



## Research paper

# Association of blood IgG with tumor necrosis factor-alpha and clinical course of chronic lymphocytic leukemia



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## ARTICLE INFO

## Article history:

Received 29 April 2018

Received in revised form 14 August 2018

Accepted 20 August 2018

Available online 30 August 2018

## Keywords:

Chronic lymphocytic leukemia

Immunoglobulin replacement therapy

IVIg

SCIG

Hypogammaglobulinemia

Immunodeficiency

Signal transduction

Cytokines

Toll-like receptors

Janus kinases

Bruton's tyrosine kinase

## ABSTRACT

The intrinsic humoral immunodeficiency of chronic lymphocytic leukemia (CLL) is often managed with immunoglobulin replacement therapy (IgRT) to maintain IgG levels in the low-normal range (6–8 g/L) but optimal targets for IgG and timing to commence IgRT are unclear. IgG levels fell near 6 g/L at rates of  $-0.85 \pm 0.14$  g/L/year in 51 patients who required treatment for CLL within  $4.5 \pm 0.4$  years from initial diagnosis and  $-0.27 \pm 0.04$  g/L/year in 40 patients with progressive disease who remained untreated after  $8.5 \pm 0.5$  years. In contrast, endogenous IgG levels remained above 8 g/L in patients with highly indolent disease ( $n = 25$ ) and TNF $\alpha$  and beta-2-microglobulin ( $\beta 2M$ ) in blood decreased when IgRT was used to increase IgG levels over 9 g/L. At 15 g/L but not 5 g/L, the IgRT product Hizentra® inhibited B cell receptor (BCR)-activation, TNF $\alpha$  production, and survival *in vitro*, particularly of CLL cells that spontaneously made little TNF $\alpha$ . These findings suggest deterioration of the humoral immune system is associated with progressive CLL and altering the dosing of IgRT to achieve higher than conventional IgG target levels may have therapeutic activity.

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## 1. Introduction

Hypogammaglobulinemia involving all three immunoglobulin (Ig) classes is intrinsic to Chronic Lymphocytic Leukemia (CLL) [14,35] and can cause significant morbidity, particularly from sino-pulmonary infections [48]. It is not entirely clear why hypogammaglobulinemia is so characteristic of CLL in the absence of immunosuppressive treatments like Fludarabine and Rituximab (Forconi et al., [14]). Explanations include direct inhibitory effects of leukemia cells on normal B cells and plasma cells or indirect effects on helper T cells [9,10,38].

Immunoglobulin replacement therapy (IgRT) is the major form of treatment for humoral immunodeficiency ([4,5]). The immunoglobulin product is precipitated from the blood of thousands of healthy donors

and consists mainly of pooled IgG preparations. A number of commercial Ig formulations are available for injection as intravenous immunoglobulin (IVIg) or subcutaneous immunoglobulin (SCIG). These formulations use different small molecule stabilizers including sugars, polyols, and amino acids and contain different amounts of antibodies. Privigen® for intravenous use and Hizentra® for subcutaneous administration are made by CSL-Behring and stabilized with L-proline. Privigen® is 20% IgG and Hizentra® is 10% IgG ([4,5]). IgRT is usually prescribed at a dose of 0.4 g/kg monthly when IgG levels fall below 6 g/L in CLL patients with histories of infections. This approach decreases infection rates but does not affect the clinical course or improve survival [15,48]. The IgRT dose can be individualized to keep patients free of infections ([1, 4,5]) and it has been suggested that IgG levels  $>7.44$  g/L provide the best protection from major infections [48]. However, optimal IgG target levels for IgRT have not been established in CLL.

Despite the importance of humoral immunodeficiency in CLL, little information is available about how Ig levels evolve in the absence of

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### Research in context

Chronic lymphocytic leukemia (CLL) is the commonest adult leukemia. Immunoglobulin deficiency is a unique and important problem in CLL that is often managed with immunoglobulin replacement therapy (IgRT). The mechanism of the immunodeficiency and optimal use of IgRT remain unclear. The results in this manuscript implicate defective transitional B cell development in the decline of Ig levels. Allowing IgG levels to fall below 8 g/L may promote growth of CLL cells and maintaining IgG levels close to the upper normal limit (15 g/L) with IgRT may slow disease progression. These findings have important implications for the care of CLL patients.

immunosuppressive drug treatment that might influence a decision to start IgRT. In this manuscript, the clinical records of 116 patients with serial Ig levels available over at least an 8 year period or until first treatment for symptomatic CLL were examined to provide such information. There is also little information about the effects of IgRT on CLL cells *in vitro* or *in vivo* that may help identify appropriate IgG target levels. To address this gap, records of 21 patients receiving IgRT were examined and experiments were performed to determine how IgRT affects CLL cells *in vitro*. The results suggest appropriate target levels for IgG in CLL may be much higher than achieved with conventional dosing.

## 2. Methods

The retrospective study population consisted of 116 patients attending the Sunnybrook CLL clinic in 2003, 2009, and 2013 with immunoglobulin quantification available from diagnosis for at least 5 years or until first treatment for CLL. CLL was diagnosed according to IWCLL criteria [19]. Twenty-five patients met criteria for indolent disease as described by Guarini [17] and 51 received treatment for CLL based on conventional criteria [19]. The remaining 40 patients were classed as “progressors”, implying most would eventually come to treatment. Information about demographics, prognostic markers [19], and treatments were obtained from electronic patient records. Serum beta-2-microglobulin ( $\beta$ 2M), cytogenetics, and CD38 expression were obtained from pathology reports. Clinical staging was based on the Rai classification [19]. Ig measurements were performed by the clinical service laboratory at Sunnybrook. The last available Ig level or the level prior to treatment for CLL was used to calculate the rate of decline of the Ig class (Fig. 1). Patient characteristics are listed in Supplementary table 1.

For the observations in Fig. 2B, records of 119 consecutive patients attending the CLL clinic in 2017 were examined only for Ig levels at diagnosis and use of IgRT. The study was approved by the Sunnybrook Research Ethics Board.

Details about reagents, cell culture, flow cytometry [47], cytokine measurements [43], immunoblotting and statistical methods [29] are provided in the supplemental Methods (available on the *Ebiomedicine* Web site).

## 3. Results

### 3.1. Temporal changes in Ig classes in CLL patients

Records of 116 CLL patients on “watch and wait” management [19] with Ig quantification recorded over at least a 5-year period from time of diagnosis were studied to provide information about the evolution of IgG, IgM, and IgA levels during disease progression. No patient received IgRT in this time. Patients were classed into three groups based on the outcome at the end of the observation period. Twenty-five were classed as having “benign” disease [17] in that they did not

progress beyond Rai stage 0/1, develop lymphocyte counts  $>30 \times 10^9$  cells/L, or require treatment in the observation period. Such patients may never need treatment for CLL in their life-time [17]. Fifty-one patients were treated for CLL in the study period on the basis of standard indications [19]. Their last recorded Ig quantification for the purposes of the study was taken just before beginning treatment. Remaining patients ( $n = 40$ ) were classed as “progressors” as their lymphocyte counts increased to  $>30 \times 10^9$  cells/L, which often portends an eventual need for treatment although none was prescribed over the observation period.

