Target it All Right But Do Not Forget the Torchbearer

Running title: Tsokos; Complement and platelet activation in injury

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Ischemia is frequent in clinical practice and extends beyond trauma to solid organ transplantation, and vasculitis to mention a few. Ischemia and a number of additional stressors condition cells and tissues to express new antigens. Previously, it was recognized that apoptosis enables the formation of blebs on the surface of the cells which may contain nuclear antigens and proteins which normally are retained within the plasma membrane. The consequences are manifold. These “new antigens” may stimulate the production of autoantibodies, which in the predisposed individual may lead to clinical autoimmunity. A majority of the produced antibodies are polyreactive and may recognize nuclear and phospholipid antigens. Should ischemia occur in sufficient magnitude, then naturally occurring crossreactive antibodies, anti-DNA or antibodies against other nuclear or phospholipid antigens will be engaged, activate complement, and facilitate local organ and, as we will discuss below, remote organ damage. It should be noted that some of these autoantibodies, particularly those of the IgM class may counteract the action of others which are pathogenic.

Certain neoantigens have been identified and exploited as potential therapeutic tools. One of them, nonmuscle myosin heavy chain type II A and C, is recognized by a natural monoclonal IgM antibody and is expressed by stressed cells. The IgM anti-myosin antibody may enhance injury and conversely, a myosin-defined peptide may protect or mitigate ischemia injury in mice. Another is annexin IV which was discovered to be recognized by an IgM monoclonal antibody. This IgM anti-annexin IV antibody can alone restore ischemia/reperfusion injury (IRI) in the IRI-resistant Rag1-deficient mouse, and infusion of recombinant annexin IV into normal animals prevents effectively IRI. Interestingly, healthy people and IRI-resistant Cr2-deficient mice have high titers of anti-annexin IV antibodies.

The IRI-enabling (auto) antibodies invariably engage complement, and promote the
recruitment of polymorphonuclear cells, platelets, IL-17 producing T cells, and other effectors of inflammation. While complement inhibition using various inhibitors appears to mitigate IRI in many animal models, it has been wisely entertained that generalized complement inhibition may increase the risk of infections and this may be catastrophic in conditions like intestinal IRI when the entry of pathogens in to the blood stream may become uncontrolled. To this end the Hollers lab has considered “targeted” delivery of complement inhibitors to “hush” complement activation only at the site of injury. At the site of injury, C3 is broken down to generate iC3b and C3dg which can be found deposited locally. iC3b and C3dg represent ligands for complement receptor 2 (CR2) and when the iC3b/C3dg binding component of CR2 was conjugated to a mouse complement-inhibitory protein (Crry), Crry was delivered only to the injured areas and IRI ceased.

In the accompanying article in this issue of Circulation, Atkinson et al found annexin IV to be expressed on transplanted hearts in mice. They constructed an anti-annexin IV single chain antibody (scFv) and conjugated it to Crry. scFv-Crry targeted the transplanted hearts, limited complement deposition to the graft and inflammation. As predicted, immunity to infection was not affected and therefore, repeated or prolonged administration of such a targeted complement inhibitor should not be associated with generalized immunosuppression. Organ transplantation, revascularization surgery, vasculitis and other conditions should be expected to benefit from this approach. It has been mentioned already that complement activation and pathways leading to IRI may not be identical in all organs and the expression of neoantigens may vary. Complement regulation by naturally occurring regulators (Crry is one of them) are expressed in certain cells and keep complement activation under control. For example, proximal renal tubules express Crry on their basolateral surfaces. During IRI, Crry expression vanishes
and if deleted genetically, the kidney suffers extensive IRI\(^{11,12}\). As more kidney transplants (~17,000 each year) than heart transplants (2,000 each year) are performed in the US, it would be interesting to know whether annexin IV is similarly expressed in the transplanted kidney and whether scFv-Cry would have a similar protective effect. Certainly, Cry being is been lost in the kidney during IRI and its replacement is needed but the targeting carrier should be identified. That complement activation is rampant in kidney transplants and many types of nephritides have been demonstrated in multiple complement-deficient mice (for example C3 deficiency mitigates injury whereas lack of complement regulators worsens injury) and various complement inhibitors avert damage invariably\(^{13}\). Atkinson et al\(^{10}\) though prompt us to consider targeted delivery of complement inhibitors, and the best approach for the kidney as well as for other molecules needs to be defined.

The nucleotide-binding oligomerization domain (NOD) proteins NOD1 and NOD2, the first members of the intracellular NOD-like receptor family, sense conserved motifs in bacterial peptidoglycan present on the cell wall of bacteria and induce proinflammatory and antimicrobial responses. NOD1 and NOD2, unlike Toll-like receptors which recognize microbial ligands at the cell surface or within endosomes, sense bacterial products in the host cytosol\(^{14}\). They are also important in the expression of kidney IRI,\(^{15}\) and antibiotic treatment limits intestinal IRI\(^{16}\). But it appears that NODs can be activated not only in the presence of bacteria but also by peptidoglycan present in antigen present cells in the CNS\(^{17}\) and possibly elsewhere.

