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The immunology of blood: connecting the dots at the neurovascular interface

Katerina Akassoglou

Gladstone Institutes and Department of Neurology, Weill Institute of Neurosciences, University of California, San Francisco, 1650 Owens St., San Francisco, CA 94158, USA.

In 1883, Eduard von Rindfleisch identified multiple sclerosis (MS) lesions around vessels “engorged with blood”, providing the first link between blood leaks and brain inflammation¹. Since then, blood extravasation has been shown to be a common thread in many devastating brain and peripheral diseases, correlating with disease severity. However, a chicken-and-egg question has posed a long-standing dilemma: does more blood leak into the brain as a consequence of disease, or does the influx of blood proteins enable disease pathogenesis? Here, I focus on my 26-year journey to connect the dots between blood, the inflammatory response, and brain disease. Together with an international team of scientists and collaborators, my work shed light on the causal role of blood leaks in toxic inflammation and tissue damage, and revealed a unique niche at the neurovascular interface for the development of imaging tools and new therapeutics^{2–4}.

TNF days

In 1994, I started my PhD work studying Tumor Necrosis Factor (TNF) in the laboratory of George Kollias at the Hellenic Pasteur Institute in Athens, Greece. The Kollias lab had just discovered that transgenic mice expressing TNF in the synovium spontaneously developed severe inflammation in their joints, indicating a direct involvement of TNF in rheumatoid arthritis (RA)⁵. I was fortunate to participate in the preclinical testing of anti-TNF monoclonal antibodies that were later approved for the treatment of RA⁶. However, as all the arthritis-related PhD projects were taken, for my thesis I was assigned to resolve the enigmatic phenotype of a paralyzed TNF transgenic mouse line without signs of arthritis. In the Kollias lab, we found that in this mouse model TNF was expressed only in the brain and spinal cord resulting in features reminiscent of MS, such as neuroinflammation, demyelination, and paralysis. The finding that expression of a cytokine in the brain was sufficient to induce demyelination and paralysis without immunizing with myelin antigens was so unexpected at the time that my short oral presentation at the IV International Society

kakassoglou@gladstone.ucsf.edu.

Competing interests

K.A. is the scientific founder and advisor of Therini Bio, Inc. K.A. is an inventor on The Regents of the University of California issued patents US7807645, US8569242, US8877195, and US8980836 covering fibrin antibodies; a co-inventor on The Regents of the University of California and Gladstone Institutes pending patent applications U.S. 15/943,474 and EP 15735033.1 covering fibrin assays of microglia activation; and a co-inventor on Gladstone Institutes issued patent US9669112 covering fibrin in vivo models, US10451611 and pending patent applications U.S. 16/572,365 and EP 15735284.05 covering in vitro fibrin assays. Her interests are managed in accordance with the Gladstone Institutes’ conflict of interest policy.

of Neuroimmunology (ISNI) Congress in Amsterdam in 1994 was featured in the meeting highlights⁷. A year later, our paper reporting spontaneous inflammatory demyelination by TNF expression in the brain was communicated by Rita Levi-Montalcini to the *Proceedings of the National Academy of Sciences*. Building upon these findings, I generated multiple transgenic lines that revealed that TNF produced by astrocytes, but not neurons was responsible for the neurologic disease⁸. We attempted to genetically rescue the TNF transgenic mice by depleting T cells, but surprisingly found that T cell depletion exacerbated features of the disease⁹. This result led us to conclude that innate immunity was responsible for demyelination, and made us question the prevailing hypothesis that T cell suppression would be therapeutic in progressive disease⁹. But if T cells were not the mediators of inflammatory demyelination in the TNF transgenic mice, what was triggering the disease?

Finding fibrin in Vienna

I presented my findings in the TNF transgenic mice to the Demyelination and Remyelination INSERM Conference Philippe Laudat at Aix-Les-Bains in 1995. After a talk in which I faced more questions than I could answer, I realized how essential it would be to master neuropathology in order to understand how innate immune activation induced brain disease. During the meeting social on a boat tour of the Rhône river, I met Hans Lassmann, an expert MS neuropathologist at the Medical University of Vienna. Dr. Lassmann was intrigued by my presentation, and we brainstormed ways to experimentally determine the mechanism of disease in the TNF transgenic mice. In the fall of 1996, with support from the European Molecular Biology Organization, I moved to his laboratory in Vienna to analyze hundreds of brains from the TNF transgenic mice. A key finding from these studies was axonal damage and oligodendroglial pathology induced by innate immune activation, suggesting that TNF transgenic mice could be a model to study MS¹⁰. To find out how this pathology started, we analyzed the brains early, before any symptoms occurred. Using electron microscopy, I noticed major changes in blood vessels and perivascular deposits, which Dr. Lassmann identified as massive blood-brain barrier (BBB) disruptions with fibrin deposits. We could detect BBB leaks as early as one week of age, before any other symptom or cell infiltration was present. Had we found the smoking gun? As fibrinogen is a marker for BBB disruption, we looked for fibrinogen immunostaining in the brains of MS patients. I was awed to discover an abundance of fibrin deposits, which often colocalized with microglia and macrophages. Eager to learn more about fibrin, I found a paper that had just been published reporting that fibrin mediates inflammation via the CD11b/CD18 integrin receptor¹¹. These observations and results, in combination with our earlier finding that astrocytes—which regulate the BBB—were the primary source of TNF promoting neuroinflammation⁸, led me to hypothesize that BBB disruption is an upstream activator of innate immune-mediated demyelination and neurodegeneration.

