γδ T Cells Are Required for Pulmonary IL-17A Expression after Ozone Exposure in Mice: Role of TNFα

Citation
Mathews, Joel A., Alison S. Williams, Jeffrey D. Brand, Allison P. Wurmbrand, Lucas Chen, Fernanda MC. Ninin, Huiqing Si, David I. Kasahara, and Stephanie A. Shore. 2014. “γδ T Cells Are Required for Pulmonary IL-17A Expression after Ozone Exposure in Mice: Role of TNFα.” PLoS ONE 9 (5): e97707. doi:10.1371/journal.pone.0097707. http://dx.doi.org/10.1371/journal.pone.0097707.

Published Version
doi:10.1371/journal.pone.0097707

Permanent link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:12406878

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos: dash.current.terms-of-use#LAA

Share Your Story
The Harvard community has made this article openly available. Please share how this access benefits you. Submit a story.

Accessibility
Introduction

γδ T cells are a key component of the innate immune response, especially at mucosal surfaces. These cells are found throughout the lung, particularly in the subepithelial region, where they may regulate other immune cells including macrophages and dendritic cells [1]. γδ T cells are an important source of IL-17A, a key cytokine involved in neutrophil inflammation [2]. In mice, the number of pulmonary γδ T cells increases following infection with certain bacteria [3]. Mice deficient in γδ T cells (TCRδ−/− mice) have attenuated pulmonary clearance of these bacteria, likely as a result of loss of IL-17A production by γδ T cells and consequent reduced neutrophil recruitment [4]. The number of γδ T cells in the lung also increases under conditions associated with oxidative stress, including smoking, bleomycin instillation, and allergen challenge [5–8]. Moreover, the pulmonary inflammation induced by such agents requires γδ T cells.

Inhalation of ozone (O₃), a common air pollutant, has a significant impact on human health. O₃ causes respiratory symptoms and reductions in lung function [9–13]. O₃ also increases the risk of respiratory infections and is a trigger for asthma [14–16]. Exposure to O₃ induces oxidative stress in the lung, damages lung epithelial cells, and causes the release of numerous cytokines and chemokines that recruit neutrophils and macrophages to the lung [9,17]. We have reported increased IL17a mRNA expression and increased numbers of IL-17A+ γδ T cells in the lungs after subacute O₃ exposure (0.3 ppm O₃ for 24–72 h) [18]. Hence, we tested the hypothesis that γδ T cells, via their ability to produce IL-17A, are involved in orchestrating the inflammatory response to subacute O₃ exposure. We examined IL-17A expression in WT and TCRδ−/− mice after exposure to air or to O₃ (0.3 ppm for 24–72 h). We also examined the effect of IL-17A neutralizing antibodies on O₃-induced inflammation. Our results indicate an important role for IL-17A+ γδ T cells in the inflammatory cell recruitment induced by subacute O₃ exposure.

TNFα, a pleiotropic pro-inflammatory cytokine, enhances the recruitment of neutrophils to the lungs in response to a variety of noxious stimuli, including LPS [19], cigarette smoke [20], and enterobacteria [21]. TNFα also required for neutrophil recruitment after subacute O₃ exposure [22,23]. However, TNFα does not have direct chemoattractant activity for neutrophils [24]. Instead, TNFα recruits neutrophils in part by inducing expression of other cytokines and chemokines [24,25]. In several pathological states, TNFα induces the expression of IL-17A [26,27]. Hence, we hypothesized that TNFα contributes to neutrophil recruitment following subacute O₃ exposure by promoting recruitment to or
activation of IL-17A+ γδ T cells in the lungs. We used two methods to test this hypothesis. First, we assessed the effect of O3 exposure on pulmonary Il17a expression and recruitment of IL-17A+ γδ T cells in WT mice and in mice deficient in TNFR2 (TNFR2−/− mice). Others have established that either TNFR1 or TNFR2 deficiency reduces the inflammatory response to subacute O3, and there is no further impact of combined TNFR1/TNFR2 deficiency [22]. Second, we examined the impact of the TNFα antagonist, etanercept, on Il17a expression. Our data suggest that TNFα is required for the recruitment of IL-17A+ γδ T cells to the lung after subacute O3 exposure.

Approximately one third of the US population is obese and another third is overweight, but our understanding of how obesity impacts pulmonary responses to O3 is still rudimentary. Such an understanding may have broad reaching implications since oxidative stress also contributes to responses to a variety of other noxious stimuli [5–8], many of which are affected by obesity [28,29]. In mice, the impact of obesity on responses to O3 depends on the nature of the exposure: the pulmonary inflammation induced by acute O3 exposure (2 ppm for 3 h) is augmented in all types of obese mice examined to date [30–33], whereas the pulmonary inflammation induced by subacute O3 exposure (0.3 ppm for 24–72 h) is reduced [34]. Given our findings of the requirement for TNFα-recruitment of IL-17A producing γδ T cells in the induction of pulmonary inflammation after subacute O3, we sought to determine if changes in the activation of γδ T cells might explain the reduced responses to subacute O3 we observed in obese Cpe mice (Cpefat/−/− mice). Data described below indicate that the reduced O3-induced neutrophil recruitment observed in obese mice is likely the result of reduced Il17a expression leading to reduced IL-17A+ γδ T cells. Given the importance of IL-17A+ γδ T cells for responses to viral and bacterial pathogens [see above], these observations might explain the altered response of the obese to bacteria and virus (see review by Peter Mancuso [35]).

**Methods**

**Animals**

This study was approved by the Harvard Medical Area Standing Committee on Animals. Male age-matched WT and TCRδ−/− mice were either purchased from The Jackson Laboratory (Bar Harbor, ME) and acclimated for 4 weeks, or bred in house. Cpe mice are deficient in carboxypeptidase E, an enzyme involved in processing neuropeptides involved in eating behaviors [36]. The breeding strategy used to generate Cpe/+/ TNFR2−/− mice from Cpe/+/ and TNFR2−/− mice (also originally purchased from The Jackson Laboratory) was previously described [37]. All mice were on a C57BL/6J background, fed a standard mouse chow diet, and were 10–13 weeks old at the time of study.

