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Targeting mechanisms at sites of complement activation for imaging and therapy

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Abstract

The complement system plays a key role in many acute injury states as well as chronic autoimmune and inflammatory diseases. Localized complement activation and alternative pathway-mediated amplification on diverse target surfaces promote local recruitment of pro-inflammatory cells and elaboration of other mediators. Despite a general understanding of the architecture of the system, though, many of the mechanisms that underlie site-specific complement activation and amplification *in vivo* are incompletely understood. In addition, there is no capability yet to measure the level of local tissue site-specific complement activation in patients without performing biopsies to detect products using immunohistochemical techniques. Herein is reviewed emerging evidence obtained through clinical research studies of human rheumatoid arthritis along with translational studies of its disease models which demonstrate that several parallel mechanisms are involved in site-specific amplification of activation of the complement system *in vivo*. Among these processes are de-regulation of the alternative pathway, effector pathway-catalyzed amplification of proximal complement activation, recognition of injury-associated ligands by components of the lectin pathway, and engagement of pathogenic natural antibodies that recognize a limited set of injury-associated neopeptides. Studies suggest that each of these inter-related processes can play key roles in amplification of complement-dependent injury on self-tissues *in vivo*. These findings, in addition to development of an imaging strategy described herein designed to quantitatively measure local complement C3 fixation, have relevance to therapeutic and diagnostic strategies targeting the complement system.

Keywords

Alternative Pathway; Complement Therapeutics; Pathogenesis; Lectin Pathway; Molecular Imaging; Arthritis; Effector Pathways

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Introduction

The complement system is a major component of innate immunity (Fearon and Locksley, 1996) and plays central roles in many protective immune processes [reviewed in (Holers, 2001; Holers, Carroll, and Holers, 2005; Ricklin, Hajishengallis, Yang, and Lambris, 2010)]. In contrast to these beneficial roles, inappropriate complement activation that is directed not to foreign pathogens but rather to self-tissues is also believed to underlie the pathogenesis of many human inflammatory and autoimmune diseases (Ricklin and Lambris, 2013). This review will focus on the human autoimmune disease rheumatoid arthritis (RA) and the use of a murine model of this process to identify and characterize specific mechanisms that underlie complement-mediated joint damage. In addition, a new approach to characterizing local complement C3 activation through molecular imaging will be introduced.

The study of RA is particularly relevant in light of new findings that suggest the complement system plays key roles at the initiation of inflammatory arthritis, where the pathophysiology of disease is likely to be different than in the chronic stages (Holers, 2013a). RA is also an especially relevant human disease to study the effects of chronic complement activation, as many previous studies have demonstrated elevated activation fragments in patients with longstanding disease (Linton and Morgan, 1999; Monach, Benoist, and Mathis, 2004; Neumann, Barnum, Tarner, Echols, Fleck, Judex, Kullmann, Mountz, Scholmerich, Gay, and Muller-Ladner, 2002).

New roles of complement in incipient human rheumatoid arthritis

RA is an important human autoimmune disease and exhibits the second highest prevalence of the autoimmune diseases (Silman, 1994; Silman and Hochberg, 1993). RA is a disease that extensively involves both the cartilage and synovium as potential targets of complement-mediated damage. With regard to the human cartilage surface, studies have demonstrated the presence of IgG-containing immune complexes as well as complement C3 activation fragments in >90% of chronic RA patient samples (Cooke, Hurd, Jasin, Bienenstock, and Ziff, 1975). The heavily infiltrated synovium is also a site of extensive complement deposition and synthesis of activation pathway proteins and receptors in patients and animal models (Linton *et al.*, 1999; Neumann *et al.*, 2002). As one potential link to complement activation mechanisms, it is now appreciated that disease-associated autoantibodies such as anti-citrullinated protein/peptide antibodies (ACPA) and rheumatoid factors (RF) are both associated with poor outcomes and are pathogenic when tested in animal models (Klareskog, Ronnelid, Lundberg, Padyukov, and Alfredsson, 2008).

