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A Role for Regulatory T Cells in a Murine Model of Epicutaneous Toluene Diisocyanate Sensitization

Carrie Mae Long[*,†,1], **Nikki B. Marshall**[*], **Ewa Lukomska**[*], **Michael L. Kashon**[‡], **B. Jean Meade**[*], **Hillary Shane**[*], and **Stacey E. Anderson**[*]

[*]Allergy and Clinical Immunology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, West Virginia 26505

[†]Immunology and Microbial Pathogenesis Graduate Program, West Virginia University School of Medicine, Morgantown, West Virginia 26505

[‡]Biostatics and Epidemiology Branch Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, West Virginia 26505

Abstract

Toluene diisocyanate (TDI) is a leading cause of chemical-induced occupational asthma which impacts workers in a variety of industries worldwide. Recently, the robust regulatory potential of regulatory T cells (T_{regs}) has become apparent, including their functional role in the regulation of allergic disease; however, their function in TDI-induced sensitization has not been explored. To elucidate the kinetics, phenotype, and function of T_{regs} during TDI sensitization, BALB/c mice were dermally exposed (on each ear) to a single application of TDI (0.5–4% v/v) or acetone vehicle and endpoints were evaluated via RT-PCR and flow cytometry. The draining lymph node (dLN) T_{reg} population expanded significantly 4, 7, and 9 days after single 4% TDI exposure. This population was identified using a variety of surface and intracellular markers and was found to be phenotypically heterogeneous based on increased expression of markers including CD103, CCR6, CTLA4, ICOS, and Neuropilin-1 during TDI sensitization. T_{regs} isolated from TDI-sensitized mice were significantly more suppressive compared with their control counterparts, further supporting a functional role for T_{regs} during TDI sensitization. Last, T_{regs} were depleted prior to TDI sensitization and an intensified sensitization response was observed. Collectively, these data indicate that T_{regs} exhibit a functional role during TDI sensitization. Because the role of T_{regs} in TDI sensitization has not been previously elucidated, these data contribute to the understanding of the immunologic mechanisms of chemical induced allergic disease.

Keywords

chemical sensitization; isocyanate; TDI; chemical allergy; regulatory T cells; hypersensitivity

[1]To whom correspondence should be addressed at CDC-NIOSH, 1095 Willowdale Road, Mailstop 4020, Morgantown, WV 26505. Fax: (304) 293-610. cmlong@cdc.gov.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

Toluene diisocyanate (TDI) is a low-molecular weight, highly reactive chemical utilized in the automobile industry and in the manufacture of polyurethane, foams, paints, and coatings. The U.S. Environmental Protection Agency reports that domestic production and importation of 2,4- and 2,6-TDI isomers rose above 1 billion pounds in 2006 (NTP, 2011). TDI is a potent sensitizer and irritant; repeat dermal and/or inhalation exposure can lead to a variety of allergic diseases including asthma, hypersensitivity pneumonitis, rhinitis, and contact dermatitis (Anderson and Meade, 2014; Bello *et al.*, 2007; Mapp, 2001) These diseases can be disabling and extremely severe, potentially resulting in lifelong illness and/or death (Fabbri et al., 1988). TDI is generally classified as primarily a respiratory, Th2-mediated sensitizer with a Th1 component (Matheson *et al.*, 2005). Although the murine local lymph node assay (LLNA) is validated for the identification of chemicals that preferentially elicit dermal allergic disease, it is not validated for chemicals that preferentially elicit respiratory disease (Anderson *et al.*, 2011). In light of this challenge, further mechanistic insight into the role of immunologically relevant cellular subsets (in addition to Th1 and Th2), involved in respiratory sensitization may ultimately contribute to the development of improved predictive models based on cellular phenotype, secreted cytokine expression, or other related parameters. Recent immunological developments such as the discovery of novel T cell subsets including Th17, T follicular helper, Th22, Th9, and T_{reg}, cells support the potential for the utilization of novel mediators of allergic disease (Liu and Wisnewski, 2003).

Classical regulatory T cells (T_{regs}) ($CD4^+$ $CD25^+$) were initially identified based on their suppressive capabilities which contributed to the maintenance of immune tolerance in mice (Sakaguchi *et al.*, 1995). Following this discovery, a transcription factor known as forkhead box p3 (Foxp3) was identified as the master transcription factor of T_{regs}, allowing for their identification and functional manipulation (Hori *et al.*, 2003). Both naturally occurring T_{regs} (nT_{regs}) that develop in the thymus and inducible T_{regs} (iT_{regs}) that develop in the periphery are involved in the maintenance of immune tolerance. Functionally, T_{regs} have demonstrated a critical role in the development of immune tolerance and can serve as effectors helping to prevent overzealous adaptive responses to foreign antigens and allergens (Sojka *et al.*, 2008; Yadav *et al.*, 2012). This suppressive function is mediated by a variety of mechanisms including, but not limited to, the control of conventional T cell proliferation through the inhibition of co-stimulation via cytotoxic T-lymphocyte associated protein 4 (CTLA4) expression and/or IL-2 consumption, immunosuppressive cytokine secretion (IL-10 and Transforming growth factor β(TGF-β), metabolic interference, and disruption of dendritic cell function (Corthay, 2009; Kimber *et al.*, 2012; Sojka *et al.*, 2008).

Although a functional role for T_{regs} has been suggested in models of chemical-induced contact hypersensitivity (Christensen *et al.*, 2015), this cellular subset has not been investigated in TDI sensitization. Vanoirbeek *et al.* (2004) observed suspected immune tolerance induced by high dose (3% TDI in acetone-olive oil) dermal sensitization followed by intranasal challenge resulting in the absence of airway hyperreactivity, contrasted with low dose (0.3% TDI in acetone-olive oil) dermal sensitization resulting in airway hyperreactivity following intranasal challenge. Although not explicitly stated in the article, this seminal study suggested a role for T_{regs} in TDI sensitization. The collection of data

regarding T_{regs} and chemical allergy is growing but remains limited. In order to elucidate the immunologic mechanisms involved in TDI sensitization, the biological functions of pertinent immune cell subsets need to be delineated. It is important to note that although a variety of T_{regs} may be involved in chemical sensitization, this study focuses on classical T_{regs} ($CD3^+$ $CD4^+$ $CD25^+$ $Foxp3^+$) due to their well-documented regulatory potential in a variety of disease states, including allergy and asthma (Robinson, 2009), and to the lack of data regarding these cells in models of low molecular weight chemical allergy. Here, we utilize a murine model of dermal TDI sensitization in order to elucidate the expression kinetics, phenotype, and functional role of T_{regs} during chemical sensitization.

MATERIALS AND METHODS

Mice

Female BALB/c mice (6–8 weeks of age) purchased from Taconic (Germantown, New York) were acclimated for 5 days and then randomly assigned to treatment group. Homogenous weight distribution was insured across treatment groups. BALB/c mice were selected on the basis of their Th2 bias, robust IgE production, and the historical use of these mice in the laboratory to investigate chemical sensitization (Anderson *et al.*, 2010). Mice were housed in ventilated plastic shoebox cages with hardwood chip bedding at a maximum of 5 animals per cage. A NIH-31 modified 6% irradiated rodent diet (Harlan Teklad) and tap water were administered *ad libitum*. Housing facilities were maintained at 68–72°F and 36–57% relative humidity, and a 12-h light–dark cycle was maintained. 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 Institutional Animal Care and Use Committee-approved protocol.

TDI sensitization model

Toluene 2,4-diisocyanate (TDI, CAS no. 584-84-9) was purchased from Sigma-Aldrich Chemical Company (Milwaukee, Wisconsin). Animals (n = 4–5) were exposed to a single dose of 0.5–4% TDI (v/v) on the dorsal surface of each ear (25 μl per ear). The highest concentration (4% v/v; the maximum sensitizing concentration with minimal toxicity) and dosing regimen was previously shown to induce sensitization (Anderson *et al.*, 2013) and sensitization was confirmed in the lower dose based on total serum IgE levels and dLN allergic cytokine mRNA expression (0.5% v/v; data not shown). Acetone was selected as the vehicle control to minimize chemical reactivity as diisocyanates react with OH groups which are present in other potential vehicles such as olive oil. Ear thickness was measured 4 days following TDI exposure using a modified engineer's micrometer (Mitutoyo Corporation, Japan) and measurements were collected in millimeters (mm). Average ear swelling was calculated as previously described in Anderson *et al.* (2012). Mice were euthanized via CO_2 asphyxiation at time points ranging from 1 to 11 days postchemical exposure.

Euthanasia, tissue collection, and processing

Animals were weighed, euthanized via CO_2 asphyxiation, and examined for gross pathology at the designated time point. Left and right auricular draining lymph nodes (dLNs; drain site of chemical application) were collected in sterile phosphate-buffered saline (pH 7.4) and

manually dissociated using the frosted ends of 2 microscope slides. dLN cellularity was determined using a Cellometer (Nexcelom Bioscience, Lawrence, Massa chusetts) with size exclusion parameters (3.5–36 μm) and a combined Acridine Orange/Propidium Iodide solution to identify viable cells. Blood was collected via cardiac puncture, placed into serum collection tubes, centrifuged, and serum was removed and stored at −80°C for subsequent total IgE analysis via ELISA. Ears were collected in 1 mL RNALater (Ambion, Pittsburgh, Pennsylvania), stored at −80°C for subsequent gene expression analysis.

