

RESEARCH ARTICLE

Identification and validation of genes encoding humoral specificity for the chemical allergen toluene diisocyanate

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Data availability statement: All relevant data are within the paper and its [Supporting information](#) files. The data underlying the results presented in the study are available from (NCBI: <https://www.ncbi.nlm.nih.gov/genbank/>).

Abstract

A panel of hybridomas specific for different isomers of toluene diisocyanate (TDI), a cross-linking chemical used in polyurethane production, has been previously described. These hybridomas were originally developed by researchers at the USA's National Institute for Occupational Safety and Health (NIOSH). We sought to determine the DNA sequence encoding these TDI-specific monoclonal antibodies, enabling identification of germline gene rearrangement resulting in chemical specificity as well as production of the mAbs recombinantly. B cell receptor sequencing (BCR-seq) of hybridoma RNA readily identified productive heavy and light chain antibody sequences. The productive light chains of all 7 hybridomas showed strong identity with different genomic variable (V) and joining (J) region sequences with few changes from germline configuration. However, the productive heavy chains contained more substantial changes in their genomic V and J-region sequences consistent with antigen-driven affinity maturation, as well as N- and P- nucleotide additions comprising their complementarity-determining region 3 (CDR3). The hybridoma-defined TDI-specific mAbs were subsequently produced recombinantly in a human embryonic kidney cell line expression system, purified, and tested for their binding capacity against albumin derivatized with TDI, related diisocyanates, and control antigen. The recombinant versions of the TDI-specific mAbs demonstrated binding capacity for different isomers (2,4 and 2,6) of TDI consistent with that previously reported for the hybridoma secreted clones; one specific for 2,4-TDI, one specific for 2,6-TDI, three that bind both 2,4- and 2,6-TDI, and two that show cross-reactivity with 4,4'-methylene diphenyl diisocyanate (MDI). None of the recombinant mAbs bound to aliphatic hexamethylene diisocyanate (HDI), its oligomer, or control antigen. Additional recombinant versions of the TDI mAbs, with identical V-regions, but different C-regions, demonstrated the dependence of antigen specificity on the V-region, but also highlighted the potential for C-region sequence to affect their detection in ELISA assays. The DNA sequences defined herein may be useful to other investigators

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wishing to generate recombinant TDI-specific mAbs as detection reagents for research or as standards for clinical serology tests.

Introduction

Diisocyanate chemicals are crucial ingredients for making polyurethane [1]. TDI is one of the most abundantly produced and consumed diisocyanates world-wide, and is preferred for generating more flexible foams [2]. Like other diisocyanates, TDI is immunogenic, and occupational exposure can result in health effects of varying severity, including rare fatalities [3–5]. The ϵ -amine groups of specific lysines in albumin have been identified as primary reaction targets for TDI's N=C=O groups as shown in supporting information S1 Fig [6–8]. However, the mechanisms by which the small reactive chemical, TDI, causes asthma remain unclear and appear to differ from common large-molecular weight antigens, impeding recognition of affected individuals and development of targeted treatment [4,9–12].

Monoclonal antibodies that specifically recognize TDI are useful reagents for research studies of TDI exposure and host responses [13,14]. Their specificity allows them to serve as chemical detectors in exposed tissue and fluid, where the chemical reacts with self-molecules, potentially altering their function and immunogenicity [14,15]. To date, a limited number of TDI-specific mAbs have been produced through investigations at the National Institute of Occupational Safety and Health (NIOSH) [14,16]. Seven different monoclonal antibodies were described with varying specificity for different isomers (2,4 vs. 2,6) of TDI and limited or no cross-reactivity with a panel of closely related chemicals [13]. In research studies to date, these TDI-specific mAbs have proven useful in tracking TDI binding to airway cells, entry through the skin, uptake by dendritic cells and transport to draining lymph nodes [13,15].

