2 patients), cirrhosis (1 patient), and granulomatosis (5 patients).

DESCRIPTIVE ANALYSES

Patients' characteristics at baseline and comparison between SF-36 in CVID and in other chronic diseases

At baseline (T0), the characteristics of patients were those reported in our study published in 2012 (7). A summary of our previous data on HRQoL is reported in **Table 1**. Being female, older, and affected by CLD and chronic diarrhea proved to be major risk factors leading to a poor quality of life. The basal mean scores for SF-36 scales were also compared to those reported (12) on patients affected by other chronic diseases (**Table 2**). HRQoL was lower than that reported in generally healthy population, with mental health scales less affected than physical scales. In CVID, better scores for PF, BP, VT, SF, RE, and MH scales were observed, while RP and GH scales showed a lower score in comparison to patients with all other disease entities, with the exception of patients affected by heart failures who showed the lowest scores. The different age range of patients in each group (older in cancer, younger in CVID) has a significant effect on profiles of SF-36 average scores. Therefore, one must be cautious in the interpretation of differences in SF-36 scores between pathologies: they cannot be entirely attributed to the "pure" effect of the disease. This consideration is even more valid in the longitudinal study.

Longitudinal variation of SF-36

Longitudinal variations in SF-36 scores were observed over the whole sample, as well as over the subsample of 66 patients attending all three sequential assessments. The scores observed considering the SF-36 mean values in the 66 patients, who were followed at all three points of observation times, are shown in **Figure 2**. Differences in the scores of SF-36 scales between T0 and T2 are statistically significant for the following scales: PF ($p = 0.03$), BP ($p = 0.05$), GH ($p = 0.02$), SF ($p = 0.002$), and RE ($p = 0.03$). However, we should consider the age dependence of all scales, especially because patients at T2 were 6 years older than at T0. Moreover, we found no differences in HRQoL scales between patients on replacement with IVIG and SCIG.

Table 1 | SF-36 mean values (SD) and clinical characteristics of CVID patients.

	n	PF	RP	BP	GH	VT	SF	RE	MH	PCS	MCS
All	96	72 (25)	47 (42)	67 (26)	39 (24)	55 (22)	69 (22)	68 (40)	66 (20)	40 (12)	43 (12)
Gender											
Male	50	78 (24)	59 (41)	73 (25)	44 (27)	62 (20)	72 (23)	74 (38)	69 (20)	43 (12)	45 (12)
Female	46	**66 (25)**	**34 (40)**	**60 (26)**	34 (20)	**47 (21)**	66 (21)	62 (42)	63 (19)	**36 (11)**	42 (12)
Age											
<50 years	52	84 (19)	56 (42)	74 (26)	43 (27)	59 (21)	69 (24)	79 (33)	68 (19)	44 (12)	44 (12)
≥50 years	44	**58 (25)**	**39 (40)**	**59 (25)**	35 (21)	51 (22)	70 (20)	**55 (43)**	64 (21)	**34 (11)**	43 (13)
Duration of disease											
≤8 years	47	73 (28)	49 (42)	69 (28)	42 (23)	55 (20)	68 (24)	72 (38)	63 (20)	41 (13)	43 (13)
>8 years	46	71 (23)	44 (42)	65 (26)	37 (27)	56 (24)	69 (21)	64 (42)	68 (20)	39 (12)	44 (12)
Co-morbidities											
CLD	62	70 (25)	41 (41)	63 (26)	37 (23)	54 (23)	65 (23)	60 (41)	63 (21)	38 (12)	41 (13)
Sinusitis	48	73 (25)	44 (43)	66 (28)	36 (23)	54 (22)	68 (22)	65 (42)	65 (22)	39 (12)	42 (13)
Diarrhea	42	65 (26)	37 (40)	60 (29)	34 (24)	51 (23)	65 (24)	54 (41)	63 (22)	36 (12)	41 (13)

SF-36 scales: PF, physical functioning; RP, role-physical; BP, bodily pain; GH, general health; VT, vitality; SF, social functioning; RE, role-emotional; MH, mental health; PCS, physical component summary; MCS, mental component summary; CLD, chronic lung disease; CVID, common variable immunodeficiency.

Bold – $p < 0.05$.

Totals may vary because of missing values.

Table 2 | Mean values of SF-36 scales for CVID patients compared with healthy subjects and with patients with different chronic diseases in Italy.

	N	PF	RP	BP	GH	VT	SF	RE	MH
Healthy subjects	608	97.3	94.3	89.2	80.2	72.2	86.4	88.0	75.8
Diabetes	98	62.9	59.7	59.8	43.6	47.6	66.9	57.6	53.4
Heart failure	129	49.5	43.1	47.6	35.4	38.3	54.8	46.3	46.6
Cancer	34	60.6	62.4	57.0	44.9	48.3	64.1	58.6	49.9
Chronic obstructive pulmonary disease	188	58.7	49.5	52.0	41.5	45.8	62.6	55.8	53.5
Mental disorders	180	65.4	49.5	51.9	44.7	40.9	55.0	43.7	42.0
CVID	96	72.4	47.3	67.2	39.3	55.2	69.5	68.3	66.3

SF-36 Scales: Physical Functioning (PF), Role-Physical (RP), Bodily Pain (BP), General Health (GH), Vitality (VT), Social Functioning (SF), Role-Emotional (RE), Mental Health (MH). SF-36 values for Italian diseases (12).

GHQ assessment and PtGA/PhGA

GHQ-12 assessment showed that more than 35% of the patients were at risk of anxiety and depression (GHQ-positive) at all observation times (**Table 3**). This percentage was increased to about 70% in females. The disease severity perception graded by patients and by physicians at the three points of observation is reported in **Table 3**. Differences between the perception of patients and physicians were more evident at T2, with higher percentage of low severity grade reported by physicians. As expected, PhGA was greater in GHQ-positive patients. Twenty-five percent of GHQ-positive patients considered their disease severity as high.

The 15 patients who were permanently GHQ-positive at all observations had constantly low mean values of scores on SF-36 scales. Patients with a highly severe perception of the disease (PtGA) and patients who were judged as seriously affected by the physicians (PhGA) reported a lower health status with respect to the others.

GENERAL HEALTH QUESTIONNAIRE DATA AND VARIATIONS OF MEAN VALUES IN SPECIFIC SF-36 SCALES

To analyze the relationship between SF-36 and GHQ-12 data, variation in GHQ status between T0 and T2 were coded as 1 when GHQ passed from 0 to 1 (GHQ-worsened) and as 0 in all other instances (GHQ-stable/improved). Changes in SF-36 scales were recorded as absolute differences between values at T0 at T2. Average variation in SF-36 scores was then compared between the group of GHQ-worsened and GHQ-stable/improved. The SF-36 scales showing variations significantly different in the two groups (GHQ-worsened and GHQ-stable/improved) were selected.

Over the 6-year observational period, the general health condition of 11/66 patients worsened, passing from "GHQ-negative" to "GHQ-positive" status, i.e., showing symptoms of psychological distress.

These patients also registered a negative score variation on SF-36 scales: Physical Functioning, Vitality, Social Functioning, and Mental Health. In a logistic regression, controlled for age

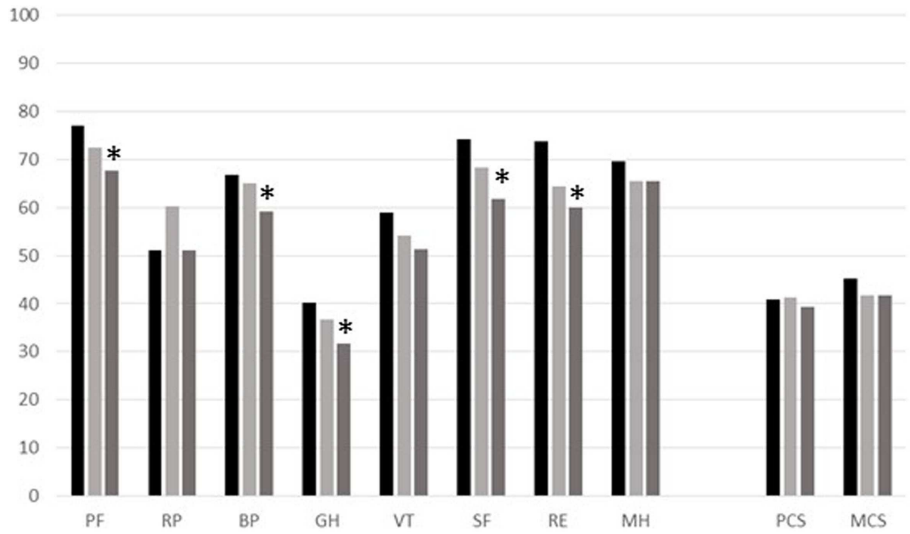

FIGURE 2 | Profile of the mean values for each scale of SF-36 for the group of 66 CVID patients observed at different times (T0, black column; T1, pale-gray column; T2, gray column). SF-36 Scales: Physical Functioning (PF), Role-Physical (RP), Bodily Pain (BP), General Health (GH), Vitality (VT), Social Functioning (SF), Role-Emotional (RE), Mental Health (MH), Physical component summary (PCS), and Mental component summary (MCS). *Significant *p*-values between T0 and T2.

Table 3 | Gender, GHQ status, and disease severity reported by patients and by physicians at each assessment (numbers and percentages).

	All	*n*.96 (T0) (%)	*n*.92 (T1) (%)	*n*.66 (T2) (%)
Sex	Males	52	51	52
	Females	48	49	48
GHQ+		36	39	37
GHQ+	Males	28	23	33
	Females	72	77	67
PtGA	Low	27	17	38
	Moderate	49	49	40
	High	24	33	22
PhGA	Low	17	16	47
	Moderate	50	51	39
	High	33	33	14

PtGA, Patient Global Assessment; PhGA, Physician Global Assessment. GHQ, General Health Questionnaire; GHQ+, GHQ-positive/"cases" or GHQ ≥4.

and gender, we noticed that a decrement of 1 point in each of the four mentioned scales increases the risk of developing anxiety/depression from 3 to 5% (**Table 4**). Thus, the GHQ-12 and SF-36 deteriorations were strictly linked. To convey an idea of the magnitude of the effect, we considered the cumulative observed variation of the Physical Functioning scale (PF) over the follow-up period: 10 points. The odds ratio of 1.05 means that a patient affected by the average negative variation of PF has an increased risk of 5% per year of psychological distress. Also VT, SF, and MH subscales were strongly influenced by GHQ status (**Figure 3**).

Table 4 | Odd ratio (OR) and *p*-values for SF-36 scales for "changes" in GHQ-12 status (from negative to positive).

SF-36 scale	OR	*p*-value
PF	1.05 (1.01–1.06)	0.012
VT	1.04 (1.01–1.05)	0.008
SF	1.03 (1.01–1.06)	0.024
MH	1.04 (1.01–1.07)	0.03

IMMUNOLOGICAL DATA AND HRQoL

In CVID, we have previously identified (15) a severe clinical phenotype, characterized by low IgA level (<7 mg/dL) and low switched memory B cells, confirming previous observations showing that the loss of function of memory B cells seems to represent the major cause of CVID-associated clinical conditions (16). Moreover, clinical improvement of CVID was observed in patients receiving high Ig dosages, >600 mg/kg/months (17, 18). These dosages might allow to keep IgG trough levels >600–800 mg/dL. We then grouped our CVID cohort on the basis of three defined parameters: IgA >7 mg/dL, IgG trough levels >600 mg/dL, switched memory >2%. At T0, 67% of the patients had IgA levels <7; 50% had switched memory B cells <2%; 61% had IgG levels >600 mg/dL (a percentage ranging from 14 to 26 has missing information on these parameters).

The analysis of the mean values on SF-36 scales in patients with or without each defined parameter showed no statistically significant differences among groups.

LOW HRQoL WAS PREDICTIVE OF MORTALITY

Patients' mortality during the follow-up was registered with exact date of death. According to this outcome, patients at T0 were

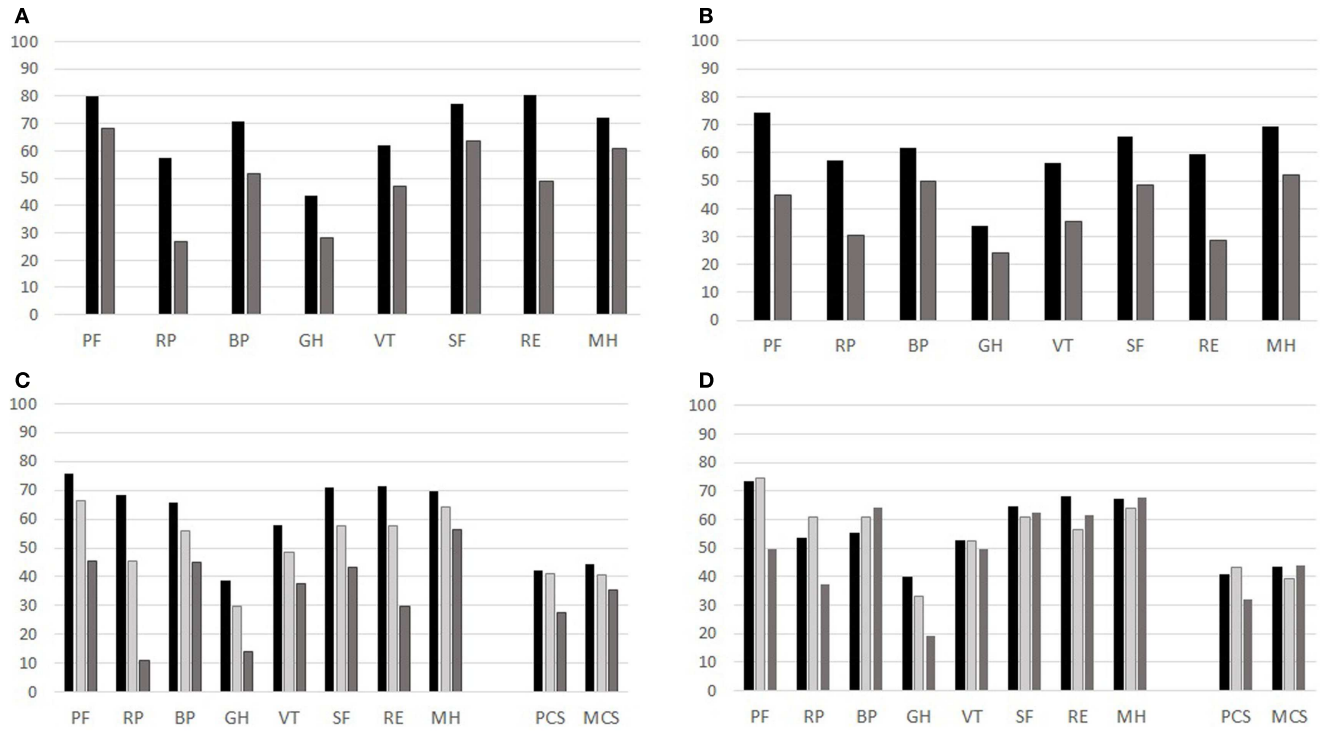

FIGURE 3 | Mean values for each scale of SF-36 for the group of 66 CVID patients observed at different times (T0, T1, T2), GHQ status (T0 and T2) and PtGA and PhGA assessments. **(A)** GHQ at T0: black column GHQ-negative; gray column: GHQ-positive. **(B)** GHQ at T2: black column GHQ-negative; gray column: GHQ-positive. **(C)** PtGA (black column: very mild/mild; pale-gray column: moderate; gray column: severe/very severe). **(D)** PhGA (black column: very mild/mild; pale-gray column: moderate; gray column: severe/very severe).

divided into two groups: survivors vs. deceased. Cox Survival analysis was performed, introducing as covariates the SF-36 scales that showed marked differences between survivors and deceased at T0. Age and gender were also included in the analysis in order to estimate the role of HRQoL in predicting mortality, as adjusted by these two variables. For the SF-36 scales independently predictive of mortality, we operated a dichotomization of values, indicating "at-risk" and "not at-risk" patients. The cut-off values were selected observationally based on points of disruption in the trend of number of death over SF-36 scores. For SF-36 scales predictive of mortality, we graphed the two groups ("at-risk" and "not at-risk") with Kaplan–Meier survival curves, whose significance was verified by Log-rank test. A new Cox survival analysis with SF-36 scales dichotomized was produced, in order to assess the specific risk of death for the patients that scored under the cut-off at T0. The most evident difference between the two groups (**Table 5**) is, not surprisingly, in terms of age: survivors are significantly younger than deceased patients. However, score values of Physical and Social Functioning as well as Role-Emotional at T0 seem to be remarkably reduced for patients that died during the study time. This result was partially confirmed when adjusted by age: except for the Role-Emotional, which has no age-independent effect on mortality, both Physical and Social Functioning score values maintain their significant predictive power.

The relative risk (RR) of death associated with PF and SF scales is 0.98 and 0.97, respectively, meaning that each point increase in Physical and Social Functioning scores, independently of age, reduced the risk of death by 2% and 3%. The two predictive scales (PF and SF) were then dichotomized based on the observed point of disruption in the trend of number of deaths per different scores of PF and SF. More specifically, a cut-off value of 50 was selected for PF and a value of 37.5 for SF. People with values higher than the cut-off were classified as "not at-risk," compared to the "at-risk" below the cut-off. In a survival analysis with these dichotomized variables, PF scores <50 were associated with a RR of 4.4 (CI: 1.7–11.8, $p < 0.03$) and SF scores <37.5 determined a RR of 10.0 (CI: 2.6–37.9, $p < 0.001$). In other words, all other variables being equal, patients with scores below the cut-off in Physical Functioning have 4.4 times the risk of dying than patients with higher scores; the same risk is 10 times higher for patients under the cut-off in social functioning. **Figures 4A,B** compare survival rates over the follow-up period, between "at-risk" and "not at-risk" patients as classified by T0 for PF/SF scores. The difference is extremely significant with the "at-risk" group survival curve always below the "not at-risk" curve (Log-rank test <0.0001). The median value was approached in both the "at-risk" groups, meaning that half of the patients have died at 48 months (SF) and 60 months (PF), whereas, the percentage of death in the "not at-risk" groups is lower than 25% at the end of the observation period (72 months).

Table 5 | Characteristics of survival and deceased patients.

Characteristics	All patients, *N* = 96	Survived patients, *N* = 78	Deceased patients, *N* = 18	*p*-Value
SF-36 scores, mean (SD)				
PCS	39.8 (12.4)	40.8 (12.5)	35.5 (10.9)	0.06
MCS	43.4 (12.2)	44.0 (12.1)	41.0 (12.9)	0.18
Physical Functioning	72.4 (25.4)	76.9 (23.4)	53.8 (25.8)	0.001
Role-Physical	47.3 (42.3)	50.3 (42.4)	34.7 (40.3)	0.08
Body Pain	67.1 (26.5)	67.5 (26.9)	65.9 (25.9)	0.41
General Health	39.3 (24.5)	40.1 (25.3)	36.0 (21.2)	0.27
Vitality	55.2 (21.8)	56.8 (21.1)	48.6 (23.8)	0.08
Social Functioning	69.5 (22.2)	71.5 (21.6)	61.1 (23.4)	0.04
Role-Emotional	68.3 (39.8)	73.1 (37.3)	48.1 (44.6)	0.008
Mental Health	66.3 (19.9)	67.6 (19.1)	60.7 (22.6)	0.09
Age, mean years (SD)	48.2 (17.0)	44.8 (15.7)	62.9 (14.7)	0.0001
Gender, men%	52.2	52.6	50.0	0.84
GHQ, cases%	26.6	28.6	27.7	0.36
IgA, >7 mg/dL%	32.9	33.3	30.0	0.83
IgG, >600 mg/dL%	61.0	63.9	40.0	0.5
SW mem, >2%	50.0	50.8	44.4	0.72

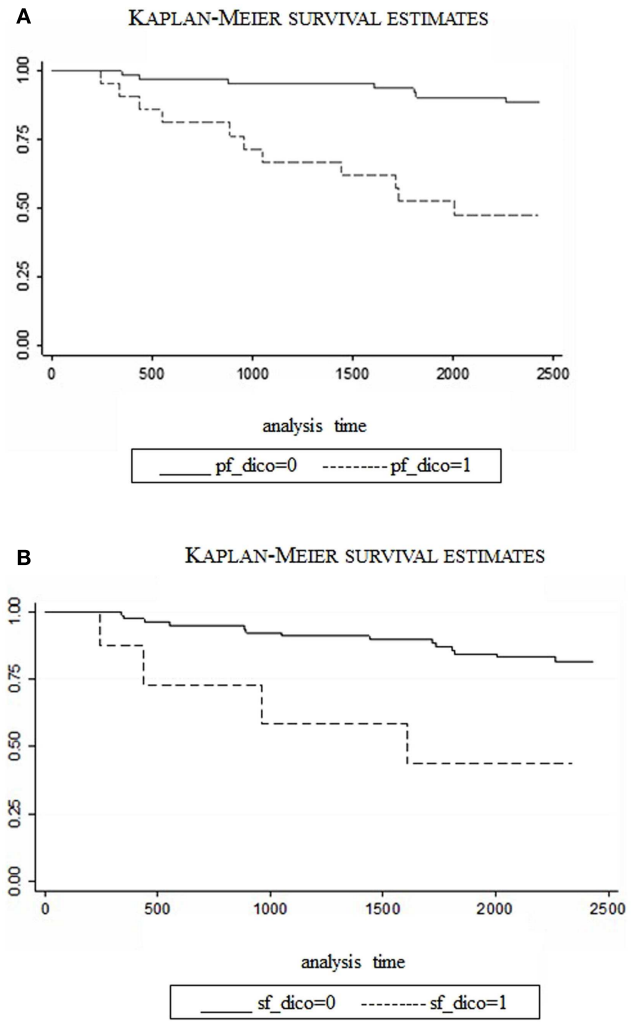

FIGURE 4 | Survival rates for CVID patients ("at-risk" vs. "not at-risk") considering SF-36 scales: Physical Functioning, cut-off at 50 (A) and Social Functioning cut-off at 37.5 (B). (dico: dichotomized).

DISCUSSION

Patient reported outcome measures in clinical practice, in particular, those evaluating HRQoL (19), have been proposed as a means of facilitating doctor–patient communication, uncovering patients' problems, as well as monitoring disease or treatment, and as a screening for functional problems (20, 21). In a previous study on HRQoL performed in our cohort of CVID patients, we showed (7) a low HRQoL in particular in physical domains: the Role-Physical and the General Health scales of the SF-36 questionnaire. Moreover, we showed that being female, older, and GHQ-positive proved to be major risk factors associated with a poor health status. Here, we extended the study time over a 6-year period. To our knowledge, this is the first longitudinal assessment of HRQoL in adult CVID patients. We confirmed that HRQoL was lower than that reported in generally healthy population with mental health scales less affected than physical scales. Moreover, Physical Role and General Health scales showed the worse scores at all observational times and were lower than those reported in patients with other chronic disease entities (12), with the exception of patients affected by heart failures. Moreover, we showed that about one-third of patients were at risk of anxiety/depression at all observation times, a percentage that reached two-thirds of patients, considering only the group of females. The crucial role of anxiety/depression symptoms on HRQoL has been proven recently also in a pediatric population affected by primary immunodeficiencies, where the disease was less likely to affect physical functioning than psychosocial functioning (22). Patients tested continuously that GHQ-positive had a perception of the disease as severe and recorded low mean values of all SF-36 scales. Thus, the GHQ-12 and SF-36 deteriorations were strictly linked. However, it is impossible to verify which might be the *primum movens*. Since patients with a decrease of the Physical Functioning, the worse SF-36 scale in CVID, increased their risk of psychological distress about 50% during the observational time, it is possible to hypothesize that the disease might be the major cause of depression/anxiety. Our data on HRQoL confirmed the data reported 10 years ago in the first multidimensional assessment on HRQoL in CVID (23) and more recently in a survey run by IPOPI (4). However, SF-36 scales showed less severe defects than those previously reported, possibly because of the over-representation of females in both studies. All studies recognized limitations in work and other daily activities as a result of declined physical health and general health. HRQoL measures ensure that treatment and evaluations are focused on the patient rather than on the disease, and may be used as a way of capturing the personal and social context of patients and linking it to the classical clinical view of the disease. However, while HRQoL measures are now quite commonly included in the protocols of randomized, controlled clinical trials and other clinical studies, their use in routine clinical practice is still quite limited; they were never used in combination with other questionnaires, limiting the

possibility to identify correct measures of intervention. In fact, our experience in the evaluation of the HRQoL in a day hospital setting for CVID patients, confirmed what has been observed in other diseases, namely that GHQ-positive patients (with minor psychiatric, non-psychotic diseases such as depression or anxiety) suffer a lot (13). Thus, in the comprehensive evaluation of the patients' status, their psychological condition and disorders such as anxiety/depression should be evaluated in a global HRQoL assessment as they yet often go unrecognized. Counseling can be useful in GHQ-positive patients, since an improvement of GHQ status might lead to a better HRQoL. On the other hand, an improvement in HRQoL can be associated with improvement in psychological well-being. Our results might be helpful in the interpretation of data currently available on quality of life in CVID patients receiving different immunoglobulin treatment options. A recent study from IPOPI (4) reported that most patients were satisfied with their current therapy. Significant differences in satisfaction were seen when comparing SCIG with IVIG administration; SCIG respondents were more satisfied with treatment than IVIG respondents. Since we have shown that the perception of disease severity was linked to the GHQ status and that GHQ-positive patients will perceive their disease as more severe, without a parallel assessment of the psychological status of patients receiving SCIG or IVIG, it might be difficult to attribute an advantage to a treatment modality vs. another. Moreover, as the authors claimed, the results of the survey done on patients affiliated to the IPOPI, might not represent all people with primary immune deficiencies treated with immunoglobulin therapy. Despite this limitation, their data indicated that the primary immunodeficiency impacted on quality of life, also taking into account the favorable effect of immunoglobulin treatment. In our study, the clinical condition, as opposed to the different treatment strategies with immunoglobulins, had a major role on the deterioration of HRQoL. Values of Physical and Social Functioning as well as Role-Emotional scales of SF-36 at T0 were remarkably reduced for patients that died during the study time. Even if survivors were significantly younger than deceased patients, with the exception of the Role-Emotional, which has no age-independent effect on mortality, both Physical and Social Functioning maintained their significant predictive power. The RR of death for PF and SF was 0.98 and 0.97, respectively, meaning that each point increase in PF and SF, independently of age reduces the risk of death by 2 and 3%, respectively. We have already shown (24) that malignancies are the major cause of death in patients with adult onset CVID: the high rate of mortality (about 20% in a 40-year follow-up) was similar to that reported in a study run in the United States of America over a similar period of follow-up (25). The high rate of mortality observed here further confirmed the need to focus our attention on early diagnosis of non-lymphoid and lymphoid cancers that influence lives and HRQoL in CVID. Monitoring health status in long-term longitudinal studies with generic tools, such as SF-36 and GHQ-12, may provide information about clinical status, therapy efficacy, and eventually a modification in physical or mental status. It is relevant to consider here the specificities of each area of the SF-36 health status and the GHQ-12 assessments, taking into consideration any effort to reduce the physical burden of the disease. However, a major limitation in the assessment of HRQoL in CVID is still the absence of a disease-specific questionnaire available to monitor quality of life in many other diseases. We are now developing and validating a CVID-disease-specific questionnaire on HRQoL necessary to complement evidence-based guidelines and policies.

AUTHOR CONTRIBUTIONS

Stefano Tabolli: designed research, analyzed data, and wrote the manuscript; Patrizia Giannantoni: performed the statistical analysis; Federica Pulvirenti: performed research, collected, and analyzed data; Fabiola La Marra: performed research, collected, and analyzed data; Guido Granata: performed research, collected, and analyzed data; Cinzia Milito: performed research, collected, and analyzed data; Isabella Quinti: designed research, analyzed data, and wrote the manuscript.

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Immunoglobulin replacement therapy in secondary hypogammaglobulinemia

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Immunoglobulin (Ig) replacement therapy dramatically changed the clinical course of primary hypogammaglobulinemias, significantly reducing the incidence of infectious events. Over the last two decades its use has been extended to secondary antibody deficiencies, particularly those related to hematological disorders as lymphoproliferative diseases (LPDs) and multiple myeloma. In these malignancies, hypogammaglobulinemia can be an intrinsic aspect of the disease or follow chemo-immunotherapy regimens, including anti-CD20 treatment. Other than in LPDs the broadening use of immunotherapy (e.g., rituximab) and immune-suppressive therapy (steroids, sulfasalazine, and mycophenolate mofetil) has extended the occurrence of iatrogenic hypogammaglobulinemia. In particular, in both autoimmune diseases and solid organ transplantation Ig replacement therapy has been shown to reduce the rate of infectious events. Here, we review the existing literature about Ig replacement therapy in secondary hypogammaglobulinemia, with special regard for subcutaneous administration route, a safe, effective, and well-tolerated treatment approach, currently well established in primary immunodeficiencies and secondary hypogammaglobulinemias.

Keywords: immunoglobulin replacement therapy, secondary hypogammaglobulinemia, chronic lymphocytic leukemia, multiple myeloma, bone marrow transplantation, solid organ transplantation, subcutaneous immunoglobulins

INTRODUCTION

Immunoglobulins (Ig) have been commercially available since the 1940s, and first carried out as replacement therapy (IgRT) in a patient with agammaglobulinemia in 1951 by Ogden Bruton. From that first experience on, Ig have been widely used in patients with primary immune deficiency (PID), as it is well known that hypogammaglobulinemia is a crucial risk factor for development of infectious events and IgRT is able to reduce this risk. By contrast, the lack of clear indications regarding the use of prophylactic IVIG in secondary antibody deficiencies (SAD) may appear paradoxical, if we consider the broad range of disorders in which hypogammaglobulinemia is an intrinsic aspect of the disease or a iatrogenic consequence (**Table 1**) and being the number of affected patients significantly higher than in PID.

In this review, we summarize the most common conditions in which SAD can be observed. We also describe evidence indicating the possibility of subcutaneous (SCIG) rather than intravenous (IVIG) Ig for the treatment for SAD.

CHRONIC LYMPHOCYTIC LEUKEMIA

Infective complications account for up to 50% of all chronic lymphocytic leukemia (CLL)-related deaths (1). Their etiology is multifactorial, due to disease-related immune defects and/or chemo-immunotherapy treatment regimens (2, 3). Hypogammaglobulinemia is the most common chronic immune defect in patients with CLL (the prevalence ranges from 20 to 70%), and correlates with duration and stage of the disease (4). The defect is usually irreversible even in patients achieving complete disease remission. A direct relationship was found between low levels of IgG and the frequency/severity of infections.

Two main strategies are indicated in UK guidelines for patients with hypogammaglobulinemia who develop recurrent bacterial infections (1): antibiotic prophylaxis and IgRT. Nonetheless, there are no standard guidelines for antimicrobial prophylaxis in CLL patients, and most recommendations are derived from small clinical trials and anecdotal reports (4, 5).

Regarding prophylactic IVIG in patients with CLL and hypogammaglobulinemia, randomized controlled studies have been performed in the 1980s ad 1990s and recently summarized (6): they suggest that the use of IVIG may be considered in patients with hypogammaglobulinemia secondary to CLL who experience recurrent infections, since IVIG could significantly decrease the number of infections and the use of antibiotics, reducing hospitalization need and loss of working days. Despite this, the authors did not found any difference in all cause mortality between the treated and control group, maybe due to the relatively short follow-up time (1 year). For these reasons, according to UK guidelines IgRT indicated in patients with a serum IgG < 500 mg/dl complaining of recurrent or severe infections (1). Similar indications are suggested in the Canadian guidelines (7) and by the panel group of the Primary Immunodeficiency Committee of the American Academy of Allergy, Asthma, and Immunology (8). In all these guidelines, an initial dose of 400 mg/kg administered intravenously 3–4 weekly is suggested, aiming at a trough level of 600–800 mg/dl (1, 7). The Ig dose should be then adjusted according to clinical response and steady state trough levels (IgG trough level > 400 mg/dl). As

in PID, higher trough levels may be beneficial in patients with underlying co-morbidities, particularly bronchiectasis. On the basis of these evidences and our experience (9), the same indications are followed in our outpatient clinic. We regularly evaluate the response to IgRT after 12 months, to assess its efficacy and to judge the need of continuing or stopping the infusions.

Attempts have been made to define the risk factors for infections in CLL, in order to select patients who could benefit from IgRT even with a pre-emptive approach. In two papers (10, 11), factors other than Ig concentration (previous chemotherapy, clinical stage, CD38 expression, genetic analysis, and IgVH mutations) have been pointed out as the main prognostic markers for the development of infections, while low IgG level seem not to be clearly associated with infections. Nonetheless, in these studies IgG levels were recorded independently from infectious events, at diagnosis or at the moment of the survey. In CLL, hypogammaglobulinemia is commonly progressive over time; thus, IgG levels evaluated at onset of the disease may significantly differ from levels observed at the time of infection onset. Dhalla et al. (12) suggested that immunization responses could be used to stratify infection risk and select patients for IgRT, but caution is recommend since interpreting immunization assays is complex, cut-off levels may vary and there are controversies in stating what constitutes an adequate response. Finally, Freeman et al. (13) suggested that screening patients with CLL for IgG subclass deficiency may be a useful adjunct in stratifying their risk for infection, since they were able to show a significant relationship between any IgG subclass deficiency and infections, regardless of the total Ig level.

MULTIPLE MYELOMA

Infection is a significant cause of morbidity and the leading cause of death in patients with multiple myeloma (MM) (14). The increased susceptibility to infections results from the interplay between antineoplastic therapies and disease-related complications (15). Moreover, novel treatments significantly prolonged survival transforming MM into a chronic condition, characterized by multiple relapses and salvage therapies, resulting in an increased cumulative immunosuppression and a higher risk for infection. The incidence of infectious events varies in different phases of MM (16), and seems to be higher during active disease and in the first months of induction chemotherapy. In early-stage MM, the most common infections involve the respiratory tract, manifesting as bronchitis and pneumonia. These infections are predominantly caused by *H. influenzae* or *S. pneumonia*, suggesting a role of hypogammaglobulinemia in their pathogenesis. In patients with advanced disease and during the neutropenic phases of intensive chemotherapy, *S. aureus* and gram-negative bacteria are more common, thus implying different underlying pathogenic mechanisms.

As in CLL, only few studies evaluated the role of IgRT in patients with hypogammaglobulinemia and MM. Chapel et al. (17) in 1994 performed a randomized, double-blind, placebo-controlled, multicenter trial of IVIG employed as prophylaxis against infection in 82 patients with stable MM; the authors demonstrated a protective role of IVIG against life-threatening infections and a significant role in reducing the risk of recurrent infections. Other two studies have been performed in patients with hypogammaglobulinemia and either CLL or MM, obtaining similar results (6). Conversely, Blombery et al. (18) showed no benefit of the use of peritransplant IVIG to reduce infectious complications in hypogammaglobulinemic patients with MM undergoing autologous stem cell transplantation.

In our opinion, regular Ig substitution should be considered in MM patients who suffer from life-threatening or recurrent infections that are reasonably thought to be caused by low levels of polyclonal Ig (16, 19). Similar indications are suggested in the Canadian guidelines about IgRT (7), where the proposed regimen consists of 400 mg/kg of IVIG administered every 4 weeks, subsequently adjusted to reach an individualized "biological" trough level. Prophylactic therapy with Ig during autologous stem cell transplantation is not recommended. Unfortunately, we observed that it may be not so easy to exactly define the level of polyclonal Ig in patients with MM, due to the presence of the monoclonal protein that interfere with Ig determinations. We suggest that clinical evaluation and anamnestic aspects are fundamental to decide if may be helpful to prescribe or not substitutive therapy in MM.

IATROGENIC HYPOGAMMAGLOBULINEMIA

As shown in **Table 2**, pharmacological history of the patient should be carefully evaluated when hypogammaglobulinemia is detected.

One of the most used drug able to induce iatrogenic hypogammaglobulinemia is the anti-CD20 monoclonal antibody

Table 1 | Main causes of secondary hypogammaglobulinemia.

Secondary hypogammaglobulinemia	
Excessive loss of immunoglobulins	Protein-losing enteropathy
	Nephrotic syndrome
	Severe burns
Malignancy	Chronic lymphocytic leukemia
	Multiple myeloma
	Good's syndrome
	Non-Hodgkin B cell lymphomas
Drug induced	See **Table 2**

Table 2 | Main causes of drug-induced hypogammaglobulinemia.

Drug-induced hypogammaglobulinemia	
Anti B cells monoclonal antibodies	Rituximab
	Belimumab
Immunosuppressants and chemotherapeutics	Steroids
	Gold salts
	Azathioprine
TKI inhibitors	Imatinib
	Dasatinib
Anticonvulsants	Carbamazepine
	Valproate
Others (sporadically described)	Ramipril
	Acetylsalicylic acid

rituximab. Firstly experimented and introduced in the clinical practice for the treatment of hematological malignancies, it has become a commonly used immune modulatory strategy for the treatment of many refractory or poorly controlled autoimmune or inflammatory disorders. Removal of CD20-expressing cell populations induces a dysregulation of immune homeostasis, impacting regulatory functions of normal B cells (20, 21). Hypogammaglobulinemia represents common negative consequences of this imbalance (22–27), and is considered the main factor that influences the increased risk of infection in patients receiving rituximab (28–30). According to recent hypotheses (31–33), a subgroup of rituximab-induced hypogammaglobulinemias should be considered as the consequence of the presence of a latent PID. In some cases, rituximab would elicit the antibody defect in genetically predisposed individuals (34).

Kelesidis et al. (35) recently summarized the available evidence about rituximab use and infections: the risk was increased in patients with hematological malignancies, while in patients with autoimmune diseases the risk was similar to other treatments. However, considering the number of confounders potentially masking or modulating the overall effects (e.g., co-morbidities, concurrent chemotherapy, presence of neutropenia, number of rituximab cycles, etc.), caution has been suggested in drawing definitive conclusions. In hematological conditions, the incidence of transient or persistent hypogammaglobulinemia following rituximab-therapy ranges from 15 to 40% (28). Female gender, fludarabine association regimens (36), and administration after autologous stem cell transplantation (37–40) have been identified as main risks factors, while maintenance therapy for follicular lymphoma is associated with a very low incidence of hypogammaglobulinemia (41). Regarding autoimmune disorders, two recent studies described a possible detrimental effects of rituximab on Ig synthesis in patients with ANCA-associated vasculitides (42, 43), and in particular in patients relapsing after cyclophosphamide treatment (43). Conversely, other studies contradict these observations: Marco et al. (44) reported that hypogammaglobulinemia could be attributed to the prior cyclophosphamide/steroid exposure rather than to the cumulative rituximab dose. Finally, the risk of hypogammaglobulinemia and infections seems to be related to the underlying autoimmune condition: in patients with rheumatoid arthritis or immune thrombocytopenia, the treatment is well-tolerated without significant risk of infections and hypogammaglobulinemia (22, 45–47).

We suggest that Ig levels should be always checked before rituximab administration and monitored for at least 6 months after the last dose. A longer period of observation should be addressed in patients presenting with low Ig levels prior to rituximab-therapy or in those who show risk factors for prolonged/severe hypogammaglobulinemia. In any case prompted IgRT should be offered, either e.v. or subcutaneous, to those patients who complain severe or recurrent infection episodes and concomitant low Ig levels.

BONE MARROW TRANSPLANTATION

Allogeneic bone marrow transplantation (ASCT) is a well established therapy in hematological malignancies. Immune defects are common consequences, persisting for years after ASCT. Arai et al. (48) recently described that the cumulative incidence of hypogammaglobulinemia (IgG < 400 mg/dl) in a cohort of 278 patients was 24.1 or 27.1%, respectively 1 or 3 years after ASCT. Risk factors were lymphoid malignancies, history of previous ASCT, usage of mycophenolate mofetil, low pre-ASCT IgG levels, and grade 2–4 aGVHD. Norlin et al. (49) described that patients with low IgG levels (<400 mg/dl) had an increased risk of transplant-related mortality compared to patients with moderately low or normal levels. Patients with low IgG levels had an increased incidence of infections as cause of death.

A recent meta-analysis (6) summarizes data on IgRT in patients undergone ASCT. When polyvalent Ig or hyperimmune cytomegalovirus CMV-IVIG are compared, there are no differences in mortality, rate of infections, CMV infections, and microbiologically documented infections. Polyvalent Ig significantly reduced the risk for interstitial pneumonitis but increased the risk for veno-occlusive disease and adverse events. Moreover, guidelines for preventing infectious complications provided by the American Society of Bone Marrow Transplantation (50) suggest that benefit is small if IVIG are used for prophylaxis of bacterial infections in patients with severe hypogammaglobulinemia (defined by authors as IgG < 400 mg/dL). The panel of experts recommends that IVIG dose and frequency should be individualized to maintain trough serum IgG concentrations >400 mg/dL. Again, IVIG are not recommended for CMV-disease prophylaxis after ASCT. Interestingly, Sundin et al. (51) showed that SCIG therapy is as effective as IVIG in maintaining IgG levels above 400 mg/dl in pediatric patients after ASCT, and SCIG were associated to less adverse events compared to IVIG.

SOLID ORGAN TRANSPLANTATION

Hypogammaglobulinemia has been reported as a complication of solid organ transplantation, particularly after heart, lung, and kidney transplantation, with an associated increased risk for infections. Moderate to severe hypogammaglobulinemia in recipients of lung transplantation has been associated with increased occurrence of infections, longer hospitalization, and acute cellular rejection. Hypogammaglobulinemia appeared to be related to 1-year all cause mortality. A recent meta-analysis by Florescu et al. (52) found a consistent prevalence of hypogammaglobulinemia (45%) and severe hypogammaglobulinemia (15%) during the first post-transplant year. The risk of infections was higher (2.46-fold) when IgG were less than 400 mg/dL; the risk of infections in patients with IgG > 400 mg/dL did not differ with respect to patients with normal Ig levels. This risk is related to the immune-suppressive treatment, being particularly high if mycophenolate mofetil is employed.

Concerning the role of IgRT in transplanted patients with hypogammaglobulinemia it has been reported that IgRT favors the reduction of infections rate (53, 54). Moreover, a single center experience (55) suggested that SCIG replacement therapy represents a well-tolerated alternative to IVIG after lung transplantation. Unfortunately, these studies are heterogeneous in terms of study populations or/and criteria of inclusion/exclusion for the beginning of IgRT and definitive conclusion cannot be drawn. Anyway, we suggest to start IgRT in patients with severe hypogammaglobulinemia, or in patients who complain recurrent infections reasonably related to low IgG levels. The proposed starting

regimen should be 400 mg/kg of IVIG administered every 4 weeks, subsequently adjusted to reach an individualized "biological" trough level.

OTHER CAUSES OF SECONDARY HYPOGAMMAGLOBULINEMIA

Conditions that frequently cause Ig loss include burns, renal, or intestinal diseases. Protein-losing enteropathy is characterized by an excessive loss of proteins into the gastrointestinal tract, resulting in some cases in symptomatic hypogammaglobulinemia. An hypoproteinemic state with hypogammaglobulinemia can be also a consequence of severe burns. Treatment of these disorders consists mainly in the management of the underlying disease, and no extensive data are available regarding IgRT.

A variety of infections has been recognized as an important cause of morbidity and mortality in patients with nephrotic syndrome. Immune defects in these patients are due to edema complications, urinary loss of complement factors and Ig, and defects in cellular immunity and secondary effects of immunosuppressive therapy. Many different prophylactic interventions have been used for reducing the risks of infection in these patients but recommendations for routine use are still lacking (56).

Hypogammaglobulinemia is present in a small percentage of patients with thymoma (Good's syndrome), usually symptomatic. Often, hypogammaglobulinemia persists after the treatment of the underlying disease. There are some reports on the efficacy of IgRT in these patients (57, 58).

HIV INFECTION

HIV infection is peculiar in its immunological profile. Patients usually exhibited hypergammaglobulinemia that encompassed all three Ig classes, but given the T cell defect they failed to mount specific antibody responses to various T cell dependent antigens, and in particular encapsulated bacteria (59). In adults with HIV, opportunistic infections are the most common manifestations of the immune deficiency; instead, children frequently experience a different immunological impairment, complaining of recurrent serious bacterial infections (bacteremia and pneumonia) (60).

On the basis of the results obtained in the NICHHD trials in 1991 (61) and several smaller studies previously performed, IVIG have been approved for preventing invasive bacterial infections in children with HIV infection (400 mg/kg every 2–4 weeks). IVIG seems to have only limited therapeutic benefit in terms of mortality reduction in the acute phase of the bacterial infections (60). The utility of IgRT has been later questioned, because antibiotic prophylaxis and especially retroviral therapy have dramatically changed the clinical course of the disease (60); nowadays IgRT is taken into account only in selective cases with recurrent bacterial infections despite antiretroviral and antibiotic therapy.

Adult patients with HIV may retain their full complement of B cells, and are less susceptible to infections with common pathogens, even if HIV can profoundly affect humoral immunity in a subset of patients. Several uncontrolled trials did not shown conclusive benefits to routine IgRT (59). These studies should not exclude the use of IVIG for adults who are infected with HIV-1 with recurrent bacterial infections despite antiretroviral and antibiotic therapy.

SUBCUTANEOUS IMMUNOGLOBULINS

Subcutaneous Ig have been widely shown to be safe, cost-effective, and greatly appreciated in terms of Health-Related Quality of Life (HRQL) in patients with PID (62–66). SCIG can be self administered at home, do not require venous access and systemic premedication, are characterized by a gradual absorption of the drug and by a decrease in the incidence of systemic adverse effects (66, 67). Local reactions are usually mild and do not affect the good tolerability of the treatment (68).

Safety and efficacy of SCIG in SAD have been assessed in few case series. We showed that SCIG are safe and effective in maintaining adequate levels of serum IgG in patients with lymphoproliferative diseases (LPDs) and hypogammaglobulinemia (9). We also observed an improvement perceived in HRQL. Similar results in term of efficacy, safety, and HRQL were obtained in children after hematopoietic stem cell transplantation (51) and in patients after lung transplantation (55).

Other potential benefits of SCIG should be considered in patients with hematological malignancies. Venous access often represents a great concern after chemotherapy treatments, and SCIG provide the possibility to avoid the use of venous accesses. The flexibility of SCIG treatment and the possibility of a home-based infusion represent a further advance for patients who usually need an high number of outpatient visits.

OPEN QUESTIONS

In this review, evidence has been provided suggesting that IgRT is effective in most patients with SAD. Nonetheless, cost-effectiveness advantage of initiating an IgRT in subjects with SAD has been criticized by some authors. To provide a conclusive reply to this criticism, a question should be definitively answered by future studies: what criteria should be used to identify patients in whom prophylactic IgRT may help to decrease infectious risk? At present IgG levels represent the only parameter employed to this aim. A better selection of patients in which IgRT is needed can lead us to limit the perhaps too extended use of IgRT in SAD.

AUTHOR CONTRIBUTIONS

All the authors participate in the definition of the work. Nicolò Compagno, Giacomo Malipiero, and Francesco Cinetto prepared the manuscript. Carlo Agostini coordinated the work and critically revised the manuscript. The final version of this work was approved by all authors.

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Is dosing of therapeutic immunoglobulins optimal? A review of a three-decade long debate in Europe

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The consumption of immunoglobulins (Ig) is increasing due to better recognition of antibody deficiencies, an aging population, and new indications. This review aims to examine the various dosing regimens and research developments in the established and in some of the relevant off-label indications in Europe. The background to the current regulatory settings in Europe is provided as a backdrop for the latest developments in primary and secondary immunodeficiencies and in immunomodulatory indications. In these heterogeneous areas, clinical trials encompassing different routes of administration, varying intervals, and infusion rates are paving the way toward more individualized therapy regimens. In primary antibody deficiencies, adjustments in dosing and intervals will depend on the clinical presentation, effective IgG trough levels and IgG metabolism. Ideally, individual pharmacokinetic profiles in conjunction with the clinical phenotype could lead to highly tailored treatment. In practice, incremental dosage increases are necessary to titrate the optimal dose for more severely ill patients. Higher intravenous doses in these patients also have beneficial immunomodulatory effects beyond mere IgG replacement. Better understanding of the pharmacokinetics of Ig therapy is leading to a move away from simplistic "per kg" dosing. Defective antibody production is common in many secondary immunodeficiencies irrespective of whether the causative factor was lymphoid malignancies (established indications), certain autoimmune disorders, immunosuppressive agents, or biologics. This antibody failure, as shown by test immunization, may be amenable to treatment with replacement Ig therapy. In certain immunomodulatory settings [e.g., idiopathic thrombocytopenic purpura (ITP)], selection of patients for Ig therapy may be enhanced by relevant biomarkers in order to exclude non-responders and thus obtain higher response rates. In this review, the developments in dosing of therapeutic immunoglobulins have been limited to high and some medium priority indications such as ITP, Kawasaki' disease, Guillain–Barré syndrome, chronic inflammatory demyelinating polyradiculoneuropathy, myasthenia gravis, multifocal motor neuropathy, fetal alloimmune thrombocytopenia, fetal hemolytic anemia, and dermatological diseases.

Keywords: IVIG, SCIG, replacement therapy, immunomodulation, dosing

INTRODUCTION

Indications for intravenous (IVIG) and subcutaneous (SCIG) immunoglobulin (Ig) therapies are steadily increasing and the annual demand has tripled in the last 15 years reaching a worldwide consumption of ~130 metric tons in 2012 (1). Shortages in supply have occurred in the past and cost pressure on health systems worldwide is growing The increased demand for therapeutic Ig has been met by increasing the number of plasma donors and by introducing new high-yield fractionation procedures, which in some cases have been accompanied by increases in rates of hemolytic anemia.

Reports involving alternative doses given in both Ig replacement and immunomodulatory indications are questioning current practice. These, together with emerging biomarkers associated with Ig-responder and non-responder status, invite close scrutiny of indications for, and methods of administering, IVIG and SCIG use in the future. Traditional dosing of Ig has relied heavily on a "per kg of bodyweight" calculation, which has now been

Table 1 | Well established indications according to "Guideline to assess efficacy and safety of normal intravenous immunoglobulin products" (CPMP/388/95 and CPMP/BPWG/859/95).

1994 "well established" indications	Dose	Frequency of injections
REPLACEMENT THERAPY IN		
Primary immunodeficiency syndromes (PID)		
• Congenital agammaglobulinemia and hypogammaglobulinemia	Starting dose: 0.4–	Every 3–4 weeks to obtain IgG trough level of at least
• Common variable immunodeficiency disorders	0.8 g/kg – thereafter:	5–6 g/l
• Severe combined immunodeficiencies	0.2–0.8 g/kg	Every 3–4 weeks to obtain IgG trough level of at least
• Wiskott–Aldrich syndrome	0.2–0.4 g/kg	5–6 g/l
Secondary immunodeficiency syndromes (SID)		
• Myeloma	0.2–0.4 g/kg	
• Chronic lymphocytic leukemia (CLL) with severe secondary hypogammaglobulinemia and recurrent infections	0.2–0.4 g/kg	Every 3–4 weeks Every 3–4 weeks to obtain IgG trough level of at least
• Congenital AIDS with recurrent infections		5 g/l
IMMUNOMODULATORY EFFECT IN		
• Idiopathic thrombocytopenic purpura (ITP) in adults and children at high risk of bleeding or prior to surgery to correct platelet count	0.8–1 g/kg or 0.4 g/kg/day	On day 1, possibly repeated once within 3 days
• Kawasaki disease	0.4 g/kg/day	For 2–5 days
• Bone marrow transplantation (BMT)	1.6–2 or 2 g/kg	For 5 days in divided doses over 2–5 days in association with acetylsalicylic acid in one dose in association with acetylsalicylic acid

brought into question [Ref. (2) and Chapel, submitted]. Given that IVIG/SCIG is manufactured from a limited resource, the possibility of dose adjustment according to lean body weight or even fixed doses titrated to effect in both replacement therapy and in immunomodulation arises (2, 3). This may become especially relevant in view of the combination of increasing numbers of patients due to improving diagnostics in developing countries, an aging population, and worldwide increases in body weight.

