CR1/2 is an important suppressor of Adenovirus induced innate immune responses and is required for induction of neutralizing antibodies

Sergey S. Seregin\textsuperscript{1}, Yasser A. Aldhamen\textsuperscript{1}, Daniel M. Appledorn\textsuperscript{1}, Nathaniel J. Schuldt\textsuperscript{1}, Aaron J. McBride\textsuperscript{1}, Mathew Bujold\textsuperscript{1}, Sarah S. Godbehere\textsuperscript{1}, and Andrea Amalfitano\textsuperscript{1,2,3,†}

\textsuperscript{1}Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824
\textsuperscript{2}Department of Pediatrics, Michigan State University, East Lansing, MI 48824
\textsuperscript{3}Department of Pediatrics, Duke University Medical Center, Durham, NC 27710

Abstract

Human complement receptors 1 and 2 are well described as important regulators of innate and adaptive immune responses, having pivotal roles in regulating complement activation (CR1) and B cell maturation/survival. In contrast, the role of the murine homologues of CR1 and CR2 (mCR1/2) have been primarily defined as modulating activation of the adaptive immune system, with very little evidence available about the role of mCR1/2 in regulating the innate immune responses to pathogens. In this manuscript, we confirm that mCR1/2 plays an important role in regulating both the innate and adaptive immune responses noted after Adenovirus (Ad) mediated gene transfer. Our results uncovered a novel role of mCR1/2 in down-regulating several, complement dependent innate immune responses. We also unveiled the mechanism underlying the complement dependent induction of neutralizing antibodies to Ad capsids as a CR1/2 dependent phenomenon that correlates with B-cell activation. These results confirm that Ad interactions with the complement system are pivotal in understanding how to maximize the safety or potency of Ad mediated gene transfer for both gene therapy and vaccine applications.

Keywords

complement receptor; innate immunity; liver; recombinant Adenovirus

Introduction

Adenovirus (Ad) based vectors offer tremendous capabilities as potent vaccine platforms, primarily reflected by their superior ability to induce both humoral and cellular immune responses to desired antigens expressed by the vectors. It is widely believed that the known
ability of Ad vectors to induce pro-inflammatory cytokine responses may contribute to Ad vector mediated induction of potent immune responses. However, strain specific neutralizing antibodies to Ads can hinder the ability to repeatedly administer the vector as a boosting immunogen, prompting multiple efforts to develop alternative serotype Ad vectors to overcome this problem. It is with these realities in mind that studies investigating the mechanisms underlying the induction of neutralizing antibodies to the well characterized Ad5 vector platform are required. Importantly, the Ad5 serotype is the only Ad serotype utilized in all human gene transfer clinical trials (>367 as per September 2008, comprising 24.9% of all gene therapy trials to date), please see http://www.wiley.co.uk/wileychi/genmed/clinical/).

Our previous studies have confirmed that the induction of neutralizing antibodies to Ads is dependent upon the presence of a functional complement system in the host 1. Although we have discovered that the protein C3 is essential in this response, the mechanisms underlying C3 dependent induction of neutralizing antibody by Ad vectors is currently unknown. Our previous results confirm that lack of Ad interactions with the C3 protein results in a diminished induction of pro-inflammatory cytokines and acute phase responses early after Ad administration, suggesting that a lack of this initial response may contribute to diminished induction of neutralizing antibody responses. This hypothesis is supported by recent studies in IFN receptor KO mice that also showed diminished induction of neutralizing antibody titers after Ad treatments 2. We have utilized Ad treated CR1/2-KO mice in this study to test this hypothesis.

It is known that complement system activation acts to optimize induction of pathogen specific antibody responses 3-5. Subsequent to opsonization by activated C3, pathogens are bound to B cells and dendritic cells via binding of pathogen bound C3 to the complement receptors (CRs). The human CR1 and CR2 receptors have well known roles in modulating both innate and adaptive immune responses. Human CR1 (hCR1) is a potent inhibitor of complement activation, having both decay-accelerating and cofactor activities. Furthermore, hCR1 has a critical role in the clearance of immune complexes, and B cell maturation, as thoroughly reviewed elsewhere 6,7. Human CR2 (hCR2) expression is restricted to the surface of B cells, follicular dendritic cells and thymocytes. When hCR2 binds C3d oponised pathogens and becomes associated with CD19, it lowers the threshold for B cell activation by up to 1000 fold 8. HCR1 and hCR2 also play a role in T cell biology, for example, crosslinking of hCR1 inhibits T cell proliferation and IL-12 production 9.

Murine complement receptors (mCRs) 1 and 2 (CD35/CD21) are products of alternative splicing from the same gene. mCR1 contains 21 complement control protein repeats (CCPRs), whereas the smaller mCR2 contains only 15 C-terminal CCPRs of mCR1 10. mCR1/2 is known to be expressed on B cells and dendritic cells 10. Therefore, the expression pattern of mCR1/2 resembles that of hCR2, but not hCR1. Similar to their human homologues, the functions of mCR1/2 related to generation of maximal humoral responses have been well described 11-15. Interestingly, mCR1/2 functionality prevents excessive myocardial tissue damage subsequent to coxsackievirus B3 infection 16, as well prevents lethal Streptococcus pneumoniae infection, a role potentially indirectly reflective of the complement inhibitory activities of the CRs 17.
While the role of murine CR1/2 protein has been extensively studied in regards to adaptive immune responses, its function in inhibiting/regulating murine complement has not been demonstrated, possibly since in most mouse models, the Crry protein was suggested to play the predominant role in controlling complement activation. We feel that the role of murine CR1/2 protein in innate immune responses (including the ones which are known to be complement dependent) may be more important than previously considered, as suggested by our present studies of Adenovirus mediated gene transfer into mCR1/2-KO mice. Our results in murine models revealed dual roles for mCR1/2; roles that include down-regulation of multiple aspects of the Ad induced innate immune responses, while also playing the major role in the complement dependent induction of neutralizing antibody responses to Ads.

Results

Murine Complement Receptor 1/2 regulates Ad mediated cytokine and chemokine release in C57BL/6 mice

To study the role of mCR1/2 protein in Ad induced innate and adaptive immune responses, we utilized mCR1/2-KO mice. These mice have been previously demonstrated to completely lack expression of CR1/2 on B cells. It is also known that CR1/2 activities also impact upon levels of activated C3, by virtue of CR1/2’s decay accelerating properties. Utilizing western blotting with C3-specific antibodies, we confirmed that mock injected CR1/2-KO mice have normal overall levels of C3 no different than wild-type mice, and equivalent amounts of C3 cleavage products were present in the plasma of virus injected WT and CR1/2-KO mice, as investigated both at 10 minutes post injection and 6 hours post injection (Supplemental figure 1). These results suggest that CR1/2-KO mice do not have significant alterations in the ability of C3 to initially interact with Ads, an interaction that we have previously confirmed mediates many Ad induced innate and adaptive immune responses.

Inflammatory cytokines and chemokines are rapidly released after systemic Ad injection. We have identified 7 cytokines and chemokines (KC or CXCL1, MCP-1, MIP-1β, G-CSF, RANTES, IL-6, IL-12p40) that become significantly elevated within hours of systemic administration of Ad vectors, some of which are elevated in a C3 dependent fashion. We investigated the role that mCR1/2 has in the induction of these cytokines by administering Ads into wild type and mCR1/2 knockout mice. Plasma samples, collected at 1 and 6 hours post intravenous Ad administration confirmed that KC and MCP-1 chemokines are rapidly released in response to Ad injections and reach maximum levels by 1 hpi. Ad injected mCR1/2-KO mice had identical levels of activation of these 2 chemokines at 1 hpi (Figure 1), suggesting that Ad induction of these very early mediators of the inflammatory responses is not dependent upon mCR1/2 functionality. Interestingly, Ad injected mCR1/2-KO mice exhibited significantly higher plasma levels of G-CSF, MCP-1 and RANTES at 6 hours after systemic Ad injection, as compared to identically treated WT mice (Figure 1). This result identifies the role of functional mCR1/2 protein as a complement regulator that suppresses complement activation, resulting in diminished production of several cytokines released subsequent to Ad administration in mice. This result may reflect a complement decay accelerating property of mCR1/2 thought to be
present in the extracellular portion of the protein, based upon analogy to the human CR1/2 homologs.

