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Soluble Fibrinogen-Like Protein 2/Fibroleukin Exhibits Immunosuppressive Properties: Suppressing T Cell Proliferation and Inhibiting Maturation of Bone Marrow-Derived Dendritic Cells[1]

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Fibrinogen-like protein 2 (fgl2)/fibroleukin is a member of the fibrinogen-related protein superfamily. In addition to its established role in triggering thrombosis, it is known to be secreted by T cells. The soluble fgl2 ($_s$fgl2) protein generated in a baculovirus expression system bound to both T cells and bone marrow-derived dendritic cells (DC) in a specific manner. $_s$fgl2 exhibited immunomodulatory properties capable of inhibiting T cell proliferation stimulated by alloantigens, anti-CD3/anti-CD28 mAbs, and Con A in a dose-dependent manner; however, it had no inhibitory effects on CTL activity. The time- and dose-dependent inhibitory effect of $_s$fgl2 on alloreactive T cell proliferation could be neutralized by a mAb against mouse fgl2. Polarization toward a Th2 cytokine profile with decreased production of IL-2 and IFN-γ and increased production of IL-4 and IL-10 was observed in $_s$fgl2-treated allogeneic cultures. Exposure of immature DC to $_s$fgl2 abrogated the expression of CD80^{high} and MHC class II^{high} molecules and markedly inhibited NF-κB nuclear translocation, thus inhibiting their maturation. $_s$Fgl2-treated DC had an impaired ability to stimulate allogeneic T cell proliferation. Maximal inhibition of proliferation was observed when allogeneic T cells were cultured with $_s$fgl2-treated DC and $_s$fgl2 protein was added in the culture. These data provide the first evidence to demonstrate that $_s$fgl2 exerts immunosuppressive effects on T cell proliferation and DC maturation. ***The Journal of Immunology*****, 2003, 170: 4036–4044.**

Proteins homologous to the carboxyl terminus of the β- and γ-chains (fibrinogen-related domain or FRED)[3] of fibrinogen, including angiopoietins, ficolins and tenascins, have been classified into the fibrinogen-related protein superfamily and have been demonstrated to exert multifaceted roles in immune responses (1–3). For example, fibrinogen can act as a "bridge" between $\alpha_m\beta_2$-bearing leukocytes to ICAM-1 on endothelial cells and the engagement of $\alpha_m\beta_2$ by fibrinogen triggers a series of intracellular signaling events and cellular responses, including cytokine secretion and NF-κB activation (4, 5). Angiopoietin-1 inhibits endothelial cell permeability in response to thrombin and vascular endothelial growth factor in vitro via the regulation of the junctional complexes, platelet endothelial cell adhesion molecule 1, and vascular endothelial cadherin (6). Two independent studies have reported that soluble tenascin blocks T cell activation induced by soluble Ags, alloantigens, or the mitogen Con A (7, 8).

Fibrinogen-like protein 2 (fgl2), also known as fibroleukin, has been demonstrated to be involved in the pathogenesis of diseases including viral-induced fulminant hepatitis and Th1 cytokine-induced fetal loss syndrome, in which fibrin deposition is a prominent feature (9–11). The gene *fgl2* was originally cloned from CTL and the encoded protein shares a 36% homology to the fibrinogen β- and γ-chains and a 40% homology to the FRED of tenascin (1, 12). The coagulation activity of fgl2 was first described in a murine fulminant hepatitis model (13, 14) and fgl2 prothrombinase was detected in activated reticuloendothelial cells (macrophages and endothelial cells) (9, 15, 16). fgl2 functions as an immune coagulant with the ability to generate thrombin directly and, thus, fgl2 appears to play an important role in innate immunity.

Human fgl2/fibroleukin expressed by peripheral blood CD4$^+$ and CD8$^+$ T cells has been shown to be a secreted protein devoid of coagulation activity (17, 18). However, the function of soluble fgl2 protein (hereafter referred to as $_s$fgl2) generated by T cells remains undefined. Recently, Kohno et al. (19) have reported that human fgl2/fibroleukin gene transcription was absent in both patients with chronic and acute adult T cell leukemia/lymphoma. Furthermore, proteins in the fibrinogen-related protein superfamily have been shown to regulate immune activation. Based on these observations, we postulate that $_s$fgl2 plays a role in acquired immune responses.

In this study, we demonstrated that $_s$fgl2 generated in a baculovirus expression system inhibited T cell proliferation induced by alloantigens, anti-CD3/anti-CD28 mAb, and Con A in a dose- and time-dependent manner, whereas it had no inhibitory effects on CTL activity. Promotion of a Th2 cytokine profile was observed in allogeneic cultures when $_s$fgl2 was added. In addition, $_s$fgl2 abrogated the LPS-induced maturation of bone marrow (BM)-derived dendritic cells

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[3] Abbreviations used in this paper: FRED, fibrinogen-related domain; fgl2, fibrinogen-like protein 2; $_s$fgl2, soluble fgl2; BM, bone marrow; DC, dendritic cell; rm, recombinant mouse; DAPI, 4′,6′-diamidino-2-phenylindole; H5, High 5.

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(DC) by inhibiting NF-κB nuclear translocation, resulting in a reduced ability to induce alloreactive T cell proliferation. Our findings demonstrated a direct immunosuppressive activity of $_s$fgl2, suggesting a potential strategy of developing $_s$fgl2 as a therapeutic agent in treating autoimmune disorders and transplant rejection.

Materials and Methods

Mice

Female 6- to 8-wk-old BALB/c (H-2^d) and AJ (H-2^a) mice were purchased from Charles River Breeding Laboratory (Wilmington, MA) and The Jackson Laboratory (Bar Harbor, ME), respectively. All mice were chow-fed and allowed to acclimatize for 1 wk before experiments.

Reagents

Recombinant mouse (rm) GM-CSF and rmIL-4 were purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). LPS (*Escherichia coli*), human fibrinogen, and Con A were purchased from Sigma-Aldrich (St. Louis, MO). FITC- or PE-conjugated mAbs used to detect cell surface expression of CD80 (16-10A1), CD86 (GL1), CD40 (3/23) and CD11c (HL3), MHC class I (H2-K^d), and MHC class II (I-A^d) were purchased from BD PharMingen (San Diego, CA). Anti-Thy-1.2, anti-Ly-2.2 Abs, and rabbit anti-mouse complement were purchased from Cedarlane Laboratories. All culture reagents were purchased from Life Technologies (Mississauga, Ontario, Canada) unless otherwise stated.

Production of purified $_s$fgl2

Mouse $_s$fgl2 with a tandem repeat of six histidine residues followed by an enterokinase cleavage site fused to its N terminus was expressed in an Invitrogen (San Diego, CA) Insect Expression System (20). Briefly, a 1.4-kb cDNA encoding mouse *fgl2* was amplified using the forward primer 5′-TGCCGCACTGGATCCATGAGGCTTCCTGGT-3′ (with the methionine start codon underlined) and the reverse primer 5′-TTATGGCTT GAAATTCTTGGGC-3′ (nt 1283–1302 relative to the ATG start codon). Amplification was performed for 25 cycles with 2 min at 96°C, 2 min at 55°C, and 3 min at 72°C. The PCR product was cloned into the *Eco*RI and *Bam*HI sites of the vector pBlueBacHis2A (Invitrogen).

