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RESEARCH ARTICLE

Expression kinetics of miRNA involved in dermal toluene 2,4-diisocyanate sensitization

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Abstract

Allergic disease is an important occupational health concern, with work-related asthma and allergic contact dermatitis being the most frequently diagnosed occupational illnesses. Diisocyanates, particularly toluene 2,4-diisocyanate (TDI), have been the leading cause of occupational asthma for many years. Understanding the mechanisms behind allergic disease is critical for treatment and prevention. Recently, the study of post-transcriptional regulation by microRNAs (miRNA) has shed light on mechanisms of allergic disease. The present studies report the expression of miRNA during the sensitization phase of an allergic response to TDI in a murine model. Female BALB/c mice were dermally exposed to TDI (0.1–15% [v/v]) or vehicle. RNA was isolated from superficial parotid lymph nodes at timepoints between 1 h and 15 days post-exposure and then miRNA expression was analyzed using array and real-time quantitative PCR analysis. Consistent changes in miRNA expression were identified for miR-21, miR-22, miR-27b, miR-31, miR-126, miR-155, miR-210, and miR-301a. Following TDI exposure, peak expression was observed by Day 4 for the majority of miRNA evaluated with trends in expression correlated to exposure concentration. Confirmed and predicted targets were identified using Diana-microT, miRanda, miRwalk, and Targetscan algorithms. Evaluation of mRNA expression of cytokine and transcription factor targets suggests that miRNA may have a central role early in TDI sensitization. Understanding the role of these miRNA and their specific mechanism of action in sensitization to TDI may provide pertinent information for the identification of other chemical sensitizers while also contributing to the treatment and prevention of allergic disease.

Keywords

Allergic disease, expression kinetics, miRNA, TDI chemical sensitization

History

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Introduction

Allergic disease is an important health concern with occupational implications such as increased healthcare costs and decreased productivity due to time off work. Allergic sensitization as a result of workplace exposure can manifest as rhinitis, conjunctivitis, hypersensitivity pneumonitis, and asthma or as skin diseases, such as contact urticaria and contact dermatitis. These allergic diseases have been shown to be caused, in part, by exposure to low molecular weight (LMW) chemicals. Isocyanates are LMW chemicals widely used in a variety of industries including the automobile industry, autobody repair, and building insulation materials. They are also used in the manufacture of flexible and rigid foams, fibers, coatings such as paints and varnishes, and spray-on polyurethane products. While isocyanates have been shown to be the leading cause of occupational asthma (OA) (Vandenplas, 2011), they have also been reported to be the causative agent in cases of contact dermatitis (Engfeldt & Ponten, 2013). It is generally accepted that the development of allergic disease is largely mediated by an imbalance of effector T-helper (T_H)-lymphocytes (T_H1 vs T_H2), with contact hypersensitivity generally classified as a T_H1 response and respiratory sensitization as a T_H2 response (Wisniewski & Borish, 2011). Numerous studies have investigated the role of the well-characterized asthmogen, toluene 2,4-diisocyanate (TDI; which is now being phased out of commercial use) in allergic disease (Johnson et al., 2007). Studies conducted in humans exposed to diisocyanates and animal models suggest that a mixed T_H1/T_H2 immune response is associated with TDI exposure with T_H2 cytokines, including interleukin (IL)-4 and IL-5, as well as T_H1 cytokines, such as interferon (IFN)-γ, being identified in the respiratory mucosa of workers following exposure (Maestrelli et al., 1995, 1997; Redlich & Karol, 2002).

Due to the prevalence and health impact of allergic disease, elucidation of mechanisms behind the immunological responses is critical for prevention and treatment. Studies have recently demonstrated a role for microRNAs (miRNA) in the development and function of the immune system (Baltimore et al., 2008; Dai & Ahmed, 2011; O'Connell et al., 2010). MicroRNA are small non-coding RNA $\approx$ 20–22 nucleotides long. Their primary function is to regulate gene expression by influencing mRNA stability and turnover by functioning as endogenous inhibitors of protein translation. Up-regulated or dysregulated miRNA regulate diverse biological processes including development, stress response, inflammation, and cancer. The central importance of miRNA

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regulation in cellular function is becoming clearer as more miRNA targets are discovered. While information regarding roles of miRNA in chemical sensitization are limited, recent research suggests involvement of miRNA in both chemical-induced contact hypersensitivity and asthma (Lu & Rothenberg, 2013; Vennegaard et al., 2012; Zhang et al., 2012).

This study evaluated miRNA expression profiles in cells isolated from the draining lymph nodes of BALB/c mice following dermal exposure to the well characterized sensitizing chemical, TDI. The results identified reproducible changes in the miRNA expression for miR-21, -22, -27b, -31, -126, -155, -210, and -301, and suggest that miRNA might play a critical role in TDI sensitization. Given their regulatory function, screening, and profiling, miRNA expression may provide mechanistic insight into TDI sensitization and ultimately identify a set of miRNA that serve as biomarkers for allergic disease.

Materials and methods

Animals

Female BALB/c mice (6–8 weeks-of-age) purchased from Taconic (Germantown, NY) were acclimated for 5 days and then randomly assigned to treatment group; all mice were weighed to ensure homogeneous distribution across treatment groups. This strain was selected because these mice are good IgE producers and have historically been used in the laboratory to evaluate chemical sensitization (Anderson et al., 2010). Mice were housed at a maximum of five per cage in ventilated plastic shoebox cages with hardwood chip bedding, NIH-31 modified 6% irradiated rodent diet (Harlan Teklad), and filtered tap water (*ad libitum*). The facilities were maintained at 68–72°F with a 36–57% relative humidity and a 12-h light–dark cycle. All animal experiments were performed in the Association for Assessment and Accreditation of Laboratory Animal Care accredited National Institute for Occupational Safety and Health animal facility in accordance with an animal protocol approved by the Institutional Animal Care and Use Committee.