**Protocol**

For comparisons of WT and TCRδ−/− mice, mice were exposed to O3 (0.3 ppm) or to air, for 24–72 hours, as previously described [18]. Mice were exposed in normal cages without the microisolator top, but with free access to water and food throughout exposure. Mice were checked daily. At least two mice were placed in each cage to limit stress. After exposure, mice were euthanized with an overdose of sodium pentobarbital. The trachea was cannulated and bronchoalveolar lavage (BAL) was performed. After BAL, the lungs were flushed of blood by injecting 10 ml of cold PBS through the right ventricle, after creating a large excision in the left ventricle. One lung was excised and used for flow cytometry. The other was excised and placed in RNAlater (Qiagen, Germantown, MD) for preparation of RNA for real time PCR. In another cohort, WT mice were injected i.p. with 100 μg of anti-IL-17A neutralizing monoclonal antibody (Ab) (Rat IgG2A, clone 50104, MAB421; R&D Systems, Minneapolis, MN) or isotype control Ab (clone 54447, MAB006; R&D Systems) in 100 μl of sterile saline 24 hours before O3 exposure. Mice were exposed to O3 for 72 hours, euthanized, and tissues were harvested as described above. In a separate series of experiments, WT, TNFR2−/−, Cpeδ−/−, and Cpeδ−/−/TNFR2−/− mice were exposed to room air or O3 (0.3 ppm) for 48 h followed by BAL and tissue harvest. In other experiments, WT and Cpeδ−/− mice were treated twice (48 h and 1 h prior to O3 exposure) with the TNFα blocking drug, etanercept (30 mg/kg s.c.) (Immunex, Thousand Oaks, CA), or vehicle. A similar etanercept dosing regimen has been shown to be effective in inhibiting TNFα in mice over the time course of O3 exposures we used (48 h) [38,39].

**Bronchoalveolar Lavage**

BAL was performed and cells counted as previously described [18]. BAL supernatant was stored at −80°C until assayed. BAL KC, IL-6, MCP-1, IP-10 and G-CSF were measured by ELISA (R&D Systems). In mice treated with anti-IL-17A, BAL cytokines and chemokines were measured by multiplex assay (Eve Technologies, Calgary, Alberta). Total BAL protein was measured by Bradford assay (Bio-Rad, Hercules, CA).

**Flow Cytometry**

Left lungs were harvested and placed on ice in RPMI 1640 media containing 2% FBS and HEPES. Lungs were digested and prepared for flow cytometry as previously described [18]. Cells were stained using the following antibodies: Alexa Fluor 647 anti-TNFα, PE anti-IL-17A (clone: TG11-18H10.1), PE anti-TCRδ (clone: GL3), PE-cy7 anti-CD45 (clone: 30-F11), and APC-cy7 anti-CD3 (clone: 17A2) (all antibodies from BioLegend). Isotype control antibodies were used to set all gates. Cells were visualized using a Canto II (BD Biosciences) and the data was analyzed using Flowjo (Tree Star; Ashland, OR).

To determine if TNFα impacted IL-12Rβ1 expression on lung γδ T cells, lungs from WT mice were digested as above and then cultured in complete RPMI media (RPMI 1640 (Corning, Tewksbury, MA), 10% FBS (Life Technologies), 2 Mm L-glutamine (Life Technologies), 100 units/ml Pen/Strep (Lonza, Hopkinton, MA) and 20 Mm Hepes (Thermo Scientific, Tewksbury, MA)). Cells were plated at a concentration of 10^6 cells/ml in 24 well plates with or without 100 ng/ml of recombinant murine TNFα (R&D Systems) [40]. Cells were harvested after 24 h, washed with PBS, and stained using the following antibodies: anti-CD16/32 (True Stain biolegend), Strept-APC (Biolegend), PE anti-CD12 (IL-12Rβ1) (BD Biosciences), Biotin anti-TCRδ (clone: GL3, biolegend), PE-cy7 anti-CD45 (clone: 30-F11) and analyzed by flow cytometry as described above.

**Real-time PCR**

RNA was extracted from lung tissue and prepared for qPCR using the SYBR method as previously described [18]. All expression values were normalized to 36B4 expression using the ΔΔCt method. The primers for Il17a and 36B4 were previously described [37]. Primers for Cd20, Il23 (p19) and Il12Rβ1 are described in Table 1. For each set of primers, melt curve analysis yielded a single peak. Il12Rβ1 expression was measured at baseline in order to tease apart the effects of genotype (deficiency of TNFα signaling versus sufficient signaling) versus O3 exposure.
Table 1. Primers used for real time PCR.

| Primers     | Sequence                   |
|-------------|----------------------------|
| Il23p19     | F: CCC ATG GAG CAA CTG CTC AC R: GTG GCC ACT GCT GAC TAG AAC |
| Ccl20       | F: AAG ACA GAT GGC CCA TGA AG R: AGG TTC ACA GCC CTT TTC AC |
| Il12Rb1     | F: GTG CTC GCC AAA ACT CGT TT R: GGA TGT CAT GTC GCC TCC CA |

doi:10.1371/journal.pone.0097707.t001

Statistical Analysis
Data were analyzed by factorial ANOVA using STATISTICA software (Statistica, StatSoft; Tulsa, OK) with mouse genotype and exposure as main effects. Fisher’s least significant difference test was used as a post-hoc test. BAL cells and flow cytometry data were normalized by log transformed prior to analysis. A p value < 0.05 was considered significant.