The present investigation sought to determine the DNA sequences of TDI-specific mAbs for two purposes. First, to better understand the molecular determinants of humoral specificity to TDI, as exposed human workers also develop chemical-specific antibodies. Second, to preserve the ability to generate additional TDI-specific mAbs recombinantly should the hybridoma lines become unavailable from NIOSH [17]. Together, the data identify murine germline genes that encode hybridoma-derived TDI-specific mAbs, and further demonstrate TDI-binding capacity of recombinant mAbs based on the assembled DNA sequences.

Materials and methods

Culture and RNA purification from TDI hybridomas

TDI hybridomas were kindly provided by Drs. Brett Green, Tanishe Ruwona, and Paul Siegel from NIOSH. Upon arrival cells were cultured to assure viability, culture supernatant was assessed by ELISA to ensure secretion of TDI-specific mAbs and their isotype. RNA was isolated from 1×10^7 cells using RNA easy kit from Qiagen (Germantown, MD) according to the manufacturer's instructions.

BCR-seq library prep and amplification/sequencing of antibody genes

A BCR library was created from 1 µg of each hybridomas' RNA and profiled using SMART-Seq BCR from TAKARA Bio (San Jose, CA) kits according to the manufacturer's instructions. To determine the complete variable regions the libraries were sequenced on an Element Aviti in 2x300bp PE configuration. Data were processed using MiXCR software (v 4.7.0) to identify the top clone with the highest read count. The DNA sequences for the mAbs have been archived in GeneBank under the following accession numbers: N1 Heavy chain (PX423944), N1 Light chain (PX423945), N3 Heavy chain (PX423946), N3 Light chain (PX423947), N4 Heavy chain (PX423948), N4 Light chain (PX423949), N5 Heavy chain (PX423950), N5 Light chain (PX423951), N6 Heavy chain (PX423952), N6 Light chain (PX423953), N7 Heavy chain (PX423954), N7 Light chain (PX423955), N8 Heavy chain (PX423956), N8 Light chain (PX423957).

mAb gene analysis

The antibody heavy- and light-chain sequences from each hybridoma were queried against IMGT®, the international ImMunoGeneTics information system, to identify homology with murine germline genes and to characterize any non-germline N- or P-nucleotide additions or deletions

Recombinant mAb production

Using the V-region sequences identified for each TDI mAb, recombinant mAbs were produced by a commercial source (Sino Biological, Paoli, PA). The V-region sequences identified as described above were fused to consensus murine IgG1, IgG2A, or IgG3 constant regions provided in the supplemental methods (see supporting information [S2 Table](#)). The antibodies were expressed in HEK293 cells and purified over a protein G affinity column. For each mAb the newly identified V-regions were fused to a defined murine constant region of the original hybridoma. For three hybridoma mAbs that used IgG3 or IgG2A isotypes, recombinant mAbs were generated with the “original hybridoma isotype” as well as a standard murine IgG1 constant region. Each mAb was purified >96% and demonstrated expected banding patterns by reducing and non-reducing SDS-PAGE (see supporting information [S3](#) and [S4 Figs](#)).

ELISA assays for binding capacity of recombinant mAbs

Microtiter plates were coated overnight at 4°C with control human albumin or human albumin conjugated with 2,4-TDI, 2,6-TDI, 4, 4'-MDI or 1,6-HDI (Sigma-Aldrich, St Louis, MO) as previously described [13,18–20]. Diisocyanate:albumin ratios of 10:1, 10:1, 8:1, and 7:1 were calculated for 2,4-TDI, 2,6-TDI, 4, 4'-MDI and 1,6-HDI-albumin conjugates respectively, based on substitution analysis with 2,4,6-trinitrobenzene sulphonic acid (Sigma-Aldrich) as previously described [19,21]. Plates were “blocked” with 3% dry milk, followed by one hour room temp incubation with test sample (culture supernatant or recombinant mAb) and washing. mAb binding was detected with HRP-conjugated anti-mouse Ig (H+L chains) from BD Biosciences (San Jose, CA). Optical density readouts at 450 and 630 (reference) nm wavelengths were obtained using an iMark ELISA plate reader (BioRad, Hercules, CA). Data analysis and curve fits for titration studies were analyzed with Graph Pad Prism software version 10.6.0.

