Gestational Hypothyroidism Improves the Ability of the Female Offspring to Clear *Streptococcus pneumoniae* Infection and to Recover From Pneumococcal Pneumonia

Pamela A. Nieto, Hernán F. Peñaloza, Francisco J. Salazar-Echegarai, Raquel M. Castellanos, Maria Cecilia Opazo, Luis Venegas, Oslando Padilla, Alexis M. Kalergis, Claudia A. Riedel, and Susan M. Bueno

Millennium Institute on Immunology and Immunotherapy, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile (P.A.N., H.F.P., F.J.S.-E., A.M.K., S.A.M.), Santiago, Chile 8331150; Departamento de Morfología, Facultad de Medicina, Universidad Andrés Bello (R.M.C.), Santiago, Chile 8370146; Millennium Institute on Immunology and Immunotherapy, Departamento de Ciencias Biológicas, Facultad de Ciencias Biológicas y Facultad de Medicina, Universidad Andrés Bello (M.C.O., L.V., C.A.R.), Santiago, Chile 8370146; Departamento de Salud Pública, Escuela de Medicina, Pontificia Universidad Católica de Chile (O.P.), Santiago, Chile 8330073; Institut National de la Santé et de la Recherche Médicale Unité Mixte de Recherche 1064 (A.M.K., S.M.B.), Nantes, France 44093; and Departamento de Endocrinología, Facultad de Medicina, Pontificia Universidad Católica de Chile (A.M.K.), Santiago, Chile 8330074

Maternal thyroid hormones are essential for proper fetal development. A deficit of these hormones during gestation has enduring consequences in the central nervous system of the offspring, including detrimental learning and impaired memory. Few studies have shown that thyroid hormone deficiency has a transient effect in the number of T and B cells in the offspring gestated under hypothyroidism; however, there are no studies showing whether maternal hypothyroidism during gestation impacts the response of the offspring to infections. In this study, we have evaluated whether adult mice gestated in hypothyroid mothers have an altered response to pneumococcal pneumonia. We observed that female mice gestated in hypothyroidism have increased survival rate and less bacterial dissemination to blood and brain after an intranasal challenge with *Streptococcus pneumoniae*. Further, these mice had higher amounts of inflammatory cells in the lungs and reduced production of cytokines characteristic of sepsis in spleen, blood, and brain at 48 hours after infection. Interestingly, mice gestated in hypothyroid mothers had basally increased vascular permeability in the lungs. These observations suggest that gestational hypothyroidism alters the immune response and the physiology of lungs in the offspring, increasing the resistance to respiratory bacterial infections. **(*Endocrinology* 157: 2217–2228, 2016)**

Thyroid hormones (THs) T_4 and T_3 are essential for the normal development of the central nervous system (CNS), skeletal muscle, and lungs, as well as for neuronal differentiation, metabolic regulation, and growth of mammals (1, 2). T_4 is the main hormone secreted by the thyroid gland, it has a long half-life but poor biological activity. It is considered a hormonal reservoir, because it is converted to T_3 by deiodinases that act locally in TH-target tissues. T_3, on the other hand, has a short half-life in comparison with T_4, but its biological activity is much higher (3).

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in USA

Received November 11, 2015. Accepted March 23, 2016.
First Published Online April 1, 2016

Abbreviations: BAL, bronchoalveolar lavage; CCL, chemokine (C-C Motif) ligand; CFU, colony forming unit; CNS, central nervous system; DC, dendritic cell; EAE, autoimmune encephalomyelitis; EBD, Evans blue dye; HBSS, Hank's balanced salt solution; hpi, hours postinfection; IFN, interferon; IM, interstitial macrophage; i.n., intranasally; MMI, 2-mercapto-1-methylimidazole; P, postnatal day; PMN, polymorphonuclear cell; qPCR, quantitative real-time PCR; TH, thyroid hormone; THYE, Todd-Hewitt broth supplemented with 0.5% yeast extract; WT, wild type.

Hypothyroidism is a TH deficiency clinically characterized by low levels of T_4 and T_3 and high levels of TSH (4). It has been estimated that 0.06%–1.2% of women develop spontaneous hypothyroidism around the world (5). During pregnancy, the fetus depends on maternal THs until his or her thyroid gland is mature (6). Maternal TH deficiency is a highly frequent condition, which results in diverse effects in the offspring such as mental retardation, lower intellectual quotient, impaired motor development, attention deficit and hyperactivity disorder, and increased risk of autism development (7–12). Rat and mouse models have been used to study the imprints of maternal hypothyroidism in the offspring. In these models, it has been reported that maternal hypothyroidism causes impaired neuronal migration in the offspring, altering the cytoarchitecture of the somatosensory cortex and hippocampus (13, 14). It has been also shown that it decreases the number of mature oligodendrocytes, affecting neuronal myelination (15), and that it impairs the synaptic function, decreasing neuronal plasticity (16). Further, adult rats gestated in mothers with TH deficiency show spatial learning impairment and increased motor activity (17).

Although the effects of gestational hypothyroidism on CNS development have been intensively studied, the effects on the immune system of the progeny have not been totally elucidated, and only few studies have addressed this question. Using a model of neonatal hypothyroidism in rats, Rooney et al found that these animals had reduced weight and decreased number of splenic and thymic B and T cells, whereas relative values of T and NK cells were increased. However, these effects were only transient, as all values returned to normal at postnatal day (P)91 (18). Nakamura et al (19) found transient changes in the total cell numbers in the thymus of the offspring gestated under TH deficiency, a decrease on B cells and an increase in the frequency of inactive and regulatory T cells. Albornoz et al (20) recently showed that adult female mice gestated in maternal hypothyroidism develop a more severe disease when induced with experimental autoimmune encephalomyelitis (EAE). The authors found higher demyelination in the spinal cord, increased $CD4^+$ and $CD8^+$ T-cell infiltration in the spinal cord, and increased oligodendrocyte death in the offspring gestated in hypothyroidism and suffering EAE (20). This study showed that gestational hypothyroidism has a long-lasting effect in the offspring, which presented an exacerbated response upon an inflammatory stimulus. However, the exact mechanism that links together this observation and the lack of THs during gestation is unknown. Whether the immune alteration produced by TH deficiency during gestation influences the activation state, expression of surface markers, and/or the response of the different immune cell populations has not been reported.

The fact that female mice gestated in hypothyroid mothers reacted to EAE with higher inflammation (20), prompted us to study whether gestational hypothyroidism also makes the offspring respond different to an infection. To address this question, we used the bacterial pathogen *Streptococcus pneumoniae* (pneumococcus), one of the major causative agents of pneumonia worldwide. This bacterium is a frequent colonizer of the upper respiratory tract of healthy children and adults (21), and although pneumococcal infections can occur through life, populations at higher risk are children under 2 years old and the elderly (22, 23). According to the World Health Organization, of the estimated 8.8 million global annual deaths among children under 5 years old in 2008, 476 000 were caused by pneumococcal infections (24).

In this study, we examined the outcome of invasive pneumococcal pneumonia in adult mice gestated in hypothyroidism and strikingly found that these mice are more resistant to die from this infection. Although mice gestated in hypothyroidism had similar bacterial load in the lungs than mice gestated in euthyroid conditions, they had lower bacterial load in blood and brain, suggesting a reduced dissemination of bacteria from the respiratory tract. Accordingly, several cytokines characteristic of sepsis were less produced by these mice, supporting the notion that *S. pneumoniae* resistance in mice gestated in hypothyroidism is due to a restriction of bacteria within the lungs. Interestingly, mice gestated in hypothyroidism had basally increased vascular permeability in the lungs and higher infiltration of inflammatory cells at 48 hours after infection, suggesting that important alterations in the lungs of these animals, in terms of immune response and physiology, occur due to gestation in hypothyroidism, which keep the infection restricted to the lungs and limit bacterial dissemination to other tissues. These results are highly novel and contribute to understand the impact of maternal THs over the response of the offspring to infections.

Materials and Methods

Animals

C57BL/6J mice were originally purchased from The Jackson Laboratory and were maintained in a specific pathogen-free animal facility at the Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile. All experimental procedures performed in this study were revised and approved by the Bioethics and Biosafety Committee of the Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile. All animal work was performed according to the Guide for Care and Use of Laboratory Animals (National Institutes of Health) and institutional

guidelines. All mice were fed with Prolab Rat/mouse/Hamster 3000, with an iodine content of 0.97 parts per million. Mice used in all experiments were 7–8 weeks old and were overseen daily by a veterinarian.