Results
O3-induced Inflammation is Reduced in TCRδ−/− Mice
In WT mice, O3 exposure caused a time-dependent increase in BAL neutrophils, macrophages, and protein (a measure of O3-induced lung injury [41]) (Fig. 1A-C), consistent with previous reports by ourselves and others [18,22,23,41,42]. Increases in BAL inflammatory cells were significantly reduced in TCRδ−/− mice after 48 (neutrophils) and 72 (neutrophils and inflammatory cells) hours of exposure (Fig. 1A,B). BAL protein was also reduced in TCRδ−/− versus WT mice after 72 hours exposure, but not at earlier times (Fig. 1C).

Several cytokines, including KC, IL-6, IP-10 (CXCL10), G-CSF, MCP-1 and IL-17A [17,18,22,23,41-44], can contribute to inflammatory cell recruitment to the lungs after O3 exposure. BAL IL-17A expression was below the limits of detection of ELISA. Consequently, we used q-RT-PCR to measure IL-17A. Il17a mRNA abundance increased after 24, 48 and 72 hours of O3 in WT but not TCRδ−/− mice (Fig. 1D). O3-induced increases in BAL concentrations of BAL G-CSF, IL-6, KC and IP-10 were each reduced in TCRδ−/− versus WT mice at 72 hours of exposure (Fig. 1E-H). For G-CSF and IP-10, there was a similar trend at 24 and 48 hours (Fig. 1E,G). γδ T cell deficiency had no effect on O3-induced changes in BAL MCP-1, although MCP-1 trended lower in TCRδ−/− versus WT mice at 72 hours.

IL-17A+ γδ T Cells are Increased by O3 Exposure
Flow cytometry indicated that the number of IL-17A+ CD45+ cells was significantly increased by O3 in WT mice. This effect was ablated in TCRδ−/− mice (Fig. 2A). Further analysis indicated that in WT mice, the numbers of IL-17A+ γδ T cells as well as the total number of γδ T cells were increased by O3 (Fig. 2B, C), as reported previously using a similar gating strategy [18].

Effect of Anti-IL-17A Treatment
Compared to isotype control, anti-IL-17A treatment of WT mice caused a significant reduction in BAL neutrophils and macrophages (Fig. 3A). Anti-IL-17A treatment also significantly decreased BAL protein (Fig. 3B) and BAL G-CSF (Fig. 3C). Given this key role for IL-17A, these data indicate that the decreased inflammatory response observed in the TCRδ−/− mice was likely due to the lack of Il17a expression (Fig. 1D) and demonstrate that G-CSF likely contributes to the effect of IL-17A on neutrophil recruitment.

Role of TNFα
BAL neutrophils were significantly lower in TNFR2−/− versus WT mice exposed to O3 for 48 h (Fig. 4A), consistent with the results of Cho et al [22]. Similar results were obtained in WT mice treated with etanercept versus vehicle (Fig. 4D). O3 exposure caused a significant increase in pulmonary Il17a expression in WT mice (Fig. 4B), consistent with results described above (Fig. 1D). However in TNFR2−/− mice, no such increase in Il17a mRNA abundance was observed (Fig. 4B). Similar results were obtained in mice treated with etanercept (Fig. 4E). Flow cytometry also indicated a decrease in IL-17A+CD45+ cells in O3-exposed TNFR2−/− versus WT mice (Fig. 5A). This change was due to decreased numbers of IL-17A+ γδ T cells (Fig. 5B). BAL G-CSF was also significantly lower in O3-exposed TNFR2−/− versus WT mice (Fig. 4C) and in etanercept treated versus vehicle treated WT mice (Fig. 4F).

The requirement of IL-23 and IL-6 for IL-17A expression in γδ T cells [45,46], suggested that reductions in IL-17A+ γδ T cells in TNFR2−/− mice might be the result of loss of TNFα-induced expression of IL-23 or IL-6. O3 increased BAL IL-6 in WT mice (Fig. 1F) and O3 also increased pulmonary Il23 (p19) mRNA abundance (Fig. 6B), but neither IL-6 nor IL-23 were affected by TNFR2 deficiency or etanercept treatment (Fig. 6A, C). In contrast, TNFR2−/− mice had reduced expression at baseline of Il12Rb1 (Fig. 6H), a component of the IL-23 receptor. A similar trend was observed in etanercept treated mice (data not shown). O3 exposure had no effect on Il12Rb1 (data not shown).

Expression of the other component of the IL-23 receptor, Il23R, was not affected by TNFR2 deficiency (data not shown). To determine if TNFα was having direct effects on Il12Rb1 expression on γδ T cells [45,46], we examined IL-12Rb1 expression on γδ T cells by flow cytometry (Fig. 6J). TNFα had no effect on the levels of IL-12Rb1 on γδ T cells as measured by MFI and did not affect the percentage of γδ T cells expressing IL-12Rb1 mRNA expression.

We also considered the possibility that TNFα might impact the recruitment of γδ T cells to the lungs. In WT mice, O3 exposure caused an increase in pulmonary mRNA expression of Ccl20 (Fig. 6E), a chemoattractant for IL-17A+ cells [47,48], whereas no such increase was observed in mice treated with etanercept (Fig. 6F), suggesting that the role of TNFα is in the CCL20 dependent recruitment of IL-17A+ γδ T cells to the lungs. Similarly, there was a trend towards reduced Ccl20 mRNA abundance in O3-exposed TNFR2−/− versus WT mice (Fig. 6G), although the effect did not reach statistical significance.

Response to O3 in Obese Mice
O6+ mice, regardless of their TNFR2 genotype or exposure, weighed almost twice as much as controls (data not shown). BAL neutrophils were significantly lower in C/Ob+ versus WT mice exposed to O3 (Fig. 4AD), consistent with our previous observations using this exposure regimen [34]. In contrast to the
substantial reduction in BAL neutrophils observed in TNFR2^{−/−} versus WT mice. TNFR2 deficiency had no significant effect on BAL neutrophils in O₃-exposed Cpefat mice (Fig. 4A). Similar results were obtained in etanercept treated WT mice (Fig. 4D). Cpe genotype had no impact on the number of BAL or lung macrophages (data not shown).

