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Role of CD38 in TNF- α -induced airway hyperresponsiveness

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Departments of ¹Veterinary Small Animal Clinical Sciences, Texas A&M University, College Station, Texas; ²Veterinary and Biomedical Sciences, ³Veterinary Population Medicine, and ⁶Pediatrics, University of Minnesota, St. Paul, Minnesota; ⁴Department of Medicine and Immunology, Mayo Clinic, Rochester, Minnesota; and ⁵Trudeau Institute, Saranac Lake, New York

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Guedes AG, Jude JA, Paulin J, Kita H, Lund FE, Kannan MS. Role of CD38 in TNF- α -induced airway hyperresponsiveness. *Am J Physiol Lung Cell Mol Physiol* 294: L290–L299, 2008. First published November 30, 2007; doi:10.1152/ajplung.00367.2007.—CD38 is involved in normal airway function, IL-13-induced airway hyperresponsiveness (AHR), and is also regulated by tumor necrosis factor (TNF)- α in airway smooth muscle (ASM) cells. This study aimed to determine whether TNF- α -induced CD38 upregulation in ASM cells contributes to AHR, a hallmark of asthma. We hypothesized that AHR would be attenuated in TNF- α -exposed CD38-deficient (CD38KO) mice compared with wild-type (WT) controls. Mice ($n = 6$ –8/group) were intranasally challenged with vehicle control or TNF- α (50 ng) once and every other day during 1 or 4 wk. Lung inflammation and AHR, measured by changes in lung resistance after inhaled methacholine, were assessed 24 h following the last challenge. Tracheal rings were incubated with TNF- α (50 ng/ml) to assess contractile changes in the ASM. While a single TNF- α challenge caused no airway inflammation, both multiple-challenge protocols induced equally significant inflammation in CD38KO and WT mice. A single intranasal TNF- α challenge induced AHR in the WT but not in the CD38KO mice, whereas both mice developed AHR after 1 wk of challenges. The AHR was suppressed by extending the challenges for 4 wk in both mice, although to a larger magnitude in the WT than in the CD38KO mice. TNF- α increased ASM contractile properties in tracheal rings from WT but not from CD38KO mice. In conclusion, CD38 contributes to TNF- α -induced AHR after a brief airway exposure to the cytokine, likely by mediating changes in ASM contractile responses, and is associated with greater AHR remission following chronic airway exposure to TNF- α . The mechanisms involved in this remission remain to be determined.

tumor necrosis factor- α

CYTOKINES PLAY A FUNDAMENTAL ROLE in the pathogenesis of asthma (46), a pulmonary disease characterized by airway inflammation, airflow obstruction, airway remodeling, and airway hyperresponsiveness (AHR) (7). Asthma has been classically thought as a T-helper type 2 (Th2)-polarized airway disease. Pivotal Th2 cytokines such as interleukin (IL)-4, IL-5, and IL-13 have been identified in the bronchoalveolar space and lung parenchyma of asthmatic patients (47, 59), and their pathological roles are confirmed in studies with murine models of the disease (38, 58, 60, 61). However, many other cytokines, some not belonging to the Th2 phenotype, have also been associated with the asthmatic inflammatory response. Examples include IL-1 β , IL-2, granulocyte/macrophage colony-

stimulating factor (GM-CSF), interferon- γ (IFN- γ), and tumor necrosis factor- α (TNF- α) (1, 5, 22, 31, 51, 52). An important target of many of these mediators is the airway smooth muscle (ASM) (23, 40, 49, 63) whereby ASM cells from asthmatic patients become hypercontractile when compared with cells from normal subjects (39) with changes in intracellular calcium ($[Ca^{2+}]_i$) homeostasis being one important underlying mechanism (3, 23, 50).

CD38, a 45-kDa multifunctional ectoenzyme present in ASM cells, has an important role in the regulation of $[Ca^{2+}]_i$ homeostasis and airway function through the synthesis and degradation of cyclic ADP-ribose (cADPR) (13, 14, 25, 45). Increased CD38 expression based on immunophenotyping has been reported in patients with asthma and other allergic manifestations (17). In human ASM cells, multiple studies have shown that the CD38/cADPR signaling mediates $[Ca^{2+}]_i$ hyperresponsiveness to contractile agonists with potential relevance to inflammatory airway diseases (13, 29, 30, 55). For example, exposure of human ASM cells to IL-1 β , IFN- γ , TNF- α , and IL-13 significantly increased CD38 expression and enzymatic activity and resulted in heightened $[Ca^{2+}]_i$ responses to a variety of contractile agonists. Additionally, the observed $[Ca^{2+}]_i$ hyperresponsiveness could be significantly attenuated by the addition of 8-bromo-cADPR, a cADPR antagonist (2, 11–13, 29, 30, 55, 57). Using CD38-deficient (CD38KO) mice, we have documented that CD38 is the major, if not the only, source of cADPR in the lungs and that the CD38/cADPR signaling in the ASM has clear implications to normal airway function (14). More recently, we also demonstrated that when these mice are challenged intranasally with recombinant murine IL-13 (mu rIL-13), the resulting AHR to inhaled methacholine is much more attenuated compared with wild-type (WT) controls (21). In that study, airway inflammation was robust and comparable in all mu rIL-13-treated mice, but the ASM reactivity to carbachol as measured in tracheal segments incubated with mu rIL-13 was significantly altered only in the presence of CD38. This indicated that CD38 was not required for the development of inflammation but was clearly an important mediator of IL-13-induced ASM hyperresponsiveness. Relaxation responses to isoproterenol were attenuated following IL-13 to a comparable degree in WT and CD38KO mice (21), suggesting that CD38 contributes to the IL-13-induced changes in the contractile capacity but not to the relaxation properties of ASM.

