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The FGL2-FcγRIIB pathway: A novel mechanism leading to immunosuppression

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Fibrinogen-like protein 2 (FGL2) is a multifunctional protein, which has been implicated in the pathogenesis of allograft and xenograft rejection. Previously, FGL2 was shown to inhibit maturation of BM-derived DC and T-cell proliferation. The mechanism of the immunosuppressive activity of FGL2 remains poorly elucidated. Here, we focus on identification of FGL2-specific receptor(s) and their ability to modulate APC activity and allograft survival. Using flow cytometry and surface plasmon resonance analysis, we show that FGL2 binds specifically to Fc gamma receptor (FcγR)IIB and FcγRIII receptors, which are expressed on the surface of APC, including B lymphocytes, macrophages and DC. Antibody to FcγRIIB and FcγRIII, or deficiency of these receptors, abrogated FGL2 binding. FGL2 inhibited the maturation of BMDC from FcγRIIB$^{+/+}$ mice but not from FcγRIIB$^{-/-}$ mice and induced apoptosis in the FcγRIIB^{+} mouse B-cell line (A20) but not the A20IIA1.6 cell line that does not express FcγRIIB. Recombinant FGL2 infused into FcγRIIB$^{+/+}$ (C57BL/6J, H-2^{b}) mice but not FcγRIIB$^{-/-}$ mice inhibited rejection of fully mismatched BALB/cJ (H-2^{d}) skin allografts. The identification of specific receptor binding has important implications for the pathogenesis of immune-mediated disease and suggests a potential for targeted FGL2 therapy.

Key words: Fcγ receptors · Fibrinogen-like protein · Immunomodulation

Supporting Information available online

Introduction

Fibrinogen-like protein 2 (FGL2), also known as fibroleukin, was first cloned from cytotoxic T lymphocytes and was classified as a member of the fibrinogen superfamily due to its homology (36%) with fibrinogen β and γ chains [1]. Predicted as a type II transmembrane glycoprotein [2], cell membrane–associated FGL2 was shown to exhibit novel prothrombinase activity when associated with cell membranes/phospholipid vesicles [3,4], which has been implicated in the pathogenesis of

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experimental and human viral-induced fulminant hepatitis [2], cytokine-induced fetal loss syndrome [5], allograft [6] and xenograft rejection [7]. The procoagulant activity was shown to depend on the serine 89 at the linear N terminal domain of FGL2 [4].

The C terminal globular portion of FGL2 lacks the prothrombinase activity and contains a classical fibrinogen-related domain (FRED), which has been suggested to possess immunomodulatory activity based on several lines of evidence. First, other fibrinogen superfamily members including fibrinogen [8], tenascin [9], angiopoietin [10] and ficolin [11] have been shown to have immunomodulatory activity. Degradation products of fibrinogen have been shown to interact with CD11b/CD18, alter Fc receptor expression, and inhibit lymphocyte proliferation in response to T-cell mitogens, allogeneic mononuclear leukocytes and anti-CD3 *in vitro* [12]. Second, FGL2 was shown to be secreted by T cells [13]. Of particular interest, *fgl2* mRNA is increased in $CD4^+CD25^+$ $Foxp3^+$ Treg along with mRNA for several known Treg suppression effector molecules [14, 15]. A marked increase of *fgl2* mRNA was also reported in immunosuppressive CD8αα intraepithelial lymphocytes [16]. FGL2 could represent one of the mediators of Treg cells that prevent autoimmune disease [17, 18]. We have shown that a recombinant FGL2 can inhibit DC-cell maturation, subsequent T-cell proliferation and polarizes T helper cell activity towards a type 2 cytokine response [19]. *Fgl2*$^{-/-}$ (knockout) mice developed autoimmune glomerulonephritis with age, and the immunosuppressive activity of Treg from *fgl2*$^{-/-}$ mice was impaired [20]. These findings collectively suggested that FGL2 is an important immunomodulatory molecule relevant to regulating certain types of immune disease.

It is logical to suspect that the immunomodulatory activity of FGL2 occurs through binding to specific receptor(s). In this paper, we report that recombinant FGL2 specifically bound to FcγRIIB and FcγRIII on APCs. FGL2 inhibited the maturation of BMDC and induced apoptosis of B cells in an FcγRIIB-dependent way. A short course of i.v. FGL2 treatment prolonged the survival of fully H-2-mismatched BALB/cJ (H-2^d) skin allografts in C57BL/6J (H-2^b) mice but not in FcγRIIB$^{-/-}$ mice (H-2^b).

Results

Recombinant FcFGL2 protein binds to APC

Recombinant FcFGL2 was generated and purified as described in the *Materials and methods*. The molecular size of the recombinant proteins was examined by SDS-PAGE, silver staining and Western blotting. FcFGL2 had a molecular weight of approximately 440 kDa under non-reducing conditions, which was confirmed by gel filtration (data not shown), and 110 kDa under reducing conditions (Fig. 1A). These data suggested that FcFGL2 exists as a tetramer, consistent with previous reports [13, 21]. The Fc tag had a molecular size of 64 kDa under non-reducing conditions and 33 kDa under reducing conditions, suggesting that Fc is dimeric.

Biotinylated FcFGL2 bound to the RAW264.7 cells (mouse macrophage cell lines), BMDC, the B-cell line A20 (which expresses only one Fcγ receptor, FcγRIIB), thioglycolate-elicited peritoneal exudates cells (>95% macrophages) from C57BL/6J mice, but FcFGL2 did not bind to the B-cell line A20IIA1.6, which does not express FcγRIIB (Fig. 1B) [22]. EL4 cells (a mouse T-cell line) did not bind FcFGL2 (data not shown). As expected, the Fc tag protein alone failed to bind to any of these cells and thus, Fc provided an appropriate negative control. Staining of A20 cells with anti-FcγRIIB/RIII monoclonal antibody (2.4G2) was detected but not in A20IIA1.6 cells as expected (Fig. 1C). Macrophages are known to express high levels of both FcγRIIB and FcγRIII, and showed a high fluorescence intensity of antibody staining.

To study whether FcFGL2 bound to FcγRIIB, FcγRIII or both, splenic lymphocytes and macrophages were isolated from FcγRIIB$^{+/+}$ and FcγRIIB$^{-/-}$ mice. B cells (anti-$CD19^+$) from FcγRIIB$^{+/+}$ mice express FcγRIIB and were stained by 2.4G2 (Fig. 2A, upper panel); this was not the case for B cells derived from FcγRIIB$^{-/-}$ mice. FcFGL2 bound only to the B cells from FcγRIIB$^{+/+}$ mice but not from FcγRIIB$^{-/-}$ mice (Fig. 2A, lower panel), confirming that FcγRIIB is a receptor for FcFGL2. In addition to B cells, thioglycolate-elicited peritoneal macrophages were isolated from FcγRIIB$^{-/-}$ mice and stained with 2.4G2 (Fig. 2B), which demonstrated that these cells express FcγRIII. FcFGL2 bound to these macrophages from FcγRIIB$^{-/-}$ mice and binding was blocked by antibody to FcγRIIB/III, indicating that FcFGL2 also binds to FcγRIII (Fig. 2B). Taken together, these data suggested that FcFGL2 binds to the low-affinity Fcγ receptors, FcγRIIB and FcγRIII on APC.

FcFGL2 binds specifically to FcγRIIB and FcγRIII

To determine whether FcγRIIB and RIII were specific receptors for FGL2, we performed antibody blocking assay and showed that binding of FcFGL2 to A20 cells, peritoneal macrophages (Fig. 3A), LPS-stimulated B cells (Fig. 3B) and RAW264.7 cells was completely blocked by antibody to FcγRIIB/III (Fig. 3C), whereas a rat anti-mouse IgG2b isotype antibody failed to block FcFGL2 binding.

