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### Inflammation in ischemia and reperfusion: From mice to men

Diepenhorst, G.M.P.

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Gwendolyn MP Diepenhorst<sup>1,2</sup>  
Thomas M van Gulik<sup>2</sup>  
C Erik Hack<sup>3</sup>

1. Department of Surgery, Surgical Laboratory, Academic Medical Center, Amsterdam, The Netherlands
2. Department of Pathology and Cardiac Surgery, ICaR-VU, VU Medical Center, Amsterdam, The Netherlands
3. Laboratory of Translational Immunology, Utrecht Medical Center Utrecht, Utrecht, The Netherlands

## COMPLEMENT-MEDIATED ISCHEMIA- REPERFUSION INJURY: LESSONS LEARNT FROM ANIMAL AND CLINICAL STUDIES

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## INTRODUCTION

Tissue ischemia is a key event in clinical conditions such as myocardial infarction and stroke, and may occur as a complication in vascular surgery and organ transplantation. The most evident treatment for this condition is the restoration of interrupted blood flow to the jeopardized tissue. However, reperfusion of ischemic tissue paradoxically exacerbates tissue damage, particularly after longer periods of ischemia. This negative effect of reperfusion is the result of an inflammatory reaction induced by the restoration of blood flow and is called ischemia-reperfusion (I/R) injury<sup>1</sup>. This phenomenon may cause irreversible damage to cells that were viable shortly before reperfusion, limiting the beneficial effect of reperfusion in conditions such as intestinal ischemia, myocardial infarction, cerebral ischemic events and the operative management of trauma. Despite high morbidity and mortality rates associated with these ischemia-related diseases as well as the increasing frequency of organ transplantation, no effective therapy or treatment of the I/R phenomenon exists to date<sup>2</sup>. As a matter of fact, I/R-injury severely hampers further improvements in the development of therapeutic strategies for diseases such as myocardial infarction. As intervention on more than one level is most probably required to prevent reperfusion injury, extensive understanding of the pathophysiology of I/R injury is necessary for the development of much needed effective therapies.

## PATHOGENESIS OF I/R INJURY: ROLE OF INFLAMMATION

Interruption of blood flow by occlusion of afferent blood vessels and subsequent reperfusion initiates an inflammatory response in the jeopardized tissue. Within minutes of reperfusion, reactive oxygen species (ROS) are generated<sup>3-7</sup>, stimulating the release of cytokines<sup>8-11</sup> and expression of adhesion molecules on damaged cells in reperfused tissues. Several hours after the onset of reperfusion<sup>2</sup>, neutrophils and other inflammatory cells are activated and adhere to damaged cell membranes<sup>12-19</sup>, further enhancing the inflammatory response. This inflammatory response ultimately leads to cell damage. However, a number of studies have demonstrated that I/R-injury may occur even in the presence of limited number of inflammatory cells, indicating that this ROS-related mechanism alone cannot be completely responsible for I/R injury<sup>6,20,21</sup>.

The inflammatory cascade triggered by reperfusion of ischemic tissue has been a focus of intensive research throughout the previous decades, as it is generally believed that intervention at this level may open new ways for therapy of tissue ischemia. Notably, this research has been performed mainly in animal models, as in vitro systems mimicking molecular and cellular mechanisms involved in I/R-injury are scarce. Amongst the inflammatory mediators involved in the pathogenesis of I/R-injury, the complement system has been demonstrated to play a major role. Activation of this cascade system of soluble plasma proteins results in the recruitment of a large number of inflammatory cells, ultimately leading to cell injury and death<sup>6</sup>. Moreover, the infiltration and activation of neutrophils and

other inflammatory cells causes vascular plugging and impediment of microvascular blood flow in the ischemic tissue (“no-reflow phenomenon”)<sup>22</sup>. In the following sections we will critically review the role of complement, and in particular address the role of the different pathways of complement in animal I/R models studies as well as in human disease conditions.

## THE COMPLEMENT SYSTEM

The complement system functions at the interface of innate and adaptive immunity and consists of a series of fluid-phase as well as some membrane-bound proteins. The complement system has long been recognized as an important mediator of innate immune defense and inflammation<sup>23</sup>. The main biologic effect of complement is to provide a potent host defence against microbial infection through pathogen recognition and opsonization. Precursor zymogens of the complement proteins circulate in inactive form to become activated locally at sites of infection. During activation, a sequence of enzymatic steps results in a huge amplification of the subsequent inflammatory response.

The complement system comprises three pathways: the classical pathway, the alternative pathway and the lectin pathway (*Figure 1*). Each pathway leads to complement activation on pathogen surfaces and is triggered by different molecules on these surfaces: host-derived IgG and IgM antibodies and the acute phase protein C-reactive protein (CRP) in case of the classical pathway; exposed poly-mannose carbohydrates in case of the lectin pathway; and the carbohydrate composition of the surface in case of the alternative pathway. Activation of the alternative pathway is a more complex process and essentially occurs when activated C3b binds to a surface where it is protected against the inhibitory effects of factors I and H. All three pathways merge at the level of the complement component C3 and ultimately converge to generate the same set of effector molecules; the complement components C5 to C9. Other known activators of the classical pathway of complement are CRP and natural IgM antibodies, which are produced without stimulation or selection by specific antigens. Activation of the classical pathway is initiated by binding of C1q, the first protein in the complement cascade, to antibodies or CRP bound to a pathogen. As a consequence C1 and downstream components (C4, C2, C3) are activated. Characterization of the lectin pathway of complement activation is relatively recent. The recognition molecules of this pathway are mannose binding lectin (MBL) and ficolin which both recognize specific carbohydrate patterns expressed on pathogen surfaces, belonging to the group of pathogen associated molecular patterns (PAMPs)<sup>24-28</sup>. Activation of the pathway is triggered by the enzymatic activity of MBL-associated serine proteases (MASPs)<sup>29</sup> after which relevant substrates are cleaved, i.e. C4 and C2 for MASP-2; C3 and C2 for MASP-130. In the alternative pathway, activated C3 is spontaneously generated in plasma and hence forms a soluble C3 convertase in the presence of factor B and factor D. This will continuously generate trace amounts of C3b in the fluid-phase. When fixed to a suitable pathogen surface this C3b escapes control by factors I and H, and by interacting with factors B, D and P, will generate activated C3b. Notably, the alternative pathway also functions as an amplification loop for classical and lectin pathway activity.