RNA isolation and reverse transcription

Ears were processed for RNA isolation using a Tissue Lyser II in Qiagen lysis buffer. Total RNA was isolated from the ears and dLN using the Qiagen RNeasy and miRNeasy kits, respectively. A QiaCube (Qiagen, Hilden, Germany) automated RNA isolation machine was utilized in conjunction with the specified RNA isolation kits. A DNase treatment was performed for removal of residual DNA. The concentration and purity of the RNA was determined using a ND-1000 spectrophotometer (Thermo Scientific Nanodrop, Wilmington, Delaware). First strand cDNA synthesis was performed using a High-Capacity cDNA Synthesis Kit (Applied Biosystems, Carlsbad, California) according to manufacturer recommendations. Ultimately, the cDNA was analyzed for mRNA expression as described in the real-time PCR (RT-PCR) methods section below.

Real-Time PCR

For analysis of mRNA expression, TaqMan Universal Fast master mix (Applied Biosystems, Calsbad, California), cDNA, and mouse-specific mRNA primers (TaqMan Custom PCR Arrays, Carlsbad, California) were combined and PCR was performed according to manufacturer protocol. Primers used include: *il-1β*, *il*-6, and *tnf-α*. Master mix, primers, and cDNA were added to a MicroAmp Fast Optical 96-well reaction plate and analyzed on an Applied Biosystems 7500 Fast RT PCR system using cycling conditions as specified by the manufacturer. β-actin was used as the endogenous reference control gene as expression was determined to be stable following chemical exposure (data not shown). RT-PCR data were collected and represented as relative fold change over vehicle control, calculated by the following formula: $2^{-\Delta\Delta Ct} = \Delta Ct_{Sample} - \Delta Ct_{Control}$. $\Delta Ct = Ct_{Target} - Ct_{\beta\text{-}ACTIN}$, where Ct = cycle threshold.

Flow cytometric analysis

Single cell suspensions were prepared from tissues and a minimum of 150 000 dLN cells were aliquoted into 96-well U-bottom plates and washed in staining buffer (PBS + 1% bovine serum albumin + 0.1% sodium azide). Cells were resuspended in staining buffer containing anti-mouse CD16/32 antibody (clone 2.4G2; BD Biosciences, San Jose, California) for blocking of F_c receptors to minimize nonspecific binding. Cells were resuspended in staining buffer containing a cocktail of fluorochrome-conjugated antibodies specific for cell surface antigens including: CCR6 (clone: 29-2L17, fluorophore: BV605, BioLegend), CD3 (500A2, V500, BD), CD4 (RM4-5, AF700, BD), CD8a (53-6.7, AF488, BioLegend), CD25 (PC61, APC Cy7, BioLegend), CD45 (30-F11, PE, BD), CD103 (2E7, PerCP Cy5.5, BioLegend), Inducible T-cell costimulator (ICOS) (C938.4A, PE Cy7, eBioscience), Neu-1 (3E12, PE, BioLegend), and Ter-119 (TER-119, FITC, eBioscience).

Following surface staining, cells were washed in staining buffer and fixed using the Foxp3 fixation buffer set (eBioscience, San Diego, California). After overnight incubation in staining buffer, cells were permeablilized using the Foxp3 fixation buffer set (eBioscience, San Diego, Calfornia) and re-suspended in permeabilization buffer containing a cocktail of fluorochrome-conjugated antibodies specific for intracellular antigens including: CTLA4 (UC10-4B9, BV421, BioLegend), Foxp3 (FLK-16s, eF450, and APC, eBioscience), Gata3 (TWAJ, eF660, eBioscience), Rorγt (Q31-378, PerCP Cy5.5, eBioscience), and Tbet (4B10, PE Cy7, eBioscience). Following staining, cells were re-suspended in staining buffer and analyzed on an LSR II flow cytometer using FacsDiva software (BD Biosciences). Data analysis was performed with FlowJo 10.0 software (TreeStar Inc., Ashland, Oregon). A minimum of 10 000 events were captured for each sample. Leukocytes were first identified by their expression of CD45. The T_{reg} subset was further identified as $CD3^+$ $CD4^+$ $CD8^-$ $CD25^+$ $Foxp3^+$. Numerical population values were calculated by applying subset frequencies to the initial cell count obtained following lymph node homogenization. Compensation controls were performed using single stained cellular suspensions and OneComp beads (eBioscience, San Diego, California) and fluorescence minus one staining controls were included to help set gating boundaries.

T_{reg} suppression assay

The suppressive ability of T_{regs} was analyzed using an *ex vivo* T_{reg} suppression assay as described by Kruisbeek *et al.* (2001) and Marshall *et al.* (2008) with some modifications. This assay evaluates the ability of naïve, conventional dLN-derived T cells (T_{cons}) to proliferate in the presence of varying numbers of T_{regs} isolated from acetone- or TDI-exposed mice. Mice were exposed to acetone (n = 7–11) or TDI (4%) (n = 4–5) as previously described and following sacrifice at 4 and 7 days postTDI (peak of the expansion) exposure the dLN and spleens were removed. T_{regs} ($CD4^+$ $CD25^+$) and T_{cons} ($CD4^+$ $CD25^-$) were isolated from the lymph nodes and $CD4^-$ accessory cells were isolated from naïve spleens using CD4 negative and CD25 positive selection-based magnetic separation kits (Stemcell, Vancouver, British of Columbia). Average T_{reg} purity is as follows for 4 days: Acetone-70.65% of $CD3^+$ $CD4^+$ cells and 4% TDI-63.05% of $CD3^+$ $CD4^+$ cells and 7 days: Acetone-91.35% of $CD3^+$ $CD4^+$ cells and 4% TDI-82.8% of $CD3^+$ $CD4^+$ cells post 4% TDI exposure. Following isolation from naïve mouse dLNs, T_{cons} were labeled with 2 μM carboxyfluorescein succinimidyl ester (CFSE). T_{cons} and T_{regs} were cultured in a 96-well U-bottom plate with anti-CD3 (0.2 μg/ml; BD Biosciences) and accessory cells at a variety of T_{con}:T_{reg} ratios (1:1, 2:1, 4:1, and 8:1). Naïve $CD4^-$ splenocytes were treated with Mitomycin C (Sigma Aldrich, St Louis, Missouri) and utilized as accessory cells. Additional controls included stimulated T_{cons} only to assess baseline proliferation, T_{regs} only, accessory cells only, and T_{cons} only with no stimulation nor accessory cells. Cells from each treatment group were pooled and added to triplicate wells of the culture plate. Seventy-two hours following plating, cells were stained with anti-CD4 and Live/Dead Violet (Life Technologies, Carlsbad, California). T_{cons} were defined as $CD4^+$ $CFSE^+$ cells and suppression was measured based on changes in the frequency of dividing $CFSE^+$ cells based on the dilution of CFSE. T_{regs} were analyzed for purity based on their expression of CD3, CD4, and Foxp3 as determined by flow cytometric analysis as previously described.

In vivo anti-CD25 antibody treatment

In order to deplete T_{regs} before and during TDI sensitization, *Invivo*MAb anti m CD25 (BioXCell, PC-61.5.3) was administered *in vivo*. The use of this antibody as a T_{reg} depletion strategy is validated *in vivo* (Felonato *et al.*, 2012; Setiady *et al.*, 2010). *In Vivo*MAb Rat IgG_1 (Bioxcell, HRPN) was utilized as a negative, isotype control. Treatment groups for this study (n = 5 mice) are as follows: isotype/acetone, isotype/0.5% TDI, isotype/2% TDI, antiCD25/acetone, antiCD25/0.5% TDI, and antiCD25/2% TDI. 200 μg of the respective antibody in USP grade saline was administered intraperitoneally at days −11 and −8 during the depletion study (see Figure 6A for study timeline). Animal weights were recorded throughout the duration of the experiment to monitor potential toxicity. In order to confirm the effectiveness of the antibody, blood was collected from the lateral tail vein at days 2 and 7, and T_{regs} were measured by flow cytometry as previously described. Baseline blood T_{reg} levels were assayed at day 12 to ensure equal pretreatment frequencies across all groups. Mice were exposed to a single dermal application of 0.5 or 2% TDI on day 0. The high dose of 2% TDI was selected in an effort to allow for a measurable increase in the sensitization response, as 4% TDI elicits a maximum sensitization response. Mice were euthanized 7 days following dermal chemical exposure. Specific measures of sensitization were evaluated, including examination of the Th2 population by flow cytometry ($CD3^+$ $CD4^+$ $Gata3^+$), IL-4 dLN mRNA levels by RT-PCR, and total IgE levels in the serum. Total serum IgE was quantified following serum separation from whole blood (centrifugation) and analysis using a Mouse IgE Ready-SET-Go! Kit (Affymetrix eBioscience, San Diego, Californnia) according to the manufacturer's protocol. Absorbance was determined using a Spectramax V_{max} plate reader (Molecular Devices, Sunnyvale, California) at 450 and 650 nm. Data analysis was performed using the IBM Softmax Pro 3.1 program (Molecular Devices, Sunnyvale, California) and the IgE concentrations for each sample were interpolated from a standard curve derived from multipoint analysis.