Next, we examined whether FGL2 bound to receptors of other fibrinogen family members. Specifically, we studied whether FcFGL2 bound to Mac-1 (CD11b/CD18) or TLR4, which are known receptors for fibrinogen. Antibodies against CD11b, CD18 and TLR4 blocked the binding of fibrinogen (data not shown) but none of these antibodies blocked the binding of FcFGL2 to RAW264.7 cells (Fig. 3C). Binding of labeled FcFGL2 was inhibited by co-incubation with 100-fold molar excess of unlabeled FcFGL2, but not with unlabeled Fc, BSA or fibrinogen (Fig. 3D). Anti-FGL2 antibody also blocked FcFGL2 binding to FcγRIIB and RIII on RAW264.7 cells (Fig. 3E).

An *in vitro* kinetic binding assay using FcFGL2 with RAW264.7 cells was performed by flow cytometry. In this assay, the binding of FcFGL2 to RAW264.7 cells reached equilibrium at 150 min and the binding affinity was calculated as 20 nM (nonlinear regression analysis, Graphpad Prism 3.02), whereas binding of the Fc tag was not detectable (Supporting Information Fig. 1).

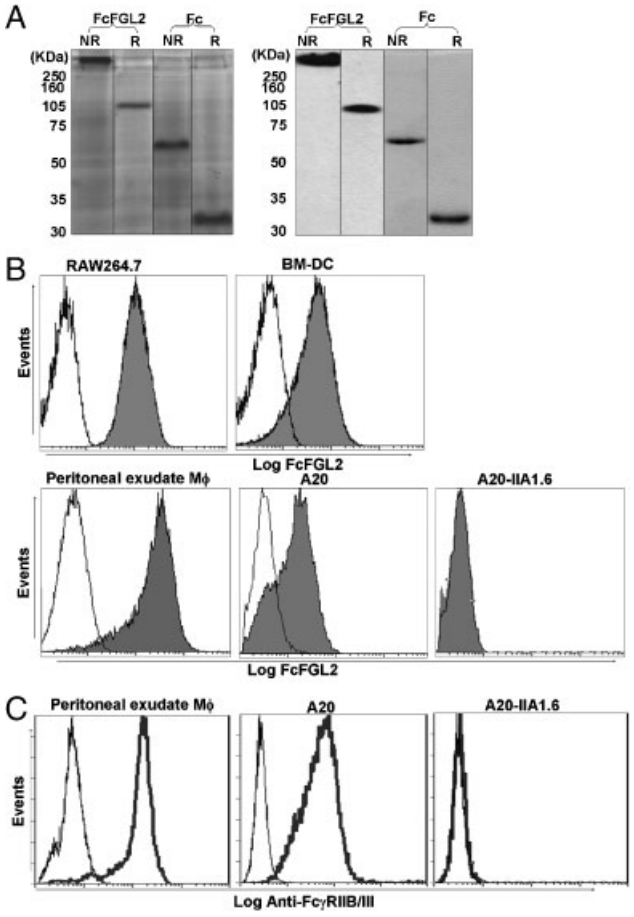

Figure 1. FcFGL2 recombinant protein binds to BMDC, macrophage and B-cell lines A20 but not to FcγIIB-deficient A20IIA1.6. (A) Recombinant mouse FGL2 with a mutated mouse IgG2a Fc tag at the N terminus (FcFGL2) and the tag alone (Fc) were produced in a CHO cell system, purified by protein A chromatography and analyzed by 10% SDS-PAGE. Left: Silver staining, 50 ng of each protein was loaded under both non-reducing (NR) and reducing (R) conditions. Right: Western blot; 1 μg of each purified protein was detected by goat anti-mouse IgG2a HRP. (B) Binding of FcFGL2 to RAW264.7 cells, BMDC, thioglycolate-stimulated peritoneal exudate macrophages from C57BL/6J mice, A20 cells (B-cell line) and A20-IIA1.6 cells (B-cell line); 1×10^6 cells were incubated with 2 μg of either biotinylated mouse FcFGL2 (shaded histograms) or biotinylated Fc as controls (open histograms), followed by Streptavidin-phycoerythrin (SA-PE). Cells were analyzed by flow cytometry. Histograms show that FcFGL2 bound to RAW264.7, DC, A20 cells and peritoneal exudate macrophages but not to A20-IIA1.6 cells, whereas Fc did not bind to any of these cells. (C) Expression of FcγRIIB/III on thioglycolate-stimulated peritoneal macrophages, A20 cells and A20-IIA1.6 cells; 1×10^6 cells were stained with 1 μg of rat anti-mouse FcγRIIB/III-FITC (thick line). Cells stained with isotype control (Rat IgG2b-FITC) are shown as the negative control (thin line). A20-IIA1.6 cells showed no staining with anti-FcγRIIB/III-FITC. Representative data from three separate experiments are shown.

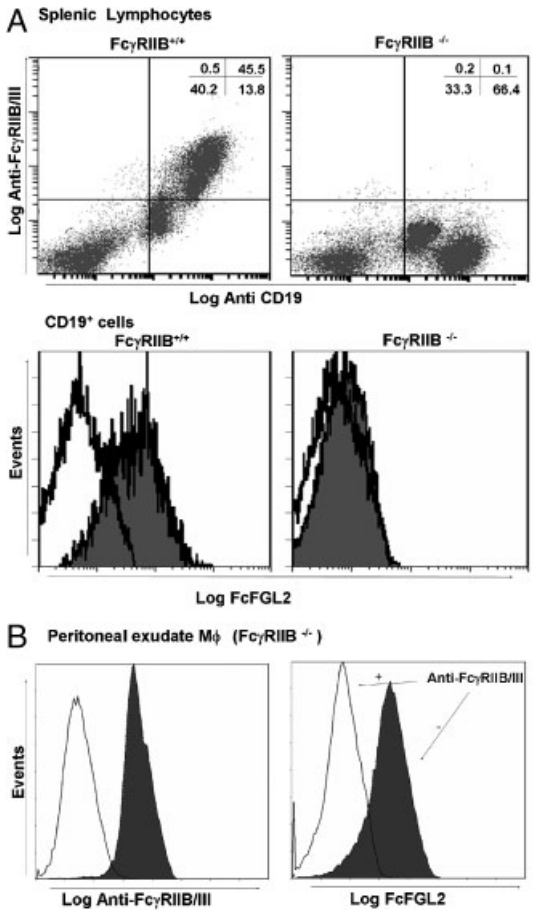

Figure 2. FcFGL2 binds both to FcγRIIB and to FcγRIII. (A) Lymphocytes were isolated from the spleen of either FcγRIIB$^{+/+}$ mice (C57BL/6J) or FcγRIIB$^{-/-}$ mice as described in the *Materials and methods* and analyzed after LPS stimulation for 24 h. The lymphocytes cells shown were gated based on their forward and side scatter properties. Upper panel: B cells (PC5-anti-CD19$^+$) from FcγRIIB$^{+/+}$ mice but not from FcγRIIB$^{-/-}$ mice were stained with anti-FcγRIIB/III antibody. Lower panel: The cells shown are gated on B cells (anti-CD19$^+$), FcFGL2 binds to B cells from FcγRIIB$^{+/+}$ mice (left) but not B cells from FcγRIIB$^{-/-}$ mice (right) (shaded histogram). B cells stained with SA-PE alone were shown as the negative control (open histogram). (B) Left panel: Thioglycolate-stimulated peritoneal exudate macrophages (1×10^6) from FcγRIIB$^{-/-}$ mice were stained with rat anti-mouse FcγRIIB/III-FITC (shaded histogram). Cells stained with isotype antibody were shown as the open histogram. Right panel: The binding of FcFGL2 to peritoneal exudate macrophages from FcγRIIB$^{-/-}$ mice (shaded histogram) was blocked by anti-mouse FcγRIIB/III antibody. All cells were analyzed by flow cytometry and representative data from three separate experiments are shown.