TDI exposure

Toluene 2,4-diisocyanate (TDI, CAS 584-84-9) was purchased from Aldrich Chemical Co. (Milwaukee, WI). In the initial microRNA array and in-house confirmation studies, animals were exposed to TDI (1% [v/v]) on the dorsal surface of each ear (25 µl/ear) for 4 consecutive days to ensure chemical sensitization. The selected concentration and dosing regimen for the repeat exposure studies was previously shown to induce sensitization in a mouse model (Franko et al., 2012). A single exposure of TDI (0.1%, 4%, or 15%) was used for subsequent studies to evaluate miRNA expression kinetics. Initial range finding studies identified 4% TDI as the minimum single dose concentration of TDI that could induce maximum sensitization in the absence of systemic toxicity (<10% loss in body weight). Acetone was selected in place of acetone/olive oil as the vehicle control to minimize chemical reactivity. Diisocyantes react with OH groups forming urethane bonds. Olive oil has poly-ols and therefore the stability of free diisocyanate in olive oil would be a concern. This vehicle control has also historically been used in our laboratory to evaluate chemical sensitization (Anderson et al., 2010; Franko et al., 2012).

MicroRNA array

To identify candidate miRNA involved in TDI sensitization, mice (five per group) were dermally exposed to acetone vehicle or TDI (1%) on Day 1 and then sacrificed by CO_2 asphyxiation on Day 5. The superficial parotid draining lymph nodes (DLN) were collected, and stimulated for 24 h *ex vivo* using α-CD3/α-CD28 (see below). RNA was then isolated using a miRCURY RNA Isolation Kit (Exiqon, Woburn, MA) according to manufacturer guidelines (see below) and submitted to Exiqon for miRCURY LNA microRNA array analysis. Exiqon Services (Vedbaek, Denmark) verified the quality of the total RNA by an Agilent 2100 Bioanalyzer profile. Total RNA (300 ng) from the sample and reference was labeled, respectively, with Hy3™ and Hy5™ fluorescent label (miRCURY LNA™ microRNA Hi-Power Labeling Kit, Hy3™/Hy5™, Exiqon) as described by the manufacturer. Hy3™-labeled samples and Hy5™-labeled reference RNA sample were mixed pair-wise and hybridized to the miRCURY LNA™ microRNA Array (6th gen - human, mouse, and rat; Exiqon) that contained capture probes targeting all miRNA for human, mouse, or rat registered in the miRBASE 17.0. Hybridization was done according to the miRCURY LNA™ microRNA Array using an HS 4800™ hybridization station (Tecan, Grödig, Austria). After hybridization, the microarray slides were scanned and stored in an ozone-free environment (level < 2.0 ppb) to prevent potential bleaching of the dyes. The miRCURY™ LNA array slides were then scanned using a G2565BA Microarray Scanner System (Agilent Technologies, Santa Clara, CA) and image analysis was carried out using ImaGene 9.0 software (BioDiscovery, Inc., Hawthorne, CA). Quantified signals were background corrected (Normexp with offset value 10) and normalized using a global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm. Based on recommendations set forth by Exiqon, significant changes were identified by difference (Diff) values of >1 compared to control and with a p-value ≤0.08 following TDI exposure. The miRNA expression profile obtained from the miRNA array was validated using real-time polymerase chain reaction (RT-PCR) in mice exposed to TDI.

miRNA expression kinetics

Additional studies were conducted to examine miRNA expression kinetics and chemical concentration dose following a single exposure to TDI. For these studies, mice (3–5 per group) were exposed to a single dose (25 µl/ear) of TDI (0.1–15%) or acetone. Animals were weighed and sacrificed via CO_2 asphyxiation at timepoints including: 0 h, 1 h, 3 h, 6 h, 2, 4, 7, 9, 11, and 15 days post-exposure. Vehicle controls (acetone) were processed at the select timepoints of 3 h, 6 h, and 4 days post-acetone exposure. DLN were collected (2 nodes/animal/4 ml phosphate-buffered saline) and dissociated using the frosted ends of two microscope slides for subsequent analysis. Serum was also collected via cardiac puncture for subsequent total IgE analysis.

RNA isolation and reverse transcription

Total RNA was isolated from the DLN using the miRCURY RNA Isolation Kit (Exiqon). A DNase treatment was performed for removal of residual DNA. The concentration and purity (OD260/OD280) of the RNA was determined using an ND-1000 spectrophotometer (Thermo Scientific Nanodrop, Wilmington, DE). First-strand cDNA synthesis (1000 ng) was performed using Exiqon's miRCURY LNA™ Universal RT microRNA PCR kit according to manufacturer recommendations. Ultimately, the RNA was analyzed for miRNA and mRNA expression.

Real-time PCR (RT-PCR)

For analysis of miRNA expression, a reaction of cDNA, mouse-specific miRNA primers (miRCURY LNA universal primers, Exiqon), and SYBR Green Master Mix Universal RT (Exiqon) was added to a MicroAmp Fast Optical 96-well Reaction Plates

and analyzed on an Applied Biosystems 7500 Fast Real Time PCR System using cycling conditions as specified by Exiqon. Samples were analyzed in duplicate. miRNA SNORD65 was used as the endogenous reference control because expression was determined to be stable following chemical exposure (data not shown). RT-PCR data were collected and expressed as relative fold change over vehicle control, calculated by the following formula: $2^{-\Delta\Delta Ct} = \Delta Ct_{Sample} - \Delta Ct_{Control}$. $\Delta Ct = Ct_{Target} - Ct_{SNORD65}$, where Ct = cycle threshold as defined by manufacturer. A Student's unpaired *t*-test was conducted on the average Ct value for each mouse at each timepoint ($n = 3$–5) compared to the average Ct value for each mouse in the acetone vehicle control group (0 h: $n = 3$–5). Statistical significance is indicated (*) at a p value < 0.05. For certain miRNA, only select timepoints were analyzed due to limitations on sample volume.

Confirmation of chemical sensitization

Chemical sensitization was confirmed by expression of allergic cytokines (IL-4, IL-5, IL-13, and IFNγ) protein expression and total serum IgE using methods previously described (Anderson et al., 2010; Franko et al., 2012).

Predicted and confirmed targets that are common among miRNA

In order to determine potential shared mechanisms or functions of identified miRNA, predicted and validated miRNA targets were compiled using Diana-microT, miRanda, miRwalk, and Targetscan algorithms (Dweep et al., 2011). The validated targets for each miRNA, as compiled by the mirwalk servers, were also determined based on reported protein:miRNA relationships that range from direct interactions to no interaction based on published literature. The list was curated to include proteins or protein families that are in some way associated with miRNA expression or function either upstream or downstream. In instances where the link was not readily evident in the associated publication, the record was not included in the results.

