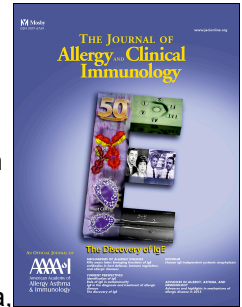


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Sleep deprivation predisposes allergic mice to develop neutrophilic lung inflammation

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Key messages

- Sleep deprivation worsened experimental allergic airway inflammation favoring lung neutrophilia
- Sleep deprivation contributes to Th17 allergen-induced lung inflammation
- Sleep deprived allergic mice are more refractory to corticoid treatment

Summary Capsule

Sleep plays a pivotal role in immune system homeostasis. Sleep disturbances influence immune-mediated allergic responses and improving sleep quality may contribute to asthma management.

Keywords:

Sleep deprivation; allergic lung inflammation; airway neutrophilia; IL-17, mouse model.

Abbreviations:

REM: Rapid Eye Movement

SD: Sleep deprivation

HS: Healthy sleep

OVA: Ovalbumin

BALF: Bronchoalveolar lavage fluid

KO: Knockout

IL-17R: IL-17 receptor

50

51 **Abstract**

52 Background: Although different studies associated sleep deprivation with
53 systemic inflammatory changes, the effect of sleep duration on the pathology
54 of allergic chronic diseases is poorly understood.

55 Objective: To evaluate the influence of sleep deprivation on allergen-induced
56 pulmonary inflammation.

57 Methods: Ovalbumin (OVA)-sensitized C57BL/6 mice were exposed to a first
58 set of intranasal OVA challenge under sleep deprivation (SD) or healthy sleep
59 (HS) conditions followed by a second OVA challenge, one week apart. Some
60 groups were subjected to corticoid treatment with dexamethasone.

61 Results: OVA-sensitized SD mice developed a more severe airway
62 inflammation than the HS allergic group. Analysis of lung parenchyma
63 revealed that the inflammation in SD allergic mice was marked by an influx of
64 neutrophils (mainly) and eosinophils and secretion of IL-6, TNF- α , and IL-17,
65 in contrast to the eosinophilic inflammation and IL-4 production observed in
66 HS allergic mice. The same cytokine profile was observed in *ex vivo* culture of
67 cervical lymph nodes cells and splenocytes, indicating that in allergic mice SD
68 favors immune responses towards a pro-inflammatory Th17 profile. This idea
69 is supported by the fact that disruption of IL-17 signaling (IL-17RA^{-/-})
70 prevented airway neutrophilia in allergic SD mice. Furthermore, SD allergic
71 mice became refractory to corticoid treatment, in contrast to HS allergic group.
72 Conclusion: Collectively, our data show that sleep quality participates in the
73 progression of allergen-induced eosinophilic lung inflammation to corticoid
74 refractory neutrophilic manifestation.

75

76 **Introduction**

77 Asthma is a complex chronic disease that affects around 235 million
78 people worldwide ¹. Asthma pathology was first described as a Th2 disorder
79 characterized by intermittent airway hyperresponsiveness (AHR), eosinophilic
80 airway inflammation, mucus hypersecretion and high IgE levels. However,
81 evidence from the clinical practice demonstrated that asthma is a
82 heterogeneous disorder that presents several phenotypes that vary from
83 mild/moderate to severe manifestations. According to the spectrum of the
84 inflammatory response found in patients, asthma severity has been classified
85 in mild/moderate, manifested by minimal or prominent eosinophilic
86 inflammation, and moderate/severe with predominant neutrophilic or mixed
87 granulocytic inflammation ^{2, 3}. Furthermore, a recent study demonstrated that
88 Th2/Th17 cells and IL-17 were present at a higher frequency in the
89 bronchoalveolar lavage fluid (BALF) of patients with severe asthma, and that
90 these cells were resistant to dexamethasone induced-cell death ⁴.

91 Allergens directly play an important role in asthma severity, but the
92 individuals' genetic and epigenetic variability mediated by several
93 environmental factors may contribute to exacerbation of asthma ⁵. In this
94 regard, recent studies have investigated the relationship between sleep
95 quality and asthma ⁶⁻⁸. Indeed, there is an association between sleep quality
96 and asthma morbidity in children ⁹.

97 Sleep quality is important in many aspects of immune system
98 homeostasis ¹⁰. A night of wakefulness or five days of sleep restriction alter
99 the Th1/Th2 balance ^{11, 12}. Because it has been described that sleep

deprivation (SD) results in a nonspecific production of inflammatory cytokines with marked neutrophilia^{13-15 10, 16}, we hypothesized that SD could be a risk factor for the development of neutrophilic asthma in allergic individuals. Hence, our goal was to determine the influence of sleep duration in the outcome of allergic inflammation. Specifically, we used the ovalbumin (OVA)-allergic lung inflammation model associated with SD and compared to healthy sleep (HS) allergic mice. We show that SD allergic mice developed a mixed, IL-17-dependent, granulocytic inflammatory response with predominant neutrophilic infiltration, and also became refractory to corticoid treatment. Hence, we present experimental data that support the idea that sleep may play a role in the progression of mild/moderate allergic inflammation towards a more severe neutrophilic manifestation.

Methods

Mice

Male C57BL/6 wild-type (n=5-7/group), OT-II transgenic and CD4 deficient mice at 6- to 8-week-old were purchased from Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia (CEDEME), UNIFESP. Interleukin- 17 receptor (IL-17RA) deficient mice were purchased from Ribeirão Preto Medical School, USP. Mice were housed under specific-pathogen-free (SPF) conditions at the Division of Immunology facility, UNIFESP. All animal protocols used in this study were approved by the UNIFESP Animal Care and Use Committee (IACUC) under protocol number #3919250314 and were in accordance with the recommendations of the Federal Law 11.794 (2008) and the Guide for the Care and Use of

Laboratory Animals of the Brazilian National Council of Animal Experimentation (CONCEA). All protocols and animal care and handling strictly followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication No.85-23, revised 1985).

