CD200-dependent and nonCD200-dependant pathways of NK cell suppression by human IVIG

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Abstract

Problem Intravenous immunoglobulin (IVIG) has been used to suppress autoimmune and inflammatory disorders by a variety of mechanisms. Recently, the CD200 tolerance-promoting signal has been found to play a role in IVIG suppression of blood natural killer (NK) cells. Further, different types of IVIG have been reported to differ in this activity, and that has been related to efficacy (and inefficacy) of treatment of women with pregnancy failure. CD200 acts by binding to CD200 receptors (C200R). The objective of this study was to determine if CD200-dependent NK suppression by IVIG involved direct binding of IVIG-associated CD200 molecules to CD200R on NK cells.

Method of study Peripheral Blood Lymphocytes isolated from human blood were used as a source of NK cells to lyse Cr⁵¹-labelled K562 target cells in vitro in 18 and 4 h assays, and three different types of IVIG were tested for

suppressive activity in the presence or absence of specific monoclonal anti-huCD200. In some experiments, CD56⁺ NK cells were purified using anti-CD56 magnetic beads. Western blotting of IVIG using a specific anti-huCD200 antibody was done. Enzyme-Linked ImmunoSorbent Assays were used to measure cytokine production in NK assays.

Results Different IVIGs showed significant differences in potency in suppressing NK cytolytic activity in vitro (mg/ml for 60% suppression, Gammagard 4.1, Gamunex 14.1, Gamimmune 20.2). For CD200-dependent suppression, Gammagard was twice as potent as Gamimmune, but equivalent to Gamunex. The presence of suppression in 4 hour assays indicated stimulation of cytokine synthesis was unlikely to explain CD200-dependent suppression. Purification of NK cells led to loss of the CD200-dependent component. Western blotting confirmed that material reactive with anti-CD200 antibody was present in Immunoglobulin G (IgG) preparations, and at a lower level in human serum that contains IgG.

Conclusions IVIGs are not all equipotent in suppressing NK cell cytolytic activity. CD200 associated with IVIG is an important component of suppression. CD200-dependent suppression appears to be mediated by a non-NK population that then acts on NK cells by direct contact rather than indirectly through release of immunosuppressive cytokines.

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Introduction

Intravenous immunoglobulins (IVIGs) has licensed and ‘off label’ efficacy in suppressing a variety of immune

inflammatory disorders [1]. There is also empirical evidence for benefit in patients with recurrent spontaneous abortion if secondary or associated with autoantibodies or elevated natural killer (NK)-lineage cells in blood [2–4]. In vitro fertilization failure may also be prevented in patients who have immune abnormalities [2]. Suppression of blood NK cell activity has been proposed as an important effect, and different IVIGs may differ in potency both in vitro and in vivo [3–5]. However, the mechanism of IVIG suppression of NK-type cells is uncharacterized. In autoantibody-mediated immune thrombocytopenia, a fragment crystallizable (Fc) dependent effect on myeloid dendritic cells has been suggested [6]. With respect to NK cytolytic activity, we recently reported that a significant proportion of IVIG suppression could be blocked by a specific monoclonal antibody against CD200 [7]. CD200 is a tolerance signaling molecule expressed on a variety of cells including activated T and B cells, brain cells, and fetal trophoblasts, and acts by binding to CD200 receptors [8, 9]. This binding results in generation of different types of immunoregulatory suppressor T cells, including CD4⁺25⁺ Treg cells, and activation of indoleamine 2,3-dioxygenase production in myeloid cells [10, 11]. When murine or human lymphocytic cells are stored at 4°C overnight, cell surface CD200 is lost and activity blocking anti-CD200 binding to fresh cells is present in conditioned supernatant [7]. Interestingly, this activity was absent when cells were stored in plasma that contains Immunoglobulin G (IgG). It was suggested that a soluble CD200 might bind to IgG which could then contribute to suppression of NK cell activity. It was unclear if this putative IgG-associated CD200 acted directly on NK cells, on target cells, or indirectly by binding to non-NK cells in peripheral blood cell preparations.