RT-PCR for mRNA expression

Relative fold-changes in mRNA gene expression were assessed via RT-PCR as previously described (Franko et al., 2012). Select predicted and/or confirmed cytokines (IL-1β, -4, -6, -10, -17, -21, IL-13 receptor, and IL-2 receptor) and transcription factor (forkhead box P3 [*foxp3*], *smad3*, runt-related transcription factor 1 [*runx1*], signal transducer and activator of transcription 3 [*stat3*], and suppressor of cytokine signaling 5 [*socs5*]) targets were analyzed. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control. A Student's unpaired *t*-test was conducted on the average Ct value obtained for each mouse at each timepoint ($n = 3$) compared to the average Ct value for each mouse in the acetone vehicle control group (0 h: $n = 3$). All primers and probes used for mRNA expression analysis were purchased from Applied Biosystems.

Results

miRNA array and identification of candidate miRNA

A miRNA array was initially conducted to identify miRNA potentially involved in chemical sensitization; results are presented in Table 1. Of the ~700 miRNA tested, 11 were identified from the array for further study due to significant changes (difference values of >1 compared to control and with p value ≤ 0.08) following TDI exposure. miRNA-21 had the greatest difference in expression based on results from the array.

Table 1. miRNA array or real-time PCR analysis ($n = 3$) following four daily consecutive dermal doses of TDI (1%).

	MiRNA array		
miRNA	Diff	p Value	Real-time PCR fold-change (±SE)
21*	2.25	0.001	7.3 ± 2.8
27b*	1.21	0.004	4.8 ± 1.4
22*	1.26	0.018	14.0 ± 5.1
301a*	1.01	0.004	20.0 ± 7.3
210*	1.33	0.082	15 ± 4.0
19a*	1.16	0.010	2.5 ± 0.6
146b*	1.26	0.015	2.7 ± 0.8
29b	1.44	0.014	1.3 ± 0.4
142-3p	1.40	0.080	1.7 ± 0.7
32	1.05	0.006	1.0 ± 0.5
33	1.18	0.019	1.3 ± 0.2

As recommended by Exiqon, for the array difference (Diff) values >1 and with p value ≤ 0.08 were considered statistically different compared to vehicle control. Real-time PCR ($n = 3$) is based on relative fold-change (±SE) compared to vehicle control. *Positive finding in array and PCR.

The miRNA identified from the array were confirmed in a subsequent experiment using real time-PCR (Table 1). Real-time PCR (2-fold change greater than control) confirmed the microRNA array results for miR-21, miR-27b, miR-19a, miR-146b, miR-22, miR-301a, and miR-210, but not for miR-32, miR-29b, miR-142-3p, and miR-33 (Table 1). Additional miRNA, i.e., miR-31, 126, and 155—identified from the literature as having a potential role in allergic processes (Mattes et al., 2009; Oglesby et al., 2010; Sonkoly et al., 2010)—were also included for subsequent analyses. However, based on the array data, no significant changes were identified for miR-155, miR-126, and miR-31 (data not shown).

Confirmation of TDI sensitization

To confirm that a single dose exposure to TDI (4%) would sensitize animals, traditional markers of sensitization were evaluated at various timepoints between 3 h and 15 days post-exposure. This level of TDI was identified to be the lowest concentration to induce maximum sensitization following a single exposure (data not shown). Consistent with the reported data on TDI sensitization, protein expression of IL-4, IL-5, IL-13, and IFNγ was significantly increased by Day 4 following a single TDI exposure (Table 2). Elevations in T_H1 (IFNγ) and T_H2 (IL-4, -5, and -13) cytokines are characteristic of the mixed T-helper cell response induced by TDI. Serum IgE levels were also significantly elevated by Day 7 post-TDI exposure (Table 2).

Expression kinetics following exposure to TDI

Using real time-PCR, the expression kinetics of 10 miRNA of interest (e.g., miR-21, -22, -155, -126, -27b, -210, -31, -301a, -29b, and -146b) was evaluated at various timepoints between 3 h and 15 days following a single TDI (4%) exposure. Consistent with the initial micro-array results (Table 1), alterations in expression were observed for miR-21, -22, -27b, -210, and -301a (respectively, Figures 1A, B, E, F, and H). In accordance with what has been described in the literature, changes in miRNA expression were confirmed for miR-31, -155, and -126 (respectively, Figures 1C, D, and G). Changes in expression were not reproducible for miR-29b and miR-146b (data not shown). The largest increase in relative fold-change (9.6 ± 0.5 at 6 h post-exposure) in miRNA expression was observed for miR-126 after TDI exposure (Figure 1D). Interestingly, this was the only miRNA that had an early peak (3–6 h) in expression that returned to

Table 2. Total IgE and cytokine expression kinetics in mice at various timepoints after TDI dermal exposure.

	0 hours	6 hours	4 days	7 days	11 days	15 days
IgE (ng/ml)	299 ± 62	305 ± 62	324 ± 86	2433 ± 205*	2250 ± 311*	2040 ± 262*
IFNγ (pg/ml)	480 ± 102	999 ± 140*	1713 ± 166*	1133 ± 71*	1223 ± 83*	661 ± 42
IL-4 (pg/ml)	7 ± 5	19 ± 4	1335 ± 17*	771 ± 51*	639 ± 20*	458 ± 36*
IL-5 (pg/ml)	17 ± 6	42 ± 6	306 ± 100*	46 ± 14	70 ± 13	62 ± 6
IL-13 (pg/ml)	4 ± 3	3 ± 2	3658 ± 636*	262 ± 88	90 ± 26	81 ± 17

Analysis of *ex vivo* simulated cytokine expression of IL-4, -5, -13, and IFNγ in DLN and serum IgE following single dermal exposure to TDI (4% v/v). Mean concentration ± standard error of three mice/group at each indicated timepoint. *Statistically different compared to vehicle control ($p < 0.05$).

baseline values by Day 4. The average peak expression timepoint was observed between 4–7 days post-exposure, returning to baseline values by Day 15 for the other miRNA depicted in Figure 1. Statistically significant increases in expression were observed for all the miRNA evaluated for at least one timepoint, with significant changes at four points for miR-21.

