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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

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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. 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 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 fgl2-treated allogeneic cultures. Exposure of immature DC to fgl2 abrogated the expression of CD80high and MHC class IIhigh molecules and markedly inhibited NF-κB nuclear translocation, thus inhibiting their maturation. 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 fgl2-treated DC and fgl2 protein was added in the culture. These data provide the first evidence to demonstrate that fgl2 exerts immunosuppressive effects on T cell proliferation and DC maturation. The Journal of Immunology, 2003, 170: 4036–4044.

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, natural text content ...
(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 fgl2, suggesting a potential strategy of developing 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-2b) and AJ (H-2h) mice were purchased from Charles River Breeding Laboratory (Wilmington, MA) and The Jackson Laboratory (Bar Harbor, ME), respectively. All mice were chow-fed 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 (GL4), CD40 (3/23) and CD11c (HL3), MHC class I (I-EK), and MHC class II (I-A^q) 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 fgl2

Mouse fgl2 with a tandem repeat of six histidine residues followed by an enterokinase cleavage site fused to its N terminus was expressed in an Insectra (San Diego, CA) Insect Expression System (20). Briefly, a 1.4-kb cDNA encoding mouse fgl2 was amplified using the forward primer 5'-TGCGGCACTGGATCCATGAGGCTTCCTGGT-3' (with the methionine start codon underlined) and the reverse primer 5'-TTATGGCTTGAATTCATTGGCGGC-3' (nt 1283-1303 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 EcoRI and BamHI 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 Pansol, 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 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 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 Lymphocyte-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-2b) 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) AJ splenic mononuclear cells (4 × 10^5 cells/100 μl) with or without fgl2 protein (ranging from 1 μg/ml to 1 ng/ml) added to the culture.

Response to Con A.

Purified T cells (2 × 10^4 cells/200 μl) were stimulated with Con A (5 μg/ml; Sigma-Aldrich) in the presence or absence of fgl2 protein (ranging from 1 μg/ml to 1 ng/ml). After 3 days, cells were pulsed with [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 × 10^4 cells/200 μl) were stimulated with immobilized anti-CD3 mAb (1 μg/ml) and soluble anti-CD28 mAb (20 μg/ml) added to the culture. [H]^thymidine (1 μCi/well) was added at the beginning of culture along with fgl2 protein. An isotype control Ab was added for comparison.

Allogenic MLR

DC (1 × 10^5) obtained from the BM of AJ mice were first stimulated with LPS as described above, irradiated, and then mixed with responder BALB/c lymph node T cells (2 × 10^3) in 96-well U-bottom microtiter plates for 48 h. Purified 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 [H]^thymidine (1 μCi/well) for 18 h as described above. In cultures used to assay cytotoxic 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 fgl2), before cells were harvested. These effector cell populations were then washed and assayed by 51Cr release assays at various E:T ratios with 51Cr-labeled 72-h Con A-activated AJ blast target cells, as described elsewhere (21). Data were expressed as a percent specific lysis at 50:1 E:T.

The effect of fgl2 on the LPS-induced maturation of BM-derived DC was examined by adding 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 fgl2 protein (1 μg/ml) for 12 h, washed, and then cultured with alloegenic DC. Proliferation was measured by pulsing after 2 days of culture with [H]^thymidine (1 μCi/well) for 18 h as described above.
Flow cytometric analysis

To examine the binding of biotinylated \( \text{fgl2} \) 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 \( \text{fgl2} \) 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 a Coulter Epics-XL-MCL flow cytometer (Beckman Coulter, Hialeah, FL) using XL software. Binding of biotinylated \( \text{fgl2} \) on T cells and DC was analyzed on CD3- and CD11c-positive cells, respectively. Cells incubated with biotinylated BSA and then streptavidin-PE were used as negative controls.

For characterization of the prepared DC population, 2 \( \times 10^6 \) 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 \( \text{fgl2} \) 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 \( \text{fgl2} \) on LPS-induced NF-\( \kappa \)B 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 rIL-4 as described (22). To examine the effects that \( \text{fgl2} \) had on LPS-induced NF-\( \kappa \)B translocation, \( \text{fgl2} \) (1 \( \mu \)g/ml) was added to the DC cultures during LPS (1 \( \mu \)g/ml) stimulation. NF-\( \kappa \)B 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 cells were then washed twice and cultured at 4°C for 30 min. Cells stained with propidium iodide were then washed three times with PBS for 5 min each, and incubated with fluorescently labeled Alexa 555 donkey anti-goat IgG secondary Ab in PBS (Molecular Probes, Eugene, OR) for 1 h at room temperature. 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 TE2000 fluorescence microscope (\( \times 100 \) 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 \( \pm \) 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 \( \text{fgl2} \) protein in a baculovirus expression system