The patient population is described in Fig. 1A and Supplementary table 1. Treated patients came to treatment within  $4.7 \pm 0.4$  years while progressors and benign patients were observed for  $8.5 \pm 0.5$  and  $7.6 \pm 0.5$  years, respectively, without any treatment. There was no difference in average ages among the different groups and a non-significant increase in proportion of males in the treated group. Rai stage at the end of the observation period was  $0.2 \pm 0.1$  for benign patients but increased to  $2.3 \pm 0.3$  and  $3.5 \pm 0.1$  for progressors and treated patients, respectively. Serum beta2-microglobulin ( $\beta$ 2M) at the end of the observation period was higher in treated patients, and their CLL cells had higher CD38 expression and more high-risk genetic lesions (del11q22 and del17p) [19] than the other groups. Progressors were intermediate between benign and treated patients with respect to  $\beta$ 2M levels, CD38 expression, and high-risk cytogenetic features (Fig. 1A).

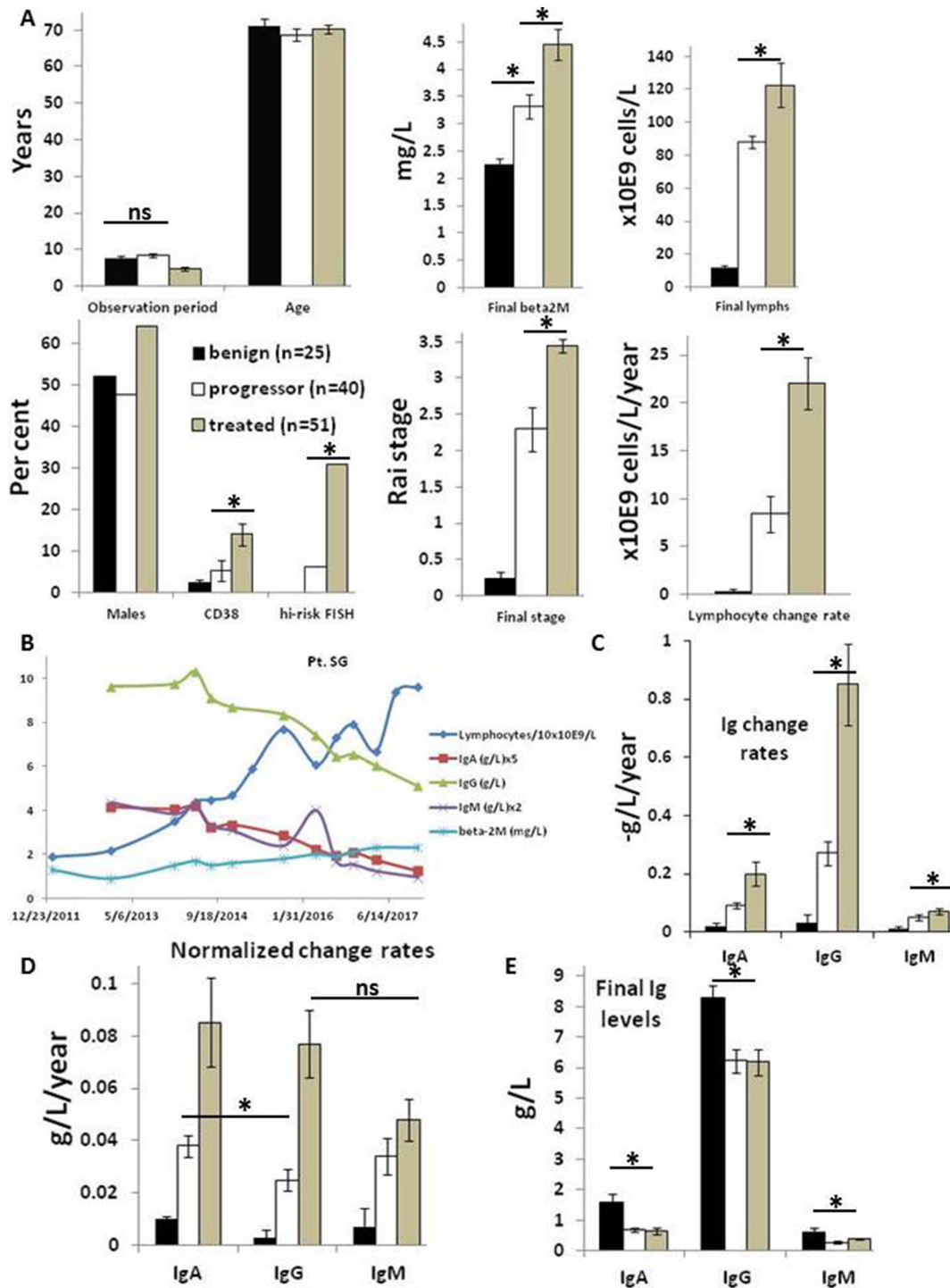
Rates of change of blood lymphocytes were calculated by subtracting the initial lymphocyte count from the final count at the end of the observation period and dividing by the years of observation (Fig. 1A; bottom right panel). This rate was higher for treated patients ( $22.1 \pm 2.7 \times 10^9$  cells/L/year) and culminated in an average final lymphocyte count of  $122.6 \pm 13.4 \times 10^9$  cells/L compared with final counts of  $88.3 \pm 3.6 \times 10^9$  cells/L for progressors and only  $11.7 \pm 1.4 \times 10^9$  cells/L for benign patients (Fig. 1A; top right panel).

### 3.2. Coordinated decline of Ig classes in patients with progressive disease

Initial inspection of the data suggested a parallel decline of all three classes of Ig with increases in  $\beta$ 2M and circulating lymphocytes, as shown in the example (Fig. 1B). The approximately linear declines allowed a rate of change for each Ig class to be calculated for each patient by subtracting the initial amount of IgA, IgG, or IgM from the amount at the end and dividing by the length of the observation period. Rates of decline for IgA, IgG, and IgM were all greater in patients who went on to require treatment for their CLL (Fig. 1C) with highest absolute changes for IgG. However, normal levels are higher for IgG (6–16 g/L) than IgA (0.7–4 g/L) or IgM (0.4–2.5 g/L). To compare directly the rates of change of the different Ig classes, absolute values were normalized by dividing them by the midpoint value of the normal range (*i.e.* 11, 2.35, and 1.2 g/L for IgG, IgA, and IgM, respectively) (Fig. 1D). With this adjustment, there was no difference between the rates of change of IgG, IgA, and IgM for the patients who required treatment.

IgG and IgM levels did not change significantly over the observation period in patients with a benign clinical course although there was a low decline ( $0.01 \pm 0.001$  g/L/year) for IgA (Fig. 1D). Rates of decline of IgG, IgA, and IgM in patients with progressive disease that did not require treatment were lower than for patients who required treatment at the end of the observation period but higher than for benign patients and IgA levels appeared to decline somewhat faster than IgG (Fig. 1C,D).

Consistent with these rates of decline, Ig levels at the end of the observation period were significantly lower in progressor and treated patients compared to patients with benign disease (Fig. 1E). Notably, IgG levels that remained above 8 g/L were associated with a benign clinical course while IgG levels that fell near 6 g/L were associated with disease progression and need for treatment (Fig. 1E).



