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T cell-derived TNF down-regulates acute airway response to endotoxin

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Acute and chronic airway inflammations caused by environmental agents including endotoxin represent an increasing health problem. Local TNF production may contribute to lung dysfunction and inflammation, although pulmonary neutrophil recruitment occurs in the absence of TNF. First, we demonstrate that membrane-bound TNF is sufficient to mediate the inflammatory responses to lipopolysaccharide (LPS). Secondly, using cell type-specific TNF-deficient mice we show that TNF derived from either macrophage/neutrophil (M/N) or T lymphocytes have differential effects on LPS-induced respiratory dysfunction (enhanced respiratory pause, Penh) and pulmonary neutrophil recruitment. While Penh, vascular leak, neutrophil recruitment, TNF, and thymus- and activation-regulated chemokine/CCL17 (TARC) expression in the lung were reduced in M/N-deficient mice, T cell-specific TNF-deficient mice displayed augmented Penh, vascular leak, neutrophil influx, increased $CD11c^+$ cells and expression of TNF, TARC and murine CXC chemokines KC/CXCL1 in the lung. In conclusion, inactivation of TNF in either M/N or T cells has differential effects on LPS-induced lung disease, suggesting that selective deletion of TNF in T cells may aggravate airway pathology.

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Abbreviations: **BAL:** bronchoalveolar lavage · **KC:** murine CXC chemokines KC/CXCL1 · **memTNF:** membrane-bound TNF · **M/N-TNF:** macrophage and neutrophil-derived TNF · **Penh:** enhanced respiratory pause · **TARC:** thymus- and activation-regulated chemokine/CCL17

Introduction

Endotoxin is a major component of pulmonary inflammation leading to acute lung injury [1] and contributes to the development and progression of chronic respiratory disease including asthma [2–6]. Pathologic changes induced by endotoxin inhalation include acute respiratory distress syndrome (ARDS), neutrophil recruitment, injury of the alveolar epithelium and endothelium with protein leak in the alveolar space [7, 8]. In mice, aerogenic exposure to endotoxin from Gram-negative bacteria induces acute pulmonary inflammation, local

TNF production, alveolar-capillary leak and augmented airway resistance as expressed by enhanced respiratory pause (Penh) [9, 10]. TNF is dispensable for neutrophil recruitment, but necessary for Penh increase in response to intranasal endotoxin [10].

Cell type-specific sources of TNF convey distinct and only partially overlapping functions [11]. In particular, TNF produced by myeloid cells confers resistance to pathogens, parasites and can control granuloma formation [12, 13], while TNF produced by B cells is essential in maintaining splenic microarchitecture [12, 14, 15]. The contribution of the different cellular sources of TNF in LPS-induced acute lung inflammation requires more detailed investigation.

TNF is synthesized as a membrane-bound precursor [16], which is subsequently cleaved by metalloproteinases including the TNF-α-converting enzyme TACE or a disintegrin and metalloproteinase [17, 18] into the secreted homotrimer TNF. Both molecular forms of TNF are biologically active. In particular, the membrane-bound TNF (memTNF) was shown to mediate cytotoxicity, host defense, polyclonal activation of B cells, induction of IL-10 by monocytes, ICAM-1 expression on endothelial cells, and chemokine expression and to support secondary lymphoid organ structure [16, 19–22].

Deregulated TNF may be deleterious, for example sustained activation of TNF signaling aggravates sepsis [23, 24], cerebral malaria [25, 26]; allograft rejection, inflammatory disorders and autoimmune diseases [27, 28]. TNF-blocking therapies have been successfully applied in rheumatoid arthritis (RA), Crohn's disease, and psoriasis [27, 29]. However, TNF-blocking therapies are accompanied by compromised host defense, and adverse effects such as reactivation of tuberculosis have been reported in RA patients treated with TNF blockers [30–33]. Selective blocking of particular molecular forms or cellular sources of TNF may have beneficial effects, as it was shown recently that membrane-associated TNF is sufficient to control acute *Mycobacterium tuberculosis* infection [34, 35].

In the present study, we addressed the role of cell-associated, memTNF in LPS-induced airway response, by comparing responses in mice producing uncleavable memTNF [22] with those in TNF-deficient (TNF-KO) mice [36]. We show that cell-associated TNF can efficiently substitute soluble TNF to convey LPS-induced respiratory dysfunction as well as acute pulmonary inflammation with recruitment of neutrophils, and vascular leakage. Using the recently developed mice with specific deletion of the TNF gene in macrophages and neutrophils or in T lymphocytes [11], we show that selective ablation of TNF in macrophages/neutrophils reduced both inflammation and bronchoconstriction, while absence of TNF in T cells augmented the pulmonary inflammatory response to LPS. Therefore, the data suggest a novel negative regulatory function of T cell-derived, possibly membrane-associated, TNF for LPS-induced inflammatory airway response and provide evidence for the involvement of T cells in innate immunity regulation.

Results

Membrane-bound TNF mediates lung inflammation and airway resistance in response to endotoxin

TNF is essential for acute LPS-induced respiratory dysfunction as shown in TNF-deficient mice [10]. We first asked whether cell-associated, mem TNF is sufficient for inducing airway response to LPS [22]. Unlike mice with a complete TNF deficiency (TNF KO), mice engineered to express uncleavable memTNF developed an acute increase of Penh, a measure of respiratory dysfunction by whole body plethysmography, in response to intranasal LPS exposure (Fig. 1A, B).

In the bronchoalveolar fluid (BAL) of memTNF mice, total BAL cells (data not shown), total protein concentration, an indicator of vascular leakage (Fig. 1C), neutrophil and macrophage numbers (Fig. 1D and E) were similar to the response observed in WT mice. However, memTNF mice showed reduced neutrophil recruitment in the tissue (by 32 ± 10%), as assessed by lung myeloperoxidase (MPO) activity measurements (Supporting Information Fig. 1A). Microscopically, lung tissue sections showed strong inflammation and cell infiltration comparable to WT (Supporting Information Fig. 1B).

Soluble TNF in the BAL was absent in complete TNF-deficient mice, and only minimal amounts of TNF were detected in memTNF mice by ELISA (Fig. 1F), which was however non-functional TNF using the WEHI bioassay (data not shown). The BAL concentrations of IL-12 p40 were not significantly altered in both TNF KO and memTNF animals (315 ± 24 pg/mL, 291 ± 28 pg/mL, and 216 ± 80 pg/mL in TNF KO, memTNF, and WT mice, respectively).

