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Coagulation Factor X Activates Innate Immunity to Human Species C Adenovirus

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Abstract

Although coagulation factors play a role in host defense for “living fossils” such as horseshoe crabs, the role of the coagulation system in immunity in higher organisms remains unclear. We modeled the interface of human species C adenovirus (HAdv) interaction with coagulation factor X (FX) and introduced a mutation that abrogated formation of the HAdv-FX complex. In vivo

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Supplementary Materials

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Materials and Methods

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genome-wide transcriptional profiling revealed that FX-binding–ablated virus failed to activate a distinct network of nuclear factor κ B–dependent early-response genes that are activated by HAdv-FX complex downstream of TLR4/MyD88/TRIF/TRAF6 signaling. Our study implicates host factor “decoration” of the virus as a mechanism to trigger an innate immune sensor that responds to a misplacement of coagulation FX from the blood into intracellular macrophage compartments upon virus entry into the cell.

Upon infection with microbial and viral pathogens, specialized innate immune sensors recognize specific pathogen-associated chemical moieties [pathogen-associated molecular patterns (PAMPs)] (1). This triggers the activation of effector mechanisms aimed at restricting spread and facilitating pathogen elimination from the host. In the context of sterile inflammation, the innate immune receptors can also recognize host moieties released from damaged cells as an intrinsic danger signal [danger-associated molecular patterns (DAMPs)] (2) to initiate tissue repair. Often, innate immune recognition of pathogens leads to a severe inflammatory host response that may be solely responsible for the morbidity and mortality associated with the infection (3–6).

Human species C adenovirus (HAdv) induces potent innate immune and inflammatory responses (7). For immunocompromised individuals, HAdv infections can be lethal (8–10). Disseminated HAdv infections are frequently associated with liver and kidney failure and a high virus burden in the blood (11). When HAdv-based vectors are injected intravenously in preclinical and clinical gene therapy trials, they induce an acute inflammatory response that may lead to morbidity and mortality (12, 13). The molecular basis for innate immune activation by HAdv remains poorly characterized. While in the blood, species C HAdv2 and HAdv5 bind coagulation factor X (FX) with high affinity (14, 15). Because coagulation factor activation may trigger systemic inflammation, we hypothesized that FX binding to HAdv may trigger virus recognition by the innate immune system and activation of an antiviral inflammatory response. As a first step in testing this hypothesis, we analyzed the interaction interface between FX and HAdv by determining a high-resolution cryogenic electron microscopy (cryo-EM) structure followed by molecular dynamics flexible fitting (MDFF) simulations (16).

In the cryo-EM structure, FX density protrudes from the top of each hexon in the HAdv5 capsid (Fig. 1, A to C, and movie S1). FX interacts with the capsid by attaching to threefold symmetric central depressions in each hexon trimer, as seen in earlier moderate resolution cryo-EM structures (14, 15). Atomic models for hexon and FX were docked into cryo-EM density (9 Å resolution for the capsid and 11 Å resolution for the capsid plus FX). MDFF simulations revealed an electrostatic interaction between FX- γ -carboxyglutamic acid (GLA) domain residue K10 and HAdv5 hexon residues E424 and T425 (Fig. 1, D to F; table S1; and movie S2). [Singleletter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.] To test the robustness of the MDFF simulated protein-protein interface, we performed a series of simulations with the FX starting orientation rotated by $\pm 10^\circ$ or $\pm 20^\circ$ about the threefold molecular axis of hexon

(figs. S1 and S2 and table S1). Despite these adjustments, the FX-GLA/HAdv5 hexon interface snaps back to form the same packing arrangement.

