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UNIVERSITY OF CALIFORNIA

Los Angeles

Immune regulation and early inflammatory predictors of adverse responses to systemic
AAV gene therapy in mice and humans

A dissertation submitted in partial satisfaction of the requirements for the degree of
Doctor of Philosophy in Molecular Biology

by

Robert Joseph Jimenez

2026

ABSTRACT OF THE DISSERTATION

Immune regulation and early inflammatory predictors of adverse responses to systemic AAV gene therapy in mice and humans

by

Robert Joseph Jimenez

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2026

Professor Melissa J. Spencer, Chair

Systemic adeno-associated virus (AAV) gene therapy has demonstrated substantial therapeutic potential for monogenic disorders but can provoke variable immune responses that reduce efficacy and contribute to serious adverse events. The mechanisms regulating these responses remain incompletely understood, and clinically useful predictors of toxicity are limited. This dissertation investigates immune regulation and early predictors of adverse responses to systemic AAV therapy through complementary preclinical and clinical studies.

The first study examined how the classical complement component C1q shapes immune responses to repeated systemic AAV administration. C1q-deficient and wild-type mice were evaluated following single or repeated AAV dosing, with and without corticosteroid immunosuppression. C1q deficiency altered the inflammatory response to repeat administration, including an enhanced monocyte-associated chemokine signature shortly after the second dose and changes in adaptive immune-cell populations and costimulatory molecule expression. Prednisone reduced the magnitude of several inflammatory responses but did not eliminate the overall response pattern. Conditioned-media experiments further demonstrated that AAV–antibody immune complexes can induce soluble inflammatory signals capable of activating additional immune cells. Together, these findings suggest that C1q regulates immune-complex handling and influences the coordination of innate and adaptive immunity following AAV exposure.

The second study longitudinally characterized immune and clinical responses in patients receiving delandistrogene moxeparvovec (Elevidys) for Duchenne muscular dystrophy. Plasma collected before treatment and during the first two weeks after dosing was analyzed for circulating vector-derived transgene DNA, anti-capsid antibodies, cytokines,

chemokines, and liver enzymes. Patients displayed rapid transgene clearance, increasing anti-AAVrh74 antibody responses, antibody cross-reactivity across AAV serotypes, and heterogeneous inflammatory profiles. Statistical modeling identified baseline and early chemokine signatures associated with subsequent elevations in gamma-glutamyl transferase, supporting the potential development of biomarkers that identify patients at increased risk before overt liver injury occurs.

Collectively, these studies identify complement-dependent mechanisms that shape AAV immunity and demonstrate that early immune profiling may help predict clinical toxicity. These findings provide a foundation for improved risk stratification and more targeted approaches to reducing immune-mediated complications of systemic AAV gene therapy.

This dissertation of Robert Joseph Jimenez is approved.

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Dedication

To my parents, Robert Jimenez and Deborah Jimenez, whose sacrifices made it possible for me to pursue goals I once could only imagine. I can never fully express my gratitude for all you have given me.

And to everyone who has supported, encouraged, and guided me along the way, this accomplishment belongs to all of us.

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2. F. Chen, **R. Jimenez***, K. Sharma, B.Y. Hsu, H.Y. Luu, A. Ravindranathan, B.A. Stohr, and H. Willenbring. Broad Distribution of Hepatocyte Proliferation in Liver Homeostasis and Regeneration. Cell Stem Cell. 2020;26(1):27-33.e4.
3. M. Questa, M. Moshref, **R. Jimenez***, V. Lopez-Cervantes, C. Crawford, M. Settles, P. Ross, and A. Kol. Chromatin accessibility in canine stromal cells and its implications for canine somatic cell reprogramming. Stem Cells Transl Med. 2020;
4. J.G. Anderson, A. Kol, P. Bizikova, B.P. Stapelton, K. Ford, A. Villarreal, **R. Jimenez***, D. Vasilatis, B.G. Murphy. Immunopathogenesis of canine chronic ulcerative stomatitis. PLoS One. 2020; 15(1):e0227386.

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Chapter 1 – Introduction Gene therapies for treating monogenic disorders

Gene therapy has evolved into a broad therapeutic field encompassing multiple strategies for correcting, replacing, or functionally compensating for disease-associated genetic defects. These approaches can be broadly categorized as *in vivo* therapies, in which genetic material is delivered directly to tissues within the patient. As well as *ex vivo* therapies, in which patient-derived cells are isolated, genetically modified outside the body, and then reintroduced as a therapeutic product¹. Within this framework, genome editing technologies such as CRISPR enable direct modification of the patient genome and offer the potential to correct, disrupt, or regulate disease-associated sequences at their endogenous loci². In contrast, gene replacement therapy does not necessarily modify the endogenous disease-causing locus but instead introduces a functional copy of a defective or missing gene into living cells to restore expression of the therapeutic gene product³.

Adeno-associated virus (AAV) vectors have become one of the most widely used platforms for *in vivo* gene replacement therapy due to their non-pathogenic nature, relatively low propensity for host genome integration, and capacity to transduce a broad range of tissues. Recombinant AAV vectors are engineered to carry therapeutic expression cassettes in place of viral coding sequences enabling delivery of functional gene copies for the treatment of monogenic disorders. This approach has been particularly impactful for inherited diseases in which restoration of a single missing or dysfunctional protein can provide meaningful clinical benefit. The translational significance of AAV-mediated gene replacement was highlighted by the 2017 approval

of Luxturna for Leber congenital amaurosis type 2 caused by biallelic *RPE65* mutations representing the first AAV-based gene therapy approved in the United States. Since that milestone, the gene therapy field has continued to grow rapidly with approximately 28 gene therapies, and 33 cellular and gene therapy products combined, approved by the U.S Food and Drug Administration as of 2026.

AAV Biology

Adeno-associated virus (AAV) is a small, non-enveloped parvovirus with a single-stranded DNA genome of approximately 4.7 kilobases flanked by 145 base pair inverted terminal repeats (ITRs). The AAV capsid is an icosahedral protein shell composed of 60 viral protein subunits, assembled from VP1, VP2, and VP3 in an approximate 1:1:10 ratio ⁴. In wild-type AAV, the viral genome contains two major open reading frames, *rep* and *cap*. The *rep* open reading frame encodes non-structural proteins required for viral genome replication, rescue, and packaging, while the *cap* open reading frame encodes the structural proteins that form the viral capsid. Efficient AAV particle formation also depends on additional assembly-associated factors that support capsid formation and viral genome packaging ⁵.

The inverted terminal repeats are essential cis-acting sequences that distinguish AAV genomes from standard transgene expression cassettes. These structures serve as origins of replication and provide the packaging signals required for incorporation of the viral genome into the AAV capsid⁶. In recombinant AAV vectors, the viral coding sequences are replaced by a therapeutic expression cassette containing a promoter, transgene, and regulatory elements designed to drive expression of the therapeutic gene product. However, the ITRs must be retained because they are required for vector

genome replication and packaging during production. As a result, recombinant AAV vectors lack the majority of wild-type viral genes but still preserve the terminal viral sequences necessary for generating vector particles.

AAV capsids exist as multiple serotypes, each with distinct structural features that influence receptor usage, cellular entry, intracellular trafficking, and tissue tropism. These serotype-specific properties allow AAV vectors to preferentially transduce different tissues, making capsid selection a central determinant of therapeutic targeting and vector biodistribution ⁷. This biological flexibility has contributed to the widespread use of AAV vectors for gene replacement therapy across a range of genetic disorders. However, the same viral structures that enable efficient delivery can also be recognized by the host immune system. Naturally, acquired exposure to wild-type AAV can generate pre-existing humoral and cellular immunity against the capsid and in vitro studies of human peripheral blood mononuclear cells have shown that AAV-seropositive individuals rapidly mount capsid-specific adaptive immune responses, including T cell activation and antibody production, upon re-exposure to AAV antigens ⁸.

Retained vector genome elements may also contribute to innate immune activation. Although recombinant AAV vectors are stripped of most viral coding sequences, the ITRs remain. These are viral-derived DNA elements and contain unmethylated CpG motifs that can be sensed by TLR9. Studies using TLR9-expressing cells have shown that CpG motifs within the AAV ITR can promote innate immune recognition, and rational engineering of CpG-free ITR sequences can reduce this immunostimulatory activity while preserving vector function ⁹. Therefore, recombinant AAV vectors are best understood as engineered delivery platforms that remove many

features of wild-type AAV replication while retaining key viral structures necessary for therapeutic delivery. These retained features, including the capsid and ITRs, are essential for AAV vector function but can also engage host immune pathways, providing a biological basis for immunogenicity and serious adverse events increasingly reported in the clinical development of systemic AAV gene therapies.

Serious Adverse Events

Systemic AAV gene therapies have enabled therapeutic delivery to broad or difficult-to-access target tissues, including skeletal muscle, motor neurons, and liver. However, systemic administration also exposes multiple organs to high vector burdens and has been associated with a growing spectrum of serious adverse events, including hepatotoxicity, thrombocytopenia, thrombotic microangiopathy (TMA), capillary leak syndrome, and cardiac toxicity^{10 11 12}. These toxicities have been most prominently reported in high-dose neuromuscular and muscle-targeted programs, including Duchenne muscular dystrophy, spinal muscular atrophy, and X-linked myotubular myopathy, with additional safety signals emerging in Danon disease, limb-girdle muscular dystrophy, Pompe disease, and liver-directed hemophilia programs ^{10; 13; 14}. Together, these clinical observations suggest that serious adverse events associated with AAV gene therapy are closely linked to the need for systemic vector delivery, the high vector doses required to achieve therapeutic benefit in target tissues, and the interaction between vector-related immune activation and disease-specific patient vulnerabilities.

Immune responses to AAV

Innate Immune Responses to AAV

The serious adverse events observed following systemic AAV gene therapy are likely the clinical manifestations of underlying molecular and cellular immune pathways that remain incompletely defined. Although recombinant AAV vectors are engineered to remove most viral coding sequences, they retain essential viral-derived structures, including the capsid and vector genome, that can be detected by the host immune system. Consequently, immune recognition of AAV vectors remains a major barrier to both therapeutic efficacy and clinical safety, particularly in the context of high-dose systemic delivery ¹⁵.

Toll-like Receptors

The innate immune system represents the earliest phase of host recognition following AAV administration. Through pattern recognition receptors, innate immune cells detect pathogen-associated molecular patterns (PAMPs) and initiate inflammatory signaling cascades that shape subsequent adaptive immune responses. In AAV gene therapy, these early sensing events are especially important because they can influence anti-capsid antibody formation, neutralizing antibody responses, capsid-specific T cell activation, and inflammation within exposed tissues ^{16 15}. Among these innate pathways, Toll-like receptors (TLRs) have emerged as important sensors of both the AAV capsid and vector genome.

AAV capsids can be recognized through TLR2-mediated signaling and it has been demonstrated that AAV vectors induce innate immune activation in human

nonparenchymal liver cells through a TLR2-dependent mechanism, leading to inflammatory cytokine and chemokine production ¹⁷. This mechanism is particularly relevant to systemic AAV administration, because intravenously delivered vectors expose the liver to substantial vector burden regardless of whether the liver is the intended therapeutic target. TLR2-mediated capsid sensing therefore provides a potential explanation for how AAV particles can initiate early inflammatory responses in liver-resident immune and stromal cells.

AAV vector genomes can also be sensed by TLR9, an endosomal DNA receptor that recognizes unmethylated CpG motifs. Following vector uptake and endosomal trafficking, the AAV genome may become accessible to TLR9, triggering MyD88-dependent signaling and type I interferon-associated innate immune activation. Zhu et al. showed that TLR9–MyD88 signaling is critical for adaptive immune responses to AAV vectors in mice, as *Tlr9*^{-/-} and *MyD88*^{-/-} animals exhibited reduced anti-transgene and AAV-neutralizing antibody responses following gene transfer ¹⁸. This finding demonstrates that TLR9 is not only involved in early innate sensing but also contributes to the development of downstream humoral immunity against AAV vectors.

The importance of CpG-mediated TLR9 activation is further supported by studies using CpG-depleted AAV vectors. Faust et al. demonstrated that reducing CpG motifs within the packaged AAV genome allowed vectors to evade immune detection and improve transgene expression, indicating that the sequence composition of the delivered genome directly influences vector immunogenicity ¹⁹. This distinction is important because CpG depletion removes the immunostimulatory motifs from the genome ultimately delivered to target and immune cells, whereas methylation of

producer plasmid DNA may not be preserved during Rep-mediated rescue, replication, and packaging of the vector genome. Thus, CpG depletion provides a more direct strategy for reducing TLR9-mediated recognition of the AAV genome.

Together, these findings demonstrate that innate recognition of AAV involves both capsid- and genome-dependent sensing mechanisms. TLR2 can detect the AAV capsid and promote inflammatory signaling, while TLR9 recognizes CpG motifs within the vector genome and shapes downstream adaptive immune responses. These innate sensing pathways provide a mechanistic bridge between AAV vector biology and the clinical immune toxicities observed after systemic administration. Because TLR activation can promote cytokine production, antigen presentation, antibody formation, and inflammatory amplification, understanding how AAV engages TLR pathways is essential for defining the molecular mechanisms that contribute to vector immunogenicity and serious adverse events.

While TLRs provide one mechanism for innate recognition of AAV vectors, they do not fully explain the vascular injury, thrombocytopenia, and thrombotic microangiopathy observed in some patients following systemic AAV gene therapy. These clinical features implicate additional innate immune effector systems, particularly the complement cascade, which links pathogen recognition, antibody binding, inflammation, opsonization, and endothelial injury. Complement is therefore positioned at the interface of humoral immunity and innate inflammation, making it a central pathway for understanding how pre-existing or induced anti-AAV antibodies may contribute to serious adverse events after systemic vector administration.

Complement System

Early mechanistic studies demonstrated that AAV vectors can interact directly with the complement system. Zaiss et al. showed that AAV2 capsids bind complement fragments, including iC3b, and that incubation of AAV with human serum increased viral uptake by THP-1-derived macrophages ²⁰. This serum-dependent uptake was associated with increased expression of inflammatory chemokines and cytokines, suggesting that complement opsonization promotes macrophage recognition and inflammatory activation in response to AAV ²¹. These findings established complement not only as a soluble antiviral defense pathway, but also as a mechanism that can enhance cellular recognition of AAV particles and amplify innate immune activation.

More recent studies have expanded this model by demonstrating that anti-AAV antibodies can amplify complement activation. West et al. showed that AAV-neutralizing antibody complexes activate complement in human serum, supporting a model in which pre-existing anti-capsid antibodies promote classical pathway activation following AAV exposure ²². Similarly, Smith et al. demonstrated that stimulation of human whole blood with AAV induced stronger innate immune responses in samples with higher anti-AAV antibody titers. Complement inhibition with APL-9 reduced both AAV uptake and chemokine responses, supporting a role for antibody- and complement-mediated amplification of AAV-induced inflammation ²³. Together, these findings suggest that pre-existing humoral immunity can do more than neutralize vector transduction; it can also convert AAV exposure into a more inflammatory event through antibody-dependent complement activation.

This antibody-dependent model is further supported by studies of AAV-induced innate immune responses in human whole blood. Xicluna et al. demonstrated that AAV-based gene therapy vectors activate complement and inflammatory cytokine production in human blood, with responses amplified by pre-existing anti-capsid antibodies ²⁴. In addition to innate inflammatory activation, this study identified changes in adaptive immune cell populations, including expansion of B and T cell subsets, suggesting that early complement activation may help shape broader immune responses to AAV exposure. These findings are consistent with a model in which AAV vectors engage a network of immune pathways rather than a single isolated innate sensor.

Clinically, the strongest evidence linking AAV, antibodies, and complement comes from studies of AAV-associated thrombotic microangiopathy. Salabarria et al. demonstrated that TMA following systemic AAV administration is dependent on anti-capsid antibodies, with complement activation initiated through the classical pathway and amplified through the alternative pathway ²⁵. In affected patients, the immune profile included increases in anti-capsid IgM and IgG, platelet decline, elevated D-dimer, C4 consumption, and increased soluble C5b-9, consistent with antibody-dependent complement activation and complement-mediated vascular injury ²⁵. These clinical observations provide a direct mechanistic link between anti-AAV humoral immunity, complement activation, and the endothelial injury that defines TMA.

Preclinical studies further support the relevance of complement activation in AAV immune responses. Using a mouse model of Duchenne muscular dystrophy, Emami et al. demonstrated that systemic AAV administration induces anti-AAV antibody responses and complement activation, establishing an in vivo model to study the

contribution of complement to AAV-mediated innate and adaptive immunity ²⁶. This is particularly important because in vitro human blood and macrophage systems can identify candidate mechanisms, while animal models allow complement activation to be studied in the context of vector biodistribution, antibody generation, inflammatory signaling, and organ-level pathology.

AAV-associated complement activation may also contribute to endothelial injury through terminal pathway activation. Generation of C5b-9, also known as the membrane attack complex, can promote endothelial activation, dysfunction, and injury when complement regulation is overwhelmed or dysregulated ^{27 28}. In the setting of systemic AAV administration, antibody-dependent classical pathway activation and alternative pathway amplification may generate inflammatory split products such as C3a and C5a, promote opsonization through C3 fragments, and contribute to terminal complement pathway activation. However, the precise mechanisms linking AAV-induced complement activation to endothelial injury, platelet consumption, and organ damage remain incompletely understood. This gap is especially important because complement may influence AAV immunity through multiple overlapping mechanisms, including enhanced vector uptake, chemokine production, antigen presentation, vascular inflammation, and adaptive immune programming.

Together, these studies support a model in which AAV vectors engage complement through both direct particle–serum interactions and antibody-dependent classical pathway activation. Complement opsonization can enhance vector uptake and inflammatory chemokine production, while anti-capsid antibodies can amplify complement activation and contribute to clinical complications such as TMA. These

observations suggest that complement is not simply a downstream marker of AAV toxicity, but an active mediator of immune recognition and inflammatory amplification following systemic vector administration. Understanding how complement shapes AAV-induced innate and adaptive immune responses is therefore critical for defining the mechanisms underlying serious adverse events and for improving the safety of systemic AAV gene therapies.

Adaptive Immunity

Humoral Immunity

Humoral immunity is one of the major barriers to successful AAV-mediated gene therapy. Because humans are naturally exposed to wild-type AAV throughout life, many individuals develop antibodies against one or more AAV serotypes prior to therapeutic vector administration. The prevalence of anti-AAV antibodies varies by capsid serotype, age, geography, and assay methodology, but serum IgG and neutralizing factors against commonly used serotypes, including AAV1, AAV2, AAV5, AAV6, AAV8, and AAV9, are frequently detected in healthy populations^{29 30}. This pre-existing humoral immunity is clinically important because anti-AAV antibodies can reduce vector efficacy, alter vector biodistribution, and amplify inflammatory immune responses following systemic administration.

Humoral immunity can affect AAV gene therapy by directly neutralizing vector particles and by promoting Fc-mediated immune activation after antibody binding. The best-established effect of pre-existing anti-AAV antibodies is reduced vector transduction, as neutralizing antibodies bind the capsid and prevent efficient entry into

target cells. In an early liver-directed AAV2-factor IX trial for hemophilia B, a neutralizing antibody titer of 1:2 was compatible with successful transduction, whereas a titer of 1:17 prevented detectable transduction at the same high vector dose of 2×10^{12} vg/kg³¹. These findings established that even modest levels of pre-existing neutralizing antibodies can substantially affect AAV vector efficacy, particularly after systemic delivery where circulating antibodies encounter vector particles before they reach the intended target tissue.

Although neutralizing antibodies are often emphasized clinically because they directly block transduction, non-neutralizing binding antibodies may also be biologically important because they can form immune complexes and redirect vector particles toward Fc receptor- and complement-mediated uptake. Fitzpatrick et al. demonstrated that neutralizing and binding anti-capsid antibodies have distinct functional consequences: neutralizing antibodies reduced target-cell transduction, whereas binding antibodies increased capsid internalization by antigen-presenting cells and, in some contexts, increased transgene expression within these immune cell populations³². These findings suggest that antibody-bound AAV particles may be diverted away from therapeutic target cells and toward immune cells capable of antigen uptake, processing, and presentation. As a result, humoral immunity can simultaneously reduce therapeutic gene delivery and increase the likelihood of downstream adaptive immune activation.

Pre-existing humoral immunity can also amplify innate immune responses through engagement of the complement system. Antibody-bound AAV particles can interact with C1q to initiate the classical complement pathway, leading to complement

deposition, opsonization, inflammatory mediator generation, and immune cell recruitment^{23 33 25}. This mechanism provides a direct link between anti-capsid antibodies and innate immune activation. In human peripheral blood mononuclear cells, recombinant AAV stimulation of cells from seropositive donors induced IL-1 β and IL-6 secretion from monocytic dendritic cells and promoted B cell differentiation into antibody-secreting cells⁸. These data support a model in which natural exposure to wild-type AAV creates a primed humoral immune environment that can respond rapidly to recombinant AAV vectors.

High anti-AAV antibody titers may be especially important in the setting of systemic AAV-associated toxicity. Salabarria et al. demonstrated that patients with elevated anti-capsid antibody titers exhibited enhanced AAV uptake into immune cells, including dendritic cells and monocytes, along with complement activation and pro-inflammatory cytokine production^{23 24}. This is consistent with prior in vitro studies showing that antibody- and complement-dependent mechanisms can increase AAV uptake by immune cells and enhance inflammatory chemokine responses^{21 23 22}. Thus, in patients with pre-existing or rapidly induced anti-AAV antibodies, vector particles may form immune complexes that promote complement activation, phagocytic uptake, antigen presentation, and inflammatory amplification.

Because humoral immunity can limit efficacy and contribute to immunogenicity, B cell-targeted immunomodulation has been explored as a strategy to reduce anti-AAV antibody responses. Rituximab is an anti-CD20 monoclonal antibody that depletes CD20-expressing B cells and is commonly used to suppress pathogenic or treatment-limiting humoral immune responses³⁴. In the context of AAV gene therapy, rituximab-

based B cell depletion has been used to reduce anti-capsid and anti-transgene antibody responses. In a human subject case study, transient B cell depletion with rituximab, combined with additional immunomodulation, was associated with reduced anti-AAV capsid immune responses and improved target tissue transduction following AAV-mediated gene transfer ³⁵. However, because rituximab targets CD20-expressing B cells but does not efficiently deplete long-lived plasma cells, CD20 blockade may be most effective when used to prevent newly induced humoral responses rather than to eliminate established anti-AAV antibody titers.

Collectively, these studies demonstrate that anti-AAV antibodies contribute to AAV immunogenicity through mechanisms that extend beyond simple vector neutralization. While neutralizing antibodies can prevent efficient transduction of target cells, antibody-bound AAV particles may also activate complement, enhance uptake by antigen-presenting cells, and promote inflammatory amplification. This antibody-dependent amplification is especially relevant to the classical complement pathway, where C1q recognition of immune complexes can initiate downstream complement activation. Furthermore, CD20-targeted B cell depletion has been shown to reduce anti-AAV humoral responses and improve target tissue transduction, supporting antibody modulation as a strategy to improve vector efficacy and potentially reduce antibody-mediated immune activation. Therefore, humoral immunity should be considered not only as a barrier to vector delivery, but also as a key determinant of the inflammatory and complement-mediated responses that shape clinical outcomes after AAV administration.

Cellular Immunity

Cellular immunity represents an additional barrier to durable AAV-mediated gene therapy. Prior exposure to wild-type AAV can generate long-lived capsid-specific T cell memory, with AAV capsid-specific CD8⁺ and/or CD4⁺ T cell responses detected in approximately 50% of healthy adults by intracellular cytokine staining ³⁶. These pre-existing responses are clinically relevant because recombinant AAV vectors retain the viral capsid, allowing therapeutic vector administration to reactivate memory T cells generated from prior natural exposure.

Early studies demonstrated that healthy subjects could harbor AAV capsid-specific CD8⁺ T cells capable of expanding after antigen stimulation. Mingozzi et al. showed that AAV capsid-specific CD8⁺ T cells are present in humans and can expand into a memory phenotype following stimulation with AAV capsid peptides ³⁷. Importantly, T cells expanded in response to peptides from multiple AAV serotypes, suggesting that switching serotypes may not reliably avoid recall T cell responses after prior AAV exposure ³⁷. This finding is important for clinical gene therapy because prior exposure to one AAV serotype may generate T cell responses capable of recognizing related capsids used therapeutically.

AAV-mediated gene transfer can also activate capsid-specific cellular immunity in treated patients. In a clinical trial of AAV1-mediated gene transfer through multiple intramuscular injections for lipoprotein lipase deficiency, Mingozzi et al. demonstrated dose-dependent activation of AAV capsid-specific CD4⁺ and CD8⁺ T cells, with capsid-specific responses detected in 4 of 8 subjects ³⁸. In one high-dose subject, this response coincided with transient muscle enzyme elevation, suggesting immune-

mediated injury to transduced tissue. Notably, these responses were directed against the AAV capsid rather than the therapeutic transgene product, highlighting capsid-specific T cells as a potential barrier to durable transgene expression after AAV administration.

The clinical relevance of capsid-specific cellular immunity is further supported by liver-directed AAV gene therapy trials. Although George et al. demonstrated sustained therapeutic FIX activity following low-dose delivery of an AAV vector encoding a high-specific-activity factor IX Padua variant, the study also highlighted capsid-specific T cell immunity as a potential threat to long-term expression. Two participants developed ALT elevations accompanied by detectable cellular immune responses to AAV capsid peptides, and one participant experienced a reduction in FIX activity, suggesting that reactivation of capsid-specific T cells can damage or eliminate transduced hepatocytes and thereby reduce the persistence of AAV-mediated gene expression ³⁹.

These clinical observations are mechanistically supported by studies showing that AAV capsid peptides can be processed and presented on MHC class I molecules by transduced cells. Li et al. demonstrated that AAV capsid antigen presentation depends on endosomal escape of the virion into the cytosol, where capsid proteins undergo proteasomal degradation and generate peptides for MHC class I presentation ⁴⁰. This provides a mechanism by which AAV-transduced cells can display capsid-derived peptides and become targets for capsid-specific CD8⁺ T cells. Following recognition of peptide–MHC class I complexes, activated CD8⁺ T cells can mediate cytotoxicity through perforin- and granzyme-dependent killing, leading to death of the transduced target cell ⁴¹. Thus, even though recombinant AAV vectors do not encode

capsid genes, the delivered capsid itself can provide antigenic material for MHC I presentation and immune-mediated clearance of transduced cells.