In this situation, the authors felt a need to reconsider the dosing issue for the so-called established indications (**Table 1**) by reviewing the literature, addressing safety and efficacy issues of new IVIG and SCIG preparations, and proposing appropriate measures where needed. The issue of class effect of different Ig preparations will be discussed. To date, switching brands during shortages or because of tender systems does not seem to have had clinically relevant effects on efficacy. However, the spectrum of side effects may differ from brand to brand, according to route of application, infusion rates, different dose levels, and the underlying disorder. In addition, product switching complicates exposure tracking in the event of a contamination incident, and alters the donor exposure profile of individual patients. Thus, dosing recommendations might have to be adjusted for certain diseases on the basis of effectiveness, safety profiles, and possibly in future on the basis of validated individual biomarkers and clinical outcome.

Switching from IVIG to SCIG in the case of chronic disorders has been the topic of recent research given similar efficacy to IVIG infusions, the lower incidence of systemic side effects, a lack of "wear-off" effect, improved health-related quality of life, better treatment satisfaction, and faster functional recovery with less time off work are frequently quoted advantages of SCIG (4, 5). The possible pharmacoeconomic benefits of SCIG are beyond the scope of this review, especially considering the various health/insurance systems and the varying prices of SCIG and IVIG in the different European countries. In general, switching from IVIG to SCIG in Europe is done in a dose-equivalent manner and not performed with a dose adjustment coefficient (DAC) as applied in the USA (~150%) (6, 7). As no official guidelines require a DAC in Europe (coreSPC, see below) additional product costs do not incur. The efficacy of DAC vs. dose-equivalent switch is a matter of ongoing debate and may require more long-term data.

As all authors of this review build on a long-standing professional experience in Europe, the current dosing recommendations reflect European practice, which has grown historically and encompasses recommendations from learned societies, national recommendations, European recommendations (8), and the European Medicines Agency (EMA). In the next section, the regulatory framework and the historical development of the EU recommendations will be briefly outlined without elaborating on subtle differences to American, Canadian, and Australian guidelines.

REGULATORY FRAMEWORK

Three main bodies regulate blood products in Europe:

- the European Directorate for the Quality of Medicines and Healthcare (EDQM), which provides standardization of quality control of medicines as elaborated in the European Pharmacopeia; it also co-ordinates the network of the Official Medicines Control Laboratories (OMCL),
- the EMA, which evaluates marketing authorization or variation applications for medicinal products within the EU in the so-called centralized procedure (CP); it composes Investigational Guidelines for clinical trials and core Summary of Product Characteristics (coreSPCs), i.e., templates for Product Information leaflets), and
- the Heads of Medicines Agencies (HMA), which is network of the heads of the National Competent Authorities (NCA) whose

organizations are responsible for the regulation of medicinal products for human use in the European economic area (EEA) in so-called mutual recognition (MR) or decentralized (DC) Procedures.

In 1994, the criteria for investigating IVIG in clinical trials were laid out in the "Guideline to assess efficacy and safety of normal intravenous immunoglobulin products" (CPMP/388/95) and a core Summary of Product Characteristics (coreSPC; CPMP/BPWG/859/95) encompassing "well established" indications for IVIG was proposed (see **Table 1**). These "well established" indications were based on certain pivotal studies and the doses administered therein; they became the cornerstone for the dosing recommendations in the coreSPC.

In 2000, analogous documents (investigational Guideline and coreSPC) were devised for subcutaneous and intramuscular immunoglobulin products (CPMP/BPWG/283/00 and CPMP/BPWG/282/00).

Over the years, these documents have undergone a number of revisions in order to encompass the developments in medical research and practice.

The current European Investigational Guideline and coreSPC for IVIG are undergoing a revision process (for Concept Paper see: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2014/08/WC500170555.pdf)

The revision of Investigational Guideline and the coreSPC for SCIG is near completion: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/12/WC500135705.pdf and http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500130466.pdf

In the EU three IVIG and two SCIG preparations have been centrally authorized (CP procedure)[1] and five IVIG and two SCIG brands were granted authorization via the MR- or DC-procedures[2]. In addition, some European countries will have nationally authorized products.

Several National Guidelines consider neuroimmunological diseases such as Guillain–Barré syndrome (GBS), chronic inflammatory demyelinating polyneuropathy (CIDP), multifocal motor neuropathy (MMN), myasthenia gravis (MG), and others as high to medium priority immunomodulatory indications for IVIG/SCIG use [Ref. (8); supporting information **Table 2**]. Progress in this field will be discussed as well as changing high-priority indications in pediatric AIDS, feto-neonatal alloimmune thrombocytopenia (FNAIT), and dermatological indications.

Owing to space limitations the majority of the medium and low-priority indications [Ref. (8); supporting information **Table 2**] were not included in this review.

Table 2 | Abbreviations used in the text.

BMT	Bone marrow transplantation
BPWP	Blood products working party
BWP	Biologics working party
CHMP	Committee for medicinal products for human use
CIDP	Chronic inflammatory demyelinating polyradiculoneuropathy
CLL	Chronic lymphocytic leukemia
CMD-human	Co-ordination group for MR- and DC-procedures
CMS	Concerned member states
CMV	Cytomegalovirus
CoE	Council of Europe
coreSPCs	Core summaries of product characteristics
CP	Centralized procedure
DCP	Decentralized procedure
EDQM	European Directorate for the Quality of Medicines and Healthcare
EEA	European economic area
EMA	European medicines agency
EU	European union
FNAIT	Fetal neonatal alloimmune thrombocytopenia
GBS	Guillain–Barré syndrome
GvHD	Graft vs. host disease
HMA	Heads of medicines agencies
HSCT	Hematopoietic stem cell transplantation
ITP	Idiopathic thrombocytopenic purpura
IVIG	Intravenous immunoglobulin
KD	Kawasaki's disease
MM	Multiple myeloma
MMN	Multifocal motor neuropathy
MRP	Mutual recognition procedure
NCA	National competent authorities
OMCL	Official medicines control laboratories
PE	Plasma exchange
PAD	Primary antibody deficiency
PID	Primary immunodeficiency syndromes
PK	Pharmacokinetic
PRAC	Pharmacovigilance risk assessment committee
RMS	Reference member state
RBC	Red blood cell
SBI	Severe bacterial Infections
SCIG	Subcutaneous immunoglobulin
SID	Secondary immunodeficiency syndromes

REPLACEMENT THERAPY

PRIMARY IMMUNODEFICIENCY SYNDROMES (PID)

In 1952, Colonel Ogden Bruton (9) noted the absence of serum Ig in an 8-year-old boy with a history of pneumonia and other bacterial sino-pulmonary infections. Bruton was also the first physician to provide specific immunotherapy for this X-linked disorder by initially administering 3.2 g of IgG subcutaneously. Assuming an approximate weight of an 8-year old to be 30 kg, this dose would correspond to 0.1 g/kg. This dosing was taken into the coreSPC,

[1]http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/landing/epar_search.jsp&mid=WC0b01ac058001d124

[2]http://mri.medagencies.org/Human/

but is currently considered within the low range of the recommended weekly dosing for SCIGs (0.1 g/kg–0.2 g/kg/week) or the monthly dosing of IVIG (0.4 g/kg–0.8 g/kg/month).

Current IVIG coreSPC indication and dosing:

Primary immunodeficiency syndromes with impaired antibody production

The recommended starting dose is 0.4–0.8 g/kg given once, followed by at least 0.2 g/kg given every 3–4 weeks.

The dose required to achieve a trough level of 5–6 g/l is of the order of 0.2–0.8 g/kg/month. The dosage interval when steady state has been reached varies from 3-4 weeks. Trough levels should be measured and assessed in conjunction with the incidence of infection. To reduce the rate of infections, it may be necessary to increase the dosage and aim for higher trough levels.

Current draft SCIG coreSPC indication and dosing:

Primary immunodeficiency syndromes with impaired antibody production

The dose regimen should achieve a trough level of IgG (measured before the next infusion) of at least 5-6 g/l and aim to be within the reference interval of serum IgG for age. A loading dose of at least 0.2-0.5 g/kg (10-40 ml/kg) body weight may be required. This may need to be divided over several days, with a maximal daily dose of 0.1-0.15 g/kg)

After steady state IgG levels have been attained, maintenance doses are administered at repeated intervals (approximately once per week) to reach a cumulative monthly dose of the order of 0.4-0.8 g/kg. Each single dose may need to be injected at different anatomic sites.

Trough levels[a] should be measured and assessed in conjunction with the incidence of infection. To reduce the rate of infection, it may be necessary to increase the dose and aim for higher trough levels.

[a] *N.b: Trough levels can be measured for a facilitated SCIG given every 3-4 weeks and for a normal SCIG given at biweekly-weekly intervals; however, in clinical practice SCIG products are sometimes given at even shorter intervals - in these cases the term "trough level" would not capture the fact that what is actually being measured is the mean level.*

Recent developments in Ig replacement therapy of PID

The demonstrated success of Ig prophylaxis via the intravenous route depends predominantly on maintaining an adequate protection against infections. According to international guidelines the Ig monthly dose of 300–600 mg/kg body weight should be administer intravenously every 3 or 4 weeks and subcutaneously once/twice a week (10–13). The trend over the past years has been to increase the monthly cumulative doses (14–17). This general rule might not be optimal for all patients affected by primary antibody deficiencies (PAD) due to high clinical and immunological heterogeneity of the underlying diseases. A recent paper (18) analyzed the clinical presentation, association between clinical features, and differences and effects of Ig treatment in a large series of European patients affected by common variable immunodeficiency disorders (CVID), the most common symptomatic PAD. Different treatment strategies applied in Europe resulted in considerable differences in Ig dosing, ranging from 0.13 up to 0.75 g/kg/month. This and previous studies suggested that a correlation between patients' antibody levels and clinical effects: patients with very low-trough levels of <4 g/l had poor clinical outcomes (15, 16, 19) whereas higher trough levels were associated with a reduced frequency of serious bacterial infections.

Thus, the aim should be to maintain an individual's effective antibody level and not to establish a universally defined immunoglobulin monthly dosage. Consequently, almost all recent studies on Ig replacement advocate that the treatment strategies should be individualized not only in terms of dosages but also with regard to treatment schedules including intervals between administrations and routes of administration (20–23). Milito et al. (24) have recently demonstrated that in PAD patients with fewer disease-associated complications the IVIG replacement could be administered with the widely used interval of 3 or 4 weeks, even administering low-IVIG replacement dosages. On the other hand, in patients with bronchiectasis and enteropathy and a severe immunological phenotype with IgG trough levels <500 mg/dl, IgA <7 mg/dl, absent response to polysaccharides, and low-switched memory B cells (<2%) the protective effect might be achieved by lowering the interval between administrations to 2 weeks and in few cases to 1 week, without increasing the cumulative monthly Ig dosage (19). Alternatively, patients with severe clinical and immunological phenotypes might be treated with higher Ig dosages at an interval of 3–4 weeks. The need to elucidate the effects of therapy on patient outcomes might allow identification of what works best in which setting and under what conditions (25, 26). Health care delivery systems are quickly changing in response to economic pressure and concerns about quality of care emerge. The system of care is itself an important determinant of patient outcomes. The promise of individualized medicine has launched a huge research enterprise to explore the personal characteristics that influence responses to therapy.

The pharmacokinetics (PK) of IgG and specific antibodies exhibited a different half-life in patients treated at different intervals between infusions (26). Trough levels of the lowest specific antibody concentrations rise if regular infusions are given and the actual trough levels in a regularly infused patient are likely to be higher than the levels of specific IgG measured by ELISA in the IVIG preparations (27, 28).

The clinical relevance of regular application of Ig has been underlined by the finding that an important determinant of the efficacy of Ig prophylaxis is the length of time an individual spends with a lower IgG level a situation, which is minimized by subcutaneous therapy (29). This time is more dependent on the patient's IgG half-life and the frequency of dosing than on the dose of Ig infused. In clinical practice, it would be ideal to perform a PK study in all patients. However, this would require a significant commitment in time and costs from the patient and the treating physician. This practical drawback has limited the use of PK information in clinical practice. Different alternative options have been attempted. The Oxford approach, for example, based on monitoring break-through infections, was to increase the IVIG dose by 0.15 g/kg/month when patients present with a serious infection, or three or more moderate infections over a year (17). This recommendation could be an alternative for patients who have persistent infections; although other factors such as protein loosing conditions, airway and intestinal inflammation, need to be assessed when defining an individual

Ig treatment schedule as the Oxford approach related treatment to CVID phenotype (17). Moreover, in PAD, several lines of experimental evidence gathered recently (30–32) provided a basis for an active role for IVIG in immunomodulation beside the main role to replace the missing antibodies. Ig has such a role in regulating autoimmune and inflammatory responses through modulating B and other cells functions (8). These new findings might help to explain the different results found in trials aimed at establishing the clinical outcome of Ig replacement in PAD patients. It is possible that some of the positive effects observed in patients treated with higher doses are not dependent only on the prophylactic role of Igs but also on their anti-inflammatory ones. In contrast to subcutaneous administration, intravenous administration might allow maintenance of the protective and immunomodulatory effects due to the serum IgG peak reached at the time of each administration.

Methods to improve IgG recovery and increase productivity have been implemented in the last few years as a response to growing clinical demand for therapeutic Igs (27). Any effects of major changes in the Ig production should be assessed in clinical and drug surveillance studies.

In addition, approval has been granted for

- high-concentration formulations of IVIG and SCIG preparations (27, 29, 33),
- rapid push administration (34),
- one SCIG product (Hyqvia) combined with the prior use of a spreading factor (hyaluronidase), thus, allowing for 3–4 weekly intervals (current indication excludes children <18 years) (35)[3], and
- one SCIG product (Hizentra) where modeling and simulation approaches were applied that led to comparable IgG exposure levels if the product were to be administered every 2 weeks using double the weekly dose during maintenance therapy (21, 22)[4].

SCIG studies in PID

A recent evidence-based review article (36), comparing IVIG and SCIG administration in PID and SID patients, encompassed 25 studies (in PID patients 2/25 studies were randomized and 17 non-randomized; for SID patients 1/25 studies was non-randomized and 5/25 were health economic studies). Of the studies that mentioned specific products, eight used an SCIG from CSL-Behring, seven a product from Baxter, and two a product from Grifols. Only 3/25 studies reported severe bacterial infections (SBI) as their primary outcome of interest for both SCIG and IVIG; no SBI occurred in these studies. In a total of five studies, the annual number of infections was investigated and no difference found between the two routes of administration; however, the definitions of infections were fairly heterogeneous. Higher IgG trough levels were found with SCIG substitution. No serious adverse events were reported in five trials that investigated this parameter, however, here again definitions and inclusion/exclusion criteria varied. Minor adverse events, consisting of local symptoms were usually mild and more frequent with SCIG substitution, as would be expected of this modality. Four studies investigating health-related quality of life showed improvement when patients switched from hospital-based IVIG to SCIG substitution at home. Of the 5/25 studies that evaluated health economics, 4 found that SCIG administration was considerably more cost effective in comparison with IVIG substitution, whereas one older study did not show this gain.

The authors caution the reader that for most efficacy and safety parameters measured in the studies the value of evidence was low and that with regard to the pharmacoeconomic evaluation due to the differences in the health care and insurance systems in the different countries the results cannot be generalized. They conclude that good studies comparing IVIG to SCIG are lacking, but it is possible to state that SCIG is safe and efficacious and at least non-inferior to IVIG. They also view the issue of switching from IVIG to SCIG as not yet solved.

SECONDARY IMMUNODEFICIENCY SYNDROMES (SID)

Patients with secondary immunodeficiency are a continuously increasing heterogeneous group (36, 37). Recommendations for treatment of antibody deficiency associated with lymphoid malignancies [e.g., CLL, multiple myeloma (MM)], post-hematopoietic stem cell transplantation (HSCT), or HIV are referred to in core SPCs, which require critical updates. Those SIDs due to medications [e.g., anticonvulsants, anti-rheumatics, MAbs (e.g., anti-CD20), chemotherapy, immunosuppression] may be suspected from the patient's medical history although usually only a subset of patients develops signs of SID.

SID patients are often less well defined than PADs as the diagnosis relies only on serum Ig levels rather than proven antibody failure, but their clinical importance is recognized increasingly. In this section, findings in patient groups, including those not included in the core SPC, are discussed.

Patients with SID are unduly susceptible to different types of pathogens depending on the type of SID: viral and/or opportunistic infections are common in patients with non-functional or absent T-cells while bacterial infections predominate in antibody failure syndromes. Severe life-threatening infections affect the respiratory or gastrointestinal tracts though chronic infections of prolonged duration also occur with agents of low pathogenicity (38, 39). These infections are an important cause of morbidity, mortality, hospital admissions, and intensive care treatment in this group of patients. The following discussion relates largely to antibody failures secondary to therapy or disease that maybe amenable to treatment with Ig replacement therapy.

Numerous studies in patients with lymphoid malignancies have shown beneficial effects of IVIG, resulting in fewer infectious episodes, reduced use of antibiotics, shorter hospital stay but no difference in overall mortality. Most of these studies report on patients in chronic state of disease, treated with IVIG doses comparable to patients with PAD.

Chronic lymphocytic leukemia (CLL)

In 1988, the Cooperative Group for the Study of Immunoglobulin in CLL published the results of a double-blind, placebo-controlled

[3]http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/002491/human_med_001647.jsp&mid=WC0b01ac058001d124
[4]http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/002127/human_med_001440.jsp&mid=WC0b01ac058001d124

study (40) in 81 CLL patients (with 50% lower than normal IgG levels and a history of >1 serious infection since onset of the illness). Those patients who received immunoglobulin therapy (0.4 g/kg every 3 weeks for ~1 year) had significantly fewer bacterial infections compared to those in the placebo group.

Current IVIG coreSPC indication and dosing:

Hypogammaglobulinaemia and recurrent bacterial infections in patients with CLL, in whom prophylactic antibiotics have failed.

The recommended dose is 0.2–0.4 g/kg every 3–4 weeks.

Current draft SCIG coreSPC indication and dosing:

Hypogammaglobulinaemia and recurrent bacterial infections in patients with CLL, in whom prophylactic antibiotics have failed or are contra-indicated.

For dosing see PID/SCIG above.

Following the original multi-center, randomized, placebo-controlled study (40) that resulted in inclusion in the coreSPC, the protective effect of IVIG in CLL and low-grade non-Hodgkin's lymphoma was confirmed by a 2 year, placebo vs. Ig cross-over study (41); in this study, serious bacterial infections correlated with low-serum IgG levels (<6.4 g/l) (41). Along the same line Boughton et al. (42) noted that 10 patients (24%) with IgG levels <3.0 g/l experienced 65% of infections in the whole CLL group. In view of newer treatments for CLL, studies on frequencies of bacterial infections before and after treatment with fludarabine, anti-CD20, or BTK inhibitors will be important. Such studies need to include documentation of IgG levels and specific antibody titers following test immunization.

Antibody deficiency, for which hypogammaglobulinemia is a surrogate, is the most important risk factor for bacterial infections; furthermore, antibody failure, as shown by test immunization, can occur in the absence of hypogammaglobulinemia (43). Failure to respond to test immunization with approved, killed vaccines is the most reliable indicator though absence of circulating antibodies after documented infections, such as HSV or VZV, are also helpful. Test immunizations in CLL patients have shown poor IgG responses against a range of antigens, including polysaccharide vaccines or conjugate pneumococcal vaccines (44). Whether those patients that do produce antibodies are even protected against bacterial infections is not clear due to lack of clinical trials (45). At present most guidelines use serum IgG levels in SID but logic requires test immunizations of patients (one protein and one polysaccharide vaccine) rather than relying on previous infections or surrogate markers.

Data from the original trials (40, 41) showed that starting doses of IVIG used in PAD (0.4 g/kg/month) were protective against bacterial infections in patients with low-serum IgG levels. Protection at a lower dose of IVIG (0.3 g/kg/month) was confirmed by Molica et al. (46) in a cross-over study in which 42 patients with serum IgG levels <6 g/l and/or a history of severe infection received either IVIG (0.3 g/kg/month) or standard care for 12 months and then crossed over to the alternative regime. Jurlander et al. (47) showed that 15 patients with low-serum IgG receiving 10 g IVIG every 3 weeks (0.2 g/kg/month) had reduced hospital admissions for infections and significantly reduced febrile episodes although neither were totally abolished.

Multiple myeloma (MM)

In 1994, Chapel et al. published a study on IVIG as a prophylaxis in 83 patients with stable MM (48). As in the case of CLL, the administration of IVIG (0.4 g/kg every 4 weeks for 1 year) led to significant reduction in life-threatening infections compared to placebo.

Current IVIG coreSPC indication and dosing:

Hypogammaglobulinaemia and recurrent bacterial infections in plateau phase MM patients who have failed to respond to pneumococcal immunization.

The recommended dose is 0.2–0.4 g/kg every 3–4 weeks.

Current draft SCIG coreSPC indication and dosing:

Hypogammaglobulinaemia and recurrent bacterial infections in MM patients

For dosing see PID/SCIG above.

The randomized placebo-controlled multi-center study in plateau-phase MM patients given IVIG 0.4 g/kg monthly for 1 year showed significant reductions in frequency and severity of infections (48). Fortunately, this trial included test immunization with polysaccharide pneumococcal vaccine before starting Ig therapy and benefit from IVIG was shown only in patients who responded poorly to pneumococcal immunization and had normal numbers of circulating neutrophils (48). Recently, antibody failure to a range of pathogens in MM and related conditions has been confirmed (49) suggesting that IVIG therapy may be justified. However, many newer treatment regimes in MM use antibiotic prophylaxis and each patient should be considered primarily on an individual basis for Ig therapy until more data is provided. Similar conclusions were drawn from a recent systemic review and meta-analysis by Raanani et al. (50) suggesting that IVIG cannot be recommended routinely for patients with CLL or MM with hypogammaglobulinemia and/or recurrent infections; instead decisions should be made on an individual basis.

Bone marrow transplantation

In 1990, Sullivan et al. (51) published the results of a double blind, placebo-controlled study in 382 bone marrow transplant recipients. The patients received placebo or 0.5 g/kg IVIG from day −7 to day +90, and continued IVIG treatment on a monthly basis until 1 year after transplantation. The endpoints were the rate of interstitial pneumonia and acute Graft vs. Host Disease (GvHD). Among the 61 cytomegalovirus (CMV) seronegative patients, none contracted interstitial pneumonia and in the group of 308 CMV positive patients the IVIG treated ones fared significantly better. Acute GvHD was reduced in the IVIG treated patients over 20 years of age but not in the younger age group.

In the initial coreSPC, the indication covered both GvHD and infection prophylaxis (mainly for CMV infection) and was thus

placed in the category of immunomodulation. However, a later placebo-controlled, double-blind study by Cordonnier et al. (52) and a meta-analysis on 30 trials including 4223 patients (53) revealed that there was no benefit on survival and frequency of infections while the risk of veno-occlusive disease (VOD) was increased. Thus, after allogeneic HSCT IVIG is currently used as a replacement therapy for hypogammaglobulinaemic patients with secondary B cell deficiency (serum IgG < 4 g/l) and no longer for GvHD.

The use of SCIG compared to IVIG has been studied in one non-randomized, retrospective study in 58 children with prolonged hypogammaglobulinemia after HSCT. All children were treated with IVIG and 12 continued on SCIG home treatment (54). While being of equivalent efficacy to IVIG, SCIG showed fewer side-effects and was the preferred treatment option.

Current IVIG coreSPC indication and dosing:

Hypogammaglobulinaemia in patients after allogeneic haematopoietic stem cell transplantation (HSCT).

The recommended dose is 0.2–0.4 g/kg every 3–4 weeks.
Hypogammaglobulinaemia in patients requiring allogeneic hematopoietic stem cell transplantation (HSCT).

For dosing see PID/SCIG above.

Congenital AIDS

In the late 1980s, the US National Institute of Child Health and Human Development (NICHHD) performed a double blind, placebo-controlled, multi-center IVIG study in 372 children with symptomatic HIV infection (55). The children were given 0.4 g/kg every 4 weeks. Median follow-up time was 17 months. The results showed that for children with CD4+ T-cell entry values >200/ml the time free of serious bacterial infection increased, serious and minor infections were reduced, as were the number of hospitalizations. However, there was no survival advantage for the IVIG group.

A further double blind, placebo-controlled IVIG study in 255 children with AIDS was published in 1994 (56). All children had received 180 mg/m^2 of zidovudine. The IVIG dose was 0.4 g/kg every 4 weeks. Patients were stratified according to their history (>1 serious bacterial infection), previous zidovudine treatment, and trimethoprim–sulfamethoxazole (TMP–SMZ) prophylaxis for *Pneumocystis jirovecii* pneumonia at entry. Median follow-up time was 30.6 months. The children who were on IVIG plus zidovudine and not receiving TMP–SMZ prophylaxis had a reduced rate of serious bacterial infections. As currently an efficient, highly active anti-retroviral therapy (HAART) is given to the mothers prior to delivery the development of congenital AIDS and pediatric HIV associated antibody deficiency is greatly reduced. In these patients, IVIG treatment is medically necessary for the prevention of bacterial infection when the following criteria are met (A) diagnosis of HIV disease, (B) patient age ≤13 years, and (C1) documented hypogammaglobulinemia or (C2) functional antibody deficiency as demonstrated by pool specific anti-titers (or recurrent bacterial infections). IVIG dose should not exceed 1.4 g/kg every 28 days. IVIG replacement therapy in HIV infected children without antibody deficiency may not be necessary; indeed it is even contra-indicated in the UK Guidelines.

Current IVIG coreSPC indication and dosing:

Congenital AIDS with antibody deficiency and recurrent bacterial infections.

The recommended dose is 0.2-0.4 g/kg every three to four weeks.

No current SCIG coreSPC indication or dosing under discussion.

Recent developments in Ig replacement therapy of SID

After anti-CD20 therapy. Anti-CD20 trials, however, have monitored both circulating B cells and serum Igs and although originally reduction of serum Igs was thought to be transient, some patients continue to have hypogammglobulinaemia and accompanying infections for prolonged periods possibly forever (57). In this recent study from Sloan-Kettering involving patients with lymphoid malignancies, low-serum IgG levels were identified in 38.5% (69/179) of patients after CD20 therapy, all of whom had normal levels initially; the risk was greater in patients who received maintenance rituximab. In 14 patients of this subset, IVIG significantly reduced the frequency of sino-pulmonary bacterial infection and pneumonias (57). Likewise, monitoring serum IgG levels and B cell numbers after anti-CD20 treatment in ANCA+ vasculitis is warranted for recognition of SID (58, 59), which may require IVIG replacement therapy.

After immunosuppressive regimes in solid organ transplantation. Floruesco et al. (60) discuss the impact of hypogammaglobulinemia on the rate of infections and survival following solid organ transplantation, in a meta-analysis that included 1756 patients from 18 studies. The study included patients with lung, kidney, heart, and liver transplants. The rate of severe hypogammaglobulinemia (IgG < 0.4 g/dl) amounted to 15%; it significantly increased the risk of CMV, fungal, and respiratory infections and was associated with higher 1-year all-cause mortality as originally described by Rubin (61). Sarmiento et al. (62) looked at 75 patients post heart transplantation of whom 10 patients developed CMV disease; those with a low-serum IgG level (IgG < 5 g/dl) were at higher risk of reactivation of CMV disease; the authors recommended CMV monitoring as a potential tool to recognize high-risk patients (62). More recently, Carbone et al. (63) reported results from 55 consecutive adult heart recipients who were subjected to an immune monitoring including measurement of specific antibodies and underwent IVIG therapy when SID was established. Eighty five percent of severe infections occurred during the first 3 months and mean time to IVIG infusion was 2.47 months. IVIG therapy resulted in improved specific antibody titers in the group on replacement therapy and a significant reduction of bacterial infections, in the substituted group of patients compared with 55 untreated patients.

During autoimmune diseases. An increased risk of invasive pneumococcal disease has been demonstrated in a retrospective

analysis of a cohort of systemic autoimmune diseases including patients with SLE, rheumatoid arthritis, hemolytic anemia, and Sjogren's syndrome (37–39, 64). In a 10-year European study of 1000 SLE patients 68 patients died (6.8%) and the most frequent causes of death were similarly divided among active SLE (26.5%), thrombosis (26.5%), and infections (25%), especially in the first 5 years after diagnosis (65). Similar results have been obtained in a multi-ethnic US cohort study: the 5-year mortality was 11.8%, active SLE (41%), and fatal infections (32%) headed the ranking (66). Infections were also frequent causes of increased hospitalization (67). Prolonged hypogammaglobulinemia requiring antibody replacement therapy occurred in 21% of ANCA+ vasculitis patients treated with cyclophosphamide followed by rituximab (58).

Antibody failure due to medications other than immunosuppressants. It has been known for a long time that anticonvulsant therapy with phenytoin or carbamazepine can cause low-serum IgG levels and recurrent infections (68, 69). Recently, valproic acid, a histone deacetylase (HDAC) inhibitor, has also been demonstrated to inhibit early B cell differentiation and activation (70) leading to hypogammaglobulinemia. Few studies performed in small patient groups with SID due to immunosuppressive medication or chemotherapy also demonstrate beneficial effects of IVIG treatment. However, robust studies looking on clinical outcome are lacking, not least as prophylactic antibiotics are used as standard measure in many centers.

Most studies in SID conditions such as CLL, MM, solid organ transplantation, and autoimmune diseases have been performed in chronic, stable disease with 0.3–0.4 g/kg IVIG every 3–4 weeks. Outcome in patients with antibody deficiency, with or without low-serum IgG levels as well as episodes of severe – potentially lethal – infection have to be included in the analysis (37–39). Present research in translational medicine including PID aims at early diagnosis to identify patients before the first (potentially life threatening) infectious complications and this applies to SID as well. Further clinical trials have been recommended in patients with SID. Patient selection in such studies will be critical and only test-immunized patients with proven antibody deficiency should be entered (one T dependent vaccine – usually tetanus toxoid is used since the assay is reliable and available in immunology laboratories and one T-independent vaccine – either pneumovax or polysaccharide *Salmonella* vaccine – both with reliable assays as used in PIDs). IVIG should be given early on during the course of an aggressive immunosuppressive therapy since lethal infections often occur at this stage.

IMMUNOMODULATORY THERAPY

IDIOPATHIC THROMBOCYTOPENIC PURPURA

In 1981, Paul Imbach and colleagues (71) treated 13 children with idiopathic thrombocytopenic purpura (ITP) (6 with acute and 7 with chronic forms of ITP, platelets counts <30,000/μl) with 0.4 g/kg/day IVIG for 5 days and could demonstrate a normalization of platelets counts (150–600 $\times 10^3$/μl) in 12/13 children within 5 days. However, the effect was transient as platelets fell to 80–400 $\times 10^3$/μl during the following 10 days.

In the years after this study, various trials followed in adults with ITP – also comparing IVIG to prednisone. In a study by Blanchette et al. (72), a reduced dose of 0.8–1.0 g/kg was given on day 1. If 48–72 h later platelet counts remained at values $\leq 20 \times 10^3$/μl a second IVIG dose was recommended. Although this protocol proved to be equally efficacious as the original of Imbach et al. (70), it was the latter one that set the stage for the regulatory adoption of the dosage regimen in ITP and other "immunomodulatory settings" for years to come.

In 2009, the International Working Group (73) standardized the terminology, definitions, and outcome criteria for clinical trials in ITP, which in turn was taken on board during the IVIG guideline and coreSPC revision process.

Current IVIG coreSPC indication and dosing:

Primary immune thrombocytopenia (ITP), in patients at high risk of bleeding or prior to surgery to correct the platelet count.

There are two alternative treatment schedules:

0.8–1 g/kg given on day one; this dose may be repeated once within 3 days

0.4 g/kg given daily for 2–5 days.

The treatment can be repeated if relapse occurs.

No current SCIG coreSPC indication or dosing under discussion.

Recent developments in ITP research

Treatment options in ITP vary with patient age (childhood vs. adults) and diagnostic status (newly diagnosed, persistent for 3–12 months, chronic beyond 12 months, or secondary to other diseases) (74, 75). Notably, in newly diagnosed ITP of childhood (<18 years) the standard of care is still IVIG at a total dose of 0.8–1.0/kg given on 1 or 2 consecutive days. In IVIG non-responders, the results can be improved by adding 20 mg/kg methylprednisolone during day 1–3 (75). In a prospective randomized trial, a 2 g/kg total dose IVIG was clearly more effective than 50 or 75 μg/kg anti-D as first-line treatment in childhood ITP (76). Interestingly, individual cases of successful treatment with very low-IVIG doses (100 or 200 mg/kg) imposed by economic constraints have been reported (77, 78). These observations warrant a systematic evaluation of an up-scaling protocol starting with doses of 0.2–0.4 g/kg IVIG. In a study from Thailand (a developing country), cost effectiveness of IVIG in childhood ITP has been proven, as compared to standard treatment of thrombocyte transfusions, corticosteroids plus immunosuppressants (79). On the other hand, health economic studies from Canada and Ireland show for adult chronic ITP patients that romiplostim, a thrombopoietin receptor agonist, seems to compare favorably with standard treatment including IVIG (80, 81).

A general observation throughout all ITP studies is an IVIG response rate of 60–75% in newly diagnosed childhood ITP. Hopefully, new biomarkers may in future be able to identify early on IVIG responders from non-responders. Thus, a recent study by Morimoto et al. (82) indicates that patients with WBC count $<7.0 \times 10^9$/l had a lower probability of thrombocytopenia-free survival (41 vs. 77%, $P = 0.003$) and a higher rate of progression

to chronic ITP (29 vs. 6%, $P = 0.040$) than those with WBC count $\geq 7.0 \times 10^9$/l. These results suggest that ITP with lower WBC count may represent a distinct subgroup requiring early on additional or other treatments than IVIG [e.g., rituximab (83)]. Similarly, in adults with ITP, the presence of anti-GPIb-IX auto-antibodies is a predictor for poor response to IVIG treatment: only 36.4% responded as compared to 80% of anti-GPIb-IX negative ITP patients (84). Less promising results came from studies correlating FcγRIIa and FcγRIIIa polymorphic variants to IVIG responsiveness and outcome (85): while the high-affinity FcγRIIIa variant 158V is possibly implicated in the pathogenesis of ITP, FcγRIIa (131R), and FcγRIIIA (158V) variants do not seem to impact on chronicity and therapeutic efficacy of IVIG, although studies on such correlations are underway. In this respect, it is interesting that the expression of an open-reading frame for the activating FcγRIIc (instead of the more common, non-expressed pseudogene) also seems to predispose to ITP (86).

KAWASAKI DISEASE

Kawasaki disease (KD) is an acute self-limiting inflammatory disorder of children, associated with vasculitis, affecting predominantly medium-sized arteries, particularly the coronary arteries. In 1984, Furusho et al. (87) treated 93 patients with KD [45 with acetylsalicylic acid (ASA) and 40 with ASA + IVIG] at a dose of 0.4 g/kg/day for 5 day. They observed a greater percentage of patients with coronary artery lesions in the ASA-alone group compared to the combined therapy (42 vs. 15%).

In 1991, Newburger et al. (88) treated 276 KD patients with ASA and IVIG 0.4 g/kg/day for 4 day vs. 273 ASA + IVIG 2 g/kg (given once). The rational to choose 2 g/kg (and not 1.6 g/kg) for the single dose was that serum IgG concentration on day 4 of the 0.4 g/kg × 4 day regime would be approximately the same. It was shown that the single large dose was more effective than the conventional regimen and equally safe. Both dosing possibilities were taken into the coreSPC (0.4 g/kg/day IVIG for 5 day + ASA and IVIG 2 g/kg + ASA).

Current IVIG coreSPC indication and dosing:

Kawasaki disease.

2.0 g/kg as a single dose is the recommended treatment worldwide.

Patients should receive concomitant treatment with acetylsalicylic acid.

No current SCIG coreSPC indication or dosing under discussion.

Recent developments in KD research

The etiology of KD is still unknown. It is assumed that unidentified infectious agents trigger a strong, self-limiting inflammation in genetically susceptible hosts. Numerous studies have been undertaken to identify susceptibility genes for KD as well as for resistance to IVIG treatment. Polymorphic variants of *FCGR2A*, *CD40*, *ITPKC*, *FAM167A-BLK*, and *CASP3* have been shown to be associated with KD (89). Similarly, gene copy number (GCN) variants of FcγR2c and FcγR3b were significantly associated with KD susceptibility and seem to influence also the IVIG treatment response (90) as does the increased expression of IL-1 pathway genes (91). Recently, Ogata et al. (92) found that sialylation levels of therapeutic IVIG are unrelated to treatment response whereas low sialylation of endogenous IgG and low-serum β-galactoside:α2-6 sialyltransferase-I (ST6Gal-I), ST6GAL1 RNA, and enzyme levels predict therapy resistance. As the authors compare only 10 IVIG responders to 10 non-responders their findings have to be met with great caution and need a rigorous confirmation.

The analysis of 3860 data sets from children with KD registered in the Taiwan National Health Insurance Data Base focused on the impact of different IVIG manufacturing procedures on the responsiveness in KD (93). They compared effects of β-propiolactone, acidification, and IgA content. Whereas β-propiolactone treatment of Ig had a relative risk of 1.45 to confer IVIG non-responsiveness and prolonged anti-platelet and anti-coagulants treatment, the relative risks for acidification and IgA content were non-significant in this respect. These findings are difficult to confirm as IVIG treated with β-propiolactone is no longer on the market.

On clinical grounds IVIG non-responders were shown to be older, had >6 days fever before the initiation of IVIG therapy; their serum levels of CRP, total bilirubin, lactate dehydrogenase (LDH), and gamma-glutamyltranspeptidase (g-GT) were significantly higher ($P = 0.002$, $P < 0.001$, $P < 0.034$, and $P < 0.038$, respectively), and their hemoglobin value was significantly lower ($P = 0.025$) than in IVIG responders (94–96). The authors defined the following predictors for IVIG non-responders: CRP level >10 mg/l, LDH level >590 IU/l, and/or hemoglobin value <10 g/l and suggested as escalating treatment options corticosteroids (97), TNF blockers (89, 98, 99), or plasma exchange (100).

Until relatively recently, corticosteroids were considered potentially detrimental in KD, as early studies showed an association with worse outcome (101). However, it is likely that this at least in part reflected an inadvertent selection bias, as those with more severe KD received corticosteroids. Corticosteroids are recommended as "rescue" therapy if there is no response to initial infusion(s) of IVIG (102). More recently, the potential role of corticosteroids as adjunct primary therapy in addition to IVIG has been addressed in randomized trials, either in unselected patients or in those considered at particularly high risk of coronary artery damage.

A multicenter, randomized, double-blind trial from the U.S. assessed primary treatment with IVIG (2 g/kg) and aspirin with or without a single dose methylprednisolone (30 mg/kg) in 199 unselected children. Addition of a single steroid dose to conventional therapy did not improve coronary artery outcomes (103).

A more recent prospective randomized, open-label, trial in Japan enrolled only those assessed by a locally derived risk score as being at particularly high risk of coronary damage (104). Patients were randomized to a prolonged course of intravenous followed by oral prednisolone (or placebo), in addition to standard therapy with IVIG (2 g/kg) and aspirin. Coronary artery outcomes during the 4-week study period were significantly better in the corticosteroid group. However, the generalizability of these findings is uncertain; in particular, the scoring system on which selective recruitment was based does not perform well in non-Japanese patients (105, 106). As approximately three-quarters of KD patients were excluded, as they did not meet enrollment criteria [including those with coronary artery dilatation at presentation

(104)], it remains unclear whether this corticosteroid regimen would benefit KD patients more broadly. Moreover, the prolonged intravenous course of corticosteroids would itself incur significant additional costs by prolonged admission and potential side effects (105).

GUILLAIN–BARRÉ SYNDROME

In the late 1990s, the indication GBS was taken on board the coreSPC as a new "established indication" after a prior authorization of a product specific indication within a variation procedure. The variation procedure showed that the data were mainly based on three published studies which each used different Ig products but revealed similarly efficacious outcomes with regard to decrease in disability grading when compared to plasma-exchange (PE) – the standard therapy at the time (107–109). In the studies by van der Meché (107) and the GBS Trial Group (109), the dosing was 0.4 g/kg × 5 day and in the study by Bril et al. (108) 0.5 g/kg × 4 day. The dosing taken into the coreSPC was 0.4 g/kg × 5 day.

Current IVIG coreSPC indication and dosing:

Guillain–Barré syndrome.

0.4 g/kg/day over 5 days.

No current SCIG coreSPC indication or dosing under discussion.

Recent developments in GBS research

Guillain–Barré syndrome is characterized by several subtypes (110). The most common form, acute inflammatory demyelinating polyneuropathy (AIDP), is characterized by segmental demyelination in peripheral nerves with acute flaccid paralysis. An axonal variant without demyelination either in the form of acute motor axonal neuropathy (AMAN) or acute motor and sensory axonal neuropathy (AMSAN) have been distinguished from AIDP. The Miller Fisher syndrome (MFS) variant is defined by the clinical triad of ophthalmoplegia, areflexia, and ataxia. High titers of anti-ganglioside IgG auto-antibodies have been described in GBS: anti-QD1a/Anti-GM1 IgG in AMAN, and Anti-QD1b IgG in MFS. Antecedent infections (*Campylobacter jejuni*, *Mycoplasma pneumoniae*, or EBV) support the hypothesis of a "carbohydrate mimicry" driven immunopathogenesis.

N-glycosylation of the Fc-portion of serum IgG was investigated in patients with GBS before and after treatment with IVIG in relation to clinical course and outcome (111). Treatment-naive GBS patients compared with age- and sex-matched controls had lower levels of galactosylation of IgG1 and IgG2. IVIG preparations contained relatively high levels of galactosylated and sialylated IgG Fc glycoforms compared with serum IgG in patients. Treatment with IVIG resulted in an increase in serum of the Fc-galactosylation and -sialylation of both IgG1 and IgG2. Multiple logistic regression analysis showed that patients with persistent low-IgG galactosylation and sialylation despite IVIG treatment had the most severe forms of GBS and needed ventilator support more often.

Guillain–Barré syndrome normally runs a monophasic disease course and immunomodulatory treatment is only needed during the acute phase of the disease. PE and IVIG are both proven to be equally effective in GBS, while corticosteroids do not confer any benefit (112–114).

The empirical dose of IVIG is 0.4 g/kg/day for 5 days, which is based on practice in other autoimmune diseases. In a small, randomized trial including 39 GBS patients, treatment with 0.4 g/kg/day for 3 or for 6 days was compared. In patients receiving six treatments, there was a non-significant trend toward a better outcome. This finding became significant in ventilated patients (115). In another randomized, open trial with 51 children with GBS 1.0 g/kg/day IVIG for 2 days was compared with 0.4 g/kg/day for 5 days, giving the same total dose to each (116). There were no significant differences in the primary or secondary outcome measures except that early relapses were significantly more common after the 2-day (5/23) than the 5-day regimen (0/23; $P = 0.049$) In one study, including 50 GBS patients, the total dose of 2 g/kg was given over 4 days and compared with plasma exchange. Both treatments were equally effective; IVIG had less adverse events (108).

Interestingly, the increase in serum IgG (ΔIgG) 2 weeks after IVIG treatment varied considerably (mean 7.8 g/l SD 5.6 g/l). Patients with low-ΔIgG recovered significantly more slowly and fewer could walk unaided at 6 months (log-rank $P < 0.001$) (117).

In the latest Cochrane Review of seven trials with a variable bias risk, IVIG was compared with PE in 623 severely affected participants. In five trials with 536 participants for whom the outcome was available, the mean difference of change in a seven-grade disability scale after 4 weeks was not significantly different between the two treatments (118). The authors concluded that IVIG when started within 2 weeks of disease onset hastens neurological recovery as much as PE. Adverse events were not significantly different with either treatment but IVIG was significantly more likely to be completed than PE. IVIG following PE did not provide significant additional benefit. GBS patients receiving a combination of IVIG and glucocorticosteroids did not recover faster than patients receiving IVIG alone (119) and adding mycophenolate mofetil to a combined treatment with IVIG and corticosteroids did not improve outcomes (120). In a small pilot study, adding IFN-β1a to IVIG treatment did not contribute to a better outcome (121).

Various studies have found that IVIG may be superior to PE, especially in GBS patients with a preceding *C. jejuni* infection and GM1 or GM1b auto-antibodies. However, none of these correlations is strong enough to guide therapeutic decisions (122). Clearly, more dose finding and biomarker research is warranted in all forms of GBS including pediatric GBS.

CHRONIC INFLAMMATORY DEMYELINATING POLYRADICULONEUROPATHY

Eight randomized controlled trials (RCT) including 332 CIDP patients using different IVIG brands and comparing the effects to either placebo, prednisone or PE showed that IVIG improves disability short-term, in one large trial the benefit of IVIG persisted for at least 24 weeks (123). Currently, five IVIG products are licensed for CIDP either purely nationally or via the MR-procedure in certain EU states or, in one case, centrally in the entire EU.

Product specific indication and dosing:

Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP)

For those IVIG products which have the indication CIDP, the dosing generally consists of a loading dose at 2 g/kg (given over 2–5 days) followed by maintenance doses of 1 g/kg (given over 1–2 days), cautioning physicians that the duration of treatment beyond 24 weeks should be subject to their discretion based upon the patient's response and maintenance response in the long-term and further that the dosing and intervals may have to be adapted according to the individual course of the disease.

No current IVIG/SCIG coreSPC indication or dosing available.

Recent developments in CIDP research

Chronic inflammatory demyelinating polyneuropathy is an immune-mediated peripheral nerve disorder characterized by motor and/or sensory symptoms and signs in more than one limb, developing over at least 2 months. The disease runs a progressive, relapsing-remitting, or monophasic course and can lead to significant disability due to walking difficulties and loss of arm dexterity. A diagnosis relies heavily on electrophysiological studies that typically show evidence of conduction block and demyelination. Apart from the typical clinical picture, the EFNS/PNS CIDP treatment guideline has defined several atypical CIDP phenotypes of which the pure sensory form is most frequently occurring. The atypical forms of CIDP may exhibit a different natural course and treatment response (124).

The key mechanisms in the pathogenesis have not been identified although several studies have highlighted the role of T-cells in CIDP and an important role for auto-reactive T-cell responses against peripheral myelin antigens such as P0, P1, P2, and peripheral myelin protein (PMP)-22 has been suggested (125–130).

The short- and midterm efficacy of corticosteroids, IVIG, and PE has been demonstrated in CIDP in several RCTs and meta-analyses (131–138). The most recent Cochrane systemic review (123) analyzed 8 RCTs with a total of 332 eligible patients. The authors concluded that IVIG improves disability for at least 2–6 weeks compared with placebo, with a number needed to treat (NNT) of 3. During this period, it has similar efficacy to PE, oral prednisolone, and intravenous methylprednisolone. In one large trial, the benefit of IVIG persisted for 24 and possibly 48 weeks. Further research is needed to compare the long-term benefits as well as side effects of IVIG with other treatments (123).

In a recent PK study in 25 CIDP patients with active but stable disease serum IgG levels before and shortly after serial IVIG infusions were remarkably constant over time. The change in IgG levels was associated with IVIG dosage, but not with treatment frequency, and both inter- and intra-patient variability was low. This indicates that these patients have reached a steady state with a constant distribution rate and turnover of IgG without accumulation over time. Constant serum IgG levels seem to be required to stabilize CIDP patients (139). A study in two CIDP patients showed that weekly dosing with IVIG resulted in higher serum IgG trough levels, which correlated with improved clinical response (140). In a retrospective cohort study, 15 CIDP patients underwent successful gradual dose reductions. Most patient started on an initial dose of 2 g/kg/course and could reduce that dose by mean 63% at an average dose interval of 7 weeks (range of dose reduction: 42.4–88%; range of treatment frequency: 2–17 weeks). There was high variability between patients in observed IgG levels (141): the lowest effective dose of IVIG per course ranged between 18 and 108 g; it did not correlate to weight, frequency of administration, disease duration, or pre-therapeutic degree of disability. These results suggest considerably lower, standardized, initiating, and maintenance doses might be effective and highlight the need for prospective dose comparative trials (142).

Post-infusion rise in IgG levels (ΔIgG) were correlated in individual patients ($P = 0.005$), but inter-patient variability was high (142). No correlations were ascertained between IgG level variation and weight, BMI, functional improvement, total dose of IVIG administered, or dose of IVIG administered per kilogram per week. Required frequency of IVIG infusions may, however, relate to patient-specific post-infusion rise of ΔIgG levels hence possibly explaining inter-patient differences in treatment frequency needs. IgG level monitoring may be helpful in establishing optimum treatment regimens in individual cases (142).

The highly variable individual IgG doses and treatment intervals were also observed and analyzed by Broyeles et al. (143). The authors suggested that physicians might be adjusting IgG dosing in CIDP according to each patient's clinical condition and treatment response. However, whether these adjustments will optimize clinical outcome while limiting overall costs has still to be seen. Not surprising that a Canadian cost-utility study using a Markov model failed to perceive IVIG as cost-effective treatment for CIDP compared to corticosteroid treatment (144). A criticism of this study may be that it was too short to capture all long-term problems encountered with corticosteroids. On the other hand, IVIG may be a short-term cost minimizing therapy compared to PE and in long-term maintenance therapy SCIG has been proven to be feasible, safe, effective, and cost reducing (145, 146). However, it should be stated that in Europe no application for a centralized authorization of a SCIG product in CIDP has as yet been submitted, while several IVIG products are authorized either nationally or via the MR or DC-procedures in numerous European countries supporting a class effect of current IVIG brands for the treatment of CIDP.

Nobile-Orazio et al. (132) compared in a RCT efficacy and tolerability of 6 month IVIG vs. IV methylprednisolone (0.5 g/day on four consecutive days given monthly for 6 months). Treatment of CIDP with IVIG for 6 months was less frequently discontinued because of inefficacy, adverse events, or intolerance than was treatment with IV methylprednisolone. The longer-term effects of these treatments on the course of CIDP need to be addressed in future studies, notably as in some patients improvement after corticosteroids seems to be more long-lasting than after IVIG (124).

Recent RCTs with rituximab (147), intramuscular interferon β-1a (148), and methotrexate (149) failed to show a beneficial effect as add-on therapy. In a review by Cocito et al. (150), analyzing 110 patients with refractory CIDP, various immunomodulatory drugs yielded similarly disappointing results. In contrast, small

open-label studies investigating mycophenolate mofetil (151) and alemtuzumab (152) showed promising results on the possibility to stop or reduce maintenance IVIG therapy.

Better understanding of the pathogenesis is needed to identify new treatment strategies and to develop biomarkers that correlate with disease activity and could help guiding maintenance treatment in these patients.

MULTIFOCAL MOTOR NEUROPATHY

Since the mid '80s MMN was identified as a treatable immune-mediated disease that responded to cyclophosphamide and IVIG (153, 154). A Cochrane Review (155) identified a total of 4 RCTs (including 34 patients) concerning the efficacy and safety of different IVIG brands in MMN. These showed that IVIG had a beneficial effect on strength and a non-significant trend toward improvement of disability. Two further open-label, non-controlled studies confirmed these results with 1 IVIG product in 20 MMN patients.

Currently, one IVIG product is licensed centrally in the entire EU; however, some other IVIG products are licensed nationally for MMN, supporting a class effect of current IVIG brands. Patients with stable clinical course on IVIG conditions can be safely switched to SCIG at the same monthly dose without risking deterioration but with an improvement of quality of life (156, 157).

Product specific indication and dosing:

Multifocal Motor Neuropathy (MMN)

In general the starting dose is 2 g/kg for 2–5 days and the maintenance dose is 1 g/kg every 2–4 weeks or 2 g/kg every 4–8 weeks. IVIG may be switched to SCIG at the same monthly dose.

No current IVIG/SCIG coreSPC indication or dosing available.

Recent developments in MMN research

Multifocal motor neuropathy is a rare focal inflammatory neuropathy characterized by slowly progressive, asymmetric distal limb weakness without sensory loss. The hallmark of electrophysiological examination is a conduction block in the absence of abnormalities in sensory nerves. Differentiation from amyotrophic lateral sclerosis (ALS) and CIDP with asymmetric onset is important as these diseases differ in prognosis and treatment (153, 158). The underlying immuno-pathological mechanisms are unknown but IgM auto-antibodies against ganglioside GM1 and galactocerebroside GalC are thought to play a role (159).