Induction of gestational hypothyroidism

Female adult C57BL/6J mice were maintained at 22°C in a room with automatic 12-hour light, 12-hour dark cycles. These mice were mated, and 1 day after mating was referred to as embryonic day 0. Pregnant mice were randomly separated in 2 groups. One group was transiently treated with 0.02% 2-mercapto-1-methylimidazole (MMI), which was added to the drinking water from embryonic day 10 to birthday. Control animals received the same drinking water but without MMI. After MMI treatment, blood samples were collected from the superficial temporal vein of the mothers, and sera were analyzed for TH levels. The day of birth was referred to as P0. The offspring from MMI-treated mothers was designated as "hypo" mice, and the offspring from control animals was designated as "untreated." Hypo and untreated mice were weaned at P30 and culled at 7–8 weeks old to perform the experiments.

Pneumococcal infection

S. pneumoniae strain D39 (NCTC 7466; National Collection of Type Cultures) was used in all infection assays. Pneumococci were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract (THYE) to midlog phase at 37°C in 5% CO_2. Aliquots were frozen in THYE 15% glycerol at −80°C until needed. For infection studies in mice, aliquots were thawed, concentrated by centrifugation at 7700*g*, and resuspended in THYE to obtain an infective dose of 3×10^7 colony forming units (CFUs) in 50 μL. Mice were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (4 mg/kg), and intranasally (i.n.) inoculated with the bacterial dose described above. To confirm the amount of bacteria administered, the inoculum was serially diluted, plated in trypticase soy agar plates with 5% sheep's blood (BD Pharmingen), incubated overnight at 37°C in 5% CO_2, and the number of CFUs was counted. For survival studies, mice were infected as described, and weight changes and clinical symptoms were daily monitored for 10 days. A clinical score was determined for each mouse using a modified version of the table published by Mook-Kanamori et al (25) (Supplemental Table 1).

Bacterial load determination

Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and then euthanized by cervical dislocation 24 or 48 hours after infection with *S. pneumoniae* D39. Blood was obtained by puncture of the superficial temporal vein. Bronchoalveolar lavage (BAL) was obtained by instillation of 0.5 mL of PBS into the trachea using a syringe, for a total of 1.5 mL. Lungs, spleen, and brain were removed aseptically and homogenized using a syringe plunger and a 70-μm cell strainer with PBS without calcium, magnesium, or phenol red. Blood, BAL and homogenized lungs, spleen, and brain were serially diluted in PBS and plated onto trypticase soy agar with 5% sheep's blood plates (BD Pharmingen). The plates were incubated overnight at 37°C in 5% CO_2, and the number of CFUs was counted.

Histology

Whole lungs were removed aseptically and fixed for at least 24 hours in 4% paraformaldehyde (Merck) in PBS, embedded in paraffin, cut with a microtome into 5-μm-thick sections, and adhered to slides. Staining was performed with Harris Hematoxylin (Leyca Biosystems) and Aqueous Eosin Y (Thermo Scientific) (hematoxylin and eosin staining), and images were captured on an Olympus BX51 light microscope at ×40 magnification. Images were evaluated and scored by a pathologist.

Protein content in BAL

BAL was centrifuged at 100*g* for 20 minutes, and the supernatant was collected and stored at −80°C until used. A total of 20 μL of direct or PBS-diluted BAL supernatant were mixed with 980 μL of Bradford reagent (Sigma), read in a Biomater 3 spectrophotometer, and interpolated to a standard curve made with BSA.

Quantitative real-time PCR (qPCR)

Total RNA was isolated from lungs using the SV Total RNA Isolation System (Promega), and RNA was deoxyribonuclease treated using a TURBO DNA-Free kit (Ambion). RNA was reverse transcribed using the Improm-II Reverse Transcription System (Promega). qPCR was performed on a AB One-Step Plus real-time PCR machine (Applied Biosystems) using a TaqMan gene expression Fast Master Mix (Applied Biosystems) and one of the following TaqMan Probes from Applied Biosystems: β-2-microglobulin (Mm00437762_m1), interferon-γ (IFN-γ) (Mm01168134_m1), IL-10 (Mm00439614_m1), IL-6 (Mm00439653_m1), TGF-β (Mm01178820_m1), and TNF-α (Mm00443260_g1). One microliter of the cDNA reaction was used as a template. For control reactions, nucleic acid-free water was added instead of the cDNA. qPCR was performed with a holding stage of 95°C for 20 seconds, and a cycling stage of 40 cycles of 95°C for 1 second and 60°C for 20 seconds. Relative expression was calculated using β-2-microglobulin as the reference gene, and fold change was calculated normalizing the values to the average of the untreated uninfected group.

Multiplex cytokine assay

Blood obtained from the superficial temporal vein was mixed with 50 μL of 0.25M EDTA, centrifuged at 400*g* for 20 minutes, and the resulting plasma was collected and stored at −80°C until used. Levels of IL-1β, IL-6, IL-10, IFN-γ, and TNF-α were determined on a Luminex 200 using a Milliplex MAP Mouse magnetic bead kit (Merck Millipore), following manufacturer instructions. Serum matrix was added to the standard curve.

Flow cytometry

Lungs were removed aseptically and homogenized using a syringe plunger and a 70-μm cell strainer with PBS without calcium, magnesium, or phenol red. Red blood cells were lysed using Ammonium-Chloride-Potassium lysis buffer. Cells were stained for surface markers using the following antibodies from BD Pharmingen: anti-CD3e (phycoerythrin) clone 145–2c11, anti-CD4 (allophycocyanin) clone RM4–5, anti-CD69 (peridinin chlorophyll-cyanine5.5) clone H1.2F3, anti-CD19 (phycoerythrin-cyanine 7) clone 1D3, anti-CD11c (allophycocyanin) clone HL3, anti-Gr1 (phycoerythrin) clone RB6–8C5, and anti-MHC-II (fluorescein isothiocyanate) clone 2G9; from Biolegend:

anti-CD8a (fluorescein isothiocyanate) clone 53–6.7 and anti-CD11b (phycoerythrin-cyanine 7), clone M1/70. Cells were incubated with the antibodies for 30 minutes at 4°C, after which they were washed and resuspended in 100 μL of staining buffer. CountBright absolute counting beads (Life Technologies) were added to quantify cell numbers. Flow cytometry data were acquired on a BD Fluorescence-activated cell sorting Canto II flow cytometer and analyzed using Flowjo Software (TreeStar). Singlets were selected from total forward scatter/side scatter events; then the following combinations of antibodies were used to discriminate between cell types: $CD3^+CD4^+$ ($CD4^+$ T cells), $CD3^+CD4^+CD69^+$ (activated $CD4^+$ T cells), $CD3^+CD8^+$ ($CD8^+$ T cells), $CD3^+CD8^+CD69^+$ (activated $CD8^+$ T cells), $CD19^+$ (B cells), MHC-$II^{neg}Gr1^{high}CD11b^+$ (polymorphonuclear cells [PMNs]), and $Gr1^{neg}CD11b^+CD11c^+$MHC-II^+ ($CD11b^+$ dendritic cells [DCs] and interstitial macrophages [IMs]).

Isolation of murine neutrophils from bone marrow

Mice were euthanized as previously described and femur and tibia from both hind legs were removed. Muscular tissue was completely detached from the bones, and the distal tip of each extremity was cut off. Hank's balanced salt solution (HBSS) without calcium and magnesium with 0.1% gelatin was forced several times through the bone with an insulin syringe to obtain the bone marrow, which was passed through the syringe to disperse cell clumps. The cell suspension was centrifuged at 300*g*, 10 minutes, 4°C, and red blood cells were lysed using Ammonium-Chloride-Potassium lysis buffer. Cells were washed, resuspended in PEB buffer (PBS [pH 7.2], 0.5% BSA, and 2mM EDTA), and counted by trypan blue staining. Neutrophils were isolated by negative selection using the Neutrophil Isolation kit from Miltenyi Biotec. Finally, cells were counted by trypan blue staining and resuspended in HBSS with calcium and magnesium with 0.1% gelatin to a concentration of 2.5×10^6 cells/mL. An aliquot from this cell suspension was used to verify purity by flow cytometry, using antibodies against CD11b and Ly6G. In all our assays purity was more than 98%.