MSA-FGL2 shows similar binding pattern as FcFGL2 to FcγRIIB and FcγRIII

To determine if binding of recombinant FcFGL2 was due to the Fc component, a mouse serum albumin tagged FGL2 (MSA-FGL2) was generated and purified as described in the *Materials and methods*. Under non-reducing conditions, MSA-FGL2 had a molecular size of approximately 600 kDa (confirmed by gel filtration), whereas under reducing conditions, it had a molecular size of 150 kDa (Fig 4A). This confirmed the tetrameric structure of FcFGL2 described earlier. In contrast, MSA had a molecular size of 67 KDa under both non-reducing and reducing conditions.

MSA-FGL2 bound to thioglycolate-elicited peritoneal exudates cells, the B cell line A20 but not to the FcγRIIB$^{-/-}$ B-cell line A20IIA1.6 (Fig. 4B). The binding of MSA-FGL2 to A20, to peritoneal macrophages and to splenic B lymphocytes was blocked by anti-FcγRIIB/RIII antibody, consistent with the

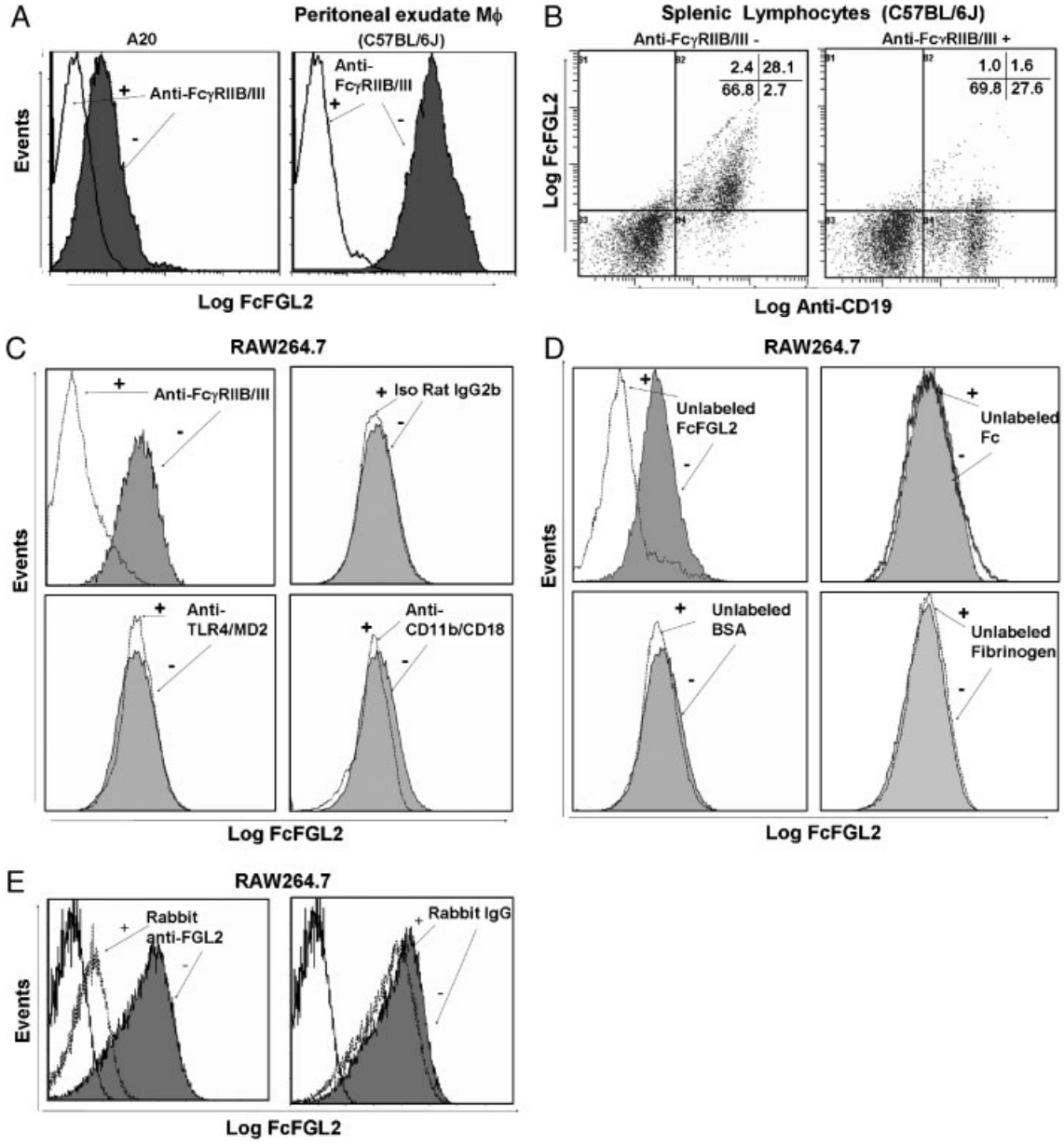

Figure 3. FcFGL2 binds specifically to FcγRIIB and to FcγRIII. (A) 1×10^6 A20 cells or peritoneal macrophages were incubated with 1 μg of either anti-FcγRIIB/III antibody (open histograms) or 1 × PBS (shaded histograms) for 5 min at room temperature. The cells were then stained with 2 μg of biotinylated FcFGL2 followed by SA-PE. Cells stained with SA-PE alone were used as the negative control and the histogram was identical to the open histogram (data not shown). (B) Anti-FcγRIIB/III antibody blocked the binding of FcFGL2 to splenic B lymphocytes. Left panel: Without anti-FcγRIIB/III antibody, B cells (anti-CD19$^+$) showed binding with FcFGL2. Right panel: The lymphocytes were pre-incubated with anti-FcγRIIB/III antibody for 5 min and then incubated with 2 μg of biotinylated FcFGL2, followed by SA-PE. The B-cell binding with FcFGL2 was completely blocked (right lower quadrant). (C) Antibody blocking assay of FcFGL2 in RAW264.7 cells. The binding of FcFGL2 to RAW264.7cells with (open histograms) or without blocking antibodies (shaded histograms), including anti-FcγRIIB/III, isotype rat IgG2b, anti-TLR4/MD2, anti-CD11b and anti-CD18 are shown. (D) Competition assay of FcFGL2 binding to RAW264.7 cells. The binding of biotinylated FcFGL2 to RAW264.7 cells with (open histogram) or without (shaded histogram) the addition of 100-fold molar excess of unlabeled FcFGL2, Fc, BSA or fibrinogen are compared. (E). Anti-FGL2 antibody blocked the binding of FcFGL2 to RAW264.7 cells. The binding of FcFGL2 to RAW264.7cells with (open histograms, dotted line) or without blocking antibodies (shaded histogram), including rabbit anti-FGL2 (left) and an isotype rabbit IgG (right) is shown. RAW264.7 cells stained with SA-PE alone are shown as the negative control (open histograms, solid curve). Representative data from three separate experiments are shown.

hypothesis that the receptor(s) for FGL2 are FcγRIIB and FcγRIII (Fig. 4C and D).

We further examined binding of MSA-FGL2 by surface plasmon resonance (SPR) analysis. A two-direction study was performed by changing the coating-injection protein combinations. First, MSA was used to coat the chip and none of the injected Fcγ receptors (RI, RIIB or RIII) showed detectable binding (Fig. 5A). When MSA-FGL2 was used to coat the chip, FcγRIIB and FcγRIII but not FcγRI showed binding (Fig. 5B). In a separate set of experiments, the Fcγ receptors (RI, RIIB or RIII) were used to