Once well established, chronic human RA appears to be a largely cytokine-driven inflammatory disorder characterized by joint damage and disability (Firestein, 2003). Nevertheless, while this is true of longstanding disease, recent studies that are focused on the very early development of human RA suggests a more complex pathogenic process (Figure 1) (Holers, 2013a; Klareskog *et al.*, 2008). In this regard, it is now apparent that subjects first break immune tolerance at a site peripheral to the joints and develop circulating RA-related autoantibodies which recognize citrullinated epitopes, a post-translational modification on self-proteins. These RA-related ACPA are present for many years in asymptomatic

individuals (Deane, Norris, and Holers, 2010) and are focused on a subset of citrullinated autoantigens including fibrinogen, vimentin, enolase and type II collagen (CII) (Klareskog *et al.*, 2008; van Venrooij and Pruijn, 2000; van Venrooij and van de Putte, 2005). Following this period of circulating autoantibodies in the absence of signs or symptoms of arthritis, it appears that when one or more of the citrullinated autoantigens develops in the joint, likely generated through a neutrophil-driven inflammatory process (Khandpur, Carmona-Rivera, Vivekanandan-Giri, Gizinski, Yalavarthi, Knight, Friday, Li, Patel, Subramanian, Thompson, Chen, Fox, Pennathur, and Kaplan, 2013; Klareskog *et al.*, 2008), or when circulating immune complexes deposit in the joint (Sokolove, Zhao, Chandra, and Robinson, 2011), a transition occurs wherein clinically apparent arthritis is now present. Through engagement of effector pathways, these autoantibodies then appear to cause joint damage (Klareskog *et al.*, 2008). Thus, in contrast with chronic well-established RA, the pathogenesis of early arthritis appears now to be very consistent with an immune complex-driven disorder similar to murine passive transfer models (Klareskog *et al.*, 2008; Monach *et al.*, 2004). In that light, studies of the effects of autoantibody disease transfer models are highly relevant, especially as mechanism-based designs for very early treatment and prevention strategies for patients in this phase of disease are being developed (Demoruelle and Deane, 2012; Klareskog, Gregersen, and Huizinga, 2010).

Consistent with this model, our own recent studies have confirmed prior findings that subjects with both longstanding chronic RA as well as in subjects who are in the early clinical initiation period demonstrate evidence of complement activation manifest by increases in the activation fragments C3a and sC5b-9 (Holers and Kinslow, unpublished). Thus, complement activation is likely to be involved in the early phases of joint and/or other tissue inflammation in humans. Notably, type II collagen (CII), the target of the pathogenic antibodies utilized in the murine studies described below, is also an autoantigen in its native, glycosylated and citrullinated forms in a substantial subset of patients with RA (Backlund, Carlsen, Hoger, Holm, Fugger, Kihlberg, Burkhardt, and Holmdahl, 2002; Uysal, Bockermann, Nandakumar, Sehnert, Bajtner, Engstrom, Serre, Burkhardt, Thunnissen, and Holmdahl, 2009).

Complement Pathogenic Mechanisms in Inflammatory Arthritis Models

Local Activation and Amplification Pathways

Several groups have worked to define the pathogenic role of antibodies and the complement system in animal models of RA (Linton *et al.*, 1999; Monach *et al.*, 2004). Herein the focus of the discussion is upon the CII antibody-induced arthritis (CAIA) model wherein a set of high affinity mAbs directed to epitopes within CII are used to induce disease in susceptible strains (Banda, Thurman, Kraus, Wood, Carroll, Arend, and Holers, 2006). Initial studies of the mechanisms underlying damage demonstrated that, while the classical and lectin pathways are engaged following antibody binding in the joint, the alternative pathway was both necessary and sufficient to develop arthritis through its apparent roles in both the initiation and amplification steps (Banda, Takahashi, Wood, Holers, and Arend, 2007; Banda *et al.*, 2006). Subsequent studies in the model revealed that mannose-binding lectin (MBL)-associated serine proteases MASP-1 and MASP-3 are required to cleave the inactive

alternative pathway member pro-factor D (FD) into active FD, which could then function in the alternative pathway C3 convertase, and that this activity was necessary to develop substantial joint damage (Banda, Takahashi, Levitt, Glogowska, Nicholas, Takahashi, Stahl, Fujita, Arend, and Holers, 2010; Banda, Takahashi, Takahashi, Stahl, Hyatt, Glogowska, Wiles, Endo, Fujita, Holers, and Arend, 2011).