Effect of chemical concentration on miRNA expression kinetics following exposure to TDI

Effects of TDI concentration of miRNA expression kinetics were also explored (Figure 2). A single exposure of 0.1%, 4%, or 15% of TDI was administered dermally to animals and the expression of miRNA (miR-21, -22, -155, -126, -27b, -210, -31, and -301a) was evaluated. Dose-dependent effects were observed for all of the miRNA evaluated, with the highest TDI concentration (15%) generating the largest expression changes. Expression trends similar to those in Figure 1 were observed. In general, following exposure to 4% TDI, the peak responses were seen at Day 4 for all miRNA except miR-126, which peaked at 6 h. Increased miRNA expression was also observed at the lowest dose of TDI (0.1%) for all miRNA investigated. At the highest level of TDI, miR-22 had average mean fold-changes >20 at 9 days post-exposure (Figures 2B and H). The peak changes in miRNA expression observed at Day 4 (Figures 1 and 2) were confirmed in a repeat study using a larger number of animals per group (Table 3). Statistically significant increases in expression were observed for miR-21, -22, -210, and -31 at 4 days post-exposure. Although not statistically significant, increases in expression were also observed for miR-155 and -301a. No changes in expression were identified for miR-27b and -126. This is consistent with the peak increase for miR-126 that was observed at 6 h (Figures 1 and 2). While the peak increase in expression of miR-27b was previously identified (Figures 1 and 2) at 4 days, it persisted until ~9 days post-exposure. The lack of increase in expression at 4 days may support a role for miR-27b later (7–9 days) in the sensitization response.

Common targets among miRNA

To explore potential shared mechanisms or functions of miR-21, -22, -155, -126, -27b, -210, -31, and -301a, databases of predicted and validated miRNA targets from multiple algorithms were utilized. Lists of validated targets (bold) or otherwise associated proteins and predicted target (italic) mRNAs for each miRNA that play important roles in immune cell development and function (either positive or negative association) are presented in Tables 4 and 5. Similar cytokine targets were identified for several of the miRNA (Table 4). For example, the prototypical T_H2 allergic cytokine IL-13 has been shown to increase in response to miR-21 and miR-155. This association was also predicted for miR-22 (IL-13RA1) and 27b (IL-13RA1). Up-regulation of tumor necrosis factor (TNF) and transforming growth factor (TGF) proteins have also been reported for several of the miRNA including miR-21, -155, and -27b, and there is a predicted role for miR-22. Numerous transcription factors were also identified and predicted as targets for these miRNA. It was also seen that miR-21, -31, -210, and -155 regulated expression of forkhead box transcription factor (*foxp3*). A positive association between runt-related transcription factor (r*unx1* and *runx2*) was also identified/predicted for the majority of the miRNA analyzed. The Signal Transducer and Activator of Transcription (*stat*) family of proteins are conserved predicted targets for the identified miRNA and also confirmed (*stat3*) to be negatively regulated for miR-21 and miR-155.

Expression analysis of predicted targets

Confirmed cytokine targets common among the miRNA were selected based on Table 4 and evaluated for changes in mRNA expression following TDI exposure. Consistent with the protein expression data, a statistically significant increase in relative fold-change in *IL-4* mRNA expression was noted at 7 days (and persisted until 15 days) post-TDI exposure (Figure 3A). Peak increases in *IL-21* and *IL-6* expression were seen at Day 4 post-exposure (Figures 3B and C), with a decreasing trend for *IL-1*β (Figure 3A). No changes in mRNA expression of *IL-13*, *IL-10*, *IL-17*, *IL-13RA*, or *IL-2RA* were observed post-TDI exposure (data not shown). Select confirmed transcription factor targets common among the miRNA were also selected based on Table 4 and evaluated for changes in mRNA expression after TDI exposure. A decrease in expression of *runx1* and *foxp3* was observed following TDI exposure (Figure 4). Statistically significant decreases in *runx1* were observed by Day 1 and persisted until Day 9 post-exposure (Figure 4A). Peak decreases in expression were observed 4–9 days post-exposure for *foxp3*. No change in *smad3*, *stat3*, or *socs5* expression was seen over the 15 days post-TDI exposure period (data not shown).

Discussion

Allergic disease is an occupational and public health concern that has been increasing in frequency over the last decade. The identification of contributing agents and elucidation of the mechanisms behind the biological responses is critical for prevention and treatment. In this study, miRNAs involved in dermal TDI sensitization were identified using a murine model. A role for miRNAs in airway inflammatory disorders including asthma and allergic rhinitis has recently been described in both human and animal models (Ariel & Upadhyay, 2012; Garbacki et al., 2011; Shaoqing et al., 2011). It has been proposed that the specific mechanism of miRNA action involves a large number of miRNA targeting multiple functionally-related mRNA to orchestrate a co-ordinated regulation of multiple steps in the pathogenesis of asthma, but these processes are not well understood.

This manuscript investigates the expression kinetics of eight miRNA potentially involved in allergic sensitization to TDI; these included miR-21, mi-R-22, miR-31, miR-126, miR-210, miR-155,