Putative recombinant viruses were generated according to the Invitrogen protocol and screened for the presence of *fgl2* by PCR followed by three rounds of viral plaque purification. The sequence of the recombinant baculovirus containing mouse *fgl2* cDNA was confirmed by an automated DNA sequencer (Applied Biosystems model 377; PerkinElmer, Norwalk, CT).

Monolayers of High 5 (H5) insect cells were infected with the recombinant baculovirus for the expression of mouse fgl2 protein. Seventy-two hours later, the infected cells were harvested by centrifugation and lysed in 6 M guanidinium hydrochloride, 20 mM sodium phosphate, and 500 mM NaCl. The soluble material was mixed with a 50% slurry of ProBond Ni-NTA resin (Invitrogen) for 1 h at 4°C. After washings, bound fgl2 protein was eluted with 8 M urea and 20 mM sodium phosphate (pH 5.3) with 150 mM NaCl. The pH of the eluted protein was adjusted to pH 7.2 immediately upon elution, and the protein was renatured by dialyzing against urea-saline buffers (150 mM NaCl, pH 7.2) with successive decreases in urea concentrations (6, 4, 2, and 1 M) and finally against TBS (10 mM Tris and 150 mM NaCl, pH 7.2). The dialyzed material was concentrated and $_s$fgl2 protein was collected by centrifugation at 14,000 rpm for 10 min to remove insoluble particulates. Protein concentrations were determined by a modified Lowry method/bicinchoninic acid BCA assay (Pierce, Brockville, Ontario, Canada).

Homogeneity of purified $_s$fgl2 was evaluated by SDS-PAGE and confirmed by Western blot probed with anti-fgl2 Ab as previously described (16). Proteins were stained directly using Coomassie brilliant blue or were transferred to nitrocellulose and probed using polyclonal rabbit anti-mouse fgl2 IgG as the primary Ab. The secondary Ab used for immunoblotting was affinity-purified donkey anti-rabbit IgG conjugated to HRP (Amersham, Buckinghamshire, U.K.) and the blot was visualized with an immunochemiluminescent kit (Amersham).

Biotinylation of $_s$fgl2 and BSA

A 1 mg/ml solution of purified $_s$fgl2 and Ig-free BSA (Sigma-Aldrich) were incubated with a 1:10 molar reaction mixture of d-biotinyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester (Biotin-7-NHS; Roche Diagnostics, Laval, Quebec, Canada) for 1 h at room temperature with gentle mixing. The reaction was then applied to a Sephadex G-25 column (Roche Diagnostics), washed with 1.5 ml of PBS, and then the biotinylated $_s$fgl2 was eluted with 3.5 ml of PBS. The eluate was collected and the protein concentration was determined by a modified Lowry method/bicinchoninic acid assay. The labeled proteins were used in binding assays as described below.

Preparation of cells

Spleen, lymph node, and BM cell suspensions were prepared aseptically. Spleen mononuclear cells were isolated by standard Lympholyte-M density gradient (Cedarlane Laboratories). All cell suspensions were resuspended in complete medium (α-MEM, supplemented with 10% FBS, 50 μM 2-ME, 1 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin).

BM-derived DC were prepared as described elsewhere (21). Briefly, BM cells were removed from femurs and tibias of BALB/c (H-2^d) mice and filtered through nylon mesh. Cells were incubated with anti-Thy-1.2 on ice for 45 min and then treated with rabbit anti-mouse complement for 1 h at 37°C. The cells were washed and cultured in 100-mm tissue culture dishes in complete medium supplemented with 10% FBS (Flow Laboratories, Mississauga, Ontario, Canada) at a concentration of 1×10^6 cells/ml with rmGM-CSF (800 U/ml) and rmIL-4 (500 U/ml). On days 2 and 4, nonadherent granulocytes were discarded and fresh rmGM-CSF and rmIL-4 were added at 36-h intervals. Immature DC were collected on day 7 and LPS (1 μg/ml) was added to the culture for 24 h to allow for maturation.

Assays for T cell proliferation

All assays were performed in 96-well U-bottom microtiter plates (Falcon Plastics, Washington, PA) in a humidified atmosphere with 5% CO_2 at 37°C.

Alloantigen stimulation. For alloantigen stimulation, BALB/c splenic mononuclear cells (4×10^5 cells/100 μl) were stimulated with irradiated (3000 rad) A/J splenic mononuclear cells (4×10^5 cells/100 μl) with or without $_s$fgl2 (ranging from 1 μg/ml to 1 ng/ml) added to the culture.

Response to Con A. Purified T cells (2×10^5 cells/200 μl) were stimulated with Con A (5 μg/ml; Sigma-Aldrich) in the presence or absence of $_s$fgl2 protein (ranging from 1 μg/ml to 1 ng/ml). After 3 days, cells were pulsed with [^{3}H]thymidine (1 μCi/well; Amersham Biosciences; Piscataway, NJ) for 18 h before harvesting and determining the incorporated radioactivity.

Response to anti-CD3 and anti-CD28. Purified T cells (2×10^5 cells/200 μl) were stimulated with immobilized anti-CD3 mAb (1 μg/ml) and soluble anti-CD28 mAb (20 ng/ml) in the presence or absence of $_s$fgl2 protein (ranging from 1 μg/ml to 1 ng/ml). After 3 days, cells were pulsed with [^{3}H]thymidine (1 μCi/well; Amersham Biosciences) for 18 h before harvesting and before determining the incorporated radioactivity.

H5 supernatants, wild-type baculovirus-infected H5 supernatants, human fibrinogen (1 μg/ml), and BSA (1 μg/ml) were added to the culture in parallel with $_s$fgl2 protein as controls as previously described (20).

In some experiments, a mAb against the "domain 2" (FRED-containing C-terminal region) of fgl2 (1 μg) was added at the beginning of culture along with $_s$fgl2 protein. An isotype control Ab was added for comparison.

Allogeneic MLR

DC (1×10^4) obtained from the BM of A/J mice were first stimulated with LPS as described above, irradiated, and then mixed with responder BALB/c lymph node T cells (2×10^5) in 96-well U-bottom microtiter plates for 48 h. Purified $_s$fgl2 protein (1 μg/ml or 1 ng/ml) was added at the beginning of cultures. Proliferation was measured by pulsing after 2 days of culture with [^{3}H]thymidine (1 μCi/well) for 18 h as described above. In cultures used to assess cytokine production, supernatants were pooled from triplicate wells at 40 h. Levels of IL-2, IL-4, IFN-γ, and IL-10 were assayed using ELISA kits (Pierce) according to the manufacturer's instructions. Where CTL induction was assayed, cultures were allowed to continue for 5 days (in the presence/absence of $_s$fgl2), before cells were harvested. These effector cells were assayed in standard 4-h ^{51}Cr release assays at various E:T ratios with ^{51}Cr-labeled 72-h Con A-activated A/J blast target cells, as described elsewhere (21). Data were expressed as a percent specific lysis at 50:1 E:T.