With regard to mechanisms of activation within the joint, FD appeared to be produced locally in synovial adipose tissue (SAT), and cultured differentiated 3T3 adipocytes, a surrogate for SAT, produced pro-FD but no mature FD. In contrast, fibroblast-like synoviocytes (FLS), a major cell type centrally involved in the inflamed joint (Bottini and Firestein, 2013), were the main source of MASP-1/3 mRNA and protein (Arend, Mehta, Antonioli, Takahashi, Takahashi, Stahl, Holers, and Banda, 2013). Using cartilage microparticles (CMPs) coated with anti-CII mAb and serum from *MASP-1/3(-/-)* mice as a source of factor B in the absence of active FD, pro-FD in 3T3 supernatants was cleaved into mature FD by MASP-1/3 in FLS supernatants. These results strongly suggest that pathogenic activation of the alternative pathway can occur in the joint through immune complexes adherent to cartilage, and that local production of alternative pathway proteins by adipocytes and FLS is likely to be sufficient to develop damage.

Additional studies of CAIA have also begun to unravel the role of the lectin pathway in promotion of complement-dependent injury. Despite previously demonstration of no effect on the development of CAIA in mice lacking MBL-A/C expression (Banda *et al.*, 2007; Banda *et al.*, 2006) [there are two MBL encoding genes and proteins in mice (Kjaer, Thiel, and Andersen, 2013)], more recent findings in several other experimental settings have rekindled interest in this pathway. The first was the publication of data demonstrating substantial increases in lectin pathway components in RA patients (Ammitzboll, Thiel, Ellingsen, Deleuran, Jorgensen, Jensenius, and Stengaard-Pedersen, 2012). The second was the emerging understanding of the complexity of the recognition and protease components of the lectin pathway and the consideration that recognition of ligands by other pattern recognition molecules [reviewed in (Kjaer *et al.*, 2013)] could engage MASPs and “bypass” the lack of MBL expression and activate C3 in the MBL-A/C deficient mice that were previously evaluated. And the third was findings in a model of aortic injury wherein IgG antibody activated the alternative pathway amplification loop through MBL-A/C engagement (Zhou, Yana, Bertrama, Hua, Springer, Thompson, Curci, Hourcade, and Pham, 2013), which further suggested a critical *in vivo* link between the pathways.

The lectin pathway is initiated by a family of pattern recognition molecules, designated the collectins, which includes mannose-binding lectin (MBL) (Ip, Takahashi, Ezekowitz, and Stuart, 2009; Kjaer *et al.*, 2013), Ficolins (Matsushita, 2010; Runza, Schwaeble, and Mannel, 2008) and collectin-K1 (CL-K1, also designated collectin 11) (Hansen, Sehman, Palaniyar, Ziegler, Brandt, Kliem, Jonasson, Skjoedt, Nielsen, Hartshom, Jorgensen, and Holmskov, 2010; Ohtani, Suzuki, and Wakamiya, 2012) that recognize diverse ligands and initiate complement activation. Protective roles of this family include the recognition and clearance of foreign organisms, such as bacteria and viruses (Gadjeva, Takahashi, and Thiel, 2004; Reid and Turner, 1994; Runza *et al.*, 2008) through recognition of certain densely displayed monosaccharides (glucose, mannose) as well as acetylated carbohydrates, such as