Therefore, cell-associated TNF is sufficient to increase Penh values and acute inflammation in response to local LPS exposure.

Contribution of M/N-TNF to airway response to LPS

To assess the contribution of TNF of hematopoietic cell origin in LPS-induced airway response, TNF KO and WT mice were lethally irradiated and reconstituted with WT or TNF KO bone marrow cells. TNF KO mice recon-

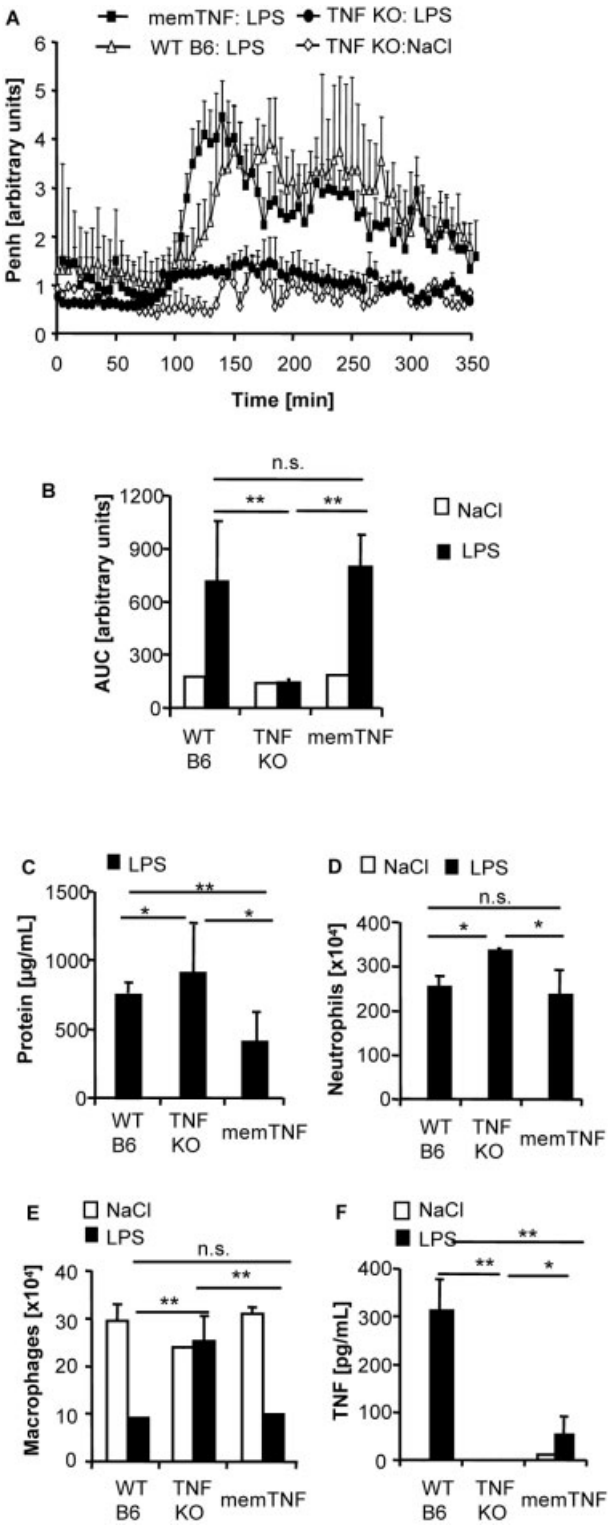

Figure 1. Membrane-bound TNF mediates bronchoconstriction and lung inflammation in response to endotoxin. WT mice (C57BL/6), mice deficient in TNF (TNF KO) or expressing only membrane bound TNF (memTNF) were challenged intranasally with 10 μg LPS and Penh was recorded for 360 min using whole body plethysmography (A). The bar graph in (B) represents the calculated area under the curve (AUC) obtained in (A). Twenty-four hours after challenge mice were sacrificed and the bronchoalveolar fluid was analyzed for protein contents (C), neutrophil (D) and macrophages (E) counts. The BAL concentration of TNF (F) was determined by ELISA. The values represent the mean ± SD of $n = 7$ mice per group pooled from two independent experiments (*$p < 0.05$; **,$p < 0.01$; n.s., not significant).

stituted with WT bone marrow (BM) cells showed a Penh response to LPS similar to WT mice, indicating that TNF of hematopoietic origin is sufficient to mediate LPS-induced respiratory dysfunction (Fig. 2). This is in line with a study by Hollingsworth and colleagues [37], showing that TLR4 on hematopoietic cells is critical for LPS-induced neutrophil recruitment to the lung. However, the partial response in WT mice reconstituted with bone marrow cells from TNF KO mice indicates that tissue-derived TNF participates at least partially in the response to LPS (Fig. 2).

We then asked which cellular source of TNF might contribute to the development of acute lung inflammation. Macrophages and neutrophils are a major source of TNF in inflammation. The M/N-TNF KO mice showed a partial reduction in LPS-induced respiratory dysfunction (Fig. 3A and B). This was accompanied by reduced total cell numbers ($2.1 \pm 0.3 \times 10^6$ to $1.6 \pm 0.5 \times 10^6$ cells), neutrophil recruitment, and protein leakage (by 24 and 25%, respectively, Fig. 3C and D) in the BAL. Macrophage counts were not significantly different in M/N-TNF KO compared to WT mice (Fig. 3E).

The reduction in neutrophil recruitment in the lung tissue was more pronounced in M/N-TNF KO mice. MPO activity was reduced by 72% as compared to WT mice ($p < 0.05$, Supporting Information Fig. 2A). Reduced cell infiltration and inflammation was confirmed microscopically in lung sections (Supporting Information Fig. 2B). Adoptive transfer of macrophages expressing only memTNF into complete TNF KO mice partially reconstituted the LPS-mediated respiratory response (by 40%, data not shown), suggesting that memTNF of macrophage origin contributes to mount an optimal response to LPS. Therefore, M/N-TNF is involved in the recruitment of neutrophils although it may not be the sole factor responsible. Furthermore, the absence of M/N-TNF could not fully explain the phenotype of complete TNF KO mice, indicating that other cellular sources of TNF are involved in the airway response.

