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Lung Gene Expression Suggests Roles for Interferon-Stimulated Genes and Adenosine Deaminase Acting against RNA-1 in Pathologic Responses to Diisocyanate

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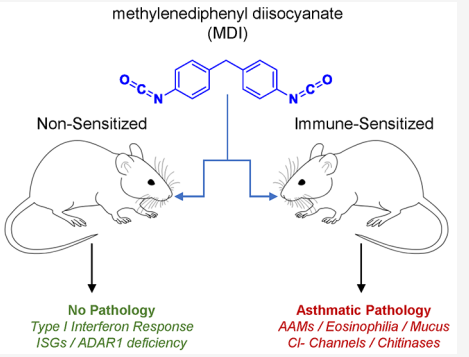
ABSTRACT: Mechanisms underlying methylene diphenyl diisocyanate (MDI) and other low molecular weight chemical-induced asthma are unclear and appear distinct from those of high molecular weight (HMW) allergen-induced asthma. We sought to elucidate molecular pathways that differentiate asthma-like pathogenic vs non-pathogenic responses to respiratory tract MDI exposure in a murine model. Lung gene expression differences in MDI exposed immune-sensitized and nonsensitized mice vs unexposed controls were measured by microarrays, and associated molecular pathways were identified through bioinformatic analyses and further compared with published studies of a prototypic HMW asthmagen (ovalbumin). Respiratory tract MDI exposure significantly altered lung gene expression in both nonsensitized and immune-sensitized mice, vs controls. Fifty-three gene transcripts were altered in all MDI exposed lung tissue vs controls, with levels up to 10-fold higher in immune-sensitized vs nonsensitized mice. Gene transcripts selectively increased in MDI exposed immune-sensitized animals were dominated by chitinases and chemokines and showed substantial overlap with those increased in ovalbumin-induced asthma. In contrast, MDI exposure of nonsensitized mice increased type I interferon stimulated genes (ISGs) in a pattern reflecting deficiency in adenosine deaminase acting against RNA (ADAR-1), an important regulator of innate, as well as "sterile" or autoimmunity triggered by tissue damage. Thus, MDI-induced changes in lung gene expression were identified that differentiate nonpathogenic innate responses in nonsensitized hosts from pathologic adaptive responses in immune-sensitized hosts. The data suggest that MDI alters unique biological pathways involving ISGs and ADAR-1, potentially explaining its unique immunogenicity/allergenicity.



INTRODUCTION

Diisocyanates used industrially throughout the world are well recognized causes of occupational asthma.[1] Methylene diphenyl diisocyanate (MDI) is the most abundantly produced and utilized diisocyanate, with continually evolving applications.[2−6] The future use of MDI, and potential for occupational exposure, is expected to increase with increasing economic demands for diisocyanate-based products.[7]

The pathogenic mechanisms that underlie MDI and other low-molecular-weight (LMW) allergen-induced asthma remain unclear. Despite clinical and pathological commonality with HMW allergen-induced asthma, fundamental immunologic differences have been well described, perhaps the most important being the lack of detectable antigen (i.e., chemical)-specific adaptive immune responses (IgE, TH2 cells) in diisocyanate asthma; an issue further clouded by uncertainty regarding the nature of the underlying diisocyanate "allergen/antigen", with evidence against conventional hapten-like activity of the chemical and continuing uncertainty of the full spectrum of reaction targets in the lung.[8−20] These unusual characteristics of diisocyanate asthma have prompted the hypothesis that its pathogenic mechanisms differ vs those triggered by common environmental allergens, which are typically HMW compounds, e.g., proteins and pollen.[21−28] Our lack of understanding of diisocyanate asthma pathogenesis substantially impedes disease prevention/screening, diagnosis, and treatment of affected workers.

Diisocyanate activation of the innate arm of the immune system has been described by several investigators and may be a differential pathway by which certain LMW allergens, especially reactive chemicals, cause asthma.[29] Pro-inflammatory myeloid and innate lymphoid cell ($\gamma\delta$ T-cells, ILC2) responses have been described in vitro with human cells and in vivo in limited murine studies.[24,26,29−31] While diisocyanates can stimulate innate immune responses, mechanisms connecting these responses to the clinical syndrome experienced by affected workers, with long-lived memory and increasing

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hypersensitivity upon repeated exposures (hallmarks of Type I Hypersensitivity) remain challenging to define.

The present investigation sought to gain new information on MDI asthma pathogenesis through the analysis of lung gene expression in mice. Studies were done in animals with and without prior immune sensitization, which exhibit differential pathology, to distinguish molecular changes associated with disease. Bioinformatic analyses were employed to predictably identify biological pathways altered by MDI exposure based on changes in gene transcription, and potential differences vs prototypic HMW allergen-induced asthma (ovalbumin). The results are discussed along with new questions and hypotheses raised by the findings, including the potential role of type I interferon stimulated genes (ISGs), and adenosine deaminase acting against RNA (ADAR-1) in response to MDI exposure.

EXPERIMENTAL PROCEDURES

Caution. Methylene diphenyl diisocyanate (MDI) is hazardous and a well-recognized immune-sensitizing chemical. Nitrile gloves, protective clothing, and goggles should be used for personal protection.