First treatment option in MMN is IVIG. It improves muscle strength by 78% and to a lesser extent the disability (39%) in most patients (153). Corticosteroids, immunosuppressants, or PE are not effective therapies for MMN, actually these treatments can even worsen the paresis (158, 160, 161). Optimal dose and intervals in maintenance treatment have not been established. Evaluating serum IgG levels in MMN patients receiving a cumulative dose of 2.0 g/kg over 5 days, a wide variation was found in total IgG and ΔIgG levels between patients. Comparing IVIG responders with non-responders, the ΔIgG levels were higher in the IVIG responders at each time point (1, 5 days, and 3 weeks after treatment) with the largest difference on day 1 after IVIG (162). In several small studies (156, 157, 160), IVIG was switched to SCIG as maintenance therapy at the same monthly dose with beneficial results. An interesting dose-reduction protocol was described by Eftimov et al. (163) when switching from IVIG to SCIG in 10 stable-phase MMN patients: 5 received 100% of the IVIG maintenance dose, the other 5 were put on 50%. All patients in the lower dose group deteriorated.

In a small randomized trial, mycophenolate mofetil has been investigated as add-on therapy but did not show any additional effect over IVIG with placebo (164). Rituximab did not reduce the need for IVIG in another small open-label study in six MMN patients (165). Cyclophosphamide especially in combination with autologous stem cell transplantation has been used in clinical practice and is recommended in some guidelines for treatment of refractory patients (160).

MYASTHENIA GRAVIS (MG)

Myasthenia gravis is an autoimmune disease with auto-antibodies interfering with neuromuscular transmission. Auto-antibodies are directed to signaling proteins at the neuromuscular junction, in particular, the nicotinic acetylcholine receptor (AChR). At least three mechanisms have been proposed to explain how anti-AChR antibodies compromise neuromuscular transmission: (i) complement binding and activation at the neuromuscular junction; (ii) accelerated degradation of AChR molecules (antigenic modulation); and (iii) functional block of AChR-binding sites (166). As in other autoimmune neuropathies IVIG has been tried as therapy besides corticosteroid, immunosuppressants, and PE. The results for IVIG are less convincing than in MMN, CIDP, and GBS and currently, there is not sufficient solid data to include MG in the core SPC's established indications.

Two small trials published in 1984 demonstrated that IVIG treatment was effective in MG patients at doses of 20 g given 6× over 2 weeks or 1–2 g/kg over 5 days (167). A study in 2005 compared 1 g/kg with 2 g/kg in MG, and found no significant difference between the two doses for the primary and secondary endpoints (168).

In the latest Cochrane systematic review, the authors analyzed all available RCTs ($n = 7$) differing in inclusion criteria and comparator treatment (169). The conclusion of this review is that there is no evidence from RCTs or from other trials to determine whether IVIG improves function or reduces the need for steroids.

FETAL NEONATAL ALLOIMMUNE THROMBOCYTOPENIA

Fetomaternal or neonatal alloimmune thrombocytopenia is the most common cause of severe thrombocytopenia in an otherwise healthy fetus or neonate. Affected babies are at risk of bleeding, and ~10–20% (170, 171) may develop intra- and/or extra-uterine intracranial hemorrhage. In 1988, Bussel and colleagues (172) described the successful use of a weekly dose of 1 g/kg maternal weight starting when thrombocytopenia developed in the fetus until delivery, in seven pregnancies at risk. Meanwhile, several observational studies have reported on further successful cases in such affected women (173–176). Based on the results of the available data, there is no doubt that IVIG (starting between 12 and 20 weeks of gestation) is currently the standard therapy (171, 173, 174, 177). This indication is primarily based on the successful

prevention of intracranial hemorrhage rather than on increasing the fetal platelet count. The question whether corticosteroids may have an additional positive effect in women treated with IVIG remains open (178).

From a regulatory perspective FNAIT would warrant further discussion.

Recent developments

Currently, different recombinant monoclonal anti-HPA-1a antibodies, which would be applied in the management of FNAIT, are under development in murine and translational models (179–182). In addition, efforts are now being made to establish a screening program that would help in identifying pregnancies at risk, and justify the prevention of immunization by vaccination or neutralization of the antibodies (180, 183).

FETAL HEMOLYTIC DISEASE

Immune antibodies to red blood cell (RBC) antigens can cause significant fetal anemia that may occur early in gestation, when fetal transfusion is difficult to perform. Based on the success observed in the treatment of FNAIT, IVIG has been used in cases with severe hemolysis that cannot be compensated by transfusion due to technical difficulties and/or highly aggressive antibodies. Although, the results are somewhat conflicting, some benefit was observed in most cases (184) using a weekly dose of 1 g/kg maternal weight, commencing from the first trimester. In a recent Cochrane study, the author's conclusion was as follows: no information is available from randomized trials to indicate whether the antenatal use of IVIG is effective in the management of fetal RBC alloimmunization, although several case studies suggest that a beneficial role in delaying the onset of fetal anemia requiring invasive intrauterine transfusion (185).

DERMATOLOGICAL DISEASES

Recommendations from the most recent European dermatology guideline for IVIG use list levels of evidence and grade of recommendations. These recommendations are briefly reiterated here. IVIGs were deemed to be efficacious in severe forms of dermatomyositis, polymyositis, and inclusion body myositis (184, 185) but are usually recommended as second-line therapy. The most convincing results have been reported in juvenile and adult myositis patients with acute, potentially life-threatening complications such as dysphagia, severe weakness, ulcerative skin leasions, and calcinosis cutis (188, 189). The authors further recommended IVIG as a second-line treatment in autoimmune blistering diseases, which are relapsing or refractory to standard therapy (190). In addition, for vasculitic syndromes with a particularly fulminant progressive disease form with multiple complications and severe side effects, IVIG therapy may be considered as a first-line treatment option. Less clear evidence exists for the use of IVIG in systemic lupus erythematosus. In numerous studies, the early administration of IVIG in toxic epidermal necrolysis (TEN) was suggested to be potentially life-saving (evidence level IV, recommendation grade C). The early administration of high-dose immunoglobulin should be considered in confirmed cases of TEN in the absence of any therapeutic alternative (187, 191).

For the various disorders, the authors recommended a dose of 2 g/kg body weight (3 g/kg in TEN) applied over a period of 2–5 days. For chronic dermatological diseases treatment, intervals should be every 4 weeks and after 6 months gradually increased to 6-week intervals.

DISCUSSION AND CONCLUSION

Over the last 40 years, the demand of therapeutic Ig has been steadily increasing worldwide. This has several reasons (i) the number of indications kept extending from PID and SID into hematology, neurology, rheumatology, intensive care, and dermatology; by now international guidelines list 10–12 high-priority indications, 15–18 medium priority, and over 20 low-priority indications. (ii) IVIG is conceived as a safe, well-tolerated, and well-accepted medicine among patients and doctors explaining why it has been tried in so many different conditions and helped surprisingly quite often. (iii) The introduction of a variety of new preparations (5 and 10% IVIG and 16 and 20% SCIG, hyaluronidase facilitated SCIG and hyperimmune IVIG) and different routes of application (IV, SC with pump or rapid push, home treatments) has made the practical therapy more flexible and easier (8).

It is the purpose of this survey to review critically and to update indications and dosing strategies of IVIG and SCIG therapies in view of increasing demand, pharmacoeconomic aspects, and emerging alternatives for the immunomodulatory indications. Moreover, we wanted to recall the regulatory recommendations laid down in the core SPC of the European Medicine Agency and complement this information with current research and development data in the field of high-priority IVIG/SCIG replacement or immunomodulatory therapies.

Dosing of IVIG/SCIG in PAD replacement therapy no longer relies on fixed monthly doses but rather on a treat-to-target strategy the goal being a maximal reduction and control of bacterial infectious episodes and avoidance of side effects. Thus, monthly dosage, route of administration (IV or SC), infusion intervals and serum Ig levels are secondary to this goal but have to be optimized for the individual patient in order to reach the best possible result (24). The flexibility to comply with patients' needs has been greatly improved. Not only can the same total weekly SCIG dose be administered at different intervals, from daily to biweekly (to monthly for the facilitated SCIG), with minimal impact on serum IgG levels (21, 22), SCIG and IVIG may also be applied in an alternating mode to increase convenience and quality of life (23). Several SCIG loading regimens rapidly achieve adequate serum IgG levels in treatment-naïve patients (22). There is reasonable evidence to calculate the loading dose on ideal body-weight (2, 3) and to increase monthly dosages by 0.15 g/kg until break-through infections are sufficiently controlled (17). Infusion-related reactions can be avoided or minimized in most cases by switching from IVIG to SCIG. While the FDA recommends the use of a DAC when switching from IVIG to SCIG (6, 7) the European experience reports satisfactory results by just maintaining the monthly IVIG dose and dividing it in weekly, biweekly, or even smaller doses (192). It is now generally accepted that Ig replacement therapy does more than just replace the antibody repertoire in PAD patients (30). It also serves as a biological response modifier with anti-inflammatory properties (31, 32) that contribute to the clinical benefit of IVIG/SCIG therapy in immunodeficient patients. The

bottom line of the current knowledge on Ig replacement therapy is optimizing the results by individualizing the treatment regimens; recently, this principle has been beautifully illustrated by case studies from Bonagura (20).

Regarding the major immunomodulatory indications for IVIG, it is striking how little evidence and structure has so far been brought into the dosing issue. Most studies still start with the 2 g/kg protocol (preferably given on 1 single day, instead of being spread over 5 days), as originally described in the ITP (71, 72) and KD (87, 88) trials. Although a down-scaling regime in ITP showed that 1 g/kg was as efficient as 2 g/kg (72), it was the latter dose that set the stage for the regulatory adoption of the dosage regimen in ITP and other "immunomodulatory settings" for years to come. There are only a few case reports dictated by economic constraints suggesting that lower doses (0.1–0.4 g/kg) may also exhibit effective immunomodulatory activity (77, 78) but no systematic dose exploration has been performed. Especially no up-scaling dosage regimen has been pursued as in Ig replacement therapy for PAD patients (17). Down-scaling attempts (141, 142) and a trend to switch from IVIG to SCIG have been made in chronic diseases such CIDP (145, 146) and MMN (156, 157, 160, 163). In the acute phases of GBS or in myasthenic crisis comparative studies between PE and IVIG showed equal effectiveness but advantages for IVIG with respect to feasibility and costs. Despite much effort that has been put into the analysis of mechanisms governing responsiveness and non-responsiveness to high-dose IVIG in ITP, KD, and the neuroimmunological indications no clear-cut picture has emerged so far (186). Some biomarkers, however, may be helpful to identify responders from non-responders and thereby try early on alternative treatment options (94–96, 111). Driven by the need to be cost-effective the pursuit of alternative treatment options is another strong trend in many studies on immunomodulatory IVIG indications. Thus, the renaissance of corticosteroids in the long-term treatment of KD (97, 103–105) and CIDP (132, 135) are examples. A recent overview of immunological treatment options in neuroimmunological emergencies by von Geldern et al. (193) nicely illustrates that IVIG is only one among several treatment choices, although a very important and effective one.

Clearly, more research is needed to clarify the mode of action of IVIG in immunomodulation and to optimize dosing and treatment intervals.

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Persistent immune activation in CVID and the role of IVIg in its suppression

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Common variable immunodeficiency (CVID) is one of the most common and clinically important primary immune deficiencies. CVID patients have poor humoral immunity, resulting in recurrent infections of the gastrointestinal and upper respiratory tracts, as well as increased incidence of some forms of cancers and autoimmune diseases. The treatment for CVID is IgG replacement, often given as intravenous immunoglobulins (IVIg). IVIg consists of monomeric IgG purified from pooled plasma from healthy donors and is used to treat an increasing number of conditions including autoimmune diseases. In the case of CVID, IVIg has mainly been seen as reconstitution therapy, providing patients with pathogen-specific antibodies. Recent evidence shows that IVIg has diverse effects on the immune system of CVID patients, and one important component is that IVIg alleviates the state of chronic immune activation. In this review, we will discuss causes and consequences of persistent immune activation in CVID, possible underlying mechanisms for how IVIg treatment reduces immune activation, and implications for our understanding of primary as well as acquired immune deficiencies.

Keywords: CVID, IVIg, iNKT cells, CD8 T cells, CD4 T cells, dendritic cells, immune activation, IgG replacement therapy

INTRODUCTION

Diagnosis of common variable immunodeficiency (CVID) is based on low levels of IgG and IgA and lack of specific IgG response following vaccination (1). Several genetic mutations associated with CVID have been identified but for a significant proportion of patients the exact cause is unknown (2). CVID patients, thus, are a heterogeneous group characterized by poor humoral immunity, resulting in recurrent infection of the gastrointestinal and upper respiratory tracts. CVID is also associated with non-infection related complications including cancers, mainly non-Hodgkin's lymphoma, and autoimmune diseases such as idiopathic thrombocytopenia purpura and autoimmune hemolytic anemia, contributing to a significantly shorter life expectancy (3, 4). It has become clear that defects in the immune system in CVID go beyond humoral immunity with significant changes and persistent activation of the cellular immune system, involving dendritic cells (DCs), CD8 T cells, CD4 T cells, invariant natural killer T (iNKT) cells, and regulatory T cells (Tregs). The treatment for CVID is IgG replacement, often given as intravenous immunoglobulins (IVIg). IVIg consists of monomeric IgG purified from pooled plasma from healthy donors and it is used to treat an increasing number of conditions (5), including autoimmune diseases. The mechanisms of action of IVIg in treatment of autoimmune diseases are numerous and controversial (6, 7). In the case of CVID, it has mainly been seen as a reconstitution therapy, providing patients with pathogen-specific antibodies, but evidence now shows that IVIg has a variety of effects on the immune system of CVID patients (8) and can alleviate the state of chronic immune activation. In this review, we will discuss the causes and consequences of innate and adaptive immune activation, and how IVIg treatment reduces immune activation in CVID focusing on data obtained *ex vivo*. We will also discuss the similarities between primary and secondary immune deficiencies and the possible implications for our understanding of those diseases.

INNATE IMMUNE ACTIVATION

Common variable immunodeficiency patients present defects in several arms of the innate immune system. Natural killer (NK) cells express a wide repertoire of activating and inhibitory receptors and are part of the innate defenses against viral infections and tumors (9). NK cells were first recognized for their cytotoxic capacity but they can also produce cytokines and have regulatory properties (10). The frequency of NK cells has been reported to be lower in CVID (11). Detailed studies of NK cells in CVID are lacking, and the effects IVIg treatment may have on NK cell frequency, phenotype, and functions are unknown. Because of their antitumor function, decline of NK cells could contribute to the increased risk of cancer in CVID patients.

Polymorphonuclear neutrophils (PMNs) are an important component of the innate immune system. In response to pathogens, PMN can rapidly migrate to the site of inflammation and have microbicidal activity by the release of proteolytic enzymes and antimicrobial peptides as well as production of reactive oxygen species (ROS) (12). In CVID, PMNs have been reported to express lower levels of CD11b, CD16b, and CD15, suggesting a maturation defect (13). The same study also reported impaired phagocytosis of *E. coli* and reduced ROS production after TLR stimulation by PMN from CVID patients. The patients in this study were all under IVIg treatment, and the effects of IVIg on PMN phenotype and function therefore remain undetermined.

However, *in vitro* experiments performed on whole blood from healthy individuals suggest that low doses of IVIg, as used for treatment of CVID, can induce CD11b expression and increase the ROS response (14).

Monocytes are myeloid-derived cells with phagocytosis and antigen presentation capacities. They can rapidly differentiate into tissue-resident macrophages and DCs after leaving the blood stream. Monocytes play an important role in various inflammatory conditions (15). In CVID, the frequency of pro-inflammatory $CD14^{bright}$ $CD16^{+}$ monocytes is elevated and these cells express higher levels of HLA-DR indicating a higher activation level (16). Another study showed that IVIg temporarily reduced the frequency of pro-inflammatory monocytes 4 h after injection and that the levels returned to baseline after 20 h (17). Furthermore, IVIg may reduce TNF production by monocytes from CVID patients, possibly by triggering of the inhibitory receptor FcγRIIb (17). Monocytes from CVID patients were also found to have increased production of ROS, and this was inversely correlated with CD4 counts (18).

Dendritic cells are professional antigen presenting cells (APCs) specialized in capturing, processing, and presenting antigens to initiate immune responses to pathogens. After TLR activation, DCs will mature and increase the expression of co-stimulatory molecules to provide the second signal needed to activate T cells. Bayry et al. showed that *in vitro* differentiation of monocytes from CVID patients into DCs (19) is defective, and that normal differentiation could be restored by natural antibodies against CD40 present in IVIg (20). However, the *in vivo* relevance of this mechanism remains to be investigated as *ex vivo* DCs from CVID patients present a different phenotype. CVID patients have reduced frequencies of plasmacytoid and myeloid DCs (21), and the residual myeloid DCs have increased expression of co-stimulatory molecules CD80 and CD83 (22). The frequency of myeloid DCs is partially restored following initiation of IVIg treatment and the expression of CD80 is significantly decreased. Moreover, myeloid DCs in treatment-naïve CVID patients display an abnormal profile of group I CD1 molecules characterized by an elevated representation of the $CD1c^{+}$ subset. In addition, the $CD1c^{+}$ and the $CD1c^{-}$ subsets of DCs have higher CD1a and CD1b expression in these patients (23), whereas CD1d is expressed at similar levels between CVID patients and controls, being present on the majority of the cells. Following the increase in IgG level after initiation of replacement therapy, the CD1c subset frequency is normalized together with the expression levels of CD1a and CD1b, while CD1d expression is unaffected. These findings suggest that IgG can regulate the expression of group I CD1 molecules *in vivo*. Earlier studies *in vitro* indicated that this effect is mediated by binding to the FcγRIIb (24). It remains to be investigated if the increased expression of CD1a in treatment-naïve CVID patients can lead to aberrant activation of the CD1a restricted T cells that are present in the normal repertoire (25).

Because they can rapidly be activated and produce cytokines without previous encounter of their antigen, iNKT cells are sometimes considered to be part of the innate immune system (26). They recognize endogenous and bacterial-derived glycolipids presented by CD1d molecules. It is believed that iNKT cells are important for the control of both bacterial and viral infections and they are also believed to be involved in immune surveillance against cancer and to have the capacity to regulate auto-immunity (26). iNKT cells are numerically reduced in CVID (27) and present elevated expression of HLA-DR, CD161, and PD-1 (22), signs of activation and exhaustion. In addition, the distribution of iNKT cell subsets defined by CD4 and CD8 is skewed in CVID, with an increase in the $CD4^{+}$ and a decrease in the $CD8^{+}$ subset reported in one cohort (28). The function of iNKT seems to be relatively preserved in treatment-naïve CVID patients, as only a trend for reduced IFNγ production was seen after stimulation with the model antigen α-Gal–Cer (29). Increased IFNγ production by iNKT cells was reported for a small number of CVID patients on going IVIg after *in vitro* expansion (30). The frequency of iNKT cells does not improve by reconstitution therapy, and HLA-DR remains elevated. However, expression of CD161 and PD-1 is reduced when CVID patients are under IVIg treatment (22), indicating that IVIg can alleviate iNKT cell activation and exhaustion in patients. Because of the important role of iNKT cells in tumor surveillance and immune regulation, it is possible that the loss of these cells is contributing to the increased risks of cancer and auto-immunity in CVID patients.

ADAPTIVE IMMUNE ACTIVATION

Treatment-naïve CVID patients present low-CD4 T cell counts, in some cases down to numbers that would be considered AIDS-defining in HIV-1 infected patients. Following IVIg initiation, CD4 counts increase in the majority of CVID patients and can reach normal levels in some cases (22, 31). It is noteworthy that a similar effect has been reported in HIV-1 patients treated with IVIg (32, 33). The mechanisms by which IVIg can normalize CD4 counts remain elusive, but Dolcino et al. reported that lower expression of LEPR, a gene important for CD4 proliferation, was normalized after IVIg treatment in CVID patients (31). CD8 T cell counts in CVID patients are in general not different from healthy controls but some patients have an expansion of this population. Therefore, the inverted CD4:CD8 ratio seen in CVID is mostly due to their low-CD4 count. CD4 T cells in CVID have elevated levels of the activation markers Ki67, CD38, and HLA-DR, as well as exhaustion markers PD-1 and CTLA-4. The expression levels of activation and exhaustion markers remained elevated for up to 1 year on IVIg treatment (22). Interestingly, another study found that IVIg treatment could reduce PD-1 expression on CD4 T cells and restore their response to bacteria (34).

Similar to the CD4 T cell compartment, CD8 T cells in treatment-naïve CVID have elevated expression of activation markers Ki67 and co-expression of CD38 and HLA-DR. IgG replacement therapy leads to reduced expression of Ki67, CD38, and HLA-DR on CD8 T cells (22), indicating that IgG replacement may help control infections or infection-associated factors that are implicated in chronic activation of the CD8 T cells. Expression of some activation markers on CD8 T cells and exhaustion markers on CD4 T cells correlate positively with age in IVIg-naïve CVID patients (22), suggesting that immune activation and exhaustion are developing progressively over time. Furthermore, the ratio of activated T cells to Tregs was found to be higher in CVID patients with auto-immunity compared to patients without auto-immunity (35). Therefore, early initiation of IgG replacement

therapy in CVID patients may be beneficial by preventing further increase in T cell activation. However, diagnosis of CVID is frequently delayed by 6–8 years after the onset of symptoms (1).

Regulatory T cells are key regulators of immune responses and they play a crucial role in limiting unwanted and persistent immune activation. Several studies demonstrated that Tregs are reduced in CVID patients (22, 36–38) and that residual Tregs appear to have reduced suppressive capacity (39). An increase in Tregs was reported 30 min after IVIg infusion in CVID patients (38). This increase seems to be only transient as no sustained effect on Treg frequency was observed between samples obtained at baseline and up to 1 year after initiation of IVIg treatment (22). The inability of IVIg to restore normal frequency and function of Tregs may contribute to the increased risk of auto-immunity in CVID patients (36).

WHAT IS DRIVING IMMUNE ACTIVATION IN CVID?

Signs of activation in both monocytes and DCs are associated with T cell activation (16, 22, 40), suggesting that persistent innate activation contribute to chronic activation of the adaptive immune system. We propose a model where recurrent and chronic infections at mucosal surfaces in treatment-naïve CVID patients result in sustained activation of monocytes and DCs, and that these cells in turn promote T cell activation (**Figure 1**). In this model, one possible mechanism by which IVIg reduces activation of T cells is by acting at the level of the APCs. *In vivo* and *in vitro* studies support the notion that IVIg reduces T cells activation indirectly by acting on APCs rather than on the T cells themselves (41–45). IVIg may act directly on APCs via Fc-receptors, or have indirect effects on APCs activation by reducing the infection burden. However, persistent activation of monocytes and DCs is seen in CVID patients even after IVIg therapy and the causes are largely unknown.

CMV infection has been associated with complications in CVID patients (46, 47) and thus CMV is a possible candidate as a cause for chronic immune activation in CVID. Resurgence of endogenous retroviruses (ERVs) in the absence of LPS-specific antibodies has been reported in a mouse model (48). IVIg preparations contain antibodies specific for both CMV (49) and LPS (50), and may therefore help reduce the pressure on the immune system by supporting immune control of CMV and by preventing activation of ERVs. Enteric virus infections were found in 25% of CVID and CVID-like patients compared to 9% in controls, and these infections were associated with increased levels of calprotectin, a marker of inflammation, and low levels of IgA (51). Therefore, enteric viruses may also contribute to increased immune activation in CVID patients. However, only few CVID patients present the CD8 T cell expansion typically associated with chronic viral infections, suggesting that other causes may be involved. Soluble CD14 (sCD14), a marker associated with monocyte activation and possibly microbial translocation, is elevated in CVID (16, 22, 52). LPS levels were found to be elevated in one cohort of treatment-naïve CVID patients and were reduced following replacement therapy (34). However, we and others were unable to detect elevated LPS levels in the circulation of CVID patients [our unpublished data and Ref. (16, 52)]. Therefore, more investigations are needed to clarify the role of microbial translocation in CVID patients. The loss of Tregs as well as iNKT cells with regulatory capacities may contribute to the persistent immune action seen in CVID. These two populations appear to not recover after initiation of IVIg

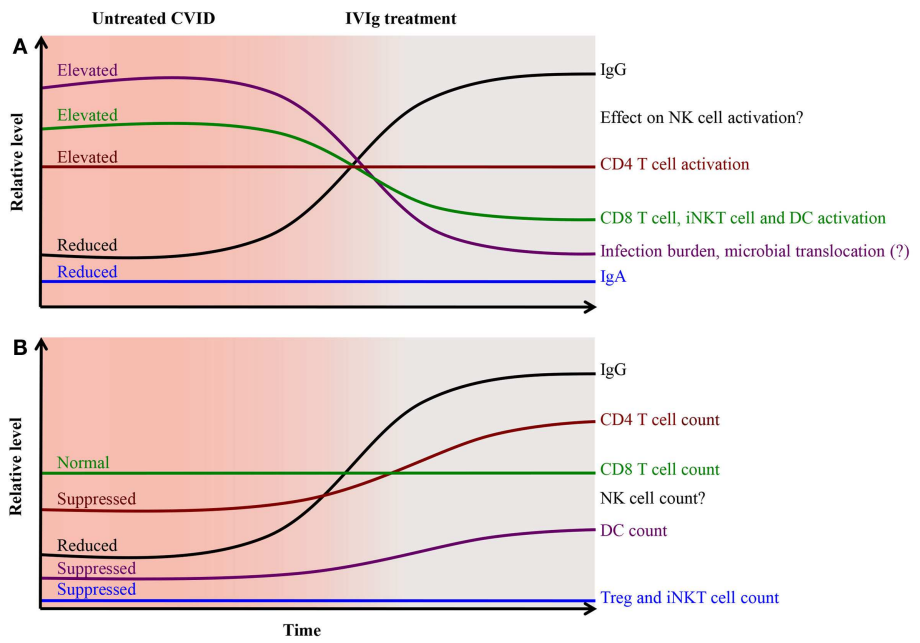

FIGURE 1 | Pathological changes and activation of cellular immunity in CVID is partially alleviated after immunoglobulin replacement therapy. IgG replacement therapy restores humoral immunity and provides better control of microbes and pathogens, reducing the infection burden on the immune system. This together with triggering of the FcR-mediated inhibitory effects on antigen presenting cells leads to reduced activation of CD8 T cells, iNKT cells, and DCs **(A)** and improvement in CD4 T cell counts and DC count **(B)**.

therapy (22), possibly providing an explanation as to why immune activation remains elevated.

SIMILARITIES BETWEEN PRIMARY AND SECONDARY IMMUNE DEFICIENCIES

Some of the immunological perturbations observed in treatment-naïve CVID patients are strikingly similar to those seen in untreated HIV-1 infection. HIV-1 infection leads to chronic immune activation characterized by increased T cell activation and exhaustion, elevated levels of sCD14 and a partial loss of CD4 T cells, DCs, iNKT cells, and Tregs. These features are also found in CVID patients. T cell activation is closely associated with HIV-1 disease progression (53) but it is unknown if similar associations exists in CVID. IL-6 is a predictor of all-cause mortality (54) and disease progression (55) in HIV-1 infection, and has been associated with opportunistic infections (56) and increased risk of cancer (57). Interestingly, several studies reported an increase in IL-6 in CVID (58); however, it has not been studied as a biomarker of disease progression or complications. CVID and HIV-1 also present a similar signature in gene expression in the intestinal epithelium with up-regulation of innate immune gene and down-regulation of lipid and carbohydrate metabolism genes and transport of micronutrients genes (59), suggesting that events at the mucosal barrier may be involved in the similarities between the two diseases. Moreover, sCD14 levels have been found to associate with immune activation in both CVID (52) and HIV-1 (60), suggesting that monocyte activation is common to both conditions. IL-2 administration in combination with antiretroviral treatment results in increased CD4 counts (61), and expansion of iNKT cells (62) and NK cells (63) in HIV-1 infected patients. IL-2 has also been studied as a complementary therapy in CVID, resulting in increased T cell responses to mitogens and soluble antigens in the absence of changes in CD4 count and NK cell frequency (64) while iNKT cell frequency was not evaluated. Because of the similarities between CVID and HIV-1 disease, it is not unexpected that IVIg treatment can have modest effects on T cell activation and CD4 count in HIV-1 infection (32, 33). Therefore, it is possible that IVIg could be beneficial as a complement therapy for patients that have residual immune activation despite successful viral control on ART.

CONCLUDING REMARKS

Intravenous immunoglobulin provides CVID patients with a partial replacement for their defective humoral immunity. However, CVID patients also present abnormalities in cellular immunity in a way similar to what is often seen in other conditions associated with chronic immune activation such as HIV-1 infection. Some of these changes are normalized by IVIg treatment, suggesting that IVIg may also be beneficial in other immune deficiencies characterized by persistent immune activation. IVIg seems to have short-lived effects on the monocyte and Treg populations, whereas the reduction of activation in DCs, iNKT cells, and T cells appear to be sustained. However, the vast majority of immunological studies of CVID have been performed on patients who are already on IVIg replacement, therefore, missing or underestimating some of the abnormalities in CVID that are corrected by IVIg. More studies of treatment-naïve CVID patients are needed to better understand what is driving immune activation in CVID and how IVIg is helping to improve cellular immunity. Furthermore, it will be important to investigate how we can restore the compartments that are not recovering after initiation of IVIg, such as Tregs and iNKT cells. Persistent loss of these cells may help explain why some CVID patients still suffer from severe inflammatory complications, such as interstitial lung disease and autoimmune enteropathy, even when on replacement therapy. Cellular therapy has been safely and successfully used to treat two CVID patients suffering from CMV intestinal disease by injection of autologous specific cytotoxic T cell lines expanded *ex vivo* (65). Thus, cellular therapy may represent a good complement to IVIg treatment to help restore a functional immune system in CVID patients.

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Manufacture of immunoglobulin products for patients with primary antibody deficiencies – the effect of processing conditions on product safety and efficacy

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Early preparations of immunoglobulin (Ig) manufactured from human plasma by ethanol (Cohn) fractionation were limited in their usefulness for substitution therapy in patients with primary antibody deficiencies (PAD), as Ig aggregates formed during manufacture resulted in severe systemic reactions in patients when given intravenously. Developments in manufacturing technology obviated this problem through the capacity to produce concentrated solutions of intact monomeric Ig, revolutionizing PAD treatment and improving patient life expectancy and quality of life. As the need for Ig has grown, manufacturers have refined further manufacturing technologies to improve yield from plasma and produce therapies, which are easier and less expensive to deliver. This has led to the substitution, partly or wholly, of ethanol precipitation by other techniques such as chromatography, and has also stimulated the production of highly concentrated solutions capable of rapid infusion. Ig products have been associated, since their inception, with certain adverse events, including infectious disease transmission, hemolysis, and thromboembolism. The introduction of standardized manufacturing processes and dedicated pathogen elimination steps has removed the risk of infectious disease, and the focus of attention has shifted to other problems, which appear to have increased over the past 5 years. These include hemolysis and thromboembolism, both the cause for substantial concern and the subject of recent regulatory scrutiny and actions. We review the development of manufacturing technology and the emerging evidence that changes for the optimization of yield and convenience has contributed to the recent incidents in certain adverse events. Industry measures under development will be discussed in terms of their potential to improve safety and optimize care for patients with PAD.

Keywords: immunoglobulins, safety, manufacturing technology, hemolysis, thrombosis

INTRODUCTION

DEVELOPMENT OF MANUFACTURING METHODS FOR THERAPEUTIC IMMUNOGLOBULIN PREPARATIONS

Immunoglobulins (Igs) may claim to be, historically, the first therapeutic plasma product, with Emil von Behring's work on diphtheria antitoxin and von Behring and Kitasato's demonstration (1) that serum of rabbits immunized with tetanus toxin contained activity against experimentally induced tetanus poison, which provided protection to non-immunized rabbits exposed to tetanus. For this work, von Behring was awarded the first Nobel Prize in Medicine or Physiology in 1901. Ehrlich (2) demonstrated that protection was correlated with the amount of antitoxin administered. Antibody preparations such as these, where protective antitoxin is generated through the immunization of animals, still have a role in the treatment of some conditions (3). Karelitz's work showing that protection against measles was localized to the globulin portion of serum (4) and Tiselius' characterization of serum proteins using electrophoresis (5) pointed to the role of gamma globulin in passive immunity. However, the manufacture of Ig solutions from a human source had to await Cohn's development of methods to separate plasma fractions on a large scale (6), using ethanol as a precipitating agent in a series of separations manipulating pH, ionic strength, and temperature. Initial clinical experience with the immune serum globulin (ISG) fraction from Cohn's scheme quickly led to limiting administration to the intramuscular and subcutaneous routes, as severe systemic reactions occurred in patients given this product intravenously.

Initially, ISG manufactured with Cohn's method was limited to prophylaxis of certain microbial diseases, including polymyelitis, measles, mumps, pertussis, and hepatitis A (7). These preparations became redundant with the development of vaccines for the respective diseases. In 1952, Bruton (8) infused a child with undetectable "gamma globulin" levels and who suffered from chronic infections. Subcutaneous injections of ISG produced measurable gamma globulin levels and completely eliminated pneumococcal

infections. Over the 1950s, human ISG became the standard treatment for patients with primary antibody deficiencies (PAD) who develop chronic bacterial infections (9). Preparations in which antibodies were enriched approximately 10- to 20-fold in 15–18% solutions were administered intramuscularly, a route, which caused problems. The intramuscular injection was painful, maximum serum levels were not reached before 24 h and could take several days, and *in vivo* recovery was usually less than 50% (10). At higher dosages, the preservative containing mercury caused increased concern (11). Intravenous administration would clearly obviate many of these problems but led to severe systemic reactions in 15–25% of patients. Patients with antibody deficiencies were particularly susceptible (12). The hypothesis that Ig aggregates in the preparations were leading to systemic complement activation (13) led manufacturers to explore ways of removing such aggregates as a way of preparing an intravenously administered product. These included digestion with enzymes such as pepsin and plasmin, leading to Ig fragments, which could bind antigens and were tolerated intravenously but which were lacking in effector functions and had very short intravascular lives. Further modifications involving chemical manipulation with β-Propiolactone, sulfonation, and alkylation resulted in intact molecules, with loss or modification of certain functions (14). By the end of the 1970s, various modifications of the original Cohn procedure resulted in a number of products containing >99% intact, monomeric Ig, well-tolerated intravenously, and able to be infused in high volumes and result in a prolonged presence of high Ig levels in the patient's blood. Coupled with the increasing range of indications for Ig in a number of autoimmune and inflammatory diseases, this ability to delivery higher dosages, and improve clinical outcomes, in immune deficient patients ushered in the current era of ever increasing usages of immune globulin therapies.

IMMUNOGLOBULIN THERAPIES – CURRENT PRODUCTS, CURRENT ISSUES

Bruton first treated agammaglobulinemia in 1952 with ISG administered subcutaneously (8), a route, which was supplanted first by intramuscular and, since the 1980s, intravenous, products. Over the past decade, subcutaneous products have been developed with an enhanced capacity to allow home care and avoid adverse events (15). More recently, most manufacturers have acquired approval to market products for both intravenous and subcutaneous routes with increased strengths, with Ig concentrations of up to 20% compared to the mainstream intravenous products of 4–5%, as well as faster infusion rates. Most of these developments have been spurred by economic considerations, aimed at minimizing time in hospitals (16), while allowing patients more freedom through home therapy. In addition, reports that increasing dosages result in a continuing improvement in clinical outcomes in PID (17) have possibly encouraged the development of more concentrated solutions. As Ig consumption has continued to increase, payer influence in therapeutic practice, particularly in USA, has become a matter of concern (18, 19). Increasingly, the techniques of mainstream pharmacoeconomics have been used to question the allocation of health care resources to Ig, although such analyses have been limited to indications other than PID so far (20, 21).

HISTORICALLY RECOGNIZED ADVERSE EFFECTS OF Ig THERAPIES

Ig therapies have been associated with a number of adverse side effects, which have been extensively reviewed in Ref. (22). The present work does not intend to reiterate these efforts, which have all drawn attention to the rarity of serious side effects. Rather, we intend to focus on effects, which, at one time or another, increased in frequency as a result of what were, retrospectively, recognized as ensuing from changes in plasma collection and manufacturing methods. We have chosen three such adverse events: pathogen transmission, thromboembolism, and hemolysis. Our aim is to demonstrate how the uniqueness of each manufacturer's process influences the product, and reinforces the concept that Ig therapies, as biological drugs, cannot be viewed as generic and interchangeable.

PATHOGEN TRANSMISSION ISSUES IN Ig THERAPIES

All plasma protein therapies had a history of transmitting pathogens before the current era characterized by robust pathogen elimination steps in the manufacture came into effect. Intramuscular Ig products transmitted hepatitis B on rare occasions (23) but were not associated with the transmission of other bloodborne viruses. The presence of antibodies to the respective viruses, which was the basis of the protective action of specific IG products, was assumed to account for the rarity of transmission by ISG. In addition, the sequential precipitation of fractions during the Cohn process was thought to partition virus away from the therapeutic Ig fraction (24). Initial experience with early intravenous preparations seemed to support this safety record.

Over the early 1980s, several IVIG preparations manufactured at pilot scale transmitted Non A-Non B hepatitis, subsequently shown to be hepatitis C (HCV). Changes in the finishing steps used to remove ethanol from the final product were initially ascribed as the cause of the different infectivity of these IV preparations compared to their IM counterparts (25), as the Cohn system common to both products was considered to have a high HCV clearing capacity (26). This was subsequently shown to be modest in the absence of a specific virus inactivating step (27). Such steps were introduced into the manufacture of IVIG from the early 1990s onward, but not in time to prevent two major outbreaks of HCV in recipients of intravenous Ig.

HCV FROM ANTI-D – "*THE ROAD TO HELL. . .*

In 1994, reports emerged of HCV transmission in Irish women, subsequently linked to the administration of anti-D Ig (28). Historically, anti-D Ig is an intramuscular preparation, which, up to the time of this incident, had a strong safety record. Investigations showed that the transmissions occurred during 1977 and 1978, following administration of an anti-D product manufactured on a small scale in the Irish blood service's facility in Dublin. The method used was a chromatographic technique developed in East Germany some years before (29), and, as in the case of the parent facility, was introduced in order to maximize the yield of anti-D from domestic plasma, and achieve self-sufficiency in this product in the absence of a domestic Cohn fractionation facility (30). Concurrently, HCV from the original product was also shown to transmit HCV (31). Genomic studies revealed concordance between the

HCV in the product and that in the infected patients, and the original contamination was traced to one infected donor contributing through plasmapheresis for therapeutic purposes (32).

This epidemic in Irish women affected some 400 recipients of a product, which has, in its classical form, had an unblemished safety record (33). A process executed by the Irish government as a result of this catastrophe led to profound reform in the blood service and its oversight (34). The Irish Blood Service, which was relatively unsupervised at the time of these events, chose, in its quest for self-sufficiency in anti-D, a manufacturing method, which was relatively new and untested through the long years of experience, which had resulted in high levels of confidence in Cohn fractionated product. Clearly, the chromatographic method had little capacity to eliminate the viral load resulting from one repeat plasmapheresis donor. Reliance on the safety history of anti-D ignored the effect of a total change in manufacture, in a modest facility with little adherence to the principles of good manufacturing practice, and where high-risk donors were accepted for contribution to the plasma pool. In the sad history of pathogen transmission through plasma products, it is difficult to find another accident of such obvious culpability. It also provides a salutary example of the difficulty in predicting the outcome of changes in established manufacturing techniques on the safety of products. It is not the only such example.

THE GAMMAGARD INCIDENT – . . .. .IS PAVED WITH GOOD INTENTIONS"

The Irish/German incident involved an intravenous product administered to healthy women. In 1994, reports emerged indicating an epidemic of HCV in recipients of polyclonal IVIG from a specific manufacturer. This product – Gammagard from Baxter Healthcare Corporation – had been licensed in USA since 1986 and had demonstrated a good safety record, including lack of evidence of transmission of Non A, Non B hepatitis (35). Despite this, an apparent epidemic of HCV occurred in recipients given Gammagard over 1993–94 (36). Gammagard – and all other plasma products – differed in one important feature in 1993 relative to 1986; in the intervening period, the characterization of HCV and the introduction of rapidly succeeding tests for anti-HCV antibody resulted in the rapid diminishment of anti-HCV from transfusion products and the raw material for plasma fractionation. While this immediately resulted in a substantial increase in the safety of transfusion products, a concurrent increase in the safety of pooled plasma products could not be assured. The safety of plasma products is primarily assured through pathogen elimination procedures, which enhance safety margins to a much higher extent than the donor selection and testing measures, which still underpin the safety of single or small pool transfusion therapies (37).

Scientific opinion at the time of the introduction of the anti-HCV test included those who speculated that the exclusion of anti-HCV antibodies from fractionation pools might decrease product safety (38). The US FDA did not require HCV antibody testing for source plasma for fractionation until experiments in their laboratories indicated that the removal of such antibodies did not affect safety from HCV (39). These experiments used plasma tested negative with the first-generation anti-HCV test, which does not exclude all antibody units, particularly units with HCV antibody specificities against HCV envelope proteins (40). Other works showed that the Cohn system did not clear HCV from the final therapeutic fraction (26). After investigating the Gammagard incident (41), the FDA scientists hypothesized that the exclusion of anti-HCV antibodies against HCV envelope proteins through the use of second and third generation anti-HCV tests had affected the portioning of HCV during fractionation, to result in free, viable virus being deposited in the Ig fraction, which otherwise would have been neutralized by antibodies to HCV (42, 43). Although the data available make this hypothesis persuasive, it is regrettable that a specific viral inactivation step, which had been under development for this product for some years had not been introduced in time to obviate any viral transmission. The step was hastily approved by the FDA and introduced in the wake of the Gammagard incident (36).

This incident continues to accentuate the point made above that the safety of Ig products cannot be assumed on the basis of historical experience, when any variation in the complex nexus of processes, which constitute the manufacture of Ig products is instituted. In this particular instance, a test, which unquestionably led to the enhancement of the safety of transfusion products was insufficiently assessed in its potential role in Ig safety, and was assumed to contribute to safety in the absence of specific viral safety measures. The rarity of viral transmission from Ig products was considered to be a good foundation for variations in their manufacture, in the absence of any scientific validation. The presumption of linearity in blood safety, i.e., assuming a capacity for predicting the future safety of plasma products on the basis of past experience, was fulfilled following these incidents through the efforts of the plasma industry and its regulatory overseers in introducing the complex nexus of measures for ensuring the safety of donors through the exclusion of high-risk groups, the screening of donations and plasma pools with nucleic acid tests to minimize the inclusion of donors in the silent "window period" of infection (44) and the implementation of dedicated pathogen reduction steps in the manufacture, which ensured that Ig therapies were safe from established and emerging pathogens. In particular, the role of pathogen reduction steps in overcoming the "Non-Linearity" of blood safety risks cannot be overemphasized (45). Early precautionistic measures implemented by regulators to minimize exposure to products potentially contaminated with prions from patients with transmissible spongiform encephalopathies (TSEs) led to product recalls and shortages (46). In the absence of a screening test, these measures could only be revised with the introduction of steps in the manufacture, which were shown to decrease prion contamination during plasma fractionation (47). The absence of infection in a patient who received Ig manufactured from a donor who subsequently developed variant Creutzfeldt–Jakob disease (vCJD) demonstrates the effectiveness of these measures (48).

The salutary experiences encountered during the evolution of Ig therapies to their current status of safety from blood borne pathogens, unfortunately, did not prevent similar problems in other areas as a result of manufacturing changes in succeeding years, as we shall now review.

THROMBOEMBOLIC EVENTS

Until recently, thromboembolic sequelae (TEEs) of Ig administration were relatively rarely reported, although their consequences were severe. With the increasing level of adverse event monitoring required of plasma therapies, increasing reports of these events have been published. Given the dramatic and often life threatening nature of these incidents, the increase in reports may be assumed to reflect an increased incidence. The interest and concern in this issue increased considerably when a major product was recalled from the North American and European markets because of an increased incidence in thromboembolic events. The product – Octagam from Octapharma – had a good safety record prior to this incident (49). The regulatory agencies and the industry collaborated in investigating the putative causes in the increased incidence of TEEs, including the Octagam problem, with the US FDA holding a workshop in 2011 whose proceedings are available (50). As a result of these investigations, the causative agent involved in the increased thrombogenicity of Ig preparations was determined to be coagulation Factor XI and XIa, generated in increased amounts following changes in the manufacturing procedures introduced over the previous years. The regulatory agencies mandated the introduction of changes in the manufacturing schemes of products determined to have increased Factor XI (51), and provided guidance and reference preparations for testing methods for FXIa in Ig (52). Further guidance was offered subsequently emphasizing the need to factor in patient characteristics in the administration of Ig (53), following epidemiological investigation, which showed that the majority of affected patients had pro-thrombotic co-morbidities (54). A high level warning on the risk of thrombosis was mandated for the product information available to patients and prescribers (55).

These regulatory measures indicated a somewhat belated recognition of the multifactorial nature of the problem of TEEs from Ig therapies. It appears definite that a previously very rare adverse event increased in reported incidence in the early 2000s. The attribution of increased FXIa levels in the product as a causal agent precedes this period (56), as do many of the manufacturing methods and variations described for the products associated with the highest TEE incidence (57, 58). These products included Octagam (Octapharma) and Vivaglobin (CSL), a 5% (for the formulation associated with TEE) intravenous and a 16% subcutaneous preparation, respectively. The variations in the methods, which are referred to in the literature describing the TEE incidents are common measures used by most manufacturers, and must be presumed, if they were introduced following initial market approval, to have been incorporated into standard practice following regulatory scrutiny and clinical studies. Any such regulatory processes failed to detect any problems, providing a sober example of the limitations of mainstream regulation and the difference between efficacy as assessed by regulators and effectiveness as reflected from the real world of therapeutic practice (59). Recalls of product only occurred for Octagam, despite an FDA investigation in which interrogation of a large US health insurance claims revealed higher risks with Vivaglobin (60). These risks were also higher in European countries but not to the levels reported in USA (61). Vivaglobin was subjected to scrutiny and warnings (62), and was withdrawn from the market concurrently with these events to be replaced with a 20% SCIG – Hizentra (63) – using a process validated to exclude pro-coagulants (64). The paucity of TEE reports following these incidents indicates that the measures introduced led to the desired effects. It would heighten the understanding of this and similar problems if investigations were to be done and reported on any changes in clinical and product administrative practices, such as infusion rates. The continuing increase in consumption of Ig therapies (65) does not indicate any modification in dosage and other issues, which are considered to play a role in TEEs (66).

HEMOLYSIS

Like TEE, intravascular hemolysis following Ig administration is a rare adverse event, which seems to be increasing in frequency (67, 68). A workshop was again convened by the FDA in January 2014 to assess this problem. Transcripts are available (69). Regulatory laboratories have determined that products with high concentrations of Ig, which are produced by revised versions of the classical fractionation scheme are preferentially associated with reactions (70) and are also associated with the highest titers of blood group antibodies anti-A and anti-B (71). One company obtained regulatory approval for updates to its safety information regarding hemolysis, specifying the role of dosage and the relationship to patient blood group and underlying disease state (72).

Investigations from the industry indicate that the apparent increase in the incidence of hemolysis may be related, as with TEEs, to changes in manufacturing methods introduced over recent years in order to increase the yield of Ig from plasma. Classical Cohn fractionation has been shown to result in a substantial reduction in isoagglutinin titer through the sequential removal through precipitation of Fraction I and, in particular, Fraction III (73, 74), and modifications of the scheme to omit these sequential precipitations or to purify Ig with different precipitating/chromatographic techniques result in products with little removal of isoagglutinins. As a result, manufacturers are assessing the feasibility of introducing further modifications in the fractionation of the current generation of products in order to remove isoagglutinins (75), as well as screening donors to exclude high isoagglutinin titer donations from the fractionation pool (76).

REFLECTIONS ON RECENT ADVERSE EVENT REPORTS

The past 20 years have seen the following developments in Ig therapies:

1. The introduction, in common with all plasma protein therapies, of measures to decrease pathogen transmission.
2. A widening of indications, in particular to treat autoimmune neuropathies such as Chronic Inflammatory Demyelinating Polyneuropathy (CIDP), GBS, and MMN.
3. A consequent drive to increase Ig yield from plasma, through modification of the classic ethanol precipitation scheme or its abandonment to other techniques.
4. A consequent increase in the cost of treating these disorders, leading to scrutiny by reimbursement agencies and pressure to cut costs.
5. The introduction of more concentrated solutions administered intravenously through high infusion rates, and subcutaneously,

at least partly fueled by the need to decrease costs through lessening hospital length of stay periods.
6. The emergence of adverse events at higher incidence than historically expected.

The contention of this work is that developments 1–5 led to development 6. We propose this as a "Popperian" hypothesis, able to be falsified through evidence. We do not assert this with the aim of ascribing blame, but rather as a route to a better understanding of Ig therapies and the need to address their benefits as well as their limitations. We propose the following aspects merit consideration.

THE DIFFICULTY IN PREDICTING OUTCOMES IN BIOLOGICAL THERAPEUTICS

Murphy has pointed out that a linear approach to predict the safety of blood-derived therapies is not desirable (77), proposing that many features of the blood system show characteristics of the chaotic behavior found in systems composed of complex interdependent components. Reflecting on the myriad of factors, which may affect the safety and efficacy of a product made, as are Ig therapies, from the blood of tens of thousands of individuals using a complex manufacturing process, the capacity for apparently minor and well-intentioned variations in donor composition and technology to result in deleterious outcomes is not to be wondered at. Hence, oft-stated claims about "zero risk" plasma therapies and "well-controlled" manufacturing processes risk being hubristic at best. Considering the scenarios, which ultimately led to pathogen transmission from blood in the past 20 years resonate, superficially, more to science fiction than to what was current empirical knowledge (78), any hypothesis proposing caution before the implementation of any potential perturbations in the blood safety landscape deserves to be treated with respect. Hence, the doubts shown on the advisability of anti-HCV testing for plasma destined for Ig production were shown to be justified, and the empirical evidence supposedly disproving this hypothesis was shown to be incomplete, unfortunately after affected product transmitted HCV. Similarly, changes in the manufacturing method intended to increase yield and lead to "self-sufficiency" resulted in infections in healthy women given anti-D, a product with an unblemished safety record when manufactured in the classical fractionation scheme. Clearly, improving blood transfusion safety through HCV antibody testing and improving anti-D access from a domestic source were well-intentioned policies, which, however, failed to take into account the specialized and complex area of plasma protein manufacture, with its vulnerability to processing changes. Similarly, the more recent events whereby adverse events hitherto rare in incidence became more common following, again, manufacturing changes whose potential effects on the product were insufficiently considered to demonstrate a failure by industry and regulators alike, in not appreciating that the rarity of such events with the classical products indicated a robustness in the manufacturing method, long derided as "bucket chemistry," which the more recent technologies failed to attain. When Oncley and co-workers had specifically reported in 1949 that Fraction III in the Cohn system contained the bulk of the isoagglutinins (79), was it to be wondered when omission of this step led to hemolytic sequelae in patients? We would contend that the evidence for safety of Ig therapies, before these changes and the subsequent problems, was evident, and "If it ain't broke, don't fix it" might have played a role in the decision-making process.

THE NEED FOR HIGHER YIELDS

As we have discussed, the driver for changes in the classical technologies has been the need for higher yields of Ig. Ig yield from the classical Cohn scheme is of the order of 3 g/L of plasma at best (14). The current generation of products using modified techniques is obtained at yields of 3.5–5.4 g/L (14, 80), compared to plasma levels of around 6.5–8.5 g/L in most of the raw material used by the manufacturers (81). We presume that further enhancements in yield are possible and that manufacturers will continue to examine the feasibility of modifications to the fractionation process. Such modifications generally require regulatory approval, which may include evidence of clinical efficacy and safety. The failure of agencies across the world to specify regulatory processes able to detect the harmful features introduced in previous modifications such as those discussed in this work does not augur well for the safety of any further changes.