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A number of studies have documented altered contractile properties of ASM upon exposure to TNF- α (6, 8, 20, 32, 36, 56). This cytokine has also been shown to be a potent regulator of the expression and enzymatic activity of CD38 in ASM cells (12, 29, 30). Recent studies from our laboratory (30, 54) have demonstrated that this involves activation of the transcription factors NF- κ B and AP-1 through mitogen-activated protein kinase (MAPK)-dependent pathways. The MAPKs mediate not only transcriptional events but also affect message stability posttranscriptionally (54). Thus, the expression and enzymatic activity of CD38 in ASM cells is highly regulated by inflammatory mediators that are linked to the asthmatic phenotype. In the study reported here, we sought to determine whether the effects of TNF- α on CD38 expression and enzymatic activity leading to $[Ca^{2+}]_i$ hyperresponsiveness previously shown in ASM cells (12, 29, 30) are operative elements during AHR *in vivo*. We found that the CD38 contribution to TNF- α -induced AHR varies depending on the length of exposure of the airways to the cytokine. As such, CD38 contributes to TNF- α -induced AHR after a brief airway exposure to the cytokine, likely by mediating changes in ASM contractile responses; but, it is associated with greater AHR remission following an extended airway exposure to TNF- α .

MATERIALS AND METHODS

Animals. Specific pathogen-free, 8- to 12-wk-old C57BL/6J WT and CD38KO mice (backcrossed 12 generations to C57BL/6J) were obtained by inhouse breeding. The original breeding pairs were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were housed in a 12-h light-dark schedule with food and water available *ad libitum*. The

study was approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Intranasal challenge with recombinant murine TNF- α . WT and CD38KO mice ($n = 6$ –8/group) were anesthetized with isoflurane and intranasally challenged with 50 ng of recombinant murine TNF- α (mu rTNF- α ; Sigma-Aldrich, St. Louis, MO) dissolved in nonpyrogenic sterile water or with vehicle control in a total volume of 50 μ l. The mice were intranasally challenged once or every other day during 1 or 4 wk. Measurement of airway responsiveness to inhaled methacholine, airway inflammation, and bronchoalveolar lavage fluid cytokine concentrations were performed 24 h following the last intranasal challenge, as described below.

Airway responsiveness to methacholine. Respiratory resistance (R_L) was measured in anesthetized (pentobarbital sodium 100 mg/kg ip), paralyzed (pancuronium bromide 6 μ g/g ip), tracheostomized, intubated, and mechanically ventilated mice as described previously (14) using whole body plethysmography (Buxco Electronics, Sharon, CT). R_L was measured in response to increasing concentrations of inhaled methacholine (0, 6.25, 12.5, 25, 50, and 100 mg/ml saline). As determined by preliminary experiments, each dose of methacholine was delivered in a volume of 2 μ l via an inline ultrasonic nebulizer during 10 consecutive breaths. During each nebulization, the respiratory rate was reduced to 100 breaths/minute with the tidal volume increased to 350 μ l, and then returned to prenebulization values. Data were collected continuously for 3 min after each nebulization and stored on a desktop computer. Peak R_L to airflow was obtained and analyzed offline. The dose-response curves were further evaluated by regression analysis to obtain indices of airway reactivity (slope) and sensitivity (lowest dose to produce bronchoconstriction) as described previously (37). Briefly, the reactivity was obtained by calculating the slope of the dose-response relationship by linear regression, whereas the sensitivity was determined as the intercept between the dose-response regression line and the baseline respiratory resistance.

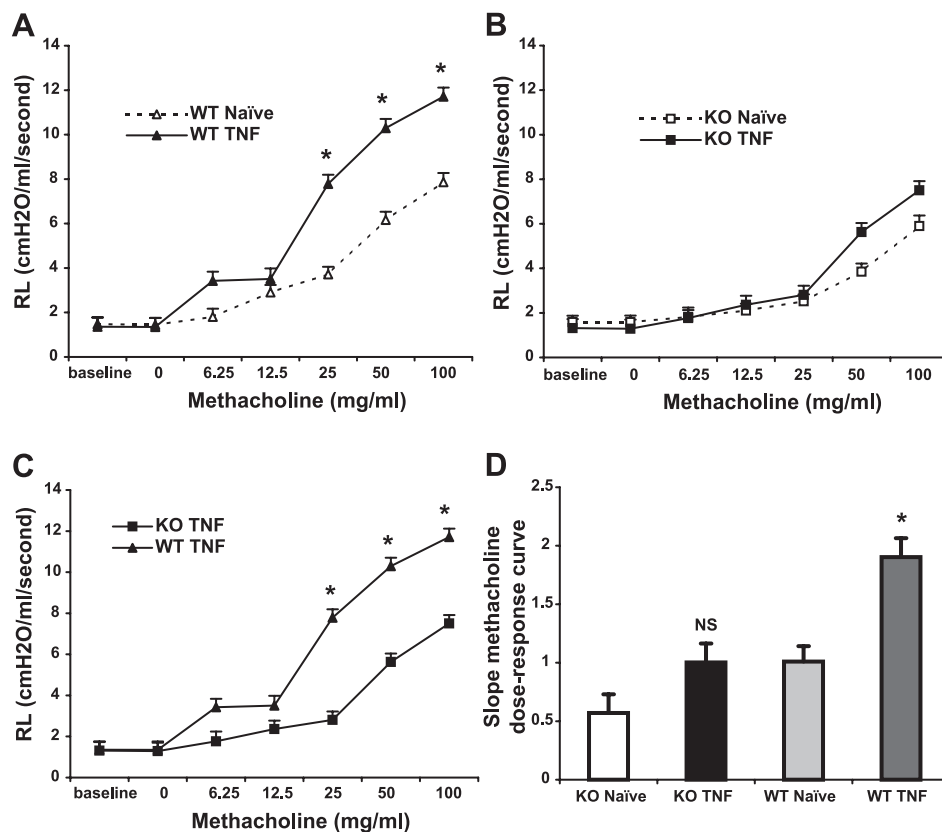


Fig. 1. A single challenge with recombinant murine TNF- α (mu rTNF- α) induces airway hyperresponsiveness in the wild-type (WT) but not in the CD38-deficient (CD38KO) mice. **A:** changes in lung resistance (R_L) in response to different doses of methacholine in WT naïve and TNF- α -treated mice. **B:** changes in R_L in response to different doses of methacholine in CD38KO naïve (KO Naïve) and TNF- α -treated (KO TNF) mice. **C:** changes in R_L in response to different doses of methacholine in the TNF- α -treated and CD38KO (KO TNF) mice. **D:** a single airway exposure to TNF- α significantly increases airway reactivity to inhaled methacholine in the WT mice but not in the CD38 KO mice. Values are shown as means $\pm$ SE ($n = 6$ –8/group). * $P < 0.05$.