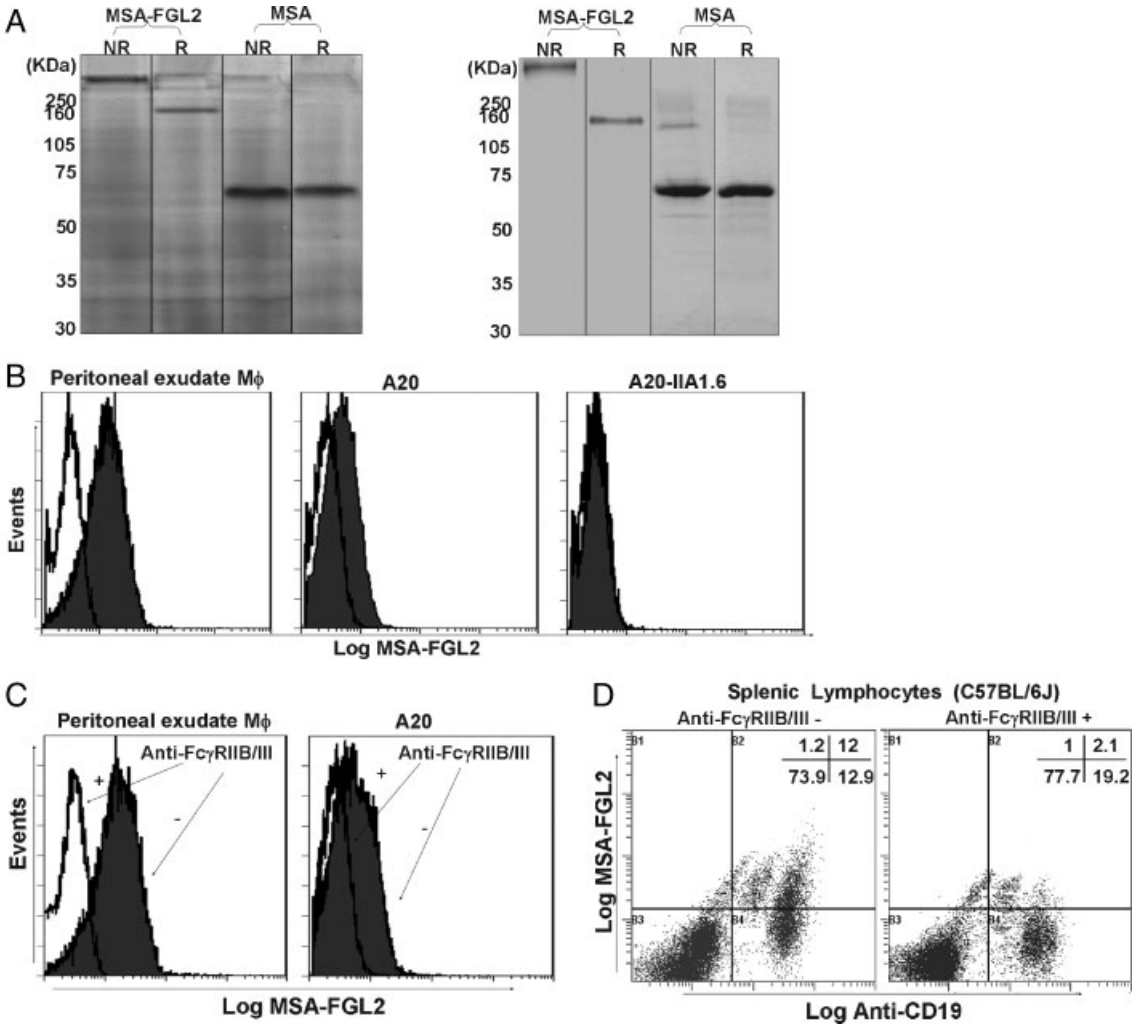

Figure 4. Recombinant MSA-FGL2 protein binds specifically to FcγRIIB and to FcγRIII. (A) Mouse FGL2 with MSA tag at the N terminus and 6× His tag at the C terminus (MSA-FGL2) was produced in a COS-7 cell system, purified by nickel column chromatography and analyzed by SDS-PAGE. Left panel: Silver staining, 50 ng of each protein was loaded under both non-reducing (NR) and reducing (R) conditions. Right panel: Western blot, 1 μg of MSA-FGL2 protein and MSA were detected by polyclonal goat anti-MSA conjugated with HRP. (B) 1×10^6 A20 cells, A20-IIA1.6 cells or peritoneal macrophages were incubated with 2 μg of MSA-FGL2 and followed by anti-His-biotin and SA-PE (shaded histogram). The cells incubated with only anti-His-biotin and SA-PE were used as the negative control (open histogram). (C) Anti-FcγRIIB/III antibody blocked the binding of MSA-FGL2 to A20 and thioglycolate stimulated peritoneal exudate cells; 1×10^6 A20 cells or peritoneal exudate cells (macrophage 95%) were pre-incubated with 1 μg of anti-FcγRIIB/III antibody at room temperature for 5 min before adding 2 μg of MSA-FGL2, followed by anti-HIS-biotin and SA-PE (open histograms). Cells without pre-incubation with anti-FcγRIIB/III antibody are shown in shaded histograms. (D) Anti-FcγRIIB/III antibody blocked the binding of MSA-FGL2 to splenic B lymphocytes. Left panel: Without anti-FcγRIIB/III blocking antibody, B cells (anti-CD19$^+$) showed binding with MSA-FGL2. Right panel: The lymphocytes were pre-incubated with anti-FcγRIIB/III antibody for 5 min and then incubated with 2 μg of MSA-FGL2, followed by anti-His-biotin and SA-PE. The B-cell binding with MSA-FGL2 was completely blocked (right lower quadrant). Cells in parts B and C were analyzed by flow cytometry and representative data from three separate experiments are shown.

coat the chips and MSA failed to bind to any of these receptors (Fig. 5C). In contrast, MSA-FGL2 bound to FcγRIIB (Fig. 5D) and FcγRIII (Fig. 5E) in a dose-dependent fashion but did not bind to FcγRI (Fig. 5F). Human IgG1 Fc was used as a positive control and bound to the FcγRI receptor (Supporting Information Fig. 2).

Intracellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 failed to bind to the chip that had been coated with MSA-FGL2, consistent with the flow cytometry data that these molecules are not receptors for FGL2 (data not shown). Mouse fibrinogen failed to bind to chips coated with any of the Fcγ receptors, as expected (data not shown). Taken together, these data strongly confirm that FGL2 binds to FcγRIIB and FcγRIII independent of the Fc tag.

FGL2 inhibits DC maturation through binding to FcγRIIB

To examine the biological effects of binding of FGL2 to the low-affinity Fcγ receptors, BMDC were isolated from FcγRIIB$^{+/+}$ and FcγRIIB$^{-/-}$ mice (C57BL/6J background) and then cultured in the presence of GM-CSF and IL-4 for seven days. Immature DC