Immunization and Airway inflammation protocol

Mice were treated with OVA as described previously¹⁷ with modifications. Briefly, mice were sensitized on days 0 and 7 with 10 µg of OVA (grade V; Sigma-Aldrich) in 1.6 mg aluminum hydroxide (Sigma-Aldrich) diluted in PBS (0.4 mL). On days 14, 15, 16, 24, 25 and 26 mice were challenged intranasally (i.n) with OVA (10 µg) in PBS (50 µL) after brief anesthesia with a mixture of isoflurane 5% and oxygen 30%¹⁸. Control mice were sensitized and challenged with PBS. On day 27 mice were euthanized, and samples were collected for further analysis. For corticoid treatment, allergic mice received daily dexamethasone I.P. injections (5mg/Kg) six hours after OVA challenges on days 24, 25 and 26.

Sleep deprivation protocol

Mice were submitted to sleep deprivation (SD) for 72 hours during the first OVA challenge (days 14,15, and 16) using the modified multiple platform method^{19, 20}. This method consists in placing 6 animals in a ventilated polycarbonate cage (38 x 31 x 17 cm) containing 12 circular platforms (3.5 cm in diameter), surrounded by water up to 1 cm beneath the surface. The animals are able to move inside the cage, jumping from one platform to the other. All mice underwent a period of training to stay on the platform before

the experimental proceedings of SD in order to attenuate the stress inherent to this protocol. This training consisted of remaining on the platforms in a box with water for 3 hours for 3 consecutive days, prior to the initiation of the SD protocol. When animals reach the paradoxical (REM) phase of sleep, they are awakened when their faces touch the water as a result of muscle atony. Previous studies in mice demonstrated that the multiple platform method completely abolished paradoxical sleep (REM) during the deprivation period, but also resulted in a mild reduction (30%) in slow wave sleep (SWS). Thus, mice can sleep about 70% of the non-REM sleep in the platforms^{21, 22}. The water in the cage was changed daily at 10 a.m., when the mice were moved to a dry cage for a few minutes. Food and water were available *ad libitum* throughout the SD period. The control non-sleep deprived groups (healthy sleep (HS)) were maintained in the same ventilated rack system. All mice were kept in 12:12-h light–dark cycles (lights on at 7 a.m.) to avoid circadian alterations.

Bronchoalveolar lavage fluid (BALF)

Mice were deeply anesthetized by intraperitoneal injection of a solution containing ketamine (0.1 g/mL) and xylazine (0.02 g/mL). The tracheae were cannulated, and lungs were washed 3 times with 0.5 mL PBS. Total and differential cell counts of bronchoalveolar lavage fluid were determined by cytopspin preparations.

Flow Cytometry

Analysis of the cellular profile in the lungs, spleen and lymph nodes (LNs) was performed by flow cytometry. Briefly, after BALF collection, lungs were perfused with 10 mL of cold PBS and removed. Lungs were minced and treated with RPMI 1640 (Gibco) containing 1 mg/mL DNase and 2 mg/mL collagenase (Sigma-Aldrich) for 20 min at 37°C. After centrifugation, the cells were submitted to a Percoll gradient ²³. Single cell suspensions from spleen and LNs were obtained by dissociation with cell strainer and red blood cell lysis using ACK solution (0.15 M NH₄Cl; 1 mM KHCO₃; 0.1 mM Na₂ EDTA). Cells were washed in RPMI 1640 (Gibco) and viability was evaluated using 0.2% Trypan Blue exclusion dye to discriminate between live and dead cells. Cell concentration was estimated using a cell counter (Countess - Invitrogen) and adjusted in cell culture medium. Cells were then stained with the following fluorochrome-conjugated antibodies: CD3 PE (clone 145-2C11); CD4 PerCP (clone RM4-5); CD8 APC (clone 53-6.7); B220 Pacific Blue (clone RA3-6B2); CD45 Pacific Blue (clone 3D-F11), CD11b APC-Cy7 (clone M1/70); Gr1 PE-Cy7 (clone RB6-8C5); IgE FITC (clone 23G3), FcεRI PE (clone MAR-1), c-Kit PerCP (clone 2B8); CD49b APC (clone DX5) (all from BD Biosciences). Online repository figure E1 shows representative dot plots of the multicolor flow cytometry panel used to identify myeloid cell subsets.

To assess intracellular cytokine (ICS) production, 1 million cells were cultured for 6 h in the presence of phorbol myristate acetate (20 ng/mL, Sigma), ionomycin (1 µg/mL, Sigma) and brefeldin (GolgiPlugTM, BD Pharmingen). Subsequently, cells were processed using Cytofix / Cytoperm (BD Pharmingen) according to manufacturer's instructions and stained with the following antibodies: CD45 Pacific Blue (clone 3D-F11); CD3 APC-Cy7

(clone 145-2C11); CD4 PerCP (clone RM4-5); IFN- γ APC (clone XMG1.2); TNF- α PE-Cy7 (clone MP6-XT22); IL-17 PE (clone TC11-18H10); IL-6 FITC (clone MP5-20F3); F4/80 PE (clone BM8); Gr1 Pacific Blue (clone RB6-8C5); CD11b APC-Cy7 (clone M1/70). One million events in a live lymphocyte gate were acquired on a FACSCanto flow cytometer (BD Biosciences) and then analyzed using FlowJo software (version 10, Tree Star, San Carlo, CA). Besides, we used the Boolean gate platform to create several combinations of cytokine producing cells resulting in distinct patterns. The frequency of cytokine-producing cells was calculated by subtracting background values. For each flow cytometry experiment performed in this paper, unstained and all single-color controls were processed to allow proper compensation.

Sorting and adoptive transfer of CD4⁺ T cells

Transgenic OT-II mice were sensitized and challenged with 10 μ g of OVA 323-339 MHC class II-restricted peptide (ISQAVHAAHAEINEAGR) (Genscript). OT-II mice were distributed into two groups, and one group was sleep deprived between days 14-16. Twenty-four hours after the last challenge (day 17) spleens of OT-II mice were collected, and CD4⁺ T cells were purified by Fluorescence Activated Cell Sorting (FACS) and then 1.5x 10⁶ cells were adoptively transferred intravenously to CD4 deficient mice (CD4 KO). CD4 KO mice were then challenged as previously described and 24 hours after the last challenge mice were euthanized.