Materials and methods

NK assays After informed consent as approved by the local Research Ethics Board, normal human peripheral blood was drawn into sterile acid-citrate-dextrose-containing tubes, and the lymphoid cells purified by centrifugation over a cushion of lympholyte H (Cedarlane Laboratories, Hornby, ON, Canada). In some experiments, the CD56⁺ population was purified by a Midi-MACs column to separate CD56⁺ from CD56[−] cells. K562 erythroleukemia cells were maintained in culture in α -MEM with 10% FBS, and 50 μ g/ml gentamicin. Log-phase cells were labeled with ⁵¹Cr as previously described [7]. Using V-bottom 96 well plastic trays, 5 $\times$ 10³ labeled K562 targets were incubated with 2.5 $\times$ 10⁵ Peripheral Blood Lymphocytes (PBL) or with an equivalent number of purified CD56⁺ NK cells in 200 μ l culture medium. After 4 or 18 h at 37°C in a humidified 5% CO₂ incubator, 100 μ l supernatant was removed and

counted in a γ counter. Percent specific lysis was determined using the standard formula and converted to 20% lytic units (LU20) as previously described [7]. At the outset of the assay, IVIG was added to achieve a specified concentration $\pm$ 2 μ g monoclonal anti-human CD200 (9A2 clone, Biocan Canada). In some experiments, PBL or purified NK cells were incubated with unlabeled K562 cells for 18 h, and the supernatant was used to test for cytokine levels.

ELISA Assays Enzyme-Linked ImmunoSorbent Assay (ELISA) kits for tumor necrosis factor (TNF)- α , interferon (IFN)- γ , interleukin (IL)-10, and transforming growth factor (TGF)- β were obtained from eBiosciences and used in accordance with the manufacturer's directions. Fifty microliter of undiluted test supernatant, or a 1/3 dilution of supernatant was added to replicate assay wells, and cytokine concentration was determined from a standard curve.

Western blotting CD200-transfected human embryonic kidney (Hek 293) cells were kindly provided by Trillium Technologies Inc., CD200⁺ Ly 5 and CD200[−] Ly2 cells were provided by Dr. David Spanner (Sunnybrook Hospital Research Center, University of Toronto, Toronto, Canada). The cells were lysed in 0.025% sodium dodecyl sulfate (SDS) and 5 μ g Human Embryonic Kidney cells and 30 μ g Ly5 or Ly2 was run on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel, along with 1 μ l of a 1/30 dilution of IVIG preparations 1 and 2 (5%, Gammagard). One microliter containing 3 μ g purified human IgG (Jackson ImmunoResearch Lab., West Grove, PA, USA), and 1 μ l of a 1:20 dilution of normal human serum (approximately 1.2–1.4 μ g IgG) was also run along with molecular size markers. After transfer to a Polyvinylidene fluoride membrane, the blot was blocked overnight with 5% milk-Tris-buffered saline, 0.1% Tween 20 at 4°C, and probed a 1:5,000 dilution of rabbit anti-CD200 antiserum for 2 h at room temperature. After washing four times haptoglobin-related goat-anti-rabbit IgG (Jackson) was applied for 1 h at room temperature to detect anti-CD200. After extensive washing, the blots were developed using the Enhanced Chemiluminescence Western blotting detection reagents from Amersham. Anti-CD200 antiserum was developed by immunizing New Zealand white rabbits with 250 μ g recombinant human CD200Fc in complete Freund's adjuvant 4 weeks apart. The serum was run over a human Fc column (Cedarlane Laboratories) to eliminate any reactivity with Fc in Western blots. A commercially available affinity-purified goat polyclonal anti-CD200 antiserum, raised against the N-terminus of human OX-2 (N-16, # sc-14381, Santa Cruz Biotechnology Inc., CA, USA) was used to confirm the result.

Fig. 1 Comparison of three different types of IVIG in ability to suppress NK cell cytotoxicity in vitro. GIM, Gamimmune; GEX, Gamunex; GGD, Gammagard. Mean and 1 standard error of mean (SEM) is shown for four replicate wells

Statistics Difference in means was determined using Student's *t* test. $P < 0.05$ was considered significant. IVIG dose-response curves were analyzed by linear regression analysis.