Preclinical studies have provided direct experimental support for this mechanism. Using an immune-competent murine model, Palaschak et al. demonstrated that AAV capsid-specific CD8⁺ T cells can infiltrate the liver and eliminate AAV-transduced hepatocytes ⁴². This work supports the proposed mechanism that capsid-specific cellular immunity contributes to transaminitis and loss of durable transgene expression following liver-directed AAV gene therapy. Together with human clinical observations, these findings support a model in which capsid-specific CD8⁺ T cells recognize transduced cells through MHC I presentation of capsid-derived peptides and mediate tissue injury or loss of vector-transduced cells.

The clinical use of immunosuppression further supports the role of cellular immune activation in AAV-associated toxicities. Corticosteroids are commonly used to manage AAV-associated liver enzyme elevations, but some cases of hepatotoxicity may be refractory to steroid therapy. More recently, Leon-Astudillo et al. described four patients with Duchenne muscular dystrophy who developed steroid-refractory hepatotoxicity five to seven weeks after AAV-mediated micro-dystrophin gene therapy. Addition of sirolimus, an mTOR inhibitor used primarily for T cell immunomodulation, supported the potential role of T cell–targeted immunosuppression in managing AAV-associated liver inflammation ⁴³. Although this case series does not definitively prove that hepatotoxicity was T cell mediated, it suggests that pharmacologic suppression of T cell activation pathways may alleviate steroid-refractory liver inflammation after AAV gene therapy.

Collectively, these studies demonstrate that cellular immunity to AAV is both common and clinically important. Pre-existing capsid-specific T cells can be reactivated by therapeutic AAV administration, recognize capsid-derived peptides presented on MHC class I, and contribute to tissue inflammation, transduced cell clearance, and reduced durability of transgene expression. This is particularly relevant for liver- and muscle-directed AAV therapies, where long-term benefit depends on the persistence of transduced cells. Therefore, cellular immunity should be considered alongside humoral immunity and innate immune activation as a key determinant of AAV gene therapy efficacy and safety. In this thesis, I explore the relationship between innate and adaptive immunity following AAV dosing, with a particular focus on how complement activation influences inflammatory signaling, humoral immunity, and T cell responses.

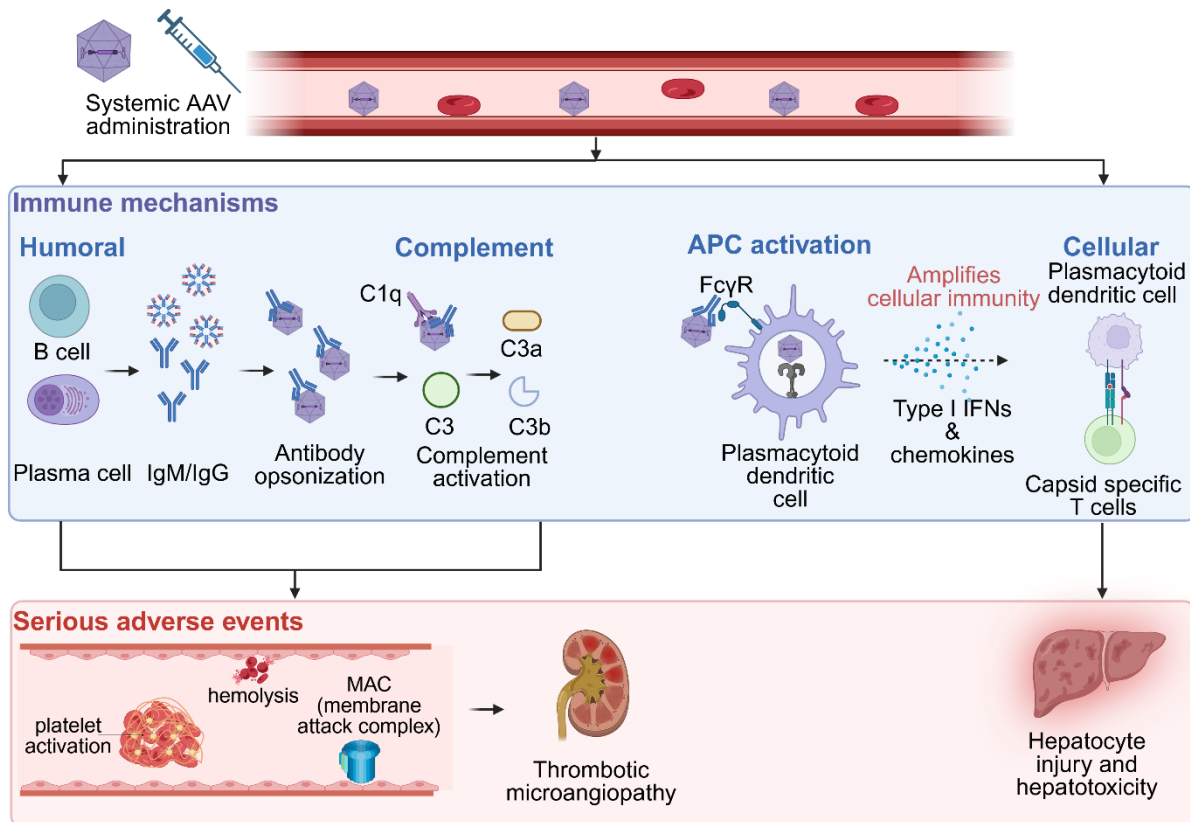


Figure 1-1. Crosstalk among humoral, innate, and cellular immune responses shapes AAV efficacy and safety.

Systemically administered AAV induces humoral, innate, and cellular immune responses. Anti-AAV IgM and IgG promote vector opsonization and immune-complex formation, leading to classical complement activation and the generation of complement cleavage products. Complement-mediated endothelial injury, platelet activation, microthrombus formation, and hemolysis may contribute to thrombotic microangiopathy and renal injury. Fcγ receptor-mediated uptake of antibody-opsonized AAV by antigen-presenting cells may enhance innate sensing and the production of type I interferons and inflammatory chemokines, thereby amplifying capsid-specific T-cell responses. Recognition of AAV capsid peptides on transduced cells can promote hepatocyte injury and hepatotoxicity. Together, these pathways illustrate how crosstalk among humoral, innate, and cellular immunity may shape the safety and efficacy of systemic AAV gene therapy.

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Chapter 2 -- Loss of Classical Complement Reprograms AAV Immunity and Drives Inflammatory T Cell Programs

Abstract

Systemically administered adeno associated virus (AAV) for gene therapy applications is associated with serious immune-related adverse events including thrombotic microangiopathy (TMA), due to uncontrolled complement activation. TMA is treated with eculizumab to block formation of the membrane attack complex, but this treatment does not block the generation of complement split products called anaphylatoxins. Given that these molecules can influence downstream immune responses, we sought to determine how eliminating the classical complement pathway in the setting of AAV dosing would impact downstream immune responses to AAV. We utilized *C1q*^{-/-} and C57 BL/6 mice given two AAV doses, four weeks apart, and conducted immune profiling. Surprisingly, the loss of C1q attenuated, but did not completely ablate, all C3 complement consumption in response to AAV dosing. In addition, C1q null mice did not show attenuated CXCL10 and CCL4 as expected after the second AAV dose. To dissect the immune responses that arise after dosing we performed single-cell RNA sequencing of spleens from AAV-dosed C1q and WT mice. C1q deficiency was associated with amplified CXCL10–CXCR3 inflammatory signaling in both CD8 T cells and T follicular helper cells compared with wild-type controls. These findings suggested a potential feed-forward mechanism mediated by CXCL10-CXCR3 signaling. Conditioned media experiments using splenocytes stimulated with AAV-antibody immune complexes support this hypothesis. Collectively, these findings indicate that ablation of C1q does not suppress most complement activation and instead leads to enhanced activation of both

CD8 T cells and T follicular helper cells through amplification of the CXCL10-CXCR3 inflammatory axis.

Introduction

Adeno-associated virus (AAV) is currently the leading platform for systemic gene delivery for monogenic disorders. Despite the promise, AAV-based therapies have been associated with serious complement-mediated adverse events, including thrombotic microangiopathy and atypical hemolytic uremic syndrome (TMA/aHUS).¹ TMA/aHUS is thought to arise from dysregulated complement activation, leading to thrombocytopenia, endothelial injury, and microvascular thrombosis, ultimately resulting in hemolysis and renal impairment. These observations highlight the critical role of complement activation in shaping the safety of AAV-mediated gene therapies.

The complement system is a central component of innate immunity that senses foreign antigens and initiates a proteolytic cascade that amplifies inflammation, promotes opsonization, and bridges innate and adaptive immune responses^{2,3,4}. Understanding how complement shapes immune responses to AAV requires considering not only its contribution to acute toxicity but also its broader role in orchestrating downstream immune responses. In the context of adeno-associated virus (AAV) gene therapy, complement is thought to be initiated predominantly through the classical pathway, with subsequent activation of the alternative pathway^{5, 6}. The classical complement pathway is initiated when C1q binds to the Fc region of IgM or IgG antibodies complexed with the AAV capsid. Upon activation, all three complement pathways converge at C3 cleavage, producing bioactive fragments such as C3a, C3b, iC3b, and C3d. Downstream, C5 is cleaved into C5a and C5b, the latter of which initiates assembly of the membrane attack complex

(MAC: C5b–C9) which inserts in endothelial cells of capillaries, leading to endothelial damage. Although complement activation is critical for pathogen clearance, dysregulated activation can contribute to host tissue damage. C3a and C5a are anaphylatoxins that promote immune cell recruitment and inflammatory mediator release, while C3b and iC3b act as opsonins that enhance phagocytosis and antigen clearance. Collectively, these anaphylatoxins provide a mechanistic link between innate and adaptive immunity.

Complement not only serves as an early innate immune defense mechanism but also regulates adaptive immunity by modulating B cell antibody responses and T cell differentiation. Complement split products such as C3d enhance B cell activation by engaging complement receptor 2 (CR2/CD21), lowering the threshold for antigen recognition and promoting class switching and somatic hypermutation⁷. In parallel, locally generated C3a and C5a can signal through receptors that influence T cell activation, survival, and differentiation^{3,8}. Antibody recognition of AAV can also enhance Fc receptor–mediated uptake on antigen-presenting cells leading to activation and pro-inflammatory cytokine/chemokine secretion, linking antibody-bound vector particles to broader innate immune activation.⁹ Mechanistically, this is supported by findings that antibody-opsonized AAV exhibits increased entry into macrophages in vitro through Fc receptor–mediated uptake¹⁰.

Therapeutic strategies to mitigate AAV-associated complement-mediated injury include the C5 inhibitor eculizumab, which has been used in patients with AAV-associated TMA to block terminal complement activation by preventing C5 cleavage into C5a and C5b, thereby limiting downstream MAC formation. However, while C5 inhibition blocks terminal pathway activity, it does not prevent the generation of upstream complement split

products such as C3a, which can continue to drive chemotaxis and modulate leukocyte activation. In addition to targeting C5, upstream inhibition at the level of C3 has also been explored. For example, the C3 inhibitor APL-9 reduces PBMC activation in vitro, as evidenced by decreased costimulatory molecule expression and reduced inflammatory chemokine production⁹. Unexpectedly, when applied in AAV gene therapy trial for Danon disease^{11,12}, this C3 inhibitor was associated with capillary leak syndrome that was fatal in two patients¹¹ which may be related to the predisposition of Danon patients to develop TMA¹². It is not entirely clear if the capillary leak observed was related to complement. However, the enhanced capillary leak observed after inhibiting C3 complement suggests that complement is highly dynamic and driven by extensive crosstalk among the three pathways, potentially limiting the effectiveness of inhibiting any single pathway in isolation.

Given that AAV initially activates the classical complement pathway, and because the role of complement in shaping immune responses to AAV is incompletely understood beyond its contribution to acute toxicity, we utilized C1q deficient mice in combination with a dual-AAV-dosing strategy previously established by Emami et al.¹³ to investigate the downstream immunological consequences of classical complement inhibition. Using multiplex cytokine/chemokine assessments, high content protein microarrays for humoral evaluation and single-cell RNA sequencing, we demonstrate that C1q blockade was insufficient to prevent C3 complement consumption and instead was associated with enhanced activation of the CXCL10–CXCR3 pathway, a key mediator of type 1 immune responses. CXCL10 is induced by inflammatory cytokines such as IFN- γ that recruits CXCR3-expressing lymphocytes to inflamed tissues and secondary lymphoid organs. By

promoting both CD8 T cell trafficking and T follicular helper cell localization, enhanced CXCL10-CXCR3 signaling in the absence of C1q likely contributes to the increased inflammatory cytokine production observed following AAV exposure.

Materials and Methods

Sex as a biological variable

Given this study focuses on gene therapy for Duchenne muscular dystrophy, an X-linked recessive disorder, only male mice were used.

Animals

C1q-deficient mice were obtained from The Jackson Laboratory. The strain used was B6(Cg)-C1qatm1d(EUCOMM)Wtsi/TennJ, commonly referred to as C1qa KO mice, JAX strain #031675. These mice carry a targeted null allele of the complement component 1, q subcomponent, alpha polypeptide gene (*C1qa*), generated through deletion of exon 3. C1qa-deficient mice were maintained on a C57BL/6 background and compared with age- and sex-matched C57BL/6J control mice. Guidelines from the Animal Research Committee at UCLA were followed in the handling and breeding of all mice. Protocols for the care and use of animals were approved by the UCLA Office of Protection of Research Subjects.

In vivo AAV9 dual-dosing study in C1q -/- and C57 BL/6 mice

For Luminex, male mice aged 8-12 weeks were systemically injected via retro-orbital administration, first with AAV9-CMV-eGFP at 1.16×10^{14} vg/kg followed by AAV9-CMV-mCherry at $\sim 1.16 \times 10^{14}$ vg/kg four weeks later. Plasma was collected prior to AAV exposure (pre), five hours after the first dose (5hrD1), two weeks after the first dose (post

1), five hours after the second dose (5hrD2), and two weeks after the second dose (post 2).

To model corticosteroid administration during AAV exposure, C1q^{-/-} and wild-type mice were treated with prednisone at 1 mg/kg once daily by intraperitoneal injection for the duration of the AAV dosing period.

For scRNA-seq experiments, 8-12 week old mice were dosed via retro-orbital administration with empty AAV9 at ~1.16E14 vg/kg. A second dose was administered using AAV9-CMV-mCherry at ~1.16E14 vg/kg two weeks after the first dose. Animals were euthanized and splenocytes harvested one and two weeks after a single AAV dose, or one week after two AAV doses.

Detection of complement components by ELISA

Mouse blood was collected in EDTA tubes and centrifuged at 6,000 rpm for 10 min at 4°C, after which the plasma layer was collected. Complement C3 was measured by ELISA using the Mouse C3 ELISA Kit (ab157711, Abcam) with plasma diluted 1:50,000 according to the manufacturer's instructions. C5b-9 was measured using an ELISA kit from Aviva Systems Biology (OKCD01374) with plasma diluted 1:30. Complement C3a was measured using the Mouse C3a ELISA Kit (NBP2-70037, Novus Biologicals) with plasma diluted 1:10. All assays were performed according to the manufacturers' instructions.

High content protein microarray for anti-AAV IgG assessment

Humoral IgG responses against AAV capsids and SpCas9 were assessed using human cellular proteome microarray (HCPM) chips generated as previously described.

Briefly, antigens were printed onto nitrocellulose-coated microarrays at four concentrations: 0.1 mg/mL, 0.05 mg/mL, 0.025 mg/mL, and 0.0125 mg/mL. Printed antigens included empty AAV2 capsid, empty AAV8 capsid and empty AAV9 capsid. Empty AAV capsids were obtained from Virovek Inc. and included AAV2-EMPTY, AAV8-EMPTY, and AAV9-EMPTY.

Plasma samples from male and female mice were evaluated across serial dilutions of 1:200, 1:2,000, 1:20,000, and 1:200,000 to determine antigen-specific IgG reactivity. Samples were analyzed at baseline prior to AAV exposure, two weeks after the first AAV dose, four weeks after the first AAV dose, and two weeks after the second AAV dose. Quantitative microarray analysis was performed by the Vaccine R&D Center at the University of California, Irvine, using their previously described workflow.

ELISA for IgM

Antigen-specific antibody levels were measured using a custom enzyme-linked immunosorbent assay. High-binding 96-well ELISA plates were coated with AAV9 empty capsid diluted in 50 mM carbonate-bicarbonate coating buffer at 1.6×10^7 vp/ μ L. Plates were washed with PBS containing 0.05% Tween-20 (PBS-T) and blocked with 5% BSA at room temperature to reduce nonspecific binding.

Serum samples were diluted 1:20 in blocking buffer and added to plates in triplicate. Samples were incubated for 2 hours at room temperature to allow antigen-specific antibodies to bind the coated capsid. Antibody isotype controls were diluted in blocking buffer beginning at 2000 ng/ μ L and serially diluted two-fold to 15 ng/ μ L. Plates were washed three times with PBS-T, followed by incubation with HRP-conjugated

secondary detection antibody specific for mouse IgG diluted 1:2000 in blocking buffer for 1 hour at 37°C.

After a final wash step, plates were developed with TMB substrate protected from light. The reaction was stopped using 1 N sulfuric acid, and absorbance was measured at 450 nm using a microplate reader. Background absorbance from blank wells was subtracted from all sample values. Antibody levels were reported as blank-corrected absorbance values at a fixed serum dilution.

Multiplex cytokine analysis using Luminex

Plasma cytokines and chemokines were quantified using a custom 27-plex ProcartaPlex mouse panel (Thermo Fisher Scientific). *The panel included IFN- α , IFN- β , IFN- γ , IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-18, IP-10/CXCL10, MCP-1, MCP-3, MIG, MIP-1 α , MIP-1 β , MIP-2 α , RANTES, and TNF- α .* Mouse plasma samples were analyzed on a Luminex 200 instrument through the UCLA Immune Assessment Core according to the manufacturer's protocol. Samples from male mice were evaluated at baseline and following AAV dosing at the following time points: pre-dose, 5 hours after the first dose, 2 weeks after the first dose, 5 hours after the second dose, and 2 weeks after the second dose.

Raw fluorescence data were processed using the cloud-based ProcartaPlex Analysis software from Thermo Fisher Scientific. Standard curves were generated using either four-parameter logistic or five-parameter logistic regression models, and assay performance was evaluated by confirming that measured standard concentrations fell within the acceptable working range, defined as 70–130% of the expected concentration.

Cytokine and chemokine concentrations were reported in picograms per milliliter. Analytes that were at or below the lower limit of quantification, such as IFN- α , were excluded from downstream analyses.

scRNAseq library construction and analysis

Single-cell RNA sequencing (scRNA-seq) was performed on splenocytes isolated as described above. Cryopreserved splenocytes were thawed and rested for 2 hr at 37°C in RPMI at a concentration of 2×10^6 cells/mL. Following rest, dead cells were removed using the Dead Cell Removal Kit (Miltenyi Biotec), and viable cells were resuspended at 1,200 cells/ μ L in DPBS containing 0.04% BSA. Single-cell libraries were prepared using the Chromium Single Cell 3' Reagent Kits v3 (10x Genomics) by the UCLA Technology Center for Genomics & Bioinformatics (TCGB). Libraries were sequenced on an Illumina NovaSeq S4 platform to a depth of approximately 20,000 reads per cell.

Raw sequencing data were processed using Cell Ranger (10x Genomics) [Seurat version 4.0] aligned to the mouse reference genome [mm10/GRCm38]. Downstream analysis was performed in Seurat v5.4.0¹⁴. Low-quality cells were excluded if they contained greater than 20% mitochondrial UMIs, fewer than 200 detected features, or more than 1,800 detected features. Gene expression counts were normalized using the NormalizeData function with default parameters. The 2,000 most variable genes were identified using FindVariableFeatures, and scaled expression values were generated using ScaleData. Dimensionality reduction, nearest-neighbor graph construction, clustering, and visualization were performed using RunPCA, FindNeighbors, FindClusters, and RunUMAP. Cell clusters were manually annotated based on canonical lineage markers and the top 50 differentially expressed genes identified using

FindAllMarkers. T cell and B cell populations were subsetted using the subset function, subclustered and reanalyzed using the same normalization, scaling, dimensionality reduction, clustering, and annotation workflow described above.

Splenocyte harvest

Spleens were mechanically dissociated in 5 mL of ice-cold PBS and passed through a 40- μ m nylon cell strainer to generate single-cell suspensions. Cells were pelleted by centrifugation at $800 \times g$ for 3 min at 4°C, and red blood cells were lysed for 2 min using Buffer EL erythrocyte lysis buffer (Qiagen). Cells were then washed twice with PBS, pelleted, counted, and cryopreserved in 90% FBS/10% DMSO at a concentration of 1×10^6 cells/mL.

Splenocyte culture and stimulation

Spleens were collected from mice and brought to single cell suspension by mechanical stimulation, filtered through a 20 micron filter, and plated in RPMI media. Splenocytes were cultured in complete RPMI supplemented with 1% heat inactivated FBS, penicillin-streptomycin, L-glutamine, and β -mercaptoethanol. Cells were cultured in complete RPMI and rested at 37°C with 5% CO₂ prior to stimulation.

For stimulation experiments, splenocytes were treated with the indicated stimuli, including AAV particles, antibody-opsonized AAV immune complexes, conditioned media, or media only controls, depending on the experiment. Cells were incubated at 37°C with 5% CO₂ for the indicated duration. At the end of culture, supernatants were collected and clarified by centrifugation prior to multiplex cytokine and chemokine analysis by Luminex.

All stimulation conditions were performed using matched cell numbers and culture volumes across experimental groups.

Results:

C1q efficiency reduces, but does not ablate, complement activation while maintaining comparable split product levels

To investigate the contribution of complement split products on downstream immune responses after AAV dosing, we compared immune outcomes in AAV-dosed C57BL/6J mice and mice deficient in C1q (*C1q*^{-/-}) on the BL/6J background. C1q is the molecule that binds to antibodies on the AAV capsid and initiates the classical complement pathway. For these studies we employed an AAV dual-dosing strategy that was previously shown to recapitulate certain aspects of patient innate and adaptive immune responses to AAV therapy¹⁵.

To evaluate complement activation across both exposures in the dual-dosing model, complement proteins were measured by ELISA at baseline, 5 hours after each AAV dose ("5hrd1" and "5hrd2"), and 2 weeks after each AAV dose ("post 1" and "post 2") (Fig. 1A). In our previous study, C3 and C4 consumption were observed only at 5hrd2, consistent with activation of the classical complement pathway¹³. In the current study, neither genotype showed C3 consumption after the first AAV dose. Unexpectedly, both WT and *C1q*^{-/-} mice showed C3 consumption at 5hrd2 (Fig. 1B). Complement activation products C5a and soluble C5b-9 were also measured, and showed no change with AAV dosing, consistent with previous findings¹³ (Fig. 1 C,D). These results indicate that loss of C1q does not fully prevent C3 consumption following dual AAV exposure, suggesting that alternative pathways or compensatory mechanisms drive complement activation in

the absence of C1q. C1q deficiency did not permit redosing (Supplemental Figure 1 A,B). GFP-positive skeletal muscle fibers were observed following the initial dose; however, no mCherry-positive fibers were detected after the second dose, indicating that AAV neutralization prevented transduction from the second AAV administration.

Classical complement ablation enhances a chemokine signature consistent with monocyte activation

To determine whether C1q ablation altered downstream inflammatory responses, we performed a 27-plex Luminex assay to measure circulating cytokines and chemokines after AAV dosing. Prior studies have shown that dual AAV dosing can induce innate immune activation only at 5hrd2 in mice¹³. Studies on humans PBMCs have demonstrated cytokine and chemokine production is enhanced through complement-dependent mechanisms^{16,17}. In contrast to the expectation that loss of classical complement initiation would reduce inflammatory signaling, C1q-deficient mice exhibited significantly increased circulating CXCL10 and CCL4 at 5hrd2 compared with wild-type mice (Fig. 2A) and a trend towards increased CCL2. This chemokine signature is consistent with AAV-immune complex uptake by monocytes followed by their activation. In contrast, IFN- γ , TNF- α , and IFN- β levels did not differ significantly between genotypes (Fig. 2A), suggesting that C1q-deficiency in AAV dosed mice was not accompanied by a broad alteration in canonical inflammatory cytokines. Our data suggest selective enhancement of monocyte-derived chemokine production following loss of classical complement.

Prior work has shown that prednisolone treatment can dampen early innate inflammatory responses following AAV dosing, including suppression of IL-1 β -associated

inflammatory signaling and innate immune transcriptional programs^{18,19}. While not specific to AAV, prior *in vitro* work has shown that prednisolone can suppress macrophage-associated inflammatory mediators, including CCL2, CXCL10, TNF- α , and CD80²⁰. To assess whether corticosteroid treatment could attenuate the chemokine response in dual dosed mice, *C1q*^{-/-} and WT mice were treated with prednisone at 1 mg/kg daily during the entire AAV dosing period. Prednisolone reduced the overall levels of CXCL10, CCL2, and CCL4 across both genotypes and in the case of CCL2, completely ablated the response. However, the overall pattern of chemokines in the blood was maintained and *C1q*^{-/-} mice retained an enhanced, albeit attenuated, response compared to WT (Fig. 2B). These findings suggest that corticosteroid treatment reduces the overall magnitude of AAV-induced chemokine production, but C1q deficiency remains associated with elevated CXCL10, CCL2 and CCL4 following repeat AAV exposure.

Comparable anti-AAV IgG responses but enhanced IgM levels in *C1q*^{-/-} vs WT

Given that anti-AAV promotes Fc γ receptor-mediated AAV-immune complex uptake and activation by antigen-presenting cells (APCs)^{10,21}, we sought to determine if differing levels of anti-AAV IgG could explain the enhanced chemokine induction in *C1q*^{-/-} mice. Anti-AAV IgG was evaluated using microarrays printed with AAV9, AAV8, and AAV2 capsids followed by exposure to serum from dual dosed mice. These assays demonstrated no significant difference in anti-AAV IgG levels between mouse genotypes (Fig. 3A). IgG reactivity was detected across AAV9 and AAV8 capsids, and neither genotype had antibodies that recognized AAV2. Thus the enhanced chemokine response in *C1q*-deficient mice cannot be explained by increased anti-AAV IgG abundance alone. In contrast, *C1q*-deficient mice exhibited moderately elevated anti-AAV IgM levels two

weeks after the second AAV dose compared with wild-type controls (Fig. 3B). In mice, FcμR/FCMR is expressed predominantly on B cells, in contrast to its broader lymphocyte expression in humans, and has been implicated in B-cell development, homeostasis, and antigen-driven immune responses^{22,23,24}. Thus, a difference in antibody levels does not explain the enhanced chemokine response in *C1q*^{-/-} mice.