At the time, fibrin was primarily studied by hematologists for its role in blood coagulation, which made my search for a laboratory in which to study its role in brain inflammation unfruitful. However, in 1997, I met Sidney Strickland at the 17th Blankenese Conference on Neurodegeneration in Hamburg, shortly after his landmark paper on the role of the fibrinolytic enzyme, tissue plasminogen activator (tPA), in excitotoxic neuronal death¹². I joined his laboratory as a postdoctoral fellow, first at the State University of New York at

Stony Brook and later at The Rockefeller University, to determine the role of the tPA/fibrin pathway in neuroinflammation. Integrating vascular and BBB research in neuroimmunology was unconventional at the time and many assumed I had changed fields. In Dr. Strickland's laboratory, I first found that tPA- and plasminogen-deficient mice had exacerbated demyelination after sciatic nerve injury, which could be rescued by fibrin depletion¹³. In collaboration with Jay Degen at Cincinnati Children's Hospital, we performed the first genetic validation of the role of fibrinogen in the nervous system¹⁴. Mice lacking fibrinogen had increased remyelination after nerve injury and decreased neuroinflammation and demyelination in the context of transgenic TNF expression^{13,15,16}. These results were in line with protective effects of anti-coagulants in autoimmune MS models¹⁷. In addition, fibrinogen-deficient mice had increased expression of the p75 neurotrophin receptor (p75^{NTR}) after nerve injury¹⁵. In the laboratory of Moses Chao at New York University, I made the serendipitous discovery that tPA expression and fibrinolysis were suppressed by p75^{NTR}, identifying an unanticipated role for neurotrophin receptor signaling in fibrin removal from the nervous system¹⁸. Fibrinogen-deficient mice were later studied in other laboratories and their protection from neuroinflammation and demyelination was confirmed in mouse models of Alzheimer's, pericyte-deficiency, and brain injury^{19–21}. But how did fibrin induce these effects on the brain's immune and repair functions?

Zeroing in on innate immunity

In 2002, in a review article entitled “*Nervous system pathology: the fibrin perspective*”, we first proposed the hypothesis that fibrin interacts with receptors on nervous system cells²². In my laboratory at the University of California San Diego and Gladstone Institutes, we discovered the cell targets for fibrinogen and fibrin in the brain and identified a causal role for BBB disruption in glial activation and neuronal dysfunction^{23–26}. We discovered that culturing microglia on fibrin-coated plates induced dramatic activation²⁶. Fibrinogen binds the CD11b/CD18 receptor via the fibrin $\gamma_{377-397}$ peptide²⁷ and genetic mutation of that peptide (*Fgg* ^{$\gamma^{390-396A}$} mice) results in fully coagulable fibrinogen which does not bind the CD11b/CD18²⁸. This allowed us to investigate the role of fibrin-induced inflammation in the brain without disrupting hemostasis. We showed that *Fgg* ^{$\gamma^{390-396A}$} mice had reduced neurological signs, inflammation, demyelination, and perivascular microglia clusters in EAE – a mouse model of MS^{26,29}. By in vivo two-photon imaging, we showed that fibrin deposition and coagulation activity occurred and correlated with microglial activation^{29–31}. Fibrin signaling via CD11b/CD18 was sufficient to induce demyelination and peripheral cell recruitment³², as well as oxidative stress via activation of NADPH oxidase⁴; a mechanism that also contributes to microglia-mediated synapse loss in Alzheimer's disease models³³. Blood clotting and oxidative stress genes are mechanistically coupled in neuroinflammation, suggesting a positive feedback loop between fibrin, inflammation, coagulation and oxidative injury³⁵. These results established that interaction of fibrin with an inflammatory receptor was necessary and sufficient to promote neuroinflammation (Fig. 1). Could we develop an approach to selectively target the inflammatory functions of fibrin without affecting its beneficial effects in hemostasis?