Lack of T cell-derived TNF exacerbates airway resistance and inflammation

T cells represent another important source of bioactive TNF, which may contribute to the LPS response in the airway. We therefore evaluated the pathology in mice deficient for TNF in T cells only. Respiratory dysfunction

upon LPS administration increased more rapidly in T cell-TNF KO mice and was significantly higher than in WT mice (Fig. 3A and B). This functional response was associated with augmented protein leakage (Fig. 3C) and cell numbers in BAL as compared to WT mice ($p < 0.01$, $3.3 \pm 0.8 \times 10^6$ *versus* $2.1 \pm 0.3 \times 10^6$ cells). The cells consisted essentially of neutrophils (Fig. 3D). This was confirmed by microscopic analysis of the lung

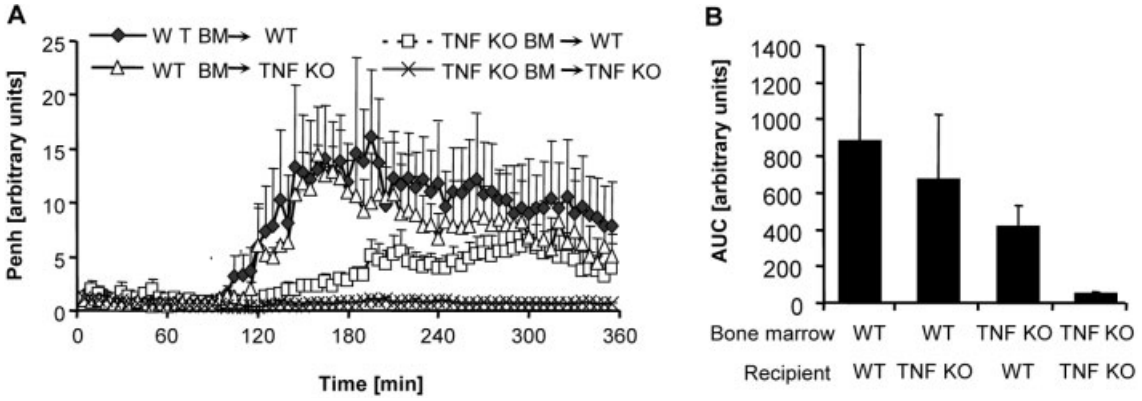

Figure 2. TNF derived from hematopoietic cells is sufficient for the LPS response. WT C56BL/6 or TNF KO mice (recipient) lethally irradiated and reconstituted with the bone marrow (BM) indicated were challenged intranasally with 10 μg LPS and Penh (respiratory discomfort) was recorded for 360 min using whole body plethysmography (A). The bar graph in (B) represents the calculated area under the curve obtained in (A).

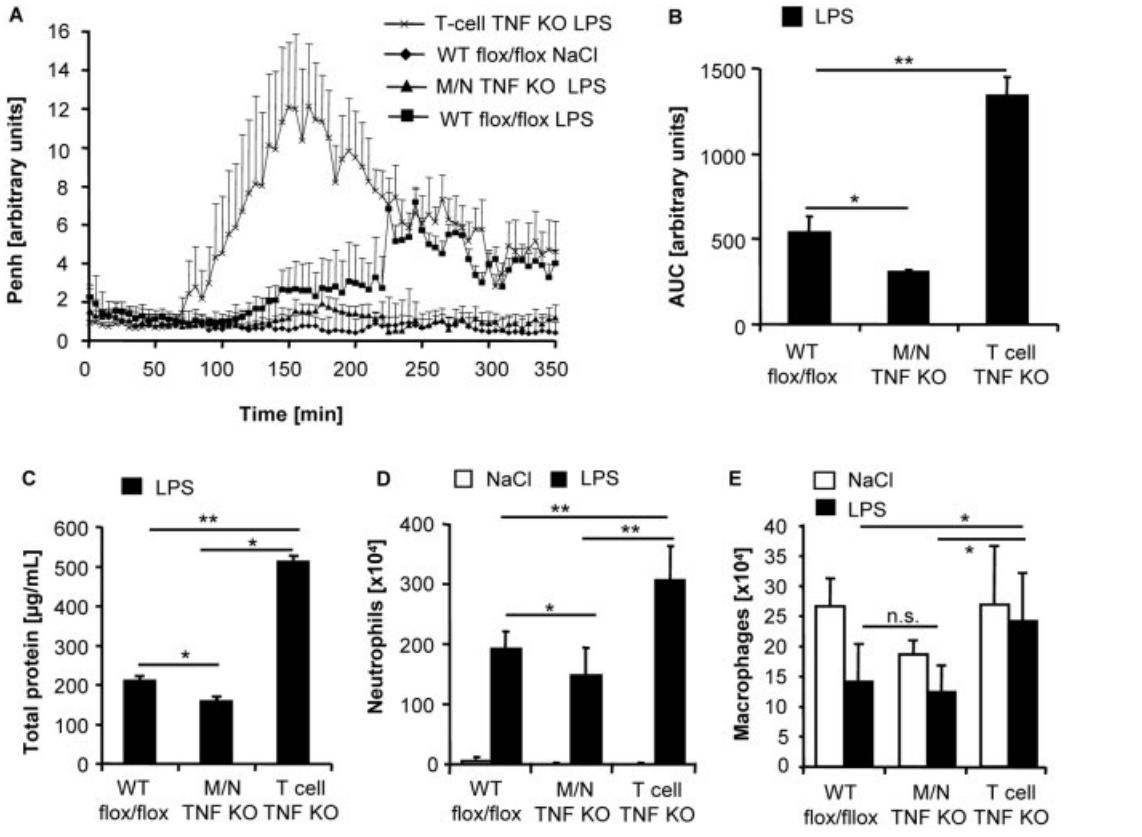

Figure 3. Absence of T cell specific TNF enhances acute bronchoconstriction. WT mice with a floxed TNF gene (WT flox/flox), macrophage/neutrophil TNF KO (M/N-TNF KO) and T cell- TNF KO mice were challenged intranasally with 10 μg LPS and acute bronchoconstriction was recorded for 360 min using whole body plethysmography (A). The bar graph in (B) represents the calculated area under the curves obtained in (A). Twenty-four hours after the challenge mice were sacrificed and the bronchoalveolar fluid was analyzed for protein content (C), and cell composition (D, E). The data represent the mean ± SD of $n = 7$ mice per group pooled from two independent experiments (* $p < 0.05$; ** $p < 0.01$; n.s., not significant).

tissue, which revealed abundant infiltration of neutrophils in the vessels, the alveolar septae and alveolar space (Supporting Information Fig. 2B).

