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MAJOR ARTICLE

A Protein Epitope Targeted by the Antibody Response to Kawasaki Disease

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Background. Kawasaki disease (KD) is the leading cause of childhood acquired heart disease in developed nations and can result in coronary artery aneurysms and death. Clinical and epidemiologic features implicate an infectious cause but specific antigenic targets of the disease are unknown. Peripheral blood plasmablasts are normally highly clonally diverse but the antibodies they encode are approximately 70% antigen-specific 1–2 weeks after infection.

Methods. We isolated single peripheral blood plasmablasts from children with KD 1–3 weeks after onset and prepared 60 monoclonal antibodies (mAbs). We used the mAbs to identify their target antigens and assessed serologic response among KD patients and controls to specific antigen.

Results. Thirty-two mAbs from 9 of 11 patients recognize antigen within intracytoplasmic inclusion bodies in ciliated bronchial epithelial cells of fatal cases. Five of these mAbs, from 3 patients with coronary aneurysms, recognize a specific peptide, which blocks binding to inclusion bodies. Sera from 5/8 KD patients day ≥ 8 after illness onset, compared with 0/17 infant controls ($P < .01$), recognized the KD peptide antigen.

Conclusions. These results identify a protein epitope targeted by the antibody response to KD and provide a means to elucidate the pathogenesis of this important worldwide pediatric problem.

Keywords. plasmablast; monoclonal antibodies; Kawasaki disease.

Kawasaki disease (KD) is a febrile illness of young childhood that has clinical and epidemiologic features of an infectious disease [1], including epidemics with geographic wavelike spread [2]. KD can result in potentially severe or even fatal coronary artery aneurysms in infants and children [3]. First described by Dr Tomisaku Kawasaki in Japan in the 1960s and now recognized worldwide, the etiology has remained elusive. The highest attack rates of KD are observed in Asian children, most likely because of genetic predisposition to the inciting agent [4]; in Japan, 1 in 65 children develop the disease by the age of 5 years [5].

The antigens triggering the immune response in KD patients have been unknown. Analysis of peripheral blood plasmablasts, plasma cell precursors, is emerging as a powerful tool in studies of pathogenesis, diagnosis, and therapeutic discovery in infectious diseases [6–14], vaccine science [14–20], and autoimmune disease [21, 22]. Multiple studies have shown that > 70% of peripheral blood plasmablasts express antibodies specific to the infectious or immunizing agent at 1–2 weeks following infection [23–26]. Identification of specific KD antigens would enable diagnostic test development and improved therapies.

Our previous studies provided support for the concept that KD patients mount an antigen-specific antibody response. We identified an oligoclonal immunoglobulin A (IgA) response in KD arterial tissues [27], and made synthetic antibodies comprised of clonally expanded α heavy chains from KD arterial tissue paired with random light chains [28,29]. These antibodies identified intracytoplasmic inclusion bodies in KD but not in infant control ciliated bronchial epithelium by immunohistochemistry [28–30]. However, these antibodies did not yield the specific antigen, likely because they did not include in vivo cognate immunoglobulin variable, diversity, joining (VDJ) and variable, joining (VJ) heavy and light chains. In the present study, we isolated and analyzed single peripheral blood plasmablasts from children with KD 1–3 weeks after fever onset, prepared monoclonal antibodies from clonally expanded plasmablasts, and used these antibodies to identify their target antigens.

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METHODS

Patients and Specimens

This study was approved by the institutional review board of the Ann and Robert H. Lurie Children's Hospital of Chicago, and patients were enrolled following informed consent. Peripheral blood was obtained from 11 KD patients on day 8–24 after fever onset (Figure 1A and Supplementary Table 1) from April 2017. Blood was also obtained from 1 healthy adult volunteer as a source of control antibodies. Sera available from additional KD children were used for serologic assays, as were sera from febrile control children and infant controls.

Flow Cytometry

$CD3^{-}CD19^{+}CD38^{++}CD27^{++}$ peripheral blood mononuclear cells were gated (Figure 1A) and single cells sorted into individual wells of 96-well polymerase chain reaction (PCR) plates.

Amplification, Sequencing, and Cloning of Immunoglobulin Variable Regions

Reverse transcription and PCR of heavy and light chain variable genes were performed according to a published protocol [31,32], and PCR products were directly sequenced. Heavy chain sequences were analyzed for variable heavy (VH) family and complementarity-determining region 3 (CDR3) sequence and clonally related sequences identified and prioritized for antibody synthesis. Light chains were cloned into human immunoglobulin κ or λ light chain expression vectors [32] and heavy chains were cloned into human γ1 and rabbit γ (pFUSEss vectors; InvivoGen) heavy chain expression vectors, to enable production of human and rabbit versions of the antibodies.

Antibody Production and Analysis

Antibodies were produced by transfection of 293F suspension cells using a 1.5:1 ratio of light chain:heavy chain DNAs and Freestyle MAX reagent, and were purified using protein A agarose beads (ThermoFisher Scientific). Immunohistochemistry was performed on KD and control infant tissues as previously reported [28, 33].

Monoclonal Antibody Reactivity With Animal Virus Peptides

A custom animal virus discovery peptide array was designed (PEPperPRINT, www.pepperprint.com) and antibodies KD4-2H4 and KD6-2B2 tested on the array. To identify common motifs of binding peptides, MEME bioinformatics analysis was performed on those peptides with the highest spot intensities (meme-suite.org/tools/meme).

Substitution Analysis

Substitution analysis was performed on viral peptides recognized by antibodies KD4-2H4 and KD6-2B2 by creating a peptide array that includes stepwise substitution of all amino acid positions of the peptide with all 20 amino acids, to determine the amino acids that yielded optimal binding to the antibody (PEPperPRINT).