**Portions of the acute and chronic cellular responses to Ad vectors are Complement Receptor 1/2 dependent**

Activation of vascular endothelium is a critical step during initiation of inflammatory immune responses, since many inflammatory cells (i.e. platelets, neutrophils, macrophages, mast cells) utilize activated endothelial cells (EC) as a means to localize to damaged sites. This response is mediated by activated EC over-expression of E-selectin, ICAM-1 and/or VCAM-1 adhesion molecules on their surface, molecules that facilitate the migration of inflammatory cell types into damaged organs. Soluble forms of these cell adhesion molecules are also produced by activated ECs. Interestingly, while the levels of sE-selectin in Ad treated WT mice were increased 3-fold, sE-selectin levels in Ad treated mCR1/2-KO mice were at least 7-fold higher, as compared to mock injected mCR1/2-KO mice, these levels were also significantly higher than levels noted in Ad treated WT mice (Figure 2). Induction of both sVCAM-1 and sICAM-1 was also slightly more robust in Ad treated mCR1/2-KO mice as compared to Ad injected WT mice, although these levels did not reach a significant difference (data not shown). We have also tested the level of ICAM-1, VCAM-1 and E-selectin mRNA transcripts in murine liver at 6 hpi after systemic Ad injection and found significantly increased levels of all 3 transcripts in mCR1/2-KO mice (Table 1). These results confirm that lack of mCR1/2 functionality results in a more robust induction of cellular adhesion molecules in Ad treated mice, suggesting that another role of mCR1/2 is to modulate the extent of complement dependent induction of EC activation.

We next wished to correlate EC activation with cellular elements known to be mobilized subsequent to Ad infections or Ad vector administrations. Acute thrombocytopenia and/or lymphopenia can occur within hours of natural Ad infections, and Ad induction of thrombocytopenia is a complement (C3) dependent event. However, at 24 hours after systemic Ad injection, mCR1/2-KO mice exhibited levels of thrombocytopenia no different than that noted in Ad injected WT mice (data not shown). Ad injected mCR1/2-KO mice trended towards elevated levels of neutrophils and reduced levels of lymphocytes at 24 hpi, though the changes did not reach statistical significance when compared to identically treated WT mice. There was also no difference in levels of Kupffer cell necrosis in virus treated WT and mCR1/2-KO mice, suggesting that mCR1/2 is not involved in Kupffer cell necrosis, an event that occurs within hours of Ad injection (data not shown).

Systemically administered Ad vectors induce significant leukocyte infiltration into the liver. The infiltration becomes apparent at 3 dpi, continuously increases with the generation of an adaptive immune response to the virus and/or antigen it expresses, and reaches maximal levels by 21-56 dpi. We have quantified monocellular (leukocyte) infiltration into the livers of Ad treated WT and mCR1/2-KO mice at 28 dpi. Both WT and mCR1/2-KO Ad injected mice exhibited significant infiltration of inflammatory cells into their livers, as compared to mock injected animals. Despite the previously noted enhanced pro-inflammatory cytokine responses, as well as enhanced activation of ECs in Ad injected
mCR1/2-KO mice, Ad injected mCR1/2-KO mice had significantly reduced periportal inflammation as well as total levels of hepatic inflammation relative to Ad injected WT mice (Supplemental figure 2ab). Portal and lobular inflammation were somewhat reduced in virus treated mCR1/2-KO mice as compared to identically treated WT mice, although these differences did not reach statistical significance. These results suggest that maximal induction of cellular inflammation after Ad vector administration is directly dependent upon mCR1/2, despite enhanced activation of the innate immune system in the same animals earlier.

**Murine Complement Receptor 1/2 downregulates Ad induction of pro-inflammatory genes in livers of Ad treated C57BL/6 mice**

We have previously confirmed that systemic administrations of Ad vectors results in a rapid and profound transcriptome response in the murine liver, responses that are mediated by Ad interactions with the murine complement system. Based in part upon those studies, we analyzed expression levels of several liver gene transcripts, including those participating in innate immune responses, i.e.: pattern recognition receptors (TLRs, NODs), TLR signaling pathways (MyD88, TRIF, TRAF6, TRAF2bp, TBK1), markers of EC activation (ICAM1, VCAM1, E-selectin), interferon responsive genes (ADAR, OAS1a, IRF7, IRF8), all listed in Table 1. Note, that none of the genes tested showed any differences in basal transcription levels between mock injected WT and mCR1/2-KO mice liver derived transcripts. However, the levels of 13 liver transcripts, although significantly induced in Ad injected WT mice at 6 hpi, were induced to statistically greater levels in Ad treated mCR1/2-KO mice. These Ad induced, but mCR1/2 suppressed genes included ADAR, ICAM1, VCAM1, E-selectin, MyD88, TBK1, TLR2, TLR3, JAK3, SOCS1. Importantly, 5 gene transcripts, that were not induced in Ad injected WT mice (as compared to Mock injected animals) also revealed significant inductions in mCR1/2-KO Ad treated mice (Table 1). These observations allowed us to conclude that mCR1/2 plays a significant role in down-regulating Ad induction of pro-inflammatory gene expression. Importantly, the Ad induction of most of these genes has also been previously shown by us to be a complement protein C3-dependent event, suggesting that mCR1/2 downregulates Ad activation of pro-inflammatory genes by suppressing and/or regulating the overall levels of Ad induced, C3 dependent activation of the complement system.

**Ad mediated transduction in vivo is not dependent upon murine Complement Receptor 1/2, but the levels of Ad derived transgene expression are complement and mCR1/2 dependent**

We next confirmed that Ad mediated liver transduction of Ad genomes was not diminished at any time point tested during our studies (Figure 3a), in part confirming our previously published results that complement does not significantly mediate Ad transduction efficacy in vivo. Ad derived transgene expression in livers of treated mice was then tested by two independent techniques: qualitatively by staining liver sections with X-Gal (Figure 3b) and by quantitative measurement of β-galactosidase activity at 6 hpi, 24 hpi and 28 dpi (Figure 3c). We found a significant reduction of β-Gal activity in mCR1/2-KO mice as compared to
WT mice both at 24 hpi and 28 dpi, but not at 6 hpi (Figure 3bc). Ad injected C3-KO mice also exhibited significantly reduced β-Gal activity both at 6 hpi and 24 hpi, a trend that we and others have noted previously.\(^1\)\(^2\)\(^3\). Our observations confirm that lack of mCR1/2 functionality does not dramatically alter the ability of Ads to transduce their genomes into the murine liver \textit{per se}, but rather results in a more rapid shutdown of CMV enhancer dependent transgene expression from Ad vectors, a complex finding elaborated upon further in the discussion section of this manuscript.

**Murine Complement Receptor 1/2 is required for generation of Ad vector specific, but not transgene specific adaptive immune responses**

Ad specific adaptive immune responses can be primed by the robustness of innate immune responses.\(^2\)\(^9\)\(^3\)\(^3\). Our results confirm that mCR1/2 protein suppresses or down-regulates many Ad induced innate immune responses, therefore we wished to determine whether enhanced induction of Ad induced innate immune responses (due to lack of mCR1/2 function) altered subsequent adaptive immune responses to the vector capsid, and/or the transgene product the Ad vector expresses. Importantly, we first confirmed that baseline IgG levels in mock injected WT and mCR1/2-KO mice were identical (data not shown), confirming previously published observations.\(^9\)\(^1\)\(^1\)\(^1\)\(^3\). However, our results demonstrated that Ad injected mCR1/2-KO mice had diminished Ad-capsid specific primary humoral responses (as compared to identically treated WT mice) both at 14 and 28 dpi (Figure 4). IgA, total IgG, IgG1, IgG2a. IgG3 subtypes of Ad capsid specific antibodies tested in Ad treated mCR1/2-KO mice were significantly reduced at least at one of the two time points tested as compared to Ad injected WT mice, moreover all were diminished both at 14 and 28 dpi in mCR1/2-KO mice (Figure 4). Additionally, Ad injected mCR1/2-KO mice did not generate significant titers of Ad-capsid neutralizing antibodies, as determined both at 14 and 28 dpi (Figure 5). These observations strongly parallel our previously published data noting that Ad injected C3-KO mice also exhibit a diminished capacity to generate anti-Ad capsid specific humoral responses, as well as neutralizing antibody responses.\(^1\)

Since B cells are known to express high levels of mCR1/2, and depend upon mCR1/2 for induction of antigen specific B cell activation, we quantified B cell activation 48 hours after systemic Ad injection into WT, C3-KO and mCR1/2-KO mice. We confirmed that the number of activated B cells (i.e.: CD19+/CD69+ splenocytes) was significantly increased in WT mice injected with Ad vectors as compared to uninfected WT mice (Figure 6). Interestingly, the levels of B cell activation were significantly and identically reduced in both Ad treated C3 and mCR1/2-KO mice, again as compared to identically injected WT mice, suggesting that the C3 dependent portion of induction of neutralizing antibody responses to Ad capsids may be entirely mediated by interactions mediated by the CR1/2 protein (Figure 6). This result positively correlates with our findings that functional mCR1/2 protein is required to generate Ad capsid specific humoral immune responses (Figures 4-5), a response that is at least in part a complement dependent event.\(^1\) The overall results suggest that induction of neutralizing antibodies to Ads is dependent upon C3 opsonized Ads interacting with mCR1/2 present on B cells, resulting in their activation.
Finally, we have previously published that the lack of complement protein C3 did not reduce the induction of transgene specific humoral responses in Ad injected mice\(^1\). Interestingly, mCR1/2-KO mice injected with Ad5-LacZ generated levels of transgene specific (β-Gal) antibodies that paralleled those noted in identically treated WT mice (Supplemental figure 3). Only β-Gal specific IgA, total IgG and IgG2a reached significant differences, and this occurred at only one of the two time points tested. These results suggest that Ad expressed, transgene specific adaptive immune responses are not dependent upon mCR1/2.