Cell culture

Cells from spleen, cervical lymph nodes (CLNs) and lungs were isolated as described before and cultured (1×10^6 /200 μ L) for 3 days in RPMI 1640 supplemented with 10% of fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), 10 mM Hepes (Gibco), 1mM sodium pyruvate (Gibco), 1% v/v non-essential aminoacids solution (Gibco), 40 μ g/mL of Gentamicin, 20 μ g/mL of Peflacin and 5×10^{-5} M 2-mercaptoethanol (Gibco) in the presence of OVA (10 μ g/ml). After the incubation period, 150 μ L of the supernatants were collected and frozen at -20°C until cytokine quantification.

Cytokine determination

Mouse cytokines in culture supernatants were detected simultaneously using the mouse Th1/Th2/Th17 cytokine cytometric bead array (CBA) kit (BD Pharmingen), according to the manufacturer's instructions. Cytokines in serum samples (IL-4, IL-5 and IL-13) were evaluated using ELISA kits Ready-SET-Go! (eBiosciences).

OVA-specific IgG1 and IgE determination

Ninety-six well plates (High Binding, Costar) were coated with 20 μ g of OVA (grade II; Sigma-Aldrich) diluted in PBS overnight. After washes with PBS-0.02% Tween 20 (PBST), wells were blocked with 5% nonfat milk and 1% BSA in PBST for 2 h at room temperature (rt). After this period, the plates were washed with PBST and incubated with mouse serum for 1 h at rt. Plates were washed with PBST and incubated with horseradish peroxidase (HRP) anti-mouse IgG1 (Southern Biotech) for 2 h at rt. After washes with PBST, the enzymatic reaction was developed by the addition of 1 mg/mL of o-

phenylenediamine (Sigma) diluted in phosphate–citrate buffer, pH 5.0, containing 0.03% (v/v) hydrogen peroxide. The enzymatic reaction was stopped by the addition of 50 μ L of a solution containing 4N H₂SO₄. Plates were read at 492 nm (OD₄₉₂) with an ELISA reader (Labsystems Multiskan MS). Anti-OVA-specific IgE were measured with the mouse anti-ovalbumin IgE ELISA Kit (Cayman Chemical) according to the manufacturer's instruction.

Corticosterone quantification

Plasma corticosterone levels were assessed by ultra fast liquid chromatography-tandem mass spectrometry (LC-MS/MS)²⁴. All samples analyses were performed on a Quattro Micro triple quadrupole mass spectrometer (Waters, Milford, MA, USA) using a Kinetex® (50 x 2.10 mm, 2,6 μ m) column at a flow rate of 0.2 mL/min which was controlled by Masslynx® 4.1 software. The lower quantification limit for corticosterone was 10 ng/mL.

Data analysis

One-way analysis of variance (ANOVA) followed by Tukey's honestly significantly different (HSD) or unpaired T-test were used to compare the possible differences between groups. Statistical analysis and graphical representation of data were performed using GraphPad Prism version 7.0 software. Significance was accepted at an alpha of 0.05.

Results

Sleep deprivation exacerbates airways and lung inflammation in allergic mice

To investigate the effect of sleep deprivation in allergic lung inflammation, mice were sensitized with OVA adsorbed to alum and challenged with OVA intranasally (on days 14,15,16 and 24, 25, 26) to induce airway inflammation. One group of mice was sleep-deprived (SD) for 72 hours during the first round of OVA challenge as outlined in Figure 1A. After the last set of OVA challenge, healthy sleep (HS) allergic mice presented significant airway inflammation, which was increased approximately 2-fold by SD (Figure 1B). Notably, the exacerbated inflammatory response in SD allergic mice was characterized by a significant influx of mononuclear cells (Figure 1C) that resulted in a shift towards predominant neutrophilic BALF inflammation (Figure 1D). In contrast, HS allergic mice presented airway eosinophilia (Figure 1E).

Consistent with the BALF findings, the analysis of lung parenchyma by flow cytometry showed a significant increase in the frequency of eosinophils in HS allergic mice (Figure 1F). The SD allergic group presented an influx of neutrophils and mast cells into the lung parenchyma, supporting the idea that sleep duration influences the pattern of the inflammatory reaction in allergic mice (Figure 1G and H, respectively). Although basophils were increased both in the HS and SD allergic mice, no significant difference was observed between these groups (Figure 1I).

Sleep deprivation drives the cytokine profile to a pro-Th17 pattern

Next, we evaluated the cytokine profile of culture supernatants after cells were stimulated *in vitro* with OVA. Figure 2 shows that lung cells from SD allergic mice produced higher levels of IL-17 and IL-6, in contrast to those

from the HS allergic group, where IL-4 production was higher. A similar cytokine profile was observed in the cervical lymph nodes (CLN) and spleens from HS and SD allergic mice, corroborating the idea that SD influences the pattern of the allergen-induced immune response. Also, SD led to a higher secretion of TNF- α by lung cells, while did not affect TNF- α production by CLN or spleen cells. When we analyzed cytokine content in serum (Figure 2B), we observed higher amounts of IL-4 and IL-13 in serum from SD allergic mice when compared to the HS allergic group, while no difference was observed in IL-5.

Despite the influence of SD in the local and systemic cytokine profile, B cell responses did not seem to be modulated by sleep duration, since antibody production, marked by high levels anti-OVA IgG1 and IgE, was similar in HS and SD allergic mice (online repository figure E2).

Sleep deprivation potentiates the migration of Th17-committed CD4⁺ T cells to the lungs

The results described above demonstrated that sleep deprivation favors the development of a pro-Th17 environment. Analysis of intracellular cytokine content (Figure 3A) revealed that lungs from SD allergic mice presented higher frequency of IL-17 and TNF- α but not of IFN- γ producing CD4⁺T cells in comparison to the HS allergic group. Boolean combinations of cytokine-producing CD4⁺ T cells indicated that SD allergic mice displayed a higher percentage of polyfunctional IL-17⁺/TNF- α ⁺ T cells. Similarly, the frequency of IL-6⁺ and TNF α ⁺-producing macrophages was higher in allergic mice subjected to SD than in the HS group (Figure 3B). We also observed

higher frequency of IL6⁺/TNF- α ⁺ double positive macrophages in the lungs of SD allergic mice. Notably, polyfunctional cells produced larger amounts of each cytokine than single cytokine producing cells, as determined by median fluorescent intensity (MFI) (data not shown). Thus, SD predisposes allergic mice to a more inflammatory allergen-induced airway reaction, marked by high levels of IL-17, IL-6 and TNF α .