Neutrophil bacterial killing assays

An aliquot of a midlog culture of *S. pneumoniae* D39 stored at −80°C was thawed and washed with HBSS with calcium and magnesium with 0.1% gelatin; 10^3 bacterial cells (in 10 μL) were preopsonized with mouse serum obtained from 6-week-old C57BL/6 mice (40 μL) for 30 minutes at 37°C. Neutrophils purified from bone marrow were then added to the bacterial suspension (10^5 cells per reaction in 40 μL) and 110 μL of HBSS with calcium and magnesium with 0.1% gelatin were added to complete a final volume of 200 μL. Reactions were incubated for 45 minutes at 37°C with end-to-end rotation and were stopped by incubation at 4°C. Viable counts of bacteria were determined by plating in trypticase soy agar plates with 5% sheep's blood (BD Pharmingen). Percent survival was determined relative to control reactions lacking neutrophils.

Lungs permeability assay

Mice were i.n. infected with *S. pneumoniae* D39 as described previously. Evans blue dye (EBD) (30 mg/kg; Sigma) was administered by lateral tail vein injection 1 hour before the end of the experiment. One hour later (48 h postinfection [hpi]), mice were euthanized and bled by cardiac puncture. Lungs were perfused by right ventricle puncture with 30 mL of PBS to remove EBD from the vascular spaces. Lungs were removed, homogenized in PBS, incubated with 2 volumes of formamide (18 h, 60°C), and centrifuged at 5000*g* for 30 minutes. The extravasated EBD in lungs was quantified spectrophotometrically measuring optical density at 620 and 740 nm, which allows for correction of contaminating heme pigments by using the formula $OD_{620}(EBD) = OD_{620} - (1.426 \times OD_{740} + 0.030)$. EBD concentration was calculated using a standard curve made with BSA.

Statistics

Survival curves were compared using a Kaplan Meier plot and log-rank test. Comparisons of mice groups at different time points (ie, hypo uninfected mice vs hypo infected mice) were made using two-way ANOVA. Sidak or Tukey post hoc tests were used for multiple comparisons, results are indicated by the letters "a" and "b" for untreated mice; and by the Greek letters "α" and "β" for hypo mice. Groups with letters in common are not significantly different from each other. Mann-Whitney *U* test was used to compare different mice groups in the same time point (ie, hypo infected mice vs untreated infected mice) and Wilcoxon test was used to compare paired samples in different time points. Adjusted *P* values for multiple hypothesis testing were calculated by the Hommel method (26) and are shown in Supplemental Table 2 (which includes the effect size and 95% confidence intervals, as well as nonadjusted *P* values). $P < .05$ was considered significant. Analyses were performed using Prism6 (GraphPad), IBM SPSS v.17.0, and R Project for Statistical Computing.

Results

Female hypo mice are more resistant to pneumococcal pneumonia

The administration of MMI during gestation resulted in decreased levels of maternal T_4 and T_3, and increased levels of TSH, indicating that pregnant mice suffered hypothyroidism as it has been previously reported (20). Mice gestated in hypothyroid mothers (hereinafter hypo mice) and mice gestated in euthyroid mothers (hereinafter untreated mice) were i.n. infected with 3×10^7 CFUs of *S. pneumoniae* D39, and weight loss and physical signs of illness were monitored for 10 days. Male untreated and hypo mice had similar average survival, close to 50% (Figure 1A). In contrast, female hypo mice had significant more survival rate than female untreated mice (50% vs 10%, respectively; $P = .0011$) (Figure 1B). Given these results, all of the following experiments were performed only with female mice.

Next, we calculated a clinical score for each female mouse, based on the table developed by Mook-Kanamori et al (25) (Supplemental Table 1). Mice were infected and the score was measured 24 and 48 hpi. The score was similar for untreated and hypo mice at 24 hpi; however, it

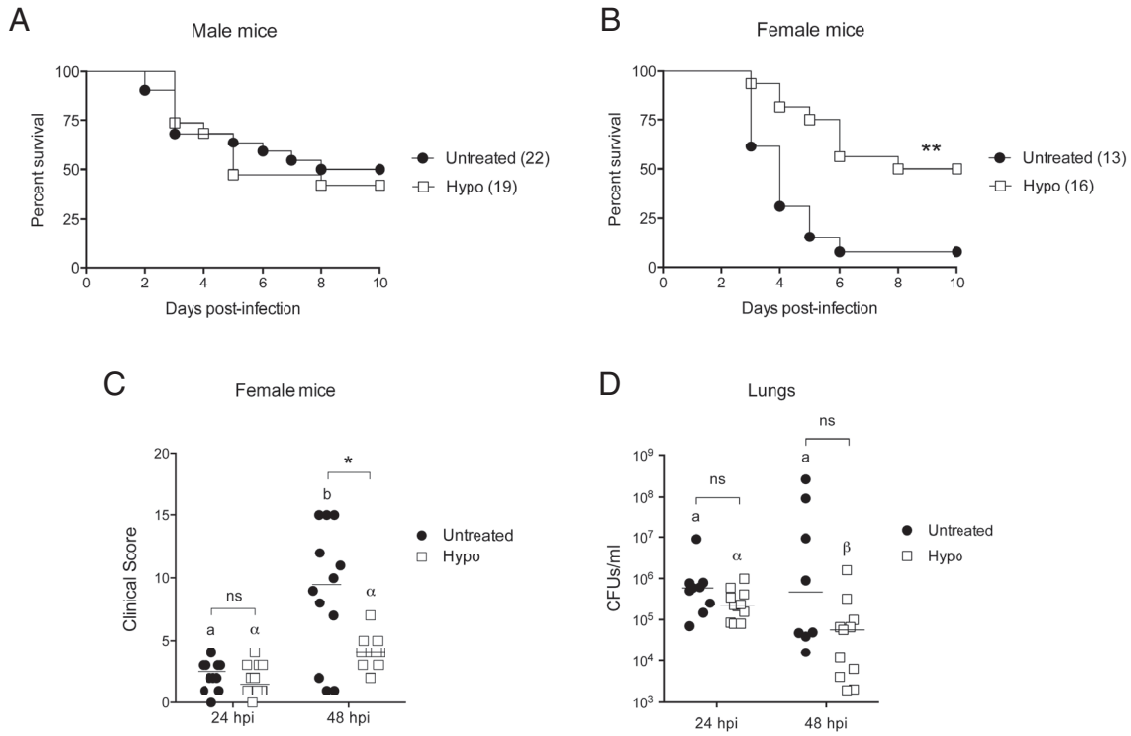

Figure 1. Female hypo mice are more resistant to die due to pneumococcal pneumonia. Untreated and hypo mice were i.n. infected with 3×10^7 CFUs of *S. pneumoniae*, and survival, weight, and clinical symptoms were daily monitored for a total of 10 days. Bacterial load was measured in lungs at 24 and 48 hpi. A, Survival curve for male mice. B, Survival curve for female mice. Combined data from 7 independent experiments are shown; total number of mice is indicated in parenthesis; **, $P = .0011$ indicates curves are significantly different by log-rank test. C, Clinical score calculated for female mice at 24 and 48 hpi. Combined data from 4 independent experiments (total n = 12 mice per group) are shown. D, Bacterial load in lungs at 24 and 48 hpi. Combined data from 3 independent experiments (total n = 8–12 mice per group) are shown. Bars represent the median. Comparisons between groups were made with Mann-Whitney *U* test; *, $P < .05$ indicates means are significantly different. Comparisons for the same group at different time points were made using a two-way ANOVA followed by Sidak test, except for clinical score values (C), in which the comparison was made using Wilcoxon test. Groups not sharing a common letter are significantly different ($P < .0001$).

was significantly lower for hypo mice at 48 hpi in comparison with untreated mice ($P = .036$) (Figure 1C).

Hypo mice show higher amounts of innate immune cells infiltrating lungs 48 hours after *S. pneumoniae* infection

To address the cause of the reduced clinical symptomatology in infected hypo mice, bacterial load, numbers of inflammatory cells, and levels of cytokine mRNA in lungs were evaluated at 24 and 48 hpi. As shown in Figure 1D, both groups had similar amounts of CFUs in lungs at 24 and 48 hpi. Further, uninfected untreated and hypo mice had similar levels of PMNs in lungs (MHC-II^{neg}Gr1$^+$CD11b$^+$ cells, Supplemental Figure 1), and as expected, both infected groups of mice had a significant increase in the amount of PMNs at 24 hpi (Figure 2A). At 48 hpi, untreated mice showed an important decrease in the amount of PMNs in lungs, which is in agreement with previous reports (26). However, hypo mice had a lesser reduction of PMNs in lungs, which resulted in significantly more PMNs than untreated mice at 48 hpi ($P = .012$) (Figure 2A). Both infected groups had no significant differences in the numbers of PMNs in BAL at 24 and 48 hpi (Figure 2B). CD11b$^+$ DCs and IMs were also characterized in lungs and BAL. These cells were selected as Gr1^{neg}CD11b$^+$CD11c$^+$MHC-II$^+$ cells (Supplemental Figure 2). The numbers of DCs/IMs in lungs were similar in uninfected untreated and hypo mice, and at 24 hpi; however, they were significantly higher in hypo mice at 48 hpi ($P = .006$) (Figure 2A). In BAL, DCs/IMs were similar in uninfected animals and at 48 hpi, but they were significantly lower in hypo mice at 24 hpi ($P = .041$) (Figure 2B). CD4$^+$ and CD8$^+$ T cells were also identified in lungs, and no differences were found between groups at any time point analyzed, neither in their numbers or in their activation state, measured by the expression of the activation marker CD69 (Supplemental Figures 3 and 4). This observation suggests that inflammatory cells reside longer in lungs of infected hypo mice than in lungs of infected untreated mice.