CLINICAL NEED OF Ig THERAPIES

An analysis of the legitimate clinical need for Ig is legitimate in order to assess the demand for plasma and ensure optimal care. An analysis of the mainstay indication – substitution therapy in patients with humoral immune deficiencies – using published evidence for the variables currently influencing clinical need determined a requirement of 72 g per thousand population for this purpose (82), a need, which exceeds the actual current usage in most countries. This indication represents around 30% of overall Ig usage in published sources (83). Around 60% of Ig is used for immunosuppressive treatment of a number of autoimmune disorders, with particularly large volumes consumed by neuropathies such as CIDP. Although much is made about the so-called "off-label" indications of Ig, there is little doubt that, in the established markets, the vast majority of prescriptions for Ig are for evidence-based indications. This is indicated by the outcomes in the Australian system, where delivery of Ig, through a single government payer, is conditional to adherence to a set of prescribing guidelines, which allocate the product strictly and solely to indications with the highest level of evidence (84). It is, therefore, likely that, as diagnosis and therapeutic practice improve, the demand for Ig will continue the inexorable rise, which has been recorded over the past 10 years.

It behooves all players in this area to scrutinize such practice. The increase in Ig consumption is based on the ability to deliver large doses of product through the intravenous route and, increasingly, through subcutaneously administered deposits. More understanding is needed on the optimal dosage regimen for the different patient groups. In immune deficiency, one hypothesis, fueled by a meta-analysis of clinical trials, proposes that continuous increases of Ig dosage to higher through levels will continue to lead to clinical benefit through decreasing pneumonia episodes (17). This hypothesis suggests that patients should attain Ig through levels to at least the mid-normal range of Ig levels. In contrast, the work of Quinti et al. (85) on a prospectively studied PID patient cohort found no benefit in the same clinical

indicator when through levels were above 400 mg/dL. We suggest that, in an era of advocacy for personalized medicine (86), focusing on the clinical condition of patients and individualizing therapy according to such data needs to replace the "guideline" driven practices, which have underpinned much of therapeutics, including the treatment of patients with Ig therapies. Milito et al. (87), using a pharmacokinetic-driven approach, have shown that stratifying PID patients according to clinical phenotype permits the individualization of Ig dosage and results in significantly lower total Ig usage than the standard dosage regimen. While large prospective multicenter studies are needed to confirm and augment this work, we suggest that agencies seeking to ensure, for financial reasons, more "rational" Ig therapy would do well to consider the funding of such studies if patient care is to be optimized.

The considerable Ig usage, in doses far exceeding those used in PID, for autoimmune disorders also requires review and more investigation. The ways in which Ig modulates the immune system in these diseases is still the subject of research and is probably multifactorial, but evidence for the role of the Fc portion of Ig appears compelling (88). Enhancing the sialylation of the Fc portion has been proposed as a specific route to more targeted Ig therapies for autoimmune and inflammatory states, allowing more potent preparations at lower dosages of total Ig (89), and a commercial development, currently lacking in detail, has been announced (90). Keeping in mind that the majority of the recently encountered adverse events have affected patients with these disorders, and that causality has been ascribed to high doses, further work on the establishment and development of this therapy is a priority and a challenge for industry and regulators alike.

SUMMARY AND CONCLUSION

Ig therapies are life-saving medicines, which have revolutionized the treatment of a large number of rare and severe chronic immune deficiency and autoimmune disorders. Industry investment in the improvement and administration of these therapies has increased greatly the number of patients, which can benefit, enhancing life expectancy and quality of life. This has also led to an increase in the adverse events associated with these medicines. Current regulatory constructs are unable to predict such events prior to the introduction of the therapies on the market place. Hence, an effort to better understand the manufacture and indications of these therapies is mandatory. Rather than treating Ig as a form of "gasoline," putting more in the patient "tank" in order to get more clinical "mileage," personalizing patient care and developing a new generation of more specific, targeted therapies should be the focus of the coming years of therapeutics. We suggest that the seeds of such approaches are appearing, and that more studies will develop these if sufficiently resourced. We propose that the current era of financial stringency should not be the excuse for rationing Ig therapies to the detriment of patient care, but should act as the spur to improving our understanding and use of these crucial natural molecules.

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Analysis of specific IgG titers against tick-borne encephalitis in patients with primary antibody deficiency under immunoglobulin substitution therapy: impact of plasma donor origin

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Immunoglobulin (Ig) replacement therapy is effective in reducing infections in patients with primary antibody deficiency (PAD). Diversity of specific antibodies is achieved by pooling plasma from over 1000 donors usually of a given geographic region. However, there is no agreement with regard to an optimal vaccination schedule for plasma donors. Especially for tick-borne encephalitis (TBE), regional vaccination rates differ widely among populations due to the epidemiology of the disease. We analyzed specific antibody titers against TBE in comparison to total IgG levels in 162 serum samples collected from 110 PAD patients substituted with polyvalent intravenous IgG or subcutaneous IgG. Some patients received different IgG products over time leading to a total number of 122 different patient-IgG product combinations. Positive TBE-specific IgG levels were detected in 35 cases when measured by standard ELISA and could be confirmed by demonstration of neutralizing antibodies in 31 cases. The detection of specific antibody levels correlated with the geographic origin of the IgG preparations. No titers were detectable in patients substituted with IgG products from North-American donors, whereas variable degrees of anti-TBE titers were observed in patients receiving products from different European countries. We suggest considering the patients' personal risk for TBE when selecting an appropriate Ig preparation. These data support regional plasma donation in order to address the diverse local infection profile.

Keywords: tick-borne encephalitis, primary antibody deficiency, CVID, IVIG, SCIG, passive immunization

INTRODUCTION

Distribution of tick-borne encephalitis (TBE) is known to show immense geographic differences leading to variable need for vaccination-induced protection of individuals (1). Patients suffering from primary antibody deficiency (PAD) are characterized by reduced or absent antibody responses following vaccination[1]. They depend on continuous Immunoglobulin G (IgG) replacement therapy to maintain a diverse antibody repertoire. Plasma-producing companies recruit plasma donors globally and the origin of plasma donors varies considerably in between commercially available IgG products even between different batches of the same product. The proof of representative specific antibody titers within the products is obligatory for authorization of each batch. Quality management within the companies often provides data for an even broader spectrum of specific antibodies, but usually not anti-TBE IgG titers. Rabel et al. reported 2012 geographic variation of neutralizing antibodies against TBE within intravenous IgG preparations (2). Seidel et al. mention passive transfer of protective anti-TBE IgG levels via IgG replacement therapy in their publication focusing on active TBE vaccination responses in 18 patients (3). However, protective antibody levels within patients have not been systematically studied so far. Epidemiological data on the prevalence of TBE infection within PAD patients do not exist, so it can only be speculated that PAD patients require analogous TBE prevention to the healthy population of their region.

Therefore, the prevalence of protective anti-TBE IgG levels in PAD patients under IgG replacement therapy was assessed in this study in order to develop a strategy for patient care in individuals at high risk to TBE exposure.

MATERIAL AND METHODS

STUDY COHORT

Analysis was performed in serum samples collected between 2003 and 2008 and supplemented by samples from 2014 from recently approved IgG products. One hundred ten patients with diagnosed PAD according to the ESID definitions[1] were included after signing informed consent according to ethical approval (vote number 239/07, Ethics Committee University Medical Center Freiburg). All patients received regular intravenous or subcutaneous IgG replacement therapy without recent change of brand. Additional

[1] http://www.esid.org

information about patient history (such as history of tick bite, meningitis in general, and previously performed TBE vaccination) and on B-cell phenotype according to Freiburg classification (4) and Euro-Class (5) was recorded. In patients receiving IVIG therapy blood samples obtained immediately prior to infusion provided trough level values, others were indicated as non-trough level measurements. Serum samples were analyzed both retro- and prospectively. In 8 patients, TBE titers were determined under two and in 2 patients under three different Ig preparations leading to a total of 122 cases of patient-preparation combinations. In 29 of the 122 cases, two or more consecutive measurements were performed over time to test for reproducibility.

LABORATORY ASSESSMENTS

Total serum IgG was measured by nephelometry using standard test kits (Dade-Behring kit, BN II nephelometer).

The SERION ELISA classic TBE Virus IgG test is a qualitative and quantitative immunoassay for the detection of human antibodies in serum, plasma, or cerebrospinal fluid directed against TBE viruses. The antibody activity is expressed in units per milliliter with a cut-off at 150 U/ml and a grey zone between 100 and 150 U/ml. The evaluation of the IgG antibody activity is referenced to the first standard serum for human IgG antibodies against TBE Virus of the Consultant Laboratory for TBE Viruses located at the Robert Koch Institute (RKI) in Berlin, Germany. Samples collected before 2013 were measured with an earlier version of the immunoassay with a different unitation. According to the manufacturer, a factor of 5.6 may be used for conversion of old values in new standard values. All results are therefore expressed in units per milliliter according to the new RKI standard. For details of the methods, we refer to the manufacturer's information.

The recombinant ED3 immune complex ELISA was performed as described before (6). Briefly, microtiter plates were coated with rheumatoid factor IgM (10 g/ml PBS + 1 mg/ml NaN3). The plates were blocked (1 h; 10 mg/ml bovine serum albumin in PBS) and washed (Tris-Tween) and stored at −80°C before use. Human sera were diluted 1:10 in PBS + 2% BSA. In each assay, two standard positive and three negative control samples were included. To 25 µl of diluted serum, 25 µl of peroxidase-labeled ED3 antigen, diluted 1:20,000, were added. The plates were incubated at 4°C over night. After washing 50 µl TMB (tetramethylbenzidine) substrate was added to each well and the reaction was stopped after 10 min by adding 50 µl of H_2SO_4 stopping solution. The reaction is read at 450 nm. The cut-off of the assay is at 0.150 OD with a gray zone between 0.100 and 0.150 OD. Results are calculated by dividing the OD of the sample by 0.150 and expressed as Signal/Cut-off (S/Co).

IgG PRODUCTS

Data on the origin of plasma donors were taken from the respective product information or were provided by courtesy of the companies. Epidemiological data concerning vaccination frequencies among different European populations have been reported for 2007–2009 by Kunze et al. and are listed in **Table 1** (7).

Up to date there exists no licensed TBE vaccine in the USA[2].

[2]http://wwwnc.cdc.gov/travel/

Table 1 | Vaccination rates of different European countries 2007–2009.

Country	Vaccination rate (%)
Austria	58 (88% incomplete)
Latvia	39
Germany	26
Estonia	20
Switzerland	17
Czech Republic	16
Sweden	13
Slovenia	13
Lithuania	10

Table 2 | Origin of plasma – assignment of product codes.

Product codes	North American	Mixture	European
IVIG	1, 2, 3, 4	5, 6, 7	8, 9
SCIG	10	11, 12	13

Due to these aspects, plasma preparations were grouped as North American, European, or a mixture of both in this analysis (**Table 2**).

RESULTS

Anti-TBE-specific IgG values were measured by a standard ELISA (Serion) in relation to total IgG levels for IVIG (**Figure 1A**) and for SCIG preparations (**Figure 1B**). Overall, 75 negative, 12 intermediate, and 35 positive results were detected with 33 negative, 7 intermediate, and 20 positive cases for IVIG preparations (**Figure 1A**) and 42 negative, 5 intermediate, and 15 positive cases for SCIG preparations (**Figure 1B**).

Products with North-American origin never achieve anti-TBE-specific IgG levels above 150 U/ml in patients (25 negative and 2 intermediate cases). For both mixed and European products in 35 out of 95 cases, positive anti-TBE titers can be detected (compared to 50 negative and 10 intermediate results). There were clear differences between single products in this group revealing the highest titers of anti-TBE antibodies in the IVIG products 5, 7, and 9 and the SCIG product 11, while there were no truly positive serum titers achieved in patients on products 8, 12, and 13. Product 6 was applied only in one case with insufficient IgG serum trough levels at the point of measurement and can therefore not be judged.

Based on our previous data (4, 8), in two cases with positive anti-TBE titers, a sustained active anti-TBE vaccination response could not be excluded because of the combination of normal switched memory B-cell counts and a history of vaccination against TBE virus.

The distribution of cases was analyzed according to the anti-TBE response for each of the 13 products (**Figure 2**). Cases were restricted to cases with IgG trough levels above 7 g/l. This confirmed the previously identified four products with mostly positive anti-TBE serum levels (5, 7, 9, and 11) and eight products with mostly negative/intermediate anti-TBE serum levels (1, 2, 3, 4, 8, 10, 12, and 13). Again, all North-American products, but also some purely European products (8 and 13) were negative,

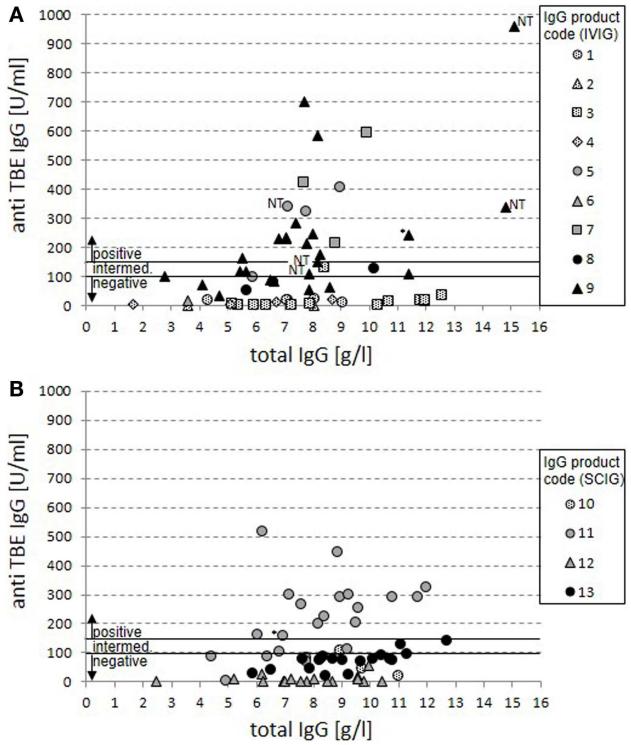

FIGURE 1 | Anti-TBE IgG vs. total IgG serum levels in patients under IgG replacement therapy. Anti-TBE IgG titers measured by standard ELISA are shown in relation to total IgG serum levels for 60 cases from patients treated with IVIG **(A)** and 62 cases from patients treated with SCIG products **(B)**. Each case of patient-product combination is presented by a single value. Product codes are assigned to symbols with dotted filling representing North-American origin of plasma, gray filling mixed origin, and black filling European origin. The anti-TBE antibody titers for positive (>150 U/ml), intermediate (100–150 U/ml), and negative (<100 U/ml) serum titers are marked. In four cases on IVIG replacement with positive anti-TBE titers, only non-trough levels were available (NT). Positive values are also marked in two cases where a sustained active anti-TBE antibody response cannot be excluded because of normal switched memory B cells and vaccination (*).

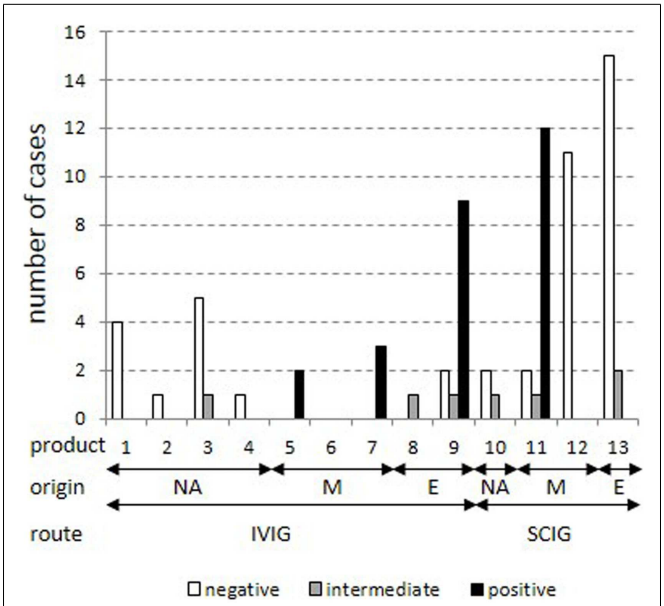

FIGURE 2 | Stratification of cases for each product in patients with IgG trough levels above 7 g/l. All cases with total IgG trough levels above 7 g/l were stratified into positive, intermediate, and negative results in the standard anti-TBE ELISA according to IgG products as coded in **Table 2**. Plasma origin is indicated as North America ("NA"), mixture ("M"), and Europe ("E").

reflecting the regional heterogeneity of the vaccination status against TBE in Europe. Unfortunately, more detailed information on the composition of mixed and European products regarding the specific country of origin and the particular mixture of single plasma batches is not routinely available through the producing companies.

To assess the reproducibility of the results, consecutive trough serum samples within 29 cases were analyzed for global anti-TBE titers (**Figure 3**). Twenty-two cases showed stable measurements at different time points with consistently negative or positive results. At overall grading in negative, intermediate, and positive results, five cases (7, 8, 10, 25, and 26) had values in the intermediate region and in two cases (12 and 14) the titers clearly varied (indicated by #), which is not explained by parallel variations in total IgG serum levels (data not shown).

Standard anti-TBE IgG ELISAs are known to have limited informative value in regard to actual protection. More informative assays detecting neutralizing antibodies on the other hand are currently rarely performed and more elaborate. We confirmed the findings of the standard ELISA in a selection of 46 cases in a test for neutralizing antibodies with an ELISA for anti-ED3 of TBE antibodies (**Figure 4**; **Table 3**). Our data show that 31 out of the 35 positive results from the standard ELISA could be confirmed by the detection of neutralizing antibodies. Standard ELISA results in the lower positive range (150–200 U/ml) changed to intermediate or even negative in three cases or were confirmed as positive in another three cases. Standard ELISA results above 200 U/ml were confirmed positive for neutralizing antibodies in all cases. Single random samples from intermediate or negative results in the standard ELISA always turned out to be negative for neutralizing antibodies as expected (**Table 3**).

DISCUSSION

Due to disease-associated impairment of vaccination responses, patients with primary antibody deficiencies depend on the quality and quantity of passive protection via the application of polyvalent IgG preparations. For most diseases including TBE, which can be prevented by vaccination, the induced protection is provided via antibodies. The impact of geographic plasma origin on the presence of distinct specific IgG titers within polyvalent IgG preparations has been described before. Rabel et al. had already reported geographic differences for TBE within IVIG products (2). In parallel, Seidel et al. found divergent levels of protective antibodies in a small number of patients with IgG replacement therapy but did not correlate their findings to the geographic plasma origin in detail (3). Our data complement the previous results by analyzing

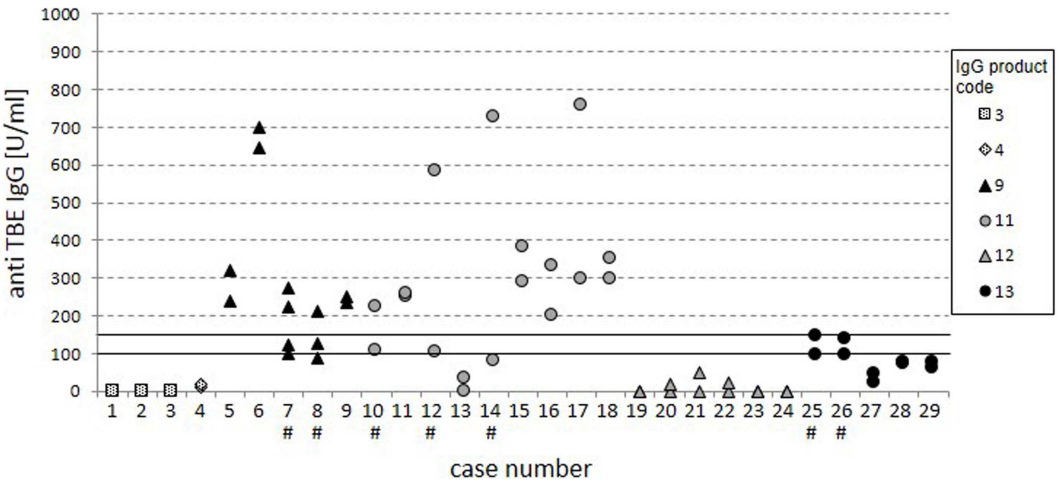

FIGURE 3 | Cases with consecutive measurements. The dot plot displays anti-TBE IgG titers from the standard ELISA for 29 separate cases with consecutive measurements. Cases with inconsistent ratings are highlighted by "#." The variation of specific anti-TBE IgG titers was not due to variation in total IgG serum levels.

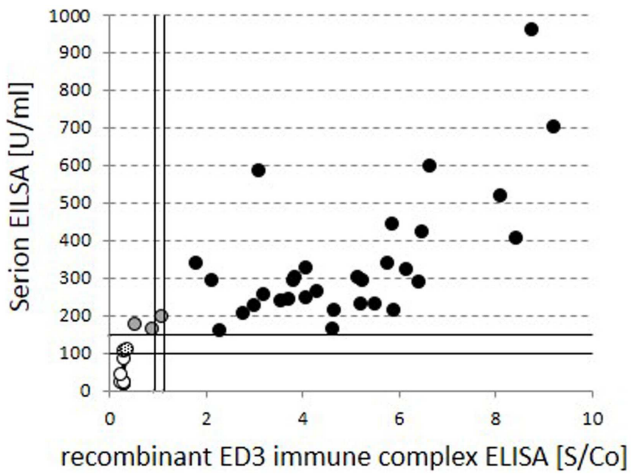

FIGURE 4 | Comparison of global vs. neutralizing anti-TBE IgG titers. Quantitative test results for global standard anti-TBE IgG titers from the standard ELISA are compared to neutralizing, anti-recombinant ED3 immune complex ELISA values within identical serum samples. Circles filled in black indicate positive and open circles indicate negative results in both tests. Gray and dotted fillings reflect deviant grading within the two assays.

TBE-specific serum titers in patients in relation to the geographic origin of the received IgG preparation.

An epidemiological proof of protection against TBE infection is impossible given the small number of CVID patients under substitution, but we could show a good correlation of high anti-TBE IgG titers in the global ELISA (>200 U/ml) and the presence of anti-ED3 neutralizing antibodies. Antibodies directed to the ED3 protein of TBE virus are highly specific for TBE virus without cross reactivity to other flaviviruses and have shown strong neutralizing activity and are thought to be protective against TBE (6, 9–11). In contrast, reactivity in global anti-TBE ELISAs using lysate of cell cultured virus, may not always be due to neutralizing antibodies and cross reactivity with other flaviviruses, e.g., yellow fever virus or dengue virus cannot be excluded (12). Our data demonstrate strong differences in TBE-specific IgG titers among patients under IgG substitution depending on the origin of plasma. In 35 patients, anti-TBE titers were positive with positive evidence of protective neutralizing antibodies in most cases.

Table 3 | Comparison of standard ELISA for anti-TBE IgG vs. neutralizing antibodies.

Serion ELISA result	Neutr. Ab neg	Neutr. AB intermed.	Neutr. AB positive	Neutr. AB not tested
75 negative	10	0	0	65
12 intermediate	2	0	0	10
35 positive	1	2	31	1

All purely North-American products were consistently negative being in line with the previously published findings from measurements within IVIG products (2). The results for mixed and European products were variable with 26 positive, 5 intermediate, and 30 negative results looking at cases with total IgG trough levels above 7 g/l. This variability among IgG preparations with European plasma contribution was highly dependent on the specific product. Most patients under replacement with products 5, 7, 9, and 11 had positive results, while serum samples from patients with products 8, 12, and 13 were negative or intermediate. It is tempting to speculate that the content of North-American plasma is of major influence in the mixed group. However, given the variability within purely European-derived plasma products, it becomes clear that the country of origin within Europe has a similarly strong influence as disclosed already by the different vaccination policies in **Table 1**. Even within a given product variable, anti-TBE serum titers were found as exemplified by product 9 suggesting that over time the composition of different batches varied

in regard to origin, mixture, and obviously specific donors within the countries. Due to lack of detailed information on the relative composition of the batches applied currently treating physicians have no chance to predict the presence of protective levels for specific products. This would not only require the disclosure of the composition of the specific batches by the plasma-producing companies but also a better estimate of an average value of national anti-TBE titers in European countries, which may vary according to changing policy over time and therefore is not feasible.

In daily practice, we suggest physicians to consider the patient's personal risk for specific infection, in this case TBE, as one aspect when selecting an appropriate IgG preparation at the initiation of IgG replacement therapy.

For patients already on IgG replacement therapy, we recommend the following approach for patients at high risk for TBE exposure:

A) Patients receiving IgG products with definitive exclusive plasma origin from North America: no anti-TBE IgG testing is necessary. The patient needs to be informed, that no passive protection can be expected. Other types of prophylaxis need to be emphasized. Whether a change of product with the option of a passive protection is justified has to be made by individual decision.
B) All others patients: standard anti-TBE ELISA testing at the time of trough levels is suitable. In serum samples with anti-TBE titers above 200 U/ml, the presence of protective neutralizing antibodies can be assumed. However, patients need to be informed about possible variations due to changes in batch composition. In case of negative or intermediate result in high-risk patients, such as lumberjacks in endemic areas, it might be helpful to contact the producing company for disclosure of the definite composition of the relevant batch. This should be added by the information whether in this individual case the patient could be provided with selective batches containing higher amounts of plasma from distinct European countries with better TBE vaccination rates.

However, this procedure still bears uncertainties due to variation between single batches of the same product. Therefore, we recommend plasma-producing companies within the European market to add measurements of anti-TBE IgG within each charge and to provide physicians treating PAD patients with these data. Alternatively, the display of a defined code within the batch numbers for the countries of plasma origin might enable treating physicians to choose products based on the national vaccination policies described above.

These data emphasize the importance of regional plasma donation to address the diverse local infection profiles in a better way. Patients with PAD lacking specific antibody responses depend on the broad passive protection of the IgG preparations provided by plasma-producing manufacturers. Optimal vaccination status of the plasma donors according to official regional recommendations can greatly contribute to the quality of the product.

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Mechanisms of action of Ig preparations: immunomodulatory and anti-inflammatory effects

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Primary immunodeficiency (PID) disorders that predispose patients to recurrent infections require immunoglobulin (Ig) replacement therapy. Ig replacement therapy has been stated as beneficial, although the optimal IgG trough level to be maintained over time in order to minimize infectious risk has not been established. The most common route of administration of Ig has been intravenously, although there are different options, one of them being the subcutaneous route. Ig replacement therapy has been a life-saving treatment for patients suffering from primary and secondary antibody immunodeficiency. The key role of regular Ig replacement in patients with antibody deficiencies is related to the ability to provide specific antibodies that could not be produced by these patients as demonstrated by the reduction of severe infections such as meningitis and pneumonia. The therapeutic benefits of Ig may also be due to an active role in various anti-inflammatory and immunomodulatory activities, which may complicate the clinical picture of PID. Anti-inflammatory activities are seen more generally when intravenous Ig is administered at high dose. The immunomodulatory and anti-inflammatory activities are important not only in the treatment of autoimmune diseases but also in patients suffering from immunodeficiency.

Keywords: immunoglobulin, immunodeficiency, immunoregulation of Ig, Ig replacement therapy, antibody defect

INTRODUCTION

Primary immunodeficiency (PID) disorders that predispose patients to recurrent respiratory, skin, and gastrointestinal infections, require immunoglobulin (Ig) replacement therapy. Common variable immunodeficiency disease (CVID) is one of the most frequent PID characterized by decreased serum levels of all Ig isotypes and recurrent bacterial infections encompassing a heterogeneous group of diseases whose unifying feature is hypogammaglobulinemia (1). The etiopathogenesis of CVID has not been yet fully elucidated. Major progress toward elucidating CVID has been achieved with the identification of defects not only in B-cells, which are directly responsible for antibody production, but also in other immune cells implicated in the generation of an effective humoral response, including antigen-presenting cells (APC) and, in a significant proportion of these patients, helper T cells (2–4). Overall, the disorder is characterized by a defective antibody production by B lymphocytes. Although the number of B-cells may be normal, patients with CVID show extremely decreased serum IgG, IgA, and, occasionally, IgM concentrations. The treatment of choice for CVID patients is replacement Ig therapy. A common route of administration of Ig has been intravenously (IVIg) although today there are different options, one of them being the subcutaneous route (SCIg). Ig replacement therapy has been a life-saving treatment for patients suffering from primary and secondary antibody immunodeficiencies, in fact, a recent published meta analysis of clinical trials in PID quantitatively confirms that trough IgG levels directly impact clinical outcomes (5). However, the optimal IgG trough level to be maintained over time in order to minimize infectious risk, has not been established and probably it should be individualized (6–8). The key role of regular Ig replacement in patients with antibody deficiencies is related to the ability to provide specific antibodies that could not be produced by these patients as demonstrated by the reduction of severe infections such as meningitis and pneumonia (9). The therapeutic benefits of Ig may also be due to an active role in various anti-inflammatory and immunomodulatory activities. In fact, clinical and immunopathological aspects of the association between CVID and autoimmune or inflammatory disorders have been extensively reported in a number of patients (10, 11). On the other hands, Ig preparations other than antibodies to superantigens and pathogens also contain numerous soluble proteins with biologic activity such as cytokines, chemokines, soluble cytokine receptors, and receptor antagonists. In fact, since they were first administered to patients with antibody deficiency disorders over 50 years ago, human intravenous Ig preparations have been used successfully to treat a rapidly increasing number of autoimmune and inflammatory disorders, among which are a series of cutaneous autoimmune and inflammatory diseases (12). Despite the identification of protective, immunomodulatory, and anti-inflammatory activities in various diseases, the benefits of Ig are not easily explained and probably depend by several mechanisms. Anti-inflammatory activities are seen more generally when intravenous Ig is administered at high dose. The immunomodulatory and anti-inflammatory activities are important not only in the treatment of autoimmune diseases but also in patients suffering from immunodeficiency. In this article, the protective and immunoregulatory mechanisms are summarized.

IMMUNOGLOBULIN PREPARATION

The major component of IVIg and SCIg preparations is the IgG fraction, which is pooled from human plasma of several thousands of donors using a procedure that varies somewhat between manufacturers, but results in a product that is a relatively pure concentrate of intact monomeric IgG, with a half-life of 3 weeks, and with small amounts of IgA and IgM. IgG subclasses (IgG1, IgG2, IgG3, and IgG4) in IVIg and SCIg products have a distribution similar to that found in normal human plasma. IgG aggregates are virtually absent from the majority of Ig preparations even if up to 1–10% of IgG can be found in dimeric form in most of IVIg preparations. The processes of purification have the potential to adversely affect the final quality and biological activity of IVIG/SCIG in terms of efficacy and safety (13). Natural antibodies and autoantibodies are prominent in commercial preparations. A wide range of specificities have been identified within Ig preparations including idiotypes of Ig itself, T cell receptor, cell surface molecules such as CD4, CD5, Fas, BAFF, cytokines, and cytokine receptors, such as IL-1; IL-6, tumor necrosis factor (TNF)-α, chemokine receptors, molecules such as sialic acid binding Ig-like lectin (Siglec)-8 and -9 or major histocompatibility complex (MHC) molecules; natural autoantibodies of IgG isotype directed against the human FcγRIII (CD16) and FcγRII (CD32) (14–16).

MECHANISMS OF IVIg IN THE CORRECTION OF HUMORAL DEFECTS

The underlying mechanisms of therapeutic effects of IVIg/SCIg in PID are not completely understood, the major aim is to prevent life-threatening bacterial or viral infections (**Figure 1**). IVIg/SCIg act mainly as a reconstitution therapy, providing patients with pathogen-specific antibodies able to protect from infectious. The unique structure of the Ig molecule ensures the large repertoire of specificities of the antibodies. To maintain the polyclonal nature of the antibody repertoire that is normally present in serum of healthy subjects naturally exposed to microbial agents or submitted to vaccines is a crucial step during the preparation of commercial Ig. Considering that IVIg preparations are generated from adults who have been vaccinated and have encountered a multitude of pathogenic microorganisms, serum IgG can comprise more than 100 million unique specificities. The clinical and functional activities can be distinguished by the infused amounts. In fact, a monthly Ig dosage of at least 400 mg/kg body weight is recommended and is most often sufficient as replacement therapy (17). The activities of Ig molecules present in IVIg/SCIg preparations, such as bactericidal effect through complement system activation, viral neutralization, inactivation of toxins, and opsonization, are crucial for the induction of an effective immune response against several microorganisms and their toxic products. As known, IgG antibodies include two functional portions as the F(ab′) fragment, which is responsible for antigen recognition, and the fragment crystallizable (Fc), crucial for activating the mechanisms of immunity by interacting with Fcγ receptors on B-cells and other cells of the innate immune system belonging to the phagocytic system. The Fc fragment is also crucial for the activation of complement and for the clearance of microorganisms (18). B-cells from several CVID patients seem to be not intrinsically defective as they express CD40 and proliferate significantly upon anti-CD40 stimulation. Interestingly, IVIg at "replacement dose" (10 mg/ml) are able to interact with B-cells, also inducing significantly higher proliferation of B-cells than anti-B cell receptor (BCR) stimulation alone. In addition, IVIg replacement is able to induce Ig synthesis *ex novo* by B-cells (19). Therefore, IVIg therapy, at least in some CVID, is able to modulate B cell functions and it is a passive transfer of antibodies. As previously mentioned, PID are a heterogeneous group of disorders that affect distinct components of the innate

Replacement therapy	**Anti-inflammatory therapy**
Fab-dependent activities Pathogen-specific antibodies Neutralization of pathogen Inactivation of toxins Opsonization Induction of B cells proliferation and Ig synthesis Increase of CD4+ T lymphocytes number Increase of myeloid dendritic cell activation	Fab-dependent activities Neutralization of autoantibodies Neutralization of pro-inflammatory cytokines Blocking of activated Complement components Blocking adhesion molecules Interference with idiotypic/anti-idiotypic network Modulation of antibody production Modulation of maturation/ function of dendritic cells
Fc-dependent activities Activation of phagocytic cells (FcγR-dependent) Complement activation Clearance of microrganisms	Fc-dependent activities Blocking of the FcRn (increased autoantibody clearance) Blocking of activating FcγR Increase of inhibitory FcγRIIb expression Immunomodulatory effects of sialylated IgG Increase of Treg cell

FIGURE 1 | Mechanisms of action of IVIg in PID.

and adaptive immune system. Defects not only in B-cells, which are directly responsible for antibody production, but also in other immune cells such as APC and T helper (Th) cells, represent the molecular basis of CVID (20, 21). It has been shown that in CVID patients the humoral defects may be associated with immunological abnormalities of T cell compartment and myeloid dendritic cells (mDC), characterized by low counts of CD4+ T cells, high expression of HLA-DR and CD38 (also on CD8+ T cells), suppressed number of mDC, highly positive for CD80 and CD83. Several of these cellular perturbations are partially corrected by the treatment with IVIg. In fact, the introduction of therapy may lead to CD4+ T cell recovery and decline in CD8+ T cells and mDC activation. These effects are likely sustained by an improved immune control of infections due to humoral reconstitution (22).

MECHANISMS OF ACTION OF IVIg IN COMORBIDITY OF PID

The use of IVIg has been firmly established for the treatment of a wide variety of autoimmune and inflammatory diseases, due to their immune-regulatory and anti-inflammatory effects (**Figure 1**). Some of these autoimmune diseases may be a comorbidity of PID, especially CVID, thus sustaining an additional role, beyond the antibody replacement, for IVIg in the treatment of immunodeficiencies. For example, the immunoregulatory functions of IVIg in PID patients explain the therapeutic effects showed in autoimmune hemolytic anemia and/or immunothrobocitopenia, probably by blocking the clearance of opsonized target cells or by suppressing antibody-dependent cell-mediated cytotoxicity. This potential was first revealed when IVIg, used to treat a patient with antibody deficiency, were able to restore platelets count when concomitant thrombocytopenia occurred (23). The way in which IVIg exert their immunomodulatory effects remain unclear, with many pathways, probably mutually non-exclusive, in the innate and adaptive immune systems being potentially targeted. At least a percentage of immune modulatory effects of IVIg are dependent upon the interaction between the Fc portion with the Fcγ receptors expressed on the surface of cells as macrophages, B-cells, natural killer (NK) cells, plasma cells, and platelets (18). For example, as previously mentioned, it has been clearly demonstrated that Fc fragment of IgG can be sufficient to ameliorate immune-mediated thrombocytopenia in humans (24), by suppressing the phagocytosis of platelets via an Fc-dependent mechanism instead of preventing autoantibodies from binding to cells (25). Studies performed both in mice and humans confirmed that IVIg infusion is able to inhibit the mononuclear phagocytic system, usually activated by immune complexes through activating of low-affinity FcγRs (26). However, there is no direct proof that IVIg block the binding of immune complexes to FcγRs. The Fc portion of Ig not only impacts the function of activating Fc receptors but also increase the expression of inhibitory FcγRIIB on macrophages (27). Recent studies in animal models of idiopathic thrombocytopenic purpura suggest that IVIg, increasing the expression of the Fcγ receptor IIB, may reset the threshold for cell activation by immune complexes (18, 25). In other words, IVIg should be able to shift the FcγR-dependent balance of activating and inhibitory signals even more toward cell inhibition of innate immune effector cells.

A mechanism implicated in immune-regulatory function of IVIg preparation is also the effect on the balance between pro- and anti-inflammatory cytokines. To this effect, antibodies to IL-1 and TNF-α have been identified in addition to a down-regulation of such cytokines (28). Furthermore, IVIg induce anti-inflammatory cytokines such as IL-10, TGF-β, and IL-1ra from monocytes/macrophages (28, 29). In our hands, in IVIg-treated PID patients the raising of IL-10 after administration of therapy was not observed in those with associated granulomatous lung disease, thus suggesting the lack of the induction of regulatory cytokines in such subgroup of patients. Dendritic cells represent an important source of pro-inflammatory and anti-inflammatory cytokines and a modulation of cytokine secretion has been shown, characterized by a decrease of IL-12 production and enhanced secretion of IL-10 has been described. DC maturation, activation, and survival are also targeted by IVIg, thus affecting the overall APC activity with subsequent inhibition of adaptive T cell response, including autoreactive cells (30). This latter effect is of potential relevance considering the beneficial action in autoimmune conditions.

Dendritic cells are professional APC with superior capacity to present both MHC-restricted and CD1-restricted antigens. DCs may adapt their CD1 antigen presentation machinery according to signals in the microenvironment. A role for IgG in regulating the expression of CD1 molecules in human DCs has been shown in *in vitro* experiments. In particular, it has been found that the level of exposure to IgG regulates the CD1 expression profile during DC differentiation, and that this is mediated by FcγRIIa. This in turn determines whether the DCs will be biased toward activation of CD1d-restricted regulatory NKT cells or T cells specific for lipid antigens presented by CD1a, CD1b, and CD1c (31). Furthermore, results obtained from patients with CVID indicate that mDCs express elevated levels of CD1a and CD1b in the presence of low levels of IgG *in vivo*, and that this aberrant expression pattern is normalized after IVIg therapy (32). These findings are important for our understanding of diseases associated with Ig deficiencies and their treatment with IVIg.

Concerning the IVIg effects on apoptosis, triggered by the interactions between CD95 (Fas) and its ligand (CD95L, FasL), controversial data are present in literature and probably both agonist and antagonist properties may be attributed to IVIg. In CVID, an increased expression of Fas on CD3+ T cells has been demonstrated and a further increase was shown after IVIg therapy (33). In the context of autoimmune disease as comorbidity of PID, the process could be important in that specific harmful T cells may be deleted through apoptosis.

The complement system is a first line of defense against invading microorganisms, but if its activation occurs under inappropriate circumstances, this system may be not beneficial leading to complement-mediated disorders characterized by cell lysis and tissue damage. Ig plays an intriguing role in complement and regulation. IVIg preparation contain pathogen-specific antibodies and autoantibodies able to activate the effector system, but on the other hand, the rest of the circulating Ig pool (mainly of IgG and IgM isotype) have the capacity to attenuate damaging effects of activated complement fragments. This latter effect seems to be

related to a scavenger action toward complement fragments that is dependent on a low-affinity interaction between the fragment in question and various Ig regions (Fc binding to C3b/C4b; Fab binding to C3a/C5a). Such immunocomplexes are then removed by the reticuloendothelial system (34).

Even if many immunoregulatory effects have been ascribed to IVIg, it remains unclear why high doses of IVIg are required to obtain this activity. Some insights were gained when the role of IgG glycosylation was addressed in animal models. Deglycosylated Ig appeared to be unable to provide anti-inflammatory protections, although glycan was found in only 1–3% of IgG preparation (35). Sialylated Fc present in low amount in IVIg binds to SIGN-R1 on macrophages leading to an increased expression of the inhibitory FcγRIIB receptors and decreased expression of FcγRs. The overall result is an anti-inflammatory and anti-phagocytic effect. The key role of the terminal sialic acid residues is confirmed by the role of the molecules CD22 and CD33, also called SIGLEC, which are expressed by B-cells and cells of innate immune system (36, 37). Their capacity to trigger cell inactivation is related to the activation of intracellular immunoreceptor tyrosine-based inhibitor motifs (ITIMs). Of note, while the importance of Fc sialylation has been clearly shown in animal models, its role in humans should be better evaluated, and important species differences may probably exist.

CONCLUSION

Immunoglobulin therapy is a consistently evolving practice that provides life-saving protection to patients with PID, particularly with antibody deficiency as well as in patients with other immune-mediated diseases. Taking into account the growing demand of Ig preparations not only for PID patients but also for subjects suffering from chronic immune-mediated diseases, more clinical trials will be required as well as basic research able to improve the knowledge on the mechanisms of action of IVIg.

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Immunomodulation by IVIg and the role of Fc-gamma receptors: classic mechanisms of action after all?

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Intravenous IgG (IVIg) contains polyclonal immunoglobulin G (IgG) from thousands of donors. It is administered at a low dose at regular intervals as antibody replacement therapy and at a higher dose as immunomodulatory treatment in various auto-immune or auto-inflammatory diseases. The working mechanism of immunomodulation is not well understood. Many different explanations have been given. During the last decade, we have focused on classical antibody binding via the Fc-domain of the IgG molecules to the common IgG receptors, i.e. the Fcγ receptors (FcγRs). Variation in the genes encoding human FcγRs determines function as well as expression among immune cells. As described here, NK cells and myeloid cells, including macrophages, can express different FcγR variants, depending on the individual's genotype, copy number variation (CNV), and promoter polymorphisms. B-cells seem to only express the single inhibitory receptor. Although these inhibitory FcγRIIb receptors are also expressed by monocytes, macrophages, and only rarely by NK cells or neutrophils, their presence is unlikely to explain the immunomodulatory capacity of IVIg, nor does the sialylation of IgG. Direct IVIg effects at the level of the activating FcγRs, including the more recently described FcγRIIc, deserve renewed attention to describe IVIg-related immunomodulation.

Keywords: IgG, Fc gamma receptors, immunomodulation, IVIg, mechanisms of action

INTRODUCTION

Intravenous IgG (IVIg) is a blood product containing polyclonal immunoglobulin G (IgG) isolated and pooled from thousands of donors. IVIg is mainly used in two clinical situations. It is administered at a low dose at regular intervals as antibody replacement therapy in primary immunodeficiencies such as agammaglobulinemia and common variable immunodeficiency (CVID), or acquired immunodeficiencies where IgG plasma concentrations have become very low. In this context, suppletion essentially functions to provide the recipient with a repertoire of protective antibodies against a range of predominantly bacterial infections.

On the other hand, IVIg can also be used at a high dose as an immunomodulatory treatment in auto-immune or auto-inflammatory diseases, of which a handful are FDA- and EMEA-approved, including Kawasaki disease and immune thrombocytopenia (ITP). In this respect, IgG administration has also been used off-label for a growing number of additional diseases, including hematologic, dermatologic, and neuromuscular disorders. A list of indications for treatment with IVIg is provided in **Table 1**. Many but certainly not all of these diseases involve (auto)-antibody responses, questioning the actual working mechanism of IVIg under conditions where auto-antibodies have not yet been shown to be involved.

Even though IVIg is widely used and has proven to be an effective treatment for many diseases, the exact immunomodulatory mechanism(s) have remained elusive. Several mechanisms by which IVIg may exert its anti-inflammatory effects have been proposed over the past decades (1), listed in **Table 2**. These mechanisms are not necessarily mutually exclusive, and may act in concert to modulate the immune system. Furthermore, different mechanisms may be at work in the different diseases for which IVIg is administered. In this review, we describe the different theories that may explain the immunomodulatory effect of IVIg, with a special interest in the actions of IVIg in ITP, being the first disease for which the usefulness of IVIg as an immune-modulatory agent was discovered (2).

Many of the theories aiming to explain the working mechanism involve Fc gamma receptors (FcγRs), which are the main receptors for IgG and therefore very likely to be involved in the working mechanisms of IVIg. Therefore, we start with an introduction of human FcγRs, followed by a description of the potential working mechanisms of IVIg, discussing how IVIg can shape immune responses by altering or interfering with FcγR expression and function.

Fc GAMMA RECEPTORS

FcγRs are receptors for (IgG), the most abundant of five classes of Ig. IgG consists of a Fab (fragment, antigen-binding) region, which determines specificity to specific antigens, and a constant region, which is the Fc (fragment, crystallizable) region, which (among other functions) mediates the effector functions of IgG, including the interactions with their major receptors, the FcγRs.

Table 1 | Overview of indications for treatment with IVIg.

IVIg as substitution therapy
Primary immunodeficiency disease
Chronic lymphocytic leukemia
Pediatric HIV infection
Common variable immunodeficiency
IVIg as immunomodulatory therapy
Inflammatory disorders
Kawasaki's disease
Transplantation
Kidney transplantation involving a recipient with a high antibody titer or an ABO-incompatible donor
Allogeneic bone marrow transplantation
Graft-versus-host disease
Hematologic disorders
Immune thrombocytopenia
Auto-immune hemolytic anemia
Auto-immune neutropenia
HIV-associated thrombocytopenia
Neonatal alloimmune thrombocytopenia
Severe anemia associated with parvovirus B19
Dermatologic disorders
Bullous pemphigoid
Epidermolysis bullosa acquisita
Mucous-membrane (cicatricial) pemphigoid
Pemphigus vulgaris
Toxic epidermal necrolysis or Stevens–Johnson syndrome
Neuromuscular disorders
Birdshot retinopathy
Chronic inflammatory demyelinating polyneuropathy
Multifocal motor neuropathy
Guillain–Barré syndrome
Lambert–Eaton myasthenic syndrome
Myasthenia gravis
Opsoclonus–myoclonus
Polyradiculoneuropathy
Refractory dermatomyositis
Refractory polymyositis
Relapsing–remitting multiple sclerosis

Table 2 | Potential immunomodulatory mechanisms of IVIg.

Fc-mediated mechanisms
1. Blockade of activating FcγR by saturation via high-dosed IVIg making them less available for auto-antibodies in oligo- or polymeric complex with their (auto)antigen
2. Upregulation of the inhibitory FcγRIIb by sialylated IgG Fc
3. Increased clearance of pathogenic antibodies by saturation of the neonatal FcR (FcRn)
4. Tipping the cellular balance from pro- to anti-inflammatory reactivity by modulating dendritic cells (DCs)
5. Reducing responses to IFN
6. Inhibition of the complement cascade by sequestering complement away from the deposited auto-antibodies
Fab-mediated mechanisms
7. Neutralization of various agents (similar to mAb), including chemokines, inflammatory cytokines, and apoptosis-inducing molecules, including FasL
8. Neutralization of auto-antibodies by anti-idiotype Abs – often claimed but never proven to effectively explain the anti-inflammatory potential

These receptors are found on almost all immune cells (**Table 3**) and, upon binding of IgG, mediate a wide range of cellular responses, such as phagocytosis of IgG-opsonized microorganisms or immune complexes, antibody-dependent cellular cytotoxicity (ADCC), activation of the NADPH oxidase, and the release of cytokines.

Importantly, as compared to many innate pattern recognition receptors, human FcγRs are quite different from their murine counterparts in the sense that no clear orthologs can be assigned. As a result, human and murine FcγRs that share nomenclature and CD numbers actually have quite different protein structures, expression patterns, and Ig binding affinities (3). Thus, functional studies on mouse FcγRs can only provide very limited information for understanding the contributions of individual FcγRs to human disease.

STRUCTURE, SIGNALING, AND EXPRESSION

Based on their affinity for monomeric IgG, FcγRs can be divided into the high-affinity FcγRI and the low-affinity FcγRII and FcγRIII (**Figure 1**). Signaling by FcγR is mediated by immunoreceptor tyrosine-based activating (ITAM) or inhibitory (ITIM) motifs that are present either in the cytoplasmic tail of the receptor itself or in non-covalently associated signaling adaptor proteins, such as the common γ-chain (FcRγ; see below). Aggregation of activating FcγR, i.e., those containing or associated with ITAMs, by binding of multivalent ligands, such as an opsonized pathogen or cancer cell, results in the phosphorylation of ITAM tyrosine residues by Src family protein tyrosine kinases (PTKs), and ultimately leads to activation of cellular responses (10). Aggregation of inhibitory FcγR, i.e., those containing ITIMs, also results in phosphorylation of tyrosine residues by Src family PTKs. In contrast to ITAMs, phosphorylated ITIMs serve as binding sites for phosphotyrosine phosphatases (PTPs), which dephosphorylate other proteins resulting in inhibition of activating pathways (11).

FcγRI (CD64) is a 72 kDa protein that has three extracellular (EC) Ig-like domains, involved in binding of IgG, a transmembrane (TM) domain and a short intracellular (IC) domain of 61 amino acids. The TM domain associates with the FcRγ-chain, an adaptor protein containing an ITAM, to induce signaling and maintain stable expression (12). FcγRI is constitutively expressed by monocytes, macrophages, and dendritic cells and its expression can be induced on neutrophils by stimulation with IFN-γ and/or G-CSF (13, 14). Although there are three genes with various transcripts (15, 16), it is generally believed that only one, the FCGR1A transcript, results in the expression of the classical FcγRIa (CD64).

FcγRII (CD32) is actually a collection of three highly homologous proteins, known as FcγRIIa, -b, and -c that all have a molecular mass of ~40 kDa. Their genes are located in one gene cluster at chromosome 1q23.3 (**Figure 2**). In contrast to FcγRI, the FcγRII proteins have only two IgG binding EC domains. On the other hand, the much larger intracellular domains of FcγRIIa,

Table 3 | Expression of FcγRs on different cell types.

	FcγRI	FcγRIIa	FcγRIIb	FcγRIIc	FcγRIIIa	FcγRIIIb
B-cells	−	−	+	−	−	−
T cells	−	−	−	−	−	−
NK cells	−	−	Genotype-dependent[a]	Genotype-dependent[b]	+	−
Dendritic cells	+	+	+	Genotype-dependent?[b]	−	−
Macrophages	+	+	+	Genotype-dependent[b]	+	−
Monocytes	+	+	Subsets	Genotype-dependent[b]	Subsets	−
Neutrophils	Induced	+	Genotype-dependent[c]	Genotype-dependent[b]	−	+
Eosinophils	Induced[d]	+	−	−	−	Induced[d]
Platelets	−	+	−	−	−	−

*Expression of FcγRs on different cell types, derived from our own data [Ref. (4–6), **Figure 3**, data not shown] as well as review of the literature (3).*

*[a] Expression of FcγRIIb occurs in NK cells in individuals with a deletion of CNR1 (**Figure 2**) (4).*

[b] Expression of FcγRIIc is dependent on SNPs in exon3 and intron7 of the FCGR2C gene, which in most individuals is a non-expressed pseudogene (4, 5).

[c] Expression of FcγRIIb on neutrophils strongly correlates with SNPs in the promoter of the FCGR2B gene [promoter haplotype 2B.4 (7), Tsang-a-Sjoe et al., submitted)].

[d] Although FcγRI and FcγRIIIb are definitely absent from eosinophils in the resting state, and are often regarded not expressed by these cells (3), some reports have described inducible expression in vitro for FcγRI and FcγRIIIb (8) and also in vivo for FcγRIII (9).