Single-cell RNA sequencing of splenocytes from *C1q*-deficient and WT mice after AAV dosing

Next we used scRNA-seq to determine how *C1q* ablation impacts the broader immune landscape and to determine whether enhanced chemokine responses in *C1q*-deficient mice was associated with broader changes in immune cell composition or transcriptional state. Single cell sequencing was conducted on splenocytes from mice receiving single or dual AAV dosing, collected at baseline, one and two weeks after a single AAV dose, and one week after a second AAV dose (Fig. 4A). Cell clusters were annotated based on canonical marker gene expression (Supplemental Figure 2A). UMAP visualization of the integrated single-cell RNA-sequencing dataset identified distinct clusters corresponding to major splenic immune populations (Fig. 4B). Analysis of cell frequencies as a proportion of total recovered cells revealed an expansion of T follicular helper cells two weeks after a single AAV dose in *C1q*-deficient splenocytes compared with wild-type controls (Fig. 4C–E). Tfh cells provide essential help to B cells during germinal center responses and support antibody production, class switching, and affinity maturation^{24, 25}. In contrast, B-cell frequencies were reduced in *C1q*-deficient mice at one and two weeks after a single AAV dose, but were more comparable to wild-type controls following dual AAV dosing (Fig. 4C–E).

C1q deficiency correlates with early upregulation of IFN γ , CXCL10 and CXCR3 in CD8 T cells and T follicular helper cells

We next performed differential gene expression analysis within subclustered CD8 T, Tfh, and B-cell populations to identify inflammatory programs that may contribute to the enhanced chemokine signature observed in C1q-deficient mice. In subclustered CD8 T cells, C1q-deficient mice showed earlier and increased expression of Ifng compared with wild-type controls at both one and two weeks following a single AAV dose (Fig. 5A, B). After dual AAV dosing, CD8 T cells in C1q-deficient mice upregulated Cxcl10 and its receptor Cxcr3 (Fig. 5A, B). CD8 T cells also showed increased Cd27 expression in *C1q*^{-/-} mice after dual dosing, consistent with a costimulatory activation program that may support T-cell survival, expansion, or effector differentiation after repeat antigen exposure^{25,26}. Because CXCL10 is a ligand for CXCR3, coordinated upregulation of Cxcl10 and Cxcr3 may support a feed-forward inflammatory amplification loop that promotes recruitment or activation of CXCR3-expressing T cells after repeat AAV exposure.

Compared with wild-type controls, *C1q*^{-/-} mice showed increased Ifng and Cxcl10 expression at one and two weeks following a single AAV dose. The Cxcl10 receptor Cxcl3 was reduced in *C1q*^{-/-} but this was increased following dual dosing (Fig. 5C, D). Cd27 levels were reduced in *C1q*^{-/-} mice at 1 week but then upregulated compared to controls at 2 weeks and after dual dosing. Together, these findings suggest that loss of C1q shifts the Tfh response toward a more inflammatory, Th1-like transcriptional program after AAV exposure.

B cells from *C1q*^{-/-} mice also exhibited time-dependent transcriptional changes following AAV exposure. After a single AAV dose, *C1q*^{-/-} B cells showed increased

expression of IgM and Il10 at both one and two weeks post-dose, with the strongest expression observed at two weeks (Fig. 5C, D). This pattern suggests an early B-cell response characterized by IgM-associated and regulatory/inflammatory features. Following dual AAV dosing, however, *C1q*^{-/-} B cells appeared to shift toward an IFN- γ -responsive inflammatory program, with increased expression of *Ifngr1* and *Stat1* compared with wild-type controls (Fig. 5C, D).

Myeloid cells from *C1q*-deficient mice showed upregulation of *Il1b* and *Ccl3* at one week and *Il1b* increased at one week post-dose that is reduced compared to WT at 2 weeks and after dual dosing (Fig. 5). *CXCL10* was enhanced in *C1q*^{-/-} monocytes after dual dosing, consistent with the increased circulating *CXCL10* detected by Luminex after dual dosing. Enhanced *CCL4* was not observed in the DEGs list of any cell type.

Additional characterization of the myeloid compartment was performed using differential gene expression volcano plots (Supplemental Figure 3 A,B). Fc receptor (Supplemental Figure 4 A,B,C), complement receptor (Supplemental Figure 5 A,B,C) and TLR-associated gene expression (Supplemental Figure 6 A,B,C) was assessed across myeloid subpopulations (Supplemental Figure 7 A,B), which demonstrated enrichment of these immune receptor programs within the macrophage-like myeloid population relative to the dendritic cell-like myeloid population (Supplemental Figures 4–7). Although *Ccl4* and *Cxcl10* were not among the differentially expressed genes emphasized in the primary analysis, *Ccl4* shows higher expression in the macrophage-like myeloid population than in the dendritic cell-like myeloid population (Supplemental Figure 8 A,B,C). Prior AAV studies have linked AAV–IgG immune complexes and complement activation to myeloid

inflammatory cytokine production^{15,17}; our findings suggest that loss of C1q does not blunt this response and may instead permit or amplify inflammatory transcriptional programs.

Collectively, these data indicate that C1q deficiency alters the kinetics and quality of the transcriptional response of immune cells to AAV. Rather than reducing AAV-induced inflammation, C1q ablation appears to shift the immune response toward coordinated myeloid and T cell inflammatory activation.

Conditioned media supports amplification of CXCL10-associated inflammatory responses in C1q^{-/-} splenocytes

To test whether the enhanced chemokine response in C1q^{-/-} mice could be propagated through soluble factors, we treated naïve splenocytes with conditioned media generated from AAV–antibody immune complex–stimulated cultures. Naïve splenocytes from both C57BL/6J and C1q^{-/-} mice were stimulated in parallel with immune complexes and cultured for 24 hr to generate conditioned media. The resulting conditioned media was then transferred to fresh naïve splenocyte cultures, and supernatants were collected for Luminex analysis (Fig. 6A). This approach was designed to test whether AAV–antibody immune complexes induce secreted mediators capable of extending inflammatory responses beyond the initially stimulated cells.

Splenocytes from C1q-deficient mice exhibited higher levels of CXCL10 and IFN- γ under both direct immune complex stimulation and conditioned media treatment (Fig. 6B). Notably, the response to conditioned media was more pronounced in C1q^{-/-} splenocytes, particularly for IFN- γ and IL1b, suggesting that C1q-deficient splenocytes are more responsive to soluble inflammatory mediators generated after AAV–antibody

immune complex stimulation. Because CXCL10 is an interferon-inducible ligand for CXCR3, and CXCR3 signaling regulates inflammatory T-cell trafficking and function, these data support a model in which IFN- γ -driven CXCL10 production may recruit or position CXCR3⁺ inflammatory T cells and reinforce a feed-forward inflammatory response^{27,28}. This inflammatory chemokine environment may further support a CXCR3⁺, Th1-like inflammatory Tfh program, consistent with prior studies describing CXCR3⁺ Tfh/Tfh1-like populations with IFN- γ -producing capacity in human and mouse immune responses^{29,30}.

B and T cell subclustering reveals early genotype-associated immune remodeling after single AAV dosing

After subsetting and subclustering T-cell populations, CD8 T cells represented a larger proportion of the T-cell compartment in C1q-deficient mice compared with wild-type controls at one and two weeks after a single AAV dose. T follicular helper cells were also proportionally increased in C1q-deficient mice two weeks after AAV dosing and exhibited a distinct transcriptional profile compared with wild-type controls (Fig. 7A, B; Supplemental Figures 9 and 10). Following dual AAV dosing, CD8⁺ T cells and T follicular helper (Tfh) cells from C1q^{-/-} mice exhibited increased expression of Cd28 and Icos (Supplemental figure 11). In subclustered CD8 T cells, limited transcriptional differences were observed one week after AAV exposure. By two weeks post-dose, C1q^{-/-} CD8 T cells showed increased Cd44, Cd48, Itgb7, and Ptprcap, consistent with an activation transcriptional profile. At the dual-dose time point, increased Ccr7 suggested altered CD8 T-cell trafficking-associated gene expression (Fig. 7C). In T follicular helper cells, C1q-deficient mice showed increased Jun, Fos, and Fosb one week after AAV dosing, with

decreased *Ccr5*, *Cxcr6*, *Ctla2a*, *Ctla2b*, and *Cd8a*. By two weeks post-dose, increased *Fos*, *Fosb*, *Klf4*, *Hspa1a*, and *Hspa1b* supported a stress-associated transcriptional state. At the dual-dose time point, genotype-associated differences were less dominated by canonical immune activation or T-cell effector genes (Fig. 7C).

After subsetting and subclustering B cells, activated B cells represented a smaller proportion of the B-cell compartment in *C1q*-deficient mice compared with wild-type controls at one and two weeks after a single AAV dose, whereas follicular B cells were proportionally increased at both time points (Supplemental Figure 12 A,B,C,D,E). After two AAV doses, population differences between genotypes were minimal (Fig. 8A, B). Following dual AAV dosing, activated and follicular B cells exhibited increased *Icosl* expression, whereas differences in other costimulatory molecules were minimal (Supplemental Fig. 13). In activated B cells, limited genotype-associated differential gene expression was observed one week after a single AAV dose (Fig. 8C). By two weeks post-dose, *C1q*^{-/-} activated B cells showed increased expression of *Il2ra*, *Irf4*, *Myb*, *Tent5c*, *Cd9*, and *Ahr*. At the dual-dose time point, transcriptional differences were less defined by canonical B-cell activation markers and instead included increased *Sec61g* and *Atp5k* (Fig. 8C). In follicular B cells, *C1q*^{-/-} mice showed increased expression of *Cd9*, *Il10*, *Il15ra*, and *Mzb1* one week after single AAV dosing. By two weeks post-dose, follicular B cells showed increased *Fcrl5*, *Cd44*, *Mzb1*, and *Il5ra* (Fig 8C). Expression of *Mzb1* appeared enriched within a specific follicular B-cell subpopulation, along with *Il10* and *Ighm* (Supplemental Figure 14 A,B,C). After dual AAV dosing, differential gene expression was less consistent with inflammatory activation and instead included increased *Sec61g*, *Atp5mpl*, and *Atp5k* (Fig. 8C). These findings suggest that genotype associated

transcriptional differences following AAV exposure are time dependent with altered immune programs occurring at specific post dosing intervals.

Discussion:

In this study, we show that loss of classical complement does not lead to broadly suppressed immunity to AAV but instead is associated with enhanced inflammatory chemokine signaling and altered B- and T-cell activation programs. Although C1q^{-/-} mice showed slightly reduced C3 consumption, it did not completely eliminate complement activation, likely reflecting the dynamic and interconnected nature of the complement cascade. The classical, lectin, and alternative pathways converge at C3 and share downstream activation products, making it possible for complement activity to persist even when one initiating pathway is disrupted. In this study, terminal pathway products C5a and soluble C5b-9 were not elevated after AAV dosing, consistent with prior murine studies showing limited detection of terminal complement activation after AAV exposure¹³. These products may be short-lived or may not persist at detectable levels at later sampling timepoints. Therefore, the absence of pronounced differences in C5a or soluble C5b-9 does not exclude transient downstream C5 cleavage or MAC formation, but suggests that sustained terminal pathway activation is not readily detected in this mouse model.

Initiation of complement activation after systemic AAV dosing has primarily been attributed to the antibody-dependent classical pathway, although alternative pathway amplification may also contribute to the overall complement response^{9,6,12}. West et al. showed that AAV-antibody immune complexes can activate complement and enhance inflammatory responses, while Xicluna et al. reported that AAV2 and AAV8 capsids

activate complement primarily through the classical pathway, with evidence of alternative pathway involvement in some seronegative donors ¹⁷. Consistent with these findings, Salabarria et al. demonstrated in patients receiving systemic AAV9 that the development of anti-capsid IgM and IgG antibodies was accompanied by activation of both the classical and alternative complement pathways, supporting a model in which antibody-dependent classical pathway activation is amplified by the alternative pathway ⁶.

In our model, ablation of C1q resulted in only a moderate reduction in C3 consumption, suggesting that complement activation may persist through classical pathway-independent mechanisms. Due to the lack of murine-specific reagents, alternative pathway activation products could not be directly assessed in the current study. In addition, C3 cleavage can occur through non-canonical mechanisms, including direct proteolytic cleavage by enzymes such as thrombin, plasmin, elastase, and cathepsin G. More recently, granzyme K has also been implicated in non-canonical C3 cleavage, highlighting additional links between cytotoxic lymphocyte activity and complement regulation ³¹. Despite reduced C3 consumption, C1q-deficient mice exhibited increased expression of inflammatory chemokines following repeat AAV dosing, including Cxcl10 and Ccl4. This profile suggests that classical complement deficiency does not simply reduce complement-dependent inflammation but instead shifts the underlying immune program toward monocyte-associated and type 1 inflammatory chemokine signaling.

The monocyte compartment may represent an important site of early inflammatory programming in C1q-deficient mice after AAV exposure. In splenic monocytes, C1q deficiency was associated with timepoint-specific upregulation of Ccl3 and Il1b after a

single AAV dose, suggesting that loss of classical complement alters early myeloid transcriptional responses within the spleen. In contrast, increased circulating CCL2, CCL4, and CXCL10 were detected by Luminex only after dual AAV dosing, indicating that the protein-level chemokine response may require repeat exposure or may arise from inflammatory activity outside the splenic compartment. CXCL10 is particularly relevant because it can recruit CXCR3-expressing T cells and is commonly associated with interferon-driven inflammatory responses. However, because the scRNA-seq data were generated from splenocytes and the Luminex assay measured soluble mediators in circulation, these findings may reflect distinct but parallel inflammatory responses rather than a single connected pathway. Therefore, C1q deficiency may promote both local splenic myeloid transcriptional changes and a broader circulating chemokine response after repeat AAV exposure.

Anti-AAV IgG levels were comparable between genotypes across the AAV serotypes tested, indicating that C1q ablation did not broadly increase capsid-specific IgG abundance. Thus, the enhanced inflammatory response in C1q-deficient mice is unlikely to be explained by increased anti-AAV IgG alone. Instead, C1q deficiency altered the kinetics and phenotype of the B-cell response. Following a single AAV dose, C1q-deficient B cells exhibited increased *Ighm* and *Ii10* expression, with peak expression two weeks post-dose, consistent with an early IgM-associated B-cell activation state. This was followed by delayed B-cell expansion, with anti-AAV IgM responses continuing to increase two weeks after the second AAV dose. Although overall B-cell transcriptional profiles became more similar between genotypes after repeated AAV exposure, C1q-deficient B cells retained important differences, including increased *Ifngr1* and *Stat1* expression,

consistent with delayed acquisition of an IFN- γ -responsive phenotype. Together, these findings suggest that C1q deficiency may initially promote IgM production on a per-cell basis and subsequently permit expansion and inflammatory polarization of the B-cell response after repeated AAV exposure.

In addition to altered B-cell responses, C1q deficiency was associated with enhanced and progressively altered CD8 T-cell inflammatory programming. Following the first AAV dose, CD8 T-cell frequencies were increased in *C1q*^{-/-} mice, accompanied by increased *Ifng* expression, consistent with an early type 1 inflammatory response. IFN- γ remained elevated after a single AAV exposure, while additional transcriptional differences emerged with redosing. In particular, *Cxcl10* became more strongly increased in C1q-deficient CD8 T cells, and *Cd27* showed a delayed increase that was apparent only after the second dose. CXCR3 expression also shifted over time: *Cxcr3* was initially higher in wild-type CD8 T cells after the first dose but became higher in C1q-deficient cells at the later endpoint. CXCL10 is an interferon-inducible chemokine that signals through CXCR3, a receptor expressed by effector CD8 T cells and involved in T-cell trafficking and positioning within inflamed tissues and lymphoid environments²⁷. CD27 is a TNF receptor superfamily costimulatory receptor that supports T-cell expansion, survival, effector differentiation, and memory formation^{25,26}, and CD27/CD70 signaling has been linked to CXCL10 production and CXCR3-dependent expansion of effector CD8 T cells³². Together, the sustained increase in *Ifng* and delayed emergence of *Cxcl10*, *Cxcr3*, and *Cd27* suggest that C1q deficiency permits an initially enhanced CD8 T-cell response to evolve into a more pronounced inflammatory and costimulatory program following repeated AAV exposure.

C1q deficiency also altered the kinetics and inflammatory phenotype of the T follicular helper (Tfh) cell response following AAV exposure. After a single AAV dose, C1q-deficient Tfh cells exhibited earlier increases in *Ifng* and *Cxcl10* expression compared with wild-type cells, consistent with an accelerated type 1 inflammatory transcriptional response. In contrast, *Cd27* expression was initially reduced one week after the first dose, but this pattern shifted following repeated AAV exposure. After two doses, both *Cxcr3* and *Cd27* were increased in C1q-deficient Tfh cells, suggesting the emergence of a more activated and inflammatory Tfh phenotype with redosing. CXCR3 is the receptor for CXCL10 and has been associated with Th1-like and Tfh1-like Tfh states in inflammatory, infectious, and vaccine settings^{33 30}. These changes occurred alongside a progressively enhanced CXCL10–CXCR3 program in C1q-deficient CD8 T cells, raising the possibility that CXCL10 produced by CD8 T cells or other inflammatory populations may contribute to the recruitment, positioning, or reinforcement of CXCR3⁺ Tfh cells within the splenic environment. Consistent with the ability of this inflammatory environment to propagate across immune populations, conditioned media from C1q-deficient splenocyte cultures induced increased IL-1 β , CXCL10, and IFN- γ . Together, these findings suggest that loss of C1q shifts Tfh cells toward an increasingly inflammatory, Th1-like state following AAV exposure and may promote coordinated CXCL10–CXCR3 signaling between CD8 and Tfh cell populations after redosing.

After subsetting and subclustering CD8 T cells, limited transcriptional changes were observed one week after AAV exposure. By two weeks post AAV dosing CD8 T cells showed increased expression of *Cd44*, *Cd48*, *Itgb7*, and *Ptprcap*, suggesting a shift toward an activation- and adhesion-associated phenotype. At the dual-dose time point,

increased *Ccr7* expression may further indicate changes in CD8 T-cell trafficking behavior. In T follicular helper cells one week after AAV dosing, C1q-deficient mice showed increased expression of *Jun*, *Fos*, and *Fosb*, suggesting induction of AP-1-associated transcriptional activity consistent with cellular stimulation or early activation. At the dual-dose time point, genotype-associated differences were less dominated by canonical immune activation or T-cell effector genes suggesting that the transcriptional differences between genotypes after dual dosing may reflect broader cellular remodeling rather than a discrete immune activation program.

In activated B cells, limited differential gene expression was observed between genotypes one week after a single dose of AAV. By two weeks post-dose, however, C1q-deficient B cells showed increased expression of activation associated genes including *Il2ra*, *Irf4*, *Myb*, *Tent5c*, *Cd9*, and *Ahr*. This pattern may indicate enhanced B cell responsiveness even in the absence of C1q. One week post single AAV dose C1q^{-/-} follicular B cells showed increased expression of *Cd9*, *Il10*, *Il15ra*, and *Mzb1* suggestive of early activation associated feature. Two weeks post single dosing follicular B cells from C1q-deficient mice showed increased expression of *Fcrl5*, *Cd44*, *Mzb1*, and *Il5ra* suggesting a shift in the activation of their B-cell state. After dual AAV dosing, differential gene expression in follicular and activated B cells did not show a strong inflammatory or immune activation signature, suggesting that B-cell immune differences between genotypes were most evident after a single AAV dose.

These findings have important implications for complement-targeted strategies in AAV gene therapy. While complement inhibitors have been used to mitigate excessive complement activation, their effects on broader immune responses remain incompletely

understood. In vitro, the C3 inhibitor APL-9 reduces PBMC activation⁹, including decreased costimulatory molecule expression and inflammatory chemokine production. However, clinical experience with complement inhibition in systemic AAV gene therapy has highlighted that blocking complement may have complex and context-dependent effects in vivo. For example, in human AAV gene therapy trials for Danon disease, prophylactic use of the C3 inhibitor pegcetacoplan (APL-9) was associated with capillary leak syndrome and death in two patients¹¹. Although the mechanisms underlying these events remain unclear, these observations underscore the need to better understand how complement inhibition affects inflammatory and adaptive immune responses after systemic AAV delivery. Consistent with this concern, our data indicate that selective disruption of classical complement does not ablate AAV-induced complement activity and may instead alter the composition and magnitude of downstream immune responses.

Overall, these findings support a model in which classical complement shapes the transcriptional programming of the immune response to AAV. Loss of C1q was associated with enhanced chemokine production, altered B-cell, CD8 T-cell, and Tfh-cell programs, and amplification of the CXCL10–CXCR3 inflammatory axis. These results suggest that complement-targeted strategies in AAV gene therapy may have complex downstream effects on adaptive immunity. Defining how individual complement pathways shape inflammatory and lymphocyte responses will be important for developing safer approaches to systemic AAV delivery while preserving effective therapeutic gene transfer.

Figures

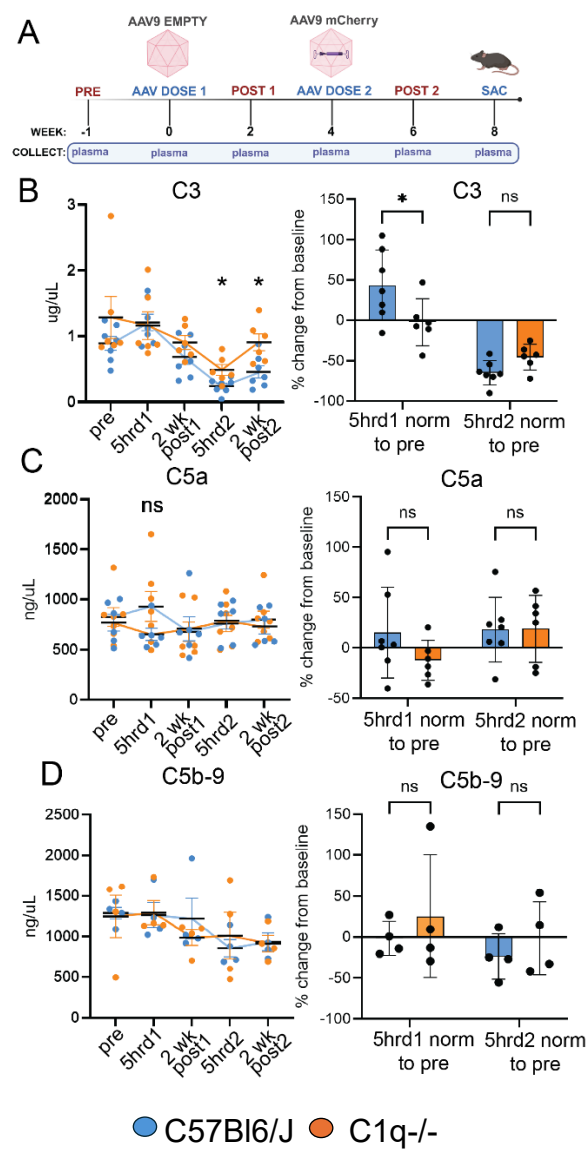
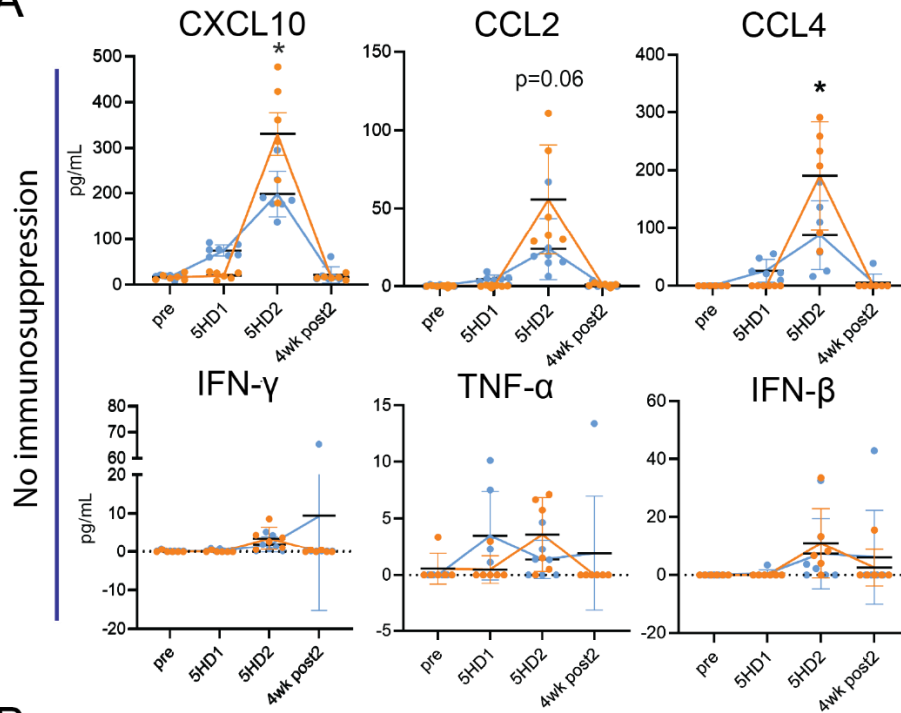


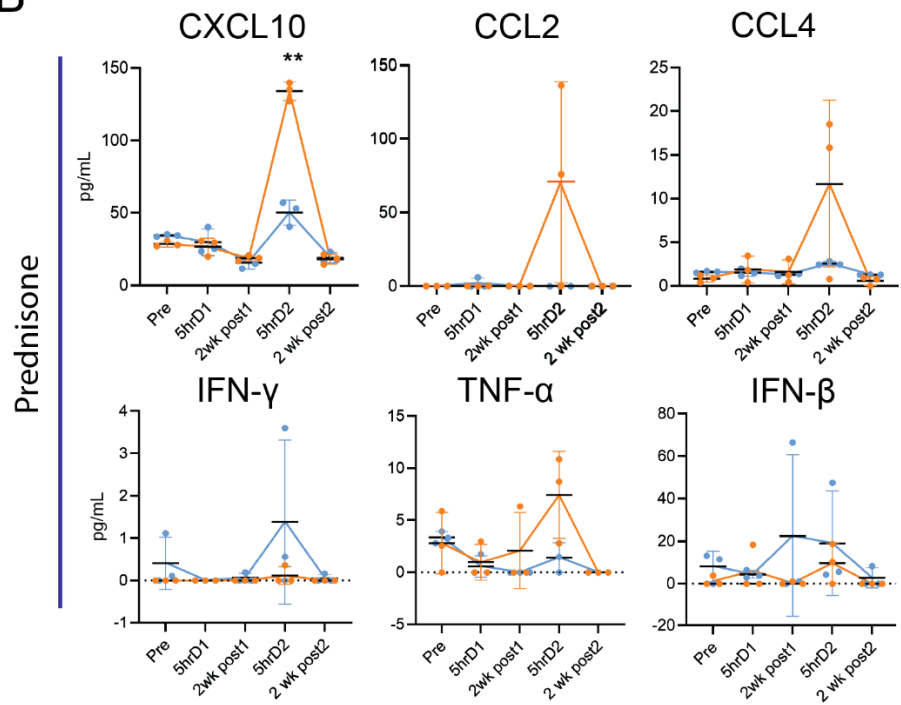
Figure 2-1: C3 consumption is retained in C1q-deficient mice despite the absence of C1q

(A) Dual AAV dosing schematic. (B) Plasma C3 levels measured by ELISA (left). Levels were quantified 5 hours after dose 1 (normalized to pre-dose) and 5 hours after dose 2 (normalized to 2 weeks post-dose 1) (right). *C57BL/6J* (n = 7); *C1q*^{-/-} (n = 6). Mann-Whitney test, **p* < 0.05. (C) Plasma C5a levels measured by ELISA (left). Levels were quantified 5 hours after dose 1 (normalized to pre-dose) and 5 hours after dose 2 (normalized to 2 weeks post-dose 1) (right). *C57BL/6J* (n = 7); *C1q*^{-/-} (n = 6). Mann-Whitney test, ns. (D) Plasma C5b-9 levels measured by ELISA (left). Levels were quantified 5 hours after dose 1 (normalized to pre-dose) and 5 hours after dose 2 (normalized to 2 weeks post-dose 1) (right). *C57BL/6J* (n = 7); *C1q*^{-/-} (n = 6). Mann-Whitney test, ns. ± SDs; ns, not significant; **p* < 0.05; ***p* < 0.01.