**Fig. 1.** Rates of decline of immunoglobulin sub-classes in CLL patients along a spectrum of disease severity. A. CLL patients were classed as benign (black bars, n = 25), treated (gray bars, n = 51), or progressor (white bars, n = 40) as described in the materials and methods. Clinical characteristics of the three groups are shown in the bar graphs with units for each descriptor indicated along the Y-axis. B. Example of a “progressor” patient, showing concordant declines in IgA, IgM, and IgG levels with disease progression indicated by increases in blood lymphocyte and  $\beta$ 2M levels. C–E. Average rates of decline (C), normalized rates of decline (D), and final levels (E) of Ig sub-classes along with standard errors are shown in the graphs. \*,  $p < 0.05$ ; ns = not significant.

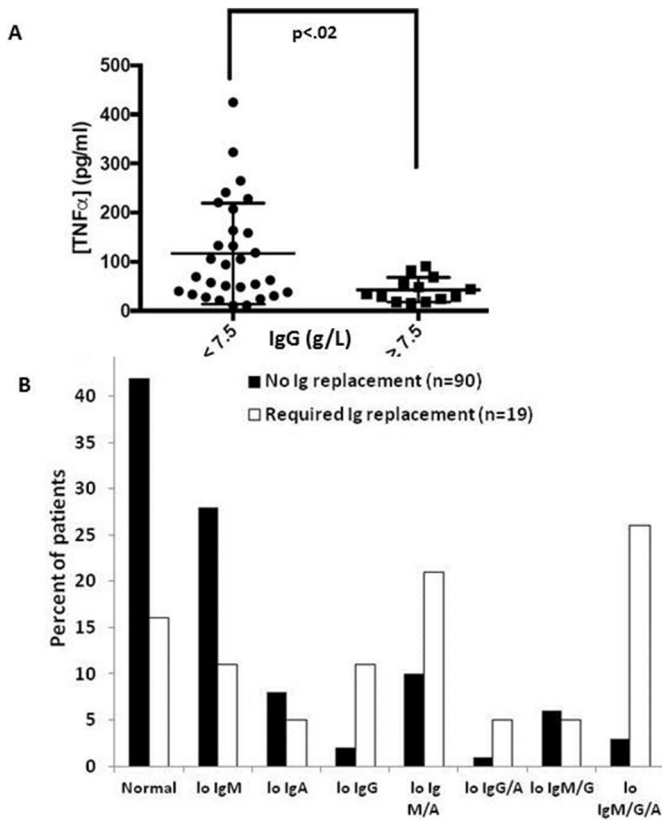
### 3.3. Inverse association of plasma TNF $\alpha$ and IgG levels

TNF $\alpha$  inhibits B cell development in the bone marrow [26] and blood TNF $\alpha$  is increased in CLL patients [12,13]. Higher TNF $\alpha$  levels are associated with more aggressive disease [12,13], although CLL cells with aggressive clinical behavior make less TNF $\alpha$  than cells from patients with indolent disease. [13,18,22]. TNF $\alpha$  in the plasma of an additional 43 patients was found to correlate inversely with IgG level at the same time-

point. Average TNF $\alpha$  levels in patients with IgG levels  $<7.5$  g/L were  $120 \pm 21$  g/L but only  $30 \pm 10$  g/L with IgG levels  $>7.5$  g/L (Fig. 2A).

### 3.4. Ig levels at diagnosis in CLL patients

The observation that Ig levels declined inexorably in most CLL patients (Fig. 1C,D) coupled with the fact that asymptomatic CLL can go undetected for a long time [32] suggested many patients may be



**Fig. 2.** Relationship of blood immunoglobulin levels with TNF $\alpha$  and need for IgRT. **A.** Plasma was collected from 43 patients. Serum IgG was determined by the Sunnybrook Clinical Laboratory and plasma TNF $\alpha$  was measured by Multiplexing LASER Bead Technology. An iterative algorithm was used to group the samples, depending on whether they contained more or <7.5 g/L IgG. **B.** Ig levels at time of initial CLL diagnosis were recorded for 109 patients including 19 who were commenced ultimately on IgRT. Low (lo) IgA, IgG, and IgM were defined as <0.7, 6.0, and 0.4 g/L, respectively.

hypogammaglobulinemic by the time they are diagnosed officially with CLL [35]. Supportive treatment for symptomatic hypogammaglobulinemia involves IGRT with IVIG or SCIG ([48]; [4,5]; [25]). Records of 119 consecutive CLL patients were examined for an initial concomitant diagnosis of hypogammaglobulinemia (Fig. 2B). Nineteen patients received IgRT at some point in the disease course. Low IgG (lo IgG), IgA (lo IgA), and IgM (lo IgM) levels were defined as below the lower limit of normal (i.e. 6, 0.7, and 0.4 g/L for IgG, IgA, and IgM, respectively).

Ig levels were in the normal ranges at the initial diagnosis for most patients (Fig. 2B). However, over 25% of the patients had an isolated IgM deficiency and a smaller number had deficiencies in IgG or IgA alone or in more than one Ig class (Fig. 2B). Notably, subnormal Ig levels at time of diagnosis appeared to predict the need for subsequent IgRT that was prescribed according to accepted guidelines [25]. Only 16% of patients who required IgRT had Ig levels in the normal range at the initial CLL diagnosis. Defects in multiple Ig classes and especially panhypogammaglobulinemia were especially associated with subsequent need for IgRT (Fig. 2B).

### 3.5. Dose-dependent effect of IgRT on $\beta$ 2M and TNF $\alpha$ levels *in vivo*

IgRT is indicated for CLL patients with low IgG levels (generally <5 g/L) and a history of serious infections requiring antibiotics [25]. Trough levels achieved with conventional administration of IgRT are usually in the low-normal range. In the course of managing hypogammaglobulinemic CLL patients with IgRT, a relationship was noticed between the level of serum  $\beta$ 2-microglobulin ( $\beta$ 2M), a marker of

CLL burden [45], and IgG level attained by IgRT as shown in the examples (Fig. 3A).

In some patients (Fig. 3A, top graphs) institution of IgRT with IVIG or SCIG was associated with an increase in  $\beta$ 2M that was sometimes followed shortly by the need to start CLL therapy. In a minority of patients,  $\beta$ 2M levels remained stable or even decreased somewhat upon institution of IgRT (Fig. 3A; bottom graphs). In one case, circulating lymphocytes declined steadily from 450 to 189  $\times 10^9$  cells/L over 6 years with IgRT as the only deliberate therapeutic maneuver (Fig. 3A; right bottom graph, Pt. SS).

These different response patterns appeared to be associated with the magnitude of IgG level achieved with IgRT. In patients with apparent disease progression despite IgRT, IgG levels only increased into the 6–7 g/L range (Fig. 3A; top graphs). In patients on IgRT with apparently better disease control, IgG levels were maintained above 12 g/L (Fig. 3A; bottom graphs).