Twenty-four hours after LPS exposure, the macrophage population decreased in the BAL of WT and M/N-TNF KO mice, but not in T cell-TNF KO or complete TNF KO mice (Fig. 3E and 1E). The sustained high

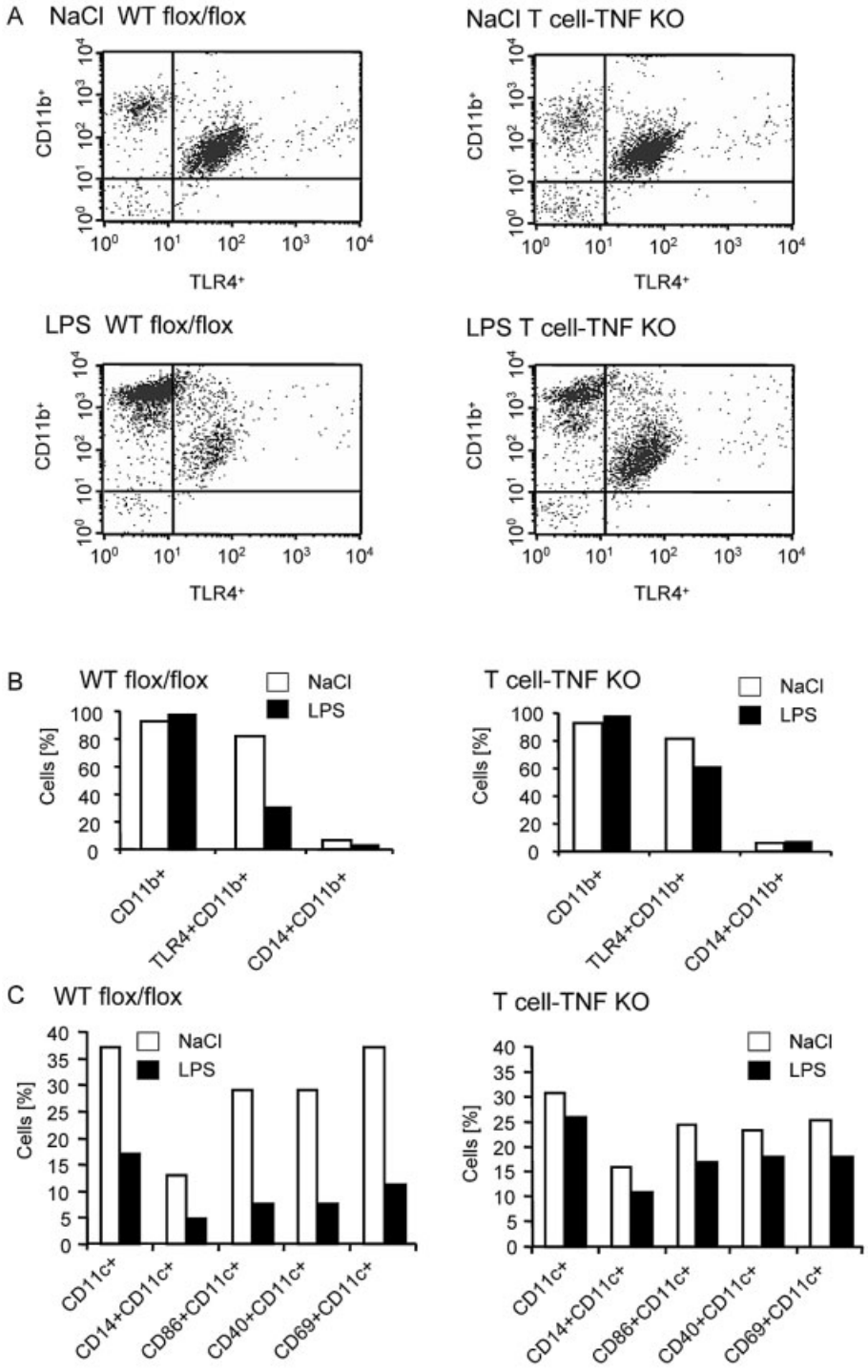

Figure 4. Increased CD11b$^+$TLR4$^+$ and CD11c$^+$ cell populations in T cell-TNF KO mice upon LPS-exposure. Flow cytometric analysis was performed on lung cells from WT mice with a floxed TNF gene (WT flox/flox) or T cell-TNF KO mice. Twenty-four hours after LPS administration lung cells were stained for CD11b (A, B) or CD11c (C) expression together with expression of TLR4, CD14, or activation markers CD40, CD86, and CD69. Percentage of positive cells is shown. Total lung cell numbers were comparable in WT and T cell-TNF KO mice (not shown). Data represent three to four mice per genotype.

macrophage number was accompanied by increased BAL neutrophil recruitment and protein concentration and was more pronounced in T cell-TNF KO (Fig. 3) than in complete TNF KO mice (Fig. 1). Flow cytometry analysis of lung cells of these mice showed identical baseline levels of $CD11b^+TLR4^+$ and $CD11b^+CD14^+$ cells in WT and T cell-TNF KO mice (Fig. 4A and B). However, 24 h after LPS challenge, the proportion of TLR4- and CD14-expressing $CD11b^+$ cells was drastically reduced in WT mice (Fig. 4A and B) but less so in T cell-TNF KO mice (Fig. 4A and B). That was also the case for a $CD11c^+CD14^+$ cell population, which was reduced in WT mice but remained elevated in T cell-TNF KO mice (Fig. 4C).

In summary, these results suggest that T cell-derived TNF may down-regulate airway resistance and pulmonary neutrophil recruitment after local exposure to endotoxins.