A



B



● C57Bl6/J ● C1q^{-/-}

Figure 2-2. C1q deficiency correlates with increased chemokine production, 5 hours after a second AAV dose

(A) Luminex chemokine panel measuring plasma chemokines before AAV dosing (pre), 5 hours post dose 1 (5HD1), 5 hours post dose 2 (5HD2), and 4 weeks post dose 2 (4wk post2). *C57BL/6J* (n = 7); *C1q^{-/-}* (n = 6). Mann–Whitney test. $\pm$ SDs; ns, not significant; * $p < 0.05$. (B) Luminex chemokine panel measuring plasma chemokines in mice treated with Prednisone (1mg/kg) before AAV dosing (pre), 5 hours post dose 1 (5HD1), 5 hours post dose 2 (5HD2), and 4 weeks post dose 2 (4wk post2). *C57BL/6J* (n = 4); *C1q^{-/-}* (n = 4). Mann–Whitney test. $\pm$ SDs; ns, not significant; * $p < 0.05$; ** $p < 0.01$.

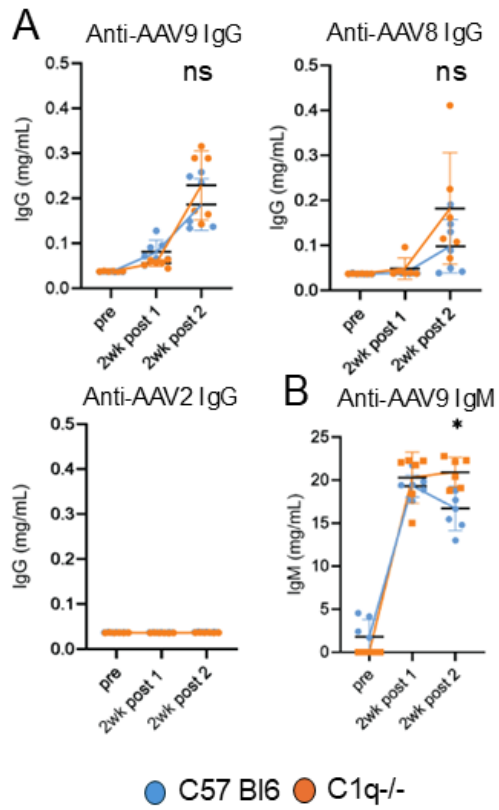


Figure 2-3: C1q^{-/-} mice generate similar anti-AAV9 IgG, but Increased IgM after dual AAV dosing in C1q^{-/-} mice

(A) Anti-AAV IgG levels measured by HCPM before AAV dosing (pre), 2 weeks after a single AAV dose (post 1) and 2 weeks after a second AAV dose (post 2). Each dot represents an individual mouse *C57BL/6J* (n = 7); *C1q^{-/-}* (n = 6). Mann–Whitney test; ns, not significant.

(B) Anti-AAV9 IgM levels measured by ELISA 2 weeks after a single dose of AAV (post 1) and 2 weeks after a second AAV dose (post 2). Each dot represents an individual mouse *C57BL/6J* (n = 7); *C1q^{-/-}* (n = 6). Mann–Whitney test; **p* < 0.05

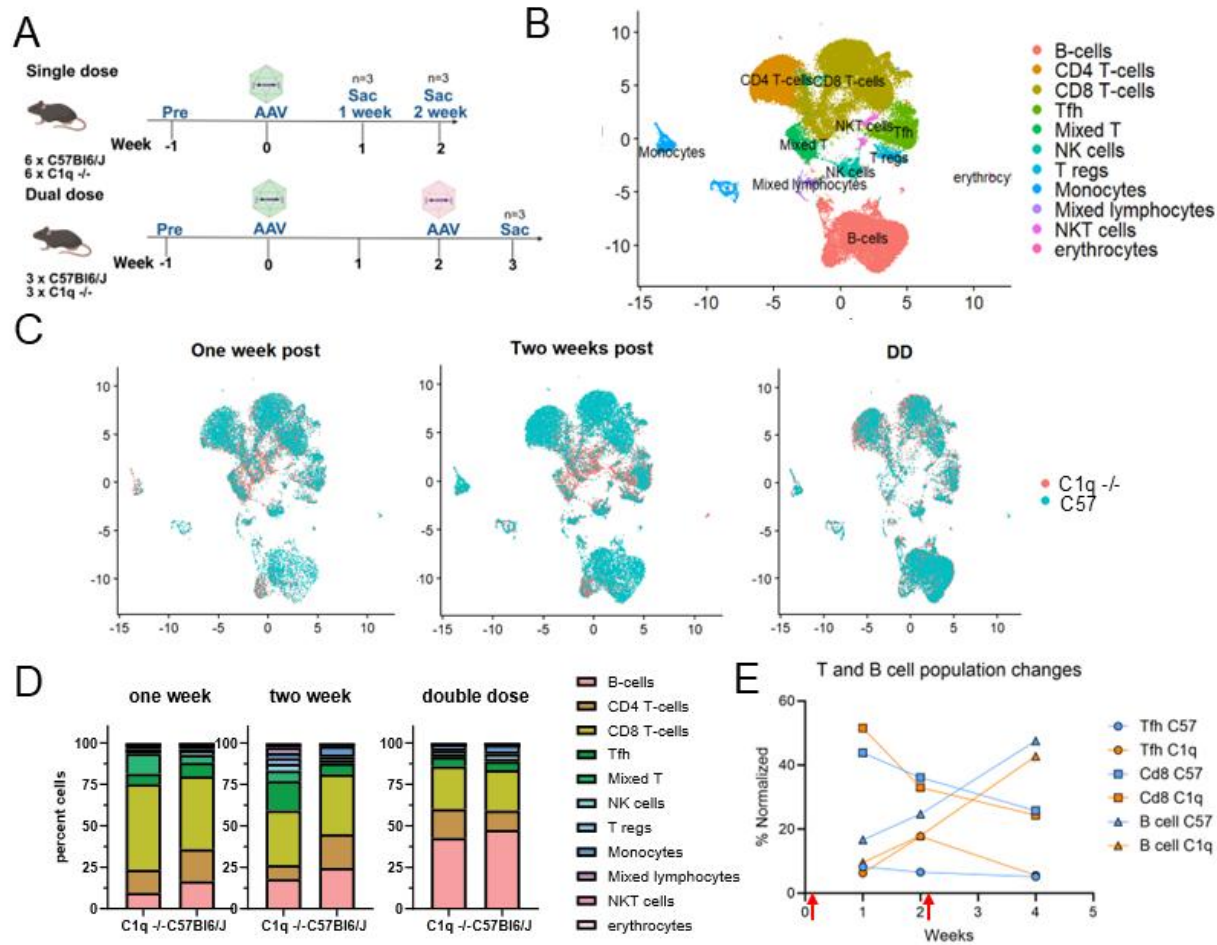


Fig 2-4: Single-cell RNA sequencing reveals altered CD8 T cell, T follicular helper cell, and B cell frequencies in $C1q^{-/-}$ mice following AAV dosing

- (A) Experimental schematic of 10x Genomics single-cell RNA sequencing (scRNA-seq) from mice receiving single or dual AAV dosing. Spleens were harvested one and two weeks post single dose (Sac) and one week post dual dose (DD).
- (B) UMAP projection of all cell populations identified by scRNA-seq of mouse splenocytes.
- (C) UMAP projections split by time point and colored by genotype (C57BL/6J, blue; $C1q^{-/-}$, pink). Time points include one week post single AAV dose, two weeks post single AAV dose, and one week post dual dosing (DD).
- (D) Proportion of each cell population across time points and genotypes normalized to number of cells collected per time point.
- (E) Line graph depicting temporal changes in T follicular helper cells, CD8 T cells, and B cells in C57BL/6J and $C1q^{-/-}$ mice. Tfh cells are represented by circles, CD8 T cells by squares, and B cells by triangles. C57BL/6J mice are shown in blue and $C1q^{-/-}$ mice are shown in orange. Red arrows indicate time points of AAV dosing.

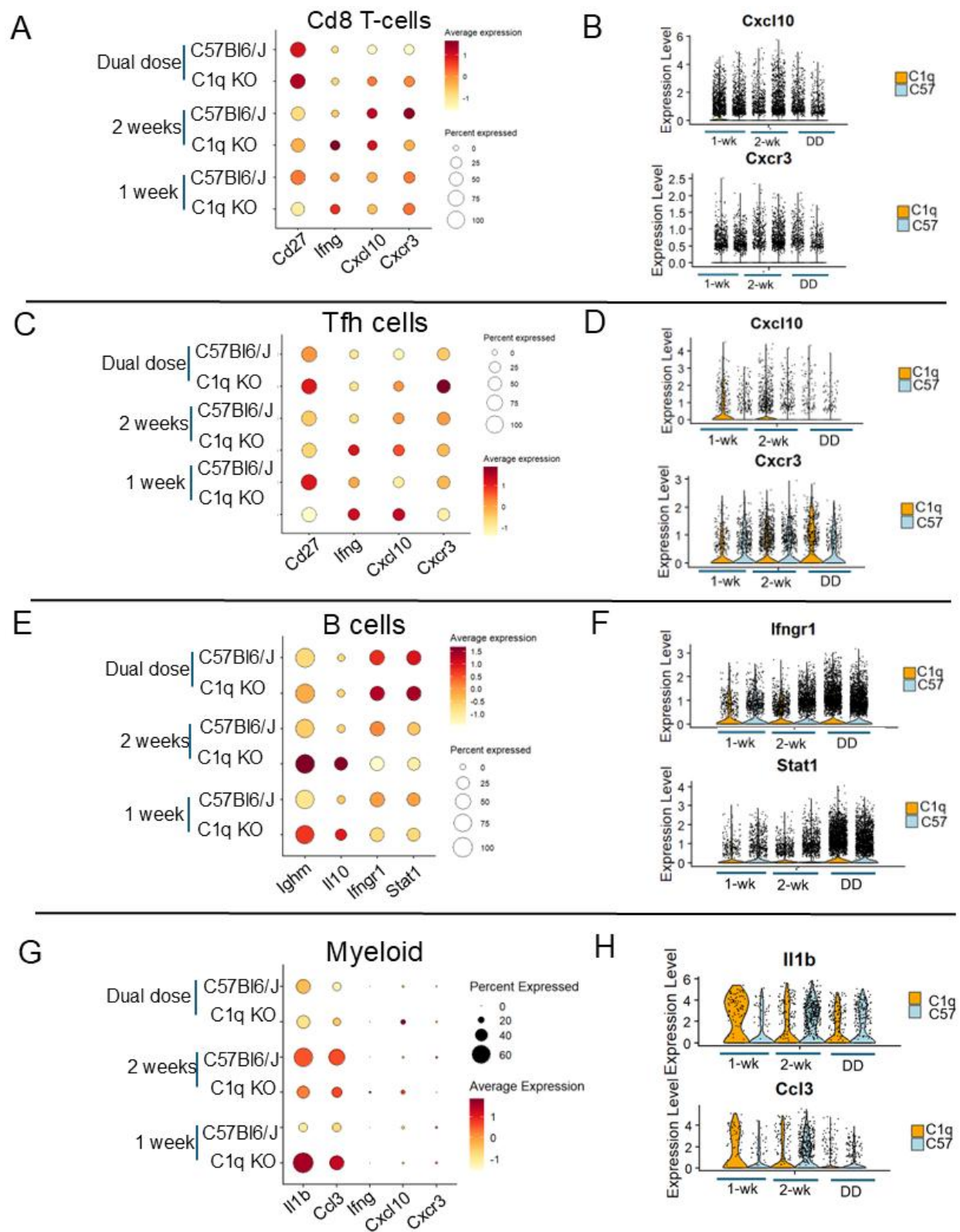


Figure 2-5: C1q deficiency correlates with enhanced inflammatory programs in CD8 T, Tfh, B and myeloid cells in response to AAV dosing

- (A) Dot plot highlighting differentially expressed genes in CD8 T cells across single-dose AAV timepoints (one week and two weeks) and following dual AAV dosing. Color intensity represents scaled average expression, and dot size indicates the percentage of cells expressing each gene.
- (B) Violin plot shows expression of *Ifng*, *Cxcl10* and *Cxcr3* across dosing regimens and between genotypes (*C57BL/6J*, blue; *C1q*^{-/-}, orange).
- (C) Dot plot highlighting differentially expressed genes in T follicular helper cells across a single dose of AAV (one week, two week) and dual dose of AAV. Color is scaled by average expression and the dot size is proportional to the percent of cells expressing the gene.
- (D) Violin plot shows expression of *Cxcl10* and *Cxcr3* across dosing regimen between genotypes (*C57BL/6J*, blue; *C1q*^{-/-}, orange).
- (E) Dot plot showing expression of *Ighm*, *Il10*, *Ifngr1* and *Stat1* in B cells from wild-type and *C1q*^{-/-} mice across AAV doses. Dot size represents the percentage of cells expressing each gene, and color intensity represents scaled average expression.
- (F) Violin plots showing single-cell expression distributions of *Ifngr1* and *Stat1* in B cells across genotype and treatment groups (*C57BL/6J*, blue; *C1q*^{-/-}, orange).
- (G) Dot plot of differentially expressed genes in monocytes across a single dose of AAV (one week, two week) and dual dose of AAV.
- (H) Violin plot shows expression of *Il1b* and *Ccl3* across dosing regimen between genotypes (*C57BL/6J*, blue; *C1q*^{-/-}, orange).

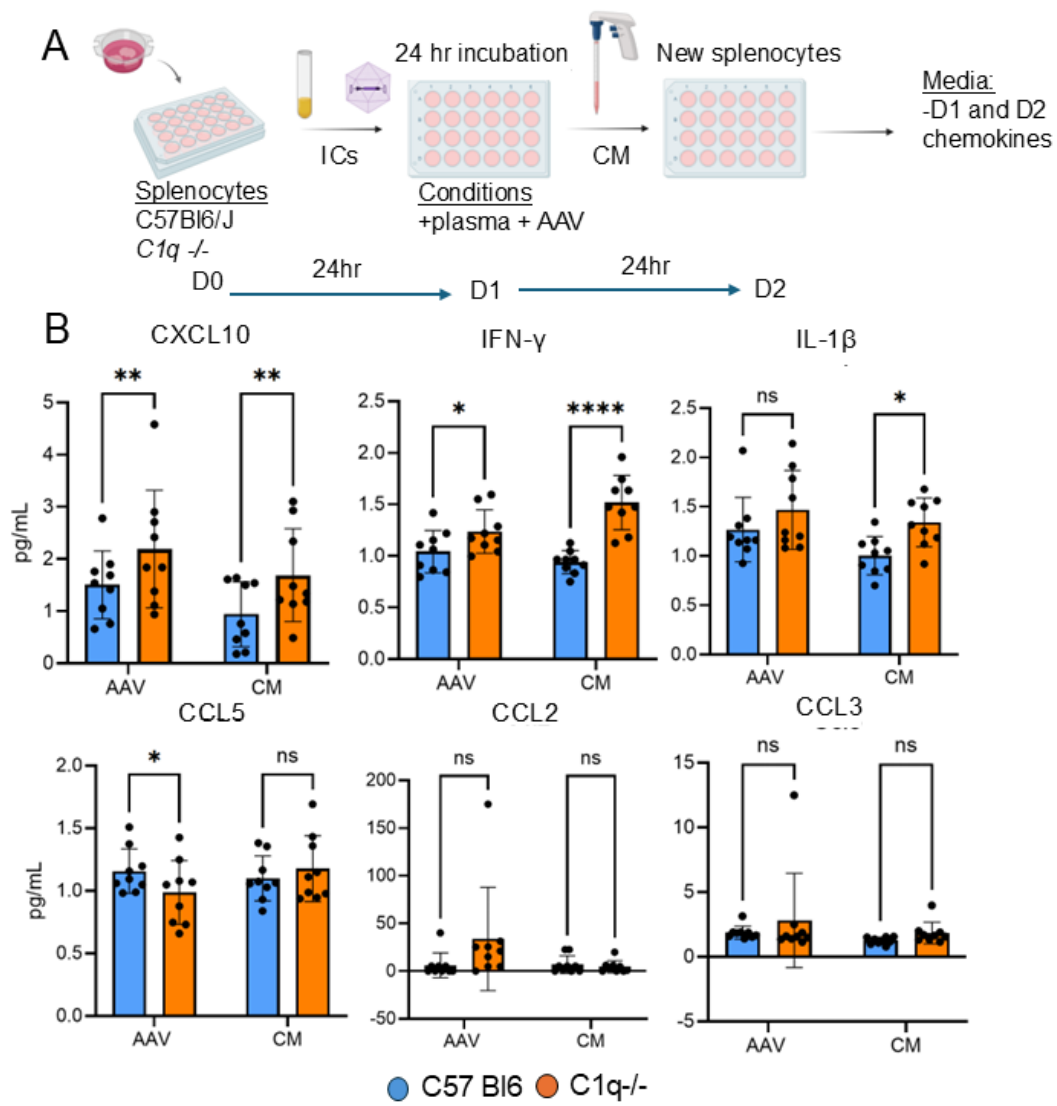


Figure 2-6: Conditioned media from C1q^{-/-} splenocytes demonstrates enhanced chemokine induction

(A) Splenocytes isolated from C57BL/6J and C1q^{-/-} mice were harvested and plated.

AAV immune complexes (ICs) were generated by incubating AAV with plasma from seropositive mice and were subsequently added to the splenocyte cultures. After 24 hours, the conditioned media (CM) were collected and transferred to freshly plated splenocytes. Media collected on days 1 and 2 were used for downstream cytokine and chemokine analysis by Luminex.

(B) Chemokine production by total splenocyte cultures stimulated with AAV-antibody immune complexes or conditioned media *in vitro*. “AAV” denotes stimulation with immune complexes, and “CM” denotes stimulation with conditioned media. C57BL/6J (blue) and C1q^{-/-} (orange). Bars represent mean ± SD. Statistical significance was determined by two-way ANOVA with Bonferroni correction for multiple comparisons; ns, not significant; * $p < 0.05$, ** $p < 0.01$.

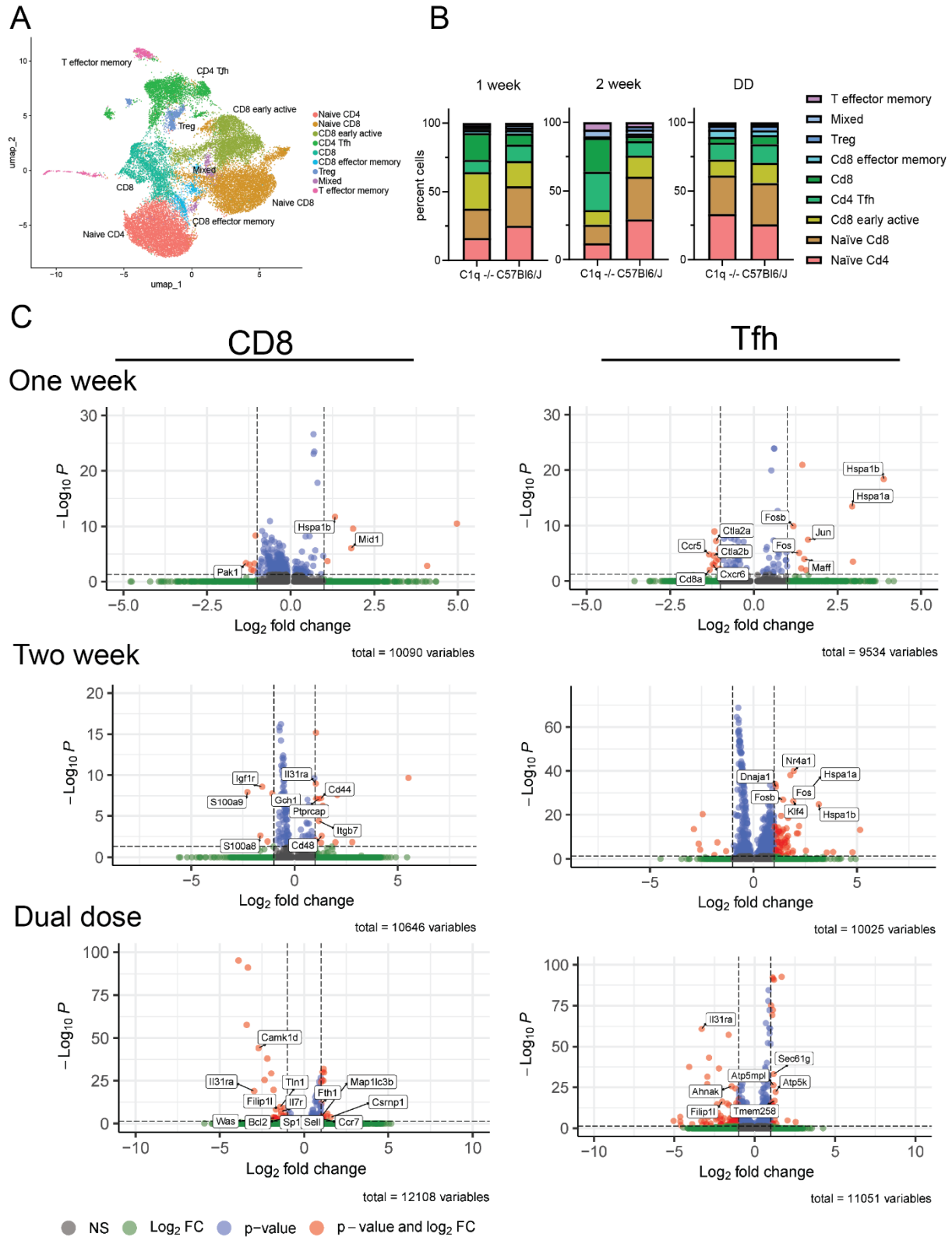


Figure 2-7: T-cell subclustering reveals genotype-associated shifts in CD8 T-cell and Tfh populations

- (A) UMAP projection of subclustered T cells identifies four distinct populations, color-coded by subpopulation.
- (B) Proportion of each T cell population across time points and genotypes (*C1q*^{-/-} and C57BL/6), normalized to the total number of cells collected at each time point.
- (C) T-cell differential gene expression volcano plots. Volcano plots showing differentially expressed genes in T cells between comparison groups, with CD8 T cells on the left and Tfh cells on the right. Each point represents an individual gene, plotted by average log₂ fold change on the x-axis and adjusted p-value on the y-axis. Gray points indicate genes that did not meet significance thresholds. Green points indicate genes meeting the log₂ fold-change cutoff only, blue points indicate genes meeting the adjusted p-value cutoff only, and red points indicate genes meeting both the adjusted p-value and log₂ fold-change thresholds. Positive log₂ fold-change values represent genes enriched in C1q KO, while negative log₂ fold-change values represent genes enriched in C57BL/6J.

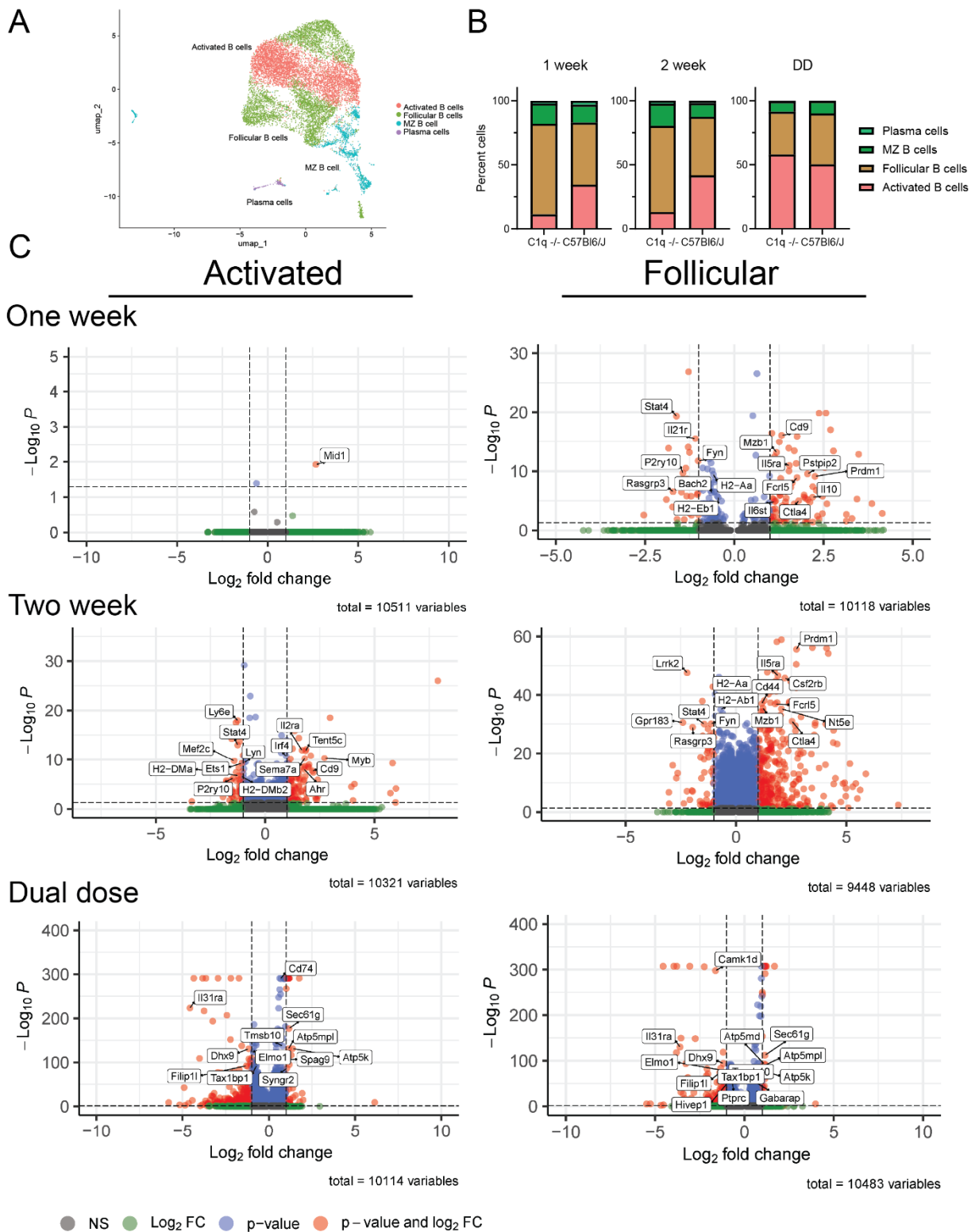


Figure 2-8: B-cell subclustering identifies distinct B-cell populations after AAV dosing

- (A) UMAP projection of subclustered B cells identifies four distinct populations, color-coded by subpopulation.
- (B) Proportion of each B cell population across time points and genotypes ($C1q^{-/-}$ and C57BL/6), normalized to the total number of cells collected at each time point.
- (C) Differential gene expression volcano plots for B-cell subpopulations, with activated B cells shown on the left and follicular B cells shown on the right. Each point represents an individual gene, plotted by average $\log_2$ fold change on the x-axis and adjusted p-value on the y-axis. Gray points indicate genes that did not meet significance thresholds. Green points indicate genes meeting the $\log_2$ fold-change cutoff only, blue points indicate genes meeting the adjusted p-value cutoff only, and red points indicate genes meeting both the adjusted p-value and $\log_2$ fold-change thresholds. Positive $\log_2$ fold-change values represent genes enriched in $C1q^{-/-}$ mice, whereas negative $\log_2$ fold-change values represent genes enriched in C57BL/6J mice.