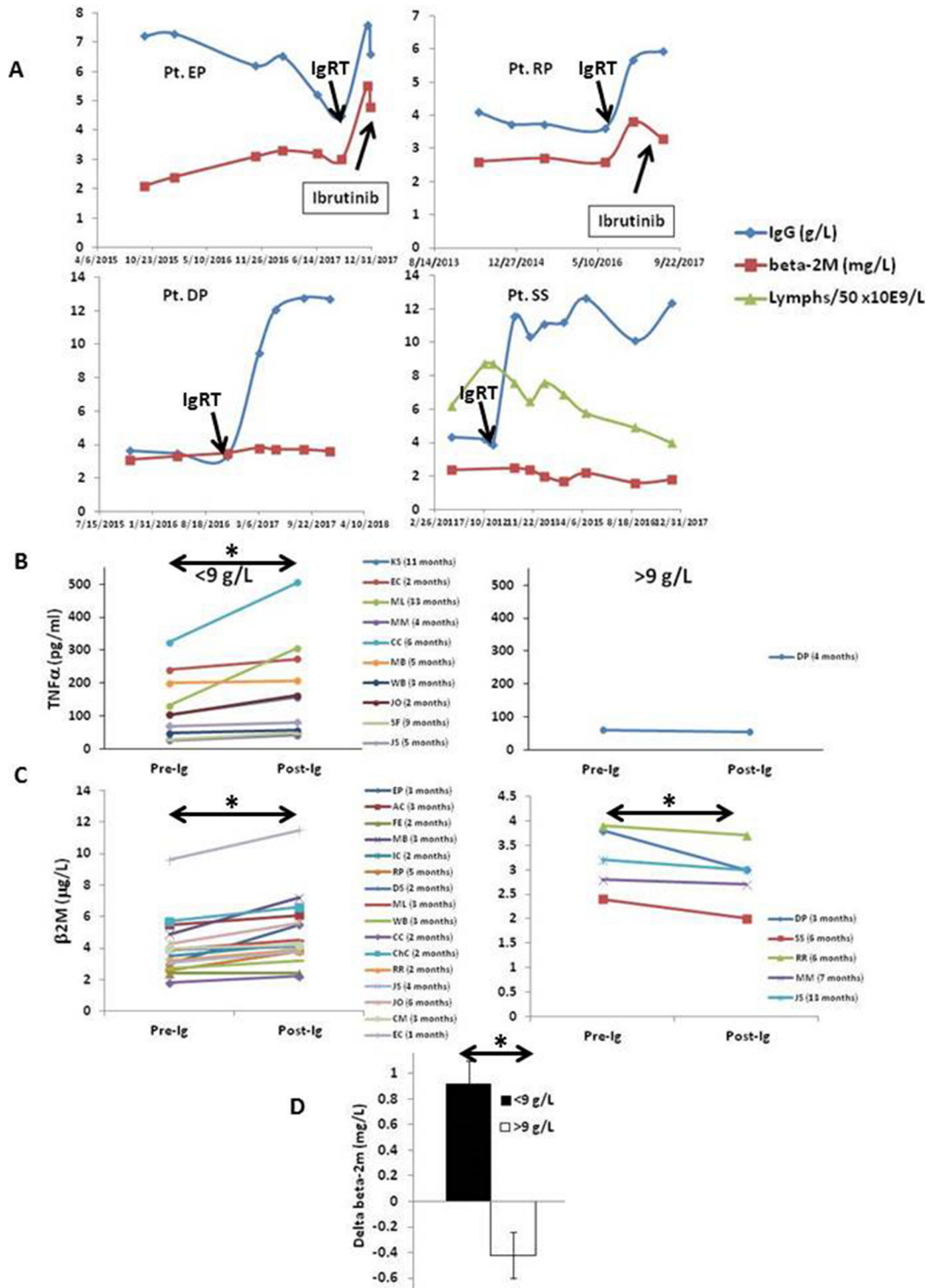
Given the apparent relationship of IgG to plasma TNF $\alpha$  (Fig. 2A), a panel of cytokines was measured in plasma samples of CLL patients that had incidentally been collected before and after commencing IgRT. Timing of the collections in relation to starting IgRT was not standardized as they had been obtained for other purposes over a three-year period. However, of the 11 paired samples available for analysis, TNF $\alpha$  levels increased after starting IgRT when replacement IgG levels were <9 g/L (Fig. 3B; left panel) but decreased in one patient when IgG levels reached >9 g/L (Fig. 3B; right panel). IL10 levels also increased in patients whose IgG levels were increased only below 9 g/L (Supplementary Fig. 1).

Due to the paucity of paired samples that had been cryopreserved and could be used to measure cytokines, the analysis was extended to include patients on IgRT with records of simultaneous IgG and  $\beta$ 2M measurements (Fig. 3C). The rationale was that  $\beta$ 2M levels are reduced by cytokine-signaling inhibitors and may serve as a partial surrogate for cytokine activity in CLL [3,16,45]. Sixteen patients were identified with IgG levels, measured within a few months of starting IgRT, that did not reach 9 g/L and five patients were identified with levels that became >9 g/L. Remarkably, serum  $\beta$ 2M increased in the former group but declined in the latter (Fig. 3C). The average increase was  $0.93 \pm 0.18$   $\mu$ g/L if IgG levels remained below 9 g/L but declined by  $0.42 \pm 0.13$   $\mu$ g/L if IgG levels were above 9 g/L (Fig. 3D). Taken together, these observations suggested target IgG levels above those that prevent infections [48] might have anti-leukemia activity as indicated by lowered TNF $\alpha$  and  $\beta$ 2M levels.

### 3.6. Dose-dependent effects of SCIG on B cell-receptor-mediated CLL cell activation *in vitro*

The effect of different concentrations of the SCIG preparation Hizentra® on the activation of CLL cells was studied *in vitro* to try to understand why higher doses of IgRT might have a beneficial effect on the clinical course. Growth of CLL cells is thought to be driven mainly by signals from B cell receptors (BCRs) or toll-like receptors (TLRs), with clinically aggressive CLL cells responding more strongly to antigens and TLR-ligands [8,27,44]. The NF $\kappa$ B pathway conveys signals from both receptor types [27] and induces CD83 on the cell surface, which can be measured by flow cytometry to provide a simple readout of BCR- and TLR-activation [41]. As shown in the example in Fig. 4A, BCR-cross-linking with anti-IgM Fab fragments up-regulated CD83 (Fig. 4A; compare first and third panels from left). Hizentra® at 15 g/L strongly decreased CD83 expression, suggesting BCR-activation was inhibited (Fig. 4A; compare third and fourth panels from left). TLR7-signaling induced by Resiquimod caused significantly higher CD83 expression and was not inhibited as strongly by this dose of Hizentra® (Fig. 4A; compare fifth and sixth panels from left).

Based on these observations, the effects of Hizentra® on BCR-activation were studied in more detail in 25 additional patient samples, using final concentrations of 4 and 15 g/L to represent extremes of