**Discussion**

Despite the fact that the role of murine Complement Receptor 1/2 (mCR1/2) in modulating adaptive immune responses has been extensively studied\(^{11,12,34,35}\), very little information is available regarding the role of mCR1/2 in regulating aspects of the innate immune system, including complement mediated responses. In this study we have investigated the role of mCR1/2 protein in regulating Ad vector induced innate immune responses and also assess what impact these roles have upon downstream adaptive immune responses to the Ad vector, and/or the transgene the vector expresses.

We have previously described the pivotal role the complement system has in generating robust innate immune responses subsequent to Ad treatment, including systemic pro-inflammatory cytokines/chemokine release, EC activation, acute thrombocytopenia, and liver transcriptome dysregulation\(^{1,21}\). In this study we have shown that many of these complement dependent responses are modulated (suppressed) by mCR1/2.

To highlight how mCR1/2 specifically down-regulates Ad dependent induction of pro-inflammatory cytokines and chemokine responses we have combined data obtained in this study with our previously published data in Ad treated C3-KO mice\(^1\) (Figure 7). Note, that MCP-1, IL-12p40, G-CSF and RANTES are all induced in a complement dependent manner (evident by the lack of induction of these cytokines/chemokines in C3-KO mice). Moreover, mCR1/2 protein down-regulates the induction of these very chemokines (MCP-1, G-CSF and RANTES) as compared to Ad treated wild-type mice. These overall results reveal that mCR1/2 plays a very important role in suppressing overall innate immune responses induced by complement system activation by Ad vectors.

This suppressive activity of mCR1/2 is not without precedent, as it has been shown that mCR1/2 is required to prevent excessive tissue damage subsequent to coxsackievirus B3 infection\(^{16}\). In addition, lack of mCR1/2 functionality results in an enhanced susceptibility (i.e.: lethality) subsequent to *Streptococcus pneumoniae*\(^{17}\). Note, that none of these studies focused on the role of mCR1/2 in innate immunity, as most studies regarding modulation of the murine complement system and the innate immune responses have focused upon the complement receptor-1 related protein (Crry)\(^{36,37}\). Those studies indirectly suggest that murine Crry has taken over some hCR1 functions, i.e.: those roles relative to mCR1/2 acting as a murine complement inhibitor\(^{10,38}\). We feel that our studies confirm a significant role for mCR1/2 in suppressing the activation of the murine complement system, and innate immune responses derived from that activation. Why the mouse has what appears to be a
redundant complement inhibiting activities (present in Crry and mCR1/2) will require future experimentation but this redundancy may have contributed as to why humans lack Crry.

Since both MCP-1 and G-CSF are known to activate macrophages \(^{39}\), and both RANTES and G-CSF activate neutrophils \(^{39}\), it might be hypothesized that lack of mCR1/2 might result in an enhanced infiltration of these inflammatory cells to the sites normally transduced by Ad vectors (i.e. liver). However, our results were not able to detect substantial increases in acute, Ad induced cellular responses in the livers of Ad injected mCR1/2-KO mice, suggesting that recruitment of these cells requires additional factors.

Pre-existing immunity to Adenoviruses remains a main hindrance for numerous applications utilizing Adenovirus based vectors as a vaccine platform. In light of this fact, it is of critical importance to study the mechanisms underlying generation of Ad capsid specific antibody responses. Increased innate immune responses are generally thought to positively correlate with enhanced inductions of humoral, and/or cellular adaptive immune responses \(^{2,9,33}\). The complement system is well known to significantly impact upon the generation of humoral responses to pathogens by facilitating interactions between the innate and adaptive response systems \(^{6-8}\).

Our previous results demonstrated that Ad vector administrations into C3-KO mice resulted in diminished cytokine and chemokine responses, and a diminished induction of neutralizing antibodies to Ad \(^{1,21}\). However, despite enhanced induction of pro-inflammatory cytokines and chemokines rapidly after Ad vector administrations into CR1/2-KO mice, we still found a dramatically reduced induction of neutralizing antibodies to the Ad vector capsid. These results positively correlated with a lack of activation of B cells in Ad injected C3-KO and mCR1/2-KO mice. The combined observations suggest that lack of induction of pro-inflammatory cytokine responses early after Ad administrations into C3-KO mice is not the reason for lack of neutralizing antibody induction in the Ad treated C3-KO mice\(^{1}\). Rather, C3 opsonized Ad interactions with mCR1/2 on murine B cells is primarily responsible for induction of neutralizing antibodies to the Ad vector capsid. This insight suggests that further investigation of this interaction may promote strategies to proactively modulate the induction of neutralizing antibodies to Ad vectors.

Zaiss at al. detected evidence of Ad induction of neutralizing antibodies in an alternative CR1/2-KO mouse model \(^{40}\). That strain of CR1/2-KO mouse has a different portion of the murine Cr2 gene disrupted, relative to the CR1/2-KO mouse strain utilized in our studies, which may be relevant to the different results. Additional technical caveats including the use of a significantly different assay system may also explain the differing results. Our multiple findings demonstrating a lack of significant induction of Ad capsid neutralizing antibodies in both Ad treated C3-KO and CR1/2-KO mice, as well as our finding that Ad induction of B cell activation is also significantly decreased in these same strains of mice strongly support our conclusion that CR1/2 does play an important role in the induction of neutralizing antibodies to Ad vectors. While Zaiss \textit{et al.} did not report on whether or not induction of neutralizing antibodies to Ads occurred in Ad treated C3-KO mice, Zaiss \textit{et al.} did find that mCR1/2 functions were also necessary for induction of neutralizing antibodies to AAV based vectors. The latter results, combined with ours, suggest that mCR1/2 may be
playing a role in the induction of neutralizing antibodies to several commonly utilized gene
transfer vectors. Interestingly, activation of type I interferons (IFNα/β), have also been
shown to play a role in the induction of Ad specific humoral adaptive immune responses,
whether these responses are due to, or a result of Ad interactions with the complement
system, and CR1/2 specifically will require future investigations 2.

In a similar vain, we found that an increased induction of Ad induced innate immune
responses in mCR1/2-KO mice did not correlate with increased induction of adaptive
immune responses to a foreign antigen expressed by an Ad vector. The reasons for this
observation are not due to a diminished ability of the Ad vector to transduce cells in
mCR1/2-KO mice, but rather appear to correlate with reduced transgene expression levels in
Ad vector treated mCR1/2-KO mice both at 24 hpi and 28 dpi. Possibly, the globally altered
cytokine and chemokine responses noted after Ad administration into CR1/2-KO mice
indirectly decreases activity of the CMV derived enhancer/promoter element utilized to
drive expression of the expression foreign transgene encoded by the vector, a possibility that
will require experiments beyond the scope of this manuscript.

We previously reported that transgene specific adaptive immune responses were higher in
Ad treated C3-KO mice as compared to identically Ad treated WT mice 1. At the least, those
results suggested that lack of complement activation does not significantly interfere with
generation of transgene specific antibody responses by Ad vectors. Thus, targeted blockade
of complement system activation that prevents C3 opsonized Ads from interacting with the
CR1/2 receptor may minimize induction of antibody responses to the Ad capsid while
simultaneously preserving or enhancing the ability of the Ad to induce beneficial immune
responses to Ad expressed foreign antigens.

Our results may also suggest a possible mechanism as to how non-covalent modifications of
Ad vectors, (such as Ad PEGylation) may allow for avoidance of induction of neutralizing
antibodies, by indirectly minimizing their induction of the complement system.41-43 We
also suggest that these findings may allow for improved use of Ad vectors as a vaccine
platform, though such studies are beyond the scope of this manuscript. However, several
previous studies indirectly confirmed roles for the complement system in generation of
robust Ad induced adaptive immune responses, in particular in Ad vaccine settings utilizing
C4-binding protein or C3d as novel adjuvants44-48.