The cytokines IFN-γ, IL-6, IL-10, TNF-α, and TGF-β, all secreted during pneumonia, were measured in lungs of both groups of animals by real-time qPCR, at 48 hpi. As shown in Figure 3, all of the cytokine mRNAs evaluated

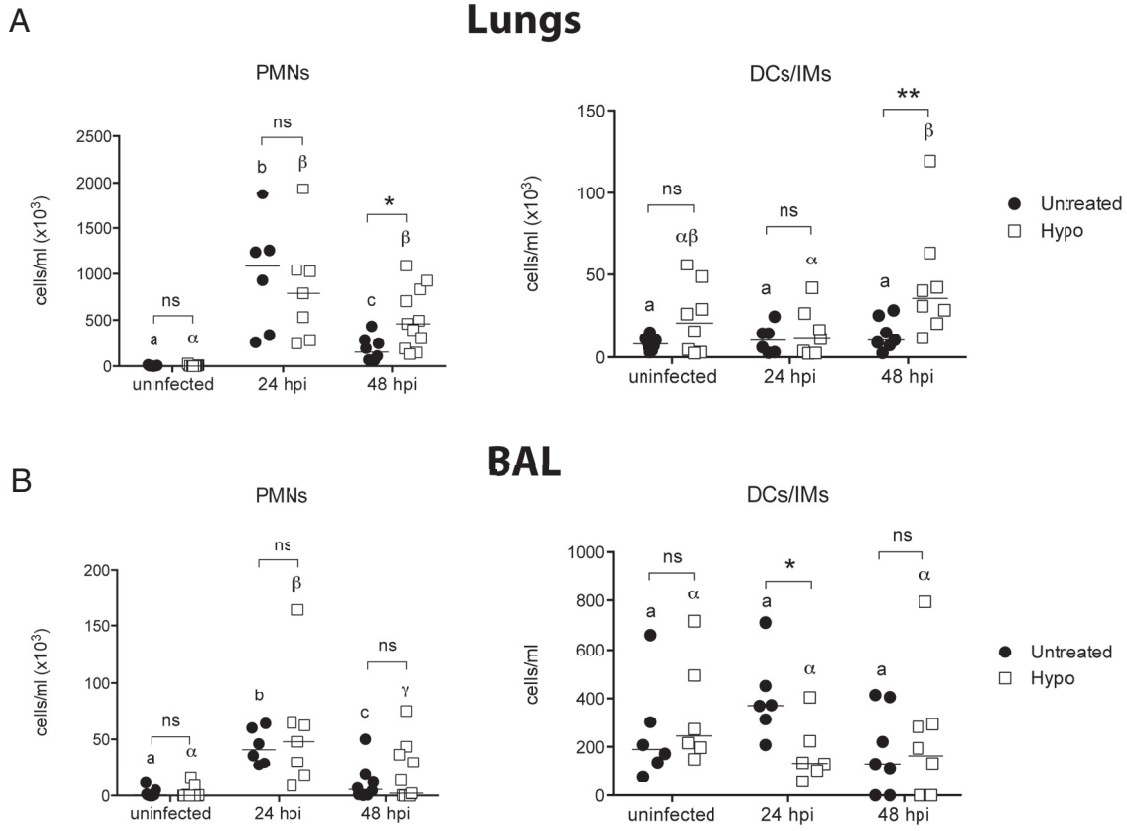

Figure 2. Hypo mice have more PMNs and CD11b$^+$ DCs/IMs in lungs at 48 hpi. Mice were i.n. infected with 3×10^7 CFUs of *S. pneumoniae* D39, and at 24 and 48 hpi, organs were harvested. Cells were analyzed by FACS, and total cell numbers were calculated using quantification beads. A, PMNs and CD11b$^+$ DCs/IMs in lungs. B, PMNs CD11b$^+$ DCs/IMs in BAL. Combined data from 2 independent experiments (total n = 6–11 mice per group) are shown. Bars represent the median. Comparisons between groups were made with Mann-Whitney *U* test; *, $P < .05$ and **, $P < .01$ indicate means are significantly different. Comparisons for the same group at different time points were made using a two-way ANOVA followed by Tukey test, groups not sharing a common letter are significantly different ($P < .05$).

were present at similar levels in uninfected untreated and hypo mice, except for IFN-γ, which was significantly higher in uninfected hypo mice ($P = .033$). In infected animals, however, only IL-6 mRNA was significantly lower in hypo mice ($P = .016$). Although no other significant differences were found between untreated and hypo mice, only infected untreated mice showed a significant increase in IFN-γ, TNF-α, and IL-10 mRNAs (a vs b) when compared with their uninfected control. TGF-β was the only cytokine mRNA less expressed in infected mice, at similar levels in both untreated and hypo.

In summary, these results suggest that hypo mice have an alteration in the recruitment/migration of inflammatory innate cells to lungs after infection with *S. pneumoniae,* and a differential expression of cytokines at 48 hpi in lungs, which correlate with the lower clinical score and mortality observed for these mice.

Hypo mice are more resistant to *S. pneumoniae* dissemination and sepsis

Given that *S. pneumoniae* D39 is an invasive strain capable of causing systemic infection, bacterial load was measured in blood and brain of untreated and hypo mice at 24 and 48 hpi. The bacterial load was significantly lower in blood ($P = .002$) and brain ($P = .008$) of hypo mice at 48 hpi (Figure 4). Furthermore, some hypo mice did not have any CFU in blood and brain at this time point. Untreated mice had an significant increase of approximately 10^4 times CFUs in blood and brain (a vs b) from 24 to 48 hpi, whereas in hypo mice, this increase was only of 10 times.

The same cytokines measured in lungs by qPCR were measured in spleen (Figure 5A) and brain (Figure 5B). In spleen, all mRNAs were found at similar levels in uninfected untreated and hypo mice, except for TNF-α, which was significantly higher in uninfected hypo mice ($P = .032$). In infected animals, IFN-γ, IL-6, and IL-10 mRNAs were significantly lower in infected hypo mice as compared with infected untreated mice ($P = .029$, $P = .008$, and $P = .008$, respectively). Instead, TGF-β mRNA was higher in infected hypo mice ($P = .008$). Additionally, only infected untreated mice showed a significant increase in IFN-γ, IL-6, and TNF-α mRNAs (a vs b) when compared with their uninfected control. TGF-β also only decreased

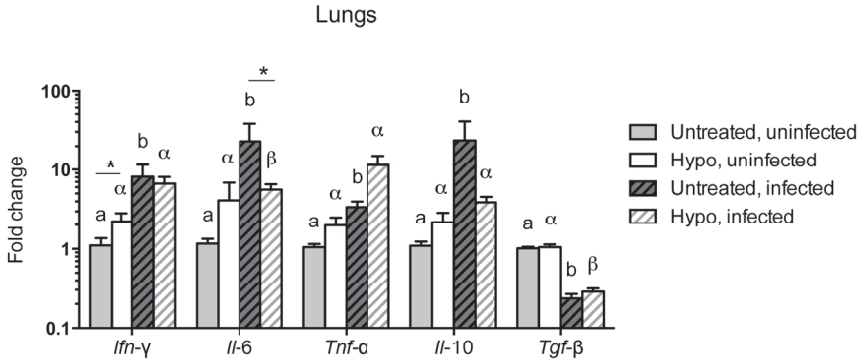

Figure 3. Uninfected hypo mice have higher expression of IFN-γ mRNA in lungs, and infected hypo mice have lower expression of IL-6 mRNA in lungs. Mice were i.n. infected with 3×10^7 CFUs of *S. pneumoniae* D39, and lungs were harvested at 24 and 48 hpi. Cytokine mRNA expression was measured by real-time qPCR. Values are relative to the average of the uninfected untreated group. Combined data from 2 independent experiments at 24 hpi (total n = 4–8 mice per group) and 3 at 48 hpi (total n = 7–11 mice per group) are shown. Data represent mean ± SEM. Comparisons between groups were made with Mann-Whitney *U* test; *, $P < .05$ indicates means are significantly different. Comparisons for the same group at different time points were made using a two-way ANOVA followed by Tukey test, means not sharing a common letter are significantly different ($P < .05$).

significantly in untreated mice with respect to the uninfected control. In the brain, TGF-β mRNA was the only cytokine that showed a significant difference between both infected groups, being higher in infected hypo mice ($P = .041$). Only infected untreated mice showed a significant increase in IL-10 mRNA (a vs b) when compared with their uninfected control, and again TGF-β mRNA only decreased significantly in untreated mice with respect to the uninfected control.