IL17a expression was significantly lower in O₃ exposed Cpefat versus WT mice (Fig. 4B,E). The number of IL-17A^{+} CD45^{+} cells was also significantly lower in O₃-exposed Cpefat than WT mice (Fig. 5A). The total number of γδ T cells and the number of IL-17A^{+} γδ T cells was also reduced in the lungs of Cpefat versus WT mice (Fig. 5B,C). O₃-induced increases in BAL G-CSF were also

---

**Figure 1.** Effect of γδ T cell deficiency on pulmonary inflammation and injury. (A–C) BAL neutrophils, macrophages, and protein; (D) pulmonary IL17a mRNA expression; (E–I) BAL G-CSF, IL-6, IP-10, KC, and MCP-1. Results are mean±SEM of 4–11 mice per group. *p<0.05 versus genotype-matched air-exposed mice. #p<0.05 versus WT mice with the same exposure. doi:10.1371/journal.pone.0097707.g001

**Figure 2.** Effect of O₃ exposure on IL-17A positive lung cells assessed by flow cytometry. (A) lung IL-17A^{+}CD45^{+}; (B) lung IL-17A^{+} γδ T cells; (C) total lung γδ T cells. Results are mean±SEM for 3–6 air-exposed and 4–11 O₃-exposed mice. *p<0.05 versus genotype-matched air-exposed mice. doi:10.1371/journal.pone.0097707.g002
lower in \textit{Cpe} vs WT mice (Fig. 4C, E) consistent with the reductions in IL-17A expression. Both BAL IL-6 and pulmonary \textit{Il23} mRNA expression were lower in \textit{Cpe} vs WT mice (Fig. 6A, C, D). Reductions in these cytokines would be expected to reduce IL-17A expression, as observed (Fig. 4B, E). Whereas TNFR2 deficiency and etanercept reduced \textit{Il17a} mRNA, IL-17A+ \textit{CD} T cells, and BAL G-CSF in lean WT mice, neither TNFR2 deficiency or etanercept affected these outcomes in obese \textit{Cpe} mice (Fig. 4B, C and 5A–C).

**Discussion**

Our data indicate a key role for IL-17A+ \textit{CD} T cells in the pulmonary inflammation induced by subacute O3. Both BAL IL-6 and pulmonary \textit{Il23} mRNA expression were lower in \textit{Cpe} vs WT mice (Fig. 6A, C, D). Reductions in these cytokines would be expected to reduce IL-17A expression, as observed (Fig. 4B, E). Whereas TNFR2 deficiency and etanercept reduced \textit{Il17a} mRNA, IL-17A+ \textit{CD} T cells, and BAL G-CSF in lean WT mice, neither TNFR2 deficiency or etanercept affected these outcomes in obese \textit{Cpe} mice (Fig. 4B, C and 5A–C).

Inflammatory cell recruitment to the lungs after subacute O3 exposure required \textit{CD} T cells (Fig. 1A, B). \textit{CD} T cells have also been shown to be required for the pulmonary inflammation observed 24 but not 8 hours after acute exposure to much higher O3 concentrations (2 ppm) [49,50], consistent with the time needed for recruitment and activation of \textit{CD} T cells. However, in those studies, the precise role of these \textit{CD} T cells was not assessed. Our data indicate that after exposure to lower concentrations of O3 for much longer periods of time, the role of \textit{CD} T cells involved IL-17A expression. Both lung \textit{Il17a} mRNA and lung IL-17A+ \textit{CD} T cells increased after subacute O3 exposure with a time course similar to that of neutrophil recruitment (Figs. 1A, 1D, 2B). Furthermore, O3-induced increases in \textit{Il17a} mRNA abundance were abolished in TCR\textit{d}−/− mice (Fig. 1D). In addition, both BAL neutrophils

**Figure 3. Effect of anti-IL-17A on O3-induced pulmonary inflammation and injury.** WT mice were injected with anti-IL-17A or isotype 24 h prior to O3 (0.3 ppm O3 for 72 h). (A) BAL macrophages and neutrophils; (B) BAL protein; (C) BAL cytokines determined by multiplex assay. Results are mean ± SEM of 5–7 mice per group. #p < 0.05 versus isotype control.

doi:10.1371/journal.pone.0097707.g003

**Figure 4. Impact of TNFR2 deficiency (A–C) or etanercept (D–F) on O3-induced inflammation in obese (\textit{Cpe}) and lean (WT) mice.** (A, D) BAL neutrophils; (B, E) \textit{Il17a} mRNA expression; (C, F) BAL G-CSF. Results are mean ± SE of data from 3–11 mice in each group.*p < 0.05 versus air-exposed mice of same genotype and treatment; #p < 0.05 versus exposure matched lean mice with same TNFR2 genotype or treatment; & p < 0.05 versus TNFR2 sufficient (A–C) or vehicle treated mice (D–F) with same exposure and Cpe genotype.

doi:10.1371/journal.pone.0097707.g004
and macrophages were reduced in mice treated with anti-IL-17A versus isotype control antibody (Fig. 3A). This ability of IL-17A+ γδ T cells to control the influx of macrophages and neutrophils is consistent with the findings in other models of lung infection and injury [4,51–54]. While our data indicate that IL-17γδ T cells are required for O₃-induced inflammatory cell recruitment, they are not sufficient. For example, O₃ is highly reactive and macrophages and epithelial cells are the initial targets of its action. These cells are the likely source of TNFα which is required for neutrophil recruitment (Fig. 4) perhaps via induction of CCL20 and consequent recruitment IL-17A+ γδ T cells (Figs. 5,6). Epithelial cells are also the likely source of CCL20. Furthermore, macrophages also produce IL-17A after O₃ exposure [18], and the role of γδ T cells may be to promote these effects. Macrophages and epithelial cells are also the likely source of other chemokines that interact with IL-17A (see below) to promote neutrophil recruitment.