Inflammatory cell numbers in the bronchoalveolar lavage fluid. Following measurement of methacholine responsiveness, the mice were euthanized, the right lung was isolated, and the left lung was lavaged three times with 0.5-ml aliquots of sterile HBSS containing 3 mM EDTA. After total inflammatory cell counting using a hemocytometer, the retrieved bronchoalveolar lavage (BAL) fluid was centrifuged at 2,000 rpm for 5 min, and the upper $\frac{2}{3}$ of the supernatant was carefully removed and stored at -80°C for cytokine/chemokine measurements. Cytospin slides were prepared and stained with Diff-Quick stain for differential cell counts (macrophages, neutrophils, lymphocytes, and eosinophils) based on cell morphology by counting 200 cells/slide. An investigator unaware of the identity of each slide performed all cell counts.

Histopathological analysis of lung sections. After the lung function experiments, the right lung was collected for evaluation of parenchymal inflammatory changes. The lung tissue was fixed in 10% phosphate-buffered formalin and prepared as described previously (21). After being stained with hematoxylin and eosin, the inflammatory infiltrate in areas representing central and peripheral airways were evaluated under light microscopy as described elsewhere (18). Briefly, peribronchiolar, perivascular, and alveolar inflammatory changes were evaluated under $\times 200$ magnifications by a pathologist unaware of the identity of the specimens, and scores indicating the degree of inflammatory changes were attributed to each area.

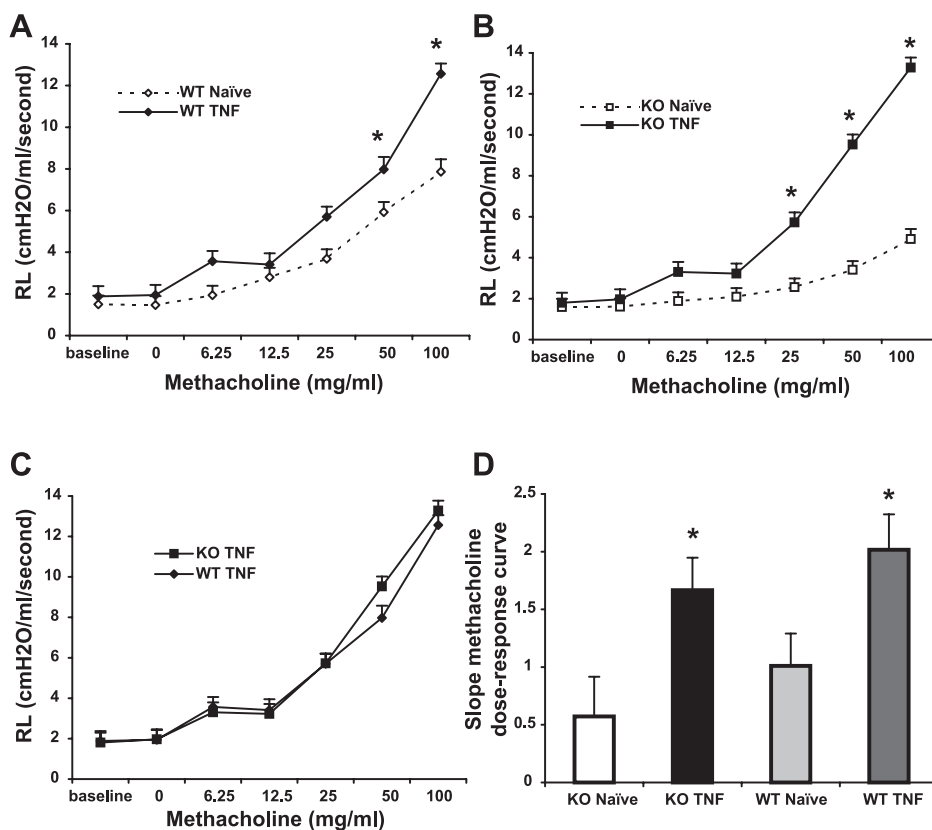
Cytokine and chemokine levels in the BAL fluid. The concentrations of IL-6, IL-10, IL-12 (p70), IFN- γ , and MCP-1 in the BAL fluid supernatants were measured with the use of a mouse inflammatory cytokine cytometric bead array kit (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's recommendations. The lower limits of detection were 20 pg/ml for MCP-1, 3 pg/ml for IFN- γ , 10 pg/ml for IL-12 (p70), 10 pg/ml for TNF- α , 10 pg/ml for IL-10, and 6 pg/ml for IL-6.

Isometric force measurement in the isolated trachea. As described previously (21), cervical tracheal segments from WT and CD38KO

mice of ~ 4 mm in length ($n = 8-10/\text{group}$) were carefully removed and incubated at 37°C in 5% CO_2 for 12–14 h with the addition of 50 ng of mu rTNF- α per milliliter of medium (DMEM with no serum and supplemented with 5.7 $\mu\text{g/ml}$ insulin and 5 $\mu\text{g/ml}$ transferrin). After incubation, the tracheas were mounted with stainless steel wires on the jaws of a horizontal myograph system (Multi Myograph System 610M; Danish Myo Technology, Denmark) for measurement of isometric force in response to carbachol. The 5-ml volume bath containing HBSS was maintained at a constant temperature of 37°C and with a continuous oxygen flow of 10 ml/min. All tracheal segments were maintained under a steady tension of 0.5 g for 45 min before baseline measurements and carbachol stimulation. With the use of a digital multiple syringe pump (WPI instruments, Longmont, CO), a carbachol solution (1 mM) was infused into each 5-ml volume organ bath at a constant rate of 5 $\mu\text{l/min}$ for 15 min. This approach produced a linear increase in the carbachol concentration within the organ bath to a maximum concentration of 15 μM at the end of the infusion. This approach allowed for the determination of ASM sensitivity and reactivity to carbachol. As such, the minimum concentration of carbachol within the organ bath at which the contraction was elicited was taken as a measure of sensitivity. Reactivity was defined as the maximum rate of rise in tension in response to the carbachol infusion.