Preparation of MDI-GSH Conjugates. Established methods for generating and characterizing MDI-GSH reaction products were followed.[32−34] Reduced glutathione, GSH (CAS No. 70−18−8), and 4,4′-methylenebis(phenyl isocyanate) (CAS No. 101−68−8) were from Sigma-Aldrich (St. Louis, MO). Two hundred 50 microliters of 10% (w/v) MDI in acetone from JT Baker (Phillipsburg, NJ) was added dropwise with stirring to 25 mL of 10 mM GSH in 200 mM sodium phosphate, pH 7.4. The reaction mixture was rotated end-overend for 2 h at 37 °C, centrifuged at 10,000*g*, 0.2 μm filtered, cooled to 4 °C and used within 2 h of preparation. Control solutions were prepared by mixing MDI and GSH solutions after individual separate reactions, during which time MDI hydrolyzes and forms low molecular weight ureas.[35,36] An aliquot of each sample was analyzed by reverse phase UV-LC-MS/MS in positive ion mode to verify bis(GSH)-MDI was the dominate reaction product based on the A_{210} and A_{245} spectra, and retention times of the 865.25 and 433.13 m/z ions reflecting singly and doubly charged species, as previously described.[32,34]

Skin Sensitization and Airway Exposures of Mice. Balb/C mice 8 weeks of age were housed under pathogen-free conditions and fed ad libitum with automated water supply and 12 h day/night light cycles. Immunologic sensitization to MDI, which is required for the development of asthma pathology following respiratory tract exposure, was accomplished via repeated skin exposure, as previously described.[32,37] Briefly, a region on the back was shaved 24 h prior to application of 50 μL of 1% (w/v) MDI in acetone on day 0 and again on day 5. For respiratory tract exposure, 50 μL of GSH-MDI reaction products or control solution (GSH mixed with MDI after separate reactions) was delivered intranasally under isoflurane sedation as previously described.[32,34,37,38] Mice received respiratory tract exposure on days 15, 16, 19, and 20. The dose and timing of MDI exposure were chosen to reflect occupational exposure and are consistent with recent field studies.[39] The 1% MDI (w/v) skin exposure dose used for inducing systemic immune sensitization is roughly equivalent to the final MDI concentration in polyol/diisocyanate mixtures used to make polyurethane foam.[1] Each intranasal exposure delivered the potential equivalent of 10 μg of MDI, approximately 50-fold below the permissible exposure limit established by the U.S. Occupational Health and Safety Administration as previously calculated.[32,40] All animal studies followed ethical guidelines established in the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press[41] and were approved by Yale University's Institutional Animal Care and Use Committee.

Bronchoalveolar Lavage (BAL) and Lung Tissue Collection. BAL (3 × 0.8 mL) and lung tissue samples were obtained from $N = 6$ mice/group (control, MDI exposed nonsensitized and MDI sensitized and exposed mice) 48 h following the last respiratory tract exposure, as previously described.[32,37,38] BAL cells were pelleted by centrifugation at 800*g*, treated with RBC lysis buffer, washed, and resuspended in PBS for cytospin and cell counting. Total BAL cell numbers were calculated using a hemacytometer, and differential counts were performed on 200 cytospun cells that had been stained with diff quick (Polysciences Inc.; Warrington, PA). Lung tissue was obtained following PBS perfusion and immediately homogenized in RLT buffer from Qiagen (Germantown, MD). RNA was purified using an RNeasy kit from Qiagen, which included on-column digestion with RNase-free DNase.

Microarray Processing. Processing of RNA and microarrays were performed by the Yale Center for Genome Analysis.[42,43] Lung tissue RNA quantity and quality was assessed respectively by microvolume spectrophotometry on the Nanodrop 2000 (Thermo Scientific) and by on-chip capillary electrophoresis on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Absorbance ratio at 260 and 280 nm was ≥1.9 and the RNA integrity number was >9 for all samples. GeneChip WT Plus Reagent Kit (Affymetrix, Santa Clara, CA) was used for the preparation of samples and generation of ss-cDNA for hybridization, using 250 ng of total RNA as template. All the reactions and hybridizations were carried out according to the manufacturer's protocol.[44] Affymetrix GeneChip Mouse Clariom-S arrays were washed using the GeneChip Fluidics Station 450 and scanned with the GeneChip Scanner 3000. Experiments were repeated with $N = 6$ age matched animals per group, with all exposures and tissue collection performed along the same timeline.

Microarray Analysis. Each data sets' quality was confirmed based on hybridization, labeling controls, internal control genes (housekeeping controls), and global array metrics. Raw probe measurements (.CEL files) were background-adjusted, normalized, and converted into summarized expression values using the signal space transformation robust multichip analysis (SST-RMA) algorithm in the Transcriptome Analysis Console (TAC) software (version 4.0.2, ThermoFisher Scientific, Waltham, MA, USA), which incorporates the limma differential expression portion of the Bioconductor package as well as modified versions of the ComBat function from its Surrogate Variable Analysis. Analysis of variance (ANOVA) was used to fit a linear model to each probeset independently of the others. An eBayes analysis was applied to correct variance of the ANOVA analysis using information from all the probesets to yield an improved estimate for the variance. Differentially expressed genes were calculated based on default settings; >2-fold difference in average fluorescence with ANOVA p value <0.05. False discovery rate p values were further calculated using the Benjamini and Hochberg method.[45] Details on data processing modules, algorithms, and statistics underlying TAC software differential gene expression analysis are available on line.[46]