In conclusion, we have confirmed the role of mCR1/2 in regulating and/or suppressing
several Ad induced innate immune responses, as well confirmed that mCR1/2 modulates the
induction of adaptive immune responses to the Ad vector. While the former observation now
confirms that in mice, mCR1/2 can play an important role in down-regulating complement
dependent innate immune responses in a manner that is independent of Crry, the latter
observations suggests that Ad vector interactions with mCR1/2 also figure prominently in
induction of neutralizing antibodies to Ads.
Materials and Methods

Adenovirus vector production and characterization

A first-generation, human Adenovirus type 5 derived replication deficient vector (deleted for the E1 genes) encoding β-galactosidase (LacZ) as a transgene (Ad5-LacZ) was used in this study. Virus construction, propagation and purification were performed as previously described. Briefly, a number of serial passages on HEK293 cells allowed high titer purification of Ad5-LacZ by sequential, cesium chloride density gradient centrifugations. Purified virus was dialyzed against 10 mM Tris (pH 8.0) and stored in 1% sucrose, 1 X PBS at −80°C until use. Viral particle (vp) and transducing unit titers (bfu/ml) were determined as previously described, and were 2.6 × 10^{12} vp/ml and 1.8 × 10^{11} bfu/ml respectively. The vp to bfu ration was ~14:1. Virus was found to be RCA free both by RCA PCR (E1 region amplification) and direct sequencing, methods as previously described. Ad vector has also been tested for the presence of bacterial endotoxin as previously described and was found to contain <0.01 EU per injection dose.

Animal procedures

Adult C57BL/6 WT and B6.129S4-C3tmlCrr (C3-KO) mice were purchased from Jackson Laboratory (Bar Harbor, ME). mCR1/2-KO mice in C57BL/6 background were a kind gift from Dr. Tedder, Duke University Medical Center. The Ad vector were injected intravenously (via the retro-orbital sinus) into 8-10 week old male C57BL/6 mice after performing proper anesthesia with isofluorane. A total of 0.75 × 10^{11} vp in 200 μl of PBS was injected per mouse. Four groups of mice were analyzed in this study: C57BL/6 Wild-type (WT) and CR1/2-KO mice, Mock injected with PBS and C57BL/6_WT or CR1/2-KO mice injected with Ad5-LacZ. For some of the control experiments C3-KO mice were utilized (N=4 for all C3-KO groups). Mice were sacrificed at different times after mock or virus treatment: 6 hours post injection (hpi), (N=6 for virus injected groups, N=4 for Mock injected groups), 24 hpi (N=4 for all groups), 28 dpi (N=5 for all groups). Plasma and tissue samples were collected and processed at the indicated time points in accordance with Michigan State University Institutional Animal Care and Use Committee. All procedures with recombinant Ads were performed under BSL-2, and all vector treated animals were maintained in ABSL-2 conditions. All animal procedures were reviewed and approved by the Michigan State University ORCBS and IACUC. Care for mice was provided in accordance with PHS and AAALAC standards.

Cytokine/Chemokine/Endothelial cells activation markers release measurement

To determine if mCR1/2 protein has any effect on Ad mediated release of cytokines/chemokines, Ad induced systemic release of pro-inflammatory cytokines/chemokines in murine plasma was measured in all groups of mice utilizing a multiplex bead array system. Plasma samples were collected at 1 and 6 hpi using heparinized capillary tubes and EDTA coated microvetttes (Sarstedt, Nümbrecht, Germany) and centrifuged at 3400 rpm for 10 min to retrieve plasma samples. Samples were assayed for 7 independent cytokines/chemokines, which we have previously shown to be rapidly induced by systemically injected Ad vectors (MCP-1, KC, MIP-1β, IL-6, IL-12p40, G-SCF, RANTES) \textsuperscript{1,19-21}. All procedures were performed exactly as previously described according to manufacturer’s instructions (Bio-

*Gene Ther.* Author manuscript; available in PMC 2014 May 30.
Rad, Hercules, CA) via Luminex 100 technology (Luminex, Austin, TX)\textsuperscript{20}. The measurement of soluble ICAM-1, VCAM-1 and e-Selectin molecules (endothelial cells activation markers) in murine plasma (collected at 6 hpi) was performed utilizing mouse cardiovascular disease panel LINCOplex kit (Millipore, Billerica, MA) as per manufacturer’s instructions.

**Complete blood count analysis and cell type differentiation**

Total blood (0.3-0.4 ml) was collected into EDTA coated 1.0 ml Lavender tubes (BD Microtainer, Franklin Lakes, NJ) at 24 hpi. For complete blood counts (CBCs), blood was analyzed on an Advia 120 Hematology System (Bayer, New York) by the Clinical Pathology Laboratory of the Diagnostic Center for Population and Animal Health at Michigan State University (East Lansing, MI). In addition, all blood samples were examined microscopically and underwent manual differential count.

**Platelet enumeration**

To access Ad vector induced thrombocytopenia in C57BL/6 WT and CR1/2-KO mice, platelets were measured 24 hpi after systemic Ad injection by using Unopette (Fisher Scientific) system as previously described\textsuperscript{20,21} as per manufacturer’s recommendations. Platelets were subsequently manually counted using Neubauer hemocytometer. Note, that platelets were counted by two independent methods.

**β-Galactosidase enzyme activity and in situ X-gal staining**

Ad mediated expression of the transgene LacZ was measured both qualitatively and quantitatively as follows. Liver sections from animals scarified at 6 hpi, 24 hpi and 28 dpi were embedded in Optimal Cutting Temperature (OCT) compound, frozen and stored at \(-80^\circ\) C until use. Frozen samples were sectioned (7 μm sections) on a Leica cryostat and were fixed and \textit{in situ} stained for LacZ expression using 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal, 1 mg/ml) as previously described\textsuperscript{30}. For quantitative assay, enzyme β-Galactosidase (β-gal) activity was measured in snap frozen liver samples. Liver samples (<0.1 g) were homogenized and total protein concentration was determined by bicinchoninic acid (BCA) assay, Pierce (Rockford, IL). β-gal activity was quantified by use of a β-gal activity detection kit (Stratagene, La Jolla, CA) according to manufacturers instructions and as previously described\textsuperscript{29}. Data were reported as Units of β-gal activity per mg of total protein.

**qRT-PCR Analysis**

To determine relative levels of a specific, liver derived RNA transcript, liver tissues were snap frozen in liquid nitrogen and RNA was harvested from \(\approx 100\)mg of frozen tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) per the manufacturer’s protocol. Following RNA isolation, reverse transcription was performed on 180ng of total RNA using SuperScript II (Invitrogen, Carlsbad, CA) reverse transcriptase and random hexamers (Applied Biosystems, Foster City, CA) per manufacturer’s protocol. RT reactions were diluted to a total volume of 60μl and 2μl was used as the template in the subsequent PCR reactions. Primers were designed using Primer Bank web based software (http://pga.mgh.harvard.edu/).
primerbank). Some primers used for amplification have been previously described 18,21. Complete list of primers utilized in this study is available in Supplementary table 1. Q-PCR was carried out on an ABI 7900HT Fast Real-Time PCR System using SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA) in a 15μl reaction. All PCRs were subjected to the following procedure: 95.0° C for 10 minutes followed by 40 cycles of 95.0° C for 15 seconds followed by 60.0° C for 1 minute. The comparative Ct method was used to determine relative gene expression using GAPDH to standardize expression levels across all samples. Relative expression changes were calculated based on comparing experimental levels of a respective liver transcript to those quantified in liver samples derived from Mock injected animals.

Antibody Titering Assay

ELISA based titering experiments were essentially completed as previously described 53. Briefly, 5 × 10^8 vp/well or 0.2 μg recombinant LacZ protein/well (each diluted in PBS) was used to coat wells of a 96 well plate overnight at 4°C. Plates were washed with PBS-Tween (0.05%) solution, and blocking buffer (3% BSA in PBS) was added to each well and incubated for 1-3 hours at room temperature. For titering of total IgG antibodies, plasma was diluted in 1:800 in blocking buffer, added to the wells, and incubated at RT for 1 h. Wells were washed using PBS-Tween (0.05%) and HRP conjugated rabbit anti-mouse antibody (BioRad, Hercules, CA) was added at a 1:4000 dilution in PBS-Tween. TMB (Sigma-Aldrich, St. Louis, MO) substrate was added to each well, and the reaction was stopped with 1 N phosphoric acid. Plates were read at 450nm in a microplate spectrophotometer. Subisotype titering was completed using a hybridoma subisotyping kit (Calbiochem, La Jolla, CA) using plasma at a dilution of 1:200 per manufacturer’s recommendations.