To evaluate whether cytokine mRNA levels in spleen correlated to protein levels in blood, we measured some of the same cytokines in plasma, using a Milliplex platform. As shown in Figure 5C, IFN-γ, IL-6, and TNF-α were significantly lower in hypo mice at 48 hpi ($P = .0113$, $P = .02$, and $P = .0250$). IL-1β was also measured and no differences were found between untreated and hypo mice. These results correlate with the mRNA quantification assays performed in spleen and suggest that hypo mice are able to restrict *S. pneumoniae* dissemination, preventing sepsis.

Because reduced amounts of proinflammatory cytokines were observed in hypo mice at 48 hpi, together with less bacterial load in blood and brain, it is possible that the amount of cytokines found in plasma depends on the amount of bacteria in blood. To address this question, plasma levels of IFN-γ, IL-6, TNF-α, and IL-10 from hypo and untreated mice at 48 hpi were plotted vs bacterial load. As shown in Supplemental Figure 5, no difference in the amount of these cytokines was observed between hypo and untreated mice that had equivalent amounts of bacteria in blood. Importantly, only untreated infected mice had more than 10^6 CFU/mL in blood (Figure 4 and Supplemental Figure 5), supporting the hypothesis that untreated mice are more susceptible than hypo mice to develop sepsis after i.n. infection by *S. pneumoniae*, which results in increased production of proinflammatory cytokines and death by sepsis.

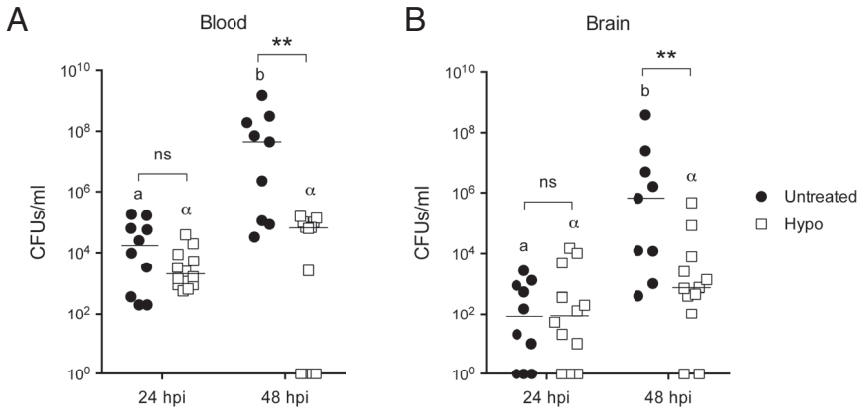

Figure 4. Hypo mice have lower bacterial load in blood and brain. Mice were i.n. infected with 3×10^7 CFUs of *S. pneumoniae* D39 and bacterial load was measured in blood and brain. A, Bacterial load in blood at 24 and 48 hpi. B, Bacterial load in brain at 24 and 48 hpi. Combined data from 3 independent experiments (total n = 8–12 mice per group) are shown. Bars represent the median. Comparisons between groups were made with Mann-Whitney *U* test; **, $P < .01$ indicates means are significantly different. Comparisons for the same group at different time points were made using a two-way ANOVA followed by Sidak test, groups not sharing a common letter are significantly different ($P < .05$).

To rule out the possibility that reduced bacterial load in blood and brain at 48 hpi is due to an enhanced ability of immune cells from hypo mice to clear bacteria, the ability of neutrophils to kill the pneumococcus was evaluated in vitro. To assess this, neutrophils were purified from bone marrow of uninfected untreated and hypo mice, cultured with preopsonized *S. pneumoniae* D39, and bacterial CFUs were counted by plating the reaction. Neutrophils derived from untreated and hypo mice were able to kill, in similar degree, approximately 50% of the initial bacterial cells (Supplemental Figure 6). This result shows that gestation under hypothyroidism does not affect the

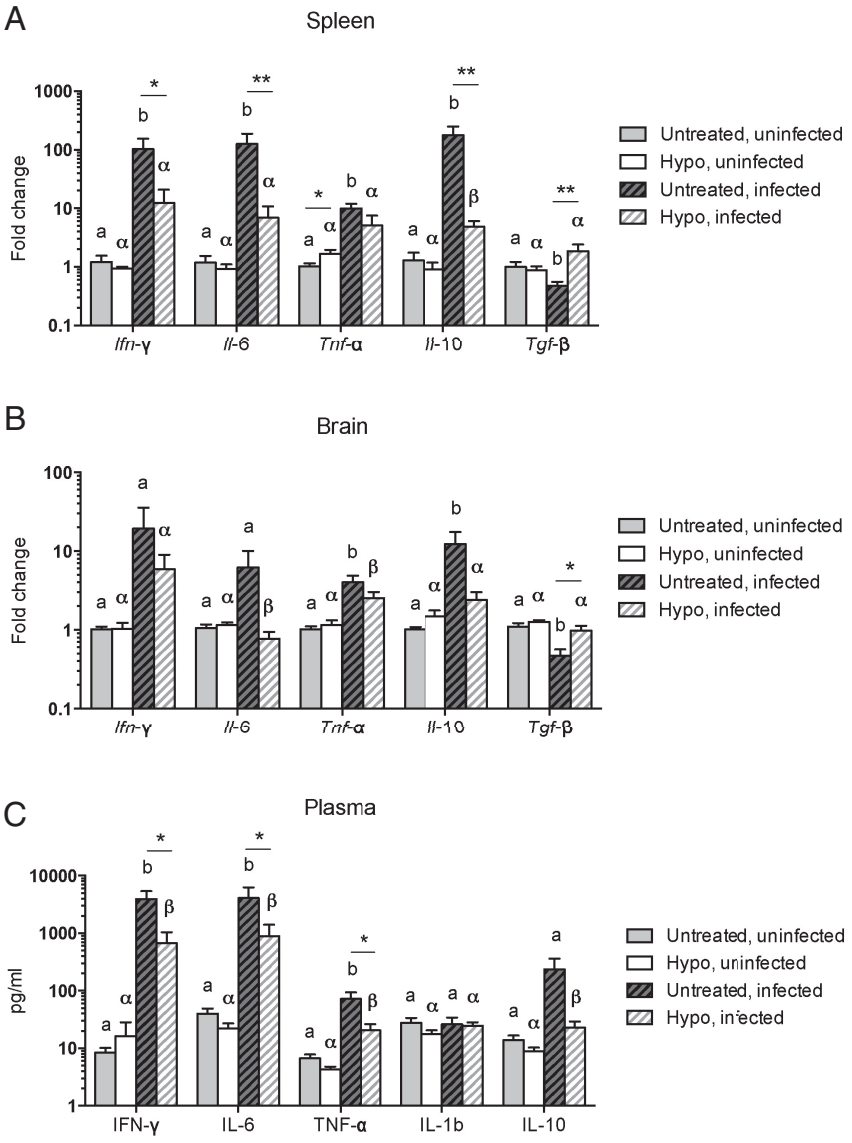

Figure 5. Hypo mice show a different cytokine profile in spleen, brain, and blood at 48 hpi. Mice were i.n. infected with 3×10^7 CFUs of *S. pneumoniae* D39, and spleen, brain, and blood were harvested at 48 hpi. Cytokine mRNA expression was measured in spleen and brain by real-time qPCR, and plasma samples were tested by multiplex analysis to calculate cytokine protein levels. Values are relative to the average of the uninfected untreated group. Combined data from 3 independent experiments (total n = 5–12 mice per group) are shown. Data represent mean ± SEM. Comparisons between groups were made with Mann-Whitney *U* test; *, $P < .05$ and **, $P < .01$ indicate means are significantly different. Comparisons for the same group at different time points were made using a two-way ANOVA followed by Sidak test, means not sharing a common letter are significantly different ($P < .05$).

functionality of neutrophils; therefore the reduced *S. pneumoniae* load in organs different from lungs cannot be explained by an increased bacterial clearance by neutrophils. In summary, these results suggest that neutrophils from hypo mice are able to respond to a bacterial challenge similar to untreated mice, and support the notion that resistance of hypo mice to *S. pneumoniae* challenge is due to reduced bacterial dissemination from lungs to other tissues.