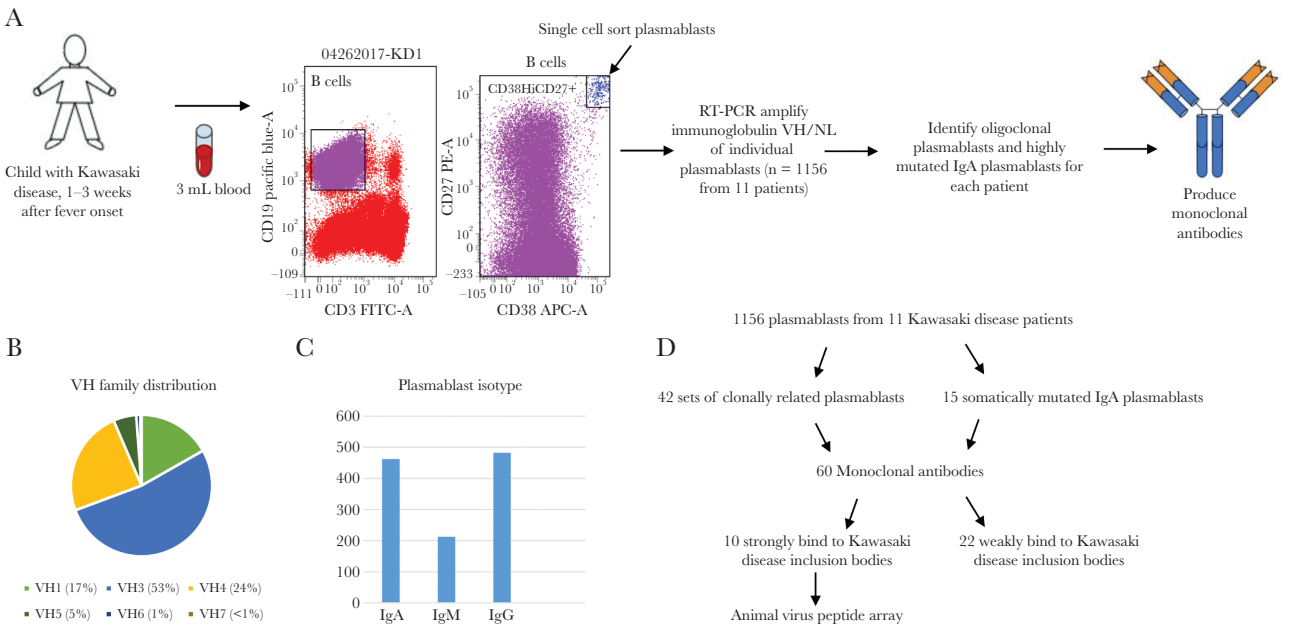

Figure 1. Experimental strategy and characteristics of PB isolated from peripheral blood of 11 children 1–3 weeks after KD fever onset. *A*, Study overview. *B–D*, Analysis of single cells from 11 children with KD. *B*, Most PB were VH3, VH4, or VH1. *C*, Number of PB by isotype; IgA and IgG PB were most commonly identified. *D*, Of 1156 PB sequenced, 42 sets of oligoclonal PB were identified for antibody production and 15 somatically mutated IgA plasmablasts were also selected for production. Of 60 monoclonal antibodies prepared, 10 strongly bound to intracytoplasmic inclusion bodies in ciliated bronchial epithelial cells of children who died of KD using immunohistochemistry and 22 were found to bind weakly. Strongly binding antibodies were tested on an animal virus peptide array. Abbreviations: IgA, immunoglobulin A; IgG, immunoglobulin G; KD, Kawasaki disease; PB, plasmablasts; RT-PCR, reverse transcription and polymerase chain reaction; VH, variable heavy; VL, variable light.

ELISA for Binding of Peptides to KD Monoclonal Antibodies

Maxisorp Nunc Immuno 96-well plates were coated with 1 μg of synthetic peptides (Anaspec) per well and incubated with rabbit KD monoclonal antibodies at 10, 1, and 0.1 μg/mL followed by horseradish peroxidase-labelled goat anti-rabbit antibody at 1:3000 (Fisher). Absorbance at 450 nm was determined on a Multiskan FC spectrophotometer after addition of ultra 3,3′,5,5′-tetramethylbenzidine followed by 1.5 M sulfuric acid solution. Absorbance of the KD peptide (KPAVIPDREALYQDIDEMEEC) assays were recorded after subtraction of absorbance with the scrambled peptide (IYPLEDMAEPKVERIDAQEDC). An OD reading >0.4 was arbitrarily determined as a positive; all negative antibodies had values of ≤0.04.

Blocking Experiments to Determine Specificity of Peptide Binding

Monoclonal antibodies showing binding to animal virus peptides were incubated with a 5-fold excess (by weight) of representative peptide or a scrambled peptide at 37°C for 45 minutes, and each mixture was then applied to KD lung tissue for immunohistochemistry.

Human Protein Array Analyses and Human Protein Immunohistochemistry

KD monoclonal antibodies human KD4-2H4 and rabbit KD6-2B2 were tested for binding to a human proteome array (HuProt v 4.0; CDI Laboratories).

Western Blot Assay Using Glutathione Sulfur Transferase-Concatemerized KD Peptide Fusion Protein

We optimized the nucleotide sequence that codes for the KD peptide sequence for expression in *Escherichia coli* and prepared a multimer with 3 copies of the peptide linked by short spacers: (glutathione sulfur transferase [GST]-3X: AGKPAVIPDREALY QDIDEMEECLD**E**AGKPAVIPDREALYQDIDEMEECLD**E**AG KPAVIPDREALYQDIDEMEECLD). The multimer sequence was cloned into the pGEX-KG plasmid (ATCC No. 77103) and fusion protein expression was induced. Western blot assays were performed following electrophoresis on 12% Tris-glycine gels (Biorad) and transfer to PVDF membrane (IPVH00010; Fisher). After blocking the membranes, serum samples from KD patients and controls (Supplementary Table 5 and Table 6) were diluted 1:5000 and incubated with membranes overnight at 4°C. Following incubation, membranes were washed and incubated with horseradish peroxidase-labelled goat anti-human IgG (A18811; Thermo Fisher) at a dilution of 1:5000 and developed using Supersignal West Femto Substrate (Thermo Fisher).