Statistical analysis

Statistical analyses were generated using SAS/STAT software, version 9.3 (SAS Institute, Cary, North Carolina) and GraphPad Prism version 5.0 (San Diego, California). For irritancy and inflammatory gene expression analysis (Figure 1), a 1-way analysis of variance (ANOVA) was conducted. If the ANOVA showed significance at $P < .05$ or less, the Dunnett's Multiple Comparison Test was used to compare values from groups of mice treated with varying concentrations of TDI to the acetone control group. Figures 2–6 and Table 2 were analyzed by analysis of variance using PROC MIXED. In some cases, data were transformed using the natural log to meet the assumptions of the analysis. Significant interactions were explored utilizing the ‘slice’ option in PROC MIXED and pairwise differences were assessed using a Fishers Least Significant Difference Test. Supplemental data was analyzed by a Student *t*-test comparing groups as indicated in the figure legends. All differences were considered significant at $P < .05$; representative significance symbols varied by figure, as indicated in the figure legend.

RESULTS

Examination of Sensitization and Skin Irritancy Potential of TDI

To confirm that a single dose exposure to TDI (0.5 and 4%) would sensitize animals, total serum IgE was evaluated following TDI exposure. Although not initially statistically significant, IgE levels appeared to increase in a dose-dependent manner, reaching significance following 4% exposure (Figure 1A). Because TDI is a known irritant (Daftarian *et al.*, 2002), the selected doses of TDI (0.5–4% v/v) were assayed for dermal irritancy potential via ear swelling measurements and ear inflammatory cytokine mRNA production quantified via RT-PCR. Average ear swelling was significantly increased four days following 2 and 4% TDI exposure (Figure 1B); however, neither 0.5 nor 1% TDI exposure induced significant increases in ear swelling (Figure 1B). Inflammatory cytokine (IL-1β, IL-6, and TNF-α) mRNA levels were significantly increased in the ear four days following 4% TDI exposure (Figs. 1C–E). These data suggest that 2 and 4% TDI exposure induce a significant irritation response in the ear compared with 0.5 and 1% TDI exposure. Based on this data, 0.5 and 4% TDI were selected as the exposure concentrations in subsequent studies to represent sensitizing concentrations that encompassed both a low dose exhibiting a lack of irritation (0.5%) along with a high dose exhibiting significant irritation (4%).

dLN Treg Expression Kinetics Reveal an Expansion of This Population During TDI Sensitization

In order to profile the expression kinetics of the T_{reg} subset during TDI sensitization we examined dLN cell populations at 1, 2, 4, 7, and 9 days postTDI exposure. T_{regs} were identified as $CD3^+$ $CD4^+$ $CD25^+$ $Foxp3^+$ cells by flow cytometry (Figure 2A). The frequency of T_{regs} was unchanged compared with control cells from acetone-treated mice during 0.5% TDI sensitization; however, T_{reg} frequency increased significantly at 4, 7, and 9 days post 4% TDI exposure (Figure 2B). T_{regs} also significantly increased in number at all time points analyzed during 0.5 and 4% TDI sensitization (Figure 2C). The peak numbers of T_{regs} in the dLN appeared to occur at day 4 post TDI exposure for both concentrations (mean ± SEM; 1.17×10^6 cells ± 0.08 (0.5%) and 1.7×10^6 cells ± 0.08 (4%)) compared with the acetone control (0.18×10^6 cells ± 0.02). The T_{reg} population remained elevated compared with the acetone control, but began to retract at days 7 (0.5% TDI- $0.88 \times 10^6 \pm 0.09$, and 4% TDI- $1.39 \times 10^6 \pm 0.1$) and 9 (0.5% TDI- $0.37 \times 10^6 \pm 0.06$, and 4% TDI- $0.79 \times 10^6 \pm 0.05$) following TDI exposure relative to their peak at day 4.

In order to better elucidate the origin of the T_{regs} involved in the TDI sensitization response, natural T_{regs} (nT_{regs}) were identified based on their expression of neuropilin-1. Interestingly, the frequency of the nT_{reg} subset as a percentage of total T_{regs} significantly decreased at 7 and 9 days during 0.5% and 4% TDI sensitization (Figure 3C). Although the frequency of this subset decreased in relation to the acetone control group, the numbers of nT_{regs} in the dLN increased during 0.5 and 4% TDI sensitization (Figure 3D). This increase was significant for 4–9 days during 0.5% TDI sensitization and at all time points measured during 4% TDI sensitization. It is important to note that both the nT_{reg} and the neuropilin-1^{neg} T_{reg} population (presumably induced T_{regs} [iT_{regs}]) were represented as co-expressing populations examined using additional markers.dLN T_{reg} subsets were further

phenotyped by flow cytometry (Table 1). The phenotyping analysis revealed a number of T_{reg} subpopulations that exhibited the potential to be functionally diverse in relation to their mechanism(s) of suppression (Table 1 and Supplementary Figure 1). An expansion in the frequency and number of the CTLA-4^+T_{reg} population was observed at all measured time points following both 0.5 and 4% TDI exposure (Figs. 3A and B). This population peaked in expression of both frequency (Figure 3A) and number (Figure 3B) at day 4 post TDI exposure. The emergence of the $CTLA4^+T_{reg}$ population during TDI sensitization suggests the engagement of CTLA-4, a negative costimulatory molecule, as a potential suppressive mechanism during this response. As expected, presumably due to compensatory mechanisms during T cell activation, there was also an increase in CTLA4 expression in $CD4^+$non-T_{regs}; however, at the peak of expression (4 days) the mean frequency of expression among all $CD4^+$ cells was 0.54% ± 0.024 for acetone, 4.6% ± 0.24 for 0.5% TDI, and 7.85% ± 0.66 for 4% TDI groups. When compared with the T_{regs} expressing CTLA4 at the same time point, these numbers reveal a sizably smaller frequency of nonT_{regs} expressing this marker, emphasizing the specificity of this marker to the T_{reg} population during this response.

In addition to the $CTLA4^+T_{regs}$ and nT_{regs}, cells expressing the homing molecules CD103, CCR6, and ICOS were analyzed during TDI sensitization. T_{reg} expression of CD103, CCR6, and ICOS increased in both frequency and number throughout 0.5 and 4% TDI sensitization (Table 2). The $CCR6^+$ population's expression frequency was highest at 7 days postTDI exposure, while the $CD103^+$ and $ICOS^+$ populations' frequency peaked at 4 days postexposure. It is important to note that while there are significant changes in these subsets, they represent a small portion of the total $CD4^+$ population, as their expression is presented on T_{regs}, which themselves constitute a minority of the total $CD4^+$ population. Although there was robust T_{reg} expression of CD103, CCR6, and ICOS as single markers, there was a significant population of dLN T_{regs} that co-expressed these molecules (Figs. 4A and B) with kinetics were similar to those exhibited by each single marker. This population likely represents a migratory subset of effector T_{regs} that have been activated during TDI sensitization. This co-expressing population expanded in the dLN in both number and percent during TDI sensitization and represented 6.34% ± 1.2 of T_{regs} following acetone exposure, 12.82% ± 1.2 of T_{regs} during 0.5% TDI sensitization and 20.7% ± 1.8 of T_{regs} during 4% TDI sensitization at 7 days postexposure (peak expression).

T_{regs} Have Potent Suppressive Ability During TDI Sensitization

Because the T_{reg} population expanded during TDI sensitization, the functional role of T_{regs} was further examined by performing a CFSE-based T_{reg} suppression assay with T_{regs} isolated from acetone or TDI-treated mice (Figure 5A). T_{regs} from acetone-treated mice were significantly suppressive at all T_{con}:T_{reg} ratios tested when isolated at 4 (Figure 5B) and 7 days (Figure 5C) postexposure. Acetone T_{reg}-induced suppression was found to be equivalent to naïve-derived T_{reg}-induced suppression (data not shown). T_{regs} from TDI-exposed mice exhibited increased suppressive ability compared with the acetone controls when isolated at both 4 (Figure 5B) and 7 days (Figure 5C) at all T_{con}:T_{reg} ratios tested. The heightened suppressive ability of T_{regs} was surprising, given the progression of sensitization

at the selected concentrations of TDI. This data suggested a functional, suppressive role for T_{regs} in TDI sensitization.