Next, CD4 deficient recipient mice (CD4KO) were adoptively transferred with purified CD4⁺ T cells from OTII-transgenic OVA-sensitized animals submitted to HS or SD regimens as outlined in Figure 3C. The airway inflammation, more specifically neutrophilia, was 3-fold higher in the CD4KO mice that received CD4⁺ T cells from SD allergic mice when compared to the group that received cells from HS allergic mice (Figure 3D and E). Furthermore, the lung parenchyma of CD4KO mice transferred with CD4⁺ T cells from the SD allergic group displayed higher frequency of neutrophils (Figure 3F). Thus, SD increases the production of inflammatory mediators committed with neutrophilic inflammation, which aggravates the pulmonary reaction in response to airborne antigens.

IL-17 signaling is essential for the allergen-induced neutrophilic inflammation associated with sleep deprivation

To evaluate whether OVA-induced neutrophilic inflammation observed in the SD allergic mice was mediated by IL-17 signaling itself or by another product of Th17 cells, we used IL-17RA deficient mice (IL-17RA^{-/-}). In contrast to WT mice, IL-17RA^{-/-} allergic mice subjected to SD did not display an exacerbated airway inflammation in BALF when compared to the HS allergic

group (Figure 4A). IL-17RA^{-/-} SD allergic mice presented higher numbers of eosinophils and impaired airway neutrophilia in contrast to the WT allergic group (Figure 4B and C). Consistent with the BALF findings, the lung parenchyma of IL-17RA^{-/-} SD allergic mice was mainly composed of eosinophils (Figure 4D and E). Our data show that during allergen-induced inflammation, IL-17 signaling is essential for neutrophil recruitment into the lungs upon SD.

Sleep deprivation induces corticoid-resistance in allergic mice

Besides neutrophilic inflammation, the resistance to steroid therapy is another hallmark of allergic diseases^{5,25}. To determine the influence of sleep duration in corticoid resistance, HS and SD allergic mice received daily dexamethasone treatment during the last set of airway OVA-challenge. Corticoid treatment inhibited the influx of cells, more specifically eosinophils and neutrophils to the airways of both HS and SD allergic groups (Figure 5A, B and C). However, dexamethasone administration failed to modulate the production of IL-17 and TNF- α by lung cells, indicating that the inflammatory response in the SD allergic mice is more resistant to corticoid effect (Figure 5D and E). No difference was observed in IL-6 levels (Figure 5F). Besides, SD also influenced the pulmonary inflammatory pattern. We also observed that SD allergic mice presented a persistent peribronchovascular infiltration, in contrast to the HS allergic group (Figure 5G).

Discussion

Sleep quality exerts an important influence on host immune system homeostasis. However, its impact in chronic allergic diseases remains elusive. The present study demonstrates that sleep deprivation is associated with increased IL-17 levels, lung neutrophilia and resistance to corticoid treatment.

Because asthmatic individuals experience disturbed sleep periods due to breathlessness, nighttime coughing, and wheezing, the disease itself impairs sleep quality, which can be implicated in further complications⁶. For example, immunity against pathogens and the efficacy of vaccination are impaired by sleep deprivation or sleep restriction^{26,27}. This phenomenon seems to be associated with a shift towards Th2 responses, impairing the protective Th1 immunity¹¹.

In the first set of experiments, it was possible to observe that allergic mice subjected to SD developed an intense mixed granulocytic inflammation with prevalence of neutrophils. The allergen-induced T helper response was marked by TNF- α and IL-17 production, in contrast to that observed in HS allergic mice, in which the pulmonary inflammation was marked by eosinophils and IL-4. In the serum, however, SD allergic mice presented higher levels of IL-4 and IL-13 than HS allergic mice. Previous works have demonstrated the presence of mixed Th2/Th17 responses in the BALF of patients with severe asthma⁴ and in a murine asthma model²⁸.

Analysis of the infiltrating T lymphocytes demonstrated that SD induced a marked increase in the frequency of Th17 cells in the parenchyma, further supporting the higher IL-17 levels observed in this group. In addition, it was

possible to observe in lungs of SD allergic mice the presence of distinct polyfunctional $CD4^+$ T lymphocytes: triple-positive $IFN-\gamma^+/IL-17^+/TNF-\alpha^+$; double-positive $IFN-\gamma^+/IL-17^+$ and a major subpopulation constituted by double-positive $IL-17^+/TNF-\alpha^+$ cells. Similarly, the pattern of cytokine-producing macrophages present in the lung parenchyma of SD allergic mice also differed from those of the HS allergic group. Although the frequency of $IL-6^+$ -producing macrophages was similar in these groups, $TNF-\alpha^+$ and double-positive $IL-6^+/TNF-\alpha^+$ cells were higher in SD allergic mice. Therefore, these data indicate that SD in allergic mice during the first airway challenge with allergen is sufficient to induce the accumulation of Th17 cells and neutrophils in the lungs.

It has been demonstrated that SD could, itself, increase the levels of inflammatory markers such as IL-6, $TNF-\alpha$, and IL-17, and systemic neutrophilia²⁹⁻³². Indeed, when euthanasia was performed 24 h after SD, non-allergic mice presented more neutrophils in the airways than HS mice (online repository figure E3). In addition, SD concomitantly to the allergen exposure impacted the development of OVA-specific inflammatory responses favoring airway neutrophilia and the development of long lasting OVA-specific Th17 cells in the lung.

To better understand the impact of SD in the specific $CD4^+$ T cell response, we adoptively transferred $CD4^+$ T lymphocytes from immunized OT-II OVA-transgenic mice to $CD4$ deficient mice ($CD4KO$). $CD4^+$ T cells transfer from SD OT-II mice to $CD4KO$ resulted in a 3-fold increase in BALF and lung inflammation when compared to $CD4KO$ mice transferred with $CD4^+$

T cells from the HS group. Thus, SD altered the pro-inflammatory potential of effector CD4⁺ T lymphocytes.