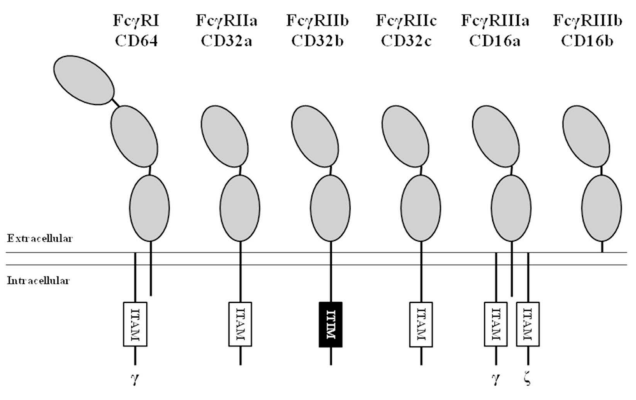

FIGURE 1 | Nomenclature and structure of human FcγRs. All human FcγRs are transmembrane proteins, except the GPI-linked FcγRIIIb. The high-affinity FcγRI contains three extracellular (EC) domains (gray ellipses), the low-affinity FcγRs contain two. All isoforms of FcγRII contain either an immunoreceptor tyrosine-based activating (ITAM, white boxes) or inhibitory (ITIM, black box) motif in their α-chain. FcγRI and FcγRIIIa associate with ITAM-containing adaptor proteins such as the Fc receptor common gamma chain (indicated by "γ") and the CD3 ζ-chain (indicated by "ζ").

-b, and -c harbor intrinsic signaling motifs. In contrast to the activating FcγRIIa and -c, FcγRIIb contains an immunoreceptor tyrosine-based *inhibitory* motif (ITIM) (17). As no other FcγR contains or associates with proteins containing ITIMs, FcγRIIb is the only inhibitory FcγR (18).

FcγRIIa is the most widely expressed isoform of FcγRII and is found on monocytes, macrophages, dendritic cells, neutrophils and platelets.

FcγRIIb is highly expressed on B-cells, where it constitutes the only surface-expressed FcγR. FcγRIIb is also expressed, albeit at much lower levels, on a subset of monocytes, on macrophages, and on dendritic cells. Expression of FcγRIIb can also be detected on neutrophils and NK cells, but only in individuals with certain genotypes [Ref. (4), Tsang-a-Sjoe et al., submitted].

FcγRIIc has long been considered not to be expressed at all, as its gene (*FCGR2C*) was thought to be a pseudogene (19, 20), and therefore, relatively little was known about the expression pattern of this receptor. In 1998, FcγRIIc was first found on NK cells of individuals with a particular haplotype of the receptor (21), but we now know that – apart from NK cells – it can also be expressed on neutrophils and monocytes in the individuals with the appropriate genotype (4, 5) (**Figure 3A**). As this activating FcγRIIc is expressed on circulating monocytes of some individuals, it may be expected that expression also occurs on (monocyte-derived) macrophages of these same individuals, but this has not been reported to date. Here, we show for the first time that monocyte-derived macrophages do indeed express FcγRIIc, at least when cultured in the presence of M-CSF (M2 phenotype), as shown by flowcytometry stainings with MoAb 2B6, which recognizes both FcγRIIb and FcγRIIc (**Figure 3C**). Although these data are difficult to interpret because of the (varying) presence of FcγRIIb on these cells, we can assume the mean difference in MFI between *FCGR2C*-ORF and *FCGR2C*-Stop donors to derive from FcγRIIc. Expression of FcγRIIc specifically was confirmed by qPCR of FCGR2C mRNA and a specific immunoprecipitation using a combination of MoAb 2B6 and a polyclonal antibody that binds FcγRIIc but not FcγRIIb (**Figure 3C**). Monocyte-derived macrophages differentiated with GM-CSF (M1 phenotype) do not seem to express FcγRIIc based on flowcytometry, although also in these cells, low levels of FCGR2C mRNA could be detected, therefore expression of FcγRIIc cannot be ruled out and may occur in later stages of differentiation of M1 macrophages. Recently, it was proposed that FcγRIIc can also be expressed on B-cells (22). However, evidence of expression of this receptor on B-cells on our own cohort of healthy donors was not confirmed on B-cells, irrespective their naïve or memory phenotype (**Figure 3B**; data not shown).

FcγRIII (CD16), similarly to FcγRII, actually represents a collection of two genes, expressed within the gene cluster at chromosome 1q23.3, each encoding proteins with two EC Ig-like domains (**Figures 1** and **2**). Due to differences in glycosylation,

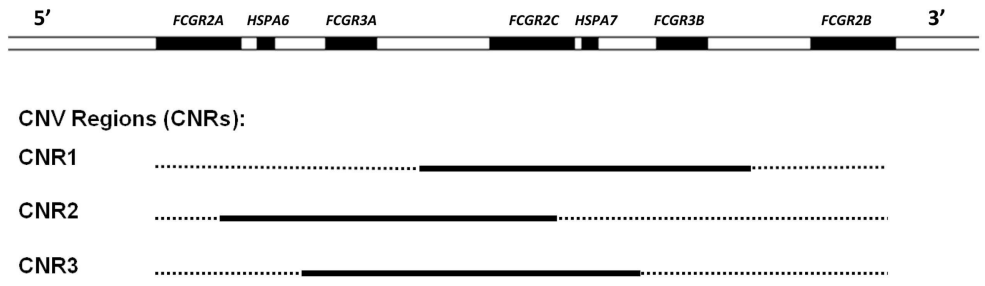

FIGURE 2 | Overview of the low-affinity FcγR gene cluster and the corresponding CNV. Three combinations of FcγR genes have been shown to occur in duplication/deletion. Black lines indicate which genes are involved in CNV.

their molecular masses are in the range of 50–80 kDa. FcγRIIIa is similar to FcγRI in its TM and IC domains. In monocytes and macrophages, this receptor associates with the FcRγ-chain, while in NK cells it associates with the CD3 ζ-chain to induce signaling (26–28). In contrast to FcγRI, association with these adaptor proteins is not only essential for maintaining stable expression but also for targeting the receptor to the cell membrane (28).

FcγRIIIb is a GPI-anchored protein, expressed only on neutrophils and eosinophils. As it does not have a TM domain, it cannot associate with FcRγ or the ζ-chain. Nonetheless, FcγRIIIb has been suggested to induce signaling, although the exact mechanism(s) is still unclear (29, 30), and current believe is that it functions mainly as a decoy receptor (31).

Recently, it has been proposed that a totally different class of receptors, the C-type lectins, may also bind the Fc-region of IVIg (32), and such receptors were termed "type II Fc receptors." In humans, DC-SIGN was proposed to bind IgG with a sialic acid sugar moiety at the Asn297 N-linked glycosylation site of IgG. However, this interaction could not be reproduced by another group (33), which found no binding at all of IgG-Fc regions to DC-SIGN, regardless of the sialylation status. Therefore, with evidence for the interaction of IgG-Fc and DC-SIGN being currently marginal and not broadly supported in the literature, in our opinion, it remains to be seen whether DC-SIGN should indeed be classified as a true IgG-Fc receptor.

GENETIC VARIATION IN FcγR: POLYMORPHISMS

The genes encoding the classical FcγRs are highly polymorphic and functionally relevant genetic variations have been described for all low-affinity FcγRs (**Table 4**).

In *FCGR2A*, encoding for FcγRIIa, a single nucleotide polymorphism (SNP) was first noticed, which results in either a histidine or an arginine at position 131 (H131R) in the IgG binding domain (EC2) (34). FcγRIIa-H131 has a higher binding affinity for IgG1 and especially IgG2, as compared to FcγRIIa-R131, but binding to IgG3 and IgG4 is similar for both variants (38). Functionally, mononuclear cells from FcγRIIa-131HH individuals produce more IL-1beta when stimulated with IgG2 than FcγRIIa-131HR and -131RR individuals (39). Similarly, neutrophils from individuals homozygous for H131 (FcγRIIa-131HH) have been shown to have increased phagocytosis and degranulation in response to serum-opsonized bacteria and increased rosette formation and phagocytosis in presence of IgG3 anti-D sensitized erythrocytes when compared to FcγRIIa-131RR individuals (36, 40).

FCGR2B also exists in two allelic variants, encoding for FcγRIIb containing either an isoleucine or a threonine at position 232 in the TM domain (35). As this SNP (I232T) does not affect the IgG-binding EC domains, it has no influence on the binding affinity. However, its localization at the TM domain results in differences in downstream signaling and subsequent inhibition of FcγRI signaling in macrophages and BCR signaling in B-cells. In particular, I232 provides stronger inhibitory signaling than T232, and this is caused by the exclusion from lipid rafts of FcγRIIb-T232 (41, 42). As FcγRIIb is the only inhibitory FcγR, it has a central role in the regulation of immune responses. The loss-of-function FcγRIIb-T232 has been linked to susceptibility and/or severity of several auto-immune diseases, particularly SLE (43–45), and also in rheumatoid arthritis (RA) (46) and ITP (47).

Inter-individual variation in FcγRIIb is also found in expression patterns and levels. Similar to the I232T SNP, the important immune-regulatory role for FcγRIIb is also reflected in the observations of aberrant expression levels of FcγRIIb in SLE, RA, ITP, and chronic inflammatory demyelinating polyneuropathy (7, 48–51). As a result of a deletion in the *FCGR* locus that includes *FCGR2C*, *FCGR3B* and is called CNR1, FcγRIIb can surprisingly also be expressed on the surface of NK cells, where it is capable to inhibit killing of target cells in ADCC (4). Expression of FcγRIIb in other cells is hardly affected by this deletion. Furthermore, two SNPs in the proximal promoter of *FCGR2B* and *FCGR2C*, a guanine or cytosine at position −386 and a thymine or adenine at position −120, form four haplotypes of which one (−386G, −120A; 2B.3) has never been found in any individual thus far. In case of *FCGR2B*, the wildtype promoter (−386G, −120T; 2B.1) has a lower transcriptional activity than one of the other haplotypes (−386C, −120A; 2B.4) [Ref. (51); Tsang-a-Sjoe et al., submitted].

In case of *FCGR2C*, only the wildtype and one other promoter haplotype (−386C, −120T; 2B.2) are found. Moreover, the 2B.2 haplotype is linked to another polymorphism in *FCGR2C* (5). This other polymorphism, a SNP in exon 3, determines whether

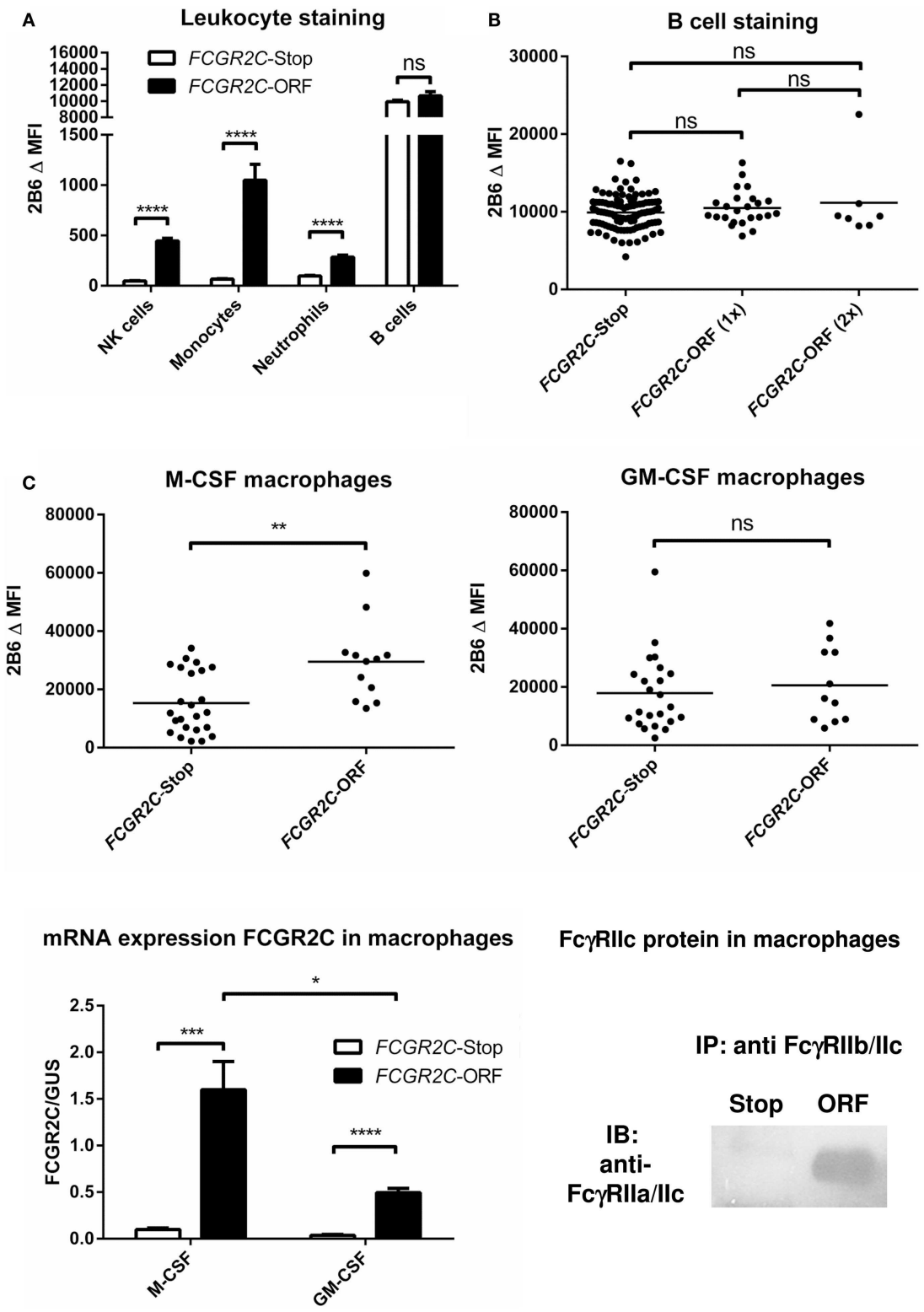

FIGURE 3 | FcγRIIc expression on various cell types in healthy human subjects.

(Continued)

FIGURE 3 | Continued
(A) Expression of FcγRIIc and FcγRIIb on circulating leukocytes. Figure adapted from van der Heijden et al. (4), now including measurements from additional individuals. MoAb 2B6 recognizes an extracellular epitope of both FcγRIIb and FcγRIIc, but since *FCGR2C*-Stop individuals cannot express FcγRIIc, the difference in MFI between *FCGR2C*-Stop and *FCGR2C*-ORF individuals can be assumed to derive from expression of FcγRIIc. *FCGR2C*-Stop (individuals with zero copies of *FCGR2C*-ORF) $n = 105$; *FCGR2C*-ORF (including individuals with one and two copies of *FCGR2C*-ORF) $n = 31$. Means + SEM are shown. **(B)** Detailed analysis of MoAb 2B6 staining on circulating B-cells, showing individual measurements, reveals no evidence of expression of FcγRIIc on B-cells. *FCGR2C*-Stop $n = 105$; *FCGR2C*-ORF(1x), individuals with one copy of *FCGR2C*-ORF, $n = 24$; *FCGR2C*-ORF(2x), individuals with two copies of *FCGR2C*-ORF, $n = 7$. **(C)** Expression of FcγRIIc on macrophages. Upper panel: MoAb 2B6 staining on monocyte-derived macrophages cultured for 9 days from 36 healthy individuals, performed as previously described (79). *FCGR2C*-Stop: $n = 24$; *FCGR2C*-ORF, individuals with one ($n = 11$) or two ($n = 1$) copies of the *FCGR2C*-ORF allele. Data are shown for M-CSF (left) and GM-CSF (right) cultured macrophages. Lower left panel: detection of FCGR2C mRNA in monocyte-derived macrophages cultured for 9 days. qPCR with FCGR2C1-specific primers was performed as previously described (5), using cDNA from M-CSF monocyte-derived macrophages as a calibration curve, as described in (79). *FCGR2C*-ORF, individuals with 1 *FCGR2C*-ORF allele, $n = 3$; *FCGR2C*-Stop, individuals with zero copies of *FCGR2C*-ORF, $n = 4$. Means + SEM are shown. Lower right panel: immunoprecipitation of FcγRIIc from M-CSF monocyte-derived macrophages confirms expression of FcγRIIc. Experiment was performed essentially as described in Ref. (4), in this case using MoAb 2B6 to capture FcγRIIc (and FcγRIIb), followed by a specific staining for the intracellular part of FcγRIIc with a rabbit polyclonal antibody against the cytoplasmic tail shared by FcγRIIc and FcγRIIa (25), in macrophages from an individual with zero copies of *FCGR2C*-ORF (Stop), and an individual with one copy of *FCGR2C*-ORF (ORF). Data are representative of three independent experiments with different individuals.
For reasons of simplicity, in this figure, individuals with the non-classical *FCGR2C*-ORF allele that is not expressed (4) [$n = 6$ for **(A,B)**, were grouped with *FCGR2C*-stop individuals. Individuals with a deletion of CNR1 (*FCGR2C* and *FCGR3B* genes], which leads to ectopic expression of FcγRIIb on NK cells (4) ($n = 14$), were left out of the analysis of NK cells in **(A)**. Statistical significance was tested by Mann–Whitney *U* test. ns ($p > 0.05$); $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$. Δ MFI: median fluorescence intensity of MoAb 2B6 minus median fluorescence intensity of isotype control. Some individuals were analyzed more than once at different time points with similar results, means are shown for these individuals.

Table 4 | Functionally relevant genetic variation in FcγR.

Gene	Type	Variants	Functional relevance
FCGR2A	SNP	H131, R131	H131 has a higher binding affinity for IgG1 and IgG2 than R131 (34)
FCGR2A	Splice site mutation	c.739 + 871A, c.739 + 871G	c.739 + 871A > G leads to splice variant FcγRIIa^{exon6*}, which shows increased cellular activation
FCGR2B	SNP	I232, T232	I232 inhibits FcγRI as well as B-cell receptor signaling more strongly than T232 (35)
FCGR2B, *FCGR2C*	Promoter haplotype	2B.1, 2B.2, 2B.3, 2B.4	2B.2 is linked with an ORF in *FCGR2C* (5). 2B.4 results in increased transcription of *FCGR2B* (7)
FCGR2C	SNP	Q13, Stop13	Q13 leads to an ORF in exon 3 and expression of FcγRIIc (21), when combined with c.798 + 1G
FCGR2C	Splice site mutation	c.798 + 1G, c.798 + 1A	c.798 + 1A leads to mis-splicing and lack of expression of FcγRIIc (4)
FCGR3A	SNP	V158, F158	V158 has a higher binding affinity for all human IgG isotypes than F158 (52)
FCGR3A	CNV	1, 2, 3, or 4 copies	Copy number relates to expression levels of FcγRIIIa and NK-cell IgG-mediated ADCC (24)
FCGR3B	Polymorphic variants	NA1, NA2, SH	NA1 phagocytizes more efficiently than NA2 (40, 57)
FCGR3B	CNV	0, 1, 2, 3, or 4 copies	Copy number relates to expression levels of FcγRIIIb and the binding and uptake of IC's

or not individuals can express FcγRIIc at all. This C > T mutation results in either an open-reading frame (*FCGR2C*-ORF, allele frequency ~10–15% in Caucasians) or a stop codon (*FCGR2C*-Stop) (5). Although expression on NK cells is low, it has been shown to be capable of inducing killing of target cells in a redirected ADCC assay (5). Classically, ORF/Stop genotyping of individuals is done based on this SNP alone. However, we have recently found that some individuals carry splice site mutations in intron 7 that introduce novel stop codons, leading to a loss of FcγRIIc expression (4), and genotyping *FCGR2C* should include these novel mutations to provide an accurate prediction for FcγRIIc expression.

The FcγRIIIa-encoding *FCGR3A* gene contains a SNP that results in either a valine or a phenylalanine at position 158 (V158F), located in the second EC domain (52). FcγRIIIa-V158 has a higher binding affinity for all human IgG classes compared to FcγRIIIa-F158 (38). In ADCC assays, NK cells from FcγRIIIa-V158 donors show increased killing of target cells that are opsonized with sub-saturating levels of Rituximab (53).

FcγRIIIb-encoding *FCGR3B* gene exists in three polymorphic variant proteins, NA1, NA2, and SH, which are also known as HNA-1a, -1b, and -1c, respectively (54, 55). FcγRIIIb-NA1 and -NA2 nucleotide sequences differ at five positions [G > C at nucleotide (nt) 141, C > T at nt 147, A > G at nt 227, G > A at nt 277, and G > A at nt 349], with four predicted amino acid differences (R36S, N65S, D82N, and V106I for NA1 and NA2, respectively). As a consequence, the NA2 variant has two additional N-linked glycosylation sites, compared to NA1. The SH variant is identical to NA2 at the five positions that distinguish NA1 from

NA2, but differs from both variants at one additional position (C > A at nt 266), resulting in an A78D amino acid change that predicts a change in the tertiary structure of the protein. Additional complexity is added by the discovery of rare individuals carrying other mutations within this gene or different combinations of these nucleotide polymorphisms (37, 56), indicating that the NA1/NA2/SH typing is incomplete. While the binding affinities for IgG1 and IgG3 appear similar between the three variants (38), neutrophils from FcγRIIIb-NA1NA1 individuals bind and phagocytize IgG-opsonized bacteria and red blood cells more efficiently than those from FcγRIIIb-NA1NA2 and -NA2NA2 individuals (40, 57).

GENE COPY NUMBER VARIATION

Besides being polymorphic, some of the low-affinity *FCGR* genes are subject to gene copy number variation (CNV). Although several large-scale studies on CNV have suggested that human *FCGR2A* and *FCGR2B* are candidate genes for CNV (58–61), our group has shown previously that this is not the case. In fact, CNV in the *FCGR* locus is restricted to *FCGR2C*, *FCGR3A*, and *FCGR3B* (24). It occurs in three different combinations: *FCGR3A/FCGR2C* (two possibilities with slightly different borders to the CNV region), and *FCGR2C/FCGR3B* (**Figure 2**).

Copy number variation translates into differences in expression levels of FcγRIIc (in case of *FCGR2C*-ORF), FcγRIIIa, and FcγRIIIb, with more gene copies leading to a higher receptor expression (and vice versa) (21, 62, 63). In case of FcγRIIIa, the level of expression on NK cells is, at least for 1 versus 2 copies, related to the level of killing of target cells in (redirected) ADCC assays (24). Increased expression of FcγRIIIb leads to higher binding and uptake of immune complexes (ICs) (64).

As is the case with polymorphic variants, CNV in *FCGR* genes is associated with several auto-immune diseases. Our group has previously shown that *FCGR2C*-ORF predisposes for ITP. The SNP in exon 3 causing an open-reading frame instead of a stop allele of *FCGR2C*, results in the expression of FcγRIIc and thus behaves as if it were CNV of *FCGR2C*-ORF. However, individuals can have an increased CNV at this locus of three *FCGR2C*-Stop alleles without increased risk since only the ORF allele was shown to predispose to ITP (5).

Although we could not find an association with various disease cohorts (24), an increased copy number of *FCGR3A* has been observed in anti-glomerular basement membrane antibody disease (anti-GBM disease) (65).

In contrast, a low copy number of *FCGR3B* has been shown to be a risk factor for SLE, even when linkage disequilibrium between *FCGR3B* CNV and FcγR SNPs that have previously been shown to be associated with SLE is taken into account (66–70). Similar associations have been reported for Sjögren's syndrome (67), systemic sclerosis (71), and RA (72, 73), although other reports have shown no association with RA (67, 74).

POTENTIAL WORKING MECHANISMS FOR THE IMMUNOMODULATORY EFFECT OF IVIg

Potential mechanisms can be divided into two categories, being dependent on either the Fc part or the Fab part of the IgG molecule. For some indications, such as ITP, clinical studies with human subjects have in fact already revealed what part of the IgG molecule is effective, as preparations with only Fab fragments of IVIg were not effective (75), whereas purified Fc fragments did have a good clinical effect (76). Thus, we know that at least for ITP, the immunomodulatory effect is Fc-mediated, although this may be different for other indications. Here, we focus most on Fc-mediated modes of action, and will briefly discuss Fab-mediated mechanisms.

Fc-MEDIATED WORKING MECHANISMS

BLOCKADE OF ACTIVATING FcγR BY SATURATION AS A RESULT OF HIGH IgG CONCENTRATIONS

Administration of IVIg greatly increases the total concentration of IgG in the recipients' plasma and extracellular fluid, and with such an increase, more FcγRs may be bound by circulating non-complexed IgG, thereby saturating the FcγRs and making them less available for auto-antibodies in oligo- or polymeric complex with their (auto)antigen. The idea that especially the low-affinity FcγRs can be blocked by their monomeric ligand *in vivo* may at first hand seem surprising, but it has been shown in the past that also low-affinity receptors bind monomeric IgG (77), indicating that some "low-affinity" FcγRs are not so low-affinity, and maybe should be better named "medium-affinity," especially in the case of FcγRIIa and FcγRIIIa (38). Greatly increasing the concentration of monomeric IgG above the normal plasma levels may shift the equilibrium toward a situation in which too many FcγRs are occupied for proper functioning – which may in part explain the immunomodulatory actions of IVIg under some of the conditions for which IVIg is used. Saturation of activating FcγRs was one of the first theories that was formulated to explain the working mechanism of IVIg (2), and this "classic" mechanism has for a long time been assumed as the most plausible explanation for the effect of IVIg in ITP (23, 78). Circumstantial evidence for this theory derives from observations that IgG preparations with increased affinity for FcγRs appear to have an increased effect (6, 79), and that in all diseases in which an immunomodulatory effect is wanted, high doses of IVIg are needed. Nevertheless, there is no formal proof for this concept, and although it has never been disproven, focus has shifted away from this theory as other explaining theories arose.

UPREGULATION OF THE INHIBITORY FcγRIIb AS A RESULT OF SIALYLATED IgG-Fc

Over the past decade, the prevailing theory for the working mechanism of IVIg in most immunomodulatory situations has become that IVIg induces an upregulation of the inhibitory FcγRIIb on effector cells. More specifically, a fraction of IVIg, i.e., the IgGs containing a sialic acid sugar residue at the end of the N-linked glycosylation site at Asn297, would be responsible for this effect by binding to SIGNR1 (mouse), or its human ortholog DC-SIGN, inducing various signaling cascades ultimately leading to the upregulation FcγRIIb. This theory has recently been excellently reviewed in Ref. (80). However, the major problem with this theory is that many findings could not be reproduced by other research groups. For instance, we have recently found that FcγRIIb is not upregulated in human macrophages in response to IVIg, but nevertheless, these macrophages respond very well to IVIg treatment,

being inhibited in phagocytosis (79). The role of FcγRIIb in ITP treatment by IVIg was also questioned in mouse studies (81). Similarly, we found that IgG-Fc sialylation was not important for the effect of IVIg on human macrophages (79), and many groups have recently published evidence that IgG-Fc sialylation of IVIg is not required for the immunomodulatory effects (82–85). As mentioned before, even the binding of sialylated IgG-Fc to DC-SIGN could not be reproduced (33). Furthermore, essentially all the evidence supporting this theory derives from murine studies, which may not be translated to the human situation, as mice and humans extensively differ in FcγR expression. Many of the murine studies describing this theory for instance use a model for arthritis, but IVIg has never proven to be a useful therapy in treating arthritic patients (86–88).

On the other hand, glycosylation may still be important, influencing the binding affinity IgG molecules to the various FcγRs. For instance, the binding affinity of FcγRIIIa is undoubtedly influenced by the level of fucosylation of the Fc-domain of IgG, a notion that may help to develop new, afucosylated IgG treatment options (89, 90). An important question will be whether the anti-inflammatory properties are directly influenced by afucosylated IgG or IgG otherwise modified in their glycosylation status.

INCREASED CLEARANCE OF PATHOGENIC ANTIBODIES BY SATURATION OF THE NEONATAL FcR

FcRn is a receptor expressed by human endothelial cells to recycle plasma IgG, extending its half-life in the circulation (91, 92); saturating this "rescue-receptor" with a high dose of IVIg may shorten the half-life of all IgG including harmful auto-antibodies. Interestingly, for a number of diseases in which IVIg therapy is beneficial, plasmapheresis, aiming to remove pathogenic auto-antibodies by replacing the patients' plasma with donor plasma is also a good option. This is for instance the case in Guillain Barre syndrome (93, 94). On the other hand, plasmapheresis is not effective in ITP (95, 96), and so apparently, rapid removal of auto-antibodies is not effective in ITP, suggesting that the effect of IVIg in ITP must be exerted in a different way.

BALANCE FROM PRO- TO ANTI-INFLAMMATORY REACTIVITY BY MODULATING DENDRITIC CELLS

Recent data have confirmed the expression of FcγRII isoforms, including FcγRIIb (97, 98) on dendritic cells (DCs), which may help to explain the subsequent steps in which inhibition of autoantibody release by B-cells, inhibition of T-helper (Th)1 and Th17 differentiation, and enhancement of $CD4^+FoxP3^+$ regulatory T cells (Treg), helps to modulate certain unwanted (auto)inflammatory responses. IVIg may be able to reset the balance at the level of DCs, involving not only the classical IgG receptors but also non-classical lectin-like surface molecules, as has been repeatedly proposed during the last decade (33, 99). We should emphasize that the relevance of such mechanisms and non-classical IgG receptors remain to be shown in humans for the IVIg-associated effects for immunomodulation.

REDUCING RESPONSES TO IFN

A recent report showed an increased expression of type I interferon response genes in ITP patients, which was rapidly reduced in patients after receiving IVIg, leading to decreased expression of FcγRIII on monocytes, thereby altering the balance between activating and inhibiting FcγRs (100). The relevance of interferons in such responses is unclear, as interferon response genes have been found in various diseases, among which is SLE, and sometimes independent of clear-cut reaction to treatment and clinical response to therapy (101, 102).

Inhibition of the complement cascade

Inhibition of the complement cascade by sequestering complement away from the deposited auto-antibodies as suggested in dermatomyositis (103). On the other hand, with the recent insight that IgG is only able to activate complement by means of generating hexamers, and not as single molecules or dimers (104), it is less likely that complement scavenging roles can realistically be involved in the anti-inflammatory IVIg-mediated effects.

Fab-MEDIATED WORKING MECHANISMS

NEUTRALIZATION OF AUTO-ANTIBODIES BY ANTI-IDIOTYPE Abs

One of the first explanations for the anti-inflammatory effect of IVIg was that there are anti-idiotypic antibodies present in the IVIg that neutralize the pathogenic auto-antibodies. This theory is often claimed but to our knowledge has only been proven to effectively explain the anti-inflammatory potential of IVIg in the case of neutralizing antibodies to coagulation factor VIII, which could be inhibited by anti-idiotypic antibodies in IVIg (105).

NEUTRALIZATION OF ENDOGENOUS CHEMOKINES, INFLAMMATORY CYTOKINES, AND APOPTOSIS-INDUCING MOLECULES

Apart from the known microbial antigen-specific binding properties, IgG preparations also contain neutralizing and clearance-enhancing antibodies that may switch a proinflammatory trigger into an anti-inflammatory condition. This suggests that healthy individuals from which plasma is collected and pooled for therapeutic IgG preparations already contain autoreactive "natural" antibodies at low levels in their blood. The infusion of such natural antibodies into the patient may be sufficient to reset certain diseases by the cross-reactive capacity of such natural "auto"antibodies (106–110).

ROLE OF Fc-GAMMA RECEPTORS IN SHAPING THE IMMUNE RESPONSE IN RELATION TO THE POTENTIAL ACTIONS OF IVIg

Clearly, the different genetic FcγR variants may not only be a risk factor for the development of some auto-immune diseases but may possibly also influence the efficacy of treatment of these diseases by IVIg. Indeed, some SNPs can be overrepresented in Kawasaki disease (KD) patients that respond well to IVIg therapy, but not in the non-responders. Among KD patients, patients who respond well to IVIg have been reported to more often carry the promoter polymorphism 2B.4 in *FCGR2B* and the FcγRIIIb-NA1 when compared to non-responders (111, 112). In both these cases, the balance between activating and inhibitory receptor signaling is altered. A shift toward the inhibitory side of the balance increases the efficacy of IVIg treatment, while a shift toward the activating side shows the opposite effect. Connections of IVIg efficacy in KD and/or other auto-immune diseases with other polymorphisms or CNV in FcγR have not been found to date. Given the

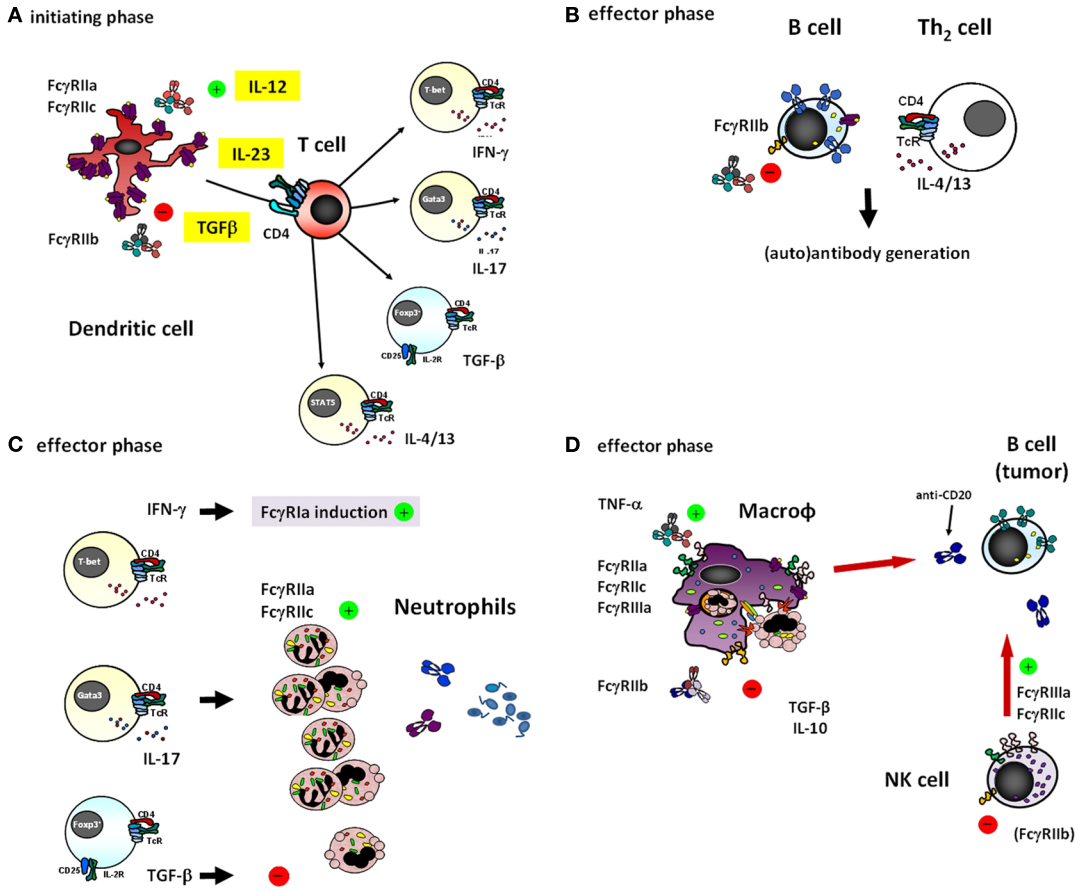

FIGURE 4 | The different ways of how IVIg may influence an immune response involving (auto)-antibodies by interfering with the function of the different FcγRs on various cell types. **(A)** dendritic cells (DCs) play a major role in the initiating phase of an antibody response, and expression and function of different FcγRs on these cells may influence this response in several ways. For instance, ligation of FcγRIIa on DCs has been shown to influence the cytokine secretion by DCs in response to various TLR ligands, promoting Th17 responses (98). Furthermore, expression of FcγRIIa on myeloid DCs was recently shown to be downregulated by IVIg in an IL-33 dependent manner, which resulted in a Th2 cytokine response (97). As the co-stimulatory signals derived from DCs determine T cell function, they are ultimately important in shaping an antibody response, as well as the extent of generating a neutrophil-driven response. T cells themselves are crucial in determining the differentiation of most B-cells necessary for the antibody response, but are not known to express any classical FcγRs or other IgG receptors themselves, suggesting that they may be influenced by IVIg in indirect ways only. **(B)** Differentiation of B-cells into antibody producing cells is a major component of an (auto)-antibody response, and involves somatic hypermutation, class switch recombination and plasma cell differentiation. B-cells express high levels of FcγRIIb, and ligation of this receptor has been shown to dampen B-cell receptor signaling. Recently, it was proposed that also FcγRIIc is expressed on B-cells in some individuals, and may influence the immune response in vaccination settings, although it appears that the expression of FcγRIIc on B-cells is very limited if present at all, especially when compared to expression of FcγRIIb (see also **Figure 3B**). **(C)** Once an (auto)-antibody is formed, innate effector cells such as neutrophils are determinants of tissue damage at the site of deposited immune complexes by release of toxic components such as reactive oxygen species or proteases. Neutrophils express FcγRIIa and FcγRIIc in some individuals, which can contribute to tissue damage and inflammation, and may be influenced by IVIg. Following the afferent immunological phase, the effector phase of activated neutrophils may be in part driven by the presence of certain Th subsets. The "inflammation-promoting" Th17 activity may be counterbalanced by Tregs inducing immunosuppressive properties by TGF-β1 as indicated by expression of neutrophil IL-10 and IL-6, indoleamine 2,3-dioxygenase (IDO), heme oxygenase-1, and "suppressor of cytokine signaling-3" (SOCS3) (113), although the production of these cytokines by human neutrophils remains controversial. **(D)** In case of natural or therapeutic antibody responses against cellular targets, these targets can be eliminated by cells of the innate immune system, for instance through antibody-dependent phagocytosis by macrophages, or antibody-dependent cellular cytotoxicity by NK cells (or monocytes and neutrophils). These responses are mediated by FcγRs on these effector cells, which could be influenced by IVIg in a direct way, i.e., saturating activating FcγRs by binding to them, or in a more indirect way as a result of upregulation of the inhibitory FcγRIIb on effector macrophages (discussed in the text).

growing number of diseases in which IVIg therapy is successfully used and the number of possible working mechanisms that involve FcγR, it does not seem unlikely that more such connections exist. The ways in which IVIg may interact with FcγRs to exert its immunomodulatory actions are multiple, since many different FcγRs are expressed by different immune cells. An overview of the potential interactions is given in **Figure 4**. One special case is the FcγRIIc, discussed in more detail below.

FcγRIIc AS COMMON DENOMINATOR IN TIPPING PRO- OR ANTI-INFLAMMATORY BALANCES?

As mentioned above, a SNP in exon 3 of *FCGR2C* determines whether or not individuals can express FcγRIIc at all (5). Although expression on NK cells is low, it has been shown to be capable of inducing killing of target cells in a redirected ADCC assay (5). We could also detect FcγRIIc expression on neutrophils and monocytes in individuals with an ORF allele (**Figure 3A**) (4). When investigating surface FcγRIIc expression on monocyte-derived macrophages skewed to either M1 or M2 phenotype, the M-CSF-cultured cells were clearly expressing FcγRIIc (**Figure 3C**).

Surprisingly, a recent report also found FcγRIIc expression on B-cells (22). Upon transfection into a murine B-cell line, the co-ligation of FcγRIIc with the BCR resulted in enhanced and more sustained tyrosine phosphorylation of the key B-cells signaling components Syk and BLNK. In contrast, the engagement of FcγRIIb with the BCR and its activation caused a reduced level of Syk and BLNK phosphorylation. Antibodies generated upon immunization in this transgene mouse model were found to be enhanced, coinciding with a higher level of B-cell activation (22). In a cohort of about 300 individuals of unknown ethnicity the levels of Ab against a neoantigen (Anthrax protein) were tested (22). At the earliest time points in the vaccine study donors homozygous for the *FCGR2C*-ORF allele [i.e., two alleles ($n = 11$)] showed higher Ab levels at 4 weeks ($p < 0.02$) but not any longer at 8 weeks (22). The more common single-ORF donors were not included.

Although interesting, direct proof of FcγRIIc expression in human B-cells is lacking, as the protein was only specifically detected in EBV-transformed B-cell lines (22). Stainings with an antibody detecting both FcγRIIb and FcγRIIc showed a difference between Stop and ORF donors (22), suggesting some expression of FcγRIIc in primary and memory B-cells. However, we performed similar stainings in a much larger cohort and did not detect such differences. Hence, we must conclude that the expression of FcγRIIc on primary B-cells is at most marginal if present at all.

In fact, the myeloid expression on macrophages and DCs of FcγRIIc may be held responsible for the earlier peak in Ab generation in *FCGR2C*-ORF-positive donors. Not only the macrophage as effector mechanism in immune responses could be relevant for adaptive immunity including Ab generation but also the afferent part of adaptive responses may be involved, as may also be suggested for FcγRIIc-expressing human DCs in *FCGR2C*-ORF individuals. Thus, we hypothesize that FcγRIIc may be a subtle but relevant genetic factor in the fine balance between health and disease, including the way the immune response will shape the adaptive repertoire as indicated by the immunization studies mentioned above (22) (**Figure 4**, overview).

CONCLUSION

The Fc-gamma Receptors constitute the major receptors for human IgG. There may be low-affinity receptors with lectin-like binding properties that have been suggested to bind a fraction of IgG depending on IgG glycosylation, such as sialylation, but definite proof awaits further study. The beneficial effects of sialylated IVIg in mice are model-dependent, and evidence that sialylation of IgG plays a role in humans has not been generated thus far. Evidence for a prominent role of DC-SIGN in mediating the anti-inflammatory activity of IVIg in humans is also lacking. Thus, clinical application of sialic acid-enriched IVIg in humans is supposed not to be superior to conventional IVIg. In fact, the classically proposed mechanism of IVIg saturating the FcγRs still appears to be the most logical explanation for the immunomodulatory effects in at least some diseases for which it is indicated, for instance in ITP. However, different mechanisms may be at work in other inflammatory diseases for which IVIg is used, and it is not unlikely that different mechanisms act in concert. Fab-mediated IVIg actions may be relevant for some indications, but clinical studies have in the past ruled out Fab-mediated mechanisms to be important for ITP. Knowledge on whether the immunomodulatory effect of IVIg for a given indication is Fc- or Fab-mediated may become very important if alternative (i.e., not donor-derived) sources of IgG are to be used in the future. When the effects of IVIg are Fc-mediated, the polyclonal aspect of IVIg is clearly not important, and recombinant IgG preparations may suffice, which can then be specifically modified to enhance function. On the other hand, for indication in which the effects are Fab-mediated, the polyclonality is likely to be very important, and recombinant preparations can only be successful if the relevant clones can be identified and expanded for therapeutic IgG production. However, the results of the ITP studies preclude further clinical trials with Fab-only or Fc-only preparations for other indications, as this may withhold patients a currently effective therapy – which clearly is unethical. Hence, it will remain difficult to determine the relative importance of Fab and Fc for indications other than ITP in the human situation.

Although the sialylation of IgG-Fc appears not to be relevant for its immunomodulatory effect, the glycosylation status of IgG may still be important for its function, as the properties of IVIg preparations can for instance be dependent on the level of fucosylation, having effect on the binding affinity to FcγRs. Further studies will help to resolve the effects of the different glycosylation moieties of IgG-Fc on the interactions with the various receptors for IgG-Fc, and the relevance of these interactions for IVIg function. Finally, FcγRIIc is one novel activating IgG receptor that may add to tip the balance of immune responses, which needs further in-depth study, using proper detection methods to obtain evidence by genotyping and biochemistry.

Concluding, an enormous increase in insight has been generated during the last decade that may help to improve IgG therapy, either as supplement or anti-inflammatory approach. Further studies related to glycosylation may be highly relevant in this respect, but the immunomodulatory effects of IVIg seem not to be determined by the level of sialylation as studied in-depth over the last decade.

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Intravenous immunoglobulin and immunomodulation of B-cell – *in vitro* and *in vivo* effects

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Intravenous immunoglobulin (IVIG) is used as replacement therapy in patients with antibody deficiencies and at higher dosages in immune-mediated disorders. Although different mechanisms have been described *in vitro*, the *in vivo* immunomodulatory effects of IVIG are poorly understood. Different studies have suggested that IVIG modulates B-cell functions as activation, proliferation, and apoptosis. Recently, it was shown that IVIG induces *in vitro* B-cell unresponsiveness similar to anergy. In accord with this, we recently reported that IVIG therapy in patients affected by common variable immunodeficiency (CVID) interferes *in vivo* with the B-cell receptor (BCR) signaling by increasing constitutive ERK activation and by reducing the phosphorylated ERK increment induced by BCR cross-linking. Moreover, we observed that IVIG induces in CVID patients an increase of circulating $CD21^{low}$ B-cells, an unusual population of anergic-like B-cells prone to apoptosis. Therefore, IVIG at replacement dose *in vivo* could prime B-cells to an anergic, apoptotic program. Here, we discuss these recent findings, which may improve our understanding of the immunomodulatory effects of IVIG, individualizing single involved molecules for more specific treatments.

Keywords: intravenous immunoglobulin, B lymphocytes, immunomodulation, $CD21^{low}$ B-cell, autoimmune disease

Originally used only in primary and secondary immune deficiencies, intravenous immunoglobulin (IVIG), as a safe and efficacious therapy, has now increasingly been used for the treatment of different autoimmune and systemic inflammatory diseases (1). One of the most intriguing properties of IVIG preparations is the spectrum of interactions with our immune system involving almost all its components (2). The effects of these interactions [mediated by both the antigen-binding F(ab′)2 fragment and the Fc fragment of the IgG molecule] vary from one disease to another. In addition to antibodies against pathogens, a broad range of naturally occurring autoantibodies with the capacity to regulate important immune functions are present in IVIG. Despite the wide use of IVIG for immunodeficiencies as well as for various autoimmune and inflammatory disorders (indicated and off-label), the exact mechanisms of their immunomodulation remain not fully elucidated and somewhat controversial.

IgG antibodies are important for protecting us from microbial infections, but IgG autoantibodies are also major pathogenetic factors in several autoimmune diseases that benefit from IVIG therapy. Typical examples of these conditions are immunothrombocytopenia, autoimmune hemolytic anemia, and chronic inflammatory demyelinating polyneuropathy (CIDP). Therefore, the same class of molecules that promotes pathology in a disease can also be used as an anti-inflammatory treatment for the very same disease and this has been referred to as the intravenous IgG paradox (2).

B lymphocytes, unique cells with an immunoglobulin as a part of the B-cell receptor (BCR), are capable of interacting with IVIG in many ways. Our knowledge of these interactions is incomplete and largely based on *in vitro* experiments or on animal models, showing profound influence of IVIG on B-cell functions. The most important effects of IVIG on B-cells interfere with the fine balance of negative and positive signals, which maintain an appropriate B-cell activation threshold, critical for immune tolerance, and autoreactivity.

IVIG AND B-CELL INHIBITORY RECEPTORS BINDING

Interaction of the BCR with the antigen results in signal transduction, which leads to the modulation of gene expression, resulting in activation, anergy, or apoptosis of B-cells. The role of co-receptors expressed on the B-cell surface is to modulate BCR signaling either positively or negatively. These co-receptors include the low-affinity receptor for IgG (FcγRIIb), CD22, and CD72, which negatively regulate BCR signaling, prevent overstimulation of the B-cells and are thus called inhibitory BCR co-receptors (3).

It has been shown that IVIG may interact with almost all these co-receptors significantly influencing B-cell fate.

IgG antibodies are glycoproteins that contain a carbohydrate moiety attached to each of the asparagine 297 residues in the two chains of the antibody Fc fragment. This glycan moiety is an integral structural component of the IgG molecule, forming part of the scaffold for FcγR binding. In addition, depending on the variable region sequences, nearly 20% of serum IgG antibodies have a F(ab′)2 fragment-attached N-linked sugar side chain (4). In 2006, Kaneko et al. for the first time demonstrated that IgG glycosylation and terminal sialic acid (SA) residues are crucial for IVIG activity in mice (5). Moreover, it was shown that only the enrichment of terminal SA residues of the Fc, but not of the F(ab′)2, fragments increased the therapeutic activity of IVIG (6).

These effects in B-cells are mostly mediated through the interaction of IVIG with CD22, a receptor belonging to the SA – binding Ig-like lectin (Siglec) superfamily. CD22 has seven immunoglobulin (Ig)-like extracellular domains and a cytoplasmic tail containing six tyrosines, three of which belong to the ITIM sequences. Unlike most other proteins from the immunoglobulin superfamily, Siglecs do not bind protein determinants but recognize exclusively sialylated carbohydrates. Sialylated glycans are usually absent on microbes but abundant in higher vertebrates and might therefore provide an important tolerogenic signal. CD22 plays a critical role in establishing signaling thresholds for B-cell activation. It is the dominant regulator of calcium signaling on conventional B2 lymphocytes (7). Séïté et al. proved that SA–IVIG colligation to CD22 promotes apoptosis via inhibiting the cascade of kinase phosphorylation in mature human tonsil B lymphocytes and in human Ramos lymphoma B-cell lines by inducing phosphorylation of ITIM (8). They also showed that only SA-positive IgG, but not SA-negative IgG bind to CD22, acting on several BCR-signaling pathways, including inhibition of the phospholipase Cγ2 cascade, sustained activation of extracellular signal-regulated kinases 1/2 (Erk1/2), p38, and down-regulation of PI3K. These changes are associated with the induction of cyclin-dependent kinase inhibitor p27Kip1, which inhibits cell-cycle progression at the G1phase and thus promotes apoptosis (8). Nevertheless, other authors, using CD22-deficient mice in models of ITP and K/BxN arthritis, could not demonstrate a role for CD22 in the immediate anti-inflammatory activity of IVIG (9).

FcγRIIb, another important B-cell inhibitory receptor, is a low-affinity single-chain receptor that carries an ITIM motif in its cytoplasmic domain, a hallmark of this inhibitory protein family. With the exception of T cells and NK cells, FcγRIIb is expressed on all cells of the immune system, and it is the only classical Fc receptor on B-cells. It regulates activating signals delivered by immunocomplexes retained on dendritic cells to the BCR (10). The inhibitory FcγRIIb on B-cells, by ITIM-dependent regulation of BCR signaling, is important in maintaining immune tolerance, thus preventing autoimmune disease. IgG immune complexes can colligate the FcγRIIb to the BCR, leading to inhibition of BCR-induced Ca2+ signals and cellular proliferation (11). It was demonstrated that FcγRIIb represents a checkpoint of human self-tolerance, probably during late stages of B-cell maturation (12). It was shown on murine and human cells that FcγRIIb controls bone marrow plasma cell persistence and apoptosis (13). Moreover, it was found that the isolated cross-linking of FcγRIIb on B-cells leads to B-cell apoptosis via ITIM- and SHIP-independent and c-Abl-family kinase-dependent pathways (14).

The study of Samuelsson et al. provided the first evidence that FcγRIIb is required for IVIG efficacy in mouse ITP (15). Animal models showed that *in vitro* and *in vivo* exposure of B lymphocytes from lupus-prone and from healthy mice to IVIG results in an increased expression of their surface inhibitory FcγRIIb receptors (16).

Tackenberg et al. found impaired inhibitory FcγRIIb expression on B-cells in CIDP with upregulation on monocytes and B-cells after clinically effective IVIG therapy, suggesting that strategies specifically targeting FcγRIIb might have therapeutic merit in this immune-mediated peripheral neuropathy (17). This is the first demonstration that IVIG *in vivo* results in the upregulation of FcγRIIB in human B-cells.

Recently, Bouhlal et al. showed that the existence of natural autoantibodies of IgG isotype directed against the FcγRIII and FcγRII. Interestingly, the immunopurified anti-FcγIII and anti-FcγII antibodies isolated from IVIG bind soluble and membrane bound FcR and inhibit rosette formation, suggesting that *in vivo* the natural anti FcR antibodies may inhibit the binding of immunocomplexes to the membrane receptors and interfere with the Fc-dependent functions (18).