Cytokines and chemokines are differently regulated by macrophage- and T cell-derived TNF

In order to assess how the cellular source of TNF may influence cytokine or chemokine production in the airways, TNF, IL-12 p40, murine CXC chemokines KC/CXCL1 (KC) and thymus- and activation-regulated chemokine/CCL17 (TARC) were measured in the BAL 24 h after LPS challenge. TNF was nearly absent in the BAL of M/N-TNF KO mice (Fig. 5A), confirming that macrophages/neutrophils are the main source of TNF in the BAL upon local LPS exposure. The secretion of neutrophil chemokine KC (Fig. 5B; $p = 0.06$ for WT *vs*. M/N-TNF KO) and TARC were reduced (Fig. 5C; $p = 0.02$ for WT *vs*. M/N-TNF KO), while IL-12 p40 production was not altered in M/N-TNF KO mice when compared to WT mice (Fig. 5D), suggesting that the absence of TNF production by macrophages/neutrophils did not lead to a systemic deregulation of inflammatory cytokines [38].

By contrast, in T cell-TNF KO the concentrations of TNF, IL-12 p40, KC and TARC in BAL were significantly

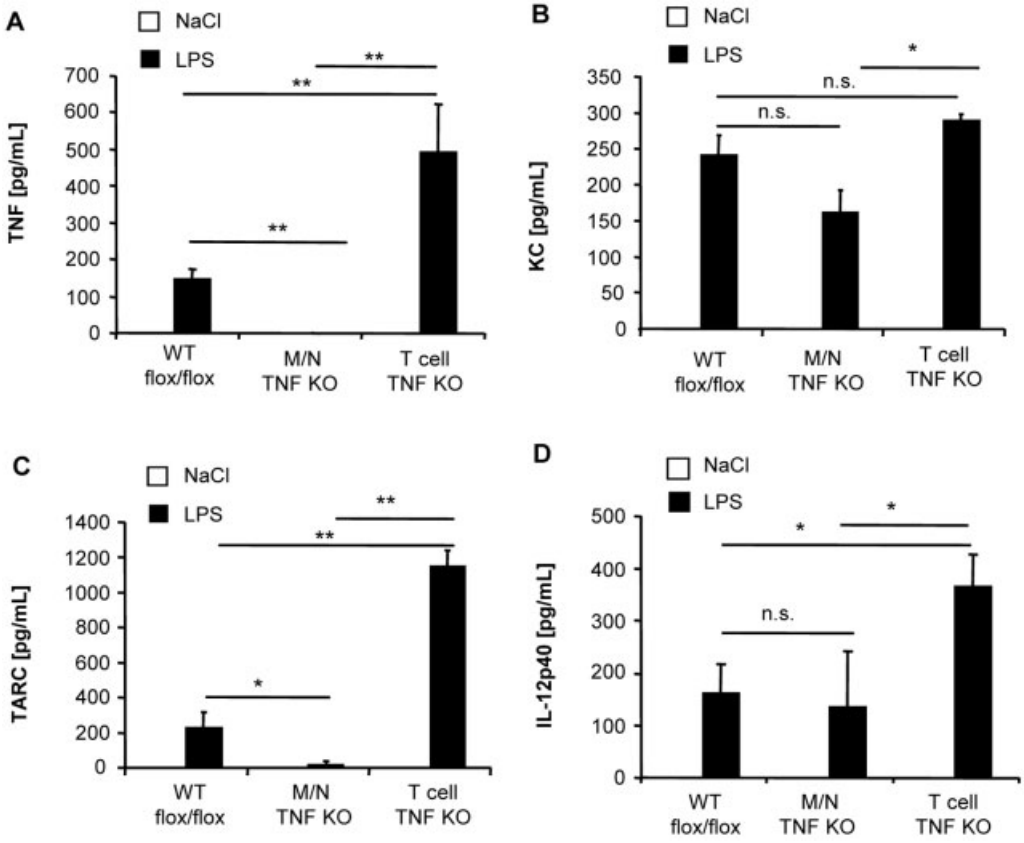

Figure 5. Increased cytokine production in airways of T cell-TNF deficient mice upon LPS exposure. Bronchoalveolar lavage fluids taken 24 h after intranasal challenge with saline or LPS (10 μg) of WT mice with a floxed TNF gene (WT flox/flox), macrophage/neutrophil (M/N) TNF KO and T cell-TNF KO mice were analyzed for their concentration in TNF, IL-12 p40, KC and TARC by ELISA. Data are representative of three independent experiments and are expressed as mean values ± SD ($n = 7$ mice per group $^*p < 0.05$; $^{**}p < 0.01$; n.s., not significant).

increased (Fig. 5A–D) in line with the enhanced inflammation and neutrophil recruitment in these mice. Increased TARC expression correlated with the increased population of $CD11c^+$ cells (Fig. 4B and 5C), which are considered the major source of TARC [39].

Analysis of pulmonary IL-12 and IL-23 mRNA expression revealed that reduced inflammation in the M/N-TNF KO mice correlated with reduced IL-12 p35 and IL-23 p19 transcripts while p40 was unaffected (Fig. 6). This implicates M/N-TNF in the regulation of p35 and p19, but not of the p40 subunit mRNA expression, which should reflect on the amounts of the heterodimeric IL-12 and IL-23, respectively. Furthermore, in T cell-TNF KO mice the pulmonary mRNA expression of IL-12 p40, IL-12 p35, and IL-23 p19 was induced upon LPS challenge, similar to WT mice (Fig. 6).