IL-17A has direct chemotactic effects on macrophages [55], which likely explains the ability of anti-IL-17A to attenuate O₃-induced increases in BAL macrophages (Fig. 3A). In contrast, IL-17A induces neutrophil recruitment to the lungs by inducing expression of other neutrophil chemotactic and survival factors. With subacute O₃ exposure, G-CSF appears to be one of these factors. In WT mice, the time courses of induction of BAL G-CSF

---

**Figure 5. Role of TNFα for IL-17A expression in γδ T cells.** Total number of (A) lung IL-17A⁺CD45⁺ cells; (B) lung IL-17A⁺ γδ T cells; and (C) total lung γδ T cells. Results are mean±SE of data from 5–6 mice in each group. * p<0.05 compared to lean mice with same TNFR2 genotype; & p<0.05 compared to TNFR2⁺⁺ Cpe genotype matched mice.

doi:10.1371/journal.pone.0097707.g005

---

**Figure 6. TNFα signaling is required for expression of Il12Rb1 and Ccl20.** (A) BAL IL-6; (B–D) Il23 (p19) mRNA; (E–G) Ccl20 mRNA; (H) Il12Rb1 mRNA; (I) MFI and (J) % of γδ T cells positive for IL-12Rb1 after stimulation with TNFα. Results are mean±SE of data from 3–11 mice in each group. * p<0.05 versus air exposed mice of the same genotype; † p<0.05 versus exposure matched lean mice with the same TNFR2 genotype or treatment; & p<0.05 versus WT; %<0.05 obese versus lean regardless of TNFR2 genotype.

doi:10.1371/journal.pone.0097707.g006
and IL17a expression were similar (Fig. 1D,E). Importantly, anti-
IL-17A and γδ T cell deficiency each caused a marked and
significant reduction in BAL G-CSF in O3-exposed mice (Fig. 1E,
3C). The data are also consistent with our previous observations
showing reductions in BAL G-CSF in O3-exposed adiponection-
deficient mice treated with anti-IL-17A [18]. The observed role of
IL-17A in G-CSF expression is in agreement with previous reports
indicating that IL-17A signaling increases the transcription and
stability of the Gsf mRNA [56,57], via effects on ERK1/2
activation [58]. G-CSF causes neutrophil release from bone
marrow and promotes neutrophil survival [59]. Since serum G-
CSF did not increase after subacute O3 exposure (data not shown),
G-CSF is unlikely to act via effects on bone marrow in this model.
Instead, G-CSF likely contributes by increasing the survival of
neutrophils recruited to the lungs in response to other factors such
as IP-10 (Fig. 1G).

TNFα is not directly chemotactic for neutrophils [24].
However, in lean WT mice, TNFR2 deficiency or the TNFα
antagonist, etanercept, reduced the O3-induced increase in BAL
neutrophils (Fig. 4A,D) consistent with previous reports [22,23,60]
indicating a role for TNFα in neutrophil recruitment induced by
subacute O3. TNFα also contributes to neutrophil recruitment in
other conditions [reviewed in [61]], though the mechanism is not
well understood. Our data suggest that at least in the setting of O3
exposure, the ability of TNFα to recruit neutrophils involves IL-
17A and that the source of this IL-17A is γδ T cells (Fig. 5). O3-
induced increases in pulmonary IL17a expression were attenuated
in TNFR2−/− versus WT mice (Fig. 4B) and in etanercept versus
vehicle treated WT mice (Fig. 4E). The number of IL-17A+ γδ T
cells in the lung was also lower in TNFR2−/− versus WT mice
exposed to O3 (Fig. 5A,B). The ability of TNFα to promote
pulmonary IL-17A expression after O3 exposure is consistent with
the role of TNFα in other pathogenic states. For example,
etanercept reduces the elevated blood and skin Th17 cells
observed in patients with psoriasis [26]. Similarly, another anti-
TNFα therapy, infliximab, reduces IL-17A in ocular fluid from
uveitis patients with Behcet’s disease [27].

To better understand the role of TNFα, we examined IL-6 and
IL-23 expression. Both these cytokines can contribute to induction
of IL-17A in γδ T cells [45,62]. Both IL-6 and IL-23 were induced
in the lungs after O3 exposure, but were not affected by TNFR2
deficiency or by etanercept (Fig. 6A,C,D), indicating that TNFα
is not required for their expression. We did observe that mRNA
expression of one of the two subunits of the IL-23 receptor,
Il12rb1, was decreased (Fig. 6H) in unexposed lungs from
TNFR2−/− mice. Similar trends were observed after etanercept
treatment (data not shown). Since others have reported that TNFα
can act directly on γδ T cells [40,63], we considered the possibility
that TNFα was acting to increase Il12rb1 expression on γδ T
cells, thus increasing their ability to respond to IL-23. However,
culture of lung cells with TNFα resulted in no change in surface
bound IL-12Rb1 on γδ T cells (Fig. 6J). Instead, our data,
suggest that effects of TNFα on Ccl20 expression (Fig. 6E,G)
account for the observed effects of TNFα/TNF blockade on IL-
17A+ γδ T cells. Ccl20 acts via CCR6, a receptor expressed by IL-
17A+ γδ T cells that promotes chemotaxis of these cells [64].
TNFα is also required for pulmonary Ccl20 expression after acute
O3 exposure (2 ppm for 3 h) [37]. A role for TNFα in Ccl20
expression has also been demonstrated in dermal lesions of psoriasis patients based on treatment with the TNFα antagonist
infliximab [65].