Depletion of T_{regs} Before and During TDI Sensitization Augments the T cell-Mediated Allergic Response

In order to analyze the functional potential of T_{regs} *in vivo* during TDI sensitization, this subset was depleted by injecting mice with anti-CD25 antibody days 11 and 8 prior to TDI exposure (Figure 6A). The high dose of 2% TDI was selected in an effort to allow for a measurable increase in the sensitization response, as 4% TDI elicits a maximum sensitization response. The basal levels of T_{regs} in the blood of mice were determined to be comparable among all groups prior to dosing (Supplementary Figure 2A) and depletion was confirmed in the blood (Sup 2A) at days −2 and 7 and in the dLN (Supplementary Figure 2B) at day 7. Mice dosed with 2% TDI that received anti-CD25 lost significantly more body weight (grams; mean decrease 5.33% 6 3.9) than mice exposed to 2% TDI and isotype control antibody (mean increase 1.01% ± 4.3), suggesting enhanced toxicity following T_{reg} depletion and high-dose TDI administration. Because local irritation is thought to influence allergic sensitization (Pauluhn, 2014) and TDI is a known irritant (Duprat *et al.*, 1976), we analyzed the dermal irritation response at the site of TDI exposure by ear swelling measurements following T_{reg} depletion. As expected, there was a dose-responsive increase (Linear trend test $P < .01$) in ear swelling following exposure to TDI for both the isotype control and anti-CD25 groups (Figure 6B). Although not statistically significant, ears from animals treated with anti-CD25 and either concentration of TDI exhibited increased swelling (12.4% ± 3.7 for 0.5%; 54.4% ± 10.7 for 2% TDI) compared with their isotype-treated counterparts (1.4% ± 6.6 for 0.5%; 35.6% ± 8.9 for 2% TDI). At day 7, dLN cellularity increased dose-responsively (Linear trend test $P < .05$) for both the isotype control and antiCD25-treated groups during TDI sensitization (Figure 6C). For both the 0.5 and 2% TDI-treated groups statistically significant increases in the dLN cellularity with anti-CD25 treatment compared with isotype were observed. Another effector $CD4^+$T cell subset, Th2, which play an important role in allergic responses, was identified by GATA-3 expression and was found to dose-responsively expand in number during 0.5 and 2% TDI sensitization for both the isotype and anti-CD25-treated groups (Figure 6D; Linear trend test $P < .01$). When comparisons were made between the isotype control and anti-CD25 treated groups this population appeared to further expand during TDI sensitization, although these perceived trends were not statistically significant. Similar observations were made for the Th2 population's frequency (data not shown). IL-4 mRNA expression levels increased dose-responsively in the dLN following 0.5 and 2% TDI exposure (Linear trend test $P < .05$; isotype and anti-CD25 groups) and were significantly augmented in groups treated with anti-CD25 compared with isotype-treated controls (Figure 6E). Similarly, dose-responsive increases in total IgE levels in the blood appeared to occur at 7 days following exposure to TDI in mice treated with both isotype control and anti-CD25, although significance was only observed for the 2% TDI anti-CD25-treated group (Linear trend test $P < .01$; Figure 6F). Statistically significant increases in serum IgE levels were observed after anti-CD25 treatment following exposure to 0.5 and 2% TDI.

Other T cell-related dLN phenotyping was performed at day 7 including CD3, CD4, and CD8 expression, along with other effector $CD4^+$Th1 and Th17 populations based on their expression of Tbet and Rorγt, respectively (Supplementary Figure 3). $CD3^+$, $CD3^+$ $CD4^+$, $CD3^+$ $CD8^+$, Th1, and Th17 cells dose-responsively expanded in number during 0.5 and 2% TDI sensitization and antiCD25 depleted animals exhibited higher numbers of cells during TDI sensitization (Supplementary Figs. 3B, D, F, H, J). dLN $CD3^+$ cellular frequency decreased in isotype and antiCD25-treated groups during 0.5 and 2% TDI sensitization compared with their respective acetone control (Supplementary Figure 3A). Interestingly, $CD3^+$ cellular frequency was significantly increased in groups treated with anti-CD25 compared with isotype controls for acetone and 0.5% TDI, indicating that antiCD25 treatment did not affect general T cell frequencies in the dLN. $CD4^+$ and $CD8^+$T cell frequency (as a percentage of $CD3^+$ cells) remained stable during TDI sensitization; however, the CD4+ frequency decreased following anti-CD25 treatment while the CD8+ frequency increased (Supplementary Figs.3C and E). The Th1 dLN population significantly expanded in frequency during TDI sensitization (Supplementary Figure 3C). This subset further expanded in mice treated with antiCD25 in frequency (2%) compared with the isotype control groups. The Th17 subset was also profiled and was not significantly altered in frequency during TDI sensitization in the isotype control treated groups; however, the frequency of Th17 cells was significantly increased above both the corresponding acetone and isotype-treated control groups during 2% TDI sensitization following anti-CD25 treatment (Supplementary Figure 3D).

DISCUSSION

Occupational exposure to sensitizing chemicals that are capable of inducing allergic disease is increasing globally and is an important public health concern. Although there continues to be progress made in the area of hazard identification, the limited knowledge of the immunologic mechanisms of sensitization induced by respiratory sensitizers such as TDI continues to complicate the development of these assays. Novel molecules and mechanisms involved in allergic disease need to be investigated in order to elucidate specific entities that can be utilized for the development of hazard identification assays for respiratory sensitizers. Due to the recognized role of T_{regs} in related allergic disease states, we chose to investigate the expression kinetics, phenotype, and functional capability of T_{regs} in a murine model of dermal TDI sensitization. To our knowledge, this is the first study that investigates the expression and functionality of T_{regs} in Th2-mediated chemical sensitization.

T_{reg} involvement has been suggested in the prevention of the development of allergic disease in both mouse and human models of allergy, specifically impacting Th2-related responses (Robinson *et al.*, 2004). Murine $CD4^+$ $CD25^+$ T cells can prevent the transition of naïve $CD4^+$T cells to Th2 cells *in vitro* (Stassen *et al.*, 2004). In a model of house dust mite antigen-induced airway inflammation decreased airway pathology and *ex vivo*-derived splenic IL-4 and IL-13 levels were observed following T_{reg} adoptive transfer (Chen *et al.*, 2003). Additionally, T_{regs} have been implicated *in vivo*-induced tolerance following inhaled ovalbumin exposure (Ostroukhova *et al.*, 2004), highlighting the importance of these cells in the prevention of allergic disease. Supporting human studies have demonstrated the expansion of T_{regs} in the nasal mucosa following allergen immunotherapy and noted their

association with the efficacy of treatment, suggesting a role for T_{regs} in the development of antigen-specific tolerance in allergic humans (Radulovic *et al.*, 2008). In addition to their direct actions on Th2 cells, T_{regs} have also demonstrated the ability to suppress allergic disease by influencing granulocytes, antibody-producing B cells, and resident tissue cells (Palomares *et al.*, 2010). In addition, T_{regs} have demonstrated involvement in maintaining tolerance and controlling the allergic response during the sensitization phase of both ovalbumin-induced allergic airway inflammation (Baru *et al.*, 2010) and hapten-induced $CD8^+$T cell-dependent contact hypersensitivity response (Christensen *et al.*, 2015).

In an effort to explore the role of T_{regs} in TDI sensitization, this population was profiled following epicutaneous TDI exposure. Several basic T_{reg} markers (CD3/CD4/CD25/Foxp3) were used to define the classical T_{reg} population but it is important to note that some T_{reg} subsets do not express CD25 and/or Foxp3 such as Tr1 (Passerini *et al.*, 2011) and Th3 (Carrier *et al.*, 2007) cells which typically exhibit TGF-β and IL-10-mediated suppression. Due to the use of CD25 as a functional tool in these studies, we have chosen to focus on classical T_{regs}. The basic T_{reg} population encompasses numerous subpopulations including, but not limited to, nT_{regs}, iT_{regs}, migratory and homing T_{regs}, and IL-10 secreting T_{regs}. Beyond the nT_{regs} and iT_{regs}, these subsets are not mutually exclusive, so while phenotyping markers may be presented alone, many of these populations are co-expressers of a variety of markers. In general, the T_{reg} populations (basic and specialized; Table 1) were identified to expand in both number and frequency, peaking around 4–7 days and beginning to retract around 7–9 days following TDI exposure. This retraction may be due to the early importance of this population in the dLN or may also be mediated by other compensatory or regulatory elements. Interestingly, the general T_{reg} population's frequency did not significantly increase following 0.5% TDI exposure (Figure 2B), potentially implicating a role for the irritant response in both the induction of T_{regs} and as a precursor to the initiation of sensitization.

Further support for a role for T_{regs} in TDI sensitization was identified following *in vivo* administration of anti-CD25 treatment. In this T_{reg} depletion study, augmentations in traditional Th2 allergic markers (dermal irritation, dLN cellularity, Th2 population, IL-4 mRNA expression, and serum total IgE production) were observed. Dose-responsive increases in dLN cellularity, IL4, Th2 cells, and IgE were also observed and were further augmented following antiCD25 treatment indicating increased sensitization in the absence of T_{regs}. dLN cellularity was measured as a gross marker of sensitization intensity, as this parameter is traditionally utilized in the standard sensitization assay known as the LLNA (Anderson *et al.*, 2011). The dLN Th2 cellular subset was examined as the main effector T cell subset traditionally thought to be involved in allergic responses, with the allergic cytokine IL-4 being primarily produced by this subset, influencing the development and homeostasis of the allergic microenvironment (Brown, 2008). IgE expression indicates the development of an allergic humoral response. Although T_{reg} depletion was identified to promote a Th2 response, the findings from the T_{reg} suppression assay suggested that T_{regs} isolated from the TDI-sensitized animals were more suppressive compared with their acetone counterparts. These data suggest a significant role for T_{regs} in the regulation of chemical-induced allergy, implying that their presence may delay or reduce the intensity of sensitization.