N-acetyl glucosamine, that are preferentially found on pathogens. In addition to this protective role, however, the lectin pathway is also known to be engaged during tissue injury either through the direct recognition of newly exposed self-ligands for MBL recognition (Collard, Montalto, Reenstra, Buras, and Stahl, 2001) or through IgG autoantibodies containing agalactosyl (G0) carbohydrates that interact with MBL (Malhotra, Wormald, Rudd, Fischer, Dwek, and Sim, 1995). After recognition, it is understood that lectin pathway activation can proceed through the serial catalytic activities of MASP-1 and MASP-2, resulting in cleavage and activation of C4 and C2, and then cleavage of C3 into C3b through the shared classical pathway C3 convertase designated C4b2a. It has also been postulated that there is direct cleavage and activation of C3 through MASP-1, bypassing MASP-2; however, the *in vitro* rate of activation is slow, and it is not known yet if this process occurs *in vivo* (Rossi, Cseh, Bally, Thielens, Jensenius, and Arlaud, 2001).

As one means to block activation of the lectin pathway in its entirety, a novel inhibitor designated MAP-44 (also called MAP-1) has been identified. MAP-44 is an alternatively spliced product of the MASP-1/3 gene that contains only the MASP recognition domains for the collectins and does not contain a protease domain (Degn, Hansen, Steffensen, Jacobsen, Jensenius, and Thiel, 2009; Skjoedt, Hummelshoj, Palarasah, Honore, Koch, Skjodt, and Garred, 2010). MAP-44 is normally present at very low circulating levels, < 2 micrograms/ml in serum. MAP-44 was shown *in vitro* to compete with MASP-1, MASP-2, and MASP-3 for binding to all of the collectins and to act as a dominant composite negative inhibitor of all three pattern recognition mechanisms (Degn, Jensen, Olszowski, Jensenius, and Thiel, 2013; Pavlov, Skjoedt, Tan, Rosbjerg, and Garred, 2012; Skjoedt *et al.*, 2010). Notably, use of recombinant MAP-44 in a murine model of myocardial infarction resulted in decreased injury (Pavlov *et al.*, 2012), although this model is distinct from CAIA in that it is MBL-dependent.

To evaluate the effect of inhibiting the lectin pathway using MAP-44, an Adeno Associated Virus (AAV) approach (Bakker, van de Loo, Joosten, Bennink, Arntz, Dmitriev, Kashentsera, Curiel, and van den Berg, 2001) was developed by constructing AAV directing the synthesis of hemagglutinin (HA)-tagged human and mouse MAP-44, along with a control AAV expressing Green Fluorescent Protein (GFP). Treatment of mice undergoing CAIA with AAV directing expression of human and mouse MAP-44 both resulted in marked inhibition of the development of CAIA (Banda, Mehta, Kjaer, Takahashi, Schaack, Morrison, Thiel, Arend, and Holers, 2014). This decrease in clinical disease activity was mirrored by a dramatic decrease in histologic injury and C3 deposition on the cartilage and in synovium, and decreased synovial IL-1 β and TNF- α expression in treated mice. These data demonstrated a previously unrecognized but apparently essential role for the lectin pathway in the development of inflammatory arthritis and severe joint damage.

Local Alternative Pathway Regulatory Mechanisms

Given the importance of the alternative pathway in local joint injury, it is also relevant to explore the role of complement regulatory mechanisms that normally would control activation in this site. Prior reviews of the topic have largely focused on the role of membrane regulatory proteins (Linton *et al.*, 1999; Liszewski, Farries, Lublin, Rooney, and

Atkinson, 1996), and thus this topic will not be reviewed. With regard to soluble regulatory proteins, though, factor H (FH)-mediated control of complement activation is increasingly understood to be centrally involved in the pathogenesis of human diseases, especially where mutations or highly informative polymorphisms of FH are associated with diseases such as atypical hemolytic uremic syndrome (aHUS) and membranoproliferative glomerulonephritis type II (MPGN Type II) (Jha, Bora, and Bora, 2007; Jokiranta, Zipfel, Fremeaux-Bacchi, Taylor, Goodship, and Noris, 2007; Kavanaugh, Goodship, and Richards, 2006)].