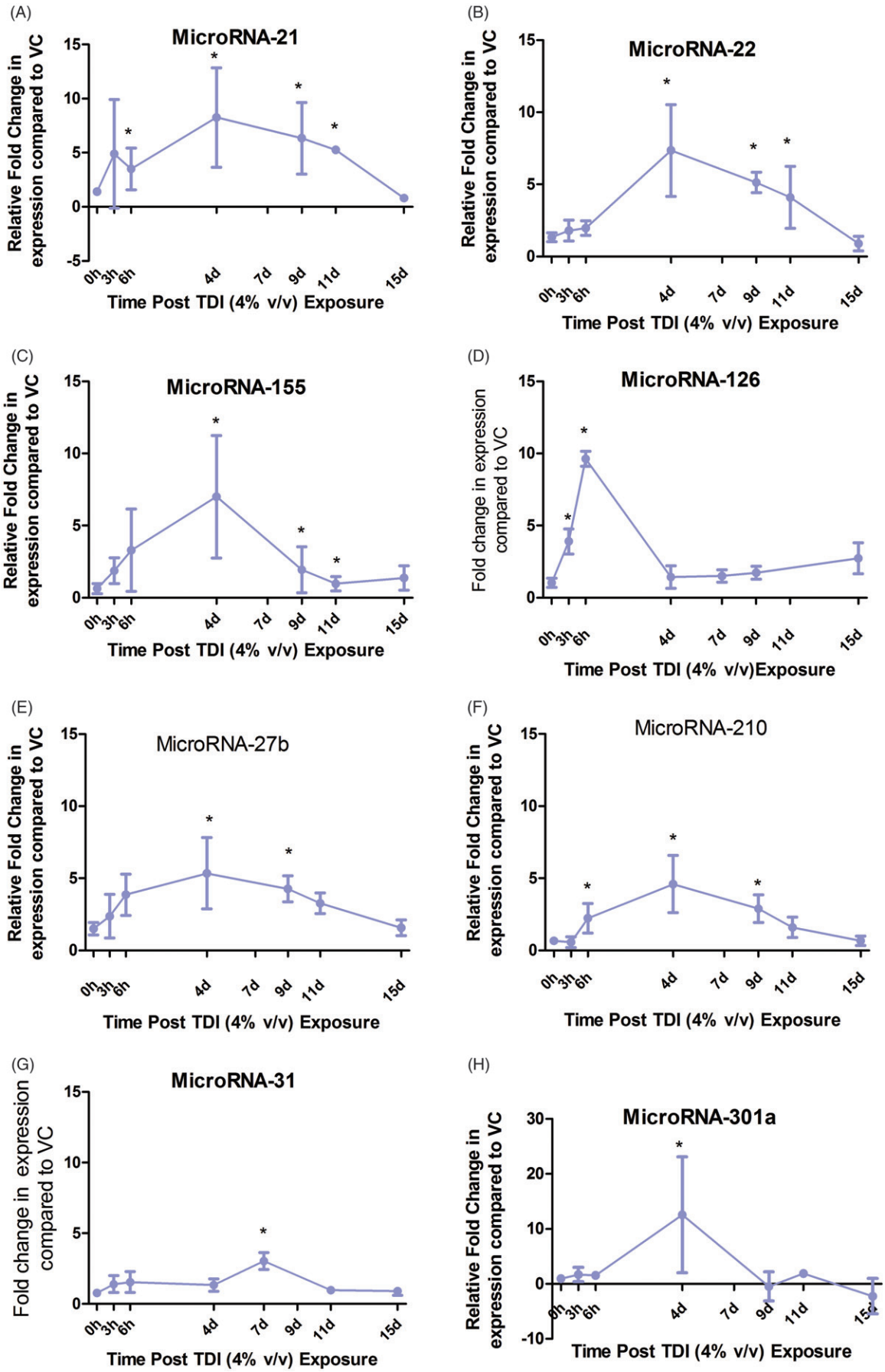

Figure 1. miRNA expression kinetics in DLN following murine TDI dermal exposure. Analysis of (A) miR-21, (B miR-22, (C) miR-155, (D) miR-126, (E) miR-27b, (F) miR-210, (G) miR-31, and (H) miR-301a expression following single dermal exposure to TDI (4% [v/v]). Bars represent mean (±SE) fold-change in gene expression of three mice/group at each indicated timepoint compared to acetone vehicle control. *Significant ($p < 0.05$) compared to vehicle control.

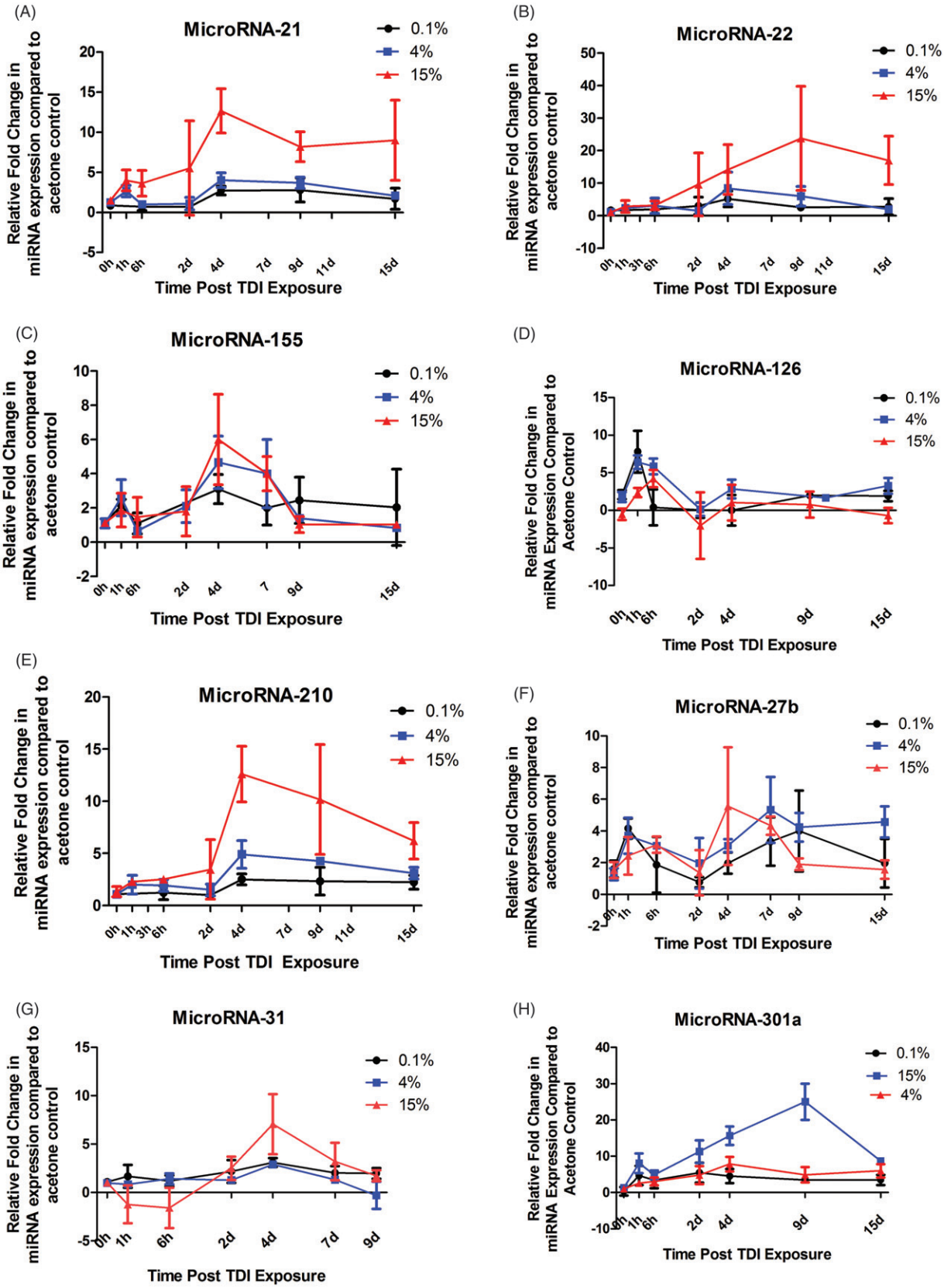

Figure 2. miRNA expression kinetics in DLN following varying dose dermal exposure to TDI. Analysis of (A) miR-21, (B) miR-22, (C) miR-155, (D) miR-126, (E) miR-27b, (F) miR-210, (G) miR-31, and (H) miR-301a expression following a single dermal exposure to TDI (0.1, 4%, or 15% [v/v]). Bars represent mean (±SE) fold-change in gene expression of three mice/group at each indicated timepoint compared to acetone vehicle control.