Our data suggest that SD predisposes allergic mice to respond to allergen stimulation with neutrophilic inflammation and increased IL-6, TNF- α and IL-17 levels. Because IL-17 is implicated in lung neutrophilia^{33, 34}, we then studied the impact of SD in IL-17RA deficient mice. In contrast to what was observed in WT allergic mice, IL-17RA^{-/-} allergic animals subjected to SD did not develop more intense airway or lung inflammatory responses when compared to HS allergic animals, further supporting a key role for IL-17 in lung neutrophilia. In addition, the inverse correlation between eosinophils/neutrophils detected in the WT mice was not observed in IL-17RA^{-/-}. There was no significant difference in the number of eosinophils in IL-17RA^{-/-} mice compared to the HS and SD allergic groups. This observation is in line with previous work that demonstrated that IL-17 signaling attenuated allergen-induced airway eosinophilia³⁵. Indeed, treatment with anti-IL-17 receptor mAb (brodalumab) showed positive results in a subgroup of patients with moderate/severe asthma while no efficacy/evidence of effect was observed in the overall study population³⁶.

Severe asthma characterized by neutrophilia is often resistant to corticoid treatment^{37, 38}, and IL-17 is associated with the resistance observed in patients with severe asthma³⁹. In animal models, transfer of effector Th17 cells to sensitized mice resulted in neutrophil migration into the lung that was not inhibited by dexamethasone, in contrast to the suppression of lung eosinophilia that resulted from Th2 cells⁴⁰.

In our model, dexamethasone treatment completely abrogated airway eosinophilia in either SD or HS allergic animals. However, the influx of neutrophils into the airways of SD allergic group was only partially inhibited by corticoid treatment. Also, dexamethasone treatment failed to suppress IL-17 production, suggesting that corticoid effect on neutrophilia may be due to its impact in granulopoiesis^{41, 42} or secretion of CXC chemokines such as cytokine-induced neutrophil chemoattractant (KC; CXCL1) and/or macrophage inflammatory protein 2 (MIP-2; CXCL-2)⁴³. Since the resistance of Th17 cells to dexamethasone has been described both in experimental and clinical settings^{44, 45, 46}, it is possible to associate decreased neutrophilia and persistent IL-17 levels. Indeed, a recent study suggests a combination of dexamethasone and anti-IL-17 treatment to alleviate steroid insensitive asthma⁴⁷.

Taken together, our findings support the idea that SD constitutes a risk factor for the evolution of mild/moderate asthma towards the moderate/severe phenotype. Although the mechanisms underlying SD-induced development of neutrophilic corticoid-resistant Th17 pattern in allergic group remain elusive, recent studies demonstrated that neutrophils recruitment to the lung, Th17 commitment and the resistance to corticoids, are under the regulation of circadian clock^{48, 49}. Thus it is conceivable that alterations in sleep regimen modulate the development of adaptive responses in allergic individuals. Although it may not be possible to completely extricate the sleep loss from the general stress effects, we did not observe a significant difference in corticosterone levels between HS and SD allergic groups (online repository figure E4).

In conclusion, sleep disturbance due to asthma symptoms might exert a deleterious influence on allergic individuals. In addition to common therapeutics, monitoring sleep quality in allergic individuals seems to constitute an important tool for the management of asthma pathology.

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Conflict of Interest Statement

The authors declare no commercial or financial conflict of interest.

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Figure legends

Figure 1. Sleep deprivation aggravates allergen-induced airway inflammation. (A) Experimental protocol; (B) Total BALF cell counts; (C) Mononuclear; (D) neutrophils and (E) eosinophils counts of cytopspin preparations from BALF cells; Frequency of (F) neutrophils, (G) eosinophils, (H) mast cells and (I) basophils in the lung by flow cytometry. Data were from

three independent experiments (n= 5-8 mice per group). Only significant differences between HS and SD allergic groups are shown, ***p<0.001, ****p<0.0001. HS, healthy sleep; SD, sleep deprived.

Figure 2. Sleep deprivation induces a pro-inflammatory Th17 profile. (A)

TNF- α , IL-4, IL-6 and IL-17 levels in culture supernatant from lungs, cervical lymph nodes and spleen cells were assessed by Cytometric Bead Array (CBA); (B) IL-4, IL-5 and IL-13 in serum were assessed by ELISA. Data were from three independent experiments (n= 5-8 mice per group). Only significant differences between HS and SD allergic groups are shown, **p<0.01; ***p<0.001, ****p<0.0001. ND, not detected; HS, healthy sleep; SD, sleep deprived.

Figure 3. Sleep deprivation leads to allergen-induced lung neutrophilic inflammation in an IL-17 dependent manner. (A) Percentage of IL-17⁺ and

TNF- α ⁺ cells in lung CD4⁺ T cells. Boolean combinations show the frequency of each response based on all possible combinations of cytokine-producing CD4⁺ T cells; (B) Percentage of IL-6⁺ and TNF- α ⁺ cells in lung macrophages. Boolean combinations show the frequency of each response based on all possible combinations of cytokine-producing macrophages; (C) Adoptive transfer scheme; (D) Total BALF cell counts; (E) Neutrophil counts of cytopspin preparations from BALF cells; (F) Frequency of neutrophils in the lungs by flow cytometry. Data were from two independent experiments (n= 5 mice per group). Only significant differences between HS and SD allergic groups are shown, * p<0.05; **p<0.01; *** p<0.001. HS, healthy sleep; SD, sleep deprived.

Figure 4. Sleep deprivation aggravates allergen-induced lung inflammation in an IL-17RA-dependent manner. (A) Total BALF cell counts; (B) Eosinophils and (C) neutrophils counts of cytopsin preparations from BALF cells; (D) Frequency of eosinophils and (E) neutrophils in the lungs by flow cytometry. Data were from two independent experiments (n= 6 mice per group). Only significant differences between HS and SD allergic groups are shown, **p<0.01, *** p<0.001. HS, healthy sleep; SD, sleep deprived. White bars, wild type mice; black bars, IL17-RA deficient mice (IL-17 RA^{-/-}).

Figure 5. Sleep deprivation induces corticoid resistance in allergic mice. (A) Total BALF cell counts; (B) Eosinophils and (C) neutrophils counts of cytopsin preparations from BALF cells; (D) IL-17, (E) TNF- α and (F) IL-6 levels in culture supernatants from lung cells were assessed by Cytometric Bead Array (CBA); (G) Representative lung section stained with hematoxylin/eosin for analysis of cellular inflammation (original magnification x 200). Data were from two independent experiments (n= 6 mice per group). Only significant differences between HS and SD allergic groups are shown, * p<0.05; **p<0.01. HS, healthy sleep; SD, sleep deprived. White bars, vehicle treated mice; black bars, dexamethasone treated mice.