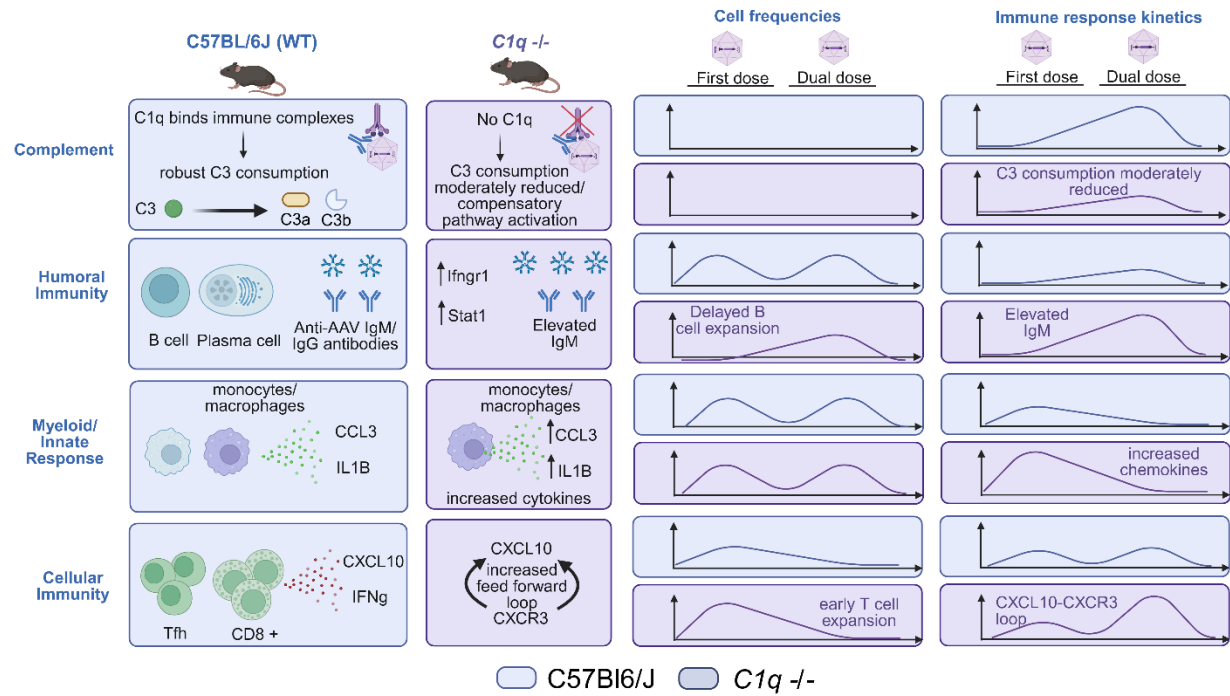
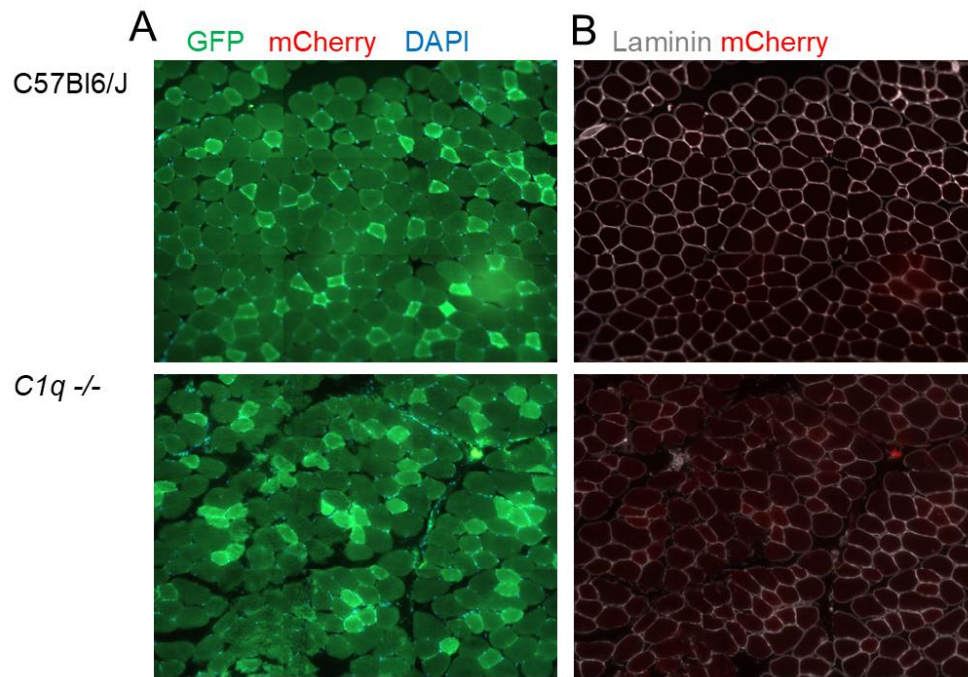


Figure 2-9: C1q deficiency reshapes immune responses following repeated systemic AAV exposure.

Schematic summary comparing wild-type and C1q^{-/-} mice across complement, humoral, myeloid, and cellular immune compartments. C1q deficiency was associated with altered response magnitude, population dynamics, and kinetics, including persistent complement activation, elevated IgM, increased inflammatory myeloid signaling, and enhanced CXCL10–CXCR3-associated cellular responses. Curves are schematic representations of relative response patterns.

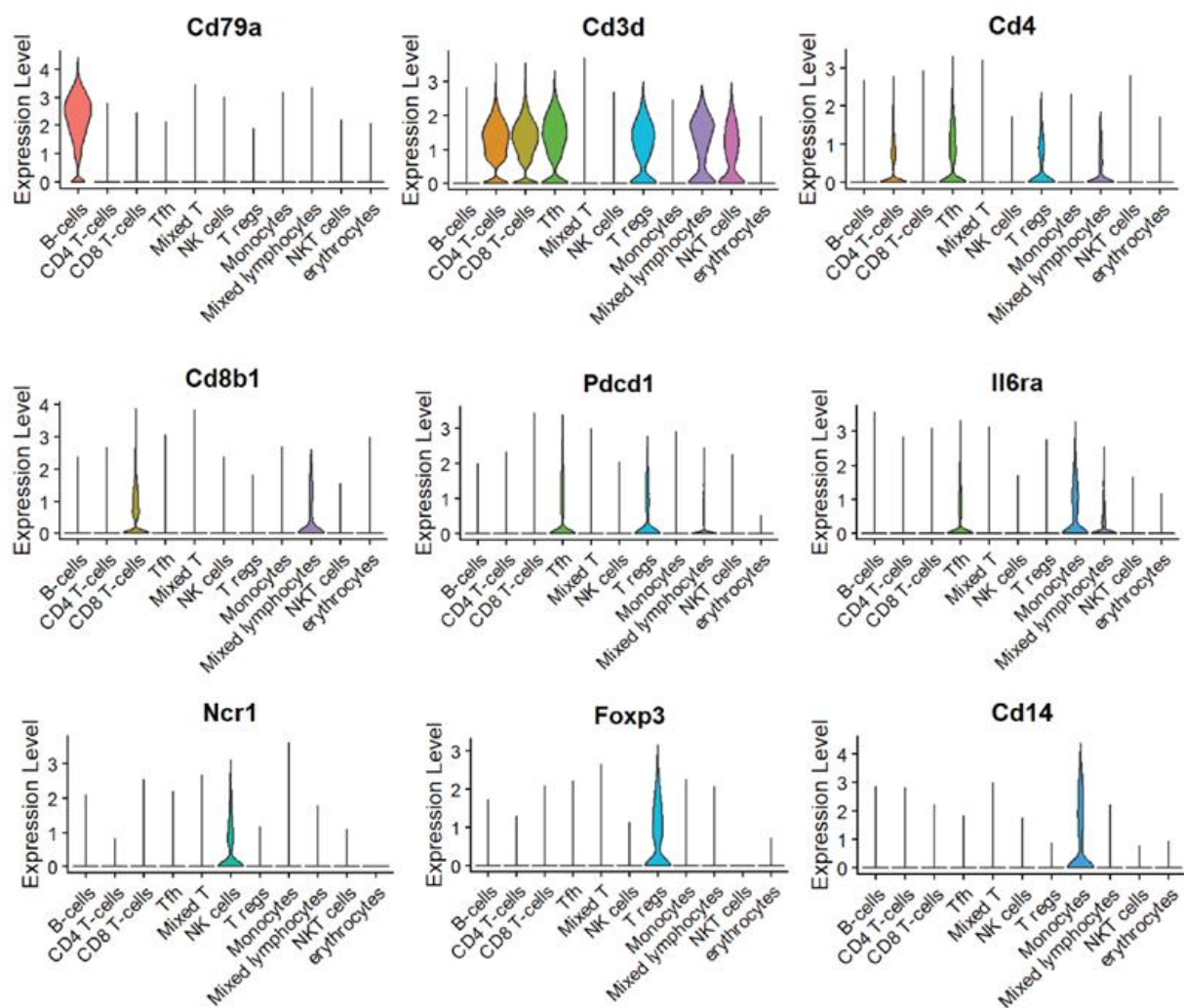


Supplemental Figure 2-1. C1q ablation does not allow for AAV redosing

(A) Quadriceps muscle sections were collected from C57BL/6J and *C1q*^{-/-} mice following a dual AAV9 dosing regimen in which mice first received AAV9-GFP and a second dose of AAV9-mCherry. Immunofluorescence microscopy was performed to assess transgene expression in quadriceps tissue. All micrographs were taken at the same magnification.

(B) GFP staining was detected in quadriceps sections from both C57BL/6J and *C1q*^{-/-} mice, with comparable GFP signal observed between genotypes. (B) In contrast, mCherry staining was undetectable in quadriceps sections from both genotypes following the second AAV9 dose demonstrating that C1q does not allow for AAV redosing.

A

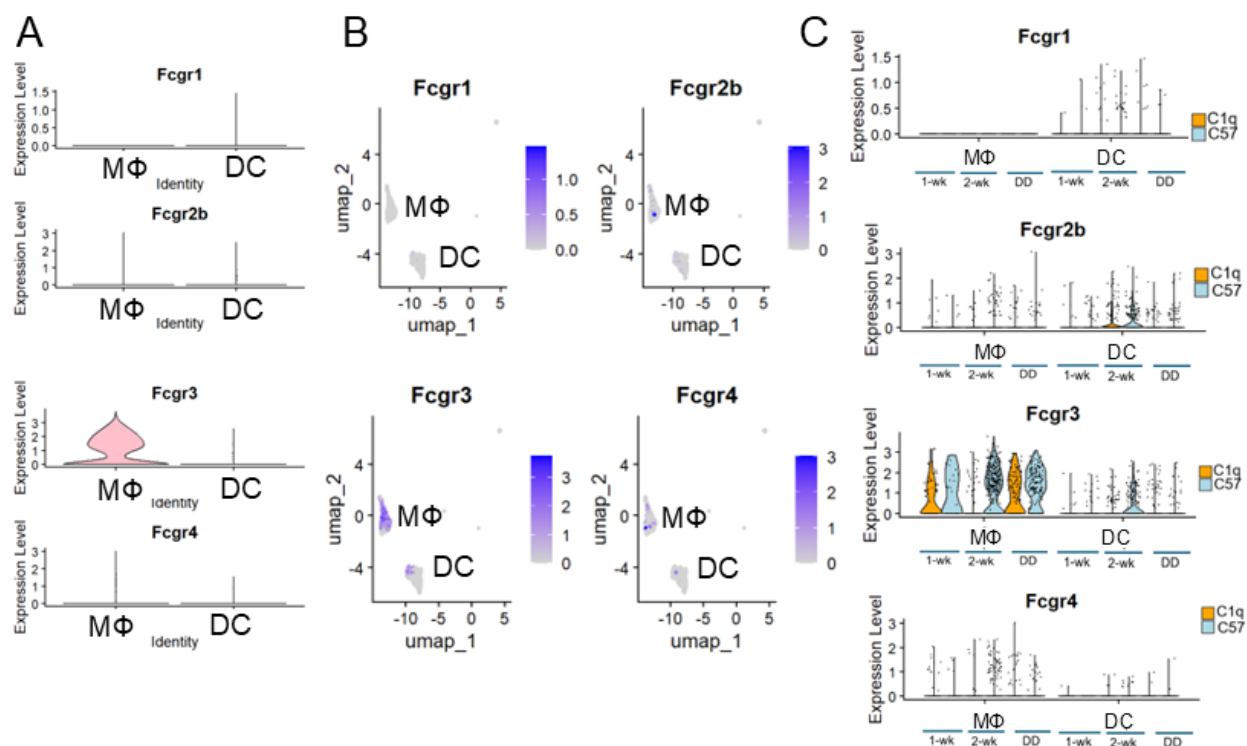


Supplemental Figure 2-2: Marker gene expression across cell clusters.

(A) Violin plots showing expression of selected marker genes across annotated cell clusters identified by single-cell RNA sequencing.

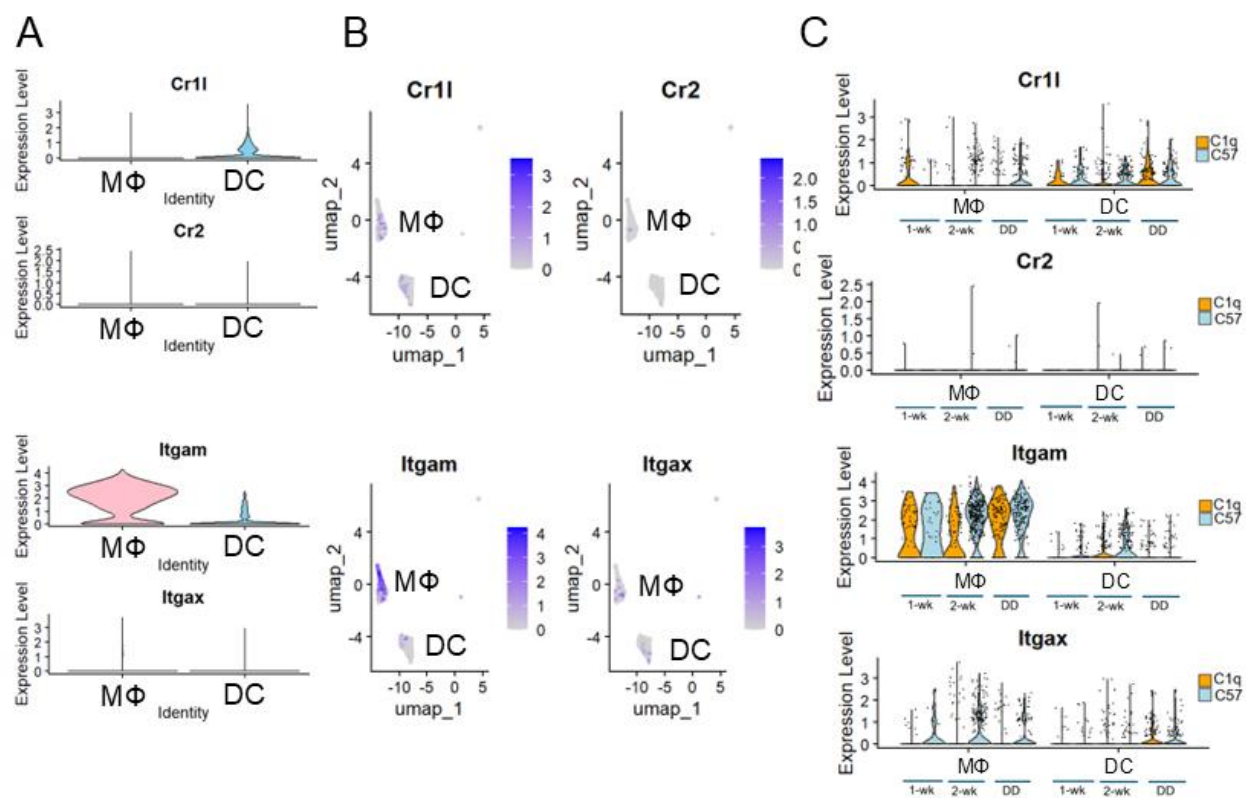
Supplemental Figure 2-3: Differential gene expression within myeloid cell populations

(A) Volcano plot showing differentially expressed genes in the macrophage cluster between C1q-deficient and C57 control mice. (B) Volcano plot showing differentially expressed genes in the dendritic cell cluster between C1q-deficient and C57 control mice. Each point represents an individual gene, plotted by average $\log_2$ fold change on the x-axis and adjusted p -value on the y-axis. Gray points indicate genes that did not meet either significance threshold, green points indicate genes meeting only the $\log_2$ fold-change cutoff, blue points indicate genes meeting only the adjusted p -value cutoff, and red points indicate genes meeting both thresholds. Positive $\log_2$ fold-change values represent genes enriched in C1q-deficient mice, whereas negative values represent genes enriched in C57 controls.



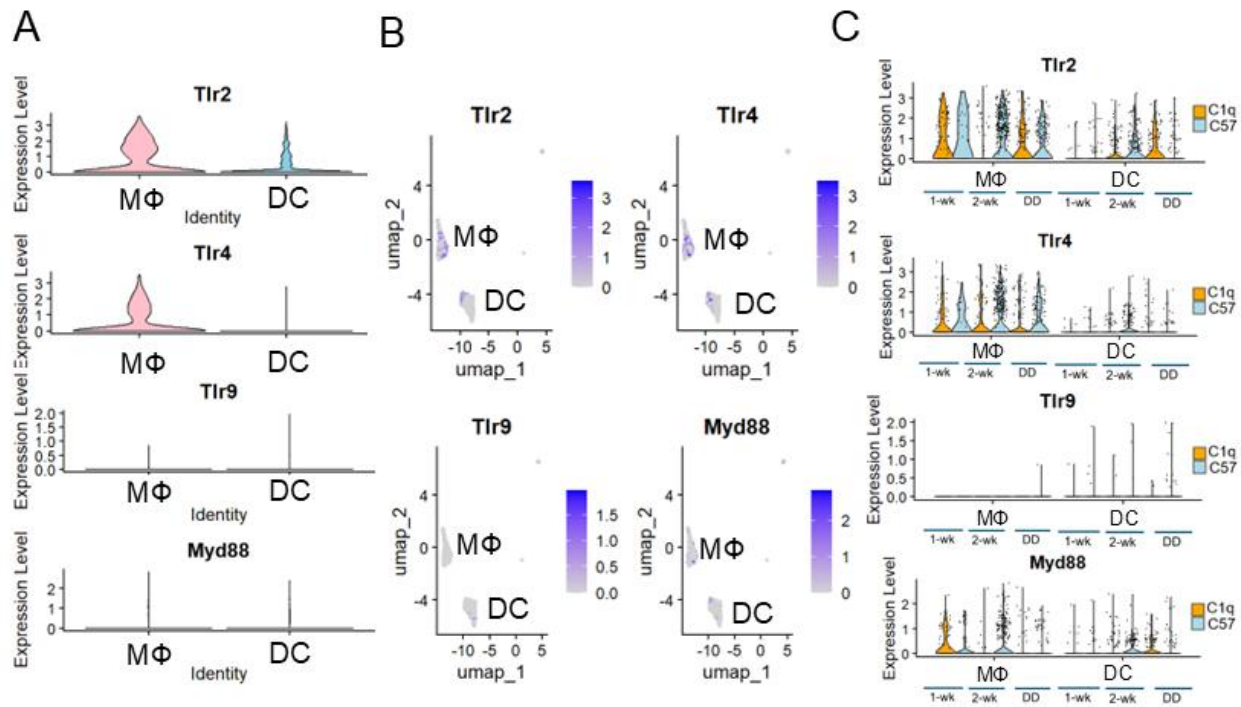
Supplemental Figure 2-4: Expression of Fcγ receptors across myeloid cell populations.

(A) Expression of Fcγ receptor genes *Fcgr1*, *Fcgr2b*, *Fcgr3*, and *Fcgr4* was evaluated across myeloid cell populations. Increased color intensity in FeaturePlots indicates higher gene expression. (B) FeaturePlots showing the spatial distribution of marker expression on the UMAP embedding. Increased color intensity in FeaturePlots indicates higher gene expression. (C) Violin plots show relative expression levels across annotated populations. These markers were used to describe patterns of Fcγ receptor expression within the myeloid compartment.



Supplemental Figure 2-5: Expression of complement receptors across myeloid cell populations.

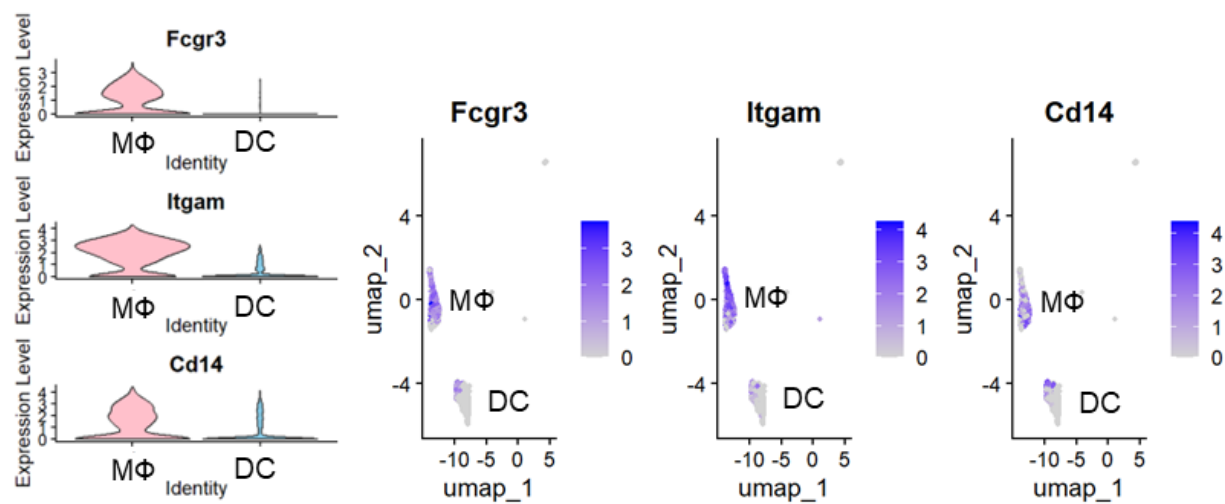
(A) Expression of complement receptor-associated genes Cr1l, Cr2, Itgam, and Itgax was evaluated across myeloid cell populations. (B) FeaturePlots show the spatial distribution of marker expression on the UMAP embedding. Increased color intensity in FeaturePlots indicates higher gene expression. (C) Violin plots show relative expression levels across annotated populations. These markers were used to describe patterns of complement receptor-associated gene expression within the myeloid compartment.



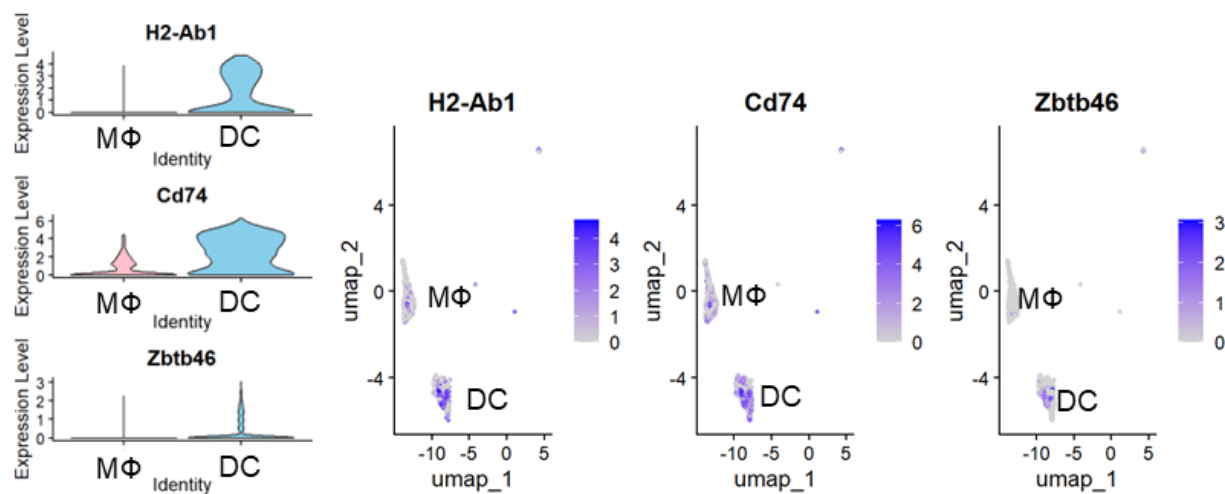
Supplemental Figure 2-6: Expression of Toll-like receptor-associated innate sensing genes across myeloid cell populations.