miR-301a, and miR-27b. These miRNA were identified using microRNA array and real-time PCR platforms. Inconsistent expression data between the two platforms emphasizes the importance of in-house confirmation when using screening tools such as arrays. While numerous candidate miRNA were identified using the array, not all (miR-29b, -142-3p, -32, and -33) were confirmed following real-time PCR. While false-positive results were an issue, false-negatives also occurred. The miRNA

miR-155, miR-126, and miR-31 that were identified from the literature and confirmed by real-time PCR to have robust changes following TDI sensitization, were not identified in the array.

Recent literature reports roles for several of these miRNA including miR-21, -31, -155, and -126 in allergic disease. Lu et al. (2009) demonstrated over-expression of miR-21 in murine models of asthma that resulted in the down-regulation of the potential target IL-12p35. These authors later reported increased T_H1 polarization in mice lacking miR-21 (Lu et al., 2011). Increases in expression of miR-21 in both human and mouse skin samples following sensitization and challenge with the chemical sensitizer diphenylcyclopropenone has also been identified (Vennegaard et al., 2012). Using peripheral blood samples of patients with allergic asthma, research investigating the role of miR-155 in allergic disease suggests that over-expression promotes $CD4^+$ T-cells to differentiate to T_H1 cells while lower expression promotes a T_H2 response (Zhang et al., 2012). In patients with atopic dermatitis, miR-155 was found to be over-expressed by $CD4^+$ T-cells resulting in decreased levels of the predicted target, CTLA-4, which is an important negative regulator of T-cell function. Other investigators found that miR-155 directly targets IL-13Rα1 and leads to reduced expression of this protein and other classical T_H2 effector cytokines such as IL-4 and IL-13 which favors T_H1 polarization (Martinez-Nunez et al., 2011). Studies investigating the function of miR-126 found that *in vivo* activation of TLR4 by house dust mite antigens lead to increased expression of miR-126 (Mattes et al., 2009). Selective blockade of miR-126 suppressed the asthmatic phenotype resulting in diminished T_H2 responses, suggesting a functional connection between miRNA expression and asthma pathogenesis. A role for miR-31

Table 3. miRNA expression following murine TDI dermal exposure.

miRNA	Vehicle, 4 days	TDI, 4 days
21	1.1 ± 0.2	6.4 ± 2.3*
22	0.4 ± 0.3	9.6 ± 5.9*
27b	1.6 ± 0.8	0.8 ± 2.1
31[a]	1.0 ± 0.8	6.8 ± 2.1*
126[a]	1.0 ± 0.2	0.9 ± 1.3
155	0.6 ± 0.5	4.1 ± 1.8
210	0.6 ± 0.4	7.0 ± 1.9*
301a	4.4 ± 1.7	79.4 ± 44.1

miRNA expression following single dermal exposure to TDI (4% [v/v]). Fold-change (±SE) of five mice/group at Day 4 post-exposure ([a]Cases where there were only four mice/group).
*Value statistically different compared to vehicle control ($p < 0.05$).

Table 4. Common predicted effector protein miRNA targets.

miR-21	miR-22	miR-31	miR-210	miR-155	miR-126	miR-301a	miR-27b
Common predicted effector protein miRNA targets							
Interferons							
IFNA1	*IFNAR1*			IFNA1			*IFNAR1*
IFNG				*IFNAR1*			*IFNAR2*
							IFNG
Interleukins, IL-receptors accessory proteins							
IL10	IL1B	*IL12RB2*		IL10	IL1B	*IL15*	IL1B
IL11	*IL13RA1*	*IL13RA1*		IL13	*IL21R*	*IL18*	*IL10*
IL12	*IL17RD*	*IL16*		IL13RA1		*IL 18BP*	*IL10RA*
IL13	*IL1R1*	*IL1R1*		IL17A		*IL1RAP*	*IL12B*
IL1B	*IL1RL1*	*IL1RAP*		IL1B		*IL23R*	*IL13RA1*
IL6	*IL27RA*	*IL1RN*		IL4		*IL28RA*	*IL16*
IL17RD	*IL28RA*			IL6			*IL17RD*
IL1RAP	*IL6R*			*IL16*			*IL1RAP*
IL9	*IL6R*			*IL17RB*			*IL24*
	IL8RB			*IL17RD*			*IL25*
				IL1F9			*IL6R*
				IL1RAP			*IL6R*
				IL1RAP			
				IL22RA2			
				IL23R			
				IL28RA			
				IL28RA			
				IL7			
Tumor necrosis factor (TNF) proteins							
TNF	*TNFRSF10D*			TNF		*TNFRSF10B*	TNF
TNFSF11	*TNFRSF1B*			TNFAIP2		*TNFRSF19*	*TNFAIP3*
TNFAIP3				TNFRSF10A		*TNFRSF1B*	*TNFRSF10D*
TNFRSF10B				TNFRSF10B		*TNFSF15*	*TNFRSF25*
TNFRSF10D				TNFSF11			*TNFSF4*
TNFRSF11B				TNFSF9			
Transforming growth factor (TGF) proteins							
TGFB1	*TGFBI*	*TGFBR3*		TGFB1	TGFBR2	*TGFBR1*	TGFB1
TGFBR1	*TGFBR1*			TGFB2		*TGFBR2*	*TGFBR1*
TGFBR2				*TGFBR1*			
TGFBR3							
Toll like receptors (TLR)							
TLR4	*TLR8*	*TLR8*		TLR4	TLR2	*TLR7*	*TLR8*
					TLR4		

List of validated (roman) targets or otherwise associated proteins and predicted (italic) target mRNA for each miRNA separated by family that play important roles in immune cell development and function. Validated targets and associated proteins may be either positively or negatively associated with miRNA expression.

