SYMPOSIUM

Fibrin(ogen)-$\alpha_M\beta_2$ Interactions Regulate Leukocyte Function and Innate Immunity *In Vivo*

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In addition to its well-characterized role in hemostasis, fibrin(ogen) has been proposed to be a central regulator of the inflammatory response. Multiple *in vitro* studies have demonstrated that this hemostatic factor can alter leukocyte function, including cell adhesion, migration, cytokine and chemokine expression, degranulation, and other specialized processes. One important link between fibrin(ogen) and leukocyte biology appears to be the integrin receptor $\alpha_M\beta_2$/Mac-1, which binds to immobilized fibrin(ogen) and regulates leukocyte activities. Although it is well established that fibrin(ogen) is a ligand for $\alpha_M\beta_2$, the precise molecular determinants that govern this interaction are only now becoming clear. A novel line of mice expressing a mutant form of fibrinogen (Fib$\gamma^{390-396A}$) has revealed that γ chain residues 390–396 are important for the high-affinity engagement of fibrinogen by $\alpha_M\beta_2$ and leukocyte unction *in vivo*. Fibrinogen $\gamma^{390-396A}$ failed to support $\alpha_M\beta_2$-mediated adhesion of primary neutrophils, monocytes, and macrophages, and mice expressing this fibrinogen variant were found to exhibit a major defect in the host inflammatory response following acute challenges. Most notably, Fib$\gamma^{390-396A}$ mice display a profound impediment in *Staphylococcus aureus* elimination by leukocytes following intraperitoneal inoculation. These findings have positively established the physiological importance of fibrin(ogen) as a ligand for $\alpha_M\beta_2$ and illustrate that the fibrin(ogen) γ chain residues 390–396 constitute a critical feature of the $\alpha_M\beta_2$ binding motif. Finally, the Fib$\gamma^{390-396A}$ mice represent a valuable system for better defining the contribution of fibrin(ogen) to the inflammatory response in the absence of any confounding alteration in clotting function.

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Crosstalk Between the Hemostatic and Inflammatory Systems

The hemostatic and inflammatory systems are activated by the same spectrum of challenges (e.g., mechanical, thermal, chemical, or toxic tissue injury and microbial infection), but these systems have historically been viewed as functioning independently. However, it is becoming increasingly clear that hemostatic and inflammatory pathways are highly integrated with considerable regulatory cross talk (Figure 1) (1–6). The capacity of inflammatory factors to regulate the coagulation and fibrinolytic systems is well recognized (1–3, 5, 7, 8). Acute inflammatory events are known to shift the hemostatic balance toward a prothrombotic state in which there is an increase in circulating levels of several key procoagulants. One established mechanism whereby inflammatory mediators can promote coagulation is by elevating levels of the cell surface initiator of the clotting cascade, tissue factor (9). The systemic activation of the coagulation and fibrinolytic systems following acute inflammatory events, such as sepsis, can lead to potentially devastating consumptive coagulopathy and disseminated intravascular coagulation (10). A reciprocal pathway whereby hemostatic factors affect inflammatory processes has been less obvious but is becoming increasingly appreciated (2, 3, 7, 11–13). The conversion of prothrombin to the active serine protease thrombin, a central event in coagulation, appears to be a critical event in the regulation of inflammatory processes. Thrombin and G-protein–coupled protease-activated receptors (PARs) coupled to thrombin have been shown to control the expression of a large number of cytokines and chemokines (e.g., interleukin-1 [IL-1], IL-6, IL-8, migration inhibitory factor, granulocyte-macrophage colony-stimulating factor, and monocyte chemotactic protein–1) in different