Neutralizing Antibody Assay

2 × 10^3 HEK293 cells were seeded in microwells in 125μl of complete media (DMEM, 10% FBS, 1% PSF). Cells were cultured overnight in a 37° C, 5% CO₂ incubator. To inactivate complement, plasma was heat inactivated for 60 min at 56° C and brought to room temperature. Dilutions were made as indicated in complete media in a total volume of 100μl for each well. 1.3 × 10^6 viral particles of Ad5-LacZ (~650 vp/cell) was next added to each dilution, mixed well and incubated at room temperature (RT) for 1 hour. 100 μl of the medium/plasma/virus mixture was applied to cells and incubated for 2-3 days. Control samples were incubated with either virus alone or complete media alone. CELLTITER 96 AQueous One solution (Promega, Madison, WI) was added to each well and incubated for 2 hours in a 37°C, 5% CO₂ incubator. 150 μl of media from each well was removed into a new microtiter plate and read at 492 nm in a microplate spectrophotometer. Blank subtracted OD492 values are reported.

B cell activation assay / Flow Cytometry

Early activation of B cells was studied by Flow Cytometry based methods. 0.75×10^{11} vp/mouse of Ad5-lacZ was injected intravenously into WT, CR1/2-KO or C3-KO mice. Forty eight hours after Ad injection splenocytes were harvested and CD19+ cells examined for expression of CD69 activation marker. Briefly, after harvesting splenocytes at 48 hpi from individual mice, RBCs were lysed by using ACK lysis buffer (Invitrogen, Carlsbad, CA).
Splenocytes were subsequently washed two times with RPMI medium 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 2 mM L-glutamine, 1% PSF (penicillin, streptomycin, fungizone), resuspended and counted. Two million cells were first washed two times with cold FACS buffer and then incubated for 15 minutes with purified rat anti-mouse CD16/CD32 Fc block (BD Biosciences, San Diego, CA). For surface staining, cells were washed one time with FACS buffer and incubated on ice for 30 minutes with the following antibodies, APC-CD3 (8 μg/ml), PerCpCy5.5-CD19 (8 μg/ml) and FITC-CD69 (10 μg/ml), (all BD Biosciences, San Diego, CA). Samples were analyzed on BD LSR II instrument and analyzed using FlowJo software (Tree Star, San Carlos, CA, USA).

Kupffer cell staining

Liver blocks, preserved in OCT compound at −80°C, were sliced into 7μM sections using a cryostat, placed on glass slides and frozen at −80°C for future use. Slides were thawed, fixed in 100% ethanol for 15min, and washed in KPBS containing 0.2% gelatin and 0.05% tween-20. Sections were permeabilized in 0.1% triton-X100 and blocked with KPBS containing 0.1% gelatin, 1% tween-20, and 5% BSA for 60 min at RT. To prevent non-specific binding of the secondary antibody, 5% rabbit serum in wash buffer was added and incubated at RT for 30 min. Rat anti-mouse F4/80 antibody (Invitrogen, Carlsbad, CA) diluted to 5.2 μg/mL in wash buffer containing 2% rabbit serum was added to the sections and incubated overnight at 4°C. Sections were washed and incubated at RT for 45 min with rabbit anti-rat Alexafluor-488 (Invitrogen, Carlsbad, CA) secondary antibody diluted 1:25,000 in wash buffer containing 2% rabbit serum. Sections were then washed, stained with DAPI (Invitrogen, Carlsbad, CA) for 5 min at RT and mounted using VECTASHIELD (Vector Laboratories, Burlingame, CA). Images were obtained using a Leica DMLB microscope, and captured using SPOT software v.3.5.9. Both DAPI and F4/80 stained images were transferred to Adobe Photoshop and converted to grayscale. The background threshold was set based on mock injected livers, and pixels were quantified. F4/80 fluorescence values were normalized to DAPI fluorescence values to control for cell number differences. Values are reported as percent relative to number of Kupffer cells identically enumerated in liver tissues derived from mock injected control mice.

Hematoxilin and Eosin staining

To study the role of mCR1/2 in Ad vector mediated hepatic inflammation at 28 dpi, Hematoxilin and Eosin (H&E) staining of mouse liver samples was performed as previously described \(^{29}\). Briefly, tissues were fixed in 10% neutral formalin for 12 hours, washed in 70% ethanol, embedded in paraffin and 6-μm sections were stained with H&E. We have adapted a previously developed semi-quantitative scoring system, which allows the level of hepatic pathology between different liver sections to be quantified and statistically compared \(^{29}\). For every mouse, 10 liver sections obtained at different portions of the liver (0-1000 μm from liver surface) were analyzed and given a numerical score (0-3) for three different categories of liver pathology:

1. **Portal inflammation:**
   
   0 – no portal inflammation
1 – low-moderate number of inflammatory cells (macrophages, lymphocytes) evident in <1/3 of portal tracts
2 – moderate number of inflammatory cells in 1/3-2/3 of portal tracts
3 – high number of inflammatory cells in over 2/3 of portal tracts

2. Periportal inflammation
0 – no inflammation
1 – low-moderate number of inflammatory cells infiltration evident around <1/3 of portal tracts
2 – moderate number of inflammatory cells infiltrated through 1/3-2/3 of portal tracts, in majority of which they take less that 50% of circumference. Minimum hepato-cellular necrosis observed.
3 – moderate-high number of inflammatory cells infiltrated through over 2/3 of portal tracts or infiltrated through over 1/3 of tracts but occupy over 50% of circumference in at least 50% of them. Significant hepato-cellular necrosis observed.

3. Lobular inflammation
0 – no inflammation
1 – minimum-moderate necrosis observed in <1/3 of lobules
2 – moderate hepato-cellular necrosis observed in 1/3-2/3 of lobules
3 – moderate-severe hepato-cellular necrosis observed in over 2/3 of lobules

Two independent researchers have scored all the slides in a blind manner and the averages of their scores were taken. The sum of scores (10 slides) for each mouse was taken and individual category scores were averaged for each group. Total inflammation index was computed by averaging the sum of all three individual category scores for each mouse.

**Ad genome copy number per liver cell**

To determine the number of Ad genome copies per liver cell at different time points post-transduction liver tissues (<0.1 g) were snap frozen in liquid nitrogen, crushed to a fine powder using a mortar and pestle and total DNA was extracted from as previously described 54. Ad genome copy numbers were assessed using Real-Time PCR based quantification. PCR reactions were performed on an ABI 7900HT Fast Real-Time PCR System using the SYBR Green PCR Mastermix as described for qRT-PCR technique. Primers generated against the Ad5 Hexon gene have been previously described 18. As an internal control for ensuring adequate DNA amplification, liver DNA was quantified using primers spanning the GAPDH gene. Standard curves were run in duplicate and consisted of 6 half-log dilutions using total genomic DNA, or DNA extracted from the purified Ad5-LacZ virus. These standard curves were used to determine the number of viral genomes present per liver cell. Melting curve analysis confirmed the quality and specificity of the PCR (data not shown).
**Western blotting**

To determine if mCR1/2 protein has any effect on levels of major complement protein C3 (or its cleavage products) in murine plasma, we have performed Western blotting utilizing murine C3 specific polyclonal antibody, which also recognizes C3 cleavage products (Abcam, Cambridge, MA). Assay was performed as previously described \(^{37}\). Briefly, 5 μl of murine plasma, collected at 10 minutes post injection or 6 hpi from all 4 groups of mice, utilized in this study was loaded on 7.5% SDS-PAGE, transferred onto PVDF membranes and probed with C3 antibody. Subsequently, the blot was probed with secondary fluorescent antibodies, scanned and quantified utilizing Licor’s Odyssey scanner \(^{55}\).

**Statistical analysis**

For every experiment, pilot trials were performed with 3 mice per group. This allowed us to determine effect size and sample variance so that Power Analysis could be performed to correctly determine the number of subjects per group required to achieve a statistical Power > 0.8 at the 95% confidence level. Statistically significant differences in toxicities associated with innate immune response (i.e. platelet counts, gene induction, etc.) were determined using One Way ANOVA with a Student-Newman-Keuls post-hoc test (p value < 0.05). Furthermore, a Two Way ANOVA with a Bonferroni post-hoc test was used to analyze the levels of cytokines at 1 and 6 hpi to determine significant differences (p value < 0.05) between groups. For antibody titering assays, liver H&E stains, β-Gal activity and Ad genomes in mouse liver, a two-tailed Student t-test was used to compare 2 groups of virus injected animals (p < 0.05). All graphs in this paper are presented as Mean of the average ± SD. GraphPad Prism software was utilized for statistical analysis.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgements**

We wish to thank Michigan State University Laboratory Animal support facility for their assistance in the humane care and maintenance of the animals utilized in this work and Michigan State University Investigative Histopathology Laboratory for performing H&E stains of liver tissues. S.S.S. was supported by American Heart Association Midwest Affiliate Fellowship 0815660G. A.A. was supported by the National Institutes of Health grants RO1DK-069884, P01 CA078673, the MSU Foundation as well the Osteopathic Heritage Foundation.