Statistical Analysis

GraphPad Prism 8 was used to plot enzyme-linked immunosorbent assay (ELISA) results. Comparison of serologic results between groups was performed using a 2-tailed Fisher exact test using the function fisher.test in R 3.6.1.

Additional detailed methods are available in the Supplementary Material.

RESULTS

Plasmablasts From KD Patients

Our approach for studying single peripheral blood plasmablasts from KD patients is outlined in Figure 1. We identified heavy chain sequences in 1156 plasmablasts derived from 11 patients. Most of the plasmablasts encoded antibodies of the VH3 family (Figure 1B); 462 were IgA, 482 were IgG, and 212 were IgM (Figure 1C).

Genetic Characterization of KD Plasmablasts Reveals an Oligoclonal Response

We identified 42 sets of clonally related plasmablasts in 10 patients (Figure 1D), typical of B-cell response to specific antigen. One patient (KD patient 7) did not have clonally related plasmablasts but did have IgA plasmablasts with many mutations from germline (Supplementary Table 2). More than 1 isotype was present in 12/42 (29%) of the clonally related plasmablast sets from these 10 patients (Supplementary Table 2). KD plasmablasts were otherwise of varying genetic composition and the clonally related heavy chain CDR3 sequences differed among patients. This result was expected based on published data showing that the VH nucleotide repertoire is highly private [34, 35]. We selected clonally related sets of plasmablasts and selected IgA plasmablasts with many somatic mutations for antibody production (Figure 1D and Supplementary Table 2). VDJ and VJ sequences of these plasmablasts and for healthy adult volunteer plasmablasts E3 and E4 are available as GenBank accession numbers MK416266-MK417513.

Generating KD Monoclonal Antibodies

We expressed the antibodies encoded by 38 clonally related sets of plasmablasts and 12 highly mutated single IgA plasmablasts in 293F cells, generating approximately 300 μg to 1 mg of human/rabbit or human/human antibodies in one 60-mL culture flask per assay. Of the 60 antibodies, 52 had entirely different VH/VL sequences and 8 were members of clonally related plasmablast sets in which the related antibodies had 1–4 amino acid mutations in the CDR3 sequence within the set.

KD Monoclonal Antibodies Bind to Cytoplasmic Inclusion Bodies in KD Ciliated Bronchial Epithelium

Our prior studies demonstrated binding of synthetic antibodies with noncognate VDJ and VJ pairs to intracytoplasmic inclusion bodies in ciliated bronchial epithelial cells of children who died from acute KD but not of infant controls who died of non-KD illnesses [28–30, 36]. Initial studies with antibodies KD1-2G11 and KD4-2H4 revealed strong binding of the antibody to KD lung tissues from the United States (n = 3) and Japan (n = 2) and not to infant control lung tissue (n = 3) (Figure 2A–2D), similar to our prior studies [28–30, 36, 37]. To test all 60 monoclonal antibodies for