Lung vascular permeability is higher in uninfected hypo mice

To address the mechanism accounting for reduced dissemination of *S. pneumoniae* from lungs to other organs in hypo mice, we evaluated whether lungs from hypo mice have any alteration in terms of functionality or morphology, before and after infection. Lung histopathology and total proteins in BAL supernatant were analyzed in uninfected mice and at 24 and 48 hpi. As seen in the representative ×40 photographs (Figure 6), untreated and hypo mice had similar histopathology at 24 and 48 hpi. Both uninfected groups have some cellular infiltrate, mainly composed of lymphocytes and macrophages and some loss in the alveolar structure in certain areas of the lungs, probably as a result of the i.n. inoculation with bacteria-free medium. Both infected groups showed marked cellular infiltration composed mainly of PMNs, along with abscesses, perivascular edema, hemorrhages, and a more pronounced loss of alveolar structure in some areas. The amount of total proteins in BAL, which gives an indication of lung inflammation and tissue damage, was similar in untreated and hypo mice at 24 and 48 hpi (data not shown). These results suggest that lungs from untreated and hypo mice do not have a different response in terms of damage or morphology upon infection.

Then, lung vascular permeability was evaluated in untreated and hypo mice (Figure 7). To measure this, EBD was administered by lateral tail vein injection 1 hour before the end of the 48 hours experiment. This dye is used to measure endothelial permeability; it binds to albumin in the blood, which in healthy conditions does not diffuse to tissues. Unexpectedly, uninfected hypo mice showed higher levels of EBD in lung tissue than uninfected untreated mice ($P = .0495$) (Figure 7). Infected animals did not have a significant increase in EBD lung concentration in comparison with uninfected mice, indicating the infection did not alter the vascular

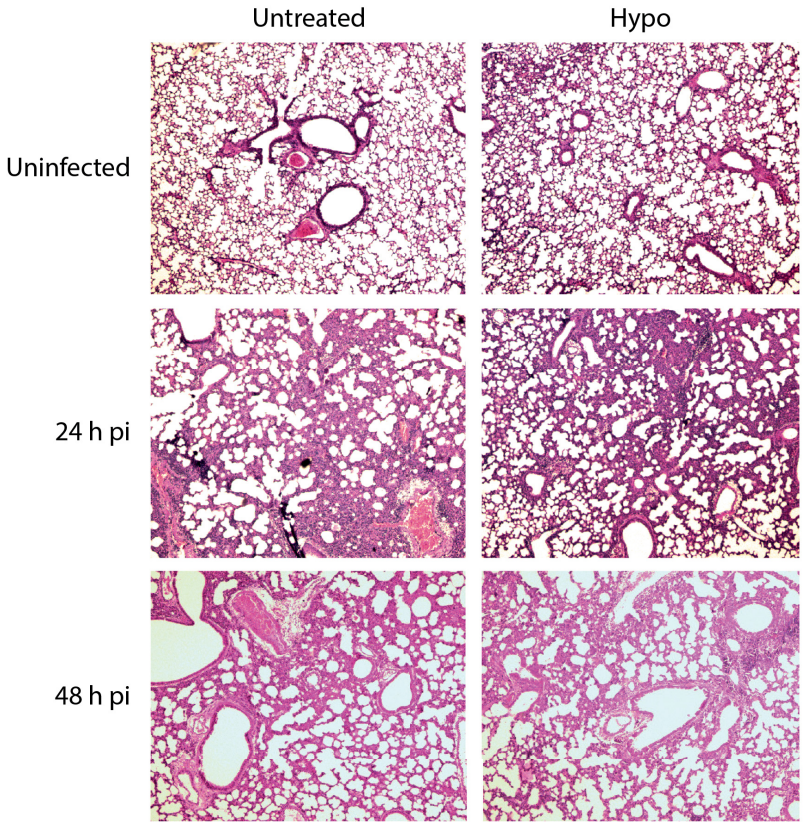

Figure 6. Untreated and hypo mice have similar lung tissue damage. Mice were i.n. infected with 3×10^7 CFUs of *S. pneumoniae* D39, lungs were removed, fixed, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin. Images were taken in an optical microscope at ×40 magnification.

permeability at this experimental time point. Although some infected untreated mice did have an increase in EBD lung levels, they were not significantly different from hypo mice at this point of study (Figure 7).

Discussion

This study is the first to report that gestational hypothyroidism imprints the female offspring with more resistance to die after an infection with *S. pneumoniae*. Even though female untreated and hypo mice showed similar response at 24 hpi, female hypo mice survived longer than female untreated mice (Figure 1B), and important differences were observed at 48 hpi: hypo mice had lower clinical score (Figure 1C), lower bacterial load in blood and brain (Figure 4), higher levels of innate cells in lungs (Figure 2A). These results and the fact that similar bacterial load was found in lungs of female untreated and hypo mice (Figure 1D) suggest that hypo mice are more efficient to prevent bacterial dissemination from lungs to other tissues.

The fact that the higher resistance to *S. pneumoniae* was only observed in females is in agreement with the work of Albornoz et al, in which only female offspring gestated in hypothyroidism had a higher disease score after EAE induction (20). This sex-dependent impact of gestational hypothyroidism was also reported by Bagheripuor et al (27), who found that male offspring gestated in hypothyroid rats had glucose intolerance, whereas female offspring did not. These findings suggest that the TH deficiency suffered during pregnancy could have affected the levels of female sex hormones of the progeny. In fact, THs have influence on sexual development; however, this interaction has been mostly studied in fish and frog species (28). In mammals, studies performed in males show that THs are important for the differentiation of Leydig cells and for the regulation of Sertoli cell development in the testes (28). There are no studies in mammals showing the effects of maternal THs in female sexual development. Rats treated with propylthiouracil to induce short-term hypothyroidism develop abnormal sexual cycling and increased levels of estradiol (29), suggesting that the induction of hypothyroidism during gestation using PTU or MMI could affect the sex hormones levels in the mother. Maternal levels of estradiol might affect the development of the progeny at least in males, whose testes are very sensitive to estrogen levels during fetal and neonatal development (30). Studies in rats have measured estradiol in female adults gestated in mothers with hypothyroidism, and they were found to be normal (27).

In this work, untreated male mice were more resistant to pneumococcal pneumonia than untreated female mice (Figure 1, A and B). In contrast, Kadioglu et al (31) found that male mice were more susceptible to this disease. However, the infective dose used in that study was lower, and it cannot be ruled out that diet, housing or breeding conditions influenced and changed the expected male/female behavior. On the other hand, other studies support our finding, showing that female mice are more susceptible to respiratory infections (32, 33).

Untreated and hypo mice had similar amounts of innate cells infiltrating the lungs at 24 hpi, but hypo mice had significantly more at 48 hpi (Figure 2A). Different hypotheses might explain this observation and need to be tested: 1) innate cells in hypo mice can normally reach the lungs,

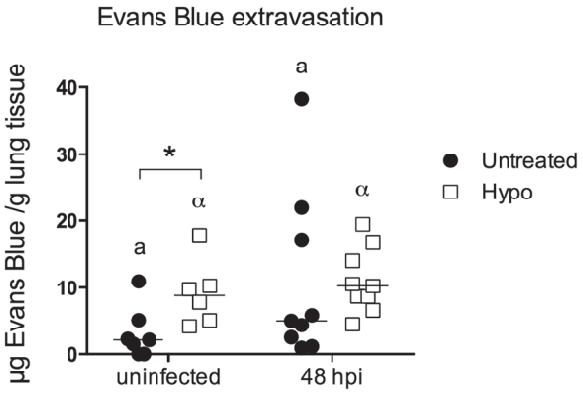

Figure 7. Hypo uninfected mice have higher basal lung permeability. Mice were i.n. infected with 3×10^7 CFUs of *S. pneumoniae* D39 and injected with 30 mg/kg weight of EBD through the tail vein 1 hour before end of the experiment. Forty-eight hpi mice were perfused, lungs were removed, homogenized, incubated in formamide, and the amount of EBD was quantified by spectrophotometry. Combined data from 2 independent experiments (total n = 5–7 mice per group) are shown. Bars represent the median. Comparisons between groups were made with Mann-Whitney *U* test; *, $P < .05$ indicates means are significantly different. Comparisons for the same group at different time points were made using a two-way ANOVA followed by Sidak test; no significant differences were found.