Results

DNA sequence of hybridoma antibodies and comparison with murine germline gene sequences

The DNA sequence of productively rearranged antibody genes were determined based on BCR-seq analysis of sub-clones of seven different hybridomas previously characterized by Ruwona et al [13]. [Tables 1](#) and [2](#) provides the amino acid sequence of the CDR3 regions, as well as the identity to germline V, D, and J-region genes each of the hybridomas. All 7 light chains are expressed in near germline configuration with 95.9–99.7% identity in the V-region and up to 100% identity in J-regions, including the one mAb (N6) that uses a lambda light chain. In contrast, the productively rearranged

Table 1. Light chain sequences: CDR3 amino acids, V-, and J-Region Genes.

Hybridoma	CRD3	VL	IDENTITY	J-REGION	IDENTITY
N1 (2E5)	CHQRSSFTF	IGKV4–70*01	99.3%	IGKJ2*01	97.1%
N3 (16C6)	CFQGSHVPPTF	IGKV1–117*01	95.9%	IGKJ4*01	100.0%
N4 (32B6)	CKQSYNLYTF	IGKV8–21*01	99.3%	IGKJ2*01	94.6%
N6 (60G2)	CALWYSNLWVF	IGLV1*01	97.6%	IGLJ1*01	100.0%
N5 (59E9)	CQHSRELPTF	IGKV3–12*01	99.7%	IGKJ4*01	100.0%
N7 (62G5)	CAQNLELPPTF	IGKV2–109*01	97.3%	IGKJ1*01	100.0%
N8 (79G7)	CAQNLELPPTF	IGKV2–109*01	97.6%	IGKJ1*01	100.0%

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Table 2. Light chain sequences: CDR3 amino acids, V-, D-, and J-Region Genes.

Hybridoma	CRD3	VH	IDENTITY	J-REGION	IDENTITY	D-REGION	IDENTITY
N1 (2E5)	CAREGNYIDFDYW	IGHV1-71-10*01	95.8%	IGHJ2*01	93.6%	IGHD2–3*01	100
N3 (16C6)	CGGLPPFYW	IGHV1–37*02	94.4%	IGHJ3*01	91.5%	IGHD6–2*01	100
N4 (32B6)	CARARPYSTYPLAMDYW	IGHV1–18*04	97.6%	IGHJ4*01	88.7%	IGHD2–5*01	93.8
N5 (59E9)	CTRSSPLLRLRGSFVDW	IGHV1-47-14*01	97.2%	IGHJ1*01	82.7%	IGHD1–2*01	100
N6 (60G2)	CARGGLGRQGFAYW	IGHV1–42*04	93.4%	IGHJ3*01	91.5%	IGHD2–4*01	88.9
N7 (62G5)	CGRSGRRGATVVLDYW	IGHV1-69-2*01	93.4%	IGHJ2*01	85.1%	IGHD3–3*01	100
N8 (79G7)	CARSGRRGQTVVLDYW	IGHV1-69-2*01	94.8%	IGHJ2*01	87.2%	IGHD1–1*01	100

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heavy chains demonstrate evidence of affinity driven somatic mutation, with a higher proportion of non-silent (vs. silent) mutations in CDR1 and CDR2 vs framework regions as well as non-germline encoded amino acids comprising the CDR3.

