A method for ameliorating autoimmune disease by passive transfer of IVIg-primed leukocytes

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Introduction

While strides have been made in the understanding of how intravenous immunoglobulin (IVIg) ameliorates autoimmune diseases by using mouse models of immune thrombocytopenia (ITP) and inflammatory arthritis, the precise cellular target of IVIg has remained elusive. To determine which cell populations interact with IVIg upstream of the inhibition of phagocytosis, we have developed a method for 'priming' specific leukocyte populations with IVIg to study their potential role in ameliorating ITP upon passive transfer, which we describe herein. Using this technique, we found that splenic CD11c+ dendritic cells reacted with IVIg, or several IVIg mimetic regimes, were primed to ameliorate ITP upon transfer to thrombocytopenic mice. We anticipate that this novel methodology could also be used to determine the mechanism of action of IVIg in other autoimmune diseases in addition to ITP.

Introduction

The precise mechanism of action of intravenous immunoglobulin (IVIg) in the amelioration of autoimmune diseases such as immune thrombocytopenia (ITP) has, until now, been difficult to ascertain (1,2). Studies using gene knockout mice have demonstrated that in the treatment of murine ITP, IVIg requires the expression of the inhibitory receptor FcγRIIB (3,4) and that IVIg requires CSF-1-dependent macrophages to increase FcγRIIB expression on CSF-1-independent macrophages (5). IVIg has also been demonstrated to affect the function and maturation of dendritic cells *in vitro* (6,7). However, the precise cellular target(s) of IVIg has remained, until recently, an enigma. In an attempt to answer these questions, we have developed a method for isolating and 'priming' splenic leukocytes or specific splenic cell populations with i) IVIg, ii) soluble immune complexes (sIC) or iii) antigen-specific antibodies plus crosslinking antibodies. Splenic white cells are used for priming as is (whole leukocytes) or purified into CD11b negative and positive or CD11c negative and positive populations using magnetic bead-coupled monoclonal antibodies. Cells are then incubated with IVIg, sIC or antigen-specific antibodies (as above) at 37^{o}C for 30 min, washed and injected into mice one day prior to injection of anti-platelet antibody to induce ITP (8). This method avoids the actual administration of IVIg and allows the researcher to isolate specific cell populations, prime them and

passively transfer them to thrombocytopenic mice in order to determine their potential role in IVIg function. Recently, using this technique we found activating Fcγ receptors on CD11c+ dendritic cells to be the target of IVIg and IVIg mimetics in the amelioration of ITP in our mouse model (9). We anticipate that this new technique could also be used to determine the mechanism of action of IVIg in other models of autoimmune disease.

Reagents

Reagents:

• IVIg, Gamimune N, 10% (Bayer Corporation)

• PBS, calcium, magnesium free, pH 7.2 (Sigma, cat. no. D1408)

• Bovine serum albumin (BSA) solution: dissolve BSA, Fraction V, ≥96% (Sigma, cat. no. A9647-50G) to 50 mg/mL in 0.2 M glycine (Sigma, cat. no. G-8898). Filter the solution using 0.20 µm syringe filter (Sarstedt, cat. no. 83.1826.102) and store at 4^{o}C.

• Normal rat IgG (Caltag, cat. no. 10700)

• Ovalbumin (Grade V, Sigma, cat. no. A5503)

• Monoclonal anti-ovalbumin (Clone OVA-14, mouse IgG1, Sigma, cat. no. A-6075)

• Rat anti-mouse FcγRIIA (tissue culture supernatant from clone 2.4G2, ATCC cat. no. HB-197)

• Goat F(ab')2 anti-rat IgG (H+L) (Caltag, cat. no. R40100)

• Rat anti-mouse PIR-A/B (Clone: 6C1, rat IgG1, Pharmingen cat. no. 550348)

• Rat anti-mouse CD41 (Clone: MWReg30, Rat IgG1, Pharmingen, cat. no. 553847): dilute 2 µg of antibody into 200 µL PBS. CRITICAL Prepare on the day of the experiment.

• Culture medium: RPMI-1640 medium (Sigma, cat. no. R8758) supplemented with 10% heat-inactivated fetal calf serum (CanSera, cat. no. CS-C08-500-U), 80 µg/mL streptomycin sulphate, 80 U/mL penicillin G, 0.2 µg/mL amphotericin B (GIBCO, antibiotic-antimycotic, cat. no. 15240-062), and 1.6 mM L-glutamine (GIBCO, cat. no. 25030-081).

• Red blood cell (RBC) lysis buffer (ACK buffer): Prepare solution containing 0.15 M NH4Cl, 10 mM KHC03, 0.1 mM Na2EDTA in distilled H20. Filter and store at 22^{o}C.