(A) Expression of Toll-like receptor-associated genes Tlr2, Tlr4, Tlr9, and Myd88 was evaluated across myeloid cell populations. (B) FeaturePlots show the spatial distribution of marker expression on the UMAP embedding. Increased color intensity in FeaturePlots indicates higher gene expression. (C) Violin plots show relative expression levels across annotated populations. These markers were used to describe patterns of TLR-associated innate immune sensing and downstream adaptor signaling within the myeloid compartment.

A



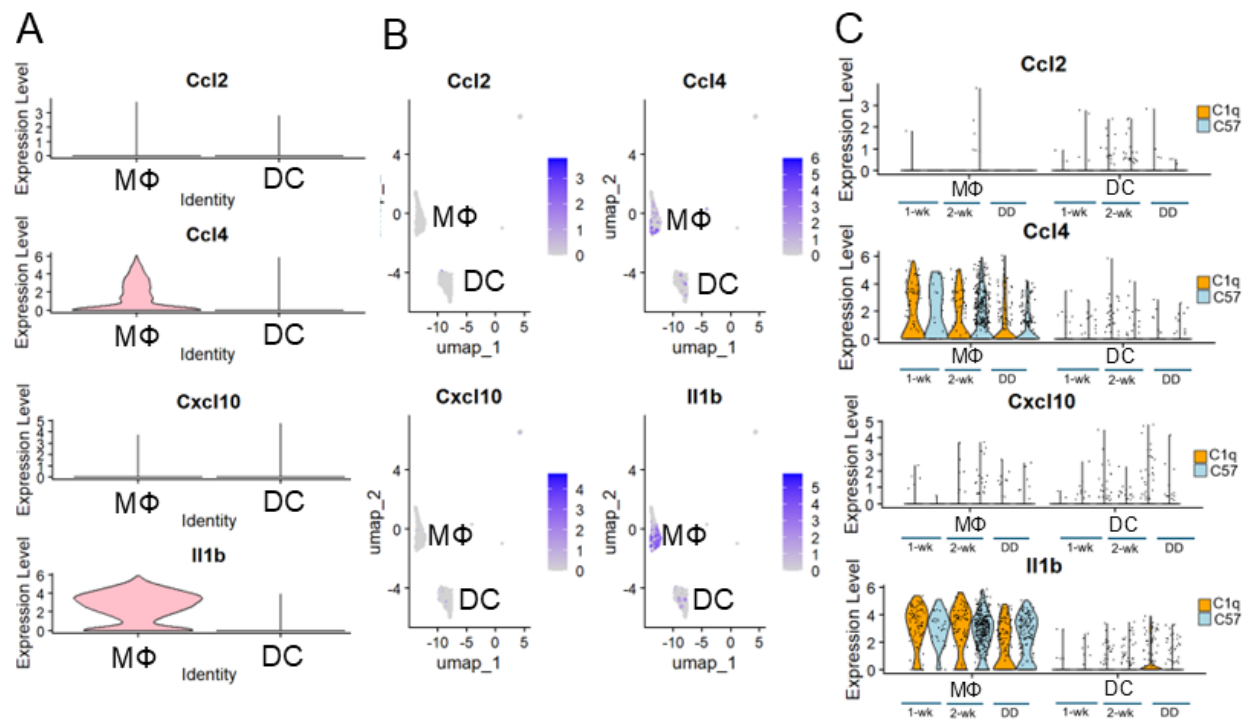
B



Supplemental Figure 2-7: Expression of macrophage- and dendritic cell-like markers across myeloid populations.

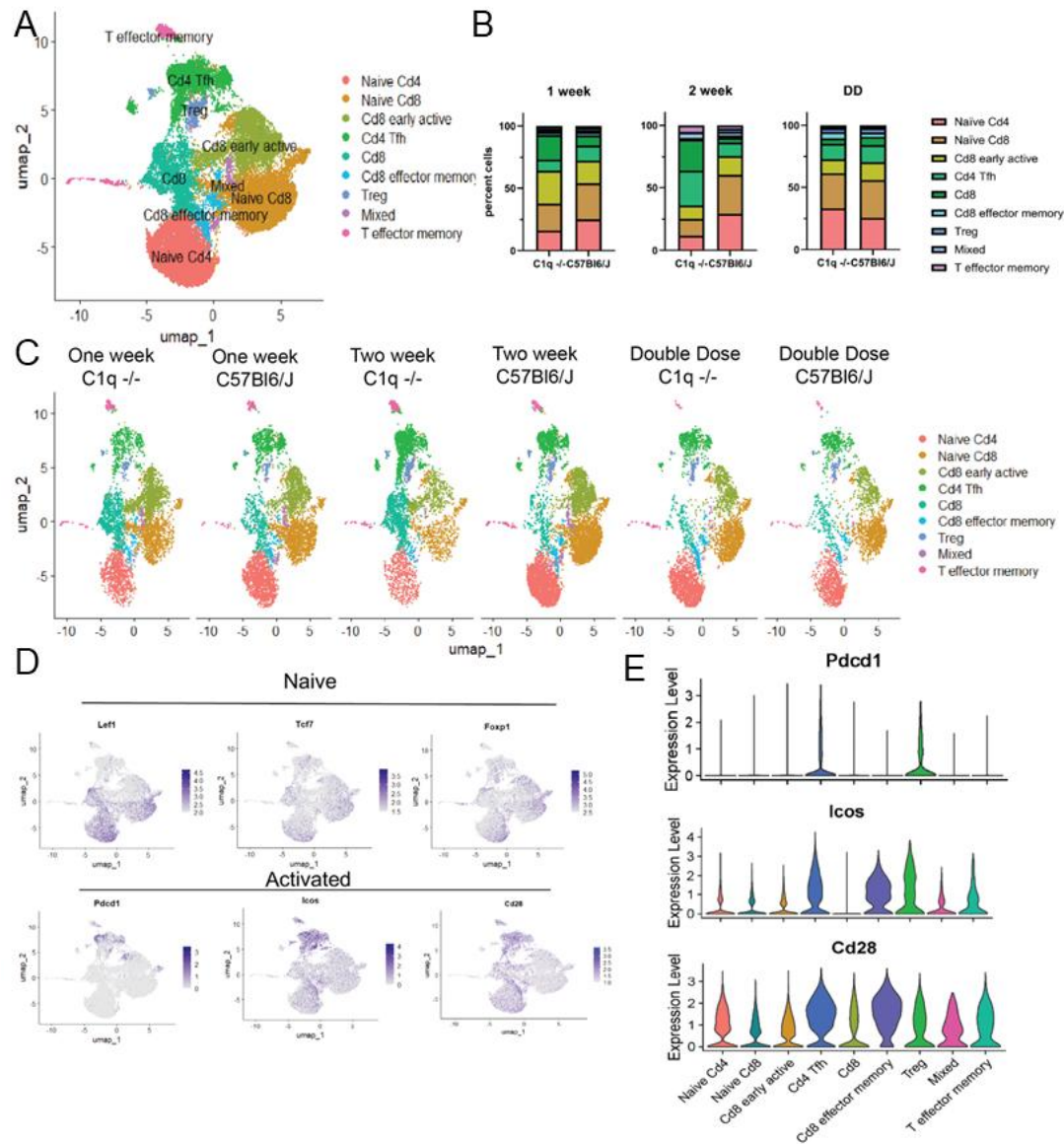
(A) Expression of macrophage-associated markers *Fcgr3*, *Itgam*, and *Cd14* was evaluated across myeloid cell populations. Violin plots on the left show relative expression levels across annotated populations, while FeaturePlots on the right show the spatial distribution of marker expression on the UMAP embedding. Increased color intensity in FeaturePlots indicates higher gene expression.

(B) Expression of dendritic cell-like markers *H2-Ab1*, *Cd47*, and *Zbtb46* was evaluated using the same visualization approach, with violin plots showing relative expression across populations and FeaturePlots showing the distribution and intensity of expression across the UMAP space. Together, these markers were used to describe macrophage-associated and dendritic cell-like transcriptional features within the myeloid compartment.



Supplemental Figure 2-8: Expression of inflammatory chemokine and cytokine genes across myeloid cell populations.

(A) Expression of inflammatory mediator genes *Ccl2*, *Ccl4*, *Cxcl10*, and *Il1b* was evaluated across myeloid cell populations. Violin plots show relative expression levels across annotated populations. (B) FeaturePlots show the spatial distribution of marker expression on the UMAP embedding. Increased color intensity in FeaturePlots indicates higher gene expression. (C) Violin plots show relative expression levels across annotated populations. These markers were used to describe patterns of inflammatory chemokine and cytokine-associated gene expression within the myeloid compartment.



Supplemental Figure 2-9: Characterization of T cell subsets and subclusters.

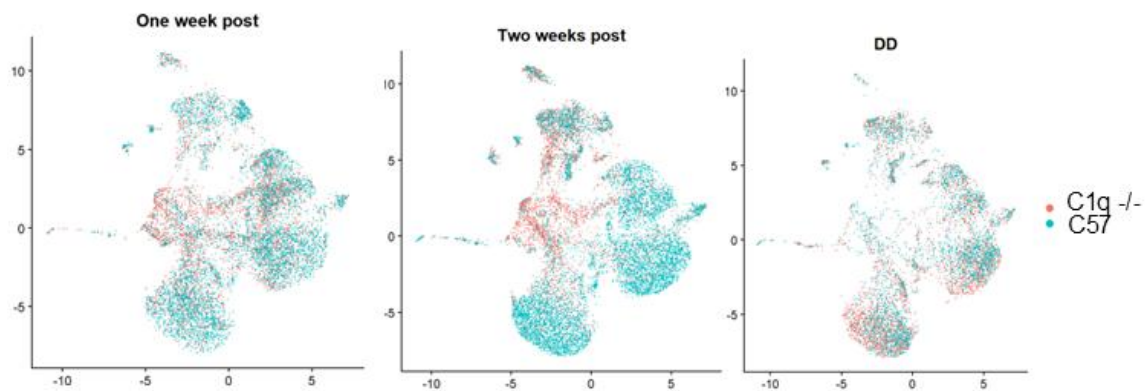
(A) UMAP projection of T cell subclusters identifies four distinct populations, color-coded by subpopulation.

(B) Proportion of each T cell population across time points and genotypes (C1q^{-/-} and C57BL/6), normalized to the total number of cells collected at each time point.

(C) UMAP projections of T cells from C1q^{-/-} and C57BL/6 mice across time points.

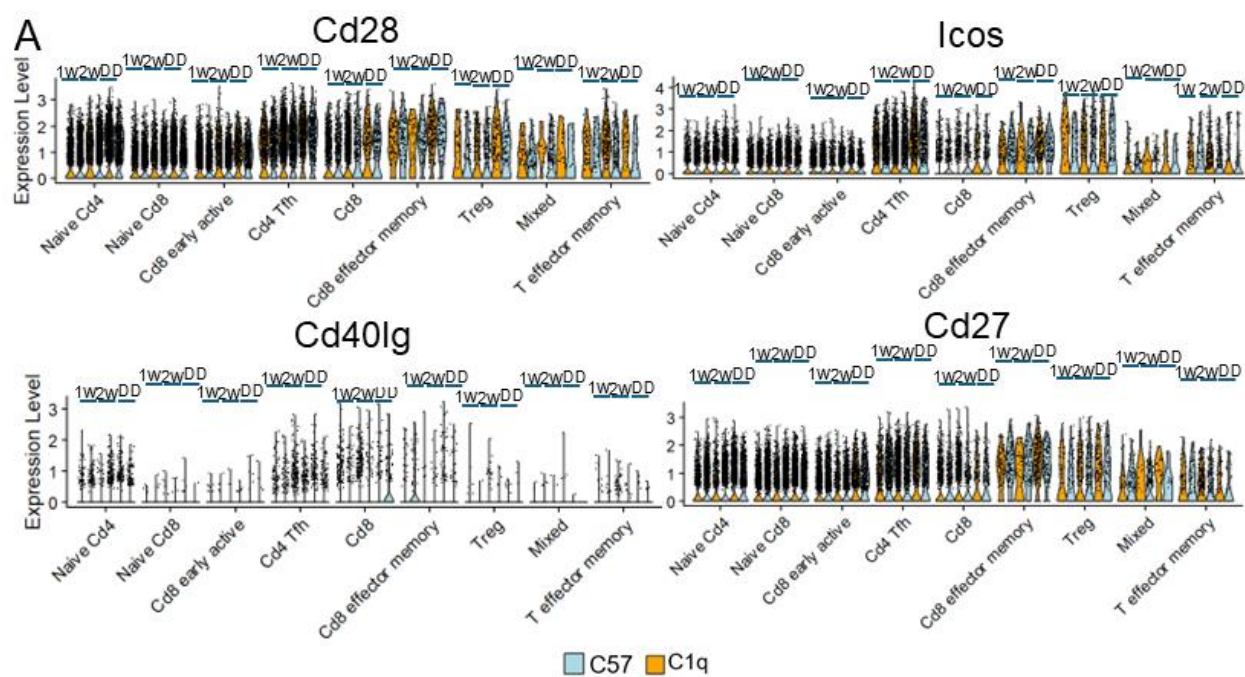
(D) Expression of marker genes used to define subset T cell subpopulations.

(E) Expression of activation-associated markers used to identify activated T cell populations.



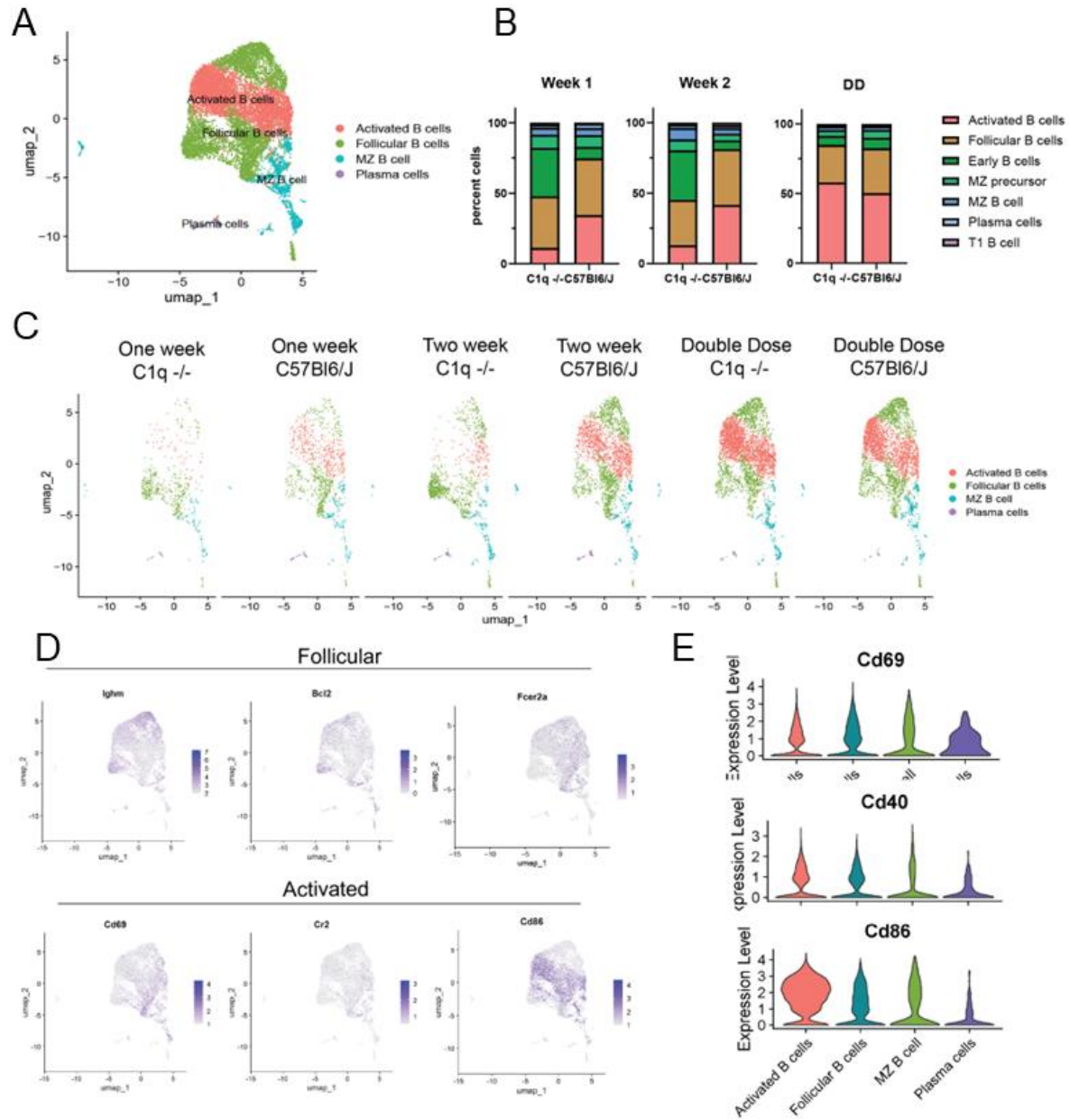
Supplemental Figure 2-10: Population shifts in T cell subsets and subclusters.

UMAP projections of T cell subclusters, color-coded by genotype (blue: C57BL/6J; pink: C1q^{-/-}) at one and two weeks following a single dose and one week following dual dosing.



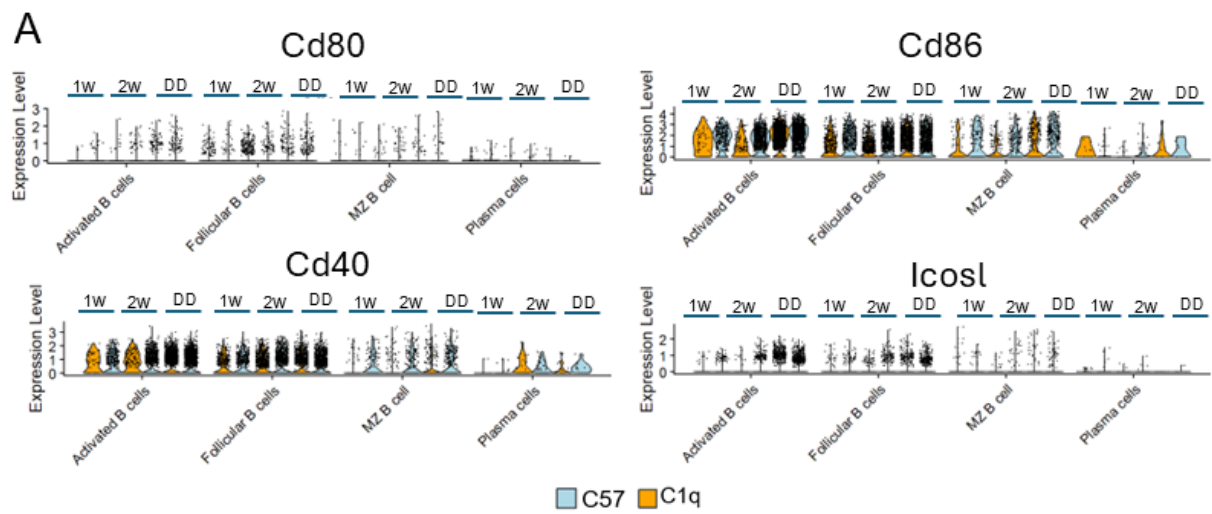
Supplemental Figure 2-11: Costimulatory molecule expression across subset and subclustered T cell populations following AAV exposure.

Expression of T cell costimulatory molecules Cd28, Icos, Cd40lg, and Cd27 was evaluated across annotated T cell subsets and subclustered T cell populations from C57BL/6J and C1q-deficient mice. Plots show relative expression levels of each marker across T cell populations, with C1q^{-/-} mice shown in orange and C57BL/6J controls shown in blue. These markers were selected to assess changes in T cell activation, costimulatory signaling, and helper-associated programs in the setting of C1q deficiency.



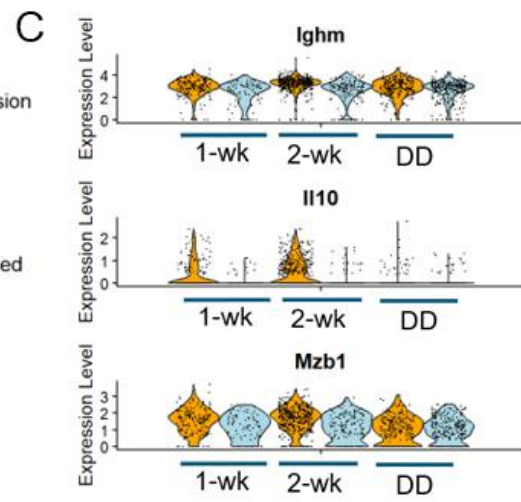
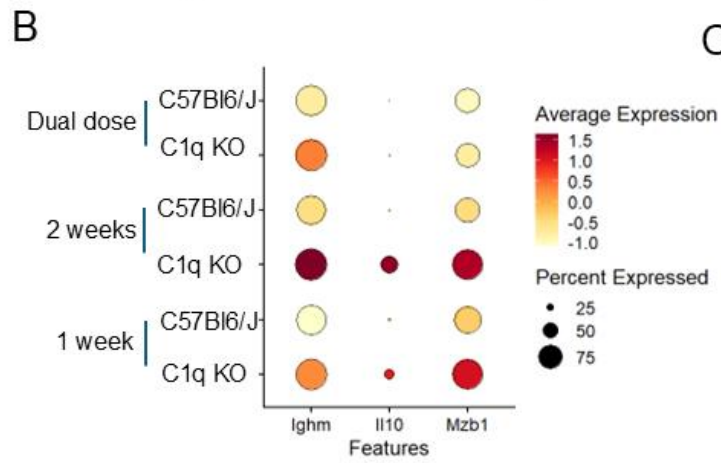
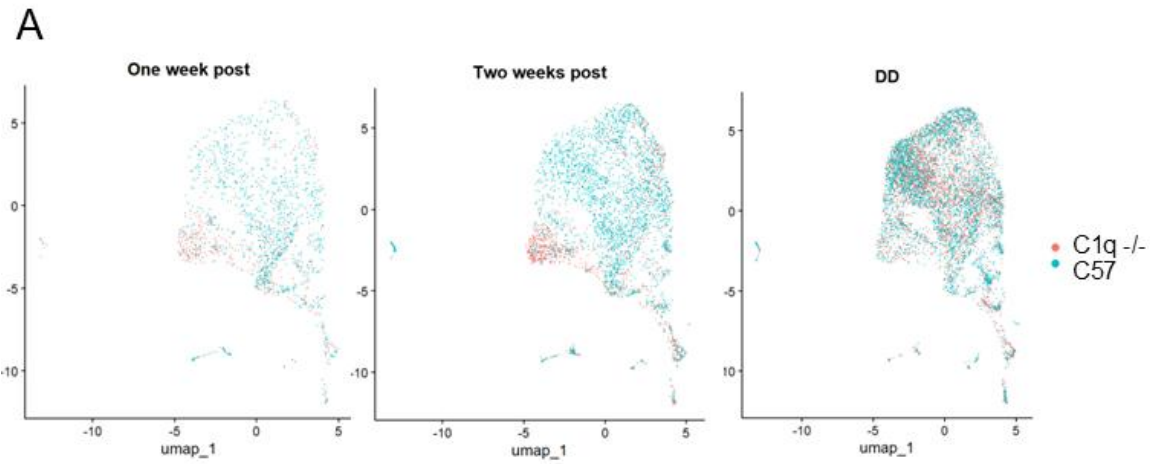
Supplemental Figure 2-12: Characterization of B cell subsets and subclusters

- (A) UMAP projection of B cell subclusters identifies four distinct populations, color-coded by subpopulation.
- (B) Proportion of each B cell population across time points and genotypes ($C1q^{-/-}$ and C57BL/6), normalized to the total number of cells collected at each time point.
- (C) UMAP projections of B cells from $C1q^{-/-}$ and C57BL/6 mice across time points.
- (D) Expression of marker genes used to define B cell subpopulations.
- (E) Expression of activation-associated markers used to identify activated B cell states.



Supplemental Figure 2-13: Costimulatory molecule expression across subset and subclustered B cell populations following AAV exposure.

Expression of B cell costimulatory molecules Cd80, Cd86, Cd40, and Icosl was evaluated across annotated B cell subsets and subclustered B cell populations from C57BL/6J and C1q-deficient mice. Plots show relative expression levels of each marker across B cell populations, with C1q^{-/-} mice shown in orange and C57BL/6J controls shown in blue. These markers were selected to assess changes in B cell activation, antigen-presenting capacity, and costimulatory signaling in the setting of C1q deficiency.



Supplemental Figure 2-14: Population shifts in B cell subsets and subclusters.

(A) UMAP projections of B cell subclusters, color-coded by genotype (blue: C57BL/6J; pink: C1q^{-/-}) at one and two weeks following a single dose and one week following dual dosing.

(B) Dot plot of differentially expressed genes in B cells across a single dose of AAV (one week, two week) and dual dose of AAV.

(C) Violin plot shows expression of Ighm, Il10, and Mzb1 across dosing regimen between genotypes (C57BL/6J, blue; C1q^{-/-}, orange).

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Chapter 3 – Early predictors of adverse responses to systemic AAV therapy

Introduction

Immune-mediated toxicities remain a major barrier to the safety, efficacy, and long-term success of adeno-associated virus (AAV)-based gene therapies. Following systemic administration, AAV vectors are recognized as foreign by the immune system, triggering coordinated innate and adaptive immune responses that contribute to many of the toxicities observed after dosing. Consequently, severe immune-mediated adverse events have emerged across neuromuscular gene therapy clinical trials and are now increasingly being recognized in routine clinical practice. These toxicities include thrombotic microangiopathy (TMA), acute liver injury and liver failure, myositis, myocarditis, and, in some cases, death^{1, 2, 3, 4}.

A recent meta-analysis of approximately 80 AAV gene replacement studies spanning multiple diseases, capsids, and dose ranges reported an overall incidence of immune-related adverse events of approximately 30%, with liver toxicity representing the most common complication (30% of all reported adverse events)⁵. These findings underscore that systemic AAV administration carries a meaningful risk of serious immune-mediated toxicity. At the same time, the potential therapeutic benefit is extraordinary because these treatments directly address the underlying genetic cause of disease. This balance between risk and benefit is particularly compelling in Duchenne muscular dystrophy, where progressive muscle degeneration is relentless and ultimately fatal. The critical challenge is therefore no longer simply developing effective gene therapies but understanding why only a subset of patients develops severe immune-mediated toxicity and identifying biomarkers that can predict risk before irreversible tissue injury occurs.