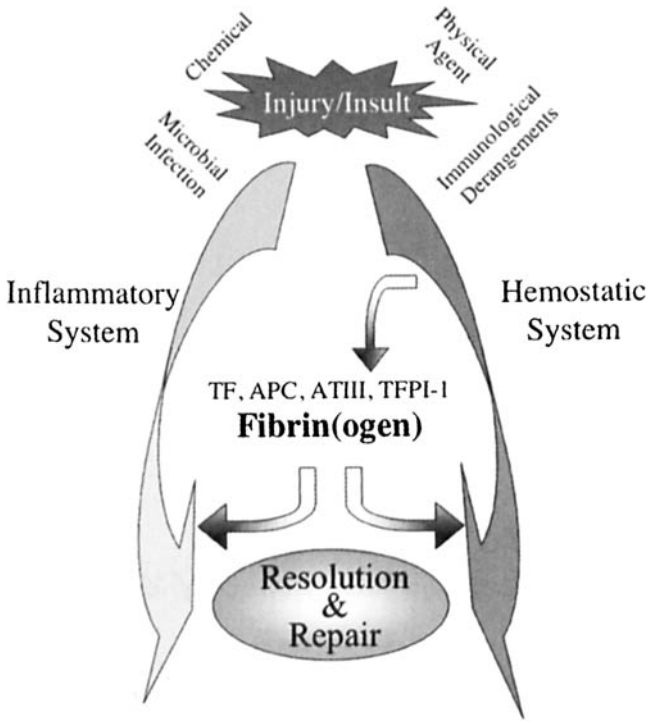

Figure 1. Regulatory crosstalk between hemostatic system components and the inflammatory system. Like the initiator of coagulation (tissue factor [TF]) and the natural anticoagulants (activated protein C [APC], antithrombin III [ATIII], and tissue factor pathway inhibitor–1 [TFPI-1]), recent evidence suggests that fibrinogen directly modulates the innate immune system.

cell types, including endothelial cells, smooth muscle cells, lung epithelial cells, and mononuclear cells (14–17). Furthermore, the activation of platelets by thrombin and other agonists is known to result in the release of a cocktail of chemokines and cytokines stored in platelet α granules (e.g., platelet factor–4, IL-8, macrophage inflammatory protein–1α, RANTES, MCP-3, CCL17, CCXL1, and CXCL5) and expression of platelet surface adhesion molecules (e.g., P-selectin and CD40 ligand) that control leukocyte trafficking and activity (18–21). The inhibitors that regulate thrombin generation or activity (tissue factor pathway inhibitor, antithrombin III, and activated protein C [APC]), have been shown to exhibit anti-inflammatory properties and enhance survival in models of bacterial sepsis (22–24). Activated protein C appears to modulate the inflammatory response by limiting thrombin-coupled events and by directly altering inflammatory regulatory pathways. Activated protein C binding to monocytic cells has been shown to alter NF-κB–mediated gene expression and induce the production of antiapoptotic gene products (2–5). Activated protein C binding to the endothelial cell protein C receptor also regulates the expression of proinflammatory cytokines and adhesion molecules critical for rolling and firm adhesion of leukocytes along activated endothelium (25).

Fibrinogen and the Leukocyte Integrin Receptor $\alpha_M\beta_2$/Mac-1

Among all known thrombin substrates, fibrinogen stands out as a central factor in hemostasis and a contributor to the inflammatory response. Fibrinogen is a classic "acute-phase reactant," with serious inflammatory challenges resulting in increased hepatic expression, the induction of extrahepatic expression, and increased circulating protein (26–29). Fibrin(ogen) deposition is a universal feature within injured tissues and inflammatory foci. Notably, fibrin appears to locally regulate inflammatory responses. *In vitro* studies (30–34) have shown that fibrin(ogen) can profoundly alter leukocyte function, leading to changes in cell migration, phagocytosis, NF-κB–mediated transcription, production of chemokines and cytokines, degranulation, and other processes. Many of the effects of fibrin(ogen) on leukocyte activity appear to be mediated by a specific receptor on leukocytes, the integrin receptor $\alpha_M\beta_2$ (35–37).

Four members of the β_2 subfamily of integrins have been identified: $\alpha_M\beta_2$ (Mac-1, CD11b/CD18, and CR3), $\alpha_L\beta_2$ (leukocyte function–associated antigen and CD11a/CD18), $\alpha_X\beta_2$ (p150,95 and CD11c/CD18), and $\alpha_D\beta_2$ (CD11d/CD18) (38, 39). The importance of this integrin family in leukocyte function is underscored by the fact that a genetic deficiency in β_2 results in a severe immunological disorder, leukocyte adhesion deficiency type 1, characterized by profound defects in leukocyte function and chronic infections (40). Based in part on the prominent expression of $\alpha_M\beta_2$ on the surface of neutrophils, monocytes, macrophages, and mast cells, this integrin is generally thought to play a pivotal role in inflammatory cell function. This view has been supported by detailed studies (41, 42) of mice with a specific genetic deficit in the α_M subunit. Although $\alpha_M\beta_2$ is likely to contribute to leukocyte trafficking, it appears to be particularly instrumental in leukocyte activation and the regulation of cell survival and apoptosis. A confounding factor in defining the general importance of this integrin in leukocyte biology has been the extraordinary number of potential ligands that have been identified. To date, more than 30 different *putative* ligands have been reported, including the endothelial intracellular adhesion molecule–1 (ICAM-1), the complement derivative C3bi, glycoprotein Ibα (αGPIb), plasminogen activator, urokinase-type plasminogen activator receptor (uPAR), plasminogen, and fibrinogen, among others (39, 43–47). Defining which of the many proposed $\alpha_M\beta_2$ ligands, if any, are biologically relevant *in vivo* has been a significant lingering problem that is just beginning to be resolved.