Typically, the development of allergic disease is thought to involve an imbalance between Th1 and Th2 responses to allergens (Akdis *et al.*, 2004), resulting in an 'overzealous' Th2 response. Although the Th2 response is an important mediator of TDI sensitization, it is important to note that TDI sensitization and resultant allergic disease also contain Th1 and Th17 components (Supplementary Figs. 3G–J) (Liu and Wisnewski, 2003). T_{regs} are capable of suppressing both Th1 (Xu *et al.*, 2012) and Th17 (Jaffar *et al.*, 2009)-mediated responses, indicating that they may be influencing these components of the TDI sensitization response. Selective suppression of the Th1 response during TDI sensitization could further enhance the development of Th2-mediated sensitization based on the Th1/Th2 imbalance hypothesis.

Although T_{regs} isolated from TDI sensitized mice were identified to be more suppressive than acetone-derived T_{regs}, this was reported based as a combined function for all populations of $T_{regs.}$ This is important to note because TDI-induced T_{regs} were identified to be phenotypically heterogeneous. Although the kinetics of the specialized T_{reg} populations generally mimic that of the basic T_{reg} population several discrepancies were identified that may provide insight into the mechanisms driving this response. The $CTLA4^{+}T_{reg}$ population was identified to increase in number and frequency earlier than the general T_{reg} population with significant increases identified one day post exposure. The early expansion of this subset was intriguing, given the lack of any data implying a role for CTLA4-mediated suppression in chemical-induced allergy, although this population has been noted to play an immunosuppressive role in the sensitization phase of the ovalbumin-induced allergic response (Hellings *et al.*, 2002). This population participates in contact-mediated suppression by binding to the B7 costimulatory complex expressed on APCs, thus inhibiting T cell activation in a highly suppressive manner (Tai *et al.*, 2012). Based on the early expansion of this subset, it is possible that these cells are interacting with APCs in the early phase of the sensitization response. nT_{regs} are thymus-derived cells whose counterparts are iT_{regs} generated in the periphery; nT_{regs} are identified by neuropilin-1 expression (Weiss *et al.*, 2012; Yadav *et al.*, 2012). Interestingly, the nT_{reg} frequency significantly decreased between 7 and 9 days following TDI exposure compared with the acetone-treated population. This decrease may be attributed to the efflux of cells from the dLN into peripheral tissues and/or the influx and expansion of iT_{regs} in the dLN. Because certain populations of T_{regs} are known to possess migratory capabilities, the T_{reg} phenotyping panel included several markers associated with T_{reg} migration and effector capabilities including CCR6, CD103, and ICOS. T_{regs} expressing any or all of these markers were defined as migratory effector T_{regs} due to the noted homing capabilities and the effector functions (Chang *et al.*, 2012; Kleinewietfeld *et al.*, 2005; Matsushima and Takashima, 2010; Vocanson *et al.*, 2010; Yamazaki *et al.*, 2008). The expansion of T_{regs} expressing CCR6, CD103, and/or ICOS may represent subsets that have acquired effector functions, such as IL-10 production, and possess migratory capabilities. Taken together, these findings suggest that T_{regs} likely utilize a variety of suppressive mechanisms as indicated by their phenotypic diversity. This could be a potential explanation for why TDI sensitized T_{regs} were identified as more suppressive than their acetone control counterparts.

These studies reveal an important role for T_{regs} in a murine model of TDI sensitization. Elucidation of the role of T_{regs} in this response will result in a better understanding of the immunologic mechanisms involved chemical sensitization. Due to the complexity of

mechanisms involved in chemical allergy, the investigation of novel cellular subsets and mediators of allergic disease is imperative for the greater understanding of these conditions and the development of hazard identification strategies for respiratory chemical sensitizers. In conclusion, we have demonstrated that T_{regs} are a phenotypically heterogeneous population that expand, suppress, and play a part in controlling the allergic response during TDI sensitization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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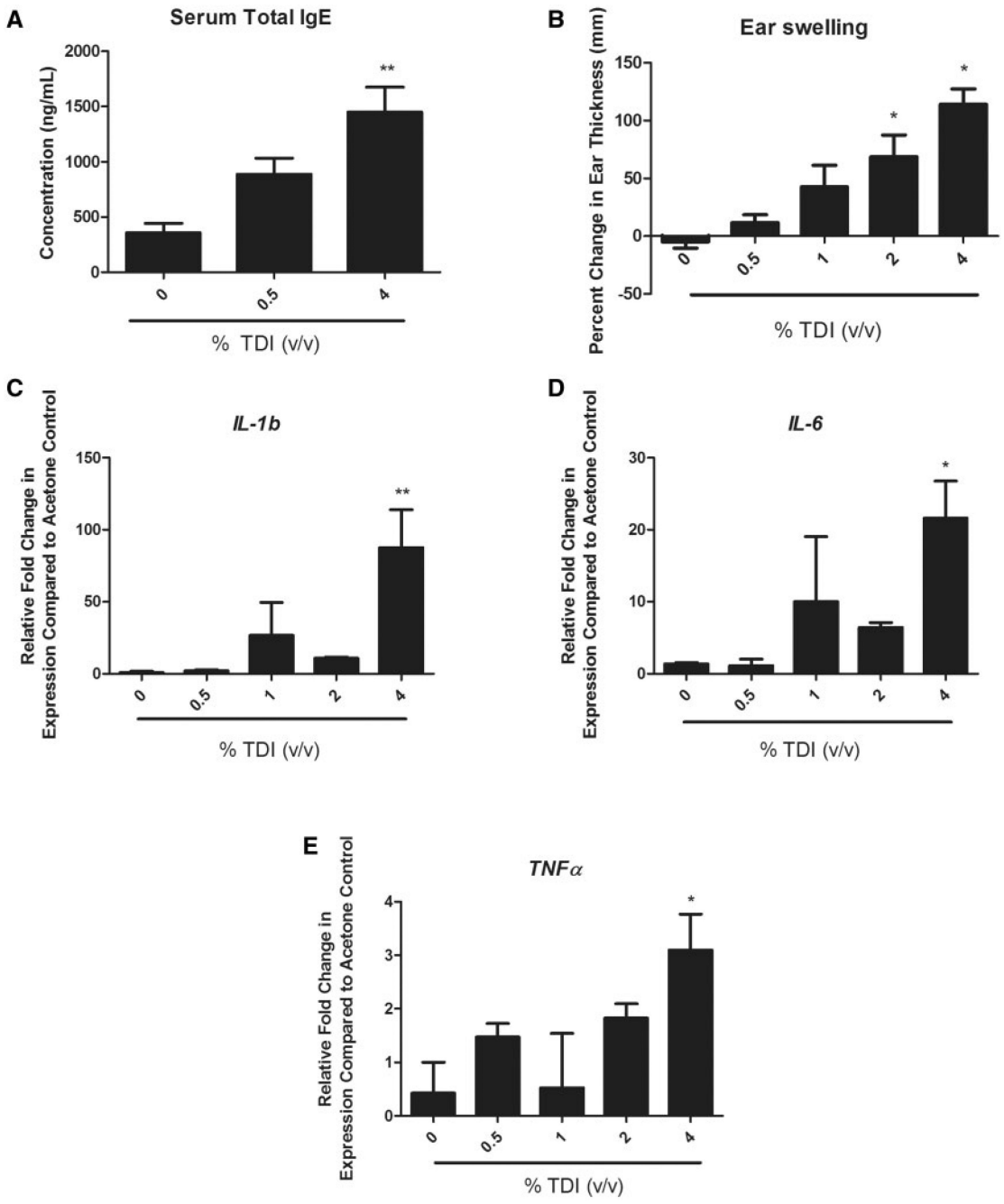

FIG. 1.

Confirmation of sensitization and evaluation of skin irritancy following dermal TDI exposure. ELISA analysis of total serum IgE levels 11 days following single TDI exposure at the indicated concentration **(A)**. Percent change in ear thickness as determined 4 days following TDI exposure **(B)**. Ear mRNA expression of the inflammatory cytokines *il-1β* **(C)**, *il*-6 **(D)**, and *tnf-α* **(E)** as determined 4 days following TDI exposure via RT-PCR. Bars represent mean (± SE) of 4–5 mice per group. Statistical significance is indicated by (*) at a *P*-value < .05 and (**) at a *P*-value < .01.