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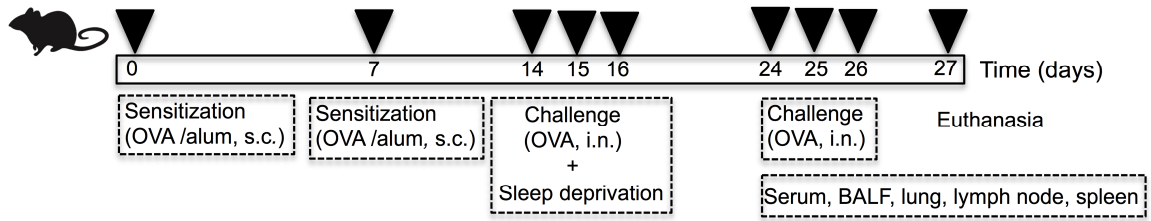
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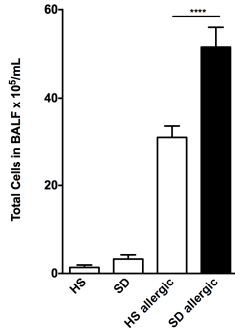
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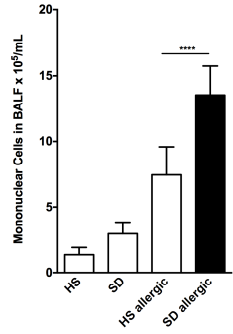
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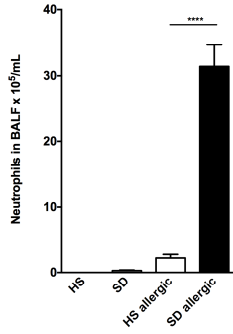
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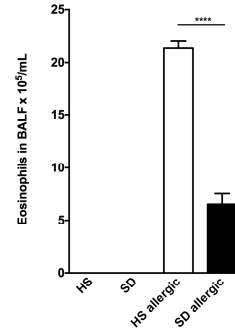
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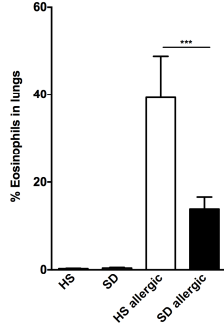
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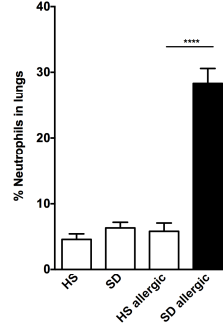
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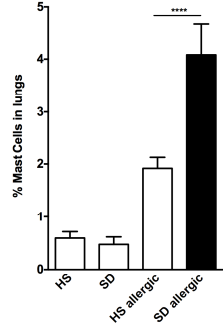
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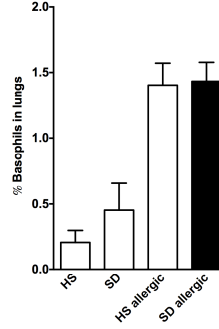
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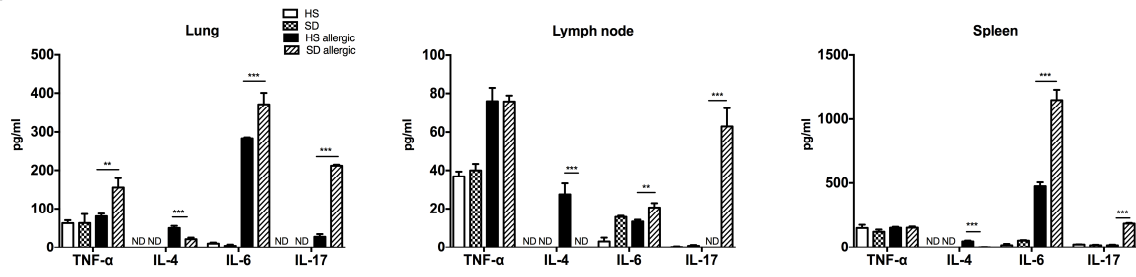
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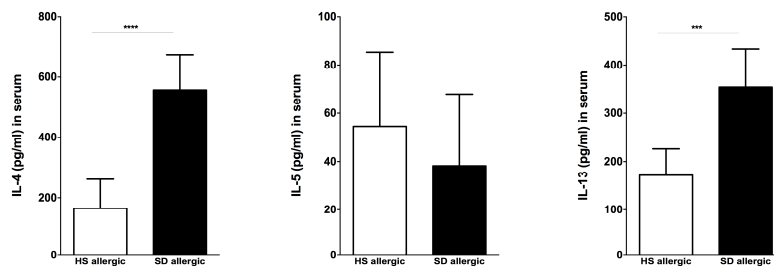
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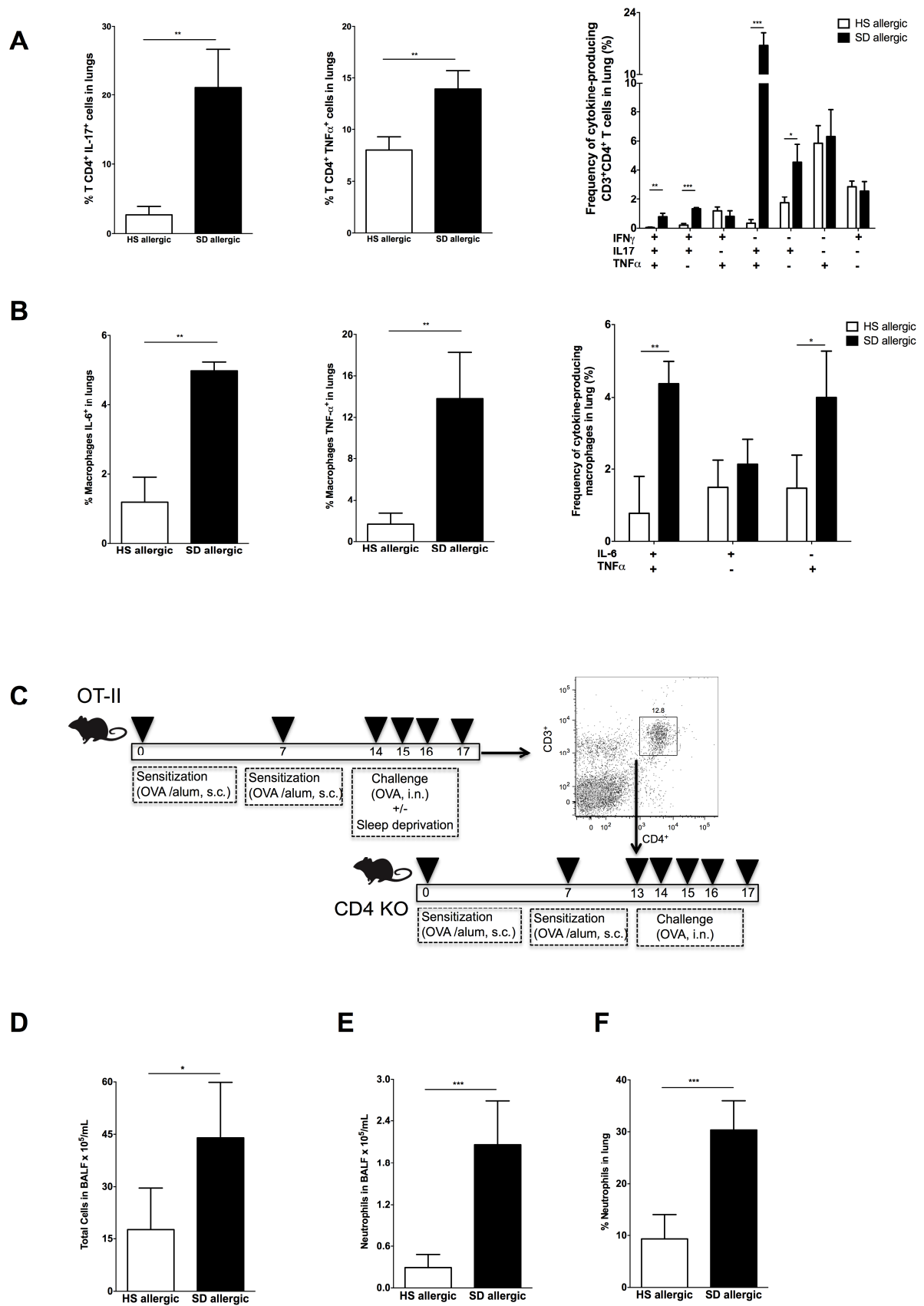