Ingenuity Pathway Analysis (IPA). IPA software (Ingenuity Systems; Qiagen China Co., Ltd.) was also used for bioinformatics analysis, specifically canonical pathway analysis and identification of predicted upstream regulators. IPA uses a network generation algorithm to segment the network map between molecules into multiple networks and assign scores for each network.[47,48] The score is generated based on hypergeometric distribution, where the negative logarithm of the significance level is obtained by Fisher's exact test at the right tail. For canonical pathway analysis, the −log (P value) > 2 was taken as threshold, the Z score >2 was defined as the threshold of significant activation, while a Z score < − 2 was defined as the threshold of significant inhibition. For upstream regulators, the P value of overlap <0.05 was set as the threshold. The algorithm used for calculating the Z scores and P values of overlap has been described previously.[49]

Data Comparison to Prototypic HMW (Ovalbumin) Asthma. We compared the present gene expression data on LMW diisocyanate chemically induced pathology with previously published data on the prototypic asthma model triggered by the HMW allergen, ovalbumin. We queried the NCBI GEO database using the keywords "ovalbumin", "asthma", "lung", "tissue", and "Balb", which identified

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47 entries. Six of these studies provided data of lung mRNA 48 h post-exposure. Of these, the study entitled "Effects of the OVA challenge in mouse model of asthma" (GSO Series GSE41665) best matched the present study design (48 h post-exposure time point, Balb/C mouse strain, paired control exposures, mRNA, similar timing of sensitization) and was used to evaluate potential differences in lung gene expression associated with asthma pathology induced by LMW vs HMW asthmagens. For analysis, all $N = 8$ each (ovalbumin exposed, control). CEL files from the 48 h time point of GSO Series GSE41665 were imported and processed using TAC to calculate the fold change of individual genes in ovalbumin exposed mice (which developed asthmatic pathology) vs controls. Two of the total ($N = 16$). CEL files from GSO Series GSE41665 that did not pass all quality control assessments by TAC software (3′ labeling controls outside threshold value) were excluded from analysis, leaving $N = 7$ samples each for ovalbumin exposed and controls. Venn diagrams, hierarchical cluster analysis, and bar graphs were used to identify and visualize individual genes with the greatest relative change in expression induced by pathologic diisocyanate vs prototypic HMW allergen exposure.

Real-Time PCR Studies. DNase treated total lung RNA from $N = 8$ animals/group was used to prepare cDNA with an iScript cDNA synthesis kit. The cDNA samples were amplified with iTaq universal SYBR green PCR supermix on a BioRad C1000 thermocycler interfaced with a CFX96 real-time PCR detection system with CFX Maestro Software, according to the manufacturer's recommendations (Bio-Rad; Hercules, CA). Changes in gene expression were calculated based on the ΔΔCt approach, with beta-actin (ACTB) as a reference gene.[50] PCR primer pairs were Bio-Rad's PrimePCR SYBR Green Assay for ZBTB16 (qMmuCID0022995), IRF7 (qMmuCED0040274), IIGP1 (qMmuCED0038266), IFI44 (qMmuCID0011454), IFIT3 (qMmuCID0041372), CLCA1 aka murine CLCA3 (qMmuCID0009928), and ACTB (qMmuCED0027505).

Statistical Analyses. For each group of mice, the mean and standard deviation of the data were initially entered, calculated in an Excel spreadsheet, and then exported to GraphPad Prism for graphing and statistical analyses. Significant differences of data that were not normally distributed were determined using the nonparametric Kruskal–Wallis test with Dunns's correction for multiple comparisons. Data that were normally distributed were evaluated by Welch ANOVA with Dunnet T3 correction for multiple comparisons. Detailed descriptions of statistical calculations for TAC and IPA programs are available online as described above.[45,51,52]

RESULTS

Lung Tissue Gene Expression in a Mouse Model of MDI Asthma. We performed paired analysis of cellular airway inflammation and lung tissue gene expression in samples from nonsensitized and MDI-immune sensitized mice exposed to MDI via the respiratory tract and control mice. Following exposure, only MDI-sensitized mice contained significantly ($P < 0.05$) increased percentages of eosinophils (Figure 1) in BAL samples. However, lung tissue from both nonsensitized and MDI-immune sensitized mice contained large numbers of differentially (>2-fold) expressed gene transcripts compared to controls. In nonsensitized mice, 534 gene transcripts were differentially expressed (254 increased and 280 decreased), while in MDI-immune sensitized mice, 218 gene transcripts were differentially expressed (181 increased and 37 decreased), as depicted in Venn diagram analysis (Figure 2) and listed in the Supporting Information (Tables S1 and S2).

Genes Differentially Expressed in All (Nonsensitized and MDI-Immune Sensitized) MDI Exposed Mice vs Controls. A core set of 53 genes was differentially expressed in all MDI exposed vs control mice, with up to 10-fold greater changes in immune-sensitized vs nonsensitized hosts (Table 1 and Supporting Information Table S3 and Figure S1). Of these, 51 were increased and 2 were decreased. Proteins

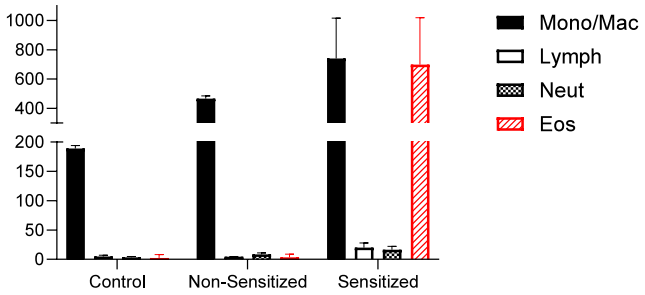

Figure 1. Airway eosinophilia induced by respiratory tract exposure to MDI. The number (Y-axis × 10^{-3}) of different cell types (key to right) collected by BAL was quantitated by differential analysis of cytospun cells. The mean and standard error are shown for $N = 6$ mice/group.