**References**

1. Appledorn DM, McBride A, Seregin S, Scott JM, Schuldt N, Kiang A, et al. Complex interactions with several arms of the complement system dictate innate and humoral immunity to adenoviral vectors. Gene therapy. 2008
2. Zhu J, Huang X, Yang Y. Type I IFN signaling on both B and CD4 T cells is required for protective antibody response to adenovirus. J Immunol. 2007; 178:3505–3510. [PubMed: 17339445]
3. Carlisle RC, Di Y, Cerny AM, Sonnen AF, Sim RB, Green NK, et al. Human erythrocytes bind and inactivate type 5 adenovirus by presenting Coxsackie virus-adenovirus receptor and complement receptor 1. Blood. 2009; 113:1909–1918. [PubMed: 19131551]
4. Muruve DA, Petrilli V, Zaiss AK, White LR, Clark SA, Ross PJ, et al. The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. Nature. 2008; 452:103–107. [PubMed: 18288107]

*Gene Ther.* Author manuscript; available in PMC 2014 May 30.
5. Xu Z, Tian J, Smith JS, Byrnes AP. Clearance of adenovirus by Kupffer cells is mediated by scavenger receptors, natural antibodies, and complement. Journal of virology. 2008; 82:11705–11713. [PubMed: 18815305]

6. Gasque P. Complement: a unique innate immune sensor for danger signals. Molecular immunology. 2004; 41:1089–1098. [PubMed: 15476920]

7. Kawano M. Complement regulatory proteins and autoimmunity. Archivum immunologicae et therapiæ experimentalis. 2000; 48:367–372. [PubMed: 11140463]

8. Morgan BP, Marchbank KJ, Longhi MP, Harris CL, Gallimore AM. Complement: central to innate immunity and bridging to adaptive responses. Immunology letters. 2005; 97:171–179. [PubMed: 15752555]

9. Kemper C, Atkinson JP. T-cell regulation: with complements from innate immunity. Nature reviews. 2007; 7:9–18.

10. Prechtl J, Erdei A. Immunomodulatory functions of murine CR1/2. Immunopharmacology. 2000; 49:117–124. [PubMed: 10904111]

11. Tedder TF, Inaoki M, Sato S. The CD19-CD21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity. Immunity. 1997; 6:107–118. [PubMed: 9047233]

12. Tedder TF, Haas KM, Poe JC. CD19-CD21 complex regulates an intrinsic Src family kinase amplification loop that links innate immunity with B-lymphocyte intracellular calcium responses. Biochemical Society transactions. 2002; 30:807–811. [PubMed: 12196203]

13. Carroll MC. The complement system in B cell regulation. Molecular immunology. 2004; 41:141–146. [PubMed: 15159059]

14. Boackle SA, Holers VM, Karp DR. CD21 augments antigen presentation in immune individuals. European journal of immunology. 1997; 27:122–129. [PubMed: 9022008]

15. Holers VM, Kulik L. Complement receptor 2, natural antibodies and innate immunity: Inter-relationships in B cell selection and activation. Molecular immunology. 2007; 44:64–72. [PubMed: 16876864]

16. Fairweather D, Frisancho-Kiss S, Njoku DB, Nyland JF, Kaya Z, Yusung SA, et al. Complement receptor 1 and 2 deficiency increases coxsackievirus B3-induced myocarditis, dilated cardiomyopathy, and heart failure by increasing macrophages, IL-1beta, and immune complex deposition in the heart. J Immunol. 2006; 176:3516–3524. [PubMed: 16517720]

17. Haas KM, Hasegawa M, Steeber DA, Poe JC, Zabel MD, Bock CB, et al. Complement receptors CD21/35 link innate and protective immunity during Streptococcus pneumoniae infection by regulating IgG3 antibody responses. Immunity. 2002; 17:713–723. [PubMed: 12479818]

18. Appledorn DM, Kiang A, McBride A, Jiang H, Seregin S, Scott JM, et al. Wild-type adenoviruses from groups A-F evoke unique innate immune responses, of which HAd3 and SAd23 are partially complement dependent. Gene therapy. 2008

19. Hartman ZC, Appledorn DM, Amalfitano A. Adenovirus vector induced innate immune responses: impact upon efficacy and toxicity in gene therapy and vaccine applications. Virus research. 2008; 132:1–14. [PubMed: 18036698]

20. Hartman ZC, Kiang A, Everett RS, Serra D, Yang XY, Clay TM, et al. Adenovirus infection triggers a rapid, MyD88-regulated transcriptome response critical to acute-phase and adaptive immune responses in vivo. Journal of virology. 2007; 81:1796–1812. [PubMed: 17121790]

21. Kiang A, Hartman ZC, Everett RS, Serra D, Jiang H, Frank MM, et al. Multiple innate inflammatory responses induced after systemic adenovirus vector delivery depend on a functional complement system. Mol Ther. 2006; 14:588–598. [PubMed: 16733096]

22. Li Y, Muruve DA, Collins RG, Lee SS, Kubes P. The role of selectins and integrins in adenovirus vector-induced neutrophil recruitment to the liver. European journal of immunology. 2002; 32:3443–3452. [PubMed: 12442326]

23. Liu Q, Zaiss AK, Colarusso P, Patel K, Haljan G, Wickham TJ, et al. The role of capsid-endothelial interactions in the innate immune response to adenovirus vectors. Human gene therapy. 2003; 14:627–643. [PubMed: 12804145]
24. Schiedner G, Hertel S, Kochanek S. Efficient transformation of primary human amniocytes by E1 functions of Ad5: generation of new cell lines for adenoviral vector production. Human gene therapy. 2000; 11:2105–2116. [PubMed: 11044912]

25. Miwa K, Igawa A, Inoue H. Soluble E-selectin, ICAM-1 and VCAM-1 levels in systemic and coronary circulation in patients with variant angina. Cardiovascular research. 1997; 36:37–44. [PubMed: 9415270]

26. Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, et al. Fatal systemic inflammatory response syndrome in an ornithine transcarbamylase deficient patient following adenoviral gene transfer. Molecular genetics and metabolism. 2003; 80:148–158. [PubMed: 14567964]

27. Manickan E, Smith JS, Tian J, Eggerman TL, Lozier JN, Muller J, et al. Rapid Kupffer cell death after intravenous injection of adenovirus vectors. Mol Ther. 2006; 13:108–117. [PubMed: 16198149]

28. Cotter MJ, Zaiss AK, Muruve DA. Neutrophils interact with adenovirus vectors via Fc receptors and complement receptor 1. Journal of virology. 2005; 79:14622–14631. [PubMed: 16282462]

29. Hu H, Serra D, Amalfitano A. Persistence of an [E1-, polymerase-] adenovirus vector despite transduction of a neoaantigen into immune-competent mice. Human gene therapy. 1999; 10:355–364. [PubMed: 10048388]

30. Everett RS, Hodges BL, Ding EY, Xu F, Serra D, Amalfitano A. Liver toxicities typically induced by first-generation adenoviral vectors can be reduced by use of E1, E2b-deleted adenoviral vectors. Human gene therapy. 2003; 14:1715–1726. [PubMed: 14670123]

31. Hartman ZC, Black EP, Amalfitano A. Adenoviral infection induces a multi-faceted innate cellular immune response that is mediated by the toll-like receptor pathway in A549 cells. Virology. 2007; 358:357–372. [PubMed: 17027060]

32. Zinn KR, Szalai AJ, Stargel A, Krasnykh V, Chaudhuri TR. Bioluminescence imaging reveals a significant role for complement in liver transduction following intravenous delivery of adenovirus. Gene therapy. 2004; 11:1482–1486. [PubMed: 15295616]

33. Hawlisch H, Kohl J. Complement and Toll-like receptors: key regulators of adaptive immune responses. Molecular immunology. 2006; 43:13–21. [PubMed: 16019071]

34. Carroll M. Role of complement receptors CD21/CD35 in B lymphocyte activation and survival. Current topics in microbiology and immunology. 1999; 246:63–68. discussion 69. [PubMed: 10396040]

35. Sato S, Miller AS, Howard MC, Tedder TF. Regulation of B lymphocyte development and activation by the CD19/CD21/CD81/Leu 13 complex requires the cytoplasmic domain of CD19. J Immunol. 1997; 159:3278–3287. [PubMed: 9317126]