The second accompanying paper by Zhang et al\(^{18}\) in this issue of Circulation reports the novel observation that human and murine platelets have NOD2 and that they can be activated by its ligand muramyl dipeptide (MDP). Interestingly, MDP did not change platelet function on its own but accentuated platelet aggregation and clot retraction caused by ATP. The potentiating
effect of MDP was abolished in NOD2-deficient mice. In functional \textit{in vivo} experiments Zhang et al.\textsuperscript{18} showed that administration of MDP greatly enhanced FeCl3-induced thrombus formation in mesenteric arterioles in normal but not in NOD2-deficient mice. Platelets treated with MDP in vitro and then transferred to platelet-depleted animals caused lethal pulmonary thromboembolism after challenge with thrombin or collagen. Similarly, platelets exposed to plasma from patients with sepsis, accelerated thrombosis. It is quite possible that platelets will be exposed to NOD2 ligands present in antigen presenting cells\textsuperscript{17} or on the surface wall of bacteria which may have entered the bloodstream in excess particularly during intestinal ischemia and accelerate damage in in organs beyond those directly affected. After mesenteric IRI, platelets lodge at increasing amounts over 24 hours in the lungs and they appear to be covered with complement\textsuperscript{7}. While we do not know if complement deposition causes or facilitates platelet activation, it is certain that the deposited complement may facilitate remote lodging whereas peptidoglycan activates them and causes thrombus formation. In that respect, the scFv-Crry construct prevents complement activation at the site of injury and by not altering immunity to bacteria, limits the possibility for MDP-mediated platelet activation.

Zhang et al.\textsuperscript{18} present a complete report on the signaling pathway utilized by NOD2 in platelets. Here there are no surprises. As in other cells, engagement of NOD2 involves the RIP2/MAPK pathway. All Erk, p38 and JNKL inhibitors abolished the NOD2 effect on platelet aggregation and thrombus contraction. Platelet activation, at least as it occurs during ischemia reperfusion, involves a number of other pathways, which should be reconsidered in the context of NOD2 engagement. Platelets from CD40 or CD40 ligand deficient mice cannot lodge in remote (lung) tissues and cause damage\textsuperscript{19} while spleen tyrosine kinase (Syk) appears to be necessary for platelet-executed remote organ damage\textsuperscript{20} and platelet factor 4 deficient mice do
not suffer local (intestinal) or remote (lung) injury. It would be interesting to know whether MDP can cause platelet aggregation in the absence of CD40, CD40 ligand, Syk, or platelet factor 4 deficiency as in the absence of MAPK. The value of this information lies with the possibility of expanding the list of drugs which can limit tissue damage facilitated by activated platelets.

Platelets have been assigned additional roles besides thrombus formation and hemostasis. When activated they produce a number of inflammatory molecules including IL-1beta which promote inflammation where they happen to lodge. Zhang et al show here that platelets stimulated through NOD2 produce IL-1beta indicating that even this mode of stimulation bestows on to platelets inflammatory capacity.

Platelets have more recently been identified to be important in the pathogenesis of autoimmune diseases like rheumatoid arthritis. When activated they release microparticles which are abundant in the synovial fluid, release IL-1 which in turn stimulates proliferation of synovial fibroblasts and the development of arthritis. In patients with systemic lupus erythematosus and lupus-prone mice, platelets are activated and express on the surface CD40 ligand through which they serve a strong costimulatory signal to B cells to produce autoantibody.

The two accompanying papers address two important points in the current practice of medicine (Figure 1). Complement becomes activated in transplanted organs and many other clinical conditions where there is an element of ischemia followed by reperfusion. Platelets traveling through the burning land will become decorated by complement proteins, become activated by peptidoglycan available either by surrounding bacteria or antigen presenting cells (possibly other cells) and probably by thrombin and collagen and then travel to other places where they will attach to the walls of vessels and initiate thromboembolism or lodge in the...
microvasculature of organs such as the lung and the CNS and compromise their function. In both places they will release IL-1 and instigate inflammation or modulate the immune system otherwise. It seems that targeted inhibition of complement activation, which is expected to limit bacterial infections and platelet activation, will result in limited local organ damage and remote organ damage will be avoided.

Conflict of Interest Disclosures: None.

References:


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20. Lapchak PH, Kannan L, Rani P, Pamuk ON, Ioannou A, Dalle Lucca JJ, Pine P, Tsokos GC. Inhibition of Syk activity by R788 in platelets prevents remote lung tissue damage after


**Figure Legend:**

**Figure 1.** Ischemia-stressed cells express annexin IV on their surface which is recognized by naturally occurring IgM anti-annexin IV antibodies which activate complement to execute local damage. C3dg and iC3b are deposited locally and they decorate passerby platelets. scFv-Crry can recognize newly expressed annexin IV and deliver the complement inhibitor Crry to the site of injury. Peptidoglycan released either by cells or from bacteria activate the NOD2-PIP2/MAPK pathway in platelets. Platelets covered with complement and activated (or made more susceptible to activation by classical platelet activators like collagen or thrombin) travel and attach to large and small vessels where they will form clogging thrombi, release IL-1 and propagate inflammation and destruction.
Activated platelets coated with complement

"The torchbearer"

Platelet

NOD2> RIP2

Peptidogly can

Annexin IV

C3b C3dg

C3

C3a

C5b-9

scFv-Crry

IL-1

C3a C5a

C5b-9
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