Validation of DNA-encoded specificity for TDI

To confirm the productively rearranged DNA sequences present in the hybridomas encode antibody that specifically recognizes TDI, recombinant mAbs were generated based on the defined V-region DNA sequences. Initially, each recombinant mAb was generated with the V-region sequence identified from the hybridoma fused to the murine constant region used by the hybridoma derived mAb; four IgG1s (N1, N3, N4 N6), two IgG2As (N7 and N8), and one IgG3 (N5). All recombinant mAbs were tested by ELISA for binding capacity to control antigen human albumin, or albumin conjugated with TDI (an 80/20 mixture of 2,4 and 2,6 isomers), as well as related chemical-albumin conjugates prepared with MDI, or HDI. As shown in Fig 1, all seven recombinant mAbs bound strongly to TDI-conjugated albumin, but not to control albumin or albumin conjugated with HDI. Five of the mAbs were specific for TDI-conjugated albumin, however two mAbs (N4 and N6) demonstrated cross-reactivity with MDI, as previously described for their respective hybridoma derived mAbs.

Titration of recombinant mAbs and recombinant isotype switching

To further characterize the recombinant mAbs specificity for different TDI isomers and cross-reactivity (if observed) with MDI, we performed titration studies. As shown in Fig 2, one mAb (N1) specifically bound 2,4-TDI, one mAb (N5) specifically bound 2,6-TDI, and the remaining five mAbs bound similarly to 2,4- and 2,6-TDI (including the two mAbs with cross-reactivity to MDI). As part of our investigation, we also generated isotype switched recombinant mAbs, to further confirm that TDI specificity depends upon the V-region sequence, and to generate recombinant mAbs with differing

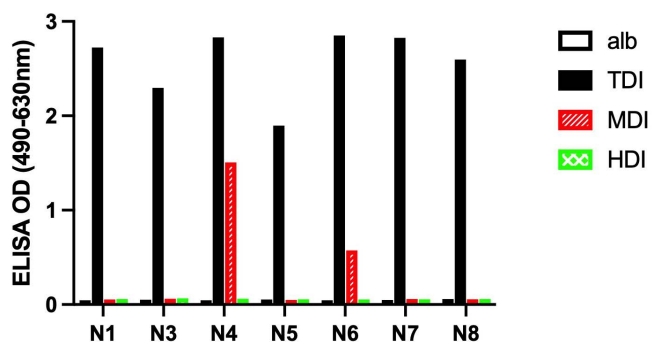


Fig 1. ELISA testing of recombinant mAbs against control protein (albumin) and albumin conjugated with different diisocyanates. Microtiter plates coated with albumin (white), or albumin conjugated with TDI (black), MDI (stripped/red) or HDI (spotted/green) were incubated with 1µg/mL of each recombinant mAb followed by HRP-conjugated anti-mouse Ig (H+L). The resulting optical density values (OD) 450-630λ are plotted on the Y-axis.

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isotypes that might be used in the future to develop “sandwich ELISAs” for TDI detection. The data demonstrate the dependence of TDI binding on the V-region sequence, with similar binding observed when the V-region of IgG2A or IgG3 mAbs were expressed with IgG1 constant regions. It remains unclear if the slight differences in ELISA OD values for mAbs with the same V-region, but different constant regions reflect differences in binding capacity, or recognition of the mAbs by the secondary reagent, which may differentially recognize different isotypes. Non-the-less the data confirm the specificity relies upon the variable and not the antibody constant regions.

Discussion

In this study, we aimed to determine the DNA sequences for a panel of TDI-specific monoclonal antibodies. We further validated that these sequences encode TDI-specificity by producing recombinant mAbs based on the newly identified sequences and testing their binding capacity. Our findings highlight several important insights into the molecular basis of TDI recognition and provide sequence knowledge to help preserve these valuable antibodies, in the event the original hybridomas become unavailable, due to funding cuts and/or loss of personnel at NIOSH [17,22].

The productive heavy and light chain sequences obtained from the TDI-specific hybridomas reveal distinct patterns of mutation and gene usage. The productive light chains showed limited deviation from germline configuration. However, the heavy chains exhibited significant somatic hypermutation, particularly in the complementarity determining regions (CDRs), with a notable concentration of non-silent mutations in the CDRs compared to the framework regions. This pattern is consistent with an antigen-driven immune response, where somatic hypermutation in the heavy chain contributes largely to the specificity and affinity for TDI [23].