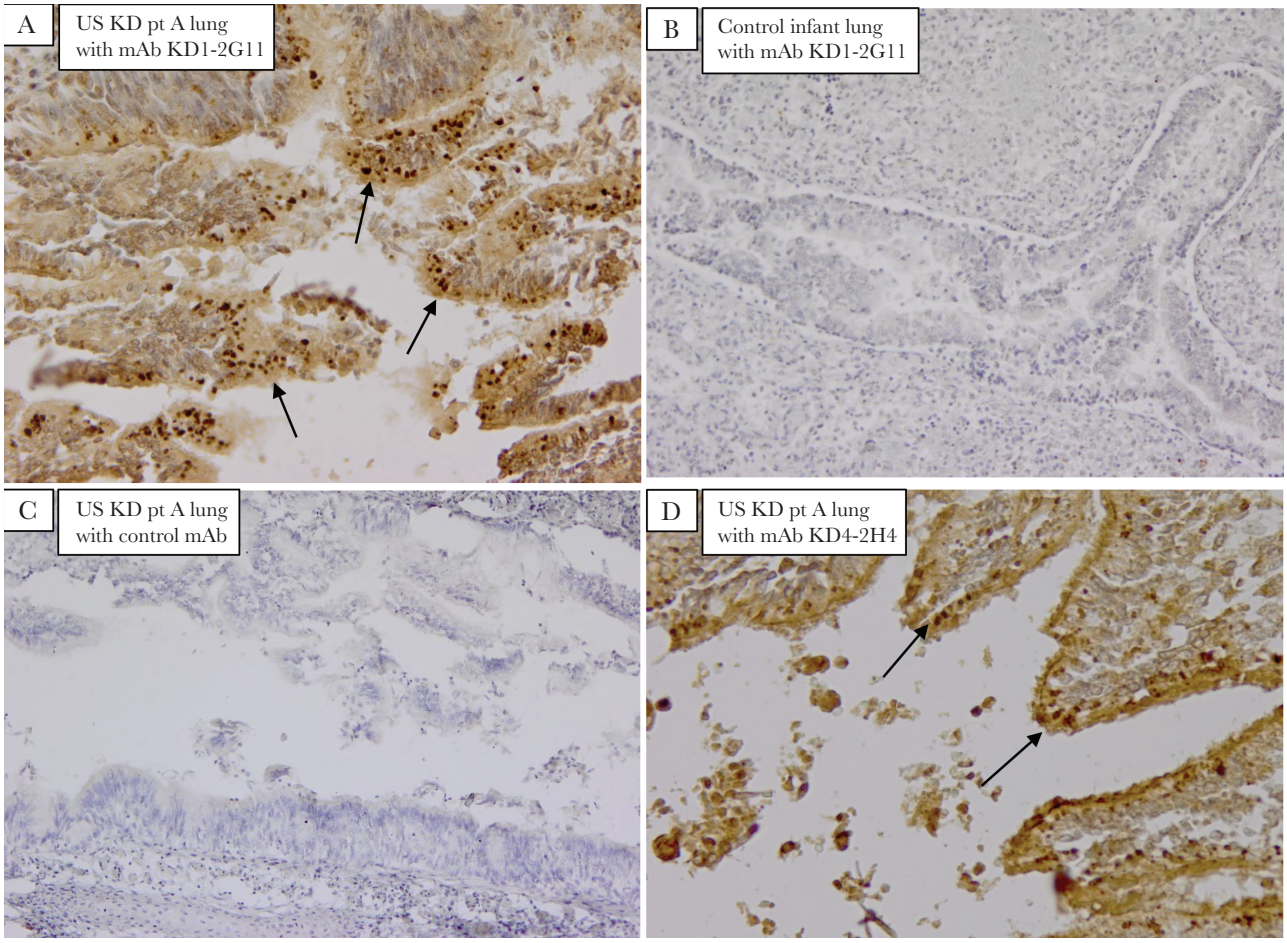

Figure 2. *A* and *D*, KD monoclonal antibodies recognize ICI in KD ciliated bronchial epithelium by immunohistochemistry (arrows). *A*, ICI are identified by monoclonal antibody KD1-2G11 in a 4-month-old male infant (KD patient A) who died of acute KD in the United States at 3 weeks after fever onset. *B*, No staining is observed using KD1-2G11 in a 3-month-old US female infant who died of influenza. *D*, ICI are identified in KD patient A using antibody KD4-2H4. *A–D*, ×20. Abbreviations: ICI, intracytoplasmic inclusion bodies; KD, Kawasaki disease; mAb, monoclonal antibody; pt, patient.

inclusion body binding, we used lung tissue from a KD child found in our prior studies to have many inclusion bodies. Strong positive staining similar to that demonstrated in our prior studies [28–30, 36, 37] was observed using 10 monoclonal antibodies (Table 1, Supplementary Table 3, and Figure 2A and 2D). Another 22 monoclonal antibodies showed weak binding to the inclusion bodies. Overall, an antibody that recognizes KD inclusion bodies was made from 9/11 (82%) KD patients. The 2 patients from whom we did not clone such an antibody were sampled at the latest time points after onset in the cohort, on days 20 and 24 after fever onset, at which time the antigen-specific plasmablast response may have abated [38].

Identifying a Protein Target of Monoclonal Antibody KD4-2H4

To determine whether KD monoclonal antibodies bind to an epitope that is shared with a known animal virus, we created a custom array (PEPperPRINT; Supplementary Table 4) of peptides reported to be B-cell epitopes of animal viruses and included in the Immune Epitome Database and Analysis Resource (https://www.iedb.org/). Monoclonal antibody KD4-2H4 showed binding to multiple similar peptides from the C-terminal region of hepacivirus NS4A (Figure 3A). MEME bioinformatic analysis was used to identify a shared motif among the top 27 peptide hits, which yielded a motif that was significant at $1.1e^{-118}$ (Figure 3B).

KD4-2H4 Binds to a Novel Peptide Epitope Demonstrated by Substitution Matrix Array Analysis

To identify the critical amino acids required for KD4-2H4 peptide binding, we tested a peptide substitution array, in which each position of the reactive peptide AIIPDREALYQEFDEME was sequentially replaced by each of 20 amino acids (PEPperPRINT). The substitution array showed that amino acids ^{9}L and ^{11}Q of this peptide were essential for antibody binding (Figure 3C). Replacement of ^{12}E by D, T, or S and of ^{13}F by I and V resulted in a marked increase in antibody binding on the array. These results indicate that the length of the reactive epitope is an approximately 8 amino acid segment, similar to the length of other linear epitopes

Table 1. Genetic Characteristics of KD mAbs That Bind Strongly to KD Inclusion Bodies

mAb	Heavy Chain	CDR3 Length, Amino Acids	No. of Mutations From Germline, Amino Acids	Light Chain	KD Patient	Original Isotype
1-2G11	IGHV3-15/JH4	15	10	IGKV1-30/JK3	1	IgA
4-2H4	IGHV3-74/JH2	14	4	IGLV2-14/JL3	4	IgG
6-1A10	IGHV3-33/JH4	20	11	IGLV1-44/JL3	6	IgA
6-2B2	IGHV3-33/JH3	12	15	IGKV1-5/JK1	6	IgA
7-1D3	IGHV3-23/JH4	11	15	IGKV1-5/JK1	7	IgA
7-2C1	IGHV3-23/JH4	14	13	IGLV5-45/JL3	7	IgA
9-1E10	IGHV1-46/JH4	12	22	IGLV1-44/JL1	9	IgA
11-1E9[a]	IGHV1-46/JH4	16	28	IGKV2-30/JK5	11	IgG
11-1C2[a]	IGHV1-46/JH4	16	15	IGKV2-30/JK5	11	IgA
11-2E10	IGHV4-59/JH3	16	16	IGKV1-33/JK3	11	IgG

Abbreviations: aa, amino acid; CDR3, complementarity-determining region 3; IgA, immunoglobulin A; IgG, immunoglobulin G; KD, Kawasaki disease; mAb, monoclonal antibody.
[a]Clonally related plasmablasts from KD patient 11.

bound by antibodies [39], and BLAST analysis showed that it does not match any known hepacivirus sequence. We designated a new peptide (AVIPDREALYQDIDEME), which includes the critical amino acid sequence in the center of the peptide, as KD peptide.