Complement activation has three main effects contributing to antimicrobial defences. Firstly, activated complement proteins, in particular the third complement component C3, covalently bind to pathogens. As a result, the uptake and opsonization of microbes is facilitated by phagocytes bearing receptors for fixed complement proteins. Secondly, small fragments of some complement proteins act as chemoattractants, recruiting inflammatory cells such as neutrophils and other phagocytes to the inflammatory site where they are subsequently activated. A particularly potent chemoattractant is C5a, which is generated during activation of the fifth complement component, C5. Thirdly, lysis of microbes occurs by generation of so-called membrane attack complexes by the terminal complement components C5 to C9, creating pores in bacterial membranes and causing lysis of microbes.

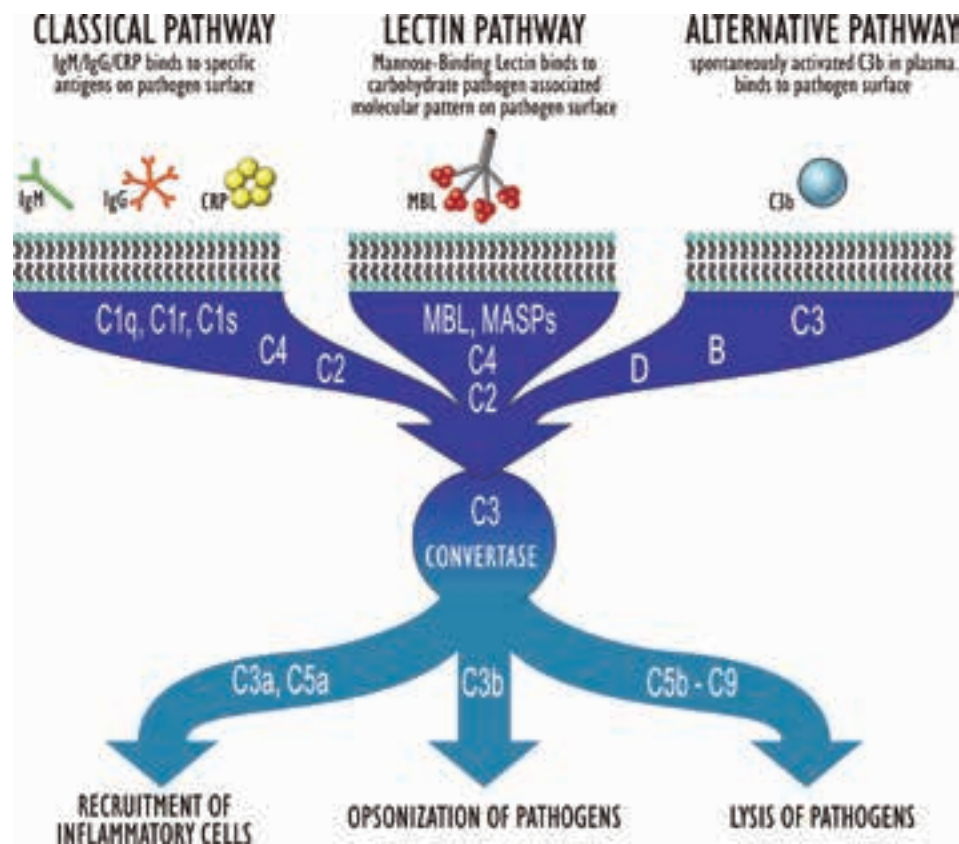


Figure 1. Schematic representation of the complement cascade and its three pathways: the classical pathway, the alternative pathway and the lectin pathway. Each of these pathways is triggered by different molecules on pathogen surfaces. Complement activation via these pathways merges at the level of C3 convertase and ultimately generates the same set of effector molecules; the complement components C5 to C9. MBL, mannose-binding lectin; CRP, C-reactive protein; MASP, MBL-associated serum protein.

These antimicrobial effects should however be discriminated from the inflammatory effects of the complement system. Based on the clinical consequences of genetic deficiencies of individual complement proteins, one may conclude that the opsonization of microbes is essential for the defence against a number of bacteria, whereas lysis mainly serves to protect the host against a number of mostly Gram-negative bacteria such as *Neisseria*. Regarding the inflammatory component of complement, the biologic effects of the anaphylatoxin C5a and to a lesser extent those of C4a and C3a, as well as the effect of sublytic amounts of membrane attack complexes appear to be dominant.

The activity of all three pathways is modulated by a number of regulatory proteins preventing tissue damage as a result of uncontrolled complement activation, such as C1 inhibitor (C1-INH), soluble complement receptor 1 (sCR1) and decay accelerating factor (DAF). These complement control proteins are employed in various animal and human studies exploring the effects of complement inhibition on I/R injury. According to literature all three complement pathways may be involved in the development of I/R injury, depending on the model, the tissue, and the time course of inflammation.

## COMPLEMENT-MEDIATED I/R INJURY IN ANIMAL MODELS

A role of complement activation in I/R injury was first demonstrated by Peter Ward and co-workers in the early 70s in a rat myocardial infarction model<sup>31</sup>. Thereafter, a key role of the complement system in mediation of tissue injury has been further established in this and other animal models<sup>32-36</sup>, including intestinal, hindlimb and kidney I/R models. Convincing evidence for such a role is provided by studies in which I/R injury is attenuated or prevented using knock-out models or intervention with complement inhibitors. Some of these studies provide further support by demonstrating immunohistochemical depositions of complement components in ischemic tissue and/or detection of complement activation products in plasma.