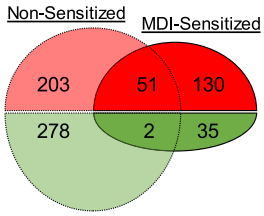

Figure 2. Similarities and differences in lung gene expression between nonsensitized and sensitized mice. Venn diagram depicting the number of lung gene transcripts differentially expressed >2-fold (up in red, down in green) in MDI exposed mice vs controls ($N = 6$/group), highlighting overlap and differences in nonsensitized vs immune-sensitized hosts as labeled.

corresponding to several of the differentially expressed lung gene transcripts were elevated in prior proteomic studies of airway fluid from MDI immune sensitized mice similarly exposed to MDI, providing orthogonal evidence supporting the present data. The gene transcripts most increased by MDI exposure have been linked to human asthma functionally and/or genetically.[53−57]

Genes Differentially Expressed in Nonsensitized, but Not MDI-Sensitized, Mice Following Respiratory Tract MDI Exposure. In total, 480 gene transcripts were differentially expressed (±2-fold, $p < 0.05$ vs controls) in nonsensitized, but not MDI immune-sensitized, mice following respiratory tract MDI exposure; 203 increased and 278 decreased (Figure 2). The top differentially expressed gene transcripts are shown in a heat map (Figure 3) and a complete list and volcano plots are included in the Supporting Information (Table S4, Figure S2). The differentially expressed transcripts are enriched in Type 1 interferon related genes, with the largest relative increase in interferon regulatory factor 7 (IRF7), a master regulator of type-I interferon-dependent immune responses.[58] The gene with the largest relative decrease in expression encodes the antibody joining (J) chain expressed exclusively by B-cells, which play a major role in HMW allergen pathogenesis via secretion of antigen-specific IgE.[59]

Genes Differentially Expressed in MDI-Sensitized but Not Nonsensitized Mice Following Respiratory Tract MDI Exposure. In total, 164 genes were differentially expressed (±2-fold, $p < 0.05$ vs controls) in MDI immune-sensitized, but not nonsensitized, mice following respiratory tract MDI exposure; 130 increased and 35 decreased. The top differentially expressed genes are shown in Figure 3 (with a

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Table 1. Top Differentially Expressed Genes in Response to Airway MDI Exposure in All Hosts (MDI-Sensitized and Nonsensitized)

rank (MS)[a]	gene symbol	fold change (sensitized)	FDR p value	rank (NS)[a]	fold change (nonsensitized)	FDR p value	fold difference (sensitized vs nonsensitized)	fold increase BAL protein[b]
1	**CLCA1**	7422.9	2.9×10^{-6}	1	995	8.9×10^{-5}	**7.5**	11.7
2	**RETNLA/ FIZZ1**	75.1	9.5×10^{-5}	3	11.7	0.005	**6.4**	6.6
3	**RNASE2A/ EDN**	70.2	4.5×10^{-11}	5	8.2	3.9×10^{-6}	**8.5**	
4	**CCL8/ MCP2**	68.3	1.1×10^{-7}	8	7.4	0.0006	**9.2**	
5	**MUC5AC**	48.5	1.1×10^{-7}	4	10.8	0.0002	**4.5**	
6	**FCGBP**	20.1	7.7×10^{-10}	25	3.5	0.0001	**5.8**	
7	**MUC5B**	17.4	0.0001	6	8.1	0.0019	**2.1**	
8	**TFF2**	16	1.0×10^{-5}	7	7.8	0.0006	**2**	
9	**SLC26A4**	13.9	1.0×10^{-5}	14	4.7	0.0184	**3**	
10	**REG3γ**	10.8	0.0824	11	5.1	0.1127	**2.1**	4.2
11	**CXCL9/ MIG**	10.5	2.8×10^{-5}	2	14.7	2.2×10^{-5}	**0.7**	
12	**PIGR**	8.7	0.0002	106	2.2	0.0584	**3.9**	3.2
−2	**DUSP1**	−2.4	0.1671	−66	−2.5	0.028	**1**	
−1	**PON1**	−4.3	0.0049	−36	−2.9	0.0439	**1.5**	

[a]Rank in relative fold change of transcript in MDI sensitized (MS) or nonsensitized (NS) mice vs controls. [b]Fold protein increase in bronchoalveolar lavage (BAL) fluid of MDI sensitized/exposed mice vs controls in prior published studies.[72]

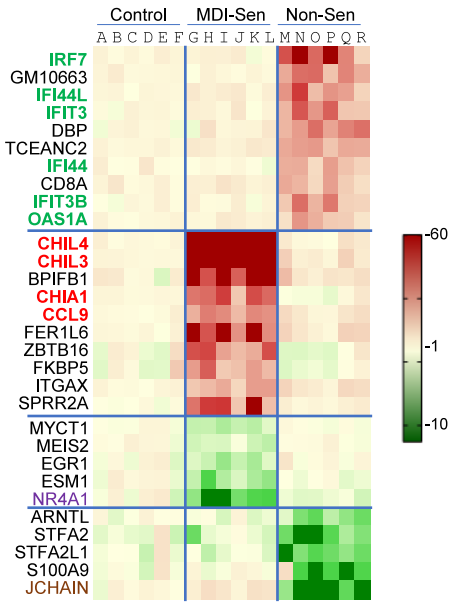

Figure 3. Heat map of differentially expressed genes in nonsensitized vs immune-sensitized mice upon respiratory tract MDI exposure. Color depicts fold change gene transcription based on fluorescence signal intensity relative to the mean of control samples (key on right). Each letter headed column shows data from a different mouse control (A−F), MDI exposed MDI-sensitized (G−L), MDI exposed nonsensitized (M−R). The data highlight gene transcripts (labeled on left) with the greatest relative change in signal intensity, with those central to type I interferon signaling in bold green font and chitinases/ chemotaxis in bold red font.