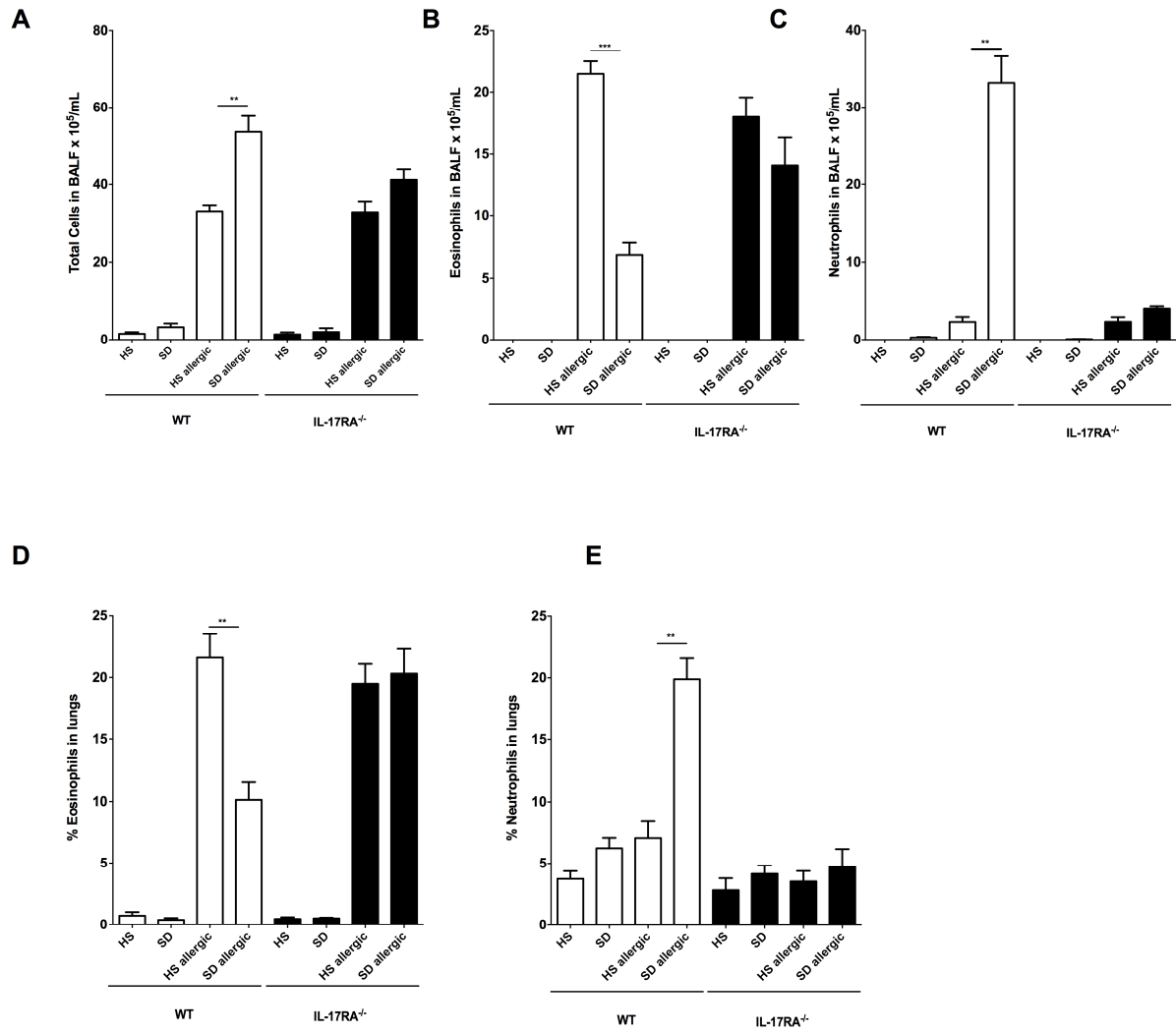
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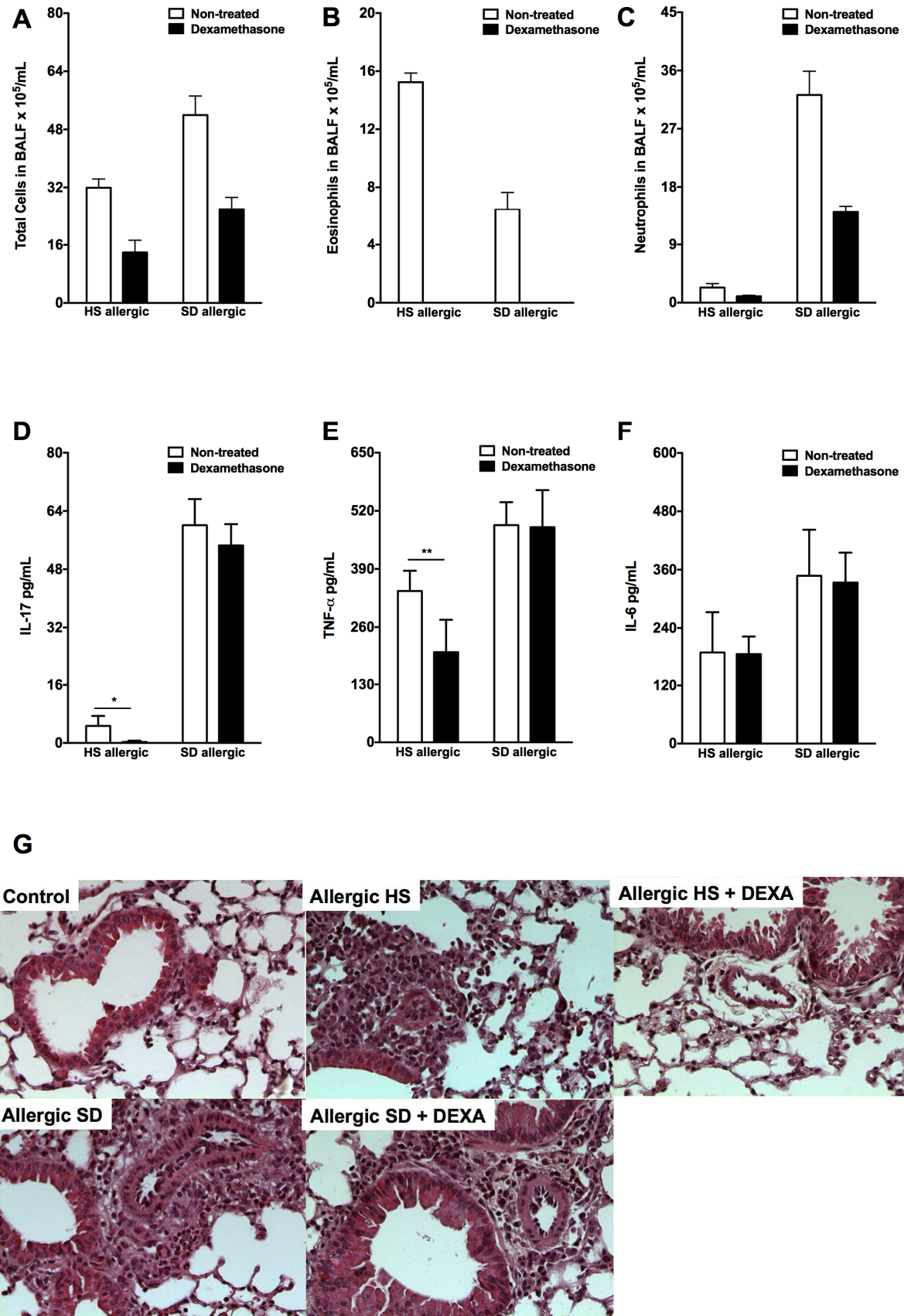