### The role of complement in I/R-injury

Attenuation of complement mediated tissue damage has been demonstrated by inhibition or depletion of complement components by a variety of regulatory proteins as well as antibodies against complement components. In both mouse and rat models of intestinal I/R, protection from local tissue injury has been reported using sCR1<sup>33,37</sup>, C1-INH<sup>35,38</sup> and complement receptor-related gene Y (Crry-Ig)<sup>39</sup>, a murine regulator of complement activation. Cardioprotective effects have been implicated in myocardial I/R models using cobra venom factor (CVF)<sup>40</sup>, C1-INH<sup>41,42</sup>, sCR1<sup>43,44</sup> and anti-C5 monoclonal antibodies<sup>45</sup>. An inhibitory monoclonal antibody to the alternative pathway component mouse factor B<sup>46</sup> as well as C5a receptor antagonists<sup>47-49</sup>, have shown to offer protection in animal models of ischemic acute renal failure. In a rat model of hepatic I/R, Jaeschke and colleagues were the first to report the role of complement activation using CVF<sup>50</sup>. Thereafter, complement inhibitors such as the C5aR antagonist<sup>51</sup>, sCR1 and C1-INH<sup>52,53</sup> have effectively reduced complement mediated tissue damage in this model. However, despite amelioration of liver

injury and mortality using complement inhibitors in this model, complete protection has never been achieved<sup>54,55</sup>.

Knock-out mice deficient in specific complement proteins have been shown to be protected from local and remote injury in I/R models of various organs. In this manner, C3<sup>-/-</sup> as well as C4<sup>-/-</sup> mice have shown protection in intestinal and hindlimb I/R models<sup>37,56,57</sup>. Similarly, C3<sup>-/-</sup> mice have demonstrated protection from injury in a renal I/R model<sup>49</sup>. Overall, these studies provide compelling evidence for a role of complement in I/R-injury by the use of complement inhibitors and knock-out animal models.

### The role of specific pathways in I/R-injury

Whereas the role of complement in animal I/R models is relatively well established, the importance of each specific pathway in the initiation of I/R injury remains controversial and largely unresolved to date. Specific inhibitors of the different pathways are lacking, and therefore hypotheses regarding the roles of individual complement pathways are mainly based on studies in knock-out mice models. The traditional view holds the classical pathway of complement responsible for a large part of complement-mediated I/R damage. Recently however, the lectin pathway has gained increasing attention. This has raised the question of the sequence of molecular events leading to complement activation in I/R-injury and in particular the relationship between the roles of the classical and the lectin pathways. The fact that the relative role of each pathway appears to differ among organs complicates matters further, as summarized in *Table 1*.

### Intestinal I/R

Indications for a role of the classical pathway in intestinal I/R injury was first provided by studies in which mice deficient in classical pathway components C3, C4, or total immunoglobulins (Rag<sup>-/-</sup>) were shown to be protected from local and remote injury<sup>37</sup>. Furthermore, reconstitution with polyclonal plasma IgM from wild-type (WT) mice specifically restored injury in Rag-1<sup>-/-</sup> mice in this model, suggesting a key role of IgM-mediated classical pathway activation of complement. Subsequent studies indicated involvement of a subset of the natural antibody repertoire in I/R initiation by demonstrating protection of mice deficient specifically in natural antibodies (Cr2<sup>-/-</sup>) and restoration of injury by reconstitution of IgM from WT mice<sup>58</sup>. Zhang and colleagues have provided a convincing line of evidence in support of this hypothesis by identifying this potentially pathogenic IgM subset as a single monoclonal natural IgM from a panel of B-1 cell hybridomas, CM22, demonstrated to specifically restore I/R injury in Rag<sup>-/-</sup> mice<sup>59</sup>. In following studies, potential target antigens to the pathogenic IgM were explored and various antibodies were developed that reconstituted damage in I/R-resistant mice. Zhang's group identified the critical self-ligand as a highly conserved region within non-muscle myosin heavy chain type II (NMHC-II) A and C and blocked intestinal I/R injury in WT mice using a synthetic peptide directed against this self-ligand (P8)<sup>60</sup>. Most recently, human natural IgM was also demonstrated to induce intestinal I/R injury in Rag<sup>-/-</sup> mice<sup>61</sup>.

A different hypothesis has been proposed by Tsokos and colleagues, based on several studies in which intestinal and lung tissue I/R damage is restored in Cr2<sup>-/-</sup> animals by murine and human antibodies specific for negatively-charged phospholipids and/or β-2 glycoprotein. Unlike Cr2<sup>-/-</sup> mice, reconstitution of I/R tissue damage in Rag-1<sup>-/-</sup> mouse required the infusion of both anti-β2 glycoprotein I and anti-phospholipid antibody<sup>62,63</sup>. Hence, anti-phospholipid antibodies were proposed to represent members of the injury inducing antibody repertoire missing in Cr2<sup>-/-</sup> mice. Interestingly, another known activator of the classical pathway with specificity for phospholipids, CRP, has also been suggested to play a role in mediation of intestinal I/R injury. In a rat model of intestinal I/R, CRP and IgM depositions were demonstrated to correlate with local complement activation<sup>38</sup>.