Sample size and statistical analysis. Sample size was determined using data from preliminary studies considering a power of 0.8 and a 95% confidence interval. Inflammatory cell counts and cytokine concentrations in the BAL fluid were analyzed with one-way ANOVA, R_L values were subjected to repeated measures ANOVA, and inflammatory scores of lung parenchyma were subjected to Kruskal-Wallis one-way ANOVA on ranks. Tukey-Kramer multiple comparison test was used to detect statistically significant differences between means. Statistical analysis was performed with commercial statistic software (NCSS, Kaysville, UT), with $P < 0.05$ considered statistically significant. Parametric data are shown as means $\pm$ SE and nonparametric data as ranks of inflammatory scores.

Fig. 2. Changes in R_L following airway challenges with TNF- α on alternate days for 1 wk in CD38KO and WT mice reveal airway hyperresponsiveness in both WT and CD38KO mice. A: changes in R_L in response to different doses of methacholine in WT naïve and TNF- α -treated mice. B: changes in R_L in response to different doses of methacholine in CD38KO naïve (KO Naïve) and TNF- α -treated (KO TNF) mice. C: methacholine-induced changes in R_L in the TNF- α -treated and CD38KO (KO TNF) mice. Note similar degrees of airway hyperresponsiveness in WT and CD38KO mice. D: airway reactivity to inhaled methacholine following 3 intranasal challenges with TNF- α increases significantly and to similar extents in both WT mice and CD38 KO mice compared with respective naïve controls. Values are shown as means $\pm$ SE ($n = 6-8/\text{group}$). $*P < 0.05$.



RESULTS

AHR to methacholine. We have previously shown that the CD38/cADPR pathway plays a role in TNF- α and IL-13-induced calcium modulation in human ASM cells (11) and that CD38 contributes to normal airway function in vivo (14). Subsequently, we demonstrated that AHR to inhaled methacholine is significantly attenuated in mu rIL-13-challenged CD38KO mice relative to WT mice (21). In the present study, when naïve animals were exposed to increasing amounts of methacholine, the changes in R_L were significantly higher in the WT than in the CD38KO mice, confirming our previous findings (14). To test the role of CD38 in TNF- α -induced AHR, the mice were intranasally exposed to the cytokine for 1 day (single exposure), 1 wk (3 times on alternate days), or during 4 wk (every other day), with the studies being performed 24 h after the last intranasal challenge.

A one-day exposure of the airways to mu rTNF- α produced a significantly greater methacholine-induced R_L (e.g., hyperresponsiveness) in the WT mice than in the CD38KO animals (Fig. 1, A–C). Analysis of the methacholine dose-response curves indicated a significant increase in airway reactivity (slope) in the WT animals, whereas airway reactivity in the CD38KO mice did not change compared with respective naïve controls (Fig. 1D). The airway sensitivity, as indicated by the minimum amount of methacholine to trigger bronchoconstriction (e.g., the intercept of the dose-response curve), did not change following exposure to TNF- α in any of the groups (not shown). Following a 1-wk exposure to mu rTNF- α , methacholine-induced

changes in R_L increased significantly in both WT and CD38KO mice compared with each naïve control (Fig. 2, A–C). The slope of the dose-response curve (e.g., airway reactivity) increased significantly and to similar magnitudes in both WT and CD38KO mice following this TNF- α exposure protocol (Fig. 2D). Airway sensitivity was not significantly changed (not shown).

Because our data indicated differences in airway responsiveness to inhaled methacholine upon a single or multiple exposures of the airways to TNF- α , the effects of a more chronic exposure were explored. The mice underwent intranasal challenges with mu rTNF- α on alternate days during four consecutive weeks. This protocol did not cause AHR in any of the groups (Fig. 3, A–C). In the WT mice, this prolonged exposure to mu rTNF- α not only did not cause AHR but significantly attenuated airway responsiveness to levels below naïve conditions (Fig. 3A). The methacholine-induced R_L did not change in the mu rTNF- α -treated CD38KO mice compared with the respective naïve controls (Fig. 3B). Comparison between mu rTNF- α -treated WT and CD38KO mice indicated similar airway responsiveness to methacholine, with the dose-response curves for R_L almost superimposed on each other (Fig. 3C). Analysis of the slope of the methacholine dose-response curves indicated a slight reduction in airway reactivity in the WT mice following this prolonged exposure to mu rTNF- α , although it was not statistically significant (Fig. 3D). As in the previous protocols, airway sensitivity was unchanged following mu rTNF- α compared with naïve controls (not shown).