36. Miwa T, Song WC. Membrane complement regulatory proteins: insight from animal studies and relevance to human diseases. International immunopharmacology. 2001; 1:445–459. [PubMed: 11367529]

37. Miwa T, Zhou L, Tudoran R, Lambris JD, Madaio MP, Nangaku M, et al. DAF/Crry double deficiency in mice exacerbates nephrotoxic serum-induced proteinuria despite markedly reduced systemic complement activity. Molecular immunology. 2007; 44:139–146. [PubMed: 16887189]

38. Turnberg D, Botto M. The regulation of the complement system: insights from genetically-engineered mice. Molecular immunology. 2003; 40:145–153. [PubMed: 12914820]

39. Moser B, Willimann K. Chemokines: role in inflammation and immune surveillance. Annals of the rheumatic diseases. 2004; 63(Suppl 2):i184–i189. [PubMed: 15479880]

40. Zaiss AK, Cotter MJ, White LR, Clark SA, Wong NC, Holers VM, et al. Complement is an essential component of the immune response to adeno-associated virus vectors. Journal of virology. 2008; 82:2727–2740. [PubMed: 18199646]

41. Tian J, Xu Z, Smith JS, Holherr SE, Barry MA, Byrnes AP. Adenovirus activates complement by distinctly different mechanisms in vitro and in vivo: indirect complement activation by virions in vivo. Journal of virology. 2009

42. Croyle MA, Chirmule N, Zhang Y, Wilson JM. “Stealth” adenoviruses blunt cell-mediated and humoral immune responses against the virus and allow for significant gene expression upon readministration in the lung. Journal of virology. 2001; 75:4792–4801. [PubMed: 11312351]
43. Ricklin D, Lambris JD. Compstatin: a complement inhibitor on its way to clinical application. Advances in experimental medicine and biology. 2008; 632:273–292. [PubMed: 19025129]

44. Green TD, Newton BR, Rota PA, Xu Y, Robinson HL, Ross TM. C3d enhancement of neutralizing antibodies to measles hemagglutinin. Vaccine. 2001; 20:242–248. [PubMed: 11567770]

45. Ross TM, Xu Y, Bright RA, Robinson HL. C3d enhancement of antibodies to hemagglutinin accelerates protection against influenza virus challenge. Nature immunology. 2000; 1:127–131. [PubMed: 11248804]

46. Draper SJ, Moore AC, Goodman AL, Long CA, Holder AA, Gilbert SC, et al. Effective induction of high-titer antibodies by viral vector vaccines. Nature medicine. 2008; 14:819–821.

47. Ogun SA, Dumon-Seignovert L, Marchand JB, Holder AA, Hill F. The oligomerization domain of C4-binding protein (C4bp) acts as an adjuvant, and the fusion protein comprised of the 19-kilodalton merozoite surface protein 1 fused with the murine C4bp domain protects mice against malaria. Infection and immunity. 2008; 76:3817–3823. [PubMed: 18474650]

48. Ricklin D, Lambris JD. Complement-targeted therapeutics. Nature biotechnology. 2007; 25:1265–1275.

49. Hodges BL, Evans HK, Everett RS, Ding EY, Serra D, Amalfitano A. Adenovirus vectors with the 100K gene deleted and their potential for multiple gene therapy applications. Journal of virology. 2001; 75:5913–5920. [PubMed: 11390592]

50. Ng P, Graham FL. Construction of first-generation adenoviral vectors. Methods in molecular medicine. 2002; 69:389–414. [PubMed: 11987791]

51. Amalfitano A, Hauser MA, Hu H, Serra D, Begy CR, Chamberlain JS. Production and characterization of improved adenovirus vectors with the E1, E2b, and E3 genes deleted. Journal of virology. 1998; 72:926–933. [PubMed: 9444984]

52. Gommmerman JL, Oh DY, Zhou X, Tedder TF, Maurer M, Galli SJ, et al. A role for CD21/CD35 and CD19 in responses to acute septic peritonitis: a potential mechanism for mast cell activation. J Immunol. 2000; 165:6915–6921. [PubMed: 11120817]

53. Hensley SE, Cun AS, Giles-Davis W, Li Y, Xiang Z, Lasaro MO, et al. Type I interferon inhibits antibody responses induced by a chimpanzee adenovirus vector. Mol Ther. 2007; 15:393–403. [PubMed: 17235319]

54. Hofstetter JR, Zhang A, Mayeda AR, Guscar T, Nurnberger JI Jr, Lahiri DK. Genomic DNA from mice: a comparison of recovery methods and tissue sources. Biochemical and molecular medicine. 1997; 62:197–202. [PubMed: 9441873]

55. Loniewski KJ, Patial S, Parameswaran N. Sensitivity of TLR4- and -7-induced NF kappa B1 p105-TPL2-ERK pathway to TNF-receptor-associated-factor-6 revealed by RNAi in mouse macrophages. Molecular immunology. 2007; 44:3715–3723. [PubMed: 17507094]
Figure 1. Murine Complement Receptor 1/2 mitigates Ad mediated cytokine and chemokine release in C57BL/6 mice

C57BL/6 WT and CR1/2-KO mice were intravenously injected with $0.75 \times 10^{11}$ vp/mouse of Ad5-LacZ vector. Plasma samples were collected at 1 and 6 hours post virus injection (hpi). Plasma samples were analyzed using a multiplexed bead array based system. Statistical analysis was completed using Two Way ANOVA with a Bonferroni post-hoc test. The N=4 for Mock (PBS) injected animals, N=6 for virus injected mice) and 6 hpi (N=4 for Mock, N=12 for virus treated mice. The bars represent Mean ± SD.

*, ** - indicate plasma cytokine values that are statistically different from those in Mock injected animals of the same treatment at the same time point (i.e. CR1/2-KO_Ad-LacZ group from CR1/2-KO_Mock group), p<0.05, p<0.001 respectively.

#, ## - indicate statistically different values in CR1/2-KO_Ad5-LacZ group compared to WT_Ad5-LacZ group at the same time point, p<0.05, p<0.001 respectively.
Figure 2. Murine Complement Receptor 1/2 reduces Ad dependent activation of endothelial cells in C57BL/6 mice
C57BL/6 WT and CR1/2-KO mice were intravenously injected with $0.75 \times 10^{11}$ vp/mouse of Ad5-LacZ vector. Plasma samples, collected at 6 hpi (N=6 for virus treated groups, N=4 for Mock injected groups) were analyzed using a multiplexed bead array based quantitative system. The bars represent Mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test. Fold difference over WT_Mock group is shown. *, ** - indicate values, statistically different from those in Mock injected animals of the same genotype, p<0.05, p<0.001 respectively. #, ## - indicate statistically different values in CR1/2-KO_Ad5-LacZ group compared to WT_Ad5-LacZ group, p<0.05, p<0.001 respectively.
Figure 3. The efficacy of Ad transduction of the liver of C57BL/6 mice is not dependent upon murine Complement Receptor 1/2, but the levels of Ad derived transgene expression are complement and mCR1/2 dependent

A) qPCR based quantification of Ad5-LacZ genomes in livers harvested from C57BL/6 WT and CR1/2-KO mice at 6 hpi, 24 hpi, 28 dpi. The bars represent Mean ± SD. Statistical analysis was completed using two-tailed Student t-test to compare 2 groups of virus injected animals. #, ## - indicate statistically different values in CR1/2-KO_Ad5-LacZ group compared to WT_Ad5-LacZ group, p<0.05, p<0.001 respectively. Note the difference in scale for different time points. B) In situ visualization of bacterial β-galactosidase in liver of Ad5-LacZ treated C57BL/6 WT and mCR1/2-KO mice. Cryosections of liver from all groups of mice were stained for β-Gal in situ as described in Materials and Methods. Representative sections for each of the groups are shown. Total magnification of 200X was used to obtain images. N=6 for all virus injected groups at 6 hpi, N=4 for all virus injected groups at 24 hpi, N=5 for all virus injected groups at 28 dpi, N=4 for all Mock injected groups at all time points. C) Bacterial β-galactosidase activity levels were analyzed in liver protein homogenates prepared at 6 hpi, 24 hpi and 28 dpi from four groups of mice: WT_Mock, CR1/2-KO_Mock, WT_Ad5-LacZ and CR1/2-KO_Ad5-LacZ. Activity levels were presented as Units per mg of total protein (see Materials and Methods). The bars represent Mean ± SD. Statistical analysis was completed using two-tailed Student t-test to compare 2 groups of virus injected animals. #, ### - indicate statistically different values in CR1/2-KO_Ad5-LacZ group compared to WT_Ad5-LacZ group, p<0.05, p<0.001 respectively.
Figure 4. mCR1/2-KO mice exhibit significantly reduced Ad vector capsid specific humoral immune responses

Three groups of mice were treated as described in Materials and Methods: WT_Mock (N=4), WT_Ad5-LacZ (N=5), CR1/2-KO_Ad5-LacZ (N=5). Plasma samples, collected at 14 dpi and 28 dpi, were analyzed for anti Ad capsid specific total IgM, IgA and IgG antibodies and various IgG subclasses. The error bars represent ± SD. Statistical analysis was completed using two-tailed Student t-test to compare 2 groups of virus injected animals. #, ## - indicate statistically different values in CR1/2-KO_Ad5-LacZ group compared to WT_Ad5-LacZ group, p<0.05, p<0.001 respectively.