TABLE 1. Summary of animal I/R models: effects on tissue injury

Effect complement inhibitors	Effect knock-out models	Responsible pathway(s)	Triggering event
<b>Intestinal I/R</b>			
Protection from local tissue injury	Protection from local + remote tissue injury		
SCR1 (mouse <sup>37</sup> ; rat <sup>37</sup> )	C3 <sup>-/-</sup> , C4 <sup>-/-</sup> , Rag <sup>-/-</sup> mice <sup>37</sup>	CLASSICAL	IgM
C1-INH (rat <sup>37,38</sup> )	Cr2 <sup>-/-</sup> mice <sup>38,61,128</sup>	AND/OR	AND/OR
Cry (mouse <sup>39</sup> )	MBL <sup>-/-</sup> and C2/B <sup>-/-</sup> mice <sup>38</sup>	LECTIN	MBL
	No protective effect		
	C1q <sup>-/-</sup> mice <sup>38</sup>		
<b>Hindlimb I/R</b>			
Protection from remote organ injury	Protection from local tissue injury	CLASSICAL	IgM
DAF (mouse <sup>31</sup> )	C5 <sup>-/-</sup> mice <sup>132</sup>	AND/OR	AND/OR
	C3 <sup>-/-</sup> C4 <sup>-/-</sup> , Rag <sup>-/-</sup> mice <sup>37</sup>	LECTIN	MBL
	Cr2 <sup>-/-</sup> mice <sup>38</sup>		
<b>Myocardial I/R</b>			
Cardioprotective effects	Protection from local tissue injury		IgM
CVF (dog <sup>65</sup> )	Rag <sup>-/-</sup> and Cr2 <sup>-/-</sup> mice <sup>67,128</sup>	CLASSICAL	AND/OR
C1-INH (rabbit <sup>62</sup> ; feline <sup>61</sup> )	(incomplete) protection local tissue injury	AND/OR	CRP
sCR1 (rat <sup>61,66</sup> )	MBL <sup>-/-</sup> mice <sup>72</sup>	LECTIN	AND/OR
anti-C5 antibodies (rat <sup>65</sup> )	No protection in		MBL
	C1q <sup>-/-</sup> mice <sup>72</sup>		
<b>Renal I/R</b>			
Protection from ischemic acute renal failure	Protection from local tissue injury in	ALTERNATIVE	
Ab to mouse factor B (mouse <sup>69</sup> )	C3 <sup>-/-</sup> C5 <sup>-/-</sup> C6 <sup>-/-</sup> mice <sup>68</sup>	AND/OR	IgM
C5a receptor antagonists (mouse <sup>69</sup> )	mu <sup>-/-</sup> mice <sup>105</sup>	LECTIN	AND/OR
	MBL <sup>-/-</sup> mice <sup>75</sup>		MBL
	βB <sup>-/-</sup> mice <sup>75</sup>	AND/OR	
	No protection from local tissue injury	(CLASSICAL)	
	C4 <sup>-/-</sup> mice <sup>68</sup>		
	Rag <sup>-/-</sup> mice <sup>54</sup>		
<b>Hepatic I/R</b>			
Partial protection	- no studies using knock-out mice -	CLASSICAL	Unexplored
CVF (rat <sup>65</sup> )			
C5aR antagonist (rat <sup>61</sup> )			
C1-INH (rat <sup>61,66</sup> )			

MBL, Mannose-Binding Lectin; CRP, C-reactive protein; C1-INH, C1 inhibitor; CVF, Cobra Venom Factor; sCR1, soluble complement receptor 1; Cry-Ig, complement receptor-related gene Y; βB<sup>-/-</sup>, factor B deficient mice; ab, antibody

Despite the strong support for classical pathway initiation of complement activation in I/R injury, more recent studies point to an important role of the lectin pathway. Stahl and colleagues demonstrated protection from local but not remote I/R injury in MBL-deficient mice as well as in complement factor 2- and factor B-deficient (C2/fB<sup>-/-</sup>) mice. Supplementation of MBL and C2 significantly restored injury and could be reversed by monoclonal anti-MBL. In the same study, C1q<sup>-/-</sup> mice, deficient in the initiating molecule of the classical pathway, were shown to sustain intestinal damage similar to WT mice, indicating C2 and MBL, but not C1q, to be essential for intestinal I/R injury. Based on these findings a novel hypothesis was proposed, according to which natural IgM interacts with MBL instead of C1q and activates the lectin pathway, ultimately leading to tissue damage. In support of this theory, MBL was shown to be unable to induce damage in the absence of IgM, and MBL and IgM were found to co-localize in the injured tissues in WT as well as Rag<sup>-/-</sup> mice reconstituted with I/R-specific IgM<sup>64</sup>. Hence, convincing evidence performed in knock-out intestinal I/R models points both in the direction of classical and lectin pathway involvement, as well as of a variety of initiating factors. The combination of these findings into one unifying theory remains a challenging matter.

#### **Hindlimb I/R**

The hindlimb or skeletal I/R model has been used in a number of mechanistic studies on complement-mediated injury. Findings in this model are comparable to those obtained in the intestinal I/R-injury model. Hindlimb I/R is strongly attenuated in mice deficient in immunoglobulins or classical pathway components C3 and C4<sup>37,57</sup>. In Rag<sup>-/-</sup> mice, injury is restored by administration of the natural IgM clone CM22,<sup>56</sup> and blocked upon administration of the synthetic peptide P8 in WT mice<sup>65</sup>. Furthermore, IgM deposition in the injured tissues was demonstrated to precede complement activation after hindlimb I/R, presenting IgM as an early effector of the I/R cascade<sup>66</sup>. In parallel with the intestinal I/R model, more recent studies have indicated involvement of the lectin pathway instead of the classical pathway in hindlimb I/R. Likewise, dependence on MBL and C2 but not on C1q was demonstrated by restoration of injury using MBL and C2 supplementation, and reversal by monoclonal anti-MBL<sup>64</sup>. Thus, additional studies in hindlimb I/R models are required alongside the intestinal I/R model to clarify its tissue injury-inducing mechanism.

#### **Myocardial I/R**

A key role of classical pathway involvement via natural IgM has also been proposed in myocardial I/R models. Protection from tissue damage has been demonstrated in Rag-1<sup>-/-</sup> and Cr2<sup>-/-</sup> mice, as well as restoration of full injury after pre-ischemic reconstitution with wild-type IgM<sup>67</sup>. In contrast to most other models, CRP has been studied quite extensively in myocardial I/R. A key finding by Pepys and colleagues was a complement-dependent 40% increase in infarct size after administration of human CRP in a rat acute myocardial infarction model<sup>67,68</sup>. More recently, a specific small-molecule inhibitor of CRP (1,6-bis(phosphocholine)-hexane) was developed which was able to abrogate this CRP-induced increase in infarct size and its resulting cardiac dysfunction<sup>67,69</sup>. Hence, complement activation by CRP appears to be an important mediator in myocardial I/R and extrapolation of these data to clinical I/R conditions may signify an essential role of CRP. However, further support for this theory awaits additional experimental evidence.