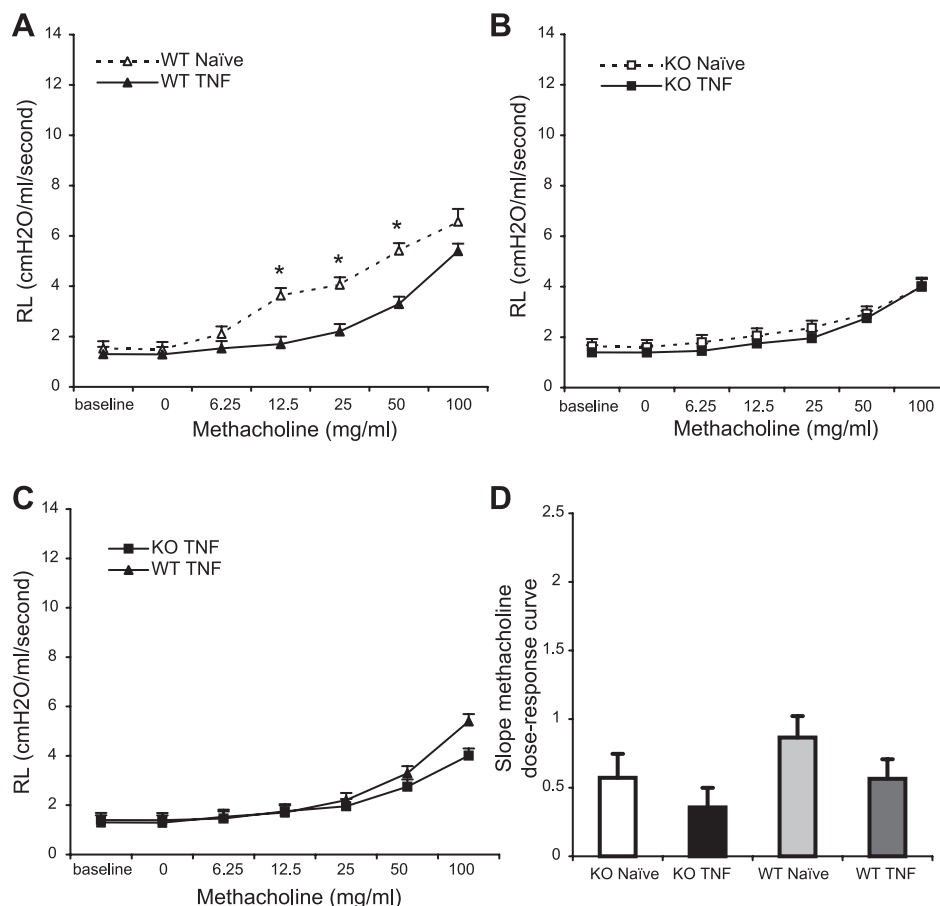


Fig. 3. A 4-wk challenge with TNF- α administered intranasally every other day caused airway hyperresponsiveness in the WT mice and did not change the responsiveness of CD38KO animals. A: changes in R_L in response to different doses of methacholine in wild-type naïve and TNF- α -treated mice. Note a significant reduction in methacholine-induced changes in R_L following TNF- α exposure compared with the naïve animals. B: changes in R_L in response to different doses of methacholine in CD38KO naïve (KO Naïve) and TNF- α -treated (KO TNF) mice. Note that TNF- α treatment did not alter airway responsiveness compared with responses in the naïve mice. C: methacholine-induced changes in R_L in the TNF- α -treated and CD38KO (KO TNF) mice. Note that the chronic TNF- α treatment suppressed airway responsiveness in the WT mice to CD38KO levels. D: airway reactivity to inhaled methacholine is slightly reduced (not significant statistically) in both WT mice and CD38 KO mice compared with respective naïve controls. Values are shown as means $\pm$ SE ($n = 6$ –8/group). * $P < 0.05$.

Isometric force measurement in the isolated trachea. The results obtained *in vivo* with a single μ rTNF- α challenge revealed the presence of increased airway reactivity and maximum responsiveness in the WT but not in the CD38KO mice. To evaluate possible changes in ASM contractility, tracheal segments from WT and CD38KO mice were incubated with μ rTNF- α for 12–14 h, and contractility responses to carbachol were measured as described above. The μ rTNF- α -treated tracheal segments obtained from WT mice presented significantly increased ASM sensitivity (Fig. 4A), reactivity (Fig. 4B), and maximum contractile responses to carbachol stimulation (Fig. 4C) compared with their respective naïve controls. However, the changes in these variables in the CD38KO mice, although visibly present, did not reach statistical significance (Fig. 4, A–C).

Bronchoalveolar inflammatory cell infiltrate. CD38 has an important role in the chemotaxis of monocytes and neutrophils to a wide range of chemoattractants (44) and is required for proper clearance of bacterial infection in the lungs (43). Hence, we sought to determine whether the observed differences in AHR were accompanied by inflammatory changes in the bronchoalveolar space between WT and CD38KO mice. Under naïve conditions, total and differential cell counts were similar in both WT and CD38KO mice (Fig. 5, A–C). No changes in total inflammatory cell numbers or differential cell counts were observed in the WT or CD38KO mice after a 1-day exposure of the airways to μ rTNF- α (Fig. 5A). When the airways were exposed to μ rTNF- α for 1 wk, lymphocyte numbers increased in the CD38KO only, whereas neutrophils increased significantly and to a similar magnitude in both WT and CD38KO mice. No change was observed in total cell numbers, macrophages, and eosinophils in both WT and CD38 KO mice, compared with naïve controls (Fig. 5B). After 4 wk of intranasal challenges with μ rTNF- α , both neutrophil and eosinophil numbers increased significantly and to similar magnitudes in both WT and CD38KO mice, whereas total inflammatory cell numbers, lymphocytes, and macrophage counts remained unchanged (Fig. 5C).

Cytokine and chemokine levels in the BAL fluid. Since our studies showed strikingly different patterns of airway responsiveness between a 1-day vs. a 4-wk exposure of the airways to μ rTNF- α but with no major differences in the number of inflammatory cells into the BAL space between WT and CD38KO mice, an array of representative cytokines and chemokines that may influence airway inflammation and responsiveness were determined in the BAL fluid. Also, to rule out possible disparities in cytokine production between the two groups of mice, given that CD38 crosslinking triggers a number of biologically relevant activities in lymphoid cells, includ-

ing the production of INF- γ , IL-2, IL-6, IL-10, and IL-12, depending on the cell type (10, 16, 19, 35), we determined the levels of these cytokines and MCP-1 in the BAL fluid. No changes were observed in the concentrations of all cytokines following airway exposure to μ rTNF- α for a single day or 4 wk compared with naïve conditions, and the only cytokine detected in the BAL fluid of any of the groups was IL-10.