Multiple KD Monoclonal Antibodies Recognize the KD Peptide by ELISA

To determine if monoclonal antibodies from multiple KD patients recognize the KD peptide, we conjugated the peptide and a scrambled version of the peptide to bovine serum albumin to improve microtiter well coating and tested all 60 KD monoclonal antibodies by ELISA. We found that monoclonal antibodies KD4-2H4, KD6-2B2, KD6-1A10, KD8-1B10, and KD8-1D4 reacted with KD peptide and not with the scrambled peptide by ELISA (Table 2 and Figure 4A). The remainder of the KD monoclonal antibodies did not show specific binding to KD peptide by ELISA. All of the antibodies reactive with the peptide by ELISA identify inclusion bodies in KD ciliated bronchial epithelium by immunohistochemistry. Therefore, of the 9 KD patients in this study whose antibodies identified KD inclusion bodies, 3 patients (33%) had plasmablasts with differing VDJ and VJ sequences (Table 2 and Supplementary Table 3) that recognize the KD peptide. These antibodies were not polyreactive against DNA, insulin, or bovine serum albumin by ELISA (Supplementary Figure 1).

Monoclonal Antibodies KD4-2H4 and KD6-2B2 Share a Common Epitope

We noted that KD4-2H4 and KD6-2B2 showed strong reactivity with KD peptide by ELISA (Figure 4A), indicating that they likely recognize the same peptide epitope. To further evaluate this, we performed substitution matrix analysis on KD6-2B2, which revealed that its binding epitope is highly similar to that of KD4-2H4 (Figure 4B).

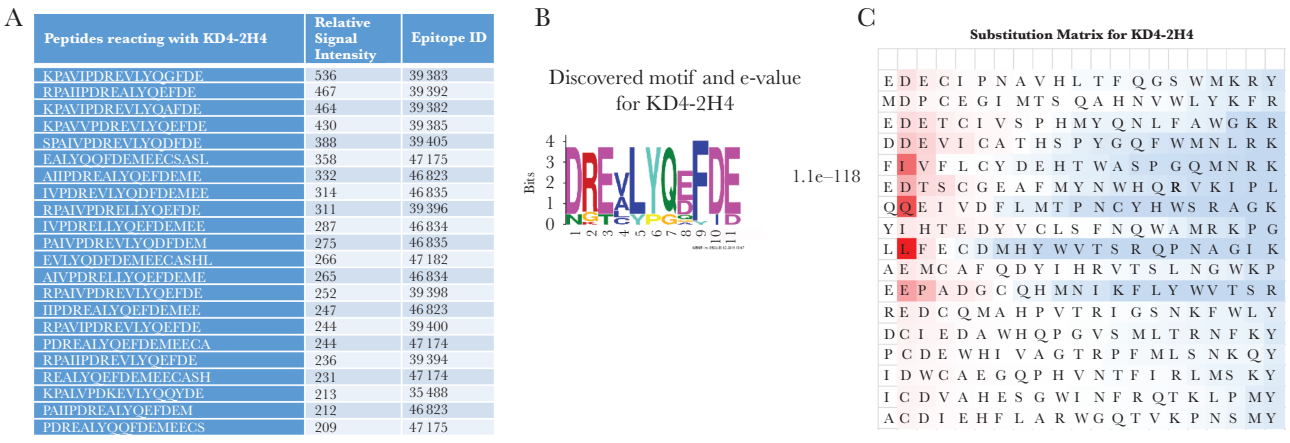

Figure 3. Identifying the peptide motif recognized by Kawasaki disease (KD) monoclonal antibody KD4-2H4 using animal virus peptide array and substitution array. *A*, Animal virus peptide array demonstrates that KD4-2H4 recognizes multiple related peptides with averaged median foreground fluorescence intensities above a cutoff value of 200; the epitope identity number from the Immune Epitope Database (www.iedb.org) is listed for each reacting peptide. *B*, The motif of KD4-2H4 binding identified as statistically significant by MEME bioinformatics analysis. *C*, Substitution matrix array of peptide AIIPDREALYQEFDEME using KD4-2H4. Preferred amino acids for binding are those on the left of the substitution matrix while nonpreferred amino acids are those on the right.

Table 2. Genetic Characteristics of KD mAb That Recognize KD Peptide

mAb	Heavy Chain	CDR3 Length, aa	No. of Mutations From Germline, aa	Light Chain	KD Patient	Original Isotype	Binding to KD Inclusion Bodies
4-2H4	IGHV3-74/JH2	14	4	IGLV2-14/JL3	4	IgG	Strong
6-1A10	IGHV3-33/JH4	20	11	IGLV1-44/JL3	6	IgA	Strong
6-2B2	IGHV3-33/JH3	12	15	IGKV1-5/JK1	6	IgA	Strong
8-1B10[a]	IGHV3-72/JH3	12	3	IGKV1-6/JK4	8	IgA	Weak
8-1D4[a]	IGHV3-72/JH3	12	1	IGKV1-6/JK4	8	IgA	Weak

Abbreviations: aa, amino acid; CDR3, complementarity-determining region 3; IgA, immunoglobulin A; IgG, immunoglobulin G; KD, Kawasaki disease; mAb, monoclonal antibody.
[a]Clonally related plasmablasts from KD patient 8.

KD Peptide Blocks Binding of KD4-2H4 and KD6-2B2 to Inclusion Bodies

To determine if the KD peptide would compete with monoclonal antibodies KD4-2H4 and KD6-2B2 for binding to KD tissues, we preincubated KD peptide with the antibodies prior to immunohistochemistry. We found that KD peptide blocked binding of the antibodies to intracytoplasmic inclusion bodies in KD ciliated bronchial epithelium (Figure 5B and 5D), demonstrating that an epitope of this peptide sequence is a specific target of the antibodies. Incubation with the scrambled peptide did not block the binding (Figure 5A and 5C). These results demonstrate that a protein with an epitope highly similar to KD peptide is present in the inclusion bodies.

Human protein array analyses and immunohistochemistry studies of monoclonal antibodies KD4-2H4 and KD6-2B2 do not yield a human protein as the target of the antibodies.