Additional recent myocardial I/R animal studies have indicated a responsibility of the lectin pathway in mediation of tissue injury. MBL was found to colocalize with C3 throughout rat ischemic tissue<sup>70</sup>, and blocking of the MBL-pathway with an inhibitory monoclonal antibody yielded a cardioprotective effect<sup>71</sup>. More recently, MBL-deficient mice have been reported to display limited injury in a murine myocardial infarction model, whereas C1q-deficient mice were not protected<sup>72</sup>. Thus, roles of both the classical and lectin pathways have been suggested in myocardial I/R, similar to intestinal and hindlimb I/R models.

#### **Renal I/R**

Whereas the alternative pathway is generally believed to play a secondary role in I/R injury in most organs, the pathway appears to play an essential role in renal I/R. This is indicated by the finding that C3-deficient mice have been shown to be protected from injury, whereas C4-deficient mice are not<sup>49</sup>. In addition, mice deficient in factor B, a necessary component of the alternative pathway, have little detectable deposition of C3 after renal I/R<sup>73</sup>. The role of the classical pathway in renal I/R is controversial. Mu-chain-deficient mice, lacking mature B cells, have shown protection from renal I/R, suggesting that immunoglobulins may be important initiators of injury in this model. However, several findings contradict this hypothesis. The mu<sup>-/-</sup> mice did not show decreased C3 deposition in the cited study and subsequent restoration of circulating IgM failed to restore injury. Furthermore, Rag-1<sup>-/-</sup> mice are not protected from local injury and neither is IgG seen deposited in the post-ischemic kidney<sup>74</sup>. In parallel with the other I/R models, more recent reports have focussed on the lectin pathway and supported the view that complement activation in renal I/R may occur via the lectin pathway. Evidence for this theory was provided by the demonstration of MBL binding to structures in the post-ischemic murine kidney<sup>48,75</sup>, as well as protection from ischemic tissue damage in MBL-deficient mice, with an increase in C3 deposition compared with WT controls<sup>75</sup>. Hence, a clear specific pathway involvement has not been elucidated in renal I/R to date.

#### **Hepatic I/R**

The number of studies exploring the role of the complement system and its individual pathways in the pathogenesis of hepatic I/R injury has been limited. Hepatocytes are responsible for biosynthesis of about 80-90% of plasma complement components and their soluble regulators and one could speculate that the local production of these soluble regulators may contribute to the limited protection from tissue injury when using complement inhibitors in hepatic I/R injury. Classical pathway-mediated complement activation has been suggested in a rat hepatic I/R model in which CRP has been demonstrated to colocalize with C3 and the membrane attack complex C5b9<sup>92</sup>. To our knowledge, a role for the lectin or alternative pathway-mediated hepatic I/R injury has not been described in literature to date.

Hence, combining these results into a unifying theory regarding I/R injury in these animal models remains a major challenge to date.

## COMPLEMENT-MEDIATED INJURY IN HUMAN STUDIES

Complement-mediated tissue injury in the human setting has been studied most extensively in myocardial infarctions. Evidence on different levels has been collected, i.e. ischemic damage and mortality after complement inhibition, immunohistochemical depositions in ischemic tissue and analysis of complement factors in plasma.

### Complement inhibitors

A number of clinical trials have aimed to attenuate myocardial tissue injury using complement inhibitors such as sCR1, C1-INH and Pexelizumab, a recombinant humanized monoclonal antibody to C5. During coronary artery surgery, a protective effect has been demonstrated using C1-INH<sup>76-78</sup>, and administration of sCR1 in cardiac surgery patients resulted in a reduction of ischemic damage<sup>79</sup>. More recently, a meta-analysis and several large phase III trials showed a reduction in mortality in coronary bypass surgery and myocardial infarction patients using Pexelizumab<sup>80-82</sup>. In several other randomized, placebo-controlled trials, however, infarct size<sup>83,84</sup> and mortality<sup>85</sup> remained unaffected following administration of the monoclonal antibody. In general, clinical trials of complement inhibitors have yielded disappointing results, as summarized in *Table 2*.

### Immunohistochemical studies

In several studies using autopsy material of myocardial infarction patients, the immunohistochemical demonstration of complement activation points to the classical pathway as the main mediator of tissue damage. Depositions and co-localization patterns are in line with activation by CRP and/or IgM: CRP was found to be co-localized with activated complement components in infarcted tissue, whereas neither component was detected in unaffected myocardial tissue<sup>86</sup>. Moreover, the extent of CRP depositions correlated with complement deposition<sup>87</sup>. A later study demonstrated co-localization of IgM with complement and CRP in infarcted myocardium<sup>88</sup>, suggesting binding of the deposited IgM to the same ligands as CRP. A limited number of human studies have been performed to investigate the role of CRP and IgM in complement-mediated I/R damage in other organs than myocardium. In human liver resections, immunohistochemical analysis of *in situ* complement activation in ischemic tissues pointed to a role of CRP<sup>89</sup>. Infarction sites of multiple organs during sepsis demonstrated co-localisation of CRP and activated complement, whereas non-infarct sites were negative for both<sup>90</sup>. Most importantly, MBL depositions have not been demonstrated in human tissue to date. In contrast to the recent emphasis on the role of the lectin pathway in animal studies, limited evidence is therefore available for the role of this pathway in the human setting.