Table 5. Common predicted transcription factors and signal transducer miRNA targets.

miR-21	miR-22	miR-31	miR-210	miR-155	miR-126	miR-301a	miR-27b
Common predicted transcription factors and signal transducer miRNA targets							
Forkhead box transcription factors (FOX)							
FOXO3	*FOXP1*	FOXP3	FOXP3	FOXO3		*FOXP1*	FOXO1
FOXP3	*FOXP4*	FOXP2		FOXP3			*FOXP2*
FOXO1		FOXP4		*FOXP4*			*FOXP4*
FOXP1							
FOXP2							
	RUNX1	*RUNXA*	*RUNX1T1*	RUNX2		*RUNX2*	RUNX1
	RUNX2		*RUNX3*	*RUNX2*		*RUNX3*	*RUNX3*
SMAD3	*SMAD4*		*SMAD4*	SMAD2		*SMAD2*	*SMAD1*
SMAD7				SMAD4		*SMAD4*	*SMAD2*
				SMAD5			*SMAD4*
Suppressors of cytokine signaling (SOCS)							
SOCS5				SOCS1		*SOCS4*	*SOCS4*
SOCS6				*SOCS5*		*SOCS5*	*SOCS6*
SOCS7				*SOCS6*			
Signal transducer and activator of transcription (STAT)							
STAT3	*STAT5A*	*STAT2*	*STAT6*	STAT1		*STAT3*	*STAT5A*
		STAT5A		STAT3			*STAT5B*
				STAT5A			
				STAT6			

List of validated (roman) targets or otherwise associated proteins and predicted (italic) target mRNA for each miRNA (separated by family) that play important roles in immune cell development and function. Validated targets and associated proteins may be either positively or negatively associated with miRNA expression.

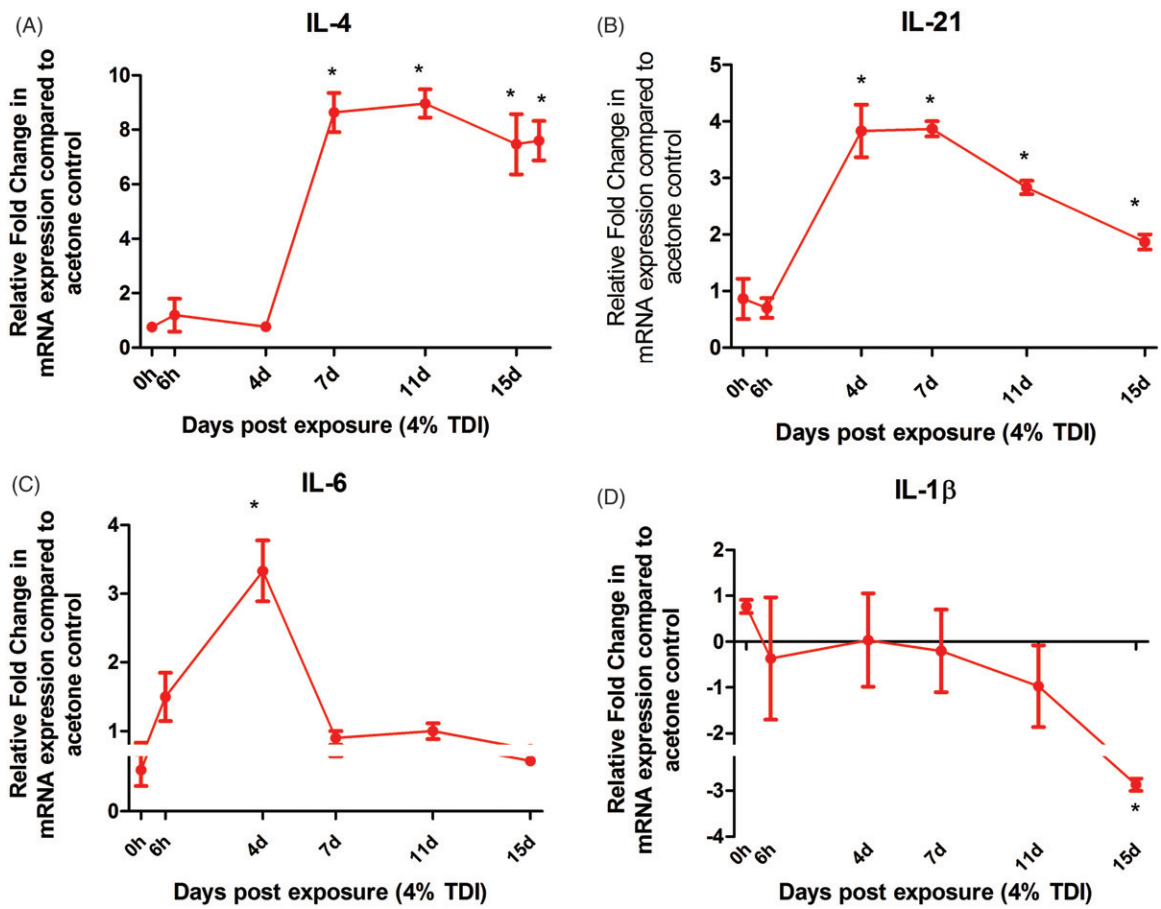

Figure 3. Differential mRNA cytokine expression kinetics in DLN following murine TDI dermal exposure. Analysis of (A) *IL-4*, (B) *IL-21*, (C) *IL-6*, and (D) *IL-1*β mRNA expression following single dermal exposure to TDI (4% [v/v]). Points on graph represent mean (±SE) fold-change in gene expression of three mice/group at each indicated timepoint compared to acetone vehicle control. *Significant ($p < 0.05$) compared to vehicle control.