Importantly, FH can function in both the fluid phase and on surfaces, and the major characteristic that controls its surface inhibitory capacity is its relative ability to bind in a tissue-specific manner to glycosaminoglycans (GAG) as well as C3b/C3d molecules that are fixed onto the target surface [reviewed in (Zipfel, Skerka, Hellwage, Jokiranta, Meri, Brade, Kraicz, Noris, and Remuzzi, 2002)]. FH exhibits in both the fluid phase as well as on surfaces both cofactor activity for factor I mediated cleavage of C3b as well as decay-accelerating activity for the alternative pathway initiation and amplification C3 convertases. FH exhibits a modular structure with the C3 regulatory sites in short consensus repeat (SCR)1-4 and a series of binding sites for ligands in the carboxy-terminal 16 SCRs. Factor H has been suggested to interact with target cells and tissues through several SCR-containing domains, including SCR5-7, which is associated by the “unfurling” of the molecule over the surface, the primary dependence of SCR19-20 interactions with GAGs and C3b/C3d for stable binding, and the elaboration of protective function blocking further C3 activation at the site (Jozsi, Manuelian, Heinen, Opperman, and Zipfel, 2004; Jozsi, Opperman, Lambris, and Zipfel, 2007).

To explore the role of FH in CAIA, its binding to tissues was blocked through administration to wild type mice undergoing disease induction of a recombinant dominant negative inhibitor containing SCRs 19 and 20 (rFH19-20), which impairs FH function and amplifies surface alternative pathway activation *in vitro* (Ferreira, Herbert, Hocking, Barlow, and Pangburn, 2006). Administration of rFH19-20, but not control rFH3-5, was found to significantly increase clinical disease activity, histopathologic injury and C3 deposition in the synovium and cartilage in both wild-type and FH(+/-) mice undergoing the CAIA model (Banda, Mehta, Ferreira, Cortes, Pickering, Pangburn, Arend, and Holers, 2013). With regard to the mechanism of this effect, *in vitro* studies demonstrated that rFH19-20 increased complement activation on cartilage extracts treated with anti-CII mAbs as well as injured FLS. These data suggested that endogenous FH makes a significant contribution to inhibition of the alternative pathway and amplification loop in CAIA, likely through localized binding to and decreasing complement activation at sites of immune complex formation and complement activation.

Because of this key role for FH revealed through the use of the non-physiologic inhibitor rFH19-20, additional studies are now focusing on control of FH actions by endogenous modulators. One such potential class of modulators is exemplified by the factor H-related (FHR) proteins. FHR proteins are part of the structurally related family that includes the larger FH protein but are encoded by genes that are physically adjacent to the FH gene on chromosome 1 and also contain short consensus repeat (SCR) domains with homology to sub-regions of FH (de Cordoba, Tortajada, Harris, and Morgan, 2012; Jozsi and Zipfel,

2008). Notably, there are an increasing number of identified protective or risk associations of deletions, rare mutations or polymorphic variants of these genes with human diseases (de Cordoba *et al.*, 2012; Jozsi *et al.*, 2008), including age-related macular degeneration where an uncommon deletion of FHR1 and FHR3 is a highly penetrant protective factor (Hughes, Orr, Esfandiary, Diaz-Torres, Goodship, and Chakravarthy, 2006).

What has been uncertain is the role of these key proteins *in vivo* (Holers, 2013b). Many prior studies of FHRs have reported a role for these proteins in directly regulating complement activation and impairing activation at specific steps of the cascade. For example, FHR2 and FHR4 were reported to demonstrate enhancement of FH cofactor activity, and FHR5 to exhibit weak cofactor and decay accelerating activity [reviewed in (Jozsi *et al.*, 2008)]. Likewise, FHR1 was postulated to inhibit C5 convertase activity and terminal complex formation (Heinen, Hartmann, Lauer, Wiehl, Dahse, Schirmer, Gropp, Enghardt, Wallich, Halbich, Mihlan, Schlotzer-Schrehardt, Zipfel, and Skerka, 2009), and FHR3 to inhibit C3 convertase activity and display anti-inflammatory activity (Fritsche, Lauer, Hartmann, Stippa, Keilhauer, Oppermann, Pandey, Kohl, Zipfel, Weber, and Skerka, 2010). However, as the structure-function relationships of FH has become increasingly understood (Morgan, Schmidt, Guariento, Blaum, Gillespie, Herbert, Kavanagh, Mertens, Svergun, Johansson, Uhrin, Barlow, and Hannan, 2011), it has become more challenging to reconcile regulatory activities of FHRs with the structural determinants, other than through unusual steric hindrance mechanisms, because they do not encode domains of similar structure to FH SCR1-4.