complete list in Supporting Information Table S5 and volcano plot Figure S2). The increased gene transcripts are dominated by molecules crucial to inflammatory responses, especially chemotaxis. Three of the four gene transcripts with the largest relative increases encode Chitinase-like molecules, which are secreted by alternatively activated macrophages among other cell types and attract eosinophils.[60−62] The most down-regulated gene transcript encodes nuclear receptor 4A1 (NR4A1) aka Nur77, known for its role in restricting metabolic activation of autoreactive T-cells and increasing asthma pathology when absent.[63,64]

Thus, differential expression of 646 total genes, 333 increased and 313 decreased (<3% of the genome), is associated with differential pathologic responses to respiratory tract MDI exposure in mice, depending on prior exposure and systemic immune sensitization.

Biological Pathways Predictably Altered by MDI Exposure Based on IPA of Lung Gene Expression of Nonsensitized and MDI-Sensitized Mice vs Controls. IPA comparison of MDI exposed nonsensitized and immune-sensitized mice vs controls identified overlapping as well as unique features in the top predicted upstream regulators and canonical pathways (Tables 2 and 3 and Supporting Information Table S6). LPS and IFN-γ were the most significantly predicted upstream regulators of the MDI response in all exposed animals but were followed differentially by IFN-α, STAT1, and TLR3, in nonsensitized mice, vs IL-13, IL-4, and TCL1A in MDI-sensitized mice, consistent with observed pathology. The most significantly affected canonical pathway for all MDI exposed mice vs controls was "granulocyte adhesion and diapedesis". However, marked differences between MDI immune sensitized vs nonsensitized animals were observed in other significantly affected canonical

Table 2. Predicted Upstream Regulators Triggered by MDI Airway Exposure

nonsensitized		MDI-sensitized	
name	p value	name	p value
LPS	1.1×10^{-28}	*IFNγ*	6.8×10^{-32}
IFNγ	2.8×10^{-22}	*LPS*	1.1×10^{-28}
IFNα	4.5×10^{-21}	**IL13**	9.3×10^{-28}
STAT1	1.9×10^{-20}	**IL4**	8.8×10^{-25}
TLR3	3.9×10^{-19}	**TCL1A**	3.2×10^{-21}

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Table 3. Predicted Canonical Pathways Altered by MDI Airway Exposure

nonsensitized	*p* value	overlap
granulocyte adhesion and diapedesis	9.2×10^{-6}	9.7% 15/154
glucocorticoid receptor signaling	1.5×10^{-5}	7.1% 22/310
communication-innate/adaptive immune cells	3.2×10^{-5}	14.1% 9/64
TREM1 signaling	6.0×10^{-5}	13.0% 9/69
antigen presentation pathway	6.1×10^{-5}	21.4% 6/28
MDI-sensitized	***p* value**	**overlap**
granulocyte adhesion and diapedesis	5.6×10^{-8}	8.4% 13/154
agranulocyte adhesion and diapedesis	1.1×10^{-7}	7.9% 13/164
complement system	1.2×10^{-7}	21.2% 7/33
dendritic cell maturation	7.5×10^{-7}	7.4% 12/162
B cell development	5.8×10^{-6}	22.7% 5/22

pathways, several driven by a combination of complement, CD80, and HLA molecules (see Supporting Information Table S6). Compared with controls, murine MHC class II H2-A gene transcripts were exclusively increased >2-fold in exposed MDI sensitized mice, while nonclassical MHC class 1b molecules (H2-T23, H2-M3) were exclusively increased >2-fold in exposed nonsensitized mice, consistent with differing antigenic underpinnings of the exposure response.

Pathway Analysis of the Molecular Response to Respiratory Tract MDI Exposure of Nonsensitized vs MDI Sensitized Mice Using TAC Software. TAC analysis, which curates genes based on Wiki pathways, predicted the involvement of slightly different biological pathways in response to MDI exposure (Table 4). The Wiki pathway most significantly affected in nonsensitized mice vs controls is that expected to result from "Deficiency in RNA Editing by Adenosine Deaminase Acting Against RNA-1 (ADAR1)".[65] In contrast, "Chemokine Signaling" is the pathway most significantly affected in MDI immune-sensitized mice.

Together, IPA and TAC analysis suggests MDI respiratory tract exposure induces marked changes in immune-related genes in both nonsensitized and immunologically sensitized hosts. Gene transcripts involved in chemokine signaling are altered in all MDI exposed mice (vs controls), but to a larger extent in immune sensitized vs nonsensitized hosts. Distinct differences in gene expression reflecting ADAR-1 deficiency occur only in nonsensitized mice while complement, dendritic, and B cell developmental pathways are more significantly impacted by exposure in immune sensitized hosts. Differences in MHC gene expression suggest further potential differences in underlying antigenic stimuli and responsive cell clonotypes in exposed nonsensitized vs sensitized mice. Based on the present gene expression and bioinformatic analysis, Figure 4 presents a hypothetical mechanism by which MDI might affect airway inflammation by inhibiting an active innate immune process for differentiating self-vs nonself.