To test whether glutamic acid E424 and threonine T425 are essential for formation of the FX-HAdv5 complex, we introduced single-amino acid substitutions in this region of the hexon by substituting the wild-type (WT) amino acid sequence TET at positions 423 to 425 for GAT, TAT, or TEA (fig. S3A). Surface plasmon resonance analysis revealed that the affinity of GAT and TAT mutants for FX remained in the low nanomolar range, whereas TEA mutant was unable to bind FX (Fig. 2A). The in vitro infection of CHO-K1 cells (which can only be infected in the presence of FX) showed that unlike all other viruses, the TEA mutant failed to infect these cells in the presence of FX (fig. S3B). In contrast, all of the mutant viruses were able to infect susceptible lung carcinoma A549 cells with equal efficacy (fig. S3). Because FX-binding-ablation abrogates HAdv5 infection of hepatocytes in vivo (14, 15, 17), we injected mice intravenously with virus mutants expressing green fluorescent protein (GFP) and analyzed GFP expression 48 hours later. This analysis confirmed that all the mutants, except for TEA, infected hepatocytes efficiently (Fig. 2, B and C, and fig. S3F). Analysis of virus interaction with tissue macrophages showed that both liver and spleen macrophages trapped all of the viruses with comparable efficacy (Fig. 2D and figs. S4 to S6).

Because tissue macrophages are the principal inducers of inflammation in response to adenovirus (18, 19), we next compared the innate immune response to TEA virus versus control WT HAdv5. The analysis of transcripts for >28,000 genes revealed that expression of 519 genes was changed more than 1.5-fold ($P < 0.001$) in the spleen of WT mice 30 min after HAdv injection. Gene ontology analysis with the CateGORizer tool (Iowa State University, Ames, IA) (materials and methods are available as supplementary materials on *Science* Online) showed that over 60% of genes transcriptionally up-regulated in response to HAdv5 were divided between four categories: stress response (23.88%), metabolism (20.90%), death pathways (11.94%), and apoptosis (8.96%) (Fig. 3A). To define the signaling pathway (or pathways) specifically activated by HAdv sensors, we identified only those genes that were coactivated after HAdv injection in both WT and *Il1r1*^{-/-} mice. This is because signaling downstream of HAdv sensors occurs independently of interleukin-1 receptor 1 (IL-1R1), and IL-1R1 triggers feed-forward amplification loops (19). We found that after applying a strict cut off criteria ($P < 0.0002$), there were 34 genes coactivated after HAdv injection in both WT and *Il1r1*^{-/-} mice (table S2). We next injected WT and *Il1r1*^{-/-} mice with TEA mutant virus and compared the transcriptional signatures induced by HAdv5 and TEA on this 34-coactivated-gene set. This analysis showed that there were numerous genes differentially activated by HAdv and TEA viruses (Fig. 3B and table S3). For *Nr4a2* ($P = 9.61 \times 10^{-7}$), *Atf3* ($P = 6.77 \times 10^{-6}$), *Fosb*, *Fosl2*, and *Ptgs2* genes, differential activation ranged between 2.2- and 5.8-fold. The P-scan analysis also demonstrated that there was enrichment for cyclic adenosine monophosphate (cAMP) response element-binding protein 1 (CREB1), nuclear factor- κ B 1 (NFKB1), and serum response factor (SRF) transcription factor binding sites within the promoters of the 34-gene set (fig. S7). However, only NFKB1 transcription factor binding sites were overrepresented in proximal promoters of the gene set differentially activated by HAdv and TEA (Fig. 3C). Using Pathway

Analysis software (Ingenuity Systems, Redwood City, CA), we further confirmed that NFKB1, CREB1, and SRF are at the center of a signaling network that responds to HAdV entry into macrophages in vivo (figs. S8 and S9).