To determine whether KD4-2H4 and KD6-2B2 recognize a human antigen, we performed human protein array analysis. The array covers approximately 80% of the canonical proteome. The only human protein that showed reactivity with both antibodies on this array was integral membrane protein 2B (ITM2B). This reactivity could be explained by a partially shared epitope between KD peptide and the ITM2B protein (-ALYQ-I-). Immunohistochemistry of KD lung with polyclonal anti-ITM2B revealed a different pattern of staining of bronchial epithelium compared with KD4-2H4 and KD6-2B2, and anti-ITM2B did not block the binding of the antibodies to inclusion bodies in KD ciliated bronchial epithelium (Supplementary Figure 2). These results strongly suggest that ITM2B is not the antigen in inclusion bodies in KD ciliated bronchial epithelium specifically targeted by these KD monoclonal antibodies. These results do not exclude a potential human antigen as the target of KD; however, the clinical and epidemiologic features of the illness do not support this explanation of disease pathogenesis [40].

KD Peptide Is Recognized by Sera From KD Patients

To determine if sera from KD patients recognize KD peptide, we performed western blot analyses for IgG antibody using GST-KD peptide multimer fusion protein and GST

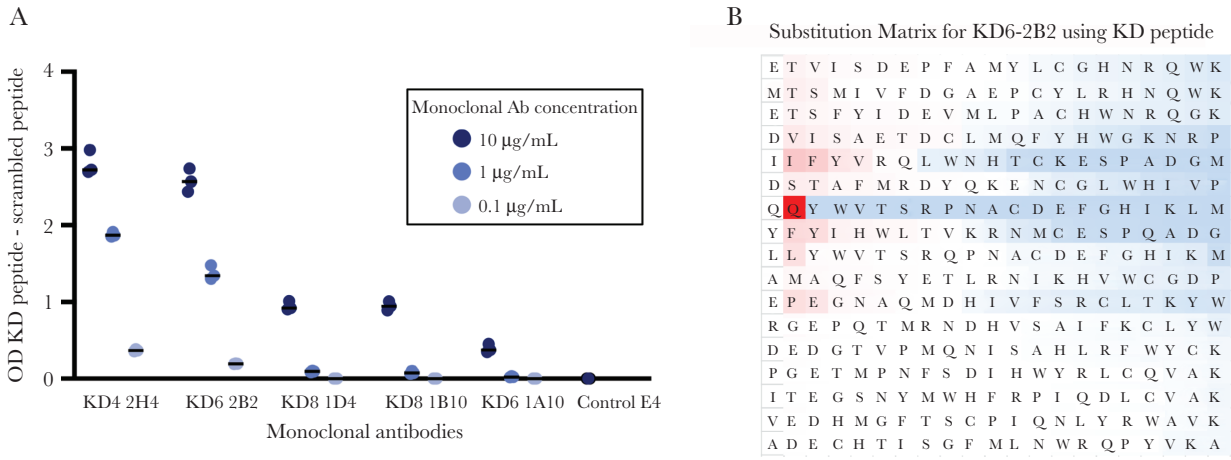

Figure 4. Monoclonal antibodies from additional KD patients recognize KD peptide. *A*, KD monoclonal antibodies bind to KD peptide by ELISA. The OD reading of a scrambled version of the peptide was subtracted as a negative control. Antibodies KD4-2H4 and KD6-2B2 bind most strongly to the peptide, followed by KD8-1D4, KD8-1B10, and KD6-1A10. Samples were assayed in triplicate at each dilution of 10, 1, and 0.1 µg/mL. Dots represent individual assay results and horizontal lines represent mean of 3 assays. *B*, Substitution matrix array of peptide AVIPDREALYQDIDEME (derived from KD peptide) using KD6-2B2 demonstrates that KD6-2B2 shares an epitope with KD4-2H4. Preferred amino acids for binding are those on the left of the substitution matrix while nonpreferred amino acids are those on the right. Abbreviations: KD, Kawasaki disease; OD, optical density.