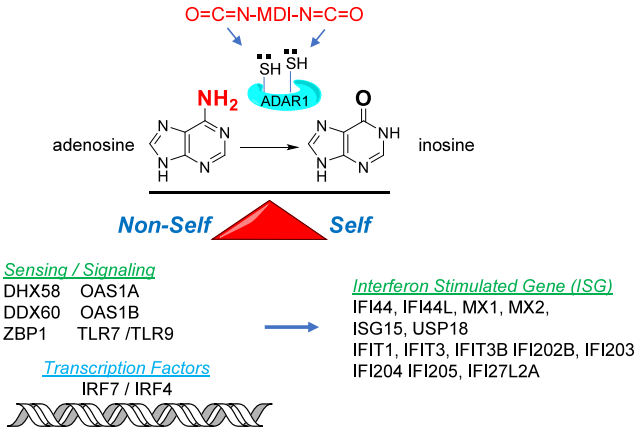

Figure 4. Hypothetical pathway through which MDI exposure may alter active immune homeostasis, based on lung gene expression changes in nonsensitized vs MDI-sensitized mice. Deamination of RNA adenosine (A) to inosine (I) by ADAR-1 is a key process for marking RNA as self (vs viral), therefore preventing innate immune activation and affecting the development and resolution of immune-mediated diseases. The active site of ADAR-1 contains two free thiols, which are preferred reaction targets for isocyanate chemicals in vivo and could be functionally compromised via nucleophilic addition reactions. Deficiency in ADAR-1 activity results in production of interferon stimulated genes via specific RNA-sensing/signaling molecules and transcription factors. In the present study, MDI exposure selective increases multiple lung gene transcripts along this pathway in nonsensitized mice but not in immune-sensitized mice, which may play a protective role acutely, but could become pathogenic with chronic exposure.

Gene Expression Changes Associated with Asthmatic Pathology Induced by MDI vs Prototypic HMW Allergen (Ovalbumin). We compared the present gene expression data with previously published data from murine studies of the prototypic HMW allergen, ovalbumin. Analyses were performed with the NCBI GEO database that best matched the present study with regard to mouse strain (Balb/C), time point (48 h) post-exposure, tissue (lung), and paired control samples (GSO Series GSE41665).[66]

Substantial overlap and similar trends in asthmagen-triggered increases in gene transcription were noted between ovalbumin and diisocyanate, especially among transcripts with the largest (>6-fold) differences, e.g., CLCA1, chitinases, and RNase2a aka eosinophil derived neurotoxin or EDN (Figure 5). However, roughly 1/2 of the (>2-fold) increased, and most of the (>2-fold) decreased gene transcripts differed in sensitized animals exposed to MDI vs ovalbumin. As shown in Figure 6, the largest increase in lung gene expression in mice with MDI vs ovalbumin-induced asthma pathology was identified for ZBTB16 and FK506BP5; transcript levels increased >5-fold in response to MDI, but unchanged or decreased in response to ovalbumin. Macrophage-lineage gene

Table 4. Most Significantly Affected Pathways Based on TAC Analysis

MDI sensitization	pathway	# GENES	UP > 2-FOLD	DOWN > 2-FOLD
nonsensitized	ADAR1 editing deficiency	27	IFI44, MX1, DHX58, IRF7, MX2, ISG15, TGTP1, IFIT3, ZBP1, NLRC5, TGTP2, IFIT1, GM4951, IIGP1, OAS1A, USP18, DDX60, GBP3, GM12185, CXCL10, XAF1, CCL5, OAS1B, F830016B08RIK	IFNA1, IKBKG, SLFN4
MDI-sensitized	chemokine signaling pathway	13	CCL22, FOXO3, CCL17, CCL9, XCR1, CCL11, CXCL9, CCL8, CCL12, CCR5, NCF1, CCL6, CXCR1	None

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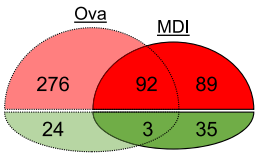

Figure 5. Similarities and differences in lung gene expression in murine models of (LMW) MDI asthma vs (HMW) ovalbumin asthma. Venn diagram depicting the number of lung gene transcripts differentially expressed >2-fold (up in red, down in green) in MDI sensitized/MDI exposed mice vs ovalbumin (OVA) sensitized/ovalbumin exposed mice as labeled. Analysis was performed using data from the GEO database (GSO Series GSE41665) that best matched the present study with regard to mouse strain and timing of sensitization and exposure. Fold changes in gene expression for each model were calculated relative to unexposed control samples from the different respective studies.