IVIG AND ANTI-IDIOTYPIC ANTIBODY BINDING

Many antibodies to self-antigens are found in IVIG and are thought to have important role in its immunonodulatory effects. Some of these self-antigens include the variable domains of other antibodies and are recognized by anti-idiotypic antibodies (19), which may bind and neutralize pathogenic autoantibodies. An additional protective mechanism provided by anti-idiotypic antibodies is mediated through their binding by F(ab′)2 to the surface IgG or IgM of B-cells, transmitting negative signals and resulting in the downmodulation of pathogenic autoantibody production and elimination of potentially autoreactive clones (20, 21). Because of the small amounts of anti-idiotypic antibody in IVIG, it is not clear whether this potential mechanism of action of IVIG is a significant immune-modulating mechanism for autoimmune and inflammatory disorders. During the last decade, the beneficial effects of IVIG in diverse conditions were improved by using target-specific IVIG (sIVIG) *in vitro* and *in vivo* in animal models on such conditions as lupus (anti-DNA-idiotype-sIVIG in lupus mice) or antiphospholipid syndrome (anti-β2GPI-idiotype-sIVIG in APS mice) as a novel approach to treat different immune-mediated conditions in a more accurate antigen-specific manner (22). On a model of rats with experimental autoimmune myasthenia gravis and on blood samples from myasthenia gravis patients, Fuchs et al. demonstrated that a minor acetylcholine receptor-specific immunoglobulin fraction present in IVIG is essential for its suppressive activity (23).

An interesting mechanism of B-cell activation mediated by IVIG through a superantigen-like binding pattern was shown by Leucht et al. (24). They demonstrated that the favored Fab V_H germline gene segments bound by IVIG were 3–23 or 3–30/3–30.5, the most frequently rearranged V_H genes among human B-cells. In a subsequent study, they provided *in vivo* functional evidence, in patients with Kawasaki disease, that a subset of IVIG selectively activated B-cells of the same V_H germline origin, confirming the B-cell superantigen proprieties (25).

IVIG IMMUNOMODULATION OF B-CELL ANTIGEN PRESENTATION AND ACTIVATION

In order to identify the cell surface molecules recognized by IVIG on human B-cells, Proulx et al. found that a significant amount of IVIG was spontaneously internalized by B-cells and interacted with intracellular targets, such as the lysosomal-trafficking regulator or nucleolin. They showed that IVIG internalization occurred in a BCR- and FcγR-independent pathway (26). More recently, the same authors demonstrated *in vitro* that IVIG in mice is able to inhibit the B-cell-mediated antigen-specific T cell

activation following either BCR-dependent or BCR-independent antigen uptake. This inhibition of antigen presentation could not be explained by a modulation of MHC II molecules expression and was shown to occur in an FcγRIIb-independent manner, suggesting that the events responsible for the inhibitory effect occur at the intracellular level (27).

De Grandmont et al. observed that the addition of IVIG in culture of CD40L-stimulated B-cells reduced their expansion and stimulated the differentiation of part of peripheral B lymphocytes into IgG-secreting cells (28). The secreted IgGs were reactive with antigens such as nucleoprotamine, dsDNA, tetanus toxin, and human IgG F(ab′)2 fragments. Maddur et al. demonstrated that IVIG *in vitro* significantly inhibited the activation of BCR-stimulated B-cells and in a dose-dependent manner inhibited the proliferation of B-cells mediated by combination of anti-CD40 MAb, IL-21, and CpG (29). On the other hand, Heidt et al. showed that IVIG *in vitro* is not capable of directly inhibiting key B-cell responses, failing to affect the proliferative capacity of both purified *in vitro* stimulated B-cells and of autonomously growing B-cell hybridomas (30).

Thus, as with other immune-modulating activities of IVIG, these apparently contrasting observations may depend on the *in vitro* systems used and the state of activation of the B-cells exposed to IVIG (21).

An additional relevant mechanism of the immunoregulatory effects of IVIG in autoimmune disorders acts through the modulation of some toll like receptors (TLR) (31, 32). IVIG in culture, by using its Fc fraction, inhibits TLR-9 and TLR7-mediated B-cell activation and suppresses TLR-induced production of proinflammatory cytokines. IVIG mimics the effects of MyD88 inhibitor by suppressing TLR-induced B-cell activation and recruits the inhibitory SHP-1 phosphatase to regulate TLR-9 activation (33). Accordingly, Kessel et al. showed that IVIG attenuates the activation of TLR-9 and decreases secretion of IL-10 and IL-6 in B-cells from SLE patients (34).

IVIG AND B-CELL ANERGY

Dussault et al. demonstrated on human B-cell lines that immunomodulation of human B-cells following treatment with IVIG involves increased phosphorylation of ERK and also Grb2-associated binder 1 and Akt, thus influencing BCR signaling (35). Other studies demonstrated that IVIG *in vitro* induces apoptosis of human B-cells through a Fas- and caspase-dependent pathway (36, 37). Besides this mechanism J. F. Séité and his group demonstrated that modulation of ERK activation in B-cells by IVIG ligation with CD22 is associated with cell-cycle arrest at the G1 phase and B-cell apoptosis (see above) (8). More recently, they showed that *in vitro* IVIG treatment of B-cells renders them refractory to BCR stimulation, suppresses the PI3K signaling pathway, and induces a long-term state of tolerance, promoting a program of long-term functional silencing similar to anergy (38). High-constitutive ERK phosphorylation is a central feature of murine models of anergy driven by constant BCR occupancy by antigen (39); in these anergic B-cells, constitutively activated ERK provides a tolerogenic signal dampening TLR-9 responsiveness (40). We showed that a subpopulation of human B-cells characterized by the reduced expression of CD21 ($CD21^{low}$ B-cells) closely resemble murine anergic B-cells (41). $CD21^{low}$ B-cells are expanded in a subset of patients with common variable immunodeficiency (CVID) and in some other immunological disorders and are characterized by high-constitutive ERK activation (41), low responsiveness to TLR-9 and BCR stimuli (42, 43), and propensity to apoptosis (44). In this regard, we observed that IVIG replacement therapy in CVID patients profoundly affects B-cell homeostasis (41). To investigate whether IVIG modulates *in vivo* ERK signaling in B-cells from CVID patients, we analyzed constitutive and BCR-induced ERK phosphorylation before and after IVIG infusion. We showed that unstimulated naive and IgM+ memory B-cells have significantly increased constitutive ERK activation after IVIG infusion, whereas BCR-induced activation, expressed as the fold increase respect to the constitutive ERK level, decreased in these cells (41). More recent observations from our group showed that IVIG infusion induces *in vivo* selective B-cell depletion in CVID patients. This effect is preceded by a profound modulation of B-cell homeostasis, where IVIG induces the down-regulation of CD21 expression promoting the generation of anergic-like, apoptosis prone $CD21^{low}$ B-cells. We found that these newly generated $CD21^{low}$ B-cells displayed the same peculiar pattern of receptors expressed by $CD21^{low}$ B-cells present before IVIG, namely, increased FCRL4 and CD11c, and reduced CD62L expression (45).

In addition, these newly generated $CD21^{low}$ B-cells, upon overnight culture, undergo spontaneous apoptosis. These observations suggest that IVIG therapy *in vivo*, even at a replacement dosage, may influence antibody responses by inducing B-cell depletion through differentiation into $CD21^{low}$ B-cells that undergo accelerated apoptosis (45).

Bayry et al. demonstrated that IVIG *in vitro* at low doses induced proliferation and immunoglobulin synthesis from B-cells of CVID patients. It seems that IVIG rectifies the defective signaling of B-cells normally provided by T cells and delivers T-independent signaling for B-cells to proliferate (46). Moreover, in accord with our data, they showed that IVIG at low does induced the phosphorylation of ERK 1/2, Akt, and p38 MAPK in B-cells of CVID patients.

In conclusion, all these observations suggest that IVIG therapy in CVID patients, in particular, in those with autoimmune manifestations, may not only replace the missing antibodies but also regulate autoimmune and inflammatory responses through the modulation of B-cell functions (46).

INTERACTIONS WITH B LYMPHOCYTES' SURVIVAL FACTORS

Intravenous immunoglobulin contains antibodies against many cytokines (47), but often the physiologic and therapeutic relevance of these antibodies remains unclear.

In humans, B-cell-activating factor (BAFF) is considered to be a master regulatory cytokine for B-cell homeostasis. BAFF serum levels are increased in a variety of B-cell related autoimmune disorders, like systemic lupus erythematosus (48), myasthenia gravis (49), and rheumatoid arthritis (50).

It was observed that natural antibodies present in IVIG could functionally neutralize cytokines, such as BAFF and proliferation-inducing ligand, important for B-cell survival (51). In fact, it was recently confirmed by two studies that IVIG treatment resulted in a significant decrease of BAFF serum level in newly diagnosed patients affected by CIDP, which all had elevated BAFF level before treatment (52, 53).

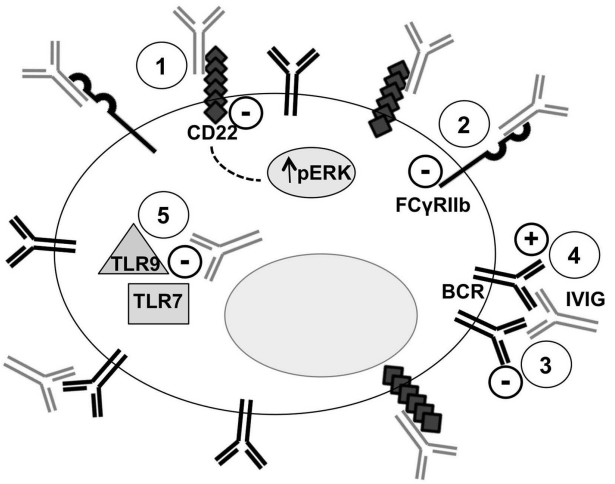

FIGURE 1 | Intravenous immunoglobulin and B-cell interactions. Five mechanisms of the interactions between IVIG and B-cells are represented. *Point 1*: binding of SA–IVIG with the inhibitory receptor CD22 acts on several BCR-signaling pathways, including sustained activation of Erk1/2, promoting apoptosis. *Point 2*: binding of IVIG with the inhibitory receptor FcγRIIb leads to inhibition of BCR-induced Ca2+ signals and cellular proliferation. *Point 3*: anti-idiotypic binding of IVIG with the BCR transmits negative signals and modulates the production of pathogenic autoantibody. *Point 4*: B-cell activation is mediated by IVIG through a superantigen-like binding pattern. *Point 5*: IVIG inhibits TLR-9 and TLR7-mediated B-cell activation and suppresses TLR-induced production of proinflammatory cytokines.

CONCLUDING REMARKS

To date, a considerable amount of data on IVIG interactions with the immune system is available. However, most of them derive from *in vitro* or in animal models studies that do not completely reflect the pathophysiological status in clinical settings. It is therefore extremely important to correlate all these data in hand and to integrate them with *in vivo* studies on human disease. Moreover, no one single mechanism is responsible for the effects of IVIG in autoimmune diseases and immunomodulation on the different cells of the immune system should be combined for a better understanding of the therapeutic effects.

B-cells play probably the most important role in the humoral immune response that, as demonstrated by the increasing amount of data, is profoundly affected by IVIG administration. A representation of some of the interactions of IVIG with B-cells is illustrated in **Figure 1**.

Despite many established aspects of IVIG–B-cell interactions, different other molecular mechanisms remain elusive. Increasing the knowledge of key molecules involved in the interaction of IVIG with B-cells may reveal which component of IVIG, whether, for example, a specific anti-idiotype antibody or an Ig fragment, is responsible for the immunomodulatory effects. This may provide the basis for the creation of more specific and tailored therapies for the different autoimmune diseases.

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Detection of impaired IgG antibody formation facilitates the decision on early immunoglobulin replacement in hypogammaglobulinemic patients

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Hypogammaglobulinemia (serum IgG lower than 2 SD below the age-matched mean) and clinical symptoms such as increased susceptibility to infection, autoimmune manifestations, granulomatous disease, and unexplained polyclonal lymphoproliferation are considered to be diagnostic hallmarks in patients with common variable immunodeficiency (CVID), the most frequent clinically severe primary immunodeficiency syndrome. In the present study, we investigated patients with hypogammaglobulinemia and no clinical or immunological signs of defective cell-mediated immunity and differentiated two groups on the basis of their IgG antibody formation capacity against a variety of different antigens (bacterial toxins, polysaccharide antigens, viral antigens). Patients with hypogammaglobulinemia and intact antibody production (HIAP) displayed no or only mild susceptibility to infections, while CVID patients showed marked susceptibility to bacterial infections that normalized following initiation of IVIG or subcutaneous immunoglobulin replacement therapy. There was a substantial overlap in IgG serum levels between the asymptomatic HIAP group and the CVID patients examined before immunoglobulin treatment. HIAP patients showed normal levels of switched B-memory cells ($CD19^+CD27^+IgD^-$), while both decreased and normal levels of switched B-memory cells could be found in CVID patients. IgG antibody response to a primary antigen, tick-borne encephalitis virus (TBEV), was defective in CVID patients, thus confirming their substantial defect in IgG antibody production. Defective IgG antibody production against multiple antigens could also be demonstrated in an adult patient with recurrent infections but normal IgG levels. To facilitate early treatment before recurrent infections may lead to organ damage, the antibody formation capacity should be examined in hypogammaglobulinemic patients and the decision to treat should be based on the finding of impaired IgG antibody production.

Keywords: hypogammaglobulinemia, IgG antibody deficiency, CVID, immunoglobulin treatment, IVIG, primary vaccination

INTRODUCTION

A considerable percentage of patients seen in clinical practice, e.g., by ENT specialists for recurrent infections (1, 2) and/or referred for immunological evaluation have hypogammaglobulinemia, usually defined as a decrease in serum IgG lower than 2 SD below the age-matched mean, with a variable decrease in IgA and/or IgM serum levels. Common variable immunodeficiency (CVID) is the most frequent clinically severe primary immunodeficiency (PID) syndrome and the most common indication for lifelong immunoglobulin replacement therapy due to predominant antibody deficiency. CVID is believed to comprise a heterogeneous group of patients that have defective antibody formation in common while other known PID syndromes should have been excluded and substantial defects in cell-mediated immunity are lacking. In view of the known heterogeneity, diagnostic criteria for CVID are more and more under debate (3). A diagnosis of CVID has considerable clinical relevance, as it invariably results in long-term immunoglobulin replacement therapy, and the question of how defective antibody formation should be demonstrated is not uniformly clear. A serum IgG lower than 2 SD below the age-matched mean is considered to be one diagnostic hallmark in patients with CVID (4, 5). Although impaired antibody responses were included as a decisive diagnostic feature for CVID in PID classification reports very early on [e.g., as stated in Ref. (6):"The *sine qua non-for* the diagnosis of CVID is defective antibody formation."], the most commonly used European Society for Immunodeficiencies/Pan American Group for Immunodeficiency (ESID/PAGID) definition of CVID (4) proposes hypogammaglobulinemia and demonstrable impairment in antibody responses as equivalent criteria, and it has even been reported that "positive vaccination responses are not contradictory to the diagnosis of CVID" (7).

In addition to hypogammaglobulinemia, the presence of clinical symptoms, such as increased susceptibility to infection,

autoimmune manifestations, granulomatous disease, unexplained polyclonal lymphoproliferation, or an affected family member with antibody deficiency, is mandatory for the diagnosis of CVID in the 2014 registry diagnostic criteria for CVID proposed by experts in the field (5), given that all other forms of primary antibody deficiency and secondary forms of hypogammaglobulinemia can be excluded. Increased awareness for PID has been raised during the last decade with the ultimate goal of an earlier diagnosis and initiation of adequate therapy. This development is certainly desirable. Thus, patients with predominantly antibody deficiency such as X-linked agammaglobulinemia (XLA) who have a long history of clinical disease, in particular, recurrent infections of the lower respiratory tract, are well known to be prone to developing organ damage such as chronic lung disease, which determines their long-term prognosis (8). However, earlier presentation of patients with suspected PID also means that more and more patients with predominantly antibody deficiency lack a long history of clinical disease, making it necessary to initiate immunoglobulin replacement therapy based on laboratory findings rather than patient history. In view of this development, a more advanced laboratory definition of patients in need of intravenous immunoglobulin (IVIG) or subcutaneous immunoglobulin (SCIG) therapy is required than the one that is given, among others, in the currently used criteria for CVID diagnosis (4, 5).

In the present study, we performed an immunological investigation in patients with hypogammaglobulinemia and no clinical or immunological signs for defective cell-mediated immunity and differentiated patients with CVID requiring immunoglobulin replacement treatment from patients with hypogammaglobulinemia receiving no immunoglobulin therapy on the basis of their IgG antibody formation capacity against a variety of different antigens (bacterial toxins, polysaccharide antigens, viral antigens). IgG antibody response to a primary antigen, e.g., tick-borne encephalitis virus (TBEV) was examined in CVID patients already receiving IVIG therapy to reevaluate their IgG antibody production capacity. To further underline the importance of defining clinically relevant antibody deficiency by measuring antibody responses rather than serum-immunoglobulin levels, a patient was presented with a massive defect in IgG antibody production comparable to that seen in CVID despite normal IgG serum levels.

PATIENTS AND METHODS

PATIENTS WITH HYPOGAMMAGLOBULINEMIA AND CONTROLS

Forty-nine patients with hypogammaglobulinemia defined as a serum IgG concentration below 500 mg/dl [median age (years) 37, interquartile range (IQR) 22–54, 26 men, 23 women], were included in a retrospective observational cohort study after the patients gave their informed consent that the anonymized data collected as part of the routine medical attendance the patients received could be included in a scientific publication. In these patients, no clinical or immunological indication of defective cell-mediated immunity (i.e., combined immunodeficiency) could be found. The patients had been referred for immunological investigation because of hypogammaglobulinemia and/or recurrent infections, recurrent fever of unknown origin, etc. (for a detailed description of the clinical symptoms in the individual patients, see **Table 1**) and were assigned to two groups depending on whether they were diagnosed as CVID according to the criteria established by the IUIS expert committee (6) and set on immunoglobulin replacement therapy or left untreated. Based on the results of the immunological workup such as determination of serum-immunoglobulin classes and IgG subclasses, IgG antibody titers to a variety of different antigens and/or antibody response after booster immunization it was found out that IgG antibody formation capacity distinguished these two groups of hypogammaglobulinemic patients, which correlated with the patients' susceptibility to infections. The clinical characterization and serum-immunoglobulin levels of the 23 hypogammaglobulinemic patients [11 women and 12 men, median age at diagnosis (years) 41, IQR 19.5–61.5] with intact IgG antibody formation [hypogammaglobulinemia and intact antibody production (HIAP)] is given in **Table 1** (A). This group of patients did not receive IVIG replacement. In 26 patients [14 men and 12 women, median age at diagnosis (years) 33, IQR 22.5–49.25, **Table 2**] CVID was diagnosed according to the criteria established by ESID (4, 5), and other PID disorders were excluded by sequence analysis (Illumina technology performed on a MiSeq bench-top next generation DNA sequencer) of PID genes listed in the 2011 IUIS classification (9). The CVID patients showed increased susceptibility to infections and [**Table 1** (B)] and were treated with IVIG or SCIG, and blood samples for determination of serum-immunoglobulin levels and serum antibody concentrations were drawn before regular IVIG or SCIG replacement therapy was started. All results presented in this study were obtained as part of the routine medical attendance that the patients received; no extra venipuncture was performed on the basis of this study. Healthy adult blood donors served as controls.

FLOW CYTOMETRY AND EXAMINATION OF HUMORAL IMMUNITY

Lymphocyte subpopulations and B cell subsets were analyzed by flow cytometry using standard protocols with commercially available directly conjugated monoclonal antibodies (anti-CD19 PerCP, Becton Dickinson Austria Ges.m.b.H., Schwechat, Austria; anti-IgD FITC, Becton Dickinson Austria Ges.m.b.H., anti-CD27 PE, eBioscience, Vienna, Austria) and a FACSCalibur (Becton Dickinson Austria Ges.m.b.H.). Data analysis was performed using CellQuest software (Becton Dickinson, Austria Ges.m.b.H.). Serum concentrations of immunoglobulins and IgG subclasses were determined by laser nephelometry using reagents purchased from Siemens-Behring Division (Siemens Healthcare Diagnostics GmbH, Vienna, Austria). Serum levels of IgG and IgM antibodies against bacterial and viral antigens were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits for IgG antibodies against tetanus (VaccZyme Tetanus Toxoid IgG EIA, The Binding Site GmbH, Schwetzingen, Germany) and diphtheria toxoid (VaccZyme Diphtheria Toxoid IgG EIA, The Binding Site GmbH), pertussis (*Bordetella pertussis* IgG ELISA – VIROTECH, Sekisui Virotech GmbH, Rüsselsheim, Germany), TBEV TECHNOZYM® FSME (TBE) lgG (CE), Technoclone GmbH, Vienna, Austria), mumps (Mumps-IgG ELISA Genzyme/Virotech, Sekisui Virotech GmbH), measles (Measles-IgG ELISA – VIROTECH, Sekisui Virotech GmbH), and rubella virus [ETI-RUBEK-G Plus, DiaSorin S.p.A., Saluggia (VC), Italy], VZV (VZV-IgG ELISA – VIROTECH, Sekisui Virotech

Table 1 | (A) Clinical characteristics and serum-immunoglobulin levels in patients with hypogammaglobulinemia but intact IgG antibody production (HIAP); (B) Clinical characteristics and serum-immunoglobulin levels in CVID patients.

Patient no.	Sex	Age at diagnosis (years)	Medical history leading to immunological evaluation	Serum immunoglobulins (mg/dl)		
				IgG	IgA	IgM
(A)						
1	M	6	Recurrent febrile episodes, hypogammaglobulinemia	360	40	95
2	M	9	Fever, diarrhea, abnormal liver function tests, hypogammaglobulinemia	291	18	26
3	M	22	Allergic rhinitis, hypogammaglobulinemia	393	64	120
4	M	25	Recurrent febrile episodes, hypogammaglobulinemia	459	115	127
5	F	37	Family history of antibody deficiency, hypogammaglobulinemia	431	162	201
6	M	34	Rec. Rhinitis, rec. gastritis, knee hurts, hypogammaglobulinemia	349	36	60
7	M	50	Nephrolithiasis, hypogammaglobulinemia	379	128	36
8	F	72	Hyperthyreosis, recurrent rhinitis	466	245	167
9	M	72	Chronic prostatitis, hypogammaglobulinemia, recurrent herpes labialis	427	530	71
10	F	89	Chronic fatigue, hypogammaglobulinemia	414	38	142
11	F	54	Chronic fatigue, hypogammaglobulinemia, recurrent sore throat, recurrent UTI	497	35	84
12	M	17	Celiac disease, herpes zoster, hypogammaglobulinemia	444	55	67
13	F	68	Chronic bronchitis, adrenal adenoma, hypogammaglobulinemia	430	139	171
14	M	8	Recurrent febrile episodes, hypogammaglobulinemia	466	112	112
15	M	14	Recurrent allergic rhinitis, recurrent herpes labialis	472	35	73
16	M	14	Recurrent rhinitis, Helicobacter gastritis, hypogammaglobulinemia	488	162	73
17	F	41	Recurrent gastritis, COPD, hypogammaglobulinemia	441	88	450
18	F	60	Polyarthralgia, hypogammaglobulinemia	437	257	234
19	F	23	Recurrent mild respiratory infections (three per year, one with antibiotic therapy)	411	93	187
20	F	69	Lichen ruber of the oral mucosa	227	89	140
21	M	53	Hypogammaglobulinemia, MGUS, DVT lower extremities with pulmonary infarction	435	54	136
22	F	51	COPD	459	38	78
23	F	63	Diabetes II, recurrent gastritis	459	91	41
Normal range (mg/dl)				815–1784	93–287	108–237
(B)						
24	M	50	Gastrointestinal protein loss, malabsorption, generalized edema, hypogammaglobulinemia, intestinal villous atrophy 3 pneumonias in the last 3 years, recurrent bacterial bronchitis, vitiligo	<50	53	183
25	M	20	3 pneumonias in the last 3 years, recurrent bacterial bronchitis, vitiligo	<50	<8	<6
26	M	27	Recurrent bacterial bronchitis ≥2 per year, recurrent folliculitis, hypogammaglobulinemia	<50	<8	41
27	F	27	Recurrent sinusitis for 3 years, 4 pneumonias, arthralgias, rhinitis	<50	<7	<7
28	M	59	One pneumonia shortly before diagnosis, recurrent sinusitis for years	<50	11	29
29	F	47	Rec. bronchitis for 10 years, bronchiectasis, pansinusitis for 8 years, 3 pneumonias	56	<7	8
30	F	46	Recurrent pneumonia (≥2 per year), total 12 pneumonias	69.4	243	345
31	M	13	Recurrent pneumonia	76	<8	50
32	M	15	Recurrent pneumonia for 6 years, malabsorption, protein-loosing enteropathy	164	7	<6
33	M	39	Chronic diarrhea, herpes zoster reactivation, gastrointestinal campylobacter jejuni infection, malabsorption	188	<7	<6
34	F	55	Recurrent pneumonia since childhood, recurrent bronchitis, otitis media, sinusitis for 5 years	199	<8	49
35	F	57	Hypogammaglobulinemia, head x-ray abnormalities suspicious of multiple myeloma	204	11	34
36	F	62	Arthritis of the hip, hypogammaglobulinemia, rec. bronchitis, rhinitis for 10 years	206	<7	51
37	F	24	Hypogammaglobulinemia, recurrent diarrhea, one pneumonia with pleuritis, genital condyloma	210	<7	33
38	F	32	Splenectomy, recurrent bacterial bronchitis, sinusitis, hypogammaglobulinemia	217	<8	33

(Continued)

Table 1 | Continued

Patient no.	Sex	Age at diagnosis (years)	Medical history leading to immunological evaluation	Serum immunoglobulins (mg/dl)		
				IgG	IgA	IgM
39	M	40	Total of 13 pneumonias, recurrent bronchitis (≥5 per year) since 1978	217	<8	47
40	F	43	Hypogammaglobulinemia, recurrent bronchitis, sinusitis since childhood	219	44	26
41	F	34	Giardia lamblia enteritis, recurrent bronchitis and sinusitis for years, vitiligo	239	<8	47
42	M	29	Pneumococcal meningitis in 1976, hypogammaglobulinemia, recurrent pneumonia, epilepsy	245	10	<7
43	M	50	Recurrent bronchitis, sinusitis for 3 years, first pneumonia 11 months ago, recurrent diarrhea	267	11	<7
44	M	28	Recurrent pneumonia since 1978 (total of six)	327	<7	84
45	F	71	Recurrent bronchitis during the last 6 years, pulmonary obstruction	367	89	67
46	M	13	Recurrent bronchitis and diarrhea since 1995	374	<8	39
47	M	22	Recurrent diarrhea during the last 15 years, intestinal villous atrophie	379	<7	36
48	F	6	Recurrent otitis media, one pneumonia since 1996	396	242	137
49	M	13	Recurrent ITP during the last 5 years, hypogammaglobulinemia	523	12	13
Normal range (mg/dl)				815–1784	93–287	108–237

Table 2 | Patients with hypogammaglobulinemia but no susceptibility to infections show normal IgG antibody responses to a variety of antigens.

Patient no.	Serum IgG antibodies against bacterial, viral and vaccination antigens							
	Tet-IgG	Di-IgG	Hib-IgG	Pn-IgG	Pn-IgM	TBE-IgG	HBs-Ab	HAV-Ab
	IU/ml		ug/ml	Reciprocal titer		VIEU/ml	IU/ml	IU/ml
1	1.44 (4.77)	0.45 (3.77)	2.97	42	58	3186	neg (8615)	
2	4.22	1.03	1.59 (>9)	26	33	158 (417)	218 (25658)	37 (7599)
3	0.43 (5)	0.21 (>1)	1.98	<20 (101)	465 (729)	na	na	na
4	2.38	0.23	1.5	210	214	2702	na	na
5	1.52	0.28	25.01	210	314	1740	na	na
6	0.97 (4.26)	<0.01	0.72 (5.86)	64	102	1050	7784	8800
7	2.1 (13.71)	<0.01 (1.04)	1.29	98 (536)	50 (945)	60 (1040)	neg (698)	neg (3832)
8	na	na	1.27 (32.68)	621	180	na	na	na
9	0.89	0.17	>9	26	270	1375	na	na
10	0.02	0.08	1.88	323	<20	na	na	na
11	1.04	0.06	6.31	96	72	403 (10542)	neg (238)	7592
12	0.31 (23.12)	0.22 (7.37)	0.97 (2.17)	235	113	1936	448	1093
13	4.11	0.01	0.34 (>9)	119 (1429)	181 (811)	312	737	>8800
14	1.88	0.3	2.34	592	714	2683	na	na
15	33	2.37	2.45	336	230	>6500	na	na
16	6.2	0.04 (>15)	8.25	363	39	2701	na	na
17	2.29	<0.01	0.77	277	298	1285	neg	>8800
18	3.49	0.17	0.25 (1.76)	30 (242)	343 (673)	1159	13	2355
19	3.46	0.85	2.11	574	825	5289	34876	1501
20	2.08	0.01	0.17	67 (371)	148 (836)	236	356	na
21	1.28 (6.12)	0.03 (0.17)	na	48 (756)	48 (2189)	26	neg	311
22	5.17	0.05	0.11	348	135	360	na	na
23	0.39 (10.18)	0.03	0.39 (>9)	33 (307)	25 (200)	734	na	na
Normal range	>0.4	>0.4	>1	>200	>100	>310	>100	>100

Values in parentheses represent IgG antibody responses 6–8 weeks following booster immunization; na = value not available.

GmbH), HSV-1 (anti-HSV-1 (gC1)-ELISA (IgG), Euroimmun AG, Lübeck, Germany) and HSV-2 1 (anti-HSV-2 (gG2)-ELISA (IgG), Euroimmun AG), hepatitis B virus (Enzygnost Anti-HBs II, Siemens Healthcare Diagnostics GmbH), hepatitis A virus (Enzygnost Anti-HAV, Siemens Healthcare Diagnostics GmbH), and *Haemophilus influenzae* type b (Hib) (VaccZyme™ Hib-IgG The Binding Site GmbH) or in-house produced ELISAs for IgG and IgM antibodies against 23-valent pneumococcal capsular polysaccharide and 4-valent meningococcal polysaccharide as previously described (10).

EXAMINATION OF IgG ANTIBODY RESPONSE TO A PRIMARY ANTIGEN IN PATIENTS WITH CVID

The major part of the CVID cohort studied has been vaccinated against TBEV before the observation period, as it is usual practice in Austria, and for this reason TBEV could only be applied as a primary antigen in a subgroup of our patients. Ten patients with CVID were vaccinated against TBEV (FSME-Immun-Inject, Baxter AG, Vienna, Austria, a commercially available licensed vaccine), a primary viral antigen for these patients. The patients were vaccinated twice (4-week interval between the two immunizations), followed by a third booster vaccination 6–12 months following the first immunization. During their participation in this vaccination study, the 10 CVID patients received IVIG lots with a relatively low TBEV-IgG antibody content. Serum IgG antibodies were determined by ELISA as previously described (10) before vaccination, 4–6 weeks after the second as well as before and 4 and 8 weeks after the third vaccination. Healthy controls with a positive TBEV vaccination history and CVID patients receiving IVIG-replacement therapy but no vaccination served as controls.

STATISTICAL ANALYSIS

Statistically significant differences between study groups were calculated using the non-parametric two-tailed Mann–Whitney *U*-test. Results are depicted using box plot diagrams, with the median represented by a cross, the interquartile range (IQR) represented by the box, 5- and 95-percentile values represented by the whiskers, and minimum and maximum values represented by circles.

RESULTS

PATIENTS WITH HYPOGAMMAGLOBULINEMIA CAN BE DIFFERENTIATED BASED ON THEIR CAPACITY TO PRODUCE IGG ANTIBODIES

The hypogammaglobulinemic patients presented in this study could be divided into two groups according to clinical and immunological characteristics. First of all, most patients with CVID showed a marked susceptibility to bacterial infections [**Table 1** (B)] that normalized following initiation of IVIG or SCIG therapy. In contrast, patients with HIAP displayed no or only mild susceptibility to infections, as can be seen from the clinical characterization depicted in **Table 1** (A), and did not require immunoglobulin replacement therapy. HIAP patients that were followed for many years did not show a worsening of their clinical condition [follow-up years, median (IQR) 3.8 (0.3–9.8), $n = 23$]. Furthermore, the study population could be differentiated on the basis of their IgG antibody formation capacity, which correlated with the presence of clinical symptoms. In HIAP patients with little or no susceptibility to infections, the intact IgG antibody response was demonstrated by measuring serum IgG antibody levels against a variety of different antigens (bacterial toxins, polysaccharide antigens, viral antigens). In addition, 14 HIAP patients received a booster vaccination against at least one of the vaccination antigens tested and showed a normal IgG antibody response thereafter (**Table 2**), while other patients in this group showed high IgG antibodies upon first examination because of relatively recent booster vaccinations that were performed according to current immunization recommendations.

Serum-immunoglobulin levels were significantly lower in CVID patients as compared to HIAP patients (**Figure 1A**), with a substantial overlap of approximately 25% in serum IgG levels between the asymptomatic HIAP group and the CVID patients examined before immunoglobulin treatment (**Figure 1A**, a). In contrast, median IgG antibody levels against pneumococcal and Hib polysaccharides (without prior vaccination) in CVID patients were more than one log range below the levels observed in HIAP patients without prior vaccination (**Figure 1B**), with very little overlap between the two groups: in the CVID group, only 5/26 had detectable IgG antibodies against 23-valent pneumococcal polysaccharide (**Figure 1B**, a) but the median titer of the group was significantly below that observed in healthy controls without vaccination, with only a very small (5%) overlap (**Figure 1B**, a). Furthermore, 95% of the CVID patients showed IgG serum antibody levels against tetanus toxoid that were below 0.4 IU/ml despite a positive immunization history (**Figure 1B**, b), while all HIAP patients with a positive tetanus immunization history (21/22 tested) had clearly detectable tetanus-IgG antibodies ($\geq$0.4 IU/ml). All CVID patients tested had Hib-IgG antibodies below 1 μg/ml (**Figure 1B**, c), antibody levels considered sufficient for long-term protection, and 75% of the patients had no detectable tetanus-IgG or Hib-IgG antibodies at all (**Figure 1B**). In the CVID group, the IgG antibody deficiency correlated with a markedly increased susceptibility to infections [**Table 1** (B)].

B-MEMORY CELL DIFFERENTIATION IN CVID AND HIAP PATIENTS

Our findings confirm previously published evidence that the majority of CVID patients or CVID patients as a group have decreased switched B-memory cells, as was originally reported by Warnatz et al. more than 10 years ago in order to identify subgroups of CVID patients (11), which was subsequently reevaluated in many publications. Recently, it was reported that low numbers of switched memory B cells correlate with infectious complications in pediatric patients with CVID (12, 13). We thus investigated whether or not patients with hypogammaglobulinemia but intact antibody production can be differentiated from CVID patients on the basis of the number of switched B-memory cells in peripheral blood. While HIAP patients showed levels of switched B-memory cells ($CD19^+CD27^+IgD^-$) that were comparable to healthy controls examined in parallel, switched B-memory cells were significantly decreased in CVID patients as a group. However, a 25–50% overlap in switched B-memory cells could be found between CVID patients and healthy controls (**Figure 2**), indicating that a considerable proportion of CVID patients have switched memory B cells within the range of healthy controls, and that low numbers of this B cell subset can also be found in healthy individuals.

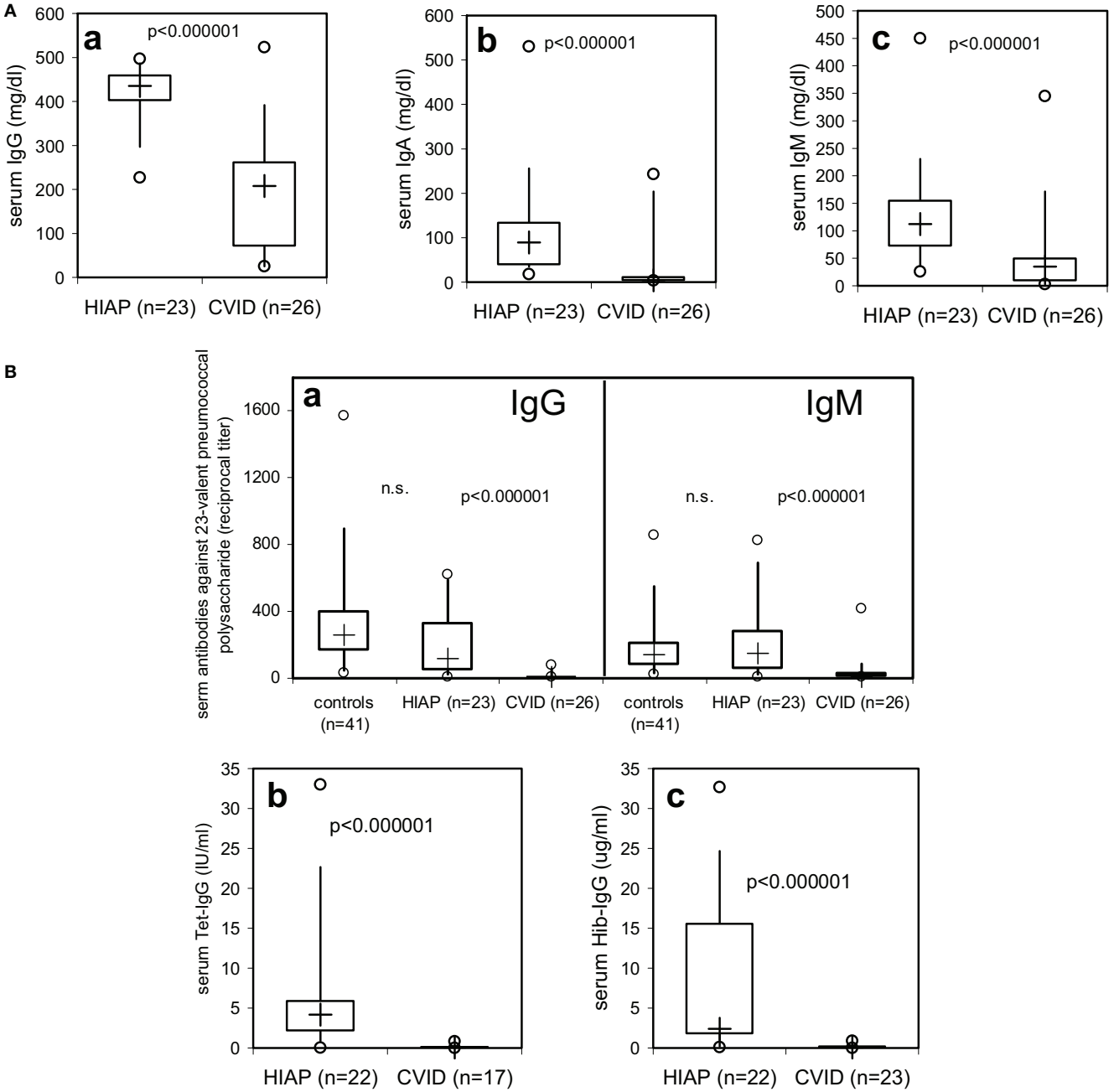

FIGURE 1 | Serum-immunoglobulin (A) and serum-IgG antibody levels against tetanus toxoid, haemophilus influenzae type b (Hib), and 23-valent pneumococcal polysaccharide (B) in CVID patients and patients with hypogammaglobulinemia but intact antibody production (HIAP). Box plot diagrams indicate the median (+), interquartile range (box), percentiles 5 and 95 (whiskers), and minimum and maximum values (circles). Statistical comparison between the two study groups was performed using the Mann–Whitney *U*-test.

IgG ANTIBODY RESPONSE TO A PRIMARY ANTIGEN IS DEFECTIVE IN CVID PATIENTS

IgG antibody response to a primary antigen, TBEV vaccine, was reevaluated in 10 CVID patients receiving IVIG-replacement therapy with lots containing low TBEV-IgG antibody levels. The results depicted in **Figure 3** confirm the presence of a substantial defect in IgG antibody production in these patients. Only 1 of the 10 vaccinated patients showed slightly higher TBEV-IgG antibody levels 2 months after the third vaccination as compared to patients receiving IVIG therapy without TBEV vaccination. In all vaccinated CVID patients, TBEV-IgG following the third vaccination was well below the minimum levels observed in healthy adult controls with a positive history of TBEV vaccination (**Figure 3**).

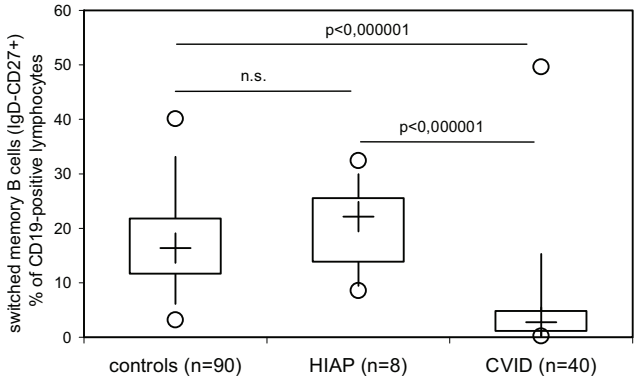

FIGURE 2 | Switched memory B cells (IgD-negative, CD27−, and CD19-positive peripheral blood lymphocytes) were determined by three-color flow cytometry in healthy adult controls, patients with hypogammaglobulinemia but intact antibody production (HIAP) and CVID patients. Box plot diagrams indicate the median (+), interquartile range (box), percentiles 5 and 95 (whiskers), and minimum and maximum values (circles). Statistical comparison between the study groups was performed using the Mann–Whitney *U*-test.

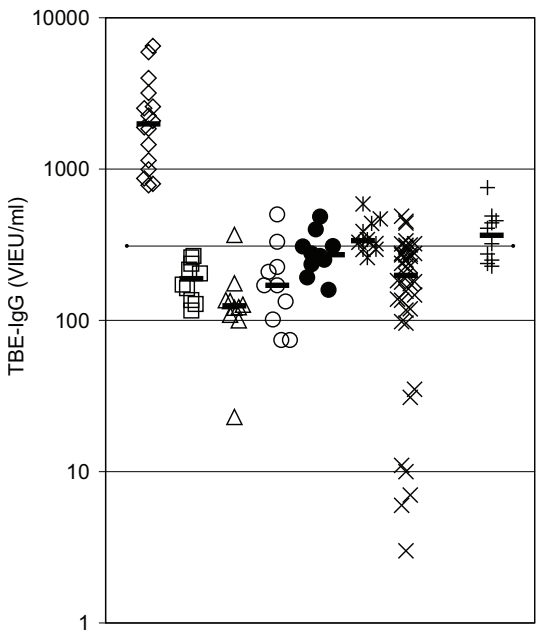

FIGURE 3 | IgG antibody response to a primary antigen is defective in CVID patients. Serum IgG antibodies against tick-borne encephalitis virus (TBEV) vaccine were determined by ELISA. Diamonds = healthy controls with a positive TBE vaccination history ($n = 16$); squares = CVID patients before TBEV vaccination ($n = 10$); triangles = CVID patients 4–6 weeks after second vaccination ($n = 10$); open circles = CVID patients before third vaccination ($n = 10$); closed circles = CVID patients 4 weeks after third vaccination ($n = 10$); asterisques = CVID patients 8 weeks after third vaccination ($n = 10$); X = IVIG-treated CVID patients without TBEV vaccination ($n = 39$); crosses = TBEV-IgG antibodies in IVIG lots (diluted to 1000 mg/dl) used during vaccination study ($n = 10$); dotted line = detection limit for positive TBEV-IgG antibodies; median values of the respective groups are indicated by horizontal bars.

DEFECTIVE IGG ANTIBODY PRODUCTION DESPITE NORMAL TOTAL SERUM IGG (CASE REPORT)

A male patient, aged 39 years, was referred for immunological evaluation because of IgA deficiency, recurrent respiratory tract infections (recurrent otitis media and sinusitis, four infectious episodes that required oral antibiotic therapy during the previous winter including his first pneumonia), lymphadenopathy of the mediastinum and hilus (bronchoscopy revealed a histologic picture compatible with the diagnosis of sarcoidosis). He had received no treatment for his lung problems yet, in particular, never any immunosuppressive therapy. Recent vaccination history included revaccinations against TBEV, dT, and hepatitis A and B. Immunological characterization revealed IgA deficiency associated with IgG2–IgG4 subclass deficiency and low to undetectable IgG antibodies against 14 of 15 antigens tested (**Table 3**). The only significant IgG antibodies found were against rubella virus as a result of previous infection, presumably during childhood. Total serum IgG levels were normal as were his serum IgM-levels. Upon revaccination with 10 different antigens (including vaccination against poliovirus serotypes I, II, and III), IgG antibody responses against seven of seven antigens tested were missing (**Table 3**; the seven antigens tested included 23-valent pneumococcal polysaccharide and 4-valent meningococcal polysaccharide). IVIG-replacement therapy was initiated, which led to a complete normalization of his susceptibility to infections during a 3.5-year follow-up. B-memory cells ($CD19^+CD27^+$ switched- and IgM-memory B cells) were absent in peripheral blood; no indication of defective cell-mediated immunity could be found. Other PID disorders were excluded by sequence analysis (Illumina technology performed on a MiSeq bench-top next generation DNA sequencer) of PID genes listed in the 2011 IUIS classification (9).

DECREASE IN DELAY OF CVID DIAGNOSIS OVER THE LAST DECADE

The results presented above indicate that the decision to start immunoglobulin replacement therapy should not be based on the finding of hypogammaglobulinemia but rather on the demonstration of defective IgG antibody formation. A more precise laboratory definition as a rationale to start immunoglobulin replacement therapy is particularly needed in early diagnosed PID patients before a massive history of infectious episodes and/or infectious complications such as bronchiectases have developed. As a result of ongoing campaigns to raise awareness for PID more and more patients with PID are diagnosed with a relatively short or, optimally, no history of clinical symptoms, in particular recurrent infectious episodes. We thus examined whether or not the time between the onset of first symptoms and the date of diagnosis of CVID became shorter in the patients diagnosed at our institution over the last 25 years. On 81 patients diagnosed with CVID according to the criteria established by ESID (4, 5) between June 30th, 1981 and September 15th, 2014, information was available to determine the time (years) between date of onset of first symptoms and date of diagnosis (**Figure 4A**) as well as the age at diagnosis (**Figure 4B**). The results presented in **Figure 4A** show that in our CVID patients diagnosed before June 1st, 2005 ($n = 42$), a median of 6.4 years (IQR, 3.6–12.3) elapsed between onset of first symptoms and diagnosis and initiation of therapy, while during the decade following June 1st, 2005 this

Table 3 | Defective IgG antibody formation against a variety of antigens in a patient with normal serum IgG levels and increased susceptibility to infections.

Age	Patient			Normal range
	At first examination	After vaccination°	Under IVIG	
	39 years	39 years 5 months	40 years 1 month	
A. SERUM-IMMUNOGLOBULIN LEVELS (mg/dl)				
IgG	851	737	1649	(790–1700)
IgA	<6	<6	<6	(76–450)
IgM	100	89	166	(90–350)
IgG1	683	573	1160	(500–880)
IgG2	102	96	400	(150–600)
IgG3	61	51	62	(20–100)
IgG4	<5	<6	<7	(8–120)
B. SERUM ANTIBODIES AGAINST BACTERIAL AND VIRAL ANTIGENS				
Tet-IgG (IU/ml)	0.14	0.19°	4.05	>0.4
Di-IgG (IU/ml)	0.08	0.05°	0.73	>0.4
pn23-antibodies (reciprocal titer)				
IgG	<20	<20°	525	>200
IgM	<20	42°	75	>100
Hib-IgG (ug/ml)	0.19	0.24°	5.64	>1
Mumps-IgG (VE)	1.6	n.a.	11.2	>11
Measles-IgG (VE)	1.8	n.a.	43.7	>11
Rubella-IgG (IU/ml)	>176.6	n.a.	>182.4	>10
Pertussis-IgG (VE)	2.8	n.a.	13.8	>10
VZV-IgG (VE)	2.6	n.a.	35.9	>11
HSV-1-IgG (VE)	20.7	n.a.	n.a.	>20
HSV-2-IgG (VE)	2.1	n.a.	n.a.	>11
TBEV-IgG (U/ml)	41	27°	444	>310
HBs-Ak (mE/ml)	neg°	neg°	1167	>100
HAV-Ak (IU/L)	neg°	neg°	4049	>100
4-Men-antibodies (reciprocal titer)				
IgG	<20	<20°	81	>100
IgM	<20	<20	<20	>50

Eight to twelve weeks after booster vaccination against hepatitis A and B, Hib, TBEV, Pn23, DiTetPertPolio and first vaccination with tetravalent meningococcal polysaccharide vaccine.

diagnostic delay was significantly shorter [median years (IQR]) 1.9 (0.6–4.5), $p = 0.000002$). The age at diagnosis was comparable between the two groups of patients diagnosed before and after 2005 (**Figure 4B**), showing that age at diagnosis in CVID patients is not a suitable parameter to determine how long clinical symptoms existed before diagnosis.

DISCUSSION

As a result of ongoing campaigns to raise awareness for PID more and more patients with PID are diagnosed with a relatively short or, optimally, no history of clinical symptoms, in particular recurrent infectious episodes. At our institution, CVID patients diagnosed during the last decade showed a reduction in diagnostic delay by a median of 70% as compared to patients diagnosed earlier. While this development is certainly desirable, it also raises the problem that diagnostic criteria (4, 5) that include mandatory clinical symptoms will not be fulfilled if the patient is treated as early in life as possible. This is especially true in those forms of primary immunodeficieny that lack a definitive diagnosis, e.g., through demonstration of a mutation in the causative gene, as is the case in the majority of patients with CVID. In the absence of clinical symptoms, the currently used diagnostic criteria for CVID mainly rely on the finding of hypogammaglobulinemia. The results presented in this study show that defective IgG antibody response to a variety of antigens rather than hypogammaglobulinemia correlate with the need for immunoglobulin replacement therapy in individual patients to normalize a preexisting susceptibility to infections. As individual patients with severe IgG antibody deficiency might show IgG antibodies against selected antigens with contact, e.g., infection early in life, as many specificities as possible should be examined. IgG antibody responses after infection, natural exposure, or booster immunization and against T-dependent and T-independent, viral and bacterial, protein and polysaccharide antigens, either vaccine-induced or infection-induced, should be tested to provide optimal diagnostic certainty when the option of a definitive diagnosis of CVID does not exist yet. Primary IgG

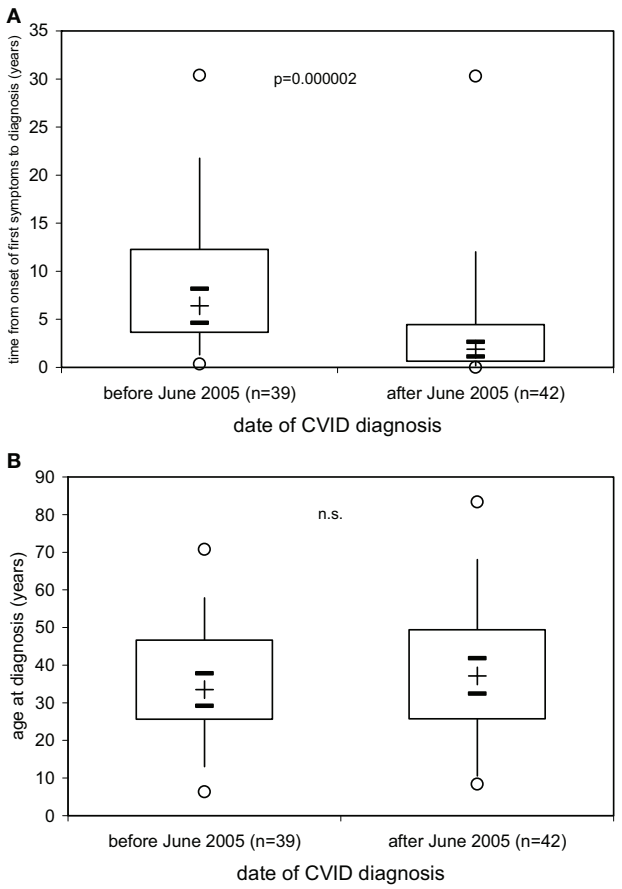

FIGURE 4 | Decrease in delay of CVID diagnosis over the last decade. The time (years) between date of onset of first symptoms and date of diagnosis. **(A)** and the age at diagnosis **(B)** is given for 81 patients with CVID divided into two groups according to whether diagnosis was made before ($n = 39$) or after ($n = 42$) June 1st, 2005. Box plot diagrams indicate the median (+), interquartile range (box), percentiles 5 and 95 (whiskers), and minimum and maximum values (circles). Statistical comparison between the two groups was performed using the Mann–Whitney *U*-test. The 95% confidence interval for the median calculated according to McGill et al. (14) is indicated by horizontal bars.

antibody responses should also be a diagnostic option in selected patients with inconclusive evaluation of booster IgG responses or to reevaluate IgG antibody production in patients already receiving immunoglobulin replacement therapy.