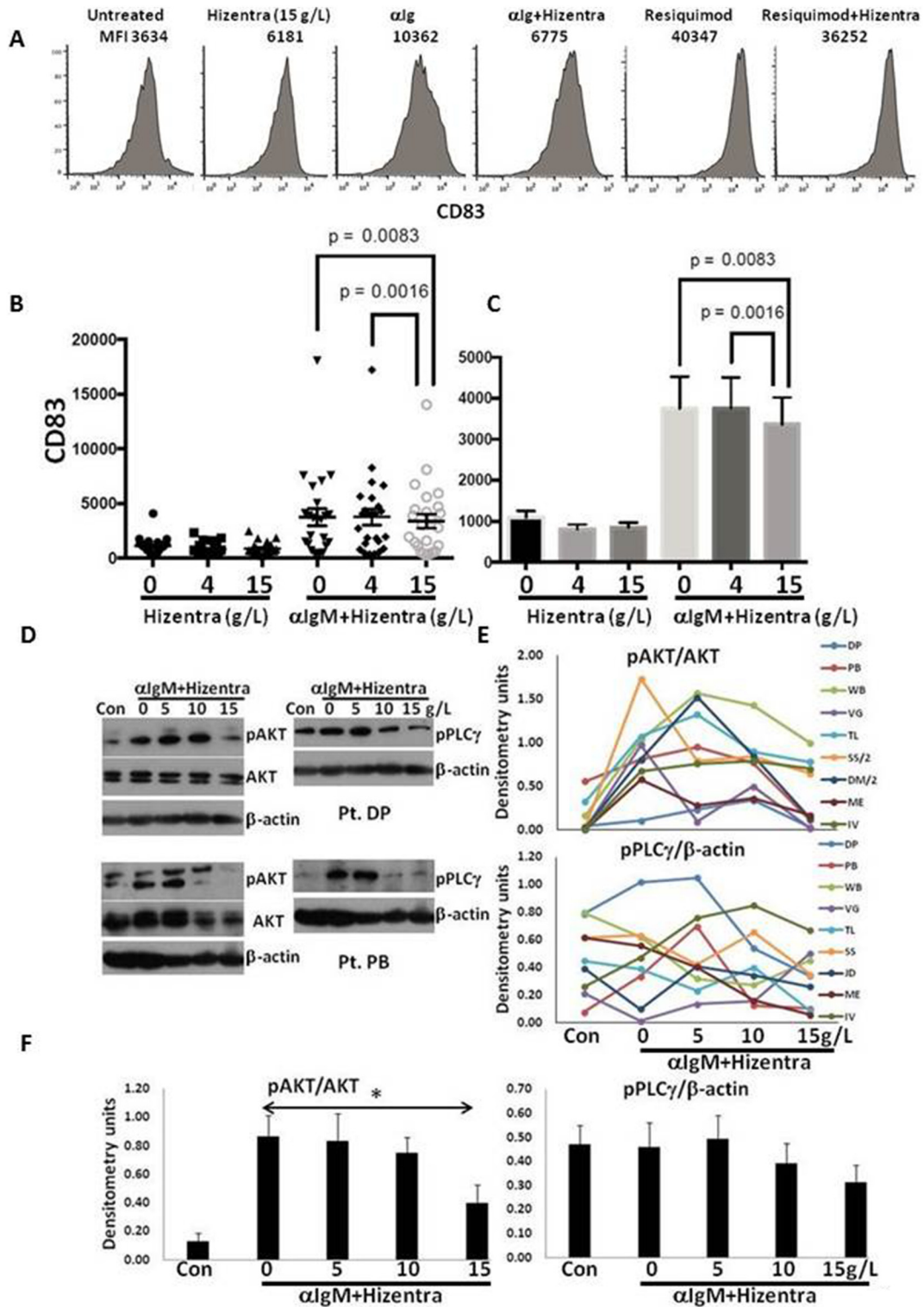


**Fig. 3.** Plasma TNFα and β2-microglobulin (β2M) changes in CLL patients following institution of Ig replacement therapy. A. Examples of time courses of IgG and β2M levels along with lymphocyte numbers following institution of IgRT to IgG levels of ~ 6 g/L (upper panels) or 12 g/L (lower panels). B,C. TNFα levels were measured in plasma samples collected incidentally from 11 patients over a 3 year period (2015–17) prior to and at varying times after starting IVIG or Hizentra (B). β2M levels before and after starting IgRT were taken from the clinical records from these and an additional 10 patients (C). The time between the two plasma collections for each patient is indicated in the figure legends. Patient samples were grouped according to IgG level achieved by IgRT at time of sample collection, using 9 g/L as a cut-off. D. Summary graph of average change in serum β2M for each group indicating an IgG level > 9 g/L is significantly associated with a lowering of β2M levels. \*, p < 0.05.

normal IgG values. Results for individual samples are shown in the left panel and summarized on the right (Fig. 4B). Hizentra® did not affect BCR-activation at 4 g/L but inhibited it significantly at 15 g/L (Fig. 4B).

BCR-cross-linking causes rapid phosphorylation of BCR-associated signaling molecules, particularly in the phospholipase C-γ2 (PLC-γ) and phosphoinositol-3-kinase (PI3K)/AKT pathways [46]. The effects

of different concentrations of Hizentra on BCR-mediated phosphorylation of AKT and PLC-γ were examined by immunoblotting in 9 patient samples (Fig. 4D–F). Consistent with the *in vivo* observations (Fig. 3), Hizentra at 10 g/L often inhibited AKT and PLC-γ-phosphorylation (Fig. 4D–F). Consistent with the flow cytometric assay (Fig. 4B), the AKT-pathway was inhibited significantly by Hizentra at 15 g/L.



**Fig. 4.** Dose-dependent inhibition of B cell receptor-signaling in CLL cells by Hizentra. Purified CLL cells were stimulated with anti-IgM antibodies (10 ng/mL) or the TLR7-agonist Resiquimod (1  $\mu$ g/ml) in the presence and absence of Hizentra at the indicated concentrations. After 4 h, CD83 expression was measured by flow cytometry. A. An example for one patient sample is shown in the top histograms, with the numbers indicating mean fluorescence intensity (MFI) of CD83-staining. B,C. The graphs show results of IgM-cross-linking for 25 different patient samples. Individual values for each treatment condition are shown on the left (B) with averages and standard deviations on the right (C). Hizentra at 15 g/L significantly ( $p < .01$ ) decreased BCR-activation in contrast to 4 g/L. D–F. After 30 min, phospho- and total AKT, phospho-PLC $\gamma$ , and  $\beta$ -actin in 9 additional samples were measured by immunoblotting and quantified by densitometry. Representative immunoblots are shown (D). Relative densitometry values for each condition are shown with the lines indicating results from individual patient samples (E). Averages and standard errors are shown in the graphs (F). \*,  $p < .05$ .

### 3.7. Dose-dependent effect of Hizentra® on cytokine production by activated CLL cells

As the BCR-transcriptional program includes chemokine, cytokine, and growth factor genes, the effect of Hizentra® on 42 different proteins in culture supernatants from 4 different purified CLL cell samples activated by IgM-cross-linking was determined after 48 h (Supplementary Fig. 2). TNF $\alpha$ , PDGF-AA, CCL4 and CCL5 were especially changed by Hizentra®, which inhibited their spontaneous release in all patient samples and BCR-mediated production from most samples (Supplementary Fig. 2).

Given the effect on TNF $\alpha$  (Supplementary Fig. 2) and its importance in CLL [12], the consequences for TNF $\alpha$  production of different concentrations of Hizentra® were measured specifically in 10 additional patient samples. Results for individual samples are shown in Fig. 5A and summarized in Fig. 5B. High concentrations of Hizentra® (15 g/L) inhibited spontaneous and anti-IgM-mediated TNF $\alpha$  production more than low concentrations. TNF $\alpha$  production was even increased by low Hizentra® concentrations (5 g/L) (Fig. 5A) in some CLL samples that spontaneously made >38 pg/ml of TNF $\alpha$ . Such cells were designated “Group 1” cells. Patient samples with lower spontaneous TNF $\alpha$  production (<38 pg/ml) were designated “Group 2” cells. Group 2 cells were more sensitive to either Hizentra® concentration and high concentrations (15 g/L) reduced TNF $\alpha$  production from activated cells almost to background levels (Fig. 5A; bottom panel, Fig. 5B).