To characterize the immunomodulatory property of \( \text{fgl2} \), \( \text{fgl2} \) 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 \( \text{fgl2} \) showed a dominant band at 65 kDa, comparable to the size of the \( \text{fgl2} \) protein previously reported (15, 16) (Fig. 1a). Purified \( \text{fgl2} \) was confirmed by Western blotting using polyclonal rabbit anti-mouse \( \text{fgl2} \) IgG (Fig. 1b). The purified \( \text{fgl2} \) protein was analyzed for its ability to induce clotting and no coagulation activity was detected (data not shown).

\hspace{-1.5cm} \text{FIGURE 1. Generation of \( \text{fgl2} \) in a baculovirus system.} \hspace{1cm}
\begin{figure}[h]
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\caption{Generation of \( \text{fgl2} \) in a baculovirus system. a. SDS-PAGE followed by Coomassie blue staining of \( \text{fgl2} \) protein purified from recombinant baculovirus-infected H5 cells showed a dominant band at 65 kDa confirmed by Western blotting (b). \( \text{fgl2} \) 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 \( \mu \)g) were analyzed on 10% SDS-PAGE followed by Coomassie blue staining (a) or Western blot analysis (b) probed with polyclonal rabbit anti-mouse \( \text{fgl2} \) 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.}
\end{figure}

\( \text{fgl2} \) binding to T cells and DC

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

\( \text{fgl2} \) inhibited \text{T cell proliferation stimulated by various stimuli}

To examine the consequence of the binding of \( \text{fgl2} \) to T cells, purified \( \text{fgl2} \) protein was initially assessed for its capacity to inhibit T cell proliferation. Fig. 3 shows that \( \text{fgl2} \) inhibited alloageneic T cell activation in a dose-dependent manner. At the highest concentration of \( \text{fgl2} \) (1 \( \mu \)g/ml) used in the cultures, 61 \( \pm \) 11% inhibition of T cell proliferation was observed. Purified \( \text{fgl2} \) 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 \( \mu \)g/ml; Fig. 3) nor BSA (1 \( \mu \)g/ml) (data not shown) had any effect on T cell proliferation stimulated by alloantigen, anti-CD3/CD28 or Con A.

\( \text{fgl2} \) inhibited the alloageneic response at early time points and the effect could be neutralized by mAb

To further explore the suppressive effect of \( \text{fgl2} \) on alloreactive T cell proliferation, \( \text{fgl2} \) was added to alloageneic cultures at different
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 sfgl2 were also mixed by pipetting at corresponding time points as controls. Fig. 4a shows that sfgl2 exhibited maximal inhibitory effect (61 ± 11% inhibition) when it was added at the initiation of allogeneic reactions (day 0). Less inhibitory effects were observed when sfgl2 was added on day 1 (39 ± 15% inhibition) with loss of inhibition when addition of sfgl2 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 sfgl2 on alloreactive T cell proliferation was next examined. As shown in Fig. 4b, a mouse mAb (1/9262 g/ml) abrogated the ability of sfgl2 to suppress alloreactive T cell proliferation, suggesting that the effect of sfgl2 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 sfgl2.

Fgl2 promoted a Th2 cytokine profile in allogeneic responses

To characterize further the effect of fgl2 on allogeneic responses, we cultured T cells with irradiated allogeneic BM-derived LPS-induced mature DC in the presence of sfgl2. A similar dose-specific sfgl2 suppressive effect on alloreactive T cell proliferation was observed as that seen in Fig. 3. Fig. 5a shows that 1 µg/ml sfgl2 resulted in a maximal 68 ± 14% inhibition of T cell proliferation. Supernatants collected from these cultures (1 µg/ml sfgl2) 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 sfgl2-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 sfgl2. Thus, percent lysis at 5 days in control cultures (no fgl2) was 30 ± 5%, whereas in fgl2-treated cultures lysis was 31 ± 3% (p = 0.74). The addition of sfgl2 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 sfgl2-untreated cultures (Fig. 5, b and c).
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 24-h exposure to sFgl2 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).

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 24-h exposure to sFgl2 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).

Fgl2 led to reduced expression of CD80high and MHC class IIhigh molecules by BM-derived DC

We next examined whether sFgl2 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 sFgl2 (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. 6a, CD11c+ cells composed the majority of both sFgl2-treated and -nontreated cells, suggesting that the addition of sFgl2 did not reduce the number or viability of LPS-treated DC. Furthermore, the expression of MHC class I and CD86 was not altered by sFgl2 (Fig. 6, b and c). A minor change in CD40 expression was observed (Fig. 6f). However, incubation of DC with sFgl2 during LPS-induced maturation significantly reduced expression of both CD80high (Fig. 6d) and CD80high (Fig. 6d) expression on DC. These findings suggest that sFgl2 inhibits LPS-induced DC maturation.