Although our understanding remains incomplete, a temporal framework for immune-mediated toxicities following systemic AAV delivery has begun to emerge. Early toxicities are thought to be driven primarily by innate immune activation and complement signaling during the first one to two weeks after dosing. As the immune response evolves, adaptive immunity becomes increasingly important, and distinct organ toxicities emerge on different timelines. Thrombotic microangiopathy (TMA) and thrombocytopenia typically occur early ^{6, 7}, acute liver injury often develops several weeks after dosing, and myositis or myocarditis may arise one month or later ^{8, 9}. This temporal pattern strongly suggests that multiple, overlapping immune mechanisms contribute to AAV-associated toxicity.

Innate immune activation not only mediates early inflammatory responses but also shapes the development of adaptive immunity. Growing evidence indicates that adaptive immune responses are major contributors to delayed toxicities, particularly liver injury. Both AAV capsid proteins and translated transgene peptides can be processed and presented on major histocompatibility complex (MHC) class I molecules, where they are recognized by CD8⁺ T cells ^{10, 11, 12}. In the liver, presentation of capsid-derived peptides may promote CD8⁺ T cell-mediated hepatocyte injury ^{13, 14}. In skeletal muscle, transgene-derived peptides may similarly be recognized as foreign, particularly in CRIM-negative individuals who lack immunological tolerance to the missing protein ⁸. Thus, adaptive immune responses may be directed against the capsid, the transgene product, or both, while the magnitude and quality of these responses are likely determined by the preceding innate inflammatory environment.

Clinically, elevations in serum transaminases often do not occur until several weeks after vector administration ¹⁵. This delayed onset is consistent with the time

required for adaptive immune responses to develop and highlights an important clinical challenge: patients may initially appear stable while pathogenic immune processes are already underway. By the time liver enzymes become elevated, substantial immune activation and tissue injury may have already occurred, underscoring the need for biomarkers capable of identifying patients at risk before overt toxicity develops.

Mouse models have informed on some aspects of underlying immune responses but have not been sufficient to predict adverse events ^{16, 17, 18}. Even primate models do not fully recapitulate human AAV immune responses ¹⁹. The commercial approval of Elevidys created a rare opportunity to study these questions prospectively in humans. We established a longitudinal biospecimen collection effort coupled to detailed clinical phenotyping. Importantly, samples are collected on a defined schedule beginning prior to dosing and extending through early post-treatment time points. Most prior studies relied on retrospective samples or sparse sampling. But immune responses after AAV dosing evolve very rapidly. If those early windows are missed, the opportunity to understand the initiating biology is lost. In addition, because adverse events vary considerably between individuals, that variability becomes highly informative biologically. Thus, a more complete understanding of the mechanisms governing these immune responses will enable more accurate prediction of toxicity, inform strategies to prevent or mitigate adverse events, and guide the development of safer and more effective AAV gene therapies.

Methods

Clinical data collection

Laboratory data were abstracted from routine clinical monitoring before pre (day 0), 4, 7, and 14 days after gene therapy administration. Extracted laboratory values

included hepatic (AST/ALT/GGT) , coagulation (platelet), and complement-associated markers when available. The timing of laboratory assessment was recorded relative to the date of vector administration, with the infusion date defined as day 0. Clinical adverse events, serious adverse events, laboratory abnormalities, changes in immunosuppression, and other post-dose interventions were recorded from clinician documentation and laboratory reports. Data were deidentified prior to analysis and summarized descriptively.

Custom enzyme-linked immunosorbent assay measuring anti-AAVrh74 IgG or IgM

Antigen-specific antibody levels were measured using a custom enzyme-linked immunosorbent assay. High-binding 96-well ELISA plates were coated with AAVrh74 empty capsid diluted in 50 mM carbonate-bicarbonate coating buffer at 1.6×10^7 vp/ μ L. Plates were washed with PBS containing 0.05% Tween-20 (PBS-T) and blocked with 5% BSA at room temperature to reduce nonspecific binding.

Serum samples were diluted 1:20 in blocking buffer and added to plates in triplicate. Samples were incubated for 2 hours at room temperature to allow antigen-specific antibodies to bind the coated capsid. Antibody isotype controls were diluted in blocking buffer beginning at 2000 ng/ μ L and serially diluted two-fold to 15 ng/ μ L. Plates were washed three times with PBS-T, followed by incubation with HRP-conjugated secondary detection antibody specific for human IgG or IgM diluted 1:2000 in blocking buffer for 1 hour at 37°C.

After a final wash step, plates were developed with TMB substrate protected from light. The reaction was stopped using 1 N sulfuric acid, and absorbance was measured at 450 nm using a microplate reader. Background absorbance from blank wells was

subtracted from all sample values. Antibody levels were reported as blank-corrected absorbance values at a fixed serum dilution.

High-content protein microarray analysis of anti-AAV capsid IgG reactivity

To evaluate cross-reactive humoral responses following Elevidys administration, plasma IgG reactivity against heterologous AAV capsids was assessed using custom high-content protein microarrays (HCPMs), as previously described by Emami et al. Briefly, empty AAV2, AAV8, and AAV9 capsids were printed directly onto nitrocellulose-coated microarrays at concentrations of 0.1, 0.05, 0.025, and 0.0125 mg/mL. Plasma samples from Elevidys-treated patients were evaluated for capsid-specific IgG binding, and antibody reactivity was quantified based on the resulting microarray signal intensity.

qPCR of Elevidys

Quantification of Elevidys Vector Genomes in plasma by qPCR

Plasma samples were thawed on ice, equilibrated to room temperature, and processed immediately. An untreated plasma sample was spiked with 10 μ L of Elevidys vector to serve as a positive control, while untreated plasma served as a negative control. Cell-free DNA (cfDNA) was extracted from 200 μ L of each plasma sample using the QIAamp MinElute Virus Spin Kit (Qiagen, Cat. No. 57704) according to the manufacturer's instructions and eluted in 30 μ L of elution buffer.

Elevidys vector genomes were quantified by TaqMan qPCR using TaqMan Universal Master Mix II with UNG (Applied Biosystems, Cat. No. 4440038). Each 20- μ L reaction contained 10 μ L of 2 $\times$ master mix, 500 nM each of forward (5'-GAGGAGCAGTGTCTGTTTAGTG-3') and reverse (5'-

CAGGTCCTGTTTCAGGCTATAC-3') primers, 250 nM hydrolysis probe (5'-CTGTCTAGCCTGCAGAACTGGCT-3'), 4 µL of extracted cfDNA, and nuclease-free water to volume. Samples were analyzed in duplicate.

Absolute quantification was performed using a standard curve generated from ten-fold serial dilutions of DNase-treated Elevidys vector (starting titer: 1.71×10^{10} vg/µL), corresponding to 6.86×10^{10} to 6.86×10^1 vector genome copies per 4-µL reaction input. Standards were included on each qPCR plate, and vector genome concentrations in unknown samples were calculated by interpolation from the standard curve using quantification cycle (Cq) values.

Longitudinal plasma cytokine and chemokine profiling following Elevidys administration

Plasma cytokines and chemokines were quantified using the Human Cytokine Magnetic 25-Plex Panel for the Luminex platform (Invitrogen, Thermo Fisher Scientific; catalog no. LHC0009M). The panel included GM-CSF, IFN- α , IFN- γ , IL-1 β , IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40/p70, IL-13, IL-15, IL-17, TNF- α , eotaxin, IP-10/CXCL10, MCP-1, MIG/CXCL9, MIP-1 α , MIP-1 β , and RANTES. Human plasma samples were analyzed on a Luminex 200 instrument through the UCLA Immune Assessment Core according to the manufacturer's protocol. Plasma samples from patients treated with Elevidys were evaluated longitudinally at the pre-dose baseline assessment (day 0) and at days 4, 7, and 14 after vector administration.

Modeling early chemokine predictors of liver enzyme status

Differences in biomarker levels between patients who did and did not subsequently develop liver injury were evaluated at baseline (day 0) and day 4, as well as for the change

in biomarker levels from baseline to day 4. Initial biomarker screening was performed using Welch's t-test, standardized effect sizes (Cohen's d), and the area under the receiver operating characteristic curve (AUC) derived from univariable logistic regression models.

Two approaches were used for biomarker selection. In the first approach, up to 10 biomarkers meeting the prespecified criteria of $P < 0.20$, Cohen's $d > 0.8$, and fewer than three missing observations were selected. In the second approach, biomarkers were required to meet the additional criterion of $AUC > 0.8$ and to have no more than one missing observation.

For each selection approach, the selected biomarkers were standardized as z scores and summarized using principal component analysis. The first two principal components were included as predictors in a logistic regression model of subsequent liver injury. Model discrimination was evaluated using the AUC.

Results

Complement activation alone is insufficient to cause thrombotic microangiopathy

Systemic AAV administration has been shown to activate the classical complement pathway, which is initiated by antibody recognition of the viral capsid and subsequently amplified through the alternative pathway^{7, 20}. Excessive complement activation can damage endothelial cells through membrane attack complex formation, promoting vascular inflammation, endothelial dysfunction, and, in severe cases, thrombotic microangiopathy (TMA). Clinically, TMA is characterized by complement activation, thrombocytopenia, endothelial injury, and microvascular thrombosis and may progress to

multiorgan dysfunction^{6, 7}. These observations suggest that severe AAV toxicities are not solely inflammatory but can also involve profound vascular pathology.

Most reported cases of AAV-associated TMA have occurred following systemic administration of AAV9 at doses exceeding 1×10^{13} vg/kg, and fewer cases have been reported following treatment with the Rh74-based vector Elevidys. To determine whether complement activation nevertheless occurs after Rh74 administration, we assessed overall complement activation by plasma C3 levels (Fig. 1A) classical complement activation by measuring C4 consumption (Fig. 1B) in prospectively collected samples from 16 Elevidys-treated individuals. All participants demonstrated evidence of complement activation, and most experienced at least transient thrombocytopenia (Fig. 1C). Despite these findings, none developed clinical TMA, indicating that complement activation alone is insufficient to drive endothelial injury and suggesting that additional host or immune factors determine progression to clinically significant disease.

Rapid anti-Rh74 antibody responses reveal two distinct response patterns

Activation of the classical complement pathway requires antibody binding to the AAV capsid. Although patients undergo pre-treatment serologic screening, anti-capsid antibodies may develop while vector remains in circulation, creating an opportunity for immune complex formation after dosing. Longitudinal analysis of anti-Rh74 antibody kinetics revealed two distinct response patterns. One group developed antibodies rapidly after vector administration, whereas the second exhibited delayed seroconversion (Fig. 2A). In contrast, anti-AAVrh74 IgM levels increased more uniformly across patients, generally beginning by day 4 after dosing (Fig. 2B). The accelerated responses are consistent with pre-existing immunological memory that was not detected by conventional

serologic screening, suggesting that current screening strategies may underestimate functional anti-capsid immunity. Alternatively, some individuals may simply show more rapid antibody secretion than others.

In addition to anti-Rh74 antibodies, many participants developed antibodies that cross-reacted with other AAV serotypes, which were evaluated by high content protein microarray. Anti-AAV9 IgG responses generally emerged later and at a lower magnitude than anti-AAVrh74 antibodies (Fig. 3A). Similar delayed, lower-magnitude responses were observed against AAV8 (Fig. 3B) and AAV2 (Fig. 3C), demonstrating immunological cross-reactivity among AAV capsids. Such cross-reactive immunity has important implications for vector re-dosing and for the future use of sequential AAV-based gene therapies.

Anti-Rh74 antibodies are associated with rapid clearance of circulating vector

We next examined the relationship between circulating Elevidys vector genomes and anti-Rh74 IgG titers. A striking inverse relationship emerged, with rising anti-Rh74 antibody levels closely paralleling the rapid disappearance of circulating vector (Fig. 4 A, B). These findings indicate that humoral immunity contributes to efficient clearance of circulating AAV within days of administration, including individuals who were classified as seronegative before treatment.

Liver enzyme elevations occur predominantly in older patients

Six of sixteen participants developed elevated serum transaminases following Elevidys administration (Fig. 5 A, B, C). Clinically significant elevations ($>2-3 \times$ the upper limit of normal) occurred predominantly in individuals older than 10 years. All participants

with transaminase elevations above the upper limit of normal received intensified corticosteroid treatment according to the clinical management protocol. These findings identify increasing age as a major clinical correlate of liver toxicity following systemic AAV gene transfer.

Longitudinal cytokine and chemokine profiling identifies early immune responses following AAV administration

To define the immune response to systemic AAV delivery, plasma samples collected longitudinally from each participant were analyzed using a multiplex Luminex cytokine and chemokine panel. While many analytes remained unchanged throughout the study period, a distinct subset of inflammatory cytokines and chemokines exhibited robust temporal changes following vector administration (Fig. 6).

Baseline immune signatures predict subsequent liver toxicity

Because elevations in hepatic laboratory markers often develop weeks after vector administration, we investigated whether baseline immune profiles could distinguish patients who subsequently developed post-dose GGT elevations above the upper limit of normal (ULN) from those whose GGT remained within the normal range. Patient E1 exhibited a borderline GGT response relative to the ULN; therefore, models were evaluated both with and without E1 as a sensitivity analysis to determine whether this ambiguous classification influenced model performance.

Two exploratory biomarker-selection approaches were evaluated. In the first model, the 10 baseline chemokines with the largest standardized between-group differences, as measured by Cohen's d, produced an AUC of 0.92 in the full cohort (Fig.

7A). Selected features included IL-13, CCL3, and IL-10 among others. In a sensitivity analysis excluding patient E1, the model achieved complete separation between groups, with a first principal component score greater than 0.65 identifying patients who developed GGT elevations above the ULN and an AUC of 1.00. In the second model, the seven baseline chemokines with the highest individual AUC values produced a combined model AUC of 0.85 in the full cohort (Fig. 7B). This feature set included CCL3, IL-10, IL-1 β , TNF- α , and CCL4. Exclusion of patient E1 increased the model AUC to 0.98. Together, these findings suggest that baseline chemokine profiles may contain discriminatory information associated with subsequent post-dose GGT elevation, although validation in a larger independent cohort is required.

Discussion

As clinical experience with Elevidys continues to expand, clinicians are increasingly faced with difficult decisions regarding treatment of older patients. The concern is that the risk-to-benefit ratio becomes less favorable in individuals with pre-existing comorbidities, including cardiomyopathy, chronic steroid-associated liver disease, hepatic steatosis, or other age-related manifestations of Duchenne muscular dystrophy. Older individuals are also more likely to have experienced natural exposure to AAV, resulting in pre-existing adaptive immune memory that may influence subsequent immune responses to vector administration. These disease-associated variables may amplify susceptibility to toxicity and are superimposed upon the substantial innate and adaptive immune activation induced by systemic high-dose AAV. Together, these factors complicate patient selection and suggest that chronological age alone is an insufficient predictor of risk. Rather, individualized assessment incorporating underlying disease

status, immune phenotype, liver health, and other host-specific factors may ultimately be required to optimize the risk-to-benefit ratio for each patient.

How Can the Risk-to-Benefit Ratio Be Improved?

Improving the safety of systemic AAV gene therapy will require advances on multiple fronts. First, reducing vector dose requirements remains a major priority. Development of more efficient capsids with enhanced muscle tropism, together with improved regulatory cassettes that maximize transgene expression while minimizing off-target activity, could substantially lower the amount of vector required to achieve therapeutic benefit. Second, continued development of next-generation dystrophin replacement strategies, including CRISPR-based genome editing, exon replacement approaches, and split-intein technologies, may ultimately permit delivery of larger, more physiologic dystrophin constructs with improved functional benefit.

Equally important is a deeper mechanistic understanding of the immune pathways that drive toxicity. Current management remains largely reactive, relying on corticosteroids or other immunosuppressive agents after clinical evidence of tissue injury has already emerged. A more complete understanding of the innate and adaptive immune mechanisms responsible for liver injury, myocarditis, thrombocytopenia, and myositis could enable rational, mechanism-based interventions that prevent these complications rather than simply treating their consequences.

Finally, increasing evidence suggests that host biology plays a central role in determining susceptibility to adverse events. Genetic background, baseline inflammatory state, prior viral exposures, immune history, liver health, and disease progression are all

likely to influence an individual's response to systemic AAV. Defining these determinants will be essential for developing personalized treatment strategies that maximize efficacy while minimizing toxicity.

Importance of Early Monitoring and Intervention

Our findings further emphasize that the period immediately following vector administration represents a critical window during which immune activation evolves rapidly. Intensive clinical monitoring during this vulnerable period is essential to detect early evidence of toxicity before irreversible tissue injury occurs. This became tragically evident in the recently reported fatal case of acute liver failure in a 15-year-old patient with Duchenne muscular dystrophy treated with Elevidys, in whom scheduled laboratory monitoring during a critical interval was missed ¹. Although a single case cannot establish causality, it illustrates how quickly severe toxicities may develop and reinforces the importance of strict adherence to post-treatment monitoring protocols.

Emerging therapeutic data also support a role for adaptive immunity in these toxicities. For example, sirolimus has been reported to improve transaminase elevations following systemic AAV administration. Because sirolimus primarily suppresses T-cell activation through inhibition of the mTOR pathway, these observations provide indirect evidence implicating T-cell-mediated mechanisms in AAV-associated liver injury. Although further studies are needed, these findings raise the possibility that targeted immunomodulatory strategies may prove more effective than broad corticosteroid therapy alone.

Predictive Biomarkers and Risk Stratification

One of the most important observations from this study is that many of the predictive immune signatures are detectable before clinically significant liver injury develops. Baseline cytokine and chemokine profiles, together with early post-dosing immune responses, distinguished patients who subsequently developed hepatotoxicity from those who did not. These findings suggest that susceptibility to toxicity is established, at least in part, before vector administration and reflects the underlying biologic state of the patient rather than solely the response to the vector itself.

Although these predictive models require validation in larger independent cohorts, they demonstrate the feasibility of identifying patients at increased risk before overt tissue injury occurs. Such biomarkers could ultimately guide patient selection, determine the intensity of post-treatment monitoring, and identify individuals who may benefit from prophylactic or intensified immunomodulatory therapy. Rather than waiting for transaminase elevations to signal established liver injury, future treatment paradigms may use baseline immune profiling to anticipate toxicity and intervene before clinically significant damage develops.

Factors That May Attenuate Toxicities

While identifying patients at increased risk is essential, an equally important goal is to develop strategies that actively reduce toxicity. Lower vector doses achieved through improved capsids and regulatory elements represent perhaps the most direct approach^{21, 22}. In addition, more selective immunomodulatory therapies targeting the specific pathways responsible for pathogenic immune activation may preserve transgene expression while limiting collateral tissue injury^{7, 23}. Improved patient selection through biomarker-based risk stratification, optimization of baseline liver health, and aggressive

monitoring during the first several weeks after dosing may further improve safety. Ultimately, combining advances in vector engineering, immune modulation, and personalized risk assessment will likely be necessary to fully realize the therapeutic potential of systemic AAV gene therapy.

Factors That May Exacerbate Toxicities

Patients enter AAV gene therapy with markedly different immune landscapes shaped by prior infections, chronic inflammation, and underlying comorbidities. Previous viral exposures may influence both baseline immunity and susceptibility to treatment-related toxicities.

The possibility that latent herpesvirus infections may also contribute to AAV-associated liver toxicity emerged from an earlier study involving five patients with Duchenne muscular dystrophy treated with Pfizer's AAV microdystrophin gene therapy ²⁴. Longitudinal peripheral blood samples collected before and after dosing underwent single-cell RNA sequencing coupled with T-cell receptor (TCR) sequencing to characterize antigen-specific immune responses. In one participant who developed TMA, expanded T-cell clonotypes were comprised almost exclusively of TCRs recognizing known HLA-A*02-restricted herpesvirus epitopes, including the cytomegalovirus (CMV) pp65 epitope (NLVPMVATV) and the Epstein–Barr virus (EBV) BMLF1 epitope (GLCTLVAML). Although preliminary and derived from a small cohort, these findings raise the possibility that reactivation of latent herpesvirus infections may amplify inflammatory responses following systemic AAV administration in susceptible individuals. If confirmed in larger studies, viral immune history and latent viral reactivation would represent additional host-specific factors that influence toxicity risk and further support

comprehensive pre-treatment immune profiling to identify patients at increased risk for adverse events.

Underlying liver health is also likely to influence susceptibility to hepatotoxicity.

Many individuals with Duchenne muscular dystrophy receive years of chronic corticosteroid therapy, which increases the risk of hepatic steatosis. These comorbidities become increasingly prevalent in older, non-ambulatory patients and may partially explain why liver toxicities appear more common in this population. Pre-existing hepatic injury or metabolic dysfunction may reduce the liver's capacity to tolerate the inflammatory and metabolic stress imposed by high-dose systemic AAV administration, thereby increasing the likelihood of clinically significant liver toxicity. Future studies incorporating liver imaging will be important to determine the extent to which underlying liver disease contributes to the observed heterogeneity in AAV-associated hepatotoxicity.

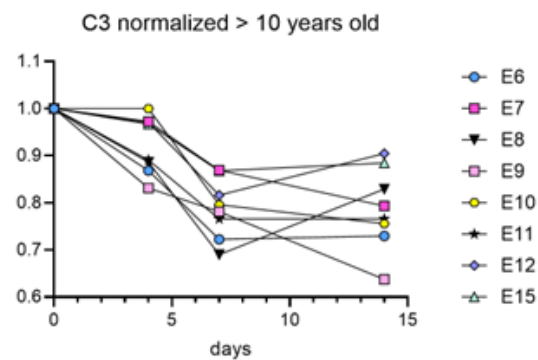
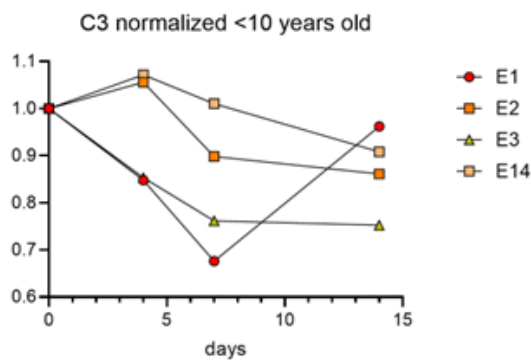
Host Biology as a Determinant of AAV Toxicity

Collectively, our findings support a conceptual shift in how AAV-associated toxicities are viewed. Rather than being solely a consequence of vector dose or transgene design, toxicity appears to emerge from a complex interaction between vector biology and the pre-existing biologic state of the host. Systemic administration of high-dose AAV provides the initiating stimulus, but the magnitude and clinical consequences of that stimulus are likely shaped by factors already present before dosing, including underlying inflammatory tone, immune history, liver health, disease progression, and genetic background. This framework helps explain why patients receiving the same vector under similar treatment conditions can experience markedly different clinical outcomes.

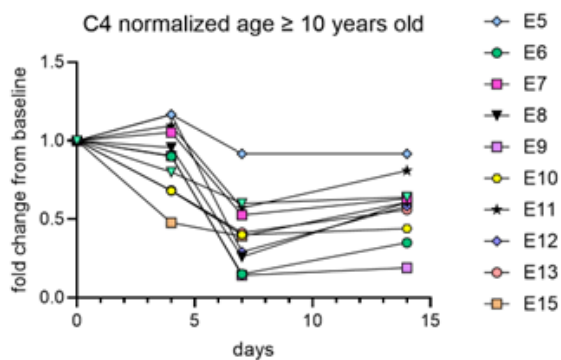
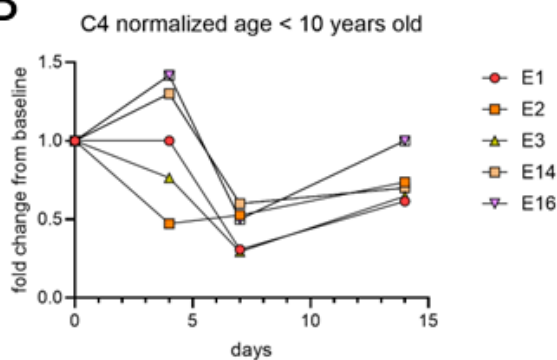
Recognizing host biology as a major determinant of toxicity has important implications for the future of systemic gene therapy. Continued improvements in vector engineering remain essential, but optimizing vector design alone is unlikely to eliminate serious adverse events. Instead, maximizing the safety of AAV therapies will likely require an integrated approach that combines next-generation vectors with biomarker-guided patient selection, individualized immunomodulatory strategies, and personalized monitoring protocols. As the field continues to expand into older patients and individuals with increasingly complex medical histories, precision medicine approaches that account for both vector characteristics and patient-specific biology may become indispensable for safely realizing the full therapeutic potential of systemic AAV gene therapy.

Figures

A



B



C

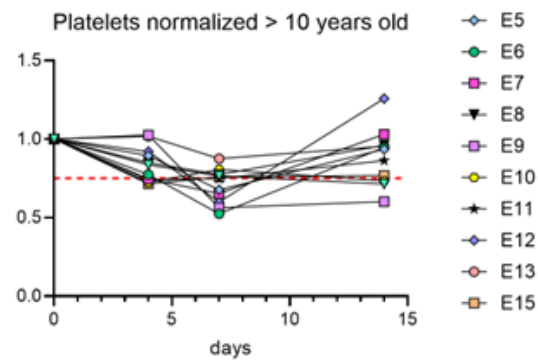
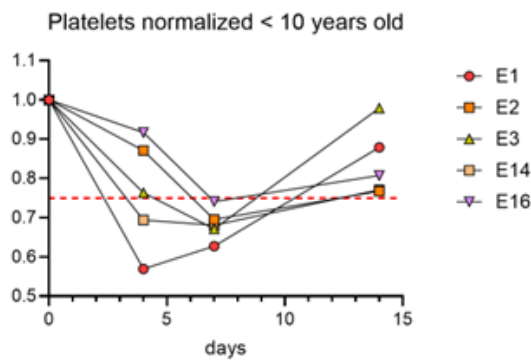


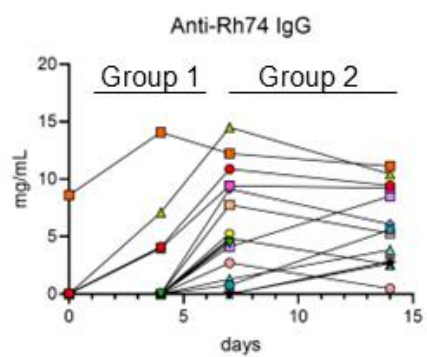
Figure 3-1. Longitudinal complement and platelet measurements following Elevidys treatment reveals overall complement activation and platelet loss.

A) Blood C3 levels normalized to each patient's pre-treatment baseline were assessed before dosing (day 0) and on days 4, 7, and 14 after treatment. Patients younger than 10 years old shown left, patients 10 years and older shown on the right. Patients without a pre-treatment measurement were excluded from the analysis.