### 3.8. Additive effects of Ibrutinib and Hizentra®

The above results (Supplementary Fig. 2, Fig. 5A,B) suggested spontaneous and BCR-activated production of TNF $\alpha$  by CLL cells could be inhibited by SCIG. The Bruton's tyrosine kinase (BTK) inhibitor Ibrutinib also lowers TNF $\alpha$  levels in CLL patients [34]. To determine if Hizentra® might inhibit BTK as well, the effect of combining Hizentra® and Ibrutinib on TNF $\alpha$  production by BCR-activated CLL cells was examined in two patient samples (Fig. 5C). As before (Supplementary Fig. 2, Fig. 5A,B), Hizentra® inhibited spontaneous and BCR-activated TNF $\alpha$  production, especially at 15 g/L (Fig. 5C, white bars). Ibrutinib strongly inhibited TNF $\alpha$  production in the presence and absence of Ig-cross-linking. Hizentra® increased the inhibitory effect of Ibrutinib (Fig. 5C), suggesting it acted through mechanisms other than inhibiting BTK.

### 3.9. Effect of Hizentra® on activated CLL cell death *in vitro*

CLL cells undergo spontaneous death, especially in serum-containing media [20,23], that can be prevented by signals through the BCR (Fig. 6A; compare first and second panels from the left). Hizentra® had variable effects on spontaneous and activated cell death that also appeared to be related to baseline production of TNF $\alpha$ . Examples are shown in Fig. 6A and the results are summarized in Fig. 6B. In “Group 1” samples with spontaneous TNF $\alpha$  secretion >38 pg/ml, Hizentra® had little effect on death and even appeared to increase relative survival (*i.e.* percent 7AAD<sup>−</sup> cells in the presence of anti-IgM and/or Hizentra® divided by percent 7AAD<sup>−</sup> cells in control cultures) (Fig. 6A; top panels and Fig. 6B; white bars). In contrast, Hizentra® increased the death of “Group 2” CLL cells with low spontaneous release of TNF $\alpha$  (<38 pg/ml) independent of Ig-cross-linking (Fig. 6A; bottom panel, Fig. 6B; black bars).

## 4. Discussion

The observations in this manuscript suggest: 1. IgG, IgA, and IgM levels fall steadily over time except in CLL patients with truly indolent disease. Rates of decline are faster for patients with aggressive disease who will require treatment (Fig. 1). 2. Plasma TNF $\alpha$  levels are inversely correlated with IgG (Fig. 2A). 3. IgRT that achieves IgG levels >9 g/L is associated with biochemical evidence of disease control *in vivo* (Fig. 3). 4.

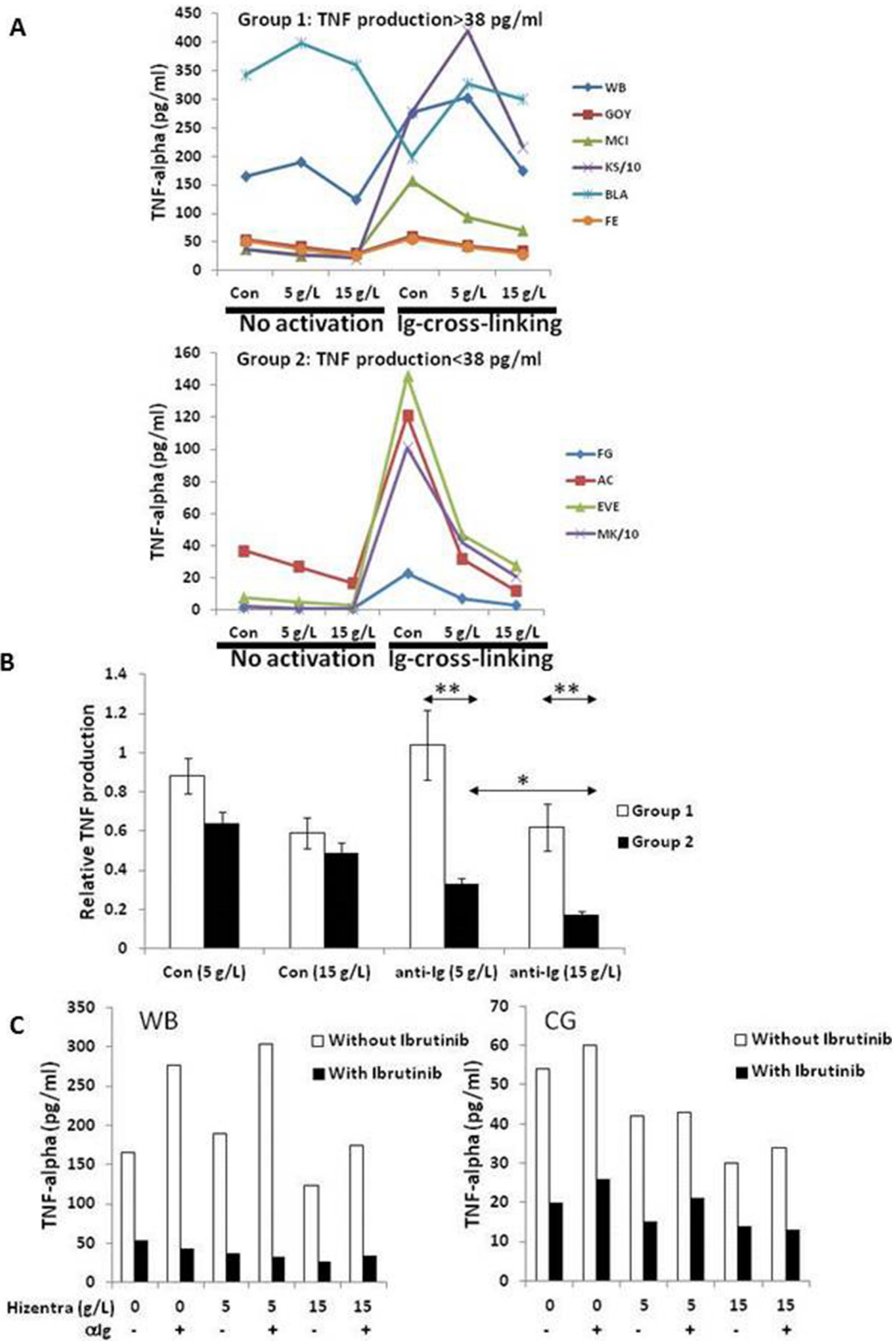
IgG concentrations at the upper limit of normal (over 10 g/L) inhibit BCR-activation and TNF $\alpha$  production and can increase cell death *in vitro* (Figs. 4, 5, 6A), especially in CLL cells with low spontaneous production of TNF $\alpha$ .

Except for patients who will never need treatment, the fact that Ig levels appear to decline in a coordinated manner (Fig. 1) suggests many patients will have experienced significant deterioration from normal by the time they receive a diagnosis of CLL. Such patients may then have “relative” hypogammaglobulinemia despite Ig levels in the low-normal range (Fig. 2B). Low IgM levels in many CLL patients at diagnosis (Fig. 2B) may not be a selective deficiency but simply reflect the narrow normal range for IgM compared to IgA and IgG, making it easier to decline below the lower limit. The rate of decline of Ig levels (Fig. 1C) might have prognostic importance in the practical management of CLL patients, much like the lymphocyte doubling time (LDT) [31].