The Fibrin(ogen) Motif Recognized by $\alpha_M\beta_2$

Fibrin(ogen) was established as a high-affinity ligand for $\alpha_M\beta_2$ more than a decade ago. In 1988, two different research groups identified $\alpha_M\beta_2$ as the cell surface receptor on monocytes and polymorphonuclear cells that supported leukocyte interaction with fibrinogen (48, 49). In 1993, it

was established that the "I-domain" within the α_M subunit, a 200–amino acid "insert" within the β-propeller structure, was central to integrin engagement of fibrinogen (47). Additional studies (46, 50, 51) of integrin site–directed mutants have further substantiated that the α_M I-domain constitutes the fibrinogen-binding motif, and this assignment has remained experimentally unchallenged. What has been far more difficult to define is the region of fibrinogen recognized by $\alpha_M\beta_2$. Synthetic peptide inhibitor studies initially pointed to a region within the fibrinogen γ chain globular domain in the neighborhood of Gly^{190}–Val^{202} (termed the "P1" site) as a region critical for the $\alpha_M\beta_2$ interaction (52). However, more detailed work revealed that mutations introduced into the P1 site did not diminish the ability of the recombinant fibrinogen γ globular domain to support $\alpha_M\beta_2$-dependent cell adhesion (53). A second search for the fibrinogen motif recognized by $\alpha_M\beta_2$ revealed another possible binding site (termed the "P2" site) in the neighborhood of 377–395 of the fibrinogen γ chain (53). Like the P1 peptide, the P2 peptide inhibited $\alpha_M\beta_2$-mediated cell adhesion to immobilized fibrinogen and directly supported saturable binding to the α_M I-domain (53). Additional analyses identified the γ residues 383–395 (referred to as "P2-C") as the core recognition motif (53). The potential importance of this region in $\alpha_M\beta_2$ binding was supported by studies (53, 54) showing that monoclonal antibodies directed against residues γ392–406 inhibited binding of $\alpha_M\beta_2$-expressing cells to immobilized P2-C and recombinant γ globular domains.

Analysis of the three-dimensional structure of the fibrinogen γ globular domain placed the P1 and P2 regions in close proximity (53, 54). This suggested the attractive hypothesis that both P1 and P2 could participate in binding to fibrinogen. Under this theory, the P2-C sequence could represent the major binding interface for $\alpha_M\beta_2$, with residues within the P1 sequence providing supplementary contact points. This hypothesis would account for (i) the robust binding activity of the P2-C peptide, (ii) the inhibitory and adhesive properties of the isolated P1 peptide, and (iii) the finding that mutations in P1 in the context of a recombinant γ-module are insufficient to ablate binding. However, this hypothesis was challenged by a study (55) describing the generation and characterization of several novel recombinant γ globular domain derivatives carrying mutations within the P1 and P2 regions. Whereas mutations in the P2 motif resulted in significantly diminished adhesion to the α_M I-domain, multiple alanine substitution mutations in the P1 region had no effect on engagement of the α_M I-domain (55). This same study concluded that the P2 domain residues γ390–395 constituted a minimal integrin recognition motif. Nevertheless, the precise fibrinogen motif recognized by $\alpha_M\beta_2$ has remained extraordinarily controversial. Recombinant γ globular domain derivatives where portions of the P2 site had been deleted were reported to retain at least partial binding activity with isolated α_M I-domain (55). Furthermore, a recombinant human fibrinogen γ chain truncation mutant lacking a portion of the P2 site was reported to retain $\alpha_M\beta_2$ binding potential (55). Multiple confounding factors are likely to account for the apparent inconsistencies within reported findings. Potential confounding factors include the use of differing assay systems, the uncertain structural integrity of deletion mutants, the use of fibrinogen fragments rather than the whole fibrinogen molecule, the use of isolated α_M I-domain rather than the whole integrin molecule, and a failure to focus exclusively on high-affinity interactions. The possibility of multiple distinct $\alpha_M\beta_2$ binding motifs on fibrinogen may also provide a partial explanation for seemingly conflicting reports. There is some support for this latter concept, including reports suggesting that the globular domain of the fibrinogen Bβ chain or the globular domain found within the uncommon (2% of total) splice variant of the Aα chain (i.e., AαE) may support $\alpha_M\beta_2$ interactions (50, 56). However, it is difficult to imagine what evolutionary pressure could preserve functionally redundant and independent $\alpha_M\beta_2$ binding motifs within a single fibrinogen molecule.