TABLE 2: Summary of clinical trials using complement inhibition

Cardioprotective strategy	Trial	No. of patients	Patients	Timing of intervention	Effect on primary end point(s)
Pexelizumab (Alexion) <sup>80</sup>	COMPLY	943	Patients with acute MI undergoing thrombolytic reperfusion therapy	Before / during thrombolysis	No difference in CK-MB-measured infarct size or 90-day composite end point of death, cardiac failure, or stroke
Pexelizumab (Alexion) <sup>81</sup>	COMMA	960	Patients with acute MI undergoing primary PCI	Before PCI	No difference in CK-MB-measured infarct size or 90-day composite end point of death, cardiac failure, or stroke
Pexelizumab (Alexion) <sup>82</sup>	PRIMO-CABG	3099	Patients undergoing CABG surgery with or without valve surgery	10 min before surgery	No difference in composite end point of death or myocardial infarction in patients undergoing CABG surgery only (n=2746). Significant 30-day risk reduction among patients undergoing CABG with or without valve surgery (n=3099)
Pexelizumab (Alexion) <sup>85</sup>	APEX-AMI	5745	Patients with acute MI undergoing primary PCI	Before PCI	No difference in 30-day mortality or 90-day composite end point of death or cardiac failure
C1-INH <sup>78</sup>	—	80	Patients with acute MI undergoing primary CABG	10 min before reperfusion (aortic unclamping) followed by continuous administration 3h after surgery	No difference in primary endpoint of early mortality. Significant difference in postreperfusion serum cTnI release
sCR1 <sup>79</sup>	—	564	High-risk patients undergoing cardiac surgery on CPB	Immediately before CPB	No difference in composite endpoint of death, myocardial infarction, prolonged intra-aortic balloon pump support and prolonged intubation

MI, myocardial infarction; PCI, percutaneous transluminal coronary intervention; CPB, cardiopulmonary bypass; CABG, coronary artery bypass graft; C1-INH, C1 inhibitor; CrV, Cobra Venom Factor; sCR1, soluble complement receptor 1

All trials were randomized, double-blind, and placebo-controlled.

### Plasma analysis

Analysis of circulating levels of complement-related components has yielded additional information regarding the involvement of complement and its individual pathways in I/R injury. Using this approach, C4 has been demonstrated to be a predictor of stroke in patients with coronary artery disease referred for coronary angiography<sup>90</sup>. Furthermore, C3 and C4 have been shown to correlate with the incidence of myocardial infarction and stroke in a population-based study<sup>91</sup>. Thus, these clinical studies are in favour of a role of complement in these cardiovascular diseases.

Plasma levels of CRP have been studied extensively in ischemic settings. In a human acute ischemic stroke study, an early rise in CRP and a following increase in the terminal complement complex in plasma correlated to the size of infarction, suggesting a possible role for CRP in complement activation and the degree of inflammation after stroke<sup>92</sup>.

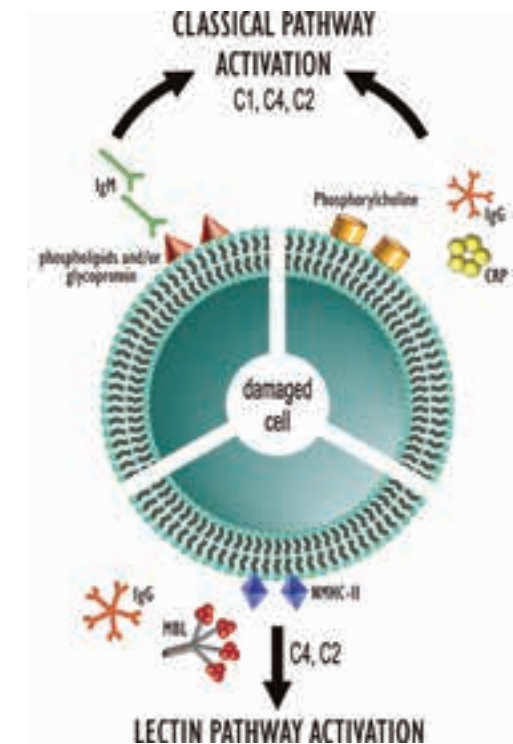
The majority of studies exploring levels of MBL in the human I/R setting have been performed in aortic aneurysm repair patients. A decrease in plasma MBL levels by approximately 40% during aneurysm repair<sup>93</sup> as well as substantial complement activation, has been documented<sup>94</sup>. Interestingly, MBL-deficient patients undergoing this procedure do not demonstrate an increase in complement activation products. In the transplantation setting, correlations between MBL levels and post-transplant outcome have been demonstrated, whereas low MBL has been found to be related to the development of post-transplant coronary artery disease and acute rejection after heart transplantation<sup>95</sup>. Moreover, the MBL-pathway has been shown to be activated in acute renal failure following renal transplantation<sup>96</sup>. Thereby, these studies suggest an involvement of the MBL pathway in human I/R-injury.

### Complement deficiencies

Assuming that the classical and/or lectin pathways of complement are essential mediators of I/R injury, individuals deficient in specific pathway components could be hypothesized to be (partially) protected from injury. Deficiency of MBL is the result of several identified polymorphisms in the promoter regions and coding sequence of the MBL gene<sup>97,98</sup>. The fact that it is the most frequent genetic complement deficiency, occurring in 10–30% of Caucasians, depending on definition<sup>99</sup>, has enabled extensive research within this population. Lectin pathway dysfunction has been associated with susceptibility to infections and disease outcome in children<sup>100,101</sup>, acute pancreatitis<sup>102</sup>, peritonitis<sup>103</sup>, liver transplantation<sup>104</sup> and sepsis<sup>105</sup>. Classical pathway deficiencies (i.e. C1q, C4 and C2) are less common but are known to predispose for development of the autoimmune disease SLE<sup>106</sup>. Increased risk of infection has also been described in this population, including increased postoperative infections after renal transplantation in patients with hereditary C4 deficiency<sup>107</sup>. The influence on susceptibility to infection is thought to result from an impaired defence to infecting micro-organisms in complement-deficient individuals. The absence of complement components severely hampers the opsonization of invading organisms, complement-mediated lysis, as well as modulation of the appropriate pro-inflammatory cytokine response. Unfortunately, very little reports are known to date concerning the extent of I/R-related tissue injury in complement-deficient individuals. Whether a defective complement system protects from human I/R injury therefore remains largely unknown.

## INITIATION OF COMPLEMENT ACTIVATION IN I/R-INJURY

The above mentioned observations in various organ models led to the hypothesis that I/R injury is initiated by recognition and binding of candidate proteins to neo-epitopes exposed on injured cells. A general mechanism may apply in which hypoxia-related events result in exposition of neo-antigens on injured membranes and subsequent recognition leads to activation of the complement system and acute inflammation, ultimately resulting in tissue injury. Based on animal as well as human studies, several potential neo-epitopes have been suggested to be involved in this process and are depicted in *Figure 2*.