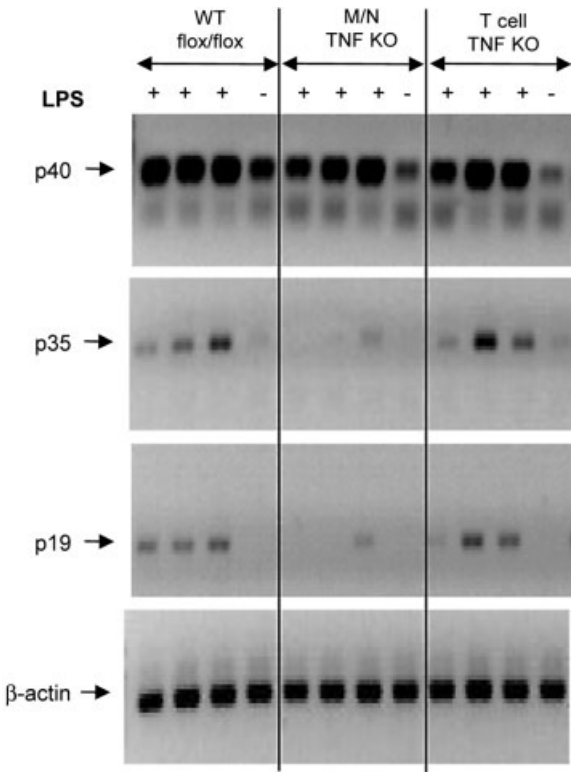

Figure 6. Pulmonary cytokine mRNA expression pattern in T cell and M/N-restricted TNF deficiency. Total RNA was extracted from the lung tissue of WT mice with the a floxed TNF gene (WT flox/flox), macrophage/neutrophil TNF (M/N-TNF) KO or T cell- TNF KO mice 24 h after LPS challenge (10 μg intranasally) and the IL-12 p40, IL-12 p35, and IL-23 p19 mRNA expression was analyzed by PCR. Ethidium bromide staining of the PCR products obtained from individual mice treated with NaCl or LPS are shown. β-Actin was amplified as a control (bottom panel).

In vitro cytokine and chemokine production by DC and macrophages

Bone marrow-derived macrophages and DC from T cell-TNF KO mice produced normal amounts of TNF *in vitro*, whereas TNF production was impaired in M/N-TNF KO-derived cells upon LPS stimulation (Fig. 7A).

The chemokine KC production was unaffected in macrophages from M/N-TNF KO mice and slightly increased in macrophages from T cell-TNF KO mice, but not in DC (Fig. 7B). Furthermore, in complete TNF KO and memTNF mice chemokine KC production was impaired in macrophages, but not in DC (Supporting Information Fig. 3). This indicates that KC is strongly regulated by TNF in macrophages, but less so in DC.

The chemokine TARC was only expressed by DC and was hardly altered by the absence of either T cell- or M/N-TNF *in vitro* (Fig. 7C). These data suggest that in T cell-TNF KO mice, the increase in TARC *in vivo* is not due to altered production by DC, but possibly due to the increased number of $CD11c^+$ cells in the lung of T cell-TNF KO mice.

IL-12 p40 production by macrophages from M/N- or T cell-TNF KO mice was not different (Fig. 7D), whereas IL-12 p40 concentrations increased twofold in DC from M/N-TNF KO but not from T cell-TNF KO mice (Fig. 7D). Similarly IL-12 p40 was increased in macrophages and DC in total TNF KO and memTNF mice (Supporting Information Fig. 3). These data suggest differential roles of TNF as a regulator of pro-inflammatory cytokines production of Th1 type such as IL-12 p40 [38] in DC and macrophages.

Discussion

Aerosol exposure to endotoxin causes acute inflammation with functional and morphological alterations in the lung. As endotoxin is a common contaminant in the environment, the understanding of the molecular mechanisms leading to pathology is of medical and therapeutic relevance. We reported earlier that TNF is critical for endotoxin-induced acute respiratory dysfunction, but dispensable for neutrophil recruitment to the lung as demonstrated in TNF KO mice [10]. Here, we show that TNF of hematopoietic origin is sufficient to induce airway resistance upon LPS challenge. Furthermore, using tissue-specific TNF KO mice we demonstrate that M/N-TNF contributes to the LPS-induced response and most importantly, we provide evidence for a novel regulatory function of T cell-derived TNF to limit the inflammatory response in the lung. In fact, T cell-TNF KO mice display augmented respiratory dysfunction, increased neutrophil recruitment into the lung parenchyma, protein leak and TNF, IL-12 p40, KC and TARC