In 2014, ESID proposed new diagnostic criteria for CVID that include either defective antibody production (defined as a poor antibody response to vaccination or absent isohemagglutinis) or low switched B-memory cells as a mandatory decisive factor (5). Our findings confirm previously published evidence that the majority of CVID patients and/or CVID patients as a group have decreased switched B-memory cells, as was originally reported by Warnatz et al. more than 10 years ago in order to identify subgroups of CVID patients (11). However, a considerable overlap (between 25 and 50%) in switched B-memory cell distribution could be found between CVID patients and normal healthy controls, suggesting that CVID patients can present with normal numbers of this B cell subset and that low numbers of switched B-memory cells can also be found in individuals with a normal IgG antibody production capacity, thus questioning the usefulness of this parameter as a rationale to start immunoglobulin replacement therapy without testing IgG antibody responses. Further studies are required to answer the question whether very low switched B-memory cells (below the range observed in healthy controls and well below the 70%-of-normal-cut off as proposed in the 2014 ESID registry diagnostic criteria (5) are invariably associated with a clinically relevant defect in IgG antibody production.

To further explore the antibody formation capacity of CVID patients, we examined the IgG antibody response to a primary antigen. Since ongoing immunoglobulin replacement therapy complicates the determination of IgG antibody responses after vaccination, we choose TBEV vaccine, an antigen suitable for primary immunization in individuals not previously vaccinated, as IgG antibody titers are frequently low in IVIG products due to the use of a large share of plasma from the US, where in contrast to middle Europe, TBEV immunization is not frequently employed (15). Previous studies proposed the use of TBEV vaccine to study antibody response to booster vaccination in patients receiving immunoglobulin replacement therapy (16), but primary TBEV antibody response has not been studied in IVIG-treated CVID patients so far. Since it is known that different IVIG lots contain different TBEV antibody contents depending on the country of plasma origin (15), we examined the lots of IVIG that were used in our patients undergoing TBEV vaccination to confirm their low TBEV antibody content. The results obtained extend our previous knowledge of a substantial defect in antibody production in CVID by showing a defective primary IgG antibody response in CVID patients under IVIG therapy. These findings confirm and extend a previous study showing defective booster antibody responses in CVID patients under IVIG treatment on the level of defective antibody forming cells examined by ELISPOT and plasmablasts examined by flow cytometry of peripheral blood B cells (17).

The concept that defective IgG antibody production rather than hypogammaglobulinemia shows the requirement for IgG replacement is supported by findings in an adult patient with IgA deficiency and IgG2–IgG4 deficiency who, despite his normal total IgG levels, displayed a marked defect in the formation of IgG antibodies against both T-dependent and T-independent antigens, associated with normalization of infectious susceptibility under IVIG-replacement therapy. While patients with agammaglobulinemia and complete lack of antibody production are well known to be susceptible to infections, equivalent clinical symptoms can develop in patients with normal levels of total serum IgG but defective IgG antibody production against clinically relevant infectious organisms such as patients with selective polysaccharide antibody deficiency (SPAD). SPAD is a well-recognized primary predominant antibody deficiency (9) that was first described by Umetsu et al. in patients with IgG2-subclass deficiency (18). It was later discovered that even patients with normal IgG subclass levels can fail to produce antipolysaccharide antibodies (19) and that IVIG-replacement therapy effectively prevents infections in these patients, despite their normal levels of total serum IgG (20). The patient described here has IgA, IgG2, and IgG4 deficiency in the presence of normal total serum IgG levels, but in contrast to previously published patients with IgG subclass deficiency and

selective anti-polysaccharide deficiency, has defective IgG antibody response against seven of seven tested vaccination antigens, i.e., against T-dependent protein antigens and T-independent polysaccharide antigens, thus closely resembling the severe antibody defect seen in CVID. Comparable to CVID, IVIG-replacement therapy led to a normalization of his susceptibility to respiratory tract infections, and his lung abnormalities (histologically classified as sarcoidosis) remained stable or even improved slightly.

In conclusion, the findings presented suggest that in order to facilitate early treatment before recurrent infections may lead to organ damage the antibody formation capacity should be examined in hypogammaglobulinemic patients and the decision to treat should be based on the demonstration of defective IgG antibody formation against all or the vast majority of multiple different antigens tested. How many different IgG antibody specificities have to be tested has to be determined in subsequent studies. A more precise definition of the laboratory findings providing a rationale to start immunoglobulin replacement in predominantly antibody deficiency is particularly helpful in early diagnosed PID patients, lacking a definitive genetic diagnosis and/or in patients with an atypical clinical presentation.

AUTHOR CONTRIBUTIONS

HW and ME were the principal investigators, provided laboratory resources, analyzed clinical and immunological data, wrote the first manuscript draft together, critically participated in all revisions of the manuscript and take primary responsibilities for the paper. VT and JL performed the vaccination study of primary antibody responsiveness, provided clinical patient data, participated in data analysis and interpretation, and critically reviewed the initial draft and all revisions of the manuscript.

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On the dark side of therapies with immunoglobulin concentrates: the adverse events

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Therapy by human immunoglobulin G (IgG) concentrates is a success story ongoing for decades with an ever increasing demand for this plasma product. The success of IgG concentrates on a clinical level is documented by the slowly increasing number of registered indication and the more rapid increase of the off-label uses, a topic dealt with in another contribution to this special issue of Frontiers in Immunology. A part of the success is the adverse event (AE) profile of IgG concentrates which is, even at life-long need for therapy, excellent. Transmission of pathogens in the last decade could be entirely controlled through the antecedent introduction by authorities of a regulatory network and installing quality standards by the plasma fractionation industry. The cornerstone of the regulatory network is current good manufacturing practice. Non-infectious AEs occur rarely and mainly are mild to moderate. However, in recent times, the increase in frequency of hemolytic and thrombotic AEs raised worrying questions on the possible background for these AEs. Below, we review elements of non-infectious AEs, and particularly focus on hemolysis and thrombosis. We discuss how the introduction of plasma fractionation by ion-exchange chromatography and polishing by immunoaffinity chromatographic steps might alter repertoire of specificities and influence AE profiles and efficacy of IgG concentrates.

Keywords: adverse events, hemolysis, thrombosis, complement, cytokines, SCIG, IVIG

INTRODUCTION – THE TINGE OF THE DARK SIDE OF THERAPIES WITH IMMUNOGLOBULIN CONCENTRATES

Since the initial clinical use of immunoglobulin G (IgG) concentrates of human origin, transmission of pathogens and non-infectious adverse events (AEs) were reported (1–7). Before the mid 90s, transmission of pathogens depended on the pool size and the fractionation methods used, particularly the polishing steps of an IgG concentrate (8). Mode of fractionation, i.e., cold-ethanol or ion-exchange chromatography, contaminants, route of application, i.e., intra muscular (IMIG), intravenous (IVIG), or subcutaneous (SCIG), the rate of increase of the exogenous IgG in the circulation of the recipient over time and, last but not least an eventually existing risk factor from patients' side (**Figure 1**) as well as incorrect handling of the concentrate are factors having a role in inducing non-infectious AEs related to administration of IgG concentrates (**Table 1**). IgG concentrates represent a defined part of the adaptive immune system, are isolated from pooled human plasma of at least 1000 donors, which contribute to the repertoire diversity in the final product. Therapies with IgG concentrates manufactured according to regulators requirements are acknowledged to be safe in general. This does not exclude the occurrence of AEs which in their majority are rare and clinically mild to moderate. Below, we like to give a few insights into various aspects and possible mechanisms of AEs.

Abbreviations: ACA, anti-complementary activity = unwanted spontaneous complement-activating capacity of an Ig concentrate; AE, adverse event; BCR, B cell receptor; BT, body temperature; C3a/C3a[desArg], anaphylatoxin C3a and its arginine deprived form which were detected together; CH50, total complement hemolytic activity 50%; cGMP, current good manufacturing practice; CVID, common variable immunodeficiency; DAT, direct antiglobulin test; DVT, deep venous thrombosis; HA, hemolytic anemia; Hb, hemoglobin; HR, heart rate; IC, immune complex; IgA, immunoglobulin A; IgA1, subclass 1 of IgA; IgA2, subclass 2 of IgA; IgAD, IgA deficiency; IgG, immunoglobulin G; IgM, immunoglobulin M; IL-1ra, interleukin 1 receptor antagonist; IL-6, interleukin 6; IL-8, interleukin 8; IMIG, immunoglobulin G concentrate for intramuscular application; IR, infusion rate; IVIG, immunoglobulin G concentrate for intravenous application; IEP, isoelectric point; ITP, immune thrombocytopenic purpura; LDH, lactate dehydrogenase; MERS, middle east respiratory syndrome; MW, molecular weight; NAb, natural antibody or autoantibody; PKA, prekallikrein activator; PE, pulmonary embolism; RBC, red blood cell, erythrocyte; SARS, severe acute respiratory syndrome; SCIG, immunoglobulin concentrate for subcutaneous application; sIgAD, selective IgAD; TCR, T cell receptor; TNFα, tumor necrosis factor alpha; V-region, variable region of antibodies, TCR and BCR; vCJD, variant Creutzfeldt-Jakob disease.

PATHOGEN SAFETY OF IgG CONCENTRATES – HOW TO EXCLUDE THE MENACES FROM A DARK AND FRIGHTENING ENVIRONMENT

Manufacturing of modern IgG concentrates has to occur in a regulatory framework and the quality standards implemented by the plasma fractionating industry (**Figure 2**). The cornerstone of the regulatory framework is current good manufacturing practice (cGMP). A pillar of pathogen safety is the validation of

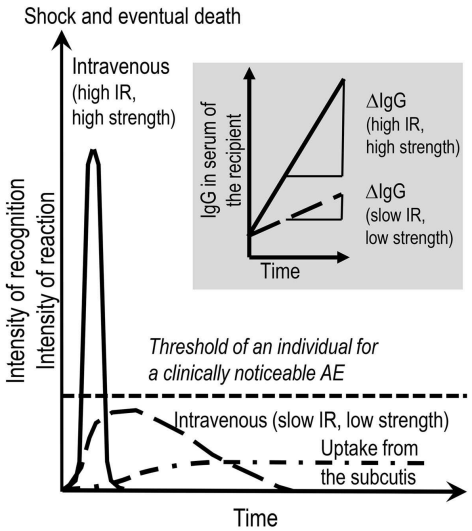

FIGURE 1 | Provoking the dark. AEs to therapeutic IgG concentrates might be evoked by several factors. These are listed in the introductory part and **Table 1**. Contemplating on the active ingredient of the concentrates, the administration of the IgG molecules inevitably results in the interactions of the exogenous IgG with the various parts of the immune system of the recipient and *vice versa*. This interaction in its principle generates an inflammatory condition. A key parameter deciding on the intensity of such a condition is the rate of increase over time of exogenous IgG in the circulation of the recipient. Insert to figure (shadowed): ΔIgG in the circulation over Δtime is dependent on the combination of infusion rate (IR) and the strength of the solution applied. Main figure: the mode of application, i.e., intravenous or subcutaneous, are additional factors being decisive for kinetics and the area under the curve of generation of pro-inflammatory mediators and for passing the individual threshold (short-dashed line) for a clinically noticeable AE. The threshold in turn depends on eventually exiting risk factors form the patient side. Although the area under the curve might be the same for low (long-dashed line) and high (solid line) IRs, at high IR, the system might not be able to cope with the extent of reactions. At low IR (long-dashed line), all the events might remain below the threshold of clinically observable AE. Subcutaneously applied IgG concentrate reaches the circulation slowly and systemic AEs are less frequent than with IVIG. In contrast, local mild to moderate AEs are more frequent with SCIG.

virus inactivation and virus elimination methods by validating an already performed step of the fractionation process or by introduction of dedicated steps (**Figure 3**). A hallmark of virus elimination introduced in the late 90s in Berne by the team of Christoph Kempf is the large-scale virus filtration technique (formerly also termed "nanofiltration") (8). Meanwhile, virus filtration became a versatile tool to eliminate a variety of pathogens, the suspected agent of variant Creutzfeldt-Jakob disease included. Thanks to the tightly implemented regulatory framework, pathogen safety of plasma products is at a level never reached before. This is well supported by the fact of reports missing in the last decade of transmission by IgG concentrates of emerging viruses (SARS coronavirus, West Nile Virus, MERS coronavirus, and others), zoonotic pathogens, or the agent of variant Creutzfeldt-Jakob disease (vCJD). Furthermore, the development of specific mass screening techniques might help to eradicate in any blood product the transmission of vCJD in the future (9).

HOW THE SHADOW MIGHT GROW – SOME BASIC INSIGHTS INTO POSSIBLE MECHANISMS OF NON-INFECTIOUS ADVERSE EVENTS

The human immune system is in charge of controlling invading organisms and mediates homeostasis. The immunoglobulin pools in mammals (IgM, IgG, and IgA) to its smaller part provide defense and to the larger part homeostasis. Human IgG has a role in both. Efficient host defense is supported by "immune antibodies." These have undergone somatic hypermutations and have in their vast majority narrow specificities and high affinities. Antibodies, generated in absence of external stimuli are termed "natural antibodies" (NAbs). They occasionally recognize self structures. In general, the V(D)J genes of NAbs are in germ-line configuration or have undergone a few somatic hypermutations only. Furthermore, they have broad specificity, are of low affinity (with exceptions) and high avidity (10). These NAbs can participate in primary host defense, i.e., at a time point when an immunologic reaction has not provided the specific antibodies, react, e.g., with repetitive structures which can be found on bacteria or viruses (11). The self-reactive NAbs, which we like to term physiologic autoantibodies, comprises various populations of antibodies such as (i) those able to interact through their complementary variable regions (V-regions) with the V-regions of circulating and membrane-bound (BCR) immunoglobulins and the T cell receptor (TCR) β-chain variable region, providing a peripheral immune network (V-connected network) (10); (ii) the NAbs reacting in a non-idiotypic manner with the hinge region of immunoglobulins (12, 13); (iii) populations of NAbs showing a wide variety of specificities toward growth factors, cytokines, or anaphylatoxin (10); (iii) and the population reacting with the soluble or membrane-bound forms of cell surface molecules having immunological importance, the last described being the Fc receptors CD16 and CD32 (14). Antibodies reacting with docking structures for viruses or bacteria can have additional first-line defense potential (15). These populations of NAbs were described having a peripheral immune network homeostatic and anti-inflammatory function (16, 17). Although the primordial humoral proteins comprising the complement and lectin-like proteins in the plasma play a definite role, another population of self-reactive NAbs reacting with, e.g., epitopes conserved over the evolution apparently has tissue homeostatic function and might support the efficient removal of roughly 10^{12} altered/senescent cells of the body per day (for references see below). The signal for research on NAbs in IVIG was the description of IgG autoantibody-mediated immune thrombocytopenia (ITP) being corrected by infusion of a polyclonal, polyspecific IgG concentrate (18, 19). This research has expanded ever since.

The populations of immune antibodies and NAbs in IgG concentrates upon infusion/injection inevitably react with occasional pathogens, toxins, or superantigens and concomitantly infusion/injection also results in recognition of a wide array of tissue antigens and V-regions of the recipient's immune system. Reactions with tissue antigens and V-regions are conveyed by the self-reactive antibodies of the many donors in the IgG concentrate. *Vice versa*, the recipient's immune system reacts with the infused IgG. A bewildering wide range of possible reactions can occur which primarily are dependent on the immune status of the recipient at the time of therapy and to a smaller part on the IgG concentrate(s).

Table 1 | The tinge of the dark.

Symptoms and signs	Frequency	IRR or total dose	PRR	System	Class, severity, and duration	Part of the product likely being involved in AEs
Fatigue	Common (SCIG as well)	No		Constitutional or systemic (generalized)	Immediate, mild, transient	
Malaise	Common	No	Yes	Constitutional or systemic (generalized)	Immediate, mild, transient	
Fever	Common	Yes	Yes	Constitutional or systemic (generalized)	Immediate, mild, transient	
Flushing	Common	Yes	Yes	Constitutional or systemic (generalized)	Immediate, mild, transient	
Chills	Common	Yes	Yes	Constitutional or systemic (generalized)	Immediate, mild, transient	
Anorexia	Common	No		Constitutional or systemic (generalized)	Immediate, mild, transient	
Myalgia	Common	Yes	Yes	Constitutional or systemic (generalized)	Immediate, mild, transient	
Arthralgia	Common	Yes	Yes	Constitutional or systemic (generalized)	Immediate, mild, transient	
Joint swelling	Common	Yes	Yes	Constitutional or systemic (generalized)	Mild, transient	
"Flu-like" symptoms	Common	Yes	Yes	Constitutional or systemic (generalized)	Immediate, mild, transient	Increase in A (dimers)
Anaphylactoid symptoms	Rare Complement activation Immune complexes (presence of acute infection)	No	Yes	Constitutional or systemic (generalized)	IgA: acute to immediate; other late, severe transient	I: IgA, very rare immune complexes
Full blown anaphylaxis	Rare Complement activation (in the presence of acute infection)	No	Yes	Constitutional or systemic (generalized)	Late, severe, hopefully transient (ICU)	I: IgA, very rare immune complexes
Headache	Common	Yes	Yes	Neurologic	Immediate, mild, transient	Increase in A
Migraine	Common	Yes	Yes	Neurologic	Transient	Increase in A
Dizziness	Common	Yes	Yes	Neurologic	Transient	Increase in A
Aseptic meningitis	Rare	No	No	Neurologic	Delayed, moderate, transient	Increase in A
Diffuse pain, muscle pain	Rare	Yes	Yes	Neurologic	Transient	Increase in A
Dysesthesia	Rare		Contributes	Neurologic		Increase in A
Weakness	Rare		Contributes	Neurologic		Increase in A

(Continued)

Table 1 | Continued

Symptoms and signs	Frequency	IRR or total dose	PRR	System	Class, severity, and duration	Part of the product likely being involved in AEs
Persistent headache	Rare		Yes	Neurologic	Delayed, moderate	Increase in A
Shortness of breath	Common	Dose	Yes	Respiratory		
Bronchospasm	Common	Yes	Yes	Respiratory		
Pleural effusion	Rare	Dose	Contributes	Respiratory	Severe, transient	
TRALI	Rare	Dose	Likely	Respiratory	Late, severe, transient (ICU)	
Hypotension	Common	Yes	Yes	Cardiovascular	Immediate, mild, transient	
Hypertension	Common	Yes	Contributes	Cardiovascular	Immediate, mild, transient	
Tachycardia	Common	Yes	Yes	Cardiovascular	Immediate, mild, transient	
Chest/back pain	Common	Yes	Yes	Cardiovascular	Immediate, mild, transient	
Arrhythmia	Rare	Dose	Contributes	Cardiovascular	Severe, hopefully transient	
Myocardial infarction	Rare	Dose	Contributes	Cardiovascular	Severe to fatal	Increase in A
Anorexia	Common			Gastrointestinal		
Nausea	Common	Yes	Yes	Gastrointestinal	Immediate, mild, transient	
Vomiting	Common	Yes	Yes	Gastrointestinal	Immediate, mild, transient	
Cramping	Common	Yes	Contributes	Gastrointestinal		
Diarrhea	Common		Contributes	Gastrointestinal		
Colitis	Rare		Contributes	Gastrointestinal	Late, severe	
Tubular swelling	Rare	Dose	Contributes	Renal	Severe, reversible; scars might remain	E: sucrose >> other sugars
Renal failure	Rare		Contributes	Renal	Delayed. severe, ICU	Increase in A (Complement deposition)
Infusion site pain, swelling, erythema	Common (SCIG more frequent)			Cutaneous	Immediate, mild, transient	SCIG: volume
Urticaria	Common		Yes	Cutaneous		Increase in A
Non-specific macular or maculopapular eruptions/eczema	Common		Yes	Cutaneous		Increase in A
Pruritus	Common		Contributes	Cutaneous		
Erythema multiforme	Rare		Contributes	Cutaneous		Increase in A

(Continued)

Table 1 | Continued

Symptoms and signs	Frequency	IRR or total dose	PRR	System	Class, severity, and duration	Part of the product likely being involved in AEs
Cutaneous vasculitis	Rare	Dose	Contributes	Cutaneous	Delayed, severe	
Hemolysis (clinically not significant)	Common	Yes	Contributes	Hematologic	Delayed, moderate, transient	
Acute hemolysis/hemolytic anemia	Rare	Yes	Yes	Hematologic	Delayed, severe	Increase in A
Thrombotic phenomena (DVT, stroke, cardial infarction)	Rare	Yes	Yes	Hematologic	Severe, ICU	Increase in A
Hyperviscosity	Rare	Yes	Contributes	Hematologic	Immediate	Increase in A
Neutropenia	Rare	Yes		Hematologic	Delayed, mild, transient	Increase in A
Blood borne infectious disease	Rare	No	No	Microbiological	Late, severe	I: blood borne viruses, spongiform encephalopathy agent
Inappropriate handling before infusion			No		Immediate, mild to severe	A: incomplete dissolution of lyophilized product; denaturation and aggregate formation due to foam
						E: lyophilized product dissolved to result in too high concentrations; lyophilized product dissolved to result in too high osmolality; low temperature of concentrate at the time of infusion

The various manifestations of AEs in recipients of polyvalent immunoglobulins. Frequency, severity, duration, timing, possible causes, risk factors.
A, active ingredient = IgG; I, impurities; E, excipients/stabilizers; "immediate", immediate reaction – within 6 h from the onset of infusion; "delayed", delayed reaction – 6 h to 1 week after infusion; "late", late reaction: weeks to months after infusion; IRR, infusion rate-related; PRR, patient-related risk factors (acute infection at the time of infusion); ICU, intensive care unit; SCIG, IgG concentrate for subcutaneous application.

The therapeutic effect achieved depends on the disease treated, and can depend on the concentration reached locally, i.e., can have agonistic or antagonistic effects (17, 20–22). In summary, it is our opinion that IgG concentrates always provide more or less the same "bouquet" of IgG specificities (similarity); however, it is the recipient's actual immune condition which decides from which IgG specificities the patient's derailed immune system is profiting (diversity).

Parameters of IgG-mediated AEs are: (i) the content in the product of biologically highly active likely beneficial ingredients that have to be kept under control (e.g., content of "dimers" devoid of remarkable complement activation *in vivo*; see below), and the content in unwanted active ingredients that have to be discarded during manufacturing (alloantibodies); (ii) impurities such as IgA (anaphylactoid reaction); (iii) activated coagulation and contact activation factors (thromboembolic events) and; (iv) excipients such as sucrose (osmotic nephrosis). Below, we like to add and contemplate on how fully native IgG molecules not harmed by the manufacturing process might add to AEs. The above mentioned inevitable interaction of the exogenous IgG with the immune system of the recipient and *vice versa* in its principle might evoke an inflammatory condition. The sum of the potentially beneficial reactions might overshoot and lead to AEs (**Figure 1**). The principle of induction of mild inflammatory

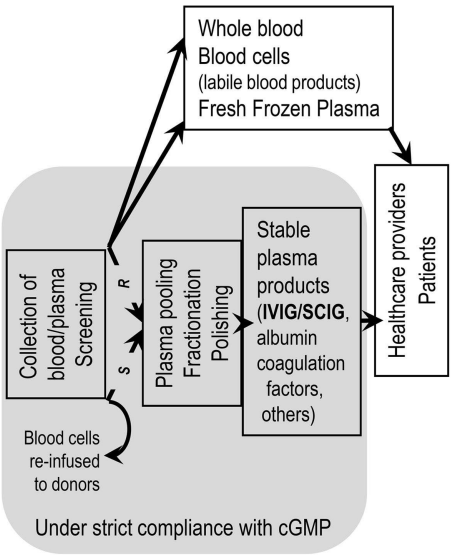

FIGURE 2 | Staying within a regulatory network is mandatory for remaining at the sunny side of the moon. Handling of blood/plasma products to obtain therapeutic goods has to be performed within a regulatory framework. Manufacturing of stable blood product has to adhere strictly to current good manufacturing practice (cGMP). Application of cGMP starts from the moment of collecting whole blood and isolation of plasma thereof (R = recovered plasma) or the machine-supported collection of plasma (S = source plasma, apheresis plasma). Application of cGMP ends at delivery of blood/plasma products to health care professionals. Not following cGMP can lead to withdrawal of a plasma product from the market from one to the other day.

conditions upon each infusion of a well-tolerated IVIG was confirmed when several dozen normogammaglobulinemic volunteers in all cases except one, showed a more or less moderate inflammatory reaction as indicated by the increase of tumor necrosis factor alpha (TNFα) at 2.5 h post initiation of infusion. The only person in the cohort not showing a measurable TNFα increase was a woman caring at home for her brother with full blown AIDS (22).

Subcutaneously applied IgG concentrate reaches the circulation slowly and systemic AEs are less frequent compared to IVIG but they are not absent (23–28) (a case of unintentional i.v. application of SCIG is not considered). In contrast, local mild to moderate AEs are more frequent with SCIG (29). In summary, the intensity of the resulting AEs is depending on the immune status of the recipient, the infusion rate (IR), e.g., how rapidly the active ingredients (the various IgG specificities), the impurities, and the excipients reach the circulation of the recipient. Thus, the i.v. application has the highest chance for the occurrence of AEs.

In the early days of IVIG therapy, complement-mediated "anaphylactoid" (i.e., immediate) and "phlogistic" (i.e., inflammatory) AEs were distinguished (30, 31). The complement-mediated AEs were considered to be caused by aggregates in the product ("spontaneous complement activation" or anti-complementary activity or ACA) or by *in vivo* formation of immune complexes (ICs, patient's condition related; e.g., subclinical infections or the unnoticed presence of anti-IgA antibodies) and therefore only IgG concentrates with low or absent ACA is accepted by authorities for human use. Below, we present one instructive case of each type of reaction.

IMMEDIATE ADVERSE EVENTS – THE RAPID ONSET OF DARKNESS

The first reports of rapid onset AEs concerned either the application of complement-activating fractions in an IgG concentrate or the *in vivo* formation of complement-activating ICs (2–4). A very rare but potentially fatal condition is the formation of IgA/anti-IgA complexes in patients being initiated on replacement therapy and having serum IgG antibodies against infused IgA not recognized before the start of the IVIG infusion (32). Prerequisite for the presence of anti-IgA antibodies is the most common primary immunoglobulin defect, i.e., selective IgA deficiency (sIgAD) or IgAD associated with diminution of other immunoglobulin classes. IgAD is defined by serum levels of <0.05 or <0.07 g/L (depending on laboratories). A marked diminution of serum IgA consistent with IgAD in various ethnic groups is estimated being 1:155 to 1:18,550 (33). The mean frequency in Caucasians is approximately 1:700 (34). Up to 40% of patients with IgAD have been reported having anti-IgA antibodies in the serum with titers ranging between 1:4 and 1:262,144. In approximately 10% of patients with common variable immunodeficiency (CVID), and occasionally in patients with other primary immunodeficiency diseases, measurable anti-IgA can be detected (35, 36). These antibodies are predominantly of the IgG class, but anti-IgA antibodies of other immunoglobulin classes have been described as well (37, 38). The reason for their emergence remains unknown.

Taken the above numbers, the infusion of human-derived products containing IgA resulting in severe anaphylactoid type AEs should be considerable. This is not the case (39). Questions about the clinical relevance of above numbers emerge as soon as blood banks (i) estimate the theoretical risk of IgA anaphylactic reactions (32); (ii) assess the relation of severe IgAD with the presence or absence of anti-IgA antibodies (40); (iii) screen donors for very low IgA levels in order to become able to provide blood and plasma-derived products free of IgA and find a considerably lower frequency than expected (41). Alternatively, the test systems may not reliably detect anti-IgA antibodies being as yet insensitive and inaccurate or – at least – do not correspond to the clinically relevant fraction of antibodies. This comes to mind when a more close look to "anti-IgA" gives "unexpected" results, including "anti-IgA" in blood donors with normal serum IgA level or "anti-IgA" that cannot be neutralized with purified IgA (42); or when blood products containing proven anti-IgA do not elicit severe AEs (43).

Among patients on replacement therapy, those with CVID may rarely develop severe immediate AEs (32). The discrepancy between anti-IgA positive patients and frequency of AEs raises the question about the nature of the many reported anti-IgA antibodies and also raises the question about the immunologic condition which allows the formation of anaphylactoid anti-IgA antibodies. There might be some logic in supposing that anaphylactoid anti-IgA cannot evolve at IgA levels otherwise fulfilling the definition of IgAD. Such a condition would constantly generate ICs which in turn could activate complement, react with immune cells, and be deposited in lung and kidney. Indeed, Horn et al. found anti-IgA

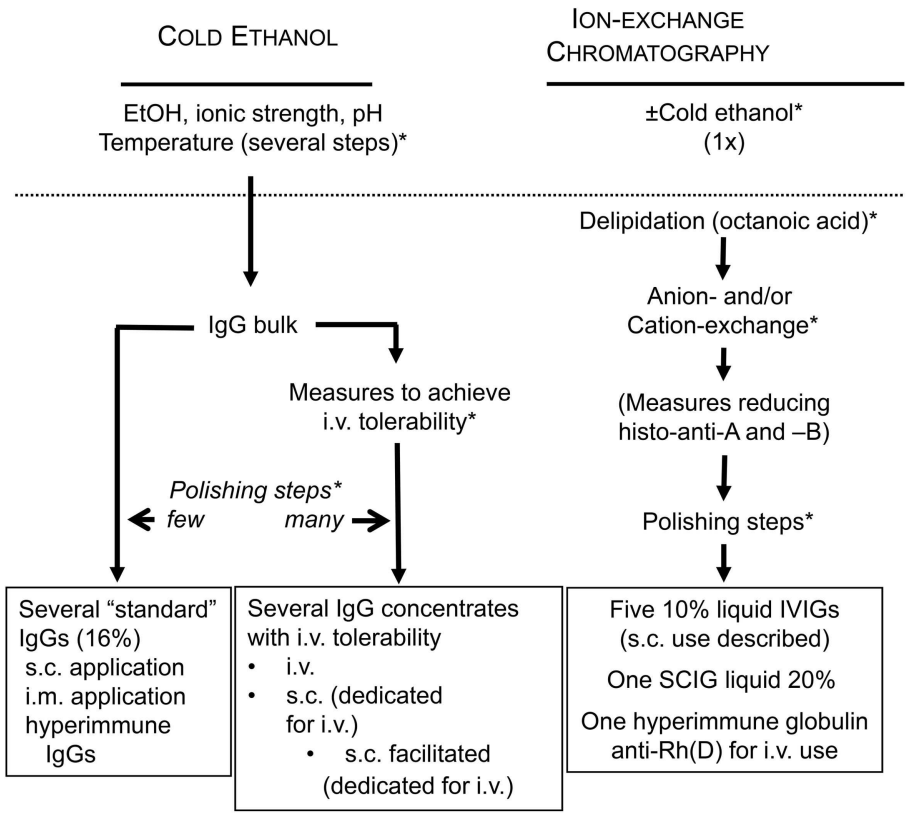

FIGURE 3 | Active measures in fractionation to stay on the sunny side of the moon. (*) depicts possible sites for validating the efficiency of already established fractionation steps or for introducing dedicated pathogen reduction and/or inactivation measures. Histo-blood group antibodies were made responsible for hemolytic AEs. Reduction of these during the manufacturing process is possible by introduction of an immunoaffinity chromatographic step in order to reduce anti-A and anti-B titers.

antibodies in CVID patients missing IgA^+ B cells and presenting with IgA levels <0.0009 g/L, a level which is more than 50- to 70-fold lower than the threshold for IgAD (44). However, a possibility for an IgA-mediated anaphylactoid reaction at measurable IgA serum levels might exist. Serum IgA contains approximately 85% subclass 1 of IgA (IgA1) and only 15% subclass 2 of IgA (IgA2). Selective deficiency of IgA2 and – although evidence is lacking – the presence of a highly specific anti-IgA2 antibody theoretically could elicit a severe AE.

The kinetics of anti-IgA after infusion of blood products have been studied in a few cases. In these patients, a fall in anti-IgA titers has been noticed followed by an increase during subsequent weeks or months. This suggests that at appropriate proportions, IgA of the infused material and anti-IgA present in the patients' serum combine with each other to form ICs. In turn, ICs activate complement that are bound and eliminated by macrophages most likely leading to cytokine release. The increase in anti-IgA titers over time indicates that the infused IgA-containing product has a booster effect (36, 37, 45). Such boosting effect together with the presence of anti-IgA before the application of an IgG concentrate can be taken as the ultimate confirmation of a supposed IgA/anti-IgA reaction. **Figure 4** depicts a well-documented case of IgA/anti-IgA reaction in a patient who progressed from sIgAD to CVID. The events during the first 12 h at occasion of the first infusion of IVIG were as follows (shadowed area in **Figure 4**): 2 min after the start of the infusion, having received eight drops of an IgG solution (IgA < 1.2 g/L; 3% solution), she experienced a flush, back pain, rigors, difficulty in breathing, and hypotension. The infusion was immediately stopped. After approximately 1 h, the reaction has weaned, and 2 h later the patient felt well again, and the infusion of total 6 g IgG could be continued without further complications. Although the patient fairly assured having never received any blood or plasma product in the past, the follow-up of her anti-IgA titers from before infusion to 1 year later confirmed a true anaphylactoid reaction mediated by anti-IgA, as the anti-IgA became undetectable immediately after the infusion and showed a boosting phenomenon during the following months. True anaphylactoid reaction was further confirmed by follow-up of total complement hemolytic activity (CH50) on the day of infusion. Interestingly enough, the CH50 value reached its nadir at the end of the infusion when the patient had no complains. Although a single case only, the events during the first infusion call for the following remarks: (i) severe AEs most likely occur at concomitant complement and cell activation with cytokine release; (ii) infusion of minute to low amounts of IVIG hours before the main infusion can "anergize" cells and stop release of pro-inflammatory

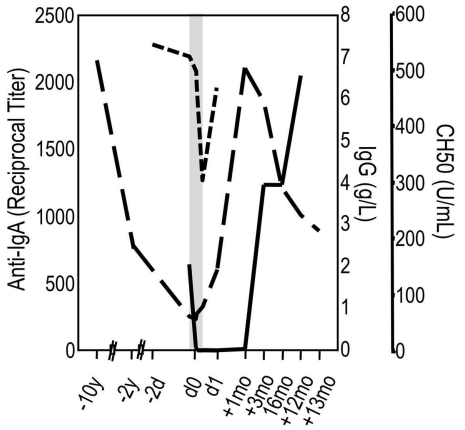

FIGURE 4 | An individual's slithering into the dark. A female patient has been suffering from recurrent airway infections since adolescence, occasionally complicated by pneumonia. At age 34, selective IgA deficiency was diagnosed. Ten years later, she was hospitalized with pneumonia. Within these 10 years, her serum IgG had dropped from 7 to 0.87 g/L (long-dashed line) and IgA was undetectable. The diagnosis was corrected into CVID, and IVIG replacement therapy was initiated (shadowed area). Two minutes after the start of the infusion of a 3% IgG solution (IgA < 1.2 g/L; 3% solution), she experienced a flush, back pain, rigors, difficulty in breathing, and hypotension. The infusion was immediately stopped and later continued without further complications (see text). The confirmation of a true anaphylactoid reaction due to anti-IgA in the serum of the patient was achieved by follow-up of anti-IgA (solid line) and CH50 (short-dashed line).

cytokines; (iii) "anergized" cells loose reactivity toward ongoing formation of ICs and complement activation products.

A non-complement-mediated anaphylactoid reaction was ascribed to the unforeseen release of elastase and other pro-inflammatory substances from neutrophils activated by the formation of *in vivo* IgA/anti-IgA complexes. Complement activation or mast cell-dependent release of vasoactive substances was excluded as pathogenic mechanisms. Although the IgA/anti-IgA complexes usually do not cause clinically relevant neutrophil degranulation within the circulation, the presence of a rare genotype encoding a novel gain-of-function IgG receptor on neutrophils may provoke premature degranulation by these complexes. This phenomenon was only relevant in hypogammaglobulinemic patients in the presence of *in vivo* IgA/anti-IgA complexes (46). The low prevalence of this genotype combined with an IgAD or CVID may add how to explain the rarity of serious anaphylactoid reactions in newly IVIG-treated patients. Authors share the opinion of Janne Björkander who at occasion of a discussion panel "Dilemmas in Diagnosis and Management of Antibody Deficiencies: Ask the Experts" held at occasion of the 58th Annual Meeting of the American Academy of Allergy, Asthma & Immunology (AAAAI), New York City, March 1–6, 2002 came to the following conclusion: a clinician has to be aware of the risk, particularly at occasion of first infusions, but otherwise IgA is not a major concern (from tape record).

PRO-INFLAMMATORY CYTOKINES – THE PHLOGISTON OF THE DARK

In the early days of Ig-therapy, the nature of the "phlogistic" AEs was obscure. However, it was already known that an AE can be prevented or its evolution halted when the patient receives a low dose of IVIG first or the infusion is stopped early and is continued several hours later. Hours later the infusion can be (re)started at high rates without further problems (**Figure 5**). One of the authors had a particular opportunity to get an insight into what a "phlogistic reaction" might be. At the occasion of a voluntary infusion of an investigational liquid IVIG, he encountered a severe flu-like AE of more than 12 h duration. Before injection, the investigational liquid preparation had passed all release criteria for human use, including spontaneous complement activation assessed by ACA and was free of prekallikrein activator (PKA). In those days, assays for cytokines in biological samples just began to become available and were included into the parameters assessed in the study. Infusion was stopped after 1 h because of a drop of pulse rate and heavy discomfort provoking the laconic comment by the proband's technician who was taking samples: "you look green." The infusion was continued after another 90 min when the heart rate had almost normalized. The infusion could be completed within an additional 3.25 h (a total of 0.4 g/kg b.w.) without further aggravation of malaise. The leukocyte count transiently had dropped to a nadir of 40% at 2 h followed by a leukocytosis peak at 8 h. Complement activation, as assessed by generation of C3a/C3a[desArg] and the formation of the terminal complement complex C5b-9, apparently did not occur: the C3a/C3a[desArg] value reached a maximum of 260 ng/mL (norm: <200 ng/mL) at 7 h while the C5b-9 value never moved outside the normal range. Instead, a sequence of rapid transient massive increases of pro-inflammatory cytokines was observed: (i) TNFα started to increase 30 min post initiation of infusion from a value of 20 pg/mL to a peak value at 2 h which was above the calibration range of the test kit of 1500 pg/mL; (ii) interleukin 8 (IL-8) increase started after 1 h from 29 pg/mL and peaked at 2.5 h with 4400 pg/mL post initiation of infusion; (iii) interleukin 6 (IL-6) secretion started after 1 h with an undetectable level and peaked at 4 h with 345 pg/mL. All pro-inflammatory cytokines fell sharply while the second part of the infusion was still ongoing. The day after infusion, the pro-inflammatory cytokine profiles were back to normal and the flu-like syndrome was gone. In contrast, interleukin 1 receptor antagonist (IL-1ra) values started increasing at 1.5 h (300 pg/mL), peaked at 4 h (>32,000 pg/mL), and decreased slowly to reach a value of 10,500 pg/mL 24 h after initiation of the infusion. Soluble TNF receptor p75 level started at 2.25 ng/mL, reached a peak with 16.5 ng/mL at the same time as IL-1ra, and 24 h after initiation of infusion was still at 10.5 ng/mL. Thus, in this normogammaglobulinemic subject similar cytokine profiles and leukocyte number changes were observed as reported for hypogammagobulinemia under replacement therapy (47, 48).

A series of further experiments with investigational and marketed IVIGs was performed. All IgG concentrates were analyzed for their molecular weight (MW) distribution. The most remarkable differences emerged in the MW range of dimers while the presence of minute amounts of higher oligomers could not be excluded with certainty. Below, we will use the term "dimers" for that fraction of IgG with higher MW. Subsequent findings indicated that levels of "dimers" >12% were responsible for complement-independent cell activation and cytokine release. The TNFα peaks assessed at 2.5 h post initiation of infusions correlated with "dimer" content of the IVIGs and mirrored a clinical score of AEs (49–51).

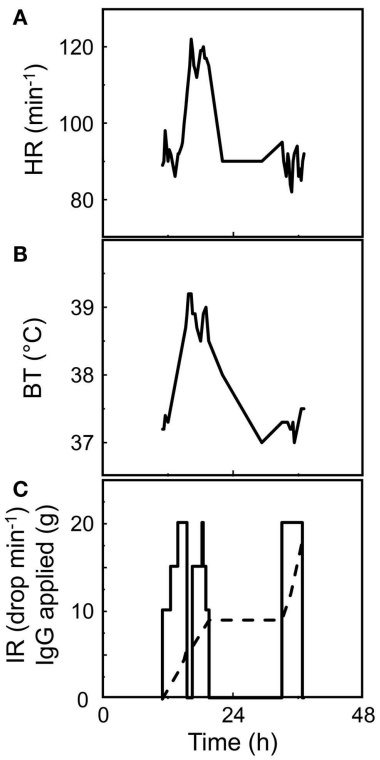

FIGURE 5 | Clinical signs of the "phlogiston" of the dark. A CVID patient received his first infusion of IVIG (a 3% solution). Despite the start of the infusion at an infusion rate [IR, **(C)**] of only 10 drops per minute and incremental increase by five drops every 30 min, rise of heart rate [HR, **(A)**], and of body temperature [BT, **(B)**] after 2 h of the initiation of infusion indicated the onset of a "phlogistic" reaction and infusion was stopped. Stop of the infusion for 30 min immediately let drop HR and BT. Restarting the infusion showed some negative effect and infusion was stopped after application of 9 g IgG [dashed line, **(C)**]. The next day the rapid infusion of additional 9 g of IgG was without consequence on BT and HR, indicating silencing of cells releasing mediators of inflammation.

A few years before a complement-independent induction of a hypotensive factor by IgG di- and oligomers was reported in animal experiments (52). A key role for macrophages in the generation of the hypotensive lipid factor was identified as platelet-activating factor, being induced by dimers and polymers (53, 54). Several years later, the dimer-mediated AEs in animal experiments were confirmed (55, 56). Yet, at the same time, the dimer content of IVIG apparently correlated with the clinical efficacy in a murine ITP model (55, 57). Variables such as "IR," "genetic background," "endogenous immunoglobulin levels," or "proportional fraction of polymers versus dimers" may impact on the balance between the phlogiston (being cytokines, active lipid substances, or a combination of factors) and the therapeutic efficacy (blocking IgG receptors on liver/spleen macrophages to prevent clearance of "opsonized" material such as platelets in ITP). As of today, reports on release of cytokines in humans in association with AEs or tolerability toward dimers remain scarce and to the best of our knowledge studies in humans of causative factors/fraction in an IgG concentrate has not been adequately addressed (47, 48, 58–61).

In IgG preparations, various forms of dimers might be present: formed through covalent binding (62) by denaturation, hydrophobic interactions of the Fc-parts, and by idiotype/anti-idiotype interactions, as part of the V-connected network of peripheral immune homeostasis (63). For a commercially viable fractionation process, pooling of donated plasma is mandatory in order to obtain a volume of starting material large enough to cover ever increasing costs for documentation, in-process, and batch-release testing as it is required by cGMP. Pooling also intends smoothing the batch-to-batch differences in antibody titers, a goal apparently difficult to achieve to levels as theory might imply (64). Consequences of pooling are on the one hand the enrichment of public/common immune antibodies while diluting out individual specificities; on the other hand, the antibodies of the immune network of an individual donor are exposed to those of many other donors. The more individuals contribute to the pool, the more complex the possible "immune-network" interactions among IgA, IgG, and IgM will become. The subsequent fractionation process has far-reaching effects on immunoglobulins from a given pool: only trace amounts of IgA and IgM are retained in the final product, i.e., IgG is deprived of its counterparts of the V-connected immune network. The IgG molecules of the homeostatic network "naked" at their V-regions can interact with each other at random combining site-interactions of single donor-derived monomeric IgG (65), otherwise not existing *in vivo*. This interaction is largely reversible. With increasing numbers of donors included into the pool, the immune network recognition among the "naked" IgG molecules of the V-connected network becomes more and more complex, and the dimer and lower oligomer content in the resulting IgG concentrate increases (66–68). In lyophilized IgG concentrates, the dimer formation is "frozen" at a low level while in liquid preparations an equilibrium between monomers and dimers is achieved over time reaching a dimer content of 12% or more if not hampered by stabilizers. Specificities, as far as they have been addressed, in the dimer fraction considerably differ from the monomeric fraction (69–73). In conclusion, the immunomodulatory efficacy of IgG concentrates in part depends on the capacity and extent to form "dimer" fractions devoid of remarkable complement activation *in vivo*. The "art" of manufacturing a liquid IgG concentrate is not to eliminate the monomeric IgG having potential for "dimer" formation but to inhibit extensive "dimerization." In summary, AEs might be associated with the induction of pro-inflammatory cytokines in absence of measurable complement activation *in vivo* where all regulatory mechanisms and removal processes of a body are at disposition. At reasonable IRs in the open system of the human body, clinically relevant systemic complement activation apparently needs oligomers formed of three or more IgG molecules.

THE MISSING OXYGEN ON THE DARK SIDE – IMMUNOGLOBULIN-INDUCED HEMOLYSIS

There are multiple reports of Ig-induced hemolytic anemia (HA) in patients receiving high doses of IVIG (60, 74–110) (**Table 2**; **Figure 6**; www.adrreports.eu). By spontaneous reporting, risk factors recognized for Ig-induced hemolysis include beside high doses (more than 100 g IVIG over 2–4 days), female gender and histo-blood group type A, B, or AB of recipients.

Table 2 | The missing oxygen on the dark side – Ig-induced hemolysis in recipients of polyvalent immunoglobulins.

Publication	Number of patients	Blood group	Monthly Ig dosage (mg/kg)	DAT	Eluted antibody	Alloantibody passively administered	Hemoglobin drop (g/L)	Outcome
Quinti et al. (110)	8	A+ (5), A- (1), O+ (2)	Low	IgG (2), IgG and C3d (4)	anti-A (5), anti-C (1), anti-C and anti-D (1)	anti-A, anti-C, anti-D	6.4, 1.5, 5.1, 1.4, 6.9, 1.1, 1, 1	Recovery (7), death (1)
Desbourouh et al. (76)	1	AB+	High	IgG and C3	anti-A and anti-B	anti-A and anti-B and anti-D	6.5	Recovery
Mohamed et al. (77)	1	A+	High	IgG	anti-A	nd	4	Recovery
Rink et al. (78)	3	A+ (2)	High	IgG	nd	nd	1.2, 3.8, 4.4	Recovery
Berard et al. (79)	4	A+ (2), B+ (1), AB+ (1)	High	IgG	anti-A (1), anti-B (1), anti-A and anti- B (1)	nd	2.9, 5.8, 5.8, 3.7	Recovery
Michelis et al. (60)	1	A+	High	IgG	anti-A	nd	3.5	Recovery
Pintova et al. (80)	2	AB+, A+	Low	IgG	anti-A	nd	6.6, 7.2	Recovery
Morgan et al. (81)	3	AB+(1), A- (1), A- (1)	High	IgG	anti-A (2), anti-A and anti-B (1)	anti-A (2), anti-A and anti-B (1)	4.8, 5.0, 1.8	Recovery
Welles et al. (82)	1	nd	High	IgG	nd	nd	4.3	Death
Canadian Group (83)	20	A (14), AB (6)	High	IgG	nd	nd	3.2, 2.8, 5.1, 5, 5.6, 5, 3.5, 4.1, 7, 3.2, 5.6, 3.2, 6.6, 2.9, 3.1, 4, 3.9, 7.8, 4.9, 4.8	Recovery (10), death (1), unknown (8)
Gordon et al. (84)	4	A+ (3), AB+ (1)	High	IgG	nd	nd	5.3, 5.5, 4.8, 4.8	Recovery
Kahwaji et al. (85)	16	A+ (10), A- (2), B+ (3), AB+ (1)	High	IgG	nd	anti-A, anti-B	5.3, 4.7, 5.6, 4.9, 5.8, 5.7, 3.3, 2.4, 3.1, 4.0, 3.6, 2.1, 2.2, 2.8, 5.3, 1.9, 2.6, 3.0	Recovery
Daw et al. (86)	16	A+ (7), AB+ (1), AB- (1), B+ (6), O- (1)	High	IgG	anti-A (6), anti-B (4)	anti-A, anti-B	1.4, 3.6, 4.3, 3.6, 3.2, 3.4, 3, 4.7, 5.1, 5, 2.4, 8, 5.2, 1.3, 3	nd
Yin et al. (87)	1	AB+	High	Negative	nd	anti-A and anti-B and anti-D	nd	Recovery

(Continued)

Table 2 | Continued

Publication	Number of patients	Blood group	Monthly Ig dosage (mg/kg)	DAT	Eluted antibody	Alloantibody passively administered	Hemoglobin drop (g/L)	Outcome
Coghil et al. (88)	1	A+	High	IgG	anti-A	anti-A	4	Recovery
Chamouni et al. (90)	1	AB+	High	IgG	nd	nd	8	Recovery
Karaaslan et al. (91)	1	nd	High	IgG	nd	nd	3.9	Recovery
Trifa et al. (92)	1	AB+	High	IgG	anti-A and anti-B	anti-A and anti-B	7.8	Recovery
Nagakawa et al. (94)	1	A+	High	IgG	nd	nd	2	Recovery
Wilson et al. (95)	12	A+ (11), O+ (1)	High (10), low (2)	IgG	anti-A (9), anti-A and anti-D (2), anti D (1)	nd	3.7, 3.8, 1, 1.6, 1.9, 2.9, 1.4, 1.9, 3, 1.8, 1, 0.9	nd
Tamada et al. (97)	2	nd	High	IgG	anti-A, anti-B	nd	nd	nd
Thomas et al. (99)	1	A+	High	IgG	anti-A	anti-A	6.3	Recovery
Comenzo et al. (100)	1	nd	High	nd	nd	nd	nd	Recovery
Okubo et al. (102)	1	A+	High	IgG	anti-A	anti-A	nd	nd
Hillyer et al. (103)	1	AB+	High	IgG	anti-A, anti-B	nd	nd	Recovery
Nicholls et al. (104)	2	nd	High	IgG	anti-A, anti-A and anti-D	nd	nd	nd
Kim et al. (105)	2	B+	High	IgG	anti-B	nd	nd	nd
Brox et al. (106)	1	nd	High	IgG	anti-A	nd	nd	nd

Clinical and immunological characteristics of patients described in case reports. Numbers in parenthesis indicate the number of patients with the given condition.

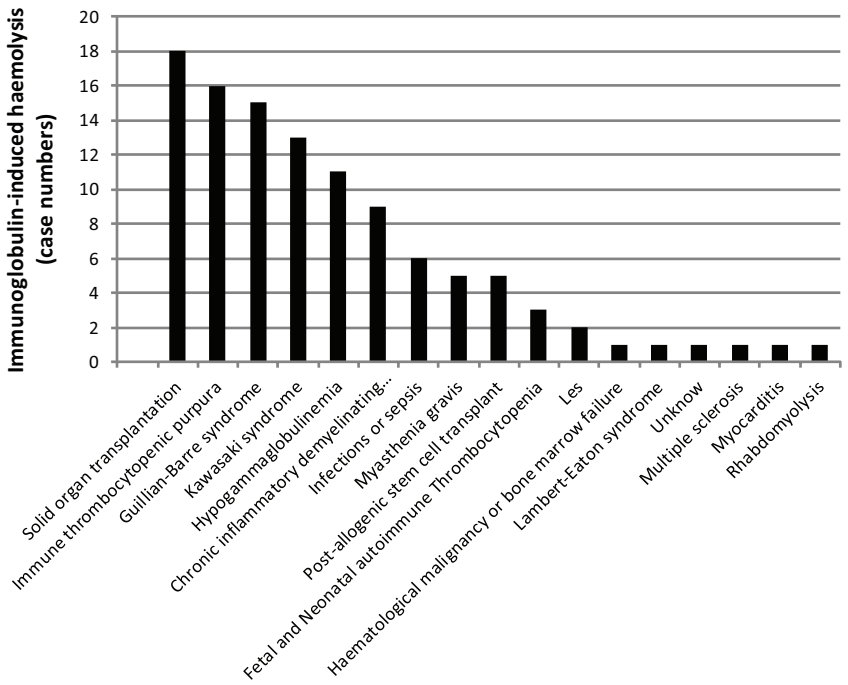

FIGURE 6 | The missing oxygen on the dark side: Ig-induced hemolysis in patients on Ig treatment. Black bars indicate the number of patients with a given clinical condition.