By synthesizing recombinant mAbs based on the V-region sequences of the original hybridomas and assessing their binding specificity through ELISA, we confirmed that these recombinant mAbs retained their selective binding profiles for TDI. Notably, the recombinant mAbs mirrored the hybridoma-derived mAb specificity, with selective binding to 2,4- and 2,6-isomers of TDI and limited cross-reactivity with MDI [13]. The results emphasize that the antigen specificity of these mAbs is predominantly determined by the V-region of the antibody, regardless of the constant region, as isotype-switched versions of the mAbs retained their specific binding to TDI. It remains unclear if slight variations in ELISA optical density values between mAbs with the same V-region, but different isotypes reflect intrinsic differences in antigen binding, or differences in secondary antibody recognition, a distinction important for designing and interpreting immunoassays. Future studies may better define the role of the C-region in modulating immune responses or detection sensitivity in different assay setups, optimizing the use of these antibodies in various contexts.

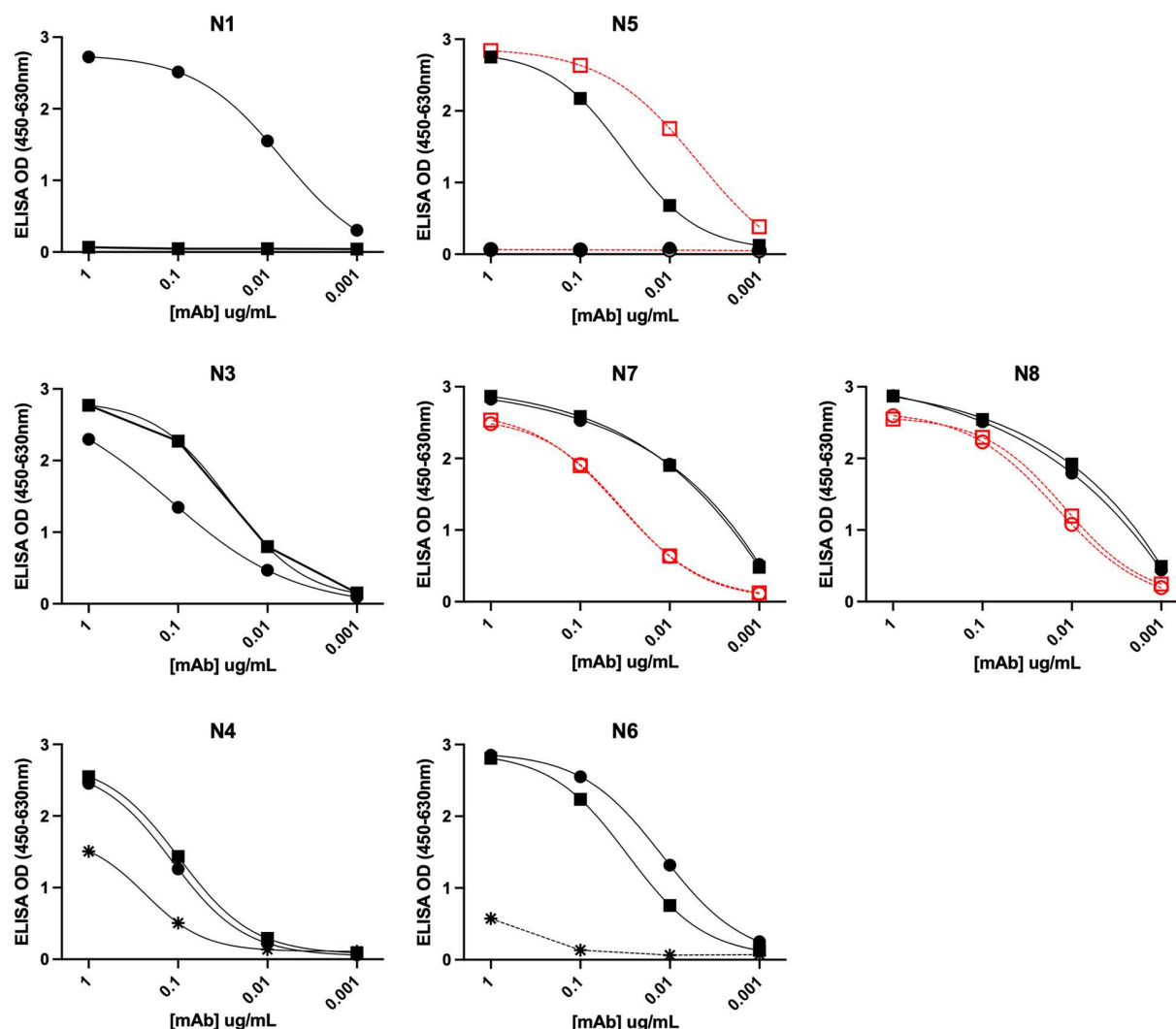


Fig 2. Titration of recombinant mAbs against different TDI isomers and MDI, including isotype-switched variants. Each of the recombinant monoclonal antibodies was further analyzed for their ability to bind to 2,4-TDI (circle) or 2,6-TDI (square) at different concentrations ($\mu\text{g/mL}$ on X-axis, ELISA OD on Y-axis). Data with solid symbols and lines are shown for recombinant mAbs generated with the constant region of the original hybridoma; N1 Heavy chain (PX423944)/Light chain (PX423945), N3 Heavy chain (PX423946)/Light chain (PX423947), N4 Heavy chain (PX423948)/Light chain (PX423949), N5 Heavy chain (PX423950)/Light chain (PX423951), N6 Heavy chain PX423952/Light chain (PX423953), N7 Heavy chain (PX423954)/Light chain (PX423955), and N8 Heavy chain (PX423956)/Light chain (PX423957). Additional data with red open symbols and dashed lines depict recombinant mAbs in which the original constant regions were switched from IgG3 (N5) or IgG2A (N7 and N8) to IgG1. For recombinant mAbs N4 and N6 binding data is also shown against MDI (asterisk).