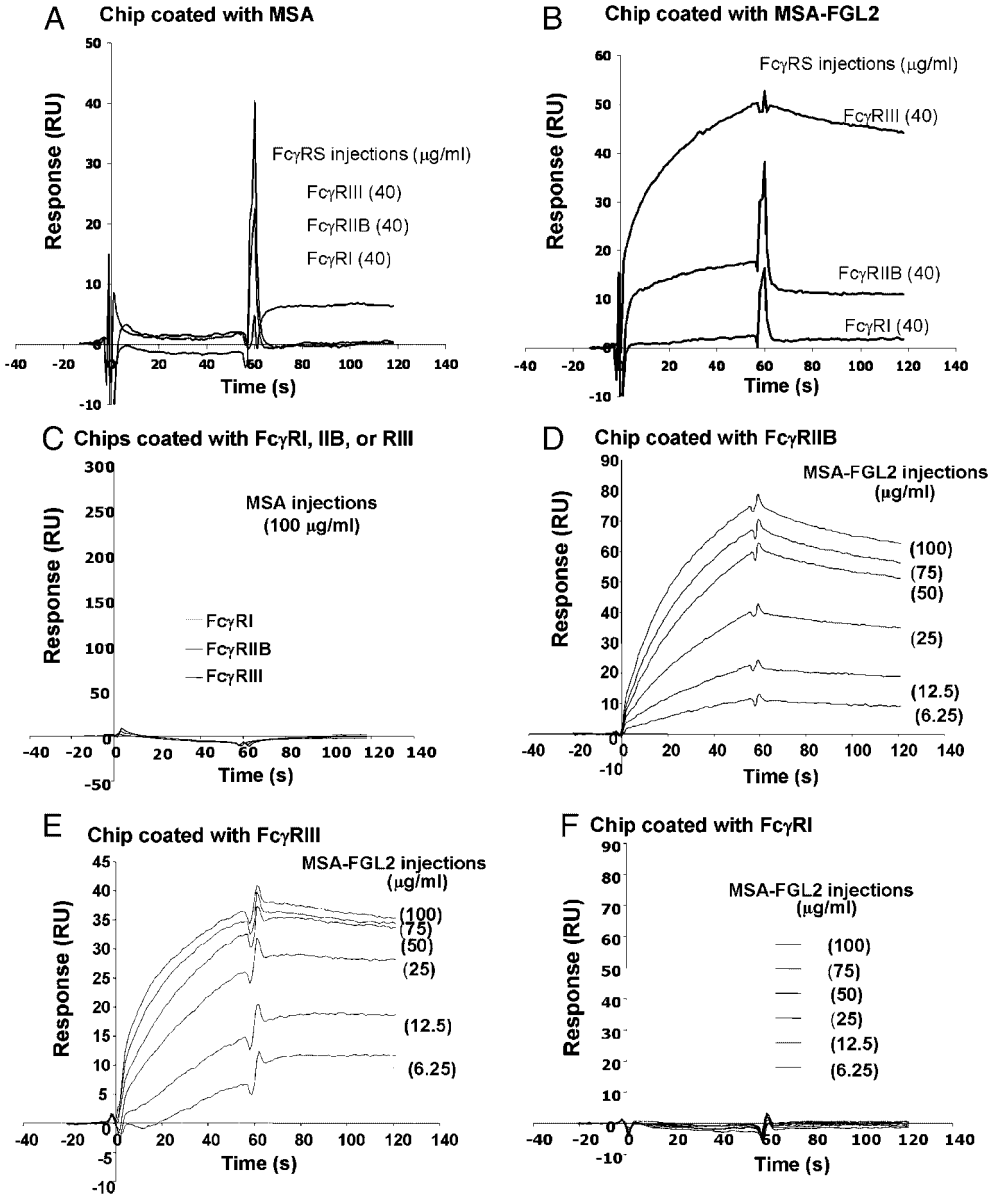

Figure 5. SPR analysis of MSA-FGL2 binding to FcγRIIB and to FcγRIII. One thousand four hundred response unit (RU) of MSA (A) or 2400 RU of MSA-FGL2 (B) was coated on the chip while 40 μg/mL of FcγRI, FcγRIIB or FcγRIII was injected, respectively. None of the three FcγR bound to MSA. In contrast, FcγRIIB and FcγRIII but not FcγRI bound to MSA-FGL2; 100 μg/mL of MSA (C) was injected onto the chip coated with FcγRI (2000 RU), FcγRIIB (1900 RU) or FcγRIII (2600 RU), respectively. MSA did not bind to any receptor. Increasing concentrations of MSA-FGL2 (6.25–100 μg/mL) were injected onto the chip coated with FcγRIIB (D), FcγRIII (E) and FcγRI (F), respectively. MSA-FGL2 did not bind to FcγRI but bound to FcγRIIB and FcγRIII in a dose-dependent manner. Representative data from three separate experiments are shown.

were stimulated for 48 h with LPS (200 ng/mL) in the presence of Fc tag or FcFGL2 (10 μg/mL). Treatment with FcFGL2 but not the Fc tag alone resulted in reduced expression of CD40, CD80, CD86 and MHC-II on DC from FcγRIIB$^{+/+}$ mice (Fig. 6A). In contrast, FcFGL2 treatment had no inhibitory effect on the expression of these DC maturation markers on DC from FcγRIIB$^{-/-}$ mice (Fig. 6B). Since BMDC from FcγRIIB$^{-/-}$ mice express FcγRIII, these data suggested that FcFGL2 inhibited DC maturation by binding and signaling through FcγRIIB.

To study the apoptotic effects of FcγRIIB homo aggregation by FcFGL2, the mouse B-cell line A20 that expresses only FcγRIIB, and A20IIA1.6 cells that are FcγRIIB deficient were treated *in vitro* with increasing doses of Fc or FcFGL2 for 12 h and examined by flow cytometry. Staurosporine, a known apoptosis inducer, was used as a positive control (Fig. 6C). The percentage of dead cells gated with lower forward scatter and higher side scatter properties were measured and plotted against the concentrations of Fc, staurosporine or FcFGL2 (Fig. 6Ci). Analysis using propidium iodide (a dead cell marker) and FAM-VAD-FMK