Regardless of what features of fibrinogen constitute the $\alpha_M\beta_2$ binding motifs, it seems clear that the structural conformation of fibrin(ogen) is critical to high-affinity $\alpha_M\beta_2$ binding. This is highlighted by the fact that soluble fibrinogen is a very poor ligand for $\alpha_M\beta_2$, whereas immobilized fibrinogen or fibrin is readily bound by the integrin. The cryptic $\alpha_M\beta_2$ binding sites on soluble fibrinogen also can be made accessible for integrin binding by certain types of proteolytic cleavage, including proteolytic events that expose the P2-C region sufficiently to permit the binding of epitope-mapped antibody (54, 55). One important inference from the conformation-dependent interaction between fibrin(ogen) and $\alpha_M\beta_2$ is that soluble fibrinogen is unlikely to be "instructive" in leukocyte activation. Rather, immobilized fibrin deposited locally at sites of injury is likely to be the most informative form of this molecule, presumably supporting local leukocyte activation events and the expression of specialized functions.

Fibrinogen Engagement of $\alpha_M\beta_2$ Through Residues $\gamma^{390–396}$ Regulates Leukocyte Function *In Vivo*

To better understand the contribution, if any, of residues N^{390}RLSIGE^{396} of the fibrinogen γ chain to the engagement of the leukocyte integrin $\alpha_M\beta_2$ in the context of intact fibrinogen, these residues were recently mutated within the endogenous fibrinogen γ chain gene in mice (35). These specific amino acids were selected for mutation based on the fact that these residues (i) were conserved between species (note that the sequence is N^{390}RLTIGE^{396} in the human molecule), (ii) were largely solvent exposed in the crystal structure of fibrinogen derivatives, (iii) were spatially far removed from the γ chain "hole" known to support fibrin polymerization, and (iv) were a consistent element within P2 peptides shown to block $\alpha_M\beta_2$ binding to fibrinogen (53, 55,

57). As an experimental approach, the generation of this fibrinogen variant offered two experimental advantages. First, the contribution of the $\gamma^{390-396}$ region to $\alpha_M\beta_2$ binding could be defined in the context of intact fibrinogen synthesized in native hepatocytes. Second, the functional importance of the fibrinogen-$\alpha_M\beta_2$ interaction *in vivo* could be evaluated without imposing any alteration in $\alpha_M\beta_2$ that might preclude $\alpha_M\beta_2$ interactions with other potential ligands or crosstalk with other receptors. Residues $\gamma^{390-396}$ were converted to a series of alanines to retain normal spacing between protein domains and the overall structural integrity of the assembled molecule. Mice homozygous for the mutation (termed "fibrinogen $\gamma^{390-396A}$") were found to be viable to adulthood, never experienced spontaneous bleeding events, carried normal levels of circulating fibrinogen, maintained normal clotting function, retained normal fibrinogen engagement by other integrin receptors (e.g., $\alpha_{IIb}\beta_3$), retained normal platelet aggregation, and exhibited normal thrombus formation *in vivo*. However, unlike wild-type fibrinogen, immobilized fibrinogen $\gamma^{390-396A}$ failed to support $\alpha_M\beta_2$-mediated adhesion of different cell types, including primary neutrophils and macrophages. Most important, the disruption in $\alpha_M\beta_2$ engagement of fibrin(ogen) via residues $\gamma^{390-396}$ was found to have dramatic consequences on the inflammatory response *in vivo*. Fib$\gamma^{390-396A}$ mice exhibited a remarkable impediment in the elimination of the microbial pathogen *Staphylococcus aureus* using an acute peritonitis model. Four major conclusions were drawn from these studies. First, fibrin(ogen) is an important regulator of inflammatory cell function and innate immunity. Second, fibrin(ogen) constitutes a physiologically relevant ligand for the leukocyte integrin $\alpha_M\beta_2$. Third, the biological consequence of a loss in the $\alpha_M\beta_2$-fibrin(ogen) interaction is not (fully) compensated by the continued availability of all other potential ligands (e.g., ICAM-1 GPIbα, uPAR, etc.). Finally, the biological importance of fibrinogen in regulating the inflammatory response can be appreciated outside of any alteration in clotting function or platelet thrombus formation.