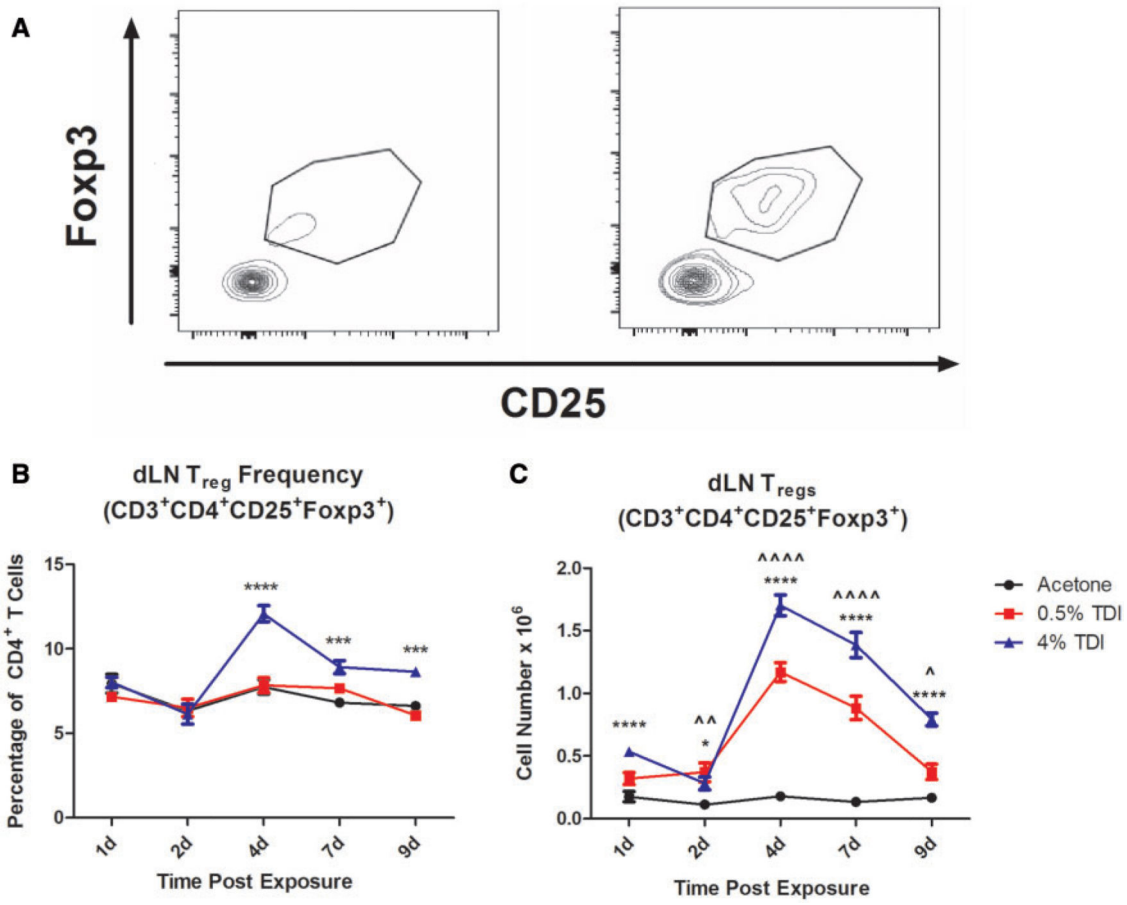

FIG. 2.

T_{reg} subset expands during dermal TDI sensitization. Flow cytometric analysis of T_{regs} following dermal TDI sensitization **(A)** T_{regs} were first gated on their expression of CD3 and CD4, then were further identified by CD25 and Foxp3 expression at indicated time points. dLN T_{reg} frequency **(B)** and number **(C)** were determined based on flow cytometry analysis and extrapolation of this data with total dLN cellularity. Graph symbols represent mean (± SE) of 5 mice per group. *P*-values are represented by (0.5% TDI) and asterisks (4% TDI) ($P < .05$). Significance is indicated as follows: $P \leq .05$ (*), $P \leq .01$ (**), $P \leq .001$ (***), and $P \leq .0001$ (****) for 4% TDI or $P \leq .05$ (^), $P \leq .01$ (∿), $P \leq .001$ (^), and $P \leq .0001$ (∿∿) for 0.5% TDI. Dermal treatment groups are indicated by the following symbols: circle, acetone; square, 0.5% TDI; and triangle, 4% TDI.

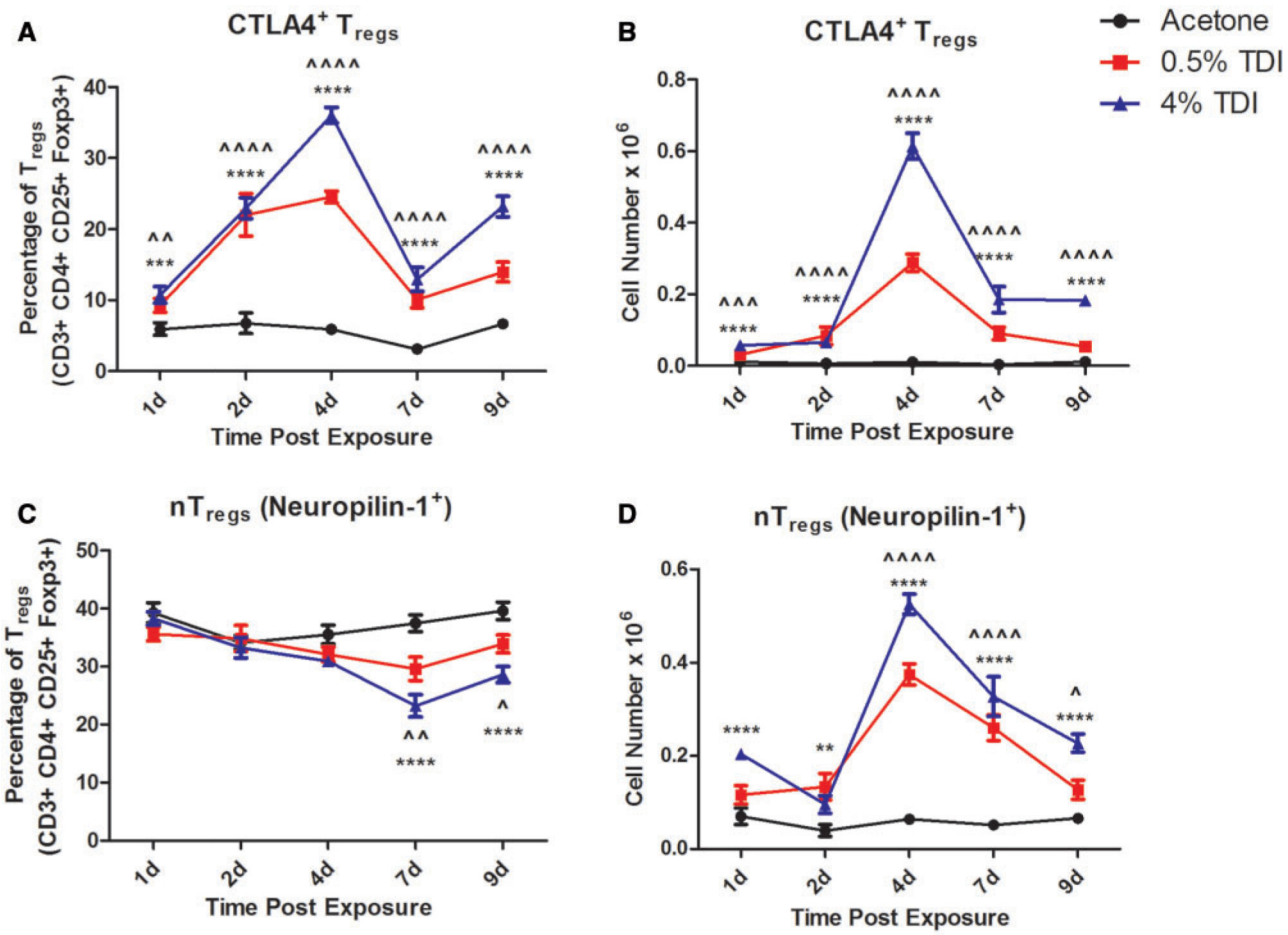

FIG. 3.

Expansion of CTLA4$^+$ and nT$_{reg}$ populations during TDI sensitization. Flow cytometric analysis of dLN CTLA4$^+$ T$_{reg}$ frequency **(A)** and number **(B)** and nT$_{reg}$ (Neuropilin-1$^+$) frequency **(C)** and number **(D)** following TDI sensitization. Bars represent mean (± SE) of 5 mice per group. *P* values are represented by (0.5% TDI) and asterisks (4% TDI) ($P < 0.05$). Significance is indicated as follows: $P \leq .05$ (*), $P < .01$ (**), $P \leq .001$ (***), and $P \leq .0001$ (****) for 4% TDI or $P \leq .05$ (^), $P \leq .01$ (^^), $P \leq .001$ (^^^), and $P \leq .0001$ (^^^^) for 0.5% TDI. Dermal treatment groups are indicated by the following symbols: circle, acetone; square, 0.5% TDI; and triangle, 4% TDI.