We observed fewer neutrophils in BAL fluid of obese Cpefa−/−
versus lean WT mice after subacute O3 exposure (Fig. 4A,D),
consistent with previous observations [34]. Reduced responses
are observed in Cpefa−/− mice not only after 48 h exposure (Fig. 4A,D),
but also after 24 or 72 h exposures [34]. Pulmonary IL-17a
expression and IL-17A+ γδ T cells were also reduced in the obese
mice, as was the total number of γδ T cells (Fig. 4). BAL G-CSF
was also lower in Cpefa−/− versus lean WT mice (Fig. 4C,F).
Moreover, O3-induced increases in BAL IL-6 and pulmonary
Il17a expression were also reduced in Cpefa−/− versus WT mice
(Fig. 6C,D). TNFR2 deficiency or etanercept treatment in Cpefa−/−
mice did not further reduce BAL neutrophils or pulmonary IL17a
expression, in contrast to what was observed in WT mice
(Fig. 4B,E). Given the already reduced numbers of total γδ T
cells in Cpefa−/− mice exposed to O3 (Fig. 5C), and our observations
indicating the key role for IL-17A+ γδ T cells in the effects of
TNFα on neutrophil recruitment, it is not surprising that TNFα
had no further effect on the response to O3 in obese mice. Taken
together, the data suggest that obesity-related reductions in
neutrophil recruitment induced by subacute O3 exposure are the
result of reduced IL-17A-dependent G-CSF release, consequent
to reduced IL-6 and IL-23 expression. However, we cannot rule out
the possibility that other factors also contributed. For example,
neutrophils from obese mice exhibit reduced chemotactic activity
towards CXCR2 ligands [66]. Such defects in neutrophil
chemotaxis would also be expected to reduce O3-induced
neutrophil recruitment in Cpefa−/− mice.

In addition to affecting responses to O3, obesity also impacts
responses to bacterial and viral infections [67–71]. As described
above, IL-17+ γδ T cells contribute to neutrophil recruitment and
pathogen clearance after certain bacterial infections [3,4]. IL-17A+
γδ T cells are also required for clearance of secondary infections
after influenza [72]. Hence, obesity-related changes in IL-17
production may have broader implications for effects of obesity on host defense. In support of this, obese mice
compared to lean mice have fewer skin γδ T cells number and the
few γδ T cells they have are dysfunctional [73], which leads to
impairment in wound healing. These decreases in γδ T cells
numbers and impairment in function of the skin in obese mice are
due to altered STAT5 signaling and chronic TNFα signaling [74].

In summary, our data indicate that γδ T cells are required for
the pulmonary inflammation that occurs after subacute O3
exposure in mice via their ability to produce IL-17A. IL-17A
then leads to G-CSF expression. Our data also indicate that TNFα
is required for recruitment IL-17A+ γδ T cells to the lungs likely
through its ability to induce Ccl20. These results emphasize the
importance of γδ T cells not only for pathogen clearance, but also
for responses to other insults that induce oxidative stress, and
describe a new role for TNFα in these events. Finally, our data
indicate that obesity-related reductions in the ability of subacute
O3 to promote neutrophil recruitment to the lungs are the result of
reduced IL-17A+ γδ T cells. These results suggest that other
conditions that impact γδ T cell recruitment or activation will also
impact responses to this common pollutant.

Author Contributions
Conceived and designed the experiments: JAM ASW JDB HS DIK SAS.
Performed the experiments: JAM ASW JDB APW LG FMCN. Analyzed
the data: JAM ASW JDB SAS. Contributed reagents/materials/analysis
tools: JAM ASW. Wrote the paper: JAM ASW SAS.
References

1. Wands JM, Rook CL, Aylington MK, Jin N, Hahn Y-S, et al. (2005) Distribution and leukocyte contacts of γδ T cells in the lung. Journal of leukocyte biology 78: 1086–1095.

2. Leung M, Cui Z-H, Hoshino H, Lovatt J, Stjordal M, et al. (1999) Neutrophil Recruitment by Human IL-17 Via C-C X-C Chemokine Release in the Airways. The Journal of Immunology 162: 2347–2352.

3. Skener MJ, Ziegler HK. (1993) Induction of murine peritoneal gamma/delta T cells and their role in resistance to bacterial infection. The Journal of Experimental Medicine 178: 971–984.

4. Cheng P, Liu T, Zhou W-Y, Zhuang Y, Peng L-s, et al. (2012) Role of gamma-delta T cells in host response against Staphylococcus aureus-induced acute lung injury. Respiratory Research 13: 8.

5. Koobart M, Tamaoka M, Gamblin A, Martin J (2007) The role of gammadelta T cells in airway epithelial injury and bronchial responsiveness after chlorine gas exposure in mice. Respiratory Research 8: 21.

6. McMenamin C, Finn C, McKeever M, Hole PG (1994) Regulation of IgE responses to inhaled antigens in mice by antigen-specific gamma-delta T cells. Science 265: 1699–1701.

7. Pociask DA, Chen K, Mi Choi S, Oury TD, Steele C, et al. (2011) γδ T Cells Attenuate Bleomycin-Induced Fibrosis through the Production of CXCL10. The Journal of Clinical Investigation 121: 2635–2648.

8. Pons J, Saulaud J, Ferrer JM, Barcelo ´ B, Fuster A, et al. (2005) Blunted γδ T-lymphocyte response in chronic obstructive pulmonary disease. European Respiratory Journal 25: 441–447.

9. Peden DB (1996) Effect of Air Pollution in Asthma and Respiratory Allergy. American Journal of Respiratory Cellular and Molecular Physiology 153: 38.

10. Levy JI, Chemerynski SM, Sarnat JA (2005) Ozone Exposure and Mortality: An Empiric Bayes Meta-regression Analysis. Epidemiology 16: 436 – 445 410.1097/ 1001.000000000000016305.

11. Levy JI, Chemerynski SM, Samart JA (2005) Oxazepam Exposure and Mortality: An Empiric Bayes Meta-regression Analysis. Epidemiology 16: 436 – 445 410.1097/ 1001.000000000000016305.

12. Triche EW, Gent JF, Holford TR, Belanger K, Bracken MB, et al. (2006) Low-level ozone exposure and respiratory symptoms in infants. Environmental Health Perspectives 114: 911–916.

13. Chiu H-F, Cheng M-H, Yang C-Y (2009) Air Pollution and Hospital Admissions for Pneumonia in a Subtropical City: Taipei, Taiwan. Inhalation Toxicology 21: 32–37.