The analyses of fibrinogen $\gamma^{390-396A}$ unequivocally show that in the context of intact fibrinogen a preeminent feature of the $\alpha_M\beta_2$ binding motif is located in the carboxy-terminal portion of the γ chain, consistent with earlier analyses focusing on the P2-C peptide. It should be emphasized that the interface between $\alpha_M\beta_2$ and fibrinogen is likely to extend well beyond N^{390}–E^{396} and that this region may not constitute the sole binding determinant. However, based on the available data, the contribution of other regions to integrin binding may only modulate the high-affinity interaction or merely support lower-affinity interactions. This view is consistent with recent comparative analyses of wild-type and $\gamma^{390-396A}$–fibrinogen interaction with $\alpha_M\beta_2$ using surface plasmon resonance (Biacore analysis). Studies using wild-type mouse fibrinogen suggest the presence of a high-affinity integrin binding site and multiple low-affinity binding sites. In contrast, studies of fibrinogen $\gamma^{390-396A}$ indicate that the high-affinity site is lost, whereas low-affinity interactions remain (J.L.D., unpublished data).

Comparative analyses of *S. aureus* clearance from the peritoneal cavity of control and Fib$\gamma^{390-396A}$ mice have provided additional support for a prevailing hypothesis that $\alpha_M\beta_2$ primarily controls leukocyte function upon arrival at sites of inflammatory challenge (35). Detailed studies of *S. aureus* infection in fibrinogen-deficient and Fib$\gamma^{390-396A}$ mice have shown that fibrinogen *per se*, and fibrinogen-$\alpha_M\beta_2$ interaction in particular, is required for the full implementation of leukocyte antimicrobial activity (X.D. and J.L.D., unpublished data). A simple extension of these observations is that, in the context of soluble inflammatory mediators, leukocyte engagement of immobilized fibrin(ogen) within inflamed or damaged tissues may be an important cue in leukocyte "target recognition," ultimately regulating the expression of specialized functions. Consistent with this view, neutrophil engagement of fibrin(ogen) via $\alpha_M\beta_2$ results in dramatic cellular changes *in vitro*, including calcium mobilization, activation of NF-κB, increased phosphorylation events, degranulation, upregulation of cell surface adhesion molecules, increased migration, and decreased apoptosis (30–34). The concept that leukocyte interaction with immobilized fibrin(ogen) is an important event in target recognition has two attractive features. First, fibrin could provide a unique, nondiffusible, or *spatially defined* signal, modulating inflammatory cell function. Second, fibrin would be found within the extracellular matrix at virtually any site of tissue damage, regardless of the underlying insult, but would be distinctly absent within normal tissues. Therefore, fibrin could flag the precise site of any challenge and provide another means to locally regulate leukocyte function. Of course, this theory does not preclude the seminal contribution of soluble inflammatory mediators (e.g., cytokines and chemokines) or a significant contribution of other $\alpha_M\beta_2$ ligands. Nevertheless, it is now clear that, even when the engagement of all other ligands remains intact, the loss of $\alpha_M\beta_2$ interaction with fibrin(ogen) compromises leukocyte function, including the ability to efficiently clear an infectious agent *in vivo*.

Although fibrin(ogen) appears to be important in innate immunity, it is not strictly required for mounting an inflammatory response. Bacterial foci clearly attract inflammatory cell infiltrates in Fib$\gamma^{390-396A}$ mice ands well as in animals entirely lacking fibrinogen, and both types of mutants appear to support partial bacterial containment. The residual capacity to eliminate bacteria in the absence of fibrin(ogen) could be in part a consequence of $\alpha_M\beta_2$-mediated leukocyte activation events via other known ligands. However, alternative leukocyte integrins undoubtedly contribute to antimicrobial function based on the finding that $\alpha_M\beta_2$-deficient animals do not exhibit a general defect in leukocyte trafficking and do not develop spontaneous infections unless the deficit extends to all β_2 integrins (41, 42, 58, 59).

The fibrinogen-$\alpha_M\beta_2$ axis can now be viewed as a potentially useful target in the development of new therapeutic strategies for the treatment or prevention of inflammatory diseases, such as sepsis and inflammatory lung, bowel, and joint disease. Like APC, fibrinogen may be a critical hemostatic component that stands at the interface between the hemostatic and inflammatory systems. In fact, the potential usefulness of fibrinogen as a target in inflammatory disease has already been underscored in studies showing that the pharmacological or genetic depletion of fibrinogen in mice can diminish the progression of arthritis (J.L.D., unpublished data; and Ref. 60). Of course, an important implication of the findings with fibrinogen $\gamma^{390-396A}$ mice is that effective anti-inflammatory strategies focusing on fibrin(ogen)-leukocyte interactions could potentially be devised that would not necessarily compromise hemostatic function. Therefore, in principle, inflammatory responses could be controlled at the level of hemostatic factors without increasing the risk of bleeding or thrombotic events.

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