• Mouse CD11c positive selection kit (StemCell Technologies, cat. no. 18758)

• CD11c purification buffer: PBS (pH 7.2) containing 2% heat inactivated fetal calf serum and 1 mM EDTA

• CD11b immunomagnetic beads (Miltenyi Biotec, cat. no.130-049-601)

• CD11b purification buffer: PBS (pH 7.2) containing 0.5% BSA and 2 mM EDTA

• Collagenase solution: dissolve collagenase IV (Worthington Biochemical, cat. no.4188) in RPMI-1640 to 43 U/mL.

Equipment

• Dialysis tubing (Spectrim Laboratories, Inc, Spectra/Por, MWCO: 12-14,00)

• Cell strainer (70 µm, BD Falcon, cat. no. 352350)

• EasySep Magnet (Stem Cell Technologies, cat. no. 18000)

• LS columns (Meltenyi Biotech, cat. no. 130-042-401)

• VarioMACS separator (Meltenyi Biotech)

Procedure

1. Preparation of dialyzed IVIg and BSA TIMING 12-16 h

i) Determine the volume of IVIg required by taking into account the number of experimental conditions and the number of mice per experimental condition (see Step 3). Use a likewise prepared BSA solution as a control.

ii) Remove the required length of the dialysis tubing (approximately 15-17 cm for 3-4 mL) and rinse in distilled H_20.

iii) Transfer the desired volume of IVIg or BSA solution into dialysis tube, tie the ends of the tubing, and transfer the tube into sterile beaker containing sterile PBS (pH 7.2).

iv) Stir in a beaker overnight at 4^oC.

2. Cell preparation TIMING 2 h

Remove spleens from appropriate mice and mechanically disrupt in 5 mL of culture medium and filter through a 70- µm nylon cell strainer.

i) Lyse RBC using 1mL of ACK buffer for 5 min.

ii) Wash cells 2x in RPMI-1640.

Alternatively, total splenic leukocytes may be replaced with specific cell type. Cell type selected will depend on the experimental set up.

(A) Purification of CD11c+ and CD11c- cells TIMING 3-4 h

(i) Prepare CD11c+ and CD11c- cell fractions from splenic leukocytes by magnetic separation using the mouse CD11c positive selection kit according to the manufacturer's instructions.

(ii) Disrupt spleen in RPMI-1640 containing 43 U/mL collagenase IV for 20 minutes at 37^{o}C.

(iii) Further disrupt the spleen and add EDTA (1mM final concentration) for 5 minutes at 37^{o}C and filter the spleen preparation through a 70 µm nylon cell strainer.

(iv) Wash splenic leukocytes with CD11c purification buffer.

(v) Resuspend the splenic leukocytes in CD11c purification buffer to 2x10(8) nucleated cells/mL and incubate with CD11c-PE at 15 µL/mL for 15 min at 22^{o}C.

(vi) After washing, incubate the splenic leukocytes in PE selection cocktail (100 µL cocktail/mL of cells) for 15 minutes at 22^{o}C.

(vii) Add magnetic particles (50 µL/mL cells) and incubated for 10 min at 22^{o}C, followed by separation using a magnet.

(viii) Collect the supernatant as the CD11c- cell fraction.

(ix) Remove the tube from the magnet, resuspend the cells as CD11c+ fraction, wash 1x in RPMI-1640.

(B) Purification of CD11b+ and CD11b- cells TIMING 3-4 h

(i) Prepare CD11b+ and CD11b- cell fractions using CD11b immunomagnetic beads according to the manufacturer's instructions.

(ii) Prepare a single-cell suspension by disrupting the spleen in RPMI-1640 and filter through a 70-µm nylon cell strainer.

(iii) After RBC lysis using ACK buffer and washing using RPMI-1640, resuspend cells (10^{8}/mL) in CD11b purification buffer containing CD11b microbeads (10 µL/10^{7}) cells).

(iv) Incubate for 15 minutes at 4^{o}C.

(v) Wash the cells in CD11b purification buffer and apply to MACS column.

(vi) Collect the effluent as CD11b- cell fraction.

(vii) Remove the column from the magnet and flush the cells from the column as CD11b+ fraction.

3. Cell priming TIMING 1 h

(A) Incubate splenic leukocytes (1.4x10^{6}/mL) with 18 mg/mL IVIg or BSA for 30 min at 37^{o}C. For CD11c+ or CD11b+ cells use 1.4x10^{5}/mL, and for CD11c- or CD11b- cells use 1.4x10^{6}/mL.

i) Wash cells 2x in RPMI-1640.

ii) Resuspend in culture medium to 5x10^{6}/mL

4. Alternative methods of leukocyte priming TIMING 2 h

(A) FcγRIIIA crosslinking

(i) Incubate splenic leukocytes (2x10^{7}/mL) with antibody 2.4G2 (2.5 µg/mL) or normal rat IgG (2.5 µg/mL) for 15 min at 22^{o}C in culture medium.

(ii) Wash cells 1x in RPMI-1640.

(iii) Incubate cells (2x10^{7}/mL) with goat F(ab')2 anti-rat IgG (25 µg/mL) for 30 min at 37^{o}C.

(iv) Wash 2x in RPMI-1640.