14. Pedersen DB (1996) Effect of Air Pollution on Asthma and Respiratory Allergy. Otolaryngology - Head and Neck Surgery 114: 242–247.

15. Charpin D, Pascal L, Birnbaum J, Amargaudia A, Sambuc R, et al. (1999) Gaseous air pollution and atopy. Clinical and Experimental Allergy 29: 1474–1480.

16. Triche EW, Gent JF, Holford TR, Belanger K, Bracken MB, et al. (2006) Low-level ozone exposure and respiratory symptoms in infants. Environmental Health Perspectives 114: 911–916.

17. Matsumoto J, Porkelis S, Kasaoka DI, Chen L, Wurmbrand AP, et al. (2013) Apoptosis of μγ-CD8+ T Cells, TNFα, and Ozone in the Context of an Inflammatory Cytokine Milieu Supports De Novo Production of Th17 cells. The Journal of Immunology 186(9): 5221–5230.

18. Bhalla DK (1999) Ozone-induced lung inflammation and mucosal barrier disruption: toxicology, mechanisms, and implications. J Toxicol Environ Health B Crit Rev 2: 31–86.

19. Backus GS, Howden R, Pospel J, Bauer AK, Cho HY, et al. (2010) Protective role of interleukin-10 in ozone-induced pulmonary inflammation. Environmental Health Perspectives 118: 1721–1727.

20. Johnson RA, Schwartzman IN, Johnston RA (2003) Responses to ozone in increased in obese mice. J Appl Physiol 95: 930–945.

21. Stote SA, Lang JE, Koshak WE, Li J, Li C, W-N Y, et al. (2009) Pulmonary responses to subacute ozone exposure in obese vs. lean mice. Journal of Applied Physiology 107: 1445–1452.

22. Miyamura M, Davies JA, Kasaoka DI, Chen L, Wurmbrand AP, et al. (2013) Augmented Pulmonary Responses to Acute Ozone Exposure in Obese Mice: Roles of TNFR2 and IL-13. Environ Health Perspect 121: 551–557.

23. Skerry C, Harper J, Klinkh M, Bishal WR, Jain SK (2012) Adjunctive TNF inhibition with standard treatment enhances bacterial clearance in a murine model of necrotic TB granulomas. PLoS ONE 7, e93680.

24. Grounds M, Davies D, Torrisi J, Shavalkerde T, White J, et al. (2005) Silencing TNFα activity by using Remicade or Enbrel blocks inflammation in whole muscle grafts: an in vivo bioassay to assess the efficacy of anti-cytokine drugs in mice. Cell and Tissue Research 320: 509–515.

25. Lahn M, Kalatazar H, Mittelstadt P, Pluem E, Vollmer M, et al. (1998) Early Preferential Stimulation of γδ T Cells by TNF-α. The Journal of Immunology 160: 5221–5230.

26. Ehrlich SF, Quesenberry CP, Van Den Eeden SK, Shan J, Ferrara A (2010) Patients Diagnosed With Diabetes Are at Increased Risk for Asthma, Chronic Obstructive Pulmonary Disease, Pulmonary Fibrosis, and Pneumonia but Not Lung Cancer. Diabetes Care 33: 55–60.

27. Johnson RA, Therman TA, Lu FL, Terry RD, Williams ES, et al. (2008) Diet-induced obesity causes inanimate airway hyperresponsiveness to methacholine and enhances ozone-induced pulmonary inflammation. Journal of Applied Physiology 104: 1727–1735.

28. Ishizaka A, Zheng H, Hogue RS, et al. (1989) Central to Acute Cigarette Smoke–induced Inflammation and Connective Tissue Breakdown. American Journal of Respiratory and Critical Care Medicine 146: 849–855.

29. Malaviya R, Ikeda T, Ross E, Abraham SN (1996) Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-α/IL-18. Nature 310: 77–80.

30. Cho H-Y, Zhang L-Y, Kleeberger SR (2001) Ozone-induced lung inflammation and hyperreactivity are mediated via tumor necrosis factor-α receptors. American Journal of Physiology - Lung Cellular and Molecular Physiology 274: L29–L46.

31. Klement D, K, Kruijff H, Williams AS, Verhout NG, Tran J, et al. (2012) Pulmonary inflammation induced by subacute ozone is augmented in adiponectin-deficient mice: role of IL-17A. J Immunol 188: 4558–4567.

32. Shimizu M, Hasegawa N, Nishimura T, Endo Y, Shirasaki Y, et al. (2009) Effects of TNF-alpha-converting enzyme inhibition on acute lung injury induced by endothox in the rat. Shock 32: 535–540.

33. Chung A, Dai J, Tai H, Xie C, Wright JL (2002) Tumor Necrosis Factor-α Is Central to Acute Cigarette Smoke-Induced Inflammation and Connective Tissue Breakdown. American Journal of Respiratory and Critical Care Medicine 166: 849–855.

34. Malaviya R, Ikeda T, Ross E, Abraham SN (1996) Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-α/IL-18. Nature 310: 77–80.

35. Cho H-Y, Zhang L-Y, Kleeberger SR (2001) Ozone-induced lung inflammation and hyperreactivity are mediated via tumor necrosis factor-α receptors. American Journal of Physiology - Lung Cellular and Molecular Physiology 280: L1357–L1346.

36. Kleeberger SR, Levitt RC, Zhang LY, Loughre M, Harkema J, et al. (1997) Linkage analysis of susceptibility to ozone-induced lung inflammation in inbred mice. Nat Genet 17: 473–478.

37. Yanamare M, Stephens JA, Hoshino H, Gouitaa M, Charpin D, et al. (1999) Neutrophil Recruitment by Human IL-17 Via C-C X-C Chemokine Release in the Airways. The Journal of Immunology 162: 2347–2352.

38. Yonemaru M, Stephens KE, Ishizaka A, Zheng H, Hogue RS, et al. (1989) Effects of tumor necrosis factor on PMN chemotaxis, chemiluminescence, and elastase activity. J Lab Clin Med 114: 674–681.