Recently, studies by Tortajada et al (Tortajada, Yebenes, Abarrategui-Garrido, Anter, Garcia-Fernandez, Martinez-Barricarte, Alba-Dominguez, Malik, Bedoya, Perez, Trascasa, Pickering, Harris, Sanchez-Corral, Llorca, and de Cordoba, 2013) and Goicoechea et al (Goicoechea de Jorge, Caesar, Malik, Paten, Colledge, Johnson, Hakobyan, Morgan, Harris, Pickering, and Lea, 2013) have challenged that model by showing that FHR1 homo- and heterooligomerizes with itself as well as with FHR2 and FHR5, but not with FHR3 or FHR4A/4B. These complexes were shown *in vitro* to compete for FH binding to C3 fragments, and to impair the ability of FH to block surface complement activation. The conclusion of the study was that FHR1, assembled into multimers either alone or in association with FHR2 and FHR5, primarily acts on surfaces to block FH binding, resulting in complement deregulation and enhanced local activation. Because the CAIA model requires the alternative pathway, is a FH-dependent model and can be strongly enhanced by treatment with recombinant FH SCR19-20 (Banda *et al.*, 2013), it is an excellent experimental means by which the roles of FHRs can be evaluated *in vivo* in a complex biological environment.

Effector-Mediated Mechanisms of Proximal Amplification

One typically thinks about complement-dependent activation solely in the context of the biochemically defined linear pathway and well characterized amplification loop [reviewed in (Lachmann, 2009)]. However, studies in the CAIA model have also led to the understanding that there are additional effector pathway-catalyzed amplification steps which occur *in vivo*. These mechanisms are characterized by the engagement of “danger signals” and other pro-

inflammatory pathways and uniformly result in more robust deposit of pathogenic antibodies and local complement activation. The conclusion from these studies is that each major complement effector pathway, C5a, C3a and the membrane attack complex (MAC), plays an important and unique role in joint injury and also promotes proximal pathway complement C3 activation (Banda, Hyatt, Antonioli, White, Glogowska, Takahashi, Merkel, Stahl, Mueller-Ortiz, Wetsel, Arend, and Holers, 2012).

In support of this model, it was found that when *C3aR*($-/-$), *C5aR*($-/-$), and C6-deficient (C6-def) mice are studied in the CAIA model, clinical disease activity was decreased by 52, 94, and 65%, respectively, as compared with wild-type mice. Comparable decreases in the levels of histopathologic injury scores as well as IgG and C3 deposition, which paralleled the clinical disease activity scores, were also found. The outcomes in each effector pathway were not identical, as a decrease in the percentage of synovial neutrophils was observed in *C3aR*($-/-$), *C5aR*($-/-$) and C6-def mice, but a decrease in macrophages was only observed in *C3aR*($-/-$) and *C5aR*($-/-$) mice. As a further example of unique aspects of the individual pathway roles, analysis of synovial mRNA obtained by laser capture microdissection revealed a decrease in TNF- α in *C5aR*($-/-$) mice and in IL-1 β in both *C5aR*($-/-$) and C6-def mice, whereas *C3aR*($-/-$) mice demonstrated no change in either cytokine. Notably, although there were differential effector mechanisms that were apparent, a decreased proximal joint IgG and C3 deposition in comparison with wild-type mice was common to mice with all three genotypes. In sum, these data suggest the existence of positive-feedback amplification pathways that is downstream of all three effectors and which promotes additional IgG deposition and C3 activation in the joint.