The effect of $_s$fgl2 on the LPS-induced maturation of BM-derived DC was examined by adding $_s$fgl2 protein (1 μg/ml) to DC cultures during LPS-induced maturation. The treated DC cultures were then washed and examined for their ability to stimulate alloreactive T cell proliferation as described above. The expression of surface molecules, including CD40, CD80, CD86, CD11c, MHC class I, and class II molecules, were measured by flow cytometric analysis. In other experiments, lymph node T cells were exposed to $_s$fgl2 protein (1 μg/ml) for 12 h, washed, and then cultured with allogeneic DC. Proliferation was measured by pulsing after 2 days of culture with [^{3}H]thymidine (1 μCi/well) for 18 h as described above.

Flow cytometric analysis

To examine the binding of biotinylated $_s$fgl2 to peripheral T cells or BM-derived DC, cells were washed twice with PBS, blocked with 10% v/v normal mouse serum for 5 min at room temperature, and then incubated with biotinylated $_s$fgl2 in PBS at 4°C for 30 min. Cells were washed extensively, stained with streptavidin-PE (BD PharMingen) at 4°C for 30 min, and then analyzed on Coulter Epics-XL-MCL flow cytometer (Beckman Coulter, Hialeah, FL) using XL software. Binding of biotinylated $_s$fgl2 on T cells and DC was analyzed on CD3- and CD11c-postive cells, respectively. Cells incubated with biotinylated BSA and then streptavidin-PE were used as negative controls.

For characterization of the prepared DC population, 2×10^5 cells were first blocked with 10% v/v normal mouse serum for 5 min at room temperature and thereafter stained with the corresponding FITC- or PE-conjugated mAb in PBS with 1% BSA at 4°C for 30 min. Cells stained with the appropriate isotype-matched Ig were used as negative controls. Cells were analyzed on a Coulter Epics-XL-MCL flow cytometer for expression of various DC markers.

To assess cell cycle and apoptosis, cells treated with $_s$fgl2 for 12 h were washed in cold PBS, resuspended in lysis buffer (0.1% sodium citrate/Triton X-100) containing 100 U/ml RNase A (Sigma-Aldrich), and stained with propidium iodide (1 mg/ml PBS) in the dark for 20 min at room temperature. The cells were then washed twice and ~10,000 data events per sample were analyzed. Gates were set, by using the untreated sample, to differentiate between G_0-G_1 (left-hand peak), S-phase (intermediate), and G_2-M (right-hand peak). Apoptotic cells appeared to the left of the G_0-G_1 phase.

Immunofluorescence microscopy

The effect of $_s$fgl2 on LPS-induced NF-κB translocation was examined as previously described (22). Immature DC were harvested on day 7 and were allowed to adhere to autoclaved glass coverslips for 6 h at 37°C in 5% CO_2 and incubated in complete medium supplemented 10% FBS, GM-CSF, and rmIL-4 as described above. To examine the effects that $_s$fgl2 had on LPS-induced NF-κB translocation, $_s$fgl2 (1 μg/ml) was added to the DC cultures during LPS (1 μg/ml) stimulation. NF-κB translocation was examined at 5, 15, 30, 60, 120, and 240 min. Cells were fixed for 30 min in PBS supplemented with 2% paraformaldehyde. The coverslips were washed three times with PBS for 10 min each, permeabilized with 0.2% Triton X-100 in PBS for 5 min, and then blocked with 5% BSA in PBS for 30 min at room temperature. The samples were stained with a goat anti-p65 polyclonal Ab (1/50 dilution in PBS; Molecular Probes, Eugene, OR) for 1 h at room temperature, washed three times with PBS for 5 min each, and incubated with fluorescently labeled Alexa 555 donkey anti-goat IgG secondary Ab (1/400 dilution in PBS; Molecular Probes) for 1 h at room temperature. The coverslips were washed three times with PBS for 5 min each and mounted on glass slides using mounting solution (DAKO from Dakocytomation, Carpinteria, CA). Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) chromosomal staining (Molecular Probes). The staining was visualized using a Nikon TE200 fluorescence microscope (×100 objective; Nikon, Melville, NY) coupled to an Orca 100 camera driven by Simple PCI (Richmond Hill, Ontario, Canada) software as previously described (22).

Statistical analysis

The results were calculated as means ± SEM. For statistical comparison, the means were compared using the ANOVA by Student's *t* test utilizing Statistix 7 software (Analytical Software, Tallahassee, FL). A $p \leq 0.05$ was considered to be statistically significant.

Results

Generation of $_s$fgl2 protein in a baculovirus expression system

To characterize the immunomodulatory property of $_s$fgl2, fgl2 protein was generated using a baculovirus expression system and purified as described in *Materials and Methods*. SDS-PAGE followed by Coomassie blue staining of the purified $_s$fgl2 showed a dominant band at 65 kDa, comparable to the size of the fgl2 protein previously reported (15, 16) (Fig. 1*a*). Purified $_s$fgl2 was confirmed by Western blotting using polyclonal rabbit anti-mouse fgl2 IgG (Fig. 1*b*). The purified $_s$fgl2 protein was analyzed for its ability to induce clotting and no coagulation activity was detected (data not shown).

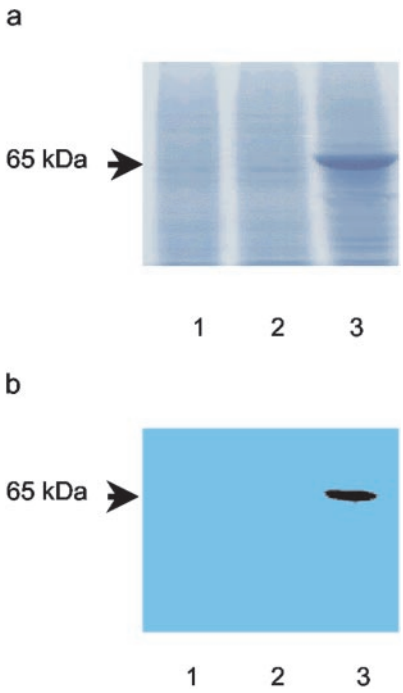

FIGURE 1. Generation of $_s$fgl2 in a baculovirus system. *a*, SDS-PAGE followed by Coomassie blue staining of fgl2 protein purified from recombinant baculovirus-infected H5 cells showed a dominant band at 65 kDa confirmed by Western blotting (*b*). fgl2 was generated using a baculovirus expression system and purified as described in *Materials and Methods*. Cell lysates prepared under denaturing conditions were incubated with Ni-NTA resin and proteins bound to Ni-NTA resin were eluted. All eluted proteins (10 μg) were analyzed on 10% SDS-PAGE followed by Coomassie blue staining (*a*) or Western blot analysis (*b*) probed with polyclonal rabbit anti-mouse fgl2 IgG as described in *Materials and Methods*. *Lane 1*, Eluted proteins from lysate of the uninfected H5 cells; *lane 2*, eluted proteins from lysate of the wild-type baculovirus-infected H5 cells; *lane 3*, eluted proteins from lysate of the recombinant baculovirus-infected H5 cells.