A significant proportion of patients receiving IVIG develop a positive direct antiglobulin test (DAT) detectable after 24 h for up to 10 days after the IVIG infusion (109, 110). However, it should be underlined that the DAT positivity due to the factors mentioned above (111, 112) is not sufficient *per se* to diagnose hemolysis and DAT positivity does not necessarily imply the presence of active hemolysis. DAT-positive mild hemolytic reactions can be easily missed and the true incidence of such reactions is difficult to document without careful clinical and laboratory follow-up.

In the majority of reports on HA, intravascular red blood cell (RBC) destruction via complement activation or extravascular RBC sequestration and removal by the reticulo-endothelial system was proposed to result from IgG alloantibodies with specificity for RBC antigens A, B, D, or C.

Hemolytic anemia induced by high-dose IVIG has an average incidence of 5.8% (85). Low-dose IgG replacement therapy is considered universally as safe, and only few cases of hemolysis following low-dose IVIG or SCIG administration have been described (80, 95, 110). A baseline WBC and RBC count prior to IVIG initiation and a close clinical and laboratory follow-up was suggested as a useful tool for early diagnosis and treatment. A possible work up might be to check hemoglobin (Hb) level prior and 48–78 h after Ig infusion. In case of a drop of Hb, the presence of DAT, an increase in unconjugated bilirubin, lactate dehydrogenase (LDH), and reduced haptoglobin level, followed by a rise in reticulocyte count should be assessed (**Figure 7**). We systematically reviewed case reports related to IVIG-induced hemolysis from 1987 to 2014 and identified 29 articles containing reports of 109 patients. Baseline characteristics of the patients are shown in **Table 2**. When available, blood group, DAT, Hb drop, and outcome are indicated. All reports showed positive DAT, except for a case of Yin et al. (87); in this case, DAT was performed 10 days after IVIG administration and the DAT negativity might have been due to a rapid removal of sensitized RBCs.

In the majority of patients, the outcome was positive: 106 out of 109 patients recovered with or without packed RBC transfusions; three patients died after HA, with the hemolytic episode representing a precipitating factor of a severe underlying condition. Elution experiments were performed and the search for blood group antibodies revealed anti-A and anti-B specificity in the majority of cases; anti-D specificity was assessed in four reports, often associated with other specificities (95, 106, 110). A search for other specificities such as anti-band 3 or anti-Gal was not performed. Only one report detected anti-C specificity in three patients; in one of them associated with anti-D irregular antibodies (110). Although studies were restricted to blood group antibodies, this finding demonstrated that polyvalent IgG preparations might contain clinically significant non-blood group antibodies, which are not part of the lot-release criteria in that their titration is not yet required by the European Pharmacopeia. Antibodies in HA, such as anti-C, may have unexpected hemolytic consequences (113–117). Beside passive transfer of alloantibodies, IgG administration also has been demonstrated to lead to unspecific enhanced erythrocyte sequestration, in particular, in patients with underlying inflammatory disorders (109, 118). In 2009, the Canadian IVIG Hemolysis Pharmacovigilance Group elaborated criteria to define an "IVIG-induced hemolysis" (83). They included a reduction of Hb levels $\geq$1 g within 10 days after Ig administration, with

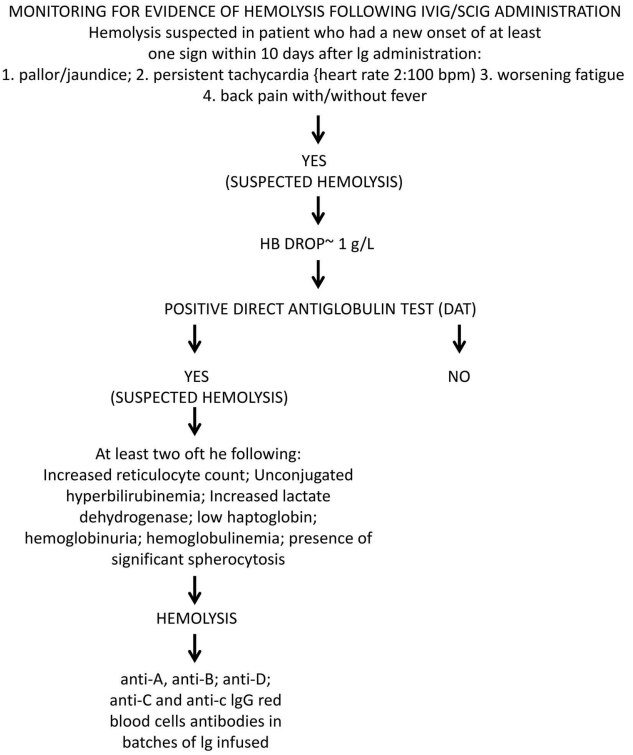

FIGURE 7 | Monitoring for evidence of oxygen missing at the dark side. Diagnostic algorithm for Ig-induced hemolysis according to criteria elaborated by the Canadian Group (83).

appearance of a positive DAT and, at least, two of the following criteria: increase in the reticulocyte count, elevation of LDH and unconjugated bilirubin serum levels, low haptoglobin, hemoglobinuria, hemoglobinemia, presence of significant spherocytosis, in the absence of alternative causes of anemia. The passive transfer of IgG alloantibodies through IgG concentrates is difficult to explain as polyvalent IgG is prepared from plasma of thousands of donors. Since immunization to RBC alloantigens can occur because of past transfusions or pregnancy, the hypothetical numbers of alloimmunized plasma donors should be rather low. Recently, other mechanisms underlying alloimmunization related to molecular mimicry have been demonstrated (119). The mechanism of high-dose IVIG-induced HA is complex and it might vary from patient to patient. IVIG cause hemolysis due to: (i) disease-associated pre-coating of RBCs; (ii) IgG with hemolysis triggered by passive transfer of IgG binding to blood group antigens; (iii) transfer of high levels of alloantibodies to RBC pre-coated at a low level only; or (iv) transfer of clinically tolerable levels of isoagglutinins plus transfer of additional RBC-reacting physiological autoantibodies. Indeed, hemolytic reactions could not be related exclusively to transfer of alloantibodies. Hence, antibodies other than histo-blood group alloantibodies (pre-)coated to RBCs might contribute to hemolysis in IgG recipients need to be identified. In addition, hemolytic episodes may possibly be precipitated by some sort of complexed/denatured IgG that co-purify with other IgG in the product (76, 109, 118, 120). Recently, a two hit mechanism for IVIG-induced hemolysis has been proposed: the passive transfer of alloantibodies through IVIG representing the first hit and the underlying inflammatory state representing the second hit (121). Nowadays all commercial Ig products have to undergo anti-A and anti-B testing and regulatory requirement ask for respective IgG antibody titers of $\leq$1:64 at 5% solution strength (w/v) (103, 104). Nevertheless, hemolysis might occur even in recipients of IgG products that meet these specifications (76). Consequently, it has been suggested that IgG recipients should be monitored for clinical signs and symptoms of hemolysis (122).

REDUCTION OF HISTO-BLOOD GROUP A AND B ALLOANTIBODIES IN IgG CONCENTRATES RAISES THE CHANCE FOR STAYING ON THE SUNNY SIDE OF THE MOON

With the detection of the immunomodulatory potential of IgG concentrates, their clinical use has continuously increased (123). To cover the need, at a first glance, an increase of the volume of plasma fractionated seems to be the most convenient option. However, this might economically not be viable because fractionation of plasma products is interconnected (124) and before increasing output of one product (e.g., IVIG), the market absorbance of the other products as well (e.g., albumin) must be ascertained. On a longer-run, a more viable option is to improve recovery. Considering recovery, the cold-ethanol fractionation apparently has reached its limits. As of today, four manufactures have invested into a "modern" fractioning technique on the basis of ion-exchange chromatography. Ion-exchange chromatography allows elevated recovery at high purity. As of today, five IVIGs, one SCIG, and one anti-D concentrate are fractionated by ion-exchange chromatography. Pharmacovigilance has shown that all chromatographically fractionated IVIG and SCIG, more or less prominently, show a tendency for elevated frequencies of hemolytic AEs. Anti-A and anti-B alloantibody titers are now lot-release criteria (see above) as they constitute the major risk parameter for hemolytic reactions mediated by IgG concentrates. To overcome the threat of end up on the dark side of the moon, two manufacturers have taken measures to reduce anti-A and anti-B titers in their IgG products. One measure chosen was adsorption of the alloantibodies by affinity chromatography (125). Reported reduction in both alloantibodies was significant and levels were similar to those in cold-ethanol fractionated immunoglobulins (126). The other measure chosen was reduction in anti-A using an automated indirect agglutination test for donor screening and exclusion of high-titer donations (approximately 5.1%) from plasma pooling and fractionation (127). This measure reduced anti-A in the IgG concentrate by one titer step. To ensure staying on the safe and sunny side, the manufacturer has announced the introduction of an alloanti-A and alloanti-B immune-affinity chromatography step into the manufacturing process (128). Preliminary results indicate depletion in anti-A and anti-B by >80% in investigational lots. Subsequently, we want to discuss possible consequences of (extensive) removal of antibodies reacting with histo-blood group antigens A and B.

REASONING ABOUT ANTIBODIES REACTING WITH TERMINAL SUGARS OF THE MAJOR HISTO-BLOOD GROUP ANTIGENS A AND B

Three facts have initiated our thinking about possible consequences of removal of histo-blood group A and B reacting

antibodies from IgG concentrates. (I) In collaboration with Hans U. Lutz, formerly Biochemistry ETH Zurich, we have observed the non-intended removal of natural anti-C3 autoantibodies regulating complement activation by large-scale immune-affinity adsorption of IgA from an IgG concentrate (129). Anti-C3 antibodies belong to the family of "NAbs" and have a particular role in homeostasis: they control activation of complement, among others, in the frame of NAb-mediated opsono-phagocytosis of altered or senescent cells, including RBCs (130–132). Thus, the intention to target one particular antibody by affinity chromatography might reduce that antibody specificity but at the same time affect other specificities as well. (II) It should be kept in mind that the blood groups A and B are in fact "histo-blood group" antigens, i.e., they are also found on white blood cells, T lymphocytes, and proteins and also can be found in soluble form (133). Alloantibodies reacting with histo-blood group antigens A and B thus have much broader tissue recognition than RBCs only. In addition, alloanti-A and alloanti-B belong to the population of NAbs recognizing non-self and most likely participate in primary host defense (134). (III) In contrast to cold-ethanol fractionation, where low titers of alloanti-A and alloanti-B are achieved on basis of their isoelectric points (IEPs), the (extensive) immune-affinity removal might affect a much wider IEP range, thereby removing broadly reacting antibodies and impairing some desirable functions of the IgG concentrate. Thus, the struggle for staying on the sunny side of the moon might have consequences for the antibody repertoire in an IgG concentrate.

Antibodies reacting with terminal di-, tri-, and tetra-saccharides belong to the large family of human anti-glycan NAbs. Histo-blood group A and B epitopes in terminal position are tetra-saccharides. Alloantibodies to these tetra-saccharides are found in the plasma of healthy individuals depending on the blood group they have. A considerable body of research into the nature of these NAbs has been performed so far, all using for isolation the corresponding terminal di- or tri-saccharides (135–137). Recently, the repertoire and epitope specificity of such immunoglobulins was addressed in depth by including the tetra-saccharide as well (138, 139). It proved that serum of healthy individuals contain respectable amounts of di- or tri-saccharide-reacting NAbs. These NAbs proved to be pseudo-anti-A and pseudo-anti-B NAbs as they are not reacting with the tetra-saccharide of histo-blood groups A and B. In contrast, alloanti-A and -B antibodies able to react with tetra-saccharides are reacting with the corresponding terminal di- and tri-saccharides. Reasoning about the biological role of these "high-titer and population conservative" anti-di- and anti-tri-saccharide NAbs and the consequence of their potential removal by immunoaffinity is outlined below.

A population of the anti-glycan NAbs are the anti-αGal NAbs which recognize Galα1-3Gal and Galα1-3(Fucα1-2)Gal epitopes. Anti-αGal NAbs have been described being xenoreactive, recognizing bacterial Galα1-3Gal (140) and having tissue homeostatic function. The daily removal of altered/senescent cells of the body is $\sim 10^{12}$. Removal is mainly mediated by apoptosis (no inflammation, no necrosis). RBCs, when they do not encounter a pathological condition, over their life span of 100–120 days remain intact although they shrink, do not undergo apoptosis but progressively become senescent, mainly due to cumulative oxidative stress. Removal of intact RBCs with a daily turnover of $\sim 2 \times 10^{11}$, corresponding to ~20 g cell mass, is effectuated by increased exposure of otherwise cryptic structures such as spectrin, band 3, or αGal epitopes. These exposed structures are recognized by low affinity, high avidity, C3-bearing NAbs, which promote the efficient removal of intact senescent RBCs (130, 141, 142). Immunoaffinity adsorption by tri-saccharides columns of di- and tri-saccharide reacting NAbs from IgG concentrates can eliminate anti-histo-blood A and B alloantibodies while it also eliminates αGal and this might have a Janus effect. The face directed to the sun tells that adsorbing αGal NAbs reacting with altered and senescent self on RBC might prevent an increase in the IgG load of RBCs over the threshold level of relevant hemolysis in individuals at risk. The face directed to the dark indicate that adsorption of tissue homeostatic antibodies might deprive an IgG concentrate of potentially beneficial antibodies. Although they are NAbs, tri-saccharide reacting antibodies can be induced by feeding bacteria bearing the corresponding carbohydrate epitopes (134). These inducible NAbs are considered to participate in primary host defense. Other antibodies possibly involved in primary host defense are the anti-αGal NAbs. They show a broad specificity and can react with a number of related αGal-terminated oligosaccharides, including those on bacteria (143). Thus, the immunoadsorption of di-and tri-saccharide reacting NAbs might diminish the potential of an IgG concentrate to mediate primary host defense. Therefore, when choosing affinity resins for immunoadsorption, there might be some aspects worth to consider.

In summary, the principles of avoiding co-fractionation through cold-ethanol fractionation (144) versus immune-affinity removal of histo-blood group alloantibodies can have an impact on the presence of homeostatic and first-line defense antibodies. According to present knowledge, only resins coated with the corresponding tetra-saccharides can ascertain the selective removal of histo-blood group alloantibodies presumably involved in HA. Resins coated with the corresponding di- and tri-saccharides also remove blood group alloantibodies, however not selectively. Such resins in addition might remove a broad range of NAbs present in IgG concentrates at relative high amounts. In the literature, the use of tri-saccharide-coated resin was reported (145–147). We have found no information available in the public domain indicating which type of resin is/will be used for reduction of the histo-blood group alloantibodies in large-scale fractionation of IgG. Furthermore, we suggest that the effect of reduction of anti-A and ant-B reacting antibodies by immune-affinity on the antibody repertoire of IgG concentrates can only be assessed by, e.g., using pathogens/commensals, which share the saccharide epitopes, that have been used to coat the affinity resins or alternatively by exposing senescent RBCs stripped off the IgGs coated *in vivo*. Finally, techniques are required, which allow detection of low affinity, high avidity NAbs.

THROMBOSIS – FALLING INTO A DARK LUNAR CRATER

IVIG administration-related AEs, including thrombosis, have been extensively described (148). Thrombotic AEs are severe AEs and patients with risk factors require a special care. Reported average incidence of IVIG-induced thrombosis ranges from 3 to 13% (149). Recognized risk factors for IVIG-induced thrombosis

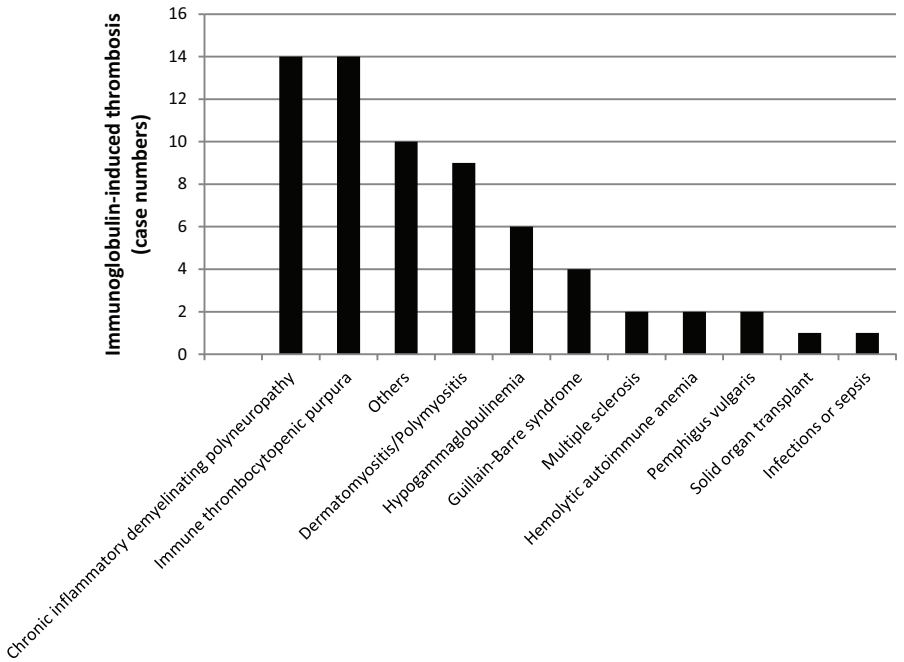

FIGURE 8 | Falling into a dark lunar crater while being on Ig treatment. Bars indicate the number of patients with a given clinical condition and Ig-mediated thrombosis.

include male gender; age >60; diabetes; renal insufficiency, dyslipidemia; hypertension; immobility; coronary disease; pre-existing vascular disease, family history of early thromboembolic disease; atrial fibrillation, high-dose and high-speed IVIG infusions. IVIG-induced thrombosis is reported both as venous events such as thrombosis stroke, pulmonary embolism (PE), deep venous thrombosis (DVT), and arterial ischemia events such as myocardial infarct and stroke. The mechanisms leading to IVIG-associated thrombosis are still not completely clear; three main mechanisms have been proposed, emphasizing the role of an increased blood viscosity causing a hypercoagulable state (150), the role of anticardiolipin antibodies passively transferred through IVIG (151), and the role of factor XIa or other biologically highly active factors passively transferred via IgG concentrates, such as PKA. Avoiding activated coagulation factors in IgG concentrates starts with appropriate anticoagulation of donated blood/plasma, i.e., careful mixing of anticoagulant and sample over the whole donation process. Alterations in an established manufacturing process neglecting appropriate controls can also lead to increased risk of transmission of activated coagulation factors. High MW proteins passively transferred by IVIG are probably contributing to this phenomenon (152). In patients with other risk factors, such as vascular disease, the increase in blood viscosity can precipitate thromboembolic events. As elderly individuals are prone for such AEs, we like to point to the possibility of elevated altered/senescent self-reacting with infused homeostatic NAbs being a possible factor facilitating thrombotic events as well. A relationship between IVIG administration and cerebral vasospasm has also been suggested by Sztajzel et al. (153); blood viscosity is a determinant for oxygen delivery to the tissues, and changes in viscosity can lead to a reduction in cerebral or myocardial perfusion.

We systematically reviewed case reports related to IVIG-induced thrombosis from 1986 to 2014 (**Figure 8**). Literature search identified 35 articles containing reports concerning 65 patients (6, 24, 149, 154–183). When data were available, diagnosis, risk factors, the number of IVIG infusion prior to thrombosis event, and outcome have been indicated. Baseline characteristics of the patients are shown in **Table 3**. High-dose IVIG induced thromboembolic events in 59 patients at low to medium IVIG doses. Marie et al. (163) observed that the frequency and type of arterial events was inversely related to the time elapsed from IVIG infusion; almost 50% (23 versus 21 reports) of arterial ischemic events occurred within 12 h following IVIG, while about 75% of venous thrombosis occurred after more than 24 h. No correlation between number of infusions and occurrence of AE was observed. The main risk factors observed in this review were hypertension (19 cases, 33% of prevalence), previous vascular disease (18%), and dyslipidemia (17%). Average mortality for thrombotic events was 10% (arterial ischemia 9% versus venous thrombosis 11%, PE representing the main venous fatal event). Predicting IVIG-induced thrombosis is difficult. Risk factors should be assessed for each patient including instrumental exams when needed. Doppler ultrasound can be useful as early diagnostic tool for thrombosis or to detect the presence of abnormal blood flow especially after prolonged immobility. IVIG should be administered at low IR to reduce the risk. The administration of antiplatelet or anticoagulant prophylaxis was suggested in patients with several risk factors (162). However, thrombotic events have been reported even after several previous uncomplicated courses of treatment.

Table 3 | Falling into a deep lunar crater – Ig-induced thrombosis in recipients of polyvalent immunoglobulins.

Publication	Number of patients	Age	Diagnosis	Ig dosage	Predisposing factors	Number of IVIG infusion prior to thrombosis event	Thrombosis (arterial or venous)	Time from the last infusion	Outcome
Vinod et al. (154)	1	>65	Guillain-Barrè	High		First	Arterial	72 h	Recovery
Sin et al. (157)	1	<65	Solid organ transplantation	High		First	Arterial	48 h	Recovery post-emergency renal transplant
Min et al. (24)	1	<65	CVID	Low (SCIG)	Hypercoagulability (oral contraceptive)	Several	Venous		nd
Rajabally et al. (149)	5	<65 (2), >65 (3)	CIDP	High	Diabetes (2), hypertension (2), immobility (4), coronary disease (2), arrhythmia (1)	First (3), several (2)	Arterial (3), venous (2)	14 days	Recovery (4), death (1)
Al-Riyami et al. (156)	1	11	ITP	High		Several	Venous	10 days	Recovery
Iroh et al. (155)	1	13	ITP	High	Estrogen treatment	First	Venous	12 h	Death
Barada et al. (158)	1	11	XLA	Low		Several	Venous	nd	nd
Lee et al. (159)	1	56	ITP	High		First	Venous	72 h	Recovery
White et al. (160)	1	43	Dermatomyositis	High		First	Arterial	2 h	Recovery
Feuillet et al. (161)	1	38	Multiple sclerosis	High	Oral contraceptives	Seventh	Venous	6 days	Recovery
Marie et al. (162)	2	51, 55	Polyarteritis nodosa (1), polymyositis (1)	High		Third, 15th	Venous (2)	2 h, 7 days	Recovery
Marie et al. (163)	6	76, 49, 63, 45, 64, 64	AHA (1), polymyositis (5)	High	Hypertension (3), hypercholesterolemia (3)	Second, sixth, several (4)	Venous (3), arterial (3)	2 days, 6 h (5)	Recovery
Geller et al. (165)	1	28	Streptococcal toxic shock syndrome	High		First	Venous	8 days	Recovery
Sheehan et al. (167)	1	43	*Pemphigus vulgaris*	High	Immobility, hypertension	First	Venous	16 days	Recovery
Hefer et al. (164)	1	85	ITP	High	Hypertension, chronic myelogenous leukemia	Second	Arterial	3 h	Recovery

(Continued)

Table 3 | Continued

Publication	Number of patients	Age	Diagnosis	Ig dosage	Predisposing factors	Number of IVIG infusion prior to thrombosis event	Thrombosis (arterial or venous)	Time from the last infusion	Outcome
Feuillet et al. (166)	1	38	Multiple sclerosis	High	Oral contraceptives	First	Venous	nd	Recovery
Vucic et al. (168)	7	57, 69, 75, 81, 79, 62, 80	CIPD (4), anti-MAG neuropathy (1), multifocal motor neuropathy (1)	High	Hypertension (3), hypercholesterolemia (3), previous stroke (2), arrhythmia (1)	Second (1), third (2), several (5)	Arterial (6), venous (1)	1 h (2), 2 days (3), 2 weeks (2)	Recovery (5)
Stamboulis et al. (169)	1	36	CIPD	High	Heavy smoker	First	Arterial	nd	Recovery
Katz et al. (170)	2	67, 65	*Pemphigus vulgaris*, dermatomyositis	High	Hypertension	Second, first	Arterial (1), venous (1)	6 h	Recovery
Zaidan et al. (171)	3	47, 65, 70	GBS (1), CIDP (2)	High	Hypercholesterolaemia (1), diabetes (2)	Third (1), several (2)	Arterial (3)	1 h (2), 1 day (1)	Recovery (2)
Brown et al. (172)	3	70, 91, 42	CVID	Low	Diabetes (1), myocardial infarction (2)		Arterial (3)	6 h	Recovery
Evangelou et al. (173)	1	54	CVID	Low	High platelets count	Several	Venous	24 h	nd
Emerson et al. (174)	2	54, 33	ITP, Evans Syndrome	High	Obesity (1)	Second	Arterial (2); venous (1)	2 h, 2 days	Death (1), recovery (1)
Alliot et al. (175)	1	63	ITP	High	Hypertension	Fifth	Venous	3 days	Death
Sherer et al. (176)	2			High			Venous		
Elkayam et al. (177)	4	60, 41, 67, 67	ITP, polymyositis, connective disease, CIPD	High	Hypercholesterolemia (2), hypertension (2)	several (3), first (1)	Arterial	4 days	Recovery
Go et al. (178)	1	52	ITP	High		First	Venous	1 day	Recovery
Turner et al. (179)	1	60	Miller-Fisher syndrome	High		Fifth	Arterial	5 days	Recovery
Harkness et al. (180)	1	40	CIDP	High	Hypercholesterolemia		Arterial and venous	7 days	Recovery

(Continued)

Table 3 | Continued

Publication	Number of patients	Age	Diagnosis	Ig dosage	Predisposing factors	Number of IVIG infusion prior to thrombosis event	Thrombosis (arterial or venous)	Time from the last infusion	Outcome
Paolini et al. (181)	1	78	ITP	High	Hypercholesterolemia	First	Arterial	7 days	Recovery
Rosenbaum et al. (182)	1	76	Miller-Fisher syndrome	High		First	Arterial	12 h	Recovery
Oh et al. (183)	1	17		High		Several	Venous	1 day	Recovery
Dalakas et al. (150)	2	62, 52		High		Second	Venous (1), arterial (2)	7 days, 2 days	Death (1), recovery (1)
Woodruff et al. (6)	4	72, 73, 62, 83	ITP	High	Hypertension (3), obesity (3), previous stroke (2), previous myocardial infarction (1)	Several (4)	Arterial (4)	2 h (3), 2 days (1)	Death (3)

Clinical and immunological characteristics of patients described in case reports.
Numbers in parenthesis indicate the number of patients with the given condition.

In such cases, patients should be examined for signs and symptoms of thrombosis during each courses of IVIG.

Immunoglobulin G concentrates are widely acknowledged to offer a safe, high-dose, long-term therapy option for a variety of diseases. AEs occur rarely and mainly are mild to moderate. Deviations from this rule of thumb are addressed by authorities and the plasma fractionation industry to achieve corrections. Above, we have reviewed two types of AE which have shown elevated frequency in the near past. We tried to give some insights which might help in reducing frequencies of AEs bed side.

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Therapeutic immunoglobulin selected for high antibody titer to RSV also contains high antibody titers to other respiratory viruses

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Specific antibodies against infections most relevant to patients with primary immunodeficiency diseases are not routinely evaluated in commercial polyclonal immunoglobulin preparations. A polyclonal immunoglobulin prepared from plasma of donors having high neutralizing antibody titers to respiratory syncytial virus (RSV) was studied for the presence of antibody titers against seven additional respiratory viruses. While donors were not selected for antibody titers other than against RSV, the immunoglobulin preparation had significantly higher titers to 6 of 7 viruses compared to those present in 10 commercially available therapeutic immunoglobulin products ($p \leq 0.01$ to $p \leq 0.001$). To consider this as a donor-specific attribute, 20 random donor plasma samples were studied individually and identified a significant correlation between the RSV antibody titer and other respiratory virus titers: donors with high RSV titers were more likely to have higher titers to other respiratory viruses. These findings suggest either some humoral antiviral response bias or more frequent viral exposure of certain individuals.

Keywords: IVIG, RSV, respiratory viruses, hyperimmune globulin, immune deficiency

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Introduction

The majority of primary immunodeficiency diseases (PIDD) include deficiencies of immunoglobulin quantity and/or quality (1, 2). Left untreated, these deficiencies lead to increased risk for recurrent upper and lower respiratory tract infections as well as for bacterial sepsis (3). While bacterial sepsis can result in immediate fatality, recurrent respiratory infections can result in chronic lung disease and bronchiectasis (4). Fortunately, the infectious susceptibilities in PIDD along with its risks can be mitigated to a substantive degree by immunoglobulin replacement therapy (5, 6). Therapeutic immunoglobulin is able to reduce incidence of infection in PIDD in large part owing to the diverse antibody specificities against pathogens contained within plasma of thousands of different healthy donors.

The quality of therapeutic immunoglobulin is monitored and evaluated in a number of ways. Intact antigen recognition by IgG in therapeutic immunoglobulin products is gaged by the presence of minimal titers of specific antibodies expected in the general population in what is referred to as potency tests. In the United States, the Food and Drug Administration requires potency tests to achieve minimal standards for Measles, Diphtheria, and Polio (FDA CFR 640.104). While these pathogens remain relevant at least to some degree – especially Measles – they are not infectious

diseases specifically relevant to PIDD patients needing immunoglobulin replacement. Commercially available immunoglobulins are therefore not standardized for specific antibody content for the most common infectious diseases to which PIDD patients are susceptible. Studies of these titers in available products have reported wide variation (7). How much specific antibody within a given dose of a therapeutic immunoglobulin preparation is needed to provide optimal resistance for PIDD patients remains an open question. One thing that is clear, however, is that increasing doses of standard polyclonal immunoglobulin are associated with decreasing incidence of infection in PIDD patients (8, 9).

Respiratory syncytial virus (RSV) is a relatively ubiquitous virus, but causes infections with severe morbidity and a resultant high mortality in PIDD and otherwise immunodeficient patients (10). In the present work, an investigational intravenous immunoglobulin (IVIG) product (RI-002) obtained from donors with high-titer antibodies against RSV was compared to commercially available polyclonal therapeutic IVIG products. RI-002 IVIG was used in a recently completed Phase III clinical trial in PIDD patients as standard replacement therapy (data currently under review). While not specifically intended for RSV activity in that trial, RI-002 prevented and treated experimental infection as well as restored pulmonary histology in normal and immune compromised cotton rats given RSV by nasal challenge (manuscript in preparation).

Since RI-002 was generated from donors with a high antibody response to RSV, we questioned whether this might reflect their being high responders to not only RSV but also other viruses. In other words, might they represent either an extreme in humoral antiviral responders or a selection of individuals highly exposed to respiratory viruses. To this end, we evaluated the antibody titers in RI-002 to several common respiratory viruses and compared them to those in other 10 commercially available IVIG preparations, which were not derived from donors with high titers against respiratory viruses.

Materials and Methods

Intravenous Immunoglobulin

Investigational IVIG (RI-002; kindly provided by ADMA Biologics) was manufactured using Cohn–Oncley Fractionation to FDA published specifications for intravenous immune globulin and included plasma from donors with high-levels of RSV antibodies. A donor with high titers to RSV was determined by RSV neutralization assay testing (MNA). IVIG was tested and released to meet the FDA guidance for the treatment of PIDD patients requiring that the donor pool consist of at least 1,000 donors and meet minimum titers for measles, polio, tetanus, and Hepatits B. Thus, RI-002 (ADMA Biologics Inc.) was manufactured to achieve standardized levels of anti-RSV potency as well as meet all standards required for a commercially available polyclonal IVIG. Of note that latter characteristic was not a feature of the previously available polyclonal RSV hyperimmune immunoglobulin. RI-002 therefore met standards for Measles, Polio, and Diphtheria titers (which are set at $\geq$0.60 CBER Reference, $\geq$0.28 CBER Reference, and $\geq$1.21 U/ml, respectively) with values of 0.73–2.70 CBER Reference, 0.83–1.24 CBER Reference, and 8.00–11.25 U/ml, respectively.

IVIG Utilized for Titer Testing

Three different batches of investigational IVIG (RI-002) produced at 3 separate times from different donor plasma pools and 10 different lots of commercially available IVIG (7 different manufactures/brands) were evaluated by ELISA to quantitate titers to [influenza A and B, RSV, parainfluenza virus serotypes 1, 2, and 3 (PIV 1-3), human metapneumovirus (hMPV), and coronavirus 229E (CoV 229E) and coronavirus OC43 (CoV OC43)] as described below. The ELISA assay was run on three separate dates in a blinded manner.

Individual Donor Sample Collection and Purification by Protein-A Chromatography

Plasma samples from 20 random donors were collected through a commercial plasma collection center and all donors provided their written consent for pathogen titer testing. All plasma and donor samples were collected in US FDA approved collection centers using approved standard operating procedures and testing methods, and all donors were compensated for their donation in accordance with FDA polices. Each donor consented to have their plasma used for various purposes including but not limited to commercial manufacturing, additional laboratory testing, reagents, etc. Plasma was purified by protein-A chromatography into their Ig fractions. Bulk recombinant Protein-A Sepharose FF resin was washed and packed in 15 ml conical and diluted with an equal volume of buffer and centrifuged at 2500 rpm for 5 min. Supernatants were removed for Protein-A chromatography and the pellets discarded. Sample supernatants were transferred to Protein-A columns corresponding to sample identification number. Flow through of approximately 6 ml was collected by gravity flow. Two CV (4 ml) of equilibration buffer were passed through the columns, combined with the flow through volume and stored at 4°C. Bound material from each column was removed with 5 ml of elution buffer (0.1 M citrate, pH 3.4) and collected in 15 ml conical tubes containing 400 µl of 3 M Tris base to bring the eluates to pH 7.8. The columns were centrifuged as described above to collect the entire elution buffer. Final volume for each elution was 6.0 ml. Recovery of Ig was calculated based upon absorbance at 280 nm/1.4 of 1:5 dilutions of sample aliquots.

The purified Ig fraction was run three separate times for determination of antibody levels using an ELISA as for the IVIG preparations. Antibody titers to each of the nine respiratory viruses were obtained from each run repeated on three separate occasions; and the results transformed to $\log_2$ scale.

Viral Titer IgG ELISA

ELISA was performed to detect virus-specific IgG for nine respiratory viruses [influenza A and B, RSV, parainfluenza virus serotypes 1, 2, and 3 (PIV 1-3), hMPV, and coronavirus 229E (CoV 229E) and coronavirus OC43 (CoV OC43)] according to published methods (11). Viral antigen preparations were created from infected whole-cell lysates for all viruses as described. Briefly, influenza viruses were grown in MDCK cells, RSV was grown Hep-2 cells, PIV 1–3 viruses were grown in Vero cells,

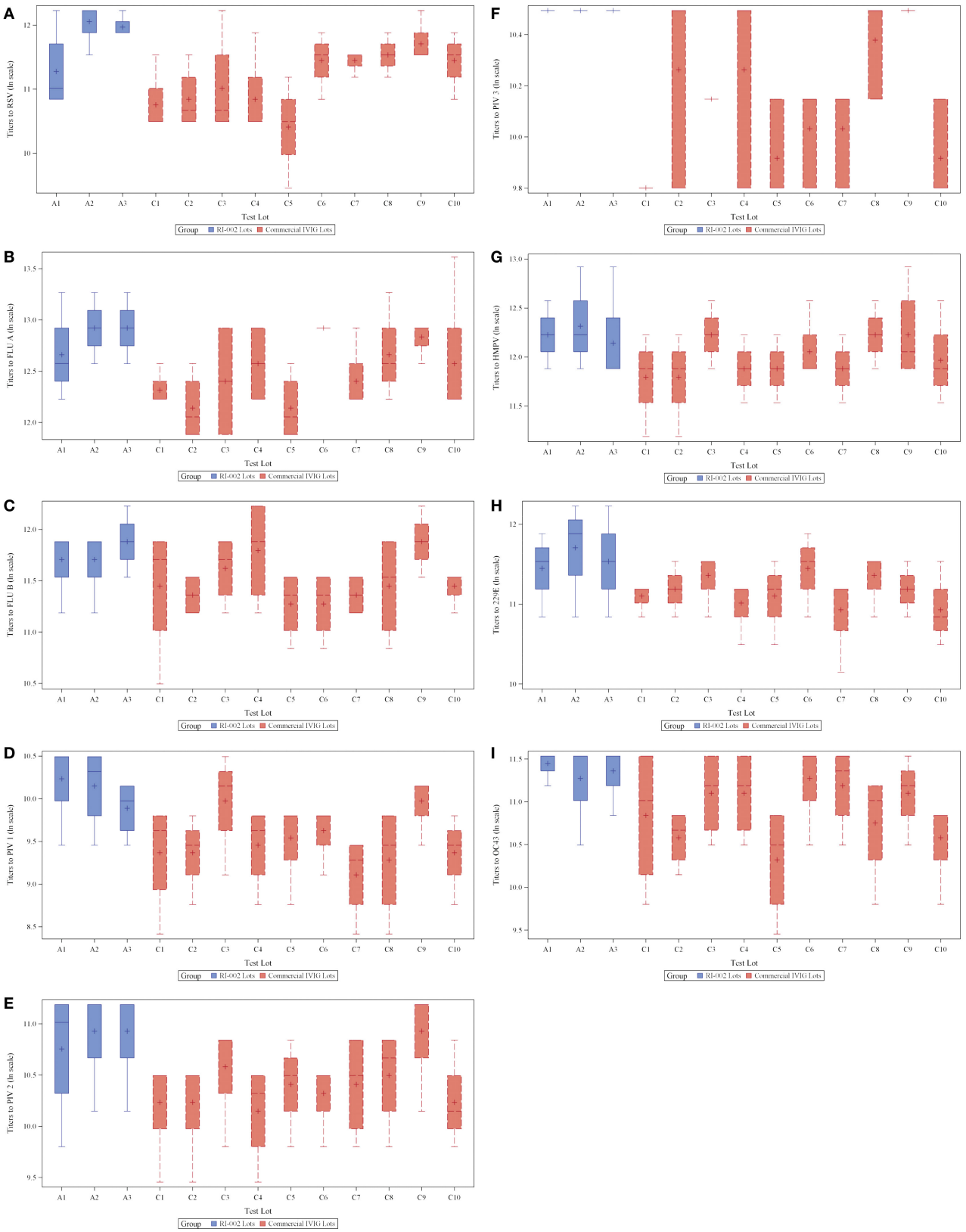

FIGURE 1 | Box plots showing 3 RI-002 lots and 10 commercial lots distribution of titers to respiratory syncytial virus (A) and titers to respiratory viruses influenza A (B), influenza B (C), parainfluenza virus serotypes 1 (D), parainfluenza virus serotypes 2 (E), parainfluenza virus serotypes 3 (F), human metapneumovirus (G), coronavirus 229E (H), and coronavirus OC43 (I).

hMPV was grown in LLC-MK2 cells, coronavirus 229E was grown in MRC-5 cells, and coronavirus OC43 was grown in HRT-18G cells. After cytopathic effect was extensive, the cells were scraped into the supernatant and centrifuged at low speed. The pellets were resuspended into distilled sterile water/0.5% NP40, sonicated, and then clarified. CoV 229E and hMPV were further purified on a 20%/60% discontinuous sucrose gradients using ultracentrifugation and bands were diluted in Tris NaCl EDTA buffer. Viral antigen preparations were stored at −80°C until use.

ELISA testing of IVIG was performed by an investigator blinded to the type of product being tested. A total of 13 IVIG products were procured and labeled A through M. All products were diluted with dilution buffer (PBS with 0.3% Tween 20 and 0.1 M EDTA) to a standard concentration of 50 mg of IgG/ml. Each viral antigen preparation was diluted to a previously determined concentration in bicarbonate buffer and coated separately on ELISA plates and stored overnight in humidified chambers at 4°C. The following day, plates were washed and eight serial twofold dilutions in duplicate of unknown IVIG product were incubated on the antigen-coated plates at room temperature in humidified chambers for 3 h (initial IVIG dilution utilized was 1:1600). Plates were then washed and bound IgG was detected with alkaline phosphatase-conjugated goat anti-human IgG followed by substrate. A known serum standard was included on each plate and the IgG titer for a specific virus was defined as the highest dilution with an optical density (OD) of 0.20.

Statistical Analysis

Titers data were tabulated with descriptive statistics, and the difference between the RI-002 and commercial IVIG preparations was presented as the ratio of geometric means (RGM) along with 95% Confidence intervals for the RGM. p values for testing the null hypothesis that the RGM equaled 1 were determined via a two-sample two-tailed Student's t-test (significance defined as $p < 0.05$).

To evaluate the correlation between the titers to RSV and the titers to another non-RSV respiratory virus at the donor level, antibody data were paired by matching the donor ID within the same ELISA Assay replicate (three separate repeats referenced as Run 1, 2, and 3). Titers to RSV from a donor were paired with the titers to another non-RSV virus of the same donor. Hence, a total of 20 pairs were created within a Run and a total of 60 pairs were created within a comparison owing to the replicates.

Linear correlation was assessed between the titers to RSV and the titers to another non-RSV virus using Pearson correlation coefficient on both linear and on $\log_2$ scale. All analysis was performed using SAS version 9.3.

Results and Discussion

To evaluate the hypothesis that therapeutic polyclonal immunoglobulin prepared from donor plasma with high titers of anti-RSV IgG may also have evidence of increased humoral immunity against other viral pathogens, RI-002 was compared in aggregate to 10 different lots of commercially available standard polyclonal IVIG products. In each case, the specific IgG against RSV antigens was evaluated by ELISA as well as the specific IgG against antigens prepared from eight other respiratory viruses. These included influenza A and B, hMPV, parainfluenza virus 1, 2, and 3, coronavirus OC42, and V299E. In each case, the mean ratio of the geometric mean of the titer of RI-002 to the commercial polyclonal IVIG preparations was higher in RI-002 (**Figure 1**). The difference in these means achieved statistical significance for all but one of the viruses (hMPV, which demonstrated a trend toward significance). In aggregate, the mean titers were 1.5-fold higher in RI-002 and ranged from 1.4 to 1.7-fold higher depending upon the particular virus (**Table 1**). Not surprisingly, RSV was the highest. While other lots of IVIG showed a wide range of titers to the other viruses and often had a high titer to one virus but low titer to other viruses, RI-002 was consistent from lot to lot and demonstrated consistently high titers to all of the viruses. Importantly, since RI-002 is standardized to high RSV titers, the repeated findings of high titers to these other viruses across the lots tested here is likely to be a consistent feature across all lots prepared in the same manner.

TABLE 1 | Titers to respiratory viruses: comparisons between RI-002 and commercial IVIG batches.

Virus	Ratio of geometric means (95% CI) (RI-002/commercial IVIG)[a]	p Value[b]
RSV	1.861 (1.249, 2.771)	0.003
PIV 1	1.792 (1.282, 2.505)	0.001
OC43	1.610 (1.127, 2.301)	0.010
PIV 2	1.601 (1.160, 2.210)	0.005
229E	1.494 (1.144, 1.950)	0.004
Flu A	1.402 (1.067, 1.843)	0.016
Flu B	1.316 (1.026, 1.688)	0.031
hMPV	1.264 (0.990, 1.613)	0.060
PIV 1 and 2	1.694 (1.250, 2.296)	0.001
OC43 and 229 E	1.551 (1.237, 1.945)	<0.001
All viruses[c]	1.529 (1.227, 1.907)	<0.001

[a] *Three randomly selected RI-002 batches and seven unselected commercial lots of IVIG from four different manufactures/brands.*

[b] *Two-group t-test for null hypothesis of no difference between the groups in geometric means (i.e., ratio of geometric means = 1).*

[c] *Pooled RSV, respiratory syncytial virus; Flu A, influenza A; Flu B, influenza B; hMPV, human metapneumovirus; PIV 1, parainfluenza virus serotypes 1; PIV 2, parainfluenza virus serotypes 2; OC43, coronavirus CoV OC43; 229E, coronaviruses CoV229E.*

While the specific levels of anti-pathogen antibodies required in IVIG to provide protection from the many pathogens that infect patients with PIDD is unknown, it stands to reason that a greater quantity of antibody to a specific pathogen will provide a greater level of protection (12). A number of variables also need to be factored into that equation, such as the actual quality (affinity and avidity) of the antigen-specific antibody as well as the inflection point past, which increasing titers become irrelevant to protection. Further study of exogenously applied immunoglobulin is required to gain the insights into both questions in a way that will translate into clinical efficacy. There is, however, evidence to suggest that having high pathogen-specific antibody in IVIG may translate into improved clinical outcomes with regards to that particular pathogen as was demonstrated for *Streptococcus pneumonia* and otitis (13).

The results, however, demonstrate that IVIG manufactured from a plasma pool derived from high titer anti-RSV plasma donors contains high titers of antibodies to other respiratory

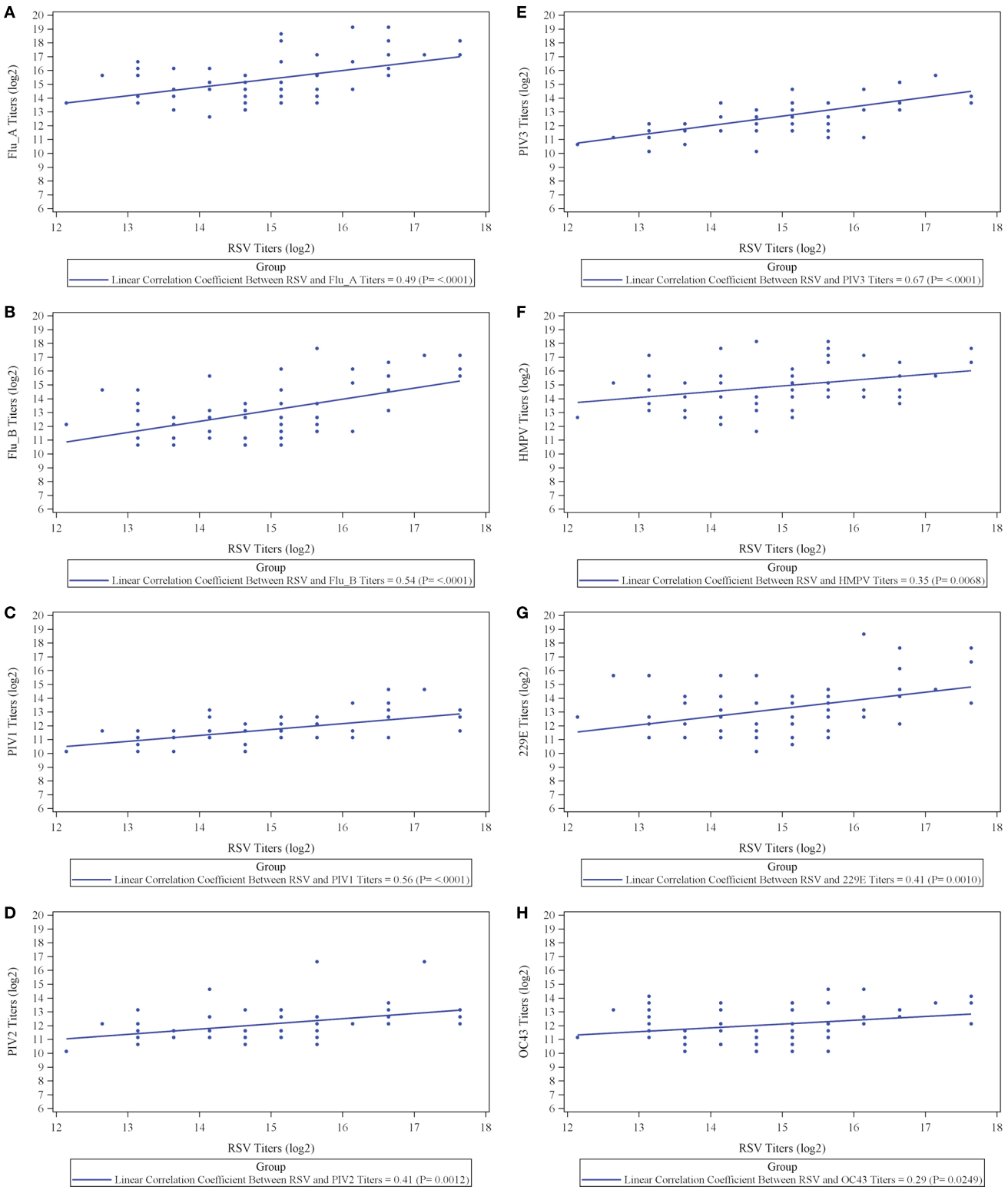

FIGURE 2 | Correlation between titers to respiratory syncytial virus (RSV) and titers to respiratory viruses influenza A (A) and influenza B (B), parainfluenza virus serotype 1 (C), parainfluenza virus serotype 2 (D), parainfluenza virus serotype 3 (E), human metapneumovirus (F), coronavirus 229E (G), and coronavirus OC43 (H).

TABLE 2 | Linear correlation coefficient between titers to RSV and titers to non-RSV virus.

Scale	Pearson linear correlation coefficients of titers to RSV and titers to other respiratory viruses							
	Flu A	**Flu B**	**hMPV**	**PIV1**	**PIV2**	**PIV3**	**OC43**	**229E**
Log2	0.49[a]	0.54[a]	0.35[b]	0.56[a]	0.41[b]	0.67[a]	0.29[c]	0.41[a]
Linear	0.49[a]	0.59[a]	0.28[c]	0.50[a]	0.24[d]	0.59[a]	0.34[b]	0.40[b]

[a] $p \leq 0.001$.
[b] $p \leq 0.01$.
[c] $p \leq 0.05$.
[d] $p > 0.05$.

viruses and suggests that there may be a direct correlation between antibody responder status of donors to RSV and their responder status to other viruses. To evaluate this, we prepared an immunoglobulin fraction from 20 randomly selected plasma donors containing high, medium, and low titers to RSV and measured their antibody levels to the other 9 respiratory virus antigens. A direct correlation was present between the RSV titers and those to all nine of the other respiratory viruses (**Figure 2**). Thus, the higher the RSV titer values, the greater the titers to other non-RSV respiratory viruses. Likewise, lower titers to RSV correlated with lower titers to the other respiratory viruses. In each case, correlation coefficients were statistically significant ($p < 0.05$) and ranged from 0.29 to 0.67 $\log_2$ scale (**Figure 2**; **Table 2**).

The exact reason that RI-002 contains elevated antibody titers to other respiratory viruses is not entirely clear as is the reason for the positive correlation between RSV titer and that to other viruses in the immunoglobulin from individual donors tested. There are, however, a number of possibilities to consider. First, it is possible that certain individuals are high humoral immune responders either in general or specifically against intracellular antigens. Given the diversity in MHC and the concomitant linkages of MHC alleles to immunity in general, this is at least plausible. Specifically, individuals having particular MHC alleles have higher humoral response after viral vaccination (14, 15). Thus, the donors who were selected based on their high responses to RSV may also have been high responders to other respiratory viruses. Whether these donors may also be high responders to pathogens other than respiratory viruses remains to be studied. Another possibility to account for the higher response of high-titer antibody RSV donors to other non-RSV respiratory viruses is that these donors may have experience to a greater diversity of viral infections. This could be a feature of their occupation or other demographic considerations that are not routinely considered in selecting plasma donors for the manufacture of therapeutic immunoglobulin. Given the diversity of high antiviral titers in both the IVIG preparation and the individual donors, we would hypothesize the former. Formal testing of elite antiviral humoral response, however, needs further study to establish this hypothesis. Moreover, whether these findings regarding the composition of a hyperimmune IVIG translate into clinical efficacy for protection against a diversity of infections, however, remains to be determined.

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