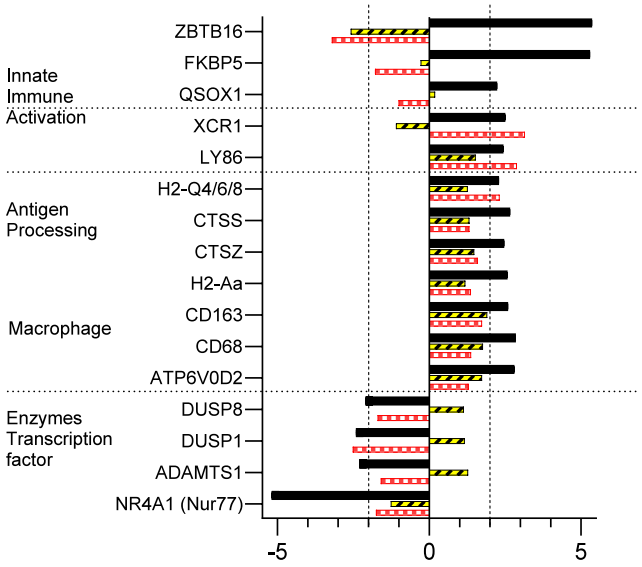

Figure 6. Individual genes differentially expressed in MDI vs ovalbumin asthma models. The fold change (relative to unexposed controls) is graphed (*X*-axis) for genes with the greatest relative differences in lung expression in mice with ovalbumin vs MDI-induced asthmatic lung pathology. Data highlight differences in MDI immune sensitized mice exposed to MDI (black) vs ovalbumin immune sensitized mice exposed to ovalbumin (yellow diagonal stripe) and nonsensitized mice exposed to MDI (red horizontal stripe).

transcripts were prominent among those differentially expressed >2-fold in response to MDI (vs ovalbumin) exposure including CD68, CD163, a marker of "alternative activation", ATP6 V0D2, a proton pump key to maintaining organelle homeostasis and limiting inflammation, and several molecules important in antigen processing; H2-Aa, MHC II molecules, and cathepsins.[67−71] The largest decrease in gene expression in response to MDI (vs ovalbumin) was for NR4A1, mentioned above, and also included DUSP1, DUSP8, and ADAMTS1, downregulated in our mouse MDI asthma pathology model in multiple strains.[72]

Together, the data demonstrate overlap in lung gene expression changes of immune-sensitized hosts exposed to MDI vs ovalbumin, as well as differences that may relate to distinct pathogenic mechanisms, including prominent involvement of the innate arm of the immune system, alternatively activated macrophages, and persistence of symptoms and inflammation despite cessation of exposure, mimicking an autoimmune-like syndrome.[73,74] The potential significance of the data is further addressed in the Discussion section.

Validation of Microarray Data for Selected Genes. To validate microarray data, beyond prior limited proteomic studies,[32] real-time PCR was performed for a panel of selected genes (CLCA1, ZBTB16, IRF7, IFIT3, IIGP1, IFI44) with tissue from additional MDI exposed animals and controls. Quantitation of gene expression for animals in each group demonstrated relative changes in nonsensitized and immune-sensitized mice consistent with those measured by microarray (Supporting Information Figure S3). CLCA1 expression was significantly elevated vs controls, upon MDI exposure of nonsensitized and MDI-sensitized mice, although levels were >10 -fold higher in sensitized mice. In contrast, ZBTB16 transcript levels were significantly increased only in MDI exposed-immune sensitized mice, while IRF-7, IFIT3, IIGP1, and IFI44 were significantly increased in MDI exposed nonsensitized but not MDI sensitized mice.

DISCUSSION

This study focuses on molecular changes associated with exposure in a mouse model of MDI (a LMW allergen) asthma pathology. Among the findings, 53 lung gene transcripts exhibited differences >2-fold in all MDI exposed mice vs controls, with up to 10-fold greater changes in immune sensitized vs nonsensitized hosts. An additional 664 gene transcripts were differentially expressed in nonsensitized vs sensitized hosts, with prominence of ISGs and activation indicators of the innate vs adaptive arm of the immune system. The MDI asthma pathology model showed substantial overlap in gene expression vs the prototypic HMW allergen (ovalbumin) asthma mouse model yet marked differences in certain immune related genes were notable, consistent with potential disparities in pathogenic mechanisms. Together, the data suggest innate immune signaling pathways involving ISGs and ADAR-1 could help explain unique aspects of MDI immunogenicity.

The mRNA most elevated by MDI exposure in nonsensitized (but not MDI immune sensitized) mice was the transcription factor IRF-7, dubbed the master regulator of type 1 interferon-dependent immune responses.[75] MDI exposure increased numerous other ISG transcripts in a pattern reflecting ADAR-1 deficiency in nonsensitized (but not sensitized) mice. This observation highlights a novel link among ISG expression, ADAR-1 deficiency, and MDI exposure, which could affect the development of adaptive immunity and asthma through mechanisms distinct from those of HMW asthmagens.

Type I IFNs (and their downstream ISGs) are generally thought to limit asthma by restricting development of TH2 cells and secretion of type 2 cytokines central to pathogenesis of HMW allergen-induced asthma.[76−81] Thus, the diisocyanate induced activation of ISGs may reflect type I interferon responses with the capacity to protect against diisocyanate asthma. Individual variability in this response could explain the clinical differences in responses to exposure.

The pattern of ISG expression in response to diisocyanate exposure in nonsensitized mice is reflective of deficiency in ADAR-1 activity.[82,83] RNA editing by ADAR1 is crucial to regulating innate immune functions, maintaining self-tolerance and preventing autoimmunity.[84,85] ADAR-1 also participates in antigen-independent sterile inflammation, as occurs with tissue

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damage, which may be especially relevant to diisocyanate exposure, given the chemicals known cytotoxicity and the lack of detectable antigen-specific IgE in asthmatic workers. Intriguingly, ADAR-1's active site contains two free thiol groups, preferred diisocyanate reaction targets in vivo, suggesting potential susceptibility to diisocyanate chemical inhibition.