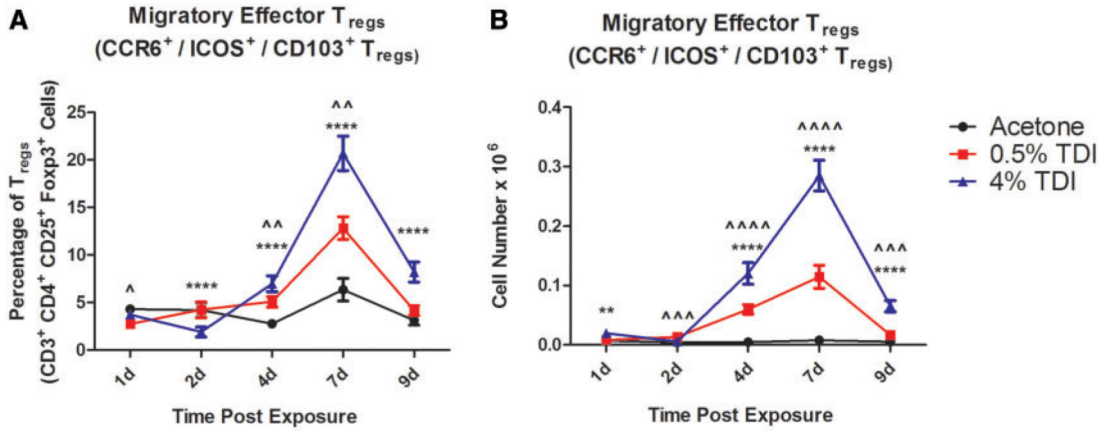

FIG. 4.

dLN migratory effector T_{reg} population expands during TDI sensitization (co-expression). dLN $CCR6^+$ $CD103^+$ $ICOS^+$ T_{reg} frequency **(A)** and number **(B)** were determined based on flow cytometry analysis and extrapolation of this data with total dLN cellularity. Bars represent mean (± SE) of 5 mice per group. *P* values are represented by (0.5% TDI) and asterisks (4% TDI) ($P < .05$). Significance is indicated as follows: $P \leq .05$ (*), $P \leq .01$ (**), $P \leq .001$ (***), and $P \leq .0001$ (****) for 4% TDI or $P \leq .05$ (^), $P \leq .01$ (∿), $P \leq .001$ (^), and $P \leq .0001$ (∿∿) for 0.5% TDI. Dermal treatment groups are indicated by the following symbols: circle, acetone; square, 0.5% TDI; and triangle, 4% TDI.

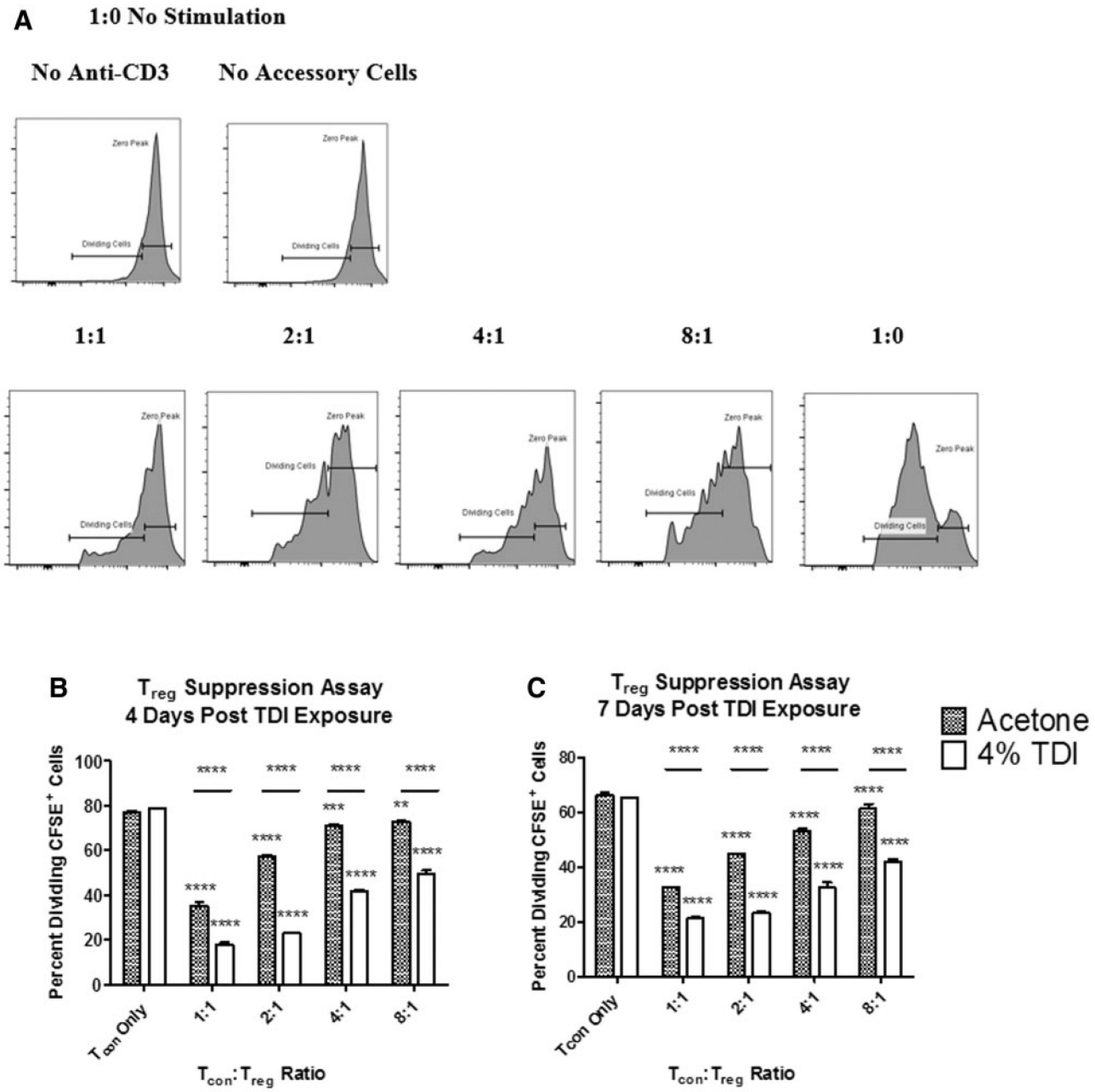

FIG. 5.

T_{reg} suppression assay reveals increased suppressive ability of T_{regs} during TDI sensitization. A CFSE-based T_{reg} suppression assay was performed and percent dividing $CFSE^+$ cells were quantified as illustrated in **(A)**. The zero peak represents the cell population that retained all original CFSE stain. The percent dividing $CFSE^+$ cells (T_{cons}) are represented in combination with T_{regs} from mice 4 **(B)** and 7 **(C)** days following TDI exposure at a variety of T_{con}:T_{reg} ratios. For **(B)** and **(C)** 3 plate replicates were utilized from groups of 4–11 mice, as described in the 'Materials and Methods' section. Significance is indicated as follows: $P \leq .05$ (*), $P \leq .01$ (**), $P \leq .001$ (***), and $P \leq .0001$ (****). P values are represented by asterisks (comparison of each treatment group to T_{con} only from the same

chemical treatment group) or horizontal bars with asterisks above (comparison of identical ratios between different chemical treatment groups).