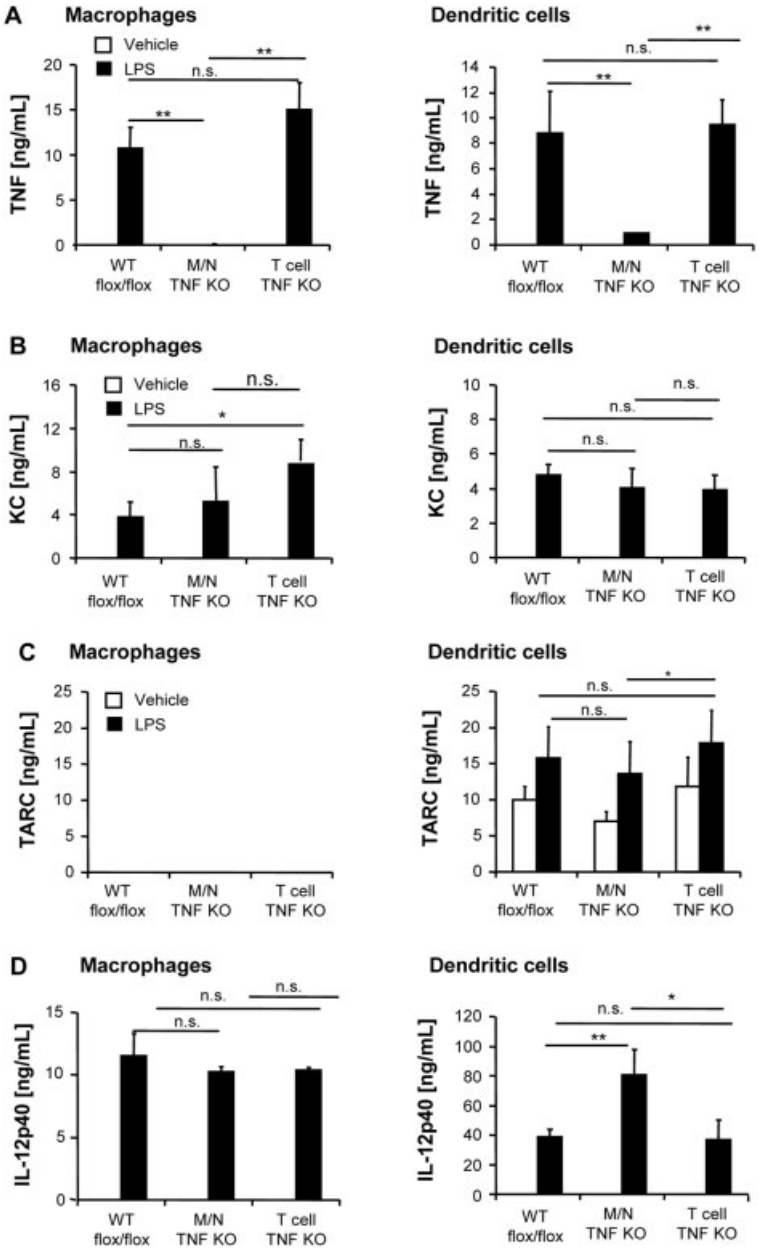

Figure 7. Effects of cell-type restricted TNF deficiency on dendritic cells and macrophage cytokine production. Bone marrow-derived macrophages and DC from WT mice with a floxed TNF gene (WT flox/flox), macrophage/granulocyte TNF (M/N-TNF) KO and T cell- TNF KO mice, were stimulated with LPS (100 ng/mL). After 24 h, the culture supernatants were analyzed for concentrations of TNF (A), KC (B), TARC (C) and IL-12 p40 (D) by ELISA. The data represent the mean ± SD of $n = 7$ mice per group pooled from two independent experiments (* $p < 0.05$; ** $p < 0.01$; n.s., not significant).

secretion in the BAL, correlating with enhanced inflammation and sequestration of a $CD11c^+$ cell population. Therefore, T cell-derived TNF is crucial in limiting acute inflammation and respiratory dysfunction, implicating that lymphocytes play a so far non-described role in regulating the innate immune response in the airways. In mycobacteria-infected TNF KO mice, an uncontrolled type 1 immune response was reported with augmented IFN-γ and IL-12 [38]. Depletion of CD4 and CD8 T cells in these TNF-deficient mice or early reconstitution of TNF-α by gene transfer reduced the frequency of antigen-specific T cells and improved their survival. Therefore, the TNF reduced T cell-mediated damage, suggesting some analogy in the protective role of TNF in mycobacteria- and endotoxin-induced inflammation [38].

The local intranasal LPS application caused local but neither systemic TNF elevations nor fever (data not shown). Increased TNF concentrations in the airways were expected to depend on TNF derived from macrophage/neutrophil cells. T cell- and macrophage-derived TNF were shown previously to cooperate in autoimmune Con A-induced hepatitis and host defense, although macrophage/neutrophil-derived, but not T or B cell-derived TNF was responsible for systemic LPS-induced acute toxicity in mice [11]. Therefore, while systemic TNF concentrations originating from T cell and macrophage sources cooperate in the infectious re-

sponse and contribute to sepsis, we show here that the local pulmonary LPS effects are partially mediated by M/N-TNF and are negatively regulated by the T cell-derived TNF exerting a beneficial, protective role.

The extent of neutrophil recruitment correlated with the chemokine KC and TARC concentrations in the BAL fluid, being low in M/N-TNF KO and high in T cell-TNF KO mice. KC, much like human IL-8 and GRO-α/β, is the classical neutrophil attracting chemokine [10, 40], while TARC is involved distinctly in the LPS-induced endotoxemia and is a chemoattractant for chemokine receptor CCR4-positive cells, including T cells, basophils, and monocytes. Injection of anti-TARC antibodies prior to LPS administration protected mice from acute liver damage, and this was accompanied by a significant reduction of CCR4 mRNA expression [41]. CCR4 KO mice exhibited significantly decreased mortality and peritoneal cell infiltration upon administration of LPS, as compared to WT mice [42]. Furthermore, the high TARC concentration correlated with the sequestration of a $CD11c^+$ cell population. Therefore, augmented TARC expression in T cell-TNF KO mice may augment the severity of LPS-induced inflammation in the lung.

DC fulfill an important regulatory function at the interface of the innate and adaptive immune system. DC, but not macrophages, produce TARC and facilitate the attraction of activated T cells. In the lung, TARC is constitutively expressed [39] and not up-regulated upon LPS stimulation. Reduced basal expression of TARC mRNA was apparent in the lung of TNF KO, M/N- or T cell-specific TNF KO mice, suggesting an effect of TNF on TARC expression (data not shown).

Both, TNF KO and T cell-specific TNF KO mice showed macrophage populations in the airway, which remained high upon endotoxin exposure and were associated with increased neutrophil recruitment. Using Rag2-deficient mice devoid of T cells, we found a similar increase of macrophages (data not shown). Using FACS analysis, this cell population in T cell-TNF KO mice was characterized as $CD11c^+$, suggesting a dendritic-like cell type. This finding is in line with increased TARC production present, as DC are described as the major source of TARC [42].

The memTNF has biological activity and may confer partial protection of the host in tuberculosis and *Listeria* infection [34, 35, 43]. We report here that memTNF is sufficient for LPS-induced respiratory dysfunction and pulmonary inflammation using a knock-in mouse model where the endogenous TNF allele was replaced by a memTNF mutated in the TACE cleavage site [22]. Hence, cell-associated memTNF may confer a full pulmonary LPS response in the absence of soluble TNF. The mechanism used by memTNF to convey LPS response may include cell-to-cell contacts of T cells, macrophages and other cells and needs further investigations. Reported biological functions of memTNF indicated a preferential TNFR2 signaling *in vitro* [44], while both TNFR1 and TNFR2 contribute to memTNF signaling *in vivo* [45–48].

Furthermore, low doses of inhaled LPS were shown to be required for allergic pulmonary responses [49], while high doses of LPS inhibited allergen-induced lung inflammation [50]. Therefore, it may be interesting to evaluate whether TNF from T cells or other cell origins will modulate the allergic response.

In conclusion, LPS-induced respiratory dysfunction and acute lung inflammation is reduced in M/N-TNF KO mice, while absence of T cell-derived TNF significantly augmented the LPS response. Therefore, the data suggest that T cell-derived TNF may have novel beneficial regulatory function in down-regulating the pulmonary LPS response.

Materials and methods

Mice

C57BL/6 WT mice, mice expressing only the uncleavable membrane form of TNF, namely Δ1–9,K11E TNF knock-in mice (memTNF) [22] on C57BL/6 background (backcross 10), TNF KO mice [36] on C57BL/6 background (backcross 10) and mice with genetic ablation of TNF in either macrophages/neutrophils, or T cells [11], and the respective control mice were bred in our specific pathogen-free animal facility at CNRS. The extent of TNF gene deletion was >98% in M/N-TNF KO mice and exhibited >98% in purified splenic T cells of T cell-TNF KO mice as described by Grivennikov and colleagues [11].