IRF-7 promotion of ISG expression occurs downstream of ADAR-1 activity and might also be involved in pathogenic responses to diisocyanate exposure. Although dispensable for TH2-mediated eosinophilic pathology triggered by conventional HMW allergens, IRF-7 plays an important role in the development of ILC2s that contribute to asthma and other lung diseases.[86,87] ISGs best known for their induction by viral infection have been linked to human asthma in several recent studies.[88] Chronic MDI exposure and resultant interferon production could mimic chronic viral infection and trigger local IL-13 overproduction by innate immune mechanisms defined by Benoit et al.[89] involving alternative monocyte/macrophage activation, a key feature of pathogenic diisocyanate responses in MDI-sensitized mice.[32]

Gene expression in the present MDI asthma pathology model showed substantial overlap with that documented in prior studies with the prototypic HMW ovalbumin allergen; however, several differences were particularly notable.

a) MDI-induced increases in the FK506 binding protein-5 (FKBP5) gene transcript. FK506 aka tacrolimus, a calcineurin inhibitor, has recently been shown to attenuate human myeloid responses to MDI in vitro.[90] FKBP5 is known to directly interact with IFI44 (also increased in MDI exposed/sensitized mice) to modify innate immune responses via IRF7 and IRF3.

b) ZBTB16 is consistently down regulated in numerous ovalbumin and TH2 cytokine (IL-13, IL4)-driven mouse asthma models, but is increased in MDI-sensitized mice following exposure.[91] ZBTB16 is a transcription factor crucial to innate immune cells that respond to "altered-self" or damage associated molecular patterns, including $\gamma\delta$ T cells responsive to hexamethylene diisocyanate (HDI).[31,92−98] Polymorphism of ZBTB16 was associated with diisocyanate asthma in genome-wide association studies of exposed workers.[99,100]

c) NR4A1, commonly known as Nur77, plays an important role in restricting autoimmunity and allergic inflammation. Reduced lung NR4A1 expression levels in MDI asthma may increase the potential for developing autoimmunity as a component of pathogenesis, which could explain ongoing disease in workers despite exposure cessation.[13,74,101]

Together, the differences in gene expression between MDI vs ovalbumin mouse asthma models are consistent with antigen-dependent differences in underlying immune mechanisms despite similar pathology, as others have previously suggested.[10,12,25,102]

The present mouse MDI asthma model's design, strengths, and limitations are critical to consider in potential translation of the findings to exposed workers. Strengths of the model include the ability to perform precisely timed exposures in age matched littermates with identical genetics and analysis of lung tissue whole genome expression. However, the nonsensitized hosts in the mouse model, being initially unexposed to MDI, may better reflect new workers than workers that remain unsensitized despite repeated exposure. The inherent limitations of the mouse model leave uncertainty if the differentially expressed genes in nonsensitized animals reflect protective responses, early steps in asthma pathology, or a combination of both. Future studies will better elucidate potential connections of diisocyanate exposure to ADAR-1 and ISG protein activity as well as gene expression.

In summary, we used lung gene expression analysis in a murine model of MDI asthma pathology to help understand molecular changes caused by respiratory tract MDI exposure, particularly those that differentiate pathologic vs non pathologic responses. The data identify previously unrecognized potential for respiratory tract MDI exposure to induce ISG expression in a pattern reflecting ADAR-1 deficiency in nonsensitized, but not immune sensitize hosts. The data also identify notable differences in gene expression changes of mice with asthma pathology due to MDI vs the prototypic (ovalbumin) HMW asthma model, consistent with potential differences in underlying immune mechanisms of pathogenesis. Overall, lung gene expression data from a mouse model of MDI asthma pathology suggest inflammatory mechanisms involving ISGs and ADAR-1 may be uniquely crucial to MDI's immunogenicity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.3c00325.

Data from the study, including tables of canonical pathways, volcano plots of microarray data, and graphs of real time PCR data (PDF)

Table S1: genes differentially expressed in MDI exposed nonsensitized mice vs controls (XLSX)

Table S2: genes differentially expressed in MDI exposed immune-sensitized mice vs controls (XLSX)

Table S3: genes differentially expressed in all MDI exposed mice (nonsensitized and MDI immune sensitized) vs controls (XLSX)

Table S4: genes differentially expressed in MDI exposed nonsensitized (but not sensitized) mice vs controls (XLSX)

Table S5: genes differentially expressed in MDI exposed immune sensitized (but not nonsensitized) mice vs controls (XLSX)

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Author Contributions

CRediT: **Adam V. Wisnewski** conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing-original draft, writing-review & editing; **Jian Liu** data curation, investigation, methodology, writing-review & editing.

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Notes

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■ ABBREVIATIONS

(ADAR1), adenosine deaminase acting against RNA 1; (ISG), interferon stimulated gene expression; (CLCA1), interferon regulatory factor 7 (IRF7), chloride activated calcium channel 1; (FDR), false discovery rate; (GSH), glutathione; (HDI), hexamethylene diisocyanate; (HMW), high molecular weight; (IPA), Ingenuity Pathway Analysis; (NCO), isocyanate; (IFI44), interferon induced protein 44; (IIGP1), interferon inducible GTPase 1; (IFIT3), interferon induced protein with tetratricopeptide repeats 3; (LMW), low molecular weight; (MDI), methylene-diphenyl diisocyanate; (NR4A1), nuclear receptor subfamily 4 group A member 1; (TAC), Transcriptome Analysis Console; (TDI), toluene diisocyanate; (ZBTB16), zinc finger and BTB domain containing 16

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