*Figure 2. Proposed models of binding of extracellular proteins to neo-epitopes expressed on injured cell surfaces. According to these theories, exposure of neo-epitopes on injured cells is triggered by an ischemic insult. Potential neo-epitopes proposed by three different groups are depicted. Molecules such as IgM, IgG, MBL and/or CRP are presumed to bind to these neo-epitopes and activate complement as a result, ultimately leading to enhanced cell injury. MBL, mannose-binding lectin; CRP, C-reactive protein; NMHC-II, non-muscle myosin heavy chain type II.*

### Non-muscle myosin heavy chain type II (NMHC-II) A and C

Zhang and colleagues identified a neo-epitope responsible for intestinal and hindlimb I/R injury by isolation of IgM-antigen complexes from Rag<sup>-/-</sup> mice reconstituted with pathogenic IgM. Separation on SDS-PAGE led to identification of a unique band in mice subjected to ischemia. Analysis of this band using tandem mass-spectrometry identified the self-antigen as a highly conserved region within non-muscle myosin heavy chain type II (NMHC-II) type A and C. Subsequently a theory was proposed in which natural IgM antibodies bind to NMHC-II via transient exposure of its N2 region on the outer cell surface of endothelium and muscle cells in the early phase of reperfusion injury. As animal studies clearly pointed to a role of MBL and the lectin pathway in I/R-mediated tissue injury, recognition of NMHC-II and deposition of IgM was suggested to either facilitate binding of MBL to IgM or to expose an MBL binding site on ischemic tissue. This binding was proposed to then lead to activation of the complement system via the lectin pathway<sup>60</sup>. However, this binding of MBL to deposited IgM with resulting complement activation has not yet been confirmed in vivo.

A number of *in vitro* studies have examined the ability of immunoglobulin binding to MBL and subsequent complement activation. Several authors report direct binding of murine MBL to murine IgM<sup>60,108,109</sup> as well as rat MBL binding to human IgM<sup>110</sup>. Moreover, immobilized human MBL has been shown to bind 20% of human serum IgM. This subpopulation of MBL-binding glycoforms were found to be enriched in GlcNAc-terminating glycans, which are ligands for MBL<sup>108</sup>. Hence, only this sub-population of suitably glycosylated IgM may have bound MBL. *In vitro* indications for lectin pathway activation via binding of immunoglobulins is provided by a number of findings. IgM, IgG, and IgA have been shown to enhance lectin pathway-mediated hemolysis<sup>111</sup> and MBL has been demonstrated to augment phagocytosis of IgM- or IgG- and C4b-opsonized erythrocytes<sup>112</sup>. Furthermore, Roos and colleagues demonstrated binding of polymeric IgA to MBL and resulting complement activation via the lectin pathway<sup>113</sup>, as well as compensation of impaired target opsonization via the lectin pathway by antibody-mediated classical pathway activation in serum of MBL-deficient individuals<sup>114</sup>.

### Phospholipids and/or $\beta$ -2 glycoprotein

Based on findings in animal studies, negatively-charged phospholipids and/or  $\beta$ -2 glycoprotein were postulated to be the epitopes responsible for induction of I/R damage by Tsokos and colleagues. This hypothesis is based on the observation that antibodies specific for these components restored I/R-induced tissue damage in knock-out animals<sup>62,63</sup>. According to this theory, natural antibodies recognize ischemia or stress altered cells produced by B cells in response to antigen-C3dg complexes crosslinking the B cell receptor and Cr2. Autoantibodies against phospholipids are suggested to bind to stressed cells, hence leading to complement activation. However, these negatively-charged phospholipids and/or  $\beta$ -2 glycoprotein has not been isolated from ischemic tissue to date.

### Phosphorylcholine

Studies performed in human myocardial tissue also point in the direction of phospholipids as stress-induced self-antigens. In immunohistochemical studies of human myocardial tissue, complement was found to co-localize with CRP and IgM in infarcted zones. As IgM and CRP demonstrated binding to the plasma membrane of cardiomyocytes in the infarcted myocardium, this membrane in particular was suggested to expose the involved ligands. Co-localisation patterns and relative staining intensities have suggested IgM and CRP to recognize similar epitopes<sup>88</sup>. CRP is known to bind phosphatidylcholine and particularly lyso-phosphatidylcholine via phosphorylcholine, and hence this chemical group can be hypothesized to be a potential epitope. Based on these findings, a hypothesis has been proposed in which the increased production of oxygen radicals together with enhanced activity of the enzyme secretory phospholipase A2 (sPLA2) which cleaves phospholipids, generates binding sites in the flip-flopped membrane of cardiomyocytes in the ischaemic myocardium. As a consequence the binding of both natural IgM and CRP is promoted, leading to activation of complement and ultimately to irreversible tissue injury. Together, these data indicate that multiple antigens may initiate antibody binding to cells exposed to I/R, as summarized in *Table 3*. Additional studies on each of these antigens are needed to evaluate their cell surface exposure during ischemia.

TABLE 3: Proposed models of complement activation in I/R tissue injury

Setting	Neo-epitope(s)	Antigen(s)	Primary evidence	Proposed mechanism	Responsible pathway
Human Myocardial infarction <sup>99</sup>	IgM and CRP	Epitopes in ischaemic cardiomyocyte membranes, hypothesized to be PC	Immunohistochemical co-localisation of IgM and CRP in infarcted zones	Ischemic myocardium: ROS production; sPLA2 activity! → binding sites generated in flip flopped cardiomyocyte membranes → natural IgM and CRP binding → complement activation	CLASSICAL
Animal Intestinal I/R <sup>11</sup>	Abs against phospholipids and/or $\beta$ -2 glycoprotein	Phospholipids and/or $\beta$ -2 glycoprotein	Specific antibodies restore tissue injury in KO models	Natural abs produced by in response to antigen-C3dg complexes crosslinking B cell receptor and Cr2 recognize stress altered cells. Auto-abs against phospholipids and/or $\beta$ -2 glycoprotein also bind stressed cells → complement activation	CLASSICAL
Animal Intestinal I/R <sup>64,107</sup>	Non-muscle myosin heavy chain type II A, C	Natural IgM and MBL	Self-antigen isolated from mice. Intestinal I/R in WT mice blocked with peptide against self-ligand (P8)	Transient exposure of N2 region of natural IgM → binding to NMHC-II on injured cells → facilitation of binding of MBL to IgM or exposition of MBL binding site on ischemic tissue → complement activation	LECTIN

sPLA2, secretory Phospholipase A2; CRP, C-reactive Protein; MBL, Mannose-Binding Lectin; abs, antibodies; ROS, radical oxygen species; KO, knock-out; PC, phosphorylcholine