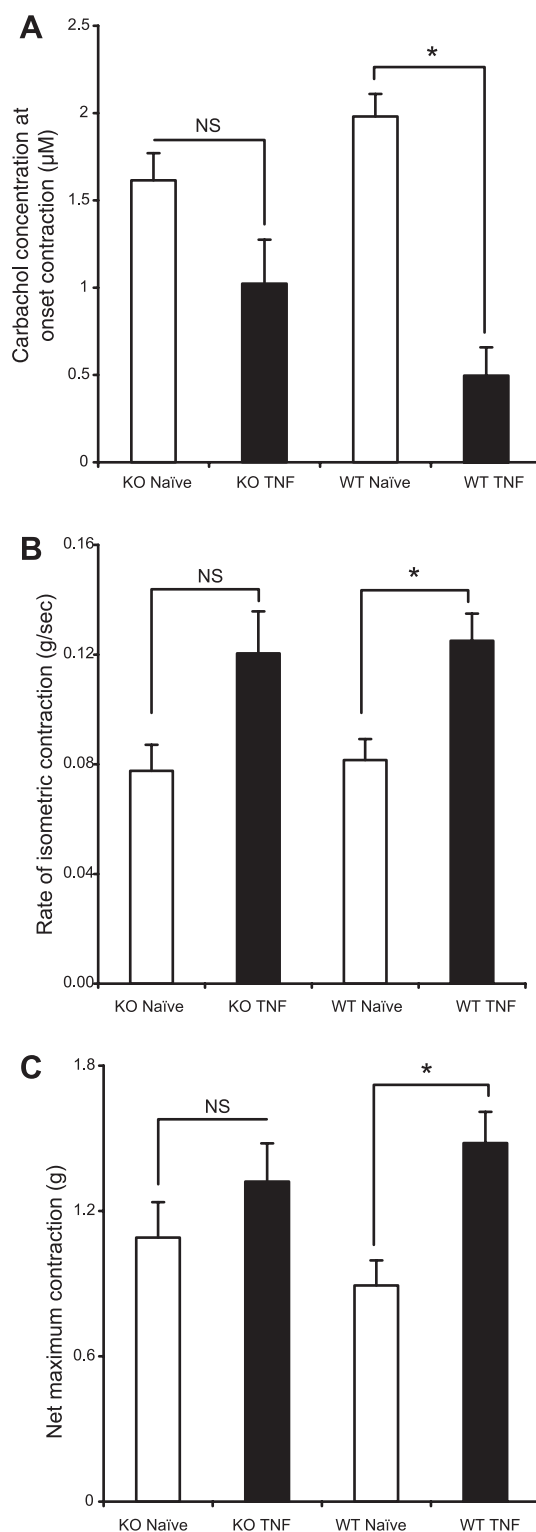


Fig. 4. TNF- α produced significantly greater changes in the contractile properties of tracheal segments from WT mice than CD38KO mice. A: minimal carbachol concentration to trigger contraction (e.g., sensitivity) is significantly reduced following TNF- α -exposure in WT mice. B: rate of isometric force responses (e.g., slope or reactivity) to continuous cumulative carbachol stimulation increases significantly following incubation with TNF- α in WT tracheal segments. C: maximal isometric force generated increases upon TNF- α treatment but significantly so only in the WT mice. The changes in these variables, although present in the tracheal segments from CD38KO mice, did not reach statistical significance. All values are shown as means $\pm$ SE ($n = 8$ –10/group). * $P < 0.05$ compared with respective naïve control; NS, nonsignificant compared with respective naïve control.

Hence, after a month of TNF- α treatment, the lungs had returned to a preinflammatory or desensitized stage at least at the level of cytokine profiles.

Histopathology of lung sections. To further clarify that the differences observed in the lung function experiments were not due to differences in lung inflammation, a careful evaluation of

the inflammatory infiltrate in peribronchiolar, perivascular, and alveolar regions was performed (Fig. 6). Compared with naïve controls, a one-day exposure of the CD38KO airways to mu rTNF- α significantly decreased inflammatory cell infiltrate in peribronchiolar and perivascular regions, whereas the alveolar inflammatory infiltrate increased. Still in these mice, extending the airway challenges with mu rTNF- α for 1 or 4 wk produced increased inflammation of all evaluated regions compared with naïve animals (Fig. 6A). In the WT mice, a 1-day exposure to mu rTNF- α caused a small (non-significant) reduction in peribronchiolar inflammation, a significant reduction in perivascular inflammation, and an increase in the alveolar inflammatory infiltrate compared with the naïve controls. As in the CD38KO mice, extending the airway challenges with mu rTNF- α increased the inflammatory infiltrate in all regions (Fig. 6B). Comparing parenchymal inflammation between CD38KO and WT mice at naïve conditions or following airway exposure to mu rTNF- α for 1 day, 1 wk, or 4 wk showed similar inflammatory infiltrate in all regions. The only exception was a smaller perivascular inflammatory reaction in the WT mice following 4 wk of exposure to mu rTNF- α than the CD38KO controls (data not shown).

DISCUSSION

TNF- α has been implicated in the asthmatic phenotype (4, 15, 42, 51, 52) and shown to increase $[Ca^{2+}]_i$ responses and contractility of ASM to a variety of agonists (8, 12, 29). Previous studies from our laboratory have demonstrated that cytokines such as TNF- α and IL-13 induce strong CD38 expression and ADP-ribosyl cyclase activity in ASM cells (11, 12). We have also shown that the modulation of CD38 expression by TNF- α occurs through activation of transcription factors such as NF- κ B and AP-1, and by RNA message stabilization through MAPK-dependent pathways (54). The upregulation of the CD38/cADPR signaling by TNF- α , as well as IL-13, results in agonist-induced calcium hyperresponsiveness in human ASM cells (11, 12, 29). One of our recent studies (21) has further revealed that AHR is significantly attenuated (although not completely eliminated) in CD38-deficient mice intranasally challenged with IL-13, a pivotal Th2-type cytokine in asthma (62). In the study reported here, we show that CD38 has a role in the TNF- α -induced AHR depending on the period of airway exposure to the cytokine.

In the present study, when the airways were exposed to TNF- α once during a 24-h period, AHR was evident in WT but not in CD38KO mice. Evaluation of bronchoalveolar inflammatory cell counts and cytokine profile showed no evidence of airway inflammation in both mice compared with naïve controls. In fact, a reduction in inflammatory cell infiltrate in