B









Online repository figure legends

Online repository figure E1. Representative dot plots of a multicolor flow

cytometry panel used to identify myeloid cell subsets. Mouse lungs were enzymatically digested and cells were isolated as described in “Materials and Methods”. After doublets exclusion, immune cells were identified by CD45 staining. Within the CD45⁺ population, mast cells were gated as C-Kit⁺FcεRI⁺ double positive; Basophils as C-Kit⁻CD49⁺FcεRI⁺; Neutrophils as Gr1⁺CD11b⁺ and eosinophils as Gr1^{low}SSC^{hi}IgE⁺FcεRI⁺. Comparison of lung cells in allergic healthy sleep (HS) and sleep deprived (SD) mice. The Gr1^{low}SSC^{hi} (gate 1) cells were further analyzed for IgE and FcεRI expression and Gr1^{hi}SSC^{int} (gate 2) were further analyzed for CD11b expression. FSC, Forward scatter; SSC, Side scatter. HS, healthy sleep; SD, sleep deprived.

Online repository figure E2. Sleep deprivation did not influence OVA-

specific humoral response. OVA-specific (A) IgG1 and (B) IgE were assessed by ELISA. Data were from three independent experiments (n= 6 mice per group). HS, healthy sleep; SD, sleep deprived.

Online repository figure E3. Sleep deprivation aggravates allergen-

induced airway inflammation. (A) Experimental protocol; (B) Total BALF cell counts; (C, D, E) Differential counts of cytopsin preparations from BALF cells; (F, G, H, I) Frequency of myeloid cells subsets in the lung by flow cytometry (J) TNF-α, IL-4, IL-6 and IL-17 levels in culture supernatant from lungs cells were assessed by Cytometric Bead Array (CBA). Data were from three independent experiments (n= 5-8 mice per group). Only significant differences

between HS and SD groups and between HS and SD allergic groups are shown, ** $p < 0.01$; *** $p < 0.001$. HS, healthy sleep; SD, sleep deprived.

Online repository figure E4. Sleep deprivation did not influence corticosterone levels. Corticosterone levels in the plasma were assessed by liquid chromatography tandem mass spectrometry (LC-MS/MS) 24 h after the last set of OVA challenge. HS, healthy sleep; SD, sleep deprived.