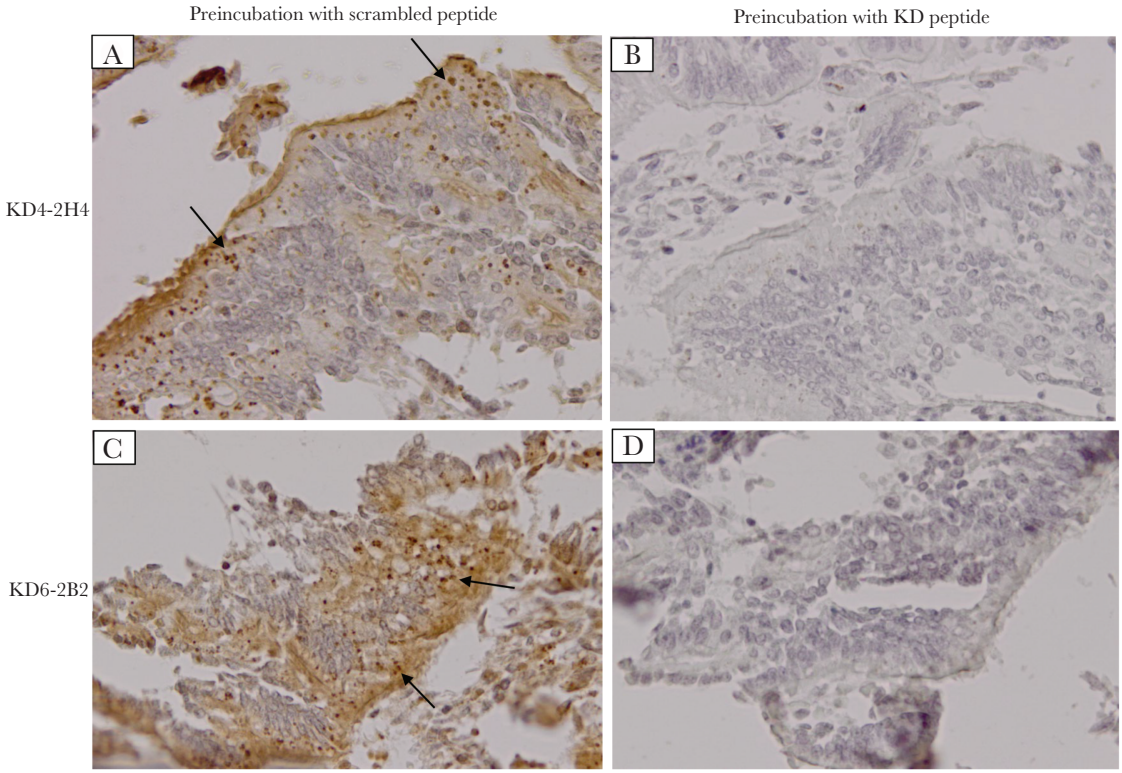

Figure 5. KD peptide blocks binding of KD monoclonal antibodies to KD ICI in ciliated bronchial epithelium of KD patient A. *A*, ICI are identified in KD patient A using antibody KD4-2H4 preincubated with scrambled peptide (arrows). *B*, ICI staining is blocked when antibody KD4-2H4 is preincubated with KD peptide. *C*, ICI are identified in KD patient A using antibody KD6-2B2 preincubated with scrambled peptide (arrows). *D*, ICI staining is blocked when antibody KD6-2B2 is preincubated with KD peptide. *A–D*, ×20. Abbreviations: ICI, intracytoplasmic inclusion bodies; KD, Kawasaki disease.

as the antigens. We screened KD and control patient sera at a dilution of 1:5000 in phosphate buffered saline, which reduced background from nonspecific binding. A minority of sera (both KD and control) exhibited nonspecific binding (reactivity with GST alone) and were excluded from the study.

Sera from 5/8 KD patients who presented on days 8–28 after fever, prior to receiving intravenous immunoglobulin (IVIG) therapy, had IgG antibody to the KD peptide epitope (Figure 6 and Supplementary Table 5). Sera from KD patient 12, obtained on day 11 after fever onset when a giant coronary artery aneurysm was diagnosed and before IVIG therapy, was seropositive in serial dilutions as high as 1:50 000.

Because clinical and epidemiologic features of KD strongly suggest a ubiquitous infectious agent, the most appropriate control group for serologic studies is infants at 5–9 months of age without KD, an age when passive maternal antibody would have abated and a low likelihood of prior infection from a ubiquitous agent would be expected. These results showed that sera from 0/17 infant controls had IgG antibody to the KD peptide epitope (Figure 6 and Supplementary Table 6) compared with 5/8 KD sera at ≥ day 8 of illness ($P < .01$).

We hypothesized that IVIG would contain antibody to the epitope, because adult donors whose blood is used to prepare this product would likely already have experienced infection with a ubiquitous agent, and the IVIG product and lot that we tested gave indeterminate results, because it appeared to react to GST alone as well as to the GST-KD peptide fusion protein.

Sera from 1/9 febrile control children ages 7 months to 10 years had IgG antibody to the epitope identified; the positive result was in a 5-year-old child (Supplementary Table 6). IgG antibody was identified in 1/11 KD patients who presented on day 4–5 of fever (Supplementary Table 5), at which time we hypothesized that an IgG response to the triggering antigen might not yet have developed. Sera from KD patient 30 was positive for IgG antibody at day 4 after fever onset. The parents of this child reported that the child was ill with neck stiffness, abdominal pain, photophobia, and arthralgias 10 days before admission, although they had only recognized fever for the preceding 4 days. Upon admission, the child was recognized to have all clinical features of KD. This raises the possibility that the duration of KD was longer than 1 week at admission.

Pretreatment sera at or before day 5 of illness, when IgG antibody might not yet have developed in response to the KD agent, was available from only 1 of the 11 patients