but once there they are prone to reside for a longer time due to altered expression of adhesion molecules in their surface; 2) innate cells of hypo mice have a longer half-life and are more resistant to die; or 3) the environment generated in lungs of infected hypo mice favors the permanence of innate cells in this tissue, due to different levels of chemokines or adhesion molecules expressed by lung parenchymal cells. A recent study from our group showed that IL-$10^{-/-}$ mice infected with pneumococcus have a similar profile, with higher numbers of neutrophils in lungs at 48 hpi, as compared with wild-type (WT) mice. However, these IL-$10^{-/-}$ mice had lower bacterial load in lungs than WT mice (34). A study with aged and young mice infected with pneumococcus showed that aged mice had more neutrophils in lungs due to a lower production of IL-10 and a concomitant increase in chemokines, such as Chemokine (C-C Motif) Ligand (CCL) 3, CCL4, and CCL5 (35). In the present study, we did not find a difference in IL-10 mRNA production between infected untreated and hypo mice; however, infected untreated mice had a significant increase in this mRNA in lungs in comparison with the uninfected untreated group (Figure 3). Therefore, it would be worthwhile to evaluate IL-10 and chemokine protein levels in lungs of untreated and hypo mice to see whether this is the cause of the higher numbers of innate cells in lungs at 48 hpi.

The lower bacterial load in blood and brain of hypo mice could be due to a higher bactericidal activity of innate cells in these mice. But the fact that PMNs from uninfected untreated and hypo mice killed the pneumococcus to a similar degree (Supplemental Figure 6) indicates that these cells per se do not have a difference in their ability to kill pathogens. In addition, with higher levels of innate cells at 48 hpi in lungs of hypo mice, it would be expected to find lower bacterial load, as in blood and brain. We cannot rule out that the conditions encountered in vivo during the infection promote a different phenotype of these cells in blood and brain and need to test this in the future.

Uninfected hypo mice had higher vascular permeability in the lungs in comparison with untreated mice (Figure 7), a difference that is lost under infection, at least at 48 hpi. This basal difference does not necessarily mean that bacteria will disseminate more easily from lungs to blood in infected hypo mice. The permeability was measured using EBD, which binds to albumin, and it is transported through endothelial cells by caveolin vacuoles (36). In inflamed and injured tissues, this transport also occurs in a paracellular manner, due to loss of cell-to-cell contact. On the other hand, bacteria can travel in the opposite direction through a variety of mechanisms, using paracellular and transcellular pathways. The pneumococcus can translocate endothelial cells with the aid of receptors such as the human polymeric Ig-receptor (37) and the platelet-activating factor receptor (38). Also, the pneumococcus has been shown to take advantage of the Toll-like receptor-mediated down-regulation of claudins, proteins that form tight junctions between adjacent cells, to translocate across the epithelium (39). Not directly involved in transport but in tissue damage instead, DCs have been shown to help *S. pneumoniae* dissemination from lungs to blood through the secretion of metalloproteinases (40). In another model of pneumococcal pneumonia, mice that overexpressed activated protein C showed similar bacterial load and tissue damage in lungs than WT mice, but less bacterial load in spleen and liver, a scenario that is similar to ours (although higher expression of activated protein C occurred along with less PMN recruitment into the lungs and BAL) (41). APC has agonistic effects on the bioactive lipid sphingosin-1-phosphatase, which plays an important role in the protection of endothelium integrity (42). Additional experiments must be conducted to explain the cause of higher basal permeability and to elucidate whether this phenomenon is related to bacterial dissemination.

Sepsis is an uncontrolled systemic response to an infection, in which both pro- and antiinflammatory mediators are released (43, 44). IFN-γ, IL-6, TNF-α, IL-1β, and IL-10 have been simultaneously detected in patients with septic shock (43, 45). We found lower expression of IFN-γ, IL-6, and IL-10 mRNAs in spleen of hypo mice (Figure 5A), with also lower levels of IFN-γ, IL-6, and TNF-α in plasma (Figure 5C). Our finding clearly indi-

cates that untreated mice are dying of septic shock, whereas hypo mice are able to recover from sepsis or maybe never develop it. TGF-β was the only cytokine analyzed that showed higher levels of mRNA in spleen and brain of hypo mice at 48 hpi (Figure 5). This cytokine, which is produced by almost all cells, has a potent suppressive action on T and B cells and its effects reduce the inflammatory damage in tissues after colonization with *S. pneumoniae* (46). We think this cytokine is increased in hypo mice because they are recovering from sepsis, and TGF-β is helping in the suppression of the immune response, along with IL-10.

Given that multiple parameters among untreated and hypo mice were compared in this study, a supplemental table that includes the effect size and 95% confidence intervals was generated (Supplemental Table 2). Based in the study by Blakesley et al (26), a multiple hypothesis testing has been performed to adjust the *P* values, using the Hommel method. However, in our study we considered the *P* values obtained before the adjustment, because there is agreement that the methodologies of *P* value adjustment are conservative and could lead to *P* values higher that 0.05, although there are in fact differences among the groups analyzed (47).

In conclusion, this work is the first evidence that a TH deficiency during gestation affects the immune response against a bacterial infection in the adult progeny. This fact adds to the already demonstrated effects of TH deficiency on CNS development and leads to think that many other processes might be affected in the progeny. The mechanisms that link TH deficiency during gestation and a better survival against a bacterial infection remain to be elucidated. The results shown in this work suggest that there might be a difference in the functionality of innate cells or in the composition of endothelial barriers. An overall assessment of the epigenetic, transcriptional and/or translational profile of mice gestated under TH deficiency would provide a broader picture and clues about which genetic pathways are affected.

Acknowledgments

Address all correspondence and requests for reprints to: Dr Susan M. Bueno, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Avenida Libertador Bernardo O'Higgins N°340, Santiago 8331010, Santiago, Chile. E-mail: sbueno@bio.puc.cl; or Dr Claudia A Riedel, Departamento de Ciencias Biológicas, Facultad de Ciencias Biológicas y Facultad de Medicina, Universidad Andrés Bello, República 217, Piso 4, Santiago 8370146, Santiago, Chile. E-mail: claudia.riedel@unab.cl.

This work was supported by Fondo Nacional de Ciencia y Tecnologia de Chile Grants 1140010, 1110604, 1100971, 1131012, 1110397, and 1130996; the Millennium Institute on Immunology and Immunotherapy Grant P09/016-F; and by Grant Nouvelles Equipes-Nouvelles Thématiques from La Région Pays De La Loire. P.A.N., H.F.P., and L.V. are supported by the Comisión Nacional de Investigación Científica y Tecnológica. A.M.K. is a Chaire De La Région Pays De La Loire, Chercheur Étranger D'excellence, France. The Millennium Institute on Immunology and Immunotherapy is a FOCIS Center of Excellence.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Cheng SY, Leonard JL, Davis PJ.** Molecular aspects of thyroid hormone actions. *Endocr Rev*. 2010;31:139–170.
2. **Williams GR.** Neurodevelopmental and neurophysiological actions of thyroid hormone. *J Neuroendocrinol*. 2008;20:784–794.
3. **Gereben B, Zavacki AM, Ribich S, et al.** Cellular and molecular basis of deiodinase-regulated thyroid hormone signaling. *Endocr Rev*. 2008;29:898–938.
4. **Roberts CG, Ladenson PW.** Hypothyroidism. *Lancet*. 2004;363:793–803.
5. **Vanderpump M.** The epidemiology of thyroid disease. *Br Med Bull*. 2011;99:39–51.
6. **Patel J, Landers K, Li H, Mortimer RH, Richard K.** Thyroid hormones and fetal neurological development. *J Endocrinol*. 2011;209:1–8.
7. **Pop VJ, Kuijpens JL, van Baar AL, et al.** Low maternal free thyroxine concentrations during early pregnancy are associated with impaired psychomotor development in infancy. *Clin Endocrinol (Oxf)*. 1999;50:149–155.
8. **Pop VJ, Brouwers EP, Vader HL, Vulsma T, van Baar AL, de Vijlder JJ.** Maternal hypothyroxinaemia during early pregnancy and subsequent child development: a 3-year follow-up study. *Clin Endocrinol (Oxf)*. 2003;59:282–288.
9. **Vermiglio F, Lo Presti VP, Moleti M, et al.** Attention deficit and hyperactivity disorders in the offspring of mothers exposed to mild-moderate iodine deficiency: a possible novel iodine deficiency disorder in developed countries. *J Clin Endocrinol Metab*. 2004;89:6054–6060.
10. **Li Y, Shan Z, Teng W, et al.** Abnormalities of maternal thyroid function during pregnancy affect neuropsychological development of their children at 25–30 months. *Clin Endocrinol (Oxf)*. 2010;72:825–829.
11. **Román GC, Ghassabian A, Bongers-Schokking JJ, et al.** Association of gestational maternal hypothyroxinemia and increased autism risk. *Ann Neurol*. 2013;74:733–742.
12. **Chang K, Shin JI.** Association of gestational maternal hypothyroxinemia and increased autism risk: the role of brain-derived neurotrophic factor. *Ann Neurol*. 2014;75:971.
13. **Pathak A, Sinha RA, Mohan V, Mitra K, Godbole MM.** Maternal thyroid hormone before onset of fetal thyroid function regulates reelin and downstream signaling cascade affecting neocortical neuronal migration. *Cereb Cortex*. 2011;21:11–21.
14. **Gong J, Liu W, Dong J, et al.** Developmental iodine deficiency and hypothyroidism impair neural development in rat hippocampus: involvement of doublecortin and NCAM-180. *BMC Neurosci*. 2010;11:50.
15. **Schoonover CM, Seibel MM, Jolson DM, et al.** Thyroid hormone regulates oligodendrocyte accumulation in developing rat brain white matter tracts. *Endocrinology*. 2004;145:5013–5020.