$_s$Fgl2 binding to T cells and DC

The binding of purified $_s$fgl2 to T cells and DC was examined using flow cytometry analysis. Fig. 2 shows that biotinylated $_s$fgl2 bound to both T cells and DC. The specific binding of biotinylated $_s$fgl2 to both cells was inhibited by nonbiotinylated $_s$fgl2 but not by fibrinogen (data not shown).

$_s$Fgl2 inhibited T cell proliferation stimulated by various stimuli

To examine the consequence of the binding of $_s$fgl2 to T cells, purified $_s$fgl2 protein was initially assessed for its capacity to inhibit T cell proliferation. Fig. 3 shows that $_s$fgl2 inhibited allogeneic T cell activation in a dose-dependent manner. At the highest concentration of $_s$fgl2 (1 μg/ml) used in the cultures, 61 ± 11% inhibition of T cell proliferation was observed. Purified $_s$fgl2 similarly inhibited T cell proliferation induced by immobilized anti-CD3 mAb with soluble anti-CD28 mAb and by Con A (Fig. 3, *b* and c) in a dose-dependent fashion. H5 supernatants, wild-type baculovirus-infected H5 supernatants, human fibrinogen (1 μg/ml; Fig. 3) nor BSA (1 μg/ml) (data not shown) had any effect on T cell proliferation stimulated by alloantigen, anti-CD3/CD28 or, Con A.

$_s$Fgl2 inhibited the allogeneic response at early time points and the effect could be neutralized by mAb

To further explore the suppressive effect of $_s$fgl2 on alloreactive T cell proliferation, $_s$fgl2 was added to allogeneic cultures at different

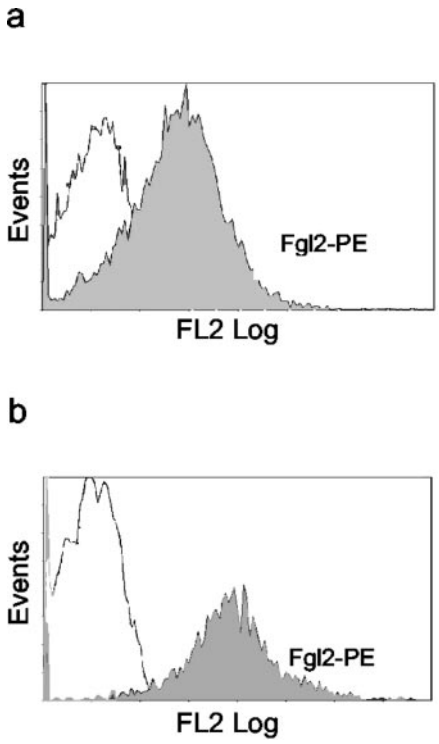

FIGURE 2. Binding of biotinylated $_s$fgl2 to T cells and BM-derived LPS-induced mature DC. T cells and BM-derived LPS-induced mature DC cells were prepared. CD3- and CD11c-positive populations were gated and the binding of $_s$fgl2 to T cells (*a*) and DC (*b*) was determined as described in *Materials and Methods* (gray area). The white area shows the binding of biotinylated BSA control.

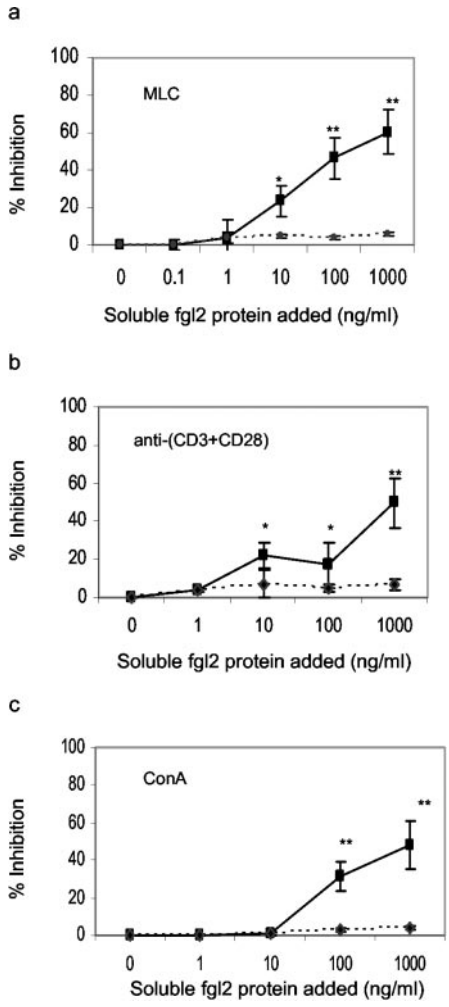

FIGURE 3. Inhibitory effects of $_s$fgl2 on T cell proliferation induced by different stimuli. Effects of $_s$fgl2 (solid line) and human fibrinogen (dotted line) on T cell proliferation induced by alloantigens (*a*), immobilized anti-CD3 mAb (1 μg/ml, *b*) with soluble anti-CD28 mAb (20 ng/ml), and Con A (5 μg/ml, *c*) with various concentrations of $_s$fgl2 or human fibrinogen added at the initiation of cultures as described in *Materials and Methods*. Proliferation was determined by [^{3}H]thymidine uptake in triplicate wells and the incorporation in $_s$fgl2-untreated control cultures (shown as 0 ng/ml soluble fgl2 added) was as follows: *a*, 14,892 $\pm$ 978 cpm; *b*, 16,890 $\pm$ 1,146 cpm; and *c*, 39,368 $\pm$ 3,560 cpm, with background 595 $\pm$ 193 cpm. The data are expressed as percentage inhibition ($_s$fgl2-untreated cultures shown as 0% inhibition) and are representative of three separate experiments. **, $p < 0.01$ and *, $p < 0.05$ compared with control groups (*far left*, no $_s$fgl2 was added).

time points (i.e., day 0, 1, 2, or 3) and mixed by pipetting to ensure proper distribution of protein to the cultures. Cell proliferation was measured as previously described. Cultures without the addition of $_s$fgl2 were also mixed by pipetting at corresponding time points as controls. Fig. 4*a* shows that $_s$fgl2 exhibited maximal inhibitory effect (61 $\pm$ 11% inhibition) when it was added at the initiation of allogeneic reactions (day 0). Less inhibitory effects were observed when $_s$fgl2 was added on day 1 (39 $\pm$ 15% inhibition) with loss of inhibition when addition of $_s$fgl2 was delayed until day 2.