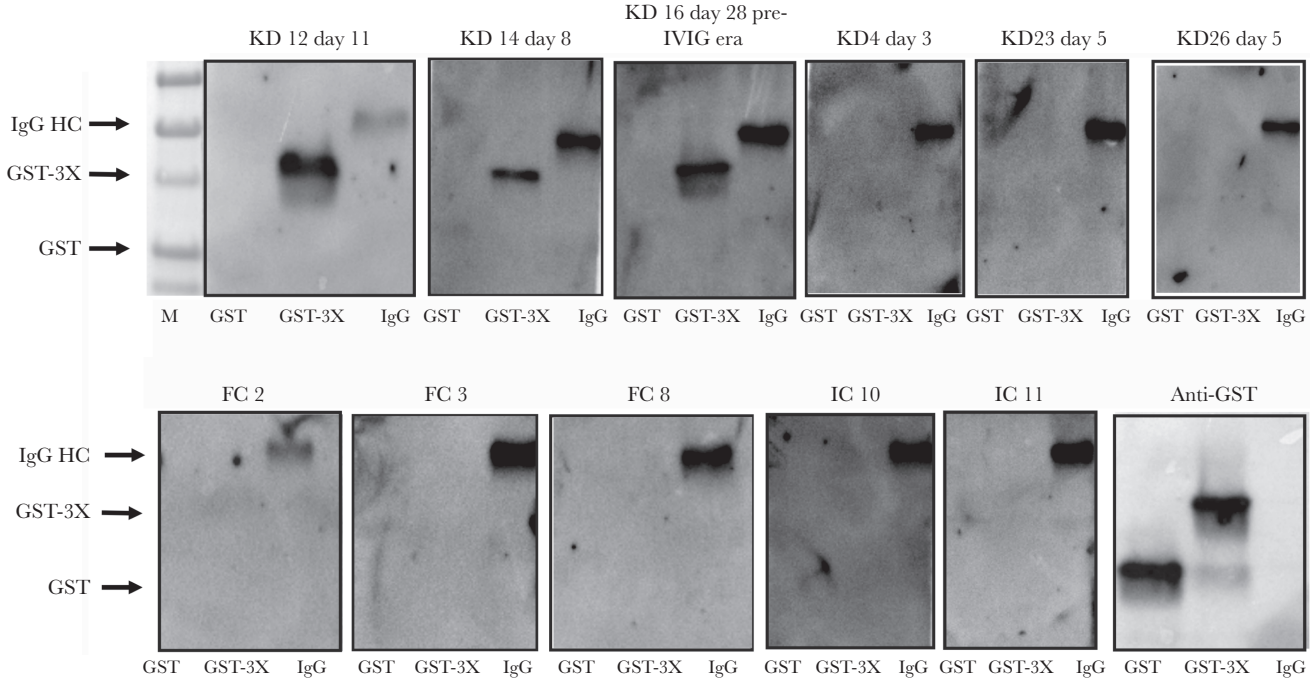

Figure 6. Western blot analysis of sera for reactivity to KD peptide fusion protein. For each blot, lane 1 contains GST alone, lane 2 contains GST-KD peptide fusion protein (GST-3X), and lane 3 contains human IgG as a positive control. Blots were stripped and polyclonal anti-GST antibody was applied to ensure that the GST fusion proteins were present; each serum sample was tested 2–5 times and a representative blot is shown. Molecular weight of IgG HC is 50 kD, GST-3X is approximately 35 kD, and GST alone is approximately 26 kD (arrows). Abbreviations: FC, febrile control; GST, glutathione sulfur transferase; IC, infant control; IgG HC, immunoglobulin G heavy chain; IVIG, intravenous immunoglobulin; KD, Kawasaki disease; M, All Blue Standard (Biorad).

whose plasmablasts were studied. Sera from day 3 of illness in KD patient 4, whose day 8 of illness IgG plasmablast-derived antibody KD4-2H4 recognized the protein epitope, was negative by western blot assay, suggesting development of antibody to this epitope between days 3 and 8 of illness (Supplementary Table 5).

When considered together, these serologic assay results support the likelihood that a KD triggering agent contains the KD peptide sequence.

Additional detailed results are available in the Supplementary Material.

DISCUSSION

Here, we identified a peptide that is recognized by antibodies that develop during acute KD, both those encoded by the plasmablast response to the disease and those identified in KD sera after the first week of illness but before administration of IVIG. This is the first discovery of a specific antigen recognized by the immune response to KD. Our report demonstrates that preparing antibodies from plasmablast responses to acute inflammatory diseases of unknown etiology can be useful in identifying the inciting antigens.

Many lines of evidence support a ubiquitous viral agent as the cause of KD in genetically susceptible children. These include the young age group affected [41], well-documented epidemics of illness [2, 42–44], the self-limited nature of the clinical illness [3], the lack of clinical response to antibiotic therapy [3, 45], the high prevalence of the condition in Japan, where 1 in 65 children develop KD by the age of 5 [41] (a prevalence rate similar to that of many ubiquitous viral infections), the upregulation of interferon response genes in KD lung and coronary arteries [36, 46], the identification of virus-like particles in close proximity to KD inclusion bodies in ciliated bronchial epithelium [36], and the prominent IgA immune response suggesting a mucosal portal of entry of the putative ubiquitous causative agent [27, 29, 33, 47].

Whether the protein epitope identified in this study derives from a previously unidentified virus remains to be determined. Although monoclonal antibody KD4-2H4 recognized related peptides of hepacivirus C NS4A, amino acid substitution array yielded optimized KD peptide, whose epitope is not present in any known hepaciviruses. Moreover, we have performed high-throughput RNA sequencing of many KD tissues, sera, and throat samples that has not yielded sequences with homology to flaviviruses ([46], data not shown). These results do not exclude a new hepacivirus as the source of the epitope but a nonhepacivirus source must also be considered. We are presently using genomic and proteomic approaches to determine the gene sequence from which the immunogenic epitope identified in this study arises. We hypothesize that the source is an RNA virus whose genome is present at very low quantity in KD

clinical samples, such as blood at the time of clinical presentation, and in the target tissues of fatal cases by the time of death occurring weeks to years after illness onset. We are working to identify the targets of KD monoclonal antibodies that bind intracytoplasmic inclusion bodies but do not bind to KD peptide, because these antibodies may bind other epitopes of the putative viral agent. We are also developing more sensitive assays for multiple antibody isotypes using fusion proteins that do not contain the GST tag that could facilitate KD diagnosis and could be evaluated in worldwide multicenter studies.

This study is limited by its investigation of the plasmablast response to KD in a single US center over a 16-month period from 2017 to 2018. However, KD monoclonal antibodies prepared in this study react with tissue samples from KD children from other geographic areas of the United States and Japan who died during different decades, and sera from KD children in Chicago from the 1980s, 1990s, and 2000s react with the identified protein epitope, suggesting that the results may be applicable to additional KD patients in other locations and over other time periods. Further multicenter collaborative studies are needed to investigate the relevance of these findings to KD patients around the world. Despite these limitations, we believe our results provide an exciting direction for etiologic studies of KD and the development of much-needed serologic tests. Identification of the etiology of KD is a pediatric research priority that will enable diagnostic test development, improved therapy, and ultimately prevention of this serious worldwide childhood illness.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Author contributions. A. R. and S. B. designed the study and carried out experiments. D. A., L. G., T. B., M. H., and Y. C. carried out experiments. K. W. performed bioinformatics analyses. N. I. assisted with experiments. K.K. performed statistical analyses. S. S. designed the study. All authors prepared the manuscript.

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Potential conflicts of interest. A. H. R., S. C. B., and S. T. S. are coinventors on a provisional patent application related to this work (serial number 62/811 930). All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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