Because nuclear factor- κ B (NF- κ B) is a transcription factor that activates numerous early response genes, including genes encoding for inflammatory cytokines and chemokines, we next analyzed whether WT HAdV5 and TEA viruses trigger differential expression of proteins associated with innate immunity. Indeed, TEA mutant failed to activate several NF- κ B-dependent cytokines and chemokines, including IL-1 β , IL-6, and MIP-1 α (macrophage inflammatory protein-1 α) (Fig. 4A). A ribonuclease (RNase) protection assay further confirmed that TEA virus failed to activate IL-1 β transcription in vivo in WT mice (Fig. 4B and fig. S10A). Deconvolution of the signaling pathway that leads to NF- κ B activation in gene-deficient mice revealed that IL-1 β transcriptional activation required TRAF6, MyD88, and TRIF (encoded by *Ticam1*) (Fig. 4C and fig. S10B). Because MyD88 and TRIF are the signaling adapters for Toll-like receptors (TLRs), we analyzed IL-1 β expression in response to HAdV challenge in mice deficient in TLR2, -4, -7 and -8, or -9. This analysis showed that transcriptional activation of IL-1 β was impaired only in *Tlr4*^{-/-} mice (Fig. 4D and fig. S10C). Analysis of inflammatory cytokines and chemokines in *Tlr4*^{-/-}, *Myd88*^{-/-}, and *Ticam1*^{-/-} mice confirmed reduced amounts of cytokines and chemokines that depend on NF- κ B (Fig. 4E). Collectively, these data demonstrate that a full-scale inflammatory response to HAdV requires TLR4/TRAF6/NF- κ B signaling, and FX binding-ablated TEA virus failed to activate this pathway in vivo. In vitro infection of primary macrophages from WT and *Tlr4*^{-/-} mice with WT HAdV5 confirmed that full cytokine and chemokine activation is dependent on the presence of FX (figs. S11 to S13). Surface plasmon resonance analysis revealed differential binding of the HAdV5-FX complex to human and mouse TLR4/MD2 complexes that were immobilized on a sensor chip in vitro (fig. S14). The impeded cytokine and chemokine activation in *Tlr4*^{-/-} mice resulted in significantly lower recruitment and retention of polymorphonuclear leukocytes (PMNs) in the splenic marginal zone as compared with that of WT mice (figs. S15 and S16A). Furthermore, WT mice or mice deficient in inflammasome components Caspase-1 and ASC (encoded by *Pycard*) (20) effectively cleared the WT HAdV5 virus from the spleen and liver, whereas *Tlr4*^{-/-} mice failed to clear the virus. The failure to clear the virus allowed for the extensive virus replication, and the amount of viral genomes in the liver 24 hours after the infection in *Tlr4*^{-/-} mice was two orders of magnitude higher, as compared with that of WT mice (fig. S16, B to D).

We next analyzed activation of a panel of cytokines and chemokines in response to a number of WT HAdV species and found that those viruses that bind FX activated a broader spectrum of cytokines and chemokines as compared with those of WT HAdV species that do not bind FX (14) (Fig. 4F). Analysis of PMN distribution in the spleen showed lack of retention of PMNs in the splenic marginal zone after infection of mice with HAdV4 and HAdV51 (do not bind FX) and efficient retention of PMNs in marginal zone after infection with HAdV2 and HAdV5 (bind FX) (fig. S17).

Thus, our results suggest that FX, a noninflammatory humoral factor of the coagulation cascade, binds to the surface of the virus and becomes a pathogen-associated molecular

pattern that, upon viral entry into the cell, triggers activation of innate immunity via the TLR/NF- κ B pathway (fig. S18). Direct binding of coagulation factors FVII and FX was recently reported for human herpes virus HSV-1 (21). The data presented here provide evidence for an evolutionary conserved link between the coagulation system and innate immunity in higher organisms, in which the coagulation system functions to facilitate direct recognition of a pathogen and activation of innate immune defenses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Cryo-EM structure of the FX-HAdv5 complex and simulation of the FX-hexon interface using MDFF. **(A)** The cryo-EM structure of HAdv5 in complex with FX. The density is shown with the hexon capsid in blue, penton base in gold, fiber in green, and FX in red. Scale bar, 100 Å. **(B)** An enlarged view of the FX-HAdv5 complex showing the network of the FX density above the hexon capsid. **(C)** The best rigid-body fit orientation of the zymogenic FX model (red ribbon) within FX cryo-EM density (transparent pink). This FX density is selected from above a hexon near the icosahedral threefold axis of the capsid. **(D)** Coordinates from a frame in the MDFF simulation that show hexon residues E424 and T425 surround residue K10 of the FX-GLA domain. The side chains of these three residues are shown in space-filling representation and colored by element. **(E)** FX-GLA domain and associated Ca_2^+ ions (green) in the central depression of the hexon trimer. **(F)** Residue K10 in the FX-GLA domain is in close enough proximity to E424 and T425 of hexon to engage in electrostatic interactions.

Fig. 2.