The ability of a mAb against the domain 2 (FRED-containing C-terminal region) of mouse fgl2 to neutralize the inhibitory effect of $_s$fgl2 on alloreactive T cell proliferation was next examined. As shown in Fig. 4*b*, a mouse mAb (1 μg/ml) abrogated the ability of $_s$fgl2 to suppress alloreactive T cell proliferation, suggesting that the effect of $_s$fgl2 protein was specific and could be prevented by this Ab. In contrast, a rabbit polyclonal Ab against the "domain 1" of fgl2 which neutralizes the coagulation activity of fgl2 failed to inhibit the immunosuppressive activity of $_s$fgl2.

$_s$Fgl2 promoted a Th2 cytokine profile in allogeneic responses

To characterize further the effect of $_s$fgl2 on allogeneic responses, we cultured T cells with irradiated allogeneic BM-derived LPS-induced mature DC in the presence of $_s$fgl2. A similar dose-specific $_s$fgl2 suppressive effect on alloreactive T cell proliferation was observed as that seen in Fig. 3. Fig. 5*a* shows that 1 μg/ml $_s$fgl2 resulted in a maximal 68 $\pm$ 14% inhibition of T cell proliferation. Supernatants collected from these cultures (1 μg/ml $_s$fgl2) showed decreased levels of IL-2 and IFN-γ, no effect on levels of IL-12, and increased levels of IL-4 and IL-10 production in comparison to supernatants from $_s$fgl2-untreated allogeneic cultures (Fig. 5, *b* and *c*). The possibility that this alteration in cytokine response was due to direct toxicity was further examined. sfgl2 did not cause nonspecific changes in cell survival of stimulated or unstimulated cells as discussed further below. In addition, we did not observe any inhibition of CTL induction in the presence of $_s$fgl2. Thus, percent lysis at 5 days in control cultures (no fgl2) at 50:1 E:T ratio was 30 $\pm$ 5%, whereas in fgl2-treated cultures lysis was 31 $\pm$ 3% ($p = 0.74$). The addition of $_s$fgl2 to the allogeneic cultures at a concentration of 1 ng/ml had no inhibitory effect on T cell proliferation, and resulted in a promotion of Th1 cytokine expression similar to that observed in $_s$fgl2-untreated cultures (Fig. 5, *b* and *c*).

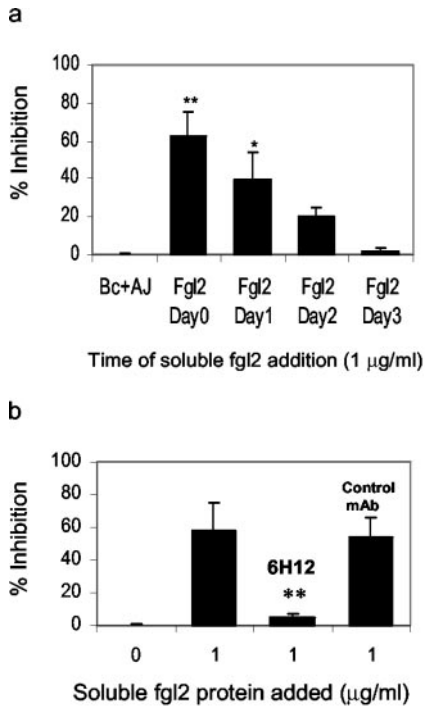

FIGURE 4. The inhibitory effect of $_s$fgl2 on alloreactive T cell proliferation. *a*, Kinetics of the effect of $_s$fgl2 on alloreactive T cell proliferation. $_s$Fgl2 (1 μg/ml) was added on days 0–3 to an ongoing allogeneic reaction as described in *Materials and Methods*. Proliferation was determined by [^{3}H]thymidine uptake in triplicate wells and the incorporation in $_s$fgl2-untreated control cultures (shown as Bc + AJ) was: 14,892 ± 978 cpm with background 523 ± 102 cpm. The data are expressed as percentage inhibition ($_s$fgl2-untreated cultures shown as 0% inhibition) and are representative of three separate experiments. **, $p < 0.01$ and *, $p < 0.05$ compared with control groups for both percent suppression and cpm (*far left*, no $_s$fgl2 was added). *b*, The inhibitory effect of $_s$fgl2 on alloreactive T cell proliferation was neutralized by an anti-mouse fgl2 mAb. One-way MLC was set up as described previously and a monoclonal anti-mouse fgl2 Ab (1 μg) was added at the beginning of cultures along with the addition of $_s$fgl2 protein (1 μg/ml). An isotype control mAb was added for comparison. **, $p < 0.01$ compared with control groups (*second left*, no anti-mouse fgl2 Ab was added).

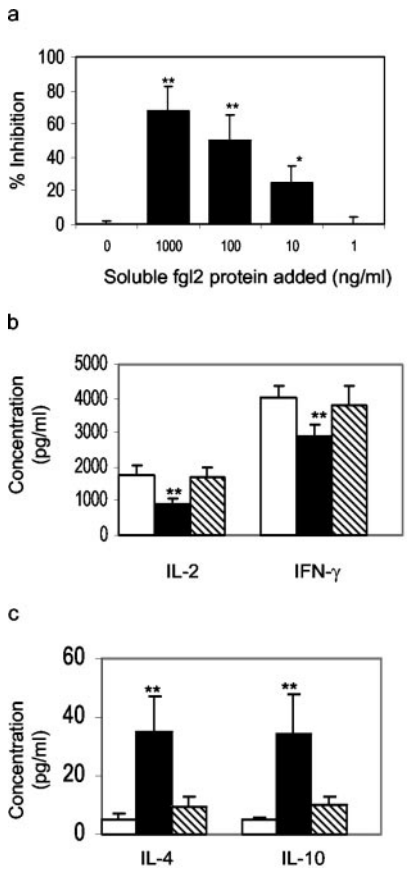

FIGURE 5. $_s$fgl2 (1 μg/ml) inhibited MLR with promotion of a Th2 cytokine profile. *a*, Lymph node T cells were cocultured with allogeneic BM-derived DC as described in *Materials and Methods*. $_s$fgl2 (1 ng/ml to 1 μg/ml) was added to the culture at the beginning (day 0) and cell proliferation was measured using [^{3}H]thymidine as described. The incorporation of [^{3}H] thymidine in $_s$fgl2-untreated control cultures (shown as 0 ng/ml $_s$fgl2 protein added) was: 5546 ± 620 cpm with background 355 ± 55 cpm. The data are expressed as percentage inhibition (fgl2-untreated cultures shown as 0% inhibition). *b* and *c*, Supernatants from the allogeneic cultures in the absence (□) or presence of 1 μg/ml $_s$fgl2 protein (■) or 1 ng/ml $_s$fgl2 protein (▨) were collected at 24 h to measure the levels of cytokine produced. The data are representative of three separate experiments. **, $p < 0.01$ and *, $p < 0.05$ compared with control groups (*far left*, no $_s$fgl2 was added).