Addition of sFgl2 during DC maturation abolished their ability to induce allogeneic responses

To further examine the effect of sFgl2 on DC maturation, we determined the ability of sFgl2-treated DC to stimulate allogeneic responses. Fig. 7 shows that DC treated with sFgl2 (1 µg/ml) during the LPS-induced maturation had an impaired ability to stimulate naive allogeneic T cell proliferation in comparison to sFgl2-untreated DC. When naive T cells were pretreated with sFgl2 (1 µg/ml) for 12 h, washed, and then cultured with allogeneic LPS-induced mature DC, no inhibitory effect on T cell proliferation was
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 Fgll2-pre-exposed DC in the presence of Fgll2 (1 μg/ml).

To examine whether Fgll2 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 Fgll2 (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 Fgll2 at all time points examined (Fig. 8).

Discussion

Our group has had a long interest in defining the regulation of induction and mechanism(s) of action of Fgll2/fibroleukin, a novel protein that is expressed by both reticuloendothelial cells (macrophages and endothelial cells) and T cells. Fgll2 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 Fgll2 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 Fgll2 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 Fgll2, 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 Fgll2/fibroleukin (Fgll2) has been described, and of particular interest to the current study is the discovery that T cells are known to express Fgll2. Nevertheless, the function(s) of Fgll2 remains unexplored. Recent studies from our laboratory and others have provided insights into the function(s) of Fgll2. Preliminary results from our laboratory using Fgll2 knockout mice suggest that T cells from Fgll2−/− mice are hyperproliferative (data not shown). A recent report from Kohno et al. (19) also suggests that Fgll2 may play a role in the acquired immune response.

To test this hypothesis, we examined the role of Fgll2 in regulating the function of APC, in particular, DC. BM-derived DC were prepared and the effect of Fgll2 on LPS-induced maturation was examined. We found that Fgll2 prevented the maturation of BM-derived DC by inhibiting the expression of CD80high and MHC class IIhigh molecules, while having no significant effects on MHC class I, CD11c, and CD86 expression. These data are consistent with the observation that Fgll2-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 Fgll2-pre-exposed DC in the presence of Fgll2 protein (1 μg/ml), suggesting that Fgll2 exerts an

FIGURE 6. Cell surface phenotype of DC generated in the absence (control) or presence of Fgll2 (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 Fgll2. 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 non-Fgll2-treated cells. The blue shaded areas are the appropriate isotype-matched Ig control.

FIGURE 7. DC treated with Fgll2 during LPS-induced maturation exhibited an impaired ability to induce alloimmune responses. DC treated with Fgll2 (1 μg/ml) during LPS-induced maturation (DCFgll2) and T cells treated with Fgll2 (1 μg/ml) prior to culturing with DC (T + Fgll2) were used to examine their ability to induce allogeneic response as described in Materials and Methods. In some cases, DC treated with Fgll2 during maturation (DCFgll2) were used to activate alloimmune T cell proliferation with Fgll2 (1 μg/ml) added to the culture (last column). Proliferation was determined by [3H]thymidine uptake in triplicate wells and the uptake in untreated cultures (shown as T + DC) was: 5546 ± 620 cpm with background 355 ± 55 cpm. The data are representative of three separate experiments. **, p < 0.01 and *, p < 0.05 compared with control groups (second left, no Fgll2 was added).
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 Fgl2 on T cell proliferation.

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

The immunosuppressive effects of 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 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 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 expression of fgl2 (28). In these patients, DC express lower levels of CD86 and an impaired ability to stimulate allogeneic T cells for expression of fgl2 (28). In these patients, DC express lower levels of CD86 and an impaired ability to stimulate allogeneic T cells for expression of fgl2 (28).

The mechanism(s) by which 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 fgl2 markedly inhibits NF-κB translocation, which may account for lack of maturation of DC as indicated by lack of expression of CD80high and MHC class II. The fact that not all DC were inhibited by fgl2 may reflect dosage requirements as well as the fact that the population of DC are not homogeneous.

In the present studies, 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 autoimmune, 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 fgl2 had no effect on CTL activity in an allo-MLC.

The actual mechanism(s) by which fgl2 promotes a Th2 cytokine differentiation is not known. However, it is known that the differentiation of naïve 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 CD80 as 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 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.

References