There are several potential mechanisms that can underlie these phenotypes. One is a contribution of bone marrow cell-derived effector pathways, perhaps through polymorphonuclear cell (PMN)-derived C5aR and/or C3aR engagement. In this regard, the alternative pathway amplification loop is believed to be promoted at sites of local injury when inflammatory cells are recruited, either through a mechanism that involves the additional generation of necrotic cells that release proteases which damage cells and result in increased complement fixation, and/or because these infiltrating cells produce C3 and properdin that increase activation specifically at that site. The presence of alternative pathway components carried in neutrophil granules and released following activation *in vitro* has previously been shown (Schwaeble and Reid, 1999), and this may play an important potential biologic role for this capacity in self tissue injury. In addition, C5a and C3a may play roles in increasing local vascular access in the joints to anti-CII mAbs as well as the engagement of additional innate immune pathways (Binstadt, Patel.P.R., Alencar.H., Nigrovic, Lee, Mahmood, Weisleder, Mathis, and Benoist, 2006; Lee, Friend, Gurish, Benoist, Mathis, and Brenner, 2002).

Another possibility that could underlie this phenotype is that activation of complement leads to changes in the expression of critical neopeptides that are recognized by an important new class of pathogenic natural antibodies. Rather than being polyreactive, as is true of the great majority of natural antibodies, these natural antibodies recognize a conserved family of neopeptides which are uniquely expressed on injured tissues and appear to serve an essential role as “danger” signals that are essential to initiate the inflammatory response which

follows. Importantly, unlike traditional “autoantibodies” directed to self-antigens, these natural antibodies do not recognize tissues in the absence of injury but only react with neoepitopes that are elaborated as part of the “danger signal” process (Carroll and Holers, 2005; Fleming, 2012). Two major examples of pro-inflammatory IgM natural antibodies have been described through the characterization of highly informative mAbs. The first recognizes non-muscle myosin heavy chain-II (NMHC) (Zhang, Alicot, Chiu, Li, Verna, Vorup-Jensen, Kessler, Shimaoko, Chan, Friend, Mahmood, Weissleder, Moore Jr., and Carroll, 2006; Zhang, Austen Jr., Chiu, Alicot, Hung, Ma, Verna, Xu, Hechtman, Moore Jr., and Carroll, 2004). The second subset recognizes annexin-4 (ANX4) (Kulik, Fleming, Moratz, Reuter, Novikov, Chen, Andrews, Markaryan, Quigg, Silverman, Tsokos, and Holers, 2009). ANX4 belongs to a family of Ca²⁺ and phospholipid-binding proteins [reviewed in (Rescher and Gerke, 2004)]; and annexins including ANX4 have been shown to be elaborated on the external cell membrane through a poorly understood mechanism (Mayran, Traverso, Maroux, and Massey-Harroche, 1996).

Anti-ANX4 IgM natural antibodies have been shown to play roles in a wide array of injury models, including intestinal ischemia-reperfusion injury (Kulik *et al.*, 2009), choroidal neovascularization following laser injury (Blanche, Maltby, Bennett, Kubes, Lee, and McNagny, 2010), and stroke injury (Elvington, Atkinson, Kulik, Zhu, Yu, Kindy, Holers, and Tomlinson, 2012). These findings suggest the likelihood of a previously under-appreciated role for this “hard-wired” recognition process in disease pathogenesis in reperfusion injury as well as non-vascular models. In addition, the potential ability to therapeutically modulate this type of natural antibody-dependent pro-inflammatory process using experimental approaches *in vivo* has yielded highly encouraging results. Specifically, the effects of pro-inflammatory natural antibodies in injury have been ameliorated by using peptides or proteins that mimic the tissue neoepitopes, including NMHC (Zhang *et al.*, 2006) and ANX4 (Kulik *et al.*, 2009), thereby blocking pathogenic natural antibody binding to the site through the recognition of neoepitopes.