Results

NK suppression by different types of IVIG

Figure 1 shows percentage suppression of lytic activity of human PBL NK activity by three different types of IVIG at 3.125 and 6.25 mg/ml concentration in vitro. It can be seen that Gamunex was intermediate in effect compared to Gammagard (GGD), the most potent, and Gamimmune (GIM), the least potent (data for GGD and GIM as in [2]). Figure 2 shows more extensive dose-response data from three independent experiments. For 60% suppression, one required 4 mg/ml of Gammagard, 14.1 mg/ml of Gamunex, and 20.2 mg/ml Gamimmune. In Fig. 3, reduction in suppression by co-presence in the assay of monoclonal anti-human CD200 antibody is shown. It can be seen that anti-CD200 reduced suppression for all IVIG types tested, and

Fig. 2 Titration of NK suppressive effects of different types of IVIG. Data represent mean $\pm$ 1 SEM of three independent experiments with four replicates in each experiment. Linear regression was performed

Fig. 3 Suppression of NK activity by IVIG in NK assays lasting 4 h and 18 h. Average suppression (and 1 SEM) is shown for same range of IVIG concentrations as shown in Fig. 2

the effect was seen in short incubation 4 h assays as well as at 18 h. Combining the 4 and 18 h assays, Gammagard has twice as much CD200-dependent suppressive activity as Gamimmune, and Gamunex had comparable activity to Gammagard. An effect at 4 h suggested that IVIG (and CD200) were not acting by induction of cytokine synthesis, as that normally takes much longer to occur. Gammagard and Gamunex appeared to contain more CD200-dependent activity than Gamimmune, but were otherwise comparable. The increased activity of Gammagard compared to Gamunex was a non-CD200-dependent effect.

Fig. 4 CD200-dependent and independent suppression of NK cytotoxic activity is lost with highly purified NK cells. **a** Suppression of unpurified PBL and CD56⁺ cells after a single Mini-MACS column purification. + α CD200 indicates monoclonal anti-CD200 added to the IVIG at outset. **b** Results with PBL and CD56⁺ cells after a second round of Mini-MACS purification. Gamunex was used at 6.25 mg/ml

Purified CD56⁺NK cells are resistant to CD200-dependent suppression by IVIG

To test whether IVIG acted directly on CD56⁺ NK cells, or indirectly via a CD56[−] cell population in PBL, CD56⁺ cells labeled with anti-CD56 magnetic beads were purified by one or two passages through a magnetic column as described in “Materials and methods”. In can be seen in Fig. 4a, that CD56⁺ cells after a single passage were just as susceptible to suppression by IVIG and to CD200-dependent suppression as unenriched cells. Flow cytometry using PE anti-CD56 showed only 76% of the first passage cells stained (data not shown). This suggested an incomplete purification, and so first passage cells were subject to a second

labeling with anti-CD56 beads and column separation. In contrast, double passaged CD56⁺ cells shown in Fig. 4b while sensitive to suppression by IVIG did not appear to show the CD200-dependent component. This suggested that the CD200-dependent component of suppression by IVIG was acting through a non-CD56⁺ cell that had been incompletely depleted after a single purification step.

Single passage CD56⁺ cells incubated with K562 target cells were also studied for cytokine production. As shown in Fig. 5a, PBL produced TNF- α , IFN- γ , IL-10 and TGF- β as measured by ELISA of 18 h culture supernatants. CD56⁺ cells, in comparison to PBL, generated less TNF- α , IL-10 and TGF- β than PBL, but more IFN- γ . IFN- γ is an

Fig. 5 Cytokine levels in supernatant of PBL + K562 and single-purification CD56⁺ cells (that are still sensitive to CD200-dependent suppression). + α indicated monoclonal anti-CD200 added; gx, Gamunex 6.25 mg/ml; gd, Gammagard 6.25 mg/ml, mean and 1 SEM shown. **a** shows the result of the four specific cytokines measured by ELISA; **b** shows Th1/(Th2+Th3) cytokine ratio

important stimulus of NK activity. IVIG that suppressed NK activity did reduce IFN- γ production by CD56⁺ cells, but not when PBL were the source of IFN- γ . Anti-CD200 increased IL-10 and TGF- β levels of PBL-K562 target cell cultures, without altering cytolytic activity, and these cytokines were also increased when anti-CD200 was added to cultures containing. There was essentially no detectable IL-10 or TGF- β in supernatants of cultures of CD56⁺ cells and K562 target cells, even when IVIG that suppresses NK activity was present. Figure 5b simplifies the analysis by computing a Th1/(Th2+Th3 ratio). Suppression of target lysis by IVIG could not be clearly related to an alteration in this ratio by IVIG. We concluded that CD200 suppression of NK activity by IVIG and its antagonism by anti-CD200 could not be related to the 4 cytokines (arising from non-CD56⁺ cells) that we tested.