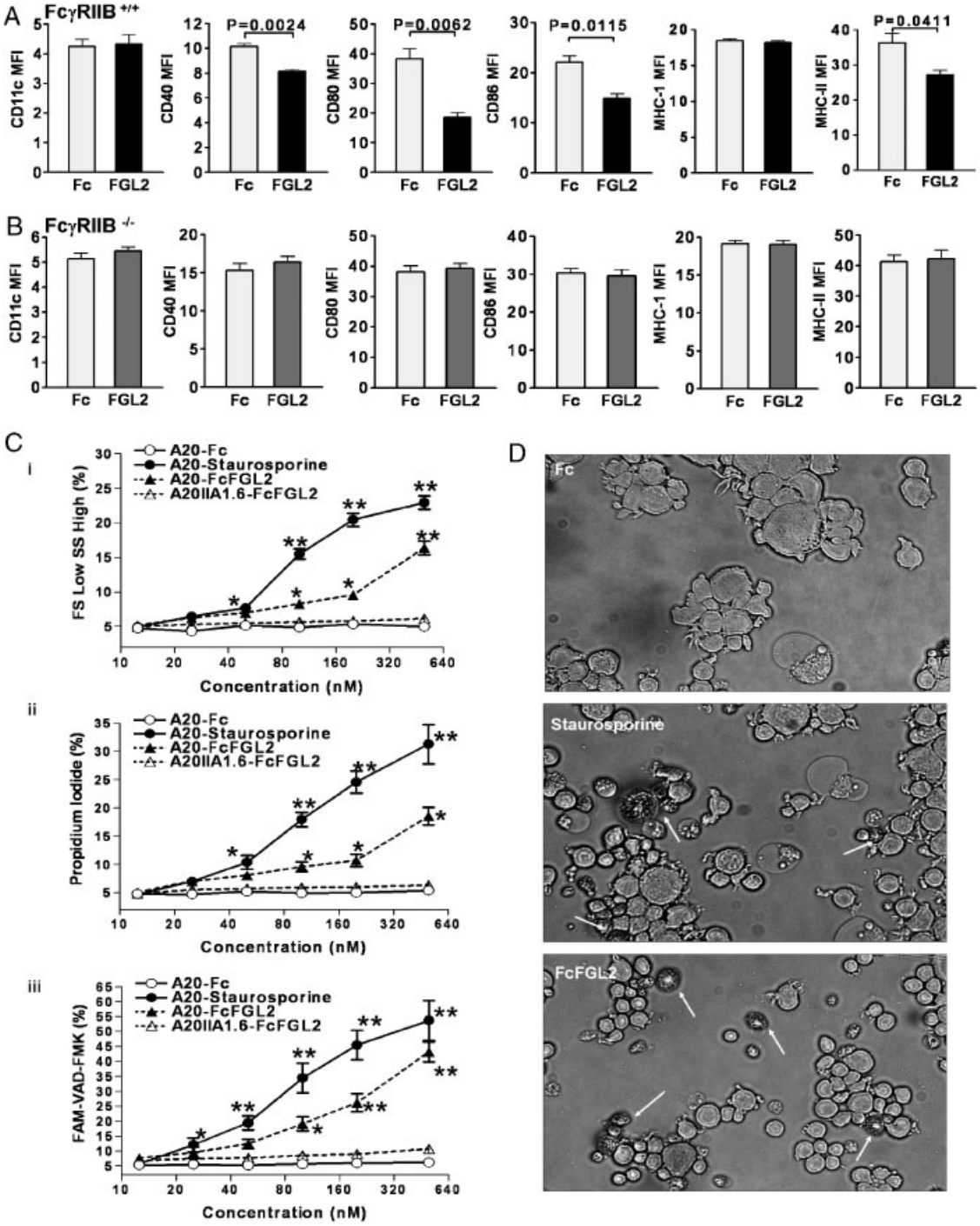

Figure 6. FGL2 inhibits the maturation of BMDC and induces the apoptosis of B cells that are FcγRIIB dependent. BM cells from B6 FcγRIIB$^{+/+}$ (A) and FcγRIIB$^{-/-}$ mice (B) were prepared and cultured for 7 days in the presence of GM-CSF and IL-4 to generate immature DC as described in the *Materials and methods*. Immature DC were stimulated with LPS (200 ng/mL) *in vitro* for 2 days to induce maturation in the presence of Fc tag (control) or FcFGL2 (10 μg/mL). The expression of surface markers including CD11c, CD40, CD80, CD86, MHC class I and MHC class II was measured by flow cytometry analysis and the results are shown as the median fluorescence intensity. (C) FGL2 induced apoptosis in A20 cells but not A20IIA1.6 cells; 2×10^5 A20 or A20IIA1.6 cells were cultured in RPMI media containing 10% FBS in 48 well plates. The cells were treated with increasing amount (12.5–500 nM) of Fc, or staurosporine or FcFGL2, respectively for 12 h, and were then stained with either PI or FAM-VAD-FMK (Carboxy fluorescein labeled valine-alanine-aspartic acid-fluoromethyl ketone an activation marker for poly-caspases), and analyzed by flow cytometry. The percentage of dead cells with high side scatter and low forward scatter properties are shown in panel (i). PI and FAM-VAD-FMK positive percentage curves are shown in panels (ii) and (iii), respectively. A20IIA1.6 cells treated with Fc or staurosporine had a similar reading as the corresponding group of A20 cells (data not shown). *p<0.01, **p<0.001 when compared with Fc treated group (Student's t-test). (D) Trypan blue staining of A20 cells after treatment with 500 nM of Fc, or staurosporine or FcFGL2 for 12 h (40 ×). White arrows indicate the dead cells. $N = 3$, error bars in all panels show mean ± SEM. The experiment was repeated three times with similar results.

Table 1. Treatment with FGL2 prolongs fully allogeneic skin graft survival

Group	Donor (H2)	Recipient (H2)	N	Treatment	Survival (days)
1	BALB/cJ (d)	BALB/cJ (d)	2	No	No rejection
2	BALB/cJ (d)	C57BL/6J (b)	10	No	7.8±1.99
3			8	FcFGL2, 20 μg i.v. on days 0, 3, 6 and 9	15±2.56 [a)]
4			7	Fc, 20 μg i.v. on days 0, 3, 6 and 9	8±2
5			7	Mouse fibrinogen, 20 μg i.v. on days 0, 3, 6 and 9	8.4±1.13
6	BALB/cJ (d)	FcγRIIB$^{-/-}$ (b)	6	No	8.5±1.76
7			6	FcFGL2, 20 μg i.v. on days 0, 3, 6 and 9	8.3±1.63

[a)] $p<0.001$ when compared with group 2 (Student's *t*-test). Survival data here were presented as mean±SD. The experiment was repeated three times with similar results.

(an apoptosis marker) are shown in (Fig. 6Cii and iii). Although less potent than staurosporine, FcFGL2 induced dose-dependent apoptosis and cell death of A20 cells. In contrast, Fc did not induce apoptosis, and FcFGL2 failed to induce apoptosis in the FcγRIIB-deficient A20IIA1.6 cells. Trypan blue staining showed that there were more dead A20 cells treated with staurosporine or FGL2 than when cells had been treated with Fc, in agreement with the flow cytometry data (Fig. 6D). These data indicate that FcFGL2 induces the apoptosis of B cells through an FcγRIIB-dependent pathway.

To study the signaling pathways downstream of binding of FGL2 to FcγRIIB/III, spleen cells from both FcγRIIB$^{+/+}$ and FcγRIIB$^{-/-}$ mice were stimulated with either rabbit anti-mouse IgG or FcFGL2 and analyzed by immunoprecipitation assays. By Western blot analysis, using a mouse anti-phosphotyrosine antibody (4G10), FGL2 stimulation did not result in phosphorylation of FcγRIIB whereas addition of rabbit anti-mouse IgG resulted in phosphorylation of FcγRIIB as expected (Supporting Information Fig. 3). The effect of ligation of FGL2 on caspases activation was also assayed by Western blot. FcFGL2 induced activation of C3, C7, and poly ADP ribose polymerase PARP of A20 cells (Supporting Information Fig. 4). Apoptosis induction of A20 cells by FcFGL2 was also confirmed by DNA ladder analysis (Supporting Information Fig. 5).

FGL2 treatment prolongs skin allograft survival in C57BL/6J

To investigate the biological significance of FGL2 binding to the FcγR *in vivo*, a series of full thickness, fully mismatched allo-skin transplants were performed in the presence or absence of treatment with FcFGL2. Survival of skin grafts from BALB/cJ (H-2^d) to B6 (H-2^b) mice that received four i.v. injections of 20 μg FcFGL2 on days 0, 3, 6 and 9 was prolonged from 7.8±1.99 to 15±2.56 days $p<0.001$ (Table 1). As long as FcFGL2 was administered, grafts were fully protected from rejection. However, after discontinuation of treatment on day 9, the grafts were rejected within 3–9 days. FcFGL2 failed to prolong the survival of BALB/cJ (H-2^d) skin grafts on FcγRIIB$^{-/-}$ B6 (H-2^b) recipients, consistent with the contention that binding of FGL2 to the inhibitory FcγRIIB receptor on APCs leads to immunosuppression in vivo. In contrast, neither fibrinogen nor Fc tag prolonged skin graft survival. Histology findings confirmed that FcFGL2 prevents graft rejection whereas untreated grafts developed acute rejection (Fig. 7).

Discussion

In this paper, we demonstrate that FGL2 specifically binds to FcγRIIB and FcγRIII on APC. By flow cytometry, recombinant FGL2 bound to A20 cells, thioglycolate stimulated peritoneal macrophages, RAW264.7 cells, BMDC and splenic B cells, whereas FGL2 did not bind to the FcγRIIB deficient B-cell line A20IIA1.6 or to naïve unstimulated splenic T cells. Binding of FGL2 was completely inhibited by anti-FcγRIIB/III antibody or unlabeled FcFGL2. The specificity of binding of FGL2 was confirmed using SPR analysis. These studies further suggest that FGL2 has a higher avidity for FcγRIIB than FcγRIII, but monomeric FGL2 will be required for assessment of affinity. Slow dissociation kinetics of MSA-FGL2 from FcγRIIB and FcγRIII is consistent with a high avidity of the oligomer. SDS-PAGE demonstrated that MSA-FGL2 forms a tetramer under non-reducing conditions. It has been documented for antibody binding to hapten arrays that the enhanced affinity observed in avidity situations is primarily due to a decreased rate of dissociation [23].

In a preliminary set of experiments, a short course of i.v. FcFGL2 treatment protected fully H-2 mismatched BALB/cJ (H-2^d) skin allografts on B6 (H-2^b) mice as long as FcFGL2 was administered, but had no protective effect on FcγRIIB$^{-/-}$ graft-bearing recipients. Upon discontinuation of treatment, all allografts were rejected within 3–5 days. Further dosing studies are now ongoing to determine whether extended graft acceptance can be achieved with long-term treatment. These results collectively demonstrate that FcFGL2 binding has immunosuppressive activity.