$_s$Fgl2 did not suppress T cell proliferation via induction of apoptosis

Others have reported that certain immunosuppressive agents suppress T cell proliferation by inducing T cell apoptosis (23). Therefore, T cell viability was examined by both trypan blue dye exclusion and propidium iodide staining of lymphocyte nuclei after a 12-h exposure to $_s$fgl2 protein. At all concentrations of $_s$fgl2 tested in this study, no significant differences in cell number and amount of apoptotic cells were detected between the $_s$fgl2-treated T cells and untreated T cells (data not shown), suggesting that the inhibitory effects of $_s$fgl2 were not an outcome of a nonspecific or cytotoxic effect.

$_s$Fgl2 led to reduced expression of CD80^{high} and MHC class II^{high} molecules by BM-derived DC

We next examined whether $_s$fgl2 had the ability to impair the maturation of BM-derived DC. To test this, immature DC were generated by culturing BM cells with GM-CSF and IL-4 for 7 days. Following the addition of LPS, in the presence or absence of $_s$fgl2 (1 μg/ml), the phenotype of these DC were examined by staining cells with various mAbs followed by flow cytometry analysis. As shown in Fig. 6*a*, CD11c$^+$ cells composed the majority of both $_s$fgl2-treated and -nontreated cells, suggesting that the addition of $_s$fgl2 did not reduce the number or viability of LPS-treated DC. Furthermore, the expression of MHC class I and CD86 was not altered by $_s$fgl2 (Fig. 6, *b* and *c*). A minor change in CD40 expression was observed (Fig. 6*f*). However, incubation of DC with $_s$fgl2 during LPS-induced maturation significantly reduced expression of both CD80^{high} (Fig. 6*d*) MHC class II^{high} (Fig. 6*e*) and CD80^{high} (Fig. 6*d*) expression on DC. These findings suggest that $_s$fgl2 inhibits LPS-induced DC maturation.

Addition of $_s$fgl2 during DC maturation abolished their ability to induce allogeneic responses

To further examine the effect of $_s$fgl2 on DC maturation, we determined the ability of $_s$fgl2-treated DC to stimulate allogeneic responses. Fig. 7 shows that DC treated with $_s$fgl2 (1 μg/ml) during the LPS-induced maturation had an impaired ability to stimulate naive allogeneic T cell proliferation in comparison to $_s$fgl2-untreated DC. When naive T cells were pretreated with $_s$fgl2 (1 μg/ml) for 12 h, washed, and then cultured with allogeneic LPS-induced mature DC, no inhibitory effect on T cell proliferation was

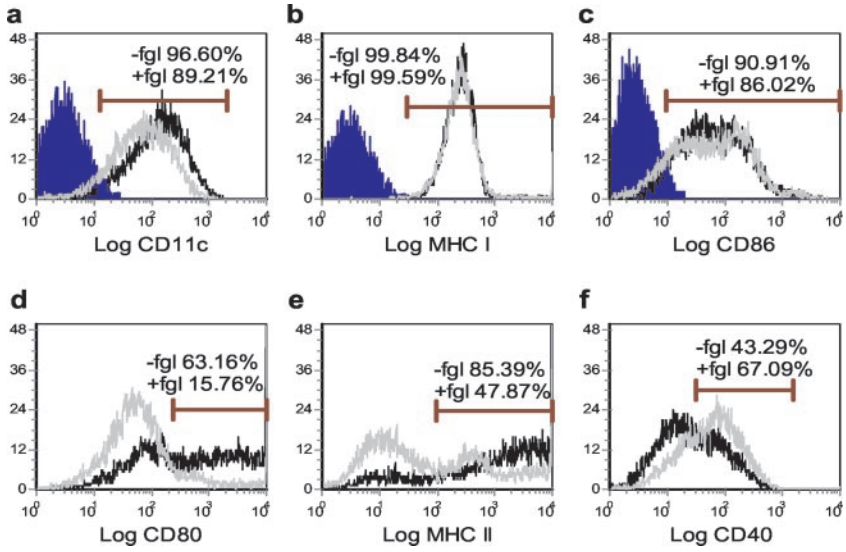

FIGURE 6. Cell surface phenotype of DC generated in the absence (control) or presence of $_s$fgl2 (1 μg/ml) during maturation. BM cells were prepared and cultured for 7 days in the presence of GM-CSF and IL-4 to derive immature DC as described in *Materials and Methods*. Immature DCs were stimulated with LPS (200 ng/ml) to reach final maturation for 2 days in the absence and presence of $_s$fgl2. The expression of surface molecules including CD11c, CD80, CD86, MHC class I and class II, and CD40 were measured by flow cytometry analysis. Results are representative of three independent experiments. Gray lines show fluorescence signals of cells treated with $_s$fgl2, and stained with the specific Abs. Black lines represent non-$_s$fgl2-treated cells. The blue shaded areas are the appropriate isotype-matched Ig control.

observed. This was compared with the levels of proliferation observed in cultures containing untreated control T cells stimulated with allogeneic LPS-induced mature DC. Maximal abrogation on alloreactive T cell proliferation was resulted when naive T cells were stimulated with $_s$fgl2-pre-exposed DC in the presence of $_s$fgl2 (1 μg/ml).

To examine whether $_s$fgl2 prevents the maturation of BM-derived DC through the NF-κB pathway, BM-derived DC were stimulated with LPS (1 μg/ml) following 7 days of incubation in GM-CSF and rmIL-4 in the presence or absence of $_s$fgl2 (1 μg/ml), which was added at the same time of LPS exposure. The DC were examined at 5, 15, 30, 60, 120, and 240 min for NF-κB translocation by immunofluorescence microscopy. To clearly determine whether there was nuclear translocation, dual staining with a primary Ab to the p65 subunit of NF-κB and DAPI nuclear staining was used. Translocation of NF-κB occurred at all times examined, but was maximal after 1 h of LPS stimulation. NF-κB translocation was significantly reduced by the presence of $_s$fgl2 at all time points examined (Fig. 8).

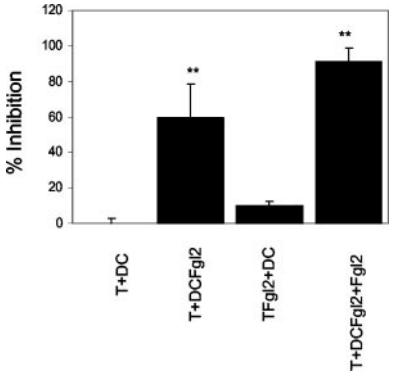

FIGURE 7. DC treated with $_s$fgl2 during LPS-induced maturation exhibited an impaired ability to induce allogeneic responses. DC treated with $_s$fgl2 (1 μg/ml) during LPS-induced maturation (DCFgl2) and T cells treated with $_s$fgl2 (1 μg/ml) prior to culturing with DC (T + Fgl2) were used to examine their ability to induce allogeneic response as described in *Materials and Methods.* In some cases, DC treated with $_s$fgl2 during maturation (DCFgl2) were used to activate allogeneic T cell proliferation with $_s$fgl2 (1 μg/ml) added to the culture (*last column*). Proliferation was determined by [^{3}H]thymidine uptake in triplicate wells and the uptake in untreated cultures (shown as T + DC) was: 5546 $\pm$ 620 cpm with background 355 $\pm$ 55 cpm. The data are representative of three separate experiments. **, $p < 0.01$ and *, $p < 0.05$ compared with control groups (*second left*, no $_s$fgl2 was added).