CD200 in Western blotting of IVIG

Evidence that CD200 molecules associated with IVIG mediate a suppressive effect on NK cells is indirect and based on inhibition by interaction with anti-human CD200 mAb. Figure 6 shows a Western blot developed using a highly specific anti-CD200 antiserum that had been absorbed to ensure absent anti-Fc activity. The two left lanes represent supernatants from CD200⁺ cells lines, and lane 3, a negative control. Two different preparations of IVIG showed a strong band at the expected molecular size for CD200 of 48 kDa. A similar amount of purified human IgG was also reactive, and to a lesser extent, human serum (which contains IgG at a lower concentration). The conditions under which we ran our PAGE clearly dissociated CD200 from the IgG carrier.

Discussion

The data in this paper show that CD200 is present in commercial IVIG preparations. CD200-dependent and non-CD200-dependent suppression of NK cytolytic activity differ significantly among different types of IVIG. CD200-dependent suppression appears to act via a cell or cells that differ from cytolytic CD56⁺ cells. CD200-dependent suppression of NK cytolytic activity did not act by suppressing Th1/Th2,3 ratios, at least where the cytokines TNF- α , IFN- γ , IL-10, and TGF- β were measured. The majority of NK suppressive activity was independent of CD200 and CD200R and appeared to represent a direct effect of IVIG on NK cells. CD200 is thought to be released spontaneous from the membranes of CD200⁺ cells in vivo, and to bind to IgG. CD200 was also detected in human serum. While we did not test human serum for CD200-dependent suppressive activity, the concentration in an assay, even with 10% serum, would normally be below the lowest level of IVIG with which we detected CD200-dependent suppression.

A number of distinct mechanism have been suggested for suppression by IVIG. Fc-dependent mechanisms have been suggested, particularly in a murine model of idiopathic thrombocytopenic purpura (ITP) and in prevention of recurrent spontaneous abortions in the CBAXDBA/2 mouse model where Fab'₂ fragments proved inactive [6, 12]. CD200 has been proposed to be released from the surface of human PBL during storage and to bind to IgG [7]. It is possible CD200 binds to Fc and is removed when Fab'₂ fragments are made. As good mouse models now exist to examine the biology of IVIG, it should be possible to determine if CD200 plays a role. The suggestion from our

Fig. 6 Western blot probed for human CD200

data that CD200 is acting indirectly is intriguing. CD200 acting on immature DC has been reported to promote development of tolerance and Treg cells (that appear to act by a short-range cell contact mechanism) [11, 13–15]. Indeed, in a mouse model of ITP, it was also not possible to show a role for cytokines in the beneficial effects of IVIG treatment [16]. Preliminary data indicates that both CD200R1 and CD200R2 are expressed in PBL and single purified CD56⁺ cell populations (Clark, Chen, Gorczynski, unpublished). More detailed reductionist studies will be required to determine which CD200R⁺ cell type is activated to suppress NK cell cytotoxicity.

In IVIG treatment of recurrent pregnancy failure, a suppression of blood NKT cells rather than NK cells was suggested as more relevant [4]. It remains to be determined how IVIG suppresses NKT cells, if this is CD200R-dependent in part, and if the effect is indirect rather than direct. Indeed, in the *in vivo* study of IVIG suppression of NKT as a correlate of pregnancy success, some patients proved resistant to suppression [4]. This was not attributable to use of a less active product (e.g. Gamimmune). Although it has been mentioned in the literature that CD200R may be expressed on NKT cells [8], confirmatory data have yet to be published. Whilst all IVIG preparations are not the same, patients also differ, and dissection of mechanism will be required to understand these differences and resistance can be overcome.

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