FcγR are widely expressed by leukocytes and have been shown to play pivotal roles in multiple immunological responses, including phagocytosis, antibody-dependent

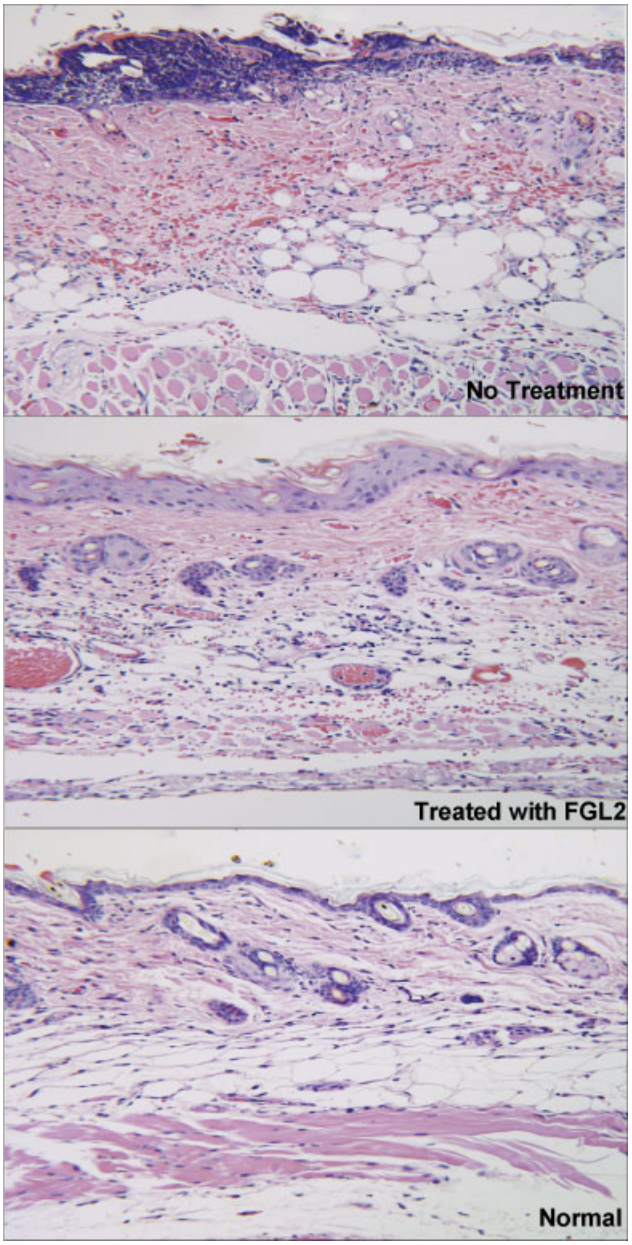

Figure 7. FGL2 treatment prolongs the survival of mouse skin allografts. Top panel: Skin grafts from untreated animals were harvested 6 days post transplantation, showing complete rejection. The dark blue area is the residual full thickness skin graft, showing a shrunken, totally necrotic and desiccated residuum. Note the absence of skin appendages. Middle panel: Full-thickness skin graft from FGL2-treated animal is near normal. Representative micrograph shows good preservation of the grafted skin. The graft is in place, intact and all layers of the skin are easily identifiable. There is mild thickening of the epidermis; hair follicles and sweat glands are all present. There is a mild inflammatory infiltrate present. Lower panel: Skin from a normal animal is shown as a normal control for comparison of histological details. Note the similarity of this graft to FGL2-treated skin graft. All skin tissue sections were stained with hematoxylin and eosin and photographed with a Leitz Laborlux S microscope. All magnifications × 200. Representative data from three separate experiments are shown.

cell-mediated cytotoxicity, inflammatory mediator release and autoimmune disease [24]. Four classes of mouse FcγR have been characterized to date, including the high (nM) affinity FcγRI/CD64, low (μM) affinity FcγRIIB/CD32, FcγRIII/CD16 and moderate (100 nM) affinity FcγRIV/CD16-2 [25]. Macrophages and DC express all four FcγR, while B lymphocytes express only FcγRIIB. In contrast, unstimulated T cells do not express any known FcγR [26]. However, FcγRIII expression has been reported on antigen-activated $CD8^+$ T cells [27] and anti-CD3-activated γ/δ T cells [28]. Thus, it is tempting to speculate that FGL2 could also directly interact with T cells under certain conditions.

FcγRIIB is a single-chain protein with signaling properties conferred by a cytoplasmic ITIM. In contrast, the activating receptors FcγRI, III and IV are all composed of two subunits, the ligand binding α unit and the ITAM-containing γ chain. FcγR provide a paradigm for the pairing of activation/inhibition membrane receptors, which could engage the same ligand but transduce opposing signals, a property shared by members of the triggering receptors expressed by myeloid cells family [29].

Currently, two different signaling pathways have been proposed for the inhibitory activity of FcγRIIB, based on the dependence of ITIM phosphorylation [30]. Co-engagement of FcγRIIB with the activating receptor leads to phosphorylation of ITIM and recruitment of SHIP, which hydrolyzes phosphatidylinositol (3,4,5)-trisphosphate, leading to release of the pleckstrin-homology domain containing proteins including Btk and phospholipase C from the membrane, blocking intracellular calcium mobilization. In contrast, homo-aggregation of FcγRIIB induces a stress signal through the transmembrane sequence in an ITIM-independent manner, eventually leading to cell apoptosis [30]. In this report, we have shown that binding of FGL2 to FcγRIIB results in B-cell apoptosis and inhibition of DC-cell maturation through a caspase-dependent mechanism, but appears to have little or no effect on ITIM phosphorylation.

In our study, FGL2 binding to BMDC that express both FcγRIIB and FcγRIII inhibited maturation, whereas binding of FGL2 to DC expressing FcγRIII but lacking FcγRIIB had no apparent effect. In contrast, LPS was able to activate these DC to a comparable extent as wild-type DC. These data highlight the immunoinhibitory role of FcγRIIB upon binding of FGL2, and are consistent with spontaneous autoimmunity in $Fc\gamma RIIB^{-/-}$ mice [31] and autoimmune glomerulonephritis in $fgl2^{-/-}$ mice [20]. FGL2 is analogous to other ligands of FcγRIIB and FcγRIII, such as C reactive protein and measles virus N protein, which bind to both the inhibitory and activating low affinity FcγR [32, 33]. In the case of measles virus infection, binding of the N protein results in inhibition of cell proliferation and apoptosis [33].

Immunoinhibition may also depend on the ratio of FcγRIIB and FcγRIII expressed by cells and on differences in ligand binding affinity to FcγR [34]. The expression of FcγRIIB or RIII can be differentially induced on myeloid cells by cytokines such as IL-4 and IFN-γ [35], and sialic acid rich IgG [36]. Promoter polymorphisms of FcγRIIB have been reported in inbred mice,

and differential expression levels of the FcγR have been linked to the occurrence of autoimmune diseases [37, 38].

Other members of the fibrinogen-related family of proteins following binding to specific receptors have been shown to have immunoregulatory activity. This binding is associated with the presence of either a P1 (amino acid residues 190–202) [39] or a P2 (residues 377–395) domain [40] within the FRED region. The P1 domain of fibrinogen-γ chain is highly conserved in fibrinogen family members including FGL2. As an example, fibrinogen promotes cellular adhesion and transendothelial migration through interactions of its FRED domain with the β2 integrin Mac-1 (CD11b/CD18) and ICAM-1 [41]. TLR4, the innate immune system receptor that transmits signaling by LPS, also binds to both fibrinogen [42] and domain A of fibronectin [43]. By flow cytometry and surface plasmon analysis, we demonstrated that FGL2 did not bind to Mac-1, ICAM-1 or TLR4. In contrast, the specific blocking of FcFGL2 to B cells and macrophages by antibody to FcγRIIB/III supports the contention that FcγRIIB/III are the receptor(s) for FGL2.

In conclusion, in this report we have demonstrated that FGL2 binds to the low-affinity FcγRIIB and FcγRIII expressed on APC. Binding of FGL2 results in immunosuppression both *in vitro* and *in vivo*. The identification of the specific cell receptor binding has important implications for the pathogenesis of immune-mediated disease and suggests a potential for targeted FGL2 therapy.