Discussion

Our group has had a long interest in defining the regulation of induction and mechanism(s) of action of fgl2/fibroleukin, a novel protein that is expressed by both reticuloendothelial cells (macrophages and endothelial cells) and T cells. fgl2 is a 432-aa protein that shares homology to the β- and γ-chains of fibrinogen with a FRED at the carboxyl terminus (aa 202–432). When fgl2 is expressed as a membrane-associated protein in activated macrophages and endothelial cells, it exhibits a coagulation activity capable of directly cleaving prothrombin to thrombin. The membrane-associated fgl2 prothrombinase with the ability to directly generate thrombin plays an important role in innate immunity.

A protein belonging to the fibrinogen-like superfamily has been shown to exhibit immunomodulatory property. Tenascin, which shares a 40% homology to the FRED region of fgl2, blocks T cell activation induced by a soluble Ag, alloantigens, or Con A. The mechanism by which tenascin blocks T cell activation remains undefined. Recently, a soluble form of fgl2/fibroleukin ($_s$fgl2) has been described, and of particular interest to the current study is the discovery that T cells are known to express $_s$fgl2. Nevertheless, the function(s) of $_s$fgl2 remains unexplored. Recent studies from our laboratory and others have provided insights into the function(s) of $_s$fgl2. Preliminary results from our laboratory using fgl2 knockout mice suggest that T cells from fgl2$^{-/-}$ mice are hyperproliferative (data not shown). A recent report from Kohno et al. (19) also suggests that $_s$fgl2 may play a role in the acquired immune response.

To test this hypothesis, we examined the role of $_s$fgl2 in regulating the function of APC, in particular, DC. BM-derived DC were prepared and the effect of $_s$fgl2 on LPS-induced maturation was examined. We found that $_s$fgl2 prevented the maturation of BM-derived DC by inhibiting the expression of CD80^{high} and MHC class II^{high} molecules, while having no significant effects on MHC class I, CD11c, and CD86 expression. These data are consistent with the observation that $_s$fgl2-treated DC had a markedly reduced capacity to stimulate T cell proliferation in an allogeneic MLR and to inhibit a Th1 cytokine response. Interestingly, further abrogation on alloreactive T cell proliferation was achieved when naive T cells were stimulated with $_s$fgl2-pre-exposed DC in the presence of $_s$fgl2 protein (1 μg/ml), suggesting that $_s$fgl2 exerts an

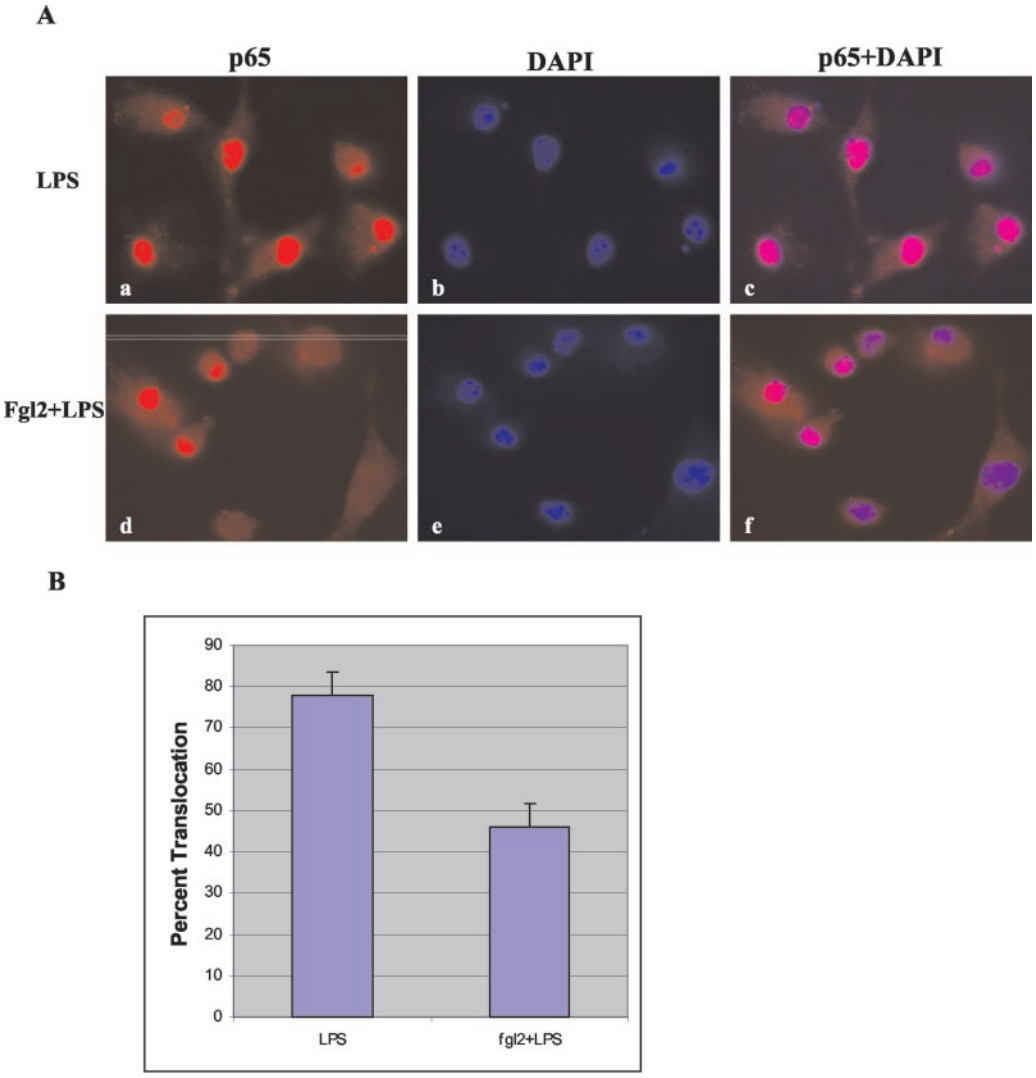

FIGURE 8. Studies of the effect of sFgl2 on translocation of NF-κB in LPS-stimulated DC. *A*, Dual immunofluorescence staining of LPS and $_s$fgl2 plus LPS-treated BM-derived DC. After 1 h of exposure to LPS or $_s$fgl2 plus LPS immunostaining was conducted as described in *Materials and Methods*. *a–f*, The same cells visualized by immunofluorescence using different filters. Cells in *a* and *d* show red fluorescence anti-p65 staining. Cells in *b* and *e* show blue chromosomal DAPI staining. *c* and *f*, An overlay of the anti-p65 and DAPI staining. The purple color represents cells that have p65 translocation. LPS-exposed cells had marked NF-κB translocation shown by the accumulation of p65 in the nucleus, confirmed by DAPI nuclear staining (*c*). Incubation with $_s$fgl2 during LPS exposure was able to inhibit NF-κB translocation shown by diffuse p65 staining and distinct blue of the DAPI staining in most of cells (*f*). *B*, Histogram showing the percent p65 NF-κB translocation in at $t = 1$ h after LPS stimulation in sfgl2-treated and -untreated groups. An average of 100 cells was counted from more than three different fields. There is a significant reduction in p65 NF-κB translocation with $_s$fgl2 treatment ($p < 0.01$).

immunosuppressive effect on T cells in addition to its effect on DC maturation.