The making of an antibody

During my postdoc at The Rockefeller University, we occasionally had joint lab meetings with Barry Coller, the inventor of ReoPro™ (abciximab), a monoclonal antibody used as a thrombolytic that potently inhibits fibrinogen binding to $\alpha_{IIb}\beta_3$ platelet integrin³⁴. Thinking back on his work, I wondered: if an antibody can specifically inhibit the binding of fibrin to platelets and reduce thrombus formation, could we develop an antibody to specifically inhibit binding of fibrin to immune cells and stop inflammation without interfering with hemostasis? As described above, the fibrinogen γ chain contains the $\gamma_{377-395}$ peptide that mediates interaction with CD11b/CD18²⁷, and this is adjacent to but distinct from the motif that mediates platelet engagement. The $\gamma_{377-395}$ peptide is considered cryptic in soluble fibrinogen, and binding to CD11b occurs only after the conversion of fibrinogen to insoluble fibrin²⁷. I hypothesized that a monoclonal antibody against the $\gamma_{377-395}$ epitope would selectively inhibit the interaction between fibrin and CD11b without affecting coagulation. This hypothesis was supported by our finding that mutating the $\gamma_{377-395}$ epitope (in *Fgg* ^{$\gamma^{390-396A}$} mice) or administering $\gamma_{377-395}$ peptide reduced neuroinflammation without interfering with in vivo clotting time²⁶. In my laboratory, we generated antibodies against the $\gamma_{377-395}$ peptide and the lead clone was 5B8, a highly selective monoclonal antibody that selectively bound fibrin and blocked fibrin-induced, CD11b-mediated activation of innate immunity without affecting fibrin polymerization⁴. As drug discovery requires broad expertise, I put together a multi-disciplinary team of thirty-four scientists in three academic institutions and pharma to fully characterize this fibrin-targeting immunotherapy⁴. Jae Kyu Ryu and Anke Meyer-Franke led the development of biochemical and cell assays to study fibrin in vitro that enabled the screening of fibrin-targeting antibody clones and their prioritization for in vivo studies⁴. In accordance with the genetic evidence in *Fgg* ^{$\gamma^{390-396A}$} mice^{26,33}, we found that the fibrin-targeting immunotherapy protected mouse models of EAE and Alzheimer's disease from neuroinflammation and neurodegeneration⁴ (Fig. 1).

As neurovascular interactions are complex and multifactorial, one might ask whether targeting a single blood protein will be enough to protect from neuroinflammation. A similar question was raised in the early nineties regarding anti-TNF therapy, as noted by Jan Vilcek, the inventor of Remicade™ (infliximab): “*To most scientists and physicians it seemed inconceivable that blocking a single cytokine could be beneficial for RA patients, when it was known that multiple cytokines are involved in the inflammatory process*”⁶. Despite this complexity, targeting individual proteins within the cytokine or angiogenic networks, such as TNF or VEGF, has indeed succeeded in the clinic. As fibrin is a global activator of innate immunity at sites of vascular damage³, these approaches could have therapeutic benefits in brain and peripheral diseases. Therefore, fibrin-targeting immunotherapy, a novel approach to selectively suppress pathogenic innate immunity at sites of vascular damage, may have therapeutic benefits in clinical applications.

What the future holds

The interface of the brain, immune, and vascular systems represents a new frontier of scientific exploration, with the potential to change the way we think about fundamental mechanisms of neurological diseases and discovery of novel therapies². “*Connecting the*

dots” will continue to depend on an unwavering focus on the discovery of new mechanisms, pursuing the integration of new technologies, and promoting collaborations within a community of experts in neuroscience, immunology, vascular biology, drug discovery, and clinical development, all the while welcoming serendipity. Developing safe and efficacious drugs against well-defined necessary and sufficient targets, such as fibrin, could expand the toolbox of therapies to target neurovascular pathology. Implementing functional multiomic approaches will further reveal new mechanisms and therapeutic targets at the neurovascular interface³⁵. As the neurovascular interface is a unique niche for the development of imaging tools and new therapies, drug discovery selectively targeting pathogenic functions of blood proteins can change the diagnosis and treatment of neurodegenerative, autoimmune and inflammatory diseases.

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Katerina Akassoglou recounts how her work on the mechanisms and functions of blood leaks in the brain led to the discovery of fibrin-targeting immunotherapy

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Photo:

Katerina Akassoglou, PhD in her laboratory at Gladstone Institutes, University of California San Francisco in 2019.

Figure 1.

Fibrin mechanisms and functions in neuroinflammation. Upon BBB disruption, fibrinogen leaks in the brain and by activation of the coagulation cascade it is converted to insoluble fibrin deposits. Fibrin activates microglia and promotes recruitment of peripheral immune cells and oxidative injury leading to tissue damage. Inhibition of the interaction of fibrin with its cellular receptors could enable the discovery of selective therapies to block the toxic effects of blood leaks in a wide range of diseases with vascular damage and inflammation.