The etiology of hypogammaglobulinemia in CLL is multi-factorial and has been ascribed to various mechanisms including defective helper T cell function and Fas-mediated killing of plasma cells by CLL cells [9,10,21,38]. The main source of immunoglobulin in CLL patients is normal B cells as paraproteins are not routinely made by CLL cells [28]. However, numbers of normal B cells, defined as CD19<sup>+</sup>CD5<sup>−</sup> blood lymphocytes, did not exhibit an obvious relationship with Ig levels at the time of the CLL diagnosis (Supplementary Fig. 3). The observation of a parallel decline in all three Ig classes (Fig. 1) suggests a major defect may occur at the transitional stage of B cell development [42]. Transitional cells exit the bone marrow to develop in the periphery and ultimately give rise to memory and plasma cells that make all classes of immunoglobulins. Studies based on quantifying kappa-deleting recombination excision circles (KRECs) concluded newly produced B lymphocytes are deficient even at early clinical stages of CLL [33]. It is not clear why transitional B cells should be impaired in the bone marrow of CLL patients. TNF $\alpha$  can inhibit human B cell development at the transitional stage [26] and the results in Fig. 2A suggest plasma TNF $\alpha$  correlates inversely with IgG in CLL patients. While TNF $\alpha$  in blood may originate from other cells and the correlation may simply reflect higher levels in patients with more advanced disease that independently develop hypogammaglobulinemia [12], perhaps bone marrow CLL cells that secrete TNF $\alpha$  suppress B cell development in the manner of senescent B cells that make TNF $\alpha$  and are implicated in the humoral immunodeficiency of aging [36].

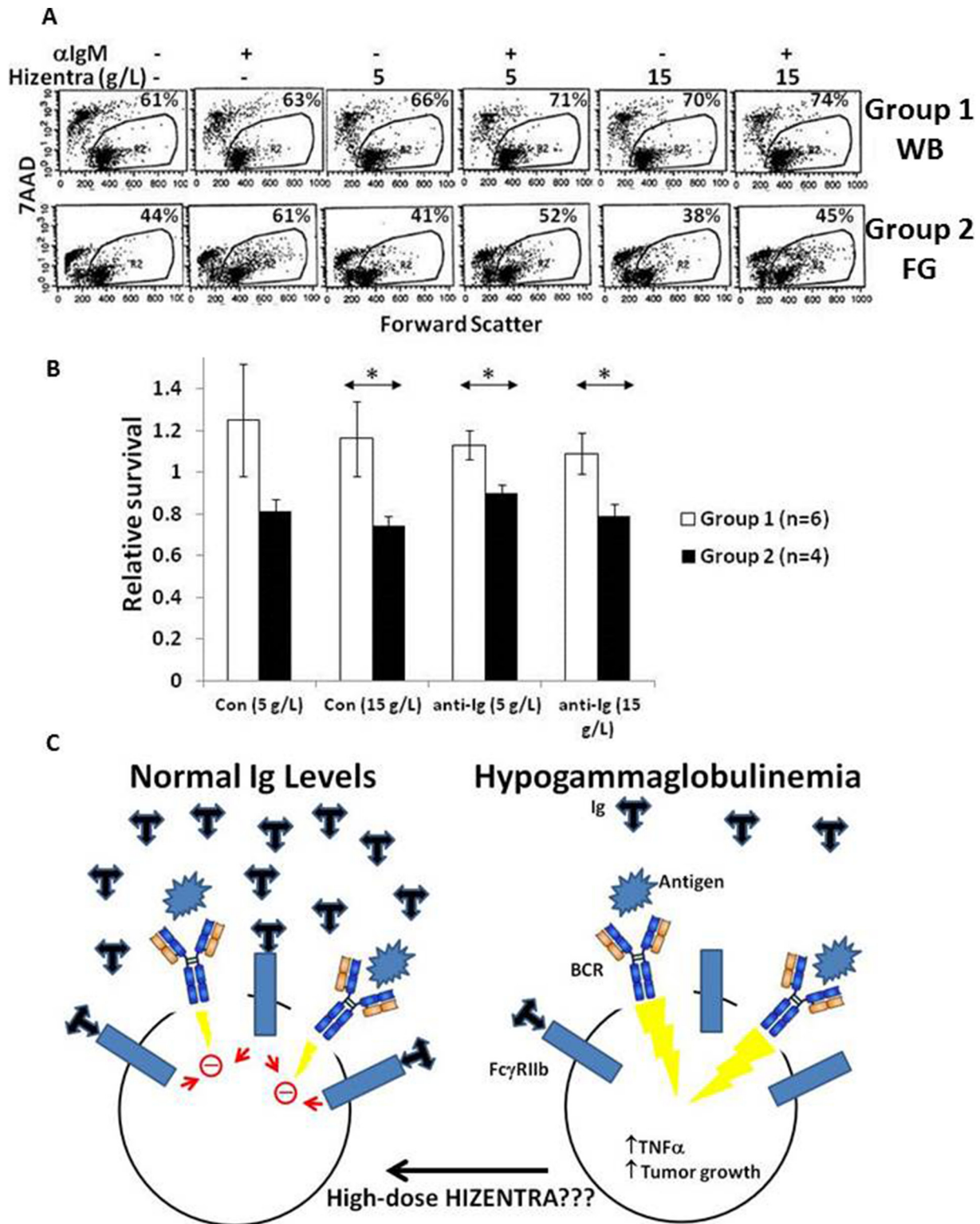
IgRT is prescribed for patients with hypogammaglobulinemia and a history of infections because it can prevent subsequent ones [15,48]. The dose of IVIG or SCIG for secondary immunodeficiency is generally the equivalent of 0.4 g/kg/month but can be titrated upward in the event of breakthrough infections ([4,5]; [1]). It is not clear if this dose is optimal for CLL patients. A review of local patient records suggests a replacement dose of 0.4 g/kg/month usually limits IgG trough levels to the low-normal range (*i.e.* 6–8 g/L) (Fig. 3), which is associated with evidence of biochemical progression (Fig. 3A,C) and increased plasma TNF $\alpha$  (Fig. 3B). However, endogenous IgG levels that remain above 8 g/L are associated with an indolent clinical course (Fig. 1E) and maintenance of IgG levels in the high-normal range (9–12 g/L) with IgRT in the absence of a paraprotein is associated with biochemical evidence of disease control (Fig. 3C,D).