A single amino acid substitution (T425A) abrogates FX binding to HAdV5. (A) Kinetic response data and dissociation constant (K_d) for FX binding to the indicated mutant viruses obtained by using surface plasmon resonance analysis (Biacore, GE Healthcare Biosciences, Pittsburgh, PA). Black indicates experimentally obtained data. Orange indicates global fits of these data to 1:1 single-site interaction model. Representative data obtained from four independent experiments are shown. (B to D) In vivo analysis of hexon-mutated viruses. (B) Histological analysis of virus-encoded GFP expression in mouse hepatocytes 48 hours after intravenous infection of mice with WT HAdV5 (WT) or mutated viruses. Representative fields are shown ($n = 5$ biological replicates). GFP expression is observed as green fluorescence on fixed liver sections. Corresponding fields in 4',6-diamidino-2-phenylindole channel (blue, nuclei-specific staining) are shown. Scale bar, 100 μm . (C) Western blotting analysis of GFP expression in the livers of mice shown in (B). The biological duplicate samples for each virus are shown. (D) Colocalization of virus particles (red) with splenic CD169⁺ and MARCO⁺ marginal zone macrophages (green) observed 1 hour after infection for indicated viruses analyzed by means of confocal microscopy. Scale bar, 10 μm . Representative fields are shown. $n = 5$ biological replicates.

Fig. 3.

FX binding–ablated virus triggers blunted transcriptional response of NFKB1-dependent genes in vivo. (A) Gene Ontology pie chart. Genes that were differentially expressed more than 1.5-fold ($P < 0.05$) in the spleens of WT mice challenged with HAdV5 or mock (saline) were identified. The CateGORizer tool was used to sort these genes in Gene Ontology categories and determine percentages of differentially expressed genes for each category. (B) Heatmap representation of the averaged gene expression levels for 34-gene set (coactivated in WT and *Il1r1*^{-/-} mice with $P < 0.0002$) when WT and *Il1r1*^{-/-} mice were infected with either HAdV5 or TEA mutant virus. In each experimental group, $n = 3$ biological replicates. The yellow and blue color legend shows log₂-transformed fold changes. Heatmap was generated by using the Bioconductor gplots package (Seattle, WA). (C) z-score map of the transcription factor binding site frequencies in proximal promoters of indicated genes was generated by means of P-scan from the analysis of binding sites for 116 transcription factors (Transfac database). The five-gene set represents a subset of genes from (B) that are the most differentially induced by HAdV5 and TEA mutant virus (1.5-fold cut off). Nr4a2, $*P = 9.61 \times 10^{-7}$; ATF3, $*P = 6.77 \times 10^{-6}$. The green and red color legend shows log₂-transformed fold z-score changes.

Fig. 4.

HAdV5 binding to FX induces NF κ B1-dependent inflammatory cytokines and chemokines downstream of the TLR4-TRIF/MyD88-TRAF6 signaling axis in vivo. (A) Mouse cytokine array panel showing differences in inflammatory cytokines and chemokines in the spleens of WT mice 1 hour after infection with HAdV5 or TEA mutant, determined by means of proteome profiler antibody array. Representative blot from four independent experiments is shown. C, mouse was challenged with saline. (B) mRNA expression of IL-1 β in the spleen of WT mice 30 min after challenge with indicated viruses. Graphs show mean $\pm$ SD, $n = 4$ biological replicates, ** $P < 0.01$. AU, arbitrary units reflecting IL-1 β to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA ratios. (C and D) mRNA expression of IL-1 β in the spleen of WT mice and mice deficient for indicated genes 30 min after challenge with HAdV5. Graphs show mean $\pm$ SD, $n = 4$ biological replicates, ** $P < 0.01$. AU, arbitrary units reflecting IL-1 β to GAPDH mRNA ratios. (E) Mouse cytokine array panel showing differences in inflammatory cytokines and chemokines in the spleens of WT and *Myd88*^{-/-}, *Ticam1*^{-/-}, *Tlr4*^{-/-}, and *Md2*^{-/-} mice 1 hour after challenge with HAdV5, determined by means of proteome profiler antibody array. Representative blot from four independent experiments is shown. C, mouse was challenged with saline. (F) Mouse cytokine array panel showing differences in inflammatory cytokines and chemokines in the spleens of WT mice 1 hour after challenge with WT human adenoviruses of indicated species, determined by means of proteome profiler antibody array. Representative blot from four independent experiments is shown. C, mouse was mock infected with saline.