Molecular imaging of tissue-fixed complement C3 activation fragments

During complement activation the C3 protein is cleaved, and C3 activation fragments are covalently attached to tissues and can serve as a durable biomarker of tissue inflammation and damage. These fragments have been exploited as addressable binding ligands for targeted therapeutics utilizing complement receptor type 2 (CR2)-linked inhibitors that direct inhibitors to the brain, intestine, eye, joints and other tissues (Holers, Rohrer, and Tomlinson, 2013). Recently, novel cross-reactive murine monoclonal antibodies to human and mouse C3d have also been generated which bind to the iC3b, C3dg, and C3d fragments in solution and on surfaces, but do not bind to intact C3 or C3b (Thurman, Kulik, Orth.H., Wong, Renner, Sargsyan, Mitchell, Hourcade, Hannan, Kovacs, Coughlin, Woodell, Pickering, Rohrer, and Holers, 2013). The same mAbs bind C3d from human, murine and cynomolgous sources also bind to tissue-bound C3 activation fragments in mice when injected systemically.

Using mouse models of renal and ocular disease, it has shown that following systemic injection the antibodies accumulate at sites of C3 fragment deposition within the glomerulus,

the renal tubular interstitium, and the posterior pole of the eye. To detect antibodies bound within the eye, optical imaging of FITC-bound mAb was used, which demonstrated accumulation of the antibodies within retinal lesions in a model of choroidal neovascularization (CNV) (Thurman *et al.*, 2013). Such imaging methods that utilize these antibodies may provide a sensitive means of detecting and monitoring complement activation-associated tissue inflammation.

Informative mAbs to tissue-bound C3 fragments could also have other biomedical uses. For example, similarly to CR2, they could be used as *in vivo* targeting methods for therapeutic agents. In addition, they could potentially modulate the biologic functions of the C3 fragments.

Conclusions and Next Steps

This review has laid out several new directions of research in the complement field that are focused on the actions which occur in the micro-environments on surfaces that lie within sites of tissue injury. These processes include effector pathway-catalyzed amplification of proximal complement activation, de-regulation of the alternative pathway through interference with FH, recognition of injury-associated ligands by components of the lectin pathway, and engagement of pathogenic natural antibodies that recognize a limited set of injury-associated neopeptides. One way to consider these processes on the cartilage and within the synovium is outlined in Figure 2. Finally, each of these concepts has potential therapeutic relevance, either as a pathway that should be blocked, a process that could be utilized to direct inhibitors to the correct sites, or as a potential biomarker that can be detected in live subjects through imaging approaches. One particular example of imaging sites of injury through detection of tissue-fixed C3 fragment deposition with monoclonal antibodies has already been demonstrated through pre-clinical studies.

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Highlights

- The complement alternative pathway and amplification loop are major mediators of injury *in vivo*
- These pathways act on tissue surfaces and are controlled by both positive and negative factors
- Distal effector mechanisms of complement exert a positive feedback on local activation
- Injury-induced ligand recognition by the lectin pathway plays a critical role in tissue injury
- Local pro-inflammatory mechanisms engaged by complement include pathogenic natural antibodies

Figure 1.

Current understanding of the step-wise phases of development of RA, starting with the disease-related genotypes, which is followed by an asymptomatic phase of circulating RA-related autoantibodies. This period leads to a clinical transition in which subjects are developing clinically apparent disease, and then finally moving into a chronic arthritis phase.

Figure 2.

Model of initiation and propagation of joint inflammation and damage in CAIA following infusion of anti-CII mAbs. Explored in this review are the importance of lectin pathway activation, the role of FHRs in controlling alternative pathway activation, and the deconstruction of the pathogenic mechanisms which are engaged following the generation of effector molecules (C5a, C3a and the MAC). These processes promote alternative pathway engagement and the amplification loop, resulting in enhanced anti-CII and natural antibody access and/or deposition followed by C3 activation. Note also the proposed role for local production of indicated factors from fibroblast-like synoviocytes[&] and adipocytes^{*}. CP: classical pathway; LP, lectin pathway.