16. **Ahmed OM, El-Gareib AW, El-Bakry AM, Abd El-Tawab SM, Ahmed RG.** Thyroid hormones states and brain development interactions. *Int J Dev Neurosci*. 2008;26:47–209.
17. **Kobayashi K, Tsuji R, Yoshioka T, et al.** Effects of hypothyroidism induced by perinatal exposure to PTU on rat behavior and synaptic gene expression. *Toxicology*. 2005;212:135–147.
18. **Rooney AA, Fournier M, Bernier J, Cyr DG.** Neonatal exposure to propylthiouracil induces a shift in lymphoid cell sub-populations in the developing postnatal male rat spleen and thymus. *Cell Immunol*. 2003;223:91–102.
19. **Nakamura R, Teshima R, Hachisuka A, et al.** Effects of developmental hypothyroidism induced by maternal administration of methimazole or propylthiouracil on the immune system of rats. *Int Immunopharmacol*. 2007;7:1630–1638.
20. **Albornoz EA, Carreño LJ, Cortes CM, et al.** Gestational hypothyroidism increases the severity of experimental autoimmune encephalomyelitis in adult offspring. *Thyroid*. 2013;23:1627–1637.
21. **Bogaert D, De Groot R, Hermans PW.** Streptococcus pneumoniae colonisation: the key to pneumococcal disease. *Lancet Infect Dis*. 2004;4:144–154.
22. **Morrill HJ, Caffrey AR, Noh E, LaPlante KL.** Epidemiology of pneumococcal disease in a national cohort of older adults. *Infect Dis Ther*. 2014;3:19–33.
23. **Jefferson T, Ferroni E, Curtale F, Giorgi Rossi P, Borgia P.** *Streptococcus pneumoniae* in western Europe: serotype distribution and incidence in children less than 2 years old. *Lancet Infect Dis*. 2006;6:405–410.
24. **World Health Organization.** Fact sheet N°331. 2014. Available at http://www.who.int/mediacentre/factsheets/fs331/en/.
25. **Mook-Kanamori B, Geldhoff M, Troost D, van der Poll T, van de Beek D.** Characterization of a pneumococcal meningitis mouse model. *BMC Infect Dis*. 2012;12:71.
26. **Blakesley RE, Mazumdar S, Dew MA, et al.** Comparisons of methods for multiple hypothesis testing in neuropsychological research. *Neuropsychology*. 2009;23:255–264.
27. **Bagheripuor F, Ghanbari M, Zahediasl S, Ghasemi A.** Comparison of the effects of fetal hypothyroidism on glucose tolerance in male and female rat offspring. *J Physiol Sci*. 2015;65:179–185.
28. **Delbès G, Levacher C, Habert R.** Estrogen effects on fetal and neonatal testicular development. *Reproduction*. 2006;132:527–538.
29. **Hapon MB, Gamarra-Luques C, Jahn GA.** Short term hypothyroidism affects ovarian function in the cycling rat. *Reprod Biol Endocrinol*. 2010;8:14.
30. **Duarte-Guterman P, Navarro-Martín L, Trudeau VL.** Mechanisms of crosstalk between endocrine systems: regulation of sex steroid hormone synthesis and action by thyroid hormones. *Gen Comp Endocrinol*. 2014;203:69–85.
31. **Kadioglu A, Cuppone AM, Trappetti C, et al.** Sex-based differences in susceptibility to respiratory and systemic pneumococcal disease in mice. *J Infect Dis*. 2011;204:1971–1979.
32. **Guilbault C, Stotland P, Lachance C, et al.** Influence of gender and interleukin-10 deficiency on the inflammatory response during lung infection with *Pseudomonas aeruginosa* in mice. *Immunology*. 2002;107:297–305.
33. **Yanke SJ, Olson ME, Davies HD, Hart DA.** A CD-1 mouse model of infection with *Staphylococcus aureus*: influence of gender on infection with MRSA and MSSA isolates. *Can J Microbiol*. 2000;46:920–926.
34. **Peñaloza HF, Nieto PA, Muñoz-Durango N, et al.** Interleukin-10 plays a key role in the modulation of neutrophils recruitment and lung inflammation during infection by *Streptococcus pneumoniae*. *Immunology*. 2015;146:100–112.
35. **Williams AE, José RJ, Brown JS, Chambers RC.** Enhanced inflammation in aged mice following infection with *Streptococcus pneumoniae* is associated with decreased IL-10 and augmented chemokine production. *Am J Physiol Lung Cell Mol Physiol*. 2015;308:L539–L549.
36. **Sun Y, Minshall RD, Hu G.** Role of caveolin-1 in the regulation of pulmonary endothelial permeability. *Methods Mol Biol*. 2011;763:303–317.
37. **Asmat TM, Agarwal V, Saleh M, Hammerschmidt S.** Endocytosis of *Streptococcus pneumoniae* via the polymeric immunoglobulin receptor of epithelial cells relies on clathrin and caveolin dependent mechanisms. *Int J Med Microbiol*. 2014;304:1233–1246.
38. **LeMessurier KS, Hacker H, Chi L, Tuomanen E, Redecke V.** Type I interferon protects against pneumococcal invasive disease by inhibiting bacterial transmigration across the lung. *PLoS Pathog*. 2013;9:e1003727.
39. **Clarke TB, Francella N, Huegel A, Weiser JN.** Invasive bacterial pathogens exploit TLR-mediated downregulation of tight junction components to facilitate translocation across the epithelium. *Cell Host Microbe*. 2011;9:404–414.
40. **Rosendahl A, Bergmann S, Hammerschmidt S, Goldmann O, Medina E.** Lung dendritic cells facilitate extrapulmonary bacterial dissemination during pneumococcal pneumonia. *Front Cell Infect Microbiol*. 2013;3:21.
41. **de Boer JD, Kager LM, Roelofs JJ, et al.** Overexpression of activated protein C hampers bacterial dissemination during pneumococcal pneumonia. *BMC Infect Dis*. 2014;14:559.
42. **Feistritzer C, Riewald M.** Endothelial barrier protection by activated protein C through PAR1-dependent sphingosine 1-phosphate receptor-1 crossactivation. *Blood*. 2005;105:3178–3184.
43. **Tamayo E, Fernández A, Almansa R, et al.** Pro- and anti-inflammatory responses are regulated simultaneously from the first moments of septic shock. *Eur Cytokine Netw*. 2011;22:82–87.
44. **Osuchowski MF, Craciun F, Weixelbaumer KM, Duffy ER, Remick DG.** Sepsis chronically in MARS: systemic cytokine responses are always mixed regardless of the outcome, magnitude, or phase of sepsis. *J Immunol*. 2012;189:4648–4656.
45. **Bozza FA, Salluh JI, Japiassu AM, et al.** Cytokine profiles as markers of disease severity in sepsis: a multiplex analysis. *Crit Care*. 2007;11:R49.
46. **Letterio JJ.** Murine models define the role of TGF-β as a master regulator of immune cell function. *Cytokine Growth Factor Rev*. 2000;11:81–87.
47. **Wright SP.** Adjusted *P* values for simultaneous inference. *Biometrics*. 1992;48:1005–1013.