An explanation for these clinical findings is provided by the observations that IgG at sufficiently high concentrations inhibits BCR-activation and cytokine production and sometimes kills CLL cells *in vitro* (Fig. 4–6). In addition to inhibiting antigen-driven BCR-signaling represented by Ig-cross-linking (Fig. 4), IgRT may also affect antigen-independent cell-autonomous BCR-signaling that has been implicated in the pathogenesis of CLL ([11]; [30]). Hizentra inhibited spontaneous release of TNF $\alpha$  by CLL cells (Supplementary Fig. 2; Fig. 5) that is ibrutinib-sensitive (Fig. 5C) and may be due to autonomous BCR-signaling. Given that BCR-signaling drives CLL progression [8], these results suggest maintaining plasma IgG levels near 15 g/L with higher doses of



**Fig. 5.** Hizentra inhibits TNF $\alpha$  production by resting and activated CLL cells in a dose- and disease subtype-dependent manner. TNF $\alpha$  was measured in the supernatants of cultures of CLL cells treated with or without anti-IgM antibodies in the presence or absence of Hizentra (5 or 15 g/L final concentration) (A,B) and the presence or absence of the BTK-inhibitor Ibrutinib (500 nM) (C). A. Individual results for 10 patient samples are graphed, grouped on the basis of spontaneous TNF $\alpha$  production of more or <38 pg/ml. B. TNF $\alpha$  levels in cultures of control and activated CLL cells in the presence of Hizentra were divided by the amounts in the control cultures without Hizentra. Averages and standard errors for each group are shown and indicate that 15 g/L Hizentra is more effective at preventing TNF $\alpha$ -production, especially by group 2 cells. C. Results in the presence of both Hizentra and Ibrutinib are shown for two patient samples. \*,  $p < .05$ ; \*\*,  $p < .01$ .





**Fig. 6.** Hizentra decreases survival of activated CLL cells from group 2 patients. Purified CLL cells were cultured in AIM-V serum-free media in the presence or absence of anti-IgM antibodies with or without Hizentra at a final concentration of 5 or 15 g/L. Percentages of viable cells that exclude the dye 7AAD were measured by flow cytometry after 5 days. A. Examples are shown in the dot-plots with the numbers representing percentages of viable 7AAD<sup>-</sup> cells. B. The summary graph shows the average and standard error of the relative survival of 6 Group 1 patient samples and 4 group 2 samples. Relative survival is defined as the percentage of 7AAD<sup>-</sup> cells with Hizentra divided by the percentage in control cultures without Hizentra. Groups 1 and 2 represent CLL cells that spontaneously make > or <38 pg/ml of TNF $\alpha$ , respectively. The results indicate group 2 samples are most sensitive to Hizentra, especially at 15 g/L. C. Schematic diagram suggesting that high-normal IgG levels may help control BCR-signaling in CLL cells. \*; p < 0.05.

IgRT may have anti-tumor activity. Indeed, it was shown almost 35 years ago that IVIG did have an anti-CLL effect when administered every 3 weeks, which would lead to higher trough levels than a conventional modern schedule of every 4 weeks [6]. IgG concentrations below

8 g/L that are associated with disease progression (Fig. 1E, Fig. 3) can sometimes even increase AKT- and PLC $\gamma$ -phosphorylation (Fig. 4D,E), cytokine production (Fig. 5) and survival of activated CLL cells *in vitro* (Fig. 6), consistent with a tumor-promoting effect.

Why some patients attain higher IgG levels than others with the same dosing strategy (Fig. 3) is unclear. Patient SS (Fig. 3A, right lower graph) was maintained on IVIG but other patients with trough levels higher than 9 g/L (Fig. 3A,B,C) were using SCIG. One possibility is that CLL cells bind IgG and lower free IgG levels in the blood. In this case, circulating CLL numbers should correlate inversely with IgG levels achieved by IgRT. However, changes in IgG levels following institution of IgRT did not exhibit an obvious relationship with blood lymphocytes (Supplementary Fig. 4). Potentially such variables as patient technique or decreased clearance of injected IgG molecules account for these variations.

The mechanism by which high IgG concentrations inhibit BCR-signaling in CLL cells is unclear. In normal human B cells, IVIG can bind the inhibitory Fc receptor CD32B, which recruits phosphatases such as SHIP-1 to prevent BCR-mediated activation of kinase cascades [40]. CD32B is the only Fc receptor expressed by CLL cells [7]. Consistent with this, the CD32 antibody AT10 [37] partially reversed the inhibitory effects of Hizentra at 15 g/L on BCR-mediated expression of CD83 in some patients (Supplementary Fig. 5). IgRT formulations also contain sialylated IgG proteins that can potentially bind CD22 on CLL cells and down-regulate BCR-signaling as described in normal human B cells ([39]).

While inhibition of BCR-signaling provides a plausible mechanism to justify using higher doses of IgRT, the effects likely also depend on the underlying biology of the CLL cells. For example, Group 2 CLL cells that produce little spontaneous TNF $\alpha$  appear to be more sensitive to IgRT than group 1 cells that make more spontaneous TNF $\alpha$  as suggested by greater inhibition of BCR-mediated TNF $\alpha$ -production and death *in vitro* (Figs. 5, 6). However, the ability of these *in vitro* assays to predict subsequent responses to high-dose IgRT *in vivo* is not clear. It is also not clear if conventional prognostic factors can predict responses to IgRT. Patients classified as “<9 g/L” or “<9 g/L” (Fig. 3B,C) were not obviously different in terms of these factors. Although it has been reported that lack of spontaneous production of TNF $\alpha$  by CLL cells is associated with clinically aggressive behavior [13,18,22], there are no obvious clinical characteristics that distinguish group 1 and group 2 patients (Figs. 5, 6), possibly due to the cut-off used for the classification. Studies with more patient samples and a prospective clinical trial are needed to determine if these *in vitro* assays and conventional prognostic and biologic properties of CLL cells can determine if some patients are more appropriate to receive high-dose IgRT than others.

Taken together, these observations suggest a model whereby progressive hypogammaglobulinemia in CLL patients constitutes a “feed-forward” oncogenic event by removing inhibitory effects on BCR-signaling once IgG levels fall below 8 g/L (Figs. 1, 3, 6C). This effect of the humoral immunodeficiency may be compounded by gut microbial products such as endotoxin that can leak through the intestinal wall and drive the growth of CLL cells by activating TLRs and BCRs, as described in common variable immunoglobulin deficiency patients [2]. Accordingly, institution of IgRT (either IVIG or SCIG) to maintain IgG levels near 15 g/L in CLL patients on a “watch and wait” management strategy whose IgG levels have fallen below 8 g/L (Fig. 1E) may have disease modifying activity resembling other BCR-signaling inhibitors such as Ibrutinib in some patients (Figs. 5, 6). This conclusion is limited by the retrospective nature of the study. For example, failure to reach IgG levels above 9 g/L with conventional Ig replacement in some patients may simply reflect rapid catabolism of IgG and represent an epiphenomena of more aggressive disease that is independent of the IgG level. A randomized prospective interventional clinical trial with Ig replacement dosed to IgG target level is needed to confirm the potential importance of maintaining high IgG levels in CLL patients. Note that increased toxicity from high IgG levels for prolonged periods of time in CLL patients is not anticipated based on the tolerability of maintenance therapies in neurological diseases that use doses of 1 g/kg administered every three weeks [24].

## Authorship contributions

DS conceived the project and wrote the manuscript. YS, RV, JH, and GW performed *in vitro* studies and helped write manuscript. AZ and PN provided reagents and helped design experiments.

## Disclosure of conflicts of interest

DS acknowledges funding from CSLBehring to support the studies reported here.

## Role of the funding source

The funding source had no role in the study design, in the collection, analysis, and interpretation of data, in the writing of the report, and in the decision to submit the article for publication.

## Acknowledgements

This work was supported by CIHR grant #374817, the Leukemia and Lymphoma Society of Canada, and CSL Behring Canada. We thank Dr. Mel Berger (CSL Behring) for helpful advice.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2018.08.045>.

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