(v) Resuspend in culture medium to 5x10^{6}/mL.

(B) PIR-A/B crosslinking

(i) Incubate splenic leukocytes (4x10^{6}/mL) with 6C1 (10 µg/mL) or normal rat IgG (10 µg/mL) for 15 min at 22^{o}C in culture medium.

(ii) Wash cells 1x in RPMI-1640.

(iii) Incubate cells (4x10^{6}/mL) with goat F(ab')2 anti-rat IgG (25 µg/mL) for 30 min at 37^{o}C.

(iv) Wash 2x in RPMI-1640.

(v) Resuspend in culture medium to 5x10^{6}/mL.

(C) Soluble immune complexes (sIC)

(i) Dissolve OVA in PBS, pH 7.2 at 5 mg/mL.

(ii) Prepare sIC by incubating 1 mg of ovalbumin with 50 µg monoclonal anti-ovalbumin or normal mouse IgG as a control for 30 min at 22^{o}C.

(iii) Incubate splenic leukocytes (5x10^{6}/mL) with sIC for 30 min at 37^{o}C.

(iv) Wash 2x in RPMI-1640.

(v) Resuspend in culture medium to 5x10^{6}/mL

5. Treatment and induction of ITP

(i) Inject 200 µl of cell suspension (5x10^{6}/mL) into the tail vein of recipient mice (Day 0).

(ii) Inject each mouse i.p. with 2 µg anti-platelet antibody (MWReg30) in 200 µL PBS, pH 7.2 (Day 1)

(iii) Bleed mice on Day 2 for platelet enumeration as described (8,10,11).

TIMING

It takes 12-16 hrs to prepare dialyzed IVIg or BSA. Cell preparation requires from 2-3 hrs depending on whether total splenic leukocytes or selected cell populations are used. Priming with IVIg requires 1 hr.

Timing

4 d

Critical Steps

Step 1

CRITICAL STEP The low pH of IVIg (4.5) may disrupt cell membrane integrity or damage cell surface antigens. Always use freshly prepared dialysed IVIg or BSA within 4 h of the end of dialysis.

Step 2

CRITICAL STEP The choice of cell type is critical to the outcome of the experiment. In the initial experiment we suggest the use of total splenic leukocytes.

Step 2A (ii)

CRITICAL STEP Prepare on the day of the experiment.

Step 3A

CRITICAL STEP To determine the amount of the cells required for the optimal affect in the

experimental model of interest, investigators should titrate the cells as well as the IVIg or BSA under the conditions described.

Step 5(i)

CRITICAL STEP Cell suspension should be mixed well immediately before injection into mice.

Anticipated Results

See Reference (9).

References

1. Bayary, J. et al. Intravenous immunoglobulin in autoimmune disorders: An insight into the immunoregulatory mechanisms. *Int Immunopharmacol* **6**, 528-34 (2006).
2. Crow, A.R., Lazarus A.H. Role of Fcγ Receptors in the Pathogenesis and Treatment of Idiopathic Thrombocytopenic Purpura. *J Pediatr Hematol Oncol* **25**, S14-S18 (2003).
3. Samuelsson, A., Towers, T.L. & Ravetch, J.V. Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor. *Science* **291**, 484-6. (2001).
4. Crow, A.R. et al. IVIg-mediated amelioration of murine ITP via Fc{gamma}RIIB is independent of SHIP1, SHP-1, and Btk activity. *Blood* **102**, 558-560 (2003).
5. Bruhns, P., Samuelsson, A., Pollard, J.W. & Ravetch, J.V. Colony-stimulating factor-1-dependent macrophages are responsible for IVIG protection in antibody-induced autoimmune disease. *Immunity* **18**, 573-81 (2003).
6. Bayry, J. et al. Modulation of dendritic cell maturation and function by B lymphocytes. *J Immunol* **175**, 15-20 (2005).
7. Bayry, J. et al. Inhibition of maturation and function of dendritic cells by intravenous immunoglobulin. *Blood* **101**, 758-65 (2003).
8. Crow, A.R., Song, S., Semple, J.W., Freedman, J. & Lazarus, A.H. IVIg inhibits reticuloendothelial system function and ameliorates murine passive-immune thrombocytopenia independent of anti-idiotype reactivity. *Br J Haematol* **115**, 679-

86. (2001).

9. Siragam, V. et al. Intravenous immunoglobulin ameliorates ITP via activating Fc γ receptors on dendritic cells. *Nat Med*, Published online: 21 May 2006 doi:10.1038/nm1416.

10. Song, S., Crow, A.R., Freedman, J. & Lazarus, A.H. Monoclonal IgG can ameliorate immune thrombocytopenia in a murine model of ITP: an alternative to IVIG. *Blood* **101**, 3708-13 (2003).

11. Siragam, V. et al. Can antibodies with specificity for soluble antigens mimic the therapeutic effects of intravenous IgG in the treatment of autoimmune disease? *J Clin Invest* **115**, 155-60 (2005).

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