*, ** - indicate values, statistically different from animals of the same group at different time point, p<0.05, p<0.001 respectively.
Figure 5. mCR1/2-KO mice exhibit significantly reduced Ad capsid specific neutralizing antibodies titer

Three groups of mice were treated as described in Materials and Methods: WT_Mock (N=4), WT_Ad5-LacZ (N=5), CR1/2-KO_Ad5-LacZ (N=5). Plasma samples were collected at 28 dpi and assayed for neutralizing antibodies using successive dilutions (see Materials and Methods). The error bars represent ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, p<0.05 was deemed a statistically significant difference.

*, ** - indicate values, statistically different from those in WT_Mock injected animals, p<0.05, p<0.001 respectively.

#, ## - indicate statistically different values in CR1/2-KO_Ad5-LacZ group compared to WT_Ad5-LacZ group, p<0.05, p<0.001 respectively.

The bottom bar graph shows endpoint neutralizing antibody titer. Note significant difference in endpoint NAb titer detected between WT and CR1/2-KO virus injected groups by two-tailed Student t-test.
Figure 6. mCR1/2 and C3-KO mice have impaired B cell activation in response to systemic Ad injection
C57BL/6_WT, C3-KO and CR1/2-KO mice were intravenously injected with 0.75×10\(^{11}\) vp/mouse of Ad5-LacZ vector. Splenocytes were isolated at 48 hpi and processed as described in Materials and Methods. Percentage of activated B cells (CD19\(^+\)/CD69\(^+\) splenocytes) was determined by flow Cytometry based methods. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, p<0.05 was deemed a statistically significant difference. The bars represent Mean ± SD.
*, ** - indicate values statistically different from those in Mock injected animals of the same treatment (i.e. CR1/2-KO_Ad-LacZ group from CR1/2-KO_Mock group), p<0.05, p<0.001 respectively.
#, ## - indicate statistically different values in virus injected complement deficient mice groups compared to WT_Ad5-LacZ group, p<0.05, p<0.001 respectively.
Figure 7. mCR1/2 protein plays a significant role in down-regulation of Ad mediated complement dependent pro-inflammatory cytokines production

This graph summarizes data obtained in this study (Figure 1) and our previously published data on Ad mediated cytokine release in C3-KO mice. Plasma levels (at 6 hpi) of pro-inflammatory cytokines and chemokines in Ad injected CR1/2-KO and C3-KO mice are normalized to the levels of WT_Ad5-LacZ. Levels higher than the ones observed in WT mice have positive fold induction (CR1/2-KO mice), whereas reduced levels compared to the levels of WT mice (C3-KO) have negative fold induction. Shaded area represents the levels of cytokines release in Ad injected WT mice.

*, ** - represent a statistically significant differences between Ad injected WT and complement (CR1/2 or C3) knockout mice, p<0.05, p<0.001 respectively.

Note, that MCP-1, IL-12p40, G-CSF and RANTES are all induced in a complement dependent manner (evident by the lack of induction of these cytokines/chemokines in C3-KO mice). Moreover, mCR1/2 protein down-regulates the induction of these very molecules (MCP-1, G-CSF and RANTES).
### Ad5-LacZ induced gene expression in a liver (fold over C57BL/6_WT_Mock)

| Gene    | C57BL/6 WT 6 hpi Mock | CRI/2 -/- 6 hpi Mock | C57BL/6 WT 6 hpi Ad5-LacZ | CRI/2 -/- 6 hpi Ad5-LacZ |
|---------|-----------------------|----------------------|---------------------------|--------------------------|
| ADAR    | 1.0 ± 0.2             | 1.0 ± 0.1            | 7.1 ± 1.2                 | 8.7 ± 1.8                |
| CXCL-9  | 1.0 ± 0.1             | 1.8 ± 0.6            | 27.9 ± 6.0                | 34.2 ± 19.9              |
| DAF     | 1.0 ± 0.4             | 1.9 ± 0.1            | 1.8 ± 0.3                 | 2.1 ± 0.6                |
| GATA-3  | 1.0 ± 0.2             | 1.4 ± 0.2            | 1.2 ± 0.4                 | **2.0 ± 0.3**            |
| ICAM    | 1.0 ± 0.5             | 1.6 ± 0.4            | 5.3 ± 1.0                 | **7.7 ± 1.6**            |
| IFNα    | 1.1 ± 0.5             | 0.9 ± 0.2            | 1.9 ± 0.5                 | 1.7 ± 0.6                |
| IFNβ    | 1.0 ± 0.3             | 1.0 ± 0.1            | 1.1 ± 0.2                 | 1.1 ± 0.2                |
| IRF-7   | 1.1 ± 0.7             | 1.2 ± 0.4            | 22.4 ± 4.9                | **22.7 ± 7.7**           |
| IRF-8   | 1.1 ± 0.7             | 2.0 ± 0.5            | 5.9 ± 0.7                 | **6.4 ± 2.4**            |
| Jak-1   | 1.0 ± 0.3             | 1.0 ± 0.2            | 1.3 ± 0.2                 | **1.6 ± 0.4**            |
| Jak-3   | 1.0 ± 0.3             | 1.2 ± 0.3            | 1.7 ± 0.3                 | **2.4 ± 0.3**            |
| MxD88   | 1.0 ± 0.4             | 1.05 ± 0.03          | 6.0 ± 1.2                 | **9.9 ± 2.0**            |
| NFKB-RelA | 1.0 ± 0.3           | 1.1 ± 0.1            | 2.2 ± 0.3                 | **2.4 ± 0.4**            |
| NOD-1   | 1.0 ± 0.3             | 1.2 ± 0.3            | 2.5 ± 0.4                 | **3.5 ± 1.1**            |
| NOD-2   | 1.1 ± 0.5             | 0.2 ± 0.1            | 2.6 ± 0.8                 | **0.6 ± 0.2**            |
| OAS-1a  | 1.0 ± 0.4             | 1.2 ± 0.5            | 20.7 ± 6.4                | **27.7 ± 6.1**           |
| SOCS-1  | 1.0 ± 0.4             | 1.1 ± 0.5            | 70.5 ± 19.1               | **115.4 ± 22.6**         |
| SOCS-3  | 1.1 ± 0.5             | 0.6 ± 0.2            | 2.8 ± 0.6                 | **3.2 ± 1.0**            |
| TBK-1   | 1.0 ± 0.2             | 1.2 ± 0.1            | 4.6 ± 0.6                 | **7.3 ± 2.2**            |
| TLR-2   | 1.0 ± 0.1             | 2.7 ± 0.3            | 169.2 ± 54.2              | **251.8 ± 71.8**         |
| TLR-3   | 1.0 ± 0.2             | 0.8 ± 0.2            | 14.8 ± 2.6                | **28.5 ± 6.1**           |
| TLR-6   | 1.0 ± 0.4             | 1.2 ± 0.4            | 6.0 ± 1.8                 | **7.0 ± 1.9**            |
| TRAF2bp | 1.1 ± 0.6             | 1.3 ± 0.5            | 18.8 ± 4.8                | **62.6 ± 9.2**           |
| TRAF6   | 1.1 ± 0.6             | 1.9 ± 0.3            | 1.8 ± 0.3                 | **2.1 ± 0.4**            |
| TRIF    | 1.0 ± 0.4             | 1.4 ± 0.2            | 1.8 ± 0.3                 | **2.5 ± 0.7**            |
| VCAM    | 1.0 ± 0.1             | 1.0 ± 0.1            | 2.0 ± 0.2                 | **3.3 ± 0.7**            |

The numbers represent Mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, p<0.05 was deemed a statistically significant difference. Note, when significant p<0.001 was observed in majority of cases. N=4 for Mock injected groups, N=6 for virus injected groups was used. Significant differences compared to C57BL/6_WT_Mock are highlighted in grey color. Significant inductions of transcriptional activation in CR1/2-KO_Ad5-LacZ group compared to WT_Ad5-LacZ group are indicated in table with black frame and boldface font. Note that there was no significant differences detected between Mock injected WT and CR1/2-KO mice.