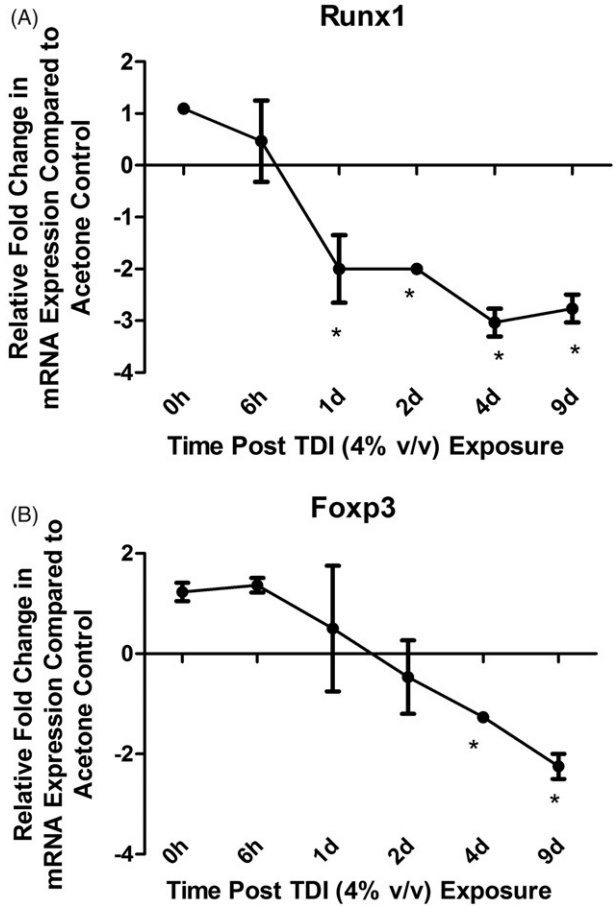

Figure 4. Differential mRNA transcription expression kinetics in DLN following murine TDI dermal exposure. Analysis of (A) *RUNX1* and (B) *FoxP3* mRNA expression following single dermal exposure to TDI (4% [v/v]). Points on graph represent mean (±SE) fold-change in gene expression of three mice/group at each indicated timepoint compared to acetone vehicle control. *Significant ($p<0.05$) compared to vehicle control.

has also been suggested in inflammatory lung disease (Oglesby et al., 2010). An increase in miR-31 expression was detected in human bronchial epithelial cells after a 24-h exposure to diesel exhaust particles.

While roles for miR-21, miR-31, miR-126, and miR-155 in allergic disease have been described, to our knowledge, these are the first studies to identify potential involvement of miR-22, miR-27b, miR-301a, and miR-210. These miRNA have been shown to be involved in other biological responses including: tumorigenesis, epigenetic modification, embryonic development, skeletal metabolism, panic disorders, and cardiomyocyte hypertrophy (miR-22); cancer cell proliferation (miR-210) (Xiong, 2012); lipid metabolism (miR-27b) (Vickers et al., 2013); and gastic and colorectal cancers (mi-301a) (Liu et al., 2013; Wang et al., 2013).

Yang & Schwartz (2012) recently reported regulation of transcription factors involved in T-cell differentiation and the development of asthma by epigenetic mechanisms including miRNA. In the present study, the target analysis conducted on the identified miRNA suggests numerous targets ranging from transcription factors to cytokines and their receptors with the potential for interaction among these factors. The majority of these targets have been shown to modulate numerous T-cell effector signaling pathways (T_H1 responses, T_H2 responses, T-regulatory (T_{reg}) cells, and T_H17 cells) involved in allergic disease (Chatila, 2008). Traditionally, sensitization to low molecular weight chemicals, such as TDI, has been reported to be a result of an imbalance in the T_H1 and T_H2 pathways. Recent work suggests a potential role for T_{reg} cells in allergic disease (Corsini et al., 2011; Kimber et al., 2012). T_{reg} cells have been suggested to impact immune responses through negative regulatory control of T-cell activation, proliferation, and differentiation. While positive and negative correlations have been made between miRNA and expression of regulatory proteins, direct interactions have also been reported. For example, miR-31 and miR-210 negatively regulate expression of the T_{reg} cell transcription factor *foxp3* by binding directly to its target site in the 3′ untranslated region of *FoxP3* mRNA (Rouas et al., 2009; Fayyad-Kazan et al., 2012).

While the transcription factors and cytokines analyzed in this manuscript are limited in comparison to the number of potential targets, statistically significant decreases in expression of *runx1* and *foxp3* were identified following TDI exposure. In addition, the suppression of *runx1* occurred at an earlier timepoint (1 day) than for *foxp3* (4 days), an outcome that is consistent with the earlier requirement for *runx1* in the regulatory T-cell pathway (Chatila, 2008). Reduced *foxp3* expression has also been recently associated with asthma and immune-mediated skin diseases (Kawayama et al., 2013; Quaglino et al., 2013). Although the exact role of T_H17 cells in allergic disease is still unclear, available data suggest that this T-cell lineage may also contribute to the pathogenesis of atopic diseases such as asthma and contact dermatitis. Further support for the role of T_{reg} cells was demonstrated in these studies by the elevations in expression of the prototypical T_H17 cytokines IL-22 and IL-6 that were statistically increased at timepoints between 4–15 days post-TDI exposure.

These finding suggest that perturbations of the T_H1/T_H2 balance in allergic disease is dependent on interactions among multiple effector T-cell pathways. The observed suppression of *runx1* and *FoxP3* was in the presence of increased IL-4 (message and protein) IL-5, IL-13, and IFNγ, suggesting that suppression of T_{reg} cells may promote a T_H2 response by suppressing the T_H1 response or through a lack of regulation of the T_H2 response. Regulation of the T-helper cell balance by T_{reg} cells has previously been suggested (Chapoval et al., 2010). It is interesting to note that, while not all miRNA are significantly altered at every timepoint, a combined effect that regulates immune function might be responsible for a complex feedback loop. T-cell activation and differentiation has been demonstrated to be dependent upon miRNA turnover (Bronevetsky et al., 2013). While the expression of the majority of miRNA peaked around 4 days, maximum increases in miR-126 occurred around 6 h post-exposure. This miRNA might be an early mediator in T-cell activation responsible for downstream activities including differentiation and activation of other miRNA.

Conclusion

These studies begin to evaluate the kinetics of miRNA expression following dermal TDI exposure in a mouse model. It is important to note that only a single dose of chemical was administered in an effort to understand miRNA expression kinetics during sensitization. However, in this model even the lowest concentrations tested induced changes in miRNA expression. These data provide evidence that interactions of multiple miRNA early in the allergic response to TDI may be critical for effector T-cell pathway commitment and for fine-tuning the T_H1 vs T_H2 balance. Identification of unique miRNA expression profiles may help to elicit the mechanisms by which exposure to TDI induces immune cell activation. Additionally, these miRNA may prove to be useful early biomarkers for predicting the type of immunologic response

likely to be induced by TDI exposure and potentially other sensitizing chemicals. In conclusion miRNA profiling may yield a powerful and important new tool for the identification of chemical sensitizers while providing valuable information for potential targets for treatment of exposed individuals.

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Declaration of interest

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