## INTEGRATED VIEW ON MOLECULAR MECHANISMS INVOLVED IN I/R INDUCED COMPLEMENT ACTIVATION

Most animal and human studies suggest involvement of the lectin and the classical pathway in I/R-mediated injury. However, combining these findings into one hypothesis as well as extrapolation to the human setting is challenging. Whilst in mouse models large effects are observed upon blocking single pathways, clinical trials evaluating complement inhibitors show small and inconsistent reductions of I/R-injury. These disappointing attempts to translate the beneficial effects shown in animal models into the human setting have raised the question whether the animal models provide relevant information for human disease. Several reasons for this disparity between findings in animal and human myocardial infarction have been proposed and discussed in literature<sup>2,115</sup>.

Firstly, the interspecies differences and species specificities in complement-mediated tissue damage may signify that conclusions from animal studies may not automatically apply to

the human situation. CRP has been described most frequently in this context, as its production and function is known to vary substantially among species. In humans and rabbits, CRP is an acute phase-protein with levels that can increase up to a 1000-fold from base-line during an acute phase response. In mice however, CRP is not an acute-phase protein and circulating levels rarely exceed 2µg/ml<sup>116,117</sup>. In rats, CRP is not a typical acute phase protein, as it circulates at levels approximately 100 times higher than human concentrations under normal and disease conditions<sup>118</sup>. Moreover, differences in pathway activities between strains have been described, such as different classical pathway activities in the murine strains C57BL/6 and BALB/c<sup>119,120</sup>. To further complicate matters, a number of studies have described species specificities of CRP in complement activation, as human CRP has been shown not to react with mouse C1q<sup>121</sup> or guinea pig C1q<sup>122</sup>.

Secondly, information regarding the protective effects of most complement inhibitors in animal I/R models must be regarded with caution, at least regarding involvement of various pathways, as most of these inhibitors are pathway non-specific. Soluble CR1, for example, binds and inhibits both C3 and C5 convertases, and thereby inhibits all complement pathways<sup>64</sup>. C1-INH has been shown to inhibit both the classical<sup>123,124</sup> and lectin complement pathways<sup>125</sup>, as well as exert a direct anti-apoptotic effect<sup>126</sup>. CVF, another agent that has frequently been used to study the role of complement in animal models, is not an inhibitor but a fluid phase activator of complement. Upon administration of this agent, strong complement activation occurs, as a result of which circulating levels of complement proteins such as C3 and C5 in particular, markedly drop. This yields a temporary deficiency state due to consumption of these factors. This initial activation process induced by CVF may have altered host responses to subsequent disease challenges and therefore complement deficiency induced by CVF may not be the same as the use of an inhibitor or a genetic (knock-out) deficiency.

Thirdly, the majority of studies exploring the role of individual pathways in I/R injury are performed in knock-out mice. However, results based on such animal studies may not reflect clinical reality as knock-out mice may develop compensatory mechanisms that influence the susceptibility to I/R injury, or mask the effect of depletion of the targeted protein<sup>127</sup>.

Importantly, it should also be noted that experimental animal models are often designed so that certain mediators, e.g. activated complement factors, are dominant in the pathophysiology. However, in human disease the relative importance of individual classes of pro-inflammatory mediators presumably varies amongst others depending on factors such as the duration and severity of the ischemia. In the pathogenesis of I/R injury other mediators than complement activation are also involved, such as the formation of cytokines and chemokines. The latter have a multitude of overlapping functions and pro-inflammatory effects with complement factors. Hence, multiple recognition molecules may be involved in mediating I/R-injury rather than a single specific complement-activation initiating factor. According to this theory, postulated by Thrane and colleagues, an inflammatory response itself may create additional antigens involved in I/R-injury<sup>128</sup>. Hence, the rapid amplification

inherent in immune cascades may complicate identification of the triggering molecular events. Notably, immune effectors such as complement are assumed to play dual roles in both causing and resolving I/R.

This questions the effectivity of a therapeutic strategy targeting one single mediator or pathway to prevent or reduce I/R injury. A more effective therapeutic intervention may therefore be to target more than one mediator simultaneously. This theory is underlined by the observation that ischemic postconditioning, which inherently targets several mediators of lethal reperfusion injury, has achieved persistent infarct size reduction in patients with acute myocardial infarction<sup>129</sup>. Hence, blocking of multiple neo-antigens that synergize in mediating I/R-injury may achieve a larger mortality reduction<sup>128</sup>.

## CONCLUSIONS

Complement activation has been shown to be an early event in I/R-injury. Therefore, inhibition of complement activation or its components may offer tissue protection after reperfusion. As results to date using complement inhibitors in clinical studies have largely been disappointing, identification of a more specific pathogenic target for therapeutic intervention appears to be warranted. For this purpose advanced knowledge of the responsible pathways for I/R-injury is required. Recent evidence from in vitro and in vivo models may indicate that the classical as well as the lectin pathways are involved in I/R-injury via exposition of neo-epitopes in ischemic membranes. However, most of these findings have been obtained in knock-out murine models. The hypotheses provided by these animal studies have for a large part remained unconfirmed in the human setting. Most importantly, involvement of MBL has not been demonstrated at tissue level in human I/R-injury to date. Thus, conclusions drawn from animal I/R studies should be extrapolated to the human setting with caution.

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