We have, in addition, explored evidence for a more direct effect of $_s$fgl2 on T cells by examining the effect of $_s$fgl2 on T cells by exploring its effect on T cell proliferation stimulated under different stimuli. In this study, we showed that $_s$fgl2 inhibited T cell proliferation induced by alloantigen, anti-CD3/CD28, or Con A. Although fgl2 shares a 36% homology to the β- and γ-chains of fibrinogen within the FRED, fibrinogen did not exhibit an immunosuppressive effect on T cell proliferation. This suggests the specificity of the immunosuppressive effect of $_s$fgl2 on T cell proliferation.

The hypothesis that $_s$fgl2 has a direct influence on T cells is supported by our findings that $_s$fgl2 suppresses T cell proliferation induced by anti-CD3/CD28 mAbs and Con A. $_s$Fgl2 may also act directly on APC to inhibit T cell proliferation in MLC. The concept of a direct action of $_s$fgl2 on T cells and APC is supported by our observations using flow cytometry analysis, which showed that $_s$fgl2 binds to DC and T cells, suggesting that the inhibitory effect of $_s$fgl2 may be through surface receptor(s). Although not the focus of this article, our finding suggests the existence of receptor(s) on DC and T cells for $_s$fgl2. Binding of $_s$fgl2 to macrophages was observed and was dependent on divalent cations (data not shown). Different affinities for $_s$fgl2 binding on macrophages, DC, and T cells were detected (preliminary observation). However, whether $_s$fgl2 exhibits its immunomodulatory properties through binding to one or different surface receptors is unclear.

The immunosuppressive effects of $_s$fgl2 on alloreactive T cell proliferation was neutralized by a mAb having no inhibitory effect on the coagulation activity of fgl2, a function which is known to reside in domain 1 of the molecule, a region distinct from domain 2 which is the FRED-containing C-terminal region. Similarly, a polyclonal Ab which possesses the ability to neutralize the fgl2 prothrombinase activity and which interacts with domain 1 of the fgl2 molecule had no inhibitory effect on the immunosuppressive

activity of $_s$fgl2. Taken together, we postulate that distinct domains of fgl2 are responsible for the prothrombinase and immunomodulatory activities of the molecule. Studies are now ongoing to define in more detail the region(s) responsible for the immunomodulatory activity.

DC themselves are professional APC which exhibit an ability to stimulate both naive and memory T lymphocytes following their maturation (24, 25). The DC maturation process involves increased expression of surface MHC class II and costimulatory molecules and occurs in vivo as DC pass from the periphery to T cell areas of secondary lymphoid tissue. BM-derived DC deficient in costimulatory molecules can induce T cells to undergo a state of hyporesponsiveness, leading to prolongation of islet and cardiac allograft survival (25, 26) and inhibition of autoimmune disease progression in a variety of animal models (27). The proposed immunosuppressive effect of $_s$fgl2 on DC maturation is further supported by the findings from hepatitis C patients with increased expression of fgl2 (28). In these patients, DC express lower levels of CD86 and an impaired ability to stimulate allogeneic T cells for IFN-γ production (29). Similarly, DC from hepatitis B patients with hepatocellular carcinoma and increased expression of fgl2 (28) showed a reduced stimulatory activity (30).

The mechanism(s) by which $_s$fgl2 alters the expression of CD80 and MHC class II was examined in the present studies. Nuclear translocation of members of the NF-κB family, particularly RelB, have been shown to be required for myeloid DC maturation (27, 31, 32). By immunofluorescence microscopy, it was shown that $_s$fgl2 markedly inhibits NF-κB translocation, which may account for lack of maturation of DC as indicated by lack of expression of CD80^{high} and MHC class II. The fact that not all DC were inhibited by $_s$fgl2 may reflect dosage requirements as well as the fact that the population of DC are not homogeneous.

In the present studies, $_s$fgl2 was shown to promote a Th2 cytokine profile (IL-4 and IL-10) during the initiation of the allogeneic response. Cytokines produced by Th2 cells have been shown to exhibit anti-inflammatory activities by regulating the development and activity of Th1 cells, which are in general associated with the development of autoimmunity, delayed-type hypersensitivity and cell-mediated immune responses (33–35). Both IL-4 and IL-10 have been shown to antagonize the development of Th1 cells, likely through decreasing expression/function of the cytokine IL-12, while promoting the differentiation of Th2 cells. In human and animal studies, polarization toward type 2 cytokine production has been associated with improved survival of allogeneic transplants (21, 35). Whether soluble fgl2 would affect graft survival in transplantation remains to be examined. Note in this report that $_s$fgl2 had no effect on CTL activity in an allo-MLC.

The actual mechanism(s) by which $_s$fgl2 promotes a Th2 cytokine differentiation is not known. However, it is known that the differentiation of naive CD4$^+$ T cells into different populations of cytokine-secreting effector cells is influenced not only by the cytokine milieu in which differentiation takes place, but by a variety of accessory molecule interactions. The interaction of CD28 with CD86 (36, 37), CD4 with MHC class II (38, 39), and OX-40 with the OX-40 ligand (40) have all been suggested to promote Th2 differentiation at the expense of Th1 differentiation, whereas CD28 interaction with CD80 on APCs such as DC have been proposed to produce a Th1 response. Thus, preservation of CD86 and loss of CD80 and MHC class II may explain the preferential bias toward the Th2 cytokine production observed.

In summary, we have reported that while membrane-bound fgl2 acts as a prothrombinase, sfgl2 is an immunomodulatory protein that has the ability to modulate T cell responses and, perhaps more importantly, to alter DC maturation to favor production of tolerogenic DC. Currently, the use of nonspecific immunosuppressive drugs to treat transplant rejection and autoimmune diseases is fraught with complications caused by drug toxicity and other adverse (immunologically) nonspecific side effects. The inhibition of CD80 interaction with CD28 has been shown to have significant immunosuppressive effects including (but not limited to) the reduction of specific Ab production, prolongation of the survival of organ transplants, and the inhibition of autoimmune diabetes and lupus. Thus, the direct immunosuppressive activity of $_s$fgl2 on T cells and its ability to prevent the expression of costimulatory molecules on LPS-stimulated DC would allow potential strategy in treating autoimmune disorders and transplant rejection.

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