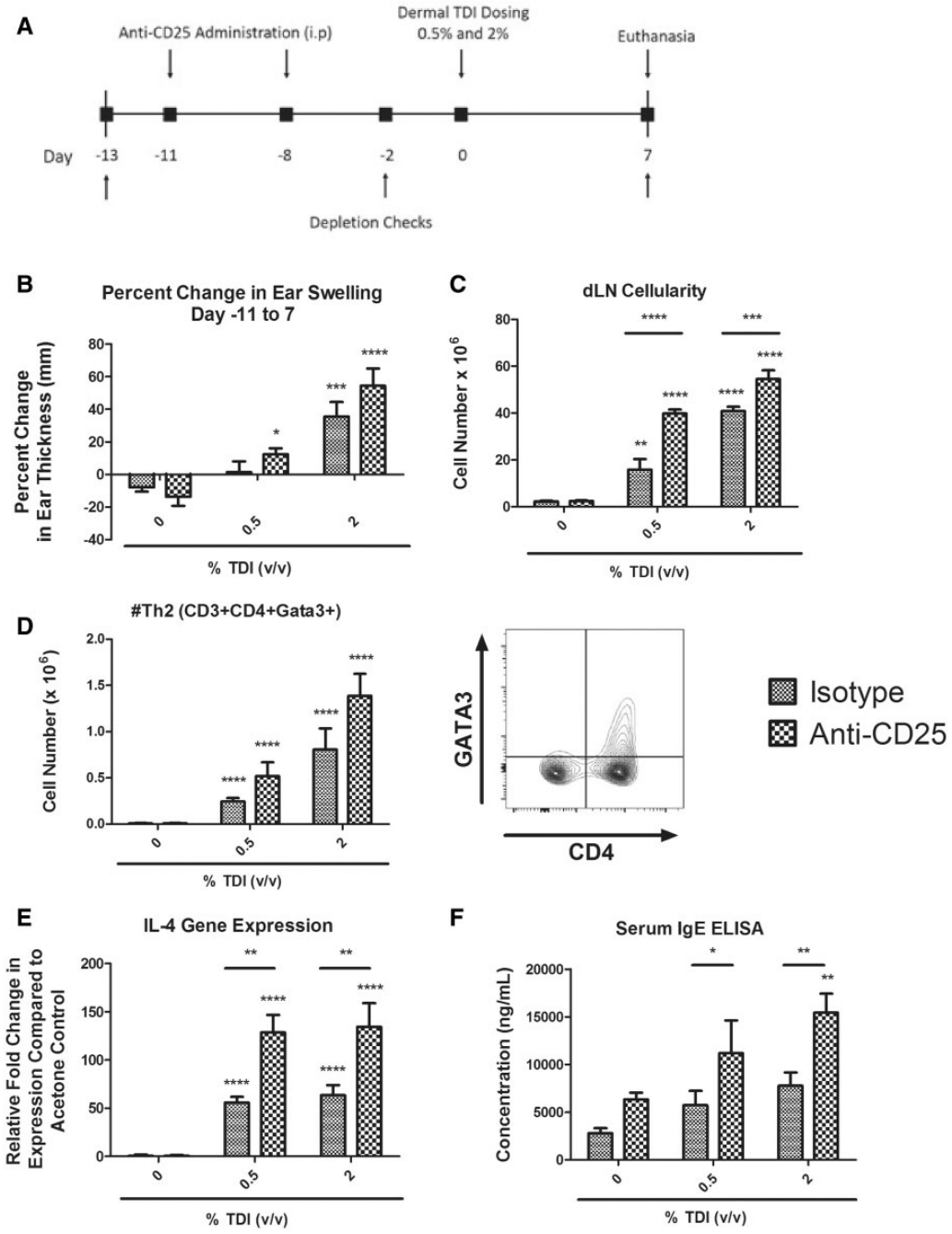

FIG. 6.

The severity of the sensitization response is intensified in the absence of T_{regs} during TDI sensitization. T_{regs} were depleted in mice prior to and during dermal TDI sensitization **(A)**. Evaluation of dermal irritancy **(B)**, dLN cellularity **(C)**, dLN Th2 population **(D)**, dLN IL-4 gene expression **(E)**, and serum total IgE **(F)** were evaluated following a single exposure of 0.5 or 2% TDI with isotype control or anti-CD25. Bars represent mean (± SE) of 5 mice per group. Significance is indicated as follows: $P \leq .05$ (*), $P \leq .01$ (**), $P \leq .001$ (***), and $P \leq .0001$ (****). P values are represented by asterisks (comparison of acetone to TDI-

exposed group from the same antibody treatment regimen) or horizontal bars with asterisks above (comparison of antibody and isotype-treated groups receiving identical chemical treatment).

TABLE 1

T_{reg} Flow Cytometry Phenotyping Marker Guide

Marker	Abbreviation	Surface or Intracellular Detection	Significance	T Cell Populations Expressing
Cluster of differentiation 3	CD3	S	Pan T cell marker	All T cells
Cluster of differentiation 4	CD4	S	$CD4^+$ T cell marker	$CD4^+$ T cells
Cluster of differentiation 25/ IL2rα	CD25	S	High affinity IL-2 receptor α, T and B cell growth factor (via IL-2 binding) (Fontenot *et al.*, 2005; Lowenthal *et al.*, 1985)	T_{regs}, some activated T cells (non-T_{regs})
Forkhead box protein 3	Foxp3	IC	Master T_{reg} transcription factor (Hori *et al.*, 2003)	T_{regs}
Chemokine (CC-motif) receptor 6	CCR6	S	Lymphocyte chemoattractant CCL20 is its ligand (Schutyser *et al.*, 2003)	Migratory Effector T_{regs} (Kleinewietfeld, *et al.*, 2005; Yamazaki *et al.*, 2008) and $CD8^+$ T cells (Kondo *et al.*, 2007)
Cluster of differentiation 103	CD103	S	Integrin involved in epithelial T cell migration and retention (Anz *et al.*, 2011)	Migratory Effector T_{regs} (Matsushima and Takashima, 2010) and tissue resident $CD8^+$ T cells (Mackay *et al.*, 2013)
Cytotoxic T-Lymphocyte-Associated Protein 4	CTLA4/CD152	IC	Member of the CD28 family that is a potent inhibitor of T cell costimulation (Frauwirth and Thompson, 2002)	T_{regs} and activated T cells (non-T_{regs})
Inducible T-cell costimulator	ICOS/CD278	S	Member of the CD28 family that has costimulatory functionality during T cell activation (Hutloff *et al.*, 1999)	Migratory Effector T_{regs} (Vocanson, *et al.*, 2010) and activated T cells
Neuropilin-1	Neuropilin-1	S	Receptor for vascular endothelial growth factors and semaphorins (Gu *et al.*, 2003)	nT_{regs} (Weiss *et al.*, 2012; Yadav *et al.*, 2012), T_{FH} cells (Renand *et al.*, 2013), and $CD8^+$ T cells (Jackson *et al.*, 2014)

TABLE 2

dLN Migratory Effector T_{reg} Population Expands During TDI Sensitization

$CCR6^+$ % (of T_{regs})	**1 day**	**2 day**	**4 day**	**7 day**	**9 day**
Acetone	11.14 ± 0.7	12.72 ± 1.03	10.86 ± 0.55	18.22 ± 1.36	9.23 ± 0.8
0.5% TDI	10.22 ± 0.68	17.7 ± 1.64**	20.58 ± 0.84****	26.54 ± 0.68****	14.82 ± 0.99**
4% TDI	10.02 ± 0.92	10.2 ± 1.1	30.7 ± 1.75****	40.42 ± 1.87****	25.02 ± 1.98****
$CCR6^+$ #(× 10^5 cells)					
Acetone	0.2 ± 0.05	0.14 ± 0.04	0.19 ± 0.02	0.24 ± 0.04	0.16 ± 0.03
0.5% TDI	0.34 ± 0.06*	0.61 ± 0.12****	2.41 ± 0.19****	2.35 ± 0.28****	0.57 ± 0.13****
4% TDI	0.533 ± 0.05****	0.3 ± 0.07**	5.25 ± 0.48****	5.59 ± 0.41****	1.99 ± 0.2****
$CD103^+$ % (of T_{regs})	**1 day**	**2 day**	**4 day**	**7 day**	**9 day**
Acetone	22.86 ± 0.75	18.88 ± 4.24	20.82 ± 0.94	28.44 ± 2	21.18 ± 0.92
0.5% TDI	18.38 ± 0.84	24.44 ± 2.99*	35.88 ± 0.84****	38.8 ± 1.1***	32.72 ± 2.23****
4% TDI	19.62 ± 1.25	16.5 ± 1.64	43.18 ± 1.78****	52.12 ± 1.36****	44.64 ± 1.45****
$CD103^+$ #(× 10^5 cells)					
Acetone	0.41 ± 0.09	0.16 ± 0.03	0.37 ± 0.04	0.38 ± 0.07	0.36 ± 0.06
0.5% TDI	0.59 ± 0.1	0.83 ± 0.15****	4.21 ± 0.33****	3.47 ± 0.45****	1.22 ± 0.23****
4% TDI	1.04 ± 0.06****	0.48 ± 0.11****	7.4 ± 0.63****	7.23 ± 0.56****	3.53 ± 0.23****
$ICOS^+$ % (of T_{regs})	**1 day**	**2 day**	**4 day**	**7 day**	**9 day**
Acetone	13.38 ± 0.36	15.92 ± 1.21	13.56 ± 0.41	18.7 ± 1.22	15.98 ± 1.24
0.5% TDI	14.06 ± 1.03	36.42 ± 2.16****	47.8 ± 1.15****	38.38 ± 1.72****	33.36 ± 2****
4% TDI	16.68 ± 1.45	33.18 ± 1.27****	62.64 ± 1.03****	55.02 ± 2.12****	45.54 ± 1.78****
$ICOS^+$ # (× 10^5 cells)					
Acetone	0.23 ± 0.05	0.17 ± 0.04	0.24 ± 0.02	0.25 ± 0.04	0.28 ± 0.5
0.5% TDI	0.46 ± 0.09**	1.31 ± 0.27****	5.61 ± 0.45****	3.42 ± 0.45****	1.27 ± 0.26****
4% TDI	0.88 ± 0.09****	0.94 ± 0.18****	10.68 ± 0.62****	7.62 ± 0.62****	3.62 ± 0.29****

Data represents mean frequency and number of each T_{reg} population 1–9 days following chemical exposure. Significance is indicated as follows:

* $P \leq .05$

** $P \leq .01$

*** $P \leq .001$

**** and $P \leq .0001$ for each group compared with the acetone control value from the matching time point.