For experiments, adult (6–8 week-old) animals of 23–25 g body weight were kept in isolated ventilated cages. The TNF "floxed" (WT) mice were used as control for all experiments involving mice with cell type-specific TNF ablation [11]. C57BL/6 mice served as control for memTNF and TNF KO mice. All protocols complied with the French Government's ethical and animal experiment regulations.

Endotoxin administration and measurement of airway resistance

LPS (10 μg) from *Escherichia coli* (serotype O111:B4; Sigma, St Louis, MO) in saline or saline alone was applied by nasal instillation in a volume of 40 μL under light ketamine-xylasine anesthesia.

The airways resistance was evaluated by whole-body plethysmography [51] over a period of 6 h after LPS application. Unrestrained conscious mice were placed in whole-body plethysmography chambers (EMKA Technologies, Paris, France). Enhanced respiratory pause (Penh) as a measure of respiratory discomfort (for detail see [10]) was registered and analyzed using Datanalyst Software (EMKA Technologies) and expressed as mean ± SEM of Penh of individual mice per group.

Bronchoalveolar lavage

BAL fluid was collected by canulating the trachea and washing the lungs four times with 0.5 mL of ice-cold PBS. After centrifugation at 400 × *g* for 10 min at 4°C, the supernatant of the first lavage was stored at –70°C for cytokine analysis. Pooled cell pellets were counted with Trypan blue solution (Sigma) in a hematocytometer chamber. For differential counts, cells were stained with Diff-Quik Staining (Merz & Dade, Dudingen, Switzerland); 2 × 100 cells were counted.

Determination of cytokines

TNF, IL-12 p40, KC and TARC protein contents in the BAL fluid and cell culture supernatants were evaluated by ELISA according to the instructions of the manufacturer (R&D Duoset, Minneapolis, MO).

Alternatively, biologically active TNF concentrations were determined by TNF-α bioactivity in murine fibroblast WEHI 164 cytotoxicity assay [52]. In brief, sample dilutions were incubated in 96-well cell culture plates with 10^4 WEHI 164 cells per well. Forty-eight hours after incubation at 37°C and 5% CO_2, cell death was assessed by using the methylthiazoletetrazolium method [53]. The absorbance was measured at 610 nm and the results were compared to a standard curve of murine TNF and expressed as pg/mL of TNF.

Determination of protein in BAL fluid

Total protein concentration in the BAL fluid was determined colorimetrically using the Bio-Rad Protein assay according to manufacturer's instructions (Bio-Rad Laboratories, Munich, Germany). Ovalbumin (Sigma) was used as standard.

Bone marrow transplantation to obtain mixed chimera

Recipient mice underwent a lethal total-body irradiation as previously described [54]. Fresh bone marrow cells (2×10^6/mouse) were injected into the lateral tail vein of the irradiated recipient mice 24 h after lethal irradiation. The reconstituted mice were used at 3 months after bone marrow transplantation.

Flow cytometric analysis (FACS)

For the cell suspension of lungs of LPS- or vehicle- (saline) challenged mice the tissue was passed through a 100-μm sieve. Cells were collected, washed and saturated with mouse serum prior to staining with fluorescence conjugated antibodies for 60 min [FITC-anti-CD14 (clone rmC5–3), PE-anti-TLR4 (clone MTS510), APC-anti-CD11c (clone HL3), FITC-anti-CD86 (clone GL1), PE-anti-CD69 (clone H1.2F3) and isotype-matched control antibodies were purchased from PharMingen (San Diego, CA)]. Cells were analyzed using a FACSCalibur-Flow cytometer and CellQuest Software (Becton Dickinson, San Jose, CA). The markers for the monovariant histogram (not shown) were set based on the negative staining control using isotype-matched control antibodies.

Total RNA extraction and semi-quantitative RT-PCR amplification

Lung tissue was shock frozen, ground under liquid nitrogen using a mortar and total RNA was extracted by using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). Of extracted RNA, 2 μg was subjected to RT reaction using Superscript II Reverse Transcriptase (Life Technologies) in a volume of 15–20 μL containing 2 U RNAGard and 100 pg random oligo hexamers. PCR reaction was performed in a volume of 25 μL containing 160 nM of each primer, 1x reaction buffer, 2 U of Taq polymerase, 10 nM dNTP, and Mg^{2+}. Primers used were for β-actin: 5′-ACATCT GCTGGAAGGTG-GACA-3′ and 5′-TTTTCCAGCCTTCCTTCTTGG-3′, IL-12 p40: 5′-AGACATTCCCGCCTTTGCA-3′ and 5′-ACATCATCAAACCA-GACCCGC-3′, IL-12 p35: 5′-ATGTGTCAATCACGCTACCT-3′ / 5′-AGGGTCATCATCAAA-GACGT-3′, IL-23 p19: 5′-GCTGGA-TTGCAGAGCAGTAA-3′ and 5′-ACTCAGGCTGGGCATCTGTT-3′. After an initial heating step to 95°C for 15 min, the amplification was performed as follows: 35 cycles 60°C for 45 s, 72°C for 1 min, 95°C for 45 s. Samples were separated on a 1.5% Agarose (Fluka, Buchs, Switzerland) containing 0.5 x TBE buffer and visualized with ethidium bromide. The samples were normalized using β-actin as an internal control.

Primary bone marrow-derived DC and macrophage cultures

Murine bone marrow cells were isolated from femurs and differentiated into myeloid DC by culturing (change on days 3, 6, and 8) at 2×10^5 cells/mL for 10 days in RPMI supplemented with 10% FCS and 4% J558L cell-conditioned medium as a source of GM-CSF as described previously [55].

For the differentiation into macrophages, 10^6 cells/mL were cultured for 10 days in DMEM (Sigma) supplemented with 20% horse serum and 30% L929 cell-conditioned medium as a source of M-CSF (change at day 7).

For experiments, 96-well microtiter plates were used. Cells (10^5/well) were stimulated with LPS (*Escherichia coli*, serotype O111:B4, Sigma, St Louis, MO, at 100 ng/mL) in medium containing 0.5% FCS for 24 h.

Statistical analysis

Data are presented as mean values and SD indicated by error bars, if not indicated differently. Statistical significance was determined by the Student's *t*-test for comparisons of two groups. The *p* values of <0.05 were considered statistically significant.

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