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The DNA sequences of these TDI-specific mAbs have been archived in GeneBank and are now accessible for researchers aiming to generate recombinant versions for various applications. The availability of these sequences ensures that new batches of recombinant mAbs can continue to be produced either for basic research into the immunological mechanisms of TDI exposure or for developing detection tools and clinical serology tests. The preservation of these clones sequences is crucial to the continuity of research and ensures the reproducibility of experiments across different labs.

In conclusion, this investigation has successfully determined the DNA sequences of TDI-specific mAbs, enabling their recombinant production and confirming their specificity. The data will permit the continued use and exploration of these mAbs in research, ensuring the scientific community has the tools necessary to study and address TDI-related occupational health risks.

Supporting information

S1 Fig. Chemical structure and reaction of TDI with epsilon amine of lysine. The structures of 2,4 and 2,6-TDI are shown along with the reaction product formed with the side chain of lysine residues.

(TIF)

S2 Table. List of constant regions fused to sequenced V-regions.

(DOCX)

S3 Fig. SDS-PAGE analysis of purified recombinant IgG 1 anti-TDI mAbs. Data are shown for mAbs N1, N3, N4 and N5 expressed with IgG1 constants regions. Note mAb N2, an IgM hybridoma anti-TDI mAb, expressed with a gamma 1 constant region, demonstrated limited stability and was not tested in this study.

(TIF)

S4 Fig. SDS-PAGE analysis of purified recombinant IgG1, IgG2A and IgG3 anti-TDI mAbs. Data are shown for mAb N5 with an IgG3 constant region, N6 (IgG1), and N7 and N8 with IgG1 and IgG2A constant regions as indicated.

(TIF)

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Author contributions

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Funding acquisition: Adam V Wisnewski.

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Supervision: Adam V Wisnewski.

Validation: Adam V Wisnewski.

Writing – original draft: Adam V Wisnewski.

Writing – review & editing: Adam V Wisnewski, Jian Liu.

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