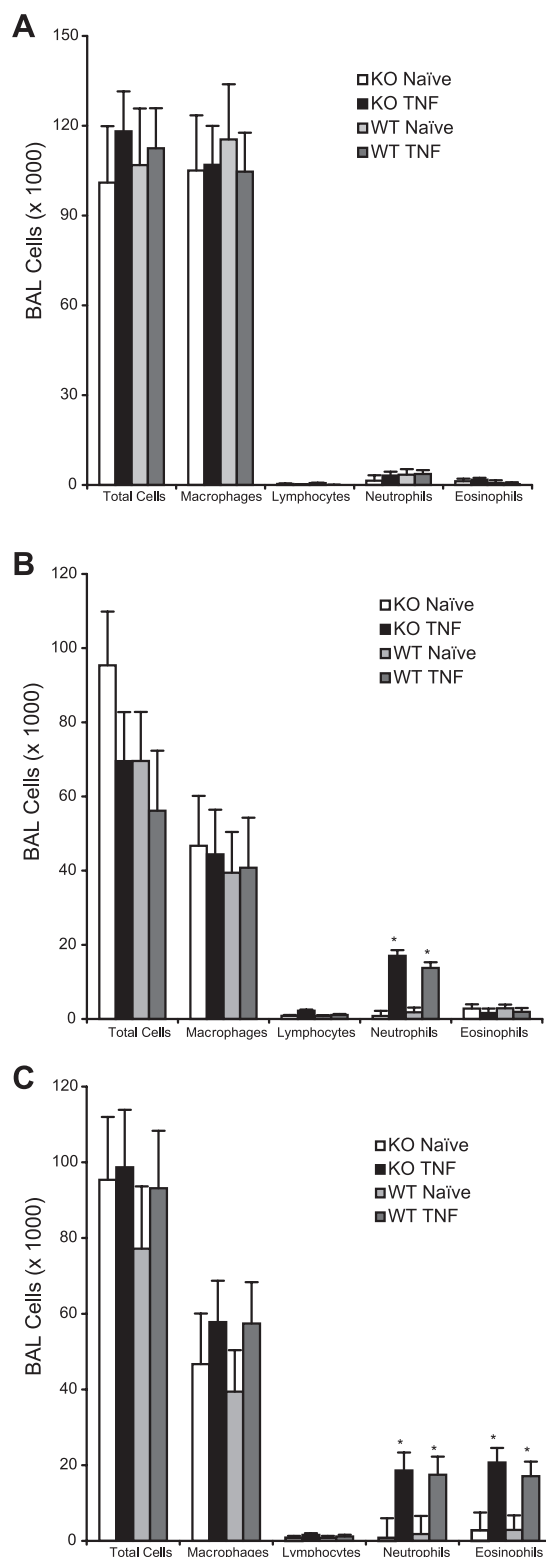


Fig. 5. Changes in bronchoalveolar inflammation following airway exposure to mu rTNF- α . A: a single intranasal TNF- α administration does not change bronchoalveolar inflammation in either WT or CD38KO mice compared with respective naïve controls. B: exposing the airways to TNF- α on alternate days for 1 wk induces significant elevation in neutrophils in the airways of both WT and CD38 KO mice, whereas lymphocytes numbers increase only in the CD38KO animals compared with respective naïve controls. C: mu rTNF- α administered to the airways every other day during 4 wk induces significant elevation in the numbers of neutrophils and eosinophils in the airways of both WT and CD38 KO mice. Data are shown as means $\pm$ SE ($n = 6-8$ /group). * $P < 0.05$ compared with respective naïve controls. BAL, bronchoalveolar lavage.

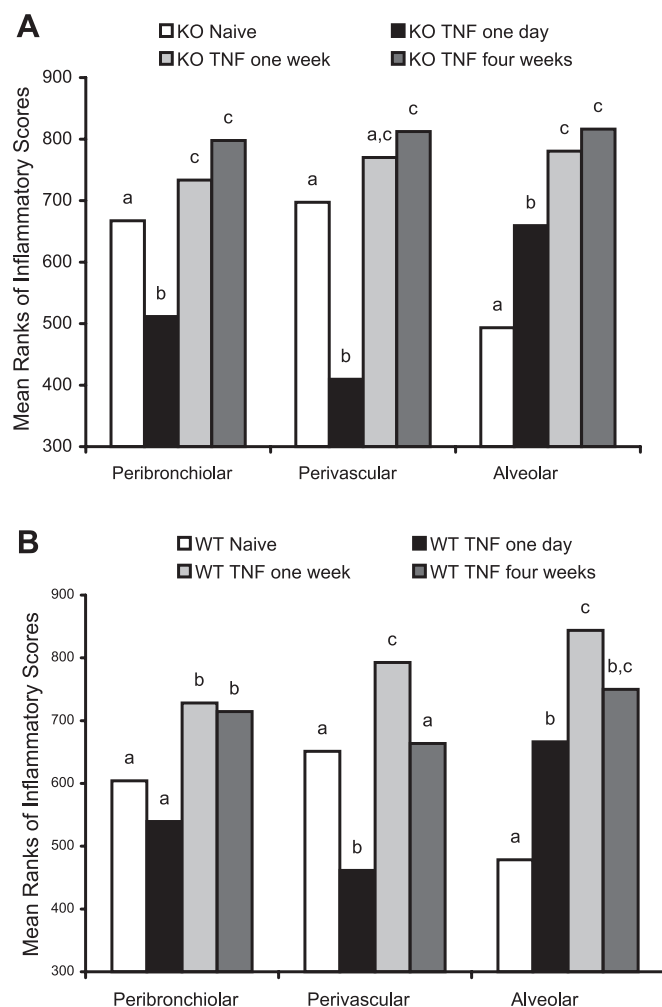


Fig. 6. The lung inflammation varies with the length of exposure of the lungs to μ rTNF- α but does not differ significantly between CD38KO and WT mice. **A:** in the CD38KO mice, lung inflammation decreased in peribronchiolar and perivascular regions but increased in the alveolar space following a 1-day exposure of airways to μ rTNF- α compared with naïve controls. All regions showed increased inflammatory infiltrate upon extending the airway challenges with μ rTNF- α for 1 or 4 wk. **B:** in the WT mice, a 1-day exposure to μ rTNF- α caused no statistically significant changes (only a slight reduction) in peribronchiolar inflammation, decreased the perivascular inflammation significantly, and increased the alveolar inflammatory infiltrate compared with the naïve controls. Prolonging the airway challenges with μ rTNF- α to 1 or 4 wk increased the inflammatory infiltrate in all regions. Data are shown as the mean ranks of inflammatory infiltrate in peribronchiolar, perivascular, and alveolar lung regions ($n = 6-8/\text{group}$). For each region, different letters above each column (a, b, c) indicate statistically significant difference ($P < 0.05$) between treatment groups.

specific regions of the lung parenchyma was observed in both WT and CD38KO mice compared with naïve controls. Airway reactivity, as assessed by changes in respiratory resistance for a given increase in the methacholine concentration (37), was significantly increased in the WT mice but not in the CD38KO controls. The AHR following such a brief exposure to TNF- α in the WT mice in the absence of changes in baseline airway resistance, airway inflammation, or changes in cytokine profiles suggests that AHR resulted from TNF- α acting directly on resident airway cells. Since the differences in airway responsiveness became apparent only during cholinergic-induced ASM contraction, the AHR in the WT mice very likely resulted

from CD38-mediated changes in the contractile properties of the ASM. The finding that TNF- α significantly increased the ASM sensitivity to carbachol in the tracheas from WT but not from the CD38KO mice supports this hypothesis, even though the *in vivo* changes in AHR, which reflect airway responses throughout the entire lung, were not fully replicated in the isolated tracheal rings. In addition, we have previously reported that TNF- α induces rapid and long-lasting changes in the expression and enzymatic activity of CD38 in human ASM cells. Such changes are maintained for at least 48 h following removal of the cytokine (29). The role of CD38/cADPR signaling in TNF- α -induced $[\text{Ca}^{2+}]_i$ hyperresponsiveness to contractile agonists in ASM cells (12, 29) and the fact that ASM cells from CD38KO mice have reduced Ca^{2+} mobilization upon agonist activation (14) indicate that a reduced $[\text{Ca}^{2+}]_i$ mobilization in the airway myocytes of the CD38KO mice is the likely mechanism underlying the lack of AHR in these mice following a brief exposure to TNF- α .