B) Blood C4 levels were normalized to each patient's pre-treatment baseline and assessed before dosing (day 0) and on days 4, 7, and 14 after treatment. Patients younger than 10 years old shown left, patients 10 years and older shown on the right. Patients without a pre-treatment measurement were excluded from the analysis.

C) Blood platelet levels were normalized to each patient's pre-treatment baseline and assessed before dosing (day 0) and on days 4, 7, and 14 after treatment. Patients younger than 10 years old shown left, patients 10 years and older shown on the right. Patients without a pre-treatment measurement were excluded from the analysis.

A



B

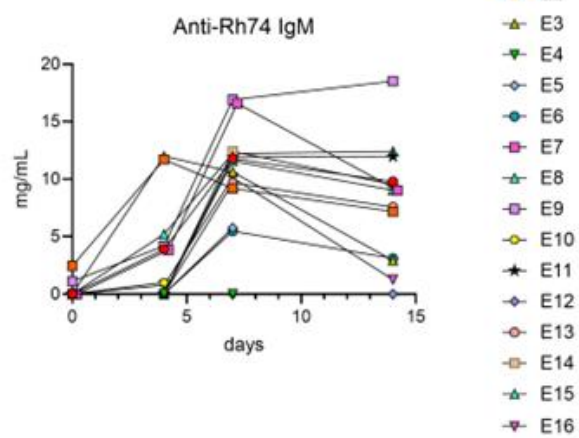


Figure 3-2. Anti-AAVrh74 antibody levels reveal two distinct response groups following Elevidys treatment.

(A) Plasma samples from patients treated with Elevidys were analyzed by ELISA for anti-AAVrh74 IgG antibodies. Longitudinal anti-AAVrh74 IgG levels identified two response patterns, consisting of a rapid-response group (group 1; left) and a delayed-response group (group 2; right). (B) Plasma samples from the same patient cohort were analyzed by ELISA for anti-AAVrh74 IgM antibodies. In contrast to the IgG response, anti-AAVrh74 IgM levels showed a more uniform increase across patients beginning approximately 4 days after dosing.

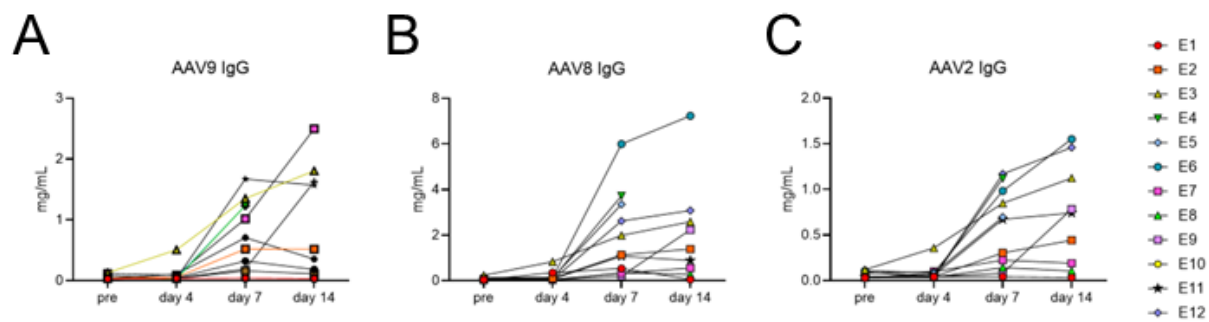
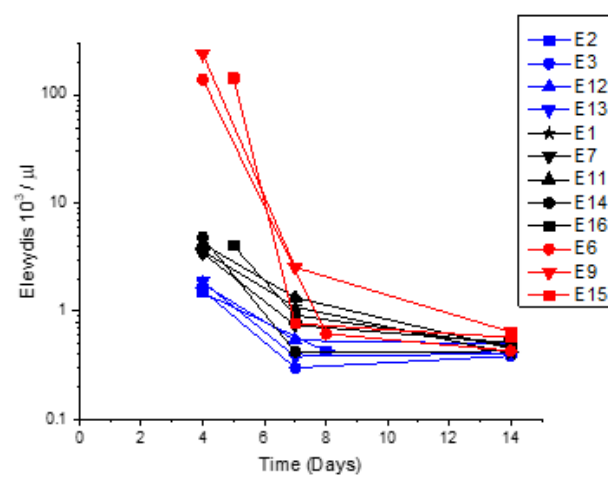


Figure 3-3. High-content protein microarray analysis reveals antibody cross-reactivity across AAV serotypes.

(A) Plasma IgG reactivity against AAV9 in 12 patients treated with Elevidys. (B) Plasma IgG reactivity against AAV8 in the same patient cohort. (C) Plasma IgG reactivity against AAV2 in the same patient cohort.

A

qPCR of μ Dystrophin transgene

B

Anti-Rh74 IgG

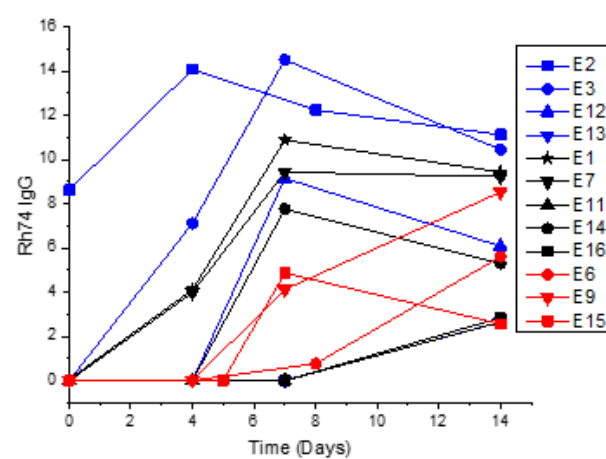
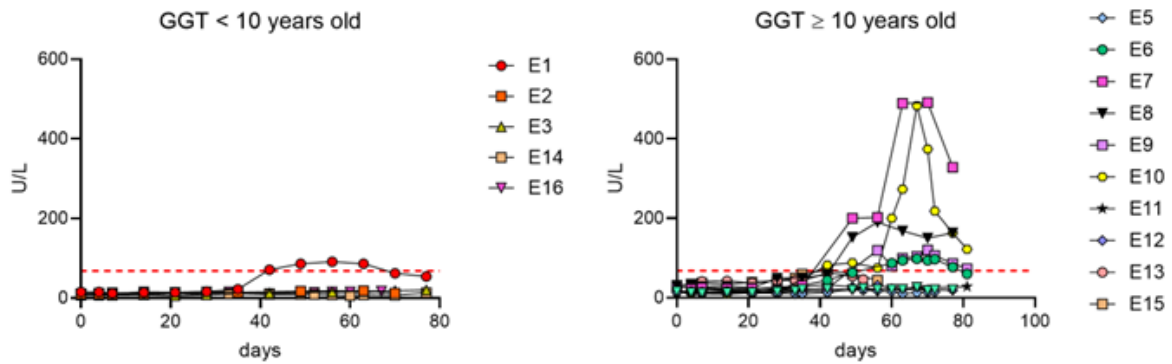


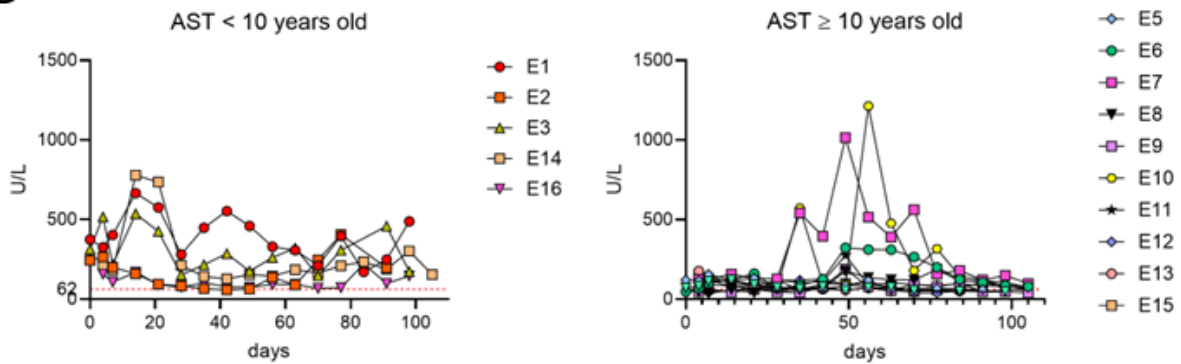
Figure 3-4. Circulating micro-dystrophin transgene levels decline rapidly as anti-AAVrh74 IgG levels increase following Elevidys treatment.

(A) Circulating micro-dystrophin transgene levels were quantified by qPCR in 15 patients at days 4, 7, and 14 after Elevidys dosing. (B) Anti-AAVrh74 IgG levels were measured in the same patients at the corresponding post-treatment time points. Patients were classified according to the kinetics of their anti-AAVrh74 IgG response: rapid responders (group 1; blue), delayed responders (group 2; red), and intermediate responders (black).

A



B



C

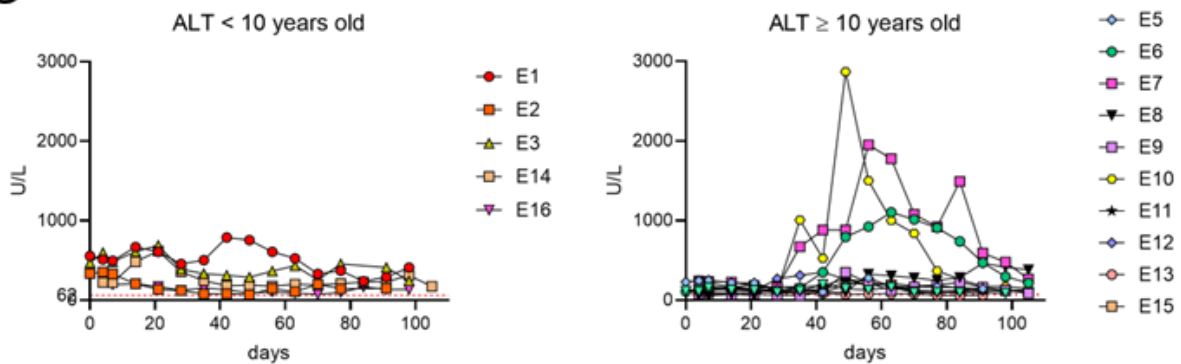


Figure 3-5. Longitudinal liver enzyme levels following Elevidys treatment.

Liver enzyme levels were measured longitudinally in patients treated with Elevidys and stratified by age, with patients younger than 10 years shown on the left and patients 10 years or older shown on the right. (A) Gamma-glutamyl transferase (GGT) levels. (B) Aspartate aminotransferase (AST) levels. (C) Alanine aminotransferase (ALT) levels. Red dashed lines indicate the upper limit of normal for each measurement.

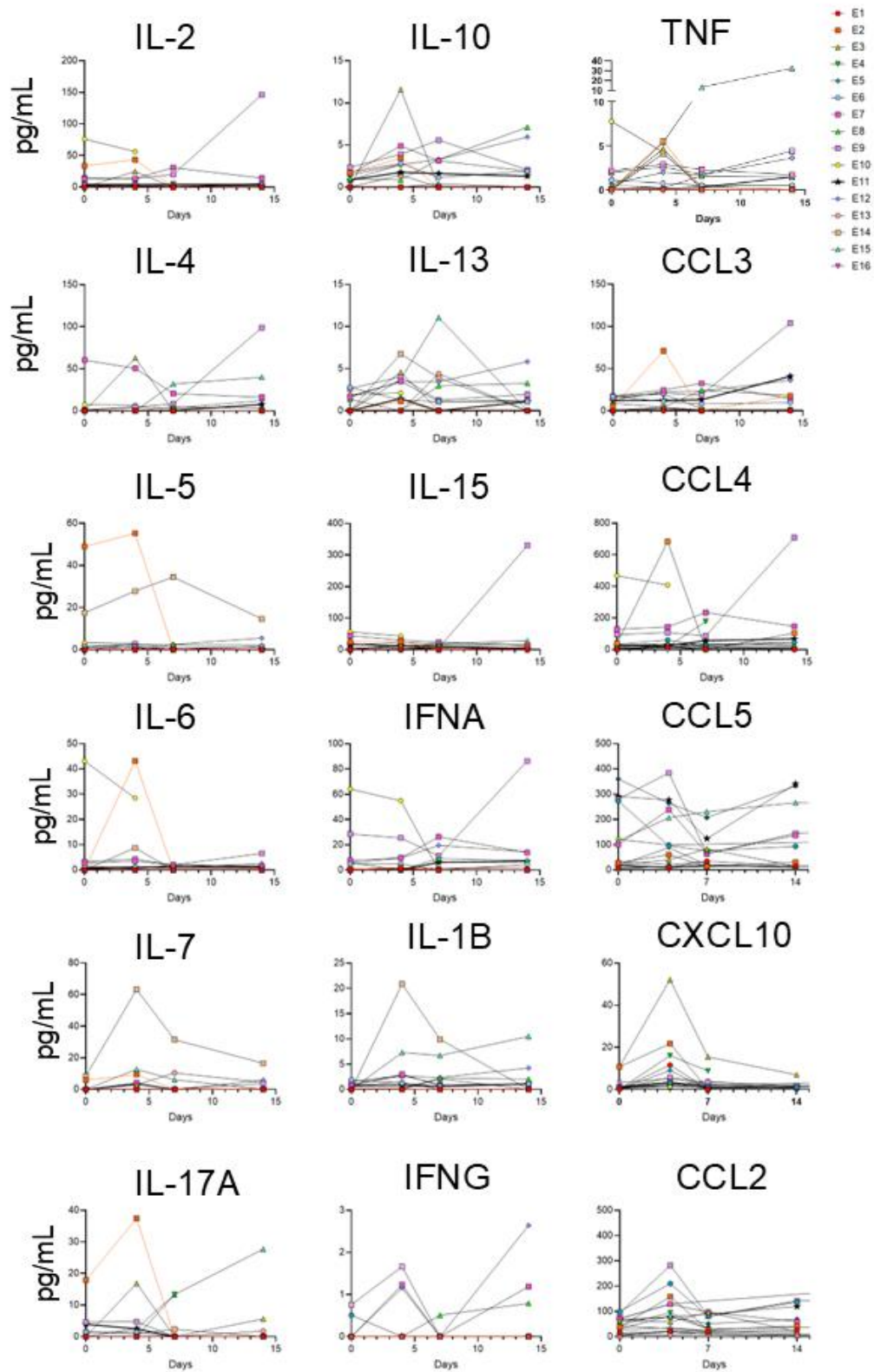
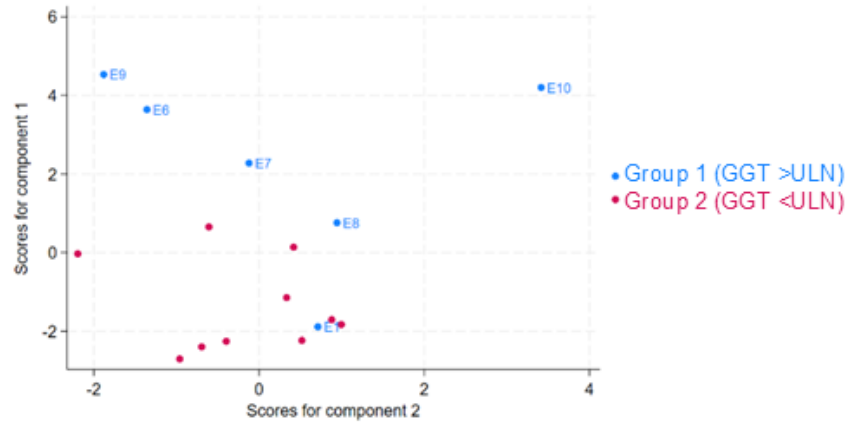


Figure 3-6. Longitudinal analysis of early inflammatory responses following Administration.

Longitudinal chemokine profiles from 16 patients treated with Elevidys. Plasma samples were collected before dosing and at 4, 7, and 14 days after administration.

A

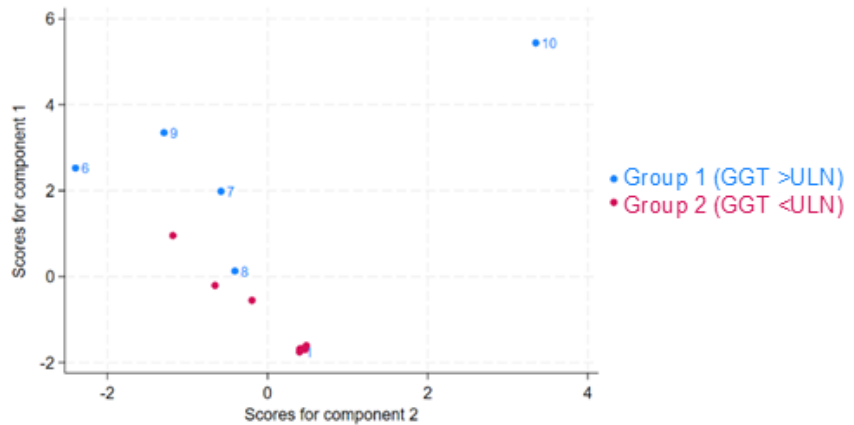
- IL13-Baseline (Day 0)
- CCL3-Baseline (Day 0)
- IL10-Baseline (Day 0)
- slope TNF (0 to 4)
- IL1b-Baseline (Day 0)
- CCL2-Day 4
- slope IL15 (0 to 4)
- TNF-Baseline (Day 0)
- slope CCL2 (0 to 4)
- slope IFNa (0 to 4)



Model	AUC (Full- includes E1)	AUC (Reduced- excludes E1)
M1 Top 10 (Cohen's D)	0.92	1.00 (PCA1 > 0.65 perfect Predictor)

B

- IL13-Baseline (Day 0)
- CCL3-Baseline (Day 0)
- IL10-Baseline (Day 0)
- IL1b-Baseline (Day 0)
- TNF-Baseline (Day 0)
- IFNa-Baseline (Day 0)
- CCL4-Baseline (Day 0)



Model	AUC (Full- includes E1)	AUC (Reduced- excludes E1)
M5 Top 7 (AUC)	0.85	0.98

Figure 3-7. Baseline chemokine profiles are associated with post-dose GGT elevation following AAV administration

(A) Patients were grouped according to whether post-dose GGT exceeded the upper limit of normal (ULN). The top 10 chemokines selected by Cohen's d produced an AUC of 0.92 in the full cohort. Exclusion of patient E1 resulted in complete group separation, with PCA1 > 0.65 identifying patients with GGT above the ULN and an AUC of 1.00.

(B) Patients were grouped according to whether post-dose GGT exceeded the upper limit of normal (ULN). The top seven baseline chemokines selected by AUC achieved an AUC of 0.85 in the full cohort. Exclusion of patient E1 increased the AUC to 0.98.

(A) Patients were grouped according to whether post-dose GGT exceeded the upper limit of normal (ULN). The top 10 chemokines selected by Cohen's d produced an AUC of 0.92 in the full cohort. Exclusion of patient E1 resulted in complete group separation, with PCA1 > 0.65 identifying patients with GGT above the ULN and an AUC of 1.00.

(B) Patients were grouped according to whether post-dose GGT exceeded the upper limit of normal (ULN). The top seven baseline chemokines selected by AUC achieved an AUC of 0.85 in the full cohort. Exclusion of patient E1 increased the AUC to 0.98.

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Chapter 4- Conclusions

Gene replacement therapy has emerged as a groundbreaking therapeutic option for previously untreatable genetic disorders. Unlike current treatments that primarily manage downstream symptoms, gene replacement seeks to address disease at its molecular origin by delivering a functional copy of a defective or missing gene. Restoration of gene expression may compensate for the underlying genetic deficiency and has the potential to produce sustained therapeutic benefit¹. Recombinant adeno-associated virus (AAV) vectors have become one of the most widely used platforms for *in vivo* gene delivery because different AAV capsids exhibit distinct tissue tropisms, enabling preferential transduction of specific cell types and target organs while supporting sustained transgene expression². Although gene therapy can address the underlying cause of monogenic disease, safe and effective dosing remains challenging, because the immune system sees the AAV vector as foreign and mounts responses to eliminate it. A more complete understanding of the immune response to AAV vectors is therefore needed to limit immune-mediated toxicities and improve long-term therapeutic outcomes³.

The expanding clinical use of AAV has revealed persistent challenges related to treatment durability, vector dose, preexisting immunity, and immune-mediated toxicities^{2, 4}. These limitations demonstrate that successful gene replacement depends not only on efficient delivery of the therapeutic transgene but also on the ability to anticipate and control host responses to both the vector and its expressed product^{5,6}. These challenges are especially relevant to neuromuscular disorders, which often affect both skeletal and

cardiac muscle, and therefore require widespread transduction to achieve therapeutic benefit ^{7,8}. Achieving this distribution generally requires systemic administration of comparatively high vector dose, thereby increasing exposure of the liver, vascular endothelium, and immune system to the AAV capsid ^{9,10}.

Gene replacement therapies for neuromuscular disorders such as Duchenne muscular dystrophy, must also overcome the 4.7 kb limited packaging capacity of AAV. This limitation requires the use of a shortened forms of dystrophin, referred to as micro-dystrophin ^{7,11}. Additional challenges include uncertainty regarding the durability of transgene expression in growing or degenerating muscle and the possibility of immune responses against newly expressed proteins or protein domains that are absent from the patient ^{4,12}.

Clinical experience has highlighted risks including liver injury, thrombocytopenia, cardiac injury, and immune-mediated myositis ^{8,10}. More recently, fatal acute liver failure was reported following AAVrh74 therapy for DMD and LGMD. These events prompted a boxed warning, restricted the Elevidys indication to ambulatory patients with Duchenne muscular dystrophy and led Sarepta Therapeutics to abandon their LGMD programs.

We first sought to define the specific contribution of complement to the immune response against AAV. To investigate this, we used a C1q-deficient mouse model to examine the contribution of the classical complement pathway. C1q is the recognition component of the C1 complex and is required for initiation of the classical complement cascade¹³. We selected this model because evidence available at the time implicated antibody-dependent activation of the classical complement pathway in immune-mediated responses following systemic AAV administration ¹⁴. Comparing C1q-deficient mice with

wild-type controls therefore allowed us to assess how loss of classical pathway initiation alters the immune response to AAV. Because classical pathway activation is initiated by antibodies bound to target antigen we combined the C1q-deficient model with a dual-dosing approach previously used by Emami et al. to investigate AAV-induced complement activation ¹⁵. In this model, mice receive two sequential doses of AAV, allowing the initial exposure to establish an AAV-specific antibody response and the second exposure to elicit a memory immune response. We hypothesized that loss of C1q, and therefore impaired activation of the classical complement pathway, would attenuate the immune response to AAV.

Contrary to our hypothesis, loss of C1q did not completely eliminate activation of the complement pathway nor did it uniformly attenuate the immune response to AAV. Instead, the immune response in C1q-deficient mice exhibited primarily enhanced kinetics of the response that included increased circulating concentrations of CXCL10 and CCL4, along with a modest increase in plasma IgM. Single-cell RNA sequencing further revealed altered inflammatory transcriptional programs within splenocytes, with particularly notable changes in Tfh and CD8 T cells and genes associated with the CXCL10–CXCR3 inflammatory axis.

One possibility is that reduced C1q-dependent clearance after the first dose allows capsid antigen to persist longer in circulation, resulting in a greater cumulative antigen burden upon redosing within an already primed immune environment. Sustained antigen processing and presentation could contribute to the heightened inflammatory and T-cell responses observed after the second dose, including the more activated T follicular helper cell phenotype. Thus, C1q deficiency may alter antigen burden between doses and thus

affect the magnitude of the subsequent immune response. Additionally, although no differences in complement split-product formation were detected at the time points examined, C1q deficiency may alter the complement pathway involved in antigen clearance, potentially resulting in differences in split product formation and downstream immune activation. Further studies would be needed to investigate this possibility. Given that complement is a highly interconnected network of more than 50 proteins, loss of a single component such as C1q may redirect the pathway and shift its kinetics, magnitude, or cellular consequences rather than abolishing the immune response altogether.

Together, these findings suggest that C1q deficiency reshapes, rather than suppresses, the immune response to AAV. These results therefore highlight the broader immunoregulatory role of C1q and demonstrate that inhibition of a single complement pathway may not be sufficient to uniformly reduce AAV-associated inflammation. Further studies will be needed to determine whether the increased CXCL10 and CCL4 responses arise directly from loss of C1q-mediated regulation or indirectly through compensatory activation of other immune pathways.

Among patients receiving Elevidys for Duchenne muscular dystrophy, there remains a limited ability to predict which individuals will develop clinically significant treatment associated toxicities that may disrupt the expected post-treatment course and required escalation of corticosteroid therapy ^{14,16}. To investigate early indicators of treatment associated toxicity, we collected blood before dosing and at days 4, 7, and 14 after AAV administration. We carried out assessments of antibodies and measured a panel of circulating chemokines and compared their baseline and early post-treatment concentrations with subsequent changes in markers of liver injury. Through this approach,

we sought to identify candidate biomarkers that are elevated before dosing or during the early treatment period that may help predict which patients are at increased risk of developing downstream hepatotoxicity.

To identify potential pretreatment biomarkers of liver toxicity, we evaluated baseline chemokine concentrations in 16 patients and compared these values with peak post-treatment gamma-glutamyl transferase (GGT) levels. IL13, CCL3, IL10, IL1B, TNF, IFN α , CCL4 emerged from this analysis as candidate biomarkers at baseline associated with subsequent peak GGT. Elevated chemokine concentrations at baseline may reflect differences in the pre-existing inflammatory state of individual patients. In DMD, ongoing cycles of muscle-fiber degeneration and regeneration promote chronic immune-cell recruitment and chemokine production, which may contribute to increased day 0 chemokine concentrations in some patients. Baseline inflammation could also be influenced by a recent or subclinical infection or by latent herpesvirus reactivation ¹⁷. Thus, increased baseline chemokines may identify patients who enter treatment with a heightened inflammatory state that could influence the magnitude or kinetics of their subsequent immune response. Interestingly, several inflammatory mediators identified in the human cohort, including CCL3, CCL4, and IL-1 β , were also observed in the mouse studies, highlighting that animal models can remain informative when their specific translational strengths and limitations are carefully defined. These findings suggest that differences in the inflammatory environment before Elevidys administration may be related to the magnitude of downstream liver enzyme elevations. However, given the limited cohort size, these chemokines should be considered preliminary candidates that require validation in larger patient populations.

Together, these studies demonstrate that immune responses to systemic AAV are shaped by both the biological context of the host and the timing of immune activation. Although animal models do not fully reproduce human responses, overlapping inflammatory signals across the mouse and patient studies indicate that they remain valuable for identifying mechanisms when they are used appropriately. A better understanding of these early immune pathways may ultimately improve the prediction, prevention, and management of adverse responses to AAV gene therapy.

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