Materials and methods

Production and purification of mouse FGL2

Recombinant mouse FcFGL2 was expressed in the mammalian CHO cell system. A mouse IgG2a Fc tag was inserted at the amino terminus of mouse FGL2 to improve protein solubility and stability. The Fc tag had been mutated to prevent CDC (E318A; K320A; K322A) [44] and antibody-dependent cell-mediated cytotoxicity effects (L235 E) [45], while retaining the binding to protein G/A [46]. A mouse IgGκ chain signal peptide was inserted 5′ of the Fc tag to promote secretion of the recombinant protein. The construct was designed in the Fc-X orientation to enhance the transcription, translation, glycosylation and secretion of the recombinant protein [47, 48]. FcFGL2 was purified using a protein A resin column (PALL, Quebec, Canada). Up to 1 mg of recombinant protein was purified from 1 L of cell culture supernatant.

A second recombinant FGL2 linked to MSA was expressed in mammalian COS-7 cells (African green monkey kidney derived ell line). MSA was fused at the amino terminus of mouse FGL2 and two additional tags, 3x Myc and 6xHis, were linked at the carboxyl terminus for detection and purification (Trillium Therapeutic, Toronto, ON, Canada). MSA-FGL2 was purified using a metal chelate ProBond resin column (Invitrogen, Burlington, ON, Canada).

SDS-PAGE and Western blot were performed as previously described [19]. Silver staining was performed using the protocol from BIO-RAD, California, USA (161-0443).

Mice

C57BL/6J (B6), BALB/cJ and FcγRIIB$^{-/-}$ mice on a B6 background were purchased from the Jackson Laboratory (Bar Harbor, MA, USA). Female mice at 2–4 months of age were used for all experiments. All mice were housed in specific pathogen-free conditions and maintained at the Toronto General Research Institute. All studies conformed to guidelines established by the University Health Network at the University of Toronto (Animal use protocol ♯903.4).

Antibodies and reagents

Rat anti-mouse CD11b, anti-CD18, anti-TLR4, FITC-anti-CD11c, PC5-anti-CD19 and FITC-anti-CD3 antibodies were purchased from eBiosciences (San Diego, CA, USA). Rat anti-mouse FcγRIIB/RIII antibody (2.4G2), streptavidin-HRP, and streptavidin-PE (SA-PE) were purchased from BD Pharmingen (San Jose, CA, USA). Polyclonal goat anti-MSA conjugated with HRP (ab19195) was purchased from Abcam (Cambridge, MA, USA). Rabbit IgG (sc-2027), Goat anti-mouse IgG2a Fc polyclonal antibody (sc-2061), donkey anti-rabbit IgG-HRP (sc-2313) and rabbit anti-6xHis antibody conjugated with biotin (sc-803 B) were purchased from Santa Cruz, CA, USA. Rabbit anti-mouse IgG were purchased from Jackson Immuno-Research Laboratories (West Grove, PA, USA). Polyclonal rabbit anti-mouse FGL2 was generated and purified in our laboratory. Concanavalin A (Con A), LPS from *Escherichia coli* (L4524), immunoglobulin-free BSA (A2058), mouse fibrinogen (F4385) and MSA (A3139) were purchased from Sigma (Oakville, ON, Canada). Recombinant mouse ICAM-1 (796-IC-050), vascular adhesion molecule 1 (643-VM-050), FcγR including FcγRI (2074-FC-050), FcγRIIB (1460-CD-050) and FcγRIII (1960-FC-050) were purchased from R&D (Minneapolis, MN, USA).

Cell isolation and culture

RAW264.7, EL4, COS-7, A20 and CHO cells were obtained from the ATCC (Manassas, VA, USA). The A20IIA1.6 B-cell line was a generous gift from Dr. James Booth (Department of Immunology, University of Toronto). Peritoneal macrophages were harvested 4 days after intraperitoneal administration of 1.5 mL of a 5% thioglycolate solution to mice.

Spleens from mice were dissected, flushed with PBS using a syringe and needle, and cut up into small pieces (0.5 cm), which were passed through a nylon filter to separate cells from connective tissue. Splenic lymphocytes were separated from

erythrocytes using a Lympholyte M (Cedarlane, Burlington, ON, Canada), and stimulated with 2 μg/mL of LPS for 24 h before analysis. Distinct populations of splenic leukocytes ($CD3^+$, $CD19^+$ and $CD11b^+$) were also purified by magnetic cell sorting (Miltenyi Biotech, CA, USA). BMDC were isolated and cultured as previously described [19].

Biotinylation of FcFGL2, Fc, BSA and fibrinogen

Purified FcFGL2, Fc, BSA and murine fibrinogen (1 mg/mL) were incubated with a 1:10 molar reaction mixture of D-biotinyl-ε-aminocaproic acid-*N*-hydroxy-succinimide ester (Biotin-7-NHS) (Roche Diagnostics, Basel Switzerland) for 1 h at room temperature with gentle stirring. The reaction mixture was then applied to a Sephadex G-25 column, washed with 1.5 mL of Dulbecco's PBS and eluted with 3.5 mL of PBS.

Flow cytometry

A Cytomics^{TM} FC 500 (Beckman Coulter, Mississauga, ON, Canada) was used for flow cytometry and the data were analyzed using Rxp analysis software. Cells were stained using a standard protocol. Briefly, 1×10^6 cells were washed twice with 1 × PBS, pH 7.4, and then blocked for 5 min with 5% BSA at room temperature. The cells were then incubated with biotin-labeled FcFGL2, biotin-labeled Fc, biotin-labeled BSA or biotin-labeled fibrinogen for 30 min at 4°C in 100 μl 1 × PBS with 0.2% BSA. The cells were washed again, and incubated with an SA-PE conjugate for 20–30 min at 4°C. After an additional two washes, the cells were analyzed by flow cytometry. In experiments utilizing MSA-FGL2, a secondary biotin-conjugated rabbit anti- × His antibody followed by SA-PE was used to detect the 6 × His tag at the C terminal of MSA-FGL2.

SPR analysis

The respective interactions of MSA-FGL2 or MSA with FcγR were studied further using SPR technology on a Biacore 3000 biosensor system (BIAcore, Piscataway, NJ, USA). The experiments were performed at room temperature in a physiologic ionic strength buffer (10 mM HEPES), 150 mM NaCl, 3 mM EDTA), and 0.01% surfactant P-20, pH 7.4); 1900 resonance units (RU) of FcγRIIB, 2000 RU of FcγRI and 2600 RU of FcγRIII were immobilized on flow cells 2–4 of a CM5 chip (BIAcore) by using the amine-coupling kit from BIAcore. Flow cell 1 was sham-activated and deactivated without the addition of protein ligand and provided a non-specific binding control for analysis of binding to the other three ligands in separate cells/channels. Binding was measured by sequentially injecting six different concentrations of MSA-FGL2 (6.25–100 μg/mL) over the four flow cells, connected in series, at a flow rate of 30 μL/min for 120 s. MSA (100 μg/mL) was used as the negative control. Following 180 s of buffer flow for dissociation, the sensor chip was regenerated with 1 M NaCl. The interactions of the MSA-FGL2 with FcγR were also studied by reversing the immobilized receptor–ligand combinations (the chip was coated with MSA-FGL2 or MSA, followed by injection of different FcγR).

Skin transplantation

Full-thickness abdominal skin from donor mice (BALB/cJ) was grafted to the dorsum of recipient B6 mice. The skin grafts (1 × 1 cm) were sutured in place and covered with a band aid (Northsafety, 3″ × 0.75″). Recipient mice received no treatment, or treatment with fibrinogen or FcFGL2, or Fc (20 μg i.v. on days 0, 3, 6 and 9). Graft survival was monitored by daily visual inspection until time of rejection, with rejection defined as complete loss of viable skin. Skin samples were harvested on day 6 post-transplantation and histology was examined by hematoxylin and eosin staining and photographed with a Leitz Laborlux S microscope.

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Abbreviations: **FGL2:** fibrinogen-like protein 2 · **FRED:** fibrinogen-related domain · **MSA:** mouse serum albumin · **RU:** resonance units · **SA-PE:** streptavidin-PE · **SPR:** surface plasmon resonance

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