While a short exposure (e.g., 24 h) allowed for evaluation of changes in airway responsiveness without the presence of inflammation in our study, asthma is frequently associated with inflammatory changes in the lung parenchyma (7), and CD38 plays a complex role in the regulation of innate and adaptive immune responses *in vivo* (9, 43). By extending the airway exposure to TNF- α to 1 wk, a bronchoalveolar neutrophilic inflammation was detected in both WT and CD38KO mice, a finding in concert with the sputum neutrophilia that occurs after inhaled TNF- α in normal and asthmatic subjects (52, 53). Histological analysis of lung parenchyma in both WT and CD38KO mice revealed similarly increased inflammatory infiltrate in perivascular, peribronchiolar, and alveolar lung regions. Hence, the absence of CD38 did not seem to impair the inflammatory response to TNF- α in the lungs of CD38KO mice, as we previously demonstrated for IL-13 (21). Under these conditions, our results showed that the CD38/cADPR signaling in ASM becomes redundant for the development of AHR. The neutrophilic inflammatory changes were paralleled by robust responses to inhaled methacholine in both the WT and the CD38KO mice. These results differ from our recent findings of significant eosinophilic inflammation and reduced AHR in IL-13-challenged CD38KO mice compared with WT controls (21). The reason for this is unclear, but neutrophils can generate an assortment of active intermediates including eicosanoids (cysteinyl leukotrienes, thromboxanes, platelet activating factor), cytokines (IL-1 β , IL-6, IL-8, TGF- β), proteases (elastase, collagenase, matrix metalloproteinase-9), reactive oxygen intermediates (superoxide, hydrogen peroxide), and nitric oxide (48), all of which may have profound effects on airway biology. In addition, TNF- α has a vast array of effects in the airways including airway remodeling (6), induction of proinflammatory genes (57), increased G protein expression (24), and changes in Ca^{2+} homeostasis or sensitization in ASM cells (2, 26). It is possible that the AHR may arise from one or more of the mechanisms described above in a CD38-independent manner.

Since chronic airway inflammation is a frequent finding in asthma (6), and at least 2 wk are required for significant epithelial cell changes and mucous hypersecretion in TNF- α -challenged mice (6), we evaluated airway inflammation and responsiveness to inhaled methacholine during 4 wk of airway exposure to TNF- α in both WT and CD38KO mice. Both mice

developed comparable neutrophilic and eosinophilic bronchoalveolar inflammation, an expected finding given that TNF- α is a chemotactic cytokine for neutrophils and eosinophils (41). There was also increased inflammatory infiltrate in perivascular, peribronchiolar, and alveolar lung regions compared with naïve conditions. These results confirmed that the lack of CD38 did not compromise lung inflammatory responses to prolonged TNF- α exposure in the CD38KO mice. Interestingly, the AHR vanished following this more prolonged exposure of the airways to TNF- α in both CD38KO and WT mice. In one study (6), BALB/c and AKR mice challenged with mu rTNF- α (50 ng/mouse intratracheally) daily for 3 wk followed by alternate day challenges for a further 3 wk developed AHR to intravenous acetylcholine administration. The mice developed BAL fluid neutrophilia, but there was no increase in eosinophil numbers. No AHR occurred when challenges were performed daily for 3 wk. The discrepancy between that study and the findings presented here is most likely related to experimental protocol and/or mouse strain used. In a model of allergen-induced AHR, TNF- α -deficient mice (C57BL/6 background) developed significantly stronger AHR compared with controls, whereas transgenic mice expressing TNF- α in their lungs failed to develop AHR (27). These results are in concert with the data presented here.

In the model of allergen-induced AHR (27), the lack of AHR in the mice expressing TNF- α within the lungs was shown to be mediated by $\gamma\delta$ T cells, which are known to downregulate AHR (27, 33, 34). The biological effects of TNF- α are mediated by two functionally distinct receptors (p55 or TNFR1 and p75 or TNFR2). Mice deficient in p55 when sensitized and challenged with ovalbumin failed to develop AHR, whereas p75 deficient mice exhibited robust AHR under similar conditions. Depletion of the $\gamma\delta$ T cells in the p55 deficient mice restored AHR, confirming that the $\gamma\delta$ T cells were the regulators of AHR in that particular model (27, 28). Whether chronic exposure to TNF- α in the WT and CD38KO mice resulted in increased recruitment of $\gamma\delta$ T cells through the p75 receptor leading to reduced AHR is a possibility that remains to be determined. A more surprising result of our study, however, was that the TNF- α -induced suppression of AHR was significantly greater in the WT mice. The methacholine-induced changes in R_L in the WT mice chronically exposed to TNF- α were even lower than changes seen in naïve WT mice. In fact, they were similar to changes seen in naïve CD38KO mice that have intrinsically lower airway responsiveness compared with WT controls (14). The above observations, together with the fact that CD38 is expressed at high epitope density in $\gamma\delta$ T cells (17), give rise to the interesting possibility of an involvement of CD38 in $\gamma\delta$ T cell function as regulators of AHR.

In summary, our study revealed that the CD38/cADPR signaling is an important mediator of AHR only to a very brief airway exposure to TNF- α , probably by changing ASM sensitivity and reactivity to contractile agonists. It becomes redundant after an intermediate period of TNF- α exposure, when there is the development of neutrophilic airway inflammation. The reduction of AHR in the WT mice to levels comparable to CD38KO mice following extended exposure to TNF- α is surprising and may warrant further investigation as to its mechanisms and relevance.

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