39. Poiber JS (1987) Effects of tumour necrosis factor and related cytokines on vascular endothelial cells. Ciba Found Symp 131: 170–184.

40. Antonio E, Volpi W, Cardilicchia E, Muggi L, Fili L, et al. (2012) Etanercept Downregulates the Th17 Pathway and Decreases the IL-17/IL-10+ Cell Ratio in Patients with Psoriasis Vulgaris. Journal of Clinical Immunology 32: 1221–1232.
53. Wozniak K, Kolls J, Wormald F (2012) Depletion of neutrophils in a protective model of pulmonary cryptococcosis results in increased IL-17A production by gamma/delta T cells. BMC Immunology 13: 65.

54. Lo Re S, Dantsiou L, Gouliou I, Van Vye C, Yakoub Y, et al. (2010) IL-17A Producing γδ T and Th17 Lymphocytes Mediate Lung Inflammation but Not Fibrosis in Experimental Silicosis. The Journal of Immunology 184: 6367–6377.

55. Sergejeva S, Ivanov S, Lottvall J, Linden A (2005) Interleukin-17 as a recruitment and survival factor for airway macrophages in allergic airway inflammation. Am J Respir Cell Mol Biol 33: 248–253.

56. Cai X-Y, Gommoll Jr GP, Juster L, Narula SK, Fine JS (1998) Regulation of granulocyte colony-stimulating factor gene expression by interleukin-17. Immunology Letters 62: 51–58.

57. Jones CE, Chan K (2002) Interleukin-17 stimulates the expression of interleukin-8, growth-related onco-gene alpha, and granulocyte-colony-stimulating factor by human airway epithelial cells. Am J Respir Cell Mol Biol 26: 748–753.

58. Hirai Y, Iyoda M, Shibata T, Kuno Y, Kawaguchi M, et al. (2012) IL-17A stimulates granulocyte colony-stimulating factor production via ERK1/2 but not p38 or JNK in human renal proximal tubular epithelial cells. American Journal of Physiology - Renal Physiology 302: F244–F250.

59. Cox G, Gauldie J, Jordana M (1992) Bronchial epithelial cell-derived cytokines (G-CSF and GM-CSF) promote the survival of peripheral blood neutrophils in vitro. Am J Respir Cell Mol Biol 7: 507–513.

60. Bauer AK, Travis EL, Malhotra SS, Rondini EA, Walker C, et al. (2010) Identification of novel susceptibility genes in ozone-induced inflammation in mice. Eur Respir J 36: 429–437.

61. Vasalli P (1992) The Pathophysiology of Tumor Necrosis Factors. Annual Review of Immunology 10: 411–452.

62. Korn T, Petermann F (2012) Development and function of interleukin-17-producing γδ T cells. Annals of the New York Academy of Sciences 1247: 34–45.

63. Ueta C, Kawasumi H, Fujisawa H, Miyagawa T, Kida H, et al. (1996) Interleukin-12 activates human gamma delta T cells: synergistic effect of tumor necrosis factor-alpha. Eur J Immunol 26: 3066–3073.

64. Kim CH (2009) Migration and function of Th17 cells. Inflamm Allergy Drug Targets 8: 221–228.

65. Brunner PM, Koszik F, Reinger B, Kalb ML, Bauer W, et al. (2013) Infliximab induces downregulation of the IL-12/IL-23 axis in 6-sulfo-LacNac (slan)+ dendritic cells and macrophages. Journal of Allergy and Clinical Immunology 132: 1184–1193.e1188.

66. Kordonowy LL, Burg E, Lenox CC, Gauthier LM, Petty JM, et al. (2012) Obesity Is Associated with Neutrophil Dysfunction and Attenuation of Murine Acute Lung Injury. American Journal of Respiratory Cell and Molecular Biology 47: 120–127.

67. Smith AG, Sheridan PA, Harp JR, Beck MA (2007) Diet-Induced Obese Mice Have Increased Mortality and Altered Immune Responses When Infected with Influenza Virus. The Journal of Nutrition 137: 1236–1243.

68. Mancuso P, Gotschall A, Phare SM, Peters-Golden M, Lakacs NW, et al. (2002) Leptin-Deficient Mice Exhibit Impaired Host Defense in Gram-Negative Pneumonia. The Journal of Immunology 168: 4018–4024.

69. Wieland CW, Florquin S, Chan ED, Leemans JC, Weijer S, et al. (2005) Pulmonary Mycobacterium tuberculosis infection in leptin-deficient ob/ob mice. International Immunology 17: 1399–1408.

70. Milner JJ, Sheridan PA, Karlsson EA, Schultz-Cherry S, Shi Q, et al. (2013) Diet-Induced Obese Mice Exhibit Altered Heterologous Immunity during a Secondary 2009 Pandemic H1N1 Infection. The Journal of Immunology 191: 2474–2485.

71. Morgan OW, Bramley A, Fowlkes A, Freedman DS, Taylor TH, et al. (2010) Morbid Obesity as a Risk Factor for Hospitalization and Death Due to 2009 Pandemic Influenza A(H1N1) Disease. PLoS ONE 5: e9694.

72. Li W, Mendoza B, Moran TM (2012) Type I interferon induction during influenza virus infection increases susceptibility to secondary Streptococcus pneumoniae infection by negative regulation of gammadelta T cells. J Virol 86: 12304–12312.

73. Taylor KR, Costanzo AE, Jameson JM (2011) Dysfunctional gammadelta T cells contribute to impaired keratinocyte homeostasis in mouse models of obesity. J Invest Dermatol 131: 2409–2418.

74. Taylor KR, Mills RE, Costanzo AE, Jameson JM (2010) Gammadelta T cells are reduced and rendered unresponsive by hyperglycemia and chronic TNFalpha in mouse models of obesity and metabolic disease. PLoS ONE 5: e11422.